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Open-complex formation by the host initiator, DnaA, at the origin of P1 plasmid replication

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Replication of P1 plasmid requires both the plasmid-specific initiator, RepA, and the host initiator, DnaA. Here we show that DnaA can make the P1 origin reactive to the single-strand specific reagents $KMnO_4$ and mung bean nuclease. Addition of RepA further increased the $KMnO_4$ reactivity of the origin, although RepA alone did not influence the reaction. The increased reactivity implies that the two initiators interact in some way to alter the origin conformation. The $KMnO_4$ reactivity was restricted to one strand of the origin. We suggest that the roles of DnaA in P1 plasmid and bacterial replication are similar: origin opening and loading of the DnaB helicase. The strand-bias in chemical reactivity at the P1 origin most likely indicates that only one of the strands is used for the loading of DnaB, a scenario consistent with the unidirectional replication of the plasmid.

Key words: DnaA/DNA–protein interactions/DNA replication/plasmid/RepA

Introduction

P1 plasmid belongs to a distinct class of replicons whose initiation and control of initiation are mediated by iterons (Nordström, 1990). The plasmid is maintained at low copy numbers characteristic of the bacterial chromosome (Prentki *et al.*, 1977; Pal *et al.*, 1986; Austin and Eichorn, 1992). Understanding the mechanism of this stringent mode of replication control has been the primary interest in studying the replication of the plasmid. There are five tandemly repeated iterons in the P1 origin, each 19 bp long (Abeles *et al.*, 1984). These iterons are the binding sites for the plasmid-encoded initiator, RepA, both *in vivo* and *in vitro* (Abeles, 1986; Sozhamannan and Chattoraj, 1993). It has been postulated that the iterated sequences help to form a specialized nucleoprotein structure that has several advantages over single DNA–protein complexes (Echols, 1990). For events like DNA replication, the structure can provide increased site specificity, a signal for initiation utilizing both the tertiary structure of proteins and the three-dimensional conformation of DNA and, finally, more opportunities for control. RepA binding bends the iterons and binding to all five iterons absorbs one positive superhelical turn of DNA, indicating that the DNA is wrapped around RepA (Mukhopadhyay and Chattoraj, 1993). How the information in the structure is used in initiation and its control is not known, but it must serve to open the strands of the origin. DNA opening is an essential step in the initiation of the theta mode of replication, as it sets the stage for loading of the helicase, DnaB (Bramhill and Kornberg, 1988b). The subsequent events in initiation are not considered relevant to the control of initiation.

From the right-handed wrapping of the origin DNA in RepA–DNA complexes, a compensatory turn of unwinding was expected, but the complexes did not show enhanced reactivity to a single-strand specific reagent, $KMnO_4$ (Mukhopadhyay and Chattoraj, 1993). The failure to observe unwinding by RepA alone drew our attention to the bacterial initiator, DnaA. The protein is required for replication of P1 plasmid *in vivo* and *in vitro* (Hansen and Yarmolinsky, 1986; Wickner and Chattoraj, 1987). The P1 origin has two sets of 9 bp sequences that are highly homologous to the boxes that bind DnaA (Hansen and Yarmolinsky, 1986; Abeles *et al.*, 1990; Stenzel *et al.*, 1991; see Holz *et al.*, 1992 for a review). One set of two tandem boxes maps at the left end of the origin and a second set of three tandem boxes maps at the right end. Either set allows replication, although optimal replication requires both of the sets (Abeles *et al.*, 1990). The origin of replication of *Escherichia coli*, *oriC*, has four discretely spaced DnaA boxes and binding of DnaA leads to opening of the origin DNA (Bramhill and Kornberg, 1988a). In the case of the plasmids R1 and pSC101, the plasmid-encoded initiator is known to stimulate the origin binding activity of DnaA, suggesting that the two proteins could interact with each other (Masai and Arai, 1987; Stenzel *et al.*, 1991; Ortega-Jiménez *et al.*, 1992).

In this study, we show that the DnaA protein stimulates the specific DNA binding activity of RepA and can open the strands of P1 origin. The DNA-opening reaction is stimulated when RepA protein was added together with DnaA. The initiators are thus used for two important functions: origin selection and DNA opening. These studies thus provide a rationale for the essential role of DnaA in P1 plasmid replication.

Results

Binding of RepA and DnaA proteins to P1 origin

The positions of the iterons and the DnaA boxes of the P1 origin are shown in Figure 1. RepA binding to the iterons has been characterized in detail (Abeles, 1986; Sozhamannan and Chattoraj, 1993). In comparison, little is known about the nature of binding of DnaA to the P1 origin. In this work, DnaA binding was studied on supercoiled DNA and assayed by DNase I footprinting. Specific protection of the DnaA boxes (coordinates 387–404) could be demonstrated on both strands of the DNA (Figure 2, lanes 3 and 7). In the presence of both RepA and DnaA, there was specific protection of both the iterons and the DnaA boxes (Figure 2, lanes 4 and 8). The two proteins could have influenced each other's

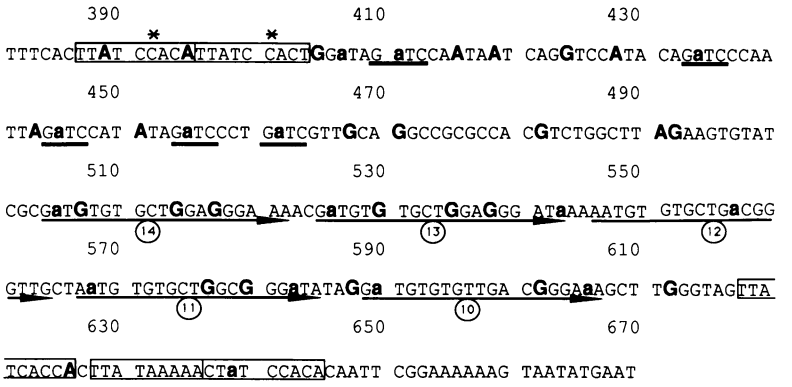

Fig. 1. Sequence of the top strand of P1 origin. Binding sites for DnaA protein are boxed; sites for Dam methylation are underlined. Arrows numbered from 10 to 14 indicate binding sites for P1-encoded RepA. Bold lower case letters represent bases whose complement in the bottom strand reacted to $KMnO_4$ in the absence of proteins, while bold upper case letters represent bases whose complement in the bottom strand of pSP102 reacted preferentially to $KMnO_4$ in the presence of DnaA and RepA (Figure 5, lanes 1–4). Asterisked bases preferentially reacted to DMS in the presence of RepA and DnaA (Figure 4).

binding as the protection was more complete. In the case of plasmid R1 the protection by DnaA is also weak and improves dramatically upon addition of the plasmid initiator (Masai and Arai, 1987; Ortega-Jiménez *et al.*, 1992). Increased RepA binding to the iterons in the presence of DnaA was independently found by band-shift assay using linear DNA (Figure 3). DnaA binding could not be reliably demonstrated by this technique (Figure 3, lane 2) but the stimulation of DnaA binding by RepA was indicated by the appearance of hypersensitive sites within the DnaA boxes upon treatment with DMS (Figure 4). From these studies we conclude that RepA and DnaA can bind to specific sites on supercoiled DNA and can weakly influence each other's binding.

$KMnO_4$ reactivity of P1 origin

In these experiments, supercoiled DNA and the two initiator proteins were used either singly or in combination and the complexes were probed with $KMnO_4$ for unwinding of the origin region. $KMnO_4$ reacts with pyrimidines (T >> C) in unstacked DNA and the reagent has been used widely to study open-complex formation in transcription (Sasse-Dwight and Gralla, 1989; Kainz and Roberts, 1992; Suh *et al.*, 1993) and in replication (Gille and Messer, 1991). In a previous study, the bases that reacted in the absence of protein were primarily thymines in 5′-ATC-3′ triplets (Mukhopadhyay and Chattoraj, 1993). In the presence of DnaA, the ATC triplet sites were no longer preferred and the reactive bases were distributed over the entire origin region (Figure 5, lane 3). More DNA had to be added to lanes 3 and 4 than to lanes 1 and 2 so that Ts of the ATC triplets appear identical in all four lanes. The change in the reaction pattern became more pronounced when RepA was added together with DnaA (lane 4). By itself RepA had an almost negligible influence on the cleavage pattern by $KMnO_4$, as was found earlier (Mukhopadhyay and Chattoraj, 1993).

pSP102, the plasmid used above, carries both sets of DnaA boxes that flank the origin. Either set suffices for initiation *in vivo* (Abeles *et al.*, 1990). In order to determine the requirement of the boxes for the observed increase in $KMnO_4$ reactivity *in vitro*, we examined pALA631, pALA657 and pALA646. Increased reactivity was most pronounced in plasmid pSP102, followed by the plasmids

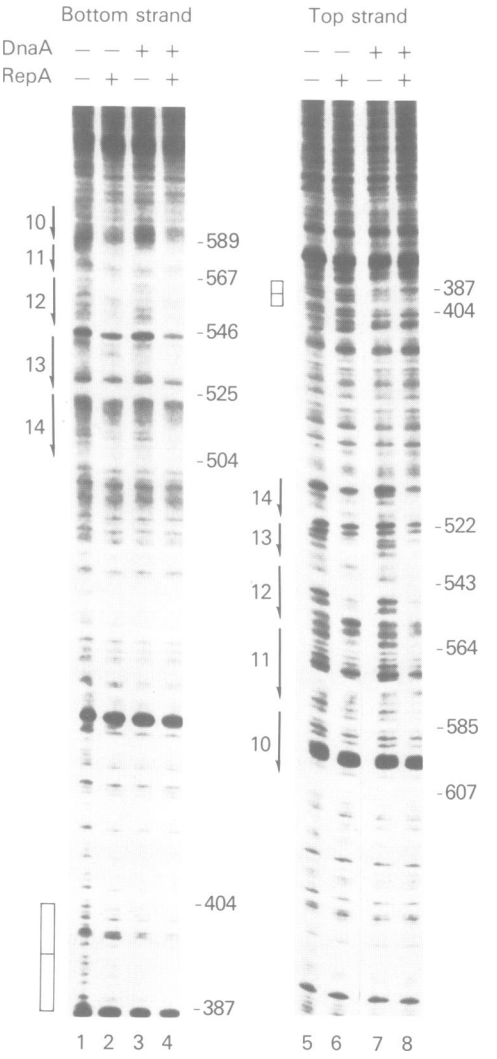

Fig. 2. DNase I footprinting showing specific binding of RepA and DnaA to supercoiled DNA of pGM23. The plasmid carries only the origin sequences from P1 (coordinates 287–611, Figure 1) but without the right set of DnaA boxes. The symbols are as in Figure 1. Note that the protection of the iterons (arrows 10–14) and the DnaA boxes (open boxes) improves when both proteins were used.

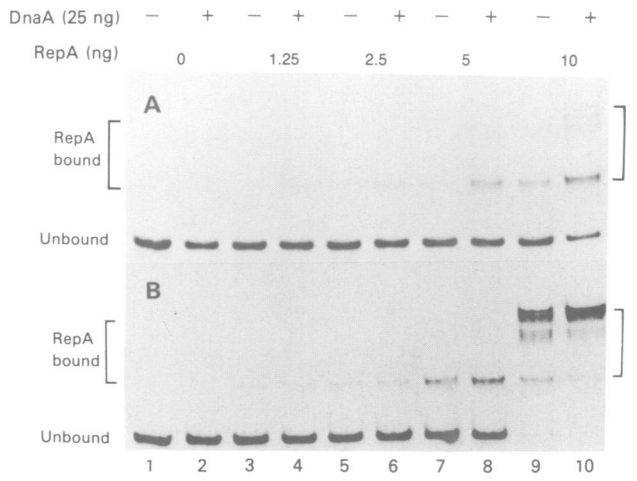

Fig. 3. Band retardation assay with linear DNA showing stimulation of RepA binding by DnaA. In **A**, the fragment is obtained from pALA631 and carries the left set of DnaA boxes and the five RepA binding sites of P1 origin sequences (coordinates 386–611, Figure 1). In **B**, the fragment is from pALA646 which has the DnaA boxes deleted. It carries P1 coordinates 419–611 (Figure 1). Note that except for the intensity, the positions of the RepA-bound bands are identical whether or not DnaA is present (e.g. the fastest moving of the RepA-bound bands in lanes 7–10, panels A and B; and all the RepA-bound bands in lanes 9 and 10, panel B). These indicate that DnaA is not present in the complexes. Binding reactions were performed in the presence of heat shock proteins, which are also absent from the RepA-bound complexes (see Materials and methods). Also note that the specific activities of the DNA fragments are not known. Therefore, comparison of the degree of binding between the panels may not be meaningful.

with the one set (pALA631 and pALA657). The plasmid with both sets deleted (pALA646) was the least reactive of all (Figure 5, lanes 5–16). In pALA646, the low level of reaction seen without DnaA boxes was also seen in DNA band retardation assays where DnaA stimulated RepA binding to a fragment lacking the DnaA boxes (Figure 3B). The degree of stimulation of RepA binding was lower than in the case where the boxes were present, as was the case in the experiments of Figure 5. The extent of the $KMnO_4$ reactivity of the four plasmids was in general agreement with the expectation from *in vivo* studies: the plasmid with both sets present was most active for replication and the one with both sets deleted did not replicate (Abeles *et al.*, 1990). From these results we conclude that DnaA can change the conformation of the strands of P1 origin but that the extent of the reaction depends on the number and location of the DnaA boxes and on whether or not RepA is present.

Somewhat surprisingly, when the plasmids were probed for $KMnO_4$ reactivity on the top strand, there was no evidence of protein-induced reactive positions in any of the plasmids for the region of DNA analyzed (Figure 6). Furthermore, the extent of the reaction was reduced specifically in the origin region in the presence of the proteins (Figure 6, lanes 3 and 4). Without any protein, the extent of reaction of the two strands was identical and the ATC triplets were preferentially reactive in both the cases (Mukhopadhyay and Chattoraj, 1993). A similar strand-bias in $KMnO_4$ reactivity was also seen with the linear form of the origin DNA in the absence of any protein (Mukhopadhyay and Chattoraj, 1993). These results indicate that the conformation of the two strands of the origin can differ in linear DNA or when DnaA and DnaA + RepA are added to supercoiled DNA.

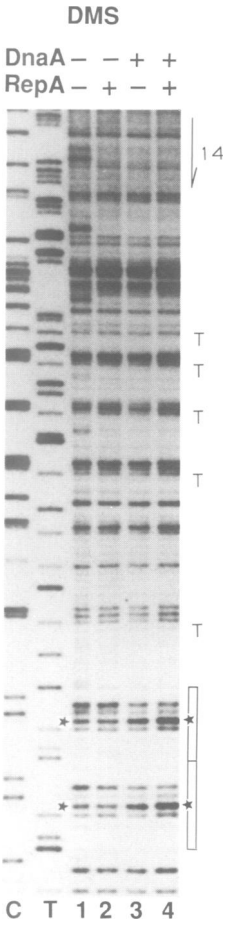

Fig. 4. DMS footprinting showing positions of hypersensitivity (marked by asterisks) within the DnaA boxes in the bottom strand of supercoiled pSP102 DNA. The Ts belong to the five GATC sites of P1 origin (Figure 1, underlined) and are used here as landmarks. DNA–protein complexes were formed here and in all subsequent figures as in Figure 2.

Mung bean nuclease reactivity of P1 origin

Mung bean nuclease reacts preferentially to single-stranded DNA and has been used in the past to probe for single-stranded regions at *oriC* (Kowalski and Eddy, 1989). In the presence of DnaA, the P1 origin region showed increased reaction to the nuclease in a DnaA box-dependent fashion (Figure 7, pSP102 versus pALA646). Both strands of the origin were reactive to the enzyme. These results demonstrate that DnaA alone can open the strands of the duplex DNA of the P1 origin. RepA alone did not increase the reactivity of the nuclease. Moreover, the increased reaction seen in the presence of DnaA alone was no longer seen when both RepA and DnaA were present together. The nuclease was most probably still active. Its reactivity was tested using the single-stranded form of M13 DNA as a substrate in the above reaction mixtures containing RepA, DnaA and supercoiled P1 DNA (data not shown). The presence of both initiators appears to block the nuclease activity specifically at the P1 origin.

Topological consequences of binding of RepA and DnaA to the P1 origin

Our studies so far indicated that DnaA alone or in combination with RepA decreases the helical stability in the origin region. In order to reveal more about the conformation

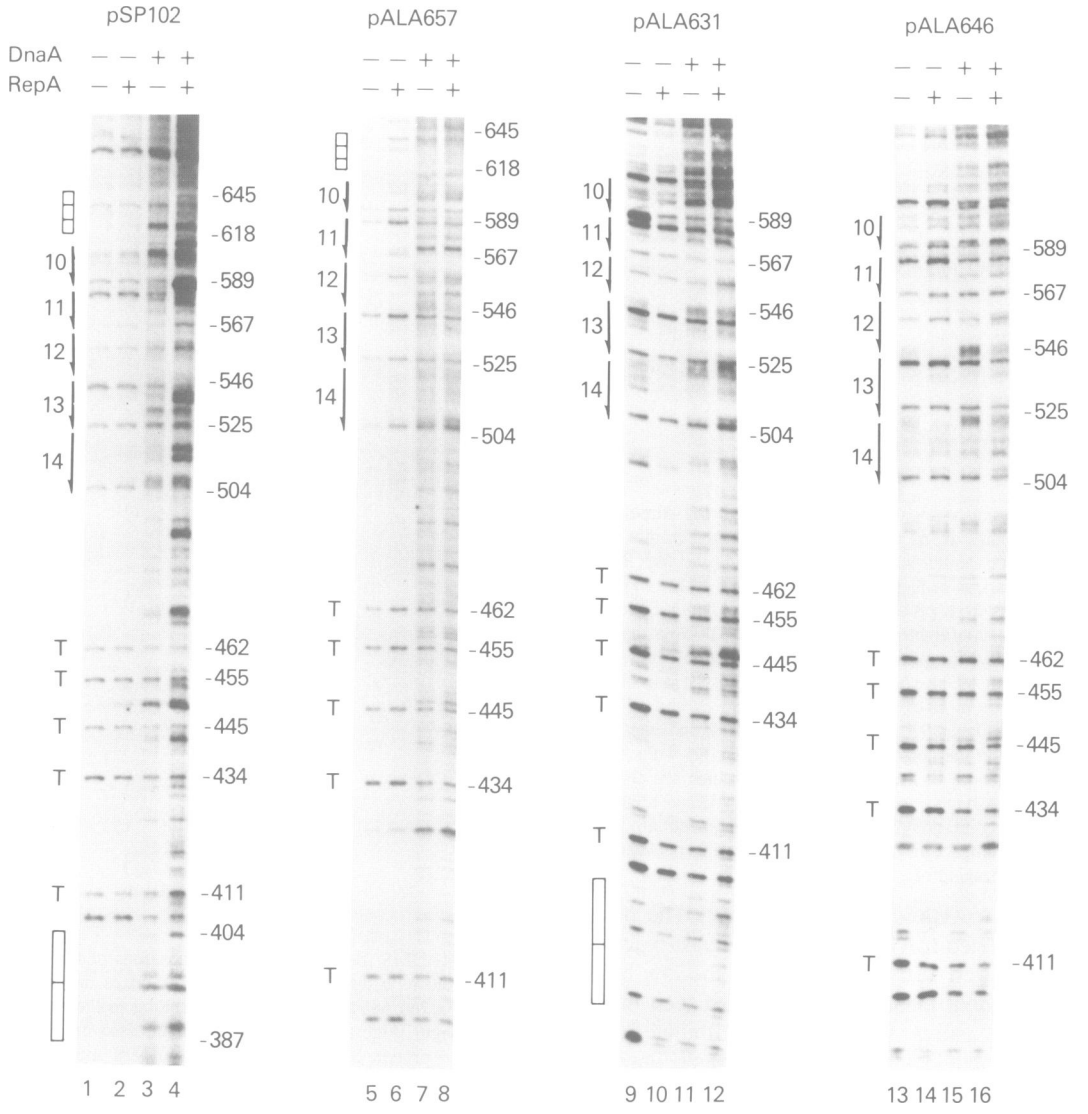

Fig. 5. Change in $KMnO_4$ reactivity of P1 origin region in the presence of RepA and DnaA. The reactions on the bottom strands are shown for four different plasmids. The plasmids differ primarily with respect to the presence and location of the DnaA boxes (identified by open boxes). The amount of DNA loaded varied: about twice as much DNA was loaded in lanes 3, 4, 11 and 12, and ~1.5-fold more in lanes 7 and 8, so that the Ts at positions 462, 455, 445, 434 and 411 show equal reactivity in all four lanes belonging to the same plasmid. Note the appearance of new bands in lanes with DnaA and DnaA + RepA.

of DNA in the nucleoprotein complexes, topological experiments were performed. These experiments were designed to measure the change in twist and/or writhe of the origin DNA induced by the protein binding. Protein–DNA complexes were formed on supercoiled DNA and the superhelical tension was released from the region of plasmid not covered by RepA and DnaA by treatment with a nicking–closing enzyme. The DNA was deproteinized and the topoisomers resolved by two-dimensional gel electrophoresis. Under the assay conditions used, binding of either DnaA or RepA created about one positive superhelical turn of DNA (0.9 and 1.5 turns respectively, Figure 8). However, when both proteins were present, the created superhelicity was less than additive (1.7 versus the expected value of ~2.4). The lack of additivity was not be due to interference of binding of one protein in the presence of the other since simultaneous binding of the two proteins to DNA could be shown by footprinting experiments (Figure 2). We suggest, instead, that the DNA between the RepA and DnaA binding sites was unwound by about one turn (1.7 − 2.4 = −0.7) and thereby compensated for some of the positive superhelicity induced by binding both proteins.

Discussion

Our studies have revealed two functions of the host initiator DnaA that could explain why the protein is essential for replication of the P1 plasmid. We find that DnaA and the P1 plasmid-encoded RepA improve each other's binding to the plasmid origin and open the strands of the origin *in vitro*. DnaA is the primary player in the DNA opening reaction, and RepA stimulates the reaction (Figure 5). One reason for this stimulation could be that the conformation of the origin DNA is altered in the presence of RepA (Mukhopadhyay and Chattoraj, 1993). A second reason could be increased DNA binding of DnaA in the presence of plasmid-specific initiators as has been found in pSC101 and R1 plasmids (Masai and Arai, 1987; Stenzel *et al.*, 1991; Ortega-Jiménez *et al.*, 1992). The DnaA-mediated stimulation of plasmid

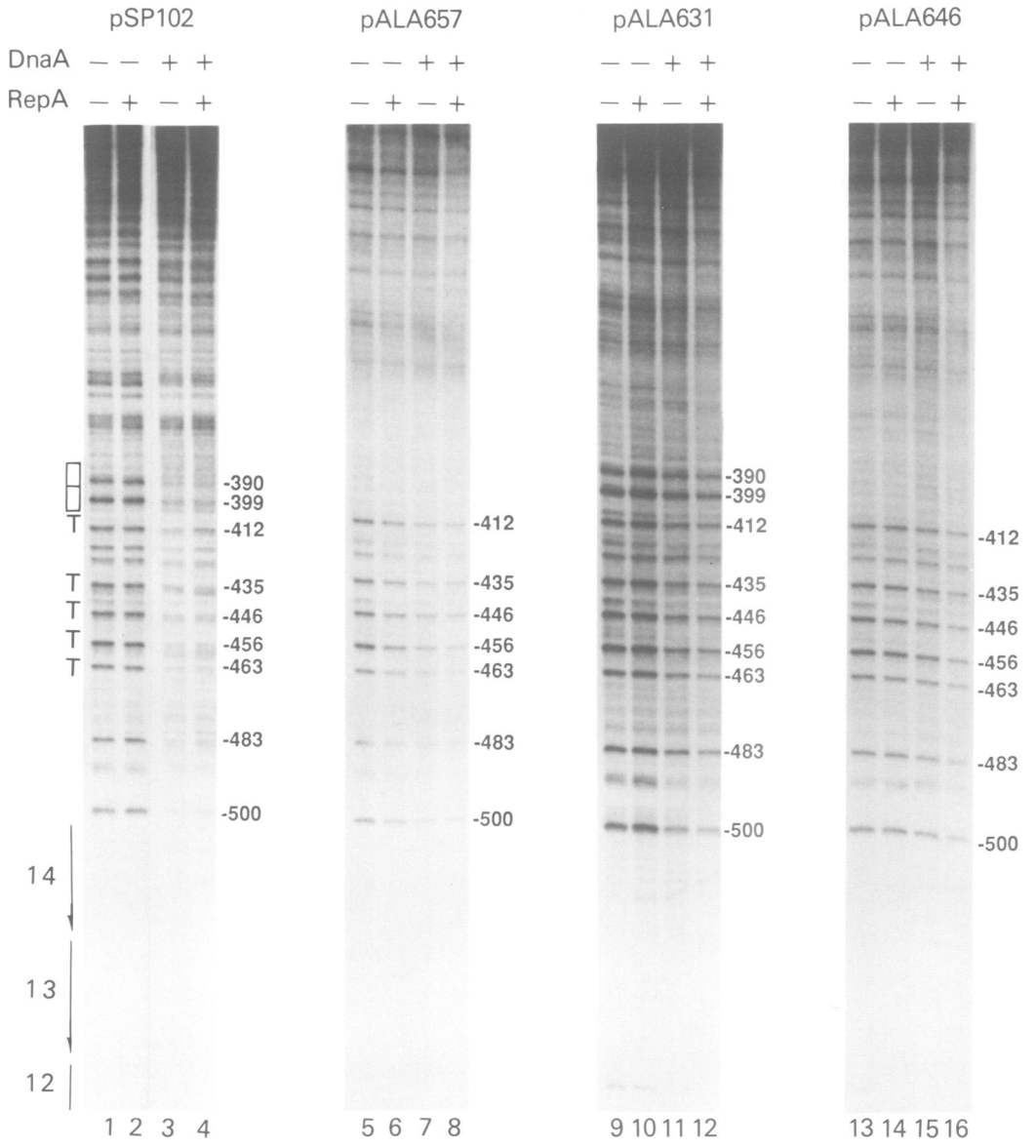

Fig. 6. The same $KMnO_4$-treated samples as in Figure 5 but probed for the top strands. Note that unlike in Figure 5, no new bands are seen in lanes with DnaA and DnaA + RepA. Instead, reactivity of the bands seen in the absence of proteins is preferentially suppressed in the origin region in the presence of DnaA and DnaA + RepA.

initiator binding and DNA opening described here, however, have not been reported earlier.

Origin opening

The sensitivity of both strands of the P1 origin to mung bean nuclease in the presence of DnaA provides the strongest evidence in favor of DNA opening at the origin by DnaA (Figure 7). In contrast, the $KMnO_4$ reaction under similar conditions was restricted to the bottom strand only (Figures 5 and 6). It was remarkable that a very similar pattern of $KMnO_4$ reactivity was seen when linear forms of the origin DNA were examined in the absence of any protein (see Figure 9 of Mukhopadhyay and Chattoraj, 1993). The bottom strand, but not the top strand, showed enhanced reactivity when supercoiled DNA was made linear. When a non-origin DNA was probed with $KMnO_4$, the reaction was poor on either strand in both supercoiled and linear forms. The strand bias can be explained if the top strand nucleotides remain stacked even after unwinding. $KMnO_4$ can attack the 5,6 double bond of the thymine ring from above or below the plane of the ring and accessibility to the bond is hindered in the B-form DNA due to base stacking (Borowiec *et al.*, 1987). If this is the case here, then the question remains why the nucleotides remain stacked in the top strand even after unwinding. In any event, it appears that the two strands of P1 origin can assume very different structures, but are prevented from doing so in supercoiled forms of the DNA in the absence of RepA and DnaA.

Our topological studies indicated that the extent of unwinding induced by RepA or DnaA or both is only about one turn (Figure 8). The unwound bubble, although small, can give rise to susceptibility to chemical and enzymatic cleavage over the entire origin if the location of the bubble varies in different molecules (Figure 9). The bubble can be attacked on both strands by the nuclease but is attacked only on the bottom strand possibly due to continued stacking of the bases of the top strand as discussed above. The presence of RepA or the two sets of DnaA boxes as opposed to one

set, enhances reactivity (Figure 5), possibly by confining the bubble to the vicinity of the origin. The inhibition of mung bean nuclease in the presence of RepA and DnaA, under the conditions of enhanced $KMnO_4$ reaction (Figures 5 and 7), can be explained due to the difference in the nature of the probes. One is a small chemical reagent while the other is a sizeable protein. It is possible that DnaA and RepA binding occluded the nuclease but still allowed reaction with the small reagent, as was found to be the case with Lac repressor (Borowiec *et al.*, 1987). The repressor binding to the operator, easily detected by dimethyl sulfate footprinting of the complexes, did very little to prevent oxidation by $KMnO_4$.

Our results also suggest the following interactions at the origin. (i) DnaA proteins bound to the left and the right set of boxes interact. The presence of one set of boxes did not influence the outcome of topological experiments (data not shown) whereas two sets did (Figure 8). This suggests that DnaA binds simultaneously to distant sites as was seen first by electron microscopy (Fuller *et al.*, 1984). The binding was shown to be cooperative in the case of pSC101 (Stenzel *et al.*, 1991). (ii) The presence of the DnaA boxes in the origin is not essential for the stimulation of RepA binding in the presence of DnaA (Figure 3B). Consistent with this observation is the finding that in R1 (Ortega-Jiménez *et al.*, 1992) and in Rts1 (Itoh and Terawaki, 1989), the requirements of the DnaA boxes are less stringent than the requirement for the DnaA protein for plasmid replication. The requirement of the boxes must be more critical in the case of P1, as in their absence both DNA opening (Figure 5,

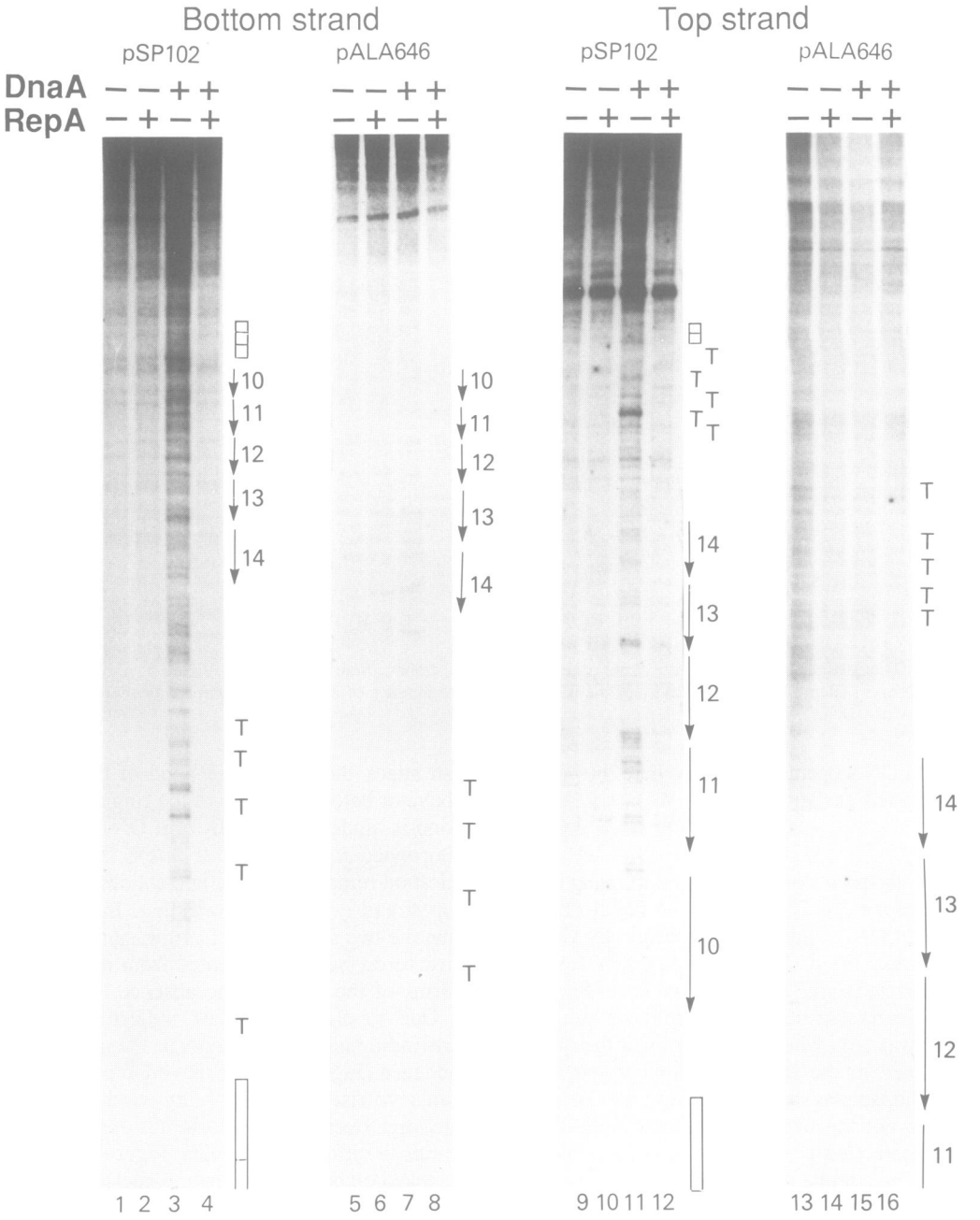

Fig. 7. Mung bean nuclease sensitivity of P1 origin region. Approximately identical amounts of DNA were loaded in each lane. Note the increased sensitivity in the presence of DnaA in lanes 3 and 11.

lanes 13–16; Figure 7, lanes 5–8 and 13–16) and replication *in vivo* were reduced severely (Abeles *et al.*, 1990).

Loading of DnaB helicase

In *oriC*, DNA opening by DnaA provides the stage for loading of the DnaB helicase (Bramhill and Kornberg, 1988a; Gille and Messer, 1991; Hwang and Kornberg, 1992). It has been proposed that DnaA could play a more direct role in the loading of the helicase (Seufert *et al.*, 1988; Masai *et al.*, 1990). When the DnaA boxes mediate loading of the helicase, the orientation of the boxes is critical for plasmids that replicate unidirectionally (Seufert *et al.*, 1988). Although the orientation dependence was tested for pBR322 replication only, the authors noted that in other DnaA dependent replicons, including P1, the orientation of the boxes relative to the direction of replication was mostly similar to that of pBR322. This idea has been further developed by Georgopoulos (1989), who suggested that DnaA may interact with one of the DNA strands more strongly, the strand on to which DnaB loading would be more productive for unidirectional replication (Figure 9). In P1, all five DnaA boxes are oriented in the same direction and, as mentioned above, their orientation relative to the direction of replication is as predicted from the results of pBR322 replication (Wickner and Chattoraj, 1987; Seufert *et al.*, 1988). These observations indicate that DnaB loads on the bottom strand of the P1 origin, the strand that reacts with $KMnO_4$ (Figures 5 and 9).

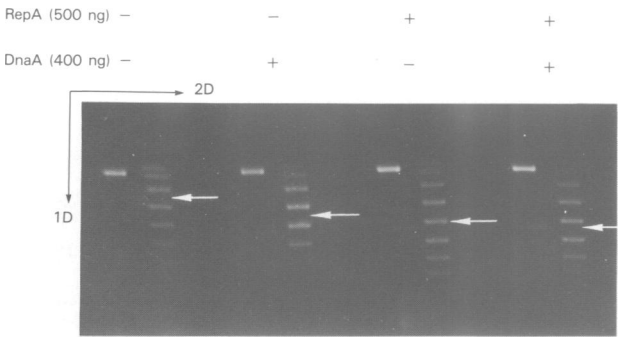

Fig. 8. Protein-induced shift of topoisomer distribution of plasmid pSP102 DNA. The top band in each distribution has 0 superhelical turns and each band differs from the next by one superhelical turn. The convexity of the distribution to the right indicates that the plasmids are positively supercoiled. The arrows show the mean of plasmid topoisomer distributions. The means were 2.5, 3.4, 4.0 and 4.2 in the presence of the following additions: none, DnaA, RepA and RepA + DnaA respectively. The bands at the top left of each distribution represent nicked circular DNA and are irrelevant in the calculation of the means.

Why are there two initiators?

If DnaA plays such a major role in the initiation of P1 and possibly other related plasmids, then what are the justifications for having a plasmid-encoded initiator and for covering as much as half of the origin with iterons to which that initiator specifically binds (Abeles *et al.*, 1990; Wickner *et al.*, 1990)? The answer most probably is that this is for correcting deviations of the copy number from a particular mean (Nordström, 1990). We think that the mechanism of host initiation control is not appropriate for plasmid stability. *oriC* plasmids, carrying the host origin, initiate their replication once per cell cycle in a synchronous fashion irrespective of the copy number (Løbner-Olsen *et al.*, 1989). The timing of initiation is controlled by the concentration of the DnaA protein. Since initiation is insensitive to origin concentration, deviations in copy number of *oriC* plasmids in newly divided cells are not corrected in subsequent division cycles (Jensen *et al.*, 1990). The copy number of *oriC* plasmids, therefore, can fluctuate from very low to very high values in individual cells of a culture and plasmid-free cells are found even when the mean copy number in the culture is high. Therefore, the *oriC* mode of replication i.e. synchronous replication once per cell cycle, is not desirable from the point of view of plasmid maintenance. By encoding their own initiation control functions, plasmids can control fluctuations in copy number.

Materials and methods

Proteins, chemicals and plasmids

The *dnaA* gene, contained in a T7 expression vector (Novagen), was purified essentially as described by Hwang and Kaguni (1988) with the modification that active fractions from heparin–Sepharose chromatography were collected as a precipitate after dialysis and resuspended in a buffer containing 4 M guanidine-HCl before chromatography on a Superose 12 gel filtration column (Pharmacia HR10/30). The specific activity of the protein, measured as

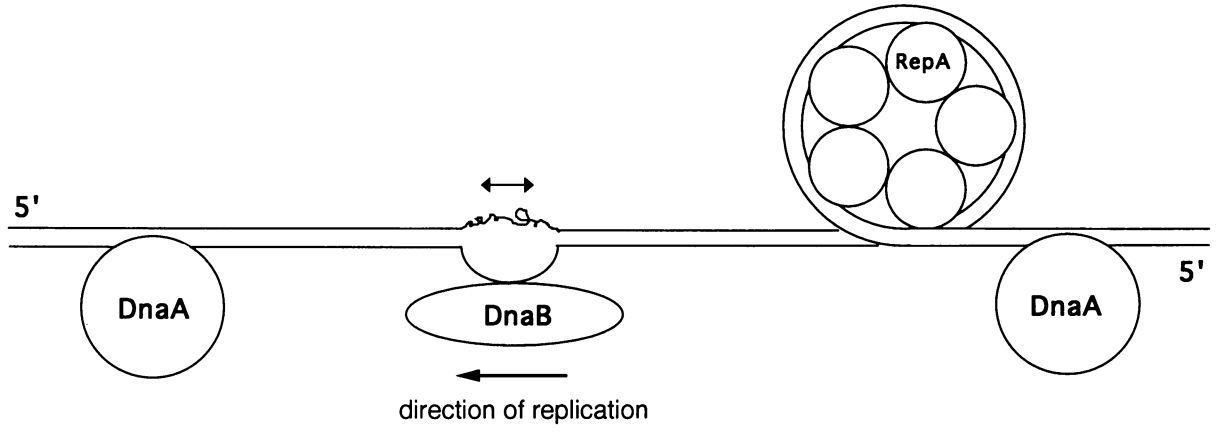

Fig. 9. Schematic of the open-complex of P1 origin DNA with RepA and DnaA showing DNA wrapping around RepA, difference in the structure of the top and bottom strands in the opened region, and binding of DnaA and DnaB on the bottom strand. The possibility that the location of the opened region can vary over the entire origin is indicated by a double-headed arrow. Not shown are the possible interactions (i) between DnaA proteins bound at the ends of the origin, (ii) between RepA and DnaA and (iii) between DnaA and DnaB.

described by Hwang and Kaguni (1988), was 4 × 10^5 units/mg. Sources of other proteins and chemicals have been described (Mukhopadhyay and Chattoraj, 1993). Plasmids used in this work have all been described before: pSP102 (Pal *et al.*, 1986), pGM23 (Mukhopadhyay and Chattoraj, 1993), pALA631, pALA646 and pALA657 (Abeles *et al.*, 1990).

DNA band retardation assay

A DNA fragment from the P1 origin region was used that included the left set of DnaA boxes only (P1 coordinates 386–610, Figure 1). Before use, the fragment was end-labeled with ^{32}P. The binding reactions were performed in 20 μl of T buffer (50 mM Tris–HCl, pH 7.4, 50 mM KCl, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 30 μg/ml BSA) supplemented with 5 mM ATP and 6% (v/v) glycerol. The mixture was incubated at 37°C for 10 min and loaded directly on to a 5% polyacrylamide gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA, pH 8.3). The gel was run at 150 V for 2 h, dried and autoradiographed. Before use DnaA was activated with DnaK (Hwang *et al.*, 1990) and RepA with DnaJ and DnaK (Wickner *et al.*, 1991). The final concentrations of the two heat shock proteins were identical in all reaction tubes whether they carried RepA or RepA + DnaA. The heat shock proteins activate the initiators before they bind to DNA as they are not found in RepA–DNA complexes (Wickner *et al.*, 1991; DasGupta *et al.*, 1993).

Topological studies

Supercoiled DNA (300 ng) of pSP102, a plasmid carrying the P1 origin (Pal *et al.*, 1986), was reacted at 37°C with DnaA (400 ng) for 5 min and then with RepA (500 ng) for another 10 min in a volume of 50 μl. In control samples carrying either DnaA or RepA, the binding reactions were allowed to proceed for 15 min. The mixtures were treated with topoisomerase I (calf thymus, Gibco-BRL) at 37°C for 15 min, deproteinized and analyzed by two-dimensional gel electrophoresis run at room temperature as described before (Mukhopadhyay and Chattoraj, 1993). Halving the protein concentration of either RepA or DnaA significantly reduced protein binding as determined by DNase I footprinting as well as by examining the effects of the proteins on topological studies. Doubling the RepA concentration led to nonspecific binding and significant inhibition of topoisomerase activity. The effect of increasing the DnaA concentration was not studied.

Probing with DNase I, $KMnO_4$, DMS and mung bean nuclease

DNA–protein complexes were formed as described above except that the reaction was scaled up to 100 μl. The complexes were probed essentially as described for DNase I and $KMnO_4$ (Mukhopahdyay and Chattoraj, 1993). DNase I reactions were stopped differently in this study by adding EDTA (final concentration 20 mM) and SDS (final concentration 0.5%). The samples were heated for 3 min at 80°C and treated with proteinase K (final concentration 50 μg/ml) at 37°C for 1 h. The DNA was extracted with phenol–chloroform and precipitated with ethanol. Details of subsequent DNA purifications were exactly as before. For DMS reactions, the DNA–protein complexes were treated with 1 μl of DMS (stock diluted 1:30 with ethanol before use) per 100 μl reaction volume for 1 min at 37°C. The reactions were stopped by adding DMS stop solution (Maxam and Gilbert, 1977) without tRNA and the DNA was precipitated with ethanol. The precipitate was washed once with 70% ethanol and resuspended in 1 M piperidine. After incubation at 90°C for 30 min the piperidine was removed by passing the sample twice through Sephadex G50 spin columns saturated with water. Digestion with mung bean nuclease (NEB, Boston; 5 units per 100 μl reaction) was for 5 min at 37°C after which the mixture was processed as described for the DNase I reactions. All modified DNAs were analyzed by primer extension as before using the following primers. Bottom strands: 5′-TAATACGACTCCCTTCC-3′ (P1 coordinates 343–359) for pSP102 and pGM23; M13 reverse (USB cat. no. 71803), 5′-TTCACACAGGAAA-3′, for pALA631, pALA646 and pALA657. Top strands: 5′-GATTGATTTATATTACT-3′ (P1 coordinates 674–658) for pSP102 and pALA657; pBR322*Hin*dIIIccw (NEB cat. no. 1205), 5′-GCAATTTAACTGTGAT-3′, for pGM23; and M13*Eco*RI(–40) (USB cat. no. 70736), 5′-GTTTTCCCAGTCACGAC-3′, for pALA631 and pALA646.

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