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Inhibition in the Joining of DNA Intermediates to Growing Simian Virus 40 Chains

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Viral DNA synthesis was inhibited for 1 h by the addition of 5-fluorodeoxyuridine (FUdR) to simian virus 40 (SV40)-infected cultures at 28 to 30 h postinfection. The subsequent addition of ^{3}H-thymidine to the inhibited cultures reverses the effect of the inhibitor, and during a 1-min labeling period there is rapid synthesis of SV40 DNA. By alkaline sedimentation analysis, it is observed that in FUdR-treated cultures there is synthesis of 4*S* SV40 DNA intermediates but there is a block in the joining of these intermediates to growing SV40 chain cultures. In addition to 4*S* fragments that are associated with replicating SV40 molecules, there is accumulation of SV40 DNA in the 6 to 8*S* region which is observed in neutral sucrose gradients. In an inhibited culture that is pulsed for 1 min with ^{3}H-thymidine and then chased for 10 min, accumulation of a Component II (Comp. II)-like material is observed. This Comp. II has the same neutral sedimentation characteristics and yields the same R_I restriction endonuclease product as does authentic Comp. II. However, in alkali it is seen that it is composed of fragmented SV40 DNA. The basis for the failure of 4*S* fragments to join to growing SV40 chains is discussed. A model in which there is a requirement for two DNA polymerases and a ligase to permit SV40 DNA chain growth is proposed which is consistent with the data presented.

We previously reported that chain growth of simian virus 40 (SV40) DNA occurred by a discontinuous mechanism (4). When infected cultures were pulsed for 15 or 30 s and then examined by alkaline sedimentation, a discrete peak of 4*S* SV40 DNA intermediates could be identified. In pulse-chase experiments they were incorporated into growing SV40 chains that were contained in replicating molecules (4). By analogy with bacterial systems, they are comparable to "Okazaki" fragments (13, 14) although they are considerably smaller (4*S*) than the Okazaki fragments observed in bacterial systems.

In the present study we have examined the consequences of a transient period of inhibition of DNA synthesis on subsequent rounds of SV40 DNA synthesis. We have observed that after a 1-h period of DNA inhibition followed by reversal of the inhibitor, there is accumulation of 4*S* SV40 DNA intermediates, but there is a failure in the joining of these to growing chains. These conditions have provided a system in which the process of SV40 DNA chain growth has been investigated.

MATERIALS AND METHODS

Primary African green monkey cells were grown in 150-mm plastic petri dishes in Eagle medium with 2 mM glutamine and 10% fetal calf serum. Confluent monolayers were infected with SV40 at an input multiplicity of 50 to 100 PFU per cell as previously described (17, 21).

FUdR treatment. At 28 or 29 h postinfection, 10^{-5} M 5-fluorodeoxyuridine (FUdR) was added to the medium. After a 1-h exposure to the inhibitor, the medium was removed and replaced with 8 ml of serum-free medium containing 100 μCi of ^{3}H-thymidine per ml. The length of the pulse period varied in different experiments and is described below. In pulse-chase experiments, the pulse medium was removed, the cell monolayer was washed three times with warm medium, and 30 ml of spent medium containing 2×10^{-6} M cold thymidine was added to each petri dish. All pulse and pulse-chase experiments were terminated by washing the cell monolayer three times with cold phosphate-buffered saline. The cells were lysed with 0.6% sodium dodecyl sulfate, and the low-molecular-weight (LMW) DNA fraction which contains the pulse-labeled SV40-replicating molecules was selectively extracted by using the procedure described by Hirt (6). The LMW DNA fraction was dialyzed and concentrated by vacuum dialysis (21).

In experiments requiring an amount of material sufficient for preparative gradients, the LMW DNA fraction was prepared from four to six petri dishes; dialyzed overnight against 0.01 M Tris, 0.01 M EDTA, and 0.05 M NaCl (pH 7.2); treated with predigested Pronase (200 μg per ml) for 1 h at 37 C; and extracted one time with an equal volume of water-saturated phenol. After exhaustive dialysis to remove the phenol, the LMW DNA was concentrated by vacuum dialysis. Treatment with Pronase and phenol did not alter the sedimentation pattern of the LMW DNA.

FUdR-cycloheximide treatment. FUdR (10^{-5} M) was added to infected cells as described above. Fifty-five minutes later, cycloheximide, at a final concentration of 100 μg per ml, was added to the same culture. Five minutes after the addition of cycloheximide, the cell monolayer was washed three times with warm medium containing 100 μg of cycloheximide per ml. The cells were then refed with 30 ml of spent medium containing cycloheximide, 100 μg/ml, and 2×10^{-5} M thymidine. Following a 30-min incubation period, the cells were washed three times with warm serum-free medium and pulsed for 1 min at room temperature with 8 ml of serum-free medium containing 100 μCi of ^{3}H-thymidine per ml. The LMW DNA fraction was extracted as described earlier.

Velocity sedimentation. The conditions for neutral and alkaline sucrose gradient velocity sedimentation were the same as those previously described (17, 21). (Details for each sedimentation analysis are given in the figure legends.)

Polynucleotide ligase assay. The conversion of SV40 Component II (Comp. II) to Comp. I, has been used to measure the level of ligase activity present in extracts of SV40-infected AGMK cells and in infected cells which have been exposed to 10^{-5} M FUdR for 1 h (16). ^{14}C-Comp. II was prepared by incubating ^{14}C-SV40 Comp. I with 5×10^{-4} μg of pancreatic deoxyribonuclease per ml until there was a 60% conversion of Comp. I to Comp. II (16). Comp. II was isolated on a neutral sucrose gradient.

Cellular extracts were prepared by lysing the cells in a hypotonic buffer containing 0.02 M Tris, 0.001 EDTA, and 0.005 M 2-mercaptoethanol, pH 8.0 (16). Extracts were stored at −70 C. Before the enzyme assay, the extracts were thawed and centrifuged at $7,000 \times g$ for 10 min, and the supernatant fluid was used in the assay.

The polynucleotide ligase reaction mixture contained 0.04 M Tris (pH 7.7), 0.01 M $MgCl_2$, 0.01 M 2-mercaptoethanol, 10^{-4} M ATP, 0.1 M KCl, 0.1 μg of ^{14}C-SV40 Comp. II, and crude cell extract (5–10 μg of protein) in a final volume of 0.1 ml. The reaction mixture was incubated at 30 C for 25 min, and the reaction was stopped by the addition of EDTA to a final concentration of 0.5 M. The proportions of Comp. I and II were determined in alkaline sucrose gradients (16).

RESULTS

Accumulation of "precursor" molecules following reversal of DNA inhibition. DNA synthesis was inhibited by the addition of FUdR to an infected culture for 1 h at 28 h postinfection. Then ^{3}H-thymidine (100 μCi/ml, 20–40 mCi/μmol), which reverses the effect of the inhibitor, was added for 1 min and the LMW fraction was obtained. The distribution of DNA in this LMW fraction is shown in Fig. 2 after velocity sedimentation for 17 h in an alkaline sucrose gradient. It is compared with an infected culture that has not been treated with FUdR and which has been analyzed in the same way (Fig. 1). In the uninhibited culture (Fig. 1) a bimodal distribution is observed. There is a distinct peak of 4S molecules, and their role as intermediates in growing SV40 chains has been described (4). The labeled DNA molecules that sediment between 6S and 16S are growing SV40 chains to which labeled 4S fragments have been attached. When FUdR has been present for 1 h prior to pulsing with ^{3}H-thymidine, a striking alteration in the alkaline sedimentation profile

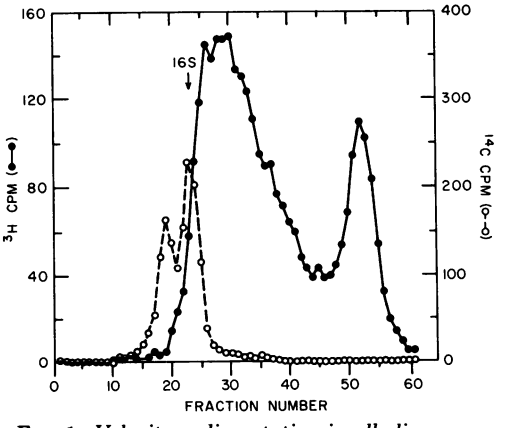

FIG. 1. *Velocity sedimentation in alkaline sucrose of the LMW DNA labeled during a 1-min pulse with ^{3}H-thymidine. An SV40-infected AGMK confluent monolayer was contained in a 150-mm Falcon petri dish. It was pulsed at 29 h postinfection for 1 min at 24 C with 8 ml containing 100 μCi of ^{3}H-thymidine per ml (20–40 μCi/μmol). The LMW DNA was selectively extracted with 0.6% sodium dodecyl sulfate according to the Hirt procedure (6). After dialysis against 0.01 M Tris, 0.01 M EDTA, 0.05 M NaCl (pH 7.2), the LMW DNA was concentrated and a portion was layered onto an 11.8-ml, 10 to 30% alkaline sucrose gradient containing 0.7 M NaCl, 0.3 M NaOH, 0.01 M Tris, 0.001 M EDTA, and 0.015% Sarkosyl. The sample was centrifuged at 10 C for 17.5 h at 40,000 rpm in an SW41 rotor. ^{14}C-SV40 DNA Comp. II was added to the gradient as a sedimentation marker and gave two peaks of radioactivity, 16S for the linear single-stranded DNA and 18S for the single-stranded circular DNA (22). Sedimentation is from right to left. Tubes were punctured at the bottom, and fractions were collected into scintillation vials, neutralized by the addition of 4 drops of glacial acetic acid, and counted as previously described (17).*

is observed (Fig. 2). There is significant DNA synthesis, but only molecules that sediment like SV40 precursor molecules are observed. In uninfected cells the LMW fraction does not contain significant amounts of labeled DNA when the cells were labeled with ^{3}H-thymidine for 1 min either in the absence of, or after treatment with, FUdR.

Continuous labeling of the LMW fraction following reversal of DNA inhibition. Two possibilities were considered as likely bases for the accumulation of 4*S* SV40 fragments in cells that had been treated with FUdR. One possibility was that during the 1-min ^{3}H-thymidine pulse that followed FUdR treatment, the predominant reaction was initiation of a new round of DNA synthesis. Under these conditions ^{3}H-thymidine would be incorporated into a population of 4*S* precursor molecules that would correspond to a specific site of initiation (3, 11, 21) or in DNA strands obtained from young replicating molecules. A second possibility was that at the time the cells were pulse labeled they contained a pool of unlabeled molecules at all stages of replication, but the 4*S* fragments which were synthesized were not joined to the growing strands. To test these proposals, at 28 h postinfection, cultures were treated with FUdR for 1 h, and then labeled with ^{3}H-thymidine for either 30 s or 1, 2, or 4 min. The LMW fraction was prepared and analyzed by alkaline velocity gradient centrifugation (Fig. 3). It can be seen that after 30 s labeled DNA sediments as 4*S* DNA and, on further labeling appears in DNAs which correspond to SV40 strands at all stages of maturation (Fig. 3). While the data in Fig. 3 clearly show the addition of 4*S* fragments to growing chains of all lengths, a comparison of Fig. 3 with Fig. 1 indicates that in the inhibited cultures a greater fraction of LMW DNA chains is present. This would suggest that initiation of new rounds of DNA synthesis may also be contributing a larger proportion of short DNA chains in the inhibited cultures than in the uninhibited infected cells. The data shown in Fig. 3 for the inhibited and reversed cultures do not permit a choice as to whether there is a decreased rate of synthesis of 4*S* fragments or a normal rate of synthesis with failure of the fragments to join to growing chains.

It has been previously shown that in neutral sucrose gradient analyses of infected cultures, after a 1- to 5-min pulse period with ^{3}H-thymidine, all of the radioactivity in the LMW fraction sediments in the 23 to 28*S* region (8, 17) and is contained in replicating SV40 DNA molecules. The neutral sucrose analyses of samples from an FUdR-inhibited culture pulsed for

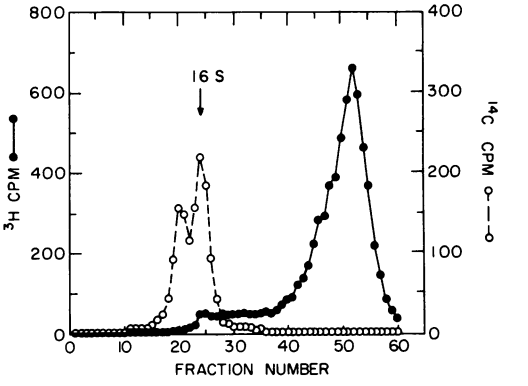

FIG. 2. *Velocity sedimentation in alkaline sucrose of LMW DNA isolated from an SV40-infected AGMK monolayer which has been treated, at 28 h postinfection, for 1 h with 10^{-5} M FUdR and pulsed for 1 min with 100 μCi of ^{3}H-thymidine per ml. The LMW DNA was extracted, dialyzed, and concentrated. A portion of the sample was sedimented in alkaline sucrose and counted. The procedures and conditions are those detailed in the legend for Fig. 1. However, the levels of incorporation cannot be compared in Fig. 1 and 2. FUdR treatment causes a depletion in the nucleotide pool. As a consequence, there is a more rapid uptake of ^{3}H-thymidine during the 1-min pulse period, since there is little dilution of the isotope which enters the cell by a pre-existing cellular pool.*

30 s or 1, 2, or 4 min and analyzed in neutral sucrose gradients are seen in Fig. 4. Two differences are noted between these samples (Fig. 4) and infected cultures which have not been treated with FUdR. One is the presence in the inhibited cultures of significant amounts of material that sediment more slowly than 8*S*. The second difference is the progressive accumulation of labeled DNA in the region corresponding to Comp. II. We have characterized the DNA sedimenting slower than 8*S* by its binding to hydroxyapatite under conditions where double-stranded DNA is absorbed to the column and single-stranded DNA is not absorbed (1). It was further characterized by the action of an exonuclease of *Neurospora crassa* which digests single-stranded DNA but does not effect double-stranded material (15). Under both conditions, 85 to 92% of this DNA behaved like single-stranded material. These data are similar to findings of Magnusson et al. (8a) in studies of polyoma replication in the presence of an inhibitor of DNA replication. In bacterial systems, single-stranded DNA is observed in corresponding regions of a neutral sucrose gradient of DNA which has been pulse labeled for 15 s (13, 14). This DNA which sediments more slowly than 8*S* has also been character-

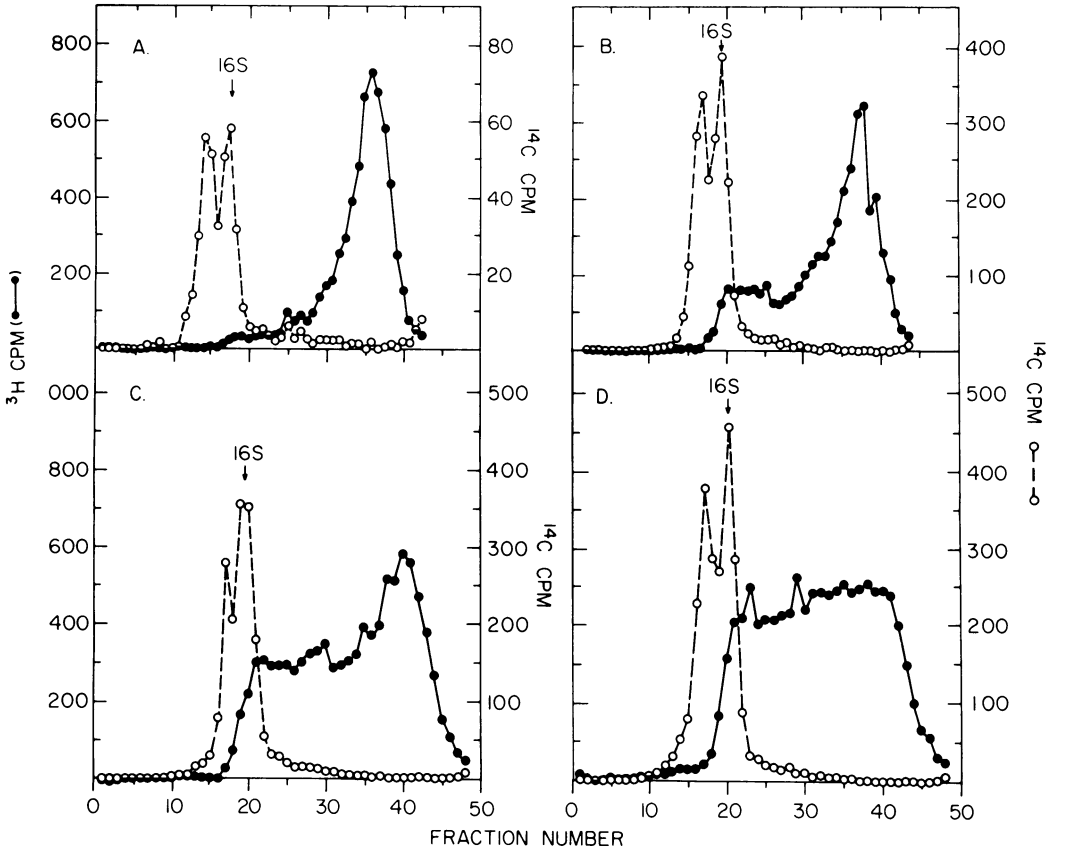

FIG. 3. *Velocity sedimentation in alkaline sucrose of LMW DNA isolated from four replicate cultures which were treated for 1 h with 10^{-5} M FUdR followed by ^{3}H-thymidine pulses of either 30 s (A), 1 min (B), 2 min (C), or 4 min (D). The conditions were the same as those described in Fig. 1. After dialysis and concentration, a portion of the LMW DNA was layered onto an 11.8 ml, 10 to 30% alkaline sucrose gradient and centrifuged at 10 C for 16.5 h at 40,000 rpm in an SW41 rotor. ^{14}C-SV40 DNA II was added to the gradient as a sedimentation marker. Sedimentation is from right to left. Fractions were collected in vials containing 0.5 mg of yeast ribonucleic acid and precipitated with 5% trichloroacetic acid. The precipitate was put on glass filter papers, washed with 5% trichloroacetic acid and with ethanol. Filters were dried and counted in Liquifluor.*

ized by hybridization to filters containing SV40 or monkey DNA. When the 6 to 8S DNA (3,390 counts/min) was added to filters containing bound DNA, 400 counts/min were hybridized to SV40 DNA and 43 counts/min to monkey DNA. Based on this partial characterization, the DNA in the 8S and under region of a neutral gradient is single-stranded SV40 DNA.

The results in Fig. 2 were obtained by analyses of all of the material contained in the LMW fraction. In Fig. 5 a characterization of the replicating molecules is shown after it has been separated by neutral sucrose sedimentation from material sedimenting slower than 22S. At 28 h postinfection a culture was inhibited for 1 h with FUdR and then pulse labeled for 1 min with ^{3}H-thymidine. The LMW sample was fractionated in a preparative neutral sucrose gradient. The fractions of the gradient which contained the replicating molecules were pooled, concentrated, and then centrifuged in an alkaline sucrose gradient. The results, seen in Fig. 5, indicate that almost all of the radioactivity contained in the replicating molecules is present in single-stranded DNA molecules that sediment as 4S molecules in alkaline gradients.

Accumulation of Comp. II in a pulse-chase experiment. Infected cultures that had been inhibited for 1 h with FUdR were pulsed for 1 min, then either harvested immediately or chased for 10 min in medium containing unlabeled thymidine, and then harvested. Results identical to those shown in Fig. 4B were obtained after neutral sedimentation analyses of the sample labeled for 1 min. The neutral sucrose analysis of pulse-labeled and chased

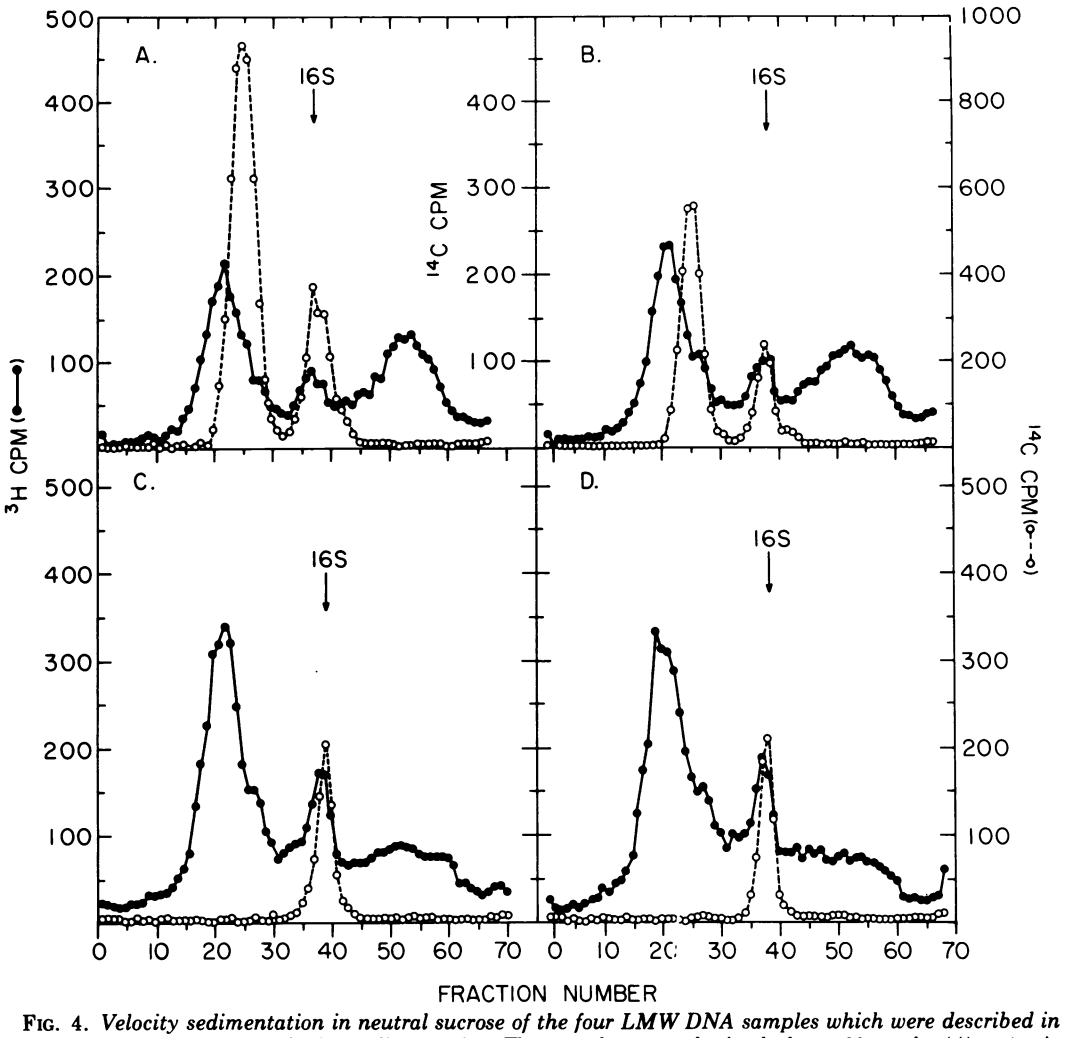

FIG. 4. *Velocity sedimentation in neutral sucrose of the four LMW DNA samples which were described in Fig. 3 and characterized by alkaline sedimentation. The samples were obtained after a 30-s pulse (A), a 1-min pulse (B), a 2-min pulse (C), or a 4-min pulse (D). Samples were layered onto an 11.8-ml, 5 to 30% neutral sucrose gradient containing 0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, final pH of 7.5. Gradients were centrifuged at 10 C for 16 h at 26,500 rpm in an SW41 rotor. Gradients A and B contain ^{14}C-SV40 DNA I and DNA II sedimentation markers; gradients C and D contain only ^{14}C-SV40 DNA II. Sedimentation is from right to left. Fractions were collected directly into scintillation vials and counted.*

DNA contained in the LMW fraction is shown in Fig. 6. Under similar pulse-chase conditions an infected uninhibited culture would contain replicating molecules and Comp. I and barely detectable amounts of Comp. II. The most striking difference in the inhibited culture is the accumulation of SV40 DNA co-sedimenting with Comp. II. From a parallel preparative gradient, a region corresponding to Comp. II was obtained and used for subsequent analyses. The material which co-sediments with authentic Comp. II is cleaved one time by the R_I restriction endonuclease and is converted to a linear duplex that sediments at 14.5*S* (Fig. 7). In this respect the material behaves in the identical manner as authentic Comp. I or Comp. II (3a). When this material is analyzed in an alkaline sucrose gradient (Fig. 8), it is clearly differentiated from Comp. II which under these conditions would be labeled almost exclusively in the 16*S* strand (3a). It is seen that the Comp. II, obtained from the pulse-chase experiment, is a highly fragmented molecule.

Sambrook and Shatkin have previously described ligase activity that is contained in extracts of uninfected and SV40-infected mon-

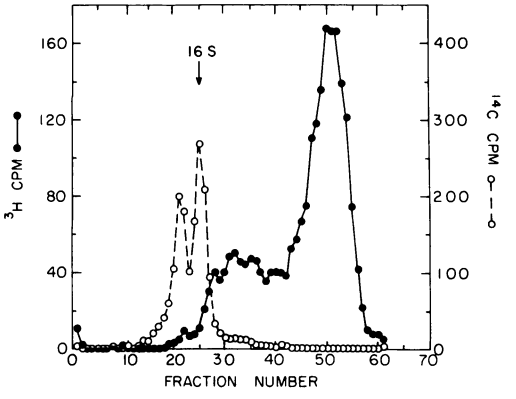

FIG. 5. *Velocity sedimentation in alkaline sucrose of SV40 replicative intermediate molecules. At 28-h postinfection, cultures contained in five petri plates were treated for 1 h with 10^{-5} M FUdR followed by a pulse of 1 min with ^{3}H-thymidine. LMW DNA was extracted and treated with Pronase and phenol as described in Materials and Methods. The sample was then dialyzed, concentrated to 0.25 ml, and layered onto an 11.8-ml, 5 to 30% neutral sucrose gradient. Centrifugation conditions were the same as those described in the legend for Fig. 4. The gradients were tapped, 0.25-ml fractions were collected, and a 10-μliter amount of each was counted. Replicating molecules which sediment in the 24 to 28S region of the gradient were pooled, dialyzed against 0.01 M Tris, 0.01 M EDTA, 0.05 M NaCl, (pH 7.2), and concentrated. The sample was layered on an 11.8-ml, 10 to 30% alkaline sucrose gradient and centrifuged at 10 C for 17 h at 40,000 rpm in an SW41 rotor. ^{14}C-DNA II was added to the gradient as a sedimentation marker. Sedimentation is from right to left. Fractions were collected directly into scintillation vials, neutralized by the addition of 4 drops of glacial acetic acid, and counted.*

key kidney cells (16). We have determined whether the DNA fragments contained in the Comp. II could be joined by the ligase that is present in the cell extracts. Under the assay conditions 40% of Comp. II (that was generated from Comp. I by the action of deoxyribonuclease) was converted to Comp. I. Under identical conditions, the Comp. II which accumulates in the inhibited cultures was not converted to Comp. I in detectable amounts, nor was there a detectable increase in the size distribution of fragmented SV40 DNA.

Protein synthesis is not required to reverse the inhibitory effects of FUdR. Two groups of infected cells were treated with FUdR for 1 h. At that time one set of plates was pulsed for 1 min with ^{3}H-thymidine. Cold thymidine (2×10^{-5} M) was added to the second set, and 30 min later the plates were washed and then pulsed for 1 min. The LMW fractions were prepared from both sets of plates at the end of the 1-min pulse period and were analyzed by alkaline sedimentation. As expected, the first set showed results like those in Fig. 2; that is, there was accumulation of 4S intermediates. However, in cells treated for 30 min with cold thymidine prior to the ^{3}H-thymidine pulse, the incorporation of ^{3}H-thymidine was the same as that observed in an uninhibited culture (e.g., Fig. 1). This established the fact that a 30-min treatment with thymidine was sufficient to reverse the inhibitory effects of FUdR. It was of interest to determine whether we could still reverse the inhibitory effects of the FUdR treatment under conditions where protein synthesis was blocked during the 30-min period that cold thymidine was present. The detailed protocol is given above. In outline, infected cells were treated with FUdR for 55 min; then for 5 min with FUdR and cycloheximide. New medium containing cold thymidine (2×10^{-5} M) and cycloheximide was added for 30 min, and finally the culture was pulse labeled for 1 min with ^{3}H-thymidine. Alkaline sedimentation analysis (Fig. 9) of the LMW fraction shows that even under conditions where protein synthesis is

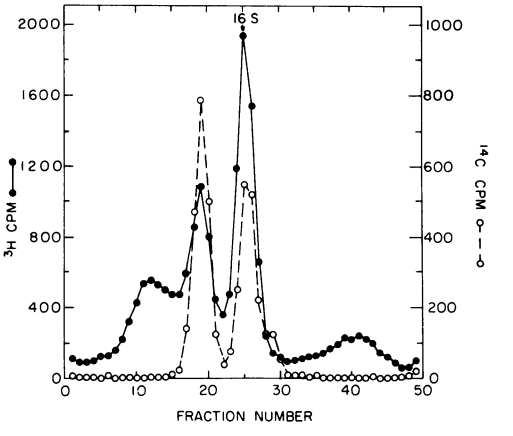

FIG. 6. *Velocity sedimentation in neutral sucrose of LMW DNA isolated from an infected culture which has been treated for 1 h with 10^{-5} M FUdR, at 28 h postinfection, pulsed for 1 min with ^{3}H-thymidine, and chased for 10 min with 2×10^{-5} M thymidine as described in Materials and Methods. The LMW DNA was obtained as previously described and layered onto an 11.8-ml, 5 to 30% neutral sucrose gradient and centrifuged at 10 C for 16 h at 26,500 rpm. ^{14}C-SV40 DNA I and DNA II have been added as sedimentation markers. Sedimentation is from right to left. Fractions were collected directly into scintillation vials and counted. In a parallel preparative gradient, the region corresponding to Comp. II (fraction 23-28) was pooled and used in subsequent analyses of fragmented Comp. II.*

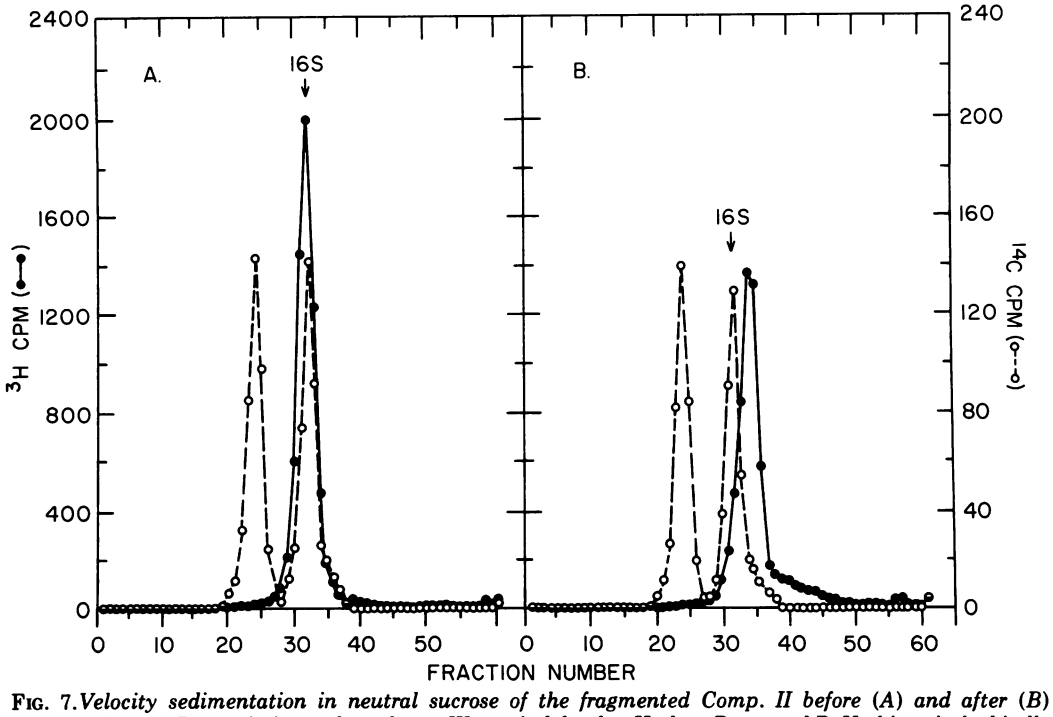

FIG. 7. *Velocity sedimentation in neutral sucrose of the fragmented Comp. II before (A) and after (B) treatment with the R_I restriction endonuclease. We are indebted to Herbert Boyer and R. Yoshimari who kindly provided us with this enzyme. The fragmented Comp. II was isolated from the LMW DNA as described in the legend for Fig. 6. A portion of the Comp. II was treated with the R_I restriction endonuclease by using the conditions described by Fareed et al. (3a). The samples were then layered onto an 11.8-ml, 5 to 30% neutral sucrose gradient, centrifuged, and counted as previously described.*

effectively blocked, thymidine is able to reverse the effects of treatment with FUdR.

DISCUSSION

The phenomenon that we have reported in the present paper is the abnormal pattern of SV40 DNA synthesis that is observed subsequent to a period of inhibition of DNA synthesis. This is observed in infected cells when DNA is labeled for periods of 30 s to 5 min after 1-h inhibition of DNA synthesis. During the period of isotope incorporation, there is rapid synthesis of 4S SV40 DNA. However, there is a failure in the joining of these 4S fragments to growing SV40 chains. We previously described the discontinuous synthesis of SV40 chains and the role of 4S DNA as an intermediate in SV40 DNA chain growth (4).

A decreased rate of synthesis in the pulse-labeled FUdR-treated cultures could cause an apparent accumulation of 4S fragments. However, pulse-chase experiments indicate that this is not the basis for their accumulation, but rather that there is a block in the joining of 4S fragments to growing SV40 chains. When FUdR-treated cultures were pulsed for 1 min

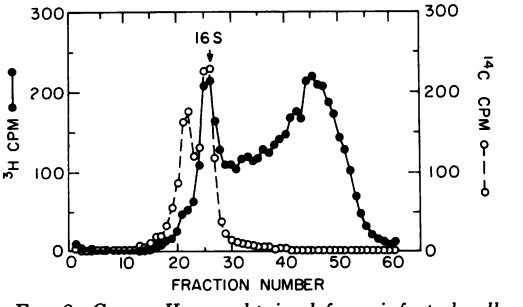

FIG. 8. *Comp. II was obtained from infected cells that had been pulse labeled for 1 min and chased for 10 min as described in the text and the legend for Fig. 6. It was analyzed by velocity sedimentation in alkaline sucrose gradients as previously described.*

and then chased for 10 min, there was some incorporation of labeled DNA into Comp. I, but the major product that accumulated during the chase period was Comp. II. In a comparable uninhibited culture labeled in this way, Comp. II would not be readily detected. The Comp. II from a pulse-chase experiment resembles authentic Comp. II in sedimentation properties in neutral sucrose gradients, and when cleaved with the R_I restriction endonuclease where it is

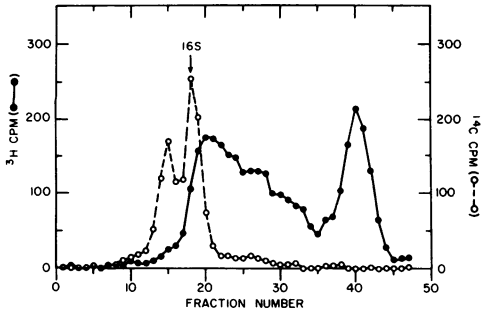

FIG. 9. *Velocity sedimentation in alkaline sucrose of the LMW DNA labeled in a 1-min ^{3}H-thymidine pulse of an infected culture which has been treated with FUdR and cycloheximide. As described in Materials and Methods, an infected cell culture which had been treated for 1 h with FUdR and for 5 min with cycloheximide was washed and refed with spent medium containing 2×10^{-5} M thymidine and 100 μg of cycloheximide/ml. After a 30-min incubation, the cells were washed and pulsed for 1 min with ^{3}H-thymidine. The LMW DNA fraction was prepared as previously described and layered onto an 11.8-ml, 10 to 30% alkaline sucrose gradient. The sample was centrifuged at 10 C for 17.5 h at 40,000 rpm. ^{14}C-SV40 DNA II was added to the gradient as a sedimentation marker. Sedimentation is from right to left. Fractions were collected directly into scintillation vials, neutralized by the addition of 4 drops of glacial acetic acid, and counted. The inhibition of protein synthesis by cycloheximide was determined by 1-min ^{3}H-amino acid pulse labeling of cultures which had been treated with FUdR and cycloheximide as described above. Incorporation of counts into trichloroacetic acid-precipitable material was inhibited 84% as compared with an infected, untreated control when pulsing was done 5 min after the addition of cycloheximine. A 1-min pulse label 10 min after the FUdR-reversal period showed that the incorporation of radioactivity into protein was 91% inhibited.*

cleaved to yield a duplex molecule that sediments at 14.5S. However, when this Comp. II is sedimented in alkali, it can be seen that it is composed of fragments of SV40 DNA. While at the beginning of the chase period there are no newly synthesized Comp. I or Comp. II molecules, significant amounts of both are present after the 10-min chase. This generation of daughter molecules occurs even though the joining of fragments to growing chains can occur to only a limited extent.

The accumulation of DNA fragments that are precursors of growing chains in the SV40-infected cells resembles results seen in bacterial systems where DNA synthesis is studied in strains lacking either ligase (9, 10, 18) or, in the case of *Escherichia coli*, DNA polymerase I (7, 12). This has also been observed when polyoma DNA synthesis has been studied in vivo, and the system has been deprived of deoxyribonucleotides (8a). In bacterial systems, in the absence of either of these enzymes DNA synthesis occurs, but there is accumulation of DNA fragments. In SV40-infected cells that have been treated with FUdR for 1 h, there is no decrease in ligase activity compared with an uninhibited infected culture. Furthermore, ligase contained in SV40-infected cell extracts is not able to join fragments contained in Comp. II under conditions in which 40% of authentic Comp. II was converted to Comp. I. These data with SV40 are consistent with a model for DNA synthesis in which 4S fragments are synthesized on parental template strands and in which gaps exist between these fragments and the growing chains to which they will be joined. By analogy with bacterial systems, there may be a requirement for the action of a second polymerase to fill the gap before ligase can join the 4S fragments to the growing chain (7, 12, 24). The presence of two separable DNA polymerases in cultured animal cells has been described (23). Alternatively, failure of ligase to join the fragments in Comp. II might be due to the absence of a 5'-phosphate ester in a nicked, but nongaped, DNA strand (5).

The primary effect that FUdR would be expected to have on the cell is to produce a depletion of pool thymidine phosphate compounds and, as a consequence of this, to block both viral and cellular DNA synthesis. Why these conditions might effect the stability of particular enzymes is not apparent. The effects of the inhibitor, FUdR, can be reversed under conditions in which thymidine is supplied for 30 min but where protein synthesis is blocked. If the inhibitor causes inactivation of an enzymatic activity, the effect seems likely to involve changes in conformation, rather than breakdown and subsequent resynthesis when the inhibitor is reversed.

In short pulses more than 84% of the radioactivity is contained in fragments less than 250,000 daltons. This would occur only if both strands of SV40 DNA were made in a discontinuous manner, or if during the pulse period we were observing only initiation of a new round of synthesis. The data presented in Fig. 3 do not support the latter idea and provide additional support for discontinuous synthesis of both SV40 strands. The FUdR-inhibited system does offer an effective way of accumulating SV40 precursor fragments for their more complete characterization.

In an analysis of temperature-sensitive mutants of SV40, there is a single complementa-

tion group for those mutants that are defective in DNA synthesis (19, 20). In the present study and in previous studies in this laboratory and in other laboratories on the mechanisms of SV40 DNA replication, a complex series of events has been described that is required for the formation of progeny DNA molecules. These include (i) initiation of synthesis at a specific site (3, 11, 21), (ii) bidirectional DNA replication (3), (iii) a discontinuous mechanism of chain growth (4; 8a) with a possible requirement for two polymerases, one required for synthesis of 4*S* fragments and the second involved in filling in the gaps in growing chains, and (iv) a mechanism which allows for nicking and sealing of parental strands (2) to permit DNA replication and yet preserve a covalently closed structure in the template strands of the replicating molecules (17). It is clear that SV40 requires a complex of integrated enzymatic activities which are provided by the cells. Based on our present understanding of SV40 replication and its limited coding potential, it is likely that SV40 replication serves as a clear reflection of the mechanism by which replication of eukaryote DNA is effected. If this is the case, the ease with which the SV40 system can be studied would make it an unusually important experimental system.

It is of interest that the single function involved in DNA replication, which is coded for by the viral genome, is required for initiation of viral DNA synthesis. One could speculate that this single function may have been preserved in a way that permits the viral genome to compete effectively with cellular DNA for the DNA replication machinery.

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