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# Relaxation of replication control in chaperone-independent initiator mutants of plasmid P1

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***Escherichia coli* chaperones DnaJ, DnaK and GrpE increase P1 plasmid initiator binding to the origin by promoting initiator folding. The binding allows initiation and also promotes pairing of origins which is believed to control initiation frequency. Chaperone-independent DNA binding mutants are often defective in replication control. We show here that these mutants have increased rates of association for DNA binding and defects in origin pairing. The increases in association rates were found to be due either to increased protein folding into active forms or to increases in the association rate constant, $k_{on}$. Since the dissociation rate constants for DNA release with these mutants are not changed, it is unlikely that the DNA binding domain is affected. The pairing domain may thus control replication and modulate DNA binding. The role of the pairing domain in DNA binding can be significant *in vivo* as the selection for chaperone-independent binding favors pairing-defective mutants.**
*Key words*: action at a distance/DNA-protein interactions/DNA replication/heat shock proteins/protein folding

## Introduction

Three chaperone proteins of *Escherichia coli*, DnaJ, DnaK and GrpE, participate in the initiation of DNA replication of phage λ (Georgopoulos *et al.*, 1990) and the low copy number plasmids P1 (Tilly and Yarmolinsky, 1989) and F (Kawasaki *et al.*, 1990). In these systems, chaperones are used at different stages of the initiation process. In λ, chaperones participate in a disassembly reaction that releases DnaB helicase from a multiprotein complex at the replication origin (Alfano and McMacken, 1989; Zylicz *et al.*, 1989). In P1 and F, the chaperones stimulate initiator binding to the origin, a reaction that is assayed easily both *in vivo* and *in vitro* (Tilly *et al.*, 1990; Kawasaki *et al.*, 1991, 1992; Wickner *et al.*, 1991a). Studies with the P1 initiator protein, RepA, indicated that the mechanism of stimulation of DNA binding could be the dissociation of RepA dimers as the species that bound DNA was monomeric (Wickner *et al.*, 1991b). Before this work, RepA was found to be dimeric by gel filtration analysis and these protein preparations were largely inactive in DNA binding (Abeles, 1986; Swack *et al.*, 1987; Wickner, 1990; Wickner *et al.*, 1991a). Recently, determination of the equilibrium constant of initiator association by analytical ultracentrifugation suggested an alternative activation mechanism: the chaperones could be refolding misfolded monomers (DasGupta *et al.*, 1993). Dilute RepA solutions, where the protein should be essentially monomeric, were still activated by chaperones for DNA binding, implying that dissociation is not enough to activate the protein.

Recent evidence suggests that in P1 and F, the chaperones may not be required for any step other than binding of the initiator to the origin (Kawasaki *et al.*, 1990; Wickner *et al.*, 1992; Sozhamannan and Chattoraj, 1993). In P1, the origin binding could be restored to levels adequate for replication proficiency in the absence of the chaperones simply by overproduction of RepA (Sozhamannan and Chattoraj, 1993). Similar results were obtained from studies *in vitro*. Addition of DnaJ and DnaK stimulated RepA binding to iterons by ~100-fold in the presence of ATP (Wickner *et al.*, 1991a). Urea treatment of RepA promoted DNA binding of RepA as efficiently as the chaperones, and also allowed replication of P1 plasmids in crude extracts lacking the chaperones (Wickner *et al.*, 1992).

Mutant initiators of P1 and F have been isolated that support plasmid replication without the chaperones (Kawasaki *et al.*, 1991, 1992; Sozhamannan and Chattoraj, 1993). These mutants often displayed a second, unselected phenotype: increased plasmid copy number. In P1, mutant initiators were screened for DNA binding in Δ*dnaJ* cells. The mutants that showed binding comparable with wild-type RepA in wild-type cells were chosen. In 10 out of 12 cases, these mutants supported plasmids at copy numbers significantly above the values obtained with wild-type RepA in wild-type cells (Sozhamannan and Chattoraj, 1993). This indicated that the mutants could be defective in replication control.

In this study, three of the mutants have been characterized *in vitro* to understand how single amino acid changes in RepA restore DNA binding to near physiological level without requiring the chaperones and increase initiation frequency beyond the physiological level. This question is important because it relates to replication control, as well as to the role of the chaperones in protein folding.

The DNA binding characteristics of the mutants were determined by measurement of equilibrium and kinetic constants of RepA-DNA complex formation. We also determined the ability of RepA to pair DNA molecules to understand how the mutants have become control defective (Chattoraj *et al.*, 1988). Pairing of origins was proposed earlier to cause steric hindrance to replisome assembly at the origin and thereby reduce initiation frequency (Pal and Chattoraj, 1988; McEachern *et al.*, 1989; Nordström, 1990; Miron *et al.*, 1992). All three mutants were defective in DNA pairing, a result which supports our prior claim that replication can be controlled by pairing of origins. The improved DNA binding was either due to increased RepA

**Table I.** DNA binding and initiator functions of mutant RepAs: effect of DnaJ and copy number control locus *incA*

| RepA source plasmids | RepA name (amino acid change) | % Autorepression | | Copy number of P1Δ*repA*Δ*incA* plasmid | | Copy number ratio of P1Δ*repA*Δ*incA* and P1Δ*repA*Δ*incA*$^+$ plasmid[a] | |
|---|---|---|---|---|---|---|---|
| | | *dnaJ*$^+$ | Δ*dnaJ* | *dnaJ*$^+$ | Δ*dnaJ* | *dnaJ*$^+$ | Δ*dnaJ* |
| pSSM1 | wt (none) | 85 | 32 | 1 | 1.0 ± 0.1[b] | 5.4 ± 1.4 | 7.4 ± 1.6[b] |
| pSSM1.4 | m4 (K163E) | 88 | 81 | 4.0 ± 0.2 | 1.8 ± 0.2 | 4.1 ± 1.3 | 4.8 ± 1.0 |
| pSSM1.5 | m5 (E184G) | 92 | 55 | 3.8 ± 0.2 | 3.0 ± 0.6 | 1.8 ± 0.1 | 1.7 ± 0.2 |
| pSSM1.9 | m9 (R141H) | 95 | 93 | 4.3 ± 0.4 | 3.0 ± 0.2 | 1.9 ± 0.2 | 2.0 ± 0.2 |

[a]P1Δ*repA*Δ*incA* plasmid was pRJM345 and P1Δ*repA*Δ*incA*$^+$ plasmid was pRJM384 (Pal and Chattoraj, 1988). Copy numbers are expressed relative to the copy number of pRJM345 in *dnaJ*$^+$ cells, which is represented by 1. BR2845 and BR4370 were used as *dnaJ*$^+$ and Δ*dnaJ* cells, respectively (Sozhamannan and Chattoraj, 1993).
[b]Source of the RepA wt plasmid was pALA162 (Swack *et al.*, 1987) when copy number was measured in Δ*dnaJ*. The plasmid produced 40-fold the physiological level, whereas the level was 3-fold in other experiments in this table. The higher level was necessary for efficient replication in Δ*dnaJ* cells for wt RepA only.

folding into active forms, resulting in an apparent increase in association of mutant RepAs and DNA, or to an increase in the association rate constant, $k_{on}$. Since the mutants were altered in a small region of the *repA* gene, the region could be crucial for directing protein folding, DNA binding and replication control. Our results also suggest that the chaperones do not unpair origins that have been paired by RepA and, therefore, may not recognize RepA in the DNA-bound form. Folding of nascent polypeptide chains may be their only role in P1 plasmid replication.

## Results

### *DNA binding of chaperone-independent mutant RepAs in vivo*

P1 plasmid has 14 RepA binding sites (Abeles *et al.*, 1984; Papp *et al.*, 1993). The sites show a high degree of homology to each other and for this reason have been called iterons (Nordström, 1990). A set of five iterons covers about half of the origin. The remaining nine iterons constitute the control locus, *incA*. RepA binding to the iterons is essential both for initiation and for control of DNA replication (Chattoraj *et al.*, 1984, 1985; Pal *et al.*, 1986). The promoter for the *repA* gene is embedded within the origin iterons. RepA binding to the origin represses the promoter and establishes an autoregulatory control loop for RepA synthesis (Chattoraj *et al.*, 1985, 1988). This autoregulation was found to be defective in cells lacking any of the three chaperone proteins DnaJ, DnaK and GrpE (Tilly *et al.*, 1990). RepA mutants were obtained that were no longer defective in repressing the *repA* promoter in Δ*dnaJ* cells (Sozhamannan and Chattoraj, 1993). The locations of mutations in 12 such mutants were mapped and they covered a stretch of 47 codons (138–184) of the 286 codon *repA* gene. Three of the mutants, called m4, m5 and m9, were chosen for further characterization. As was suggested by autorepression studies (Table I), all three mutants were competent in DNA binding, as determined by dimethyl sulfate (DMS) footprinting in Δ(*dnaK-dnaJ*) cells (Figure 1, lanes 6–10). The level of binding was comparable with that obtained for wild-type RepA in *dnaK*$^+$*dnaJ*$^+$ cells (Figure 1, lanes 1–5). We conclude that the mutants no longer require the chaperones for near physiological levels of binding to the origin.

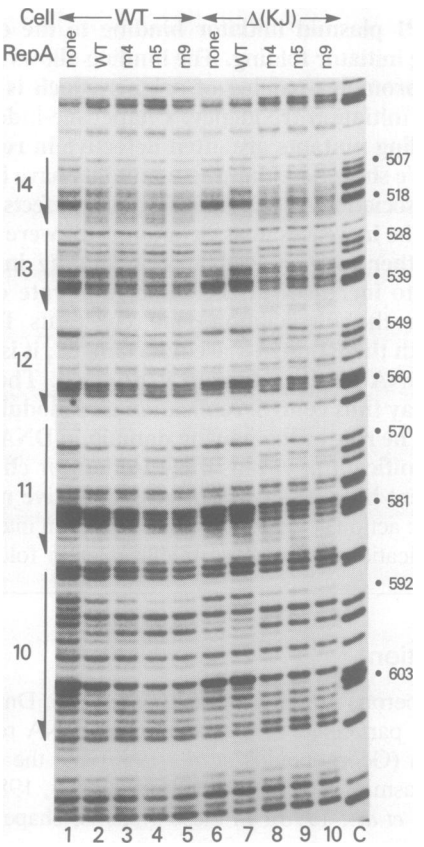


**Fig. 1.** DMS protection of top strand of P1 plasmid origin by RepA in wild-type and in Δ(*dnaK-dnaJ*) cells. RepA, wild type (WT) and mutants (m4, m5 and m9) were supplied *in trans* in similar amounts. Lanes with no RepA are labeled none. Arrows to the left represent iterons no. 14 to no. 10. Protected guanines are shown by dots and by their P1 co-ordinates. Note that the protection is significant for all four proteins in WT cells (lanes 1–5), but only for the mutants in Δ (KJ) cells (lanes 6–10).

### *DNA binding of mutant RepAs in vitro*

A 58 bp DNA fragment carrying a single 19 bp iteron (iteron no. 14, Figure 1) was used. The iteron matches the consensus sequence of all 14 iterons of P1 (Abeles *et al.*, 1984). The binding was monitored by band-shift assays, as was used previously to determine the equilibrium and kinetic parameters of RepA–iteron interactions (DasGupta *et al.*,

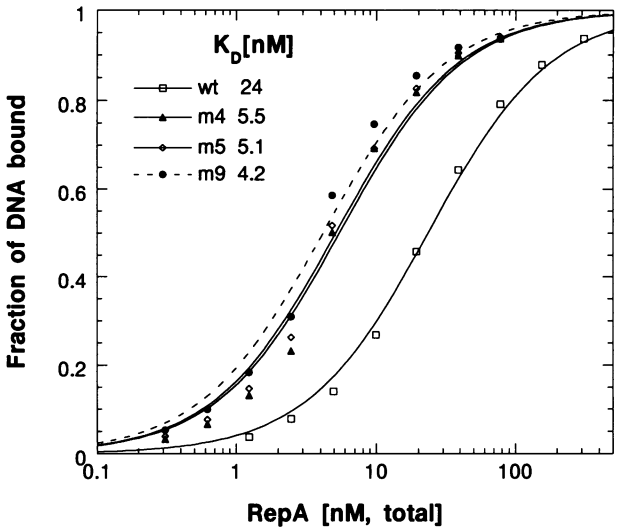


**Fig. 2.** Binding curves showing the fraction of input DNA present as complexes with wild-type and mutant RepAs (m4, m5 and m9) as determined by band-shift assay. RepA concentration represents the total protein and not active species. The points were fitted to the equation $y = Kx/(Kx + 1)$, to obtain $K_D$ values ($=1/K$; Wyman and Gill, 1990).

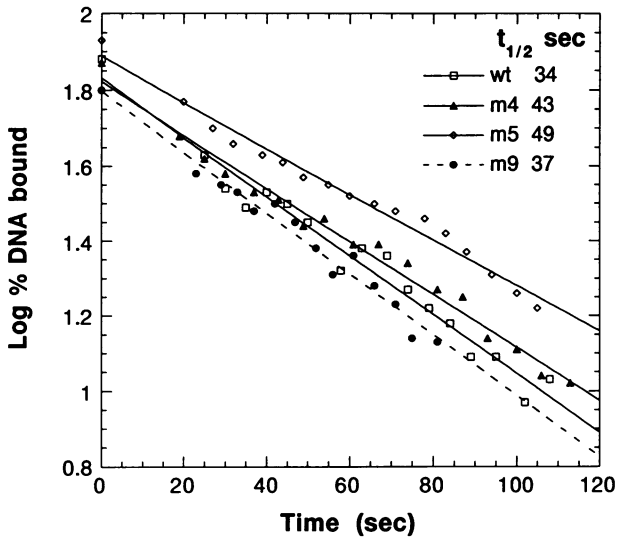


**Fig. 3.** Dissociation kinetics of RepA−iteron complexes in the presence of a 500-fold molar excess of cold competitor DNA as monitored by band-shift assay. The points were fitted to straight lines and times taken for 50% of the complexes to dissociate ($t_{½}$) were calculated from the slopes.

1993). The binding occurred at lower RepA concentrations in the case of all three mutants (Figure 2). The data points were fitted to theoretical lines representing binding of a monomeric RepA to a single iteron. In all four cases, good fits were obtained ($r^2 = 0.985-0.996$) and the overall $K_D$ for RepA−iteron interactions was lower in the case of the mutants by ~5-fold compared with the wild type.

The decrease in overall $K_D$ could be the result of changes in one or both of its kinetic components, $k_{on}$ and $k_{off}$, or it could reflect an increase in the active fraction of the protein since the protein concentrations were not corrected for the presence of inactive species in Figure 2. The overall reaction can be described as follows:

$$\mathrm{RepA}_{\mathrm{inactive}} \underset{k_{-1}}{\overset{k_{+1}}{\Leftrightarrow}} \mathrm{RepA}_{\mathrm{active}} + \mathrm{DNA} \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} \mathrm{RepA}_{\mathrm{active}} \cdot \mathrm{DNA}$$

$$K_{D,overall} = \frac{k_{off}}{k_{on}}\left(1 + \frac{1}{K_1}\right)$$

$$\text{where } K_1 = \frac{k_{+1}}{k_{-1}}$$

We first determined the dissociation rate constant, $k_{off}$, since it did not require the knowledge of active fractions. The dissociation followed simple first-order kinetics and the rates were identical in the case of all four proteins (Figure 3). The mean $t_{½}$ (the time taken for 50% of the complexes to dissociate) values were 41 ± 6, 40 ± 4, 51 ± 3 and 40 ± 2 s from four sets of experiments using two different protein preparations. We infer that the nature of RepA−iteron contacts has not changed significantly in the mutants, and

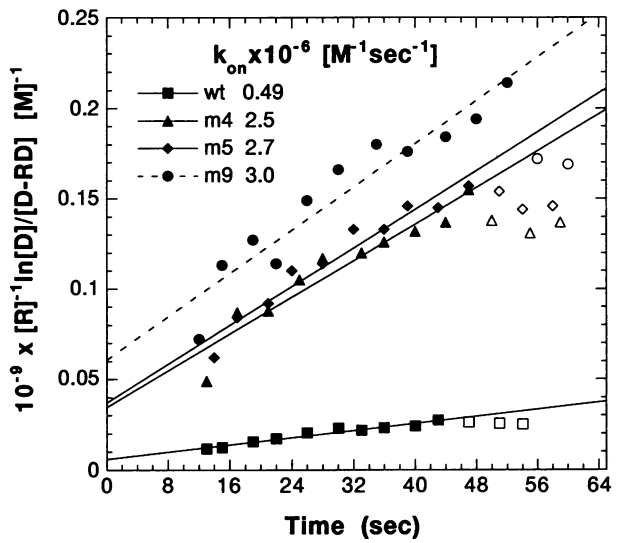


**Fig. 4.** Association kinetics of RepA−iteron complex formation. DNA sites [D] = 1.3 pM and RepA [R]= 20 nM for wild type (wt) and 5 nM for the mutants. [R] represents total RepA concentration and not active species. [RD] represents bound DNA at different times, as determined by band-shift assay. The ordinate function was modified from equation 7 of Riggs *et al.* (1970), since [D], [RD] << [R]. Initial data points (filled symbols) were fitted to straight lines and slopes were taken as $k_{on}$.

that the mutational changes were outside of the DNA binding domain of RepA.

From this similarity in dissociation rate constants, we predicted that the association rates would be similarly increased relative to the wild type in all three mutants. Measurement of relative association rates supported this expectation (Figure 4). To determine whether $k_{on}$ has increased, we needed to know the active fraction of the proteins (Figure 5A). In two separately prepared protein preparations, mutant m4 was barely increased in the active fraction, m9 was increased 2-fold and m5 could be increased

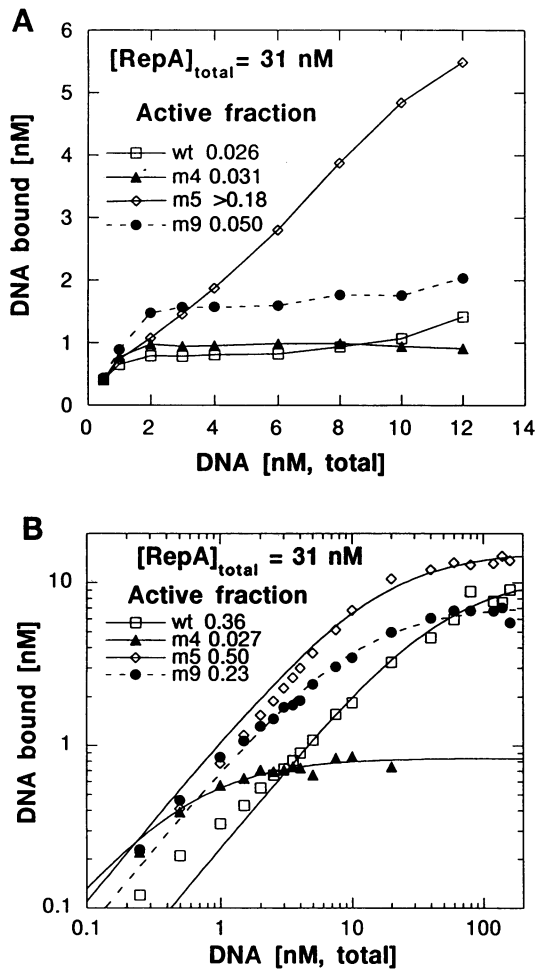


**Fig. 5.** Active fraction of RepA preparations. A fixed amount of total RepA (31 nM) was titrated with varying amounts of iteron DNA in the case of all four proteins and the amount of DNA that could be complexed with RepA was monitored by band-shift assay. The nature of the titration curves differed at low (**A**) and at high (**B**) DNA concentrations. In panel A, except for m5, the relatively flat regions (2–6 nM range) were used to deduce active fraction. In panel B, points were fitted to the equation $y = y_{max}.x/(x + D_{½})$, where $D_{½}$ = total DNA concentration at half-maximal binding (DasGupta *et al.*, 1993). The active fraction was calculated from $y_{max}$, the maximum DNA bound. From repeat experiments, the active fraction values varied within a 2-fold range, partly due to repeated freeze–thawing of the proteins.

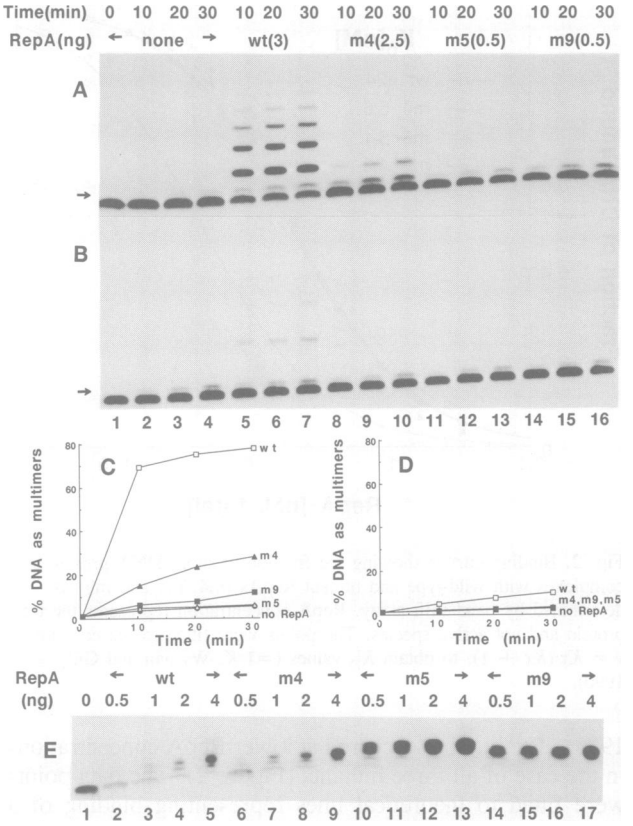


**Fig. 6.** Kinetics of ligase-mediated multimerization of blunt-ended DNA fragments. In (**A**) a 239 bp fragment carrying five origin iterons was used and in (**B**) a 395 bp fragment of *repA* was used as non-specific DNA. In panels A and B, agarose gel electrophoresis of ligation products is shown in the absence of RepA (lanes 2–4) and in the presence of wild-type (lanes 5–7) and mutant RepAs (lanes 8–16). Arrows indicate monomer circles which run just above the unligated fragments. (**C**) and (**D**) Plot of percent input DNA present as linear multimers in panels A and B with time. (**E**) Band-shift assay using proteins that were activated with chaperones under the conditions of the experiments of panels A and B. At these protein concentrations, non-specific DNA shows no binding to RepA by band-shift assay (data not shown). DNA was loaded onto running 1% agarose gels in the experiments of this figure. This has caused the band positions to vary across the gel. The use of agarose gels to study DNA binding was necessitated due to the presence of PVA in the binding reaction. In the presence of PVA, DNA did not enter into polyacrylamide gels easily.

as much as 7-fold. The increase in active fraction was sufficient to explain the increase in the rate of association for m5. However, the titration curve for m5 appeared to be biphasic, as if the DNA probe was causing some of the increase in apparent active fraction. At higher DNA concentrations, the titration curves for m9 and wild-type proteins were also biphasic and essentially overlapping (Figure 5B). This suggests that DNA binding to inactive RepA may occur at high DNA concentrations. From these results, we can conclude that at least for m4, and to some extent m9, the association rate constant, $k_{on}$, must have increased. The magnitude of this rate constant depends on several parameters, such as three-dimensional diffusion in solution, one-dimensional diffusion along the DNA and, finally, the facility with which an encounter with the iteron site leads to stable complex formation. In the case of m4 and m9, any one of these processes could have changed to increase $k_{on}$. We conclude that the reduction in overall $K_D$ is due to increases in active fraction and/or to increases in $k_{on}$ and there is no significant change in the interactions of the mutant proteins with the iteron sequences *per se*.

### *RepA-mediated pairing of iteron DNAs*

Since the mutant RepAs maintained P1 replicons at high copy numbers, they could be altered in the domain involved in the control of initiation frequency. This possibility is made more likely by the results of the binding studies that suggested that the DNA binding domains are not altered in the mutants. We tested the control properties of the mutants by examining their ability to pair DNA, and thereby catalyze the formation of DNA multimers in the presence of ligase. This assay has been used for similar purposes in the case of plasmid R6K (McEachern *et al.*, 1989; Miron *et al.*, 1992). Wild-type RepA formed multimers of both fragments carrying iteron no. 14 (Figure 2) and of similar length

fragments of non-specific DNA at comparable efficiencies. To improve the iteron specificity of multimerization, it was necessary to activate the RepA proteins with chaperones. The addition of polyvinyl alcohol and DNA fragments with all five origin iterons, as opposed to fragments with single iterons, greatly stimulated the fraction of multimers in the case of wild-type RepA. Under these conditions, multimer formation was significantly less for all three mutants, m5 and m9 being more defective than m4 (Figure 6). The rate of multimerization depended on the fraction of DNA present as RepA–DNA complexes. Therefore, the extent of binding was kept similar by adjusting the concentration of RepA. The experiments were also repeated with a constant concentration of RepA (2 ng in 50 $\mu$l = 1.2 nM). The fraction of probe DNA bound was ~0.67, 0.78, 0.97 and 0.95 for the wild type, m4, m5 and m9, respectively (Figure 6E). The corresponding fractions of multimers were 72, 52, 7.6 and 46%, respectively, at 30 min time points (data not shown). These results indicate that the mutants are defective in pairing, m4 being somewhat less defective than the other two. We suggest that this defect is responsible for the higher copy number of the mutants.

### *Chaperones do not reduce RepA-mediated DNA pairing*

We wished to determine whether the chaperones, in addition to promoting DNA binding, could facilitate P1 plasmid replication by unpairing DNA pairs and thereby relaxing replication control. We showed earlier that the copy control locus *incA* can reduce the copy number of a P1 replicon up to 8-fold (Pal *et al.*, 1986). According to the pairing model, the copy number of *incA*-deleted plasmids is controlled by pairing between origins. In *incA*-carrying plasmids, there is the opportunity for *incA*-origin pairing, which further reduces copy number. We expected that if the chaperones could unpair the paired complexes, then the potency of *incA* control would increase in the absence of the chaperones, resulting in a greater difference in copy number between plasmids with and without the *incA* locus. The results do not support this expectation: the copy number ratio of *incA*$^-$ and *incA*$^+$ plasmids was not significantly changed in wild-type and in Δ*dnaJ* cells, the values being 5.4 ± 1.4 and 7.4 ± 1.6, respectively (Table I).

The copy numbers could not be determined using an identical set of plasmids in the wild type and in Δ*dnaJ* cells as the '3-fold' RepA source (pSSM1), optimal for the wild-type cells, did not support replication in Δ*dnaJ* cells and the '40-fold' RepA source (pALA162), optimal for the Δ*dnaJ* cells, did not support replication in the wild-type cells. [The numbers refer to multiples of the concentration of RepA present in wild-type cells carrying wild-type P1 plasmids.] RepA sources were chosen to allow the *incA*-deleted plasmid pRJM345 to replicate at nearly identical copy number in the two strains. With the mutant RepAs, the copy number measurements could be done with identical RepA sources in both the wild-type and *dnaJ* strains. As expected, the mutants were significantly defective in control: they showed reduced differences between Δ*incA* and *incA*$^+$ plasmids (Table I). The copy number ratios decreased in *dnaJ*$^+$ cells from 5.4 to 4.1, 1.8 and 1.9 for m4, m5 and m9, respectively. Although the extent of *incA* control defects varied from mutant to mutant, the defect was maintained in Δ*dnaJ* cells in all three cases. We conclude that the chaperones do not play a direct role in replication control. Consistent with this conclusion is the observation that DnaJ and DnaK allowed DNA pairing *in vitro* since the ligation assays were carried out in their presence (Figure 6).

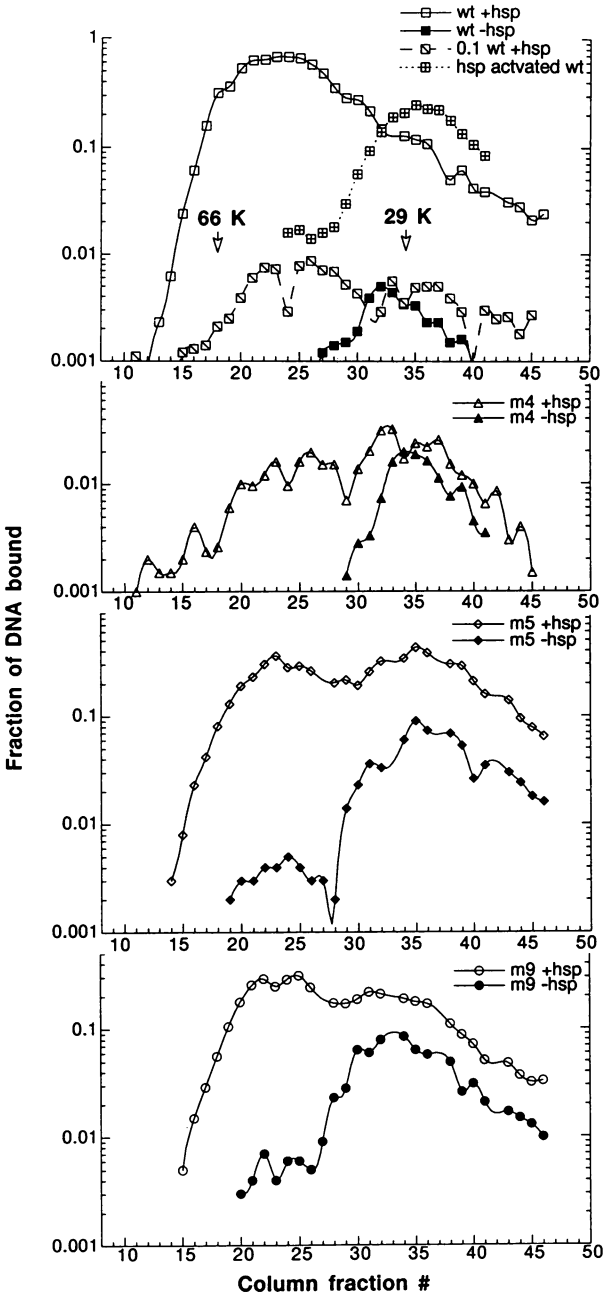


**Fig. 7.** Gel filtration of RepA to determine the distribution of RepA that bind independently and that require activation by chaperones. Each fraction of wild-type (wt) and m4, m5 and m9 mutant RepAs was analyzed by band-shift assay before (filled symbols, −hsp) and after activation with chaperones (open symbols, +hsp). Arrows in the top panel show the peak positions of elution of the protein standards carbonic anhydrase (29K) and BSA (66K). Wild-type RepA was also analyzed using 0.1 $\mu$g of total protein; at this low concentration, binding activity was seen only after activation [top panel, 0.1 wt +hsp). Wild-type RepA was also activated before loading on the column and assayed without adding chaperones [top panel, heat-shock protein (hsp)-activated wt].

### *Chaperone activation of RepA mutants*

In order to determine whether the binding activity of the mutant forms of RepA could be activated by chaperones *in*

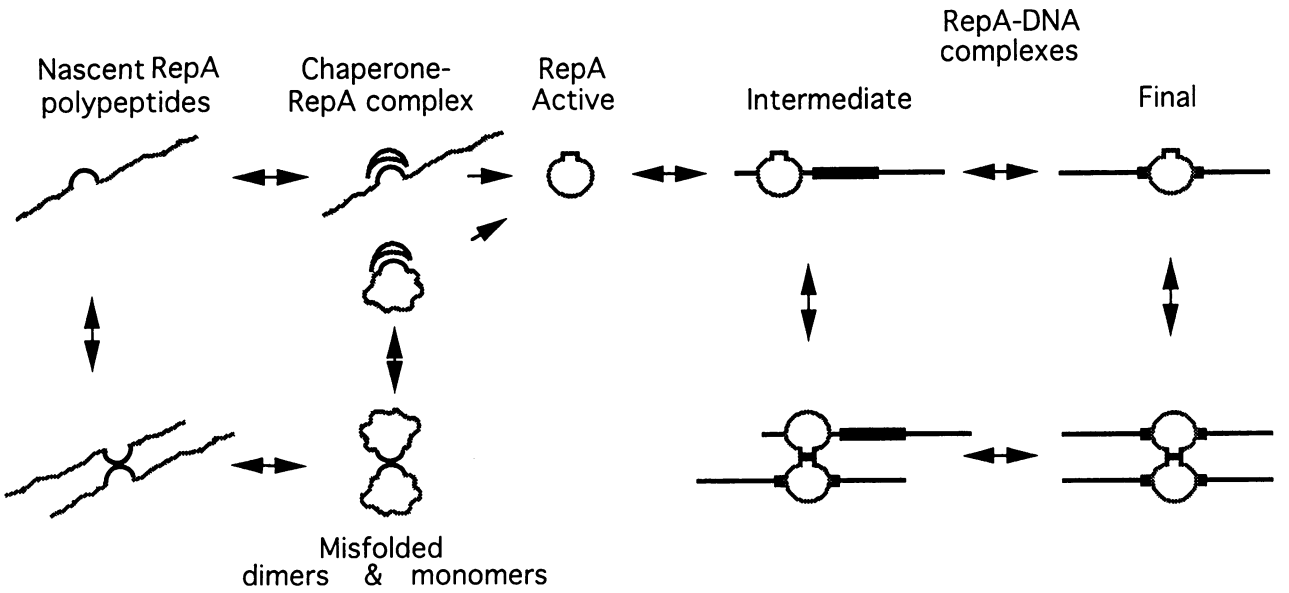


**Fig. 8.** Model showing a small stretch of RepA polypeptide (semicircular arc) that in nascent chains and in misfolded forms is recognized by the chaperones. The arc is made rectangular in DNA binding proficient (active) molecules to indicate that the region is not recognized by the chaperones in active molecules. The same stretch participates in the pairing (dimerization) of both misfolded and active forms of RepA, and in pairing of DNA–RepA complexes. The pairing can also occur when one or both of the proteins is bound to DNA non-specifically (the specific site is shown by a rectangular box) and this can affect the rate of final RepA–DNA complex formation.

*vitro*, the proteins were first fractionated by gel filtration. The distributions of the already active and chaperone-activatable species were identified by assaying the fractions in the absence and presence of chaperones (Figure 7). The binding activity of the mutants, specifically m5 and m9, in the absence of the chaperones was significantly higher, as expected from earlier results (Figure 2). The poorer binding of m4 was unexpected, but the proteins were handled differently in the experiments of Figures 2 and 7: some inactivation could have occurred during gel filtration of the dilute protein solution in the absence of bovine serum albumin (BSA). The binding activity in the presence of the chaperones was distributed over a broad region in all cases. The majority of the activatable binding species had higher Stokes radii than the already active species. Activation in the case of m4 was severely defective. The poor activation suggests that the amino acid change is in a domain that may be involved in recognizing the chaperones.

The species that eluted around fraction no. 34 are expected to be monomeric, assuming the proteins to be globular in shape. Since activation was seen in this region, the results support our earlier proposal that the monomeric form of the protein can be activated by chaperones (DasGupta *et al.*, 1993). To confirm further this notion, wild-type RepA was fractionated at a 10-fold lower concentration so that more protein was present in monomeric form. At this lower concentration, the binding activity was comparable in the two peak regions, indicating that the species that eluted later were not due to trailing of the species that eluted earlier. The possible reasons for the broad distribution of the activatable species could be due to misfolded monomers and dimers with variable Stokes radii.

## Discussion

In this paper, we have studied three initiator mutants of plasmid P1 that no longer require the chaperones DnaJ, DnaK and GrpE for efficient DNA binding. Although only the chaperone-independent DNA binding phenotype was selected, the mutants showed defects in replication control: they allowed P1 replicons to be maintained at higher copy numbers than could be obtained with the wild-type protein. We find that all three mutants are defective in DNA pairing *in vitro*. The magnitude of the defect was correlated with the efficiency of *incA* control *in vivo*: m4 was the least defective in both the assays (Figure 6; Table I). The pairing defect can thus account for the higher copy number and the results support our hypothesis that in P1, replication can be controlled by DNA pairing (Pal and Chattoraj, 1988). In plasmid R6K, where iteron pairing is also believed to control replication, a high copy initiator mutant has been shown to be defective in iteron pairing (Miron *et al.*, 1992). We can be more confident now about the validity of the pairing model in the control of replication by iterons.

The DNA binding properties of the mutants show that they have increased rates of association for DNA binding, but unchanged dissociation rates. The similarity in dissociation rates suggests that the DNA–protein contacts that contribute to the binding energy are most likely not changed in the mutants and the DNA binding domain of the mutants may still be intact. The increase in association rates was due to increases in either active fractions or in $k_{on}$, or a combination of both, depending on the mutant.

How can selection of a chaperone-independent DNA binding phenotype result in the selection of pairing-defective proteins that increases the association rates for DNA binding? One likely possibility is that the nascent polypeptides can dimerize with each other using the pairing domain and this can prevent initiator folding into active forms (Figure 8). When the dimerization is reduced either by interactions with the chaperones or by mutation in the pairing domain, the polypeptides have more opportunity to fold into the native form. In this model, the pairing domain serve as the target of the chaperones and their binding protects the protein from

misfolding (Beckmann *et al.*, 1990; Landry and Gierasch, 1991). This model, although adequate to explain increases in active fraction, does not explain how $k_{on}$ could have increased. A clue to the mechanism is obtained from studies on the *lac* repressor which, like RepA, can both bind and pair DNA. The latter property of the protein has been shown to affect the rate constants of DNA binding (Fried and Crothers, 1984; Ruusala and Crothers, 1992). Like the *lac* repressor, the pairing of RepA can influence the association rates, but the effect is to decrease them (Figure 8). Our ligation kinetics experiments in the absence of chaperones clearly indicated that the pairing can occur between RepAs bound to DNA non-specifically. Thus, the domain used in pairing DNA-bound RepAs can be important for protein folding and DNA binding, in addition to replication control. In the absence of chaperones, where the fraction of active monomers is low, it must be easier to improve DNA binding by reducing the activity of the pairing domain than to increase the affinity of the DNA binding domain. These results argue that the pairing reaction can contribute to DNA binding significantly *in vivo*.

Our results also indicate that chaperones do not actively participate in the unpairing of paired complexes, although such activity of chaperones was anticipated from other studies (Alfano and McMacken, 1989; Zylicz *et al.*, 1989; Hwang *et al.*, 1990; Wickner *et al.*, 1991b). *In vivo*, the chaperones had no influence on the control activity of the initiator proteins (Table I) and *in vitro* the ligation kinetics results were obtained in the presence of chaperones. The simplest explanation for these results is that the chaperones do not recognize RepA in the DNA-bound form and, therefore, do not have any unpairing activity.

Why have the plasmids developed a dependence on chaperones? It could be that the proteins that need to dimerize for function run the risk of doing so prematurely during nascent polypeptide synthesis and, therefore, might require the help from chaperones to prevent untimely interactions. It remains to be seen how real the problem is and how generally the chaperones are used to solve the problem. There are several examples of regulatory switches that involve modulation of dimerization. The modulation is done (i) by cofactors such as arabinose, as in the case of the *E.coli* transcription repressor/activator AraC (Lobell and Schlief, 1990) or by a protein cofactor, as in the case of a mammalian homeodomain (Mendel *et al.*, 1991) or bZIP proteins (Wagner and Green, 1993); (ii) by phosphorylation, as in the case of BglG transcription antitermination protein (Amster-Choder and Wright, 1992); and (iii) by DNA, as in the case of LexA repressor (Kim and Little, 1992) or glucocorticoid receptor proteins (Luisi *et al.*, 1991). As our studies suggest, the chaperones may provide yet another handle on this type of regulation.

## Materials and methods

### *DMS footprinting in vivo*

BR4391 and BR4392 were used as wild-type and Δ(*dnaK-dnaJ*) cells, and plasmids pBR322, pSSM1, pSSM1.4, pSSM1.5 and pSSM1.9 were used as sources for a no RepA control, wild-type, m4, m5 and m9 mutant RepAs, respectively (Sozhamannan and Chattoraj, 1993). This reference also has the details of the footprinting procedure.

### *Band-shift assays*

An end-labeled 58 bp fragment carrying iteron no. 14 was used at 50 pM concentration (DasGupta *et al.*, 1993). Binding buffer contained 20 mM Tris−HCl (pH 8), 100 mM NaCl, 40 mM KCl, 10 $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 50 μg/ml BSA, 50 μg/ml calf thymus DNA and 5% glycerol. RepA proteins (wild type and the mutants) were synthesized from overproducer sources using the vector pT7/T3α-19 (BRL) and finally purified to >95% using a Mono-S FPLC column (Pharmacia). Band intensities were quantified using a Fujix BAS2000 image analyzer. Other details of DNA binding, dissociation rate constant and active fraction determinations have been described (DasGupta *et al.*, 1993). Association rates were measured by mixing RepA and DNA (35 μl each) by gentle vortexing at time zero. The mixture was picked up with an EDP pipette (Rainin) and at different times 4 μl were dispensed into 0.65 × 0.15 cm wells of a 5% polyacrylamide gel running at 13 V/cm.

### *Ligation kinetics*

Intermolecular ligation was done using a 239 bp *Eco*RI−*Hin*dIII fragment from pALA631 carrying P1 residues 386−610 which include the five origin iterons of P1 (Abeles *et al.*, 1990). A 395 bp *Eco*RI−*Hin*dIII fragment from pSP120 carrying the N-terminal segment of P1 *repA* gene was used as a control for non-specific DNA (Pal and Chattoraj, 1988). The fragments were made blunt ended with Klenow polymerase using [α-$^{32}$P]dCTP and the other three cold dNTPs, and specific activities were determined (DasGupta *et al.*, 1993). The binding reactions were performed in 50 μl volumes in 1× T-buffer [50 mM Tris−HCl (pH 7.4), 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 30 μg/ml BSA] supplemented with 5 mM ATP, 20 mM DTT, 4% polyvinyl alcohol (PVA) at 30°C for 10 min. A 5 μl aliquot was withdrawn to monitor the percentage of DNA present as RepA−iteron complexes and to the remaining portion 4 U of T4 DNA ligase (BRL) were added. Aliquots of 10 μl were withdrawn at 10 min intervals and the reactions were stopped by adding an equal volume of a 2× solution (20 mM EDTA, 1% SDS and 0.02% bromophenol blue). The entire sample was loaded onto a 1% agarose gel in TBE buffer and run at 10 V/cm. RepA (≤4 ng) was activated with DnaJ (50 ng) and DnaK (125 ng) in 1× T-buffer supplemented with 5 mM ATP, 20 mM DTT and 4% PVA at room temperature for 40 min. The entire mixture was used for DNA binding and ligation reactions as described above. The gel was dried under vacuum and quantified as in band-shift assays.

### *Gel filtration*

One microgram of total protein in 50 μl buffer [50 mM Tris−HCl (pH 7.4), 200 mM NaCl, 200 mM KCl, 0.1 mM EDTA, 1 mM DTT and 5% glycerol] was applied to a Superose 12HR FPLC column (Pharmacia) and 100 μl fractions were collected. Aliquots of 8.5 μl were assayed for DNA binding in a total volume of 25 μl so that the salt concentration could be brought down to the level used in the band-shift assays. When chaperone activation of column fractions was desired, the binding mixtures included 50 ng DnaJ, 125 ng DnaK, 5 mM ATP and 10 mM Mg acetate. Wild-type RepA (1 μg) was also activated with chaperones (2 μg DnaJ, 6 μg DnaK) for 90 min at room temperature in the presence of 5 mM ATP and 10 mM Mg acetate in a volume of 50 μl, and the entire mixture was applied to gel filtration columns as above and the fractions assayed for DNA binding without adding chaperones. Binding activity was determined by band-shift assays as described above.

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