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In vivo studies of DnaA binding to the origin of replication of *Escherichia coli*

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We have shown that DnaA, a protein required for initiation of DNA replication in *Escherichia coli*, binds to three of four DnaA binding sequences in the replicative origin *oriC* (boxes R1, R2 and R4). Protein–*oriC* DNA interactions in minichromosomes carried by wild-type and *dnaA* mutant strains were demonstrated by *in vivo* footprinting using dimethylsulfate treatment of intact cells. The same characteristic enhancement/protection pattern was seen in wild-type minichromosomes or mutants defective in *oriC* function but carrying the four DnaA boxes. Minichromosomes in *dnaA* (Ts) mutants showed no protein binding at non-permissive temperatures and reduced binding even at permissive temperatures. *In vivo* footprints of the wild-type strain were identical to those obtained *in vitro* using purified DnaA proteins and *oriC* DNA. Transcription into *oriC* affected the binding of DnaA protein to the DnaA boxes. These findings suggest that the protein causing the *in vivo* footprints at *oriC* is DnaA.

Key words: DnaA protein/*Escherichia coli*/minichromosomes/*oriC* DNA

Introduction

Replication of the *Escherichia coli* chromosome begins at a fixed location, *oriC*, and proceeds bidirectionally to a termination site (Bird *et al.*, 1972). The initiation of each cycle requires *de novo* synthesis of protein and RNA (Lark, 1966, 1972) and is the regulated event of chromosomal replication (Maaløe and Kjeldgaard, 1966). Several models have been proposed to explain the regulatory event: the replicon theory (Jacob *et al.*, 1963), the initiator accumulation model (Maaløe and Kjeldgaard, 1966), the inhibitor dilution model (Pritchard *et al.*, 1969) and the autorepressor model (Sompayrac and Maaløe, 1973). Although these models hypothesize different types of control, all suggest the existence of a protein which serves to trigger initiation either at a saturating concentration (initiator) or upon dilution (inhibitor). The accumulated results suggest that a likely candidate for both initiator and inhibitor is DnaA, the *dnaA* gene product (Hansen and Rasmussen, 1977; Atlung *et al.*, 1985, 1987; Braun, 1985).

The *dnaA* gene maps at 83 minutes on the *E.coli* chromosome and encodes a protein of 52 kd (Yuasa and Sakakibara, 1980; Hansen and von Meyenburg, 1979). The DnaA protein can be purified as a monomer, is highly sequence specific and has a strong tendency to self-aggregate under low-salt conditions (Fuller and Kornberg, 1983). It recognizes a 9-bp sequence, the DnaA box, which has the consensus TTAT(C/A)CA(C/A)A. The DnaA box is found in four positions in *oriC* (Hansen *et al.*, 1982), and is required for association of DnaA protein with *oriC* (Fuller *et al.*, 1984). A DnaA box is also found in the promoter regions of both the *16kD* gene, which lies adjacent to *oriC*, and in the *dnaA* gene itself. DnaA binding at these sites has been shown to result in negative control of transcription from the *16kD* promoter into and through the origin (Lother *et al.*, 1985) and in autoregulation of the *dnaA* gene (Atlung *et al.*, 1984, 1985; Braun *et al.*, 1985).

The DnaA protein is essential for *E.coli* initiation of replication both *in vivo*, as shown by the use of conditional mutants (for review see von Meyenburg and Hansen, 1987) and *in vitro* in replication systems that employ *oriC*-containing plasmids (Kaguni and Kornberg, 1984). *In vitro* studies of DnaA binding to the *E.coli* origin have led to different results. Fuller *et al.* (1984) showed that when added to *oriC* DNA in saturating concentrations, DnaA protects a 250-bp region from DNase I digestion. They also found that DnaA aggregates of ≥ 20 monomers bound to restriction fragments of the origin. Matsui *et al.* (1985) showed that DnaA gives major footprints at only three of the four DnaA boxes within *oriC* (R1, R2 and R4).

In an attempt to localize physiologically relevant sites of protein binding in *oriC*, we studied minichromosomes with an *in vivo* footprinting method that employs dimethylsulfate (DMS) treatment of intact cells (Nick and Gilbert, 1985; Miller and Malamy, 1986). Using this technique, we have found evidence for *in vivo* association of DnaA to three of the four DnaA boxes in *oriC*.

Results

In vivo footprinting of the E.coli origin

Treatment of DNA with DMS, *in vivo* or *in vitro*, results in methylation at the N-7 position of guanines in the major groove (Singer, 1975). Proteins associated with DNA alter the accessibility of DMS to guanine bases to decrease or enhance their methylation, resulting in a characteristic footprint.

Escherichia coli strain CM993, containing the *oriC* plasmid pCM959 (Figure 1), was grown in M9 medium plus casamino acids. Cells were harvested during exponential growth and treated with DMS for 2 min. Methylated plasmid DNA was isolated, cleaved with piperidine and run on a sequencing gel, along with DNA methylated *in vitro* to give a typical Maxam–Gilbert G-track.

The effects of *in vivo* methylation of the *E.coli* origin of replication are shown in Figure 2. In the 'lower' strand, guanines 194 of R2 and 268 of R4 were protected and

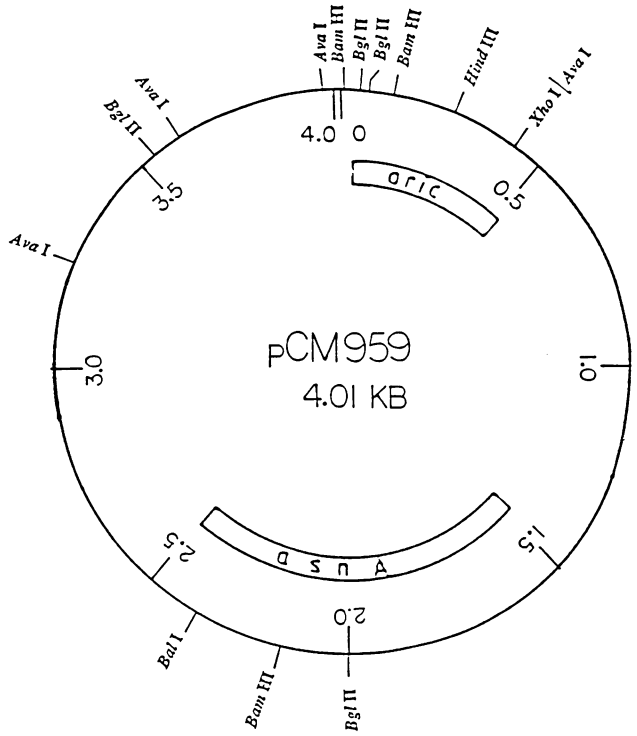

Fig. 1. Parent *oriC* plasmid used for *in vivo* footprinting. Plasmid pCM959 was obtained from K.von Meyenburg. It contains *oriC* and *16kD*, and is selected for providing asparagine to a cell mutant in asparagine production. Restriction sites at *oriC* are *Bam*HI, *Hin*dIII and *Ava*I.

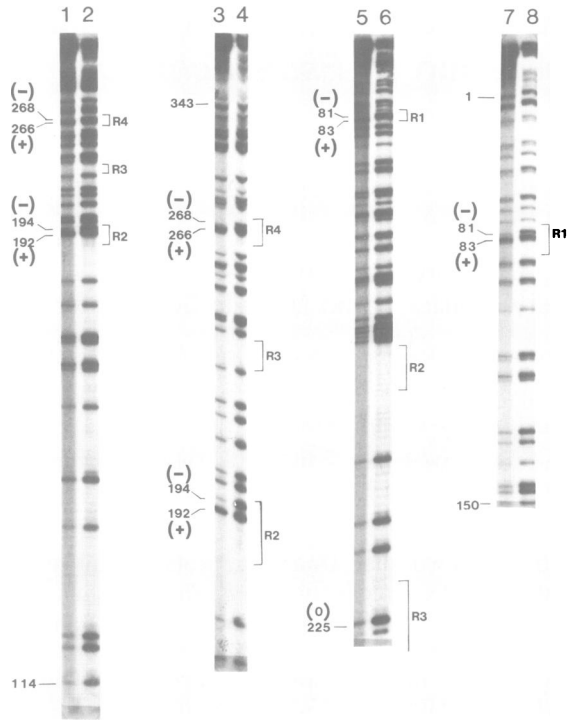

Fig. 2. *In vivo* footprinting of *oriC*. **Lanes 1–4**. Plasmid DNA was cut with *Bam*HI, ^{32}P-labeled, and cut with *Ava*I to obtain a 325-bp fragment (bp +92 to +417) labeled at the *Bam*HI site (bp +92). **Lanes 5–8**. Plasmid DNA was cut with *Hin*dIII, ^{32}P-labeled and cut with *Ava*I to obtain a 292-bp fragment (bp +244 to −46) labeled at the *Hin*dIII site (bp +244). **Lanes 1, 3, 5** and **7** show *in vivo* methylated DNA. **Lanes 2, 4, 6** and **8** show *in vitro* methylated DNA. The DNA in **lanes 3, 4, 7** and **8** was electrophoresed for longer periods than that in **lanes 1, 2, 5** and **6**. Brackets indicate the positions of DnaA boxes. Protected (−), enhanced (+) and unaffected (○) bases are indicated, together with their position.

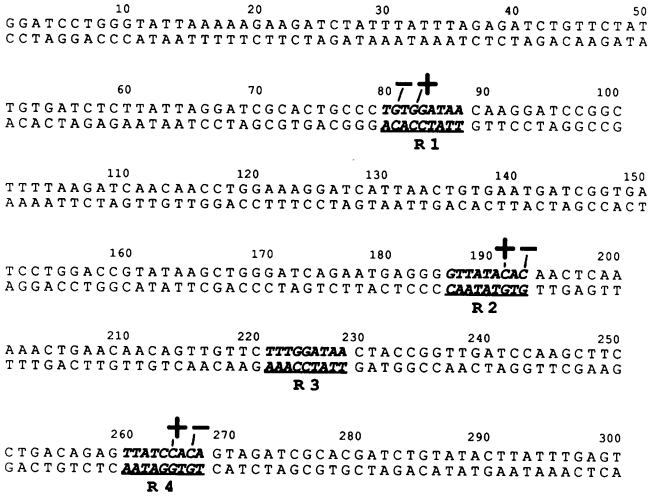

Fig. 3. Protein-binding regions in *E.coli oriC*. This map summarizes the *in vivo* footprinting results shown in Figure 2. The numbers of the bases are indicated. Shaded regions show the DnaA recognition sites R1, R2, R3 and R4. Protections and enhancements from Figure 2 are indicated by minus and plus signs respectively.

DnaA mutant protein has a decreased in vivo affinity for the oriC DnaA boxes

Minichromosomes require DnaA protein for their replication and are not stably maintained in *dnaA* (Ts) mutants. In such mutants, the minichromosome copy number is extremely low and plasmid DNA cannot be isolated in sufficient quantities for *in vivo* footprinting analysis. To produce sufficient DNA to study the effect of the *dnaA* mutation on DnaA interactions with *oriC*, we used the high copy number plasmid pFHC1427, a chimeric derivative of pBR322. Plasmid pFHC1427 carries an inactive *oriC* due to deletion of a 16-bp *Bgl*II fragment (positions 22−38), but contains the four DnaA boxes of *oriC*. We carried out *in vivo* footprinting of plasmid pFHC1427 in the wild-type strain (RB210) and its *dnaA* (Ts) derivatives TC1926 *dnaA46*, TC1927 *dnaA5* and TC1929 *dnaA2041* (Figure 4). As shown in lane 1, the protection/enhancement pattern of the DnaA boxes R2 and R4 was the same as in the wild-type (Figure 2). Thus, we conclude that the *in vivo* methylation pattern does not depend

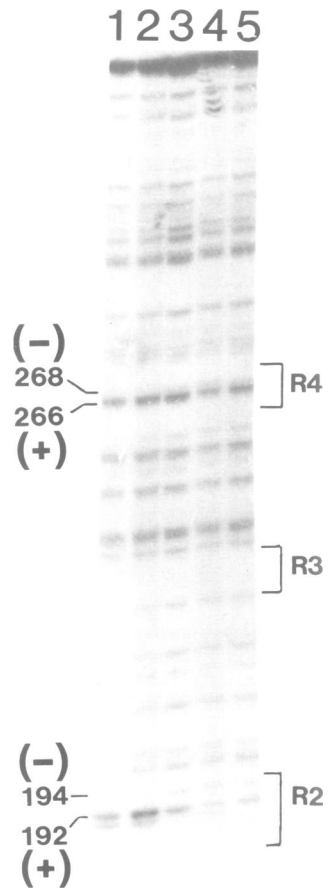

Fig. 4. *In vivo* footprinting in *dnaA* (Ts) mutants. All lanes represent a 325-bp *Bam*HI/*Ava*I fragment (position +92 to +417) labeled at the *Bam*HI site (+92). All samples are from plasmids methylated *in vivo* at 20°C as described in Materials and methods. **Lane 1**, pFHC1427 in RB201 (wild-type *E.coli*). This is a pBR322 chimeric derivative that can replicate in *dnaA* mutants; **lane 2**, pFHC1429 (as pFHC1427, but with a 4-bp insertion that inactivates the *16kD* promoter) in RB210; **lane 3**, pFHC1427 in TC1926 (*dnaA46* Ts); **lane 4**, pFHC1427 in TC1927 (*dnaA5* Ts); **lane 5**, pFHC1427 in TC1929 (*dnaA204* Ts). Protection is indicated by minus signs and enhancement by plus signs. Brackets indicate the sites of DnaA boxes R2, R3 and R4. Box R1 was not included in this analysis.

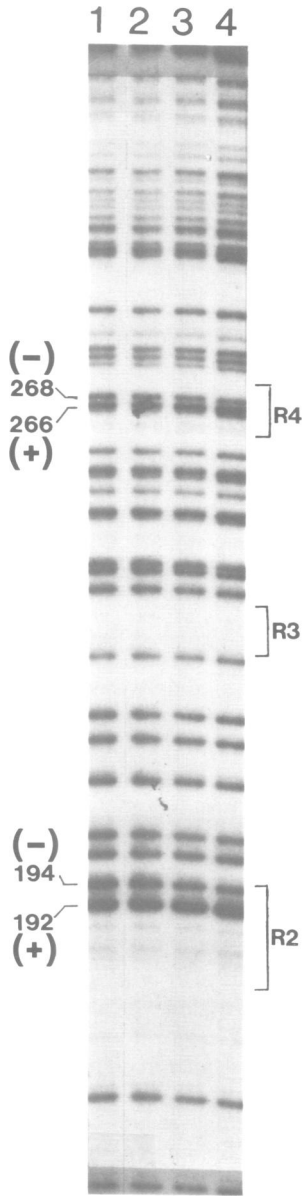

Fig. 5. *In vitro* footprinting. All lanes represent a 325-bp *Bam*HI/*Ava*I fragment of *oriC* DNA (position +92 to +417) from purified pCM959 DNA labeled at position +92 (as in legend to Figure 2). **Lane 1**. The above fragment was methylated *in vitro*, and cleaved with piperidine. **Lane 2**. Ten nanograms of the fragment was combined with 75 ng DnaA protein in binding buffer (see Materials and methods) and incubated at 30°C for 15 min prior to methylation. Remaining treatment was as in lane 1. **Lane 3**. Ten nanograms of DNA was combined with 150 ng DnaA protein and treated as in lane 2. **Lane 4**. Ten nanograms of DNA was combined with 250 ng DnaA protein and treated as in lane 2. Enhancement is indicated by plus signs, protection by minus signs. Brackets indicate sites of DnaA boxes R2, R3 and R4.

on an active *oriC*. In the three *dnaA* (Ts) mutants (lanes 3, 4 and 5) both protection and enhancement were significantly reduced. Strains *dnaA5* (lane 4) and *dnaA204* (lane 5) indeed showed no protein binding and could not be distinguished from an *in vitro* methylated sample (not shown). The *dnaA46* strain (lane 3) showed reduced protein binding to DnaA boxes at 20°C. When methylation was carried out at a non-permissive temperature, 35°C, the weak binding observed at 20°C disappeared (not shown). These results strongly suggest that DnaA protein is responsible for the *in vivo* footprint in *oriC* in the wild-type strain.

We also tried to determine whether the *in vivo* footprint is affected by the intensity of transcription into the *oriC* region. We used plasmid pFHC1429, identical to pFHC1427 except that its *16kD* promoter was inactivated by a 4-bp insertion into the *Mlu*I site (bp 778). With no transcription into *oriC* (Figure 4, lane 2), DMS reactivity of the guanine at position 192 was enhanced compared with the plasmid with an intact *16kD* promoter (Figure 4, lane 1) and protection at positions 194 and 268 was still observed.

In vitro footprinting with purified DnaA protein

We determined if the same *in vitro* footprinting pattern seen previously using DNase I (Fuller *et al.*, 1984; Matsui *et al.*, 1985) was also seen with DMS methylation. *Bam*HI fragments (+92)−*Ava*I(+417) labeled at the *Bam*HI site were isolated from purified pCM959 and treated with increasing amounts of DnaA protein. As seen in Figure 5, the higher concentration (250 ng) of DnaA protein induced

the following changes: protection at bases 194 (box R2) and 268 (box R4) and enhancement at bases 192 (box R2) and 266 (box R4). We also found changes in box R1 (protection at base 81 and enhancement at base 83) but none in box R3 (not shown). These results support the conclusion from the *in vivo* experiments, that DnaA protein affects the methylation pattern at the DnaA boxes of *oriC*.

Discussion

We have used an *in vivo* footprinting technique to localize sites of protein binding at *oriC*. We have taken as evidence for protein binding the variations in the methylation pattern between *oriC* plasmid DNA treated with DMS *in vivo* and deproteinized DNA treated *in vitro*.

We have shown that *in vivo* DMS treatment of minichromosomes in wild-type cells affects methylation at three distinct sites within *oriC*. These sites coincided with three of the four DnaA boxes within *oriC*, which were previously thought to be recognized by DnaA protein (Hansen *et al.*, 1982). These DnaA boxes were only weakly affected by *in vivo* DMS treatment of *dnaA* (Ts) mutant cells even when methylation was carried out at 20°C. In the *dnaA46* (Ts) mutant, DnaA protein binding was significantly lower than in the wild-type. Binding appeared to be even lower in the *dnaA5* (Ts) and *dnaA204* (Ts) mutants, where *in vivo* methylated DNA was indistinguishable from an *in vitro* methylated control. *In vitro* footprinting using purified DnaA protein added to *oriC* DNA showed methylation patterns identical to those obtained *in vivo*.

Previous *in vitro* footprinting of *oriC* using DnaA protein produced two different results: (i) Matsui *et al.* (1985) observed that *dnaA* boxes R1, R2 and R4 were protected from Dnase I; and (ii) Fuller *et al.* (1984) showed Dnase I protection of a 250-bp region which encompassed all DnaA boxes within *oriC*. Our *in vitro* footprinting results coincide with those found by Matsui *et al.*. The results of Fuller *et al.* may be explained by their use of higher DnaA concentrations. We may speculate that the reasons that DnaA box R3 showed no protein binding are that (i) the *in vivo* DnaA concentration is low and (ii) the consensus sequence required for DnaA binding may be more restrictive than previously thought, i.e. TTAT(C/A)CACA rather than TTAT(C/A)-CA(C/A)A (see Figure 3). Alternatively, binding of DnaA protein at R3 may be transient, implying that it may have a specific regulatory role.

We have shown that *oriC*, which is not actively initiating, gives an *in vivo* footprint similar to that of a functional *oriC*. Helmstetter and Leonard (1987) found that *oriC* plasmids and the chromosomes of their hosts initiate replication synchronously, and that *oriC* plasmid replication takes a small fraction of the time required for chromosomal replication. Since we performed our *in vivo* experiments on non-synchronized cells containing low copy number minichromosomes, we can assume that during a 2-min *in vitro* methylation period only a small fraction of plasmids were initiating replication. Because *in vivo* footprinting showed that DnaA boxes of both active and inactive *oriC* were markedly protected, our results imply that, in minichromosomes, DnaA is bound to R1, R2 and R4 boxes in *oriC* throughout most of the cell cycle. This suggests that mere binding of DnaA to the origin at R1, R2 and R4 is insufficient to initiate replication. Further aggregation of DnaA molecules, as proposed by Fuller *et al.* (1984), may be necessary to effect initiation.

We have begun to investigate changes in DnaA binding due to the presence or absence of a functional *16kD* promoter. Preliminary results, shown in Figure 4, suggest that in the absence of transcription from the *16kD* promoter, DnaA binds slightly more efficiently to *oriC* DNA. This finding supports the suggestion by Lother *et al.* (1985) that transcription from the *16kD* promoter is negatively regulated by the DnaA protein *in vivo* and *in vitro*. These authors suggest that DnaA binding to the *16kD* promoter prevents active transcription into *oriC*, thereby allowing events which initiate replication.

In vivo footprinting has provided clear indications of DnaA binding to *oriC* in minichromosomes under the limited conditions we have used. Further experiments are in progress to extend these findings to *oriC* in the *E.coli* chromosome.

Materials and methods

Bacterial strains

The following *E.coli* K-12 strains were used: CM993 (F-, *thi, asnA, asnB, recA*, pCM959) were obtained from K.von Meyenburg (Meijer *et al.*, 1979). RB210 = MC1000 (Casadaban and Cohen, 1980), lysogenized with lambda RB1 (Braun *et al.*, 1985), was obtained from Andrew Wright. *dnaA* (Ts) derivatives of RB210 were obtained from Tove Atlung and included TC1926 (*dnaA46*), TC1927 (*dnaA5*) and TC1929 (*dnaA204*).

Plasmids

pCM959 (*asnA, oriC+, 16kD+*) was obtained from K.von Meyenburg (von Meyenburg *et al.*, 1978). pFHC1427 (*Ap^r oriC, 16kD+*) is an *oriC*-deletion derivative (*Bgl*II: +24−*Bgl*II: +40; Figure 1) of the *oriC*/pBR322 chimeric plasmid pFHC1150 (von Meyenburg *et al.*, 1984). pFHC1429 (*Ap^r, oriC, 16kD*) is a derivative of pFHC1427 in which the *16kD* gene promoter has been inactivated by filling the *Mlu*I site (+778) located between the −10 and the −35 promoter sequences.

In vivo methylation

The procedure used is derived from that described by Miller and Malamy (1986). Bacterial strains carrying the different plasmids were grown overnight at 30°C in L broth or M9 media (Maniatis *et al.*, 1982) plus 50 μg/ml ampicillin, reinoculated in 25 ml to an OD_{600} of 0.05, and grown to an OD_{600} of 0.8 at 37°C (wild-type) or 30°C (temperature sensitive). Cells were harvested during exponential growth, pelleted by centrifugation at 10 000 r.p.m. for 10 min at 25°C and resuspended in 1 ml of 50 mM sodium cacodylate, pH 8.0, 1 mM EDTA. After incubation at 20°C for 10 min, DMS was added to 0.5%, and incubation continued for 2 min. To balance the increased nucleophilicity at increased temperatures, DMS incubation was reduced to 70 s when performed at 35°C. Methylation was terminated by 30-fold dilution in MPBS (150 mM NaCl, 4 mM Na_2HPO_4, 2 mM KH_2PO_4). Cells were pelleted at 10 000 r.p.m. and washed in MPBS (4°C). Plasmids were isolated via Triton X-100 lysis or alkaline lysis procedures (Maniatis *et al.*, 1982), phenol extracted, precipitated from ethanol and resuspended in TE (10 mM Tris−HCl, 1 mM EDTA pH 8.0). Purified DNA was cut with appropriate restriction endonucleases, end-labeled using radioactive α-dNPT and Klenow fragment, and restricted again to produce singly end-labeled fragments (for details, see legend to Figure 2). The desired 100−500-bp fragment was isolated from a 5% polyacrylamide gel and eluted with 0.6 ml elution buffer (500 mM ammonium acetate; 10 mM magenesium acetate; 1 mM EDTA; 0.1% SDS). DNA was precipitated from elution buffer with ethanol and suspended in H_2O. Samples were treated with 1 M piperidine at 90°C for 30 min, precipitated with *n*-butanol and 1% SDS and resuspended in formamide dye (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) to give 5000 c.p.m./1.5 μl. Samples were heated at 90°C for 3 min and electrophoresed on 8% 1.5-mm sequencing gels.

In vitro methylation

In vitro methylated samples were prepared as the normal G reaction described by Maxam and Gilbert (1980). *In vitro* footprinting was carried out as follows: DNA fragments labeled at one end were incubated with different amounts of HPLC-purified DnaA protein (obtained from K.Sekimizu and

A.Kornberg) (Fuller and Kornberg, 1983) for 15 min at 30°C in binding buffer (40 mM Hepes pH 7.6, 5 mM magnesium acetate, 2 mM dithiothreitol). Mixtures were then treated with 10% DMS for 90 s, purified and processed as the *in vitro* controls above.

Acknowledgements

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References

Atlung,T., Clausen,E.S. and Hansen,F.G. (1984) In Huebscher,U. and Spadari,S. (eds), *Proteins involved in DNA replication*. Plenum Publishing Corp., New York, pp. 199–208.

Atlung,T., Clausen,E.S. and Hansen,F.G. (1985) *Mol. Gen. Genet.*, **200**, 442–450.

Atlung,T., Løbner-Olesen,A. and Hansen,F.G. (1987) *Mol. Gen. Genet.*, **206**, 51–59.

Bird,R., Louarn,J., Martuscelli,J. and Caro,L. (1972) *J. Mol. Biol.*, **70**, 549–566.

Braun,R.E. (1985) *Studies on the structure, function and regulation of the DNA replication gene dnaA of Escherichia coli K-12*. Ph.D. dissertation. Tufts University, Boston.

Braun,R.E,. O'Day,K. and Wright,A. (1985) *Cell*, **40**, 159–169.

Casadaban,M.J. and Cohen,S.N. (1980) *J. Mol. Biol.*, **138**, 179–207.

Fuller,R.S. and Kornberg,A. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5817–5821.

Fuller,R.S., Funnell,B.E. and Kornberg,A. (1984) *Cell*, **38**, 889–900.

Hansen,F.G. and Rasmussen,K.V. (1977) *Mol. Gen. Genet.*, **155**, 219–225.

Hansen,F.G. and von Meyenburg,K. (1979) *Mol. Gen. Genet.*, **175**, 135–144.

Hansen,F.G., Hansen,E.B. and Atlung,T. (1982) *EMBO J.*, **1**, 1042–1048.

Helmstetter,C.E. and Leonard,A.C. (1987) *J. Bacteriol.*, **169**, 3489–3494.

Jacob,F., Brenner,S. and Cuzin,F. (1963) *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 329–348.

Kaguni,J.M. and Kornberg,A. (1984) *Proc. Natl. Acad. Sci. USA*, **33**, 183–190.

Lark,K.G. (1966) *Bacteriol. Rev.*, **30**, 3–32.

Lark,K.G. (1972) *J. Mol. Biol.*, **64**, 47–60.

Lother,H., Kölling,R., Kucherer,C. and Schauzu,M. (1985) *EMBO J.*, **4**, 555–560.

Maaløe,O. and Kjeldgaard,N.O. (1966) *Control of Macromolecular Synthesis*. W.A.Benjamin Inc., New York.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Matsui,M., Oka,A., Takanami,M., Yasuda,S. and Hirota,Y. (1985) *J. Mol. Biol.*, **184**, 529–533.

Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.

Meijer,M., Beck,E., Hansen,F.G., Bergmans,H.E.N., Messer,W., von Meyenburg,K. and Schaller,H. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 580–584.

Miller,J.F. and Malamy,M.H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1433–1437.

Nick,H. and Gilbert,W. (1985) *Nature*, **313**, 795.

Pritchard,R.H., Barth,P.T. and Collins,J. (1969) *Symp. Soc. Gen. Microbiol.*, **19**, 263–297.

Singer,B. (1975) *Prog. Nucleic Acids Res. Mol. Biol.*, **15**, 219.

Sompayrac,L. and Maaløe,O. (1973) Autorepressor model for control of DNA replication. *Nature New Biol.*, **241**, 133–135.

von Meyenburg,K., Hansen,F.G., Riise,E, Bergmans,H.E.N., Meijer,M. and Messer,W. (1978) *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 121–128.

von Meyenburg,K., Hansen,F.G., Atlung,T., Boe,L. Clausen,G., Hansen,E.B., Jørgensen,B.B., Jørgensen,F., Koppes,L., Michelsen,O., Nielsen,J., Pedersen,P.E., Rasmussen,K.V., Riise,E. and Skovgaard,O. (1984) In Schaechter,M., Niedhardt,F.C., Ingraham,J. and Kjeldgaard,N.O. (eds), *Molecular Biology of Bacterial Growth*. Jones and Bartlett, Boston, pp. 282–297.

von Meyenburg,K. and Hansen,F.G. (1987) In Niedhardt,F.C., Ingraham,J.L., Brooks Low,K., Magasanik,B., Schaechter,M. and Umbarger, H.E. (eds), *Escherichia coli and Salmonella typhimurium. Cellular and Molecular Biology*. American Society for Microbiology, Washington, DC, pp. 1555–1577.

Yuasa,S. and Sakakibara,Y. (1980) *Mol. Gen. Genet.*, **180**, 267–273.

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