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In Vitro DNA Synthesis by an α-Like DNA Polymerase Bound to Replicating Simian Virus 40 Chromosomes

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Simian virus 40 chromosomes carry out replicative DNA synthesis in vitro which is sensitive to aphidicolin and to *N*-ethylmaleimide, resistant to 2′,3′-dideoxythymidine-5′-triphosphate, and proportional to the amount of chromosome-associated α-like polymerase. Thus, an α-like DNA polymerase (α polymerase or δ polymerase) is responsible for in vitro DNA synthesis.

There is a large body of experimental evidence which suggests that an α-like DNA polymerase (either true α, which lacks an exonuclease, or δ, which has an associated exonuclease but resembles α in other respects [1, 6, 12]) is responsible for mammalian chromosomal DNA replication (2, 9, 17). However, this evidence does not exclude the possibility that an additional DNA polymerase may play a minor but essential role in the replication process.

Replicating simian virus 40 (SV40) chromosomes provide a valuable model system for studying mammalian DNA replication. Except for the initiation step, SV40 DNA replication is probably carried out by the same enzymes used for cellular chromosomal DNA replication (2). Replicating SV40 chromosomes can be extracted from the nuclei of infected cells and then partially purified by gradient centrifugation. The partially purified chromosomes are capable of limited DNA synthesis (4, 5, 19, 22), which appears to be replicative for the following reasons: (i) the DNA synthesized is SV40 DNA, not contaminating cellular DNA (4; see Fig. 1); (ii) the synthesis occurs in SV40 replicative intermediate molecules, not in form II or III molecules (4; see Fig. 1); (iii) the two size classes of DNA strands synthesized in vitro (4 to 16S and 2 to 4S strands) correspond to the two size classes detected during very short labeling periods in vivo (continuous strands and Okazaki pieces) (4, 5, 19, 22; see Fig. 3); and (iv) the 2 to 4S DNA synthesized by purified chromosomes in vitro is completely converted into 4 to 16S DNA and then partially converted into intact form I DNA when chased in vitro in the presence of soluble cell proteins, just as Okazaki pieces are chased into longer DNA strands in vivo (4, 5, 19, 22; M. A. Waqar, L. R. Davis, and J. A. Huberman, manuscript in preparation).

The ability of partially purified SV40 chromosomes to carry out replicative DNA synthesis suggests that some of the enzymes involved in DNA replication in vivo may remain bound to the replicating chromosomes during purification. In fact, the following enzyme activities have been detected in relative abundance in sucrose or glycerol gradient fractions containing replicating SV40 chromosomes: DNA polymerase α or δ (3, 7, 13, 14, 16, 22, 24), DNA polymerase γ (3, 17), thymidine kinase (26), a single-stranded DNA-binding protein (14), T antigen (18), uracil-DNA glycosylase (10), and poly(ADP-ribose) polymerase (15).

We wanted to determine whether the replicative DNA synthesis carried out in vitro by partially purified SV40 chromosomes is due to an α-like polymerase or, alternatively, whether this replicative DNA synthesis is due either to the chromosome-associated γ polymerase (3, 17) or to a different polymerase. To distinguish between these possibilities, we tested the sensitivity of in vitro DNA synthesis to the inhibitors aphidicolin, *N*-ethylmaleimide, and 2′,3′-dideoxythymidine-5′-triphosphate (ddTTP). The polymerases α, β, and γ respond in markedly different ways to these inhibitors (2, 9, 11, 17).

The sensitivity of in vitro DNA synthesis to aphidicolin was tested in the experiment described in the legend to Fig. 1. In this experiment, the products of in vitro DNA synthesis were analyzed by gel electrophoresis, ethidium bromide staining (left panels), and autoradiography (right panels). Notice that radioactivity was incorporated into molecules which migrated as a smear between the form I position and a position near the top of the gel. SV40-replicating DNA molecules migrated as a smear in this range (20, 21, 23; Waqar et al., manuscript in preparation). There was no significant incorporation into form I or form II DNA molecules despite their molar excess. Thus, the observed synthesis is not

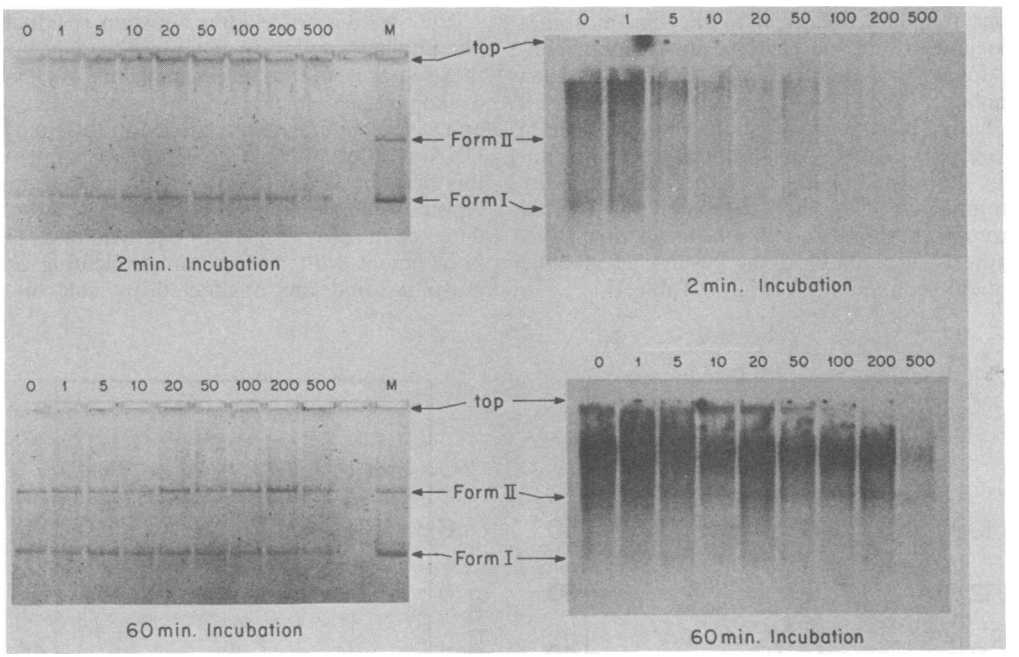

FIG. 1. Sensitivity of in vitro DNA synthesis by isolated SV40 chromosomes to aphidicolin. SV40 chromosomes were extracted (23) from 20 plates of infected CV-1 cells, and the replicating chromosomes were selectively purified by two cycles of glycerol gradient sedimentation (22), pooling fractions containing material which sedimented faster than the bulk of the nonreplicating chromosomes. The replicating chromosomes were concentrated and then incubated under conditions for in vitro DNA synthesis (5) with [α-^{32}P]dTTP (410 Ci/mmol) and with an increasing concentration (0 to 500 μg/ml) of aphidicolin, as indicated by the numbers over the lanes. Reactions were carried out for 2 or 60 min, as indicated. In additional experiments (not shown), reactions were carried out for 5, 10, and 20 min. The reactions were stopped with 0.6% sodium dodecyl sulfate–10 mM EDTA. Gel electrophoresis and fluorescent staining of the DNA with ethidium bromide were done as described previously (23). The left panels show the negative version of the fluorescence distribution. After staining, the gels were washed four times (2 h each) with cold 1 M HCl, followed by an additional overnight wash to remove unincorporated dTTP. The gels were rinsed with cold water to remove the acid and then dried on a gel drier. The dried gels were autoradiographed with Kodak XAR-5 film and an intensifying screen for 3 days. The developed autoradiograms are shown in the right panels.

simple repair synthesis, nor does this synthesis occur on aberrant replication structures (8).

When incorporation was carried out for a short time (2 min), it was strongly inhibited by aphidicolin, with 50% inhibition at an aphidicolin concentration between 10 and 20 μg/ml (determined by scanning). This concentration is similar to, but slightly higher than, the concentration required for 50% inhibition of conventionally purified (25) α polymerase in our laboratories (4 μg/ml).

In vitro DNA synthesis reaches a plateau after 5 to 15 min (4, 5). The same plateau could be reached at the reduced rates of incorporation produced by aphidicolin inhibition when a longer incubation time was used (data not shown). Thus, when incubation is carried out for 60 min, synthesis appears to be relatively resistant to aphidicolin (Fig. 1; M. A. Waqar, A. Soltyk, J. Plummer, M. J. Evans, and J. A. Huberman, Fed. Proc. **40**:1904, 1981).

In other assays we have found that in vitro DNA synthesis is sensitive (5% residual synthesis) to 5 mM *N*-ethylmaleimide, although not quite as sensitive as conventionally purified (25) α polymerase (0 to 2% residual synthesis). In addition, in vitro DNA synthesis is resistant to 20 μM ddTTP in the presence of 0.2 μM 2′,3′-deoxythymidine-5′-triphosphate (dTTP) (82 to 103% residual synthesis). Conventionally purified α polymerase is 70 to 80% resistant (in conventional α polymerase assays [25]) with these concentrations of ddTTP and dTTP. Thus, the response of in vitro DNA synthesis to the three inhibitors is very similar to the response of α polymerase and very different from the responses of β and γ polymerases (2, 9, 11, 17). Therefore, by the criterion of response to these three inhibitors, in vitro DNA synthesis is due to an α-like polymerase.

Is the α-like polymerase which is responsible for in vitro DNA synthesis (assayed with endog-

enous template) identical to the chromosome-associated α-like polymerase detected in conventional α polymerase assays (with exogenous template)? To answer this question we used increasing NaCl concentrations (Fig. 2; Table 1) or high pH (Table 1) to dissociate the chromosome-associated α-like polymerase from the chromosomes. We then incubated the treated chromosomes under conditions for in vitro DNA synthesis and analyzed the results by alkaline gradient sedimentation (Fig. 3; Table 1).

The striking correlation between residual α-like polymerase and residual in vitro DNA synthesis suggests that the chromosome-associated α-like polymerase detected in assays with exogenous template is responsible for this in vitro DNA synthesis (Table 1). Also consistent with this hypothesis are the observations that minor chromosome-associated polymerase activities detected in assays for β and γ polymerases (Fig. 2) sediment with mature, nonreplicating chromosomes and are unaffected by salt or pH

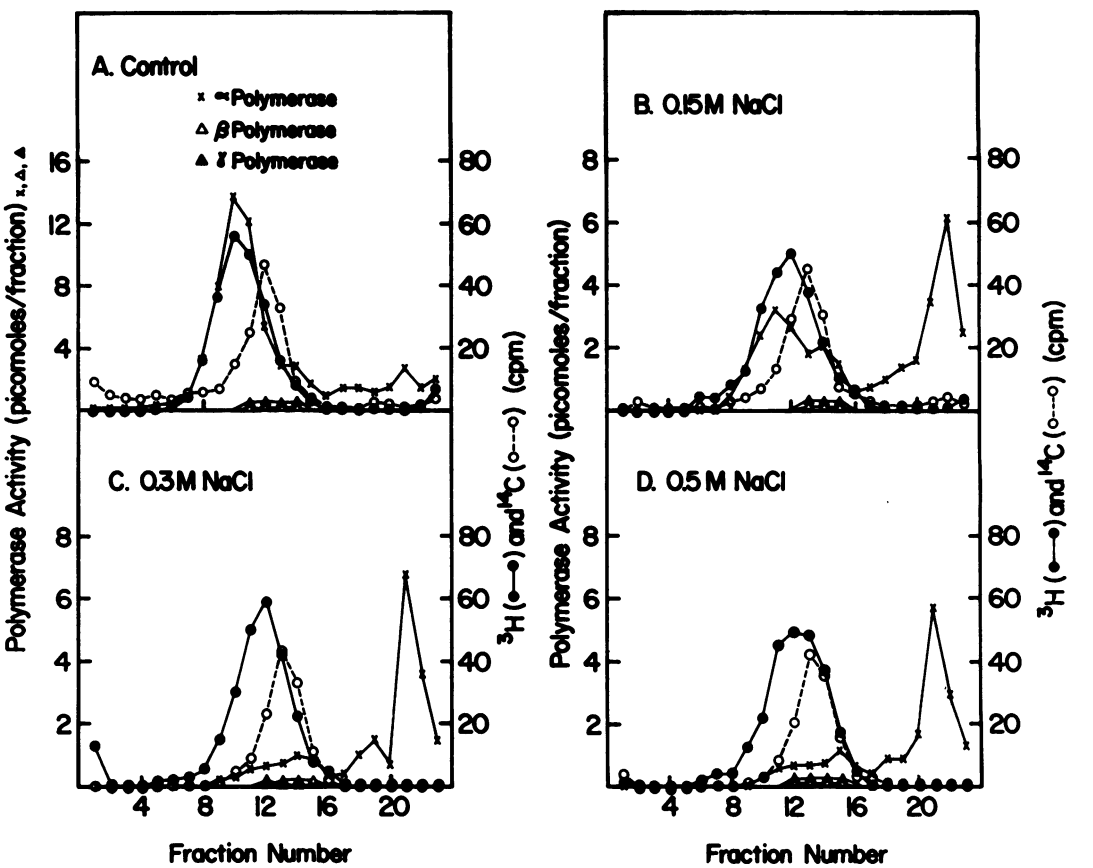

FIG. 2. SV40 chromosome-associated α-like DNA polymerase dissociated from the chromosomes by increasing concentrations of NaCl. Ten plates of SV40-infected CV-1 cells were labeled with [^{14}C]thymidine from 30 to 48 h after infection (0.3 μCi per plate; 57 Ci/mol). At 48-h postinfection, the medium was removed and replaced with 0.2 ml of fresh medium containing 50 μl of [^{3}H]thymidine (50 Ci/mmol), and incubation was continued at 37° for 5 min. The ^{3}H pulse was stopped by removal of the medium and addition of 10 ml of ice-cold phosphate-buffered saline. Chromosomes were immediately extracted and centrifuged through a glycerol gradient (22). Pooled replicating chromosomes were dialyzed against 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–5 mM KCl–1 mM EDTA–0.1 mM $MgCl_2$–0.5 mM dithiothreitol–5% glycerol, all at pH 7.0, and then were concentrated twofold by dialysis against dry Sephadex G-200. The concentrated chromosomes were split into aliquots, and concentrated NaCl was added to the final concentrations indicated in the figure. The chromosomes then were loaded onto 10 to 30% glycerol gradients in the same buffer. Centrifugation was for 135 min at 39,000 rpm in an SW41 rotor. Fractions were collected from the bottom. The ^{14}C label (○) shows the distribution of nonreplicating SV40 chromosomes; the ^{3}H label (●) shows the distribution of replicating SV40 chromosomes. Samples (25 μl) from each fraction were assayed for DNA polymerases α, β, and γ as described previously (25).

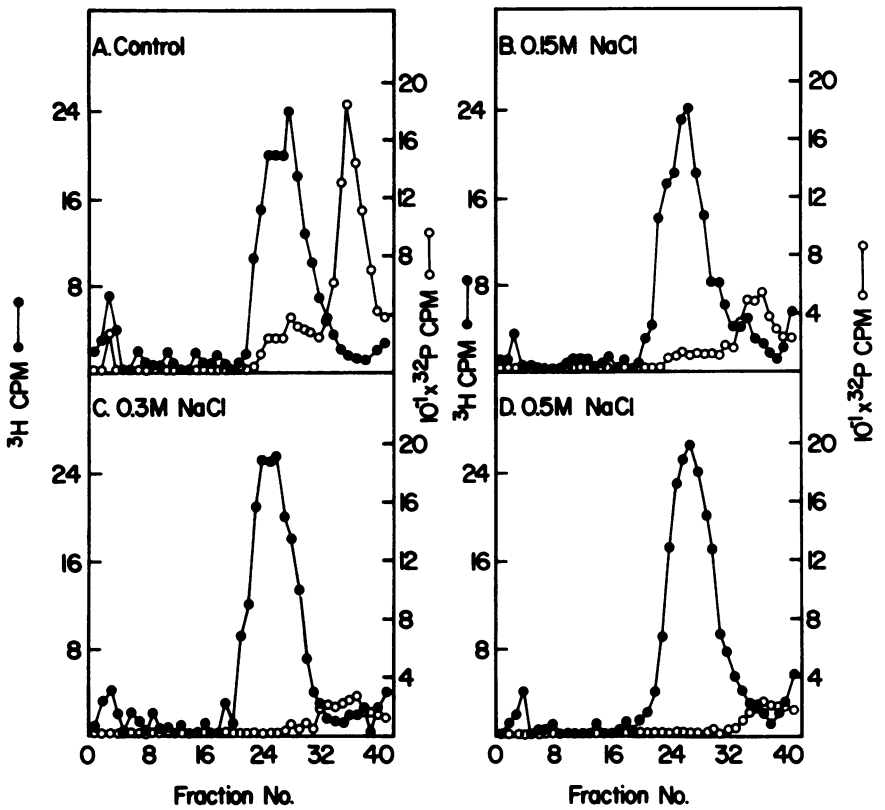

FIG. 3. Decrease in the extent of in vitro DNA synthesis by isolated SV40 chromosomes by exposure of the chromosomes to increasing concentrations of NaCl. Fractions containing replicating chromosomes were pooled from each of the gradients shown in Fig. 2 and concentrated. The chromosomes were assayed for in vitro DNA synthesis (5) with [α-^{32}P]dTTP (127.1 Ci/mmol). DNA synthesis was terminated by the addition of sodium lauryl sarcosinate (Sarkosyl) to 1%. Replicating SV40 DNA was partially purified by centrifugation through neutral sucrose gradients (5) and then was denatured and centrifuged through alkaline sucrose gradients (5). Fractions were collected from the bottoms of the alkaline gradients. Acid-precipitable radioactivity was measured for samples (25 μl) from each fraction. Notice that the ^{3}H profile shows the size distribution of nascent DNA strands labeled in vivo (leading edge at 16S), whereas the ^{32}P profile shows the distribution of DNA strands labeled in vitro (a peak at 2 to 4S due to Okazaki pieces and a shoulder with leading edge at 16S due to longer nascent strands).

treatment. The fact that other laboratories have detected a small amount of γ polymerase associated with replicating SV40 chromosomes (3, 13) may be due to minor procedural differences. Our results provide no indication that γ polymerase may play a role, even a minor one, in SV40 DNA replication.

Our findings do not distinguish between true α polymerase and δ polymerase because both enzymes respond to aphidicolin, *N*-ethylmaleimide, and ddTTP in a similar fashion. We attempted to determine whether any exonuclease activity cosediments in sucrose gradients with the α-like polymerase dissociated from SV40 chromosomes by salt treatment (see Fig. 2). So far our experiments are inconclusive; we cannot exclude the possibility that minor exonuclease activity may cosediment with this polymerase at 7.5 to 8S (data not shown). Therefore, the question of whether α polymerase or δ polymerase is responsible for in vitro DNA synthesis carried out by partially purified replicating SV40 chromosomes is a question which remains for the future.

TABLE 1. Correlation of the amount of chromosome-associated α-like DNA polymerase with the extent of DNA synthesis carried out in vitro[a]

Treatment of chromosomes	Polymerase activity (% of control)	In vitro DNA synthesis (% of control)
None	100	100
0.15 M NaCl	49	39
0.3 M NaCl	19	19
0.5 M NaCl	16	15
pH 10.0	20	17

[a] Pooled replicating chromosomes from each gradient in Fig. 2 (and from a similar gradient in which the pooled replicating chromosomes were adjusted to pH 10.0 with 27 mM glycine-sodium hydroxide buffer before sedimentation through a glycerol gradient at pH 7.0) were assayed for both α polymerase activity and in vitro DNA synthesis (Fig. 3). The in vitro DNA synthesis measurements were corrected for variation in recovery of material by expressing the results as the ratio of in vitro-incorporated ^{32}P counts per minute to in vivo-incorporated ^{3}H counts per minute. Control levels were as follows: 12.8 pmol of dTMP incorporated in the α polymerase assay and a $^{32}P/^{3}H$ ratio of 5.60 for in vitro DNA synthesis.

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