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### **Title**

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PROTEOLYTIC REMOVAL OF THE CARBOXYL TERMINUS OF THE  
T4 GENE 32 HELIX-DESTABILIZING PROTEIN ALTERS THE  
T4 IN VITRO REPLICATION COMPLEX\*

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## SUMMARY

The proteolytic removal of about 60 amino acids from the carboxyl terminus of the bacteriophage T4 helix-destabilizing protein (gene 32 protein) produces 32\*I, a 27,000 dalton fragment which still binds tightly and cooperatively to single-stranded DNA. The substitution of 32\*I protein for intact 32 protein in the seven-protein T4 replication complex results in dramatic changes in some of the reactions catalyzed by this in vitro DNA replication system, while leaving others largely unperturbed:

1) Like intact 32 protein, the 32\*I protein promotes DNA synthesis by the DNA polymerase when the T4 polymerase accessory proteins (gene 44/62 and 45 proteins) are also present. Consistent with its increased DNA affinity, the 32\*I protein is active even at low helix-destabilizing protein concentrations where the intact 32 protein is ineffective. The host helix-destabilizing protein (E. coli ssb protein) cannot replace the 32\*I protein for this synthesis.

2) Unlike intact 32 protein, 32\*I protein strongly inhibits DNA synthesis catalyzed by the T4 DNA polymerase alone on a primed single-stranded DNA template.

3) Unlike intact 32 protein, the 32\*I protein strongly inhibits RNA primer synthesis catalyzed by the T4 gene 41 and 61 proteins and also reduces the efficiency of RNA primer utilization. As a result, de novo DNA chain starts are completely blocked in the complete T4 replication system, and no lagging strand DNA synthesis occurs.

4) The 32\*I protein does not bind to either the T4 DNA polymerase or to the T4 gene 61 protein in the absence of DNA; these associations

(detected with intact 32 protein) would therefore appear to be essential for the normal control of 32 protein activity, and to account at least in part for observations 2) and 3), above.

We propose that the carboxyl-terminal domain of intact 32 protein functions to guide its interactions with the T4 DNA polymerase and the T4 gene 61 RNA priming protein. When this domain is removed, as in 32\*I protein, the helix-destabilization induced by the protein is inadequately controlled, so that polymerizing enzymes tend to be displaced from the growing 3'OH end of a polynucleotide chain and are thereby inhibited.

In vitro DNA synthesis is efficiently catalyzed by a multienzyme complex composed of seven highly purified proteins encoded by bacteriophage T4 (1,2). The replication complex reconstructed from these proteins closely resembles that formed in vivo. For example, the isolation of mutants in each of these proteins with major defects in T4 DNA synthesis indicates that each of these proteins has a central role in DNA replication (3,4). Moreover, these proteins coordinately function in vitro to propagate a replication fork on duplex DNA templates at a rate (2,5) similar to that measured in vivo (6), while maintaining an extremely high fidelity of correct base insertion (7,8). Finally, Okazaki fragments appear to be initiated in vitro by the same pentaribonucleotide primers which serve this role in vivo<sup>1</sup> (9,10,11,12).

In this report, we focus on the central function of the gene 32 protein, the T4 helix-destabilizing protein (H-D protein)<sup>2</sup> in this replication complex. The 32 protein binds tightly and cooperatively to single-stranded DNA, thus perturbing the helix-coil equilibrium (13). Apparently similar proteins are known to serve an essential role in other replication systems including those of E. coli (14,15), T7 bacteriophage (16), fd bacteriophage (17,18), and adenovirus (19; for a recent review of H-D proteins see 20). Compared to the other essential components of the T4 replication apparatus, the 32 protein is required in unusually high concentrations, reflecting its structural rather than enzymatic role in replication (13).

By controlled proteolysis, modified 32 protein molecules which lack discrete portions of either the carboxyl terminus or the amino terminus (or both) can be obtained (21,22). Each of these large fragments of



32 protein has uniquely altered DNA binding properties compared to the intact protein (21-25). The removal of an acidic region (the A peptide), composed of approximately the first 60 amino acids at the carboxyl terminus of 32 protein, produces an "activated" 32 protein designated as 32\*I. The affinity of 32\*I protein for single-stranded DNA is 2 to 4-fold greater than that of the intact H-D protein (26). More strikingly, the midpoint of the helix-coil transition ( $T_m$ ) for double-helical T4 DNA is reduced by 70°C in the presence of 32\*I protein (in 10 mM  $\text{Na}^{+2}$ ) (27), whereas intact 32 protein does not melt the T4 DNA duplex under the same conditions due to a kinetic (rather than a thermodynamic) block to the denaturation (28).

In this report, we examine how the substitution of 32\*I for intact 32 protein in the T4 multienzyme complex alters the normal replication reactions. We find that the T4 DNA polymerase can no longer utilize a primed, single-stranded DNA template in the presence of 32\*I protein. This inhibition is alleviated by the addition of a complex of three proteins called T4 polymerase accessory proteins (the 44/62 plus 45 proteins). The presence of 32\*I protein also strongly inhibits the de novo initiation of DNA chains, which requires the synthesis and the utilization of the RNA primers made by the T4 gene 41 and 61 proteins on single-stranded DNA templates. As a result, only one of the two strands of a double-stranded DNA template (the leading strand template at the fork) is copied in the presence of 32\*I protein, even with all seven T4 replication proteins present. This modified T4 H-D protein still appears to be recognized in a specific manner by some of the T4 replication proteins, since replacing the 32\*I protein by the E. coli H-D protein results in an almost complete suppression of all DNA synthesis by the T4 in vitro system.

## MATERIALS AND METHODS

Intact DNA Templates - The DNAs from bacteriophages PM-2, T4, T7,  $\phi$ x 174 and fd were isolated by phenol extraction after the respective virions were purified by equilibrium sedimentation in CsCl (29). The T7 DNA isolated contained about one randomly located nick per genome. The DNA from bacteriophage  $\lambda$  was the generous gift of Dr S. Mickel. Double-stranded, circular  $\phi$ x 174 RF was isolated from intracellular viral DNA by standard procedures (30).

Modified DNA Templates - Singly nicked, circular bacteriophage PM-2 DNA was prepared by a limited DNase I digestion in the presence of ethidium bromide at a concentration of approximately 1 mole dye per mole DNA base-pair (31). Exonuclease III digestion of bacteriophage  $\lambda$  DNA was monitored by following the hyperchromicity at 260 nm and was terminated by heating the sample for 10 min at 65°C, when 14% of the DNA had been degraded.

A  $\phi$ x 174 partial duplex was prepared by hybridization of the Hae III Z2 duplex restriction fragment (1071 base pairs (32)), with a 10-fold molar excess of single-stranded virion DNA. The restriction fragment had been isolated by preparative electrophoresis on a polyacrylamide slab gel followed by electroelution of the appropriate band. Following hybridization, the primed DNA circle was purified by hydroxylapatite chromatography (BioRad DNA grade) (33), followed by sucrose gradient sedimentation in the presence of 1 M NaCl.

Preparation of 32\*I - The 32\*I proteolytic cleavage fragment was prepared by a modification of the procedure of Hosoda and Moise (21). When cooperatively bound to single-stranded DNA, the carboxyl terminal (A) region of 32 protein is readily digestible by proteases, whereas the amino terminal (B) region is protected (21,22). Therefore, 32\*I was obtained efficiently by direct proteolysis of 32 protein bound to single-stranded DNA cellulose. A cleared lysate fraction, prepared as described (21) from 50 g of E. coli infected with the T4 double mutant amN134 (gene 33<sup>-</sup>)- amBL292 (gene 55<sup>-</sup>), was loaded onto a single-stranded DNA cellulose column (2.8 x 16 cm) at 4°C in BII buffer (20 mM Tris-HCl, pH 8.1; 1 mM Na<sub>3</sub>EDTA, 1 mM β-mercaptoethanol, 10% (w/v) glycerol and 0.1 M NaCl). The column was washed with successive steps of BII buffer containing 0.2, 0.4, and 0.6 M NaCl to elute contaminating DNA binding proteins, as well as noncooperatively bound 32 protein. The column was then washed with BII buffer containing 0.1 M NaCl. The DNA-bound 32 protein was treated with chymotrypsin for 4 hr by washing the column at a flow rate of 70 ml/h with this buffer containing 2 µg/ml chymotrypsin (CHY-5, 37 U/mg, Sigma). The column was then eluted with successive steps of BII buffer containing 1 mM L-1 tosylamide-2 phenyl ethylchloromethyl ketone and 0.1 M, 0.2 M, 0.4 M, 0.6 M and 2.0 M NaCl. Chymotrypsin emerged in the 0.2 M salt step, and a second modified 32 protein (32\*III), produced by the cleavage of both the carboxyl and amino termini, eluted in the 0.4 and 0.6 M salt washes. Forty-five mg of 32\*I protein was obtained from the 2.0 M, high salt wash. This fraction was purified further by DEAE cellulose chromatography as described (21), and the peak fraction eluting at 0.18 M NaCl and containing 80% of the applied protein was used after concentration and dialysis against storage buffer. Trailing fractions from the main peak had detectable nuclease activity

and, therefore, were not used. The [ $^3\text{H}$ ]32\*I protein was prepared from a 250 ml culture of T4-infected E. coli cells grown in the presence of [ $^3\text{H}$ ] mixed amino acids (Schwarz-Mann). The labeled 32 protein obtained from an initial single-stranded DNA cellulose column was combined with nonradioactive 32 protein (2 mg) as carrier and reapplied to a second DNA cellulose column for proteolysis as described above.

Enzymes - The T4 replication proteins, corresponding to the products of genes 43, 41, 44/62 and 45, were purified in this laboratory to greater than 90% homogeneity, as described elsewhere (34,35). The T4 gene 61 product was purified to greater than 50% homogeneity by a new procedure.<sup>3</sup> The gene 32 protein, isolated as "32-PS" according to Bittner et al. (36), was greater than 99% homogeneous as determined by SDS polyacrylamide gel electrophoresis.

None of the replication protein preparations used had contaminating endo or exodeoxyribonuclease activities detectable under the conditions of the DNA synthesis assay (see refs. 34 and 36 for nuclease assay conditions). Nuclease free bovine serum albumin was prepared as described (36) for use in enzyme dilution buffers. Pancreatic DNase I and bacterial alkaline phosphatase were both purchased from Worthington, and exonuclease III was obtained from New England Biolabs. The E. coli H-D protein was the generous gift of Dr A. Kornberg.

DNA Synthesis Assay - The complete seven-protein DNA replication reaction contained the purified T4 replication proteins 43 (2  $\mu\text{g}/\text{ml}$ ), 45 (20  $\mu\text{g}/\text{ml}$ ), 44/62 complex (20  $\mu\text{g}/\text{ml}$ ), 41 (15  $\mu\text{g}/\text{ml}$ ), 61 (0.20  $\mu\text{g}/\text{ml}$ ), and 32 or 32\*I or both (200  $\mu\text{g}/\text{ml}$  unless otherwise specified); ribo and deoxyribonucleoside triphosphates (0.5 mM rATP; 0.2 mM each of rCTP, rGTP and rUTP; 0.167 mM each of dATP, dGTP, dCTP and [ $^3\text{H}$ ]dTTP at a final

specific activity of 100-200 cpm/pmole in the reaction mix); DNA template (1 to 13  $\mu\text{g/ml}$  of a particular DNA, as noted in the figure legends); and DNA synthesis buffer (67 mM potassium acetate, 33 mM Tris acetate, pH 7.8, 10 mM magnesium acetate and 0.5 mM DTT). To study partial reactions catalyzed by subsets of the replication enzymes, specific proteins were deleted as indicated in the figure legends. The replication reactions were incubated at the given temperature and terminated by TCA precipitation onto glass fiber filters. The radiolabeled, acid-insoluble product was then quantitated by standard liquid scintillation counting techniques.

Although 32\*I protein is a stronger helix-destabilizing protein than the intact 32 protein, the helix-coil transition induced by 32\*I protein is suppressed at moderate  $\text{Mg}^{+2}$  or  $\text{K}^{+}$  concentrations (21). Under the ionic conditions used for the DNA synthesis assays (10 mM  $\text{Mg}^{+2}$  and 67 mM  $\text{K}^{+}$ ), the duplex conformation of the natural DNA templates used here remains stable at 30°C even in the presence of high concentrations of 32\*I protein. Nevertheless, when partially single-stranded templates were employed, the reactions were carried out at 24°C to minimize destabilization of the base-paired 3'-OH primer termini. The 32\*I protein undergoes a thermally induced conformational change detectable at 45°C, in contrast to the intact protein where denaturation is not detected at temperatures below 51°C (21). However, the 32\*I protein is quite stable at 30°C or below.

RNA Primer Assay - The oligoribonucleotide synthesis assay was performed as described elsewhere (1,11). The reaction mixture contained single-stranded fd DNA (8  $\mu\text{g/ml}$ ), 41 protein (30  $\mu\text{g/ml}$ ), 61 protein

(0.1  $\mu\text{g/ml}$ ), ribonucleoside triphosphates (rCTP, rGTP, and rUTP each at 200  $\mu\text{M}$  and [ $^3\text{H}$ ]rATP at a concentration of 200  $\mu\text{M}$  and a specific activity of 1000 cpm/pmol), and DNA synthesis assay buffer. When added, 32 protein or  $^{32}\text{I}$  protein was used at 100  $\mu\text{g/ml}$ . The reaction was incubated at 30°C, and aliquots were removed and spotted onto DEAE paper (Whatman DE-81) and washed as described (37). The filters were dried and counted in a toluene-based liquid scintillation cocktail.

Chemicals - Enzyme grade sucrose was obtained from Schwartz-Mann. Ribo- and deoxyribonucleotides were purchased from Sigma, and the radiolabeled nucleotides were purchased from Amersham.

Electron Microscopy - The reaction products were spread by a modified Kleinsmidt technique directly from a formamide hypophase onto a carbon-coated copper grid without prior deproteinization, as previously described (38). After shadowing with platinum, these grids were examined in a Philips EM-300 electron microscope.

## RESULTS

Seven highly purified T4 bacteriophage encoded proteins constitute the present in vitro T4 DNA replication system: the helix-destabilizing protein (gene 32 protein), the DNA polymerase (the 43 gene protein), the polymerase accessory proteins (45 protein plus a tight complex of 44 and 62 proteins), and the RNA priming proteins (41 protein and 61 protein). The properties and enzymatic activities of all of these proteins have been reviewed elsewhere (1,2).

The product made by this seven-protein replication system using a nicked, double-stranded DNA as template is primarily double-stranded DNA (5,36,39). The synthesis can be considered as the sum of two half-reactions, corresponding to leading-strand and lagging-strand DNA syntheses:

(i) Leading-strand DNA synthesis initiates at a nick (or gap) in the template DNA. Subsequent polymerization ensues in the 5' to 3' direction, with concomitant displacement of the parental template strand. This synthesis occurs in the absence of the RNA priming proteins, but requires the five other T4 replication proteins (43, 32, 44/62 and 45), as well as ATP hydrolysis (1,40). This "five-protein" reaction proceeds at near physiological salt concentrations and maintains the proper replication fork geometry (although no lagging strand DNA synthesis occurs).

(ii) Lagging-strand DNA synthesis requires de novo chain initiation on the single-stranded parental DNA template strand displaced by leading-strand synthesis. The T4 gene 41 and 61 proteins synthesize

short RNA primers on this template (1,2,11,12), which are used by the DNA polymerase to start the Okazaki fragments of DNA made on the lagging strand. The additional enzymes required to seal Okazaki fragments are not included in the seven-protein T4 system, and therefore the lagging strand DNA product remains nicked or gapped after its synthesis.

#### Addition of $^{32}\text{I}$ Protein Uncouples Leading and Lagging-Strand

Synthesis - The kinetics of DNA synthesis observed for a normal five-protein reaction are shown in Fig. 1, panel a. Here the template is double-stranded, circular PM2 DNA which has been randomly nicked once per circle (see Methods). By using such a template, initiation is limited to one event per molecule. Replication proceeds in a "rolling circle" mode to generate single-stranded tails which are much longer than unit length (5). The addition of the RNA priming proteins (41 protein and 61 protein) to this five-protein reaction stimulates incorporation approximately two fold, even without the ribonucleotide substrates rGTP, rCTP, and rUTP. This primer-independent stimulation requires only the presence of 41 protein and its rGTP (or rATP) hydrolysis (2). It arises from an acceleration of the rate at which a fraction of the replication forks synthesize DNA on the leading strand, and it is thought to be due to the action of the 41 protein as a DNA helicase (2).<sup>4</sup> As shown here, the addition of all four ribonucleoside triphosphates to the seven-protein reaction yields about a further 2-fold increase in DNA synthesis, consistent with concurrent de novo primed lagging-strand DNA synthesis in the reaction (Fig. 1a).

When  $^{32}\text{I}$  replaces 32 protein, the resulting "five-protein/ $^{32}\text{I}$ " reaction on singly-nicked PM2 DNA is slightly more efficient than



that obtained with intact 32 protein (Fig. 1b). As shown in Table I, 32\*I protein stimulates this five-protein reaction at a lower H-D protein concentration than does the normal 32 protein. For example, when the H-D protein concentration is reduced to 50  $\mu\text{g/ml}$ , the five-protein/32\*I reaction yields 3-4 fold more synthesis than obtained in the five-protein/32 reaction. Conversely, very high concentrations of 32\*I protein are somewhat less effective than a comparable amount of 32 protein.

In marked contrast to the normal five protein reaction, the five-protein/32\*I reaction is not stimulated by the addition of 4l protein, or even by the addition of both RNA priming proteins (4l and 6l) and all four rNTPs (Fig. 1b). This results suggests that 32\*I protein interferes with both the normal de novo initiation of Okazaki fragment synthesis on the lagging strand, and with the 4l protein-induced acceleration of the leading-strand synthesis rate at a replication fork.

Electron microscopic examination of the reaction products supports this interpretation. As expected, the products of the five protein reaction employing either 32 protein or 32\*I protein appear to be the same, a double-stranded DNA circle with a long single-stranded tail (data not shown). As previously observed (5,38), the product of the normal seven protein reaction (with 32 protein present) is a rolling circle with a predominantly double-stranded tail, linked by a single-stranded connection between the circle and the tail (shown in Fig. 2, panel a). Additional single-stranded regions are sometimes observed both in the middle and, more frequently, at the end of the tail. In contrast, the product of the seven-protein reaction employing 32\*I protein is a rolling circle with only a long single-stranded tail (Fig. 2, panel b). Double-stranded tails resembling those shown in panel a were not detected.

The DNA Synthesis Catalyzed by only the 43 and 32 Proteins is Blocked by 32\*I Protein - The 43 protein (DNA polymerase), when supplemented by only the 32 protein, promotes very limited of synthesis on a nicked, double-stranded DNA template, as shown in Fig. 3. Nossal (41) has shown that the product of this reaction is similar to that obtained with the E. coli DNA polymerase I, being richer in A-T residues than the template and containing rapidly renaturable regions indicative of both template strand switching and slippage by the T4 polymerase. The presumed role of 32 protein in this reaction is to facilitate helix penetration, and a similar role may be served alternatively by the 44/62 plus 45 protein complex (Fig. 3). But the incorporation obtained with all five proteins present can be seen to be very much greater than the sum of the two partial reactions (also see refs. 5 and 42).

Since the 32\*I protein is a more effective destabilizer of the DNA double helix than is intact 32 protein (21,27), we anticipated that DNA synthesis catalyzed by only the 43 and 32\*I proteins on a nicked, double-stranded template might be unusually efficient. However, as shown in Fig. 3, no DNA synthesis is obtained in the presence of only these two proteins. This difference between the 32\*I protein and intact 32 protein is abolished once the polymerase accessory proteins (44/62 and 45 proteins) are added to the reaction, since the amount of synthesis obtained in the five-protein/32\*I reaction is, if anything, somewhat greater than that obtained with intact 32 protein (Fig. 3; see also Fig. 1).

The 32\*I Protein also Inhibits DNA Polymerase Travel on Single-Stranded DNA Templates - When provided with a base-paired 3'OH primer terminus, the T4 DNA polymerase catalyzes synthesis on single-stranded

DNA templates, in a reaction which provides a model for lagging strand DNA synthesis. As shown in Fig. 4a, when exonuclease III eroded bacteriophage  $\lambda$  DNA serves as such a DNA template, the synthesis catalyzed by 43 protein supplemented with 32 protein is initially more rapid and produces more product than the reaction catalyzed by 43 protein alone. However, the substitution of 32\*I protein for intact 32 protein abolishes all of the incorporation catalyzed by 43 protein alone, analogous to the effect obtained on duplex DNA templates (see Fig. 3 above). The reaction is maximally inhibited when the concentration of 32\*I protein is sufficient to coat all of the available single-stranded DNA, and reducing the 32\*I protein concentration to half this level allows a limited extent of synthesis. Some incorporation is also observed if the polymerase and DNA template are first preincubated together in the absence of 32\*I protein and nucleotide substrates, such a preincubation has no effect on the control reaction employing intact 32 protein.

In a similar experiment, a restriction fragment-primed, single-stranded DNA circle ( $\phi$ x 174 DNA primed by the Hae III Z2 fragment) was used as the template (Fig. 4b). On this template, the DNA polymerase alone synthesizes a product equivalent to copying 20% of the available single-stranded region. While the DNA polymerase is again strongly inhibited when 32\*I protein is added, the addition of the polymerase accessory proteins is seen to remove this inhibition completely. When the reaction products made after 25 min of incubation were sized by alkaline agarose gel electrophoresis (using fluorography to permit photographic detection), full genome sized linear product strands were observed only in such five-protein reactions employing either 32 or 32\*I proteins. The partial reactions employing polymerase alone (or polymerase supplemented with

32 protein or with 44/62 and 45 proteins) resulted in products of approximately one third and one half genome length, respectively.

In summary, 32\*I protein can replace 32 protein when the polymerase functions with its accessory proteins on a pre-primed 3'OH end. However, in the absence of the 44/62 and 45 proteins, the DNA polymerase is strongly inhibited by the 32\*I protein.

The 32\*I Protein Blocks the de novo Initiation of DNA Chains on Single-Stranded DNA Templates - Replication on a single-stranded circular DNA template requires de novo priming. Thus, in the T4 in vitro replication system, all seven purified replication proteins and ribonucleotide (and deoxyribonucleotide) substrates are essential to observe replication on an fd DNA template. The eventual product of the reaction is the same as that obtained with a singly nicked duplex circular template - a double-stranded circle with a long tail containing alternating single-stranded and double-stranded regions (38). The synthesis is extremely efficient; for example, in the reaction shown in Fig. 5, approximately 7 copies of the template were obtained in 9 min at 30°C. However, when 32\*I was substituted for 32 protein, all synthesis was abolished. This inhibition is competitive, as shown by the gradual increase in synthesis as the ratio of 32 to 32\*I protein in the reaction mix is increased (Fig. 5). With an equal concentration of the two forms of 32 protein present, 80% inhibition is seen, and the block is completely relieved only when the ratio of 32 to 32\*I reaches 4:1.

The observed inhibition of de novo initiations could arise because DNA bound 32\*I protein (i) blocks the initial polymerization event which

makes RNA primers or (ii) blocks primer utilization by destabilizing the DNA-RNA oligomer helix. To select between these possibilities, the synthesis of RNA oligomers was examined directly. The production of uniquely sized ribopentanucleotides can be detected in a reaction requiring only 41 protein, 61 protein and single-stranded circular DNA (1). As shown in Fig. 6, this synthesis is only slightly decreased by the addition of 32 protein, while RNA primer synthesis is inhibited 3 to 4 fold by the presence of 32\*I protein. The fact that a much greater than 4-fold inhibition of the seven-protein/32\*I reaction was observed on the fd DNA template (Fig. 5) suggests that primer utilization is also reduced by the 32\*I protein.

The Structure of Single-Stranded DNA Complexes Formed with 32 and 32\*I Proteins - To examine the DNA-protein complexes formed at the salt concentrations used in the replication reaction, single-stranded circular  $\phi$ x174 DNA was incubated with either an excess of 32 protein or an excess of 32\*I protein, or with a mixture containing equal concentrations of both proteins. The composition of the DNA complexes formed was analyzed following sedimentation through sucrose gradients. As shown in Fig. 7b, the DNA protein complex formed in the presence of both 32 and 32\*I proteins contains 5 times more 32\*I than 32 protein, consistent with the increased DNA binding affinity of 32\*I protein. Note that the fully saturated 32\*I protein- $\phi$ x DNA complex sediments faster than the corresponding fully saturated 32 protein-DNA complex (Fig. 7a). Since the binding stoichiometries are approximately the same (24-26), the 32 I\* complex would appear to be more compact. The intermediate sedimentation

rate of the mixed complex in Fig. 7c reveals that each  $\phi$ x DNA molecule contains both types of protein. However, previous results suggest that these two proteins are unlikely to be homogeneously intermixed on the DNA (43).

43 Protein Has a Reduced Affinity for 32\*I Protein - It has been shown in several instances that a specific H-D protein stimulates its homologous DNA polymerase (14,16,20,44). Moreover, a direct association of the 32 protein with the T4 gene 43 polymerase has been shown by cosedimentation of the two proteins through sucrose gradients (44). To test whether the removal of the A peptide from 32 protein alters its intrinsic affinity for 43 protein, a mixture of the DNA polymerase and either intact 32 protein or 32\*I protein was sedimented through sucrose gradients. As shown in Fig. 8, the T4 DNA polymerase alone sediments as a sharp peak which moves more slowly than an alkaline phosphatase marker (Fig. 8a), while both the intact 32 protein (Fig. 8c) and the 32\*I protein (Fig. 8b) self-associate and therefore sediment heterogeneously across a broad region of the gradient. The sedimentation rate of the DNA polymerase is increased dramatically in the presence of 32 protein, and it now cosediments with the bulk of the 32 protein oligomeric complex (Fig. 8c and d). However, when co-sedimented with 32\*I protein, the DNA polymerase sediments at a rate which is indistinguishable from the rate measured in the absence of 32 protein (compare Figs. 8a and 8b). Thus, the removal of the carboxyl terminus from 32 protein has reduced its affinity for the 43 protein to less than  $10^4 M^{-1}$ , the limit of detection by this assay (45).

The E. coli H-D Protein Will Not Substitute for the 32 Protein - Is it possible that the 32\*I protein has lost the capacity to interact specifically with any of the T4 replication proteins, and that its activities in these assays arise solely from its single-strand DNA binding? To test this possibility, we examined the ability of the host E. coli H-D protein (ssb protein) to function in the T4 DNA synthesis reactions.

As previously demonstrated (14), on a primed single-stranded DNA template, the E. coli H-D protein itself neither stimulates nor inhibits the T4 DNA polymerase. As shown in Table II, when the E. coli H-D protein is substituted for 32 protein in a five-protein reaction on a double-stranded DNA template (a reaction dependent upon the presence of 32 protein), very little DNA synthesis is observed. Yet, when both intact 32 protein and E. coli H-D protein are present in equivalent concentrations, the reaction proceeds as if no E. coli protein were added (Table II); thus the E. coli H-D protein neither functions itself, nor competes with 32 protein in the T4 replication complex. Also shown in Table II is the fact that the E. coli H-D protein will not support DNA synthesis by the T4 system on anfd DNA template. (In fact, the addition of the E. coli H-D protein inhibits the limited, de novo primed DNA synthesis normally obtained without 32 protein present). However, once again the E. coli protein is no longer inhibitory once 32 protein is added.

All of the data on the E. coli H-D protein is thus mutually consistent, revealing that this protein is completely without activity (either

positive or negative) once all of the components of the T4 replication apparatus are present. This observation is of course relevant to the fact that T4 DNA replication normally takes place in a cell which already contains the E. coli H-D protein.

#### DISCUSSION

The T4 gene 32 protein seems to serve two interrelated roles in DNA replication: a functional role involving DNA helix destabilization and a structural role in which the 32 protein-coated DNA serves as the foundation for the assembly of replication enzyme complexes (46). It has been suggested that 32 protein has two protruding regions -- domain A at the carboxyl terminus and domain B at the amino terminus -- which are involved in 32 protein interactions with itself and with other replication proteins, and that such interactions are important in determining the over-all activities of the replication complex (21 23). In this paper, we have shown that substitution of 32 protein by 32\*I protein, a chymotryptic cleavage product lacking the A domain, modifies the replication complex so that it retains some activities while losing others. Before presenting our model for the roles of the A domain in DNA replication, we will first briefly survey some previous data concerning the alterations in 32 protein caused by removal of the A (or B) domain.



The Structure of the 32 Protein Terminal Domains - Chymotrypsin

cleavage of 32 protein releases an intact A domain peptide 54 amino acid residues long (47).<sup>5</sup> Sequence analysis of this A-peptide (47)<sup>5</sup> and the entire 32 protein<sup>6</sup> show that the most carboxyl-terminal 26-29 residues of the A domain are extremely acidic and hydrophilic (containing seven to eleven acidic amino acids, eight to nine serines, and no basic residues), while the remaining 25 or more amino acids are less acidic and more hydrophobic. At the amino terminus, the B domain can be cleaved off as a basic region of 21 amino acid residues (47-48).<sup>5,6</sup>

The Role of the A and B Domains in 32 Protein-DNA Interactions - The

32 protein can bind to a single-stranded DNA lattice either in an isolated site mode (i.e., as an individual molecule) or cooperatively in a contiguous site mode. The B domain, but not the A domain, appears to be essential for cooperative interactions between contiguously bound 32 protein molecules, because proteolytic cleavage products lacking this region (either 32\*II (minus B) or 32\*III (minus A and B)) neither self-associate<sup>7</sup> nor bind cooperatively to polynucleotides (24,25).

Although the A domain is not required for cooperative interactions, its conformation changes upon cooperative DNA binding, since it becomes more readily digestible by chymotrypsin (21,22), as well as newly susceptible to staphylococcal protease cleavage at three discrete sites (47).<sup>7</sup>

Although their cooperative DNA interactions are very similar, the 32\*I DNA complex is more compact than the 32 protein complex, as indicated both by our sedimentation studies (Fig. 8) and by electron microscopic length measurements (where the average base-base distances are 2.9Å and

3.9Å, respectively. Since these two proteins appear to cover the same size site upon DNA binding (24-26), these results suggest that the two DNA-protein complexes may have different helical pitches. Circular dichroism measurements also indicate that the polynucleotide conformation in the two complexes is similar, but not identical (27).

When an excess of intact 32 and 32\*I proteins compete for binding sites on a single-stranded DNA molecule, the resulting DNA complex contains a 1:5 ratio of 32 to 32\*I proteins (Fig. 8). Consistent with this result, Newport et al. find that the association constants of 32\*I protein for polynucleotides are 2-4 fold greater than observed for 32 protein (26). Likewise, Hosoda and coworkers have shown that 32\*I protein induces denaturation of poly [d(AT)] at lower temperatures than does intact 32 protein (27). This reduction in melting temperature is quantitatively accounted for by the increased binding constant of 32\*I protein relative to 32 protein.

#### 32 Protein: Active Helix-Destabilization Protein or Passive DNA

Binder? - The 32 protein-mediated melting of duplex DNA is the result of its strong cooperative and selective binding to single-stranded regions, which shifts the helix to coil thermodynamic equilibrium (13,28). Does 32 protein actively promote duplex disruption during DNA replication? Recent results indicate that the rate of chain elongation by the five protein complex on double-stranded DNA templates is directly proportional to the free 32 protein concentration (2). However, alone, intact 32 protein does not melt natural double-stranded DNAs, even when the DNA molecule has been gapped to provide a sufficiently long single-stranded region to

nucleate cooperative 32 protein binding (49). In marked contrast, 32\*I protein dramatically reduces the melting temperature of natural DNAs. The 2-4 fold increase in intrinsic DNA affinity of this modified 32 protein is not enough to account for its vastly increased potency for destabilization of intact double-helices. Instead, the 32\*I protein is also likely to have a greatly increased affinity for small defects in the DNA double helix.

We propose that the helix-invasion potential of 32 protein must be controlled to prevent random, widespread denaturation of intracellular DNA and that this is why such invasion is normally blocked by the A domain. However, it is tempting to speculate that a 32\*I-like activity is functional ahead of a replication fork, where special protein-protein interactions may in effect "lift up" the A domain, and thereby activate helix invasion by the intact 32 protein.

32 Protein Interactions within the Replication Complex - The position of a 32 protein molecule relative to the 3' end of the growing DNA chain must somehow determine its mode of action, and different binding properties would seem to be required at different relative positions. Ahead of the growing DNA chain, the 32 protein acts to disrupt intra-strand base pairing on the template DNA strand, thereby increasing the rate of movement of the DNA polymerase-accessory protein complex (1).<sup>9</sup> However, as the replication complex approaches, it is important for 32 protein to let other components make proper contact with the template DNA (including the primase, the DNA polymerase, the

RNA primer, and the growing 3' end of the DNA). It is this latter property which appears to be disturbed in 32\*I protein.

It has been shown that 32 protein interacts strongly with 61 protein in the absence of DNA. These two proteins cosediment during sucrose gradient sedimentation and coelute upon gel filtration chromatography. No such association was found between 32\*I protein and 61 protein.<sup>3</sup> As shown previously (44) and verified in this report (Fig. 8), intact 32 protein also associates directly with the T4 DNA polymerase (43 protein). As for the 61 protein, this association requires the presence of the A domain on 32 protein. These observations suggest that there may be a common mode of interaction between 32 protein and these two different replication proteins.

We propose that the failure of 43 protein alone to utilize a primed DNA template coated with 32\*I protein is due to a 32\*I-induced destabilization of the 3'OH primer terminus. The polymerase accessory proteins would appear to overcome this inhibition by binding to the 3'OH terminus and "clamping down" the polymerase (2,26).<sup>9</sup> It is possible that the inhibition of RNA primer synthesis by the 32\*I protein arises in a completely analogous fashion; that is, that the 32\*I protein, but not the intact 32 protein, knocks the RNA primer synthesizing enzyme (RNA primase) off of the growing 3'OH oligonucleotide end. If 61 protein is this primase, as we suspect, it is striking that the 61 protein interacts with the same domain on 32 protein as does the DNA polymerase. In both cases, such a polymerase (or primase):32 protein interaction via the A domain may serve to "tame" the 32 protein, preventing it from disturbing the base-paired 3'OH end of the polynucleotide at which the polymerizing enzyme

functions. A schematic summary of our current view of 32 protein control via interactions at its A domain is presented in Fig. 9.

As an aside, it is possible that H-D proteins in some replication systems may be designed to block DNA synthesis at the level of priming. In particular, adenovirus DNA replication proceeds via leading-strand synthesis only, lagging-strand synthesis being completely blocked (50), perhaps by the 72,000 dalton adeno-2 DNA binding protein (19).

The meaning of the observed 32 protein interactions with the remaining proteins of the T4 DNA replication complex, the polymerase accessory proteins (44/62 and 45 proteins) and the gene 41 protein, are less clear. In the presence of ATP, the accessory proteins will form a tight complex with a 32 protein-covered single-stranded DNA, but not with the same DNA without 32 protein (42). At the same time, 32 protein inhibits the stimulation of the 44/62 ATPase by single-stranded DNA (42,51). With respect to 41 protein, the marked stimulation of the five protein polymerization rate on duplex DNAs which requires 41 protein and its GTP (or ATP) hydrolysis activity (2) is prevented by either high concentrations of 32 protein or by low concentrations of 32\*I protein. It seems likely that these 32 proteins compete with 41 protein for its binding site on DNA. Further studies will be necessary before we can interpret these various observations with respect to the interactions of 32 protein within the replication complex itself.

#### Evidence for Helix Invasion by 32 Protein Ahead of the Replication

Fork - When the 32 protein actively promotes strand displacement during leading-strand synthesis at an in vitro replication fork, it must

eventually bind cooperatively to the displaced parental template strand. But does it also bind to the template strand ahead of the polymerase? If it does, then how many molecules are involved?

We have shown in this report that 32\*I protein substitution for intact 32 protein affects model reactions for both leading-strand and lagging-strand DNA synthesis in exactly the same way, greatly depressing the synthesis by DNA polymerase alone, while maintaining the same level of synthesis as the intact 32 protein when the polymerase accessory proteins are present (Figs. 3 and 4). These results suggest that the relative positions of the 32 (or 32\*I) protein, the DNA polymerase, and the 44/62 and 45 proteins are similar in both leading- and lagging-strand DNA synthesis reactions. It therefore seems likely that, as on the lagging strand, there are one or more 32 protein molecules on the leading strand template ahead of the polymerase, and thus that the parental DNA helix is unwound ahead of the replication fork for at least 10 or more base pairs.

#### ACKNOWLEDGMENTS

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## FOOTNOTES

- 1 C.-C. Liu and B.M. Alberts, J. Biol. Chem., submitted.
- 2 The abbreviations used are: H-D protein, helix-destabilizing protein; RF DNA, replicative form circular duplex DNA; SDS, sodium dodecyl sulfate; EDTA, ethylene diamine tetracetic acid; DEAE, diethyl aminoethyl; TCA, trichloroacetic acid; DTT, dithiothreitol.
- 3 R.L. Burke, C.-C., Liu, and B.M. Alberts, manuscript in preparation.
- 4 J. Barry and B.M. Alberts, manuscript in preparation.
- 5 I. Kubota, J. Hosoda, H. Moise and A. Tsugita, manuscript in preparation.
- 6 Williams, K.R., LoPresti, M.B., Setoguchi, M., and Konigsberg, W.H., Proc. Natl. Acad. Sci., USA, submitted.
- 7 H. Moise, T. Goto and J. Hosoda, manuscript in preparation.
- 8 J. Hosoda and R.C. Williams, unpublished results.
- 9 Huang, C.C., Hearst, J.E., and Alberts, B.M., J. Biol. Chem., submitted.

Table I

The efficiency of the five-protein/32\*I replication complex compared to the five-protein/32 complex as a function of the H-D protein concentration

The amount of DNA synthesized in a five-protein reaction employing the indicated concentration of either 32\*I protein or 32 protein was measured at 30°C using a double-stranded T7 DNA template at 12.5 µg/ml. Reactions were carried out as described in Methods. The amount of DNA product obtained after a 25 min incubation for the five-protein reaction with 32\*I protein concentrations of 50, 100, 200, and 300 µg/ml was 10.0, 10.3, 15.3, and 10.2 µg/ml, respectively.

Time (min)	Ratio of DNA synthesis (32*I reaction/32 Reaction)			
	Concentration of 32*I and of 32 protein (µg/ml)			
	50	100	200	300
5	4.0	1.4	1.1	0.6
10	3.8	1.6	1.0	0.9
15	2.0	1.9	0.7	0.9
25	2.8	1.5	1.5	0.7



Table II

Substitution of the *E. coli* helix-destabilizing protein for 32 protein  
in the T4 in vitro DNA replication system

Relative incorporation in a five- or seven-protein DNA synthesis reaction was measured after a 25 min incubation at 37°C. A). A double-stranded T7 DNA template was used at 6.2 µg/ml. When used alone, the 32 protein or *E. coli* H-D protein was present at 100 µg/ml; when mixed, each protein was added at 78 µg/ml. A relative incorporation of 1.0 corresponds to 3.6 copies of DNA product synthesized per template molecule, or 68 nmoles/ml. B). A single-stranded circular fd DNA template was used at 3.0 µg/ml and 32 protein or *E. coli* H-D protein (or both) were present at 78 µg/ml each. A relative incorporation of 1.0 corresponds to 3.1 copies of DNA product synthesized per template molecule, or 28 nmoles/ml.

DNA Template	Reaction	Relative DNA synthesis observed with indicated helix-destabilizing protein			
		T4	<i>E. coli</i>	T4 + <i>E. coli</i>	None
A). ds T7	five-protein	0.32	0.09	0.21	0.01
	seven-protein	(1.0)	0.09	1.1	-
B). ss fd	seven-protein	(1.0)	0.05	1.2	0.25

## FIGURE LEGENDS

Fig. 1. DNA synthesis on a singly-nicked, double-stranded circular PM-2 DNA template in the presence of either 32 or 32\*I protein. The reaction mixture (see Methods) contained PM-2 DNA as template at 39.4 nmoles/ml. The ribonucleotides rGTP, rUTP and rCTP were omitted, except in the indicated "+ r G,U,C" reactions. The 41 and 61 proteins were omitted in the five-protein (5P) reaction. The number of DNA copies synthesized per initial DNA template molecule is indicated on the right hand ordinate.

Fig. 2. Selected electron micrographs typical of the products of the complete seven-protein in vitro replication system in the presence of either a) 32 or b) 32\*I protein. The synthesis was performed as in Fig. 1 for the complete seven-protein reaction, using singly-nicked PM-2 DNA circles as template. The reaction was incubated for 5 min at 30°C and stopped by the addition of Na<sub>3</sub>EDTA to 10 mM and NaOH to 6 mM final concentrations. The DNA was spread by a modified Kleinsmidt technique (38) and viewed with a Phillips Model 300 electron microscope.

Fig. 3. DNA synthesis on a nicked, double-stranded T7 DNA template by subsets of the T4 replication proteins. The reactions were performed as described in Methods in the presence of the proteins indicated, using the T7 DNA template at 41.7 nmoles/ml.

Fig. 4. Effect of 32\*I protein on DNA synthesis on a preprimed single-stranded DNA template.

(A). DNA synthesis on an exonuclease-III-treated bacteriophage  $\lambda$  DNA template catalyzed by T4 DNA polymerase (43 protein), with or

without 32 protein or 32\*I protein. The replication reaction was carried out at 23°C for the indicated times, using 72 nmoles/ml of exoIII-treated  $\lambda$  DNA as template (corresponding to a concentration of single-stranded DNA regions of 11.5 nmoles/ml (3.8  $\mu$ g/ml)). The assay components were the same as in Methods, except that synthesis was catalyzed by subsets of the proteins as shown; in addition, the 32 and 32\*I protein concentrations were varied as indicated, with "(X1)" representing a concentration of 50  $\mu$ g/ml (about the amount needed to completely cover the DNA single-strands).

(B). DNA synthesis on a single-stranded  $\phi$ x174 DNA template primed with a restriction fragment. Incubation was at 24°C for the indicated times, using 4.2 nmoles/ml of HaeIII Z2 primed  $\phi$ x174 DNA as template (corresponding to 3.4 nmole/ml of available single-stranded template). All assays were performed as in Methods, except that 43 protein was used alone or with the additional proteins indicated.

Fig. 5. DNA synthesis on an unprimed single-stranded fd DNA template catalyzed by the complete seven-protein system in the presence of 32 protein, or 32\*I protein, or both proteins. The replication mixture was incubated at 30°C for the indicated times, using 12.7 nmoles/ml (4.2  $\mu$ g/ml) fd DNA as template. The assay components for the complete seven-protein reaction were those noted in Methods, except that the 32 and 32\*I protein concentrations were varied as indicated, with the ratios given on the figure being the weight ratios of 32:32\*I:DNA present in the reaction mixture. A 13 to 15-fold weight excess of 32 protein is needed to completely coat single-stranded DNA (26,28).

Fig. 6. Synthesis of RNA oligonucleotides by the T4 gene 41 and 61 proteins. Reactions were incubated at 30°C for the times indicated using 30.3 nmoles/ml fd DNA as template. The assay components are those given in Methods.

Fig. 7. Analysis by sucrose gradient sedimentation of 32 protein and 32\*I protein complexes with single-stranded  $\phi$ x DNA. A volume of 200  $\mu$ l contained 7.2  $\mu$ g of  $\phi$ x viral DNA added to either (a) 180  $\mu$ g of 32 protein, (b) 180  $\mu$ g of 32 protein and 180  $\mu$ g of [<sup>3</sup>H]32\*I protein (12,000 cpm), or (c) 180  $\mu$ g of [<sup>3</sup>H]32\*I protein. After incubation at 24°C for 1 hr, 180  $\mu$ l of this mixture (in a buffer composed of 20 mM Tris-HCl, pH 8.1, 10 mM MgCl<sub>2</sub>, 70 mM NaCl, 1 mM  $\beta$ -mercaptoethanol, 0.5 mM Na<sub>3</sub>EDTA, 10% glycerol and 100  $\mu$ g/ml bovine serum albumin) was overlaid on a 5 ml preformed 5-30% (w/v) linear sucrose gradient in the same buffer and centrifuged at 45,000 rpm for 150 min at 4°C in a SW50.1 rotor. Fractions of 150  $\mu$ l were collected from the bottom of the tube and aliquots were either counted or analyzed by SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie Blue and the amount of protein in each band was determined by scanning densitometry, using known amounts of each protein as standards. The distribution of 32\*I protein calculated from the densitometric analysis agreed well with the results from [<sup>3</sup>H] counting. The amount of each protein is expressed as  $\mu$ g per (150  $\mu$ l) fraction.

Fig. 8. Analysis by sucrose gradient sedimentation of the binding of T4 DNA polymerase to H-D-proteins. Samples (140  $\mu$ l) in sedimentation buffer [20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1mM  $\beta$ -mercaptoethanol,

0.5 mM Na<sub>3</sub>EDTA, 10% (w/v) glycerol, and 50 µg/ml of bovine serum albumin] contained 25 µg of an E. coli alkaline phosphatase marker, 10.2 µg of T4 DNA polymerase and either a) no further additions, b) 147 µg of 32\*I protein, c) 147 µg of 32 protein, or d) 49 µg of 32 protein. After incubation at 30°C for 30 min, 100 µl of this mixture was overlaid on a 5 ml preformed 5-30% (w/v) linear sucrose gradient in the same buffer. The samples were centrifuged for 20 h at 41,000 rpm at 4°C in a SW50.1 rotor. Fractions were collected from the bottom of the tube for analysis by SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue and analyzed by scanning densitometry for 32 protein, 32\*I protein, T4 DNA polymerase, and alkaline phosphatase bands. The percent recovery of the total applied protein in each fraction is indicated. In other experiments, intact A peptide obtained from the digestion of 7.5 µg of 32 protein was incubated with 18 µg of DNA polymerase and the mixture was sedimented through a similar sucrose gradient. Under these conditions, the intact A peptide did not cosediment with, nor alter the band position of the DNA polymerase (data not shown). The significance of this result is unclear, as the A peptide obtained may have been denatured during isolation (it could be impossible to obtain "native" A peptide if the most stable conformation of the isolated fragment differs substantially from that of the covalently bound A peptide region).

Fig. 9. Schematic view of some 32 protein interactions.

I. Favorable electrostatic contacts between the core region of 32 protein and the phosphates of the DNA backbone are postulated to

be masked by the acidic A domain when an intact 32 protein molecule x is bound to a short oligonucleotide (26).

II. Cooperative DNA binding involves favorable interactions between adjacent 32 protein molecules, which change 32 protein conformation, including that of the A domain (21,22,26), thereby allowing additional electrostatic contacts with the DNA backbone to be made (26).

III. A model for the control of 32 protein activity via protein-protein interactions at the A domain is shown on the left, where the 43 protein (or 61 protein) is postulated to alter the DNA binding of 32 protein to prevent its destabilization of the growing 3' polynucleotide chain end. On the right, we illustrate the same situation in the presence of 32\*I protein, where no such control by a polymerase is possible.

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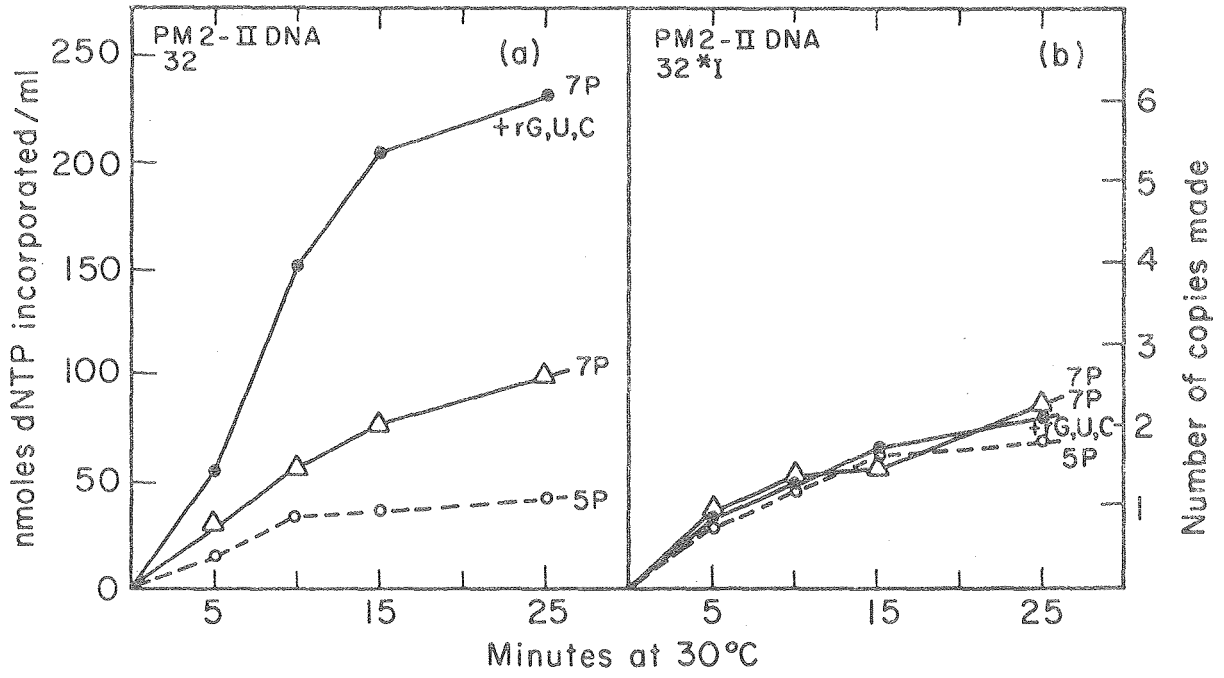
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Fig. 1

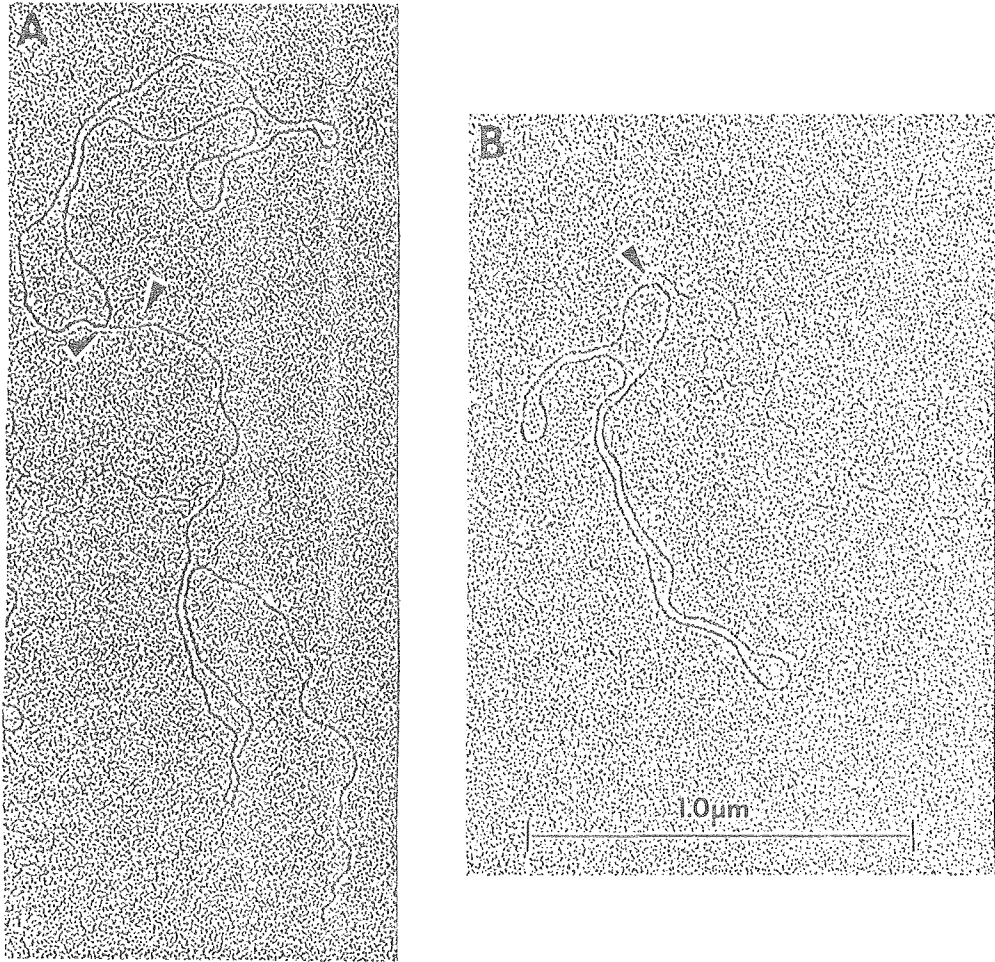


Fig. 2

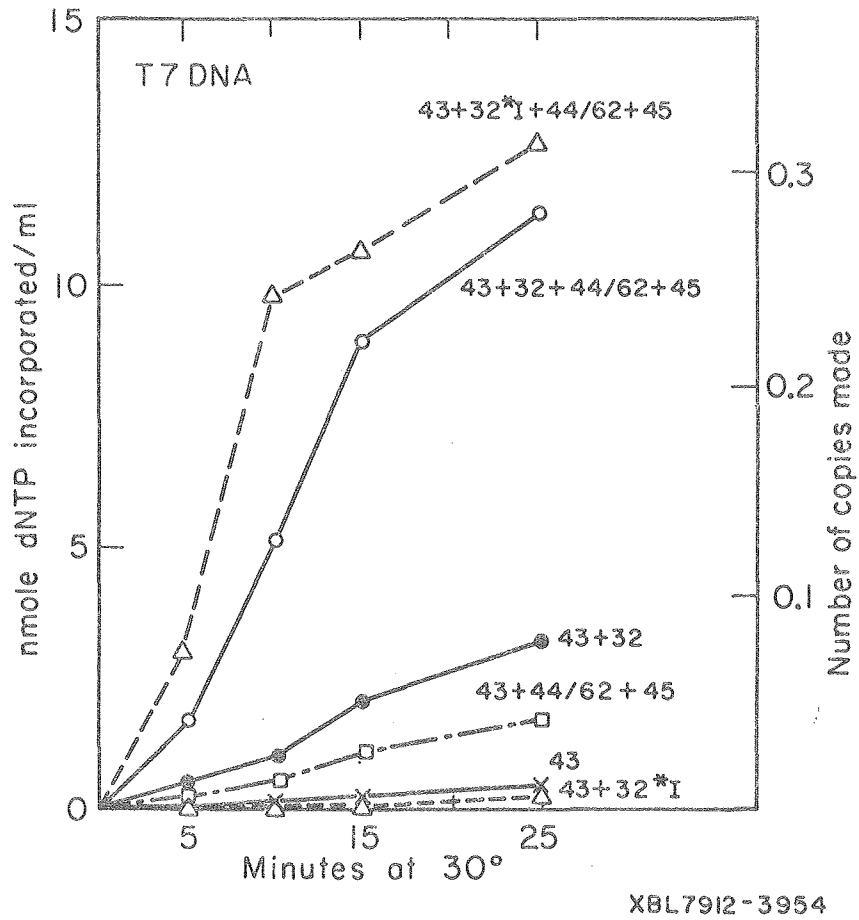


Fig. 3

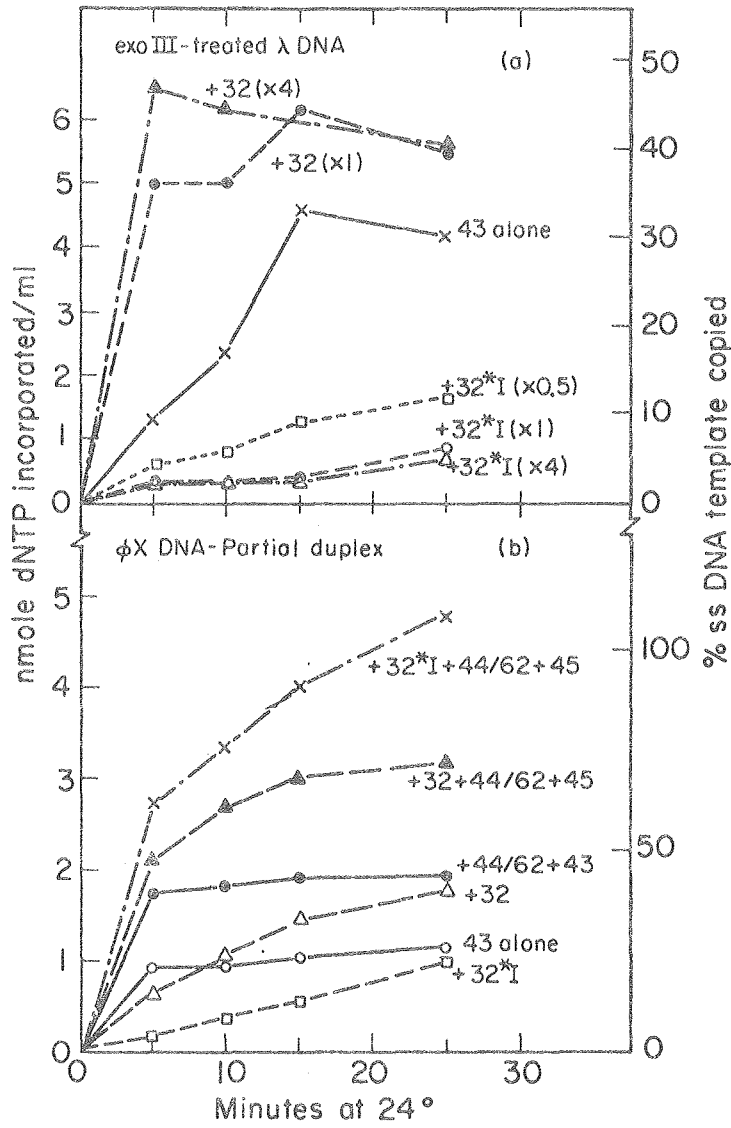
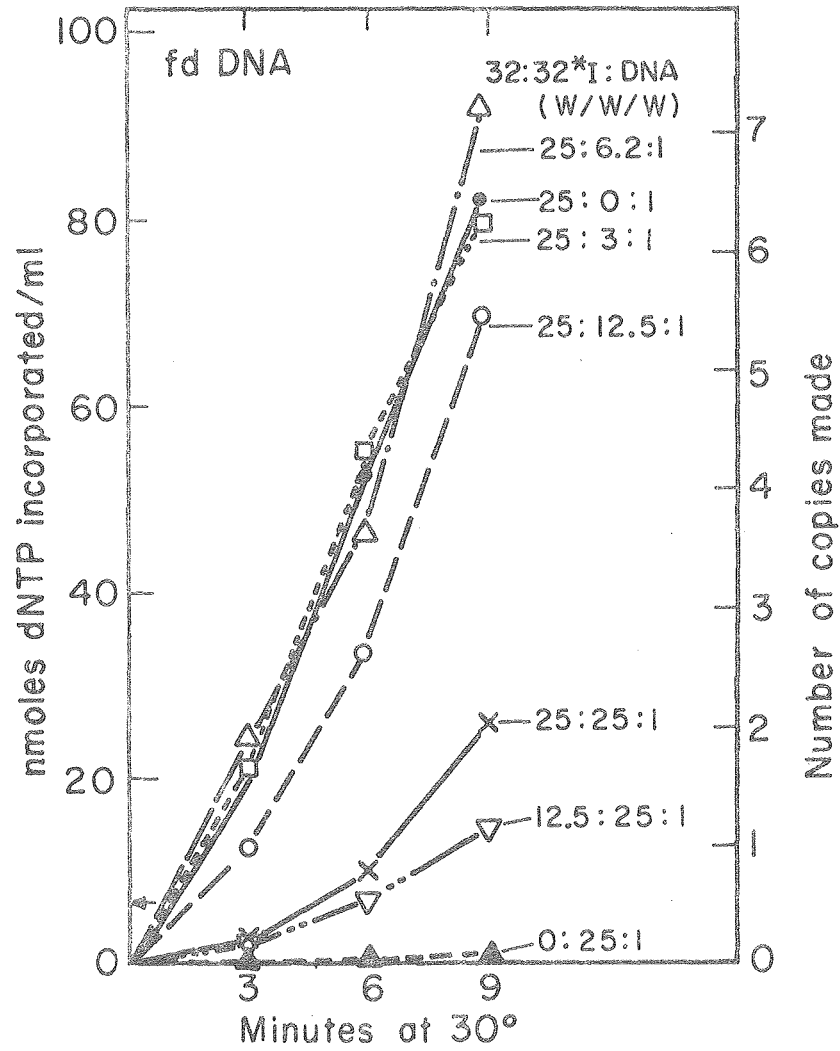


Fig 4

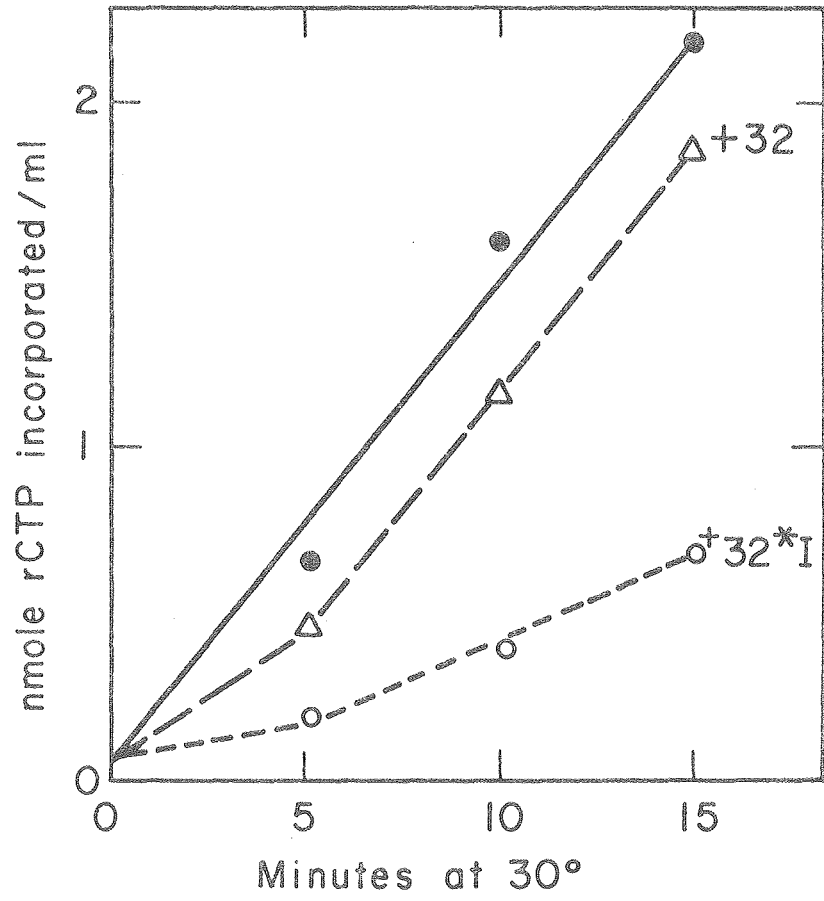
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Fig. 4



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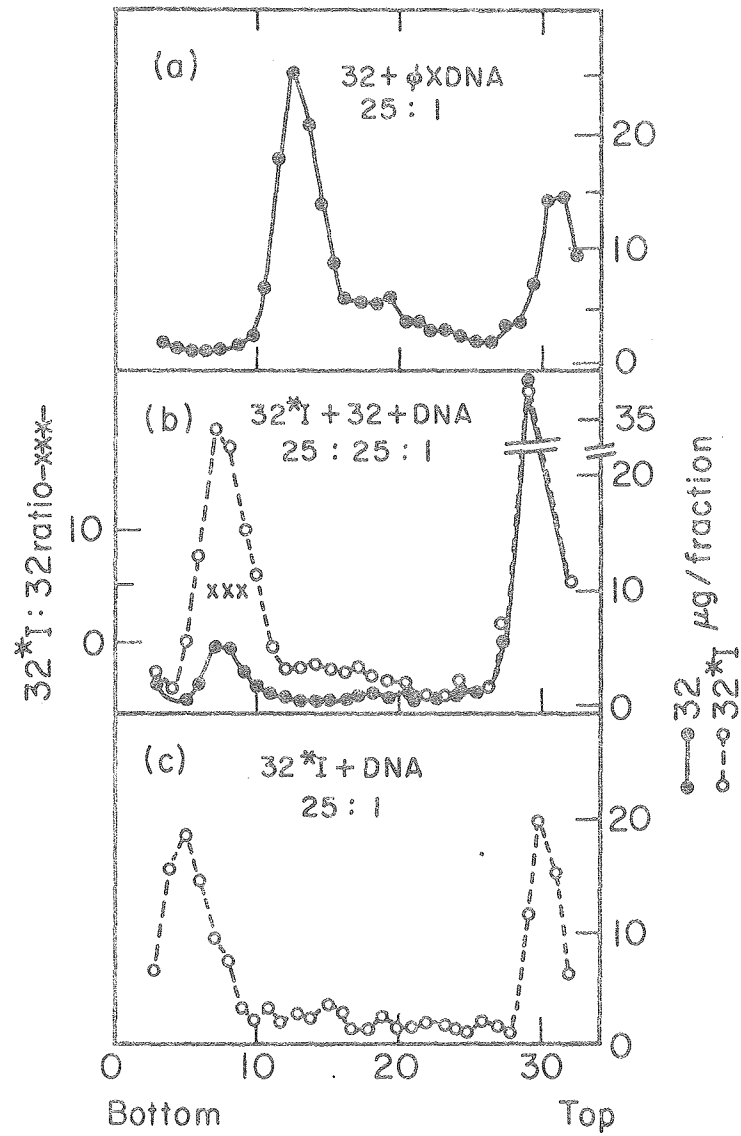
Fig. 5



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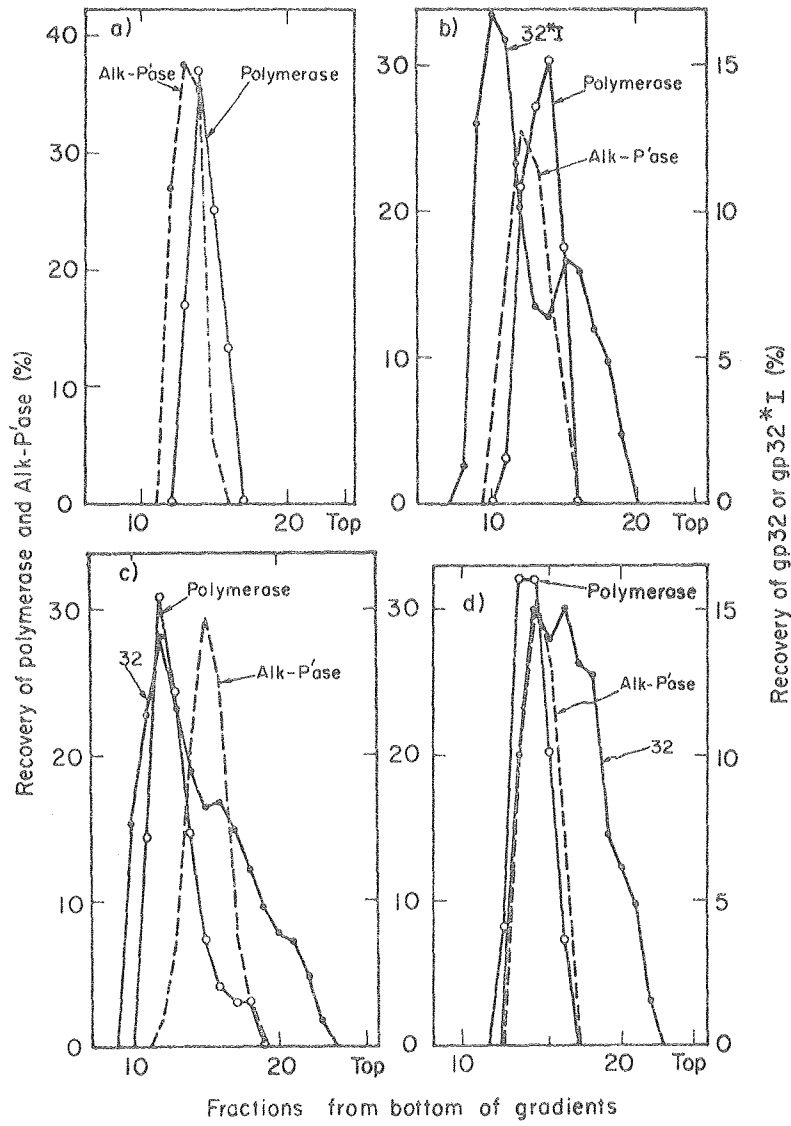
Fig. 6





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Fig. 7



XBL 785-3102

Fig. 9

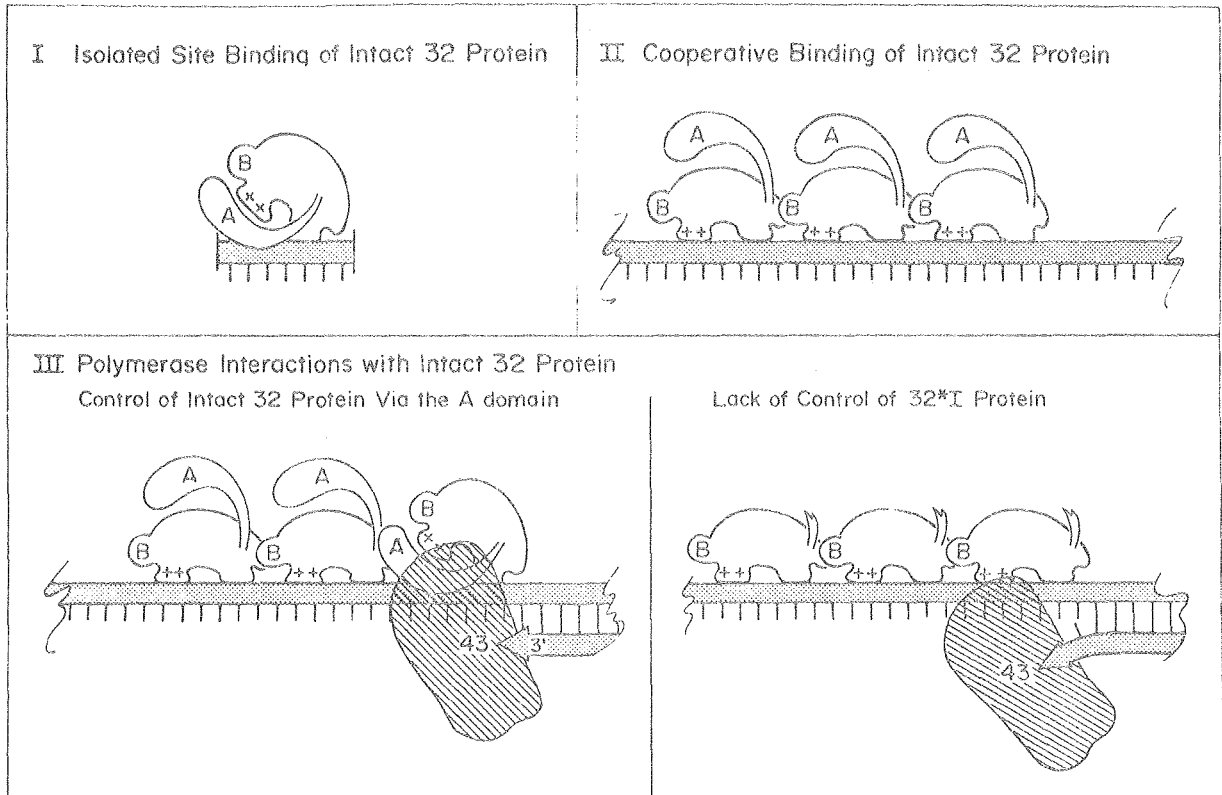


Fig. 9

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