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PROTEINS IN GENE DUPLICATION AND EXPRESSION

Function of gene 32-protein, a new protein essential for the genetic recombination and replication of T4 bacteriophage DNA[1]

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A WIDE VARIETY OF PROTEINS must interact with DNA inside living cells in order to make possible the basic genetic processes. Before DNA replication, genetic recombination, DNA repair, and selective gene expression can be fully understood at the molecular level, all of these proteins will have to be individually isolated and characterized. To facilitate this difficult task, we have developed a method which we call DNA-cellulose chromatography (3, 4).

The DNA-cellulose chromatography relies upon the fact that many proteins which function in association with intracellular DNA recognize purified DNA as a substrate and bind tightly to it in vitro. We have used this method to isolate and identify the protein specified by bacteriophage T4 gene 32 (3). The product of gene 32 is known from earlier studies to be required for normal replication and genetic recombination of bacteriophage T4 DNA (23, 43, 73, 74). This report largely concerns our progress in the characterization of "32-protein," emphasizing current ideas concerning the mechanisms of DNA replication and recombination as they appear relevant to its possible mode of action.

DNA-CELLULOSE CHROMATOGRAPHY

When DNA-free, crude protein extracts, adjusted to physiological salt concentrations, are passed over a column consisting of DNA adsorbed onto cellulose, DNA-binding proteins are retained. If desired, subfractions of DNA-binding proteins with a specific affinity for the type of DNA on the column may be selected; in this case an excess of a competing heterologous DNA is added to the extract before exposing it to the DNA-cellulose. Use of this procedure causes proteins which bind to DNA without regard to nucleotide sequence to wash through the column, bound to the DNA in the extract (3).

The DNA-cellulose used in our experiments is made by extensively drying a concentrated solution of DNA with a purified grade of cellulose (3). Other procedures are available for immobilization of DNA on a variety of different chromatographic matrices (5, 7, 9, 15, 30, 31, 47, 53, 76, 82). Most of these preparations either appear not to be suitable for protein fractionations or have not yet been tested for this purpose. Exceptions are the partial fractionation of *Escherichia coli* deoxyribonucleases on a DNA-agarose gel (53) and, most notably, the successful preparative-scale purification of *E. coli* DNA polymerase on DNA fixed to cellulose by irradiation with ultraviolet light (47).

By using DNA-cellulose chromatography followed by polyacrylamide gel electrophoresis (16, 59), we have found that more than 15 different proteins from the bacterium *E. coli* bind tightly to *E. coli* DNA. These proteins include the previously well-characterized DNA-associated enzymes, *E. coli* DNA polymerase (22) and RNA polymerase (63). To determine the intracellular location of the remaining unidentified proteins, the DNA-binding proteins in the DNA-less minicells and the DNA-containing whole cells of *E. coli* K12 mutant strain P678-54 (2) have been compared by DNA-cellulose chromatog-

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raphy of doubly labeled extracts (Alberts and Frey, unpublished results). We find that the fraction of total soluble protein binding to double-stranded *E. coli* DNA-cellulose is greatly reduced in minicells; most of these proteins therefore partition into the DNA-containing daughter cell at the time of cell division. Thus, at least in *E. coli*, the DNA-binding proteins detected by DNA-cellulose chromatography appear to associate with DNA inside the cell as well as in vitro.

The DNA-binding proteins thus far observed share a common property: essentially all of them may be reversibly eluted from a DNA column at sufficiently high ionic strengths. This strongly implies that electrostatic attractions play a crucial role in their interaction with DNA (40). Many DNA-binding proteins carry a net negative charge in the physiological pH range (3); they may nevertheless share a positively charged groove designed to fit the DNA phosphate-sugar chain.

We have chosen to devote much of our work to an analysis of the bacteriophage-induced DNA-binding proteins which can be selectively labeled with radioisotopes after infection of *E. coli* with bacteriophage T4. This system was chosen for detailed investigation of basic genetic processes because of its relative simplicity. In addition, a wide range of amber and temperature-sensitive mutant bacteriophages is available (23). Under nonpermissive conditions, a bacteriophage carrying an amber mutation synthesizes only a fragment of the protein corresponding to the mutated gene (70). Such a fragment of a DNA-binding protein might be expected to lose the ability to bind to DNA. By comparing the polyacrylamide gel electrophoretic pattern of DNA-binding proteins made during mutant and wild-type bacteriophage infections, it is therefore possible to identify a missing protein band as the product of a particular bacteriophage gene. The defective physiology of a mutant-infected cell combined with detailed studies of the DNA interaction of the corresponding purified protein should reveal new types of functions essential to genetic processes.

TOTAL DNA-BINDING PROTEINS FROM T4 BACTERIOPHAGE-INFECTED *E. coli*

To identify T4 bacteriophage-induced DNA-binding proteins by DNA-cellulose chromatography, bacteriophage-infected *E. coli* cells have been labeled with radioactive protein precursors. Adding such precursors from 35 to 45 min after infection at 25 C in this system labels proteins made late in the bacteriophage infectious cycle. These include the structural proteins of the phage head, tail, and tail fibers as well as the phage-induced lysozyme and proteins thought to be involved in packaging the DNA into phage heads (81). In contrast, labeling from 5 to 20 min after infection at 25 C labels only the group of "early" phage proteins which are thought to be primarily involved in the provision of precursors and enzymes for DNA replication and recombination. *E. coli* proteins are not labeled during either time period due to the complete turnoff of host protein synthesis shortly after T4 bacteriophage infection (37).

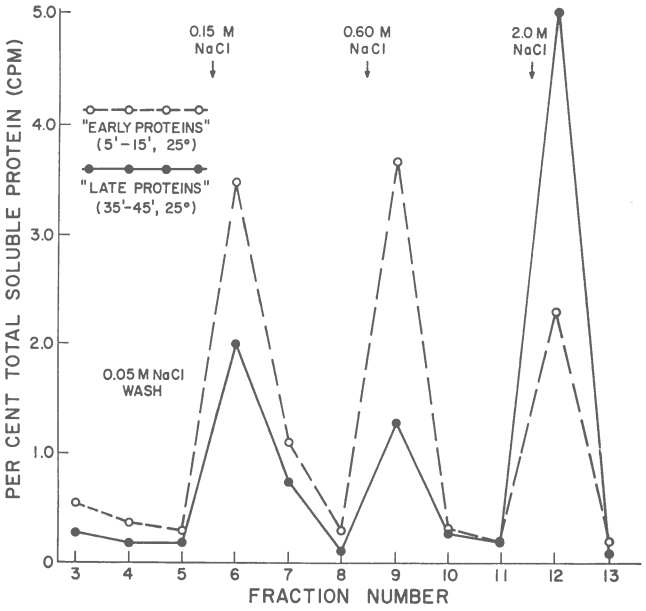


FIG. 1. Chromatography of doubly labeled, T4-infected, crude cell extracts on a mixed (1, 1) native T4 plus denatured calf thymus DNA-cellulose column. Methods were as previously described (3), with pancreatic DNase I treatment of the cell sonicate utilized for DNA removal. Three milliliters of extract from 8×10^{10} infected cells was applied to a 1-ml column containing about 1 mg of DNA. Calf thymus rather than T4 denatured DNA-cellulose was used, because it is difficult to prevent the T4 DNA single strands from renaturing during the drying procedure used to adsorb DNA to cellulose. Only about 0.2% of the total labeled protein applied to a control, DNA-free cellulose column appeared in column eluates.

The DNA-binding proteins can be conveniently obtained from the infected cells by breaking them open by sonication, destroying endogenous DNA with pancreatic DNase treatment at 10 C, centrifuging out particulate matter, dialyzing away the Mg^{2+} needed for DNase activation, and then passing the DNA-free crude extract through a DNA-cellulose column in a buffer containing 0.05 M NaCl. After washing the column with additional buffer, bound proteins are routinely eluted by raising the salt concentration in 3 steps of 0.15 M, 0.60 M, and 2.0 M NaCl (3, 4).

To compare the bacteriophage-induced DNA-binding proteins synthesized at early and late times of infection, we made a special extract by combining an equal number of infected cells containing leucine-^{3}H-labeled "late" phage proteins with cells containing leucine-^{14}C-labeled "early" phage proteins. Such a doubly labeled extract was chromatographed on a mixed DNA-cellulose column containing not only double-stranded T4 DNA, but also single-stranded DNA, thus enabling detection of a wider variety of DNA-binding proteins than otherwise possible. The elution pattern obtained from this column is illustrated in Fig. 1. A total of 8% of the soluble late bacteriophage protein and 10% of the soluble early bacteriophage protein bound to DNA in this experiment. This is a five- to eightfold greater percentage of protein binding to DNA than found in identical experiments with un-

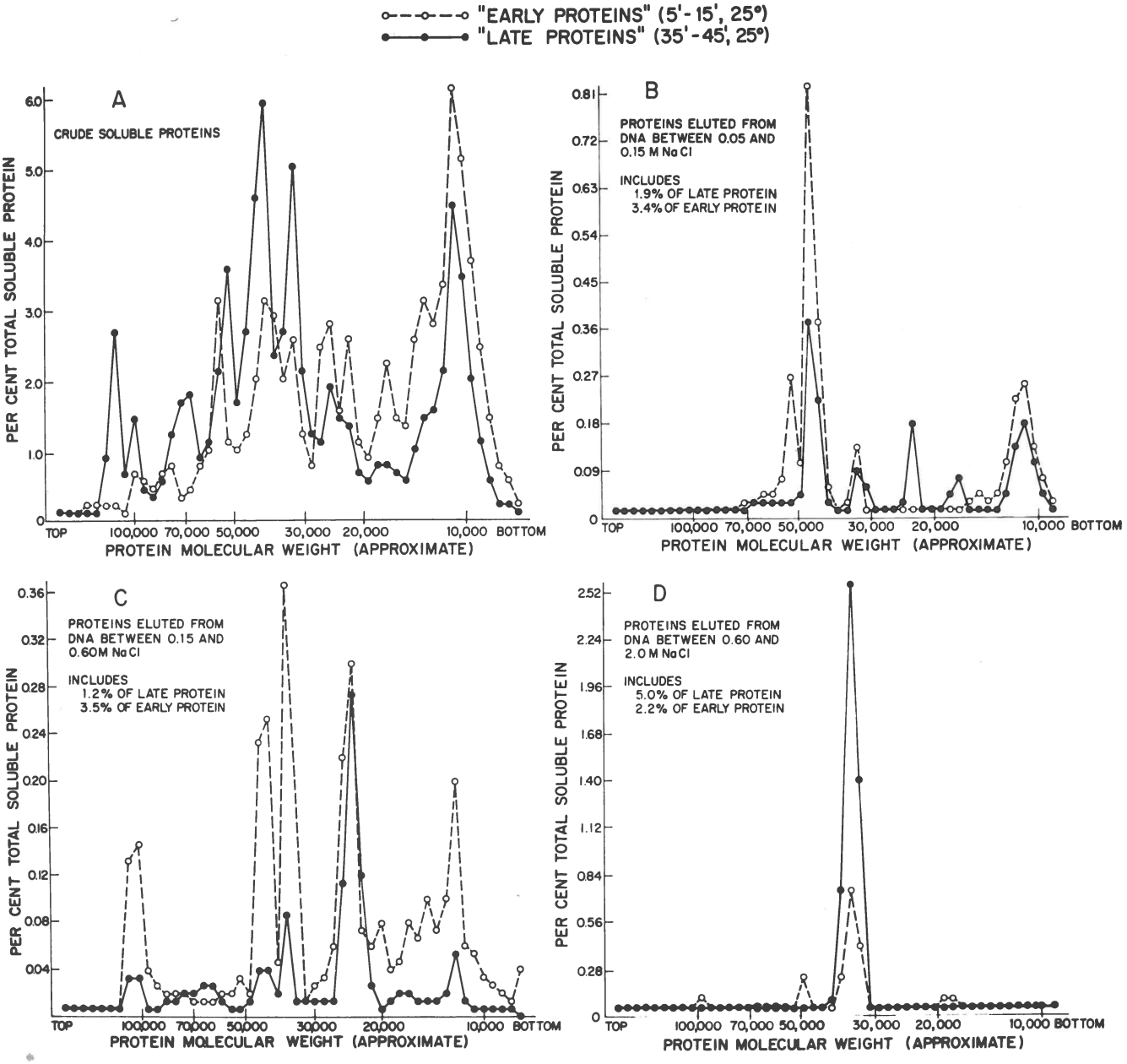


FIG. 2. Sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis of the doubly labeled T4 bacteriophage-induced DNA-binding proteins from Fig. 1. In this experiment, the "early proteins" were labeled with leucine-^{14}C and the "late proteins" with leucine-^{3}H (New England Nuclear Corp.). Controls in which these isotopic labels are reversed are indistinguishable. After concentration to 0.2 ml (containing at least 20,000 cpm) by vacuum dialysis, the DNA-binding peaks in Fig. 1 were made 1% in SDS plus 0.14 M in β-mercaptoethanol, heated to 100 C for 2 min, and subjected to electrophoresis for 16 hours through 10 cm x 0.5 cm polyacrylamide gels (10% acrylamide, 0.3% ethylenediacrylate) containing 0.1% SDS (66). For analysis, 2 mm-thick gel slices cut with stacked razor blades were incubated in one dram vials for 16 hours at 23 C in 0.5 ml 0.50 M NaOH. Four milliliters of Bray's scintillation fluid (8), containing 20 g/liter of Cab-O-Sil (Cabot Co.), were then added, and the samples thoroughly mixed and counted in a scintillation counter. The data shown have been corrected for channel overlap.

infected *E. coli*, in agreement with the much larger fraction of genes in the T4 system whose expression is known to be needed for DNA-related functions. Such a correlation is further evidence that most proteins that bind tightly to DNA in vitro under our ionic conditions were formerly associated with DNA inside the cell.

A separation of the different T4 bacteriophage-induced DNA-binding proteins made at early and late times during infection was obtained by electrophoresis of the column fractions obtained in Fig. 1 through polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS). This electrophoretic system has the advantage that the approximate molecular weight of each protein component can be determined directly from its migration

rate (66). Electrophoretic profiles obtained by scintillation counting of dissolved gel slices are displayed in Fig. 2. In Fig. 2 *A*, the many soluble proteins of the unfractionated extract labeled at early and late times of infection are compared. It is clear that very few of the major bacteriophage-induced proteins in a crude extract are synthesized during both of these periods of the infectious cycle (37).

The T4 bacteriophage-induced DNA-binding proteins from Fig. 1 are analyzed in the electrophoretic diagrams of Fig. 2 *B*, *C*, *D*. About 20 distinct protein bands can be seen. Most of these proteins appear to be synthesized in substantial amounts only during the early labeling period. However, four DNA-binding proteins are made exclusively at late times of infection, while at least three seem to be made during both the early and late labeling periods. We are currently screening available mutants in order to identify as many of these DNA-binding proteins as possible as the products of known bacteriophage genes.

CHARACTERIZATION OF T4 GENE 32-PROTEIN

Analysis of the altered DNA-binding proteins made after infection with mutants in gene 32 of bacteriophage T4 reveals that the major DNA-binding protein eluting with 2.0 M NaCl in Fig. 1 is the product of this gene (4). As can be seen in Fig. 2 *D*, "32-protein" has a molecular weight of about 35,000 daltons and is made in large quantities throughout the infectious cycle. It has a net negative charge at pH 7. Bacteriophage mutants lacking this protein fail to make joint DNA molecules after mixed infection with density labeled DNA's (43, 73, 74); they are therefore thought to be blocked in an early stage of genetic recombination. In addition, gene 32 amber mutants are severely deficient in DNA synthesis, requiring 40 min at 37 C to approximate even a single round of replication (43). Studies with purified 32-protein should therefore help to illuminate the unknown details of the reaction during which two recombining DNA molecules recognize that they are homologous and pair; in addition, they might reveal important new features of the mechanism of DNA replication.

With the relatively high 32-protein concentration present in the experiment shown in Fig. 1, 32-protein was bound strongly to the DNA on the column and could be removed only with the 2.0 M NaCl-containing elution buffer. If a lower concentration is present, or if native DNA-cellulose alone is used, 32-protein elutes from the column in the 0.60 M NaCl step instead (3). This concentration-dependent affinity of 32-protein for single-stranded DNA-cellulose indicates that individual molecules of 32-protein bind in cooperative units to single-stranded DNA and suggests a model in which the protein monomers line up in close juxtaposition along a DNA strand, as illustrated schematically in Fig. 3. The essential correctness of this model has been demonstrated by recent experiments in which the interaction of ^{3}H-labeled 32-protein with T4 DNA has been measured by velocity sedimentation of the complex through stabilizing sucrose gradients (Alberts and Frey, unpublished experiments). These studies confirm the cooperative nature of the 32-protein interaction with single-stranded DNA, and indicate saturation with 32-protein only at a 12:1 weight ratio of protein to DNA. Thus, approximately one protein monomer can be bound per every 10 DNA nucleotides. Since physical studies indicate that 32-protein is asymmetric, and roughly 120 A long in its longest dimension (3), each bound protein molecule could readily form stabilizing interactions with its immediate neighbors, as suggested in Fig. 3. From available data, we can estimate that the degree of cooperativity involved is sufficient to cause 32-protein to bind in clusters on the DNA even under conditions of large DNA excess (unpublished results).

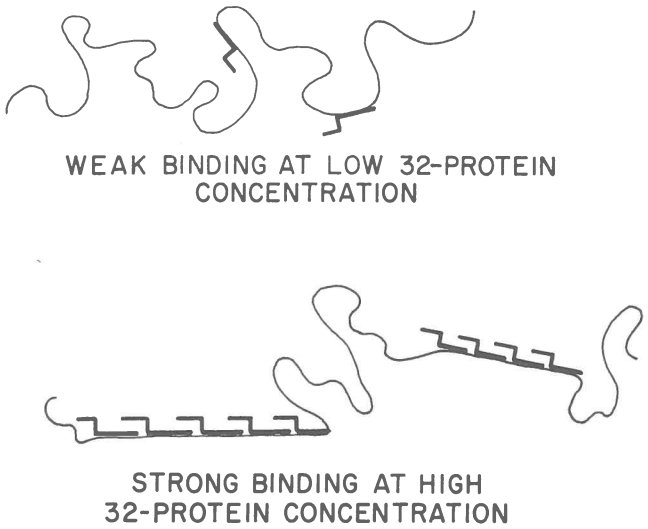


FIG. 3. A model for the cooperative binding of 32-protein to single-stranded DNA. Because 32-protein binds much more strongly to single-stranded than to double-stranded DNA, and one protein monomer can be bound per every 10 DNA nucleotides, 32-protein should loosen the DNA double-helix inside of the cell. Moreover, if binding actually occurs in the suggested manner, 32-protein would unravel the short, imperfectly paired double-helical "hairpins" which are thought to predominate in single-stranded nucleic acids (26). Under physiological conditions of salt and temperature, these regions of weak secondary structure act as kinetic barriers and prevent denatured DNA molecules containing complementary nucleotide sequences from renaturing in vitro (71). With purified DNA, renaturation is facilitated by any condition such as high temperature (50), organic solvents (72), or mild alkali (18), which preferentially melts out the imperfect helices in single-stranded DNA while leaving intact the more stable, perfect double helix formed between matching complementary strands. Provided the binding of 32-protein to single-stranded DNA leaves the bases suitably exposed, this binding could serve to speed up renaturation in vivo in a manner analogous to the action of these mild denaturing conditions in vitro. The existence of some substance in cells which acts in this way to facilitate renaturation is implicitly assumed in most models of genetic recombination.[2]

[2] In agreement with this postulated function, we have found recently that T4 DNA single-strands renature rapidly at 37 C only when they are covered with 32-protein (Alberts and Frey, ms in preparation).

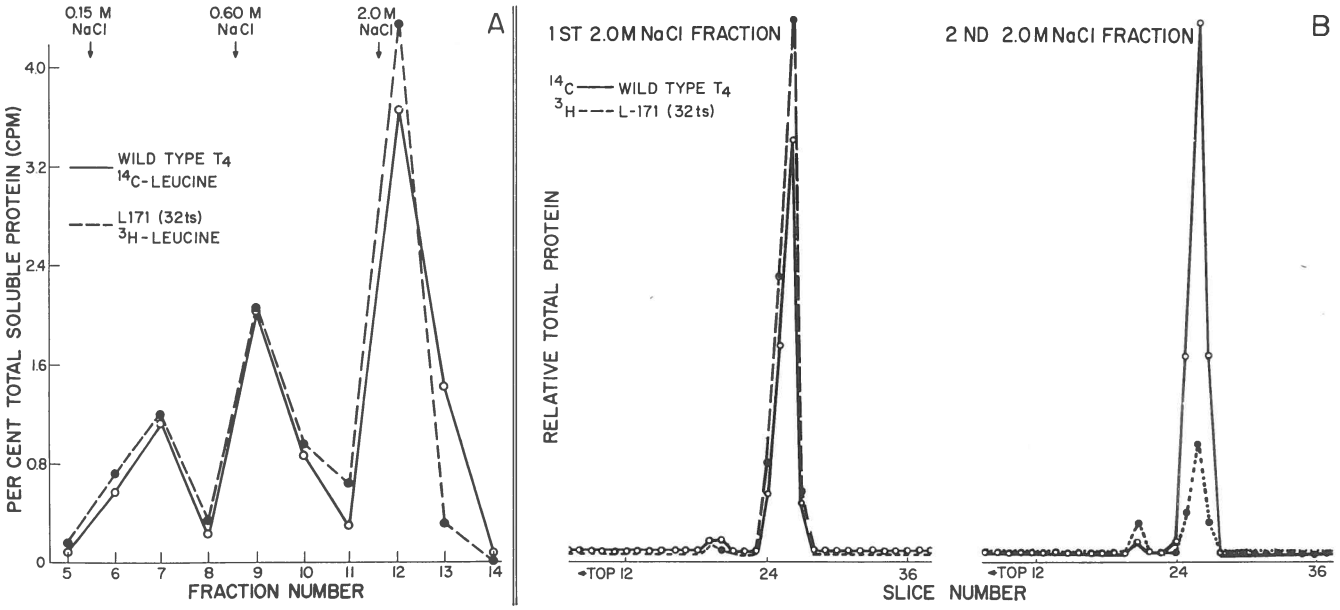


FIG. 4. Comparison of the binding affinity of normal and temperature-sensitive 32-protein for single-stranded DNA-cellulose. At 35 min after infection at 25 C, 4×10^{10} wild-type infected cells were labeled for 10 min with leucine-^{14}C and 4×10^{10} ts L171 infected cells were labeled for 10 min with leucine-^{3}H. Extracts were made as in Fig. 1 from the mixed cells and chromatographed on denatured calf thymus DNA-cellulose (0.7 ml packed volume containing about 1 mg of DNA). *A:* elution profile from the DNA column. Fractions were collected every 15 min, with a flow rate of 2 ml/hr. *B:* SDS-containing polyacrylamide gel electrophoresis of the 2.0 M NaCl-containing fractions as described in Fig. 2. The major band is 32-protein.

There is evidence that, as a result of its DNA binding, 32-protein plays a direct structural role in genetic processes. One indication to this effect is the finding that the altered 32-protein synthesized in cells infected with a temperature-sensitive gene 32 mutant binds more weakly than the normal protein to DNA. In the experiment shown in Fig. 4 *A*, a mixed extract of mutant (^{3}H-labeled) and wild-type (^{14}C-labeled)-infected cells was chromatographed on single-stranded DNA-cellulose at 4 C. Since a relatively high protein concentration was used, both mutant and wild-type 32-proteins mainly eluted in the 2.0 M NaCl-containing fraction; however, it is clear from Fig. 4 *B* that the mutant protein was more readily removed from the DNA as the salt concentration increased.

A more convincing argument for a structural role stems from our finding that there are about 10,000 molecules of 32-protein made in a normal infected cell (see Fig. 2 *D*). All of this protein appears to be stable and to accumulate, since the DNA-binding ability of the 32-protein labeled at 10 min after infection remains constant during a 40-min chase (Alberts and Frey, unpublished results). Nevertheless, it has been shown by Snustad that the quantity of 32-protein made in these cells directly limits the number of progeny phage produced (69; Snustad, personal communication). A requirement for such large quantities of 32-protein strongly suggests that it plays a structural rather than a simple catalytic role in DNA function. It is also consistent with the fact that the synthesis of 32-protein continues throughout the entire infectious cycle.

What might the function of 32-protein be in genetic

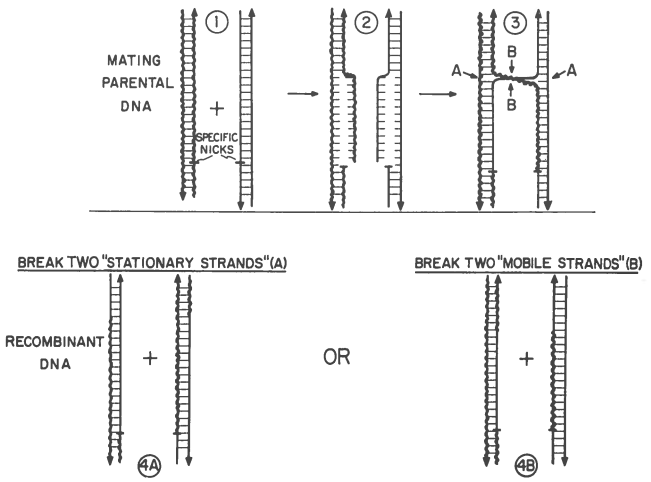


FIG. 5. A model for reciprocal genetic recombination. It is assumed that DNA is nicked at a limited number of sites by an endonuclease with sequence specificity; starting from these sites, complementary strands exchange partners as indicated. Similar models have been suggested by Whitehouse (79). A temporal correlation between the nicking of intracellular T4 DNA and DNA strand exchanges has been observed in T4-infected cells (44), and it has been postulated that some of the nucleases whose activities increase after T4 infection play a role in recombination (27, 65). (From Holliday (36).)

systems? By virtue of its strong cooperative binding to single-stranded DNA, 32-protein might serve to open up local regions of DNA double-helix. Such local "denaturation" might in turn be required in vivo for both genetic

recombination and DNA replication. For example, most theoretical models which are proposed to explain the reciprocal nature of genetic recombination (51), such as the Holliday model (36) illustrated in Fig. 5, require a protein with this function in order to proceed efficiently at 37 C. It has been demonstrated by Tomizawa (73) that T4 mutants in gene 32 fail to form recombinant DNA molecules and that these molecules normally have a structure of the type shown in Fig. 5.

Rapid DNA replication may require local denaturation in the region of the replication fork, particularly if replication in vivo proceeds in a discontinuous manner on *both* DNA template strands, as has been postulated recently (55, 58, 61; see also 56). In each infected cell, there is enough 32-protein made to complex with about 100,000 DNA nucleotides, or about one-fourth of a single T4 DNA molecule of 130 × 10^6 daltons. At late times of infection there is a pool of free DNA equal to about 20 molecules/cell (42); these molecules contain a total of 60 replication forks (78). There would therefore only be sufficient 32-protein to cover completely about 1,700 DNA nucleotides at each replication fork. If generation of each new replication fork must wait for about this number of 32-protein molecules to become available, the observed direct dependence of the quantity of phage progeny produced on 32-protein level (69) could be simply explained.

It should be pointed out that DNA replication does not appear to be required for recombination of DNA molecules in bacteriophage T4-infected cells (74), nor are recombination-deficient mutants in another bacteriophage system (phage λ) blocked in their DNA replication (67). It is therefore likely that 32-protein is a new type of protein which catalyzes a reaction required for both of these genetic processes.

As the beginning of an attempt to discover the role of 32-protein in DNA replication, we have examined the rate of replication after a shift from 25 to 42.5 C of cells infected with temperature-sensitive phage mutants in gene 32 (3). The result of pulse labeling with thymidine-^{3}H at various times after the shift up is shown in Fig. 6 (P. Mendis, Senior Thesis, Princeton, 1969). The rate of DNA replication is seen to drop drastically, reaching a very low value within 12 min at 42.5 C. The fact that none of the four independently isolated, temperature-sensitive gene 32 mutants that we have tested[3] stop DNA replication immediately might indicate that 32-protein is needed only for initiation or termination of a round of DNA replication. However, it is equally possible that these temperature-sensitive 32-protein molecules denature only slowly, especially since they may be relatively heat-resistant until released from the DNA. Studies on the structure of the intracellular T4 DNA (25) which accumulates after the temperature shift, as well as examination of the behavior of additional temperature-sensitive mutants, are needed to define further the role of

[3] Temperature-sensitive mutants in gene 32 were obtained from Dr. R. S. Edgar.

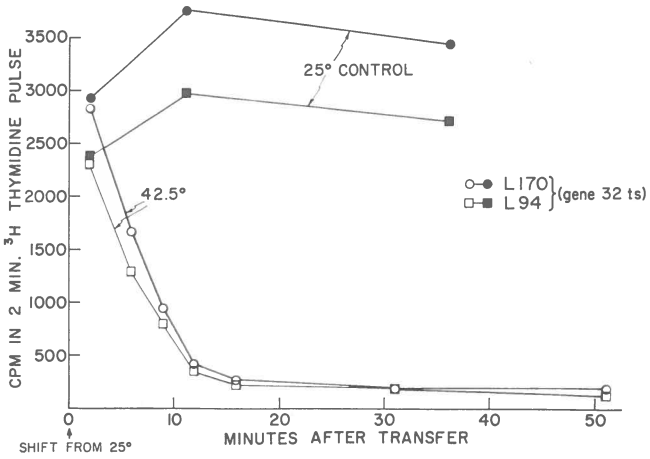


FIG. 6. Dependence of the rate of T4 DNA synthesis on the continued function of gene 32-protein. *E. coli* B3 at 3 × 10^8 cells/ml in M_9 minimal medium containing 0.3% casein hydrolysate and 20 μg/ml thymidine were infected at 25 C with a multiplicity of 5 phage per bacterium. After 25 min at 25 C, the infected cells were diluted fivefold into prewarmed media (without additional thymidine). At various times, thymidine-^{3}H was added for 2 min to an aliquot. At the indicated time, incorporation was stopped with an equal volume of cold 10% trichloroacetic acid, and the sample was collected on a membrane filter and counted. Cells infected with wild-type phage continue to synthesize DNA normally at 42.5 C in this experiment.

32-protein in DNA replication. As a guide to these and other studies, a general model for DNA replication has been explored which places special emphasis on a possible role for proteins which bind in a cooperative manner to DNA.

Models for DNA Replication

A COOPERATIVE DNA-BINDING PROTEIN AS THE INITIATOR FOR DNA REPLICATION

The DNA-cellulose chromatography should help in the discovery of as yet unknown proteins which play important roles in DNA replication. It is, therefore, useful to consider what is known about the synthesis of DNA in order to try to deduce from available data possible roles for such proteins in the replication process.

The timing and rate of initiation of DNA replication in bacteria are carefully controlled so as to be perfectly coordinated with the increase in cell mass and the time of cell division (14, 49). As first demonstrated by Sueoka and his co-workers, each round of DNA replication begins at a fixed point on the bacterial chromosome (1, 11, 12, 80, 84), and is triggered independently of completion of the previous round of replication (14, 57). The unknown initiation event requires protein synthesis (46, 48), and is observed to occur whenever the *cell mass per chromosome origin* reaches a fixed threshold value (19).

The above facts, and many others which have been reviewed recently (45), can be explained by postulating that a specific initiator protein is made in proportion to the overall rate of protein synthesis (19). However, any more explicit model for initiation must be reconciled

with the fact that very few functional units of initiator are made in each cell, and explain the apparent inability of this protein to participate in more than a single initiation event.

The above restrictions are readily accommodated by a hypothetical model which postulates that initiator protein binds tightly to DNA at the origin of replication, and that the triggering of replication requires the cooperative action of X molecules of initiator protein at this point. It follows from these assumptions that: *1*) Initiator protein need not be destroyed during each initiation event, as it would be automatically titrated out by the new origins created (to an average of 0.5 X molecules bound per origin). *2*) Initiator protein need not be synthesized in a small, well-regulated number of copies per cell, since the number of molecules required for its action (X) could be large (threshold effect). *3*) Synthesis of initiator protein would not have to be controlled by a special signal, but could be governed simply by the overall rate of protein synthesis. Initiation of DNA replication would then occur whenever the cell mass per chromosome origin was just sufficient to provide an average of X initiator molecules per origin. If the value X is large, premature initiations (due to the expected statistical fluctuation in the number of initiators bound per origin at subthreshold levels) would be rare. Initiation of replication on both daughter templates during dichotomous replication (57) would be simultaneous, provided the binding site for initiator is temporarily destroyed as replication begins.

The above model is closely related to some of the models for the initiation process which have previously been proposed by others (20, 49, 60); however, it has the apparent virtue of being both more explicit and less complicated than these earlier models. It will now be demonstrated that this general scheme can be reasonably incorporated into a more specific model for DNA replication, based on ideas originally proposed by Yoshikawa (83).

A TEMPLATE-PRIMED MODEL FOR INITIATION OF DNA REPLICATION

The overall morphology of replicating DNA was first revealed by radioautographic studies of the *E. coli* chromosome (10). The essential features are schematically illustrated at the top of Fig. 7: the replicating molecule is circular with the two daughter helices joined to the parental strands at *both* of their extremities. Recently, similar structures have been visualized in the electron microscope for purified replicating phage λ (75), mitochondrial (41), and polyoma DNA's (35). Since these structures survive extensive treatment with detergents, RNase, pronase, and trypsin (35, 41, 75), CsCl centrifugation (35, 75), and phenol (41), the most reasonable interpretation is that they contain only normal DNA phosphodiester linkages.

A semiconservative mode of DNA replication requires only that the two daughter helices be joined at the point of active DNA synthesis (replication fork). The fact that

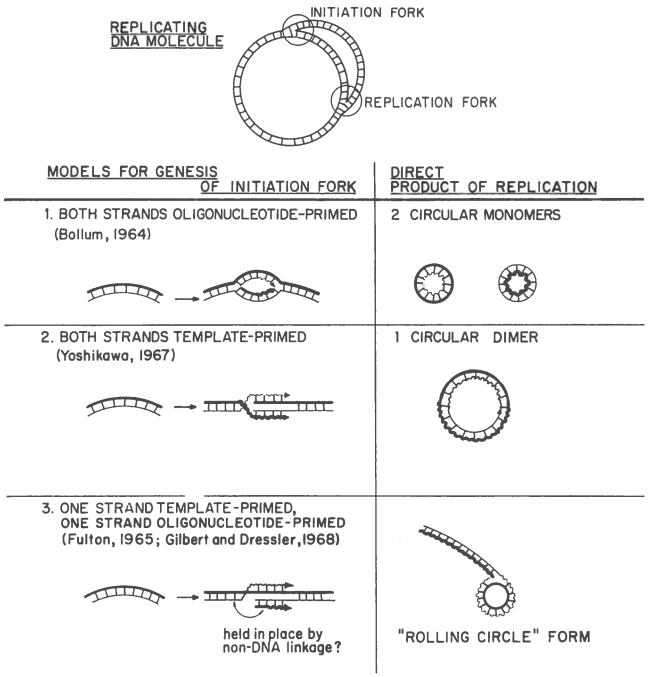


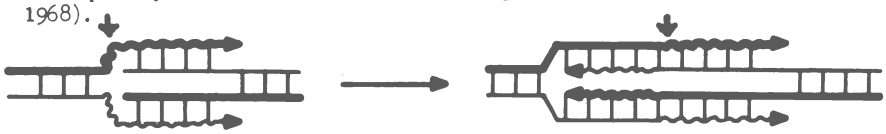
FIG. 7. Possible models for genesis of the initiation fork observed in replicating DNA molecules. The structures on the right represent the expected products of a complete round of DNA replication.

a second point of union is also observed (the "initiation fork" in Fig. 7) must tell us something about the mechanism of initiation and termination of each replication cycle. In Fig. 7, three possible types of models for initiation of DNA replication on a circular double-stranded template are schematically illustrated. Because none of the known DNA polymerases appear to be able to initiate DNA chains de novo (reviewed in 62), these models are designated as: *1*) "both strands oligonucleotide-primed" (6, 33); *2*) "both strands template-primed" (83); and *3*) "one strand template-primed plus one strand oligonucleotide-primed" (28, 29), respectively. However, it would make no difference to the considerations which follow if strands designated as oligonucleotide-primed in Fig. 7 were actually initiated de novo. Likewise, although for convenience figures are drawn as though the mechanism of polymerization on both template strandsis continuous, polymerization could be discontinuous on one or both template strands (58) without invalidating this discussion.

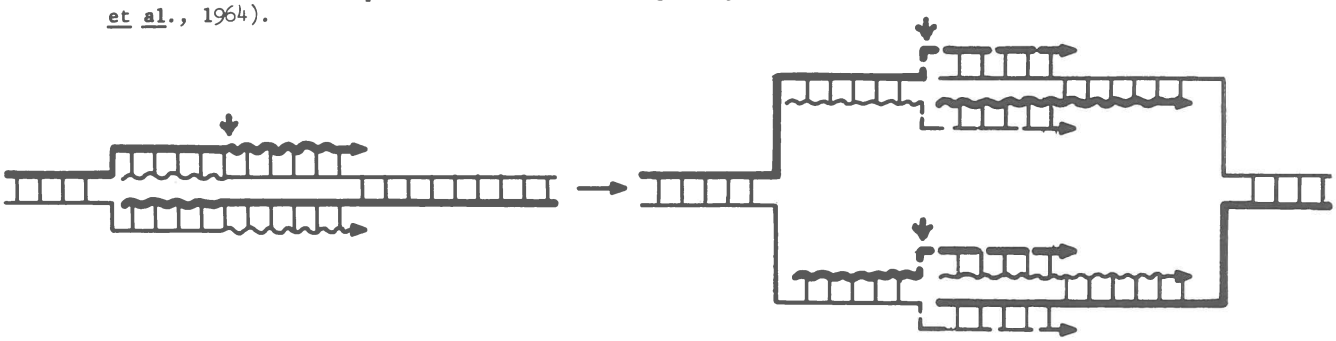
As indicated in Fig. 7, only *models 1* and *2* satisfactorily account for the existence of an initiation fork consisting solely of DNA. *Model 3* can therefore be tentatively eliminated. Of the remaining possibilities, only the template-primed model can account for the single strands of greater than one genome length observed during bacteriophage DNA replication (17, 24, 68). For this reason, and because the consequences of this manner of initiation are both unusual and interesting, *model 2* will be explored in greater detail.

A template-primed model for DNA initiation was ori-

1) A wave of DNA synthesis will propagate in both directions from the origin if the initiation fork is subsequently used as a second site for replication ("bidirectional replication", Huberman and Riggs, 1968).



2) Limited nucleotide addition at the initiation fork regenerates the original initiator region and allows reinitiation of DNA replication before finishing the preceeding round ("dichotomous replication", Sueoka et al., 1964).



3) Wheras a single replication origin per circular DNA of length N generates a dimer (circle of length 2N), two separate origins per circular DNA give back separate monomers.

FIG. 8. Several possible consequences of a mechanism of initiation of DNA replication in which both strands are template-primed. Note that the net result of two closely spaced, template-primed initiations per genome (after DNA ligase action (62)) is an initiation fork identical to that produced by *model 1* in Fig. 7.

ginally suggested by Yoshikawa on the basis of physical linkage detected between daughter and parental DNA strands in *Bacillus subtilis* (83). As Yoshikawa pointed out, one complete round of replication yields a circular dimer with half old and half new strands of double their original length (Fig. 7). The necessity of segregating only monomeric products to the daughter cells can be solved in at least two ways. One possibility is that monomers are regenerated at the end of each replication cycle by site-specific reciprocal recombination (21, 77) across the circular dimer to yield two separate circular monomers. This mechanism of segregation could also yield the concatameric DNA forms observed by Vinograd and his collaborators (39). Another possible mechanism for segregation is to initiate DNA replication by template priming at *two* separate locations on each monomeric circular genome, as illustrated at the bottom of Fig. 8. The duplicate replication origins could be so closely spaced as to be indistinguishable in most experiments designed to detect them; nevertheless, their presence would result in the formation of two circular monomers instead of a circular dimer after completion of a round of replication.

The above property of template-primed models means that any breakdown in the normal segregation mechanism can indefinitely perpetuate the circular dimer form of a DNA genome, since this form will contain two identical replication origins per DNA circle. It is striking that the circular dimer form of DNA does in fact predominate in mitochondria under special conditions (13, 54; see also 32, 64).

With the minor variations shown in Fig. 8, a template-primed model readily allows for both "bidirectional" (38) and "dichotomous" (57) modes of DNA replication. Dichotomous replication in this model is equivalent to simultaneous reinitiation at both replication origins of a circular dimer; therefore, the direct products of dichotomous replications (in the absence of segregation) will always be circular dimers.

One of the major problems with template priming on both DNA strands is that initiation of replication might appear to necessitate a transient double-strand breakage. In this case, a special non-DNA linkage would be needed to keep the broken ends together until replication begins. This difficulty can be avoided by insisting on a staggered start for replication on the two DNA strands. However, beginning replication in this way is inconsistent with the known semiconservative mode of DNA replication (52), *unless a displacement reaction between old and new DNA strands*

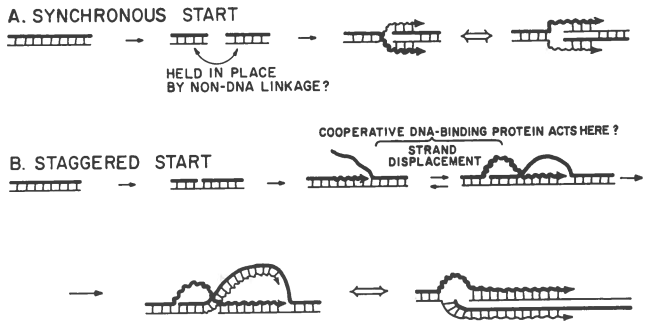


FIG. 9. Possible models for template-priming of initiation of DNA replication. *A:* synchronous starting necessitates a double-strand break at the origin and is therefore unlikely. *B:* a staggered start avoids double-strand breakage. It requires a strand displacement before initiation can begin on the second template strand. Since DNA replication (as opposed to DNA repair) is triggered only when polymerization begins on *both* template strands, replication could be regulated through control of the strand-exchange reaction shown.

occurs between initiations on the first and second template strands. The most satisfactory model of this type is schematically diagramed in Fig. 9. It is important to note that the type of strand displacement reaction shown clearly can occur inside of cells, since it is one of the basic reactions required to explain genetic recombination (see Fig. 5).

According to the template primed model just discussed, DNA replication might be controlled in bacteria by an initiator protein which binds cooperatively to DNA near the origin of replication, as postulated in the previous section. One possibility is that in bacteria a sequence-specific DNA-binding protein is made which catalyzes strand exchange only at the origin, as indicated in Fig. 9 *B*. Unlike bacteriophage systems, in bacteria this function could probably not be accomplished by the same protein that catalyzes general genetic recombination, since rigid control of initiation must be maintained. This hypothesis is readily testable, since *E. coli* initiator protein should be among the proteins binding to *E. coli* DNA-cellulose columns.

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