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Effect of *Xenopus laevis* oocyte extract on supercoiled simian virus 40 DNA: Formation of complex DNA*

(amphibian oocytes/*in vitro* DNA synthesis/electron microscopy/catenated molecules)

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ABSTRACT **A soluble cell-free extract derived from stage 6 *Xenopus laevis* oocytes is described. From supercoiled simian virus 40 DNA the extract produces nicked circles (having a single-strand scission), linear molecules of full unit size, shorter length fragments, and various forms of complex DNA.**

Several reports have described bacterial cell-free systems which are capable of mimicking the intact cell in replicating DNA (1, 2) and excising or integrating specific sequences (3, 4). An important characteristic of such extracts is that they are soluble; it is therefore possible to fractionate them into various components and to attempt to finally identify the role of each component.

Although the basic rules of DNA replication (5) seem to apply both to prokaryotes and eukaryotes, a soluble cell-free system able to replicate eukaryotic DNA has not yet been described.

Systems consisting of nuclei from cells infected by polyoma virus and their respective cytoplasms have been shown to replicate *in vitro* the viral DNA (6, 7). The particulate nature of these systems is such that it allows for the fractionating of only the cytoplasmic components; moreover, such systems function on an endogenous template.

We have been interested for several years in investigating various aspects of DNA metabolism in *Xenopus laevis* oocytes and eggs (8, 9), and in this paper we describe a soluble extract derived from *Xenopus laevis* oocytes. The extract is challenged with supercoiled simian virus 40, (SV40) DNA. The reasons for choosing this DNA are 2-fold: (*a*) SV40 DNA is simple and sufficiently characterized (1) to allow us to infer what the extract action may be through an examination of the products; (*b*) SV40 DNA is very similar to polyoma DNA, which when injected into *Xenopus laevis* eggs, is known to stimulate the incorporation of thymidine into trichloroacetic-acid-insoluble material (10). Moreover, Lasky and Gurdon (11) have shown, using the same system, that if heavy-light supercoiled polyoma DNA is injected, the density of part of the input DNA shifts to that of light double-stranded DNA, suggesting synthesis of new DNA strands.

When labeled supercoiled SV40 DNA is incubated with our extract, simple forms like open circular and linear molecules and a variety of complex forms are produced. The incorporation of labeled deoxytriphosphates directed by unlabeled SV40 DNA is also analyzed. This system should prove useful for the analysis of the process of DNA replication in eukaryotes.

Abbreviations: SV40, simian virus 40; TEMG, Tris–EDTA–mercaptoethanol–glycerol; E.M., electron microscope.

* The term *complex DNA* is used in this paper to refer to all structures described except supercoiled, open circular, and linear SV40 DNA

MATERIALS AND METHODS

Materials. Deoxynucleoside 5-triphosphates were obtained from Schwarz/Mann, Orangeburg, N.Y. Ribonucleoside 5-triphosphates were purchased from P. L. Biochemical Inc. [*Methyl*-^{3}H]thymidine (6.7 Ci/mmol) and [^{3}H]deoxyguanosine 5-triphosphate (8.5 Ci/mmol) were purchased from New England Nuclear.

Preparation of Nuclear and Cytoplasmic Extracts from *Xenopus laevis* Stage 6 Oocytes (12). Stage 6 oocytes were collected from the ovaries of adult frogs after defollicolation by collagenase treatment according to Schorderet-Slatkine (13). Germinal vesicles were isolated from the oocytes with watchmaker's forceps under a dissecting microscope in Barth's solution (14).

Each enucleated cytoplasm was immediately transferred into ice-cold TEMG buffer [50 mM Tris·HCl at pH 7.5; 1 mM ethylenediaminetetraacetate, 1.4 mM 2-mercaptoethanol, 20% (weight/volume) glycerol]. The germinal vesicles were directly transferred with a capillary pipette to a 0.6 ml nitrocellulose tube. All subsequent operations were carried out at 0–4°. About 100 nuclei were collected in 0.1 ml of Barth's solution; 0.5 ml of TEMG buffer was added and the nuclei were broken by pipetting a few times. The suspension was centrifuged in a Spinco SW 50.1 rotor with adaptors at 40,000 rpm for 60 min at 4°. The supernatant was collected and stored at −70°. The protein concentration of the nuclear extract, determined by the Lowry method, varied from 0.02 to 0.04 mg/ml in different preparations.

Cytoplasms from about 100 oocytes were collected in 1.5 ml of TEMG buffer and homogenized in a glass douncer. The homogenate was centrifuged at 8000 × *g* for 10 min and the supernatant, clean of the floating lipid layer, was brought to 1.2 ml of TEMG and centrifuged in two 0.6 ml nitrocellulose tubes as described above for the nuclear extract. The supernatant was stored at −70°. The protein concentration of the cytoplasmic extract in different preparations varied between 1.0 and 1.6 mg/ml.

Preparation of SV40 DNA. Confluent African green monkey CV-1 cells were infected at a multiplicity of 40 plaque-forming units per cell with plaque-purified SV40 virus and the DNA was extracted by the Hirt procedure (15) about 40 hr post-infection. Labeled SV40 DNA was prepared by adding [^{3}H]dThd (10 μCi/ml) to the infected cells between 24 and 40 hr after infection.

The supercoiled SV40 DNA was purified from the Hirt supernatant by isopycnic centrifugation in neutral cesium chloride (density 1.60 g/cm^3) containing 200 μg/ml of ethidium bromide. The fractions containing supercoiled DNA were pooled, dialyzed against Tris–KCl–EDTA buffer (10 mM Tris·HCl at pH 7.5, 0.15 M KCl, 1 mM EDTA), precipitated with ethanol, and redissolved in distilled water. The

supercoiled SV40 DNA was finally purified by sedimentation on a 15–30% neutral sucrose gradient. The fractions corresponding to the peak of the material sedimenting at 21 S were pooled, precipitated with ethanol, dissolved in distilled water, and stored at −20°. In some preparations the SV40 DNA was purified from the Hirt supernatant by phenol extraction and ethanol precipitation and purified directly on a sucrose gradient.

Neutral Sucrose Gradients. Sucrose linear gradients (5–20% and 15–30%) contained 0.1 M NaCl, 10 mM Tris·HCl at pH 7.5, 10 mM EDTA at pH 8.0. Centrifugation conditions are specified in the legends under the figures. Each gradient was layered on a 0.5 ml 2 M sucrose cushion.

Alkaline Sucrose Gradients. Sucrose linear gradients (5–20%) contained 0.25 M NaOH, 0.75 M NaCl, 10 mM EDTA. Centrifugation conditions are specified in the legends under the figures.

Incubation of SV40 DNA with Nuclear and Cytoplasmic Extracts. Unless otherwise stated, assays contained in a total of 100 μl: 50 mM Tris·HCl at pH 7.5, 100 μM dATP, dCTP, dGTP, dTTP, 20 μM ATP, GTP, UTP, CTP, 6 mM $MgCl_2$, 6 mM dithiothreitol, SV40 [^{3}H]DNA (10^4 cpm/μg) in amounts specified under the figures, 25 μl of nuclear and 25 μl of cytoplasmic extracts. After incubation for 30 min at 30° the reaction was stopped by addition of sodium dodecyl sulfate and EDTA at the final concentrations of 0.5% and 15 mM respectively, and the mixture was applied to a gradient. Occasionally the incubation mixture was extracted with phenol before being centrifuged. When unlabeled SV40 DNA was used as template dGTP was substituted with 20 μM [^{3}H]dGTP.

Electron Microscopy of SV40 DNA Molecules. Ethanol-precipitated SV40 DNA from the sucrose gradient fractions or from phenol-extracted reaction mixture was dissolved in 0.1 M Tris·HCl at pH 7.9 and spread by the aqueous procedure of Davis *et al.* (16). The grids were stained with uranyl acetate and rotary shadowed at 6–8° with platinum. Micrographs were taken under a Philips 300 Electron Microscope.

RESULTS

Action of the oocyte extract on SV40 DNA

To produce the extract, stage 6 oocyte nuclei were manually isolated and separate extracts from nuclei and from enucleated cytoplasms were prepared as indicated under *Materials and Methods*. The nuclei–cytoplasms extract consists of equal volumes of nuclear and cytoplasmic extracts. In our experiments we used labeled supercoiled SV40 DNA. This well-characterized DNA allows for the detection of activities in the extract by studying the fate of the input supercoiled DNA using sedimentation in sucrose gradients and the electron microscope (E.M.).

Fig. 1 shows that the input supercoiled SV40 DNA, sedimenting at 21 S, when incubated for 30 min at 30° with the nuclei–cytoplasms extract in the presence of the standard mixture (see *Materials and Methods*) is converted to: (*a*) Species sedimenting at the bottom of the gradient. The sample was deproteinized before being applied to sucrose gradient, and therefore the sedimentation properties of this material suggest that it contains oligomeric and/or catenated SV40 DNA (17, 18). (*b*) Species sedimenting at 16 S, like open circular or relaxed circular structures which may be the product of nicking or of relaxation. (*c*) Species sedimenting at approximately 14.5 S like full-length linear SV40 DNA.

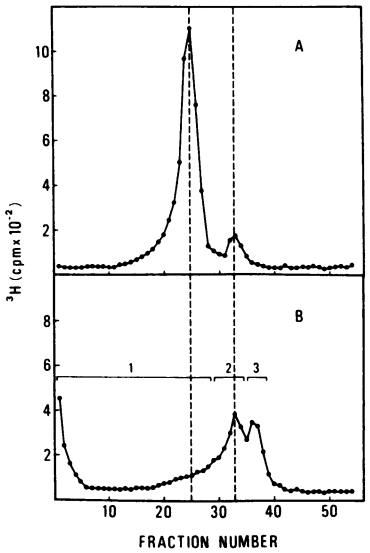

FIG. 1. SV40 [^{3}H]DNA component I (supercoiled, 21 S) (3 μg, 28,000 cpm) was incubated under the conditions specified in *Materials and Methods* without (A) and in the presence of (B) nuclei-cytoplasms extract. Sedimentation was on 15–30% neutral sucrose gradients in a Spinco SW41 rotor at 35,000 rpm at 4° for 16 hr. A 30 μl aliquot from each 0.120 ml fraction was precipitated with trichloroacetic acid and its radioactivity was determined. The remaining DNA was pooled into fractions 1, 2, and 3 as shown, precipitated with ethanol, and examined with the E.M.

We found that the presence of Mg^{++} in the incubation mixture was required for the formation of all the species described above. The species sedimenting at the bottom of the gradient were not observed when either a nuclear or a cytoplasmic extract was used separately instead of the nuclei–cytoplasms extract.

Analysis of the products

To further investigate the nature of the various products the fractions of the gradient were combined in three different pools, marked pool 1, 2, and 3, respectively, as indicated in Fig. 1B.

Pool 1, when analyzed by E.M., showed an extraordinary repertoire of structures, such as: figure 8 dimers, catenated dimers, circular dimers, catenated trimers, Cairns' structures (Fig. 2e), complex multimers, and circular monomers with tails. Table 1 shows the list of the structures found and their frequency of occurrence.

Total DNA from the incubation mixture observed at the E.M. before sucrose gradient fractionation contained 15% complex DNA computed as monomeric units, 35% open circles, 30% full-length linear molecules, and 5% fragments.

The material contained in pool 1, treated with the restriction enzyme *Eco*RI, which is known to cut SV40 DNA at a unique site (12), was converted to species sedimenting sharply at 14.5 S, like full-length linear SV40 DNA (data not shown). This finding indicates that the DNA contained in pool 1 consists of complex SV40 DNA and that there is no other kind of DNA associated with the viral molecules. Fig. 2 shows some typical examples of the complex forms of SV40 DNA present in pool 1. Fig. 2g shows a lariat in which the contour length of the tail is within the normal range of SV40 unit length monomer. This structure could arise by breakage of a Cairns' structure (19). We have also found lariats with tails shorter than monomer unit length (Fig. 2f) and lariats with tails longer than monomer unit length. The

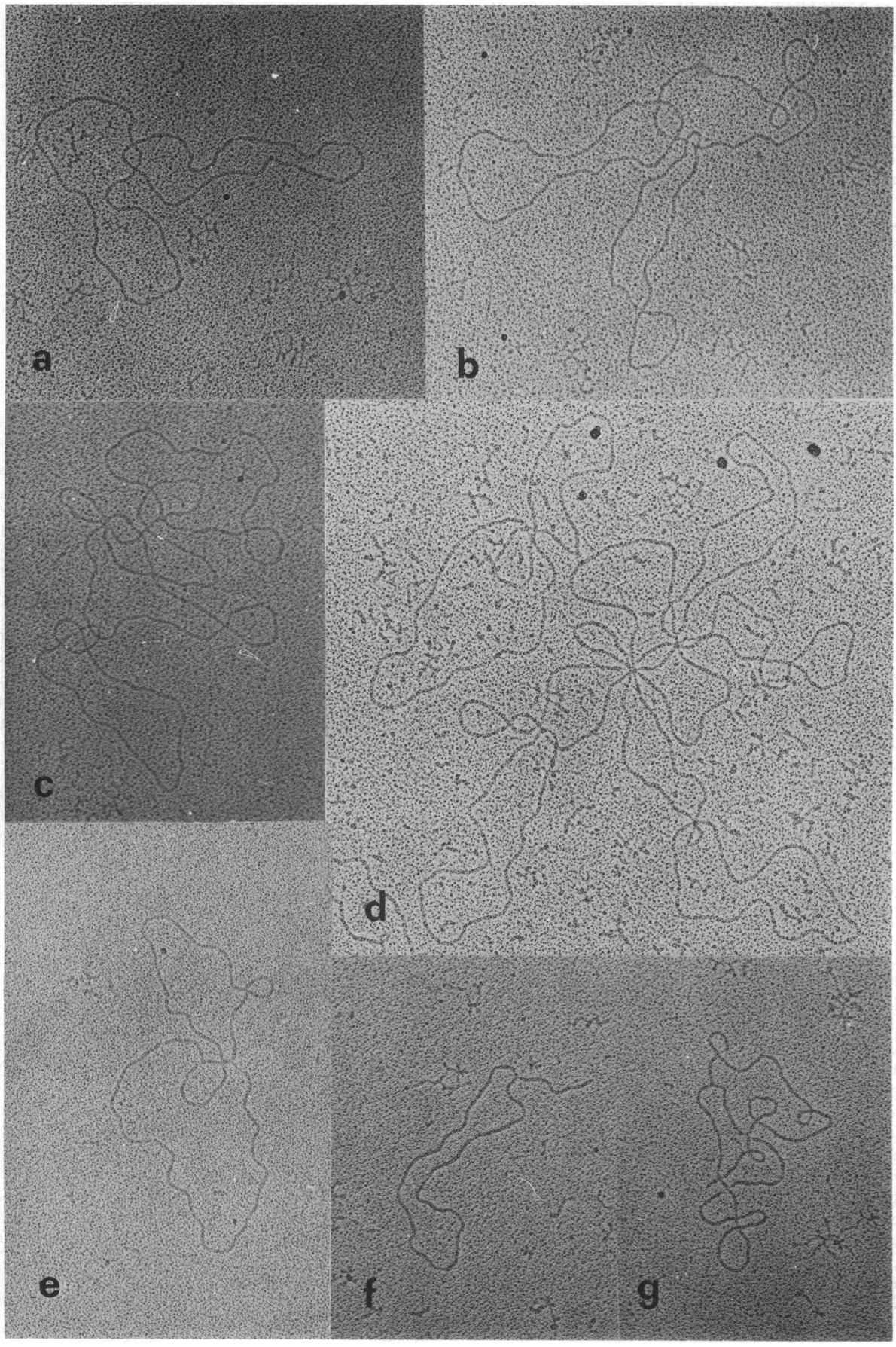

FIG. 2. Electron micrographs of complex SV40 DNA: (a) catenated dimer; (b) catenated trimer; (c) catenated tetramer; (d) complex concatemer; (e) Cairns' structure; (f) rolling circle; (g) rolling circle. ×15,000.

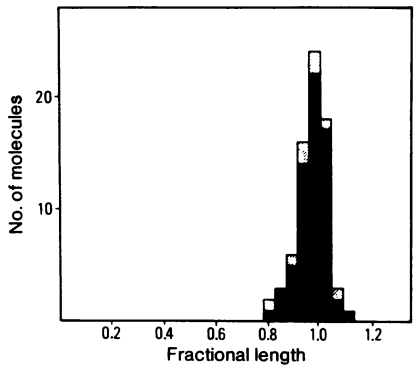

FIG. 3. Histograms of length measurements of open circular SV40 DNA molecules contained in pool 2 (diagonal stripe) and linear molecules contained in pool 3 (black), mounted on the same grid.

latter type of structure is most easily explained by the occurrence of displacement synthesis. However, the exact mechanism by which these various forms of SV40 DNA are generated is not yet known.

As shown in Fig. 1A, the heavy sedimenting material constituting pool 1 is not present in the template DNA which has been incubated without the extract. The input DNA was also examined by E.M. after nicking by pancreatic DNase and only two out of 2000 molecules observed could possibly consist of complex DNA.

The Cairns' structures are the most interesting forms we have observed, since it is reasonably certain that they are intermediates in replication (19, 20). However, in these experiments we have observed too few of them to conclude that they are formed *de novo* during incubation.

Pool 2 material when examined by E.M. was confirmed to consist of circular structures; their contour length measurements are reported in Fig. 3. Pool 2 material was analyzed in an alkaline sucrose gradient and Fig. 4 shows that two peaks, sedimenting at 18 S and 16 S, were found in approximately equimolar amounts. The material sedimenting at 18 S and 16 S corresponds to single-strand circular and single-strand linear SV40 DNA, respectively; therefore, we can conclude that the material sedimenting at 16 S in a neutral sucrose gradient (Fig. 1B) consists mainly of open circular molecules derived by the action of a nicking enzyme.

E.M. of pool 3 material confirmed that 95% of the molecules consist of full-length linear SV40 DNA; 5% of the molecules consist of fragments shorter than unit length. To establish that the linear molecules contained in pool 3 are really full length we have examined a mixture of pool 2 and pool 3 in the E.M. Comparative length measurements of the linear molecules with the circular ones on the same grid established that the linear products have the same contour length as the circular viral DNA (Fig. 3).

Table 1. Electron microscopy of molecules found in pool 1

Structure	No. molecules observed
Figure 8	20
Concatenated dimers	100
Dimers	50
Concatenated trimers	50
Concatenated tetramers	5
Concatenated pentamers	5
Concatenated hexamers	5
Complex concatemers	200
Rolling circles	15
Cairns' structures	5

Complex structures were counted following the criteria established by Gordon *et al.* (25).

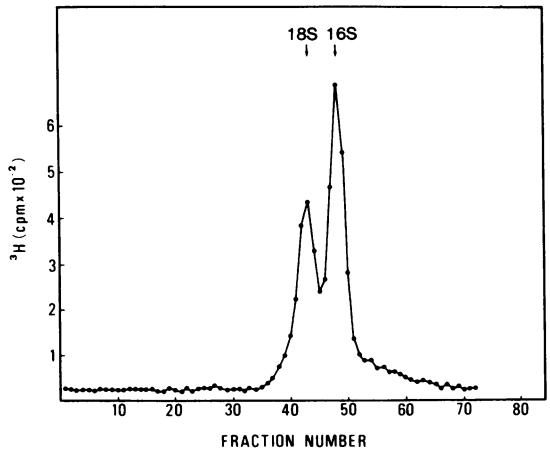

FIG. 4. Alkaline sucrose gradient (5–20%) of pool 2 material (see Fig. 1B). Pool 2 material derived from a gradient like the one shown in Fig. 1B was ethanol precipitated, dissolved in 100 μl of 0.25 M NaOH, 0.1 M NaCl, 1 mM EDTA and centrifuged in a SW 41 rotor at 33,000 rpm at 4° for 15 hr. Fractions (80 μl) were collected, acid precipitated, and counted. Arrows indicate the position of 18S single-strand circular and 16S single-strand linear molecules run separately as markers.

The purification of the activity (activities) responsible for the formation of the open circular DNA and of the full-length linear is not yet complete. It is also desirable to locate on the SV40 map (21) the site(s) at which the viral DNA is cut to generate the full-length linear molecules. Denaturation mapping (22) of full-length linear molecules produced by partially purified enzyme(s) indicates that the sites of cleavage are not randomly distributed.

Incorporation of labeled deoxynucleoside triphosphates

In another series of experiments we have incubated unlabeled SV40 supercoiled DNA with a mixture in which one of the deoxynucleoside triphosphates was radioactive. Our cell-free system is completely DNA dependent, i.e., there was no detectable incorporation of deoxynucleoside triphosphates into acid-insoluble material when DNA was not added. Fig. 5A shows a neutral sucrose gradient profile of the labeled product. Radioactive material can be seen to sediment in the heavy region of the gradient, as well as at the position of relaxed circles and of linear molecules. In other experiments we observed also a broad peak of radioactivity sedimenting in lighter parts of the gradient. E.M. observation of pools of the various fractions of the gradient confirmed the presence of the same type of molecules described in the experiment referred to in Fig. 1. We do not know if some of these structures are preferentially labeled.

Fig. 5B shows the results of an analysis in alkaline sucrose of a 30 min incorporation product. We observed a peak sedimenting at 16 S corresponding to full-length linear strands as well as material sedimenting in the lighter part of the gradient and material sedimenting at the bottom. The latter, analyzed in an alkaline sucrose gradient run for a shorter time

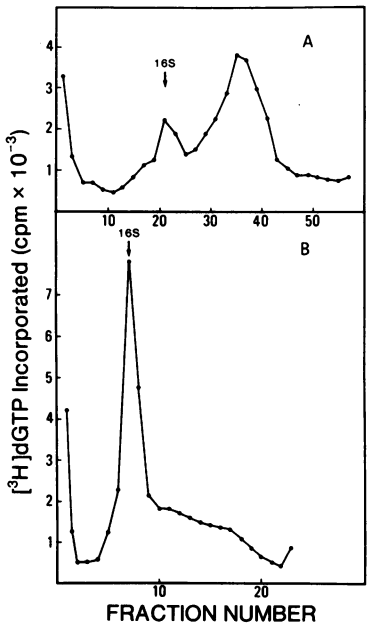

FIG. 5. Incorporation of [^{3}H]dGTP directed by unlabeled SV40 DNA. (A) About 8 μg of nonradioactive SV40 DNA I was incubated under standard conditions in a total volume of 200 μl for 60 min at 30° and the incubation mixture was applied to a 5–20% neutral sucrose gradient centrifuged in an SW 41 rotor at 35,000 rpm at 4° for 14 hr. A 100 μl aliquot from every other 250 μl fraction was acid precipitated and counted. The remaining fractions were pooled, ethanol precipitated, and examined with the E.M. (B) Unlabeled SV40 DNA I (1 μg) was incubated under standard conditions for 30 min at 30° and the incubation mixture was applied to a 5–20% alkaline sucrose gradient and centrifuged in a SW 50.1 rotor at 31,000 rpm at 21° for 12 hr. Fractions were acid precipitated and counted.

(data not shown), consists of species sedimenting at 53 S corresponding to closed circles of SV40 DNA and faster sedimenting species corresponding presumably to complex DNA. When we analyzed in alkaline sucrose gradient 10 min products we observed a distinct peak sedimenting at 4–5 S. Attempts to chase the 4–5S material into larger pieces gave variable results.

DISCUSSION

The soluble extract described in this paper appears to produce nicked circles, linear molecules of full unit size, shorter length fragments, and various forms of complex DNA from SV40 supercoiled DNA.

Complex DNA could originate by replication or by recombination.

It would obviously be extremely interesting if the catenated molecules originated by replication. Our system might be very efficient in initiation and chain elongation, but inefficient in termination and separation of replicated structures. This situation would lead to the formation of very complex multimers like the ones we have described.

It may be possible to manipulate the system in order to observe a more relevant proportion of Cairns' structures.

It has still to be determined which is the best template to feed the system.

We have performed a few experiments using chromatin (23) instead of supercoiled SV40 DNA. We have studied the incorporation of labeled deoxynucleoside triphosphates and our constant finding has been that labeled small pieces (4–5 S) of newly synthesized DNA are released from the template and subsequently degraded. It is possible that the proper template to use in an *in vitro* system consists of relaxed circles obtained by "untwistase" treatment (24).

We would like to stress again that our results do not exclude the possibility that complex DNA is formed by recombination. The fact that our system is soluble should permit the fractionation of the various components and the investigation of their relative roles.

When this manuscript was in preparation, Benbow and Laskey informed us that they succeeded in initiating polyoma DNA synthesis *in vitro* with a *Xenopus laevis* egg extract.

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