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A Multienzyme System for Priming the Replication of ϕX174 Viral DNA*

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Synthesis of the oligonucleotides that prime replication of ϕX174 single-stranded DNA employs complex protein machinery of the host cell which is probably used by the cell to replicate its own chromosome. Primer synthesis depends on at least five proteins (DNA binding protein, *dnaB* and *dnaC* proteins, protein i, and protein n) and ATP to form a replication intermediate and another protein, primase (*dnaG* protein), to assemble the oligonucleotide by template transcription. The data in this paper show that ribo- and deoxyribonucleoside triphosphates can serve as substrates and form hybrid primers when present together. Both RNA and DNA primers were initiated with ATP. At least three of the four base-pairing nucleoside triphosphates were required for the transcription that generates effective primers. Over 90% of the RNA and DNA transcripts were extended into complementary strands by DNA polymerase III holoenzyme. At optimal triphosphate concentrations, the rate and extent of primer formation were greater from ribonucleoside triphosphates than from deoxyribonucleoside triphosphates. Uncoupled from DNA replication, the length of RNA primers was 14 to 50 residues, the DNA primers 4 to 20 residues. The fingerprint pattern of an RNase digest of RNA primers has a complexity suggestive of transcription from many sites on the ϕX174 template. The multienzyme priming system is highly specific for ϕX174 DNA as template.

Replication *in vitro* of bacteriophage ϕX174 single-stranded DNA to the closed circular duplex replicative form (RF I) requires the participation of at least nine *Escherichia coli* replication proteins: DNA binding protein, *dnaB* protein, *dnaC* protein, protein i, protein n, primase (*dnaG* protein), DNA polymerase III holoenzyme, DNA polymerase I, and DNA ligase (1-4). This enzymatic conversion may be described as a sequence of these reactions: (I) coating of the viral DNA with DNA binding protein; (II) relatively slow enzymatic conversion of the DNA, in the presence of ATP, Mg^{2+}, *dnaB* protein, *dnaC* protein, and proteins i and n, to a replication intermediate (2, 5, 6) which contains bound, but apparently actively moving, *dnaB* protein (7-9); (III) transcription of the replication intermediate complex by primase to produce short polynucleotide primers (5, 7, 8); (IV) elongation of the primers by DNA polymerase III holoenzyme (7); (V) excision of the primer fragment by DNA polymerase I and filling of gaps remaining in the complementary strand; and (VI) strand sealing by DNA ligase (4).

In this report we describe some of the properties of a multienzyme system, reconstituted with extensively purified proteins, for priming ϕX174 DNA replication *in vitro*. Six *E. coli* replication proteins are required for the synthesis of primer transcripts on ϕX174 DNA. Both ribonucleotides and deoxyribonucleotides can be incorporated into the primers. Synthesized at multiple sites on the ϕX174 chromosome, the primers appear to reflect the capacity of primase to recognize a locus containing the *dnaB* protein as a signal for strand initiation (7).

MATERIALS AND METHODS

Materials – Materials were from sources previously described (9-11).

Nucleotides – [γ-^{32}P]ATP and [γ-^{32}P]dATP were prepared by the procedure of Maxam and Gilbert (12). [γ-^{32}P]GTP was prepared by Mr. L. Bertsch of this department. ^{3}H- and α-^{32}P-labeled rNTPs and dNTPs were from New England Nuclear Co. or from Amersham Corp.; unlabeled nucleotides were from P-L Biochemicals.

Enzyme Preparations – All *E. coli* replication proteins were purified from strain HMS83 (13) grown at 37°C and harvested as previously described (9). Proteins, assayed as before (3), were: DNA binding protein (Fraction 3b, 25,000 units/ml, 15,300 units/mg (14)); *dnaB* protein (Fraction V, 120,000 units/ml, 1×10^6 units/mg (9)); *dnaC* protein (Fraction VI,[1] 23,000 units/ml, 130,000 units/mg); protein i (Fraction V,[1] 370,000 units/ml, 1.3×10^6 units/mg); protein n (Fraction VI,[1] 34,000 units/ml, 120,000 units/mg); primase (Fraction VI, 140,000 units/ml, 1.1×10^6 units/mg (15)); and DNA polymerase III holoenzyme (Fraction V, 18,000 units/ml, 520,000 units/mg (16)). RNase A, RNase T_1, and pancreatic DNase I were from Worthington Biochemical Corp. Homogeneous *E. coli* RNA polymerase was donated by M. Chamberlin (University of California, Berkeley).

γ-Globulins – Production of rabbit antibodies directed against DNA binding protein (14), protein i (5), *dnaB* protein (9), and copol III* (17) has been described previously. Antibodies directed against

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primase (Fraction VI) and protein n (Fraction VI) were elicited by a procedure detailed earlier (5). Antibody preparations were purified to obtain an electrophoretically homogeneous γ-globulin fraction by the following procedure: Serum was diluted with 4 volumes of 0.15 M NaCl and precipitated at 0°C by the addition of 0.258 g $(NH_4)_2SO_4$/ml (45% saturation). The precipitate was collected by centrifugation and homogenized in 0.5 volume of 45% saturated $(NH_4)_2SO_4$ in 0.15 M NaCl. The precipitate was collected by centrifugation, dissolved in 0.1 volume of 0.02 M potassium phosphate buffer (pH 7.0), and desalted by filtration through a Sephadex G-25 column (4 volumes) equilibrated in the same buffer. The desalted protein fraction was applied to a DEAE-cellulose column (2 volumes, Whatman DE52) equilibrated in 0.02 M potassium phosphate buffer (pH 7.0). The pass-through protein was collected, then stored at −20°C.

Nucleic Acids – DNA from phages φX174, G4, and M13 were isolated by phenol extraction of the purified virus particles. Calf thymus DNA was from the Worthington Biochemical Corp. Sources of other DNAs have been described (15). *Drosophila* [^{32}P]tRNA was provided by Mariana Wolfner of this department. The [^{32}P]d(pApG)$_{2-5}$ series was prepared by Kirk Fry (this department) from the unlabeled oligonucleotides (Collaborative Research Inc.) using T4 polynucleotide kinase and [γ-^{32}P]ATP (12) and fractionating by electrophoresis in 20% polyacrylamide containing 7 M urea. Each fraction eluted from the gel was concentrated by adsorbing to DEAE-Sephadex and then eluting.

Buffers – Buffer A is 50 mM Tris·Cl (pH 7.5), 10% (w/v) sucrose, 20 mM dithiothreitol, and 200 μg/ml of bovine serum albumin. Buffer B is 120 mM $MgCl_2$, 10 mM ATP, 40 mM spermidine·Cl and 0.04 M Tris (pH 7.5). Buffer C is 10 mM imidazole·Cl (pH 7.0), 5% (w/v) sucrose, 20 mM KCl, 10 mM dithiothreitol, 0.1 mM ATP, 10 mM $MgCl_2$, and 70 μg/ml of bovine serum albumin. Buffer D is 10 mM Tris·Cl (pH 7.5), 0.2 M NaCl, 5 mM $MgCl_2$, and 4 mM spermidine·Cl.

Glassware – Glassware was siliconized with 2% Dri-Film (Pierce Chemical Co.) in chloroform.

Assay of RNA and DNA Primer Synthesis – For RNA primer synthesis, components were added at 0°C in the following order: 12 μl of buffer A, 0.8 μg of Rifampicin, 3.7 μg of DNA binding protein, 40 units of *dnaB* protein, 80 units of *dnaC* protein, 40 units of protein *i*, 35 units of protein *n*, 140 units of primase, 1.1 nmol (as nucleotide) of φX[2] DNA, 5 μl of buffer B, 4 nmol each of [^{3}H]CTP, GTP, and UTP, (each at 2,000 to 10,000 dpm/pmol) or [^{32}P]NTPs (5,000 to 50,000 dpm/pmol), and water to 40 μl. Incubation was at 30°C; reactions were terminated by chilling the mixtures to 0°C. For DNA primer synthesis, the same assay procedure was used except that CTP, GTP, and UTP were replaced with 2 nmol each of [^{3}H]dATP, dCTP, dGTP, and dTTP (each at 20,000 dpm/pmol) or [α-^{32}P]rNTPs (10,000 to 100,000 dpm/pmol).

When the isolated φX174 replication intermediate (5) was used as the template, primer synthesis was initiated by addition of primase (120 units/nmol of intermediate), appropriate rNTPs and/or dNTPs, and Rifampicin to 10 μg/ml. Replication intermediate was produced using the components for RNA primer synthesis, in a final volume of 25 μl, except that primase and CTP, GTP, and UTP were omitted and only 6 μl of buffer A was used. After 30 min at 30°C, the intermediate was isolated free of unassociated protein by filtering the reaction mixture through Bio-Gel A-15m (equilibrated in buffer C at 25°C) and collecting the excluded volume.

The amount of RNA and DNA primer chains synthesized was determined by applying aliquots of reaction mixtures to DEAE-cellulose filter paper circles (Whatman DE81). Unincorporated NTPs were removed by washing with ammonium formate-sodium pyrophosphate (15) and radioactivity on the filters was determined (15). The shortest oligonucleotide detectable by this assay was determined by measuring the binding of [^{32}P]d(pApG)$_{2-5}$ to the paper under these conditions (Table I). The results suggest that oligonucleotide chains with a triphosphate terminus and 4 residues or longer in length would be retained by the paper in this procedure. Nucleotide residues incorporated per φX174 SS template were calculated to include incorporation of unlabeled ATP, assuming that 25% of the residues in both the RNA and DNA primer transcripts were AMP. Because of quenching of ^{3}H (up to 80%) in short oligonucleotides (<10 residues) adsorbed to DE81 paper, [^{32}P]NTPs were used instead, on occasion.

Assay of DNA Replication – DNA synthesis was assayed as described previously (14). Extension of primer transcripts into DNA chains was accomplished by the addition of 90 units of DNA polymerase III holoenzyme/1000 pmol of primed φX SS. Incubation was for 3 to 5 min at 30°C in the presence of either 50 μM each of dATP, dCTP, dGTP, and 18 μM [^{3}H]dTTP (600 to 1200 dpm/pmol) or 10 to 50 μM each of [α-^{32}P]dATP, dCTP, dGTP, and dTTP (500 to 5000 dpm/pmol). The DEAE-cellulose filter assay (see above) was used.

TABLE I

Binding of oligonucleotides to DEAE-cellulose paper

Each oligonucleotide in the series [^{32}P](d(pApG)$_{2-5}$ was applied to two DE81 paper circles; one paper was washed with the ammonium formate-sodium pyrophosphate procedure (15), the other with 0.01 M Tris·Cl (pH 7.5) to determine the amount of radioactivity applied.

^{32}P-Oligonucleotide	Adsorbed
	%
d(pApG)$_2$	12
d(pApG)$_3$	54
d(pApG)$_4$	80
d(pApG)$_5$	89

Other Procedures – Unincorporated NTPs were removed from reaction products by filtration through Bio-Gel A-15m columns equilibrated at 4°C with either buffer C or buffer D. Volumes applied were less than 10% of the column bed volume. The void volume fractions, containing the φX SS template and products annealed to it, were located by measurements of ^{3}H or ^{32}P.

Electrophoresis of polynucleotides in polyacrylamide gels containing 7 M urea or 98% formamide was performed according to the method of Maniatis *et al.* (18). Two-dimensional ionophoresis of RNase digest on cellulose acetate and DEAE-cellulose was the method of Barrell (19), as was RNase treatment of primer transcripts. ^{32}P-labeled material was autoradiographed on Cronex 4 (DuPont) or Kodak XR-5 X-ray film, sometimes with the aid of a Kodak intensifying screen.

RESULTS

Ribonucleoside Triphosphate Incorporation into Primer Depends on Six Proteins – Synthesis of oligoribonucleotide transcripts proceeded linearly for 20 to 30 min or more when φX viral DNA was incubated with the four rNTPs and purified preparations of DNA binding protein, *dnaB* protein, *dnaC* protein, protein i, protein n, and primase (Fig. 1); as many as 300 ribonucleotide residues were incorporated per φX circle. Each of the components was required for this RNA primer synthesis (Table II); spermidine and rifampicin had no demonstrable effect.

Incorporation of dNTPs into Primer is Catalyzed by Same Six Proteins – Incorporation of rNTPs was reduced by the presence of unlabeled dNTPs due to competitive utilization of dNTPs as substrates (8). dNTP incorporation was generally about 3- to 4-fold slower than rNTP (Fig. 1); up to 80 dNMP residues were incorporated per input circle. Components required for synthesis of DNA primers were those required for RNA synthesis (Table II), except that spermidine enhanced DNA synthesis.

ATP, which is absolutely required for formation, stability, and activity of the φX replication intermediate complex (5, 7), was therefore present during DNA transcript synthesis. (This complex is the activated form of the template essential for transcription by primase (7).) AMP was incorporated along with dNMPs in the oligonucleotide product. Alkaline digestion of the DNA primers, which produces cleavages at rNMP linkages, converted 90% to a form not retained by DEAE-cellulose paper. Thus, most of the product contains AMP residues interspersed within stretches of 5 to 6 nucleotides. Under identical alkaline treatments, over 99% of φX [^{32}P]RNA primer and ^{32}P-labeled *Drosophila* tRNA, but less than 1% of

[2] The abbreviations used are: φX, φX174; SS, single-stranded, circular DNA; NTP, nucleoside triphosphate; RF I, covalently closed, circular, duplex replicative form DNA.

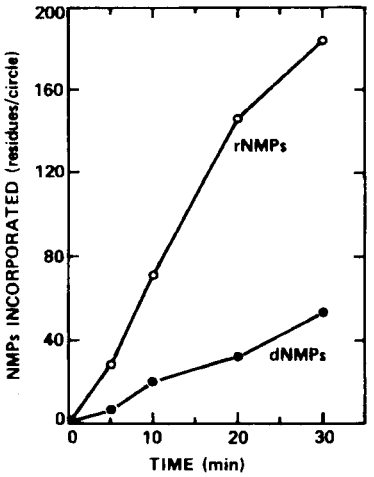

FIG. 1. Incorporation of NTPs into primers. Assays are described under "Materials and Methods" using [^{3}H]rNTPs and [^{32}P]dNTPs.

TABLE II

Requirements for rNTP and dNTP incorporation

RNA synthesis assays (30 min, 30°C) are described under "Materials and Methods"; [^{3}H]CTP, GTP, and UTP (each at 3300 dpm/pmol and 75 μM). DNA synthesis, 20 min, 30°C; [^{3}H]dCTP, dGTP, and dTTP (each at 20,000 dpm/pmol) and dATP were each 50 μM.

Component omitted	rNMP incorporated	dNMP incorporated
	residues/circle	
None (complete)	200.	45.
DNA binding protein	4.4	1.2
dnaB protein	1.9	0.6
dnaC protein	1.0	0.0
Protein i	13.	4.5
Protein n	0.0	0.0
Primase	1.4	0.0
ϕX174 DNA (SS)	0.0	0.0
ATP	0.0	0.0
Mg^{2+}	1.3	0.3
Spermidine	210.	13.
Rifampicin	180.	43.

the ^{32}P-labeled complementary strand ϕX DNA, became nonadsorbable by DEAE-cellulose paper. Evidence that the DNA transcript did not contain significant levels of ribonucleotide residues other than AMP was their complete resistance (>99%) to RNases A and T_1, neither of which cleaves at AMP residues; *Drosophila* tRNA under these conditions was completely digested to oligoribonucleotides.

Influence of rNTP and dNTP Concentration on Primer Synthesis – Varying the NTP concentrations with ATP kept at 0.5 mM (Fig. 2) yielded apparent K_m values of 10 μM for rNTPs (Lineweaver-Burk plot) and 2 μM for dNTPs (data not shown). When rNTPs and dNTPs were present together at equal concentrations, incorporation of dNTPs predominated (Table III), but the reverse was true when rNTPs were in severalfold excess over the dNTPs, as occurs in the whole cell. The presence of dNTPs inhibited rNTP incorporation to an extent greater than that expected simply from the dilution of rNTPs by competing substrate. On the other hand dNTP incorporation was stimulated by the presence of higher rNTP concentrations.

Size of RNA and DNA Primers – [^{32}P]RNA and DNA transcripts were synthesized on isolated ϕX replication intermediate. Transcripts at least 11 residues long, and thus large

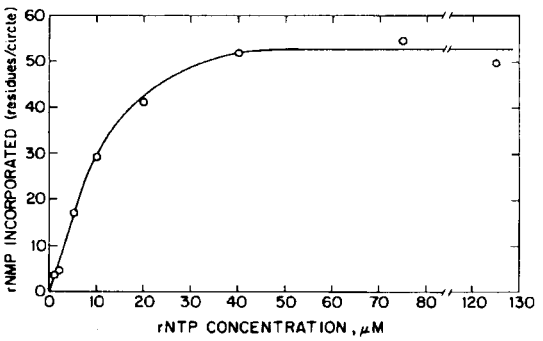

FIG. 2. Effect of rNTP concentration on rate of incorporation. Measurements using isolated ϕX replication intermediate as template are described under "Materials and Methods"; 5-min incubation.

TABLE III

Competition of dNTPs and rNTPs for primer synthesis

Assays used isolated ϕX174 replication intermediate as template and are described under "Materials and Methods," except that [^{3}H]rNTPs (CTP, GTP, and UTP) and [α-^{32}P]dNTPs (dCTP, dGTP, and dTTP) and unlabeled dATP were all present. Incubations were for 20 min at 30°C.

dNTP	rNTP	dNMP incorporated	rNMP incorporated
μM	μM	*residues/circle*	
0	50		164
5	5	18	6
20	20	22	7
50	50	37	16
10	50	27	67
40	125	72	87

enough to remain stably annealed to the ϕX SS template, were recovered in the void volumes and sized by electrophoresis in a 20% polyacrylamide gel in 7 M urea (Fig. 3). Recovered DNA transcripts were 11 to 21 nucleotides long[3] (Fig. 3, *Slot A*). Inasmuch as most of the DNA transcripts did not remain hybridized to the DNA template during gel filtration (see legend to Fig. 3), it appears that they are generally shorter than 11 residues. The RNA transcripts were 14 to 50 residues long, a major portion being near 40 nucleotides (Fig. 3, *Slot C*). ATP, known to initiate the ϕX RNA transcripts (7), was traced with a γ-^{32}P label to chains 15 to 40 residues long (Fig. 3, *Slot D*). RNA transcripts made on the isolated ϕX replication intermediate were significantly longer than those made with the single mixture of all required proteins (15 to 25 nucleotides long) (7).

ATP Initiates RNA and DNA Primers – In the absence of DNA replication, [γ-^{32}P]ATP residues were incorporated into RNA primer transcripts, about 5 to 8 per input ϕX circle (7) (legend to Fig. 3) or near one ATP per oligonucleotide chain (7). GTP was not detected at the 5′-termini of primer fragments (< 1 [γ-^{32}P]GTP residue/3000 ribonucleotides incorporated). Although primase can incorporate dNTPs into polynucleotide chains, it apparently cannot initiate a chain with a dNTP; incorporation of [γ-^{32}P]dATP was not detectable (<0.01 molecule/primer) when isolated ϕX replication intermediate was transcribed with primase in the presence of the four

[3] We have assumed that all product oligonucleotide chains have a triphosphate terminus and that chains with a triphosphate terminus migrate at the same rate in polyacrylamide gels (at pH 8) as chains 2 residues shorter which have a a monophosphate terminus.

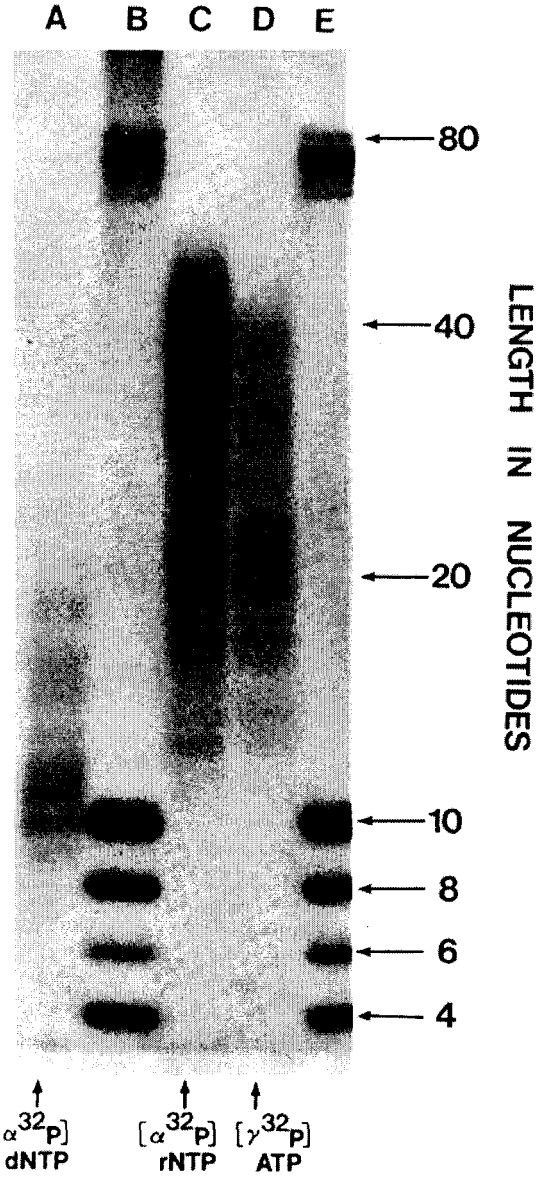

FIG. 3. Electrophoresis of RNA and DNA primer transcripts. Preparation and isolation of ϕX replication intermediate is described under "Materials and Methods." To 3 nmol of intermediate (in 100 μl of buffer C) were added 15 μl of H_2O, 15 μl of buffer A, 1 μg of rifampicin, 360 units of primase, and additional components as follows: *Slot A*, spermidine·Cl, 1.5 mM; ATP, 250 μM; [α-^{32}P]dNTPs (18,400 dpm/pmol), 25 μM. *Slot B*, *Drosophila* [^{32}P]tRNA and [^{32}P]d(pApG)$_{2-5}$ markers. *Slot C*, ATP, 250 μM; [α-^{32}P]rNTPs (23,100 dpm/pmol), 45 μM. *Slot D*, [γ-^{32}P]ATP, 70 μM (192,000 dpm/pmol); [^{3}H]CTP, GTP, and UTP (each 50 μM and 4600 dpm/pmol). *Slot E*, *Drosophila* [^{32}P]tRNA and [^{32}P]d(pApG)$_{2-5}$ markers. Primer synthesis was for 20 min at 30°C. Incorporation (nucleotides/input ϕX circle) for the reaction samples applied to *Slots A*, *C*, and *D*, were, respectively, 51, 210, 130. Incorporation of [γ-^{32}P]ATP residues (*Slot D*) was 8/input circle. Unincorporated NTPs were removed by filtering the reaction mixtures through Bio-Gel A-15m equilibrated in buffer D. (In parallel experiments, approximately 60% of applied DNA template is found in the column void-volume). Recoveries of labeled primers were: DNA primers (*Slot A*) 16%, RNA primers (*Slot C*) 45%, and 39% of ^{3}H label and 24% of ^{32}P label in [γ-^{32}P]ATP-labeled RNA primers (*Slot D*). To each void volume sample were added 40 μg of *E. coli* tRNA, EDTA to 5 mM, 0.1 volume of 3 M sodium acetate (pH 5.5), and 2 volumes of 95% ethanol. After 30 min at −78°C, the precipitates were collected by centrifugation (10 min, 20,000 × *g*), washed with cold 95% ethanol, and resuspended in 25 μl of deionized formamide. Transcripts were fractionated by electrophoresis in a 20% polyacrylamide gel containing 7 M urea (12, 18), and were located by autoradiography of the frozen gel at −85°C.

rNTPs (each at 50 μM) and 10 μM [γ-^{32}P]dATP (2.3 × 10^6 dpm/pmol).

Primers are Transcripts of Multiple Regions of DNA Template – The base sequences of the primers were determined by digesting [^{32}P]RNA transcripts (210 residues/input ϕX circle) with RNase T_1. Oligoribonucleotides produced were separated by two-dimensional ionophoresis. The complexity of the fingerprint pattern obtained (Fig. 4) indicates that the primers were synthesized at multiple (probably random) sites on the ϕX chromosome. A similarly complex fingerprint pattern was obtained from RNA transcripts produced with an average of only 10 residues incorporated per input circle (data not shown), indicating that there is no strongly preferred site on the ϕX template for RNA transcription by this multienzyme system.

Priming Capacity of RNA and DNA Transcripts – Virtually all (95 to 100%) of the RNA transcripts synthesized *in vitro* which remained annealed to the isolated template strand become covalently linked to complementary strand DNA following incubation of the primed ϕX DNA with DNA polym-

FIG. 4. T_1 ribonuclease fingerprint of ϕX primer RNA. Primer [^{32}P] RNA was synthesized and isolated as described in Fig. 3 legend, *Slot C*. RNA was digested to completion by RNase T_1; resulting oligonucleotides were fractionated by bidimensional ionophoresis on cellulose acetate and DEAE-cellulose paper and autoradiographed (19, 20).

erase III holoenzyme and the four dNTPs (7). The same observation has now been made with DNA transcripts. As judged by comigration of primer and full length complementary strands during electrophoresis of replicated DNA under denaturing conditions in polyacrylamide gels (containing 7 M urea or 98% formamide) or agarose gels (containing 5 mM methylmercuric hydroxide (21)), over 90% of each the RNA and DNA transcripts served as primers of ϕX complementary strand synthesis (data not shown).

Ribo- and Deoxyribonucleotides Required for Effective Primer Formation – Primase was incubated for 2 min (at 30°C) with the ϕX replication intermediate, ATP, Mg^{2+}, spermidine, and one or more of the other NTPs. Further transcription was prevented by addition of anti-primase γ-globulin. Extent of replication after subsequent incubation with DNA polymerase III holoenzyme and [^{3}H]dNTPs was a measure of the effectiveness of priming. All four rNTPs were required for maximal priming, but significant levels were obtained when either CTP or UTP was omitted (Table IV); omission of GTP had a profound effect.

Although dCTP, dGTP, and dTTP could each substitute for the analogous rNTP, their effectiveness as a group was rather poor (Table IV); substitution of dGTP for GTP in certain mixtures led to significant reductions in the effectiveness of priming. These results may be due to the smaller size of the DNA transcripts (see Fig. 3) and their failure to remain annealed to the template, inasmuch as DNA transcripts of 12 residues or longer were excellent primers. For example, in a coupled priming-replication reaction, optimal replication (and presumably optimal priming) was sustained without added CTP, GTP, and UTP. It would appear then that transcripts too short to remain stably hybridized can nevertheless be utilized as effective primers, provided that DNA polymerase III holoenzyme and the four dNTPs are also present during their synthesis.

Effect of Specific Antibodies on Synthesis of RNA and DNA Primers – Antibodies directed against DNA binding protein, *dnaB* protein, protein i, protein n, and primase have been used to confirm that the ϕX replication intermediate is the form of template used for synthesis of primer transcripts (7). As expected, each of these antibodies strongly inhibited RNA primer synthesis when added before the replication intermediate was formed (Table V). Neither nonimmune γ-globulin nor antibody directed against the copol III* (β) subunit of holoenzyme blocked primer synthesis under these conditions. Once the replication intermediate complex had been formed, primer synthesis also became resistant to anti-protein i and anti-protein n antibodies. The persistent sensitivity of primer synthesis to antibodies directed against *dnaB* protein, primase, and DNA binding protein indicates that these proteins probably participate directly in synthesis of primer transcripts. The relative effects on DNA primer synthesis of these γ-globulin preparations, added before or after intermediate formation, were identical with their influence on RNA primer synthesis (data not shown). The effects of these specific antibodies on ϕX complementary strand DNA synthesis using templates primed with RNA transcripts (Table V) indicated little or no inhibition by anti-protein i, anti-protein n, anti-*dnaB* protein, or anti-primase, as expected. There were, however, strong inhibitions of ϕX SS replication by antibodies directed against DNA binding protein and copol III*.

Template Specificity of Priming System for ϕX – Although the ϕX system can synthesize RNA on the circular chromosome of the ϕX-like phage G4 (Table VI), this synthesis

TABLE IV

NTPs required for priming DNA synthesis

To replication intermediate (see "Materials and Methods") in 300-pmol aliquots contained in 7.5 μl, the following were added: Rifampicin to 10 μg/ml; 3 μl of buffer A, 40 units of primase, rNTPs (other than ATP) and dNTPs (as indicated) to 40 μM, and H_2O to 18 μl. The mixture was incubated 2 min at 30°C (first stage), then chilled to 0°C, and primase was inactivated by addition of 50 μg of anti-primase γ-globulin. After 10 min at 0°C, 30 units of DNA polymerase III holoenzyme were added along with [^{3}H]dTTP (1200 dpm/pmol) and unlabeled dATP, dCTP, and dGTP, each at 50 μM. The DNA synthesis reaction (second stage) was for 4 min at 30°C; ^{3}H incorporated into an acid-insoluble form was determined.

Priming (first stage)								DNA synthesis (second stage)
rNTPs added				dNTPs added				
								pmol
A								3
A	C							5
A		G						8
A			U					4
A	C	G						58
A	C		U					10
A		G	U					60
A	C	G	U					91
A	C		U			G		85
A		G			C		T	91
A		G			C			44
A		G					T	41
A	C					G	T	56
A	C					G		5
A			U			G		12
A					C	G	T	38
A				A	C	G	T	20
A				A		G	T	10
A				A	C		T	7

TABLE V

Antibody effects on RNA primer synthesis and DNA synthesis

Addition of 75 μg of γ-globulin "at outset" was made to a mixture of components for making replication intermediate but lacking DNA (see "Materials and Methods"). After 15 min at 0°C, ϕX DNA was added and the mixtures were incubated 25 min at 30°C to form the replication intermediate; then primase and [^{3}H]rNTPs (2000 dpm/pmol) were added and the mixtures incubated for an additional 10 min at 30°C to allow primer synthesis. Addition of γ-globulin "after intermediate formation" was made to the ϕX replication intermediate at the same time as primase. After 15 min at 0°C, [^{3}H]rNTPs were added and the mixture incubated for 10 min at 30°C. For DNA synthesis, γ-globulins were added after RNA primers had been synthesized using unlabeled rNTPs; DNA polymerase III holoenzyme was added at the same time. After 15 min at 0°C, [^{3}H]dNTPs were added and followed by a 10-min incubation at 30°C. Incorporation of ^{3}H into an acid-insoluble form was measured.

γ-Globulin added			
γ-Globulin	RNA primer synthesis		DNA synthesis
	At outset	After intermediate formation	After priming
	residues/circle		*pmol*
Nonimmune	34	33	124
Anti-protein i	3.9	33	127
Anti-protein n	2.6	31	109
Anti-DNA binding protein	3.5	4.1	4
Anti-*dnaB* protein	0.7	0.5	105
Anti-primase	4.2	3.4	120
Anti-copol III*	36	34	17

TABLE VI

Template specificity of ϕX DNA priming system

RNA primer synthesis was measured (see "Materials and Methods") using 1 nmol of the indicated DNAs as template.

Template	Ribonucleotide incorporation
	pmol rNMP/nmol template
ϕX SS	39.1
G4 SS	4.8
M13 SS	<0.5
Pf-1 SS	<0.5
ϕX RF I	<0.5
ColE1	<0.5
E. coli	<0.5
E. coli (denatured)	<0.5
Calf thymus (denatured)	<0.5
λ	<0.5
λ (denatured)	<0.5

requires only two of the six proteins: primase and DNA binding protein (7, 15, 22). Whether or not *dnaB* protein, *dnaC* protein, and protein i and n are present, RNA primer synthesis on G4 viral DNA terminates after approximately 25 to 30 nucleotides are incorporated per DNA circle. This multienzyme system could not initiate primer synthesis on any of the other DNA templates tested (Table VI).

DISCUSSION

A multienzyme system reconstituted with six purified *E. coli* proteins has the capacity to synthesize primer transcripts on ϕX viral DNA. Each of the replication proteins, in addition to ϕX DNA, ATP, Mg^{2+}, and the nucleoside triphosphates, was essential for polynucleotide synthesis. Earlier attempts to isolate these primers have failed for lack of proteins sufficiently purified from nucleases and other inhibitory factors. For example, a preparation of *dnaB* protein, purified over 3000-fold and highly active for *in vitro* replication of ϕX SS, was so contaminated with nuclease activity as to prevent detection of net primer synthesis.

Primers formed from a mixture of ribo- and deoxyribonucleoside triphosphates are co-polymers of these nucleotides. When the ribonucleotide concentration was high relative to deoxyribonucleotide, as *in vivo*, both the rate and extent of ribonucleotide incorporation were significantly higher than those for deoxyribonucleotide. RNA primers were on the average about 4 times as long as the DNA primers and, as such, were much more stably annealed to the template. An enhanced longevity of the priming capacity of RNA transcripts may explain a preference by the priming system for RNA over DNA for optimal replication in two-stage assays (see Table IV). With primer transcripts of sufficient length, no preference was shown by DNA polymerase III holoenzyme for extension of either DNA or RNA transcripts.

Both RNA and DNA primers appear to be initiated solely with ATP; neither GTP nor dATP was able to substitute as the initiating nucleotide. ATP is also required during the priming reaction to stabilize the ϕX replication intermediate (5) (containing template-bound *dnaB* protein and its ATPase activity (7, 9)). Because ATP is required during DNA primer synthesis, AMP residues were interspersed in the DNA transcripts.

The magnitude of RNA synthesis by the reconstituted system in the absence of coupled DNA replication was far in excess of that required for priming of complementary DNA strand synthesis. The potency of the priming system is illustrated by its capacity to synthesize RNA on ϕX circles (coated with DNA binding protein) at a rate over 20 times that of *E. coli* RNA polymerase.[1] As much as 6% of the ϕX template was transcribed into primer RNA (in the absence of DNA replication) in the form of multiple primers initiated at numerous sites (7) (Fig. 4).

With the use of antibodies directed against the proteins required for primer synthesis, we have confirmed our earlier finding (7) that primase will transcribe only those ϕX DNA circles which have been converted to the intermediate form (Table V). In addition to primase, both *dnaB* protein and DNA binding protein participate in primer synthesis. The other three proteins required for primer synthesis, namely, protein i, protein n, and *dnaC* protein, apparently function in the synthesis of the nucleoprotein replication intermediate complex required for primase action (Table V).[4] Among these proteins, only DNA binding protein has a role in the DNA synthesis that follows priming (Table V) (23).

In spite of the capacity of this multienzyme priming system to transcribe multiple sites in ϕX174 DNA, it cannot initiate transcript synthesis on other template DNAs (Table IV). This remarkable template specificity presumably reflects the capacity of one or more of the proteins which participates in the transfer of *dnaB* protein onto ϕX DNA to recognize a specific locus in this viral chromosome. The stimulation of the DNA-dependent ATPase activity of protein n (replication factor Y of Hurwitz and co-workers (2)) specifically by ϕX DNA[5] (24) suggests such a role for this host replication protein.

The presence of *dnaB* protein in the ϕX replication intermediate complex enables this priming system to make repeated initiations on the circle. A previous study (7) indicated that *dnaB* protein, once fixed in the intermediate, migrates along the binding protein-coated DNA strand, perhaps utilizing ATP hydrolysis to energize its movement. The single *dnaB* molecule in the intermediate participates (in the absence of DNA replication) in initiating several primers which were found at regular intervals along the template (7). *dnaB* protein may act as a mobile signal for primase action and, as such, promotes primer synthesis at almost any site on the ϕX template. Assuming a similar function for *dnaB* protein in replication of the bacterial chromosome, we propose that *dnaB* protein migrates in a processive fashion along the newly exposed "lagging" strand behind the replicating fork (in the direction of fork movement) (7). *dnaB* protein or the secondary structure of templates it generates, is recognized by primase for initiation of primer synthesis for nascent (Okazaki) fragments of the discontinuously synthesized strand of the *E. coli* chromosome. The *dnaB* protein·template complex would be a signal to primase that this single-stranded DNA is engaged in replication rather than prepared for recombination, repair, or another metabolic process involving single-stranded DNA.

The recent finding that *dnaB* protein and DNA polymerase III, but not primase, appeared to be tightly associated with replication forks on an *E. coli* chromosome (25) is consistent with such a model for dnaB protein function. *dnaB* protein, once bound, might travel with the replication fork without dissociating until the round of replication was completed. However, premature dissociation of the *dnaB* protein from the replicating DNA might be a lethal event if there were no means for rapidly restoring *dnaB* at or near the replication

[4] Lack of an antibody directed against *dnaC* protein has prevented an analysis of *dnaC* protein function in ϕX SS replication as described for *dnaB* protein.

[5] Y. Shlomai and A. Kornberg, unpublished data.

fork. It seems plausible that *dnaC* protein and proteins i and n could participate in such a process based on their roles in the replication of ϕX174 DNA. Because of the processive nature of *dnaB* protein action, bacterial mutants defective in transferring *dnaB* protein to the replication fork might be expected to yield substantial residual DNA synthesis under restrictive conditions, but rounds of replication already in progress would not all be completed. Such mutants might have been erroneously classified as "leaky" elongation-defective mutants or possibly as replication origin initiation mutants. It is interesting to note that several *dnaC* mutants with this phenotype have been described (26).

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