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Parental strand recognition of the DNA replication origin by the outer membrane in *Escherichia coli*

John Herrick, Renée Kern, Sibajyoti Guha, Ahmed Landoulsi, Olivier Fayet[1], Abderrahim Malki and Masamichi Kohiyama[2]

Institut Jacques Monod, CNRS, Université Paris 7, 2 Place Jussieu, 75251 Paris cedex 05 and [1]Laboratoire de Microbiologie et Génétique Moléculaire, CNRS, 118 Route de Narbonne, 31062 Toulouse, France
[2]Corresponding author

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The outer membrane of *Escherichia coli* binds the origin of DNA replication (*ori*C) only when it is hemimethylated. We report here the results of a footprinting analysis with the outer membrane which demonstrate that its interaction with *ori*C occurs mainly at the left moiety of the minimal *ori*C, where 10 out of 11 Dam methylation sites are concentrated. Two regions, flanking the Integration Host Factor (IHF) sites, are preferentially recognized at the minimum membrane concentration at which *ori*C plasmid replication is inhibited *in vitro*. We have identified the putative proteins involved in hemimethylated *ori*C binding and cloned one of the corresponding genes (*hob*H). The purified LacZ–HobH fusion protein specifically binds *ori*C DNA at the same preferential sites as the membrane. A mutant of the *hob*H gene reveals partial asynchronous initiation of DNA replication.

Key words: *ori*C DNA/hemimethylation/HobH/outer membrane

Introduction

The replicon theory of Jacob *et al.* (1963) predicted the attachment of the *Escherichia coli* replication origin, *ori*C, to the cell envelope. The theory proposed that such an interaction might serve as a control mechanism both for the initiation of chromosomal replication and for the segregation of newly completed daughter chromosomes. DNA–membrane attachment has been the focus of several studies in the past 30 years since the theory was first presented (for a review see Firshein, 1988). Several publications have indicated participation of the outer membrane in *ori*C attachment (Wolf-Watz and Masters, 1979; Hendrikson *et al.*, 1982; Nicolaidis and Holland, 1978; Chakrabarti *et al.*, 1992). A clear picture of this interaction, however, did not emerge until the report of Ogden *et al.* (1988), which demonstrated *in vitro* the specific binding of *ori*C by an outer membrane preparation of *E.coli* K12. The authors further demonstrated that *ori*C binding by the preparation depended on the methylation state of the DNA. It was observed that the outer membrane fraction recognized *ori*C only when it was hemimethylated and not when it was unmethylated or fully methylated. Prior to this report, sequence analysis revealed that the 245 bp minimal *ori*C fragment contained an unusually high density of the Dam methylation site, GATC (Hirota *et al.*, 1979; Messer *et al.*, 1979). Furthermore, it was shown that these sites are highly conserved in the origins among the enterobacteria (Zyskind *et al.*, 1983). The physiological significance of this preponderance of Dam methylation sites in *ori*C remains obscure, but there is an apparent correlation between the density of GATC sites and the rates at which these regions of the chromosome are re-methylated. Campbell and Kleckner (1990) observed that *in vivo*, GATC rich regions of the chromosome remain hemimethylated, following passage of the replication fork, for one quarter of a generation, while more sparsely distributed GATC sites are rapidly re-methylated. More recently, methylation was also found to play a role in DNA replication in eukaryotes, suggesting a broad range of regulatory functions for methylation among diverse species (Leonhardt *et al.*, 1992). In *E.coli*, these recent results have been interpreted as providing evidence, in partial agreement with the replicon theory, of a transient, *in vivo* attachment of *ori*C to the outer membrane mediated by the activity of the Dam methyltransferase.

One important question is what are the biological consequences of *ori*C DNA sequestration by the membrane? Two functions have been reported: both a synchronization of the initiation of DNA replication (Bakker and Smith, 1989; Boye and Lobner-Olsen, 1990) and a suppression of *ori*C plasmid replication *in vitro* (Landoulsi *et al.*, 1990). Boye and Lobner-Olsen (1990) investigated the effects of varying levels of Dam methylase on *ori*C membrane attachment and observed a perturbation in the number of chromosomes per cell when Dam methylase was either under- or overexpressed. Previously, the authors reported an increase in the population of cells with an odd number of chromosomes in *dam* mutants, which they interpreted as evidence of asynchronous initiations occurring in these cells.

Previous results have indicated the existence of a number of different proteins implicated in the *ori*C–membrane interaction (Chakrabarti *et al.*, 1992; Hughes *et al.*, 1992; Schaechter and von Freiesleben, 1993). However, none of the corresponding genes has been identified or isolated and so their potential role in the initiation of DNA replication remains, at present, unknown.

We report here the results of a DNase I footprinting analysis of the *ori*C–membrane complex which demonstrate the precise manner in which the outer membrane fraction recognizes the origin of replication. We have also isolated a gene coding for a protein implicated in the *ori*C–membrane interaction. A mutant of this gene permitted us to define an *in vivo* function for the protein in the initiation of DNA replication in *E.coli*.

Results

DNase footprints of hemimethylated oriC bound to outer membrane

Filter binding experiments initially demonstrated that only hemimethylated *ori*C DNA forms a complex with the outer membrane (Ogden *et al.*, 1988; Landoulsi *et al.*, 1990; Hughes *et al.*, 1992). In order to determine the site, or the base sequence of *ori*C DNA, which binds to the outer membrane, we have employed the technique of DNase footprinting using pancreatic DNase I. The regions protected by the membrane comprise mainly the left moiety of *ori*C (bases 1–190, base numbering as in Messer *et al.*, 1979) (Figure 1A), in which 10 out of 11 GATC sites of the minimum *ori*C are located (Oka *et al.*, 1980). Analyses from different laboratories (Buhk and Messer, 1983; Assai *et al.*, 1990) have shown that the AT-rich region just outside the left end of minimal *ori*C is also necessary for replication initiation. Our results show that this region also interacts with the membrane. The right moiety (up to base 244) showed no significant protection. More precise analysis of the protected sequences revealed that all 10 Dam sites are protected by the outer membrane, whereas intervening regions are weakly protected (Figure 1B). Binding is therefore clustered at the hemimethylated GATC sites in a non-sequence-specific manner. The fact that fully methylated *ori*C DNA was not protected by the outer membrane (Figure 7A) indicates that known *ori*C-specific proteins, such as the DnaA protein (Fuller *et al.*, 1984), IHF (Filutowicz and Roll, 1990), Fis (Gille *et al.*, 1991), IciA (Hwang and Kornberg, 1990) or Rob (Skarstad *et al.*, 1993), are not implicated in this interaction. In addition, heat-denatured methylated *ori*C DNA was not protected by the membrane, demonstrating that the non-methylated complementary strand is necessary for binding.

Differences in protection between the methylated and non-methylated strands of hemimethylated oriC

One of the objectives of our analysis was to determine if the outer membrane can discriminate between the parental strands (methylated) and the daughter strands (non-methylated) of the origin DNA. We therefore prepared, according to the strand separation method of Maxam and Gilbert (1980), a radioactive 5′ methylated strand which was then hybridized to its unmethylated complementary strand. Likewise, a radioactive 5′ non-methylated strand was similarly prepared and hybridized to the methylated complementary strand. The same operation was repeated for the isolated 3′ radioactive strands. The strands were labelled previously with ^{32}P by Klenow polymerase either at the 3′ *Xma*I site (−46) or at the 3′ *Hin*dIII site (+244) situated at opposite ends of *ori*C. In this manner, four different probes were prepared: M^+, a *Xma*I labelled methylated strand probe, M^-, a *Xma*I labelled non-methylated strand probe and M^+ and M^- probes labelled at the *Hin*dIII site (see 'methylation state' in Figure 2). All four probes were digested by DNase in the absence of membrane to control for any substantial bias in DNase activity due to the methylation state. The probes were subsequently digested after complex formation with the outer membrane and the footprints were compared (Figure 2). Efficient protection was observed using the M^+ probes

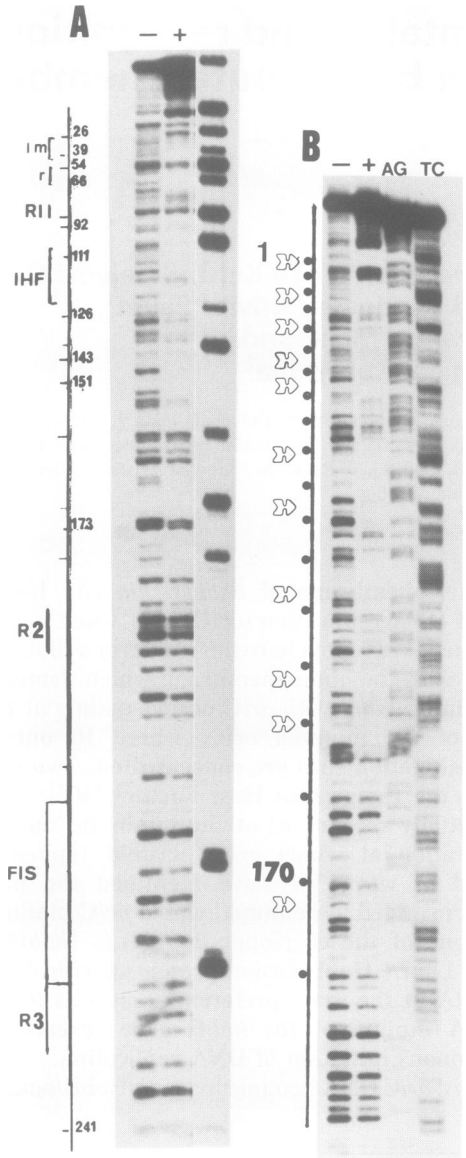

Fig. 1. DNase footprinting of *ori*C DNA complexed with the outer membrane fraction. (**A**) The 290 bp hemimethylated *ori*C probe was prepared by hybridizing a methylated labelled strand to its complementary unlabelled non-methylated strand. Methylated and non-methylated *ori*C-containing plasmid pOC42 was digested with *Xma*I (−46) and *Hin*dIII (+244; for *ori*C numbering see Messer *et al.*, 1979) and the methylated fragment was labelled at the 3′ end of the *Hin*dIII site with [^{32}P]dCTP by Klenow polymerase (see Materials and methods). Size markers (right margin) were prepared from pBR322 digested with *Hpa*II and labelled by end-filling with [^{32}P]dCTP. The numerals in the left margin are a schematic presentation of *ori*C with base numbers corresponding to GATC sites. IHF and FIS mark the locations of IHF and FIS protein binding sites. R1, R2 and R3 represent the DnaA boxes 1, 2 and 3, respectively. The 13mer AT-rich repeats are shown as l (left), m (middle) and r (right) (Bramhill and Kornberg, 1988). The (+) sign above the lanes indicates the presence of 10 μg outer membrane preparation in the reaction mixture, while the (−) sign indicates its absence. (**B**) The methylated *ori*C fragment *Xma*I (−46)−*Hin*dIII (+244) labelled with [γ-^{32}P]ATP selectively at the 5′ end of the *Hin*dIII site was hybridized to its unmethylated complementary strand as in (A). Marker base sequence was determined by limited cleavage at purines (AG) and pyrimidines (TC) by the method of Maxam and Gilbert (1980). The arrows mark the Dam methylation sequence. Dots mark every 10 bp. Before DNase digestion, the probe was pre-incubated with (+) or without (−) 10 μg membrane.

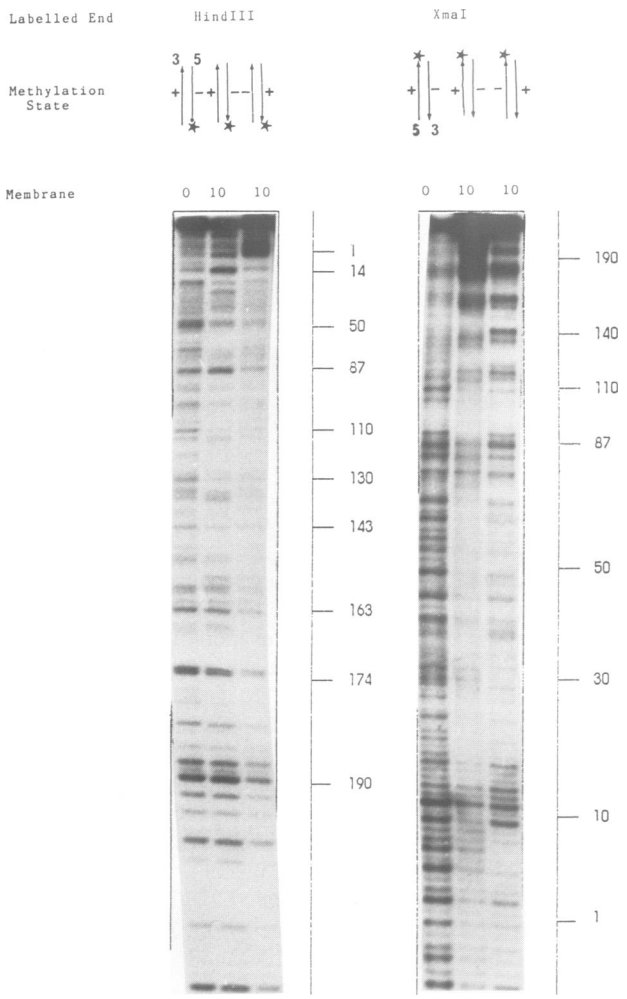

Fig. 2. Preferential protection of parental strands by the membrane. The 290 bp *ori*C fragments (*Xma*I−*Hin*dIII) were labelled with [^{32}P]dCTP (stars) by an end labelling reaction either at the 3′ *Hin*dIII site or the 3′ *Xma*I site. They were denatured and the radioactive 5′ and 3′ strands were separated before hybridization to the complementary strand with the opposite methylation status. The hemimethylated probes from left to right were: (*Hin*dIII labelled fragments) 5′ M^- labelled strand (star) with complementary M^+ cold strand; 5′ M^- labelled strand with 3′ M^+ strand; 5′ M^+ labelled strand with 3′ M^- strand; (*Xma*I labelled fragments) 5′ M^+ labelled strand with 3′ M^- strand; 5′ M^+ labelled strand with 3′ M^- strand; 5′ M^- labelled strand with 3′ M^+ strand. Membrane (10 μg) was (10) or was not (0) added.

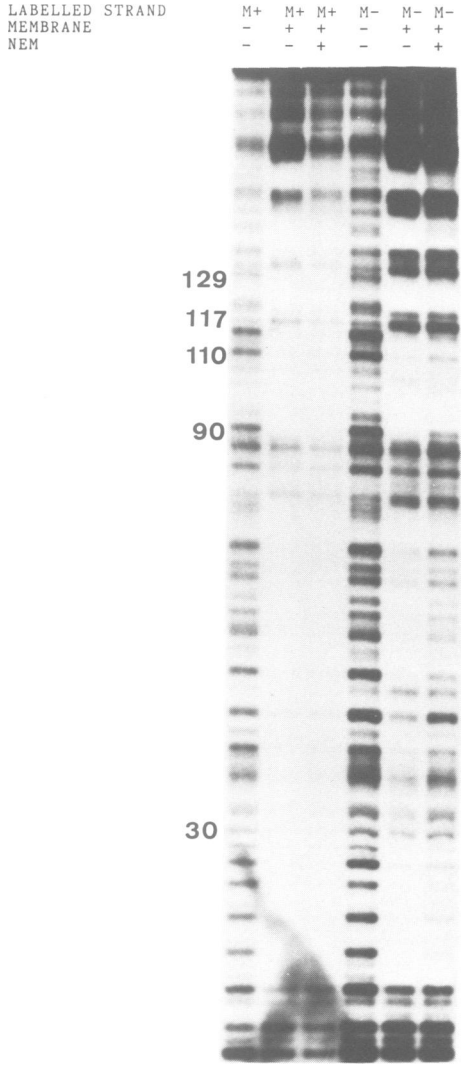

Fig. 3. Effect of NEM treatment on *ori*C footprinting in the presence of outer membrane. Outer membrane preparation 10 μg (with + or without −) was first reacted with hemimethylated *ori*C probe and then incubated at 37°C for 5 min in the presence of 1 mM NEM (with + or without −) followed by addition of 1 mM dithiothreitol before DNase I digestion. M^+, the methylated strand labelled at the *Xma*I site; M^-, the non-methylated labelled strand.

labelled at either site. However, with the M^- probes, weaker protection of these regions by outer membrane was observed. Also apparent are certain protected bands that are specific to the M^+ probes (for example, around 10, 90 and 110). There is therefore an asymmetry in membrane binding between the methylated strand and the non-methylated strand, with somewhat stronger protection of the methylated parental strands. The protection observed on the non-methylated strands further demonstrates that the methylated strand alone is not sufficient for membrane binding but that both strands are necessary for the membrane to recognize the probe. The sensitized bands (9, 14, 87 and 167) are observed with the M^- probes in a more distributed way than with M^+ probes, further indicating an asymmetry in the interaction between the membrane and the M^+ and M^- strands of hemimethylated *ori*C DNA. These observations were confirmed by treatment of the *ori*C−membrane complexes with *N*-ethylmaleimide (NEM). The results indicate that the methylated strand probes bound by the membrane are more resistant to NEM treatment than the non-methylated strand probes (Figure 3). This further indicates the preferential binding of the membrane to the parental strands of hemimethylated *ori*C DNA.

Preferential membrane attachment sites of oriC

As shown in Figure 1B, the GATC sites in the left moiety are protected by the membrane. Among the 10 sites, some may possess more affinity for the membrane than the others. In order to define more clearly the preferential site of *ori*C attachment, we have examined the profiles of footprints with varying concentrations of the outer membrane. We observe clearly in Figure 4A that DNase protection occurred at a low concentration of membrane

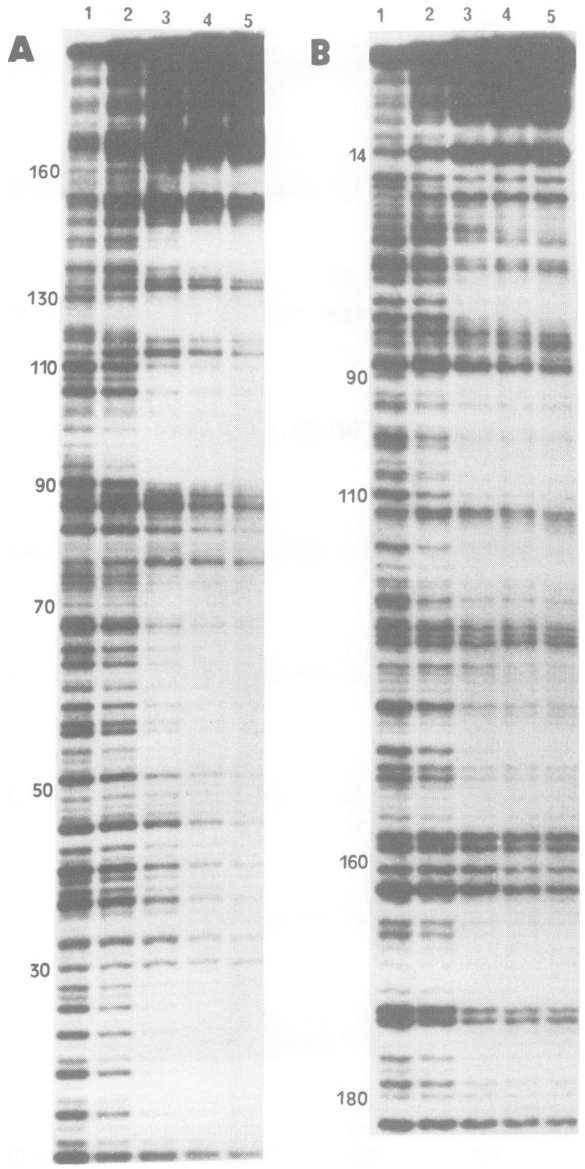

Fig. 4. Membrane concentration-dependent footprinting of hemimethylated *ori*C probe. Probes were prepared as described in Figure 2. Labelling was either at the 3′ *Xma*I site of the methylated strand (**A**) or at the *Hin*dIII site of the methylated strand (**B**). *ori*C probe (2 ng) was reacted with varying concentrations of membrane followed by DNase digestion. Lane 1, 0 μg membrane; lane 2, 2.5 μg; lane 3, 7.5 μg; lane 4, 15 μg; lane 5, 30 μg.

(2.5 μg) in two regions at bases 90–110 and 120–130 with respect to the probe labelled on the methylated strand at the 3′ *Xma*I site. The same concentration of membrane complexed with the methylated strand probe labelled at the 3′ *Hin*dIII site showed that regions 90–110 and 120–130 were again preferentially protected (Figure 4B). The probes with labelled non-methylated strands did not show significant patterns of protection except at high concentrations of membrane (>7 μg). These results also demonstrate that the lowest concentration of membrane at which the protection occurs corresponds to the minimum concentration at which *ori*C plasmid replication is inhibited *in vitro* (Landoulsi *et al.*, 1990). Besides these sites, a somewhat less strongly protected region (15–30) was observed at a low concentration of membrane (Figure 4A).

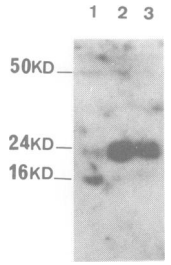

Fig. 5. Southwestern blotting of *E.coli* strain PC2 membrane proteins with hemimethylated *ori*C DNA. Lane 1, 60 μg total membrane; lane 2, 30 μg outer membrane; lane 3, 15 μg outer membrane. Total membrane and outer membrane fractions were obtained from a synchronized culture of *E.coli* strain PC2 according to Ogden *et al.* (1988). ^{32}P-Labelled hemimethylated *ori*C probe was prepared as described in Materials and methods.

Location and characterization of membrane proteins reacting with oriC

We subsequently identified the proteins in membrane preparations involved in hemimethylated *ori*C binding. We have analysed both total and outer membrane preparations (Ogden *et al.*, 1988) using the technique of Southwestern blotting (Bowen *et al.*, 1980). The hemimethylated *ori*C probe reacted predominantly with a 24 kDa protein, while other proteins corresponding to 50 kDa and 16 kDa with minor binding activities were also detected in the total membrane (Figure 5). Further purification of the outer membrane coincided with an exclusive increase in the binding activity of the 24 kDa protein. This protein did not react with the non-methylated *ori*C probe, as was shown previously (Hughes *et al.*, 1992). A similar finding has been reported by Schaechter and von Freiesleben (1993).

Isolation of the hobH gene (hemimethylated origin binding)

The high affinity of the 24 kDa protein for hemimethylated origin DNA permitted the expression cloning of the corresponding gene fragment from a λgt11−*E.coli* K12 genomic library. The bank was screened in the presence of 1000-fold calf thymus DNA as a competitor according to the method of Singh, with modifications (Singh *et al.*, 1988). Southwestern blots were performed on the crude extracts of both induced and non-induced lysogens of positive clones, two of which showed exclusive affinity for the hemimethylated probe even in the presence of 100-fold cold methylated *ori*C plasmid DNA.

The 1.2 kb *Eco*RI fragment, present in a λgt11::H phage (Figure 6A) was radiolabelled and used to probe an ordered collection of 476 hybrid λ phages. Phages 1E4 and 12D1, in the nomenclature of Kohara *et al.* (1987), gave a positive signal: they carry the 92 min region, around coordinate 4350 kb on the physical map. Further characterization revealed that they contain a 1.2 kb *Eco*RI fragment identical to the insert λgt11::H. In order to clone the entire *hob*H gene, a 4.5 kb *Bam*HI fragment from phage IE4 was inserted into the *Bam*HI site of pBluescript SK$^+$, in both orientations: the *Bam*HI site on the *uvr*A side in phage IE4 results from ligation of a *Sau*3A site (coordinate 2804 bp in *uvr*A; Husain *et al.*, 1986) into the *Bam*HI site of the vector. The nucleotide sequence revealed a coding region of 175 amino acids with a high

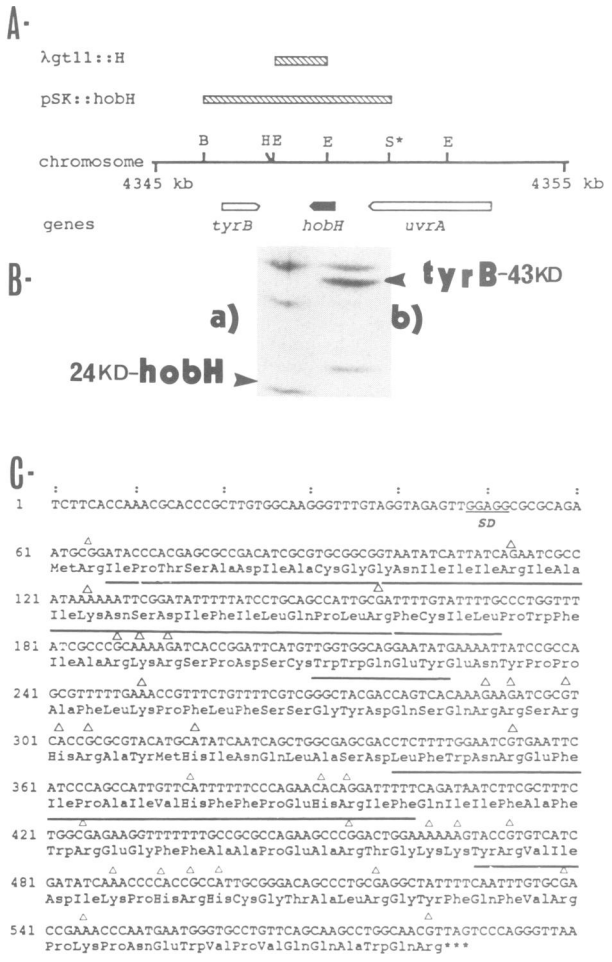

Fig. 6. Chromosomal localization and nucleotide sequence and expression of the *hobH* gene. (**A**) The letters B, E, H and S* indicate the restriction sites for *Bam*HI, *Hin*dIII, *Eco*RI and *Sau*3A (not unique). The regions cloned either in phage λgt11 or in plasmid pBluescript SK^+ (Stratagene) are indicated by hatched boxes and the known genes by boxes with a pointed end giving the direction of transcription. Base numbering is from Kohara *et al.* (1987). (**B**) pSK-*hob*H was expressed *in vivo* in the presence of [^{35}S]methionine and the labelled proteins were analysed (see Materials and methods). (a) Expression from *uvr*A side. (b) Expression from *tyr*B side using oppositely inserted *hob*H gene. (**C**) The nucleotide sequence of the region between the known *uvr*A and *tyr*B genes was established by the dideoxy chain termination method (Sanger *et al.*, 1977) using either T7 (US Biochemical) or Vent (Biolabs) DNA polymerase according to the supplier's instructions; it can be obtained from the EMBL data bank under accession number Z226592. That part of it containing the *hob*H gene and the deduced protein sequence are presented here. The *Eco*RI site at position 355 defines the fusion point of the HobH protein with the C-terminus of β-galactosidase in λgt11::H. The regions with a hydrophobic index of >8 (Kyte and Doolittle, 1982) are underlined. Triangles, basic amino acids.

frequency of both hydrophobic (in the N-terminal region) and basic amino acid residues (in the C-terminal region). A possible Shine−Dalgarno sequence is evident 8 bp upstream from the ATG start codon. The pBluescript SK−*hob*H construct was expressed in the *E.coli* strain BL21 (DE3) by inducing the T7 RNA polymerase gene integrated into the host chromosome (Studier *et al.*, 1990). Induced proteins were labelled *in vivo* with [^{35}S]methionine, separated by SDS−PAGE and autoradiographed. A 24 kDa protein consistent with the expected molecular weight of the product of the *hob*H gene was expressed from the *uvr*A side of the subcloned *Bam*HI fragment (Figure 6B,a), while a 43 kDa protein corresponding to *tyr*B (Fotheringham *et al.*, 1986) was expressed from the fragment subcloned in the opposite orientation (Figure 6B,b).

HobH protein oriC binding site

In order to demonstrate that the HobH protein interacts specifically with hemimethylated *ori*C DNA at a particular sequence, footprinting experiments were realized with LacZ−HobH fusion protein (LacZ fused to 75 C-terminal amino acids of HobH) purified to near homogeneity after overexpression (Figure 7B; see Materials and methods). Clear protection occurred at regions 90–110 and 120–130 with the M^+ probe labelled at the 3′ *Xma*I site, while weak protection occurred in the region 15–30 (Figure 7A). In addition, the zone (bases 163–174) in Figure 2 specific to the M^+ strand was also protected by the LacZ−HobH protein (Figure 7C). LacZ protein purified according to the same method as the fusion protein did not react with the hemimethylated *ori*C probe (Figure 7C).

The binding of the LacZ−HobH protein to the *ori*C probe was inhibited when NEM was added before formation of the membrane complex, but was not significantly altered when added after complex formation (Figure 7A and C). A comparison between the footprints obtained with the fusion protein and the membrane revealed some striking differences in their respective patterns of protection outside the preferential binding sites. This might be due to the absence of the N-terminal region of HobH or to other membrane factors absent in the preparation of the fusion protein.

Asynchrony of replication initiation of the hobH mutant

A mutant was constructed by introducing a kanamycin resistance gene ligated to the gene fragment coding for the N-terminal moiety of *hob*H. The mutant was examined for synchrony of initiation using flow cytometry. The mutant culture, after treatment with rifampicin and ampicillin to prevent residual growth, contained a significant number of cells with odd numbers of chromosomes (Figure 8A), whereas the wild-type culture, under the same conditions, contained bacteria with two or four chromosomes (Figure 8B). This observation indicates that the mutant presents a significant degree of asynchrony in the initiation of DNA replication. The *hob*H mutant is therefore asynchronous with respect to initiation, with an index of asynchrony calculated (Boye and Lobner-Olesen, 1990) to be 0.6, in comparison with 0.1 for the wild-type. Thus, the degree of asynchrony of the mutant corresponds to that of the mutant *dna*A205, but is below the level of the *dam* mutant (Skarstad *et al.*, 1988).

Discussion

DNA methylation on hemimethylated substrates plays an important role in both eukaryotic (Leonhardt *et al.*, 1992) and prokaryotic DNA replication. The data presented here demonstrate that hemimethylated *ori*C DNA interacts with outer membrane proteins in a specific and precise manner. It has emerged clearly from these results that the region protected by the membrane from pancreatic DNase diges-

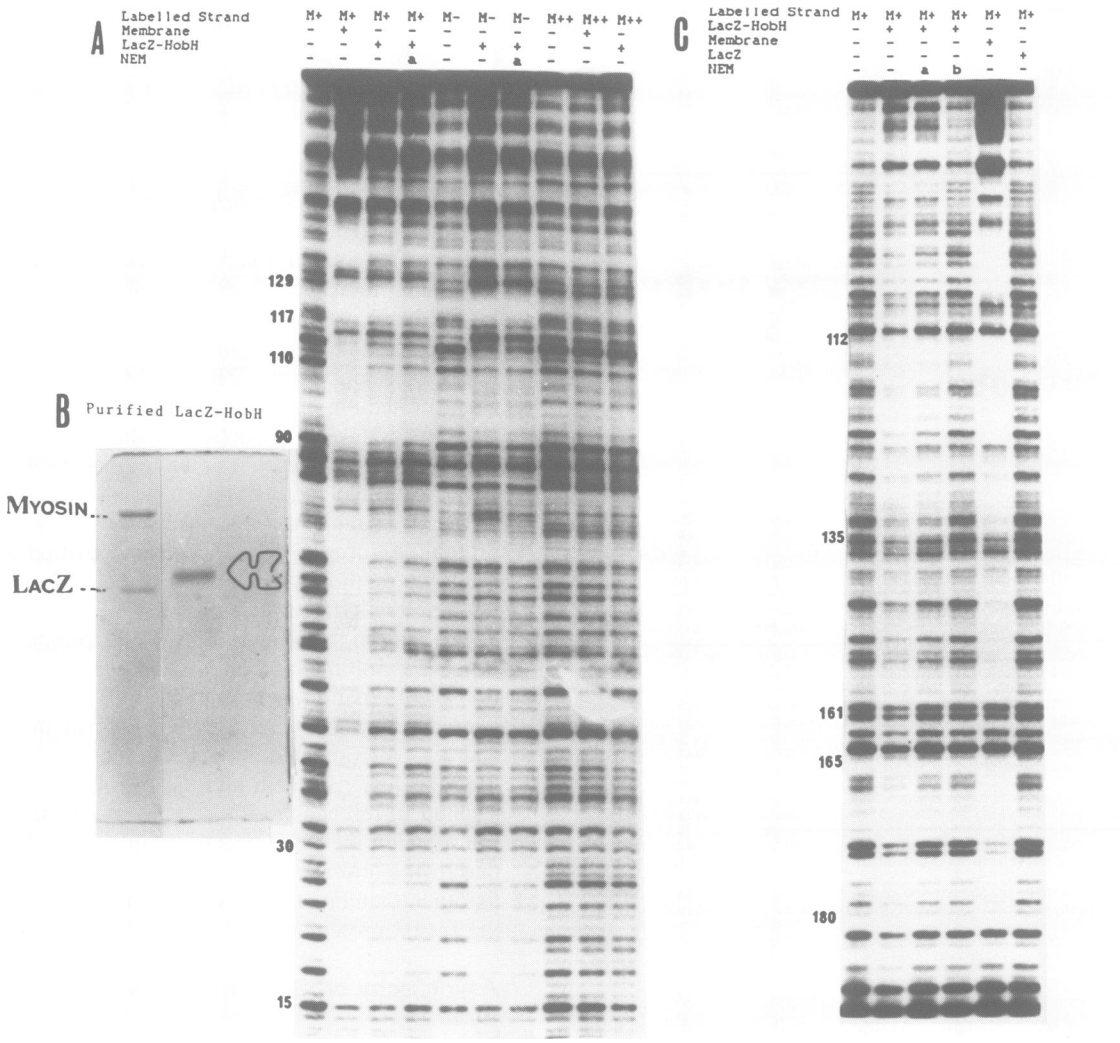

Fig. 7. DNase footprinting of *ori*C and NEM sensitivity of LacZ−HobH. (**A**) The *ori*C probe ^{32}P-labelled at the *Xma*I end of either the methylated (M^+) or the non-methylated (M^-) strand was used for footprinting experiments in the presence of 6 μg purified LacZ−HobH. M^{++} is *ori*C methylated on both strands prepared by hybridizing an M^+ radioactive strand to an M^+ complementary strand. NEM is *N*-ethylmaleimide (1 mM), added after *ori*C LacZ−HobH complex formation for 5 min at 37°C, before DNase digestion as in Figure 3. The membrane was added at 10 μg. (**B**) Coomassie blue staining of SDS−PAGE with the purified LacZ−HobH (5 μg, arrow) and marker proteins. (**C**) The experimental conditions and symbols are the same as in (A) except that the DNA strand was labelled at the *Hin*dIII 3′end. The LacZ protein was added at 12 μg. NEM was added either before *ori*C (b) or after (a), as in (A).

tion consists of the left moiety of the minimum *ori*C, where 10 out of 11 methylation sites are located (Oka *et al.*, 1980). In this widely protected region, the preferential binding sites, found at low concentrations of membrane, are 90–110 and 120–130. The integrity of the sequence in the region 90–110 has been shown previously to be essential for the binding of *ori*C to membrane fractions (Kusano *et al.*, 1984). This suggests that the region 90–110 may be the primary membrane attachment site on *ori*C *in vivo*.

Outer membrane attachment at these two neighbouring sites may then be sufficient to inhibit initiation of DNA replication at *ori*C (Landoulsi *et al.*, 1990). We have observed, consistent with this suggestion, that the concentration at which *ori*C plasmid replication is inhibited corresponds to the minimal concentration at which binding occurs. These two preferential binding sites are also recognized by the HobH protein, suggesting that HobH is a component of the outer membrane which binds to *ori*C. The purified LacZ−HobH fusion protein attaches to these sites only on hemimethylated DNA, with a somewhat enhanced affinity for the methylated strand. The fusion protein, in contrast to the membrane, does not significantly protect the 13mer region, nor the regions between 140 and 170. This difference may be due to the absence of the N-terminal moiety of HobH from the fusion protein or equally may imply the participation of other factors in *ori*C binding.

The results reported here also show that the outer membrane binds hemimethylated *ori*C with a preference for the parental strand. Enhanced affinity of the membrane for the parental strands is clearly demonstrated by the fact that NEM is accessible only to the complexed non-methylated strand in the 13mer region and not to the methylated strand. DNA bending at *ori*C by DnaA protein and IHF stimulates initiation of DNA replication (Skarstadt *et al.*, 1990; Woelker and Messer, 1993). We propose a mechanism according to which the membrane inhibits re-

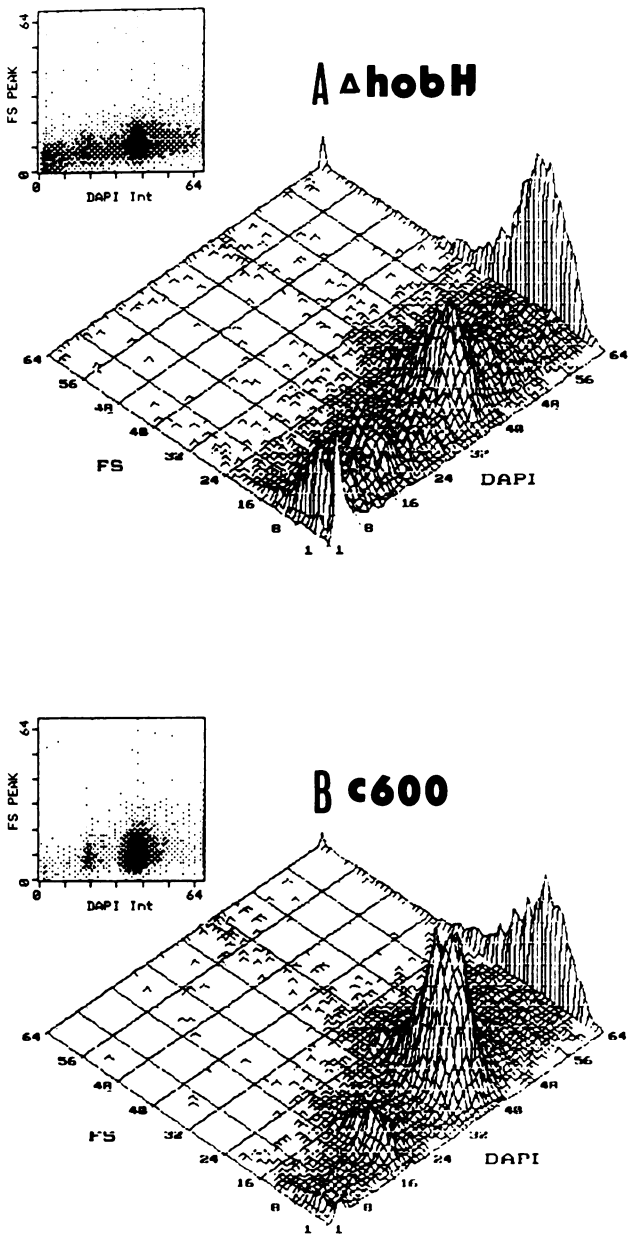

Fig. 8. Flow cytometric analysis of DAPI-stained, rifampicin-treated cells. (**A**) 13 *hob*H mutant. A LB culture of *hob*H mutant was analysed after rifampicin treatment (see Materials and methods). The left panel shows a dot plot of the distribution of cells according to cell size (FS, light scattering) and DAPI staining intensity (DAPI axis). The right panel shows the same distribution of cells as in the left panel represented as 3D peaks. (**B**) Parental strain C600. Left and right panels represent, respectively, the cluster and 3D peaks of the stained cells. Note the position of two chromosomes at around 16 (DAPI) and four chromosomes at 32, determined according to a minimal medium culture of the same strain.

initiation by binding hemimethylated *ori*C templates at the preferential sites flanking the IHF consensus sequence. This binding in turn prevents DNA bending by DnaA protein and IHF, which is essential for efficent initiation at *ori*C. The outer membrane acts as a methylation blocking factor which retards the activity of Dam methyltransferase. However, the loose binding of the non-methylated strands indicates that *ori*C sequestration occurs via the methylated parental strands. The non-methylated daughter strands are therefore partially accessible to Dam methyltransferase and the time required to re-methylate these sites could determine the period of *ori*C sequestration.

The asynchrony observed in the *hob*H mutant confirms the suggestion that the hemimethylated *ori*C–membrane interaction is essential for synchronizing the replication of multiple chromosomes in the cell. The asynchrony index of the *hob*H mutant is, however, less than that of the *dam* mutant, but is comparable with the *dna*A (ts) initiation mutant *dna*A205 (Skarstad *et al.*, 1988). The asynchrony phenotype allows for two possible interpretations: a loss in the timing of initiation during the cell cycle and/or anomalous re-initiations occurring at newly replicated origins. If asynchrony is due to the absence of *ori*C–membrane attachment in *dam* mutants, then the intermediate degree of asynchrony observed in the *hob*H mutant indicates either a partial or relaxed *ori*C–membrane interaction or that this interaction is due to more than one component producing the high degree of asynchrony in *dam* mutants. Indeed, the asynchrony observed in the *dam* mutant may reflect both a loss in the timing of initiation and multiple aberrant initiations occurring at newly replicated origins (Boye and Lobner-Olesen, 1990). The number of extra origins present, however, in these cells is only ~2-fold more than wild-type, which alone seems unlikely to account for the significant asynchrony of *dam* mutants. Additionally, DnaA protein is reduced 3-fold in *dam* mutants (Braun and Wright, 1986; Landoulsi *et al.*, 1989). This may also contribute to the pronounced asynchrony phenotype of this mutant. The asynchrony observed in the *hob*H mutant, on the other hand, may only reflect apparently random timing of initiation.

Alternatively, other factors may be involved in the *ori*C–membrane attachment necessary for a high level of initiation synchrony (Schaechter, 1990). The results of Southwestern blotting analysis of the membrane and footprinting with the LacZ–HobH fusion protein support this possibility. The absence of clear protection by the fusion protein in the AT-rich 13mer region and other regions, although protected by the membrane (140–150, 114–120), suggests that binding *in vivo* by another factor to these GATC sites may account for the difference between *hob*H and *dam* mutants in their indices of asynchrony. Our results also indicate that *ori*C–membrane attachment occurs as a post-initiation event following replication of the origin. HobH and outer membrane do not bind single-stranded methylated *ori*C DNA but require a double-stranded hemimethylated substrate. These observations are consistent with HobH and the membrane playing an important role in the observed non-random replication of multiple chromosomes in *E.coli*.

During revision of the manuscript, the isolation of a second component involved in *ori*C sequestration has been reported. The gene, called *seq*A (Lu *et al.*, 1994) codes for a protein with a calculated molecular weight almost identical to that encoded by the *hob*H gene. It is possible that this protein also binds hemimethylated *ori*C DNA, but genetic studies indicate that the protein acts at an early step in initiation, perhaps in a cooperative manner with the DnaA protein. The biochemical studies presented here demonstrate that the components associated with the outer membrane fraction bind hemimethylated *ori*C DNA at a late step in the initiation process and previous results

indicate that this binding occurs in a manner that is exclusive to DnaA protein. These two proteins may therefore represent different stages in the process of *ori*C sequestration.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strains PA3092 and GM2199 (*dam*13::Tn9; Marinus *et al.*, 1983) transformed with the plasmid pOC42 were the sources of methylated and unmethylated plasmids, respectively. The plasmid pOC42 contains the whole *ori*C region within a *Pst*I fragment inserted in the *Pst*I site of the plasmid pBR322 (Messer *et al.*, 1980). Strain PC2 [dnaC(Ts) *leu thy* Str^r] was used to isolate the membrane fractions according to Ogden *et al.* (1988). *Escherichia coli* strain BL21-DE3 (Studier *et al.*, 1990) was used to transform the pBluescriptSK-*hob*H plasmids. The strain C600 was used for the deletion by integration of the truncated *hob*H gene. The plasmid pBM14 (Guha, 1988) was used to prepare the kanamycin resistance gene-containing plasmid for integration into the chromosome. The plasmids pEX2 and pBluescript SK were obtained from Boehringer (Mannheim) and Stratagene, respectively.

DNA fragments

The plasmid pOC42, either methylated or unmethylated, was digested sequentially with the restriction enzymes *Xma*I and *Hin*dIII and the 290 bp fragment, isolated by polyacrylamide gel electrophoresis, was end-filled with Klenow DNA polymerase in the presence of [^{32}P]dCTP and the three other deoxyribonucleotides.

Separation of DNA fragments and complementary strands

Double-stranded fragments were separated on slab gels (20×17 cm× 0.8 mm) containing 4% polyacrylamide (acrylamide:bis 19:1). Restriction enzyme digests, mixed with the marker dyes bromophenol blue and xylene cyanol, were loaded onto the gel and electrophoresis was carried out in 89 mM Tris–borate and 1 mM EDTA buffer at 200 V for 4–5 h. The gel was stained with ethidium bromide (1 mg/ml) and the fluorescent bands corresponding to the fragment were removed.

5′ and 3′ strands were separated on slab gels (40×17 cm×0.8 mm) containing 5% polyacrylamide (acrylamide:bis 30:0.6). Labelled DNA fragments were dissolved in buffer containing 96% formamide and marker dyes. The solutions were heated to 100°C for 5 min in a boiling water bath and loaded immediately onto the gel. Electrophoresis was in Tris–borate–EDTA buffer at 250 V for 15 h. The gel, without drying, was exposed to an autoradiographic film (Fuji) for 30–60 min at room temperature and the gel strips corresponding to the autoradiographic bands were isolated.

Gel strips containing single-stranded DNA were crushed in Eppendorf tubes in the extraction buffer (Maxam and Gilbert, 1980) and centrifuged. The supernatant containing the DNA was made 1 M and 1% with respect to NaCl and SDS and precipitated with 2 vol of absolute ethanol. The precipitate, after centrifugation, was washed with 70% ethanol and dried. The purified DNA fragments were dissolved in SSC (0.15 M NaCl, 0.015 M sodium citrate) and stored at −20°C.

Preparation of hemimethylated oriC DNA fragments

Hemimethylated DNA fragments were prepared by mixing one of the two isolated ^{32}P-labelled, for example, unmethylated single strands (dissolved in 1× SSC) with a 2 M equivalent of unlabelled double-stranded methylated DNA. In some experiments, two different (M^+ and M^-) single-stranded *ori*C DNAs were isolated and hybridized in equimolar amounts. The mixture, after boiling for 5 min at 100°C, was brought to 70°C and allowed to cool slowly to room temperature and stored at −20°C. Methylated, ^{32}P-labelled, single-stranded DNA was similarly hybridized to the unmethylated DNA. A small portion of the hemimethylated DNA was analysed by gel electrophoresis to determine hybridization efficiency.

Membrane binding and DNase protection

Outer membrane fraction was isolated as described (Landoulsi *et al.*, 1990). The binding of DNA to the membrane fraction was carried out in a total volume of 10–15 µl. The incubation, for 10 min at 37°C, was in 10 mM HEPES (pH 7.4), 100 mM KCl, 1 mM EDTA, 2.5–30 µg membrane (see figure legends), 0.6–2 ng ^{32}P-labelled hemimethylated DNA (10–15×10^3 c.p.m.) and 800 ng calf thymus DNA as competitor. After incubation, 3 µl 10× DNase buffer (1 mM Tris–HCl, 2 mM $MgCl_2$, 10 mM $CaCl_2$, 1 M KCl, 10 mM DTT, 1 mM EDTA, 4 µg bovine serum albumin) was added and the volume was brought to 30 µl. The solution was incubated in the presence of 10 ng DNase (Boehringer) for 1 min at 37°C and the reaction was stopped by the addition of 200 µl of a mixture of 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1% SDS, 5 µg *E.coli* tRNA. The mixture was deproteinized by incubation with proteinase K (12 µg) at 45°C for 30 min. The digested DNA was precipitated and resuspended in 96% formamide containing the marker dyes. The digested DNA preparations were loaded onto a 8% acrylamide gel (7 M urea) buffered in 89 mM Tris–borate and 1 mM EDTA.

Southwestern blotting

Total and outer membrane fractions were prepared from synchronized cultures of *E.coli* strain PC2 according to Ogden *et al.* (1988). Total membrane (60 µg) and outer membrane (30 µg) proteins were separated by 15% SDS–PAGE (Laemmli, 1970) and transferred onto Hybond (Amersham) nylon filter (0.45 µm) at 4°C in Tris–glycine transfer buffer (Towbin *et al.*, 1979). Filters were blocked by incubation in binding buffer (10 mM HEPES–KOH, pH 7.4, 100 mM KCl, 1 mM EDTA, 5% skimmed milk) for 1 h at 4°C, then briefly washed and incubated overnight at 4°C with either hemimethylated or unmethylated *ori*C DNA probes (10^{-10} M) in the presence of a 1000-fold excess of competitor DNA. Radiolabelled hemimethylated or unmethylated *ori*C probes were prepared by primer extension (5′-GGGCCG-3′) from the *Sma*I end on the denatured 463 bp *Sma*I–*Xho*I restriction fragment of *ori*C derived from methylated and unmethylated pOC42 plasmid DNA. Modified T7 DNA polymerase (Biolabs) was used to extend the primer, which yielded [^{32}P]dATP-labelled probes of ~2×10^8 c.p.m./µg. Filters were washed after incubation with probe for 10 min in binding buffer, dried and autoradiographed.

Purification of HobH–LacZ fusion protein

The HobH–LacZ fusion protein was purified from *E.coli* strain N4830 (*his*, *bio*, λ del cI857) harbouring the truncated *hob*H gene (75 amino acids) inserted at the *Eco*RI site of *lac*Z of the pEX2 recombinant plasmid (Stanley and Luzio, 1984). A 100 ml culture in LB medium was grown at 30°C to an OD_{595} of 0.5. Cells were then induced to synthesize LacZ–HobH fusion protein by a temperature shift to 42°C for 4 h and harvested. The cell pellet was suspended in 10 ml 50 mM Tris–HCl (pH 7.5), 10% sucrose, sonicated for 3×1 min on ice and centrifuged at 12 000 *g* for 10 min. The pellet was resuspended in 10 ml TNE buffer (25 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, 10% sucrose). The suspension was frozen and thawed three times in liquid nitrogen and centrifuged at 12 000 *g* for 10 min. The pellet was then resuspended in TNE buffer plus 10% Triton X-100 on ice and centrifuged at 15 000 r.p.m. for 15 min. This pellet was resuspended in 8 M urea in 10 mM Tris (pH 7.4). The suspension was left on ice for 1 h and then centrifuged at 15 000 r.p.m. for 15 min. The supernatant was diluted 20-fold and dialysed overnight at 4°C against 1000 vol of binding buffer and concentrated in an Amicon concentrator. LacZ was similarly prepared from a control culture expressing *lac*Z without insert.

In vivo analysis of [^{35}S]methionine-labelled protein expressed from the hobH gene

The *hob*H gene from the *E.coli* gene bank of Kohara *et al.* (1987) was subcloned into the plasmid vector pBluescript SK$^+$ (Stratagene) as a 4.5 kb *Bam*HI fragment. From this vector the gene was expressed under the control of the T7 RNA polymerase promoter. The pSK–*hob*H recombinant plasmid was used to transform the *E.coli* strain BL21-DE3 (Studier *et al.*, 1990), which expresses the T7 RNA polymerase gene under the control of the inducible *lac-uv*$_5$ promoter. Transformed cells, containing the *hob*H gene in both sense and antisense orientations, were grown at 37°C to an OD_{595} of 0.6 and T7 polymerase was then induced by adding 0.4 mM isopropylthiogalactoside. After 30 min, rifampicin (100 µg/ml) was added and incubated for a further 30 min. [^{35}S]L-methionine (ICN) was then added (20 µCi/ml) and after 10 min incorporation, the cells were harvested, washed and lysed in sample buffer for electrophoresis (Laemmli, 1970). Electrophoresis was on 10% polyacrylamide gel containing SDS at 40 V overnight.

Construction of the hobH mutant

The plasmid pBM14 (Guha, 1988) was used to construct a plasmid which can be integrated into the *E.coli* chromosome. The plasmid pBM14 contains the replication origin, the ampicillin resistance gene (Ap^r) of the plasmid pBR322 and the kanamycin resistance (Km^r) gene from the transposon Tn5. A 1.3 kb *Eco*RI DNA fragment obtained

from the plasmid pSK-*hob*H, which contains the sequence corresponding to the N-terminal part of the HobH protein, was ligated to the unique *Eco*RI site of the plasmid pBM14. This plasmid was then digested with the enzyme *Apa*LI. The three fragments obtained were separated by agarose gel electrophoresis. The biggest, 5.1 kb fragment contained the Km^r gene and the 1.3 kb *hob*H fragment, but was without the replication origin and most of the Ap^r gene. This fragment, after self-ligation, was used to transform *E.coli* strain C600. Selection was for kanamycin resistance (50 μg/ml) at 30°C. Several colonies were obtained. DNA extracted from these clones were also checked for the absence of free plasmid. Chromosomal DNA from the Km^r clones, after *Bam*HI digestion, was probed for plasmid integration by Southern hybridization. One clone, designated '13*hob*H', had the Km^r gene inserted with the 1.3 kb fragment split from the 1.2 kb sequence which codes for the C-terminal half of the HobH protein.

Flow cytometry

An overnight culture of the mutant '13*hob*H' at 30°C was diluted in fresh LB medium (100 μg/ml kanamycin) to an OD_{600} of ~0.05 and cultured at 37°C. The parental strain C600 was used as control. At an OD_{600} of 0.2, 1 ml culture was treated with rifampicin (150 μg/ml) and ampicillin (10 μg/ml). The culture was continued for a further 90 min and the cells were centrifuged. The cell pellet was suspended in 1 ml 10 mM Tris (pH 7.4),10 mM $MgCl_2$ (freshly filtered) and 4 ml chilled methanol was added. After 20 min on ice, the suspension was centrifuged. The pellet was resuspended in 5 ml Tris−Mg^{2+} and stained with 0.5 ml DAPI (3 μg/ml). The stained cells were analysed in an Elite Flow Cytometer (Coulter Electronics). Inhibition of cell proliferation by rifampicin was ascertained by spectrofluorimetry of DAPI-stained DNA. After 30 min, no further increase in DNA content was observed.

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