The escherichia coli chromosome replication initiator protein, DnaA

Mutational analysis of the DNA binding domain and analysis of amino acid substitutions in DnaA5 and DnaA46

Nyborg, Malene

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The *Escherichia coli* Chromosome Replication Initiator Protein, DnaA.

Mutational Analysis of the DNA Binding Domain and Analysis of Amino Acid Substitutions in DnaA5 and DnaA46

Marlene Nyborg

Department of Life Sciences and Chemistry

Roskilde University

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Forord

Denne Ph.D afhandling er udført ved Roskilde Universitetscenter i perioden 1995-1999. Jeg vil først og fremmest takke min vejleder Tove Atlung for særdeles god vejledning og diskussioner og for at være positiv og inspirerende når resultaterne lader vente på sig. Endvidere takkes Ole Skovgaard og Ole Michelsen for hjælp med diverse computer programmer/problemer, Benny Pedersen for diskussioner af *in vitro* assays, Flemming G. Hansen for stor hjælp med flow cytometret og alle fra 'Mandagsmøde-sjakket' for gode diskussioner og råd. Til sidst vil jeg gerne takke Kirsten Olesen for med højt humør at have holdt mig med selskab i laboratoriet, hjulpet mig med diverse tekniske opstillinger og ikke mindst for at have hjulpet mig med sekvenseringen af de mange *dnaA* mutanter.

Denne afhandling er delt op i Part I og Part II. Part I er introducerende og inkluderer Chapter 1 (The initiation of chromosome replication and controlling elements) og Chapter 2 (The DnaA-DnaA box interaction), mens Part II inkluderer det eksperimentelle arbejde, som er delt op i to kapitler. Chapter 3 indeholder undersøgelser det DNA bindende domæne af DnaA proteinet (The DNA binding domain of the DnaA protein) og Chapter 4 indeholder undersøgelser af DnaA(A184V) proteinet og andre DnaA proteiner (Analysis of the A184V, H252Y and G426S substitutions in the DnaA protein). Afhandlingen indeholder endvidere publikationen : Two types of cold sensitivity associated with the A184→V change in the DnaA protein.

Marlene Nyborg

Roskilde Universitetscenter, April 2000.

Summary

The experimental work presented in this thesis involve mutational analysis of the DNA binding domain of the DnaA protein and analysis of the A184V substitution in the ATP area of domain III and other amino acid substitutions found in the DnaA5 and DnaA46 proteins.

To analyse the DNA binding domain, more than 100 functional DnaA proteins with amino acid substitutions in the DNA binding domain were constructed and studies *in vivo* by complementation analysis of the high temperature sensitive *dnaA46* phenotype by induction of plasmid born mutant *dnaA*(IV) alleles. The results indicate that the whole domain is involved in DNA binding. However, there seems to exist more residues in the first half and the very C-terminal region that can be substituted with non-closely related amino acids with no apparent effect on DnaA activity. Based on alignment of the DNA binding domain of the DnaA protein with proteins with known binding motifs, it is suggested that helix 3 and 4 in the DNA binding domain of the DnaA protein carries a modified helix-turn-helix motif. The results of the *in vivo* complementation analysis carried out in this study support this proposal. A more extensive analysis was carried out with 20 mutant DnaA proteins. The study included *in vivo* and *in vitro* binding analysis. The results of these analyses indicate that many of the mutant DnaA proteins retained relatively high affinity for *oriC*, but reduced specificity. DnaA proteins with the highest affinity, but lowest specificity had amino acid substitutions in α-helix 1 and in the following basic loop. A DnaA protein with a K397E substitution had higher affinity and specificity for *oriC in vitro* than the $DnaA^+$ protein had, and a strain harbouring the *dnaA*(K397E) allele on the chromosome overinitiated chromosome replication at 32°C and 42°C with a DnaA(K397E) concentration of only one fourth of the DnaA concentration in a wild type strain. Higher DnaA(K397E) concentrations seemed to inhibit initiation of chromosome replication probably due to too tight binding of the *oriC* area by DnaA(K397E) proteins.

The A184V substitution in the ATP area of domain III of the DnaA protein and other amino acid substitutions found in the DnaA5 and DnaA46 proteins were studied *in vivo* and *in vitro*. Multicopy *dnaA*(A184V) and *dnaA5* strains, which are cold sensitive, initiated chromosome replication extensively at non-permissive temperature, but the initiations were not elongated and DNA synthesis stopped. On the contrary, multicopy *dnaA46* strains (and the *dnaAcos* mutant), which are also cold sensitive, overinitiated

chromosome replication at non-permissive temperature, but (some of) these initiation were elongated and resulted in an increased DNA concentration per mass especially in the multicopy *dnaA46* cells. Multicopy *dnaA*(H252Y) and *dnaA*(G426S) strains are temperature resistant like multicopy *dnaA*$^+$ strains. However, *in vitro* binding analysis indicates that both the DnaA(H252Y) and DnaA(G426S) proteins have reduced affinity and specificity for *oriC* as the DnaA46 and DnaA5 proteins, while the DnaA(A184V) protein retained as high affinity and specificity for *oriC in vitro* as the DnaA$^+$ protein. A strain harbouring the *dnaA*(A184V) allele on the chromosome overinitiated chromosome replication at 42°C under conditions where the DnaA concentration was only one fourth of the DnaA concentration in a wild type strain. At 32°C, the initiation of chromosome replication decreased severely with increasing DnaA(A184V) concentrations.

Resumé

Det eksperimentelle arbejde, som præsenteres i denne afhandling omfatter en mutationsanalyse af det DNA binding domæne af DnaA proteinet og en analyse af A184V substitutionen i det ATP bindende domæne og andre aminosyre substitutioner fundet i DnaA5 og DnaA46 proteinerne.

For at undersøge det DNA bindende domæne blev mere end 100 funktionelle DnaA proteiner med aminosyre substitutioner i det DNA bindende domæne konstrueret og studeret *in vivo*. Analysen bestod i at undersøge hvor godt inducerbare plasmidbårne *dnaA*(IV) alleler komplementerede den høj-temperatur sensitive *dnaA46* mutant. Resultaterne indikerer, at hele domænet er involveret i DNA binding. Der er imidlertid flere aminosyrer i den første halvdel og i den helt C-terminale del, som kan erstattes uden nogen tilsyneladende effekt på DnaA aktiviteten. Ved at sammenligne aminosyre sekvensen af det DNA bindende domæne af DnaA proteinet med aminosyre sekvenser fra andre proteiner med kendte bindingsmotiver er det foreslået, at helix 3 og 4 i det DNA bindende domæne af DnaA proteinet kan have et modificeret helix-turn-helix bindingsmotiv. Resultaterne af *in vivo* komplementationsanalysen støtter dette forslag. Der blev foretaget en mere indgående undersøgelse af omkring 20 mutante DnaA proteiner. Undersøgelsen inkluderede *in vivo* og *in vitro* bindingsanalyser. Resultaterne af disse undersøgelse indikerer, at mange af DnaA proteinerne stadig har relativ høj

affinitet til *oriC*, men til gengæld er bindingen mindre specifik. De DnaA proteiner med den højeste affinitet men lavest specificitet bærer aminosyre substitutioner i α-helix 1 og i det efterfølgende basiske loop. DnaA proteinet med K397E substitution har højere affinitet og specificitet til *oriC* sammenlignet med DnaA$^+$ proteinet. Når *dnaA*(K397E) allelen sættes ind på kromosomet overinitieres kromosom replikationen ved 32°C og 42°C, selvom DnaA(K397E) koncentrationen kun er en fjerdedel af hvad DnaA koncentrationen er i en vild type stamme. Det ser ud til, at højere DnaA(K397E) koncentrationer hæmmer initieringen af kromosom replikationen. Dette skyldes måske at DnaA(K397E) proteinerne binder *oriC* området med for høj affinitet.

A184V substitutionen i det ATP bindende område i domæne III og andre aminosyre substitutioner fundet i DnaA5 og DnaA46 proteinerne blev undersøgt *in vivo* og *in vitro*. Multi-kopi *dnaA*(A184V) og *dnaA5* stammer er kulde sensitive. De initierer kromosom replikationen voldsomt ved ikke-permissive temperaturer, men initieringerne bliver imidlertid ikke elongeret, hvorved DNA syntesen stopper. Multi-kopi *dnaA46* stammer (og *dnaAcos* mutanten) er også kulde sensitive. Her overinitieres kromosom replikationen også, men nogle af initieringer elongeres, hvilket resulterer i et højere DNA indhold pr. masse. Multi-kopi *dnaA*(H252Y) og *dnaA*(G426S) stammerne er temperatur resistente ligesom multi-kopi *dnaA*$^+$ stammer. *in vitro* bindingsanalyser tyder dog på, at både DnaA(H252Y) og DnaA(G426S) proteinerne samt DnaA46 og DnaA5 proteinerne har reduceret affinitet til *oriC*, mens DnaA(A184V) proteinet har samme høje affinitet og specificitet til *oriC* som DnaA$^+$ proteinet. Når *dnaA*(A184V) allelen sættes in på kromosomet resulterer det i overinitiering af kromosom replikationen ved 42°C, selvom DnaA(A184V) koncentrationene kun er en fjerdedel af hvad DnaA koncentrationen er i en vild type stamme. Ved 32°C ser det ud til, at initieringen af kromosom replikationen ophører med stigende DnaA(A184V) koncentrationer.

Part I

Introduction

Chapter 1

Initiation of chromosome replication and controlling elements

Life is dependent on cell division and therefore dependent on replication of the chromosome, which is regulated at the level of initiation (Cooper and Helmstetter, 1968; for review see von Meyenburg and Hansen, 1987; Weigel and Messer, 1996; Skarstad and Boye, 1994). Initiation of chromosome replication is precisely coupled to the growth rate so that the bacterial genome is initiated once every cell cycle. Cooper and Helmstetter (1968) defined two constants based on experiments with *E.coli* B/r: the C period, which is the time between initiation and termination of the DNA replication (app. 40 min.), and the D period, which is the time between termination of DNA replication and cell division (app. 20 min.). If the C+D period is shorter than the doubling time, there will be a gap between cell division and initiation, and the cell has one origin and one terminus, prior to an initiation. Yet, if the C+D period is longer than the doubling time, replication reinitiates before termination of the previous initiation, and the cell has more origins than termini depending on the doubling time. However, since the cell mass increase with increasing growth rate (Schaechter *et al.*, 1958), the cell mass per replication origin, at the time of initiation, is constant and independent of the growth rate of the cells (Donachie, 1968). Experimental results with fast growing *E.coli* B/r have shown agreement about the length of the C and D period being app. 40 and 20 min., respectively (Helmstetter, 1996; Skarstad *et al.*, 1983). However, slowly growing *E.coli* B/r strains have a longer C period (Skarstad *et al.*, 1983; Churchward and Bremer, 1977). The same tendency is observed with *E.coli* K12 strains (Allman *et al.*, 1991; for review see Helmstetter, 1996). More recent results with *E.coli* B/r and *E.coli* K-12 strains grown with different growth rates have shown that the C-period decrease from about 70 min. at a growth rate of 0.6 doublings per hour to 33 min. at a growth rate of 3.0 doublings per hour (Bipatnath *et al.*, 1998). The reason why the length of the C period increases with decreasing growth rate, and what controls the DNA synthesis velocity, is not clear. The controlling elements may be directly or indirectly growth rate regulated, and it could be a protein that affects the DNA polymerase and/or the DNA conformation.

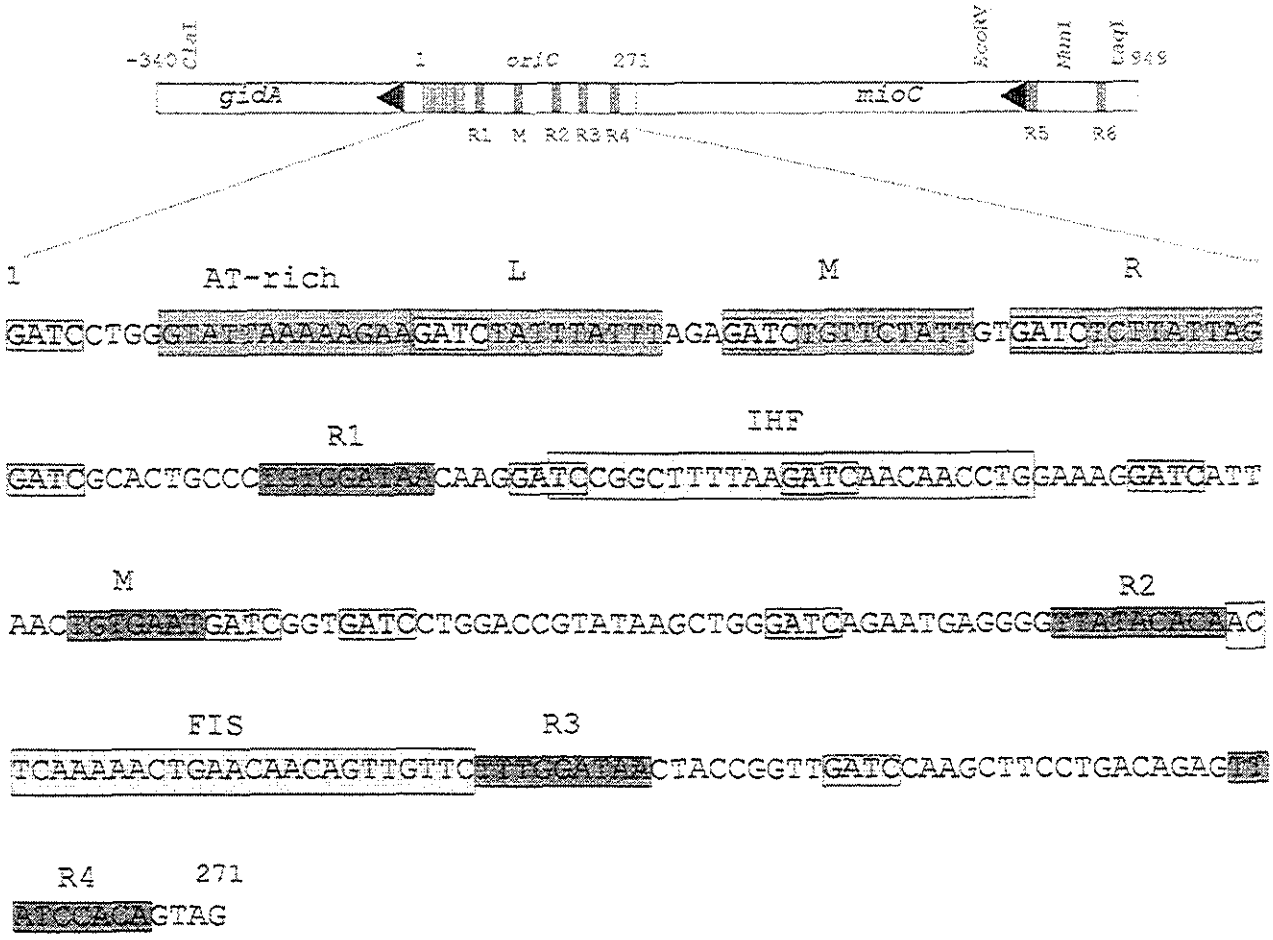

Fig. 1.1. The *E.coli* chromosomal origin and surrounding genes. DnaA boxes: red, GATC sites: yellow, AT rich 13-mers: green and IHF and FIS binding sites: grey

The chromosome replication and participating proteins

The chromosome replication is initiated at a fixed site on the chromosome, *oriC*, and after initiation, the replication proceeds bidirectionally towards the terminus, *terC* (Bird *et al.*, 1972). Thus, the DNA replication can be divided into three events: initiation, elongation and termination. The minimal region required for initiation is 245 bp long (Fig. 1.1. Oka *et al.*, 1980), and it contains a number of interesting features. There are three AT rich 13-mers on the left border of DnaA box R1 (see below), 11 GATC sites, which are methylated by the Dam methyltransferase, an event that may participate in the regulation of the initiation (Ogden *et al.*, 1988; Russell and Zinder, 1987. See below) and five DnaA boxes with the consensus $TT^{A}_{T}TNCACA$ (Schaper and Messer, 1995), to which the DnaA protein binds (Fuller *et al.*, 1984; Samitt *et al.*, 1989). Bramhill and Kornberg (1988A and 1988B) have proposed a model for the initiation event based on *in vivo* and *in vitro* experiments (Fig. 1.2): 20-40 DnaA proteins bind to the DnaA boxes within *oriC* (Fuller *et al.*, 1984; Crooke *et al.*, 1993; Samitt *et al.*, 1989) to form an **initial complex**. In the presence of ATP and HU/IHF (Hwang and Kornberg, 1992) it

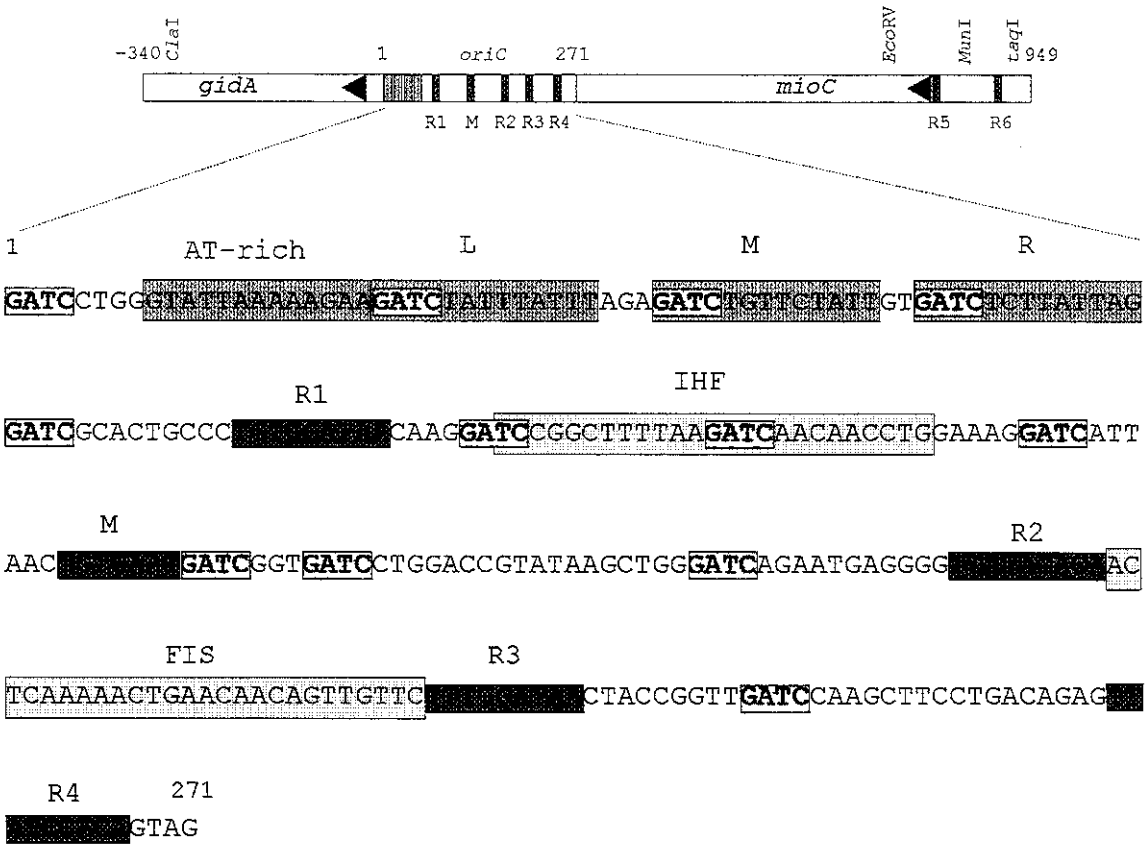

Fig. 1.1. The *E.coli* chromosomal origin and surrounding genes. DnaA boxes (R1, M, R2, R3 and R4): red, GATC sites: yellow, AT rich 13-mers: green and IHF and FIS binding sites: blue

The chromosome replication and participating proteins

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melts the AT rich 13-mers to form an **open complex** (Bramhill and Kornberg, 1988B; Gille and Messer, 1991) to which the DnaB-DnaC complex is guided through interactions between DnaA and DnaB (Marsazalek and Kaguni, 1994) to form a **prepriming complex** (Funnell *et al.*, 1987; Baker *et al.*, 1987). The function of the DnaB protein might be to unwind the DNA bidirectionally, and together with SSB and gyrase, to generate two forks, after which the DnaG primes the chain elongation carried out by the DNA polymerase. From this it is evident that both the initiation, but also the elongation and termination of the chromosomal replication is an extremely ordered process that requires high degree of coordinated interactions between the involved proteins. In addition, other DNA binding and bending proteins, than mentioned in the model, may contribute to the overall conformation of the chromosome that is required for a proper coordination (for review see Azam and Ishihama, 1999).

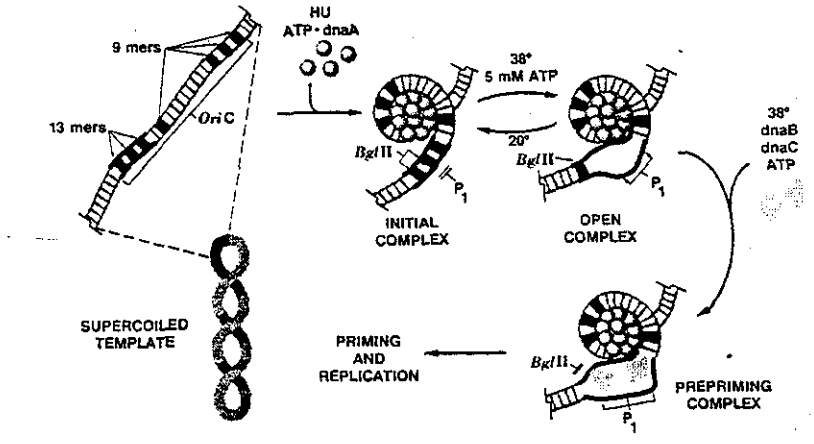


Fig. 1.2. The scheme for *oriC* initiation proposed by Bramhill and Kornberg (1988A, 1988B).

The model proposed by Bramhill and Kornberg (1988A, 1988B) may be simplified and it does not include the importance of, for instance, the RNA polymerase, which is required *in vivo*. High-temperature sensitive *rpoA, B, C, D* mutants have 'slow stop' phenotypes (for review see McMacken *et al.*, 1987), and rifampicin, which inhibits the RNA polymerase (Lark, 1972), prevents new initiations from *oriC*, but allows already ongoing replication forks to proceed to the terminus. This strongly suggests that the RNA polymerase activity is required in the initiation event. In addition, comparison of the amount of DNA synthesised in cultures treated with rifampicin and chloramphenicol has indicated that the rifampicin sensitive step follows the chloramphenicol sensitive one (Lark, 1972; Messer, 1972; Hansen, 1995). A number of promoters are located

around and within *oriC*. Some are negatively regulated (P_{mioC} and $P_{ori\text{-}L}$) whereas others are positively regulated (P_{gidA} and $P_{ori\text{-}R}$) by DnaA (Asai *et al.*, 1992; Løbner-Olesen *et al.*, 1987; Szalewska-Palashz *et al.*, 1998). The function of the RNA polymerase is still unknown. However, analysis of transcriptions from surrounding promoters on the initiation from plasmid born *oriC*, it has been suggested that transcription of *gidA* activates replication by induction of negative superhelicity behind the polymerase, and that this superhelicity facilitates unwinding of the DNA duplex required in the formation of the open complex (Ogawa and Okazaki, 1991; Asai *et al.*, 1992). On the contrary, transcription from the *mioC* promoter had a negative effect on the initiation process at least when the transcription frequency was too high (Ogawa and Okazaki, 1991). Studies of the effect of transcription of *gidA* and *mioC* on the initiation from the chromosomal *oriC* have indicated that the transcriptions have only minor (or no) effect on the initiation frequency (Løbner-Olesen and Boye, 1992; Bogan and Helmstetter, 1996), and may only be needed under sub-optimal conditions (Bates *et al.*, 1997). This is in spite of the observation that the *mioC* and *gidA* transcription fluctuate during the cell cycle; the *mioC* transcription is shut off while *gidA* transcription peaks prior to an initiation (Ogawa and Okazaki, 1994; Bogan and Helmstetter, 1996; Theisen *et al.*, 1993). The term transcriptional activation may therefore not involve transcriptions of *gidA* and *mioC*, but instead transcriptions from promoter within *oriC*. Alternatively, as suggested by Bates *et al.* (1997), transcriptional activation could include transcriptions of genes in general. When rifampicin is added, all transcriptions are shut down, and this may severely alter the structure of *oriC*. Thus, the rifampicin effect may be non-specific and not due to the inhibition of the *mioC* and *gidA* promoters. von Freiesleben and Rasmussen (1992) have found that when the level of supercoiling is altered in *gyrB* and *topA* mutants, the replication is initiated asynchronously, and thus, the DNA topology influences the initiation event. Specific *rpoB* mutations suppress the high-temperature sensitive phenotype of certain *dnaA* mutants (Atlung *et al.*, 1984; Bagdasarian *et al.*, 1977; Szalewska-Palashz *et al.*, 1998), and this may indicate that the RNA polymerase and the DnaA protein interact. Alternatively, *rpoB* mutations may result in an altered transcription frequency from different promoters including the *dnaA* promoter. It has been shown that a mutation in the *rpoC* gene, which encode the β' subunit of the RNA polymerase, resulted in a significant increase in the DnaA concentration (Petersen and Hansen, 1991). Thus, although the σ subunit remained intact, its affinity to various promoters seemed affected by amino acid substitutions in other subunits. The hypothesis that the RNA polymerase and the DnaA protein interact is further supported

by the observation that rifampicin resistant initiations can take place in certain *dnaA* mutants, maybe because the interaction with mutant DnaA proteins prevent rifampicin from binding to the β-subunit of the polymerase (Hansen, 1995; Tippe-Schindler *et al.*, 1979; Hanna and Carl, 1975). Thus, the results could indicate that the RNA polymerase participates in the initiation of chromosome replication in a more complex manner, and it may both interact with the DnaA protein and transcriptionally activate *oriC*.

In the model proposed by Bramhill and Kornberg (1988) (Fig. 1.2), it is obvious that the DnaA protein is a key protein in the initiation event. In addition, several experiment indicate that the DnaA concentration determines when the initiation takes place, and thus, the DnaA protein may be the regulator of the chromosomal replication (Hirota, 1970; Løbner-Olesen *et al.*, 1989; Hansen and Rasmussen, 1977; Atlung *et al.*, 1987; for reviews see Skarstad and Boye, 1994; von Meyenburg and Hansen, 1987; Messer and Weigel, 1996). Most of the experiments consist of analysis of 1) mutant *dnaA* strains 2) strains in which the DnaA protein or DnaA box concentration has been changed, and 3) measurements of DnaA concentrations in various strains under different growth conditions.

Early experiments with the high-temperature sensitive *dnaA46* and *dnaA5* mutants showed that the mutants stop initiation of chromosome replication at non-permissive temperature. This, in itself, does not indicate that the DnaA protein regulates the initiation, but can be explained by a lost affinity to the DnaA boxes in *oriC*. The more interesting observation was, however, that the strains accumulated initiation capacity at non-permissive temperature, which resulted in a burst of initiations upon return to permissive temperature (Hirota, 1970; Hansen and Rasmussen, 1977; Hanna and Carl, 1975; Tippe-Schindler *et al.*, 1979; Hansen and Atlung, 1995A; Hansen, 1995). This strongly suggests that the mutant DnaA proteins accumulated at non-permissive temperature, and reactivation, maybe by conformational changes of the proteins (see below), at permissive temperature resulted in overinitiations. Thus, other initiation - and replication participating proteins did not seem to be limiting. The observed initiation burst upon return to permissive temperature did not require protein synthesis. This may be explained by the finding that the *dnaA* promoter, which carries a DnaA box (Hansen *et al.*, 1982), is autoregulated (Atlung *et al.*, 1985; Braun *et al.*, 1985), and this lead to an increased mutant DnaA concentration at non-permissive temperature (Braun *et al.*, 1985) probably due to a decreased DnaA box affinity. Alternatively, the concentration of a negatively acting protein on the initiation decreases when chloramphenicol is added (Tippe-Schindler *et al.*, 1979). The mutant DnaA proteins may have an altered and

inactive conformation at high temperatures. This is suggested in the light of the observation that oversupply of GroELS proteins suppresses the temperature sensitive *dnaA46* mutant. GroELS proteins interact with misfolded proteins, and may stabilise the unfolded state of proteins. Thus, the suppression of the temperature sensitive *dnaA46* mutant by an oversupply of GroELS protein could indicate that the DnaA46 protein is misfolded and therefore 'rescued' - or activated - by GroELS proteins (Katayama and Nagata, 1991).

Overproduction of wild type DnaA protein also result in overinitiation of chromosome replication with a resulting decrease in initiation mass (Atlung *et al.*, 1987; Atlung and Hansen, 1993; Løbner-Olesen *et al.*, 1989; Skarstad *et al.*, 1989). The overinitiations are, however, stalled or aborted and therefore, do not lead to an increased DNA content of the cells (Churchward *et al.*, 1983; Atlung *et al.*, 1987). This observation strongly suggests that the DnaA protein is the limiting factor under normal conditions. The reason why the overinitiations are aborted or stalled, and therefore do not lead to replicated chromosomes, is still not understood. Atlung *et al.* (1987) suggest that a certain factor is missing, for instance a DNA unwinding protein. In the opposite situation where the DnaA concentration has been lowered, the initiation mass increases (Løbner-Olesen *et al.*, 1989). The presence of additional DnaA boxes, either from *oriC* (Christensen *et al.*, 1999) or from the *datA* locus (Kitagawa *et al.*, 1996; 1998) lead to an increased initiation mass and increased transcription from the *dnaA* promoter (Hansen *et al.*, 1987). Thus, presence of additional DnaA boxes resembles the situation where the DnaA concentration was lowered, and the results are in full agreement with the generally accepted idea that DnaA regulates when the initiation takes place. As will be discussed below, the results also indicate that the DnaA boxes on the chromosome participate in the regulation of initiation by titrating DnaA proteins away from *oriC* most of the cell cycle (Hansen *et al.*, 1991B).

In contrast to other *dnaA* mutants which have a increased initiation mass, a cold sensitive *dnaA* mutant, *dnaAcos*, seems to overinitiate chromosome replication *in vivo* and *in vitro* at non-permissive temperature, and thus, have a decreased initiation mass (Kellenberger-Gujer *et al.*, 1978; Katayama and Kornberg, 1994; Katayama *et al.*, 1997; Frey *et al.*, 1984). The observed overinitiation is probably not a result of increased DnaAcos concentration, since it is only 65% of the DnaA concentration in an isogenic $dnaA^+$ strain (Katayama and Kornberg, 1994). Thus, the DnaAcos protein may be in an initiation-competent form for a longer period of time as compared with the $DnaA^+$

protein or, alternatively, the DnaAcos protein is inert to negative regulation (Katayama, 1994. See below).

The experimental results discussed above, and the observation that the initiation mass 'follows' the DnaA concentration (Hansen *et al.*, 1991; for review see Herrick *et al.*, 1996) strongly suggests that the DnaA protein is a key molecule in the initiation process, although there are still some disagreements about the regulatory function (see below).

The DnaA protein also seems to influence the initiation synchrony. Flow cytometric analysis of rifampicin treated *dnaA*$^+$ strains display discrete peaks corresponding to 2^n initiated origins per cell (i.e. 1, 2, 4, 8, 16 etc.) depending on the growth rate (Skarstad *et al.*, 1986). Thus, all origins are initiated simultaneously within one cell cycle. On the contrary, *dnaA* mutants initiate the replication very asynchronously especially *dnaA* mutants that carry a mutation that gives rise to an A184V substitution in the DnaA protein (i.e. DnaA46, DnaA5, DnaA601 and DnaA604, Hansen *et al.*, 1992) (Skarstad *et al.*, 1988). Asynchronous initiation is also observed in *dam* mutants (Boye and Løbner-Olesen, 1990) and other mutants encoding proteins involved in the structural arrangement of the chromosome (see below).

Regulation of initiation

The initiation of chromosome replication is not a single step event, and therefore its regulation may be a combination of different mechanisms and may not solely depend on the DnaA protein. Any proposal of a control mechanism must satisfy several requirements. First, the signal must be cyclical and have the same periodicity as the cell cycle, and secondly, the signal must ensure that each origin is initiated once and only once per cell cycle. The initiation can be considered to happen when i) a certain initiator has accumulated (the Autorepressor Model by Sompayrac and Maaløe, 1973) or ii) the concentration of an inhibitor is reduced sufficiently due to the increase in cell volume (the Inhibitor Dilution Model by Prichard *et al.*, 1969) or when iii) the concentration of the initiator has increased while the concentration of the inhibitor has decreased (the Initiator Titration Model by Hansen *et al.*, 1991B).

The DnaA protein seems to fit the requirement as an initiator well for several reasons. As discussed above, there are no doubts that the DnaA protein influence the initiation mass. When the DnaA concentration (or activity) increases, the initiation mass decreases, and vice versa (Løbner-Olesen *et al.*, 1989; Atlung *et al.*, 1987; Atlung and Hansen, 1993; Kellenberger-Gujer *et al.*, 1978; Hirota, 1970). In addition, the *dnaA*

gene transcripts, and thus, the DnaA concentration, may very likely fluctuate during the cell cycle. First, the *dnaA* gene is autoregulated, and the transcription can be repressed down to 25% of the normal expression and derepressed up to 400% (Atlung *et al.*, 1985; Braun *et al.*, 1985). Secondly, Dam methylation of the GATC sites in the promoter stimulates *dnaA* transcription (Braun and Wright, 1986), and thus, its hemimethylated state following replication (Campbell and Kleckner, 1990) may have a repressing effect in itself. In a *dam*$^-$ strain, the DnaA concentration was found to be 3-fold lower than in the isogenic *dam*$^+$ strain (Landoulsi *et al.*, 1989). Third, the hemimethylated *dnaA* promoter may be sequested in the membrane like *oriC* (Ogden *et al.*, 1988), and thus, the *dnaA* gene will not be transcribed following its replication. This is consistent with experimental results that indicate that the $mRNA_{dnaA}$ concentration is highest at the time of initiation and lowest following a replication (Theisen *et al.*, 1993; Campbell and Kleckner, 1990; Ogawa and Okazaki, 1994). Fourth, results have indicated that the *dnaA* transcription is growth rate regulated (Chiaramello and Zyskind, 1990).

Whether or not the initiation mass is independent of growth rate has been discussed for some time, and a constant initiation mass (Donachie, 1968) seemed to be important for proper understanding of the cell cycle and control of the initiation. However, there does not really exist any problem with an initiation mass that depends on the growth rate, as long as the 'initiator', the DnaA protein (or another protein for that matter), also depend on the growth rate, and thus, settles the initiation mass. In fact, at different growth rates, the initiation mass will only be constant if the initiator is synthesised as a constant fraction of total protein, and there does not seem to be a reason why that should be the case. What seems more important is that the initiation mass within a population is constant, and that has not been disproved. The initiation mass of a whole cell population seems to depend on the strain in question. The initiation mass of *E.coli* K-12 was found to increase with decreasing growth rate (Wold *et al.*, 1994), whereas in *E.coli* B/r the opposite was found (Churchward *et al.*, 1981). More recent experiments with *E.coli* K-12 and B/r showed that the initiation mass was constant at different growth rates, but 25% higher in *E.coli* K-12 (Bipatnath *et al.*, 1998). The DnaA concentration has, however, not been measured together with the initiation mass in any of the published results, and thus, it is not known if (or how) the DnaA concentration depends on the growth rate in the strains discussed above. Instead, The DnaA concentration has been measured in other strains and whether or not its concentration depends on the growth rate also seem to depend on the strain. The DnaA concentration in *E.coli* K-12 strains was found to be independent of the growth rate (Hansen *et al.*, 1991A) or increasing

with increasing growth rate (Chiaramello and Zyskind, 1989; Polaczek and Wright, 1990). In *E.coli* B/r it was found to increase with decreasing growth rate (Hansen *et al.*, 1991A). By combining the data of the initiation mass at different growth rates in *E.coli* K-12 (Wold *et al.*, 1994) and B/r (Churchward *et al.*, 1981) and the DnaA concentrations in *E.coli* K-12 and B/r strains at different growth rates (Hansen *et al.*, 1991A), Herrick *et al.* (1996) observed a good correlation between the initiation mass and the reciprocal DnaA concentration. The Initiator Titration Model (Hansen *et al.*, 1991B) includes the Autorepressor- and Inhibitor Dilution Model (Sompayrac and Maaløe, 1973 and Pritchard *et al.*, 1969, respectively). In the model, the 'initiator' is the DnaA protein and the 'inhibitor' is the DnaA boxes on the chromosome. Right after replication, the free DnaA concentration is low, because the DnaA box concentration exceeds the DnaA concentration. At a certain point, all the DnaA boxes are saturated with DnaA proteins. This will result in an increase in the free DnaA concentration and subsequent interaction with the DnaA boxes (R3) within *oriC*. Introduction of additional DnaA boxes, either from *oriC* or from the *datA* locus, clearly titrate DnaA protein as discussed previously (Christensen *et al.* 1999; Kitagawa *et al.*, 1996, 1998) indicating that the DnaA boxes on the chromosome will do the same. The Initiator Titration Model requires a significant numbers of DnaA boxes or, alternatively, areas to which the DnaA protein has high affinity. The DnaA box consensus $TT^{A}{}_{T}TNCACA$ would statistically result in about 140 DnaA boxes present on the chromosome. This may not be enough when considering that the average number of DnaA proteins per cell is larger; according to Hansen *et al.* (1991B) in the order of 1000-1500 DnaA proteins per cell, according to Schaefer and Messer (1991) about 1600 and according to Sekimizu *et al.* (1988) between 800 and 2100 DnaA proteins per cell. Schaefer and Messer (1991) quantified the relative DnaA binding affinity to different DnaA boxes *in vivo*, and defined a new DnaA box consensus sequence to which the relative affinity was greater than 0.2: (T/C) (T/C) (A/T/C) **T** (A/C) **C** (A/G) (A/C/T) (A/C). Data base research revealed that these DnaA boxes are distributed uniformly over the genome with a frequency of 3.3 DnaA boxes per 10 kb, which gives 1600 DnaA boxes per genome equivalent. Whether all DnaA boxes actually titrate DnaA proteins may seem unlikely. Instead, there seems to exist high affinity loci on the chromosome that may be responsible for the DnaA titration. These areas are the *datA* locus (Kitagawa *et al.*, 1996, 1998; Roth and Messer, 1998), the *ompT-appY* region, the *mutH* gene, and the *narU* gene (Roth and Messer, 1998). These loci are distributed uniformly on the chromosome. Thus, a likely explanation why *de novo* protein synthesis is required

before another round of initiation can take place is that the DnaA boxes on the chromosome titrate the free DnaA proteins. Alternatively, or in addition, the requirement for *de novo* protein synthesis may be that once the DnaA protein has participated in an initiation event, it cannot be re-used (Zyskind and Smith, 1992). Mahaffy and Zyskind (1989) have proposed a model partly based on the observation that the DnaA concentration increases with increasing growth rate (Chiaramello and Zyskind, 1989) and therefore is growth rate regulated (Chiaramello and Zyskind, 1990). The authors suggested that the DnaA protein can exist in an active ATP form and an inactive DnaA-ADP form, and that the concentration of the active DnaA proteins is independent of the growth rate. This proposal is partly supported by the observation that when DnaA proteins are purified from a DnaA overproducing strain, about half of the proteins exist in an aggregated form, which contains phospholipids. This aggregated form is unable to initiate replication *in vitro* unless it is activated by either DnaK or phospholipase A_2 in the presence of ATP (Hwang *et al.*, 1990). Recently it has been shown that the β subunit of DNA polymerase III accelerates hydrolysis of ATP bound to DnaA *in vitro* (Katayama *et al.*, 1998) and *in vivo* (Kurokawa *et al.*, 1999) resulting in a DnaA-ADP form that is unable to initiate replication. It is suggested that this negatively regulation, called RIDA (regulatory inactivation of DnaA), of the DnaA activity is the key control mechanism of the replication cycle (Katayama *et al.*, 1998). The authors agree with the model proposed by Mahaffy and Zyskind (1989) and thus, *de novo* protein synthesis is suggested to be required due to an (ir)reversible inactivation of the DnaA protein. A DnaA protein with an E204Q substitution has been shown to have decreased intrinsic ATPase activity although the affinity for ATP and ADP was intact *in vitro*. This mutant DnaA protein may overinitiate replication *in vivo* when it is overproduced in a wild type strain and this has led to the proposal that the intrinsic ATPase activity negatively regulates chromosomal replication (Mizushima *et al.*, 1997), and therefore is part of the RIDA system (Katayama *et al.*, 1998). However, overproduction of wild type $DnaA^+$ proteins also result in overinitiation of replication (Løbner-Olesen *et al.*, 1989; Atlung and Hansen, 1993; Skarstad *et al.*, 1989), which may suggest that intrinsic ATPase activity is not a negative regulator of the initiation process, but, if any, then rather of the elongation process. This seems in agreement with the observation that overproduction of the β-subunit of polymerase III suppresses the lethal phenotype of multicopy *dnaA*(E204Q) strain (Makise *et al.*, 1999). Recent results have shown that exponentially growing wild type cells have abundant DnaA-ADP and that only 30% of the DnaA protein exists in an ATP form. In addition, in a synchronised

culture, it was found that the DnaA-ATP form oscillates and that the concentration reaches maximum (80% DnaA-ATP) upon initiation in presence of *de novo* protein synthesis (Kurokawa *et al.*, 1999). Many of the authors cited above believe in the RIDA hypothesis partly because of experimental results that indicate that the DnaAcos protein is inert towards negative regulation *in vitro* (Katayama, 1994) and therefore, overinitiates replication. However, recent *in vivo* analysis of the initiation frequency in the cold sensitive *dnaAcos* mutant at non-permissive temperature indicated that the mutant does not overinitiate replication extensively, although the DNA content increased slightly (Nyborg *et al.*, 2000). Thus, the DnaAcos protein may not be inert towards negative regulation *in vivo*. In addition, although the results may indicate that the DnaA protein is re-activated by ATP binding, which is essential for open complex formation *in vitro* (Bramhill and Kornberg, 1988B), it has been shown that not all DnaA proteins have to exist in a DnaA-ATP form in order to participate in the initiation *in vitro* (Yung *et al.*, 1990; Crooke *et al.*, 1992). In addition, several other mutant DnaA proteins have been shown not to bind, or have severely reduced affinity for ATP and ADP *in vitro* (Hwang and Kaguni, 1988; Hupp and Kaguni, 1993; Carr and Kaguni, 1996). Yet, they are able to initiate chromosome replication *in vivo*, and no overinitiations are observed (at least not when the *dnaA* allele exists as a single copy on the chromosome). Thus, the function of ATP and ADP binding to DnaA *in vivo* may be important in the (fine)tuning of the replication control, and probably not the ultimate controlling element. Interestingly, a new six base pair sequence has been identified as a target for the DnaA protein in its ATP, but not ADP form (Speck *et al.*, 1999). These sites are, among others, located in the three 13-mers (Fig. 1.1), and they are suggested to be important in the formation of the open complex. Although this remains to be further investigated, the results indicates that so-called ATP/ADP switch may contribute to an even more sophisticated control mechanism.

As mentioned previously, all origins in the cell are initiated once and only once per cell cycle. All high-temperature sensitive *dnaA* mutants initiate the replication asynchronously, especially those *dnaA* mutants with a mutation that give rise to a A184V change in the ATP binding domain of the DnaA protein (Skarstad *et al.*, 1988). However, the DnaA protein is probably not the only protein responsible for synchronous initiations, since *dam*, *him* (IHF), *fis*, *gyrB* and *topA* mutants also initiate the chromosome replication asynchronously (Boye and Løbner-Olesen, 1990; von Freiesleben and Rasmussen, 1992). The way the cell ensures that all origins are initiated at the same time in the cell cycle has been proposed by Løbner-Olesen *et al.* (1994). In

the so-called Initiation Cascade model, which partly relies on the Initiator Titration model (Hansen *et al.*, 1991), one (or more) origin(s) are initiated when the DnaA concentration reaches a certain level. The subsequent release of DnaA proteins from that particular origin results in a local increase in the DnaA concentration leading to the initiation of another origin in the cell and so fourth. This initiation cascade will ensure that all origins are initiated (almost) simultaneously within the cell cycle. The existence of a mechanism that blocks secondary initiations was proposed by Russell and Zinder (1987), who found that transformation of a *dam* strain with methylated minichromosomes resulted in an accumulation of hemimethylated minichromosomes. To explain this, the cell membrane has been drawn into the picture. Actually, it was proposed long time ago by Jacob *et al.* (1963), in the Replicon Model, that the replication origin is an integrated part of the membrane. Upon initiation, which was thought to be stimulated positively by an initiator, the membrane would grow in between the replicating origins resulting in chromosome separation. *oriC*, which contains a high number of GATC sites (Fig. 1.1), remains hemimethylated *in vivo* from eight to ten minutes after initiation (Ogden *et al.*, 1988; Campbell and Kleckner, 1990), and this relatively long period of time required for methylation is suggested to be due to sequestration of *oriC* on the cell membrane. Thus, the binding on the membrane may prevent initiations of already initiated origins shortly after initiation. At the time *oriC* is re-methylated; the number of 'newborn' DnaA boxes on the chromosome has increased sufficiently to titrate the free DnaA proteins preventing initiation at any origin. Accordingly, *dam* mutants do not initiate replication synchronously since *oriC* remains unmethylated and therefore, does not bind on the membrane. The cell seems to initiate replication from random origins until the increase in DnaA box concentration has pulled DnaA proteins away from *oriC*. A protein named SeqA has been suggested to be involved in sequestration, because a *seqA* mutant allows methylated minichromosomes to replicate in *dam* strains, and furthermore, the observed re-methylation delay of *oriC* (Ogden *et al.*, 1998; Campbell and Kleckner, 1990) is reduced in the *seqA* mutant (Lu *et al.*, 1994). However, the function of SeqA may be more complex. Based on the observation that SeqA may act directly on DnaA, it has been speculated that the SeqA protein regulates the exchange rate of ADP with ATP, and that when SeqA is absent, this exchange is much faster (von Freiesleben *et al.*, 1994). More recent *in vitro* experiments indicate that SeqA affects the DNA conformation and thereby inhibits open complex formation (Torheim and Skarstad, 1999). Thus, although there seems to be

agreement about SeqA affecting the initiation of replication negatively, the function of SeqA and the sequestration mechanism remains to be resolved.

The asynchronous initiations observed in *him* (IHF), *fis*, *gyrB* and *topA* mutants might be a result of changed DNA conformation. The IHF and FIS proteins bind *oriC* (Fig.1.1 and below), and this probably affects the DNA topology. Gyrase and topoisomerase affect the local - and global - superhelicity. Thus, although the DnaA protein may settle the initiation mass, factors involved in the structural arrangement of *oriC* can affect the DnaA-DnaA box affinity, and thereby the precise timing and synchrony of the initiation.

The DnaA boxes R1, R2 and R4 have bound DnaA proteins throughout most of the cell cycle *in vivo* (Samitt *et al.*, 1989; Cassler *et al.*, 1995). According to these results, the binding of DnaA proteins to R3 could be the final event for initiation to happen. The FIS protein binds to *oriC* between R2 and R3 (Fig. 1.1), and the DNA bends upon FIS binding (Gille *et al.*, 1991). *in vivo* and *in vitro* footprinting experiments have indicated that the FIS site is protected throughout most of the cell cycle. However, at the time of initiation, the FIS site was no longer protected, whereas DnaA box R3 was. It has been suggested that binding of FIS occluded the binding of DnaA to R3 and thereby prevents initiation of replication (Cassler *et al.*, 1995; Gille *et al.*, 1991). However, recent *in vitro* experiments indicate that the FIS protein does not occlude the DnaA protein. Instead, the inhibitory effect of FIS seemed to be due to absorption of negative superhelicity required in the initiation process (Margulies and Kaguni, 1998). The IHF protein, on the contrary, seems to bind *oriC* at the time of initiation coincident with the filling of DnaA at R3 (Cassler *et al.*, 1995). Based on recent *in vitro* footprinting experiments, it has been shown that IHF directs the DnaA protein from stronger to weaker binding sites in *oriC* (Grimwade *et al.*, 2000). The authors also find new sequences that do not resemble the DnaA box consensus to which DnaA binds *in vitro* in presence of IHF. Based on mutational analysis of DnaA boxes in *oriC*, it has been suggested that DnaA box R3 is not required for replication from *oriC*, since scrambled or inverted R3 resulted in $oriC^+$ behaviour with respect to replication of minichromosomes (Langer *et al.*, 1996). Instead, the DnaA box R3 is suggested to play a regulatory role in the fine-tuning of the initiation in the cell cycle. However, the results indicate that R3 is dispensable for minichromosomes replication, and therefore its regulatory role seems of less importance. It would be interesting to see if R3 is important for synchronous initiations from *oriC*.

In spite of the contribution of many proteins in the initiation and replication process, experimental data, some of which have been discussed above, strongly points at the

DnaA protein as the key protein in the control of the initiation. The maybe strongest evidence is that the DnaA concentration is proportional to the number of origins in the cell and inversely proportional to the initiation mass. Binding of ATP to (some of) the DnaA proteins may be crucial, but whether the hydrolysis of ATP is a controlling component needs more evidence.

Chapter 2

The DnaA-DnaA box interaction

DNA-protein interactions are very important in the genetic life of the cell. They are required in many events including transcription, repair, recombination and, not least, chromosome replication. As discussed in the previous chapter, the DnaA protein seems to be the protein that, upon binding to *oriC*, trigger the initiation event leading to chromosome replication. Therefore, it is of special interest to understand the interaction between the DnaA protein and its target, the DnaA boxes. Unfortunately, the three-dimentional structure of the DnaA-DnaA box complex is not known yet (neither is the structure of the DnaA protein). It has not been possible to crystalise the protein for X-ray analysis, and although the NMR technique has improved with respect to protein sizes, the DnaA protein is still too large for a proper analysis.

The aim of this chapter is to discuss the DNA-protein interaction, in general, and to describe the different interaction types in structure-known DNA binding proteins. Focus will be put on those interaction types - or binding motifs - the DnaA protein may be classified under. The discussion is mainly based on review articles and books, and is therefore not a detailed discussion of all DNA binding proteins and their specific structures.

The DnaA protein and binding motifs

DNA binding proteins can be classified into several groups according to their DNA binding motif. One of the well characterised motifs is the **helix-turn-helix motif**, which was the first motif to be characterised (for reviews see Harrison, 1991; Brennan and Matthews, 1989). The DNA binding domain of the DnaA protein consists of five α-helixes separated by loops (Fig. 2.1).

Fig. 2.1. Amino acid sequence (one-letter code) of the C-terminal DNA binding domain of the DnaA protein. α-helixes 1-5 are indicated by bars.

A guess would be that the DnaA protein carries a helix-turn-helix motif and that this motif is responsible for the DNA contact. The Lac repressor, Tpr repressor, catabolite activator protein (CAP) and the FIS protein (inversion stimulation factor) bear, among other procaryotic proteins, the helix-turn-helix motif (see Fig. 2.2). The proteins that carry this motif do not generally share structural similarities, and this contributes to the unique recognition of different DNA sequences by the various helix-turn-helix proteins. The length of the turn (loop) in the procaryotic group is four recidues, and a glycine occurs at the second position (Brennan and Matthews, 1989). A core of hydrophobic recidues allows the two helixes to pack together and form a compact tertiary structural domain. The DNA contact takes place through base contact by residues in the second helix that penetrates the major groove of the DNA. In the major groove, but also in the minor groove, the edges of base pairs expose hydrogen bond donor and acceptor groups. This hydrogen bond pattern is unique for each base pair in the major groove, but not in the minor, and this is probably the reason why amino acid residues often recognise and interact with the bases in the major groove. As mentioned, this interaction may be in shape of hydrogen bonding, but it can also have a hydrophobic character and involve van der Waals interactions with the non-polar surface of C5 of pyrimidines. Although the second helix in the helix-turn-helix is referred to as the recognition helix, it should be stressed that if the helix, or the whole helix-turn-helix motif, is separated from the rest of a larger DNA binding domain, DNA binding capacity is lost (Pabo and Sauer, 1992). In addition, experiments called 'helix swap' experiments where amino acid residues in one 'recognition' helix is substituted with residues that match another 'recognition' helix only works in special cases. Thus, specific recognition includes more than interactions between residues in the second helix of the helix-turn-helix motif and bases in the major groove (reviewed by Harrison and Aggerwal, 1990). Accordingly, in the λ repressor, for instance, other amino acids than the ones that are part of the helix-turn-helix motif contact the DNA backbone.

According to secondary structure predictions, the DnaA protein may have a short strech of coiled structure, probably four or five amino acids long, between helix 4 and 5 of the DNA binding domain (Fig. 2.1 and 3.3), but no glycine is seen among these residues. Instead, the amino acids present in this small coiled area are a relatively unconserved glutamic acid, a serine and a histidine, and therefore the presence of a well conserved turn among the different DnaA proteins seems unlikely (Fig.2.1 and Fig. 3.4).

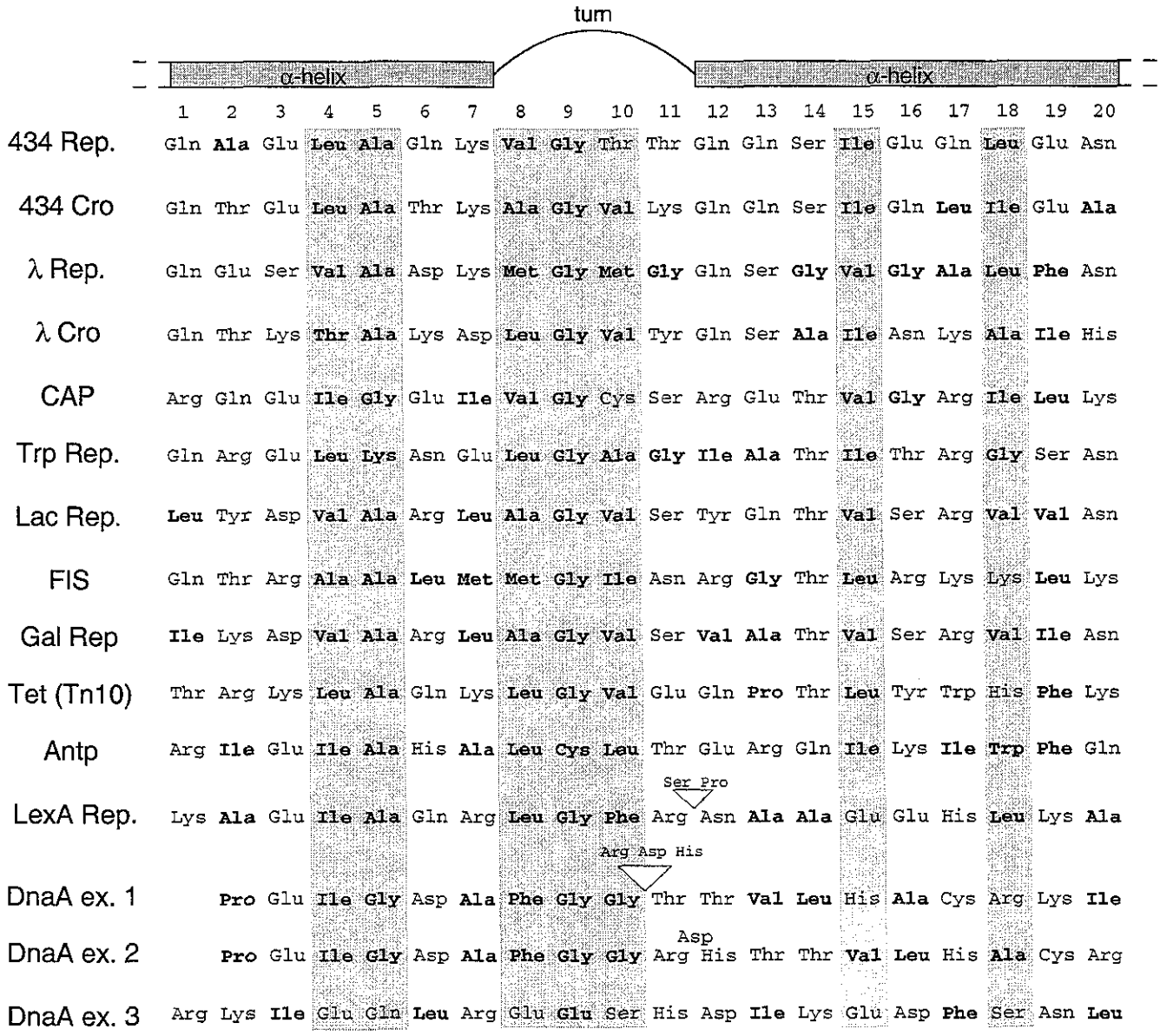


Fig. 2.2. Alignment of parts of the DNA binding domain of the DnaA protein and proteins with known helix-turn-helix motifs. DnaA ex. 1 and 2 show helix 3 and 4 and DnaA ex. 3 show helix 4 and 5 (see Fig. 2.1). Hydrophobic amino acids are bold and conserved amino acids are shaded. The amino acid that induce the turn (mostly glycine) is located as residue no. 9 in the helix-turn-helix motif. Amino acid no. 20 interacts with the DNA backbone (from Harrison and Aggerwal, 1990; Pabo and Sauer, 1992; Lamerichs *et al.*, 1989).

The coils, or turns, between the other helixes in the DnaA protein are longer. However, if the length of the turn is 'allowed' to be longer than four residues, then the helix-turn-helix family can be extended to include many eucaryotic protein as well (reviewed by Luisi, 1995). In addition, the *E.coli* LexA repressor contains such a modified helix-turn-helix motif with a longer turn (Lamerichs *et al.*, 1989. Fig. 2.2). As the procaryotic proteins, the eucaryotic proteins use the second helix in the motif as the DNA contacting part, but whereas the procaryotic proteins form homodimers, or tetramers,

and bind to palindromic DNA sequences, the eucaryotic proteins normally bind as monomers to non-palidromic sites. If the DnaA protein binds in a helix-turn-helix manner, it could seem that the interaction has a more eucaryotic character, since the DnaA box is non-palindromic, and thus, the turn between the two helixes may be longer than four residues. The eucaryotic helix-turn-helix proteins also have glycine(s) in the turn separating the helixes (reviewed by Travers, 1993). Therefore, the alignment indicates that helix 3, the following turn, and helix 4 may constitute a (modified) helix-turn-helix motif in the DnaA protein (Fig. 2.2). This also seem consistent with the observation that this area is highly conserved among DnaA proteins from different organisms (Fig. 3.1 and 3.2), and the loop (turn) between helix 3 and 4 contains two highly conserved glycines. In addition, it is possible for the helixes to make a hydrophobic core due to the presence of a number of hydrophobic amino acids.. Comparison of the tertiary structure of proteins with helix-turn-helix motifs has shown that residue 1-7 and 12-20 forms the helixes and the residues at positions 4, 8, 10, 15 and 18 are normally hydrophobic and participate in formation of the tertiary structure through van der Waals interactions. Residue number 5 is usually glycine or alanine. A small amino acid is required at this position, since larger side chains interfere with the peptide backbone in the N-terminal part of the following helix. Residue 9 in the turn is almost always a glycine as mentioned previously, but serine, cystein and glutamic acid have been found at this position (reviewed by Harrison and Aggerwal, 1990). According to the secondary structure prediction of the DNA binding domain of the DnaA protein (Fig. 3.3), the alanine at position 7 (A428) is helical, and so is the histidine (H434) and threonine (T435) in the end of the loop shown in 'DnaA ex. 1' (Fig. 2.1). If they are included in the helix as indicated in 'DnaA ex. 2', the hydrophobic amino acids follow the pattern with hydrophobic amino acids at positions 15 and 18. Amino acid no. 20, typically a hydrogen bond doner or acceptor, in the helix-turn.-helix motif contacts the DNA backbone (Harrison and Aggerwal, 1990; Pabo and Sauer, 1992). This observation supports the proposal that 'DnaA ex. 2' is the correct alignment of the DnaA protein, because amino acid no. 20 in this case is a well conserved arginine (R442, Fig. 2.1), which will be able to interact with the DNA backbone through hydrogen bonding. 'DnaA ex. 3' (Fig. 2.2) shows the amino acid sequence of α-helix 4 and 5 of the DNA binding domain of the DnaA protein with Glu Glu Ser His as the loop (turn) residues (discussed previously). This area of the DnaA protein does not fit the helix-turn-helix properties discussed above well. In addition, the residues in the loop (turn) area and helix 5 (supposed to be the 'recognition' helix) is not well conserved

among DnaA proteins from different organisms (see Fig. 3.1 and 3.2). Therefore, this area probably does not constitute a helix-turn-helix motif. Neither does helix 2 and 3. The helixes do not seem to contain hydrophobic amino acids at the right positions, and residue no. 5, which is supposed to be a small hydrophobic amino acid (reviewed by Harrison and Aggerwal, 1990), is a large charged amino acid (E416, Fig. 2.1). In addition, the loop between the helixes does not contain any glycine, and the serine located there is not placed at the right position. Helix 1 and helix 2 with the basic loop in between do not fit the requirements for a helix-turn-helix motif either. The loop is very long (19 residues) and it does not contain any glycines (Fig. 2.1). However, there are two well conserved serines, but they are located far away (10 and 14 residues, respectively) from the first helix. The presence of the high number of basic amino acids in this loop is, however, conspicuous and they may be important in the interaction with the DNA backbone. Instead of a helix-turn-helix motif, this area of the DnaA protein could be part of a **basic helix-loop-helix motif** (bHLH) as suggested previously (Roth and Messer, 1995; Schaper and Messer, 1997). The motif has, however, sofar only been observed in eucaryotic proteins. The basic helix-loop-helix (bHLH) proteins usually recognise short palindromic sequences so that each protein monomer binds one half site (reviewed by Littlewood and Evan, 1998). In bHLH proteins, a basic area of about 15 residue lies N-terminal to a 15 residue long amphipathic α-helix. A region (the loop) consisting of 9-20 residues separates this helix from another helix of similar length. It is the helix-loop-helix motif that mediates dimerisation and the basic region that interacts with the DNA (Harrison, 1991). The feature of the helixes is that they contain leucine or similar hydrophobic residues on the same side of the helix so that it can interact with a similar helix in a parallel orientation thereby forming a dimer. Fig. 2.3 shows an alignment of various bHLH bearing eucaryotic proteins. The possible bHLH motif of the DnaA protein is also shown. In some aspects, the DnaA protein fits the bHLH group: 1) the protein has a number of basic amino acids and α-helix 2 and 4 and the loop between could constitute the helix-loop-helix, 2) the α-helixes all contain hydrophobic amino acids that seem to be located on the same side of the helix (Fig. 2.1 and 3.3). However, in other aspects the DnaA protein does not fit the group: 1) The DnaA box is not palindromic as often observed, 2) there exists an α-helix (helix 3) in the potential loop between helix 2 and 4 and 3) the DnaA protein does not follow the pattern of conserved amino acids observed in other bHLH proteins, and finally 4) results obtained by Weigel *et al.* (1999) and others (Sutton and Kaguni, 1997A,C)

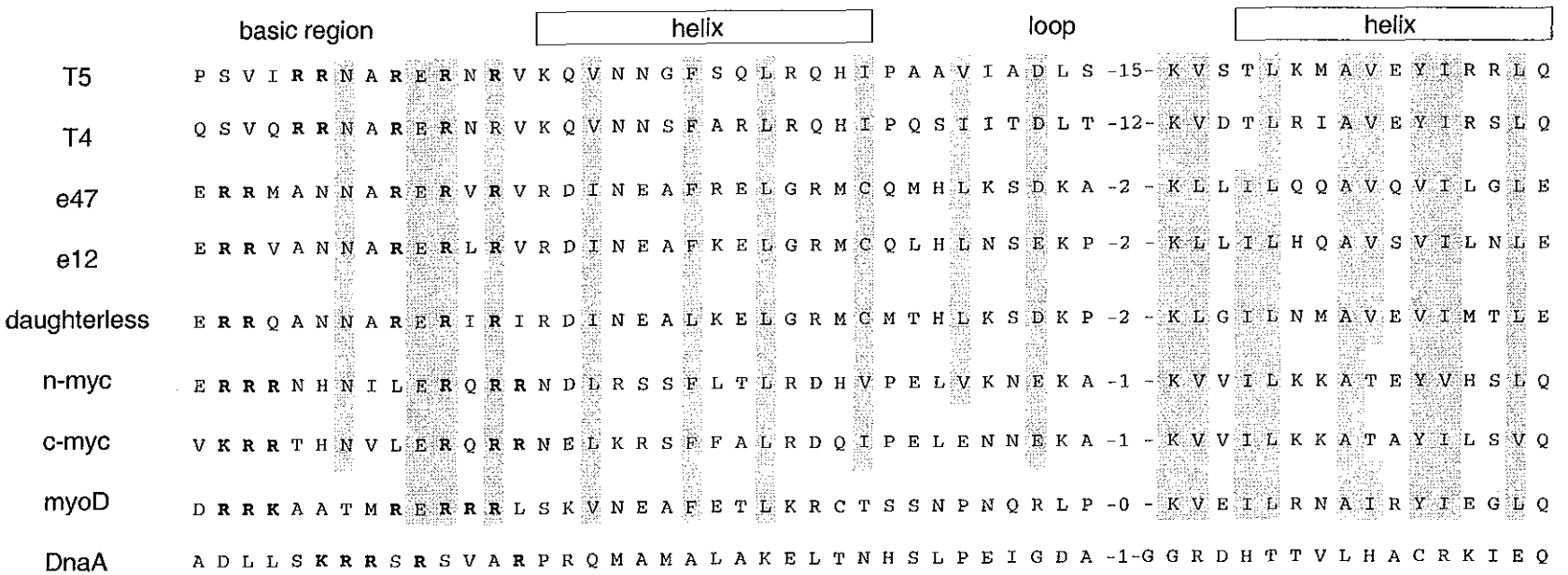


Fig. 2.3. Alignment of proteins with known bHLH motifs. The DnaA protein covers the basic loop and helix 2 and 4 (see Fig. 2.1). Basic amino acids are bold and conserved amino acid shaded (Pabo and Sauer, 1992).

indicate that it is the N-terminal part of the DnaA protein that is responsible for dimerisation, and that area lies 400 amino acids away from the basic region in the DNA binding domain. Thus, if the basic area in the DNA binding part of the DnaA protein is responsible for the DNA contact, the binding mode may have a new, and yet undefined, character. For instance, helix 1 and 2 and/or helix 4 may interact with each other through van der Waals interactions (in a leucine zipper fashion), allowing the basic loop to penetrate and interact with the DNA (Schaper and Messer, 1997). The basic loop could, however, also have a rather different function. If helix 3 and 4 constitute a helix-turn-helix motif, the basic region could particicipate in bending the DNA through electrostatic attraction/interactions between the basic residues and the DNA backbone. In this scenario, the basic loop would be important for DnaA function, but it would not be responsible for the specific DnaA box recognition.

A second very large class of specific DNA binding proteins is the **zinc-bearing DNA binding domains**, which is stabilised structually by tetrahedrally co-ordinated Zn^{2+} ion(s). In all of the so far characterised groups of zinc containing proteins, the zinc ion always complex with cysteines and sometimes also histidines. Considering that the DNA binding domain of the DnaA protein only carries one not very conserved cysteine, and that zinc is not required in *in vitro* replication systems, it does not seem likely that the DnaA protein should possess any zinc dependent conformations. **The zipper group** is, like the zinc-containing proteins, so far composed of eucaryotic proteins. The DNA binding domain of the DnaA protein may contain primary and secondary structures that could allow it to be classified under the zipper group (see below). The zipper consists of leucines or other hydrophobic residues that are part of α-helixes, either from two different proteins or from two helixes within one protein. These hydrophobic amino acids are separated by three or four residues, which corresponds to one helical turn, and the hydrophobic residues are therby located on one side of the helix. The amino acids can make hydrophobic interactions (the zipper) with a parallel α-helix that carries the same pattern of hydrophobic amino acids. The leucine-zipper is often used in the dimerisation of DNA binding proteins. In cases where protein dimers bind the DNA, the DNA sequence is palindromic. That is not the case with the DnaA box. The leucine zipper may, however, exist between two α-helixes within the same DnaA protein, for instance between helix 4 and 5 or maybe helix 1 is involved in a leucine zipper.

Other DNA binding structures clearly exists, although the helix-turn-helix and the zinc finger motifs are the best characterised. A number of DNA binding proteins use

residues in **β-sheets** (either parallel or anti-parallel sheets (MetJ)) in the interaction with bases in the major groove of the DNA helix. The DNA binding domain of the DnaA protein probably does not contain any β-sheets why such an interaction type seems rather unlikely. Some DNA binding proteins seem to have **unique binding motifs**. The *Eco*RI restriction enzyme, for instance, recognise its site through amino acids located in the ends of parallel α-helixes. As with the other binding motifs, it is bases in the major groove that is in contact with the protein. The DnaA protein could in principle also possess such a binding motif. The *Eco*RV restriction enzyme and Dnase I use loops as the recognition element that penetrates into the major groove of the recognition site. Again, this could be a possible binding motif of the DnaA protein. The RNase H bound to a DNA-RNA hybrid shows that, unlike any of the other proteins or motifs discussed so far, contact is mediated through a loop that penetrates the minor groove. Yet other proteins use both α-helixes and β-sheets to penetrate and contact bases in the minor and major groove (Klenow fragment of DNA polymerase I, for instance).

From this discussion it appears that many different binding motifs exist, and that the DnaA protein may possess either one of them, or a combination of already characterised motifs. Based on secondary structure analysis, it seem unavoidable that contact is mediated through either α-helixes and/or loops, and that the DnaA protein possess a potential helix-turn-helix motif including helix 3 and 4. In the experimental part of this thesis, the effect of different amino acid substitutions in the DNA binding domain will be discussed, and this may lead to a better understanding of the DnaA-DnaA box interaction.

Amino acids in contact with DNA

All amino acid residues seem to be able to interact with the DNA either through hydrogen bonding or van der Waals interactions. Thus, it is not possible to conclude that a certain area of a DNA binding protein will or will not interact with the DNA on basis of the primary structure of the protein. Amino acid residues can interact with the suger phosphate backbone of the DNA or with the base pairs. The interaction with the DNA backbone is often through hydrogen bonds to nonesterified phosphate oxygens from peptide -NH groups, neutral $-NH_2$ groups of glutamine and asparagine and from -OH groups of serine and threonine. Hydrogen bonds from lysine and arginine to the phosphates appear with only modest frequency (reviewed by Harrison and Aggerwal, 1990). This may seem difficult to understand, because such an interaction could be

expected to be stronger than ordinary hydrogen bonds due to the electrostatic character of the interaction. Base pairs in major groove, and minor groove, can be contacted by various sidechains that donate or accept hydrogen bonds. In addition, non-polar hydrophobic interactions are often present and they participate in the specific distinction between cytidine and thymine.

Except for tryptophan, the DNA binding domain of the DnaA protein consists of all twenty amino acids, and the area that may constitute the helix-turn-helix motif (Fig. 2.2) includes both hydrophobic residues and hydrogen bond donor and acceptor amino acids. It is not possible on the basis of analysis of the interaction between other DNA binding proteins and their DNA targets to determine which amino acids in the DnaA protein that may be responsible for the interaction with the DnaA boxes. As mentioned previously, part of the experimental work in this thesis includes analysis of various amino acid substitutions in the DNA binding domain of the DnaA protein, and this analysis may shed light on which amino acids that are likely to be involved in the DnaA box recognition and binding.

The conformation of the DnaA boxes

When analysing protein-DNA interactions, it may not be correct to consider the protein as the active part and the DNA as the passive part in the interaction and recognition. Actually, it may be just as correct to say that a certain DNA sequence recognises a certain protein with the correct structure. The DNA molecule is not a uniform unit, but the structure of it changes throughout the chromosome. The conformation depends on the actual DNA sequence and the base pair interactions leading to several types of comformational flexibilities (Fig. 2.4). Accordingly, the DNA sequence may have a B-form shape or a A-type shape, or the DNA sequence may bend or curve (reviewed by Luisi, 1995; Travers, 1993). Following parameters are important in the final conformation of DNA fragment (Fig. 2.3): 1) The propeller twist: Favorable stacking of bases affect the propeller twist moving the base pairs away from a planar configuration. A consequence of this is that the GC base pair has lower propeller twist than the AT basepair due to the higher number of hydrogen bonds. 2) The Helical twist: The twist angle vary corresponding to a winding or unwinding of the DNA sequence. Changes in the axial flexibility leads to changes in the helical axis from B-type DNA to A-type DNA or vice versa. B-DNA has a twist of 36° per base pair equivalent to 10 base pairs per turn, whereas A-DNA contains 11 base pairs per turn. As the twist decreases, the minor groove becomes wider and shallower, while the major groove becomes narrower

and deeper. If the twist increases, the opposite happens. Thus, along the DNA molecule, the size of the minor and major groove changes, and this will have an effect on the

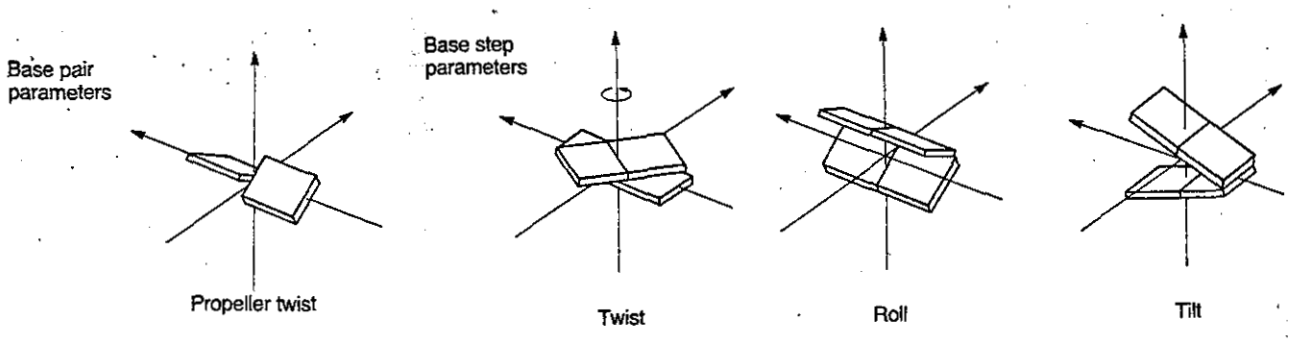


Fig. 2.4. Definition of terms defining the geometry of base pairs and base steps. The axis of the double helix is represented by the vertical line (Travers, 1993).

interaction with proteins. 3) The roll: A departure from a planar stacking of base pairs about the long axis is termed roll. A roll can be directly related to the bending of a DNA molecule and 4) The tilt: Tilt is a departure from the planar stacking of the base pairs about the short axis. B-DNA has zero tilt, but in A-DNA the average tilt is +20°. This opens the minor groove and the local helical axis follows a superhelical path around the global helical axis.

It is important to emphasize that one parameter discussed above cannot change without affecting the other parameters. For instance, charges of neighbouring guanine residues in poly(dG).(dC) result in a repulsion which forces adjacent base pairs to slide relative to each other resulting in a displacement of the base pairs from the double helical axis. Thus, the propeller twist, the helical twist, the roll and the tilt angle is affected. The result is that the DNA conformation of (dG).(dC) polymers has a A-type character with a wider minor groove. This is in contrast to poly(dA).(dT) sequences, which has a typical B-DNA conformation with zero tilt, zero roll and a helical twist of 36° equivalent to 10 base pairs per turn. These base pairs can stack with more overlap, and thus, there is no displacement of the base pairs from the double helical axis. Purine-pyrimidine and pyrimidine-purine base steps are restricted conformationally, because of the (large) purines opposing each other. This effect, called purine-purine clash, may lock the conformation of the DNA molecule, which could be required in the interaction with its 'target-protein'. It is also important to emphasise that the environment influences the conformation of a certain DNA sequence. For instance, binding of proteins to nabouring DNA sequences, mRNA transcription, and the local concentration of ions

will undoubtly affect the DNA conformation. Thus, due to all these parameters, of which some have been mentioned in this chapter, it seems difficult to determine the exact structure of a certain DNA sequence, including the DnaA box sequence, on basis of the sequence.

The DnaA box sequence differ among the various DnaA boxes, but in general they contain a high number of A and T (Table 2.1), indicating that the overall conformation of the DnaA box has a B-DNA character.

Table 2.1. The sequence of a number of DnaA boxes found in the *oriC* region and in the *dnaA* promoter

DnaA box	Sequence	Affinity [1)]
R1	TTG**TTATCCACA**GGG	High
matsui	CGA**TCATTCACA**GTT	Low
R2	GGG**TTATACACA**ACT	Medium
R3	TAG**TTATCCAAA**GAA	Low
R4	GAG**TTATCCACA**GTA	High
R5	TAC**TTTTCCACA**GGT	Medium
dnaA promoter	GAT**TTATCCACA**GGA	High

1) The affinitiy of the $DnaA^+$ protein to the DnaA boxes has been determined by several authors using *in vivo* and *in vitro* measurements. The affinities are therefore only expressed qualitatively as high, medium and low, where high is in the order of K_d=1-2 nM, medium in the order of 30-50 nM and low in the order of > 200 nM (Bläsing, 1999; Schaefer and Messer, 1991; Schaper and Messer, 1995).

The most stringent DnaA box consensus sequence is $TT^A/_TTNCACA$ (Schaper and Messer, 1995). It seems that the DnaA protein has the highest affinity to the sequence TTATCCACA, and deviation from this sequence results in decreased affinity of DnaA to the box. If C_8 is substituted with A (as in R3) the affinity is severely reduced, which indicates that the maybe relatively locked structure of CACA (due to purine-purine clashes, see above) is important in the interaction with DnaA. Substitution of T_2 with C (as the M box) also seem to have severe effects on the affinity, whereas substitutions of A_3 (R5) or C_5 (R2 box and M box) seem to be less deleterious. Schaefer and Messer (1991) have quantified the relative DnaA binding affinities to different DnaA boxes by meauring the ability of the DnaA-DnaA box complexes to block *in vivo* trancription. On basis of their analysis, they were able to suggest a more relaxed DnaA box consensus sequence to which the relative affinity is greater than 0.2: (T/C) (T/C) (A/T/C) **T** (A/C) **C** (A/G) (A/C/T) (A/C). Further analysis has been carried out by Speck *et al.* (1997), who determined which thymines in the DnaA box that are important for the interaction

with DnaA. The authors found that T_2, T_4, T_7 and T_9 are required for DnaA binding, which indicates that hydrophobic interactions with the methyl groups of the DnaA box are imporatant. Interestingly, these thymines are located opposite each other in pairs allowing interaction from two sides of the DnaA protein. However, it is probably not only the major grooves to which the DnaA protein mediates contacts, but also the minor groove (Schaper and Messer, 1995). Thus, it seems that except for the nucleotide in the middle of the DnaA box, the rest are important for specific binding. The binding of DnaA to the DnaA box induces a bend *in vitro* of about 40° (Schaper and Messer, 1995), and this may be important for a correct structure of the DNA that allows the formation of a higher order nucleoprotein complex.

According to the discussions above the DnaA box has been analysed using different experimental approches. It seems that all nine nucleotides in the DnaA box (and maybe even additional 5' and/or 3' nucleotides. Margulies and Kaguni, 1996; Weigel *et al.*, 1997) are important for proper DnaA binding, the only exception being the middle nucleotide. The interaction between DnaA and its target may include contacts in major and minor groove through hydrogen bonding and hydrophobic interactions.

Part II

Experimental work

Chapter 3

The DNA binding domain of the DnaA protein

Introduction

The DnaA protein is highly conserved among different bacteria. On basis of the degree of sequence homology, the protein has been divided into four domains (Fujita *et al.*, 1990; Messer and Weigel 1996. See Fig. 3.1). However, more recent analysis of the secondary structure and function of the DnaA protein has re-defined the domains as follows (Messer *et al.*, 1999). **Domain I** covers amino acids 1-86 of the *E.coli* DnaA protein. Experimental results strongly suggest that this area is responsible for the DnaA oligomerisation *in vivo* and *in vitro* (Weigel *et al.*, 1999). In addition, amino acids 24-86 was found to be important in loading DnaB (Messer *et al.*, 1999). **Domain II**, which covers amino acids 87- 134 of the *E.coli* DnaA protein, is very dissimilar in sequence and length among the various DnaA proteins. It is possible to remove parts of the domain and/or insert new sequences without any obvious effect on the DnaA protein (Schaper and Messer, 1997). Thus, domain II may constitute a flexible loop of the DnaA protein. **Domain III** covers amino acids 135-373 of the *E.coli* DnaA protein. The area includes a second DnaB interaction site from amino acid 154-206 (Marszalek and Kaguni, 1994), and the ATP binding domain from amino acid 168-236 (Saraste *et al.*, 1990). As discussed previously, ATP is required in the initiation process *in vitro* (Bramhill and Kornberg, 1988), and DnaA has a high affinity to both ATP and ADP (K_d=3·10^{-8} M and 10^{-7} M, respectively. Sekimizu *et al.*, 1987). In addition, analysis of DnaA(R334H) and DnaA(R342H) proteins, which render the chromosomal replication cold sensitive, has indicated that they are defective in duplex opening at non-permissive temperature (Takata *et al.*, 2000). R334 and R342 are part of a larger area that has some homology to the NtrC protein (Roth and Messer, 1995), which is a prokaryotic enhancer protein that stimulates the transcriptional opening of the DNA duplex. The finding that domain III is responsible for both ATP binding and duplex opening is conspicuous and may be consistent with the finding of several DnaA-ATP binding sites in the 13-mers, which may be important in duplex opening (Speck *et al.*, 1999). Thus, the function of ATP may be that it facilitates open complex formation. **Domain IV** covers the area from amino acid 374-467 of the *E.coli* DnaA protein. A small area (372-381) in the

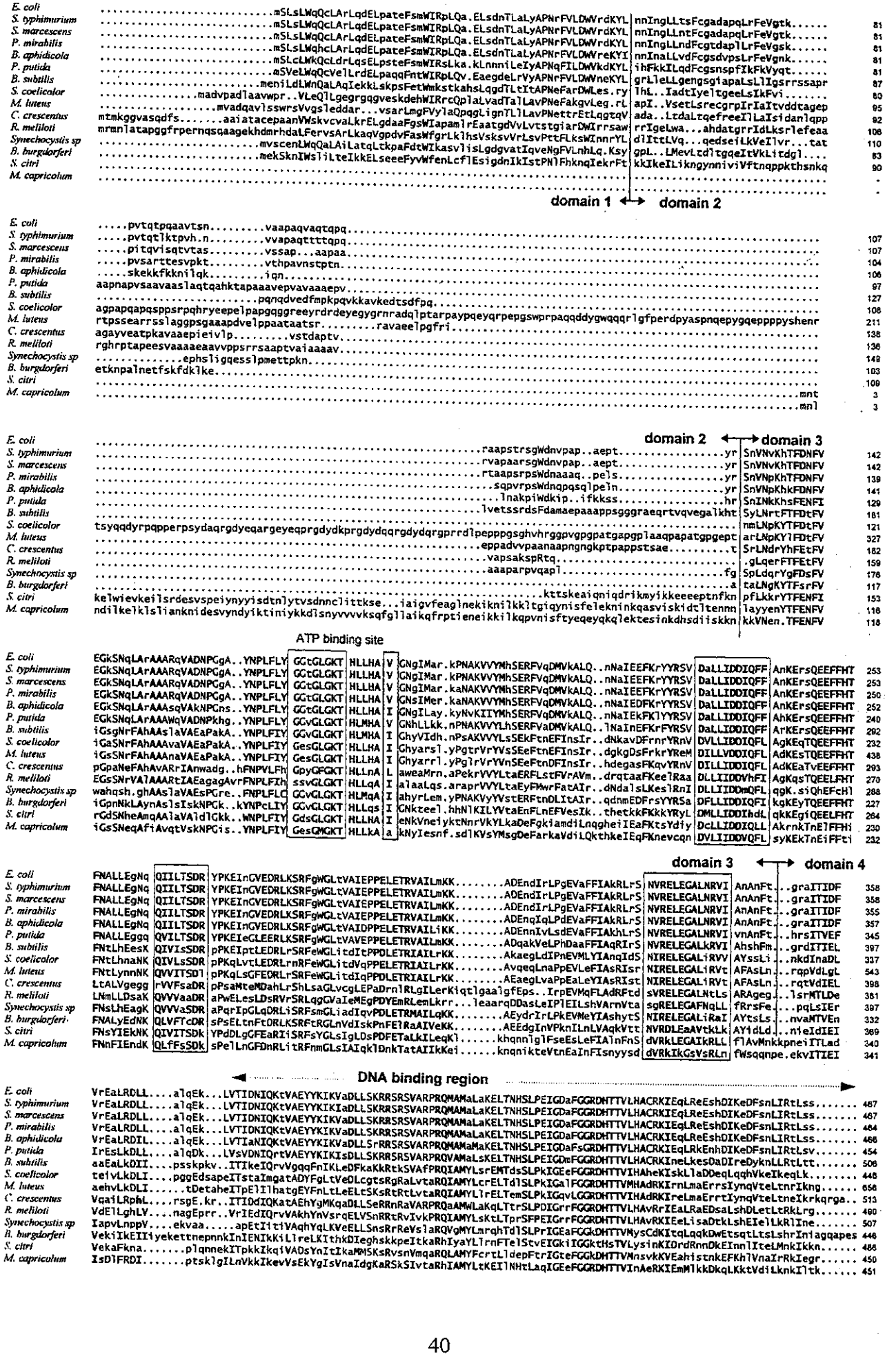

domain 1
domain 2
domain 2
domain 3
ATP binding site
domain 3
domain 4
DNA binding region

Fig. 3.1. Comparison of amino acid sequences of DnaA proteins from 15 different bacteria. Division into domain is according to Fujita *et al.* (1990). Boxed areas indicate regions of homology of DnaA proteins with the NtrC family of transcriptionsfactors (Roth and Messer, 1995). The ATP binding site and the DNA binding regions are indicated. Uppercase letters are used if 9 amino acids are identical, and boldface uppercase letters are used if 12 amino acids are identical or show a conservative exchange (Messer and Weigel, 1996).

beginning of the domain has been shown to be involved in the interaction with membrane components, which may be important in the nucleotide turn-over (Garner and Crooke, 1996). The last 94 amino acids of the DnaA protein (374-467) are required and sufficient for DNA binding (Roth and Messer, 1995).

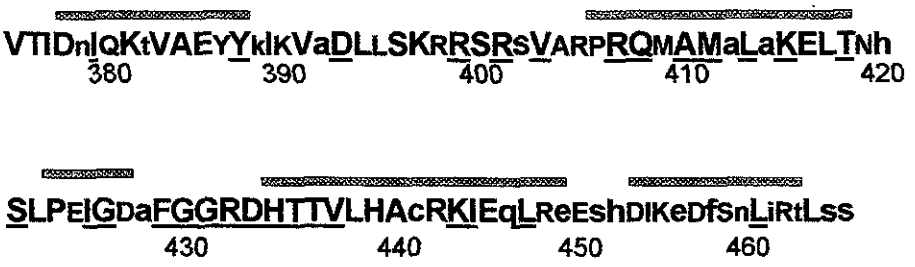


Fig. 3.2. Conservation of the DNA binding domain (domain IV) of the *E.coli* DnaA protein based on alignment of 15 DnaA proteins from different bacteria (Fig. 3.1). Horizontal bars represent α-helixes. <50% identity: not conserved (lowercase letters); >50% identity: conserved (uppercase letters); >66% identity: well conserved (large uppercase letters) and >86% identity: highly conserved (underlined large uppercase letters).

Domain IV, which is well conserved, has a highly conserved area from F429 to V437 (Fig. 3.2), and it is predicted to consist of five α-helixes separated by loops (Fig. 3.3).
The secondary structure of the DNA binding domain is rather conserved among different organisms, even though some DnaA proteins have completely different primary structures (Fig. 3.4). Thus, the secondary structure, and probably the tertiary structure seems very important for DNA binding function. The DNA binding domain of the DnaA protein is of great importance for DnaA function, since it is the binding of DnaA to DnaA boxes in *oriC* that settles the initiation of replication as discussed in Chapter 1 and 2. However, little is known about the structure of the domain and which amino acids that are responsible for the DNA contact. As discussed in Chapter 2, the DNA binding domain may possess a helix-turn-helix motif or a helix-loop-helix motif (Schaper and Messer, 1997) or maybe a completely different yet unknown and

undefined binding mode. The aim of this work is to shed more light on the DNA binding domain by analysing DnaA proteins carrying amino acid substitutions. This chapter is divided in Experimental procedures, Results and Discussion with a further subdivision of Experimental procedures and Results into section I, II and III.

```
PHD results (normal)

AA          VTIDNIQKTVAEYYKIKVADLLSKRRSRSVARPRQMAMALAKELTNHSLPEIGDAFGGRD
PHD_sec       HHHHHHHHHHHH EEEE         HHHHHHHHHHHHHHHHHH      HHHHHH
Rel_sec     991799999997612233111266751587678999999999974137569888628842
SUB_sec     LL.HHHHHHHHHH.........LLLL.HHHHHHHHHHHHHHHHHH...LLHHHHHH.LL..

P_3_acc     eebeebeebbbe bebebeebeeee eeebbebbbbbbbbbeebbeebbeebbeebbbee
Rel_acc     00241702165203131321311321021322502998385124131341276214101O
SUB_acc     ...e.b...bb.....................b..bbb.bb..b....b..bb..b....


AA          HTTVLHACRKIEQLREESHDIKEDFSNLIRTLSS
PHD_sec     HHHHHHHHHHHHHHHH   HHHHHHHHHHHHHH
Rel_sec     44553189999999737747999999999998389
SUB_sec     ..HH..HHHHHHHHH.LL.HHHHHHHHHHHH.LL

P_3_acc     bbbbb bbeebeeb eeeeebeeebeebeeebee
Rel_acc     15585061116230042133323143261322 36
SUB_acc     .bbbb.b...b....e........b..b.....e
```

Fig. 3.3. Secondary structure and solvent accessibility predictions of the DNA binding domain of the *E.coli* DnaA protein using the PHD server: www.embl-heidelberg.de/predictprotein/predictprotein.html. **AA**: amino acid sequence. **PHD sec**: PHD predicted secondary structure, where H: α-helix, E: β-sheet and L: loop. **Rel sec**: reliability index for PHD sec prediction (0=low to 9=high). **SUB sec**: subset of the PHD sec prediction. **P_3 acc**: PHD predicted solvent accessibility, where b: 0-9% (buried) and e: 36-100% (exposed). **Rel acc**: reliability index for PHD acc prediction (0=low to 9=high). **SUB acc**: subset of the PHD acc prediction.

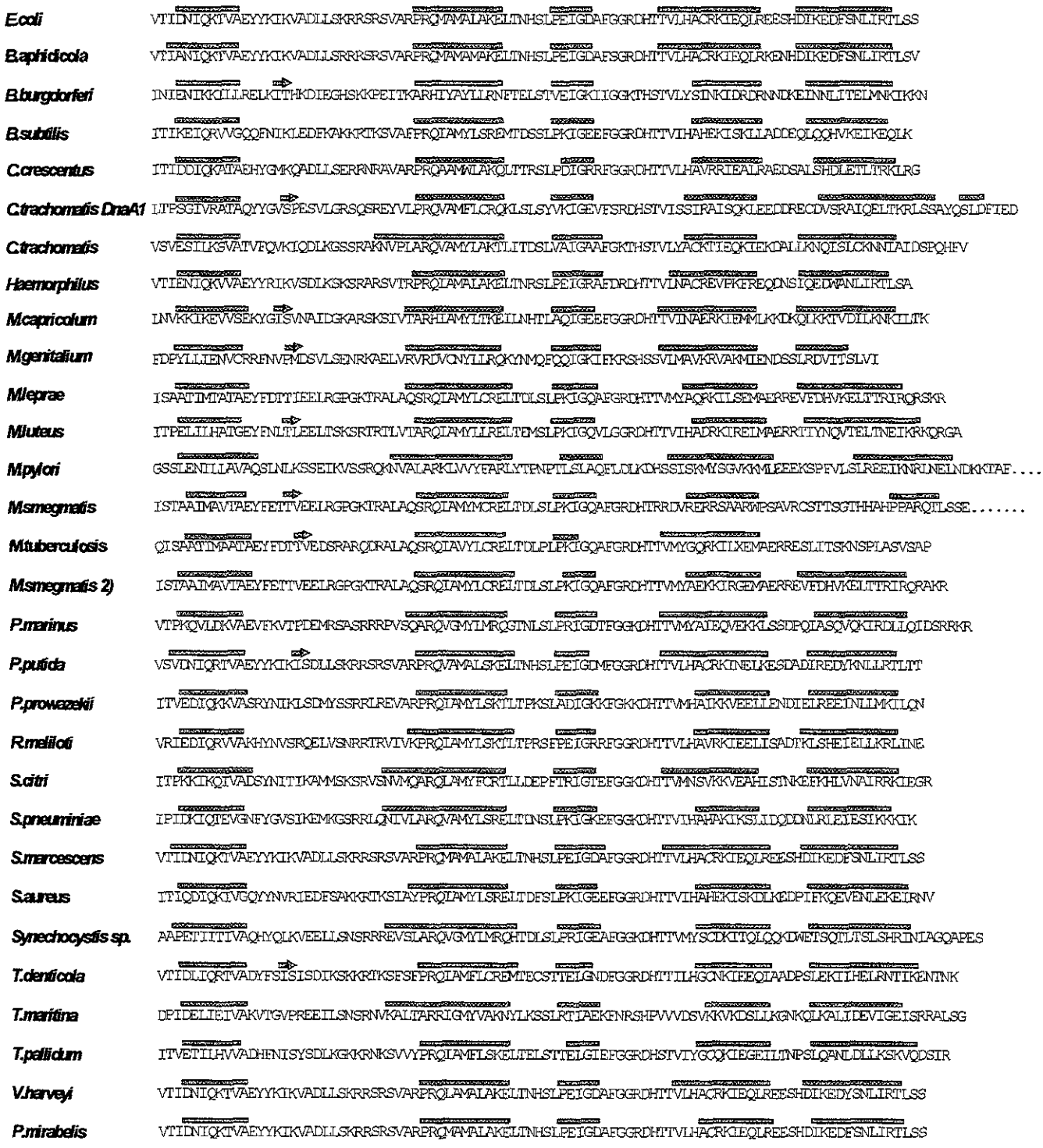


Fig. 3.4. Secondary structure predictions of the (proposed) DNA binding domain of DnaA proteins from various organisms. The PHD server with following address was used in the prediction: www.embl-heidelberg.de/predictprotein/predictprotein.html. Horizontal bars represents α-helixes and arrows β-sheets.

Experimental procedures

General procedures

P1 transduction and preparation

Preparation of P1 lysate was carried out essentially as described by Miller (1992): Overnight cultures grown in LB medium were diluted 20 times in LB medium supplemented with 10 mM $CaCl_2$ and grown for 1-2 hours. 100 µl cell suspension was incubated 20 min. at 37°C with different amounts of P1 lysate (typically 1, 10 and 100 µl 10^5 P1 phages/ml). 5 ml top-agar supplemented with 5 mM $CaCl_2$ was added, and the suspension was plated on LB plates. The following day a few drops of chloroform was added to the supernatant (P1 phages) which was kept at 5°C. P1 transductions were also carried our essentially as described by Miller (1992). 100 µl of an overnight culture grown in LB medium supplemented with 5 mM $CaCl_2$ were incubated 20 min. at 37°C with different amounts of P1 lysate (typically 1, 10 and 100 µl 10^5 P1 phages/ml). 100 µl Na-citrate was added to a final amount of 1% and the suspension was plated on selective plates and incubated overnight. When kanamycin resistance was selected, the bacteria + phage suspensions were incubated with 1 ml LB supplemented with 1% NaCi for 3 hours, centrifuged and plated on selective plates.

Competent cells and transformation

Cells were made competent and transformed with plasmids essentially as described by Sambrook *et al.* (1989): An over night culture of the strain to be transformed was inoculated 1:25 in LB and grown until $OD_{450} \sim 1.0$. 10 ml was harvested and 4 ml 50 mM $CaCl_2$ was added. After 20 min., the cells were centrifuged again and dissolved in 2 ml 50 mM $CaCl_2$. After 1 hour, the cells were competent for about a week. 200 µl competent cells was incubated with an appropriate amount of plasmid (depending on the type of transformation, ranging from 10 ng to 0.5 µg) and incubated on ice for 30 min. The transformation mixture was heat shocked at 42°C for 5 min. 1 ml LB was added and the transformation mixture was incubated at room temperature or 30°C for 30-120 min. depending on the selection.

Growth medium

The growth medium was either LB medium (10 g NaCl, 10 g Difco Bacto tryptone, 5 g Difco yeast extract and 20 g Difco agar per litre) or AB medium (Clark and Maaløe, 1967) supplemented with 0.2% glucose, 2 µg/ml thiamine and 1% casamino acids (Difco). When required, the growth medium was supplemented with 50 µg/ml kanamycin (Calbiochem), 10 µg/ml tetracycline (Sigma), 100 µg/ml ampicillin (Sigma), different concentration of IPTG (Sigma) and/or X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside. Apollo).

Mini-scale plasmid preparation

Plasmid was prepared as described by Sambrook *et al.* (1989) combined with a further improvement by using a PCR purification kit from Boehringer Mannheim: 2 ml over night culture was harvested and dissolved in 100 µl 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0. After app. 5 min. 200 µl 0.2 M NaOH, 1% SDS was added. After 5 min. incubation, the samples were incubated on ice with 150 µl 3 M NaAc pH 4.8 for 15 min. The samples were centrifuged, and the supernatant was transferred to 0.8 ml 96% EtOH. The DNA was precipitated and dissolved in 100 µl 0.5 mM EDTA, 2 mM Tris-HCl pH 8. 0.2 µl 25 mg/ml RNase was added and the samples were incubated at room temperature for 15-30 min followed by a column purification using the PCR purification kit from Boeringer Mannheim.

Immunological procedures

Samples were prepared for immunoblot analysis essentially as described previously (Hansen *et al.*, 1991A). Cells were harvested and dissolved in standard loading buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and boiled for 5 min. 12% resolving, 4% stacking Tris-HCl Ready Gels from BioRad was used in the protein separations. After electrophoresis, proteins were transferred to Immun-Blot PVDF Membranes (BioRad) by using a semi-dry blotting procedure (Kyhse-Andersen, 1984). Protein transfer was for 2 hours. The membrane was blocked over night in TBS buffer (0.05 M Tris (pH 10.4), 0.15 M NaCl) containing 2% Tween. The following day, the membrane was washed 3 times with TBS buffer containing 0.05% Tween and incubated with primary DnaA antibody (Landoulsi *et al.*, 1989) in TBS, 0.05% Tween and Blocking reagent (Boeringer Mannheim) or streptavidin conjugated alkaline phosphatase (BioRad) in TBS, 0.05% Tween for 1-2 hours. After three times washes in

TBS, 0.05% Tween, the filters were incubated with alkaline phosphatase conjugated pig immunoglobulin to rabbit immunoglobulins (DAKO) for 2 hours. After three additional washes, the filters were stained with NBT (4-nitro blue tetrazolium chloride) and XP (5-bromo-4-chloro-3-indolyl-phosphate) (Boehringer Mannheim) as described previously (Blake *et al.*, 1984). For all immunoblots DnaA protein was used as standard concentration marker by using experimental results indicating that 1 ml culture from a *dnaA*$^+$ strain at OD_{450}=1 corresponds to 25 ng DnaA/ml (Hansen *et al.*, 1991A). The western immunoblots were quantified by using a BioRad model GS-700 imaging Densitometer.

Enzyme reactions

All restriction enzyme reactions were carried out in KAc buffer (0.1 M KAc, 25 mM Tris-acetat (pH 7.5), 10 mM MgAc, 0.1 mg/ml bovine serum albumin, 1 mM β-mercaptoethanol) and incubated at the temperatures recommended by the supplier (New England Biolab., Amersham and Promega). Ligase (MBI Fermentas) was used in ligations reactions that were carried out in ligase buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.025% NP40), 20 mM dithiothreitol (DTT), 1 mM ATP. The ligation mix was incubated at room temperature for 2 hours or more.

Standard PCR reaction

1 µl linearized DNA (app. 0.05 ng) or cells corresponding to one colony was incubated with 2.5 mM $MgCl_2$, 0.8 µM of each primer, 0.2 mM of each dNTP, thermo prime DNA polymerase buffer and 1 unit Thermo prime DNA polymerase (KEBO) to a total volume of 25 µl. The cycle program was 94°C for 1 min., 50-52°C for 1 min., and 72°C for 2 min.

Section I

Construction and analysis of multicopy plasmid born *dnaA* alleles encoding DnaA proteins with amino acid substitutions in Domain IV

Strains

Table 3.1. Strains used in this work

Strain	Genotype	References/source
CSH66	Δ(*lac*) *thi* (lC1857S7 p*lac5* $i^- z^+ y^-$)	Miller (1972)
MC1000	*araD139*, Δ(*ara,leu*)7697, Δ*lac*, X74, *galU*, *galK*, *strA*	Casabadan and Cohen (1980)
MT102	a, *hsdR*	Atlung *et al.* (1989)
RUC663	a, *hsdR*, *dnaA46*, *tnaA*::Tn10	from Ole Skovgaard
TC4300	a, P_{mioC}-*lacZ*, $Kana^R$	from Tove Atlung.
MN247	a, *hsdR*, P_{mioC}-*lacZ* in λ att	P1(TC4300) x RUC663
TC1929	a, *dnaA204*, λRB1	Hansen and Atlung (1995A)
MN978	a, *dnaA204*, P_{mioC}-*lacZ* in λ att	P1(TC4300) x TC1929

a) genotype as MC1000

MN247 and MN978 were constructed by P1 transduction of RUC663 and TC1929, respectively, with P1 stock grown on TC4300 (General procedures). Kanamycin resistance was selected at 32°C. MN247 was further verified by transformation with pMN242 and pMN251 (p*dnaA*$^+$ and p*dnaA204*, respectively. See below). The MN247/pMN242 transformant was white on LB with 40 μg/ml X-gal, whereas the MN247/pMN251 transformant was blue. TC1929 carries a P_{dnaA}-*lacZ* fusion in λ attachment site, and is therefore blue on X-gal plates even in presence of pMN242 (the *dnaA* promoter is not repressed 100% by DnaA. Atlung *et al.*, 1985; Braun *et al.*, 1985. See chapter 1). However, TC1929 is λ lysogen whereas MN978 is not. Thus, TC1929 transductants were cross-streaked with λ phages on LB plates supplemented with 10 mM $MgCl_2$ and 0.2% maltose.

Plasmids

Table 3.2. Plasmids used in this work

Plasmid	Genotype or amino acid change in DnaA	IPTG (μM)[1)]
pBEX5BA	pBR322, pMK2 casette ($P_{A1\text{-}04/03}$, *lacI*), biotin-target, amp^{R} [2)]	
pMN69	pBEX5BA without *Bst*EII site in *lacI*	
pBP42	pBEX5BA carrying *dnaA*$^{+}$ [3)]	
pMN236	pMN69 carrying *dnaA*$^{+}$	
pMN242	as pMN236, without 150 bp after *dnaA* gene	10
pFHC539	pBR322 derivative carrying *dnaA* gene [4)]	
pMN251	I389N (DnaA204)	30
pMN277	L461V	10
pMN283	T418A	10
pMN287	A392T, M409I	10
pMN291	T375A	10
pMN413	Y386N	30
pMN416	A410V	30
pMN420	A384E	30
pMN422	L395P, R399Q	30
pMN424	A384V, Y386H	30
pMN429	V403M, L417Q	30
pMN430	M411T (DnaA211)	30
pMN512	H420Q, A428V, H439L, E445G	30
pMN516	N419Y, F429L	30
pMN521	E424G, L447S, K455E	30
pMN522	V383E, L395H, S402T, S421R, Q446R	30
pMN599	R432S, K390E	30
pMN600	I376V, F429L	10
pMN603	Y387C	10
pMN604	Y387C, K390E, K397R, I444V	10
pMN723	Y386H	30
pMN724	V383M (DnaA205)	30
pMN725	I389F, L394I	30

Plasmid	Genotype or amino acid change in DnaA	IPTG (μM)[1)]
pMN726	K390I	10
pMN727	P406L, L413P, K443R	10
pMN728	G431S, S451C, A384V	10
pMN729	K415N, L422Q	30
pMN730	I376N, M409I	30
pMN731	K415I	30
pMN732	M411I	10
pMN734	S402L, I444T, E450G, I454T	30
pMN735	V391A, L465S	30
pMN736	S400P, F429L	30
pMN737	A384V, E385G, D457G	30
pMN738	E385A, R442H, F458S	30
pMN739	N419S, I444V, H452R, L465S	30
pMN740	M409T, K415T	30
pMN741	L413Q	30
pMN742	Y386C, S402L	30
pMN743	K381E, K388E, L395P	30
pMN744	H420P	30
pMN747	L438P	30
pMN748	E385G, K415E	30
pMN749	N378D, M411T	10
pMN752	K415E	10
pMN753	L395P, R398H, H452R	10
pMN756	T382S, P406L	10
pMN757	T436S, L447S, D457V	10
pMN758	I454N	10
pMN760	I454T, F458S	10
pMN762	Q380R, I389T	10
pMN764	I376V, K415Q, K443R	10
pMN781	F429V	10
pMN783	L395P, E456G	10

Plasmid	Genotype or amino acid change in DnaA	IPTG (μM)[1)]
pMN784	E424G, I425V	10
pMN786	V383A	10
pMN787	D393Y, N460D, I462F, L465M	10
pMN788	I462F, I379T	10
pMN789	Y387N, K397R	10
pMN790	L394H, D427Y, E445G	10
pMN791	A414V, L417Q	10
pMN793	E416D, L465S	10
pMN796	F429S	10
pMN799	D453A	10
pMN801	A410T	10
pMN802	Q380R, K415R, I389V, L422Q, Q446R	10
pMN803	M411V, D453N	10
pMN804	E416G, C441R	10
pMN805	K390E, M411V	10
pMN806	A392V, I425S, E456G	10
pMN810	I389T	10
pMN819	N378Y, F429L	10
pMN822	K381R, R448H	30
pMN823	K415E, K455E	30
pMN833	K397R, I425T	30
pMN834	F429Y, T436S, E445G, K455Q	30
pMN835	E416G, I389TS	30
pMN836	S400P, C441G	30
pMN845	K381E	10
pMN846	Y386C, K443R	10
pMN847	K397E	10
pMN848	K390E, L417M, L465S	10
pMN849	E445G, L461F	10
pMN851	I376L, M409T	10
pMN852	M409V, T418N	10

Plasmid	Genotype or amino acid change in DnaA	IPTG (μM)[1)]
pMN853	K390E, I444A, E445G	30
pMN854	V391D, S402L, Q446R, E449K, S451G	30
pMN856	A384E, D453G	30
pMN859	V383A, E449G	10
pMN861	M409T, S451G, E456K	10
pMN862	T382A, S459P	10
pMN864	Y387S	10
pMN865	Y387H, S459T, F458L	10
pMN866	I376T, E385G, Q446L	10
pMN867	K381R, K388M, V403E	10
pMN900	T418A	10
pMN901	S467P	10
pMN903	K397E, L447M	10
pMN904	Y387H	10
pMN905	Y387H, D427N, R463K	10
pMN907	K397E, A428V, D453G, S459L	10
pMN909	K397M	10
pMN912	V391D, I462V	10
pMN913	Y386H	10
pMN914	K388Q	10
pMN918	K381R, L395P	10
pMN919	I376T, Y386F	10
pMN921	K381E, L447M	10
pMN922	K390E	10
pMN923	L395P, A414V, E416V	10
pMN925	L395P	10
pMN926	K397E	10
pMN928	A392E, M411V	10
pMN929	K415R, N419S, H439R	10
pMN931	I376T	10
pMN934	N378D, K397E, I444V	10

Plasmid	Genotype or amino acid change in DnaA	IPTG (μM)[1)]
pMN950	V437M	30
pMN951	N419T, M409T	30
pMN952	Y387C	30
pMN953	N378D, N419S, I425T	30
pMN954	I379V, K381R, M409I, L417Q, K455E	30
pMN955	R401C, K443R	30
pMN957	V437L, E445G	30
pMN958	I425T, D433N	30
pMN959	Q380R, F458S	30
pMN960	L438P	30
pMN963	N378Y, L465S	30
pMN964	Y387C	30
pMN965	A410S, A414V, E416G	30
pMN967	I379T	30
pMN968	I379T, S400T	30
pMN969	I379T, H452R	30
pMN971	E424G	30
pMN972	L395P	30
pMN974	Q380R, V383A, E445G	30
pMN975	D377G, L422M, S466P	30
pMN976	N460D, H439R, S459P, I462T	30
pMN977	V391A, C441G	30
pMN980	D377G, C441S, S400P, R398C	30

1) The various MN247 transformants harbouring pMN242 and mutant derivatives (pMN277-980) were selected on LB plates supplemented with indicated amount of IPTG (see below)
2) From Walter Messer. pBEX5BA is constructed from pLEX5BA (Diederich *et al.*, 1994)
3) From Benny Pedersen
4) Hansen *et al.* (1992)

pMN69 (Fig. 3.5) was constructed by removing the *Bst*EII site from pBEX5BA using a 'overlapping' PCR technique. The *Eco*RV-*Sph*I fragment from pBEX5BA (flanking the *Bst*EII site, Fig. 3.5) was cloned into *Eco*RV+*Sph*I digested M13BM20. JM101 was transfected with the recombinant M13 DNA and double and single stranded DNA was prepared as described by Sambrooke *et al.* (1989). The double stranded DNA was used

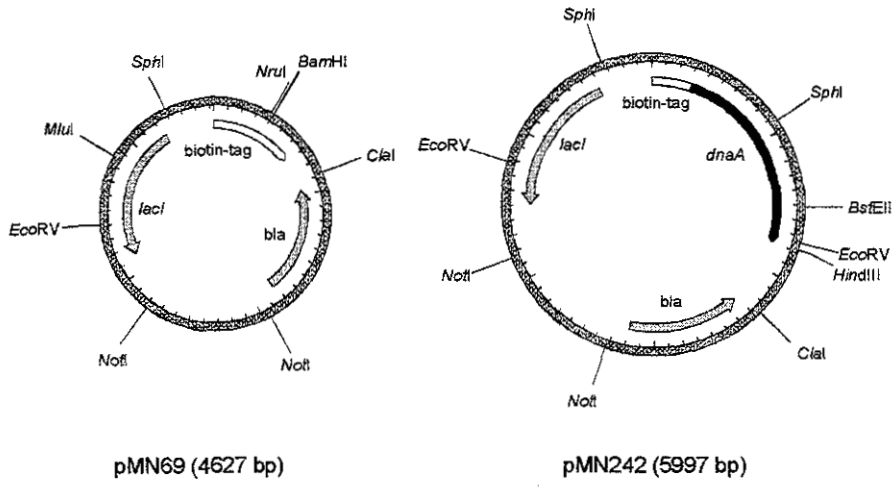


Fig. 3.5. pMN69 and pMN242 were constructed as described in the text.

to identify the correct clones and the single stranded M13 DNA was used in two parallel PCR reaction. Reaction 1 included two primers with the following sequences: CAGCGCGATTTGCTGGTGCCCCAATGCGACCAGATGCTCCACGCC (overlaps the *Bst*EII site) and AACAGCTATGACCATG (standard reverse M13 sequencing primer). Reaction 2 included two primers with the following sequences: GCATCTGGTCGCATTGGGGCACCAGCAAATCGCGCTGTTAGCGGG (overlaps the *Bst*EII site) and TGACCGGCAGCAAAATG (standard -20 sequencing M13 primer). Both PCR reactions were run as standard PCR reactions (General procedures). The two primers that overlap the *Bst*EII site both carry a silent mutation that destroys the restriction site, but leaves the LacI product intact. The PCR fragments from PCR reaction 1 and 2 (675 bp and 301 bp, respectively) were purified from the gel using a gel purification kit from Qiagen. Both PCR fragments were used as template in a second PCR reaction that was an amplification of the whole fragment using the flanking M13 standard primers (see sequence above). The PCR reaction was run as a standard PCR reaction. The resulting PCR fragment (675+301 bp) that no longer carried the *Bst*EII site, was digested with *Eco*RV and *Sph*I and re-cloned into pBEX5BA. Transformation of CSH66 with pMN69 gave only white colonies, and thus the plasmid born *lacI* gene was functional as expected.

pBP42 was constructed by Benny W. Pedersen and Peter Danø (see Pedersen, 1995) as follows: the first 200 bp of the *dnaA* gene from p*dnaA116* was PCR amplified using primers with the following sequences: *Bam*HI-*dnaA*: CTGGATCCGCAGAAACTGGT TAGC and *Nde*I-*dnaA*: GGAATTCCATATGTCACTTTCGCTTTGGC. The PCR reaction was run as a standard PCR amplification. The PCR fragment was digested with

*Nde*I and filled with Klenow fragment of DNA polymerase I. The polymerase was inactivated and the PCR fragment was digested with *Bam*HI. pBEX5BA was digested with *Nru*I and *Bam*HI and ligated with the PCR product. The correct clone was verified by sequencing using a Thermo Sequenase cycle sequencing kit from Amersham with the *Nde*I-*dnaA* primer as sequencing primer. Finally, the *Eco*RI-*Pst*I fragment from p*dnaA116* was cloned into the pBEX5BA derivative carrying the first part of the *dnaA* gene.

pMN236 is a 3187 bp *Mlu*I-*Cla*I fragment from pBP42 (carrying the *dnaA* gene) ligated to the 2973 bp *Mlu*I-*Cla*I fragment from pMN69.

pMN242 (Fig. 3.5) was constructed as follows: Two primers flanking the sequence encoding the DNA binding domain was used to PCR amplify this area - both in order to construct mutations in the area (see below) and in order to construct a wild type derivative. The primers have the following sequences: '*Bst*EII': GCGCGACTTGCTGG CATTGC ; '*Hin*dIII': CGTTCTACGGTAAATTTCAAAGCTTTACG. The '*Bst*EII' primer anneals just upstream the *Bst*EII site in the *dnaA* gene, and thus, close to the DNA binding domain encoding sequence. The '*Hin*dIII' primer anneals in the very end of the *dnaA* structural gene. This primer has a *Hin*dIII site build in so that the PCR fragment can be cut and ligated with the *Bst*EII-*Hin*dIII fragment of pMN236. Thus, pMN242 and pMN236 are identical except for the 150 bp between the end of the *dnaA* structural gene and the *Hin*dIII site in pMN236, which are missing in pMN242. The PCR was run as described below, but without $MnCl_2$.

pMN242 derivatives carrying mutations in the sequence that encodes the DNA binding domain (pMN277-980) were constructed by using a mutagenic PCR technique as follows: The PCR amplification was run under conditions that reduces the fidelity of the Taq polymerase (Leung *et al.*, 1989): 0.06 ng linear pMN236 was mixed with Taq polymerase buffer, 6 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.2 mM of each dNTP, 0.8 μM of the '*Bst*EII' and '*Hin*dIII' primers and 4 units taq polymerase (Perkin Elmer). The cycle program was 1 min 94°C, 1 min 49°C, and 1 min 72°C. The PCR fragments were digested with *Bst*EII and *Hin*dIII and ligated to the gel purified *Bst*EII + *Hin*dIII digested pMN242 fragment. The point mutation frequency was 7%.

Transformation, selection and sequencing mutant dnaA alleles

The ligation mix was used to transform MN247 (*dnaA46*, P_{mioC}-*lacZ*, see table 3.1). Transformants were selected at 42°C on LB plates with ampicillin and 10 or 30 μM IPTG. These IPTG concentrations were chosen because MN247 harbouring pMN242

(p*dnaA*$^+$) formed colonies on 10, but not 30 μM IPTG plates, whereas the presence of pMN251 (p*dnaA204*) required 30 μM IPTG in order to complement the heat sensitive *dnaA46* phenotype and form colonies. After transformation, the colonies were re-streaked and plasmids were purified (General procedures). The area of the *dnaA* gene that encodes the DNA binding domain was sequenced using a Thermo Sequenase Sequencing kit from Amersham with the '*Bst*EII' primer as sequencing primer. Further analysis was carried out with those *dnaA* mutants that had one and two mutations (see below).

Complementation analysis

MN247 carrying different multicopy mutant *dnaA* alleles were grown over night in LB medium at 32°C. The over night cultures were diluted 10, 10^2, 10^3, 10^4 times in a microtiter tray and app. 5 μl was plated on LB plates with 100 μg/ml ampicillin and 40 μg/ml X-gal and 0, 6, 10, 30 and 100 μM IPTG. The plates were incubated at 30, 39 and 42°C. Complementation analysis was carried out with two independently isolated MN247 transformants from each transformation. Complementation and growth was evaluated visually the following day by counting and measuring the number and sizes of colonies. Complementation of the heat sensitive *dnaA46* (MN247) and *dnaA204* phenotype (MN978) was also determined by re-transformations and plating on LB plates with ampicillin and different amounts of IPTG.

Verification of the dnaA allele on the chromosome

The presence of *dnaA46* allele on the chromosome of the MN247 transformants was verified to rule out the possibility that the *dnaA* genes on the multicopy plasmids had recombined with the one on the chromosome. This was done by PCR amplification of the chromosomal *dnaA* gene with primers that flank the allele specific *dnaA46* mutations. The forward primer anneals upstream the *dnaA* structural gene and can therefore anneal to the chromosomal *dnaA* but to the plasmid *dnaA*. The primers have the following sequences: FLF1: GCCAATTTTTGTCTATGGTG FLR2: TTGAGAAGGACTCTAG CAAG. The chromosomal *dnaA* allele from two colonies of each MN247 transformant were PCR amplified as described in General procedures. One of the *dnaA46* mutations disrupts a *Sph*I recognition site in the *dnaA* gene. The PCR fragments were therefore digested with *Sph*I to verify the presence of the *dnaA46* allele on the chromosome.

Stability measurements of DnaA fusion proteins

Cells were grown in LB medium at 32°C for 5-8 generations. At OD_{450} = 0.5, 1 mM IPTG was added to the culture. After 1 hour incubation, 200 μg/ml chloramphenicol was added and the culture was divided in two portions; one portion was incubated at 42°C and the other remained at 32°C. Samples were taken at times corresponding to 1, 2, 3 and 4 generations after chloramphenicol addition. Aliquots corresponding to 100 μl culture OD_{450} = 1.0 was run on SDS-PAGE gels and immunoblotted as described in General procedures.

Secondary structure prediction

Secondary structure prediction of the wild type DnaA protein was obtained from the PHD server: www.embl-heidelberg.de/predictprotein/ predictprotein.html, which includes algorithms developed by Rost and Sander (1994). Alternatively, the secondary structure was obtained from a secondary structure prediction program developed by Claus A. Andersen (1998). Both programs are a two level system based on neural network on each level. The secondary structures of all DnaA proteins with amino acid substitution constructed in this work and listed in Table 3.2, were obtained using the CBS program. When the helix prediction reliability was 0.7 or higher, it was accepted as the likely secondary structure. Otherwise, it was interpreted to be a coil. According to the prediction servers used in this work, the DNA binding domain of the *E.coli* DnaA protein probably does not contain β-sheets.

Section II

The DnaA-DnaA box interaction

Strains and plasmids

Table 3.3. *E.coli* strains and plasmids used in this work

Strain	Genotype / plasmid (amino acid change in DnaA protein)
MN978	*dnaA204*, P_{mioC}-*lacZ*
MN981	MN978/pBEX5BA
MN987	MN978/pMN242 ($DnaA^+$)
MN982	MN978/pMN724 (V383M, DnaA205)
MN994	MN978/pMN251 (I389N, DnaA204)
MN983	MN978/pMN430 (M411V, DnaA211)
MN984	MN978/pMN864 (Y387S)
MN985	MN978/pMN950 (V437M)
MN986	MN978/pMN960 (L438P)
MN988	MN978/pMN972 (L395P)
MN989	MN978/pMN971 (E424G)
MN990	MN978/pMN955 (R401C, K443R)
MN991	MN978/pMN859 (V383A, E449G)
MN995	MN978/pMN424 (A384V, Y386H)
MN996	MN978/pMN735 (V391A, L465S)
MN997	MN978/pMN758 (I454N)
MN998	MN978/pMN796 (F429S)
MN999	MN978/pMN847 (K397E)
MN1000	MN978/pMN900 (T418A)
MN1001	MN978/pMN913 (Y386H)
MN1002	MN978/pMN967 (I379T)
MN1003	MN978/pMN977 (V391A, C441G)
pTAC1257	pBR322 derivative carrying *oriC* and *mioC*

The constructions of strains and plasmids are described in Experimental procedures section I (Table 3.1 and 3.2). Transformation of MN987 was carried out as described in

General procedures. pTAC1257 was obtained from Tove Atlung: The *Taq*I (upstream *mioC*) -*Cla*I (in *gidA*) fragment was cloned into pTAC909, which is a pBR322 derivative with a deleted *Hin*dIII site. Thus, pTAC1257 carries the whole *oriC* area with DnaA box R1, M, R2, R3, R4, R5 and R6 (see Fig. 1.1 in Chapter 1).

Buffers

Binding buffer I: 50 mM HEPES-KOH pH 7.6, 100 mM K-Glutamate, 1 mM DTT and 1mM ATP. **Binding buffer II**: as Binding buffer I supplemented with 15% glycerol, 0.4% Triton X-100, 2 mM EDTA and 5 mg/ml BSA. **TBS**: 50 mM Tris-HCl pH 10.0, 150 mM NaCl. **PBS**: 140 mM NaCl, 3 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.4.

Overproduction of DnaA fusion proteins

MN981-MN1003 were inoculated directly from a fresh over night LB plate with 100 μg/ml ampicillin in liquid LB medium. 500 μg/ml ampicillin was added to the growth meduim continuously (app. every 1 hour) to avoid growth of plasmid free cells. The cultures were grown exponentially at 32°C for approximately eight generations and at OD_{450} = 0.5, 1 mM IPTG (Sigma) was added. After 1-2 hours induction, the cells were harvested and washed twice in binding buffer I. The cell pellets were dissolved in binding buffer I to a 10^2 up-concentration (i.e. 100 ml bacterial culture was dissolved in 1 ml binding buffer I). The cell suspensions were incubated 30 min. on ice with 1 mg/ml lysozyme before sonication (5 x 10 sec.). The sonicated cells were centrifuged 20 min. at 20.000 G and the supernatant was kept in the freezer until it was used in binding experiments.

Production of 'hot' and 'cold' oriC fragment

pTAC1257 was PCR amplified with the following pBR322 primers: pBR-1: CGAGGCCCTTTCGTCTTC, pBR-2: CTGCGTTAGCAATTTAACTGTG. The PCR amplification was run as a standard PCR reaction (General procedures). PCR fragments used as 'cold' *oriC* was purified using a Boeringer Mannheim PCR purification kit, whereas when it was used in preparation of 'hot' *oriC*, it was gel purified using a Qiagen gel purification kit. Thermo sequenase sequencing kit from Amersham was used to prepare 'hot' *oriC*. This procedure consists of a labelling step and an elongation step, and the procedure was carried out essentially as described by the supplier. Labelling step: 6 ng/μl gel purified PCR fragment (see above) was mixed with reaction buffer, 29

nM of primer pBR-1 and pBR-2, 0.17 μM dGTP and dTTP, 0.29 μCi/μl ^{35}S-dATP and 2 units Thermo Sequenase DNA polymerase per 17.5 μl reaction mix. The cycle program was 95°C 15 sec. and 57°C 30 sec. for 50 cycles. Elongation step: the labelled PCR fragment was mixed with 80 μM dNTP in reaction buffer (dNTP was used and not deoxy-NTP's purchased from supplier). 1 unit Thermo Sequenase DNA polymerase was added per 30 μl reaction mix. The cycle program was: 95°C 30 sec., 57°C 30 sec. and 72°C 2 min. for 10 cycles. The labelled PCR fragment was purified using a PCR purification kit from Boeringer Mannheim. To make sure that the labelling procedure was successful, the 'hot' *oriC* DNA was run on a 0.7% agarose gel, which was dried and exposed on a Kodak film. The 'hot' *oriC* concentration was in the order of 1-2 ng/μl and counted between 10.000 and 20.000 cpm/ng *oriC* in a Liquid Scintillation Counter. The DNA concentration was determined by using a λ *Bst*EII marker with known concentration. In addition, the DNA concentration was determined by measuring OD_{260} (OD_{260}=1 corresponds to 50 μg DNA/ml, Sambrooke *et al.*, 1989).

Binding fusion DnaA proteins to Dynal Streptavidin coated magnetic beads

20 μl streptavidin coated magnetic beads (Dynal) and 5 μl sonicated bacterial lysate was used in each binding assay. The total amount of magnetic beads to be used in one experiment (typically 2-300 μl per mutant DnaA protein) was washed once with PBS buffer and once with the binding buffer I. The total amount of bacterial lysate was added and the mixture was incubated for one hour at room temperature with vigorous shaking. The supernatant (i.e. proteins that do not bind to streptavidin) was removed and kept for quantification of bound DnaA protein on SDS-PAGE gels and immunoblots (see below). The magnetic beads with bound fusion DnaA protein was washed twice with binding buffer I and divided into 20 μl portions. The supernatant was removed and the magnetic beads were ready to be used in *oriC*-DnaA saturation experiments, competition experiments and protein concentration determinations (see below, and Fig. 3.6).

oriC-DnaA saturation

20 μl binding buffer with seven different *oriC* concentrations was added to the magnetic beads - DnaA suspensions. The *oriC* concentrations, and the percentage of 'cold' *oriC*, are shown in table 3.4.

Table 3.4. *oriC* concentrations used in saturation experiments.

[*oriC*] (nM)	'cold' *oriC* (%)
0.126	76
0.252	88
0.378	92
0.63	95.2
1.26	97.6
2.52	98.8
5.04	99.4

oriC was allowed to bind to the DnaA protein for one hour at room temperature. The supernatant was removed and the magnetic beads - DnaA suspensions were washed several times with binding buffer I. Each *oriC* concentration was assayed in duplicate, and a sample was always removed from each concentration (corresponds to $[oriC]_{total}$. Fig. 3.6). The wild type DnaA protein was included in each experiment.

Competition assay

20 µl binding buffer I with either 0.63 nM or 1.26 nM *oriC* and increasing amount of salmon sperm DNA was added to the magnetic beads - DnaA suspensions in order to measure the specificity of the mutant DnaA proteins towards *oriC*. The salmon sperm DNA was sonicated to a fragment size of 500 bp in average, and 10, 50, 125, 250, 500 and 1000 fold excess was added simultaneously with the *oriC* fragment. Each salmon sperm DNA concentration was assayed in duplicate, and the wild type DnaA protein was included in each experiment.

Quantification of bound oriC

The concentration of *oriC*-DnaA complex was quantified by subtracting the number of counts in the supernatant after incubation with the magnetic bead-DnaA suspensions ($[oriC]_{free}$) from the number of counts in the *oriC* solutions that had not been incubated with magnetic bead-DnaA ($[oriC]_{total}$). A liquid scintillation counter was used. It was tried several times to elute the *oriC* by adding 1.5 M NaCl (Roth and Messer, 1995), but it was never possible to recover all the counts (i.e. $[oriC]_{total} - [oriC]_{free} >> [oriC]_{bound}$). Counting the bound *oriC* together with the magnetic beads was not successful either, because the magnetic beads quenched too much to be counted properly.

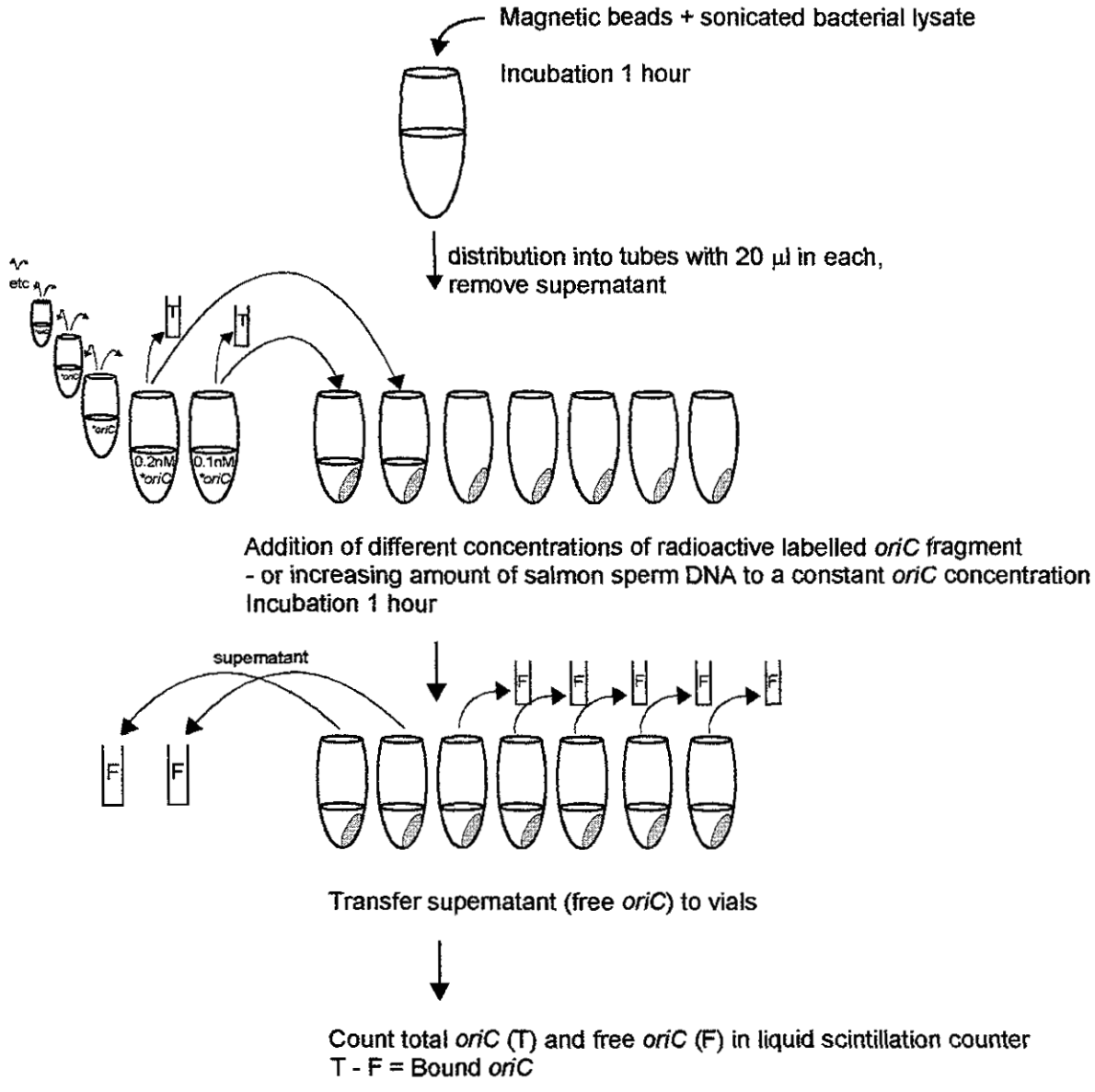


Fig. 3.6. Flow diagram of *in vitro* assay used to determine binding constants and specificity towards *oriC*. F: free *oriC* and T: total *oriC*. See text for explanations.

Besides, the $[oriC]_{total}$ - $[oriC]_{free}$ = $[oriC]_{bound}$ approach seemed valid, since the magnetic beads do not bind *oriC* themselves, and the pBEX5BA lysate do not bind *oriC* either.

Determination of DnaA concentrations

The amount of DnaA protein that had bound to the magnetic beads was quantified using three approaches: 1) the bacterial lysate (before and after incubation with magnetic beads) was run on SDS-PAGE gels and Western blotted as described in General procedures. 2) A sample was taken from each mutant DnaA protein and processed in a

Western-like manner (Benny W. Pedersen, unpublished results): 150 µl TBS, 2% Tween was added to the magnetic bead-DnaA pellet and the samples were incubated over night with vigorous shaking. The samples were washed with TBS and DnaA antibody in TBS, 0.05% Tween was added. After one hour incubation, the samples were washed several times with TBS, 0.05% Tween. 100 µl 10^{-3} dilution Pork-anti-Rabbit Immunoglobulins (DAKO) were added and the samples were incubated one hour at room temperature. The samples were washed several times with TBS, 0.05% Tween and once with 150 mM Tris-HCl pH 8.8. 200 µl Tris-HCl pH 8.8 was added and the samples were pre-incubated at 37°C. 200 µl 18.6 mg/ml PNPP (p-nitrophenyl-phosphate disodium, Sigma) was added at t = 0, and the samples were incubated at 37°C for 30-60 min. The supernatant was transferred to 800 µl 1 M NaOH and A_{410} was measured with PNPP as reference. A pBEX5BA sample was always included in the protein measurements. The amount of protein is expressed as units as follows: units DnaA protein = (A_{410}(DnaA)- A_{410}(pBEX5BA) x 1000) / t (min). Samples were taken in duplicate. Unfortunately, it was not easy to reproduce the results. The amount of DnaA proteins, expressed as units, differed more than 2-fold when comparing otherwice similar samples. Therefore, the DnaA concentrations were estimated by 3) plotting [*oriC*-DnaA] as a function of $[oriC]_{free}$ (see below).

Binding constants

in vitro dissociation constants (K_d) and total DnaA protein concentrations were determined in the saturation experiments (see above) by plotting bound *oriC* as a function of free *oriC*. GraFit (Erithacus Software) was used to make the curve fits and estimate the binding constants using the equation for the binding of one ligand (in this case one *oriC* fragment per DnaA protein): [DnaA-*oriC*] = $[oriC]_{free} \cdot [DnaA]_{total} / (K_d + [oriC]_{free})$. The total DnaA concentration can be read as the horizontal asymptote at saturation and K_D when half of the total amount of DnaA protein has bound *oriC*.

in vivo affinity: β-galactosidase assay

Over night cultures were diluted and grown exponentially for app. 6 generations at 32°C in AB medium (Clark and Maaløe, 1967) supplemented with 0.2% glucose, 2 µg/ml thiamine and 1% casamino acids. At OD_{450} = 0.1 the cultures were divided in five portions. Four portions were incubated at 42° with 0, 10, 30 and 100 µM IPTG and the fifth portion was incubated at 32°C with no IPTG. 3 x 1 ml samples were taken different

times after the shift, added to 200 μg/ml chloramphenicol and kept at 0°C. At the end of the experiment, the samples were shaken with toluene. The following day the β-galactosidase assay was carried out essentially as described by Miller (1972). Enzyme units was calculated as follows: units β-galactosidase = $(1000 \cdot OD_{420})/t \cdot v \cdot OD_{450}$, where t = min. incubation with ONPG and v = volume of the culture.

South-Western analysis

On a low Bis SDS-PAGE gel, the fusion protein always appear as a double band after, but not before, sonication (Fig. 3.17 in Results, section II). This indicates that the fusion protein is degraded. To measure if both the intact and degraded protein bind *oriC*, South-Western analysis was carried out essentially as described by Sutton and Kaguni (1997B). Sonicated lysate corresponding to 0.58 pmol DnaA protein per lane was run on a SDS-PAGE gel, and the proteins were blotted onto a Immun-Blot PVDF Membrane (BioRad). The filter was then incubated in 30 ml binding buffer II at 4°C for one hour. ^{35}S-*oriC* fragment was added to the filter to a final concentration of 50 ng/ml and incubated over night at 4°C. The filter was washed twice in binding buffer II and exposed on a Kodak film. Bound DNA was stripped from the filter in 154 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.25% SDS. The filter was then processed as in Western blotting (General procedures).

FX_a cleavage

The fusion DnaA protein was cleaved with FX_a (Promega) in 100 mM NaCl, 20 mM Tris-HCl pH 8.0 according to recommendations from supplier. Cleavage of fusion DnaA protein was verified on SDS-PAGE gels and Western blotting as described in General procedures.

Section III

Integration and analysis of mutant *dnaA* alleles in λ attachment site of the chromosome

Strains and plasmids

Table 3.5. Strains and plasmids used in this work

Strain	Source or relevant genotype	Reference/source
WM1718	*dnaA46*, *recBC*	from Christoph Weigel
MC1000	*araD139*, Δ(*ara,leu*)7697, Δ*lac*, X74, *galU*, *galK*, *strA*	Casabadan and Cohen (1980)
MT102	a, *hsdR*	Atlung *et al.* (1989)
RB210	a, λRB1 (p*dnaA-lacZ*)	Braun *et al.* (1985)
TC3422	a, *hsdR*/pTAC3422 (*int* plasmid), cam^R	Atlung *et al.* (1991)
TC3482	a, λRB1, *dnaA*::cat, *rnh373*	Hansen *et al.* (1991A)
TC1929	a, λRB1, *dnaA204*	Hansen and Atlung (1995A)
NK5525	F', *proA81*::Tn10, rnh^+, IN(*rrnD-rrnE*)	from *E.coli* Genetic Stock Ceter (#7089)
NK6034	Hfr, *car-96*::Tn10, del(*gpt-lac*)5, *relA1*, *spoT1*, *thi-1*	from *E.coli* Genetic Stock Ceter (#6181)
MN1127	a, λRB1, *dnaA*::cat, *rnh373* , *car-96*::Tn10	TC3482 x P1(NK6034)
MN1128	a, λRB1, *dnaA*::cat, *rnh373*, car-96::Tn10/pUF747	this work
MN1184	a, *dnaA*::cat, *rnh373*, car-96::Tn10/pUF747, P_{MT}-biotin-$dnaA^+$ in λ att	this work
MN1191	a, *dnaA204*, P_{MT}-biotin-*dnaA*(L395P) in λ att	this work
MN1193	a, *dnaA204*, P_{MT}-biotin-*dnaA*(K397E) in λ att	this work
MN1195	a, *dnaA204*, P_{MT}-biotin- *dnaA*(A384V,Y386H) in λ att	this work
MN1197	a, *dnaA204*, P_{MT}-biotin-*dnaA*(C441G,V391A) in λ att	this work
MN1240	a, *dnaA204*, P_{MT}-biotin-$dnaA^+$ in λ att	this work
MN1210	a, *dnaA*::cat, P_{MT}-biotin-*dnaA*(L395P) in λ att, rnh^+	this work
MN1211	a, *dnaA*::cat, P_{MT}-biotin-*dnaA*(C441G,V391A) in λ att, rnh^+	this work
MN1213	a, *dnaA*::cat, P_{MT}-biotin-*dnaA*(K397E) in λ att, rnh^+	this work

Strain	Source or relevant genotype	Reference/source
MN1215	a, *dnaA*::cat, P_{MT}-biotin-*dnaA*(A384V,Y386H) in λ att, *rnh*$^+$	this work
MN1242	a, *dnaA*::cat, P_{MT}-biotin-*dnaA*$^+$ in λ att, *rnh*$^+$	this work
pTAC3463	*AttP-aphA* DNA integration casette	Atlung *et al.* (1991)
pMN1122	as pTAC3463, *Not*I adaptor in *Eco*RI site	this work
pUF747	P_{BAD}-*rnh*$^+$	From Ulrik von Freiesleben

a) genotype as MC1000

Construction of pMN1122 was carried out as follows: pTAC3463 was digested with *Eco*RI and ligated with 10 times molar excess of *Not*I adaptors with the following sequences : AATTCAGGCCTGCGGCCGCC and AATTGGCGGCCGCAGGCC. After transformation, plasmids were prepared and the correct clone, carrying a *Not*I site, was identified. pUF747 was constructed by PCR amplification of the *rnh*$^+$ gene with following primers: CAGGAAGTCTACCAGCCATGGTTAAACAGGTAG and CCCAAGCTTCAAGCCACGAATTCGCCAGG. The PCR fragment was digested with *Nco*I and *Eco*RI and cloned into the pBAD*hisA* vector (Invitrogens).

MN1127 was constructed by P1 transduction of TC3482 with a P1 stock grown on NK6034 (General procedures). Tetracycline resistance was selected, and the transductants were screened for arginine and uracil requirements. MN1127 was transformed with pUF747 to construct MN1128. The transformation was carried out as described in General procedures.

Construction of MN1128, TC1929 and TC3482 derivatives with *dnaA* alleles in λ attachment site were carried out as follows (Fig. 3.7): pMN242 and pMN242 derivatives (Table 3.2) were digested with *Not*I and *Cla*I. The fragment carrying the *dnaA* gene (Fig 3.5) was gel purified using a gel purification kit from Qiagen, and ligated to the gel purified *Not*I-*Acc*I fragment of pMN1122. This ligation results in ligated circles that lack origin of replication and they are therefore not replicated in the cell. Kanamycin resistant colonies only appear if the circular DNA molecules have integrated on the chromosome. TC3422, carrying the *int* plasmid, was transformed with the ligation mix and kanamycin resistant colonies were selected. Integration of the DNA molecules in λ attachment site of the chromosome in kanamycin resistant cells was verified by PCR amplification with primers that anneal to the *aphA* and attR sequence, respectively (Kana: CCGAGTACGTGCTCGCTCG, AttR: GGCGCAATGCCATCTGG).

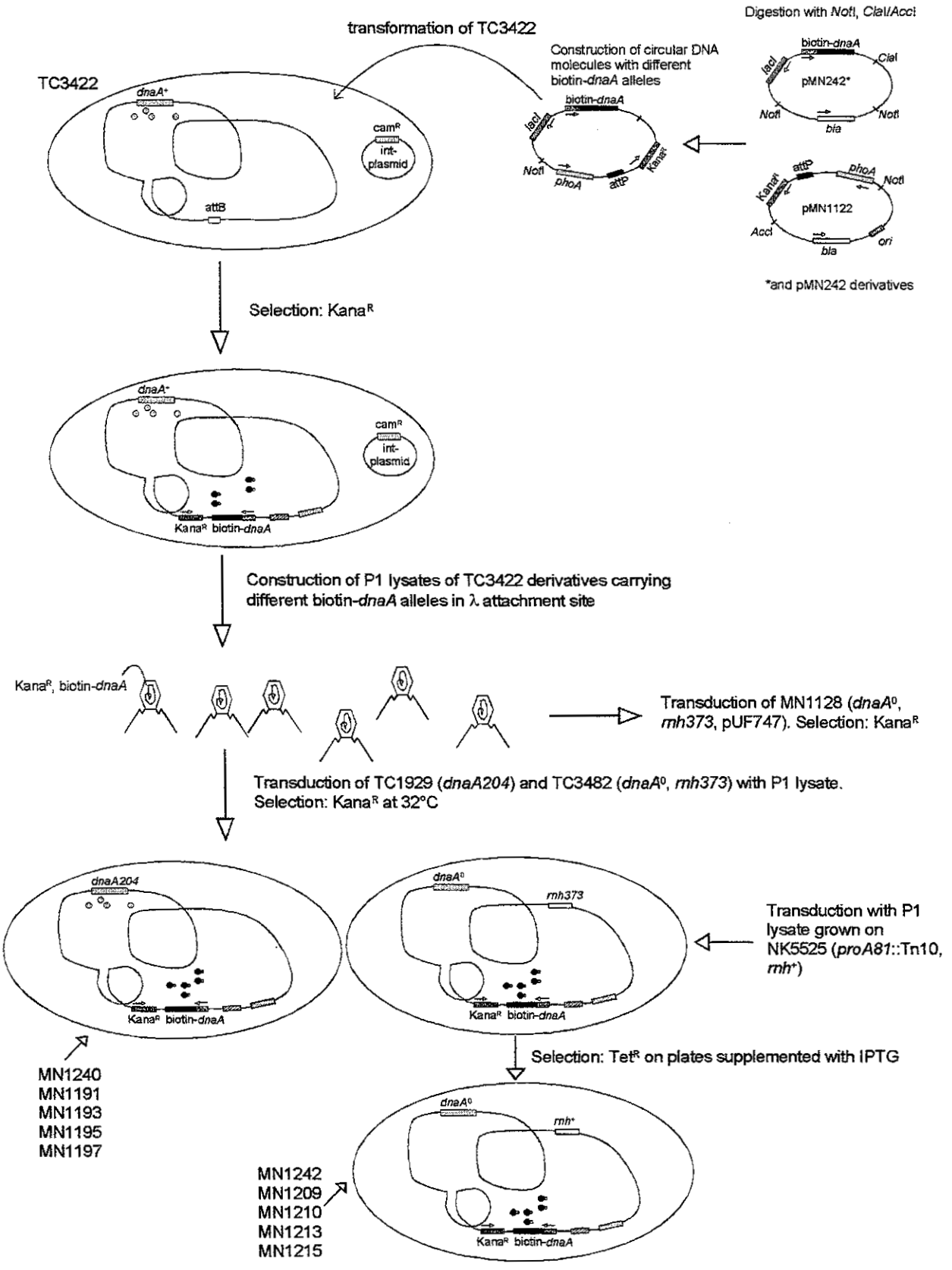


Fig. 3.7. Constructions of strains harbouring different biotin-*dnaA* alleles in λ attachment site. See text for explanations.

The PCR reaction was run as a standard PCR reaction (General procedures). P1 lysates were prepared from TC3422 derivatives carrying the different *dnaA* genes in λ attachment site and used to transduce MN1128, TC3482 and TC1929 (General procedures). Kanamycin resistance was selected on LB plates at 32°C. TC3482 transductants were screened for *rnh* depended high-temperature sensitivity. In addition, to avoid selection of cells that had recombined the whole plasmid into λ attachment site, the TC3482 transductants were screened for cam^S. TC1929 transductants were screened for cam^S for the same reason as above, and for growth at high temperature (42°C) in presence of different amounts of IPTG due to induction of the *dnaA* gene in λ attachment site. The *rnh373* mutation in the TC3482 transductants was removed by P1 transduction with P1 lysate grown on NK5525 (*proA*::Tn10, rnh^+). Tetracycline resistant transductants were selected on LB plates supplemented with 0, 30 and 75 μM IPTG. The distance between *proA* and *rnh* is 0.6 min. (Bachmann, 1990) and thus, co-transduction is approximately 33% (Wu, 1966). Tetracycline resistant transductants were screened for rnh^+ phenotype (no high-temperature sensitivity) on LB plates supplemented with IPTG. Growth of all transductants were IPTG dependent - or sensitive (see Table 3.13).

Using WM1718 to transfer dnaA mutants to their normal chromosomal location

pMN242 and a selection of pMN242 derivatives were transformed into WM1718 and plated on LB plates supplemented with 10 or 30 μM IPTG and 100 μg/ml ampicillin, and incubated at 42°C. The following day, four colonies from each transformation were streaked at 42°C on LB plates without ampicillin and without IPTG. This treatment selects recombinants that have recombined the *dnaA46* allele out of the chromosome. The streaking procedure was repeated five times to allow growth of plasmid free cells. Chromosomal DNA from ampicillin sensitive and temperature resistant cells were PCR amplified (General procedures) using primers that A) flank the allele specific *dnaA46* mutations and B) flank the sequence that encodes the DNA binding domain (for primer sequences see Experimental procedures, Section I: FLF1, FLR2 and *Bst*EII , *Hin*dIII). Absence of *dnaA46* allele on the chromosome was verified by *Sph*I digestion of PCR fragment A. A number of *dnaA* alleles that were selected for recombination into their normal chromosomal location carry mutations that introduce or remove recognition sequences for various restriction enzymes (Table 3.6). The correct isolate could

therefore be identified by enzyme digestions of PCR fragment B from the various strains.

Table 3.6. Changes in the restriction map of mutant *dnaA* alleles

plasmid	***dnaA***	**altered restriction pattern (+/- new site)**
pMN859	*dnaA*(E449G, V383A)	+ *Bso*FI (V383A), - *Ear*I (E449G)
pMN796	*dnaA*(F429S)	+ *Hga*I
pMN971	*dnaA*(E424G)	+ *Nci*I
pMN967	*dnaA*(I379T)	+ *Dde*I
pMN758	*dnaA*(I454N)	- *Eco*RV
pMN900	*dnaA*(T418A)	+ *Cac*8I
pMN955	*dnaA*(R401C, K443R)	- *Bsl*I (R401C), + *Bsl*I (K443R)
pMN960	*dnaA*(L438P)	+ *Ban*I
pMN913	*dnaA*(Y386H)	- *Sca*I
pMN972	*dnaA*(L395P)	no change
pMN424	*dnaA*(A384V Y386H)	- *Rsa*I (A384V), - *Sca*I (Y386H)
pMN977	*dnaA*(C441G, V391A)	+ *Sac*I (V391A), - *Mwo*I (C441G)
pMN847	*dnaA*(K397E)	no change

Growth of MN1128 and MN1184

The strains were inoculated from a fresh LB plate to liquid AB medium supplemented with 0.2% glycerol, 2 μg/ml thiamine, 1% casamino acids and 10 μg/ml uracil. 500 μg/ml ampicillin was added to the cultures every 30-40 min. It was important to avoid growth of plasmid free cells, because they would be able to initiate chromosome replication from *oriK*'s in presence of arabinose. The cultures were grown exponentially at 37°C for 5-8 generations, and at OD_{450} = 0.1, 0.2% arabinose was added together with 10, 30 and 100 μM IPTG. Samples were taken every 30 min. from the cultures to measure the number of initiated origins (see below).

Growth of TC1929 derivatives carrying different IPTG inducible dnaA alleles in λ attachment site

Cells were grown exponentially at 32°C in AB medium supplemented with 0.2% glucose, 2 μg/ml thiamine and 1% casamino acids. At OD_{450} = 0.1, the culture was shifted to 42°C for 1 hour. The culture was diluted 20 times and divided in ten portions; five portions were further incubated at 42°C and the rest were shifted back to 32°C. IPTG was added to final concentrations of 0, 10, 30, 50 and 100 μM to the cultures at 42 and 32°C. Samples were taken from the cultures before and 1, 2, 3 and 4 hours after IPTG addition to measure the number of initiated origins and DnaA concentrations (see below).

Growth of rnh^+ derivatives of TC3482 ($dnaA^0$) carrying different IPTG inducible dnaA alleles in λ attachment site

Strains were grown exponentially at 32°C and 42°C in AB medium supplemented with 0.2% glucose, 2 μg/ml thiamine, 1% casamino acids and 0 or 30 μM IPTG dependending on the strain. At OD_{450}=0.5, the cultures were diluted 10 times in the same media supplemented with 0, 30 and 100 μM IPTG and samples were taken from the cultures after 2, 3 and 4 hours to measure the number of initiated origins and DnaA concentrations (see below).

Number of initiated origins using flow cytometry

1 ml samples were incubated minimum three hours with 300 μg/ml rifampicin (kindly denoted by Ciba Geigy A/S) and 36 μg/ml cephalexin (Sigma). The cells were harvested and dissolved in 100 μl 10 mM Tris-HCl pH 7.4. 1 ml cold 77% ethanol was added to fixate the cells. The cells were kept at 5°C over night or longer. Between 0.1 (if OD_{450}=0.5) and 1 ml fixed cells were harvested and dissolved in 250 μl 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 90 μg/ml mitramycin and 20 μg/ml ethidium bromide. The cells were kept dark and on ice until they were analysed on a BioRad flow cytometer.

DnaA protein concentration

The DnaA concentration was determined as described in General procedures.

Results

Section I

Construction and analysis of multicopy plasmid born *dnaA* alleles encoding DnaA proteins with amino acid substitutions in domain IV

Modified PCR conditions were used to construct pMN242 derivatives with mutations in the DNA binding domain of the DnaA protein. The method is described by Leung *et al.* (1989), who has shown that the frequency of point mutations increased from 0.4% to 2% by adding 0.5 mM $MnCl_2$ and five times reduced amount of dATP relative to the other dNTP's. If the amount of dATP was not reduced, the mutation frequency decreased to 1.4%. Under both conditions, the number of transitions and transversions was fifty-fifty. In this study, it was not possible to run a mutagenic PCR reaction with decreased amount of dATP relative to the other dNTP's, whereas the DNA yield in the presence of Mn^{2+} was unaffected. However, in spite of the non-optimal mutation conditions, a higher mutation frequency - about 7% - was obtained. This resulted in PCR fragments that carried many mutations and therefore, were not further analysed. In addition, most of the mutations (75%) were transitions (Table 3.7 and 3.9). This is not in agreement with results obtained by Leung *et al.* (1989). The different results may be due to differences in the Taq polymerase activity. The mutagenic PCR fragments were digested with *Bst*EII and *Hin*dIII and cloned into pMN242 (Fig. 3.5), and used to transform MN247, which carries a *dnaA46* allele on the chromosome and a P_{mioC}-*lacZ* fusion in λ attachment site. The transformants were selected at 42°C on LB plates supplemented with ampicillin and 10 or 30 μM IPTG. Selection at 42°C ensured that only functional DnaA protein encoding *dnaA* alleles were selected, due to the temperature sensitive phenotype of the *dnaA46* host. 10 and 30 μM IPTG were chosen, because the p*dnaA*$^+$ transformant formed colonies in the presence of 10, but not 30 μM IPTG, whereas the p*dnaA204* transformant required 30 μM IPTG in order to complement the *dnaA46* phenotype. Thus, this selection resulted in selection of both not so harmful mutations (DnaA activities close to that of the wild type DnaA protein) and more harmful mutations that require more IPTG to complement the *dnaA46* allele.

Plasmids from 74 colonies selected on 10 μM IPTG and 59 colonies selected on 30 μM IPTG were prepared and the *dnaA* sequence encoding domain IV was sequenced.

Table 3.7. The number of times a particular amino acid change was observed in different plasmid isolates, and the mutation(s) causing it.

Amino acid change	Number	Mutation	Amino acid change	Number	Mutation
T375A	1	A→G	K390E	5	A→G
I376V	2	A→G	K390I	1	A→T
I376N	1	T→A	V391A	2	T→C
I376L	1	A→C	V391D	2	T→A
I376T	3	T→C	A392T	1	G→A
D377G	2	A→G	A392V	1	C→T
N378D	3	A→G	A392E	1	C→A
N378Y	2	A→T	D393Y	1	G→T
I379V	1	A→G	L394I	1	C→A
I379T	4	T→C	L394H	1	T→A
Q380R	4	A→G	L395P	8	T→C
K381R	4	A→G	L395H	1	T→A
K381E	3	A→G	K397R	3	A→G
T382A	1	A→G	K397E	5	A→G
T382S	1	A→T	K397M	1	A→T
V383A	3	T→C	R398H	1	G→A
V383E	1	T→A	R398C	1	C→T
V383M	1	G→A	R399Q	1	G→A
A384V	3	C→T	S400P	3	T→C
A384E	2	C→A	S400T	1	T→A
E385G	3	A→G	R401C	1	C→T
E385A	1	A→C	S402T	1	T→C
Y386N	1	T→A	S402L	3	C→T
Y386C	2	A→G	V403M	1	G→A
Y386H	3	T→C	V403E	1	T→A
Y386F	1	A→T	P406L	2	C→T
Y387C	4	A→G	M409I	3	G→A
Y387N	1	T→A	M409T	4	T→C
Y387S	1	A→C	M409V	1	A→G
Y387H	3	T→C	A410V	1	C→T
K388E	1	A→G	A410T	1	G→A
K388M	1	A→T	A410S	1	G→T
K388Q	1	A→G	M411T	2	T→C
I389F	1	A→T	M411I	1	G→A
I389T	3	T→C	M411V	3	A→G
I389V	1	A→G	L413P	1	T→C

Amino acid change	Number	Mutation	Amino acid change	Number	Mutation
A414V	3	C→T	T436S	2	A→T
K415N	1	A→T	V437M	1	G→A
K415I	1	A→T	V437L	1	G→C
K415T	1	A→C	L438P	2	T→C
K415E	3	A→G	H439L	1	A→T
K415Q	1	A→C	H439R	2	A→G
K415R	2	A→G	C441R	1	T→C
E416D	1	A→T	C441G	2	T→G
E416G	3	A→G	C441S	1	T→A
E416V	1	A→T	R442H	1	G→A
L417Q	3	T→A	K443R	4	A→G
L417M	1	C→A	I444V	3	A→G
T418A	2	A→G	I444T	2	T→C
T418N	1	C→A	I444A	1	A→G,T→C
N419Y	1	A→T	E445G	7	A→G
N419S	3	A→G	Q446R	3	A→G
N419T	1	A→C	Q446L	1	A→T
H420Q	1	C→A	L447S	2	T→C
H420P	1	A→C	L447M	2	T→A
S421R	1	T→A	R448H	1	G→A
L422Q	2	T→A	E449K	1	G→A
L422M	1	C→A	E449G	1	A→G
E424G	3	A→G	E450G	1	A→G
I425V	1	A→G	S451C	1	A→T
I425S	1	A→G	S451G	2	A→G
I425T	3	T→C	H452R	3	A→G
D427Y	1	G→T	D453A	1	A→C
D427N	1	G→A	D453N	1	G→A
A428V	2	C→T	D453G	2	A→G
F429L	3	T→C	I454T	2	T→C
F429L	1	T→A	I454N	1	T→A
F429V	1	T→G	K455E	3	A→G
F429S	1	T→C	K455Q	1	A→C
F429Y	1	T→A	E456G	2	A→G
G431S	1	G→A	E456K	1	G→A
R432S	1	C→A,T→C	D457G	1	A→G
D433N	1	G→A	D457V	1	A→T

Amino acid change	Number	Mutation	Amino acid change	Number	Mutation
F458S	3	T→C	I462F	2	A→T
F458L	1	T→C	I462V	1	A→G
S459P	2	T→C	I462T	1	T→C
S459T	1	T→A	R463K	1	G→A
S459L	1	C→T	L465S	5	T→C
N460D	2	A→G	L465M	1	T→A
L461V	1	T→G	S466P	1	T→C
L461F	1	A→T	S467P	1	T→C

The obtained amino acid changes in the DnaA protein are shown in table 3.2 (Experimental procedures, section I), and Table 3.7 shows the number of times the same amino acid substitution was observed in different isolates. The table also shows the type of mutation that caused the amino acid substitutions. The silent mutations are not included. An amino acid change at a given position (i.e. E456G) normally appeared 1-3 times, with few exceptions as the L395P change (and K397E and L465S). These areas may be mutational hot spots.

If the mutations are separated according to the amino acid change, with no regard to the position, it is clear that the amino acid changes Ile→Thr, Gln→Arg, Lys→Glu, Glu→Gly, Tyr→His, Phe→Leu, Phe→Ser and all the Met substitutions are the most common amino acid changes (Table 3.8).

Table 3.8. The number of times an amino acid change was observed, with no regard to the position, relative to the total number of that particular amino acid

Amino acid change	Number	relative to total [1)]
Thr→Ala	4	0.67
Thr→Ser	1	0.17
Thr→Asn	3	0.5
Ile→Val	9	1.3
Ile→Asn	2	0.29
Ile→Leu	1	0.14
Ile→Thr	18	2.6
Ile→Phe	4	0.57
Ile→Ser	1	0.14
Ile→Ala	1	0.14
Asp→Gly	5	0.83
Asp→Tyr	2	0.33
Asp→Asn	3	0.5
Asp→Ala	1	0.17
Asp→Val	1	0.17
Asn→Asp	3	1.0
Asn→Tyr	3	1.0
Asn→Ser	3	1.0
Asn→Thr	1	0.33
Gln→Arg	7	2.3
Gln→Leu	1	0.33
Lys→Arg	9	1.3
Lys→Glu	20	2.9
Lys→Met	2	0.29
Lys→Gln	3	0.43
Lys→Ile	2	0.29
Lys→Asn	1	0.14
Lys→Thr	1	0.14
Val→Ala	3	0.60
Val→Glu	2	0.40
Val→Met	3	0.60
Val→Leu	1	0.20
Ala→Val	10	1.4
Ala→Glu	3	0.43
Ala→Thr	2	0.29
Ala→Ser	1	0.14
Glu→Gly	20	2.9
Glu→Ala	1	0.14

Amino acid change	Number	relative to total [1)]
Glu→Asp	1	0.14
Glu→Val	1	0.14
Glu→Lys	2	0.29
Tyr→Asn	2	1.0
Tyr→Cys	6	3.0
Tyr→His	6	3.0
Tyr→Phe	1	0.33
Tyr→Ser	1	0.33
Leu→Ile	1	0.11
Leu→His	2	0.22
Leu→Pro	10	1.1
Leu→Gln	5	0.56
Leu→Met	5	0.56
Leu→Ser	7	0.78
Leu→Val	1	0.11
Leu→Phe	1	0.11
Arg→His	2	0.33
Arg→Cys	2	0.33
Arg→Gln	1	0.17
Arg→Ser	1	0.17
Arg→Lys	1	0.17
Ser→Pro	7	0.88
Ser→The	3	0.38
Ser→Leu	4	0.50
Ser→Arg	1	0.13
Ser→Cys	1	0.13
Ser→Gly	2	0.26
Pro→Leu	2	1.0
Met→Ile	4	2.0
Met→Thr	6	3.0
Met→Val	4	2.0
Phe→Leu	5	2.5
Phe→Val	1	0.50
Phe→Ser	4	2.0
Phe→Tyr	1	0.50
Gly→Ser	1	0.33

1) The number of times an amino acid substitution was observed, irrespective of position, relative to the total number of that particular amino acid.

This observation may, however, partly be because they are results of T→C or A→G transitions, which are the most common mutations (Table 3.9), which is in contrast to

observations done by Leung *et al.* (1989), who found a transition:transversion ratio of 1:1.

Table 3.9. The number of different mutations

Mutation	Number / % of total number of mutations
T→C	72 / **25**
T→A	23 / **8.1**
T→G	4 / **1.4**
C→T	18 / **6.3**
C→A	9 / **3.2**
C→G	0 / **0**
A→G	106 / **37**
A→T	22 / **7.7**
A→C	8 / **2.8**
G→A	19 / **6.7**
G→T	3 / **1.1**
G→C	1 / **0.4**

DnaA proteins with one and two amino acid substitutions were analysed further (see below), whereas those with 3, 4 or 5 amino acid substitutions were not due to difficulties in the later interpretations of the effects of individual amino acid substitutions.

Secondary structure predictions of DnaA proteins carrying one and two amino acid substitutions in the DNA binding domain

Secondary structure predictions of the various DnaA proteins constructed in this work, and the $DnaA^+$ protein, were carried out using a program developed by Claus A. Andersen (1998), referred to as the CBS program. The program is a two level system with neural network at each level. The more commonly used PHD server that includes algorithm developed by Rost and Sander (1994), referred to as the PHD program, was used to predict the secondary structure of the wild type DnaA protein for comparison (Fig. 3.8). This program is also based on neural networks. When both programs are used to predict the secondary structure of the DNA binding domain of the $DnaA^+$ protein two differences in the secondary structure predictions appear: A) the probability for a longer

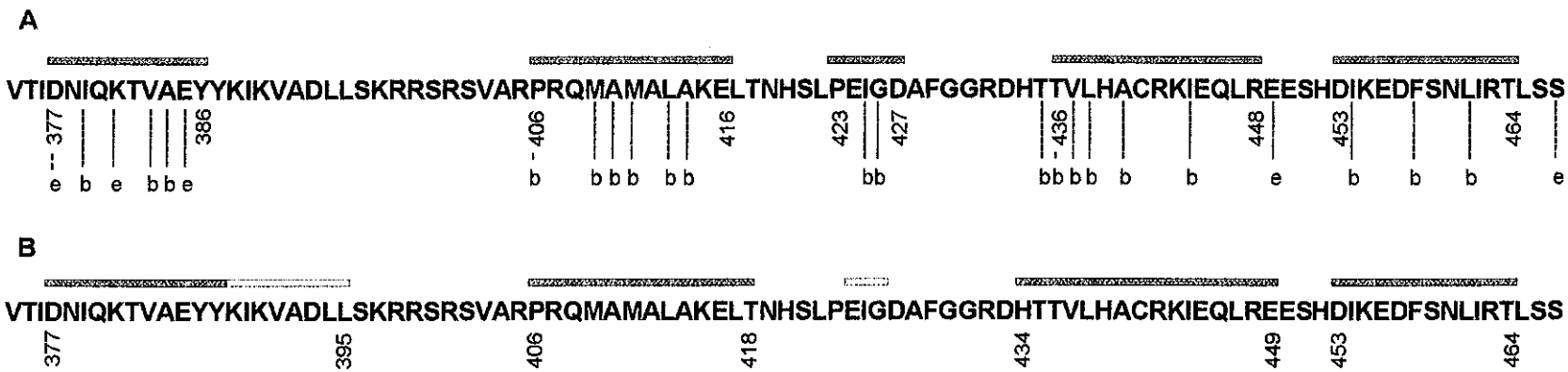


Fig. 3.8. Secondary structure prediction of the DNA binding domain of the DnaA protein (shown in one letter code) using the PHD server (A) and the CBS server. Horizontal bars represents α-helixes and numbers refer to the amino acid position in the DnaA protein. Exposed (e) and buried (b) amino acids are indicated. See also Fig. 3.3.

helix 1 is higher when the CBS program is used (0.61-0.64) compared to the PHD program (probability only 0.1-0.4) and B) the existence of helix 3 from P423-D427 has lower probability (0.65) using the CBS program than when the PHD program is used (0.8-0.9). The different amino acid changes constructed in this work will be used in the discussion of these contradictions (see below).

Complementation of the heat-sensitive dnaA46 allele by induction of multicopy mutant dnaA alleles

dnaA alleles with one and two mutations were further analysed in the *dnaA46* background (MN247). Dilutions of over night cultures were plated on LB plates supplemented with different concentrations of IPTG (0, 6, 10, 30, 100 μM) and incubated at 30, 39 and 42°C. The following day, growth and ability to complement the high temperature sensitive *dnaA46* phenotype were estimated by measuring the number and sizes of the colonies. The results of the complementation of the heat-sensitive *dnaA46* phenotype by induction of different mutant *dnaA* alleles are shown in Fig. 3.9. Growth data at 30°C are not shown, because they were quite similar among the different strains with few exceptions that will be mentioned in the text (see below). Generally, there was good growth at 30°C, which is expected due to the presence of DnaA46 proteins synthesised from the chromosomal *dnaA* gene. However, there was generally no growth on LB plates supplemented with 100 μM IPTG. The high IPTG concentration was not toxic itself, because the pBEX5BA (no *dnaA*) transformant grew on these plates (see below), which indicates that high DnaA concentrations are lethal.

pBEX5BA

MN247 (*dnaA46*) harbouring pBEX5BA (no *dnaA* allele) formed colonies at 30°C independently of the IPTG concentration (data not shown), whereas no colonies appeared at 39 or 42°C due to the heat sensitive phenotype of the host (Fig. 3.9).

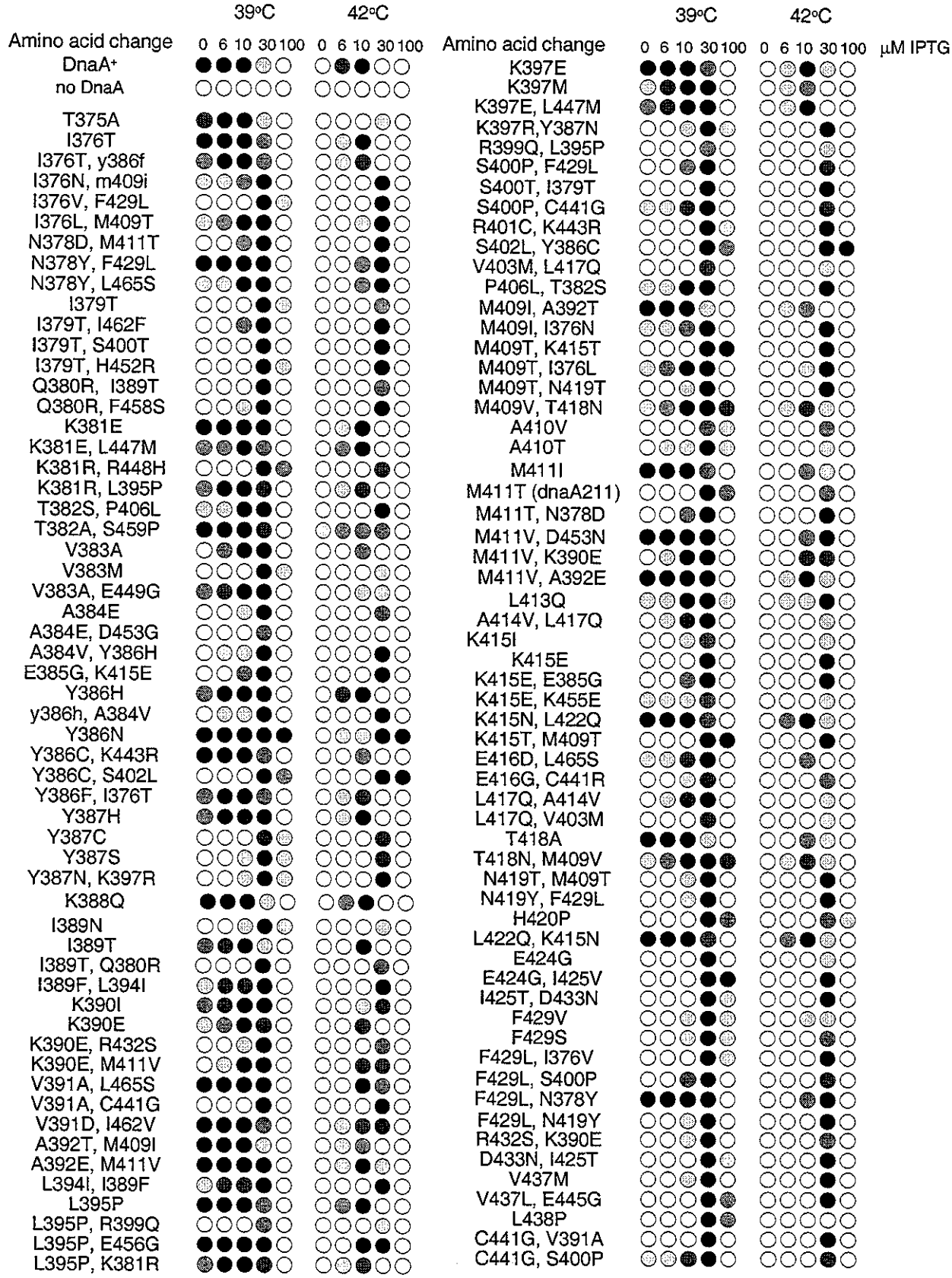
pdnaA$^+$

MN247 (*dnaA46*) harbouring p*dnaA*$^+$ formed colonies at 42°C in the presence of 6 and 10 μM IPTG. Higher IPTG concentrations seemed to inhibit growth at 42°C (Fig. 3.9). The growth at 30 and 39° was less affected by high IPTG concentrations. The DnaA$^+$ protein is not temperature sensitive, and thus, growth of the p*dnaA*$^+$ transformant was expected to be independent of the temperature. The fact that the p*dnaA*$^+$ transformant formed colonies on 30 μM IPTG plates at 30 and 39°C, but not at 42°C, may therefore

be due to the presence of some DnaA46 activity at the lower temperatures. This also seems consistent the observation that the p*dnaA*$^+$ transformant formed colonies without IPTG induction at 39°C, but not at 42°C (Fig. 3.9). The amount of DnaA$^+$ protein that was synthesised from the *lac* promoter under non-induced conditions was enough, together with the (in)active DnaA46 protein, to initiate chromosome replication and allow growth at 39°C. This was also observed in a *dnaA204* background (data not shown). Leakiness of the *lac* promoter was also observed in the β-galactosidase assays, where the specific activity in the p*dnaA*$^+$ transformant was lower under non-induced conditions than in most of the other transformants (Results, Section II).

What is the effect of the biotin-target on the DnaA protein?

Although the biotin target is fused to the N-terminal part of the DnaA protein, and therefore has maybe only minor effect on the DNA binding, its presence could have some effect on the protein activity *in vivo*. As discussed previously, the N-terminal domain of the DnaA protein seems to be responsible for protein-protein interactions, which are important in the assembly of the initiation complex (Weigel *et al.*, 1999). Bläsing (1999) have shown that *dnaA46* cells harbouring p*dnaA116*, which carry a *dnaA*$^+$ allele (without the biotin tag) under the same *lac* type promoter control as used in this work, formed colonies at 42°C with 5 and 10 μM IPTG, which is in agreement with the observations done in this work. However, Bläsing (1999) also observed (reduced) complementation at 42°C without IPTG induction, and that was not observed in this work at 42°C, only at 39°C. This could indicate that the biotin tag somehow reduces the DnaA activity slightly, but it could also be due differences in the strain backgrounds resulting in differences in the transcription frequency from the *lac* promoter under non-induced conditions and/or differences in the DnaA46 concentrations. In general, the results indicate that the DnaA protein fused to the biotin tag is functional, and has an activity comparable to the wild type DnaA protein.


39°C
42°C
Amino acid change
0 6 10 30 100 0 6 10 30 100
DnaA+
no DnaA
T375A
I376T
I376T, y386f
I376N, m409i
I376V, F429L
I376L, M409T
N378D, M411T
N378Y, F429L
N378Y, L465S
I379T
I379T, I462F
I379T, S400T
I379T, H452R
Q380R, I389T
Q380R, F458S
K381E
K381E, L447M
K381R, R448H
K381R, L395P
T382S, P406L
T382A, S459P
V383A
V383M
V383A, E449G
A384E
A384E, D453G
A384V, Y386H
E385G, K415E
Y386H
y386h, A384V
Y386N
Y386C, K443R
Y386C, S402L
Y386F, I376T
Y387H
Y387C
Y387S
Y387N, K397R
K388Q
I389N
I389T
I389T, Q380R
I389F, L394I
K390I
K390E
K390E, R432S
K390E, M411V
V391A, L465S
V391A, C441G
V391D, I462V
A392T, M409I
A392E, M411V
L394I, I389F
L395P
L395P, R399Q
L395P, E456G
L395P, K381R
39°C
42°C
Amino acid change
0 6 10 30 100 0 6 10 30 100 µM IPTG
K397E
K397M
K397E, L447M
K397R,Y387N
R399Q, L395P
S400P, F429L
S400T, I379T
S400P, C441G
R401C, K443R
S402L, Y386C
V403M, L417Q
P406L, T382S
M409I, A392T
M409I, I376N
M409T, K415T
M409T, I376L
M409T, N419T
M409V, T418N
A410V
A410T
M411I
M411T (dnaA211)
M411T, N378D
M411V, D453N
M411V, K390E
M411V, A392E
L413Q
A414V, L417Q
K415I
K415E
K415E, E385G
K415E, K455E
K415N, L422Q
K415T, M409T
E416D, L465S
E416G, C441R
L417Q, A414V
L417Q, V403M
T418A
T418N, M409V
N419T, M409T
N419Y, F429L
H420P
L422Q, K415N
E424G
E424G, I425V
I425T, D433N
F429V
F429S
F429L, I376V
F429L, S400P
F429L, N378Y
F429L, N419Y
R432S, K390E
D433N, I425T
V437M
V437L, E445G
L438P
C441G, V391A
C441G, S400P

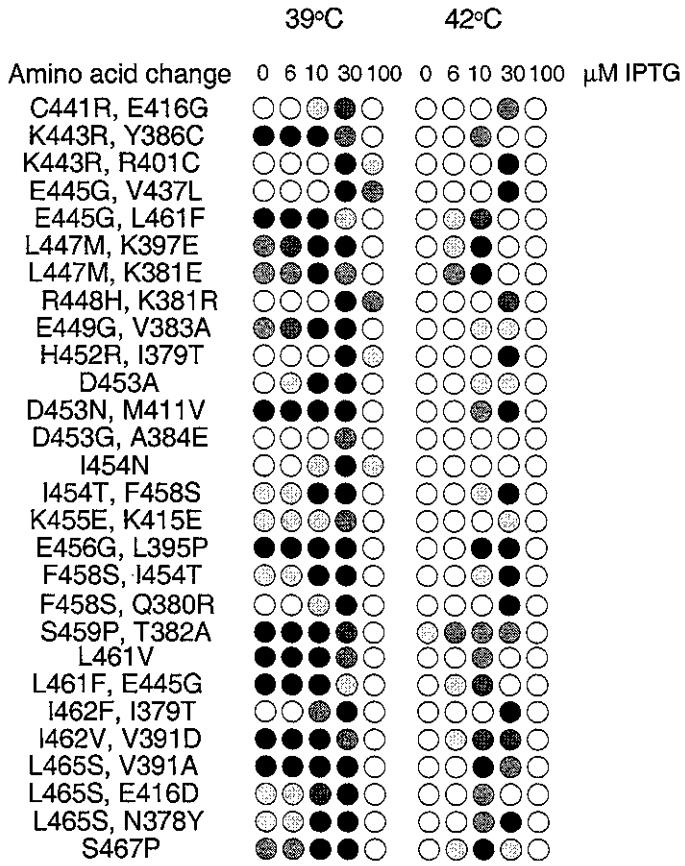


Fig. 3.9. Complementation of high-temperature sensitive *dnaA46* phenotype by IPTG induction of plasmid born mutant *dnaA* alleles. The strains were diluted and plated on LB plates supplemented with different amounts of IPTG and incubated at 30°C (data not shown), 39°C and 42°C. The following day growth was determined visually by counting the number and measuring the sizes of the colonies. Following symbols are used to describe the growth of the various strains:

● : good growth

◍ : slightly reduced growth

◍ : reduced growth

◌ : severely reduced growth

○ : no growth

First and last part of the DnaA protein may not be directly involved in DNA binding

The result of the complementation analysis may seem rather complex (Fig. 3.9) and generally, there does not seem to exist areas of the DNA binding domain that are not, if not directly then indirectly, involved in the DNA interaction. There are, however, amino acids scattered over the domain that probably are not in direct contact with the DNA. This is based on the observation that certain amino acids can be substituted with non-closely related amino acid(s) apparently without affecting the DnaA activity. These amino acids are: I376, K381, Y386, K388, K390, V391, L395, K397, M411, T418, E456, F458, L461, L465, S467 (Fig. 3.9 and 3.10).

Fig. 3.10. Amino acids that can be substituted with non-closely related amino acid(s) may not be in direct contact with the DnaA box (*). See text for explanations. The horizontal bars represent α-helixes. Only the amino acid sequence of domain IV is shown.

The first half of domain IV and the very C-terminal region seems to contain more amino acids that may not be in direct contact with the DNA, compared to helix 3 and 4 and the loop in between. The relatively high number of amino acids in helix 5 that may not be in direct contact with the DNA is consistent with the observation that this area mainly consists of not conserved amino acids (Fig. 3.2).

Neutral mutations

The PAM250 matrix was used to define neutral amino acid substitutions. The matrix is calculated from a protein evolution model that involves amino acid frequencies and pair wise substitution frequencies observed in existing alignments of proteins. A match involving a rarely occurring amino acid counts more than a match involving a common amino acid does, and if two different amino acid sequences are aligned, a mismatch between two easily exchangeable amino acids gives a higher score than a mismatch between two unrelated amino acid. A neutral substitution was defined to be a change that counts 50% of that particular amino acid, and that the score goes 'both ways'. In addition, further restrictions concerning charge and size were included. The following amino acid changes has been regarded as neutral: Arg ↔ Lys, Asp ↔ Glu, Gln ↔ His, Ile ↔ Val, Leu ↔ Met, Phe ↔ Tyr, Ser → Thr. Unfortunately, this PAM250 matrix

was not consulted in the beginning of this work, and therefore, the A→V substitution has been considered neutral as well (and, as it turned out, in many cases it seems acceptable. See below).

The effect of amino acid changes in the DNA binding domain on DnaA activity

The effects of the various amino acid substitutions constructed in this work on the activity of the DnaA protein is discussed in the following sections. Activity is based on complementation analysis of the high-temperature sensitive *dnaA46* strain shown in Fig. 3.9. In some cases, the result of the complementation analysis is rather difficult to interpret due to the presence of double mutations, and it is not easy to draw much information. Therefore, focus is put on those amino acid changes where the effect on the DnaA protein is more clear. The secondary structure was predicted for all DnaA proteins (data not shown).

The classical dnaA mutants

The well conserved **V383** is substituted with a hydrophobic methionine in the DnaA205 protein. This change seems to affect the DnaA function severely, and the high-temperature sensitive *dnaA46* phenotype was only complemented weakly. The DnaA205 protein is unstable at higher temperatures (see Table 3.10), and this may partly explain the weak complementation. On the other hand, Hansen *et al.* (1992) showed that the presence of multicopy *dnaA205* alleles (under *dnaA* promoter control) resulted in temperature resistant growth. Thus, it would be expected that at a certain IPTG concentration, good complementation was observed. The reason why that is not observed may be that the correct IPTG concentration was not used and therefore, the DnaA205 concentration in the cell never equalled that in the multicopy *dnaA205* strain used by Hansen *et al.* (1992). The V383 can be substituted with the also very hydrophobic, but smaller alanine without affecting the DnaA protein much. Since they are all hydrophobic, and none of the amino acid substitution seems to change the secondary structure (data not shown), the results indicate that at this position it is important, for steric reasons, that the amino acid is not larger than valine. **I389** is substituted with asparagine in the DnaA204 protein, and that amino acid change seem very deleterious, whereas when it is substituted with threonine, the function of the DnaA protein is unaffected. The I389N substitution may result in a longer helix 1 (data not shown), which of course can have an effect on the DnaA protein. The isoleucine is very hydrophobic, whereas both the threonine and asparagine are neutral, but can act as

a donor and/or acceptor in hydrogen bonding. Thus, the negative effect of asparagine compared to threonine may lie in differences in the functional groups of the two amino acid and/or differences in the molecular structures. As with the multicopy *dnaA205* (above) and *dnaA211* strains (below), a certain IPTG concentration would be expected to result in good complementation of the *dnaA46* phenotype by the DnaA204 protein, since a multicopy *dnaA204* strain with the *dnaA* allele under P_{dnaA} control is temperature resistant (Hansen *et al.*, 1992). A **M411T** substitution is found in the DnaA211 protein. As with the two other classical *dnaA* mutants, the DnaA211 protein complements the *dnaA46* phenotype rather weakly, although better than DnaA204 and DnaA205 proteins do. When M411 is substituted with isoleucine, the DnaA protein is not affected severely, which is consistent with the nature of the amino acids; methionine and isoleucine are hydrophobic whereas threonine has a more hydrophilic character. The results indicate that at this position a strictly hydrophobic amino acid is required and even the presence of a hydroxyl group disrupts the structure. A large area of helix 2, in which the M411 lies, is predicted to be protected from the solvent (Fig. 3.3 and 3.8), which is in agreement with the results in this work.

Helix 1 (D377 → Y386/L395)

The well conserved **T375** can apparently not be substituted with alanine without affecting the DnaA protein severely. The protein only complements the *dnaA46* phenotype weakly. Threonine can, in contrast to alanine, act as donor and acceptor in hydrogen bonding, and this ability may be important at this position. The nabour, **I376**, can, however, be substituted with the non-closely related threonine without affecting the DnaA protein. Thus, T375 cannot be substituted with a hydrophobic amino acid, whereas its hydrophobic neighbour I376 can be substituted with a threonine. This does seem a little obscure, but may indicate that the structure of the DNA binding domain is very sensitive towards certain amino acid changes. None of the amino acid substitutions discussed above seem to change the secondary structure of the binding domain (data not shown). **N378** is substituted with aspartic acid and histidine, but both substitutions co-exist with other substitutions, which make the N378 substitutions difficult to interpret. The **I379T** substitution probably changes the secondary structure of the DnaA protein by shortening helix 1 in the N-terminal (the helix is predicted to start at T382 in the DnaA(I379T) protein, data not shown), and this may be responsible for the effect the substitution has on the DnaA protein. However, the substitution of the highly conserved isoleucine could be deleterious in itself. The accessibility predictions indicate that I379

is protected from the solvent (Fig. 3.3 and 3.8), and thus, a change into threonine would be expected to have an effect on the existing hydrophobic van der Waals interactions between I379 and other hydrophobic amino acids, and thereby change the structure of the protein. The **Q380R** substitution co-exists with I389T (and F458S), a substitution that apparently has no effect on the DnaA protein, although it may extend helix 1 (see *classical mutations*). Thus, the Q380R change, which probably has no effect on the secondary structure, is responsible for the reduced DnaA function observed with the DnaA(Q380R, I389T) protein. Glutamine is a polar, but neutral residue, whereas arginine is strongly basic and thus, always positively charged. This positive charge together with the multiple hydrogen bonds donating capacity makes the arginine a surface amino acid. However, Q380 is not predicted to be located on the surface of the DnaA protein (the probability for it being exposed or buried is zero, Fig. 3.3), and if the amino acid is located inside the protein, a substitution with arginine would be expected to have dramatic effect on the structure of the protein. **T382** is probably not located on the surface of the protein either, because it can be substituted with alanine without any apparent major changes of the DnaA function, or the secondary structure (the T382A substitution co-exists with S459P). This is consistent with the observation that T382 is not conserved and often substituted with hydrophobic amino acids in other organisms (Fig. 3.1). On the contrary, **K381** may very likely be interacting with water, and thereby located on the surface, since it can be substituted with glutamic acid without any apparent effect on either secondary structure or DnaA function. The K381 is located n+4 amino acids away from the predicted exposed D377 and E385, which places the hydrophilic amino acids on the same side of the α-helix. The **A384V** substitution co-exists with the harmless Y386H substitution (see below). Substitution of the well-conserved alanine has an effect on the protein. Depending on the prediction server (Fig. 3.4), the secondary structure may be changed as follows: helix 1 is divided into two smaller α-helixes, the first cover the area T382→A385 and the second the area K388→L395. It does, however, not seem to be the changes in the secondary structure that is responsible for the effect of the amino acid substitution, since the A384E substitution probably does not change the secondary structure, but has a even more deleterious effect on the DnaA function. It seems more likely that at this position, a small hydrophobic amino acid is required. This is consistent with the accessibility predictions, which predicts that A384 is buried (Fig. 3.3 and 3.8). As mentioned above, the **Y386H** substitution has no effect on the DnaA function, although it may interrupt helix 1 with a small stretch of coil consisting of 2-3 amino acids around the substitution,

like the A384V substitution (and the Y386N substitution). Y386 is conserved, but other residues are seen at this position (mainly histidine, but also glutamine, serine, lysine and glutamic acid, Fig. 3.1). A substitution with arginine has an effect on the DnaA protein. Both histidine and arginine can act as donor and acceptor in hydrogen bonding, but the orientation is of course not identical. This may indicate that Y386 participates in hydrogen bonding and that histidine, but not arginine, has the correct orientations of the atoms. However, Y386 can also be substituted with phenylalanine without any severe effects, and although phenylalanine may have weakly polar properties, it cannot be considered as having hydrogen bonding capacity. **Y387** is a highly conserved amino acid. It can, however, as Y386 be substituted with histidine without any apparent effect on the DnaA function (although helix 1 may be interrupted from K388 to V391), whereas substitutions with cysteine, serine or arginine reduce the activity (substitutions with arginine may interrupt the helix from Y386 to A392. This amino acid substitution co-exists with K397E, a substitution that apparently does not reduce the DnaA activity, see below). They can all act as donor and acceptor in hydrogen bonding, but as discussed above, tyrosine and histidine are somewhat similar in shape which may be required for a correct orientation and angle of the hydrogen bonds between tyrosine (or histidine) and another residue. The tyrosine is predicted to be buried, although with low reliability, which indicate that the hydrogen bonding is between two residues and not between tyrosine and water. This is also consistent with the apparent sensitivity towards substitutions with other amino acids with hydrogen bonding capacity (Cys, Ser and Arg), because in contrast to other amino acid residues, water molecules may adapt new orientations more easily. **V391A** (well conserved) and **A392E** (not conserved) may also interrupt helix 1, but as the other helix interrupters, these DnaA proteins complement the *dnaA46* phenotype almost as well as the $DnaA^+$ protein does (the A392E substitution co-exists with M411V. The latter substitution may not have any effect on the DnaA protein, since the M411I substitution, which exists as single mutant, does not). The V391D and A392T substitutions very likely reduce the size of helix 1 by several amino acids (the helix stops at Y386). This does not seem to have any severe effect on the DnaA protein either (the A392T change co-exists with M409I, an amino acid change with a relatively high score according to the PAM250 matrix, and thus, is likely not to have severe effect on the DnaA protein). The A392 is not conserved among different organisms, and often substituted with hydrophilic amino acids, which is consistent with the results of the complementation analysis.

Summary of α-helix 1: According to the alignment and secondary structure predictions of the DNA binding domain of DnaA proteins from various organisms (Fig. 3.4), it seems quite conspicuous that the length of helix 1 is rather well conserved in spite of the fact that this area does have dissimilarities in amino acid sequence. If the helix goes all the way to L395, as predicted by the CBS server, there are a number of amino acid changes, constructed in this work, that interrupt the helix without any apparent effect on the DnaA activity. This seems contradicting with fact that the length of helix 1 is well conserved, and indicates that the first helix actually does stop around Y386 as predicted by the PHD server, and thus, the many amino acid substitutions that appeared to interrupt helix 1, actually do not. Instead, they are located in the following loop area. The amino acid changes that may shorten the helix N-terminally (I379T and A384V) or extend the helix (I389N, DnaA204) do indeed seem to affect the DnaA activity.

Helix 1 may be located on the surface of the DnaA protein, with D377, K381 and E385, which are located on the same side of the α-helix, exposed to the solvent, and the rest, especially I379, V383 and A384 pointing away from the solvent and probably participating in hydrophobic interactions with other residues of the DnaA protein, maybe in a leucine-zipper fashion (Chapter 2). Except for I376, K381 and Y386 it does not seem possible on basis of this complementation analysis to point at amino acids in helix 1 that are more likely to be interacting with the DNA target than others. On the other hand, this area of the DnaA protein does seem sensitive towards amino acid changes, in general, although the secondary structure is only rarely changed. This indicates that the amino acids in the helix, and not only the helix itself, help stabilise the DnaA structure and/or are responsible for DNA contact.

Loop I, the basic loop (Y387 → R405)

Generally, amino acid changes in especially the first part of this area of the DnaA protein seem, at least at first glance, to have minor effect on the function of the protein, since many of the DnaA proteins complement the *dnaA46* phenotype as well as the $DnaA^+$ protein does. The DnaA(**K388Q**) protein complements the *dnaA46* phenotype as well as the $DnaA^+$ protein does. Thus, it seems that the positive charge of lysine is of less importance, whereas the ability to participate in hydrogen bonding may be more important. K388 is not very conserved; some DnaA proteins have asparagine at this position, which is consistent with the observation that glutamine is a non-harmful substitution. Yet, other DnaA proteins have a glycine at this position. Glycine and asparagine are often located in turns, and may therefore be considered as helix breakers.

Maybe the most important function of the first amino acids following helix 1 is to ensure that the helix does not continue. The conserved **K390** is probably located on the surface of the protein, since a substitution with glutamic acid does not affect the protein whereas a substitution with isoleucine reduces the DnaA function. This 'surface hypothesis' is consistent with the observation that the **K388E** (see above), **K397E** and **R398H** substitutions do not seem to affect the DnaA protein (the R398H co-exists with two other substitutions. See Table 3.2 (pMN753): the triple mutant was isolated on 10 μM IPTG). None of the amino acid substitutions seem to change the secondary structure (data not shown). They are not highly conserved, and several organisms have other hydrophilic amino acids at these positions (Fig. 3.1). The hydrophobic amino acids **V391** and **A392** can be substituted with other amino acids, either with hydrophobic or hydrophilic character without any major effects on the DnaA protein (and without any apparent changes in the secondary structure). The alanine is not very conserved and DnaA proteins from other organisms have serine, glutamic acid, glutamine, lysine or asparagine at this position. This is consistent with the observation that both the A392T and A392E substitutions have no effect on the DnaA protein. The V391 is not well conserved *per se*, but in other organisms substituted with isoleucine (closely related) or leucine (related), and only very few organisms have glutamine, arginine or histidine at this position. Therefore, the V391A substitution (co-exists with the non-harmful L465S substitution, see below) would be expected to have maybe only minor effect on the DnaA protein, whereas the V391D substitution (co-exist with I462V, a neutral substitution) would be expected to have some effect. That is also observed. Thus, V391 may be protected from the solvent in agreement with the accessibility prediction (Fig. 3.3). The **L395P** substitution may introduce a very short stretch of β-sheet like structure around K390 (data not shaown). However, this does not seem to have any effect on the DnaA protein, since the DnaA(L395P) protein complements the *dnaA46* phenotype as well as the $DnaA^+$ protein does. This is consistent with the observation that secondary structure predictions of DnaA protein from different organisms indicate that some of them may have a small area of β-sheet like structure between helix 1 and 2 (Fig. 3.4). The highly conserved **R399** cannot be substituted with glutamine without affecting the DnaA protein (the R399Q exists as double mutant with L395P, a substitution without any effect on the protein, see above), indicating that the the arginine has other functions than to be interacting with water. The substitution does not seem to change the secondary structure of the area (data not shown). Thus, at this position, a positively charged arginine seems to be required for full DnaA activity. This also seems true for

the highly conserved **R401**. Although the effect seems smaller, this amino acid cannot be substituted with cysteine without affecting the DnaA activity. The amino acid substitution does not seem to change the secondary structure of the DnaA protein (data not shown). A substitution with cysteine may, however, not be informative when considering the function of the arginine in the basic loop due to the non-closely related natures of arginine and cysteine. Unfortunately, no other mutants with R399 nor R401 substitutions were isolated. Bläsing (1999) substituted these arginines with alanines, and found that especially the R399A substitution had severe effect on the DnaA protein, although neither substitutions destroyed the DnaA activity completely. The **S400T** substitution affects the DnaA protein (the substitution co-exists with I379T, a substitution that does not affect the function of the DnaA protein, see *helix1*, above). This seems obscure, since other organisms have the closely related threonine at this position. The substitution does not seem to change the secondary structure (neither does the S400P substitution, data not shown). The DnaA(**S402L**) protein complements the *dnaA46* phenotype with 100 μM IPTG as one of the few. The substitution probably does not alter the secondary structure of the protein (data not shown). In addition, the serine is not very conserved and some organisms have alanine or valine at this position. Thus, the apparant deleterious effect of the S402L substitution may not be due to reduced *oriC* affinity *per se*, but more likely due to an unstable DnaA(S402L) protein. This was, however, not further investigated.

Summary of loop I (basic loop): Except for the L395P substitution, none of the amino acid changes seem to change the secondary structure of the DnaA protein. The basic amino acids K388, K390 and K397 can all be substituted with the oppositely charged glutamic acid, indicating that the function of these amino acids are to interact with the solvent and thereby keep the loop on the surface of the protein. In this work, R398 was substituted with histidine without any effect on the DnaA protein. Other organisms have serine at this position, indicating that the R398, like the lysines discussed above, is required for solvent interactions. The highly conserved R399 and R401 may have other functions than to interact with the solvent. The R399Q substitution has severe effect on the protein. Unfortunately, it is not possible to determine whether these basic amino acids participate in the DNA interaction due to the few amino acid substitutions at these positions. Bläsing (1999) constructed and analysed DnaA proteins with R399A and R401A substitutions. These amino acid substitutions clearly have an effect on the protein, although they are, especially the DnaA(R401A), able to complement the *dnaA46* phenotype. In general, the solvent accessibilities are predicted with low

reliability, but there seems to be more exposed than buried amino acids, indicating that this loop is located on the surface of the protein.

Helix 2 (P406 →E416)

Helix 2 probably starts with the not so well conserved **P406**. A substitution of this amino acid with leucine has some effect on the DnaA protein, although it was possible to obtain good growth on 30 μM IPTG plates. The P→L substitution may extend helix 2 resulting in helix start at S402. It does not, however, seem to be the likely reason for the effect on the DnaA protein since helix 2 in DnaA proteins from other organisms may start at that position (Fig. 3.4). In this work, the highly conserved amino acids **R407** and **Q408** were not substituted in any of the isolated *dnaA* mutants. This could indicate that substitutions of these amino acids have a severe effect on the DnaA protein. However, Bläsing (1999) isolated several R407 substitutions (R407H, R407A, R407C) and a single Q408 substitution (Q408R). Although they all (except R407A) seem to have severe effect on the DnaA activity, the proteins are still functional to some extend (except the DnaA(R407H) protein, which did not complement the *dnaA46* phenotype at all. This is unexpected considering the related characteristics of Arg and His),. **M409** can be substituted with isoleucine and valine without any effects on the DnaA protein. This is consistent with the observation that other organisms have these amino acids at this position. The highly conserved **A410** cannot be substituted with neither valine nor threonine without affecting the DnaA function severely. These amino acid substitutions do not seem to change the secondary structure (data not shown), which indicates that at this position a small hydrophobic amino acid is required. The highly conserved **M411** can be substituted with the related amino acid isoleucine without any remarkable effect, whereas a change into threonine (DnaA211) has a much more severe effect (see *classical dnaA mutants*). Like the A410 substitutions, the M411 substitutions probably do not change the secondary structure of the DnaA protein (data not shown). Thus, at this position a hydrophobic amino acid also seems to be required. Surprisingly, the DnaA(**L413Q**) protein complements the *dnaA46* phenotype relatively well (although 30 μM IPTG is required). The highly conserved leucine in helix 2 is predicted to be protected from the solvent (Fig. 3.3 and 3.8), and therefore, substitution with glutamine could be expected to have a more severe effect on the DnaA function than observed. The double mutants DnaA(**A414V**, **L417Q**) and DnaA(**E416G**, C441R) may have a shortened helix that stops at K415 (and not E416), which may account for the reduced DnaA activity. However, it may seem wrong to attach much weight into a single amino

acid difference, but when considering the secondary structure from other organisms (see Fig. 3.4) it is obvious that the helix start position may differ among the different organisms, but it stop at a position corresponding to the *E.coli* E416. The DnaA(**K415E**) protein complements the *dnaA46* phenotype rather well with 30 μM IPTG, whereas the DnaA(**K415I**) does not. None of the amino acid substitutions seem to change the secondary structure of the protein (data not shown), which indicates that a charged amino acid is preferred at this position. Whether it is differences in charges or simply the substitution of the highly conserved lysine that is responsible for the after all reduced activity of DnaA(K415E) cannot be answered, but the results indicate that the lysine has functions other than to be interacting with water.

Summary of α-helix 2: According to the accessibility prediction, many amino acids in helix 2 are protected from the solvent, indicating that a part of this helix is located inside the protein with other hydrophobic amino acids in the surroundings, and very little space around A410. This hypothesis is consistent with the observation that the A410V and M411I substitutions have a less severe effect on the DnaA protein than the A410T and M411T substitutions. DnaA(L413Q) protein complements the dnaA46 phenotype with 30 μM IPTG indicating that the neutral, but polar glutamine somehow fits the presumed hydrophobic core. Bläsing (1999) also isolated the L413Q substitution and found that the protein complemented the *dnaA46* phenotype poorly and only in the presence of 100 μM IPTG.

Generally, the amino acid substitutions in this area of the DnaA protein do not change the secondary structure to large extends indicating that the helix structure can not be changed without damaging the DnaA protein. The length of helix 2 corresponds to between 2.5 and 3.5 α-helical turns among the different organisms (see Fig. 3.4). None of the DnaA proteins seems to have an equal number of helical turns, which may be an important factor in the direction of the following loop. Since helix 2 is likely to be located inside the protein, this helix may not interact directly with the DNA target.

Helix 3 and small loops surrounding it (L417-T435)

The **L417Q** substitution seems to have severe effect on the DnaA protein (the substitution co-exists with V403M, which is expected to have minor effect due to the related character of valine and methionine). The neighbours of the well conserved L417 are hydrophilic, but never the less a hydrophobic amino acid seems to be required at position 417. **T418** is highly conserved among DnaA proteins from different organisms

(Fig. 3.1 and 3.2). Therefore, the observation that a substitution with alanine has no apparant effect on the DnaA protein seems contradicting. The reason could be that helix 2 is predicted to be extended to N419 in the DnaA(T418A) protein (data not shown). On the contrary, the non-conserved **H420** cannot be substituted with proline without affecting the DnaA protein severely. The substitution may not change the secondary structure (data not shown) although the probability that helix 3 exists is reduced (see below), and other organisms have either negatively changed or hydrophobic amino acids at this position, which makes it difficult to understand why the H420P substitution is harmfull. The *dnaA46*/p*dnaA*(H420P) strain forms colonies on 100 μM IPTG plates. This could indicate that the DnaA(H420P) protein is unstable, and explain why it apparently complements the heat sensitive phenotype weakly. The highly conserved **S421** is not substituted in any of the isolated mutants constructed in this work. However, Bläsing (1999) isolated both a DnaA(S421G) and DnaA(S421N) version of the DnaA protein and they both complemented the *dnaA46* phenotype well.

The probability that helix 3 exists is reduced in the DnaA(**L422Q**, K415E) protein with no apparent consequences. The DnaA(**E424G**) protein may also lack helix 3 (with high reliability) and this may account for the apparent very severe effect the amino acid substitution has on the DnaA protein. The **I425** and **G426** amino acids are both highly conserved among different organisms (see Fig. 3.1 and 3.2). Never the less, I425 can be substituted with threonine and still complement the *dnaA46* phenotype well with 30 μM IPTG (the I425T co-exists with D433N, which is expected not to have negative effects on the DnaA protein. This assumption is based on observations done by Bläsing (1999), who found that the DnaA(D433G) and DnaA(D433A) proteins complemented the *dnaA46* phenotype even better than the $DnaA^+$ protein did). The highly conserved **G426** is not substituted in any of the mutants isolated in this work or by Franca Bläsing. This could indicate that a glycine is required at this position, maybe as a α-helix breaker. However, the G426 is substituted with serine in the DnaA5 protein (the G426S substitution co-exists with the A184V substitution in the DnaA5 protein). This has some effect *in vitro* (see Chapter 4), but multicopy P_{dnaA}-*dnaA*(G426S) strains are temperature resistant like multicopy P_{dnaA}-$dnaA^+$ strains, and not cold sensitive like the multicopy P_{dnaA}-*dnaA5* and P_{dnaA}-*dnaA*(A184V) strains (Nyborg *et al.*, 2000). The secondary structure is not affected by the G426S substitution, and thus, if the function of G426 is to break the helix, a serine seems to have the same effect. The **F429** is the first amino acid in a highly conserved area of the DnaA protein that covers amino acids from F429 to V437. In this work, many different F429 substitutions were isolated, both as single-

and double mutants. They all have an effect on the DnaA protein. Phenylalanine may contribute to the tertiary structure by stacking with other amino acids (tyrosine or tryptophane). The highly conserved **G430** and **G431** are not substituted neither in this work nor in wotk done by Bläsing (1999). This indicates that these glycines are important for DnaA function, maybe because they induce a turn of the coiled structure. **R432** is substituted with leucine in the cold sensitive *dnaX* suppressor DnaA721 protein (Walker mutant). In this work, it is substituted with serine, which also seems to affect the activity of the DnaA protein. Whether the protein is cold sensitive was not further investigated. The highly conserved **D433** can be substituted with non-closely related amino acid with no effect (or if any, then positive) on the DnaA protein (Bläsing, 1999). It was not possible in this work to isolate any substitutions of the highly conserved **H434** or **T435**. However, Bläsing (1999) isolated H434Y, H434A and T435P substitutions, and although the complementation ability is reduced, it is far from abolished. T435 is substituted with lysine in the second Walker mutant *dnaA71*. This mutant is cold sensitive like *dnaA721*. In addition, Sutton and Kaguni have analysed the DnaA(T425M) protein in vitro, and they found that T435 may participate in the specific recognition of DnaA boxes (see Section II, and Discussion)

Summary of α-helix 3 and loops surrounding it: It was not possible in this work to substitute many of the highly conserved amino acids, indicating that they cannot be substituted without deleterious effects on the DnaA protein. The function and importance of helix 3 is difficult to interpret. Some amino acid substitutions (E424G and maybe H420P) indicate that the helix cannot be removed without severely affecting the DnaA protein, yet another substitution (R422Q) does not seem to have any effect on the DnaA protein, although the protein may lack helix 3 (however with lower reliability than the E424G substitution, data not shown). The presence of highly conserved G430 and G431 that were not substituted neither in this work nor in Bläsing's (1999) may indicate that DnaA activity depends on helix 3, that on the other hand must be broken and followed by a turn before the next helix starts. As discussed in Chapter 2, this area could be part of a modified helix-turn-helix motif . The results of the complementation analysis do not argue against this proposal.

α-Helix 4 and 5 (T436 → T467)

The highly conserved **V437** is substituted with the related methionine, and that has some effect on the DnaA protein although good complementation was obtained with 30 μM IPTG. V437 is predicted to be buried, and a substitution with methionine may

disrupt the hydrophobic interactions with other residues. The DnaA(**L438P**) protein does not complement the *dnaA46* phenotype at 42°C, which may be due to the predicted reduction of the length of the helix that starts at A440 and not T436. However, the length helix 4 differs among DnaA proteins from different organisms (Fig. 3.4). L438 is predicted to be protected from the solvent, but proline is also hydrophobic so this is probably not the reason for the deleterious effect of the substitution. The well conserved **H439** and **A440** are not substituted in any of the isolated mutants. The A440 is predicted to be protected form the solvent. The **C441G** substitution require 30 μM IPTG in order to complement the heat sensitive *dnaA46* phenotype (the substitution co-exists with V391A, which has no apparent effect on the DnaA protein). The secondary structure may be influenced rather dramatically (data not shown): a stretch of β-sheet like structure may be substituting the first part of the α-helix, which is followed by a loop, allowing the α-helix to start at K443. C441 is not very conserved. However, none of the other organisms has glycine at this position. The L438P substitution (see above) has a more severe effect on the DnaA protein than the C441G substitution has, indicating that the small changes in the secondary structure caused by the L438P substitution are not alone responsible for the deleterious effect on the DnaA protein. The well conserved **R442** and the highly conserved **K443** and **I444** are not substituted with any non-related amino acids, which makes the function of the amino acids difficult to interpret. It does however indicate that amino acids with special characteristics are required at these positions. I444 is predicted to be buried (Fig. 3.3 and 3.8). The **E445G** substitution co-existing with L461F (see below) complements the *dnaA46* phenotype well, and that is in spite of the observation that the E445G substitution may extend the small loop between helix 4 and 5 with seven amino acids from I444 → D453 (data not shown). **Q446** is not substituted in any of the analysed DnaA proteins, which is obscure because this glutamine is not conserved among different organisms (Fig. 3.1). The highly conserved **L447** is substituted with the closely related methionine, and this substitution has no effect on the DnaA protein. The **R448H** substitution probably does not change the secondary structure (data not shown), but it has an effect on the DnaA protein. Since the R→H substitution is an exchange of one polar amino acid to another, the results indicate that the size of the amino acid and the orientation of the positive charge may be important at this position. The **E449G** substitution seems to extend the loop between helix 4 and 5, with five amino acids (Q446 → D453). This could be the reason why this substitution has severe effect on the DnaA protein. The **D453G**

substitution may also extend the loop between helix 4 and 5 by three amino acids (E450 → K455), but in contrast to the E449G substitution, the D453G substitution has severe effect on the DnaA activity (the D453G substitution co-exists with A384E. The latter also exists as single mutant, and that amino acid change has some effect on the DnaA protein (reduced growth 30 μM). However, when the D453G change is added to A384E, the effect on the DnaA protein is much more deleterious, Fig. 3.9). When **D453** is substituted with alanine the loop between helix 4 and 5 may disappear, and this seems to have just as deleterious effect on the DnaA protein as when the loop is extended (see above). However, the reason for the apparent damaging effect of D453A could also be that a charged amino acid is required at this position, since this amino acid is predicted to be exposed to the solvent. When D453 is substituted with asparagine, the activity of the DnaA protein is close to that of the $DnaA^+$ protein. This substitution probably does not change the secondary structure of the DnaA protein. The intact secondary structure in addition to an exchange with a somewhat related amino acid is very likely the reason why the DnaA(D453N) protein is a fully functional protein. The **I454T** substitution also affect the DnaA activity, and the *dnaA46* transformant requires 30 μM IPTG in order to form colonies at 42°C (the substitution co-exists with F458S that probably does not affect the protein). The DnaA(I454T) protein may have an extended loop (E450 → K455) between helix 4 and 5 (data not shown), which could be the reason for the reduced activity of DnaA(I454T). *Synechocystis sp.* has threonine at this position (the secondary structure is not changed, probably due to the general different primary structure of this DnaA protein, Fig. 3.4), indicating that it is not the presence of threonine at this position that affects the DnaA protein *per se*, but the changes in the secondary structure. When I454 is substituted with asparagine, a substitution that apparently has no effect on the secondary structure (data not shown), no complementation at 42°C was observed. This strongly suggests that a hydrophobic amino acid is required at this position. This is consistent with the observation that other organisms have hydrophobic amino acids at this position (Fig. 3.1), and the residue is therefore predicted to be protected from the solvent (Fig. 3.3 and 3.8). The **K455E** substitution co-exists with K415E. The latter substitution, which also exists as a single mutant, has an effect on the DnaA protein, but good complementation was obtained with 30 μM IPTG. As discussed in *'helix 2'* (see above) this could indicate that a positively charged amino acid is preferred at this position. When the K445E substitution is added to the K415E substitution, almost no complementation was observed. This

strongly suggests that K455, like K415, is required at this position for other reasons than to be involved in solvent interactions. However, the function of the positively charge of K445 becomes unclear when considering its conservation among different organisms; tyrosine, glutamine, serine and asparagine at this position seem to result in functional DnaA proteins (Fig. 3.1). These amino acids are all neutral, but polar to some extent, and able to participate in hydrogen bonding. So far, several amino acid substitutions indicate that the small loop between helix 4 and 5 is of great importance - not only is it important that it exists, but the length of it also seems to be critical. Therefore, it is rather contradicting when the E445G (see above) and **E456G** substitutions are predicted to have an extended loop between helix 4 and 5 (data not shown) without any apparent effect on the activity of the DnaA protein. Generally, amino acids from **S459** to **S467** of the DnaA protein can be substituted with non-closely related amino acids without any severe effects on the DnaA protein. This seems consistent with the observation that this area is less conserved among different organisms (except for L461, Fig. 3.1 and 3.2). The **L465S** and **S467P** substitutions may shorten helix 5 slightly without any apparent effect on DnaA activity. In this work, the highly conserved **L461** is substituted with the hydrophobic amino acids valine and phenylalanine, which are all somehow related. Bläsing (1999) isolated a DnaA(L461S) version, and found that this protein did not complement the *dnaA46* phenotype at all, indicating that a strictly hydrophobic amino acid is required at this position. Accordingly, L461 is predicted to be buried with high probability.

Summary of α-helix 4 and 5: In general, this area of the DNA binding domain of the DnaA protein includes few single substitution and many double substitution, and this makes it relatively difficult to interpret the results. However, there seems to be a tendency that the presence of a coiled structure between helix 4 and 5 is important, and that the length of it is critical. Helix 4 is more sensitive towards amino acid substitutions than helix 5, which is in agreement with how conserved the regions are. Those amino acids that are predicted to be protected form the solvent with high reliability cannot be substituted with polar amino acids without affecting the DnaA protein. These amino acids include V437, A440, I444, I454, F458 and L461. The hydrophobic amino acids are located on the same side of the α-helix (or close to). The two hydrophobic amino acids I454 and F458 are substituted with threonine and serine, respectively, in one of the double mutants. This DnaA(I454T, F458S) protein complements the *dnaA46* phenotype better than DnaA(I454N) and DnaA(F458S, Q380R), which indicates that there could be (weak) interactions between I454 and F458 that is maintained to some extend in the

double mutant where both amino acids are substituted with polar, hydrogen bonding residues. Helix 4 and 5 are probably located on the surface of the DnaA protein with a hydrophobic and hydrophilic side and a hydrophilic coil in between. Helix 4 could be responsible for DNA contact and as mentioned previously be part of a modified helix-turn-helix motif.

The complementation analysis discussed above was carried out in a *dnaA46* strain. The DnaA46 protein binds *oriC* at 42°C (Hwang and Kaguni, 1988; Carr and Kaguni, 1996), and therefore, some of the DnaA proteins constructed in this work might be able to complement the heat sensitive phenotype due to 'help' from the DnaA46 proteins in the cell (maybe especially at 39°C). A number of pMN242 derivatives carrying mutant *dnaA* alleles were therefore transformed into an isogenic *dnaA204* strain (MN987). A detailed complementation analysis was not carried out. Instead, the transformation mix was plated on 10 and 30 μM IPTG plates and incubated at 30 and 42°C. The results of this analysis were identical in the *dnaA46* and *dnaA204* strain backgrounds (data not shown).

Verification of the dnaA allele on the chromosome

Presence of *dnaA46* on the chromosome of the MN247 transformants was verified to rule out the possibility that the *dnaA* genes on the multicopy plasmids had recombined with the one on the chromosome. This was done by PCR amplification of the chromosomal *dnaA* gene with primers that flank the *dnaA46* mutations. The forward primer anneals upstream the *dnaA* structural gene and can therefore anneal to the chromosomal *dnaA* but not to the plasmid *dnaA*. Amplification and subsequent *Sph*I digestion of PCR fragments from the various strains showed that they all carried a *dnaA46* allele on the chromosome (data not shown). Later, experiments indicated that it was difficult (impossible) to recombine the *dnaA46* allele out of the MN247 chromosome even when the strain was under recombination 'pressure' (see Experimental procedures, section III and results, section III): the MN247/pMN242 (p*dnaA*$^+$) was plated and incubated at 42°C without IPTG. This treatment allowed selection of recombinants since the host is high-temperature sensitive. The potential recombinants were further streaked 4-5 times on LB plates without ampicillin to allow growth of plasmid free cells. When the chromosomal *dnaA* gene from ampicillin sensitive and high-temperature resistant colonies was PCR amplified and *Sph*I digested, it turned out that they apparently still had a *dnaA46* allele on the chromosome and

therefore had not recombined with the *dnaA* gene on the plasmid. Thus, the MN247 transformants formed colonies at high temperature due to formation of suppressor mutations that were not further analysed. In the complementation analysis (Fig. 3.9), most of the observed colonies at 39 and 42°C did not carry suppressor mutations, since the number of colonies at permissive and non-permissive temperature was identical. In some cases, a few colonies appeared in the 10^1 dilution spot. The cells in these colonies were probably carrying suppressor mutations and were of course not interpreted as a *dnaA46* complementation by the plasmid born *dnaA* allele.

DnaA amino acid substitutions have effect on stability

So far, the DnaA amino acid substitutions have been analysed with no regard to protein stability. Thus, an apparently weak complementation of the high temperature sensitive *dnaA46* phenotype by a mutant DnaA protein (i.e. low affinity to *oriC*) and low *in vivo* affinity to the DnaA box R5 and R6 in the *mioC* promoter (Section II) could be due to degration of the protein and not reduced affinity *per se*. Therefore, the stability of various proteins was estimated at 32 and 42°C. MN987 (*dnaA204*) strains harbouring plasmid with different *dnaA* alleles were grown at 32°, IPTG was added to induce the *lac* promoter and after app. one hour, chloramphenicol was added to stop protein synthesis. Samples were taken different times after drug addition, and the relative amounts of protein was estimated by Western blotting. Fig. 3.11 shows the degration of the $DnaA^+$, DnaA(K397E) and DnaA(R401C, K443R) protein, and Table 3.10 summarises the results with all tested proteins.

Stability of $DnaA^+$ is independent on temperature as expected. After 4 generations with chloramphenicol, there is app. 60% protein left (Fig. 3.11). The stability of the

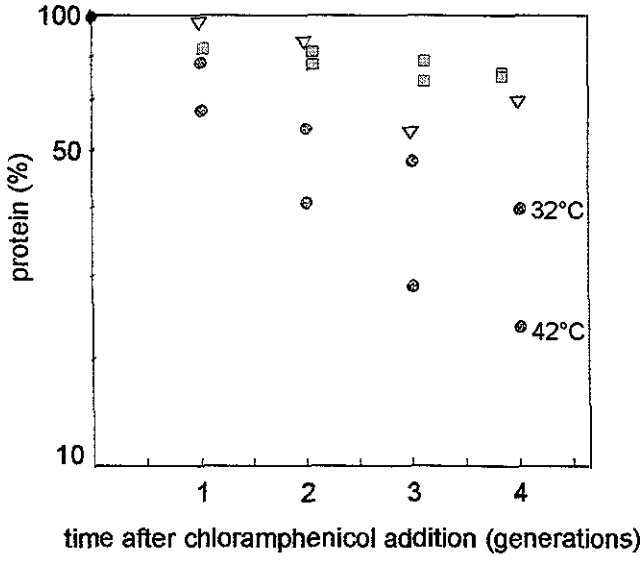


Fig. 3.11. Degration of $DnaA^+$ (white symbols), DnaA(K397E) (grey squares) and DnaA(R401C, K443R) (grey circles). Cells were grown exponentially and IPTG was added. After 1 hour, chloramphenicol was added and the amount of protein measured different times after drug addition. The degration is expressed as percentage protein left compared to t=0 (black circle) as a function of time after drug addition (expressed as generation times)

DnaA(K397E) protein is also independent on the temperature. In addition, this protein seems even more stable than the wild type protein. On the contrary, the DnaA(R401C, K443R) protein is more unstable than the $DnaA^+$ protein, especially at 42°C, where the half-life is 2.8 generations (Fig. 3.11 and Table 3.10).

Table 3.10. Half-lives of wild type and mutant DnaA proteins.

DnaA protein	$T_{½}$ (generations) [1)]	
	32°C	42°C
$DnaA^+$	> 4	> 4
I379T	> 4	> 4
DnaA205	2.2	1.4
A384V,Y386H	> 4	> 4
Y386H	> 4	> 4
Y387S	1.5	1.5
DnaA204	1.9	1.5
L395P	4.0	2.1
K397E	> 4	> 4
R401C, K443R	> 4	2.8
DnaA211	> 4	2.1
T418A	> 4	> 4
E424G	3.25	1.75
F429S	> 4	4.0
V437M	2.4	1.6
L438P	2.2	1.8
C441G, V391A	> 4	3.1
E449G, V383A	2.2	1.8
I454N	> 4	2.0
L465S, V391A	> 4	2.8

1) The half-life ($T_{1/2}$) was determines by adding chloramphenicol to IPTG induced MN987 strains harbouring plasmids carrying various *dnaA* alleles. Samples were taken several times after drug addition (see experimental procedures). $T_{1/2}$ is expressed as generation times.

The DnaA(I379T), DnaA(Y386H), DnaA(T418A), DnaA(K397E), DnaA(A384V, Y386H) and DnaA(F429S) proteins have stabilities comparable to that of the $DnaA^+$

protein (table 3.10). Thus, the reduced complementation of the *dnaA46* phenotype by the DnaA(I379T), DnaA(A384V, Y386H) and DnaA(F429S) proteins seems to be due to actual lowered affinity (or specificity) to *oriC*. The DnaA(Y386H), DnaA(T418A) and DnaA(K397E) protein are also as stable as the $DnaA^+$ protein, which is expected since they complement the *dnaA46* phenotype as well as the wild type protein does (Fig. 3.9). The DnaA(L395P) protein is degraded twice as fast at 42°C than the wild type protein, but this does not seem to affect the overall DnaA concentration in the exponentially growing cell, since the protein complements the *dnaA46* phenotype well. Thus, the stability of the DnaA(L395P) protein may represent the lower limit for protein stability without it causing a significant effect on the DnaA concentration and growth of the cell. Alternatively, the DnaA(L395P) protein has a higher affinity to *oriC* at 42°C than the $DnaA^+$ protein has. The phenotype of the multicopy *dnaA46*/p*dnaA211* strain seems to be due to reduced affinity (or specificity) to *oriC*, and not due to instability of the protein (Table 3.10). On the contrary, the phenotypes of the multicopy *dnaA204*, *dnaA205*, *dnaA*(E424G), *dnaA*(E449G, V383A), *dnaA*(V437M), *dnaA*(Y387S) and *dnaA*(R401C, K443R) strains may partly be because the proteins are more unstable, especially at 42°C.

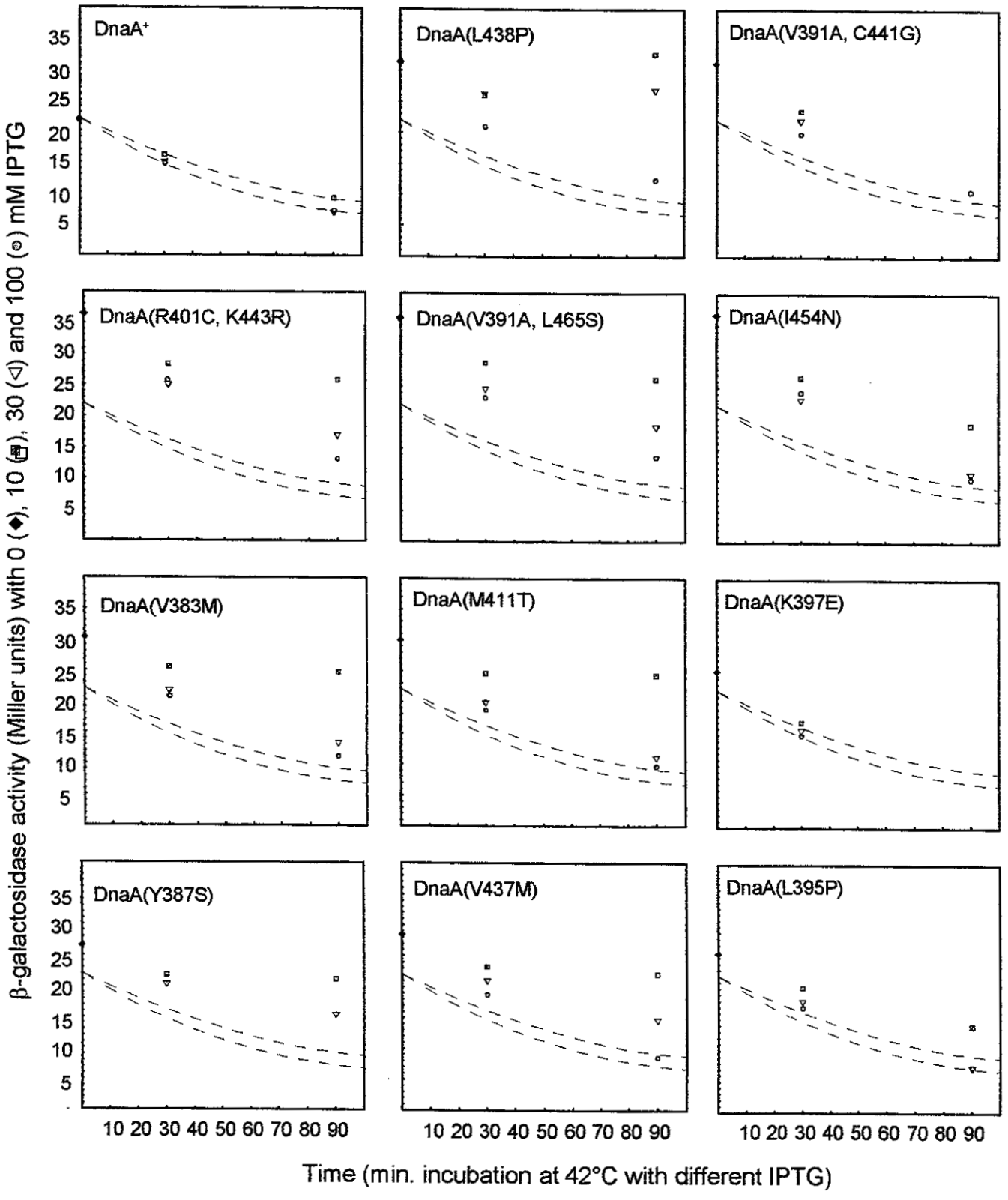
Section II

The DnaA box-DnaA interaction

in vivo affinity to DnaA box R5 and R6 in mioC promoter

The *dnaA46* and *dnaA204* strains used in this work and discussed in section I have a P_{mioC}-*lacZ* fusion in λ attachment site. The *mioC* promoter carries the DnaA boxes R5 and R6 (see Fig. 1.1), which makes it possible to measure the relative affinities of the various DnaA mutant proteins constructed in this work for box R5 and R6 by measuring the β-galactosidase activities under different growth conditions. β-galactosidase assays were carried out several times in the *dnaA46* strain background, but it was not possible to reproduce the results. Therefore, the β-galactosidase assays were carried out in the *dnaA204* strain, in which the results were reproducible. Fig. 3.12 shows the β-galactosidase activities in various MN987 strains harbouring pMN242 (p$dnaA^+$) and pMN242 derivatives under different growth conditions, and Table 3.11 summarises the

results. When no IPTG was added, the MN987/pMN242 (p*dnaA*$^+$) strain had lower β-galactosidase activity than any of the other strains carrying plasmid born mutant *dnaA* alleles. This indicates that the *lac* promoter is leaky and the (small) amount of DnaA$^+$ protein that is synthesised is sufficient to repress the *mioC* promoter 1.5 fold at 42°C, but not sufficient to complement the high temperature sensitive phenotypes of *dnaA46* and *dnaA204* (Section I, Fig. 3.9). Generally, the DnaA proteins analysed in this *in vivo* affinity experiment, retained 50-100% DnaA box affinity at 32°C and 42°C (Table 3.11). Even the DnaA(I454N), DnaA(E424G), DnaA(L438P), DnaA(E449G, V383A), DnaA204 and DnaA205 proteins that appeared to have either severely reduced or absolutely no affinity to *oriC* (Fig. 3.9). Presence of DnaA(Y386H), DnaA(K397E), DnaA(T418A) and DnaA(L395P) proteins resulted in β-galactosidase activities comparable to those of the wild type DnaA protein (Fig. 3.12). This is consistent with the observation that these DnaA proteins complemented the high temperature sensitive *dnaA46* and *dnaA204* phenotypes as well as the DnaA$^+$ protein did (Fig. 3.9). However, they all had a slightly higher activity at 32°C when no IPTG was added, which indicates that the affinity to the DnaA boxes in the *mioC* promoter is somewhat lower than that of the wild type DnaA protein (Table 3.11). The β-galactosidase activities of the rest of the MN987 strains shown in Fig. 3.12 and Table 3.11 had higher activities than the p*dnaA*$^+$ strain at 32°C and 42°C. Cells in which DnaA(I379T), DnaA(Y387S), DnaA(V437M), DnaA(F429S), DnaA(A384V, Y386H) and DnaA(V391A, C441G) proteins were expressed had slightly reduced repression of the *mioC* promoter, which is consistent with the observation that 30 μM IPTG was required for complementation of the heat-sensitive *dnaA46* and *dnaA204* phenotypes. The DnaA(R401C, K443R) and DnaA(V391A, L465S) proteins, on the contrary, had reduced *mioC* promoter repression, and that is in spite of the observation that especially the DnaA(L465S, V391A) protein complemented the *dnaA46* and *dnaA204* phenotypes as well as the DnaA$^+$ protein did (Fig. 3.9). The classical *dnaA* mutant proteins (DnaA204, 205 and 211) also had reduced *mioC* promoter affinity. However, the DnaA204 and DnaA205 proteins seem to bind the R5 and R6 box in the *mioC* promoter with higher affinity than expected from the complementation data, where the high temperature sensitive *dnaA46* and *dnaA204* phenotypes were only weakly complemented.


DnaA+
DnaA(L438P)
DnaA(V391A, C441G)
DnaA(R401C, K443R)
DnaA(V391A, L465S)
DnaA(I454N)
DnaA(V383M)
DnaA(M411T)
DnaA(K397E)
DnaA(Y387S)
DnaA(V437M)
DnaA(L395P)
β-galactosidase activity (Miller units) with 0 (◆), 10 (□), 30 (◁) and 100 (○) mM IPTG
Time (min. incubation at 42°C with different IPTG)

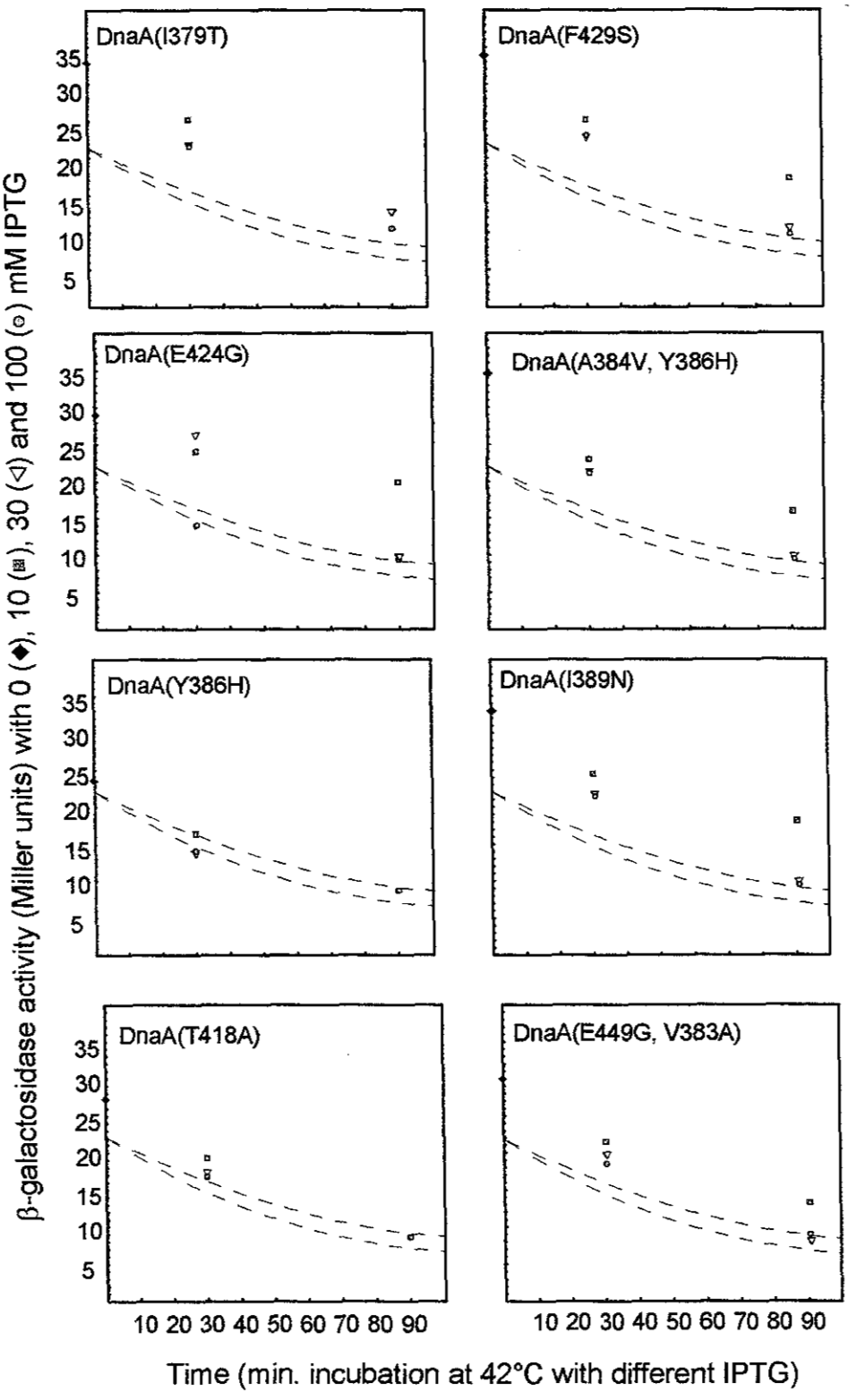


Fig. 3.12. β-galactosidase assays were carried out to estimate the relative affinities of various DnaA proteins for DnaA box R5 and R6 *in vivo*. The strains (MN987 background) were grown exponentially at 32°C. At OD_{450}=0.1 the cultures were divided in five portions. Four portions were incubated at 42°C with indicated amounts of IPTG and the fifth portion was incubated at 32°C with no IPTG. Samples were taken 30 and 90 min. after IPTG induction and β-galactosidase activities determined as described in experimental procedures. The dashed line represents the $DnaA^+$ β-galactosidase activities.

Table 3.11. Relative β-galactosidase activities in various MN987 strains (*dnaA204*, P_{mioC}-*lacZ* in λ att) harbouring multicopy plasmids with different *dnaA* alleles.

DnaA	32°C [1]	Fold repression (42°C) [4]
pBEX5BA [2]	33.6	
$DnaA^{+}$ [2]	22.9	
$DnaA^{+}$ [3]	0.69	2.04
I379T	1.0	1.32
DnaA205	0.94	1.30
A384V	1.07	1.43
Y386H	0.72	2.0
Y387S	0.82	1.52
DnaA204	1.07	1.35
L395P	0.78	1.64
K397E	0.76	1.92
R401C	1.11	1.16
DnaA211	0.92	1.33
T418A	0.83	1.72
E424G	0.90	1.37
F429S	1.05	1.32
V437M	0.88	1.41
L438P	0.95	1.27
C441G,V391A	0.95	1.39
E449G,V383A	0.87	1.54
I454N	1.11	1.23
L465S,V391A	1.11	1.15

1) The β-galactosidase activities were constant at 32°C without IPTG.

2) β-galactosidase activity expressed as Miller-units (see Experimental procedures).

3) Values are relative to the β-galactosidase activity in the pBEX5BA strain grown at 32°C without IPTG.

4) Fold repression of *mioC* promoter after 30 min. incubation at 42°C with 10 μM IPTG compared to the activity in the strain harbouring pBEX5BA. See Fig. 3.12 and Experimental procedures

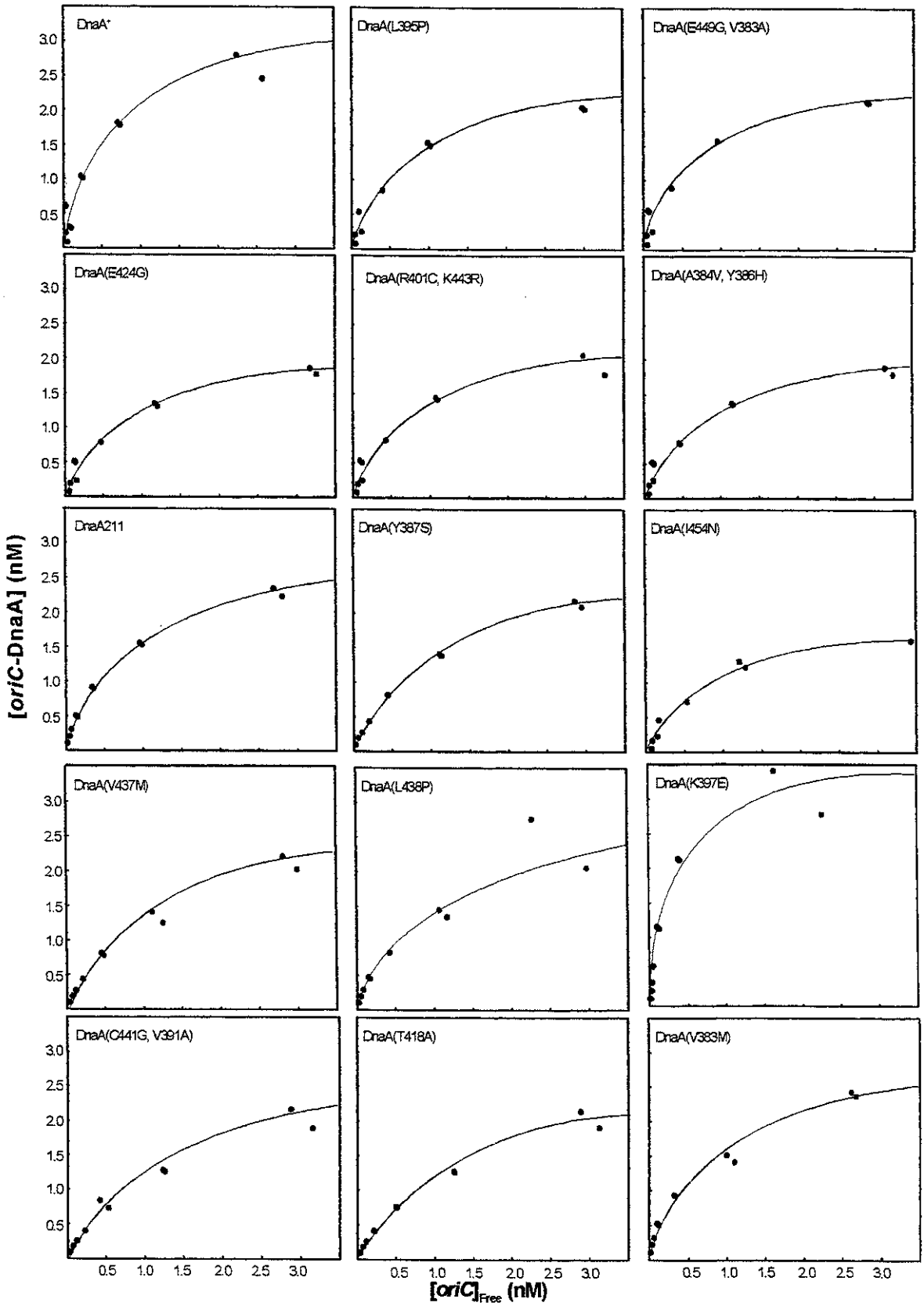
In several cases, there seem to be a nice correlation between the ability to complement the *dnaA46* and *dnaA204* phenotypes and the affinity to the DnaA boxes in the *mioC* promoter, and that is in spite of differences in the DnaA box sequences (Fig. 1.1 and Table 2.1). The mutant DnaA proteins that require 30 μM IPTG to complement the high temperature sensitive phenotype retain 60-70% of the affinity for R5 and R6 compared to the $DnaA^+$ protein, and those DnaA proteins that complement the high temperature sensitive phenotypes as well as the $DnaA^+$ protein does also repress the *mioC* promoter to almost the same extend as the $DnaA^+$ protein does. The observation that some mutant DnaA proteins have a high *mioC* promoter affinity and yet are unable to complement the *dnaA46* phenotype, and vice versa, have reduced affinity to the *mioC* promoter in spite of good complementation of the *dnaA46* phenotype is interesting and may indicates that these amino acids participate in the specific recognition and are responsible for the different affinities for the DnaA boxes in *oriC*. As discussed in Chapter 1 and 2, different affinities to DnaA boxes in *oriC* has been observed, and it has been suggested that DnaA proteins bind R3 late in the cell cycle and that this interaction is the triggering event (Samitt *et al.*, 1989; Cassler *et al.*, 1995). DnaA(E424G), DnaA(L438P), DnaA(E449G), DnaA(I454N), DnaA205 and DnaA204 seem to have retained 60-70% of the affinity to R5 and R6 and almost no *oriC* affinity *in vivo*, whereas DnaA(L465S) has reduced R5 and R6 affinity and intact *oriC* affinity. The se amino acids are located close to or in helix 1 and the last half of domain IV.

in vitro affinity to DnaA boxes in oriC and in mioC promoter region

The affinities of mutant DnaA proteins constructed in this work for the DnaA boxes in the *oriC* region (DnaA boxes R1, M, R2, R3, R4, R5 and R6. Fig. 1.1), and the specificity of the interaction, were determined *in vitro*. The main line in these assays were: 1) production of DnaA proteins (with and without amino acid substitutions) fused to a biotin-tag, 2) binding of these fusion proteins to streptavidin coated magnetic beads and 3) addition of known concentrations of radioactively labelled DNA fragment carrying *oriC* (and known concentration of 'cold' salmon sperm DNA). The principle in this approach is shown in Fig. 3.6.

oriC-DnaA binding constants

The affinities of the various DnaA proteins for DnaA boxes in the *oriC* area were estimated by adding increasing amounts of *oriC* to a constant concentration of DnaA


DnaA+
DnaA(L395P)
DnaA(E449G, V383A)
DnaA(E424G)
DnaA(R401C, K443R)
DnaA(A384V, Y386H)
DnaA211
DnaA(Y387S)
DnaA(I454N)
DnaA(V437M)
DnaA(L438P)
DnaA(K397E)
DnaA(C441G, V391A)
DnaA(T418A)
DnaA(V383M)
[oriC-DnaA] (nM)
[oriC]Free (nM)
0.5
1.0
1.5
2.0
2.5
3.0

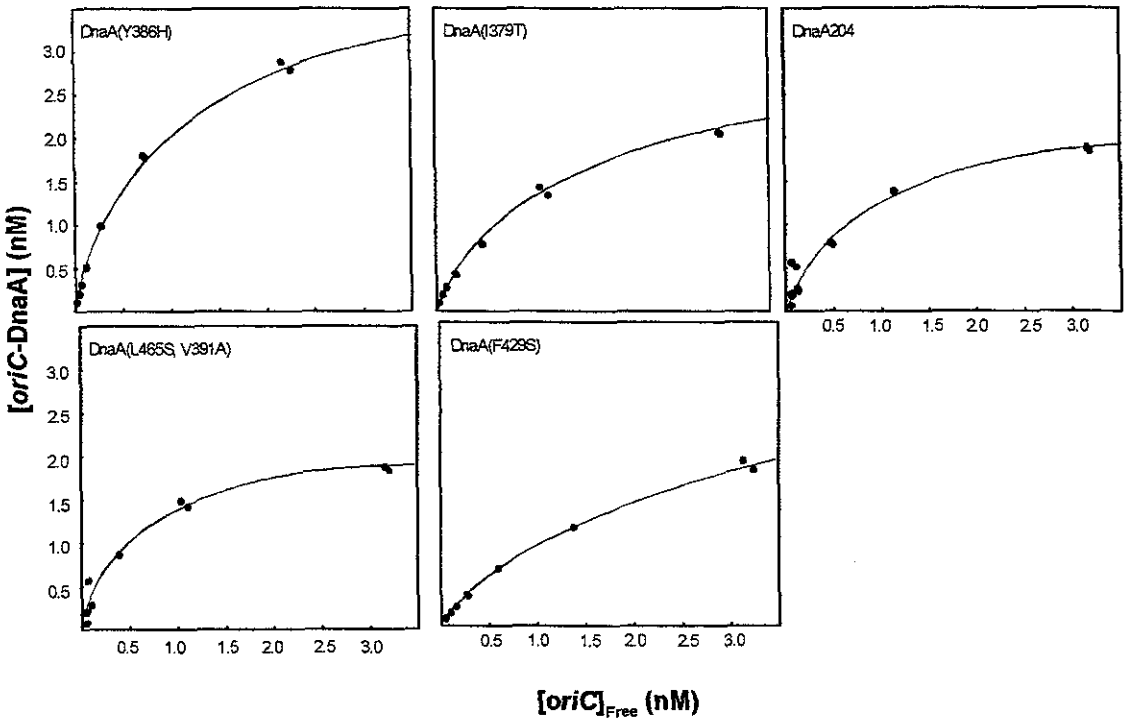


Fig. 3.13. Increasing amount of radioactively labelled *oriC* fragment was added to a constant DnaA concentration to estimate K_d as described in the text. The experimental approch is shown in Fig. 3.6.

protein, and subsequently plotting the complex concentration (*oriC*-DnaA) as a function of the free *oriC* concentration (Fig. 3.13). This approach makes it possible to read the total DnaA concentration as the horizontal asymptote at saturation. The K_d can be read as the value when half of the DnaA proteins have bound *oriC*, since [DnaA-*oriC*] = $[oriC]_{free} \cdot [DnaA]_{total} / (K_d + [oriC]_{free})$. GraFit was used to make the curve fits and do the calculations shown in table 3.12.

The results indicate that many of the proteins have the same high affinity to *oriC in vitro* as the $DnaA^+$ protein has (Fig. 3.13 and Table 3.12). The DnaA(K397E) protein has the highest affinity - three fold higher than the $DnaA^+$ protein, and the DnaA(F429S) protein has the lowest affinity - three fold lower than the $DnaA^+$ protein. The $DnaA^+$ protein has a $K_d = 0.75 \pm 0.14$ nM. This is in agreement with previously published affinity of $DnaA^+$ for *oriC in vitro* (Schaefer and Messer, 1991). The classical DnaA mutant proteins DnaA204, DnaA205 and DnaA211 have the same affinity for *oriC in vitro* as the $DnaA^+$ has. This may be expected considering that the *in vitro* experiments were carried out at room temperature. The DnaA(A384V, Y386H), DnaA(Y386H), DnaA(L395P), DnaA(R401C, K443R) and DnaA(I454N) proteins also have as high affinity for *oriC* as the $DnaA^+$ protein has (Fig. 3.13 and Table 3.12), while DnaA(E424G) has a slightly decreased and DnaA(E449G, V383A) a slightly increased

Table 3.12. DnaA concentrations and K_d (nM) of different mutant DnaA protein for DnaA boxes in the *oriC* area (includes DnaA box R1, M, R2, R3, R4, R5 and R6).

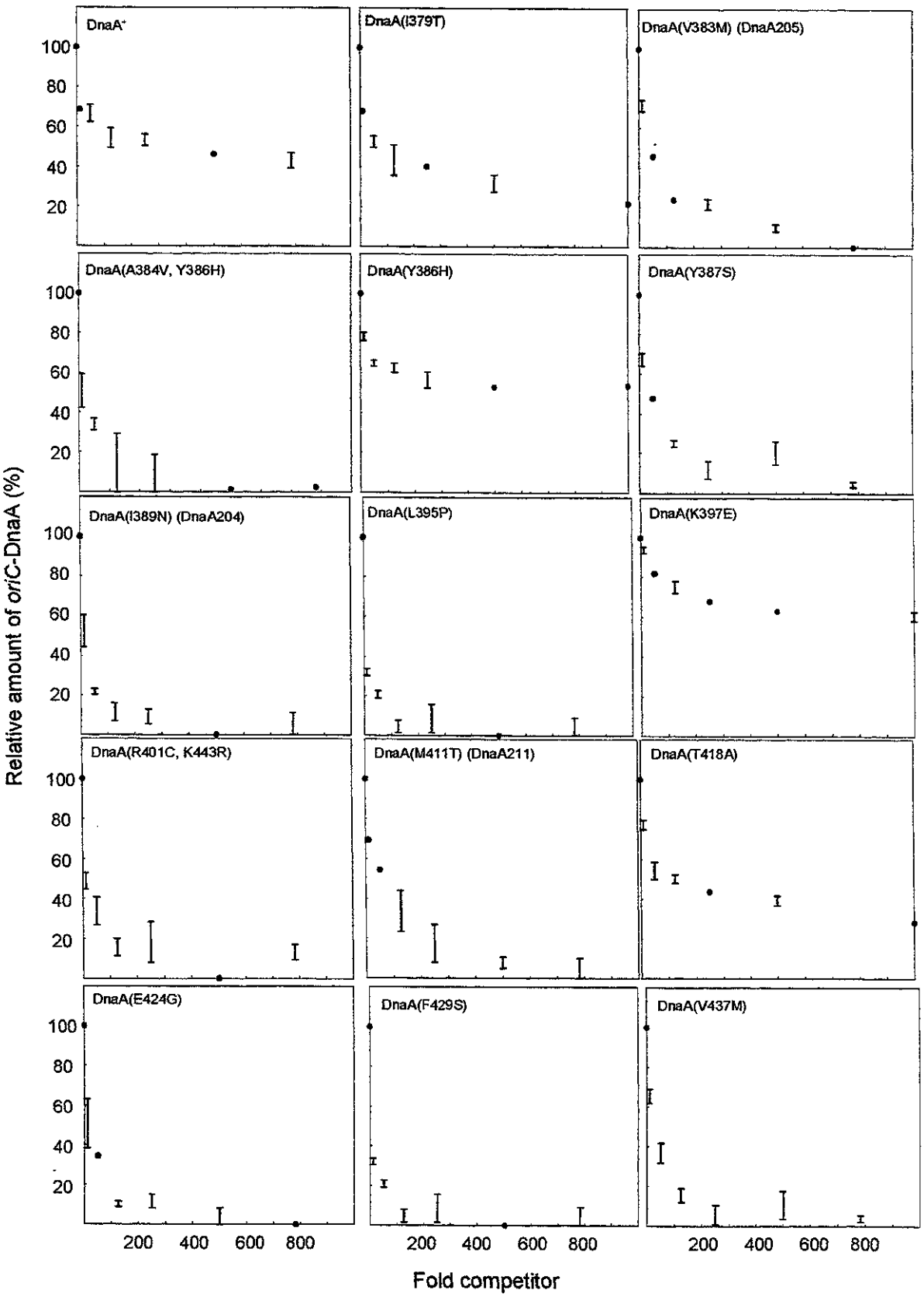
DnaA protein	[DnaA] (nM) [1)]	K_d (nM) [1)]	Affinity to *oriC* (norm.)
$DnaA^+$	3.22 ± 0.31	0.75 ± 0.14	1.00
I379T	2.86 ± 0.09	1.20 ± 0.09	0.63
V383M (DnaA205)	2.88 ± 0.22	0.71 ± 0.14	1.06
A384V, Y386H	2.28 ± 0.15	0.77 ± 0.13	0.97
Y386H	3.86 ± 0.07	0.82 ± 0.04	0.91
Y387S	3.00 ± 0.08	1.21 ± 0.08	0.62
I389N (DnaA204)	2.34 ± 0.20	0.81 ± 0.18	0.93
L395P	2.56 ± 0.18	0.73 ± 0.13	1.03
K397E	3.46 ± 0.16	0.24 ± 0.03	3.13
R401C, K443R	2.40 ± 0.17	0.76 ± 0.14	1.01
DnaA211	2.86 ± 0.10	0.74 ± 0.06	1.01
T418A	3.04 ± 0.20	1.56 ± 0.21	0.48
E424G	2.30 ± 0.15	0.87 ± 0.14	0.86
F429S	3.20 ±0.14	2.33 ± 0.19	0.32
V437M	2.98 ±0.20	1.28 ± 0.19	0.59
L438P	3.42 ± 0.52	1.28 ± 0.42	0.59
C441G, V391A	2.92 ± 0.23	1.42 ± 0.24	0.53
E449G, V383A	2.64 ± 0.21	0.66 ± 0.14	1.14
I454N	1.96 ± 0.12	0.81 ± 0.13	0.93
L465S, V391A	2.14 ± 0.15	0.51 ± 0.11	1.47

1) Values are calculated from curvefits of data in Fig. 3.13 (see experimental procedures and text).

affinity for the DnaA boxes in the *oriC* area. On the contrary, DnaA(T418A), DnaA(F429S), DnaA(I379T), DnaA(C441G, V391A), DnaA(Y387S), DnaA(V437M) and DnaA(L438P) proteins all have reduced affinity to *oriC in* vitro. The DnaA(L465S, V391A) and DnaA(K397E) proteins have higher affinity to the *oriC* area *in vitro* than the $DnaA^+$ protein has, especially the DnaA(K397E) protein.

Further analysis was carried out to determine the specificity of the DnaA-*oriC* interactions. This was done by adding increasing amount of salmon sperm DNA together with a constant *oriC* concentration. As shown in Fig. 3.14, most of the mutant DnaA proteins do not bind *oriC* with the same specificity as the $DnaA^+$ protein does.

The DnaA(K397E) and DnaA(Y386H) proteins have, however, somewhat increased specificity towards *oriC*, whereas the DnaA(T418A), DnaA(C441G, V391A), DnaA(E449G, V383), DnaA(F429S) and DnaA(I379T) proteins still have high specificity towards *oriC*, although lower than what is observed with the $DnaA^+$ protein. The rest of the DnaA proteins have reduced specificity toward *oriC*; the DnaA(A384V, Y386H), DnaA(E424G), DnaA204 and especially DnaA(L395P) have severely reduced specificity towards *oriC*. The results are rather interesting, and indicates that even though the affinity for *oriC* is reduced, the specificity towards *oriC* can still be intact (DnaA(F429S) for instance), and vice versa, a high affinity to *oriC* does not automaticly mean that the interaction is specific towards *oriC* (DnaA(L395P)). Fig. 3.15 summarises the results of the *in vivo* and *in vitro* binding experiments. The DnaA proteins with the highest *in vitro* affinity but low specificity are the classical DnaA mutant proteins DnaA205 and DnaA204 and DnaA(A384V, Y386H), DnaA(L395P) and the DnaA(R401C, K443R) protein (Fig. 3.15). These amino acid substitutions are located in α-helix 1 and in the basic loop, and this may suggest that this part of the DNA binding domain participate in the specific recognition of the DnaA box. Generally, the *in vivo* affinities of mutant DnaA proteins for the DnaA box R5 and R6 boxes are lower relative to that of the $DnaA^+$ protein compared to the *in vitro* affinities for the *oriC* area (which includes DnaA box R1-R6. See Fig. 1.1). It is also conspicuous that the *in vivo* affinities in many cases 'follow' the specificity, that is, when the *in vivo* affinity is low so is the *in vitro* specificity. This indicates that the observed reduced affinity to R5 and R6 *in vivo* may be due to a titration of DnaA proteins by the rest of the chromosome. Thus, there seems to be correlation between the *in vivo* and *in vitro* binding experiments.


DnaA+
DnaA(I379T)
DnaA(V383M) (DnaA205)
DnaA(A384V, Y386H)
DnaA(Y386H)
DnaA(Y387S)
DnaA(I389N) (DnaA204)
DnaA(L395P)
DnaA(K397E)
DnaA(R401C, K443R)
DnaA(M411T) (DnaA211)
DnaA(T418A)
DnaA(E424G)
DnaA(F429S)
DnaA(V437M)
Relative amount of oriC-DnaA (%)
Fold competitor
100
80
60
40
20
200
400
600
800

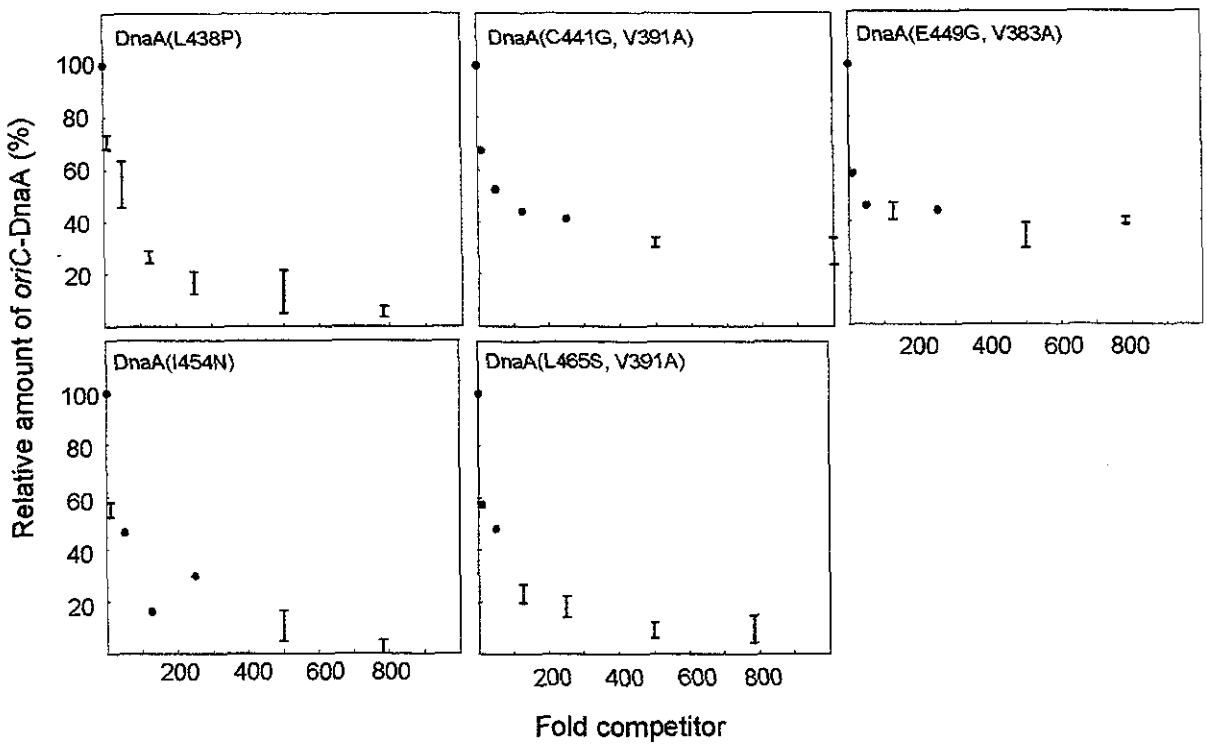


Fig. 3.14. The specificity of various mutant DnaA proteins towards the *oriC* area. Specificity was determined by adding increasing amounts of salmon sperm DNA together with a constant amount of radioactively labelled *oriC*. Values are expressed as percentage *oriC* bound as a function of fold competitor DNA relative to when no competitor was added. See Experimental procedures, Section II and Fig. 3.6.

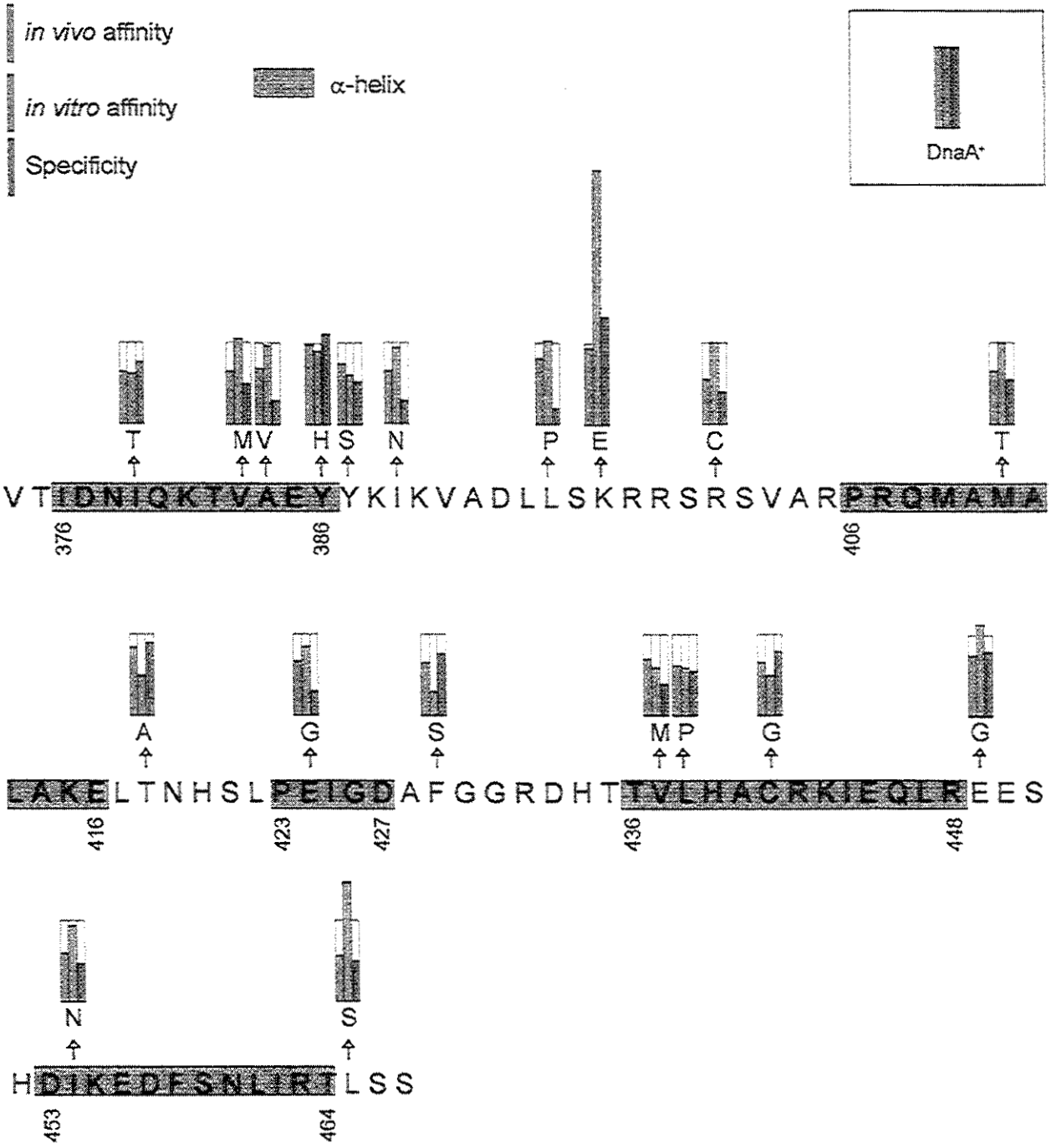


Fig. 3.15. Summary of *in vivo* and *in vitro* binding data. The values are relative to the affinity and specificity of DnaA$^+$ for *oriC in vitro* and the affinity for DnaA box R5 and R6 *in vivo*.

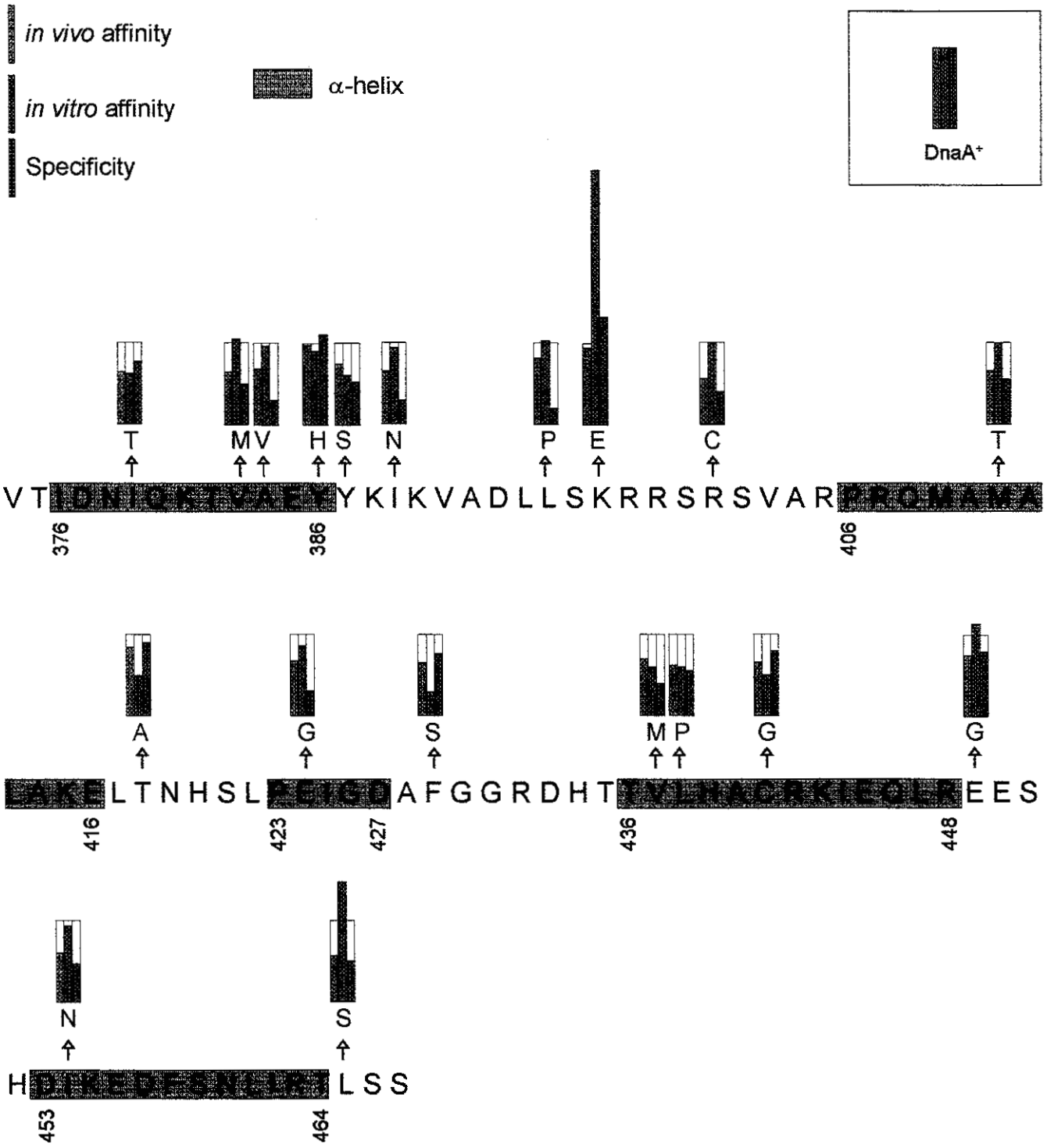


Fig. 3.15. Summary of *in vitro* and *in vivo* binding data. The values are relative to the affinity and specificity of DnaA$^+$ for *oriC in vitro* and the affinity for DnaA box R5 and R6 *in vivo*.

The N-terminal part of the fusion protein is cleaved off upon sonication

On low-Bis SDS-gels, it is quite noticeable that after sonication (but not before) the fusion proteins always appear as a double bands (Fig. 3.16). The relative amounts of intact and cleaved DnaA fusion protein seemed to depend on both the mutant DnaA protein and the sonication procedure (Fig. 3.17). The problem with truncated fusion proteins was that it could be (part of) the C-terminal region of the DnaA protein, and

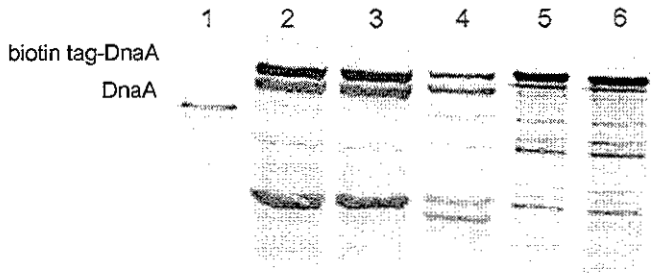


Fig. 3.16. Western blot of DnaA proteins before and after sonication. Lane 1: DnaA marker. Lane 2: Sonicated DnaA$^+$ lysate. Lane 3: Sonicated DnaA$^+$ lysate incubated over night at 37°C. Lane 3: Sonicated DnaA$^+$ lysate incubated with FX_a for 1 hour at 37°C. Lane 5: DnaA$^+$ lysate before sonication. Lane 6: DnaA(L395P) lysate before sonication.

thus, the DNA binding domain, that was cleaved off. In cases where far the most of the fusion protein was truncated, it would be problematic to carry out *in vitro* binding experiments, partly because there would not be much protein to work with, and partly due to lack of knowledge about the amount of *oriC* that might bind to the truncated fusion proteins in spite of the missing part of the DNA binding domain. Comparison of Western blots using streptavidin conjugated antibodies and DnaA antibodies clearly indicated that the truncated protein still had intact biotin tag (data not shown), and therefore, it seemed less likely that it was the N-terminal part of the fusion proteins that were cleaved off. However, the lysine that carries the biotin-tag is located at amino acid position 88 in the biotin-tag, and therefore, the N-terminal part of the fusion protein might be cleaved off, leaving K88 and thus the biotin tag on the truncated protein. South-Western analysis was carried out to determine whether both the intact and truncated DnaA fusion protein bind *oriC* (Fig 3.17). The sonicated lysate was run on an SDS-PAGE, and blotted onto a filter. The filter was then incubated with radioactively labelled *oriC* (the same as in the binding experiments), exposed, stripped and then processed as in Western blotting (see Experimental procedures). By quantifying the

amount of bound *oriC* and the amount of protein, it was possible to conclude that both the intact and the truncated DnaA fusion protein bind *oriC* with equal affinity, and thus, it seems that it is part of the biotin-tag that is cleaved off. What caused the cleavage was not further analysed. It is not a process that continue beyond the sonication period, since over night incubation at 37°C of the sonicated bacterial lysate did not result in increased amount of truncated fusion protein (Fig. 3.16).

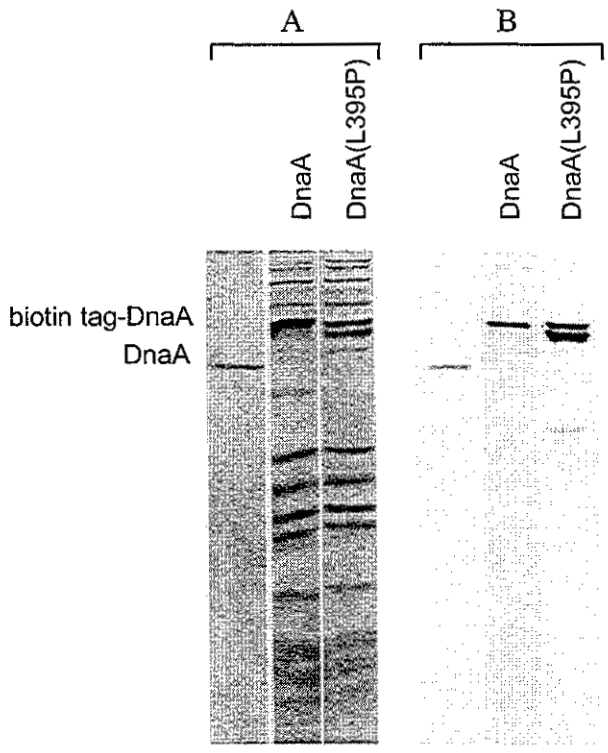


Fig. 3.17. South-Western analysis. Sonicated lysates were run on SDS-PAGE and blotted onto a filter. The filter was incubated with radioactively labelled *oriC*, exposed (A), stripped and then processed as in Western blotting (B). See Experimental procedures). Lane 1: DnaA marker. Lane 2: Sonicated $DnaA^+$ lysate. Lane 3: Sonicated DnaA(L395P) lysate.

In addition, cleavage of sonicated fusion proteins with FX_a resulted in a single band, which also suggests that the N-terminal part of the fusion protein is cleaved off (Fig. 3.16).

Section III

Integration and analysis of mutant *dnaA* alleles in λ attachment site of the chromosome

To further analyse the effect of amino acid substitutions on the function and activity of the DnaA protein, it was tried to insert several *dnaA* mutant genes into their normal chromosomal location, and later into the λ attachment site of the chromosome. Several different approaches were used, and they will be described in the following. The last attempt was successful, and the effect of the A384V, L395P, K397E and C441G substitutions will be discussed (and the A184V substitution in Chapter 4).

Recombination into chromosomal location using dnaA46, recBD strain

A *dnaA46*, *recBD* strain (kindly from Christoph Weigel) was used to insert mutant *dnaA* genes into their normal chromosomal location. The strategy was rather straightforward, and it consisted of re-streaking of high temperature resistant *dnaA46*, *recBD* recombinants (or, as it turned out, revertants). First, several pMN242 derivatives carrying various *dnaA* mutant alleles were transformed into the *dnaA46*, *recBD* strain and plated at high temperature (42°C) on rich media supplemented with ampicillin and IPTG. The following day several colonies from each transformation were streaked on rich media supplemented with ampicillin but without IPTG and incubated at 42°C. Lack of IPTG in the growth media generally resulted in reduced and 'sick' growth of the transformants, but (some of) the colonies that appeared as 'healthy' should consist of cells in which the high temperature sensitive *dnaA46* allele had recombined out of the chromosome. These high temperature resistant colonies were re-streaked at 42°C on rich media without ampicillin and IPTG several times to allow growth of plasmid free cells. Verification of the chromosomal *dnaA* alleles of high temperature resistant and ampicillin sensitive cells were carried out by PCR amplification of both the last part of the *dnaA* gene encoding the DNA binding domain, and the area that includes the *dnaA46* mutations followed by restriction enzyme digestions (see Experimental procedures, section III). The principle in this technique works well, and it was very straightforward to substitute the *dnaA46* allele with a $dnaA^+$ allele (data not shown). However, it was not possible to insert new mutant *dnaA* alleles into the chromosome. It

was unproblematic to recombine the *dnaA46* mutations out of the chromosome, but never possible to recombine the C-terminal mutations into the chromosome. The *dnaA* gene of 30 potentially high-temperature resistant and ampicillin sensitive *dnaA*(I379T) and *dnaA*(Y386H) strains were PCR amplified and digested with *Dde*I and *Sca*I, respectively (see Experimental procedures, section III). All were *dnaA*$^+$. It was expected to be possible to insert the *dnaA*(Y386H) allele on the chromosome because the DnaA(Y386H) protein seems very wild type like with respect to complementation of the *dnaA46* allele, affinity to the *mioC* promoter, *in vitro* affinity to *oriC* and specificity towards *oriC* (see sections I and II). Thus, it seems that there was not enough DNA for the recombination event. Alternatively, (some of) the DnaA proteins encoded by the mutant *dnaA* alleles are high temperature sensitive as the DnaA46 protein, and therefore, selection at 42°C would not select correct recombinants. However, lowering the temperature from 42°C to 39°C did not result in recombinants with the desired dnaA allele either.

Analysis of dnaA^0 strains carrying mutant dnaA genes in λ attachment site and harbouring a plasmid with P_{BAD}-rnh$^+$ fusion.

If the mutant *dnaA* genes cannot be introduced in their right chromosomal position, the second best seemed to be to insert them into the λ attachment site in a strain that does not carry a *dnaA* gene (*dnaA*^{0}). Such a strain has been constructed. It is called TC3482 and it carries a chloramphenicol cassette in the *dnaA* gene (Hansen *et al.*, 1991. See Experimental procedures). The cell survives due to a mutation in the *rnhA* gene, a gene that encodes RNase H. RNase H normally removes RNA-DNA hybrids, and thus, when it is absent those hybrids are allowed to exist, and the chromosomal replication can initiate from DnaA independent *oriK*'s (for review see Messer and Weigel, 1996). The experimental strategy was to insert various mutant IPTG inducible *dnaA* alleles into the λ attachment site of a *dnaA*^{0} strains harbouring a plasmid with the *rhnA* gene under arabinose promoter control and analyse the initiation frequency from *oriC* by adding arabinose to the growth medium. The construction of the various strains went well, but unfortunately, it turned out that the P_{BAD}-*rnh*$^+$ plasmid probably did not synthesise enough RNase H to prevent *oriK* initiations. This was first under suspicion when colonies, although very small, appeared on LB plates supplemented with arabinose, and later concluded from the observation that when strains carrying the P_{BAD}-*rnh*$^+$ plasmid were grown in media supplemented with arabinose and treated with rifampicin (which also inhibits initiations from *oriK*), the DNA distributions were similar to the ones in a

cell population that did not carry the P_{BAD}-*rnh*$^+$ plasmid. The *oriK* initiations in presence of arabinose was not a result of growth of plasmid free cells, because the growth medium was supplied with 500 μg/ml ampicillin every 40 min.

Analysis of mutant dnaA alleles in a dnaA204 and dnaA^0 background

A selection of mutant *dnaA* alleles and the *dnaA*$^+$ allele were inserted into a strain that carried a *dnaA204* allele on the chromosome (TC1929), or alternatively, into the *dnaA*^{0}, *rnh373* strain (TC3482) mentioned above. Both strains can grow at 32°C independently on the *dnaA* allele in λ attachment site. However, at 42°C, the *dnaA204* strains were IPTG dependent (- or sensitive) as expected (Table 3.13). The *rnh373* mutation was transduced out of the *dnaA*^{0} *rhn373* strains carrying different *dnaA* alleles in λ attachment site (Fig. 3.7) making these strains IPTG dependent (- or sensitive) (Table 3.13). The various strains were grown in the presence of different amounts of IPTG as described in Experimental procedures, section III, samples were taken, treated with rifampicin (+cephalexin) and the chromosome configurations were analysed using flow cytometry. Samples were also taken to measure the DnaA concentration using Western blotting as described below and in Experimental procedures (section III and General procedures).

Table 3.13. Growth of *dnaA204* and *dnaA*^{0} strains harbouring IPTG inducible *dnaA* alleles in λ attachment site [1)]

Strain	*dnaA* (chromosome / λ att)	32°C				42°C			
[IPTG] (μM)		0	30	75	100	0	30	75	100
MN1240	*dnaA204* / *dnaA*$^+$	+++	+++	+++	ND [2)]	-	+++	+++	+++
MN1242	*dnaA*^{0} / *dnaA*$^+$	-	+++	ND	+++	-	+++	ND	ND
MN1191	*dnaA204* / *dnaA*(L395P)	+++	+++	+++	+	-	+++	+++	+++
MN1210	*dnaA*^{0} / *dnaA*(L395P)	-	+++	ND	+++	-	+++	ND	ND
MN1193	*dnaA204* / *dnaA*(K397E)	+++	-	-	-	+++	-	-	-
MN1213	*dnaA*^{0} / *dnaA*(K397E)	+++	-	ND	ND	+++	-	ND	ND
MN1195	*dnaA204* / *dnaA*(A384V, Y386H)	+++	+++	+++	+++	-	+++	+++	+++
MN1215	*dnaA*^{0} / *dnaA*(A384V, Y386H)	-	+++	ND	ND	-	+++	ND	ND
MN1197	*dnaA204* / *dnaA*(C441G, V391A)	+++	+	-	-	-	+++	+++	+++
MN1212	*dnaA*^{0} / *dnaA*(C441G, V391A)	-	+++	ND	ND	-	+++	ND	ND

1) Growth was estimated as the ability to form colonies on LB plates supplemented with/without IPTG. +++ : good growth, ++ : reduced growth, + : severely reduced growth, - : no growth.
2) Not determined

The DnaA concentration

DnaA protein concentrations were estimated using Western blotting. Quantifications are based on experimental results where it was found that *E.coli* K-12 *dnaA*$^+$ strains have 25 ng DnaA/ml culture at OD_{450}=1 (Hansen *et al.*, 1991A). Thus, the standard DnaA marker used in all Western blots consisted of *dnaA*$^+$ cells with known density. Fig. 3.18 shows the DnaA concentration in various strains at 32 and 42°C as a function of the IPTG concentrations.

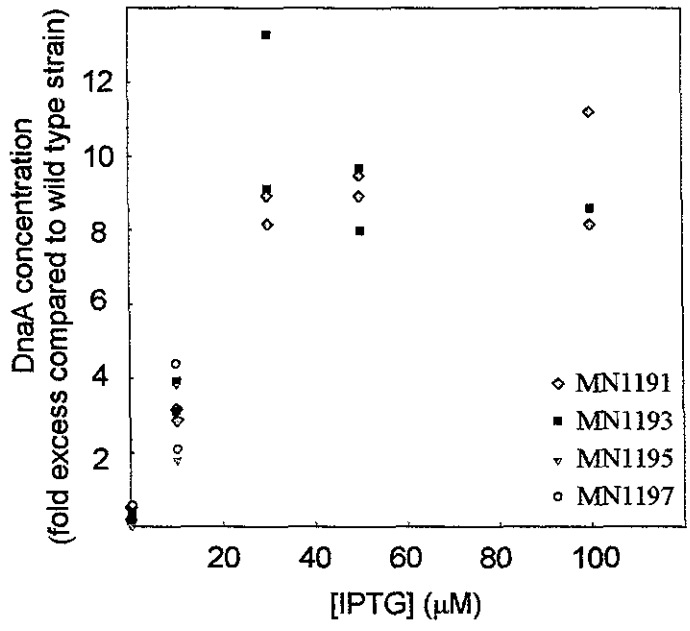


Fig. 3.18. DnaA fusion protein concentration expressed as fold excess DnaA protein compared to the DnaA concentration in a *dnaA*$^+$ strain (OD_{450}=1 ⇒ 25 ng DnaA/ml. Hansen *et al.*, 1991A). *dnaA204* strains carrying different mutant *dnaA* alleles in λ attachment site were grown exponentially at 32°C. At OD_{450} = 0.1 the cultures were shifted to 42°C for 1 hour and then diluted 20 times and divided in eight portions: four portions were further incubated at 42°C and the rest were shifted back to 32°C. Indicated amounts of IPTG was added to the cultures at 32°C and 42°C, and samples were taken different times after IPTG addition to measure the DnaA concentration. The DnaA concentration was independent on temperature (data not shown) and on the particular strain.

The results indicate that the *lac* promoter is induced to the same degree at 32 and 42°C (data not shown) independently on the *dnaA* allele in λ attachment site (Fig. 3.18). Even without IPTG induction, some DnaA protein is synthesised, and the DnaA concentration in the non-induced cells corresponds to approximately 25% of the DnaA concentration in a wild type *dnaA*$^+$ cell (Hansen *et al.*, 1991A). Leakiness of the *lac* promoter is consistent with the observation that MN1193 and MN1213 (*dnaA*(K397E) in λ

attachment site of *dnaA204* and *dnaA*^{0} strains, respectively) were able to grow at 32 and 42°C without IPTG induction (Table 3.13 and text). With 10 μM IPTG induction, the DnaA concentration increased to what corresponds to a two to four fold higher DnaA concentration compared to a wild type *dnaA*$^+$ strain, and with 30, 50 and 100 μM IPTG, the cells contained between eight and ten fold excess DnaA protein compared to a wild type *dnaA*$^+$ strain. The results clearly indicate that the *lac* promoter is transcribed at maximum speed with IPTG concentrations above 30 μM. This is consistent with the observation that the DNA distributions look identical with 30, and especially 50 and 100 μM IPTG induction (see below). The results also indicate that induction with IPTG concentrations between 0 and 10 μM would result in DnaA concentrations closer to the expected DnaA concentration in strains with the mutant *dnaA* alleles in the normal chromosomal position.

dnaA$^+$ *in λ attachment site*

Flow cytometric analysis of MN1240 (*dnaA204* with *dnaA*$^+$ allele in λ attachment site) indicates that when the strain is grown at 32°C without IPTG induction the cells contain 1, 2, 3, 4 initiated origins and a small number of cells have 5 and 6 origins (Fig. 3.19). This DNA distribution is similar to the one published previously (Hansen and Atlung, 1995), and indicates that the uninduced *lac* promoter does not synthesize enough DnaA$^+$ protein to increase or otherwise affect the initiation frequency, and that is in spite of the observation that the biotin-DnaA concentration is app. 25% of the DnaA$^+$ concentration in a wild type strain (Fig. 3.18). While the replication is initiated asynchronously without IPTG induction, only small amount of IPTG results in synchronous initiations and the DNA distributions show cells with primarily 2, 4 and 8 origins (Fig. 3.19). Without IPTG, the majority of the cells contain 2 initiated origins (28%), whereas when IPTG is added, cells with 4 origins are in majority (61%). The DNA distributions are identical with 10, 30, 50 and 100 μM IPTG induction (data not shown), which indicates that excess biotin-DnaA (up to ten fold higher DnaA concentration than a wild type strain) does not lead to an increased number of initiation forks that is able to proceed to the terminus. This is consistent with the observation that overproduction of the DnaA$^+$ protein does not result in an increased DNA content although the replication is overinitiated (Atlung *et al.*, 1987; Atlung and Hansen, 1993. See Chapter 1). At 42°C, the number of origins moves towards one per cell when no IPTG is added to the growth media.

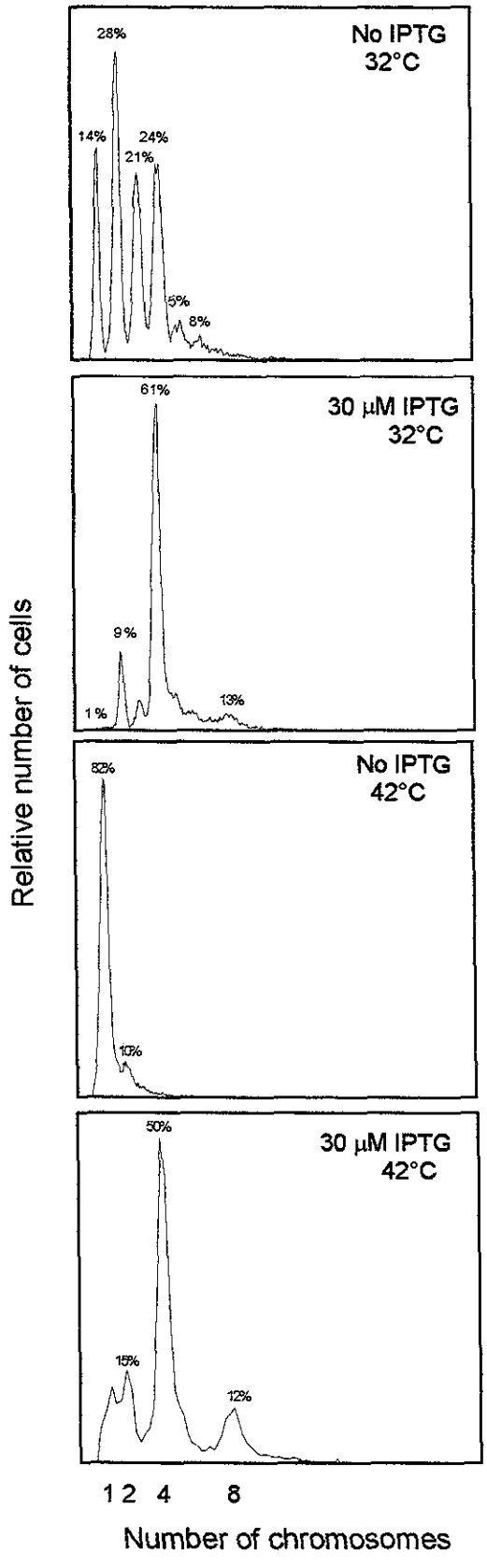


Fig. 3.19. MN1240 (*dnaA204*, biotin tag-*dnaA*$^+$ in λ attachment site) was grown exponentially at 32°C. At OD_{450} = 0.1 the culture was shifted to 42°C for 1 hour and then diluted 20 times and divided in ten portions: five portions were further incubated at 42°C and the rest were shifted back to 32°C. 0, 10, 30, 50 and 100 μM IPTG was added to the cultures at 32°C and 42°C, and samples were taken different times after IPTG addition, treated with rifampicin and analysed using flow cytometer as described in Experimental procedures. The histograms show the DNA distribution after 3 hours incubation at indicated temperatures with indicated amounts of IPTG.

Thus, without IPTG the biotin-DnaA concentration is not sufficiently high to allow initiation of chromosome replication, which is consistent with the data at 32°C and with the observation that MN1240 only forms colonies at 42°C in the presence of IPTG (table 3.13). When 10, 30, 50 and 100 μM IPTG is added, chromosome replication is initiated, and the cells contain 1, 2, 4, and 8 origins. At 42°C there is a large number of very big cells that contain 1 (and 2) chromosomes. The size of these cells seems to increase with increasing incubation time (Fig. 3.20). The existence of a fraction of big cells has been observed previously in the same *dnaA204* strain background (Hansen and Atlung, 1995A).

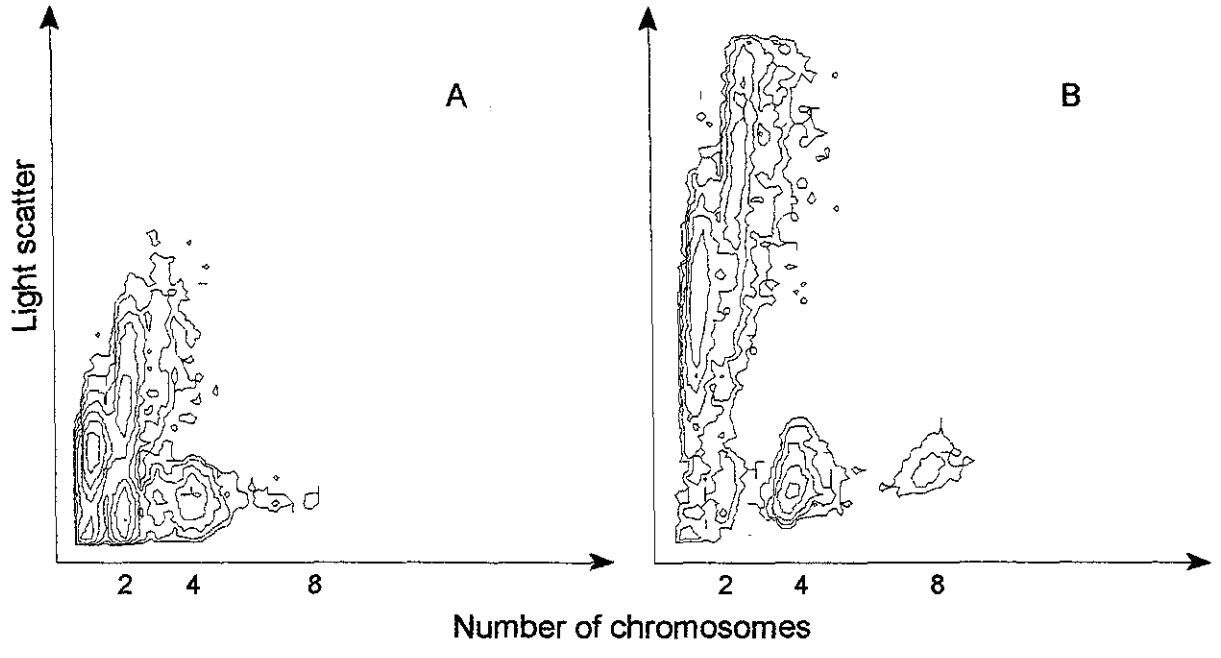


Fig. 3.20. Contour blots of MN1240 cells (*dnaA*$^+$ in *dnaA204* background) grown at 42°C with 30 μM IPTG. A: after 2 hours growth and B: after 3 hours growth.

The cells do not seem to be able to initiate chromosome replication although the growth medium is supplemented with IPTG, and it seems as if this population of cells is stalled in a locked situation. The population of cells grew exponentially, but more slowly than at 32°C. This is unexpected and may be explained by the presence of these large cells not responding to IPTG.

MN1242 (*dnaA*$^+$ in *dnaA*^{0} strain background) was able to initiate chromosome replication at both 32 and 42°C with only 3 μM IPTG induction, and the number of origins per cell depended on the temperature and only slightly on the amount of IPTG (Fig. 3.21). Thus, it seems that enough DnaA$^+$ protein is synthesised from the 3 μM

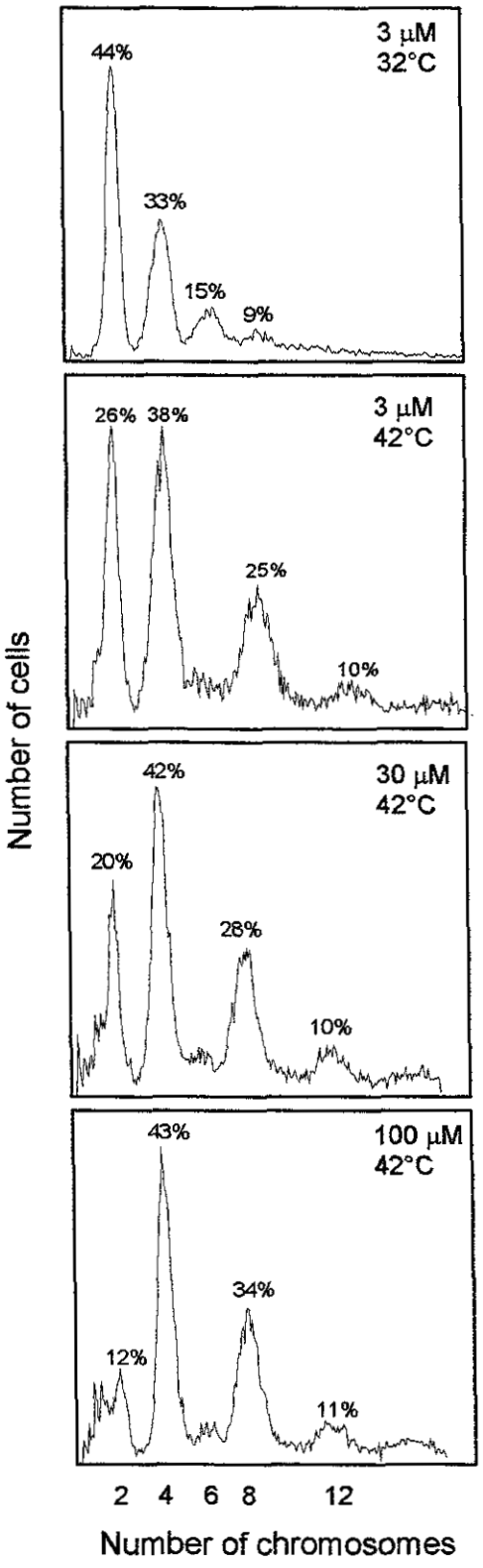


Fig. 3.21. DNA distributions in MN1242 (*dnaA*^{0}, *dnaA*$^+$ in λ attachment site). Strains were grown exponentially at 32°C and 42°C in medium supplemented with 30 μM IPTG. At OD_{450}=0.5, the cultures were diluted 10 times in the same medium supplemented with 0, 30 and 100 μM IPTG and incubated at the same temperature. Samples were taken from the cultures after 2, 3 and 4 hours, treated with rifampicin and analysed using flow cytometry as described in Experimental procedures. The histograms show the DNA distribution after 3 hours incubation at indicated temperatures with indicated amounts of IPTG.

IPTG induced *lac* promoter to allow initiation of chromosome replication. This is consistent with DnaA protein concentration data (Fig. 3.18), which indicates that with 3 μM IPTG induction, the DnaA concentration may be close to the DnaA concentration in a normal wild type strain. At 32°C, cells contain primarily 2 and 4 origins, but also 6 and 8. The DNA distribution did not change with increasing IPTG concentrations (data not shown). The replication is not initiated as synchronously as in the *dnaA204* strain background (Fig. 3.19). At 42°C, far more cells have 8, even 12 origins compared to 32°C. At the high temperature, the chromosome distribution may change slightly and move towards a higher number of origins per cell with increasing IPTG concentrations (Fig. 3.21). The effect is, however, not dramatic consistent with the observations in the *dnaA204* strain background. In the *dnaA*^{0} strain background, there is no fraction of large IPTG unsusceptible cells as observed in the *dnaA204* strain background. This indicates that the phenomena may be a consequence of DnaA$^+$ and DnaA204 interactions.

dnaA(K397E) on the chromosome

Strains harbouring the *dnaA*(K397E) allele in λ attachment site are able to form colonies at 32 and 42°C without IPTG induction (Table 3.13). Actually, the presence of IPTG seems to be very deleterious and the strains do form colonies even in the presence of only small amounts of the inducer (10μM). Flow cytometric analysis, like the ones discussed above, was carried out to determine the chromosomal configuration of these strains. With no IPTG induction, the MN1193 cells (*dnaA204* background) have more origins per cell, on average, than the isogenic *dnaA*$^+$ strain has (Fig. 3.22). The replication is initiated rather asynchronously and the cells have (1) 2, 3, 4, 5, 6, 7 and 8 origins, and not all origins are able to finish the replications. This indicates that a biotin-DnaA(K397E) concentration of only one fourth of the DnaA concentration in a normal *dnaA*$^+$ strain, results in initiations of chromosome replication. After 1 hour growth at 32°C in medium supplemented with IPTG, the DNA distribution does not change significantly compared to when no IPTG is added. However, after 4 hours growth with IPTG induction, the DNA distributions clearly indicate that the number of initiated origin decrease with increasing IPTG concentrations (Fig. 3.22), and the cells contain primarily only one, two or three origins per cell with 100 μM IPTG induction. At 42°C, the same tendency is observed, maybe even more pronounced. With no IPTG induction, the DNA distributions are comparable to the ones at 32°C, but with IPTG induction, the number of origins per cell seem to decrease faster, and after 4 hours, the

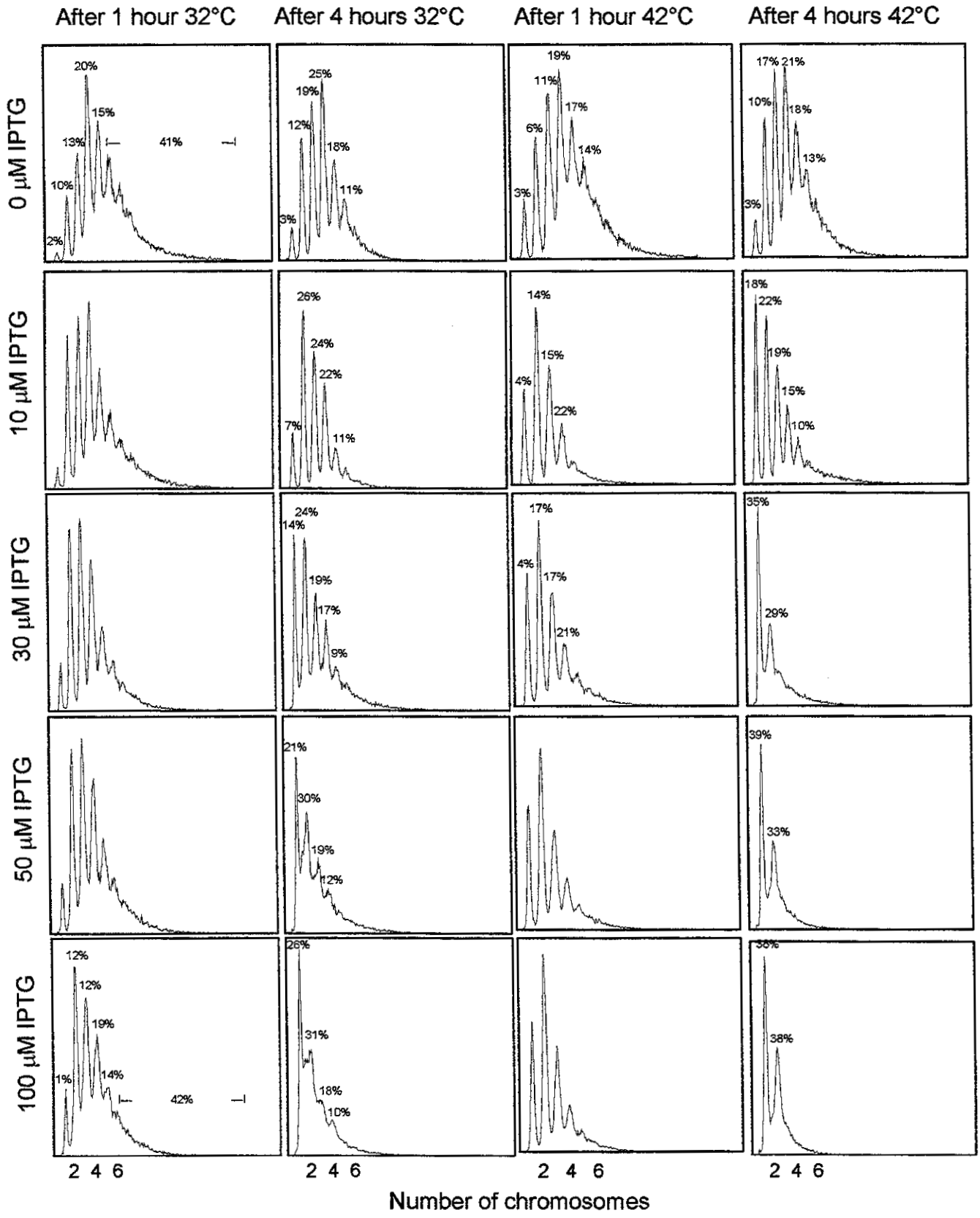


Fig. 3.22. MN1193 (*dnaA204*, *dnaA*(K397E) in λ attachment site) was grown exponentially at 32°C. At OD_{450} = 0.1 the culture was shifted to 42°C for 1 hour and then diluted 20 times and divided in ten portions: five portions were further incubated at 42°C and the rest were shifted back to 32°C. 0, 10, 30, 50 and 100 µM IPTG was added to the cultures at 32°C and 42°C, and samples were taken different times after IPTG addition, treated with rifampicin and analysed using flow cytometer as described in Experimental procedures. The histograms show the DNA distribution after 1 and 4 hours incubation at indicated temperatures with indicated amounts of IPTG.

cells have only one or two origins with 30, 50 and 100 μM IPTG induction (Fig. 3.22). The result of this analysis could indicate that the DnaA(K397E) protein has an increased affinity to *oriC* and/or decreased affinity to (some of) the DnaA boxes on the rest of the chromosome (see Discussion). The results also indicates that MN1193 (and MN1213, see below) does not form colonies at neither 32 nor 42°C in presence of IPTG (Table 3.13) due to an apparent cease of initiation of chromosome replication. Alternatively, the initiation of replication actually continues, but the forks do not pass the *oriC* area as observed with other *dnaA* mutants (Nyborg *et al.*, 2000) and in strains in which the $DnaA^+$ protein is overproduced (Atlung *et al.*, 1987; Atlung and Hansen, 1993). Flow cytometric analysis of MN1213 (*dnaA*(K397E) in $dnaA^0$ background) did not display discrete peaks (data not shown). However, there seems to be a tendency that the number of origins per cell at 32° moves towards one, which is consistent with the observations in the *dnaA204* strain background (data not shown).

dnaA(L395P) on the chromosome

The high temperature sensitive *dnaA204* phenotype is complemented when the IPTG inducible *dnaA*(L395P) allele is inserted into λ attachment site and IPTG is added to the growth medium (Table 3.13). Consistent with this observation, MN1210 (*dnaA*(L395P) in $dnaA^0$ background) forms colonies only in the presence of IPTG (Table 3.13). Flow cytometric analysis of MN1191 (*dnaA204* background) indicates that at 32°C, the number of origins per cell is rather constant and independent of the IPTG concentration, although there may be a tendency that the number of origins decrease after 4 hours incubation with the highest IPTG concentrations (Fig. 3.23). Without IPTG induction, the DNA distributions at 32°C are comparable to the ones in the isogenic $dnaA^+$ strain (MN1240, Fig. 3.19), but unlike MN1240, MN1191 does not begin to initiate replication synchronously with IPTG induction. At 42°C, MN1191 does, eventually, seem to stop initiation of replication without IPTG induction, but with only small amounts of IPTG the replication is overinitiated rather dramatically, especially after 4 hours. With the highest IPTG concentrations there seem to be too many initiated origins per cell to make a completion of the many replication forks possible (Fig. 3.23). After one hour growth with IPTG induction at 42°C, a portion of the cells has only one origin, but unlike the isogenic $dnaA^+$ strain (Fig. 3.20), these cells have a normal size (data not shown). In spite of the apparent dramatic increase in the number of origins at 42°C in presence of IPTG, MN1191 forms normal colonies on LB plates supplemented with

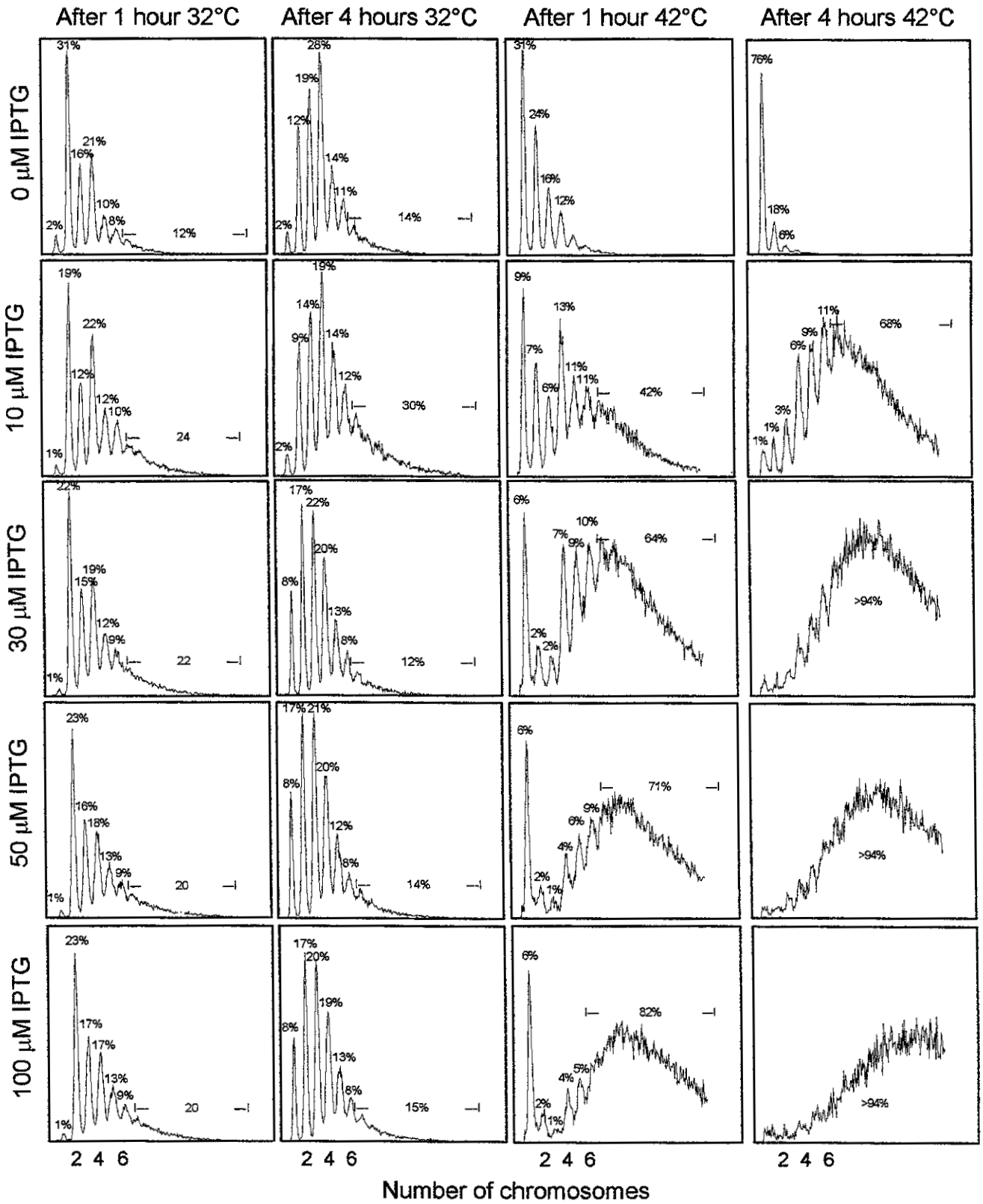


Fig. 3.23. MN1191 (*dnaA204*, *dnaA*(L395P) in λ attachment site) was grown exponentially at 32°C. At OD_{450} = 0.1 the culture was shifted to 42°C for 1 hour and then diluted 20 times and divided in ten portions: five portions were further incubated at 42°C and the rest were shifted back to 32°C. 0, 10, 30, 50 and 100 µM IPTG was added to the cultures at 32°C and 42°C, and samples were taken different times after IPTG addition, treated with rifampicin and analysed using flow cytometer as described in Experimental procedures. The histograms show the DNA distribution after 1 and 4 hours incubation at indicated temperatures with indicated amounts of IPTG.

IPTG. Unfortunately, it was difficult to grow MN1210 (*dnaA*^{0} background) in liquid medium, and therefore, there are no flow cytometric data of this strain.

dnaA(A384V, Y386H) on the chromosome

Results of growth analysis and DNA distributions in rifampicin treated strains harbouring the *dnaA*(A384V, Y386H) allele in λ attachment site are similar to strains harbouring the *dnaA*(L395P) allele (see Table 3.13 and Fig. 3.24). The DnaA(A384V, Y386H) protein also seems to overinitiate replication at 42°C, whereas at 32°C, the number of origins per cell decreases slightly with increasing IPTG concentrations (Fig. 3.24). MN1195 (*dnaA*(A384V, Y386H) in *dnaA204* background) forms colonies at 32°C with and without IPTG, whereas at 42°C IPTG is required (Table 3.13). Thus, like the isogenic *dnaA*(L395P) strain, the apparent high number of origins at 42°C in presence of IPTG does not seem to have any influence on colony formation. Unlike any of the other *dnaA* mutants studied in this work, MN1215 (*dnaA*(A384V, Y386H) in *dnaA*^{0} background) shows discrete peaks after rifampicin (+ cephalexin) treatment (Fig. 3.25). However, the DNA distributions in MN1215 do not change with increasing IPTG concentrations at neither 32 nor 42°C. This is not consistent with observed overinitiation of replication observed at 42°C in the *dnaA204* strain background.

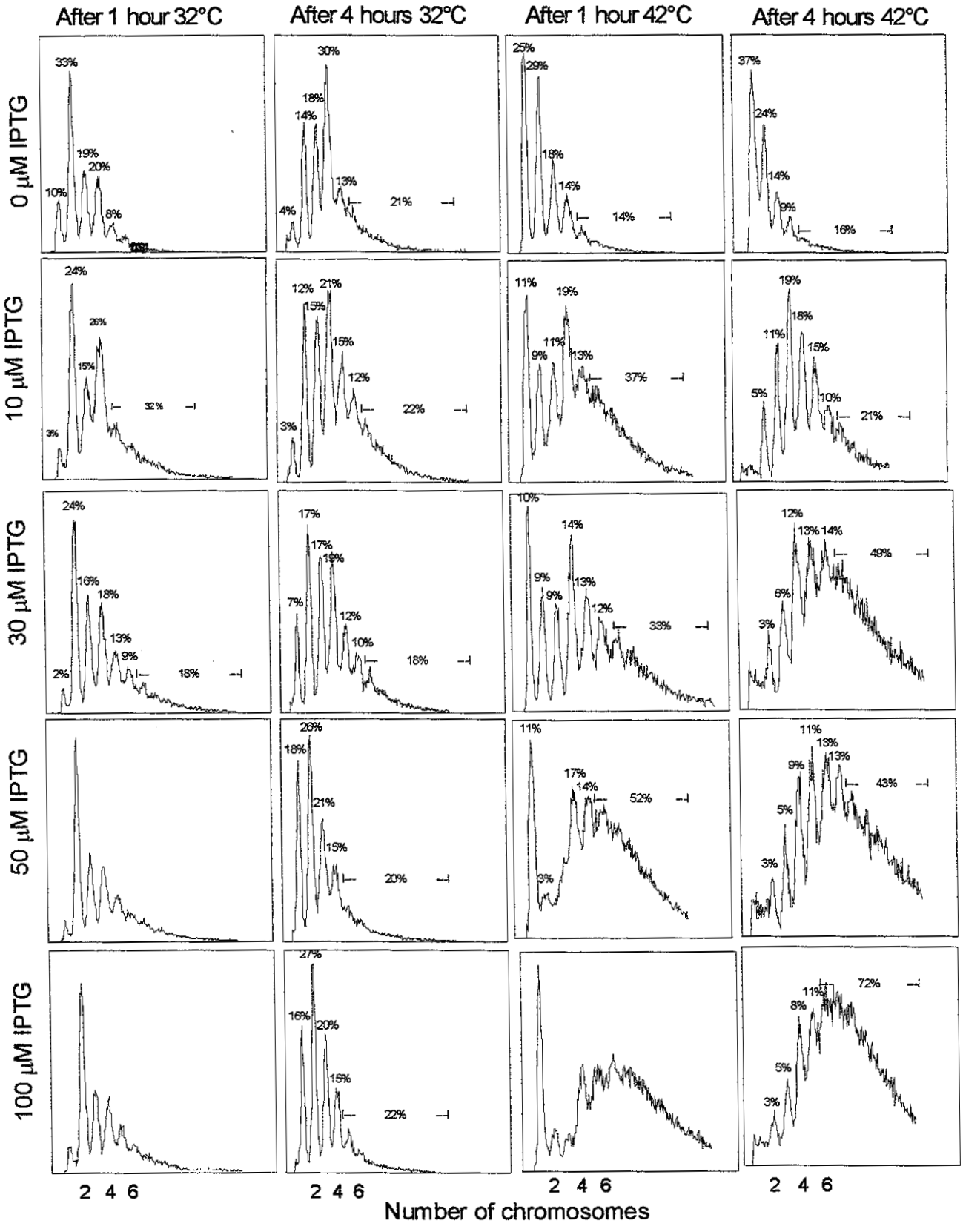


Fig. 3.24. MN1195 (*dnaA204*, *dnaA*(A384V, Y386H) in λ attachment site) was grown exponentially at 32°C. At OD_{450} = 0.1 the culture was shifted to 42°C for 1 hour and then diluted 20 times and divided in ten portions: five portions were further incubated at 42°C and the rest were shifted back to 32°C. 0, 10, 30, 50 and 100 µM IPTG was added to the cultures at 32°C and 42°C, and samples were taken different times after IPTG addition, treated with rifampicin and analysed using flow cytometer as described in Experimental procedures. The histograms show the DNA distribution after 1 and 4 hours incubation at indicated temperatures with indicated amounts of IPTG.

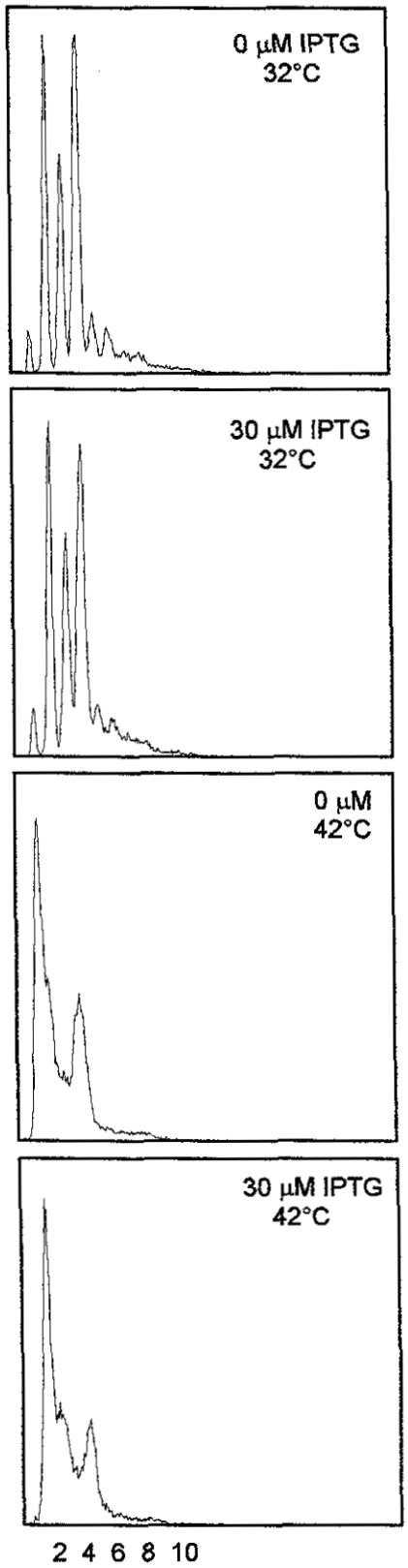


Fig. 3.25. DNA distributions in MN1215 (*dnaA*^{0}, *dnaA*(A384V, Y386H) in λ attachment site). Strains were grown exponentially at 32°C and 42°C in medium supplemented with 30 μM IPTG. At OD_{450}=0.5, the cultures were diluted 10 times in the same medium supplemented with 0, 30 and 100 μM IPTG and incubated at the same temperature. Samples were taken from the cultures after 2, 3 and 4 hours, treated with rifampicin and analysed using flow cytometry as described in Experimental procedures. The histograms show the DNA distribution after 3 hours incubation at indicated temperatures with indicated amounts of IPTG.

dnaA(C441G, V391A) on the chromosome

dnaA204 harbouring the IPTG inducible *dnaA*(C441G, V391A) allele in λ attachment site (MN1197) behaves differently from the other *dnaA* mutants discussed in this work. At 32°C, the number of origins decreases with increasing IPTG concentrations and after 3 hours growth in medium supplemented with 30, 50 and 100 μM IPTG, the cells contain primarily one and two origins (Fig.3.26). MN1197 forms small colonies on LB plates supplemented with 30 μM IPTG, but do not form colonies with 75 or 100 μM IPTG (table 3.13), which is in agreement with the DNA distributions at 32°C with IPTG induction. At 42°C, the chromosome replication is initiated asynchronously, but with a somewhat normal frequency resulting in cells with primarily 2, 3, 4 and 5 origins per cell after three hours incubation with IPTG (Fig. 3.26). The results indicate that the replication is initiated with a constant frequency, and thus, does not increase with increasing DnaA(C441G, V391A) concentrations. According to the DnaA concentration measurements, the DnaA(C441G, V391A) concentration exceeds the DnaA concentration in a wild type strain more than two fold with only 10 μM IPTG induction. Therefore, the initiation of chromosome replication may seem independent of the DnaA(C441G, V391A) concentration, because the DnaA protein concentration is high. As with the other *dnaA* mutants, it would be interesting to measure if the replication is actually overinitiated with increasing DnaA concentrations, but somehow stalled after having passed the *oriC* region as seen when the wild type DnaA protein is overproduced (Atlung *et al.*, 1987; Atlung and Hansen, 1993. See Chapter 1). The DNA distributions in MN1212 (*dnaA*(C441G, V391A) in $dnaA^0$ background) do not show discrete peaks (data not shown).

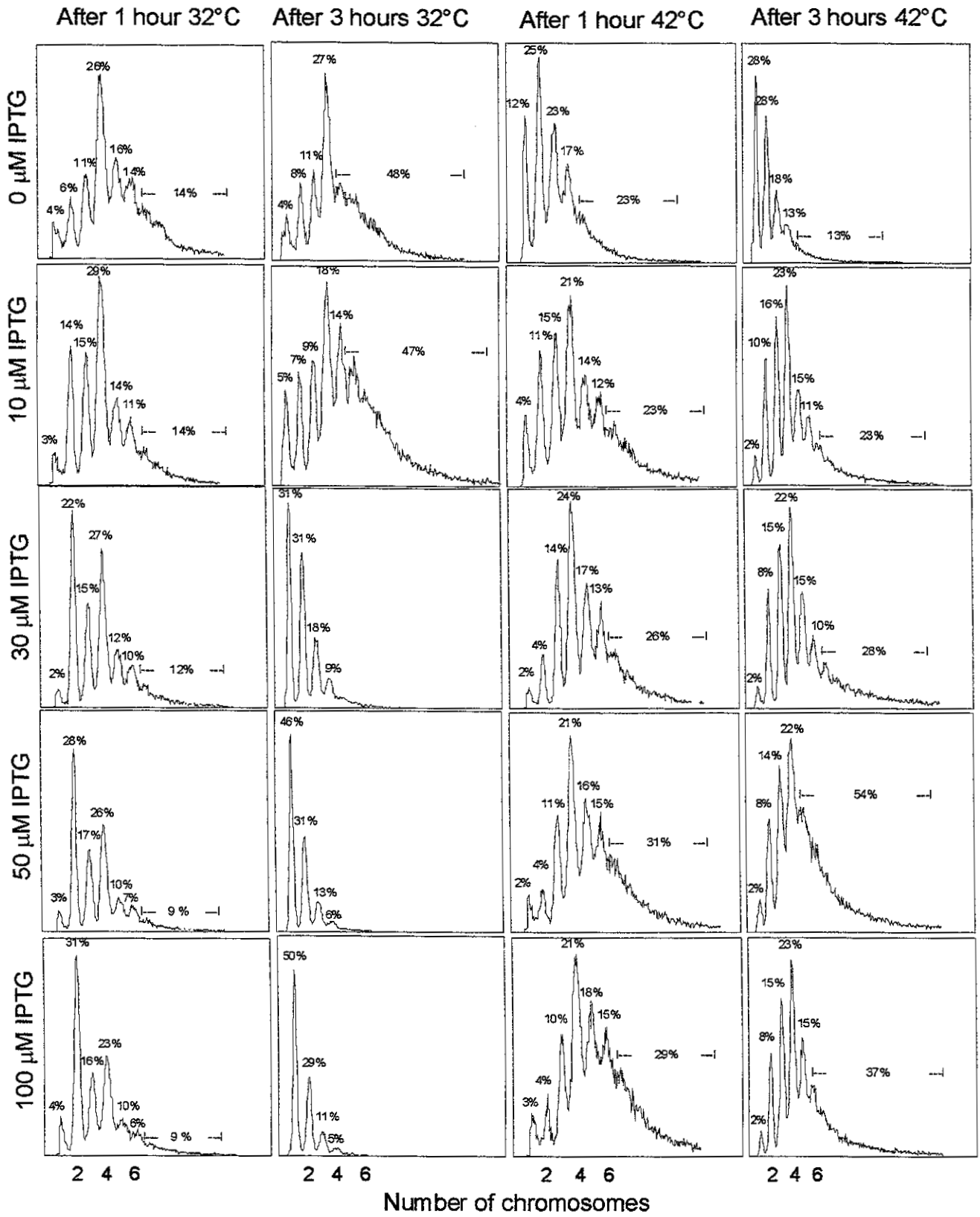


Fig. 3.26. MN1197 (*dnaA204*, *dnaA*(C441G, V391A) in λ attachment site) was grown exponentially at 32°C. At OD_{450} = 0.1 the culture was shifted to 42°C for 1 hour and then diluted 20 times and divided in ten portions: five portions were further incubated at 42°C and the rest were shifted back to 32°C. 0, 10, 30, 50 and 100 µM IPTG was added to the cultures at 32°C and 42°C, and samples were taken different times after IPTG addition, treated with rifampicin and analysed using flow cytometer as described in Experimental procedures. The histograms show the DNA distribution after 1 and 4 hours incubation at indicated temperatures with indicated amounts of IPTG.

Discussion

In this study, more than 100 different DnaA proteins with amino acid substitutions scattered over Domain IV were constructed and studied. The study consisted of a complementation analysis of the high temperature sensitive *dnaA46* and *dnaA204* alleles by induction of multicopy mutant *dnaA(IV)* alleles. Growth at different temperatures on plates supplemented with various amounts of IPTG was determined visually after over night incubations. The result of the complementation analysis, which is shown in Fig. 3.9, indicates that the whole area participates in DNA binding, either directly or indirectly. However, there seems to be a relatively higher number of amino acids in the first half of domain IV and in helix 5 that can be substituted with non-closely related amino acids without any apparent effect on the DnaA protein (Fig. 3.10). The following summarises the results of the complementation analysis carried out in this work. The results are compared to those obtained by Bläsing (1999) and Sutton and Kaguni (1997).

The length of helix 1 seems important for DnaA function. This is based on the observation that amino acid changes that either shorten the helix N-terminally (I379T and A384V) or extend the helix (I389N, DnaA204) have (severe) effects on the DnaA activity. Helix 1 may be located on the surface of the DnaA protein, with D377, K381 and E385, which are located on the same side of the α-helix, exposed to the solvent, and the rest, especially I379, V383 and A384 protected from the solvent and probably participating in hydrophobic interactions with other residues of the DnaA protein. It does not seem possible on basis of this complementation analysis to point at amino acids in helix 1 that are more likely to be interacting with the DNA target than others. On the other hand, this area of the DnaA protein seems sensitive towards amino acid changes, in general, although the secondary structure is only rarely changed. This indicates that some of the amino acids in helix 1, and not only the helix itself, help stabilise the DnaA structure and/or are responsible for DNA contact.

All lysines (K388, K390 and K397) in the basic loop can be substituted with the oppositely charged glutamic acid without any apparent effect on the DnaA protein. This could indicate that these amino acids are not in direct contact with the DNA, but instead interacting with the solvent and thereby keep the loop on the surface of the protein. This is in agreement with the observation that these lysines are not highly conserved and substituted with other polar amino acids in DnaA proteins from other organisms (Fig.

3.1 and 3.4). The results also indicate that R398, as the lysines discussed above, is required for solvent interaction. On the contrary, the highly conserved arginines R399 and R401 cannot be substituted with glutamine or cysteine, respectively, without affecting the DnaA protein. Especially the R399Q substitution has severe effect on the DnaA protein. These observations are in agreement with results obtained by Bläsing (1999), who constructed DnaA proteins with R399A and R401A substitutions, and found that the R399A substitution, in particular, had deleterious effect on the DnaA protein *in vitro*. In fact, it was found that DnaA(R399A) did not bind DnaA boxes R2, R4 and R5+R6 *in vitro*, which led to the suggestion that R399 may be one of the residues interacting with the DNA (Bläsing, 1999). This proposal is not in conflict with results obtained in this study, but it should, however, be noted that the presence of multicopy *dnaA*(R399A) alleles complemented the high temperature sensitive *dnaA46* phenotype *in vivo*, and thus, the DnaA(R399A) protein probably has some affinity to the DnaA boxes in *oriC in vivo* (Bläsing, 1999). The basic loop has been proposed to be part of a basic helix-loop-helix motif (Schaper and Messer, 1997). Alignment of the DNA binding domain of the DnaA protein with other proteins that carry a basic helix-loop-helix motif (Fig. 2.3) indicates that if the DnaA protein carries such a motif, it may be rather modified due to the poor similarity to known helix-loop-helix proteins.

According to the accessibility prediction, many amino acids in helix 2 are protected from the solvent, indicating that a part of this helix is located inside the protein with other hydrophobic amino acids in the surroundings. This hypothesis is consistent with the observation that for instance the A410V and M411I substitutions have less severe effects on the DnaA protein than the A410T and M411T substitutions have. The results also indicate that there may be very little space around A410. It was not possible to construct DnaA proteins with R407 and Q408 substitutions, and this may indicate that substitutions of these residues have severe effects on the DnaA protein. Bläsing (1999) isolated several R407 and a single Q408 substitution, and found that the DnaA proteins were severely affected especially *in vitro*. *in vivo*, however, there seemed to exist some affinity to the DnaA boxes, and the DnaA(Q408R) protein actually repressed the *mioC* promoter (DnaA box R5 and R6) almost as well as the $DnaA^+$ protein did (Bläsing, 1999). However, Bläsing suggests that both R407 and Q408 interact with the DNA. The proposal that R407 participate in DNA binding is in agreement with results obtained by Sutton and Kaguni (1997), who found that the DnaA(R407H) protein retained only 20% of the affinity for *oriC in vitro* compared to the affinity of the $DnaA^+$ protein. Sutton and

Kaguni (1997) also found that substitution of the not conserved A412 in helix 2 with proline resulted in lost affinity to *oriC*. This does not seem to be due to changes in the secondary structure (data not shown).

It was not possible in this work to obtain substitutions of many of the highly conserved amino acids in helix 3 and the loops surrounding it. This may indicate that they cannot be substituted without deleterious effects on the DnaA protein. The presence of highly conserved G430 and G431 that were not substituted neither in this work nor in previously published work may indicate that DnaA activity depends on a turn between helix 3 and 4. Based on alignment of domain IV of the DnaA protein with proteins that carry a helix-turn-helix motif, it was suggested in Chapter 2 (Fig. 2.2) that this area could be part of a modified helix-turn-helix motif, and the results of the complementation analysis presented in this study do not argue against this proposal. Sutton and Kaguni (1997) constructed a DnaA(G426D) protein, and found that it retained only 12% of the affinity to *oriC*. This seems contradicting with results presented in this study, where it was found that the DnaA(G426S) protein (the secondary amino acid substitution in the DnaA5 protein) retained 70% of the affinity for *oriC in vitro*. None of the amino acid substitutions seems to change the secondary structure (data not shown).

In general, helix 4 and 5 have few single substitutions and many double substitutions, and this makes it relatively difficult to interpret the results. However, there seems to be a tendency that the presence of a coiled structure between the two parts of the helix is important, and that the length of it is critical. Helix 4 is more sensitive towards amino acid substitutions than helix 5, which is in agreement with how conserved the regions are (Fig. 3.2). Amino acids in the beginning of helix 4 that are highly conserved are not substituted in any of the DnaA proteins constructed in this work. This strongly suggests that they are required for DnaA function, and they may be interacting with the DNA. Those amino acids that are predicted to be protected form the solvent with high reliability cannot be substituted with polar amino acids without affecting the DnaA protein. These amino acids include V437, A440, I444, I454, F458 and L461. The hydrophobic amino acids are located on the same side of the α-helix (or close to). The two hydrophobic amino acids I454 and F458 are substituted with threonine and serine, respectively, in one of the double mutants. This DnaA(I454T, F458S) protein complements the *dnaA46* phenotype better than DnaA(I454N) and DnaA(F458S, Q380R), which indicates that there could be (weak) interactions between I454 and F458

that is maintained to some extend in the double mutant where both amino acids are substituted with polar, hydrogen bonding residues. Helix 4 and 5 are probably located on the surface of the DnaA protein with a hydrophobic and hydrophilic side and a hydrophilic coil in between. Helix 4 could be responsible for DNA contact and as mentioned previously be part of a modified helix-turn-helix motif (Fig. 2.2). Bläsing (1999) also suggests that relatively many amino acids in helix 4 interact with the DNA. These include H434, T435, T436 and A440. The proposal is mainly based on *in vitro* binding analysis where it was found that substitutions of these residues severely reduced the affinity for DnaA box R2, R4 and R5+R6. However, *in vivo* complementation analysis of the *dnaA46* phenotype by induction of multicopy mutant *dnaA* alleles showed that substitutions of these residues in helix 4 resulted in good complementation of the *dnaA46* phenotype (Bläsing, 1999). Thus, it seems that the mutant DnaA proteins are somehow activated *in vivo*, or alternatively, the DnaA46 proteins present in the cells in the *in vivo* experiments bind the DnaA boxes, and thereby participate in the initiation of replication. Sutton and Kaguni (1997B) isolated DnaA proteins with T435M, V437M and A440V/T substitutions in helix 4. They find that they all, except for the DnaA(T435M) protein, have retained only app. 10% of the affinity for *oriC in vitro*. However, the DnaA(A440T) protein was nearly wild type like *in vivo* (Sutton and Kaguni, 1997C), which suggests that A440 does not interact with the DNA. The DnaA(T435M) retained 50% of the affinity for *oriC in vitro*, but the binding was found to be non-specific, and thus, it is suggested that T435 participate in the specific recognition of *oriC* (Sutton and Kaguni, 1997B). T435 is substituted with lysine in the cold sensitive *dnaX* suppressor DnaA(71) (Gines-Candelaria *et al.*, 1995). Binding analysis indicates that the affinity of DnaA(T435K) for various DnaA boxes are reduced *in vitro*, but more than 50% of the affinity is retained *in vivo* (Bläsing, 1999).

Dissociation constant of the DnaA$^+$-oriC interaction

In this study, the *in vitro* dissociation constant (K_d) of DnaA$^+$ for DnaA boxes in the *oriC* area (including R5 and R6) was estimated to be 0.75 ± 0.14 nM. This is in good agreement with results obtained by Schaper and Messer (1995), who measured the dissociation constant of DnaA bound to the whole *oriC in vitro* by using gel retardation assay and found that K_d (DnaA-*oriC*) = 0.4 ± 0.2 nM. The dissociation constants of DnaA binding to individual DnaA boxes were lower, ranging from 0.9 to >200 nM, which is in agreement with results obtained by Bläsing (1999). However, the

dissociation constant determined in this study is 10 fold lower than obtained by Kitagawa *et al.* (1996). They measured the dissociation constant *in vitro* by using nitrocellulose filter assays, and found that K_d (DnaA-*oriC*) was 8.6 nM. Majka *et al.* (1999) studied the interaction of DnaA from *Streptomyces lividans* to its target and found that the dissociation constant was in the order of 1.25 to 12 nM depending on the number of DnaA boxes. Thus, the *in vitro* dissociation constant of the DnaA-*oriC* complex obtained in this study is qualitatively similar to previously published *in vitro* dissociation constants that were determined using other methods.

in vivo and in vitro binding data of DnaA proteins with amino acid substitutions in domain IV

In this study, further work was carried out with a selection of mutant DnaA proteins. The analysis included *in vivo* and *in vitro* binding assays. In addition, the stability of the various proteins was determined *in vivo*. Table 3.14 summarises the results. The following discussion is an attempt to compare the different characteristics of the various DnaA proteins. However, it does seem problematic primarily because the experiments were not carried out under the same conditions. One of the important differences between the *in vivo* and *in vitro* binding data is that the *in vivo* binding data are based on experiments carried out at 42°C (to avoid too much influence of the chromosomally encoded DnaA204 or DnaA46 proteins), while the *in vitro* binding data have been calculated from experiments carried out at room temperature. Therefore, an apparent disagreement between the *oriC* affinities determined *in vivo* and *in vitro* might be because the activity of the DnaA protein in question is temperature dependent. In addition, the *oriC* fragment used *in vitro* include DnaA box R1, M, R2, R3, R4, R5 and R6, while the *mioC* promoter used in estimation of *in vivo* affinities only carries DnaA box R5 and R6. Since the initiation of chromosome replication probably does not require binding to R5 or R6, the *in vivo* affinity for *oriC* (= the ability to complement the heat sensititive *dnaA46* and *dnaA204* phenotypes) involve binding to R1, M, R2, R3 and R4.

The DnaA(**I379T**) protein is as stable as the $DnaA^+$ protein, and thus, the apparent reduced *in vivo* activity seems to be due to reduced *oriC* and *mioC* promoter affinity. This is also consistent with the *in vitro* data. The substitution may change the secondary structure by shortening helix 1 N-terminally. Bläsing (1999) isolated an I379S version of the DnaA protein, and this substitution seems more severe than the substitution with

Table 3.14. Summary of *in vivo* and *in vitro* binding data

DnaA	*dnaA46/dnaA204* complementation[1]	*in vivo* affinity to *mioC* promoter (norm.)[2]	*in vitro* affinity to *oriC* (norm.)[3]	*in vitro* Specificity (norm.)[4]	Stability 32°C T½ (generations)[5]	Stability 42°C (T½) (generations)[5]
DnaA$^+$	+++++	1.00	1.00	1.00	> 4	> 4
I379T	++	0.65	0.63	0.77	> 4	> 4
DnaA205 (V383M)	+	0.64	1.06	0.52	2.2	1.4
A384V, Y386H	+++	0.70	0.97	0.33	> 4	> 4
Y386H	+++++	0.98	0.91	1.11	> 4	> 4
Y387S	++	0.75	0.62	0.53	1.5	1.5
DnaA204 (I389N)	+	0.66	0.93	0.34	1.9	1.5
L395P	+++++	0.80	1.03	0.21	4.0	2.1
K397E	+++++	0.94	3.13	1.33	> 4	> 4
R401C, K443R	+++	0.57	1.01	0.39	> 4	2.8
DnaA211 (M411T)	++	0.65	1.01	0.56	> 4	2.1
T418A	++++	0.84	0.48	0.88	> 4	> 4
E424G	+	0.67	0.86	0.33	3.3	1.8
F429S	++	0.65	0.32	0.78	> 4	4.0
V437M	+++	0.69	0.59	0.41	2.4	1.6
L438P	0	0.62	0.59	0.57	2.2	1.8
C441G, V391A	+++	0.68	0.53	0.80	> 4	3.1
E449G, V383A	++	0.76	1.14	0.79	2.2	1.8
I454N	0	0.60	0.93	0.49	> 4	2.0
L465S, V391A	++++	0.56	1.47	0.50	> 4	2.8

1) Complementation of high-temperature sensitive *dnaA46* and *dnaA204* strains harbouring different multicopy IPTG inducible mutant *dnaA* alleles. 5 +: good complementation like DnaA$^+$ (complementation with 6 and 10 μM IPTG), 4 +: slightly reduced complementation, 3 +: reduced complementation (requires 30 μM IPTG), 1-2 +: severely reduced complementation, 0: no complementation. See Results, section I. 2) The β-gal activity was measured in a *dnaA204*, P_{mioC}-*lacZ* strain harbouring plasmids with different IPTG inducible *dnaA* alleles. Fold repression (= affinity to DnaA box R5 and R6) is normalised to that of the DnaA$^+$ protein. See Results, section II. 3) K_d was calculated by GraFit using the formula for the binding of one ligand. The values are expressed as affinities to DnaA boxes in *oriC* normalised to the affinity of the DnaA$^+$ protein. See Results, section II. 4) Specificity was determined by adding increasing amounts of salmon sperm DNA together with a fixed amount of *oriC* DNA to the various DnaA proteins. The specificity is normalised to that of the DnaA$^+$ protein. 5) Stability was determined by measuring the amount of DnaA protein different times after chloramphenicol addition. The proteins half-lives are expressed as generation times. See Results, section I.

threonine both *in vivo* and *in vitro*. The **DnaA205**, **DnaA204** and **DnaA211** proteins are similar. They are all unstable *in vivo* compared to the DnaA$^+$ protein, especially at 42°C, and this may partly explain why the proteins apparently complement the *dnaA46* phenotype and bind the *mioC* promoter weakly. The affinity for *oriC in vitro* is unaffected, whereas the specificity is reduced. The intact affinity *in vitro* may be expected considering that the experiments were carried out at room temperature. However, if the specificity is reduced *in vivo* as observed *in vitro* then it is expected to observe an apparent reduced affinity for *oriC in vivo*, because the chromosome titrates the DnaA proteins. Bläsing (1999) did not analyse these proteins *in vitro*. However, the affinity of DnaA205 for the *mioC* promoter at 37°C was similar to that of the DnaA$^+$ protein, whereas the DnaA211 protein was found not to repress the *mioC* promoter at all. The DnaA204 protein had reduced affinity to the *mioC* promoter as obtained in this study. The DnaA(**R401C, K443R**) protein has (slightly) reduced affinity for *oriC* and the *mioC* promoter *in vivo*, while the affinity *in vitro* is unaffected. The protein has retained 30-40% of the specificity towards *oriC*, and this may be the reason for the reduced complementation of the *dnaA46* allele and reduced *mioC* promoter affinity, because as discussed above, the DnaA proteins bind elsewhere on the chromosome. R401 is one of the highly conserved basic residues in the basic loop, and the *in vivo* and *in vitro* binding data indicates that R401 is required for the specific recognition of *oriC* and not for obtaining a high affinity *per se*. Bläsing (1999) found that the DnaA(R401A) protein bound as well to the DnaA box R5 and R6 in the *mioC* promoter as the DnaA$^+$ protein did. However, *in vitro* results indicated that the protein had between 25 and 100 fold reduced affinity depending on the DnaA box. The DnaA(**Y386H**) protein behaves like the DnaA$^+$ protein in all aspects, and thus, Y386 probably does not interact with the DNA. DnaA(**Y387S**) and DnaA(**V437M**) proteins have reduced *in vivo* and *in vitro* affinities, reduced specificity and reduced stability. The substitutions probably do not change the secondary structure of the DnaA protein, and thus, the reduced function of DnaA(Y387S) and DnaA(V437M) may be a consequence of substituting the highly conserved tyrosine and valine. According to the complementation analysis, substitution of Y387 with histidine has no effect on the DnaA protein, which indicates that the shape of the amino acid is important at this position. Sutton and Kaguni (1997) analysed DnaA(V437M) *in vitro* and found that the protein only retained 9% of the affinity to *oriC*, which is an even more severe effect than observed in this study.

DnaA(**T418A**) has retained most of the *in vivo* affinity while the *in vitro* affinity is reduced. This protein has high specificity towards *oriC*, which may explain why the *in vivo* affinities are unaffected. Helix 2 is predicted to be slightly extended in the DnaA(T418A) protein. The DnaA(**E424G**) protein complemented the *dnaA46* and *dnaA204* phenotypes poorly, whereas the protein retained 67% of the affinity to DnaA box R5 and R6 *in vivo*. This may be due to different affinities to different DnaA boxes *in vivo* (see below). The affinity for *oriC in vitro* is only slightly reduced, whereas the specificity is more severely affected. This seems consistent with the *in vivo* results and indicates that the DnaA(E424G) protein has reduced specificity towards DnaA boxes in *oriC* and not towards the *mioC* promoter. According to the secondary structure predictions, the DnaA(E424G) protein may not have a helix 3. Therefore, it is expected that the substitution has effect on the DnaA protein. In fact, an even more severe effect could be expected if this helix were part of a helix-turn-helix motif as suggested in Chapter 2 (see Fig. 2.2). However, the affinity for *oriC* is dramatically reduced *in vivo* in agreement with the proposal. The DnaA(**F429S**) protein has the lowest affinity for *oriC in vitro* than any of the other proteins studied in this work. Accordingly, the *in vivo* affinity to *oriC* is reduced, while the *in vivo* affinity for the *mioC* promoter and the *in vitro* specificity towards *oriC* is less affected. DnaA(**L438P**) does not complement the *dnaA46* phenotype at 42°C, and thus, the protein apparently has no affinity for *oriC in vivo* at high temperatures. The *in vivo* and *in vitro* affinities and the specificity, is approximately 60% of the values of the DnaA$^+$ protein. The L438P substitution may reduce the length of helix 4 N-terminally, and this may be the reason for the observed severe effects on DnaA activity *in vivo*, especially if helix 4 is part of a helix-turn-helix motif. The secondary structure is affected in the DnaA(**E449G, V383A**) protein where the small loop between helix 4 and 5 may be extended with a few amino acids. This may be the reason for the reduced affinity of DnaA(E449G, V383A) for *oriC in vivo*. The *in vivo* affinity to DnaA box R5 and R6 is less affected, and the *in vitro* affinity not affected at all. In fact, the results indicate that DnaA(E449G, V383A) has slightly higher affinity to *oriC in vitro* than the DnaA$^+$ protein has and the protein has retained 80% of the *in vitro* specificity towards *oriC*. DnaA(**I454N**) does not complement the *dnaA46* and *dnaA204* phenotypes at 42°C indicating that the protein is high temperature sensitive. However, the protein binds DnaA box R5 and R6 *in vivo*, and the affinity for *oriC in vitro* seems unaffected. The DnaA(**L465S, V391A**) protein has 50% higher affinity for *oriC in vitro* than the DnaA$^+$ protein has. However, a higher affinity is not

reflected *in vivo* where the *mioC* promoter is two fold derepressed and the *dnaA46* phenotype not complemented as well as when $DnaA^+$ proteins are present. This may partly be explained by the observation that the protein has lost 50% of the specificity towards *oriC*, and therefore these DnaA proteins may have a tendency to bind elsewhere on the chromosome.

The DnaA(384V, Y386H), DnaA(L395P), DnaA(K397E) and DnaA(C441G, V391A) proteins

In addition to the *in vivo* and *in vitro* binding experiments discussed above, the initiation frequency was analysed in a number of strains carrying different *dnaA* alleles in λ attachment site. The strains that were analysed carry a *dnaA204* allele in the normal chromosomal position and a $dnaA^+$, *dnaA*(384V, Y386H), *dnaA*(L395P), *dnaA*(K397E) and *dnaA*(C441G, V391A) allele in λ attachment site. The results of the *in vivo* and *in vitro* binding data and the initiation frequencies at different temperatures under different growth conditions are summarised below (see Table 3.14 and 3.15).

Those *in vivo* binding results that are based on complementation of the *dnaA46* phenotype and binding of the *mioC* promoter by IPTG induction of multicopy *dnaA*(A384V, Y386H) alleles indicate that the protein has reduced *in vivo* affinity for the *oriC* and the *mioC* promoter at 42°C, while the *in vitro* affinity is unaffected (Table 3.14). The proteins have retained 30-40% of the specificity towards *oriC*, and this may be the reason for the reduced complementation of the *dnaA46* allele and reduced *mioC* promoter affinity *in vivo*, because the DnaA proteins bind elsewhere on the chromosome. However, when the IPTG inducible *dnaA*(A384V, Y386H) allele is inserted into λ attachment site on the chromosome of a *dnaA204* strain (MN1195), the strain seems to overinitiate replication at 42°C, whereas at 32°C, the number of origins per cell decreases slightly with increasing IPTG concentrations (Fig. 3.24 and Table 3.15). Thus, a reduced affinity for *oriC in vivo* is not reflected in MN1195 where the *dnaA*(A384V, Y386H) allele is present in single copy. On the contrary, the affinity for *oriC in vivo* seems increased at 42°C and maybe also at 32°C. MN1195 forms colonies at 32°C with and without IPTG, whereas at 42°C IPTG is required (Table 3.13 and 3.15). The apparent high number of origins at 42°C in presence of IPTG does not seem to have any influence on colony formation. The results indicate that if the *dnaA*(A384V, Y386H) allele were inserted into its normal chromosomal position, the strain would be temperature resistant.

Table 3.15. Summary of growth and initiation frequency observed in *dnaA204* strains harbouring different IPTG inducible *dnaA* alleles in λ attachment site [1)]

Strain	*dnaA* in λ att.	32°C				42°C			
		No IPTG		+ IPTG		No IPTG		+ IPTG	
		Colony formation	Initiation frequency	Colony formation	Initiation frequency	Colony formation	Initiation frequency	Colony formation	Initiation frequency
MN1240	*dnaA*$^+$	+++	Normal [2)] Asynchronous	+++	Normal, Synchronous	-	Stop [3)]	+++	Normal, Synchronous
MN1193	*dnaA*(K397E)	+++	Slightly overinitiated, Asynchronous	- [4)]	Stop	+++	Slightly overinitiated, Asynchronous	-	Stop
MN1191	*dnaA*(L395P)	+++	Slightly overinitiated, Asynchronous	+++	Overinitiated	-	Stop	+++	Overinitiated
MN1195	*dnaA*(A384V, Y386H)	+++	Slightly overinitiated, Asynchronous	+++	Overinitiated	-	Stop	+++	Overinitiated
MN1197	*dnaA*(C441G, V391A)	+++	Slightly overinitiated, Asynchronous	+	Stop [5)]	-	Stop	+++	Normal, Asynchronous

1) The summary is based on data presented in Table 3.13 and in Fig. 3.19 - 3.26.
2) Normal initiation frequency at 32°C without IPTG induction means that the cells contain 1, 2, 3, 4 chromosomes and a small number contains 5 and 6 chromosomes after rifampicin treatment. See Fig. 3.19. With IPTG induction, normal initiation frequency means that the cells co ntain no more than 8 chromosomes per cell. Whether the chromosome replication is initiated synchronously or not is indicated.
3) The cells seem to stop initiation of replication.
4) - : No coloni formation
5) This strain overinitiates replication with 10 μM IPTG, but with higher IPTG concentrations, the initiation of replication stop (Fig. 3.26)

The DnaA(L395P) protein has retained (almost) all *in vivo* and *in vitro* affinity for *oriC*, but seems to have lost the specificity towards *oriC in vitro* (Table 3.14). The amino acid substitution probably introduces a β-sheet like structure in the loop, and this may be one of the reasons for the lost specificity observed *in vitro*, which on the other hand is not reflected in the *in vivo* experiments. *dnaA204* carrying the IPTG inducible *dnaA*(L395P) allele in λ attachment (MN1191) site resembles the strain with the *dnaA*(A384V, Y386H) allele. With only small amounts of IPTG, the replication is overinitiated extensively at 42°C, while it is relatively unaffected at 32°C, although the replication is initiated asynchronously (Fig. 3.23 and Table 3.15). Thus, although the DnaA(L395P) protein seems very $DnaA^+$ like *in vivo* when the *dnaA*(L395P) allele is present in multicopy, the single copy version reveals large differences between the DnaA(L395P) and $DnaA^+$ proteins. The DnaA(L395P) protein might have increased affinity for *oriC* at 42°C, which is not detected *in vitro* due to the *in vitro* experiments were carried out at room temperature. MN1191 forms colonies in presence of IPTG at 32°C and 42°C, which indicates that if the *dnaA*(L395P) allele was inserted into its normal chromosomal position, the strain may be temperature resistant.

When present in multicopy, the results indicate that the DnaA(K397E) protein has *in vivo* affinities comparable to those of the $DnaA^+$ protein, while *in vitro*, the affinity is three fold higher and the specificity 1.3 fold higher compared to $DnaA^+$. The strain harbouring the IPTG inducible *dnaA*(K397E) allele (MN1193) cannot form colonies in the presence of IPTG (Table 3.13 and 3.15). Flow cytometric analysis of rifampicin treated cells indicates that the strain stops initiation of replication in presence of IPTG, while it initiates chromosome replication asynchronously without IPTG induction (Fig. 3.22 and Table 3.15) where the DnaA concentration is only one fourth of the DnaA concentration in a $dnaA^+$ strain (Fig. 3.18). The results strongly suggest that the DnaA(K397E) protein has increased *oriC* affinity *in vivo* as well as *in vitro*. The reason why this strain cannot initiate replication in presence of IPTG could be that too high DnaA(K397E) concentrations result in accumulation of a large number of DnaA(K397E) proteins at *oriC* that will not 'fall off', which makes it impossible for the replication machinery to elongate the initiation. If the *dnaA*(K397E) allele was inserted into its normal chromosomal position, the strain may not survive due to the high *oriC* affinity.

The DnaA(C441G, V391A) protein has reduced affinity for *oriC* and the *mioC* promoter *in vitro* and *in vivo* (Table 3.14). The protein is slightly unstable at 42°C, but that cannot account for the observed effect the substitution has on the DnaA protein. A strain that carries the IPTG inducible *dnaA*(C441G, V391A) allele in λ attachment site (MN1197) has a different chromosome configuration compared to the other strains. At 32°C, colony formation is IPTG sensitive (table 3.13), while at 42°C, growth is independent on the IPTG concentration. Flow cytometric analysis of rifampicin treated cells indicates that at 32°C, the initiation of replication stops in presence of high DnaA(C441G, V391A) concentrations (Fig.3.26 and Table 3.15), while at 42°C, the chromosome replication is initiated asynchronously, but with a somewhat normal frequency. If the *dnaA*(C441G, V391A) allele were inserted into the normal chromosomal position, the results indicate that the strain may be cold sensitive. An explanation for the observed effect on the DnaA protein may be that helix 4 is predicted to start at K443 (data not shown), and thus, a big part of helix 4 may actually be missing in the DnaA(C441G, V391A) protein.

Summary of binding data

The DnaA proteins with the highest *in vitro* affinity but low specificity are the classical DnaA mutant proteins DnaA205 and DnaA204 and DnaA(A384V, Y386H), DnaA(L395P) and the DnaA(R401C, K443R) protein. These amino acid substitutions are located in α-helix 1 and in the basic loop, and this may suggest that this part of the DNA binding domain participates in the specific recognition of the DnaA box. In Chapter 2 it is proposed that helix 3 and 4 constitute a helix-turn-helix motif based on amino acid alignments of proteins with known helix-turn-helix motifs (Fig. 2.2) and that the basic loop between helix 1 and 2 may participate in DNA bending through electrostatic attraction between the basic residues and DNA backbone. These residues are therefore suggested not to be important for the specific recognition of the DnaA box. However, the *in vitro* affinity and specificity constants indicate that it may in fact be residues in helix 1 and in the basic loop that are responsible for the specificity towards DnaA box sequences. Thus, this may point towards the already published proposal that the DnaA protein possess a basic helix-loop-helix motif (Roth and Messer, 1995; Schaper and Messer, 1997). However, substitutions of the basic residues in the loop seem less deleterious than expected, except for R399 substitutions. On the contrary, substitution of K397 with glutamic acid actually seems to increase the DnaA activity *in*

vivo and *in vitro*. In this work, it was not possible to substitute the highly conserved amino acids in helix 4 (proposed 'recognition helix', Chapter 2), and these residues may therefore interact with the DNA. This seems in agreement with results obtained by Bläsing (1999) and Sutton and Kaguni (1997), who suggest that H434, T435, T436, V437 and A440 may interact with the DNA. In addition, no DnaA mutants with substitutions of the highly conserved glycines G431 and G432 in the loop between helix 3 and 4 has been isolated and analysed.

In several cases, there seem to be a nice correlation between the ability to complement the *dnaA46* and *dnaA204* phenotypes and the affinity for the DnaA boxes in the *mioC* promoter, and that is in spite of differences in the DnaA box sequences (Fig. 1.1 and Table 2.2). The mutant DnaA proteins that required 30 μM IPTG to complement the high temperature sensitive *dnaA46* phenotype retained 60-70% of the affinity for R5 and R6 compared to the $DnaA^+$ protein, and those DnaA proteins that complemented the high temperature sensitive *dnaA46* phenotypes as well as the $DnaA^+$ protein did also repressed the *mioC* promoter to almost the same extend as the $DnaA^+$ protein did. The observation that some mutant DnaA proteins have a high *mioC* promoter affinity and yet are unable to complement the *dnaA46* phenotype, and vice versa, have reduced affinity to the *mioC* promoter in spite of good complementation of the *dnaA46* phenotype is interesting. It may indicate that these amino acids participate in the specific recognition and are responsible for the different affinities for the DnaA boxes in *oriC*. As discussed in Chapter 1 and 2, different affinities to DnaA boxes in *oriC* has been observed, and it has been suggested that DnaA proteins bind R3 late in the cell cycle and that this interaction is the triggering event (Samitt *et al.*, 1989; Cassler *et al.*, 1995). DnaA proteins with the following amino acid substitutions E424G, L438P, E449G, I454N, V383A (DnaA205) and I389N (DnaA204) seem to have retained 60-70% of the affinity to R5 and R6 and almost no *oriC* affinity *in vivo*, while the DnaA(L465S) protein has reduced R5 and R6 affinity and intact *oriC* affinity. The amino acids are located close to or in helix 1 and the last half of domain IV.

Chapter 4

Analysis of the A184V, H252Y and G426S substitutions in the DnaA protein

Introduction

The *dnaA*(A184V) mutation is found in several *dnaA* mutants. However, the mutation never exists alone, but always in the presence of secondary intragenic mutations (Hansen *et al.*, 1992). It has been shown that multicopy *dnaA*(A184V), *dnaA5* and *dnaA46* strains are cold sensitive (Nyborg *et al.*, 2000; Hansen *et al.*, 1992), whereas multicopy *dnaA*(H252Y) and *dnaA*(G426S) (secondary mutations from *dnaA46* and *dnaA5*, respectively) strains are temperature resistant like multicopy $dnaA^+$ strains. The multicopy *dnaA*(A184V), *dnaA5* and *dnaA46* strains (and the *dnaAcos* mutant) were further analysed, and it was found that the mutants could be divided into two groups. The first group, which included the multicopy *dnaA*(A184V) and *dnaA5* strains, initiated chromosome replication extensively at non-permissive temperature, but the initiations were not elongated and DNA synthesis stopped. Group two, which included the multicopy *dnaA46* strain and the *dnaAcos* mutant, also overinitiated chromosome replication at non-permissive temperature, but (some of) these initiation were elongated and resulted in an increased DNA content in the cells, especially in the multicopy *dnaA46* cells (Nyborg *et al.*, 2000).

In this study, the affinity and specificity of DnaA(A184V), DnaA5, DnaA46, DnaA(H252Y) and DnaA(G426S) for *oriC* was analysed *in vitro*. In addition, strains carrying the *dnaA*(A184V) allele in λ attachment site were constructed and the initiation frequencies determined using flow cytometry.

Experimental procedures

E.coli strains and plasmids used in this work

Table 4.1. Strains and plasmids used in this work.

Strain or plasmid	Genotype	Reference/source
MC1000	*araD139*, Δ(*ara,leu*)7697, Δ*lac*, X74, *galU*, *galK*, *strA*	Casabadan and Cohen (1980)
TC1929	a, *dnaA204*, λRB1	Hansen and Atlung (1995A)
MN1229	a, *dnaA204*, P_{MT}-biotin-*dnaA*(A184V) in λ att	This work
TC3482	a, $dnaA^0$, *rnh373*	Hansen *et al.*(1991A)
MN1209	a, $dnaA^0$, P_{MT}-biotin-*dnaA*(A184V) in λ att	This work
p*dnaA*(H252Y)	pFHC539, *dnaA*(H252Y)	Nyborg *et al.* (2000)
p*dnaA*(G426S)	pFHC539, *dnaA*(G426S)	Nyborg *et al.* (2000)
p*dnaA*(A184V)	pFHC539, *dnaA*(A184V)	Nyborg *et al.* (2000)
p*dnaA46*	pFHC539, *dnaA46* (A184V, H252Y)	Hansen *et al.* (1992)
p*dnaA5*	pFHC539, *dnaA5* (A184V, G426S)	Hansen *et al.* (1992)
pMN242	pBEX5BA, $dnaA^+$	b)
pMN1007	as pMN242, *dnaA*(A184V)	this work
pMN1150	as pMN242, *dnaA*(H252Y)	this work
pMN1151	as pMN242, *dnaA46*	this work
pMN1152	as pMN242, *dnaA*(G426S)	this work
pMN1153	as pMN242, *dnaA5*	this work

a) genotype as MC1000
b) For construction of pMN242 see Chapter 3, Experimental procedures, section I and Fig. 3.5

MN1229 and MN1209 were constructed as described in Chapter 3, Experimental procedures, section III and shown in Fig. 3.7. pMN1007 was constructed by ligation of the 753 bp *Bsi*WI-*Age*I fragment from p*dnaA*(A184V) with the *Bsi*WI + *Age*I digested fragment from pMN242. Both fragments were gel purified before ligation. The correct clone no longer carried the *Sph*I recognition site in the *dnaA* gene due to the *dnaA*(A184V) mutation. pMN1150 and pMN1151 were constructed as follows: p*dnaA*(H252Y), p*dnaA46* and pMN242 were digested with *Eco*RI and *Bst*EII. The 1058

bp fragment carrying the *dnaA46* mutation(s) from p*dnaA*(H252Y) and p*dnaA46* was cloned into pMN242 to construct pMN1150 and pMN1151, respectively. pMN1152 and pMN1153 were constructed by PCR amplification of p*dnaA5* using primers that flank the DNA binding domain (for primer sequence see Chapter 3, Experimental procedures, section I. Primers are called '*Bst*EII' and '*Hin*dIII'). The PCR reaction was run as a standard PCR reaction (Chapter 3, General procedures) with an annealing temperature of 49°C. One of the primers anneal just upstream the *Bst*EII site prior the DNA binding encoding sequence, and the other anneal just after the *dnaA* gene and has a *Hin*dIII site build into it. The PCR fragment was digested with *Bst*EII and *Hin*dIII and cloned into pMN242 to construct pMN1152 or into pMN1007 to construct pMN1153. The part of the *dnaA* genes that encode domain IV in pMN1152 and pMN1153 were sequenced using a Thermo Sequenase Sequencing kit from Amersham with the '*Bst*EII' primer as sequencing primer (see Chapter 3, Experimental procedures, section I).

oriC-DnaA affinity and specificity

See Chapter 3, Experimental procedures, section II and Fig. 3.6.

Secondary structure predictions

The PHD server with the following address was used to predict the secondary structure of the DnaA proteins in this study: www.embl-heidelberg.de/predictprotein/predictprotein.html.

Stability of biotin-tag DnaA proteins

See Chapter 3, Experimental procedures, section I.

Growth of dnaA204 and dnaA^0, rnh$^+$ strains with dnaA(A184V) allele in λ attachment site

See Chapter 3, Experimental procedures, section III.

Results

in vitro affinity and specificity

Strains harbouring pMN242 (p*dnaA*$^+$) and pMN242 derivatives carrying *dnaA*(A184V), *dnaA5*, *dnaA46*, DnaA(H252Y) and *dnaA*(G426S) alleles were induced with IPTG and the supernatant of the sonicated and centrifuged bacterial lysates were mixed with streptavidin coated magnetic beads as described in Experimental procedures. Radioactively labelled *oriC* was added with or without increasing amounts of salmon sperm DNA, and the concentration of the DnaA-*oriC* complex was determined as described in Experimental procedures and shown i Fig. 3.6.

Fig. 4.1 (A and B) shows the concentration of the DnaA-*oriC* complex as a function of the concentration of free *oriC* (used in estimation of K_d) and as a function of fold excess salmon sperm DNA, respectively. Table 4.2 summarises the results. The table also includes *in vivo* stability data. Generally, the experiments clearly indicate that the DnaA proteins have high affinity and specificity for *oriC in vitro*. The DnaA5 and DnaA46 proteins have the lowest affinity for *oriC* compared to the other proteins, whereas the DnaA(A184V) protein has the same high affinity for *oriC* as the DnaA$^+$ protein has. Thus, it seems that the combination of the A184V substitution and the H252Y or G426S substitution has the most dramatic effect on the affinity for *oriC in vitro*. The specificity towards *oriC* is reduced in the DnaA5 and DnaA(G426) proteins, whereas it is only slightly affected in the DnaA(A184V), DnaA(H252Y) and DnaA46 protein proteins. In addition to the A184V substitution, the DnaA5 protein has an amino acid substitution in the DNA binding domain of the DnaA protein (G426S), and therefore, both the DnaA5 and DnaA(G426S) proteins could be expected to have altered affinity and specificity for *oriC* compared to the DnaA$^+$ protein. This is in agreement with the results of this *in vitro* analysis and with results obtained by Carr and Kaguni (1996). G426 is highly conserved among DnaA protein from different organisms (Fig. 3.2). In addition, it is located in helix 3 which is suggested to be part of a helix-turn-helix motif (Chapter 2 and 3). Therefore, a substitution with serine could be expected to have a greater effect than observed in this study. The G426S substitution does not change the secondary structure of the DnaA protein according to the secondary structure prediction, and this may account for the only minor effect the substitution has on the DnaA protein.

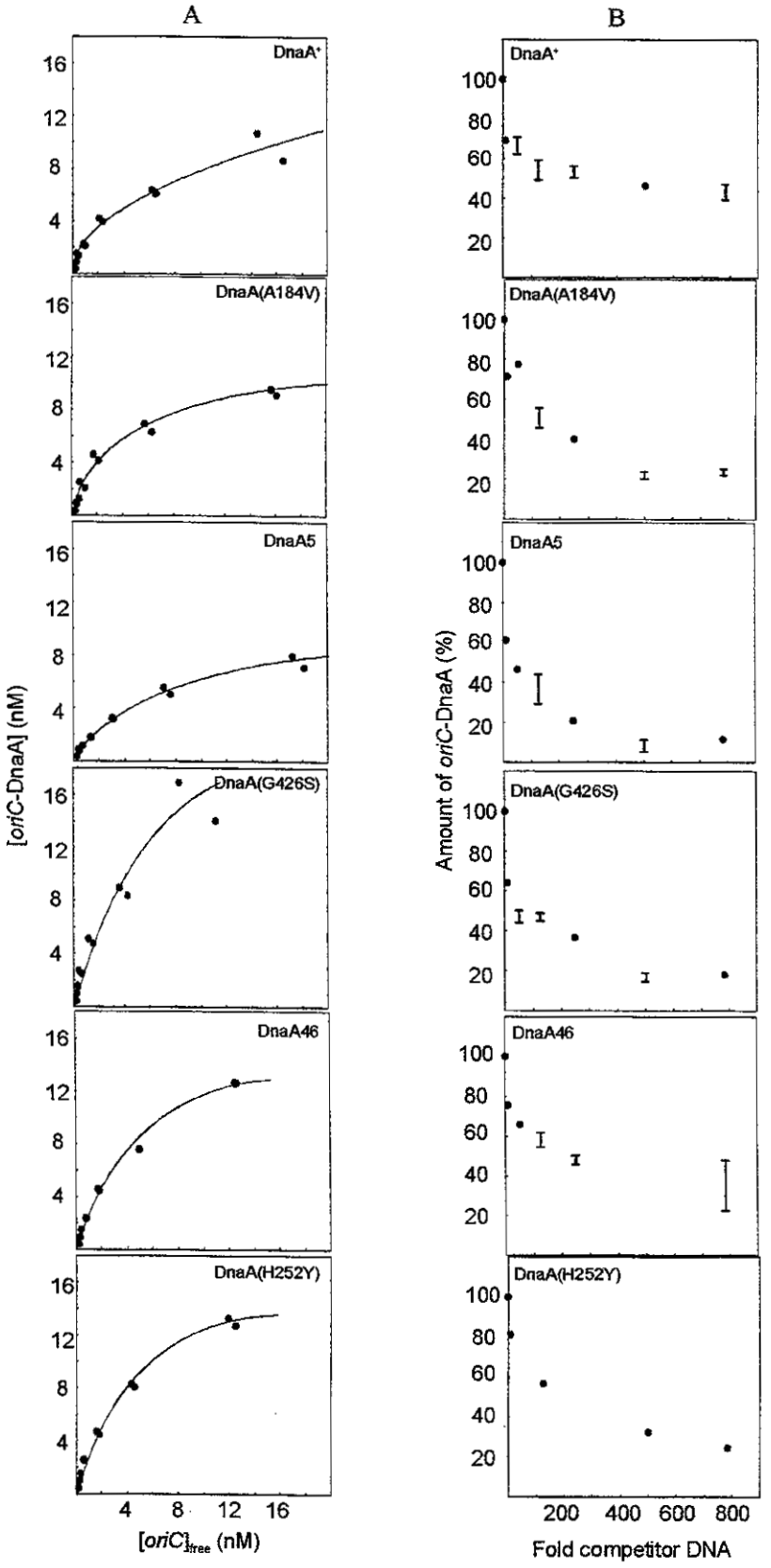


Fig. 4.1. *in vitro* affinity and specificity of indicated DnaA protein for *oriC*. A) Increasing amounts of radioactively labelled *oriC* was added to a constant DnaA concentration in order to calculate K_d (Table 4.2). B) Increasing amounts of salmon sperm DNA was added together with a fixed amount of *oriC* to estimate the specificity towards *oriC* .

Table 4.2. *in vitro* affinity and specificity of indicated DnaA proteins to *oriC*

DnaA protein	Affinity (norm.) [1)]	Specificity (norm.) [2)]	Stability 32°C [3)]	Stability 42°C
$DnaA^+$	1.00	1.00	>4	>4
DnaA46	0.61	0.97	>4	3.0
DnaA5	0.56	0.69	>4	3.25
DnaA(A184V)	1.00	0.86	>4	>4
DnaA(H252Y)	0.77	0.87	>4	>4
DnaA(G426S)	0.70	0.55	>4	>4

1) The dissiciation constant K_d was estimated from the curve fit of data shown in Fig. 4.1(A) (see Experimental procedures, Chapter 3, section II)
2) Specificity was estimated by adding increasing amounts of salmon sperm DNA together with a fixed amount of *oriC*. The values summarises the data shown in Fig. 4.1(B).
3) *in vivo* stability of the proteins was determined by adding chloramphenicol to the growing cultures, and measure the biotin-DnaA concentration various times after the drug addition using SDS-PAGE and Western blotting. The values are expressed as $T_{1/2}$ (generation times)

The result does, however, indicate that G426 may not interact with the DNA directly. The DnaA46 protein only carries amino acid substitutions in domain III of the DnaA protein (Hansen *et al.*, 1992), and since none of the substitution neither change the secondary structure nor are part of the DNA binding domain it may seem contradicting that the affinity (and specificity) is reduced in the DnaA46 and DnaA(H252Y) proteins. It indicates that the structure and function of the DNA binding domain depends on other parts of the DnaA protein. The results are, however, not in agreement with results obtained by Carr and Kaguni (1996), who found that the DnaA46 and DnaA(H252Y) proteins had higher and the same affinity to *oriC in vitro*, respectively, as the $DnaA^+$ protein had. Of all the DnaA proteins analysed in this study, the DnaA(A184V) protein seems to have *in vitro* binding constants closest to those of the $DnaA^+$ protein. The A184V substitution is located close to (or in) the ATP binding area of the DnaA protein, and therefore, it could be expected not to have any effect on DNA binding. Thus, the observed deleterious effect of the A184V substitution (Hansen *et al.*, 1992; Carr and Kaguni, 1996; Nyborg *et al.*, 2000) does not seem to involve DNA binding problems.

Stability of the proteins

The DnaA(A184V), DnaA46, DnaA5, DnaA(H252Y) and DnaA(G426S) proteins are all stable both at 32 and 42°C, although the DnaA46 and DnaA5 proteins have a slightly reduced $T_{1/2}$ at 42°C (table 4.2). This high stability of the heat sensitive DnaA46 and DnaA5 proteins at non-permissive temperature is consistant with the observation that when *dnaA46* and *dnaA5* strains are grown at permissive temperature, shifted to non-permissive temperature (42°) for 1.5 hours, and then shifted back to permissive temperature, the chromosome replication is initiated immediately (Hanna and Carl, 1975; Tippe-Schindler *et al.*, 1979; Hansen, 1995). The DnaA(A184V) protein is also stable at both 32 and 42°C, and thus, sofar there does not seem to be any reason why the *dnaA*(A184V) mutation would force the cell to pick up other intragenic mutations in order to survive.

dnaA(A184) allele on the chromosome

dnaA204 and *dnaA*^{0}, *rnh*$^+$ strains harbouring the *dnaA*(A184V) allele in λ attachment site were constructed and grown as described in Chapter 3, Experimental procedures, section III and shown i Fig. 3.7. Samples were taken from the exponentially growing cultures, treated with rifampicin (+cephalexin) and analysed using flow cytometry (Fig. 4.2). Generally, the DNA distributions do not look as nice as the distributions in the isogenic *dnaA*$^+$ strain (Fig. 3.19 and 3.21), and the *dnaA*^{0} strain carrying the *dnaA*(A184V) allele did not display discrete peaks (data nor shown). The replication is initiated in MN1229 (*dnaA*(A184V) in *dnaA204* background) at 32 and 42°C without IPTG induction (Fig. 4.2). This is consistent with the observation that the strain forms colonies at 42°C without IPTG induction (Table 4.3). The replication is initiated somewhat synchronous at both temperatures and most of the cells contain four and eight origins. At 32°C, the initiation frequency clearly decrease with increasing IPTG concentrations, and after 4 hours growth in medium supplemented with 100 μM IPTG, half of the cells contain either one or two chromosomes. This is consistent with the results of experiments with multicopy P_{dnaA}-*dnaA*(A184V) strains. These strains are cold sensitive and it was shown that DNA synthesis stopped when the cultures were shifted to non-permissive temperatures in spite of an overinitiation of chromosome replication (Nyborg *et al.*, 2000). However, MN1229 forms colonies at 32°C on LB plates supplemented with 100 μM IPTG, although the colonies are small (Table 4.3). This apparent contradiction may

Table 4.3. Growth of *dnaA204* and *dnaA*^{0}, *rnh*$^+$ strains harbouring *dnaA*(A184V) allele in λ attachment site [1)]

Strain	*dnaA* in λ att	30°C					42°C				
[IPTG] (μM)		0	30	75	100	150	0	30	75	100	150
MN1240	*dnaA204* / *dnaA*$^+$	+++	+++	+++	+++	+++	-	+++	+++	+++	+++
MN1242	*dnaA*^{0} / *dnaA*$^+$	+	+	+	+++	+++	+	+	+	+++	+++
MN1229	*dnaA204* / *dnaA*(A184V)	+++	+++	+++	++	++	++	++	++	+++	+++
MN1209	*dnaA*^{0} / *dnaA*(A184V)	+	+	+	+	+	+	+	+	+	+

1) Growth was estimated as the ability to form colonies on LB plates supplemented with different amounts of IPTG. +++ : good growth; - : no growth.

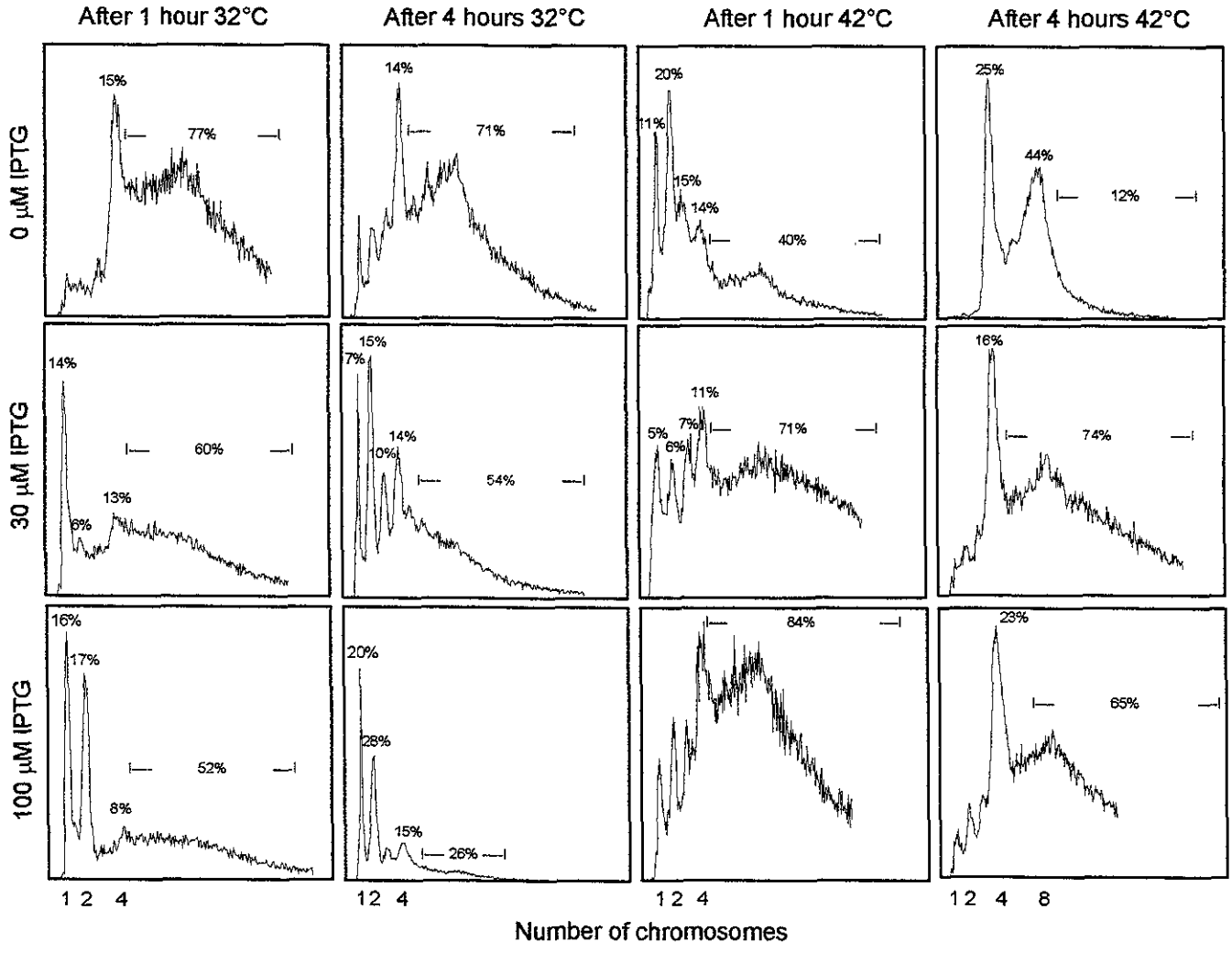


Fig. 4.2. MN1229 (*dnaA204*, *dnaA*(A184V) in λ attachment site) was grown exponentially at 32°C. At OD_{450} = 0.1 the culture was shifted to 42°C for 1 hour and then diluted 20 times and divided in ten portions: five portions were further incubated at 42°C and the rest were shifted back to 32°C. 0, 10, 30, 50 and 100 μM IPTG was added to the cultures at 32°C and 42°C, and samples were taken different times after IPTG addition, treated with rifampicin and analysed using flow cytometer as described in Experimental procedures (Chapter 3, section III). The histograms show the DNA distribution after 1 and 4 hours incubation at indicated temperatures with indicated amounts of IPTG.

be a result of differences in growth rates due to the different growth media. If it is assumed that replication is (over)initiated at 32°C, but not elongated, then growth on LB plates, which is fast, may allow the cell to 'follow' the increased DnaA activity. At 42°C, the replication is initiated synchronously after 4 hours growth without IPTG induction, and the cells contain primarily four and eight origins (Fig. 4.2). With IPTG induction, the cells seem to overinitiate replication extensively, and the replication forks are not able to finish during the incubation with rifampicin probably due to lack of sufficient amount of

nucleotides etc. Without IPTG induction, the DnaA concentration is only one fourth of the DnaA concentration in a normal *dnaA*$^+$ strain (Fig. 3.18), whereas 30 μM IPTG induction results in DnaA concentrations from two to four fold higher than the DnaA concentration in a *dnaA*$^+$ strain. This indicates that only small amounts of DnaA(A184V) protein is enough to initiate chromosome replication, and that a DnaA(A184V) concentration corresponding to what it would be if the *dnaA*(A184V) allele was inserted into its normal chromosomal position, might result in overinitiation of chromosome replication at 42°C and probably also at 32°C. The overinitiations at 32°C may not pass the *oriC* area as observed with multicopy *dnaA*(A184V) strains (Nyborg *et al.*, 2000), and thus, the strain is likely to be cold sensitive. MN1209 (*dnaA*(A184V) allele in *dnaA*^{0} strain background) grows on LB plates at 32 and 42°C both with and without IPTG, but the colonies are small. This independency of IPTG (or sensitivity), may indicate that the strain carries unknown supressor mutations, but several independently isolated strains were isolated and tested for growth on plates supplemented with different amount of IPTG. They all grew with and without IPTG.

Discussion

We have previously shown that multicopy *dnaA46*, *dnaA5* and *dnaA*(A184V) strains are cold sensitive, and that the chromosome replication is overinitiated at non-permissive temperatures. These overinitiations are, however, only elongated in the multicopy *dnaA46* strain and not in the multicopy *dnaA5* and *dnaA*(A184V) strains. We have also shown that multicopy *dnaA*(H252Y) and *dnaA*(G426S) strains are temperature resistant and thus, have the same phenotype as multicopy *dnaA*$^+$ strains.

In this study, *in vitro* binding assays indicate that the DnaA5 and DnaA46 protein have retained 50-60% of the affinity to DnaA boxes in *oriC* compared to the DnaA$^+$ protein, DnaA(H252Y) and DnaA(G426S) have retained 70-80% and the DnaA(A184V) protein has the same affinity for DnaA boxes in *oriC* as the DnaA$^+$ protein has. DnaA(A184V) has also retained high specificity towards *oriC*, whereas especially DnaA5 and DnaA(G426S) have reduced specificity, which may be due to the G426S substitution located in the DNA binding domain of the DnaA protein. Generally, the protein have a relatively high affinity for *oriC*, and this is in agreement with results obtained by Carr and Kaguni (1996). However, they observed a slightly increased affinity of both DnaA(A184V) and DnaA46 for *oriC* at 30 and 42°C, and that only the DnaA(G426S)

had a decreased affinity at these temperatures. The dicrepancy between the results obtained by Carr and Kaguni (1996) and the results in this study may be due to different incubation temperatures. In addition, the proteins used in this work all have a biotin-tag fused to the N-terminus of the DnaA protein. Sutton and Kaguni (1997) analysed the DnaA(G426D) protein and found that it retained only 12% of the *in vitro* affinity to *oriC*. The amino acid substitution does not change the secondary structure, and thus the results indicate that substitution of G426 with serine is tolerable while substitution with aspartic acid has deleterious effect on the DnaA function. The results of this work indicate that the DnaA(A184V) protein does not differ significantly from the DnaA$^+$ protein with respect to *oriC* interaction *in vitro*. The *in vivo* stability of DnaA(A184V) is also very similar to that of the DnaA$^+$ protein both at 32 and 42°C. Thus, the dominance of the multicopy *dnaA*(A184V) over the single *dnaA*$^+$ allele on the chromosome published previously (Nyborg *et al.*, 2000) may not be due to altered affinity or specificity of the DnaA(A184V) protein for *oriC*. The result of the experiment also indicate that the so-called secondary DnaA46 and DnaA5 substitutions have an effect on the DnaA protein *in vitro*. The proteins have reduced *oriC* affinity, and especially the DnaA(G426S) has reduced specificity towards *oriC*. The observation that the combination of the A184V substitution and the H252Y or G426S substitutions has the most severe effect on the affinity for *oriC* may seem difficult to explain. However, if the A184V substitution is the most deleterious one, then its presence may lead to other DnaA substitutions that reduce the affinity of the DnaA protein for *oriC*, and thereby reduce the initiation frequency from *oriC* allowing (better) growth. As shown in Fig. 4.2, the chromosome replication is probably overinitiated at 42°C and most likely also at 32°C (although these initiations are not elongated). It would be interesting to measure the number of origins per mass in isogenic *dnaA46*, *dnaA5*, *dnaA*(H252Y) and *dnaA*(G426) strains.

Recapitulation

The DnaA protein is the key protein in the initiation of the chromosomal replication of *Escherichia coli*. The DnaA protein binds to DnaA boxes in origin of replication, *oriC*, to form an initial complex. In the presence of ATP it melts part of the *oriC* to which the rest of the replication machinery can bind and elongate and eventually terminate the chromosomal replication. Based on analysis of secondary structure and function, the DnaA protein has been divided in four domains. The N-terminal domain I is responsible for protein-protein interactions, domain II constitute the flexible loop, domain III carries the ATP binding area and domain IV, which include the last 94 amino acids of the DnaA protein, constitute the DNA binding domain. Domain IV is predicted to consist of five α-helixes separated by loops, and this structure is highly conserved among DnaA proteins from a large number of organisms.

The experimental work presented in this thesis involve 1) mutational analysis of the DNA binding domain of the DnaA protein and 2) analysis of the A184V substitution in the ATP area of domain III and other amino acid substitutions found in the DnaA5 and DnaA46 proteins.

To analyse the DNA binding domain, more than 100 functional DnaA proteins with amino acid substitutions in the domain IV (fused to a biotin tag) were constructed and studies *in vivo* by complementation analysis of high temperature sensitive *dnaA46* and *dnaA204* phenotypes by induction of plasmid born mutant *dnaA*(IV) alleles. The result of the complementation analysis indicates that the whole area participates in DNA binding, either directly or indirectly. However, the complementation analysis indicates that there are a relatively higher number of amino acids in the first half of domain IV and in helix 5 that can be substituted with non-closely related amino acids without any apparent effect on the DnaA protein. These include I376, K381, Y386, K388, K390, V391, L395, K397, M411, T418, E456, F458, L461, L465 and S467. None of the mutant DnaA proteins complement the high temperature sensitive phenotype better than the $DnaA^+$ protein does. The secondary structures of the mutant DnaA proteins were determined, and when compared with the results of the complementation analysis, the results suggest that the secondary structure of domain IV cannot be changed without affecting the function of the DnaA protein severely. This seems consistent with the highly conserved secondary structure observed in a large number of DnaA protein form other organisms.

In agreement with solvent accessibility predictions, the complementation analysis indicates that helix 1 may be located on the surface of the DnaA protein, with D377, K381 and E385, which are located on the same side of the α-helix, exposed to the solvent, and the rest, especially I379, V383 and A384 protected from the solvent and probably participating in hydrophobic interactions with other residues of the DnaA protein. Generally, helix 1 is sensitive towards amino acid substitutions even though they do not change the secondary structure of the protein. This indicates that amino acids in the helix, and not only the helix itself, help stabilise the DnaA structure and/or are responsible for DNA contact. All lysines (K388, K390 and K397) in the basic loop between helix 1 and 2 can be substituted with the oppositely charged glutamic acid without any apparent effect on the DnaA protein with respect to complementation. This could indicate that these amino acids are interacting with the solvent. On the contrary, the highly conserved arginines R399 and R401 cannot be substituted without affecting the DnaA protein, which indicates that these residues may interact with the DNA. According to the accessibility prediction, many amino acids in helix 2 are protected from the solvent, indicating that a part of this helix is located inside the protein with other hydrophobic amino acids in the surroundings. This hypothesis is consistent with the complementation analysis and may indicate that residues in helix 2 does not interact with the DNA, because of the buried nature of the helix. However, except for M411, other residues are sensitive towards substitutions, and in addition, it was not possible to construct DnaA proteins with R407 and Q408 substitutions, which suggests that residues in helix 2 may in fact be interacting with the DNA. It was not possible in this work to substitute many of the highly conserved amino acids in helix 3 and the loops surrounding it. This may indicate that they cannot be substituted without deleterious effects on the DnaA protein. The presence of the highly conserved G430 and G431 that were not substituted either may indicate that DnaA functionality depends on a turn between helix 3 and 4. Alignment of DnaA with proteins harbouring a helix-turn-helix motif suggests that helix 3 and 4 of the DNA binding domain of the DnaA protein could constitute a modified helix-turn-helix motif. The results of the complementation analysis are in agreement with this proposal. Generally, helix 4 and 5 carry few single substitutions and many double substitutions, which could indicate that amino acid substitutions in this area require secondary substitutions elsewhere in order to maintain DnaA activity. There seems to be a tendency that the presence of a coiled structure between the two parts of the helix is important, and that the length of it is critical. Helix

4 is more sensitive towards amino acid substitutions than helix 5, which is in agreement with how conserved the regions are. Thus, many amino acids in helix 5 can be substituted with non-closely related amino acids without affecting the function of the DnaA protein. On the contrary, amino acids in the beginning of helix 4 that are highly conserved are not substituted in any of the DnaA proteins constructed in this work. This strongly suggests that they are required for DnaA activity, and they may be interacting with the DNA. In addition, those amino acids that are predicted to be protected form the solvent with high reliability cannot be substituted with polar amino acids without affecting the DnaA protein severely. The hydrophobic amino acids are located on the same side of the α-helix (or close to). The two hydrophobic amino acids I454 and F458 are substituted with threonine and serine, respectively, in one of the double mutants. This DnaA(I454T, F458S) protein complements the high temperature sensitive phenotype of the host better than when the substitutions exist alone, which indicates that there could be (weak) interactions between I454 and F458 that is maintained to some extend in the double mutant where both amino acids are substituted with polar, hydrogen bonding residues. Helix 4 and 5 are probably located on the surface of the DnaA protein with a hydrophobic and hydrophilic side and a hydrophilic coil in between. Helix 4 could be responsible for DNA contact and as mentioned previously be part of a modified helix-turn-helix motif.

Further work was carried out with a selection of twenty DnaA proteins with amino acid substitutions in domain IV. The analysis included *in vivo* and *in vitro* binding studies and *in vivo* stability measurements. In addition, the IPTG inducible *dnaA*(A384V), *dnaA*(L395P), *dnaA*(K397E) and *dnaA*(C441G) alleles were inserted into the λ attachment of a *dnaA204* and *dnaA*(null) strain and the initiation frequencies determined at different temperatures under different growth conditions using flow cytometry. The relative *in vivo* affinities of the various DnaA proteins for DnaA box R5 and R6 was determined by measuring the β-galactosidase activities in multicopy p*dnaA*(IV) strains harbouring a P_{mioC}-*lacZ* fusion in λ attachment site. The *in vitro* affinities and the specificity towards *oriC in vitro* was determined by using streptavidin coated magnetic beads to which the biotin-tagged DnaA proteins were bound. To determine K_d different amounts of *oriC* was added and to determine the specificity towards *oriC*, increasing amounts of competitor DNA was added together with the *oriC* fragment. The *oriC* fragment included DnaA box R1, R2, R3, R4, R5 and R6. The results of the *in vivo* and *in vitro* affinity assays indicate that DnaA proteins with amino

acid substitutions in Domain IV have affinities comparable to those of the wild type DnaA protein (K_d = 0.75 ± 0.14 nM), and the variation of the affinities only differed three-fold. There seems to be a tendency that amino acid substitutions in the area from T418 to C441 result in lower *in vitro* affinities than amino acid substitutions elsewhere in the domain. Generally, the *in vivo* affinities were lower than the *in vitro* affinities, and the *in vivo* affinities seem to 'follow' the specificity. Thus, a protein with high *in vitro* specificity towards *oriC* (which also included the R5 and R6 box) also had high *in vivo* affinity to the *mioC* promoter. The DnaA proteins that had high affinity, but low specificity towards *oriC* are located in helix 1 and in the basic loop and include DnaA205, DnaA(A384V, Y386H), DnaA204, DnaA(Y387S), DnaA(L395P) and DnaA(R401C, K433R). This indicates that this part of the DNA binding domain is responsible for the specificity towards *oriC*. The DnaA(L395) protein, in particular, had low specificity, whereas the *in vivo* and *in vitro* affinities were comparable with those of the DnaA$^+$ protein. Strains carrying *dnaA*(A384V, Y384H) and *dnaA*(L395P) alleles in λ attachment site of a *dnaA204* strain overinitiated chromosome replication extensively at 42°C, but maintained normal initiation frequency at 32°C as compared with the isogenic *dnaA*$^+$ strain. The DnaA concentration in these strains was from two to four fold higher than the DnaA concentration in a normal wild type strain. This indicates that especially the DnaA(L395P) protein has increased affinity for *oriC* at 42°C in the light of the observation that the protein is more unstable than the DnaA$^+$ protein at 42°C. The *in vitro* experiments were carried out at room temperature, and therefore an increased affinity at 42°C could not be observed. Unlike the isogenic *dnaA*$^+$ strain, the replication in *dnaA*(A384V, Y386H) and *dnaA*(L395P) were initiated asynchronously at both 32 and 42°C. Interestingly, the DnaA(K397E) protein, which has substituted one of the basic residues in the basic loop between helix 1 and 2 with an oppositely charged amino acid, has a three fold higher affinity for *oriC in vitro* than the DnaA$^+$ proteins has, and the binding is more specific. The higher affinity is, however, not reflected in the *in vivo* affinity experiments with strains harbouring multicopy *dnaA*(K397E) alleles. However, when the IPTG inducible *dnaA*(K397E) allele is inserted into the chromosome in λ attachment site of a *dnaA204* and *dnaA*(null) strain, the strains initiate replication (asynchronously) without IPTG induction even though the DnaA concentration is only one fourth of the DnaA concentration in a wild type *dnaA*$^+$ strain. When the *dnaA*(K397E) allele is induced to a DnaA concentration corresponding to two fold higher than in a wild type strain, initiation of chromosome replication seems to stop, and

cells with only one chromosome accumulate. The replication may, however, be overinitiated with increasing DnaA(K397E) concentration, but subsequently aborted or stalled and therefore not detected in the experiments carried out in this work. The results are in agreement with the *in vitro* data and indicate that the DnaA(K397E) protein has increased *oriC* affinity. The results also indicate that a strain with the *dnaA*(K397E) allele at its normal chromosomal position may not survive due to the high *oriC* affinity. The DnaA(C441G, V391A) protein has retained app. 60% of the *in vivo* and *in vitro* affinity and 80% *in vitro* specificity for *oriC*. When the *dnaA*(C441G, V391A) allele is inserted into the λ attachment site of a *dnaA204* strain, the replication is initiated asynchronously, but with a somewhat normal frequency at 42°C, whereas initiation of replication seems to stop at 32°C when the DnaA concentration is high (more than two fold higher than in a normal wild type $dnaA^+$ strain). The results indicate that a strain with the *dnaA*(C441G, V391A) alleles at its normal chromosomal position will be cold sensitive.

The amino acid substitutions found in the DnaA5 and DnaA46 proteins were separated into DnaA(A184V), DnaA(H252Y) and DnaA(G426S) proteins and analysed *in vivo* and *in vitro*. Multicopy *dnaA*(H252H) and *dnaA*(G426S) strains (secondary amino acid substitutions from DnaA46 and DnaA5, respectively) were temperature resistant like multicopy $dnaA^+$ strains, while multicopy *dnaA*(A184V), *dnaA46* and *dnaA5* strain were high temperature resistant, but cold sensitive. The multicopy *dnaA*(A184V), *dnaA5* and *dnaA46* strains (and the *dnaAcos* mutant) were further analysed, and it was found that the mutants could be divided into two groups. The first group, which included the multicopy *dnaA*(A184V) and *dnaA5* strains, initiated chromosome replication extensively at non-permissive temperature, but the initiations were not elongated and DNA synthesis stopped. Group two, which included the multicopy *dnaA46* strain and the *dnaAcos* mutant, also overinitiated chromosome replication at non-permissive temperature, but (some of) these initiation were elongated and resulted in a increased DNA content in the cells, especially in the multicopy *dnaA46* cells (Nyborg et al., 2000).

In this study *in vitro* binding assays as the ones discussed above indicate that the DnaA5 and DnaA46 proteins have retained 50-60% of the affinity to DnaA boxes in *oriC* area compared to the $DnaA^+$ protein, DnaA(H252Y) and DnaA(G426S) have retained 70-80% and the DnaA(A184V) protein has the same high affinity to DnaA

boxes in *oriC* as the DnaA$^+$ protein has. DnaA(A184V) has also retained high specificity towards *oriC*, whereas especially DnaA5 and DnaA(G426S) have reduced specificity, which may be due to the G426S substitution located in the DNA binding domain of the DnaA protein.

The results of this work indicate that the DnaA(A184V) protein does not differ significantly from the DnaA$^+$ protein with respect to *oriC* interaction *in vitro*. The *in vivo* stability of DnaA(A184V) is also very similar to that of the DnaA$^+$ protein both at 32 and 42°C. Thus, the dominance of the multicopy *dnaA*(A184V) over the single *dnaA*$^+$ allele on the chromosome may not be due to altered *oriC* affinity or specificity of the DnaA(A184V) protein. The result of the experiment also indicates that the so-called secondary DnaA46 and DnaA5 substitutions have an effect on the DnaA protein *in vitro*. The proteins have reduced *oriC* affinity, and especially the DnaA(G426S) has reduced specificity for *oriC*. The observation that the combination of the A184V substitution and the H252Y or G426S substitutions has the most severe effect on the affinity for *oriC* may seem obscure. However, if the A184V substitution is the most deleterious substitution, then its presence may lead to other DnaA substitutions that reduce the affinity of the DnaA protein for *oriC*, and thereby reduce the initiation frequency from *oriC* allowing better growth. When the IPTG inducible *dnaA*(A184V) allele is inserted into λ attachment site of a *dnaA204* strain, the chromosome replication is overinitiated at 42°C even without IPTG induction where the DnaA concentration is only one fourth of the DnaA concentration in a normal *dnaA*$^+$ strain. At 32°C the initiation frequency seems to decrease severely with increasing IPTG induction and cells with one and two chromosomes accumulate. The replication may, however, be overinitiated at 32°C as observed with multicopy *dnaA*(A184V) strains, but the initiation are stalled or aborted. The results indicate that a *dnaA*(A184V) strain with the *dnaA* allele at its normal chromosomal location is cold sensitive.

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Author:

Tove Atlung Ole Skovgaard Flemming G. Hansen

Signature:

Universitetsvej 1, Postboks 260, DK- 4000 Roskilde.
Telefon nr. 45 46 74 2470 (direkte nr.) Fax nr. 46 74 3011

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Two types of cold sensitivity associated with the A184→V change in the DnaA protein

Marlene Nyborg,[1] Tove Atlung,[1]* Ole Skovgaard[1] and Flemming G. Hansen[2]
[1]Department of Life Sciences and Chemistry (17.2), Roskilde University, PO Box 260, DK-4000 Roskilde, Denmark.[2]Department of Microbiology, Technical University of Denmark, 2800 Lyngby, Denmark.

Summary

Multicopy *dnaA*(Ts) strains carrying the *dnaA5* or *dnaA46* allele are high-temperature resistant but are cold sensitive for colony formation. The DnaA5 and DnaA46 proteins both have an A184→V change in the ATP binding motif of the protein, but they also have one additional mutation. The mutations were separated, and it was found that a plasmid carrying exclusively the A184→V mutation conferred a phenotype virtually identical to that of the *dnaA5* plasmid. Strains carrying plasmids with either of the additional mutations behaved like a strain carrying the *dnaA*$^+$ plasmid. In temperature downshifts from 42°C to 30°C, chromosome replication was stimulated in the multicopy *dnaA46* strain. The DNA per mass ratio increased threefold, and exponential growth was maintained for more than four mass doublings. Strains carrying plasmids with the *dnaA*(A184→V) or the *dnaA5* gene behaved differently. The temperature downshift resulted in run out of DNA synthesis and the strains eventually ceased growth. The arrest of DNA synthesis was not due to the inability to initiate chromosome replication because marker frequency analysis showed high initiation activity after temperature downshift. However, the marker frequencies indicated that most, if not all, of the newly initiated replication forks were stalled soon after the onset of chromosome replication. Thus, it appears that the multicopy *dnaA*(A184→V) strains are cold sensitive because of an inability to elongate replication at low temperature. The multicopy *dnaA46* strains, on the contrary, exhibit productive initiation and normal fork movement. In this case, the cold-sensitive phenotype may be due to DNA overproduction.

Received 28 September, 1999; revised 25 November, 1999; accepted 1 December, 1999. *For correspondence. E-mail atlung@ruc.dk; Tel. (+45) 4674 2402; Fax (+45) 4674 3011.

Introduction

The DnaA protein is a key protein in the initiation of chromosome replication from *oriC* in *Escherichia coli* (von Meyenburg and Hansen, 1987; Skarstad and Boye, 1994; Messer and Weigel, 1996). The protein binds to the DnaA boxes within *oriC* (Fuller *et al.*, 1984; Samitt *et al.*, 1989) and melts the AT-rich 13-mers in the presence of ATP, HU and IHF (Bramhill and Kornberg, 1988). Several *dnaA* mutants carry a C→T mutation within the *SphI* recognition site of the *dnaA* gene, giving rise to an Ala→Val change at amino acid 184 (Hansen *et al.*; 1992). This is close to the 'P-loop' motif (GX_4GKT from amino acid 172 to amino acid 179; Saraste *et al.*, 1990; Messer and Weigel, 1996) likely to be responsible for the binding of ATP to the DnaA protein that is necessary for DNA synthesis *in vitro* (Sekimizu *et al.*, 1987). The DnaA(A184→V) protein is temperature sensitive in DNA synthesis *in vitro*, and has an activity similar to the wild-type DnaA protein at permissive temperature (Carr and Kaguni, 1996). So far, all *dnaA* mutants harbouring the *dnaA*(A184→V) mutation carry secondary mutation(s) within the *dnaA* gene (*dnaA5*, *dnaA46*, *dnaA601*, *dnaA604* and *dnaAcos*) (Braun *et al.*, 1987; Hansen *et al.*, 1992), indicating that the A184→V change is not tolerated by the cell when present alone. *dnaA*(Ts) mutants transformed with plasmids carrying the same *dnaA* allele become high-temperature resistant (Hansen *et al.*, 1992). However, introduction of *dnaA* alleles harbouring the *dnaA*(A184→V) mutation resulted in cold-sensitive phenotypes (Hansen *et al.*, 1992). Cold-sensitive phenotypes are also observed with the multicopy *dnaA*(G177→D) strain (Sutton and Kaguni, 1997) and with the *dnaAcos* mutant, which was isolated as an intragenic *dnaA46* suppressor (Kellenberger-Gujer *et al.*, 1978) and which carries two mutations in addition to the two *dnaA46* mutations (Braun *et al.*, 1987). It has been suggested that the *dnaAcos* mutant is cold sensitive because of overinitiation and subsequent overproduction of DNA at non-permissive temperature (Kellenberger-Gujer *et al.*, 1978; Katayama and Kornberg, 1994). The cold sensitivity associated with amino acid changes in the proposed ATP binding region of the DnaA protein (Kellenberger-Gujer *et al.*, 1978; Katayama and Nagata, 1991; Hansen *et al.*, 1992; Sutton and Kaguni, 1997) may be a result of reduced ATP and/or ADP affinity (Katayama, 1994; Carr and Kaguni, 1996). The wild-type DnaA protein binds ATP and ADP with high affinity

(K_d = 0.03 μM and 0.1 μM respectively; Sekimizu *et al.*, 1987), and small amounts of the ATP version are needed in the formation of the open complex (Crooke *et al.*, 1993). Based on analysis of different *dnaA* mutants that over-initiate DNA replication, it has been suggested that in the wild-type cell the DNA replication is negatively regulated by the hydrolysis of the DnaA-bound ATP (Katayama, 1994; Mizushima *et al.*, 1997) and that the DNA polymerase III β-clamp and the IdaB protein stimulates this hydrolysis (Katayama *et al.*, 1998; Kurokawa *et al.*, 1998). The reason why overinitiation of DNA replication and DNA accumulation is lethal is not clear, but it has been suggested that DNA overproduction inactivates a protein that is important for cell division (Katayama *et al.*, 1997).

The aim of this work was to investigate whether the cold-sensitive phenotype of strains harbouring multicopy *dnaA* genes with the A184→V mutation is due to over-initiation of replication leading to DNA overproduction at non-permissive temperature or whether the lack of growth at non-permissive temperature is due to lack of initiations. The *dnaAcos* mutant was included in this study for comparison.

Results

Cold sensitivity of multicopy dnaA*(Ts) strains is caused by a mutation giving an alanine to valine change in amino acid 184 of the DnaA protein*

Multicopy *dnaA*(ts) strains, i.e. strains carrying the same *dnaA*(Ts) allele on the chromosome and on a high copy number plasmid, are high-temperature resistant (Hansen *et al.*, 1992). Four such strains are, in addition, cold sensitive. This cold sensitivity coincides with an A184→V amino acid change caused by a C→T transition, which destroys a *Sph*I restriction site close to (in) the region that encodes the ATP binding domain of the DnaA protein. The finding that the four mutants *dnaA5*, *dnaA46*, *dnaA601* and *dnaA606* all had another mutation in the *dnaA* gene led to the proposal that the A184→V change was the primary defect of the DnaA protein and that the other mutation was secondary and might have been created to compensate for a deleterious effect of the primary mutation (Hansen *et al.*, 1992).

To study the multicopy-induced cold sensitivity in more detail, plasmids were constructed that carried *dnaA* genes that had only one of the mutations present. These plasmids and a plasmid carrying the wild-type *dnaA* gene were transformed into strains carrying the *dnaA*$^+$, *dnaA5* or the *dnaA46* allele. The transformants were tested for growth at different temperatures, and it was found that strains carrying plasmids with a H252→Y or G426→S change in the DnaA protein (secondary mutations from *dnaA46* and *dnaA5* respectively) behaved exactly as the strains carrying a plasmid with the wild-type *dnaA* gene (Table 1). In contrast, the strains carrying the plasmid with the mutation causing the A184→V amino acid change in the DnaA protein were extremely cold sensitive (Table 1). To investigate the cold sensitivity in more detail, we carried out temperature shift experiments with radioactive labelling and measured DNA content and number of initiated origins by measuring the DNA content in rifampicin-treated cells (Hansen and Rasmussen, 1977).

The multicopy dnaA46 *strain and the* dnaAcos *mutant accumulate DNA at non-permissive temperature*

The *dnaAcos* mutant, which was isolated as an intragenic *dnaA46* suppressor, is cold sensitive (Kellenberger-Gujer *et al.*, 1978) and carries a Q156→I and Y271→H change in addition to the two *dnaA46* mutations (Braun *et al.*, 1987). It has been suggested that the cold-sensitive phenotype is due to overinitiation of DNA replication at non-permissive temperature (Kellenberger-Gujer *et al.*, 1978; Katayama and Kornberg, 1994).

Our results indicate that the *dnaAcos* mutant and the *dnaA46*/p*dnaA46* strain continue initiation of chromosome replication and DNA production at non-permissive temperature (Fig. 1). After 1.5 h incubation at non-permissive temperatures (30°C or 32°C), the DNA per mass increased by a factor of 1.4 and 3.6, respectively, compared with permissive temperature (42°C) (Table 2). The DNA per mass was the same at 30°C and 42°C in the *dnaA*$^+$/pBR322 strain (Table 2). This result clearly indicates that DNA accumulates in the *dnaA46*/p*dnaA46* strain as a result of overinitiation of chromosome replication. A qualitatively similar result was obtained in a *dnaA*$^+$ background (data not shown). A 1.4-fold increase in the DNA content of the *dnaAcos* mutant at non-permissive temperature is not as high as those reported previously (Kellenberger-Gujer *et al.*, 1978; Katayama and Kornberg, 1994). The DNA content did not increase further when the strain was shifted to other non-permissive temperatures (30°C, 34°C and 36°C; data not shown). We obtained identical results with another *dnaAcos* isolate from the transduction. Flow cytometric analysis of rifampicin (+ cephalexin)-treated *dnaAcos* cells showed that the mutant does not end up with fully replicated chromosomes, as does the isogenic wild-type strain (data not shown). The observed 1.8-fold DNA increase in the rifampicin-treated *dnaAcos* cells at non-permissive temperature could be due to rifampicin-resistant initiations rather than overinitiations *per se*. This also seems more consistent with the results of the marker frequency analysis (see below). Rifampicin-resistant initiations have been observed with other *dnaA* mutants (Hanna and Carl, 1975; Tippe-Schindler *et al.*, 1979; Hansen, 1995) and they may be a result of a direct

Table 1. Phenotype of strains harbouring multicopy *dnaA* alleles[a].

		Plasmid[b]				
Strain	*dnaA*	No plasmid	p*dnaA*$^+$, p*dnaA*(H252→Y) p*dnaA*(G426→S)	p*dnaA46*	p*dnaA5*	p*dnaA*(A184→V)
FH1218	*dnaA*$^+$	Temp^R	Temp^R	Cs (<35°C)	Cs (≤ 36°C)	Cs (≤ 37°C)
FH1220	*dnaA46*	Ts	Temp^R	Cs (<35°C)	Cs (≤ 36°C)	Cs (≤ 37°C)
FH1219	*dnaA5*	Ts	Temp^R	Cs (<35°C)	Cs (≤ 36°C)	Cs (≤ 37°C)

[a]Colony formation was tested on LB ampicillin plates. [b]All plasmids are pBR322 derivatives carrying different *dnaA* alleles (see *Experimental procedures*; Hansen *et al.*, 1992). Temp^R, cells form colonies at all tested temperatures (30°C, 35°C, 36°C, 37°C and 42°C); Ts, temperature sensitive; Cs, cold sensitive. The brackets indicate at which temperature the cells do not form colonies.

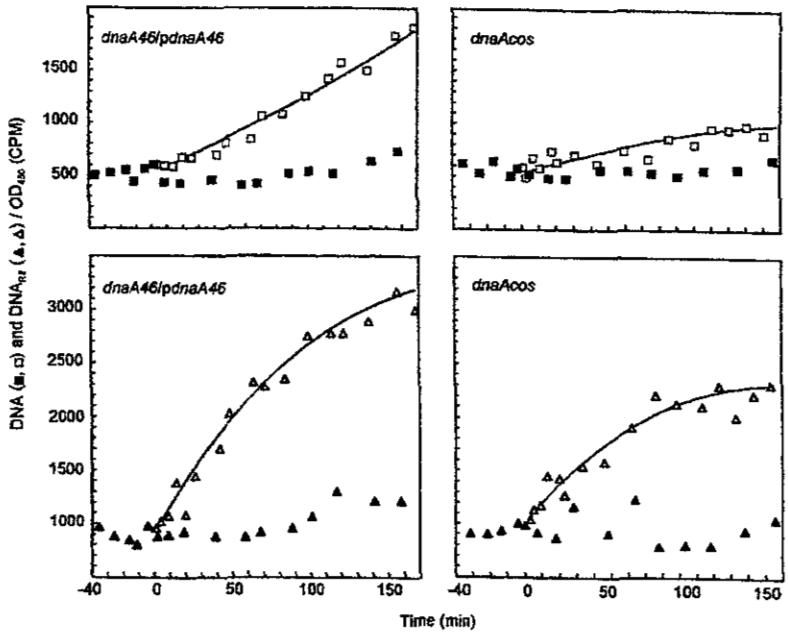

Fig. 1. DNA and origins per mass for FH1220/p*dnaA46* and MN57 (*dnaAcos*). The strains were grown exponentially at 42°C (black symbols). At $t = 0$, the cultures were shifted to 30°C (FH1220/p*dnaA46*) or to 32°C (MN57) (white symbols). Samples were taken from the cultures at permissive and non-permissive temperatures to measure OD_{450}, actual DNA content and number of origins (see *Experimental procedures*).

interaction between the DnaA protein and the RNA polymerase, which prevents rifampicin from binding to the β-subunit of the polymerase.

The multicopy dnaA*(A184→V) and* dnaA5 *strains stop DNA synthesis at non-permissive temperature*

In contrast to the multicopy *dnaA46* strains, the multicopy *dnaA*(A184→V) strains appear to stop initiation of replication at non-permissive temperature, while DNA synthesis continues and the amount approaches that in the rifampicin-treated samples (Fig. 2). The same result was obtained with the multicopy *dnaA5* strain (data not shown). When the multicopy *dnaA*(A184→V) strain is shifted back to permissive temperature after 1.5 h incubation at non-permissive temperature, the cells restart DNA synthesis immediately (Fig. 3). At 42°C, the DNA increase is three times higher in the rifampicin-treated *dnaA46*/p*dnaA* (A184→V) strain than in the *dnaA*$^+$/p*dnaA*(A184→V) strain (330% and 110% respectively; Table 2). Marker frequency analysis (see below) of the two strains indicates, however, that it is the *dnaA*$^+$ strain that has the highest number of origins (Table 2). This contradiction may, as with the *dnaAcos* mutant, be explained by the presence of rifampicin-resistant initiations in the *dnaA46* strain background.

Flow cytometric analysis of rifampicin (+ cephalexin)-treated multicopy *dnaA*(A184→V) strains did not display discrete peaks at either permissive or non-permissive temperatures (data not shown).

The multicopy dnaA*(A184→V) strains overinitiate replication at non-permissive temperature*

The results of the temperature shift experiments discussed above were unexpected considering that the heat-sensitive DnaA5 and DnaA(A184→V) proteins are able to initiate replication at low temperature, as shown in *in vivo* or *in vitro* experiments. Also, it would be expected that the *dnaA*$^+$ strains (i.e. wild-type *dnaA* gene on the chromosome)

Table 2. Comparison of DNA replication phenotypes of *dnaA*$^+$, *dnaAcos* and mutant *dnaA* multicopy strains.

Strain	FH1218/pBR322		FH1218/p*dnaA*(A184→V)		FH1220/p*dnaA*(A184→V)		FH1220/p*dnaA46*		MN57	
dnaA	*dnaA*$^+$/–		*dnaA*$^+$/p*dnaA*(A184→V)		*dnaA46*/p*dnaA*(A184→V)		*dnaA46*/p*dnaA46*		*dnaAcos*	
Temperature	42°C	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C	32°C
τ (min)	40	55	50	–[a]	40	–	47	64	42	56
DNA/OD_{450}[b]	1.0	1.0	1.3	–	1.2	–	0.9	3.2	0.9	1.3
ΔG (%)[c]	74	84	110	–	330	–	89	63	60	110
oriCterC	3.2	3.4	4.5	7.5	3.1	9.4	2.2	4.4	3.0	3.3
C/τ[d]	1.7	1.8	2.2	–	1.6	–	1.2	2.1	1.6	1.7

[a]Not determined. The cells were not growing exponentially. [b]Values are relative to the *dnaA*$^+$/pBR322 strain in which DNA per mass was constant and the same at 30°C and 42°C. [c]The DNA increase (ΔG) is the difference between the DNA content in cells treated with or without rifampicin. [d]Calculated from *oriC/terC* = $2^{C/\tau}$.

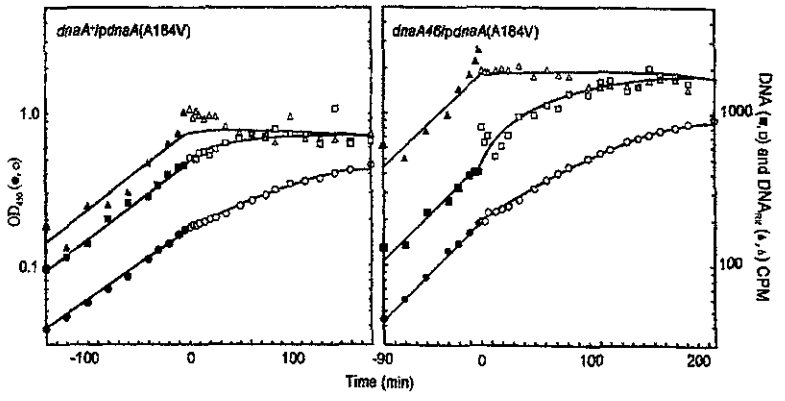

Fig. 2. Effect of temperature downshift on DNA synthesis in multicopy *dnaA*(A184→V) strains. FH1218/p*dnaA*(A184→V) and FH1220/p*dnaA*(A184→V) were grown at 42°C (black symbols). At $t = 0$, the cultures were shifted to 30°C (white symbols). OD_{450}, DNA content and number of origins were measured (see *Experimental procedures*).

would be able to initiate replication at any temperature because of the presence of wild-type DnaA proteins. In addition, earlier experiments have indicated that the *dnaAcos* mutant overinitiates replication several-fold more than that observed in our experiments (Kellenberger-Gujer *et al.*, 1978; Katayama *et al.*, 1997). Therefore, it could be that the strains used in our work do (over)initiate replication at non-permissive temperature, and that these initiations are not elongated and as a result are 'undetectable' in the radioactive precursor incorporation experiments.

Southern blot marker frequency analysis was carried out to test this hypothesis. The probe consisted of a mixture of 10 fragments, of which seven anneal to the *oriC* region of the chromosome (Table 4; Fig. 4). The results of the marker frequency analysis clearly indicate that the multicopy *dnaA*(A184→V) strains overinitiate replication at non-permissive temperature (Fig. 5). Two hours after the temperature shift, the *dnaA*$^+$/p*dnaA*(A184→V) and *dnaA46*/p*dnaA*(A184→V) strains reach an *oriC/terC* value of 7.5 and 9.4 respectively (Table 2). This is a 1.7- to 3.0-fold increase compared with the *oriC/terC* ratio at 42°C. The *pgk/terC* ratio never reaches 1 (Fig. 5), indicating that not only do the *oriC* proximal replication forks stall but also those that are half-way around the chromosome.

The multicopy *dnaA46* strain has a twofold increase in the *oriC/terC* ratio at non-permissive temperature (Table 2). Thus, although the DNA increase in this strain is dramatic, there are still forks ready to 'take off'. The *dnaAcos* mutant, however, does not overinitiate replication at non-permissive temperature (Table 2). The *oriC/terC* ratio is the same, and is independent of the temperature, in the *dnaAcos* and the isogenic *dnaA*$^+$ strain (Table 2).

Discussion

In this work, we have shown that multicopy *dnaA*(A184→V) strains are high-temperature resistant and are cold sensitive. The cold-sensitive phenotype is more pronounced in these strains compared with multicopy *dnaA5* and *dnaA46* strains, which indicates that the secondary mutations in *dnaA5* and *dnaA46* (G426→S and H252→Y respectively) partially compensate for the A184→V change (Table 1). In addition, the temperature-sensitive *dnaA5* and *dnaA46* strains grow at all temperatures in the presence of multicopy *dnaA* genes carrying the secondary mutations from *dnaA5* and *dnaA46* (Table 1).

To understand why overproduction of the heat-sensitive DnaA46, DnaA5 and DnaA(A184→V) proteins result in

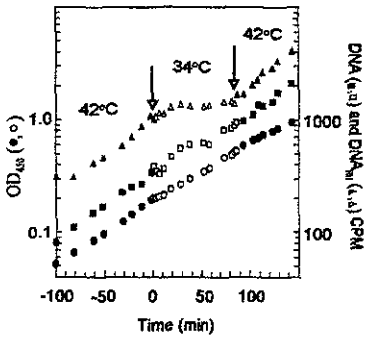

Fig. 3. The multicopy *dnaA*(A184→V) strain restarts DNA synthesis immediately after return to permissive temperature. The FH1220/p*dnaA*(A184→V) strain was grown at 42°C and was shifted to 34°C at $t = 0$. After 90 min incubation at non-permissive temperature, the strain was returned to 42°C. Symbols as in Fig. 2 (see *Experimental procedures*).

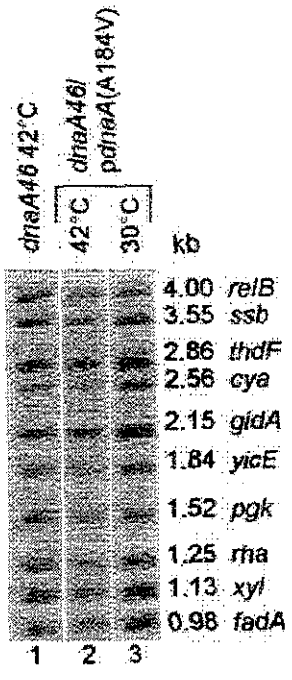

Fig. 4. Southern blot marker frequency analysis. Strains were grown exponentially at 42°C and were shifted to 30°C. Samples were taken from the cultures at permissive and non-permissive temperatures, chromosomal DNA was prepared and was triple digested with *Eco*RI–*Eco*RV–*Hin*dIII. The DNA was blotted onto a membrane and was probed with a mixture of 10 probes (see Table 4). The intensities of the bands were quantified, normalized and used to determine ratios relative to *terC*, as shown in Fig. 5. Lane 1, *dnaA46* (run out at 42°C); lane 2, FH1220/p*dnaA*(A184→V) at 42°C; lane 3, FH1220/p*dnaA*(A184→V) 60 min after temperature shift to 30°C (see *Experimental procedures*).

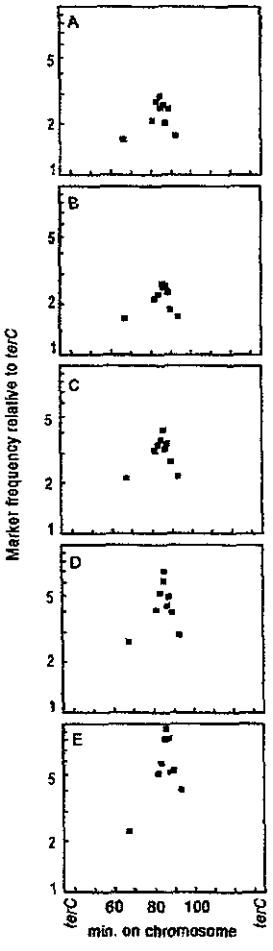

Fig. 5. Southern blot marker frequency relative to *terC*. Chromosomal DNA was prepared and processed as described in Fig. 4 legend and the *Experimental procedures*.
A. *dnaA*$^+$/pBR322 at 42°C.
B. *dnaA46*/p*dnaA*(A184→V) at 42°C.
C. *dnaA46*/p*dnaA*(A184→V) 30 min after the temperature shift to 30°C.
D. *dnaA46*/p*dnaA*(A184→V) 60 min after the temperature shift.
E. *dnaA46*/p*dnaA*(A184→V) 120 min after the temperature shift.

cold-sensitive phenotypes, we carried out temperature shift experiments and measured DNA content and number of initiated origins at permissive and non-permissive temperatures. The last parameter was estimated by measuring the increase in DNA content after rifampicin treatment and by Southern blot marker frequency analysis.

All of the cold-sensitive strains discussed in this work were found to overinitiate DNA replication at non-permissive temperature. The experiments categorized the strains into two groups: group 1, to which the multicopy *dnaA*(A184→V) and the multicopy *dnaA5* strains belong, and group 2, to which the multicopy *dnaA46* strains and the *dnaAcos* strain belong. Multicopy strains in the first group initiated replication at non-permissive temperature, but all replication forks were stalled and thus DNA synthesis stopped (Fig. 2). The *oriC*/*terC* ratio increased by a factor of 1.7–3.0, depending on the bacterial background (Table 2). The amount of

Table 3. Strains used in this work.

Strain	Genotype	References
FH1218	*trp-3, his-4, pyrB*::Tn*5, thi-1, galK2, lacY1, mtl-1, ara-9, tsx-3, ton-1, rpsL8, supE44* λ(c^+)	Hansen *et al.* (1991)
FH1219	As FH1218, *dnaA5*	Hansen *et al.* (1991)
FH1220	As FH1218, *dnaA46*	Hansen *et al.* (1991)
MN57	As FH1218, *tnaA*::Tn*10*, *dnaAcos*	This work
MN60	As FH1218, *tnaA*::Tn*10*	This work
KA441	*dnaAcos, tnaA*::Tn*10*	Katayama and Komberg (1994)

DNA synthesized after the temperature shift suggested that already ongoing replications forks finished, but the marker frequency analysis showed that existing forks must also stall because the *pgk/terC* ratio (marker located halfway around the chromosome) also increased slightly (Fig. 5). Strains in group 2 also initiated replication at non-permissive temperature but (some of) these initiations did continue and resulted in a higher actual DNA content, especially in the multicopy *dnaA46* strains, which have a more than threefold higher DNA content at non-permissive temperature. The DNA content in the *dnaAcos* mutant only increased 1.4-fold when shifted to non-permissive temperature (Table 2; Fig. 1). The DNA increase in *dnaAcos* at non-permissive temperature has previously been reported to be more dramatic (Kellenberger-Gujer *et al.*, 1978; Katayama *et al.*, 1997). The different results may be due to different growth conditions and/or the differences in the strain background.

dnaA mutants have previously been reported to become cold sensitive when the *dnaA*(A184→V) allele is involved; either as a single copy on the chromosome as the *dnaAcos* mutant (Kellenberger-Gujer *et al.*, 1978) or the *dnaA46* mutant with GroE chaperone overproduction (Katayama and Nagata, 1991), or as multicopy *dnaA* genes (Hansen *et al.*, 1992). Also, a multicopy *dnaA*(G177→D) strain is cold sensitive (Sutton and Kaguni, 1997), and because both amino acid changes are located close to, or in, the presumed ATP binding region of the DnaA protein the mutations may alter the ATP binding affinity and/or ATP hydrolysis. *In vitro* nitrocellulose binding assays have indicated that the DnaA5, DnaA46, DnaA(A184→V) and the DnaAcos proteins do not bind ATP (Hwang and Kaguni, 1988; Hupp and Kaguni, 1993; Katayama, 1994; Carr and Kaguni, 1996). However, the four mutant proteins bind *oriC* with an affinity similar to that of the wild-type DnaA protein *in vitro*, at both permissive and non-permissive temperatures (Katayama, 1994; Carr and Kaguni, 1996). Although recent *in vitro* experiments showed that the DnaA(A184→V) protein could be activated for lower affinity ATP binding by monomerization (Carr and Kaguni, 1996), the function of ATP binding and hydrolysis is still unclear. The importance of ATP binding may be more indirect, in the sense that ATP hydrolysis is required for a negative regulation of the initiation (Mizushima *et al.*, 1997; Kurokawa *et al.*, 1998). As suggested previously for the DnaAcos protein (Katayama, 1994), the lower affinity of the four mutant proteins for ATP may render them inert to negative regulation through ATP hydrolysis and thus give rise to overinitiation of replication when the mutants are shifted to low temperature, where the mutant proteins have a higher activity that may be due to a more normal conformation. There are, however, other *dnaA* mutations located in the ATP binding region (G172→A and V185→A, Skovgaard *et al.*, 1998; G175→D and P43→L–G177→S, Sutton and Kaguni, 1997) that do not confer multicopy cold sensitivity, although the mutant proteins are active for chromosome initiation. These mutations might therefore be less deleterious for ATP binding or hydrolysis than the A184→V mutation.

It has recently been shown *in vitro* that the hydrolysis of ATP bound to DnaA is strongly stimulated by replicating DNA polymerase III holoenzyme (Katayama *et al.*, 1998). We propose that *in vivo* the ATP hydrolysis may be needed to help release DnaA protein bound to DnaA

Table 4. Primers for PCR fragments used as probes in Southern blot marker frequency analysis.

Gene (min)	Primer sequences
relB (34.8)	GTTGAAGTACTTGAGTCACC CATTCAGACTTGAATGCGTG
ssb (92.0)	CGCGTTTACACTTATTCGAACGAT CTGGCAGATGCTTTGATCATCCACC
thdF (83.5)	AATTGAGGCAT(Δ1)GCGCAGGTTTG TGTGGCGCCATGCGCGGTGCTAA
cya (85.9)	ATGCTGCATTACTGGCATCA GCTGCCGATTCCTCGACTGA
gidA (84.3)	AAGAATGGCTGGGATCGTGG ATCGAGGTTACTGCGGATCA
yicE (82.1)	GATCCAGTTGACTGGTGTTG CATTGAGCGAAAGCGATCCG
pgk (63.3)	CAATTAACCAATCAGTCGCA CATGGTGAATCCTCTCGTTG
rha (88.2)	AGCACTGGATGAGGTGATCA GATATCGGCGTCATCCAGGC
xyl (80.3)	AGCAGCTCGATTACCGTACG TTCCACAGACCTACCATCAG
fadA (86.7)	GACGATAAAGGCTGCTTTGT CGCTCATGCCAGAACGGAAC

boxes around the chromosome and thus allow the replication fork to proceed at normal speed. Overproduction of wild-type DnaA protein gives rise to a marker frequency curve (Atlung and Hansen, 1993) very similar to that observed in the multicopy *dnaA*(A184→V) strains (Fig. 5). In the experiments with overproduction of DnaA$^+$, the cells were, however, in balanced growth and thus the replication forks were proceeding, albeit at a low average velocity, whereas the forks were completely blocked in the multicopy *dnaA*(A184→V) strains (Fig. 2). We hypothesize that this difference could arise from the inability of the replisome to displace the DnaA(A184→V) protein because of its lack of ATP binding and thus hydrolysis. This hypothesis is fully compatible with the dominance of the multicopy *dnaA*(A184→V) over the single *dnaA*$^+$ allele on the chromosome. The immediate resumption of DNA synthesis observed after shift back to permissive temperature (Fig. 3) also strongly suggests that the stalling of the replication forks is mediated directly by the mutant DnaA protein. In this scenario, the secondary mutation in *dnaA46* might reduce the DNA-binding affinity of the DnaA(A184→V) protein sufficiently to allow forks to get through, whereas the secondary mutation in *dnaA5* does not have this effect.

It is obvious why the lack of DNA synthesis is lethal in the multicopy *dnaA5* and *dnaA*(A184→V) strains, but less obvious why the multicopy *dnaA46* and the *dnaAcos* strains are cold sensitive. Katayama *et al.* (1997) isolated suppressors for the *dnaAcos* mutant and found that overproduction of a protein named CedA stimulated cell division without affecting over-replication. Therefore, the cold sensitivity might be due to a cell division defect and not DNA overproduction. The overproduction of DNA in the multicopy *dnaA46* strain is, however, rather dramatic, and may therefore be inhibiting in itself. It would be interesting to see whether CedA also suppresses the cold-sensitive phenotype of the multicopy *dnaA46* strain.

Experimental procedures

E.coli *strains*

Strains used in this work are shown in Table 3. MN57 and MN60 were constructed by P1 transduction (Miller, 1992) of FH1218 with a P1 stock grown on KA441.

Plasmids

A plasmid carrying the *dnaA*(A184→V) gene was constructed as follows: pFHC539 (*dnaA*$^+$) and a pFHC539 derivative carrying the *dnaA5* allele (Hansen *et al.*, 1992) were digested with *Bsm*I, which separates the *dnaA*(A184→V) mutation and the second *dnaA5* mutation on two different DNA fragments. The small *Bsm*I fragment from the *dnaA5* derivative of pFHC539, carrying the *dnaA*(A184→V) mutation, was ligated to the large *Bsm*I fragment from pFHC539. The *dnaA*(A184→V) mutation destroys a *Sph*I recognition sequence, which allowed selection of correct clone by *Sph*I digestions. The absence of the second *dnaA5* mutation was verified by sequencing using the Thermo Sequenase cycle sequencing kit from Amersham and a primer with the sequence GCGCGACTTGCTGGCATTGC. Plasmids carrying the *dnaA*(H252→Y) and *dnaA*(G426→S) alleles (secondary mutations from *dnaA46* and *dnaA5* respectively) were constructed essentially as p*dnaA*(A184→V). In these cases, the *Bsm*I fragment with the *dnaA*(A184→V) mutation from p*dnaA5* and p*dnaA46* (pFHC539 derivatives) was substituted with a wild-type fragment. The *dnaA*(G426→S) allele was verified by sequencing as above. The *dnaA*(H252→Y) allele was verified by the single-stranded conformational analysis method (SSCP; Yap and McGee, 1992). Primers with the following sequences were used to PCR amplify a 314 bp fragment carrying the C→T mutation, giving rise to the H252→Y substitution: CGAGCGCTTTGTTC and TCATCAGGATCGCC. The PCR fragment (50 ng) was boiled for 10 min in 0.25 M NaOH, mixed with loading buffer (Yap and McGee, 1992) and run on an 8% polyacrylamide gel overnight. The gel was stained with 0.4 μg ml^{-1} ethidium bromide.

Radioactive labelling

Overnight cultures were inoculated and grown exponentially at 42°C for at least five generations in AB medium (Clark and Maaløe, 1967) supplemented with 0.2% glucose, 2 μg ml^{-1} thiamine, 10 μg ml^{-1} uracil, 0.5% casamino acids and 10 μg ml^{-1} tryptophan. MN57 was grown in the same medium, but with the FN18 amino acid mix (Neidhardt *et al.*, 1977) instead of casamino acids and tryptophan. When FH1218 strains were grown, 100 μg ml^{-1} ampicilin was added continuously to the medium to avoid growth of plasmid-free cells. At $OD_{450} \approx 0.5$, the cultures were diluted 25 times in [^{14}C]-uracil-supplemented medium (0.002 Ci/g uracil) and after three or four generations of balanced growth ($OD_{450} \approx 0.2$) at 42°C half of the culture was shifted to non-permissive temperature (between 30°C and 36°C). During the experiment, samples were taken in order to measure cell mass (OD_{450}), DNA content and DNA content after overnight incubation with 300 μg ml^{-1} rifampicin. The number of initiated origins can be measured by adding rifampicin (Hansen and Rasmussen, 1977) because rifampicin blocks initiation but allows already ongoing replication forks to finish. The DNA samples were incubated with NaOH to a final concentration of 0.25 M for 1 h at 45°C to hydrolyse the RNA. TCA was added to a final concentration of 5% and the DNA was measured as [^{14}C]-uracil incorporated into alkali-stable TCA-precipitable material in a liquid scintillation counter.

At the end of each temperature shift experiment, samples of the cultures were plated and were incubated overnight at 42°C and at non-permissive temperature (30–36°C) to verify the cold-sensitive phenotype.

Marker frequency analysis using Southern hybridization

Cells were grown exponentially for eight generations in AB medium (Clark and Maaløe, 1967) supplemented with 0.2%

glucose, 2 μg ml^{-1} thiamine, 10 μg ml^{-1} uracil and FN18 amino acid mix (Neidhardt *et al.*, 1977). At OD_{450} ≈ 0.2 ,half of the culture was shifted to 32°C. Triple samples were taken at 42°C and at 32°C and chromosomal DNA was prepared essentially as described by Grimberg *et al.* (1989) with modifications (Atlung and Hansen, 1993). The chromosomal DNA was triple digested with *Eco*RI, *Hin*dIII and *Eco*RV, and the DNA fragments were separated on a 0.7% agarose gel. The DNA was transferred overnight to a nylon membrane (Amersham), as described by Sambrook *et al.* (1989). The membrane was prehybridized with denatured salmon sperm DNA for more than 1 h at 65°C and was hybridized overnight at 60°C with [^{35}S]-dATP-labelled probe. The probe consisted of 10 purified PCR fragments from different positions on the chromosome (Table 4). The DNA fragments were labelled using the megaprime DNA labelling system from Amersham. After hybridization, the membrane was washed once for 5 min in 2× SSC/0.5% SDS, once for 15 min in 2× SSC/0.1% SDS and twice for 30 min in 0.1 × SSC/0.5% SDS at room temperature. The membrane was either exposed on a Kodak film and marker frequencies determined by measuring the intensities of the different bands using a Bio-Rad model GS-700 imaging densitometer or the marker frequencies were determined using an Instant Imager (Packard). To normalize the bands, we determined marker frequencies of *Hin*dIII–*Eco*RI–*Eco*RV triple-digested chromosomal DNA from FH1220 (*dnaA46*) that was incubated at non-permissive temperature for 2 h to allow run out of replication. Flow cytometric analysis confirmed that these cells contained fully replicated chromosomes.

Flow cytometric analysis

Samples were prepared as described previously (Christensen *et al.*, 1999).

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