The Role of the Bacteriophage T4 Gene 32 Protein in Homologous Pairing*

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The gene 32 protein of the bacteriophage T4 is required for efficient genetic recombination in infected Escherichia coli cells and strongly stimulates in vitro pairing catalyzed by the phage uvsX protein, a RecA-like strand transferase. This helix-stabilizing factor is known to bind tightly and cooperatively to single-stranded DNA and to interact specifically with the uvsX protein as well as other phage gene products. However, its detailed role in homologous pairing is not well understood. I show here that when the efficiency of uvsX protein-mediated pairing is examined at different gene 32 protein and duplex DNA concentrations, a correlation between the two is found, suggesting that the two interact in a functionally important manner during the reaction. These and other data are consistent with a model in which the gene 32 protein binds to the strand displaced from the recipient duplex during pairing, thereby stabilizing the heteroduplex product. An alternative model in which the gene 32 protein replaces UvsX on the invading strand, thereby freeing the strand transferase to bind to the displaced strand, is also considered.

The role of single-stranded DNA binding proteins in homologous recombination has been a source of keen interest. In vitro studies have demonstrated that such proteins are able to significantly stimulate the rate of strand exchange between homologous single and double-stranded DNAs catalyzed by “strand transferase” proteins (1–4), a process central to the recombination cycle. This observation is presumably of relevance to the situation in vivo, since mutations in the genes that encode single-stranded DNA binding proteins greatly depress recombination frequencies in many organisms (5). Therefore, an appreciation of the biochemical role of these proteins is central to an understanding of genetic recombination.

Despite considerable effort, the mechanistic basis of single-stranded binding protein action in vitro is not clear. One popular proposal is that they function to break up intramolecular secondary structure in the single-stranded substrate that impedes the binding of strand transferases such as the recA protein of Escherichia coli. This hypothesis has been supported by a number of studies. For example, Tsang et al. (6) showed that preincubation of the single-stranded DNA and the recA protein in buffers of low magnesium concentration effectively substitutes for the presence of the E. coli SSB protein and strongly stimulates subsequent recA protein-mediated pairing with a homologous duplex. Under these conditions, intramolecular base pairs are unstable and presumably do not impede polymerization of the recA protein along the DNA. In agreement with these results, Kowalczykowski and Krupp (7) have demonstrated directly that the SSB protein stimulates RecA binding to DNA with secondary structure. A somewhat different role for the SSB protein in presynapsis has been proposed by Morrical et al. (8), who argued that the SSB protein somehow stabilizes recA protein-single-stranded DNA complexes. However, Kowalczykowski et al. (9) have shown that the SSB and recA proteins compete for single-stranded binding sites. Indeed, the enhanced activity exhibited by some mutant recA proteins appears to correlate with their increased ability to compete with SSB protein for DNA (10). In addition, other experiments suggest that whatever the role of the SSB protein in presynapsis, it also influences later stages of the reaction (11). It seems, therefore, that the role of the single-stranded binding protein is complex and that it may function in more than one stage of the reaction.

We have been studying the bacteriophage T4 in vitro system for homologous strand exchange. In its simplest form, it consists of the uvsX protein (12–15), which is functionally similar to RecA, and the gene 32 protein, a helix-stabilizing protein (16) which strongly stimulates the uvsX protein-promoted homologous pairing reaction (12). In addition, it has been shown that the uvsX protein (17–19) and dda (20) gene products also function as part of the protein machine for strand exchange in vitro. The former is an accessory factor that stabilizes uvsX protein-single-stranded DNA filaments, while the latter is a DNA helicase that stimulates the rate of protein-mediated branch migration. Affinity chromatography experiments conducted by Alberts and co-workers (21, 22) have shown that numerous specific protein-protein interactions exist within this complex. Of particular significance here is the finding that the gene 32 protein binds specifically to uvsX columns (the converse is also true). The observation that the E. coli SSB protein stimulates the UvsX-mediated pairing reaction to a much lesser degree than the T4 helix-stabilizing protein has been taken as evidence that these protein-protein interactions are of functional importance in strand exchange (12). It should be noted, however, that the DNA binding chemistry of these two factors is considerably different under so-called “high salt” buffer conditions (23) such as those used for the homologous pairing assays (12), so this interpretation must be considered tentative. In any case, this lack of generality distinguishes the T4 system from the recA protein-mediated process, which is stimulated by single-stranded binding proteins from many sources (24).
To better understand the mechanism of homologous pairing, it would be desirable to understand the molecular basis of the gene 32 protein’s stimulatory effect. Previously, Forsmova and Alberts (12) demonstrated that the 32 and uvsX proteins compete for single-stranded DNA, as determined by monitoring the uvsX protein’s DNA-dependent ATPase activity in the presence of gp32. Interestingly however, the concentration of gene 32 protein that yields the greatest stimulation of homologous pairing severely depresses the rate of uvsX protein-catalyzed ATP hydrolysis. Furthermore, Harris and Griffith (29) have shown that, unlike RecA, the uvsX protein is not greatly troubled by secondary structure in single-stranded DNA. These experiments do not support the idea that the main function of gp32 is to aid in formation of the presynaptic filament. Rather, they suggest that it has a different, or at least additional, role in the homologous pairing process. In this report, I present evidence that is consistent with models in which the gene 32 protein either binds to the displaced strand of the recipient duplex itself, thereby stabilizing the product, or displaces the uvsX protein from the invading strand, allowing it to capture the displaced strand. Both schemes are consistent with the known properties of the gene 32 protein and rationalize how the protein can inhibit presynapsis yet stimulate the overall pairing reaction.

**MATERIALS AND METHODS**

**Proteins and DNAs**—The uvsX (13) and gene 32 (26) proteins were purified as described previously. M13 single- and double-stranded DNA were isolated by the procedure of Messing (27). Single-stranded DNA concentrations are reported as nucleotides, double-stranded DNA concentrations as base pairs. A 2527-nucleotide linear single-stranded M13 DNA fragment was obtained by cleavage of the viral DNA with HaeIII restriction endonuclease (New England Biolabs) and gel purification of the band corresponding to the largest digestion product.

**Homologous Pairing Reactions**—These assays were carried out as described previously (28). Aliquots of reactions quenched by addition of EDTA to 20 mM and SDS to 1% were electrophoresed through a 1% agarose gel. The DNA bands were detected by either photographing the ethidium bromide-stained gel or by autoradiography of the dried gel. All reactions were conducted at 37 °C and were initiated by addition of ATP to 2 mM. The concentrations of the proteins and DNAs are given in the figure legends.

**RESULTS**

Protein-mediated pairing between homologous single-stranded circular and double-stranded linear DNAs is commonly used as an *in vitro* model reaction. The uvsX protein alone promotes this reaction only at very high concentrations (Fig. 1). It is interesting that a reasonable yield (50%) of paired product is achieved only when the uvsX protein concentration reaches 3.75 μM. Assuming a site size of four nucleotides per UvsX monomer, this is enough protein to cover 15 μM nucleotides, a concentration very similar to that of the single-stranded DNA employed (15.4 μM).

Fig. 2 illustrates the results of a series of experiments in which the extent of uvsX protein-mediated homologous pairing between single-stranded circular and double-stranded linear DNAs was assayed with different amounts of the gene 32 protein at a uvsX protein concentration that does not support the reaction in the absence of the helix-stabilizing factor. A critical concentration of gp32 is eventually reached that supports efficient pairing. This experiment was then repeated at higher duplex DNA concentrations; the single-stranded DNA concentration was kept constant. In each case, there was sufficient single-stranded DNA to pair with all of the duplex substrate. If the gene 32 protein functions solely at the presynapsis stage, then the result of the titration experiments should be independent of the duplex DNA concentration since the amount of single-stranded DNA remains constant. However, this is clearly not the case (Fig. 2). At higher duplex DNA concentrations, more gene 32 protein is required for efficient pairing. There is a correlation between the concentrations of the recipient DNA and the T4 helix-stabilizing protein, suggesting that gp32 binds to the duplex in a functionally significant manner at some point during the reaction. Since gp32 is known to have a low affinity for double-stranded DNA, it seems likely that this interaction is between the gene 32 protein and the duplex strand that becomes single-stranded when it is displaced by the invading DNA (Fig. 3A). The gene 32 protein is known to thermodynamically stabilize single strands with respect to a duplex (16). This interaction could be important in stabilizing the product of the pairing reaction and help to drive the overall reaction in the forward direction. A related possibility is that the gene 32 protein displaces UvsX from the invading strand, thereby allowing it to capture the displaced strand (Fig. 3B). These experiments cannot distinguish between these possibilities.

In order to test these ideas, the formation of displacement loops (D loops) from homologous single-stranded linear and double-stranded circular DNAs was explored. A preparation

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1 The abbreviations used are: gp32, gene product 32; SDS, sodium dodecyl sulfate; D-loop, displacement loop.
The rationale for this experiment is as follows. If protein-amined. Single-stranded DNA was present in molar excess. effect of increasing gene 32 protein concentrations was ex-
dernificant stabilization of the displaced strand is important insufficient to mediate the reaction alone was chosen, and the nicked was employed. Again, a uvsX protein concentration is required for efficient pairing of the supercoiled DNA. These observations strongly suggest that there is a functionally significant interaction between the helix destabilizing protein or the uvsX gene product and the duplex DNA in the course of the homologous pairing reaction. These data are consistent with the models shown in Fig. 3. The first scheme proposes that the role of the gene 32 protein is to bind directly to the displaced strand, an interaction that stabilizes the heterodu-
plex product (Fig. 3A). A related possibility is that the uvsX protein stabilizes the displaced strand and the presence of the gene 32 protein is required to blanket the unpaired, invading single-stranded DNA which would otherwise compete for UvsX. Indeed, the observation that the uvsX protein alone can efficiently carry out the pairing reaction at concentrations high enough to blanket all of the single-stranded DNA sug-
metal to note that they are by no means mutually exclusive. These models are attrac-
tive in that they rationalize why the gene 32 protein optimally stimulates the overall pairing reaction at concentrations that severely inhibit formation of extensive uvsX protein-single-stranded DNA filaments. The steady state concentration of free gene 32 protein must be sufficiently high to bind, at least transiently, the duplex strand that is displaced during syn-
thesis even in the presence of excess single-stranded substrate, or, alternatively, to occupy the invading strand, freeing the strand transferase for this duty.

All of the reactions in Fig. 2 exhibit a common critical concentration for gp32 (about 1.1 μM) below which little or no pairing occurs. Above this level, the yield of heteroduplex products is dependent on the gp32 concentration. The site size of a gene 32 protein monomer is 8–10 nucleotides, so 1.1 μM is enough helix destabilizing protein to cover about 9–11 μM single-stranded nucleotides. The 1 μM uvsX protein present can bind about 4 μM nucleotides. Therefore, efficient pairing is not observed until there is enough total protein to cover almost all of the single-stranded substrate (15.4 μM). This dependence of the pairing activity on the degree of single-stranded DNA saturation was also observed when only UvsX was present (Fig. 1). This behavior is consistent with the models discussed above. The product will only be formed efficiently if there is sufficient DNA binding protein free in solution to capture the displaced strand, whether it be the uvsX or gene 32 protein. The important point is that the gene 32 protein clearly is important in the events that follow presynapsis, whether its role is direct (binding to the displaced strand) or indirect (freeing UvsX to capture this strand).

Unfortunately, these experiments do not provide an obvious rationalization for the specific effect of the T4 helix destabilizing protein. One possibility is that there is a contact be-
tween the gene 32 protein on the displaced strand and the uvsX protein filament on the invading strand, but there is no

**Fig. 3.** Two models for the mechanism of action of the gene 32 protein. **A,** the gene 32 homologous pairing reaction, thereby stabilizing the product in a functionally important manner. **B,** an indirect model in which the gene 32 protein displaces the uvsX protein from the invading strand, thereby freeing the recombinase to capture the displaced strand. In both cases, the presynaptic filament is presumed to contain both T4 factors, as proposed by Harris and Griffith (25) based on electron microscopic investigations.

**Fig. 4.** Pairing of supercoiled DNA is less dependent on the gene 32 protein than pairing of nicked duplex DNA. Reactions containing 1 μM uvsX protein, 15.4 μM of a 2527-nucleotide linear single-stranded M13 HaeIII digestion product and 12.3 μM M13 double-stranded DNA (a mixture of supercoiled and nicked species) and the indicated amount of gene 32 protein were incubated for 5 min at 37 °C. a, ethidium bromide-stained gel of the quenched reactions following electrophoresis through a 1% agarose gel. b, quanti-
tation of the gel shown in a obtained by densitometry of the photo-
graphic negative. •, supercoiled; □, nicked DNA.

of duplex circles that was about 50% supercoiled and 50% nicked was employed. Again, a uvsX protein concentration insufficient to mediate the reaction alone was chosen, and the effect of increasing gene 32 protein concentrations was ex-
ained. Single-stranded DNA was present in molar excess. The rationale for this experiment is as follows. If protein-
dependent stabilization of the displaced strand is important in the homologous pairing process, then this effect should be relatively less pronounced for D-loops formed from super-
coiled DNA than products formed from the nicked duplex, since the former are stabilized considerably by relief of super-
helical tension but the latter are not. Both substrates are present in the same tube and undergo the same reaction, allowing a direct comparison to be made between them at different gene 32 protein concentrations. The results are shown in Fig. 4. A markedly lower gene 32 protein concentra-
tion is required for efficient pairing of the supercoiled DNA. At high gp32 concentrations however, both substrates are incorporated into heteroduplex products.
direct evidence for this. Therefore, the functional significance of the uvsX-gene 32 protein-protein interaction remains unclear. Finally, it is also not clear how relevant these studies are to the mechanism of action of the recA and SSB proteins of E. coli, although correlations between pairing efficiency and the degree of single-stranded DNA saturation in reactions containing only the recA protein have been noted (28). In addition, electron microscopic studies of recA protein-mediated pairing reactions containing the SSB protein have shown that the latter does bind to the displaced strand (29). However, the functional significance of the latter observation is not clear. It is also important to keep in mind that some important differences in the detailed biochemical properties of the T4 and E. coli proteins have been reported (23, 28-33). Further studies of the bacterial and phage in vitro recombination systems will hopefully resolve these matters.

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