JOURNAL OF VIROLOGY, May 1973, p. 682-693

Vol 11, No. 5
Printed in U.S.A.

Preferred Site for Initiation of RNA Transcription by *Escherichia coli* RNA Polymerase Within the Simian Virus 40 DNA Segment of the Nondefective Adenovirus-Simian Virus 40 Hybrid Viruses Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$

B. SAYEEDA ZAIN, RAVI DHAR, SHERMAN M. WEISSMAN, PAUL LEBOWITZ, AND ANDREW M. LEWIS, JR.

Departments of Medicine, Human Genetics, and Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06851, and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014

Received for publication 7 December 1972

The DNA of simian virus 40 (SV40) was transcribed into RNA by *Escherichia coli* RNA polymerase at 18 to 24 C after synchronization of the initiation of RNA synthesis. After a brief synthetic period the RNA product contained relatively large amounts of sequences derived from a limited segment of SV40 DNA. The source for this pulse-labeled RNA was found to be a portion of the segment of SV40 DNA included within the nondefective adenovirus (Ad)-SV40 hybrid viruses, Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$. After synthesis with [γ-^{32}P] ATP, Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ DNA transcripts contained an initial sequence missing from Ad2 transcripts. This sequence was identified as an initiation sequence for polymerase transcription of the SV40 DNA. Thus, there is a preferred site for initiation of in vitro transcription on the segment of SV40 DNA common to the nondefective Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ hybrid viruses.

The derivation of nucleotide sequences for DNA oncogenic viruses would be of interest for many reasons, related both to the general properties of the transcription process and mRNA in animal cells and to the specific information that might be obtained about the ordering, spacing, and structure of genes for proteins directed by these viruses.

Direct analysis of DNA nucleotide sequences has been difficult because of a lack of base-specific DNases. However, this may be partly overcome by transcribing the DNA into radioactive RNA and analyzing the transcript. The methods for RNA sequence analysis have now advanced to the point where it is possible to derive extensive sequences from RNA of the length of single genes. The small DNA virus, simian virus 40 (SV40) is a favorable subject for sequence analysis because its biological properties have been thoroughly studied, and its DNA is transcribed asymmetrically and extensively in vitro by purified preparations of *Escherichia coli* RNA nucleotidyl transferase (E.C.2.7.7.6) (13, 32). The availability of nondefective adenovirus-SV40 hybrid agents containing various portions of the SV40 genome (18, 19) has made it possible to isolate, by nucleic acid hybridization techniques, segments of RNA corresponding to various lengths of SV40 DNA. Our studies have utilized the Ad2$^+$ND$_3$ and Ad2$^+$ND$_1$ hybrids. The former contains approximately 2 to 5% and the latter 10 to 18% of the total SV40 genome (10, 17; T. J. Kelly, Jr., and A. M. Lewis, Jr., manuscript in preparation). This SV40 DNA is covalently inserted as a single continuous segment near one end of the adenovirus DNA (T. J. Kelly, Jr. and A. M. Lewis, Jr., manuscript in preparation). Whereas cells infected with Ad2$^+$ND$_3$ do not express detectable SV40-specific antigens, cells infected with Ad2$^+$ND$_1$ express the early SV40 U antigen (20). By transcribing SV40 DNA into complementary RNA and annealing this RNA to the DNA of Ad2$^+$ND$_1$ hybrid virus, we have been able to isolate a segment of RNA transcribed from the SV40 DNA which appears to direct the formation of the SV40 U antigen. This RNA is small enough to permit extensive sequence

analysis. During the course of our experiments we have observed that, under specific conditions, there is a preferred site for initiation of RNA transcription on SV40 DNA which lies relatively near one end of the SV40 genetic material in the $Ad2^+ND_1$ virus.

MATERIALS AND METHODS

The $Ad2^+ND_1$ and $Ad2^+ND_3$ viruses (three passages from clone isolation) were grown in either 32-oz (0.95 liter) bottle cultures of Vero cells or in suspension cultures of KB cells. Bottle cultures of Vero cells were infected with approximately 10 PFU/cell in 10 ml of Eagle minimal essential medium containing 250 units of penicillin per ml, 250 μg of streptomycin per ml, and 2 mM glutamine supplemented with 2% agamand 8S in neutral sucrose gradients (5 to 20% linear 4-h adsorption period, the cultures were refed with 20 ml of EMEM-2. Cells and fluid were harvested by scraping when 50 to 75% of the cell sheet exhibited cytopathic effects. The cells were pelleted by low-speed centrifugation (500 $\times$ *g*), suspended in either 60 ml of 0.01 M Tris-hydrochloride buffer (pH 7.8) or Tris-buffered saline (pH 7.4) (TBS), and frozen at -70 C. One liter of a suspension culture of KB cells containng 2×10^5 cells/ml was infected with 10^9 PFU of the hybrid pool. At 48 h the cells were pelleted and suspended in 60 ml of buffer or TBS and stored at -70 C.

The cell suspensions were frozen and thawed three times, brought to a final concentration of 1.0% sodium deoxycholate and 0.1% trypsin, incubated at 37 C for 30 min, and centrifuged onto a cushion of cesium chloride solution (density 1.40 g/ml) in an SW25.1 rotor at 55,000 $\times$ *g* for 90 min at 4 C. The virus band at the interface was collected and twice banded in CsCl (density 1.34 g/ml) using an SW39 rotor at 100,000 $\times$ *g* for 24 h. After 5 to 6 h of dialysis against buffer, the virions were stored at -70 C.

Extraction of the virus DNA has been described elsewhere (10). Samples of $Ad2^+ND_1$ DNA so prepared were found to contain intact strands of DNA by sedimentation through both neutral and alkaline sucrose gradients.

SV40 derived from strain 777 was passed in Vero cells at a multiplicity of infection of less than 2 PFU/cell. DNA was extracted from infected cells by the Hirt procedure (12). Super-coiled viral DNA was then isolated by centrifugation in a cesium chloide density gradient containing ethidium bromide. The ethidium was removed by isoamyl alcohol extraction, followed by exhaustive dialysis against sterile 0.1 $\times$ SSC (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate, pH 6.7). The preparation of *E. coli* RNA polymerase, general procedure for RNA synthesis, separation of RNA from triphosphates, nuclease digestion, and oligonucleotide mapping have been described elsewhere (15). [α-^{32}P]nucleoside triphosphates with specific activities in excess of 70 Ci/mmol were prepared as described previously (15) or obtained commercially (New England Nuclear Corp.). [γ-^{32}P]ATP with specific activities of 20 to 30 Ci/mmol was also obtained from New England Nuclear Corp.

Standard syntheses of RNA from SV40, adenovirus 2 (Ad2), $Ad2^+ND_3$, or $Ad2^+ND_1$ DNA were performed in reaction mixtures of 64 μliters containing 0.18 M KCl, 0.033 M Tris-hydrochloride (pH 7.9), 6 mM mercaptoethanol, 3.3 mM $MgCl_2$, 0.165 mM ATP, GTP, CTP, and UTP, 1 to 3 μg of DNA, and 5 μg of RNA polymerase holoenzyme. In each reaction only one nucleoside triphosphate was labeled. Incubations were performed at 37 C for 30 min. Syntheses utilizing [γ-^{32}P]ATP were terminated by the addition of sodium dodecyl sulfate (SDS) to 0.25% and an equal volume of water-saturated phenol. Transcriptions utilizing (α-^{32}P) nucleoside triphosphates were terminated by further incubation for 10 min with 1.5 μg of rifamycin and 0.18 μg of pancreatic DNase (E/C.3.1.4.5.), followed by SDS and phenol treatment as above.

Limited syntheses of SV40 complementary RNA were performed by modifications of the methods of Blattner and Dahlberg (1). The reaction mixture was constituted as for standard syntheses, but with the omission of $MgCl_2$, the reduction of KCl to 0.095 M, and the addition of EDTA to 2.5 mM. After preincubation for 0.5 to 2 min, transcription was initiated by the addition of $MgCl_2$ to a concentration of 6.1 mM and allowed to proceed for 5 to 60 s. Limited transcriptions were carried out with preincubation and synthesis at temperatures ranging from 18 to 24 C. In general, the cleanest results were obtained when the preincubation and synthesis were both carried out at 20 C or lower. These reactions were terminated by addition of an equal volume of phenol saturated with 15 mM EDTA.

RNA was separated from radioactive triphosphates on Sephadex G-100 columns (15) and precipitated with ethanol in the presence of 2% potassium acetate, pH 5.1, and 100 to 200 μg of *E. coli* tRNA, which had been purified from commercial preparations by three phenol extractions. Precipitated RNA was then utilized for either nucleic acid hybridization or oligonucleotide mapping procedures.

Nucleic acid hybridization was performed by the method described by Gillespie and Spiegelman (8). The DNA to which the transcript RNA was to be annealed was taken up in 2 ml of 0.01 $\times$ SSC, heat denatured at 97 C for 5 min, and rapidly chilled. The filters were presoaked in 6 $\times$ SSC for 30 min, mounted on the membrane filtration assembly (Millipore Corp.), and washed slowly with 10 ml of 6 $\times$ SSC. Three milliliters of 10 $\times$ SSC was added to the heat-denatured DNA, and the solution was filtered slowly. The filters were washed slowly with 10 ml of 6 $\times$ SSC and rapidly with 100 ml of 6 $\times$ SSC. They were then transferred to preheated scintillation vials, dried overnight at room temperature, and baked for 3 h at 80 C in a vacuum oven. Each filter was then incubated in a closed scintillation vial with RNA dissolved in 0.75 ml of 2 $\times$ SSC, 0.1% SDS. This solution contained, typically, 0.05 to 10 μCi of radioactive RNA and 100 μg of nonradioactive carrier *E. coli* tRNA. At the end of an 8-h incubation period, the filter was removed, rinsed with 10 ml of 2 $\times$ SSC on each side, and washed with 100 ml of 2 $\times$ SSC. The filter was incubated at room temperature for 40 min with 2 ml of 2 $\times$ SSC containing either 0.4 μg of

pancreatic RNase per ml (I-A, E-C 2.7.7.6) or 1 to 1.5 IU of T1 RNase per ml (E.C. 3.1.4.8) if the retained RNA was to be analyzed by further pancreatic or T1 RNase digestion, respectively. The filter was rinsed and washed again with 100 ml of 2 × SSC. It was then incubated in 2 ml of a solution of 0.15 M sodium iodoacetate, 0.1 M sodium acetate in 2 × SSC, pH 5.1, at 54 C for 40 min. At the end of the incubation period, the filter was rinsed with 10 ml of 2 × SSC and washed as mentioned above. To elute the RNA from the bound DNA, the filter was boiled in 1.5 ml of 0.01 × SSC containing 100 μg of carrier *E. coli* tRNA for 15 min and the solution was rapidly chilled. The radioactive RNA was then precipated by the addition of 0.1 vol of 20% potassium acetate, pH 5.1, and 2 vol of ethanol. In general, the radioactive RNA from extensive syntheses annealed to and eluted from $Ad2^+ND_1$ DNA had a sedimentation coefficient of between 5 and 8S in neutral sucrose gradients (5 to 20% linear sucrose gradients in a buffer containing 0.1 M $LiCl_2$, 0.01 M Tris [pH 7.9], 1 mM EDTA, 0.5% SDS, centrifuged for 3 h at 50,000 rpm in the Spinco SW50.1 rotor at 20 C).

RESULTS

RNA was synthesized by incubation of precursors and enzyme with SV40 DNA for 30 min at 37 C. This resulted in incorporation of about 20 to 30% of the radioactive precursor into polynucleotide. The radioactive SV40 complementary RNA was then annealed to $Ad2^+ND_1$ DNA, after which the bound RNA was treated with RNase and eluted. After precipitation with ethanol, the RNA was digested with T1 or pancreatic RNase and mapped by two-dimensional electrophoresis and chromatography (3, 4) (Fig. 1). The resulting maps showed a discrete and highly reproducible set of oligonucleotides. The same products were obtained even when eightfold greater levels of RNase were used to treat the DNA filters prior to elution of the RNA. Omission of RNase treatment gave maps with a much higher background. Detailed sequence analysis has been performed on all these products and will be reported elsewhere. Clean oligonucleotide maps were also obtained when the RNA was annealed to $Ad2^+ND_3$ DNA rather than $Ad2^+ND_1$ DNA (Fig. 2). Comparison of the two oligonucleotide maps showed that essentially all the oligonucleotides contained in $Ad2^+ND_3$ DNA were also contained in $Ad2^+ND_1$, but many of the $Ad2^+ND_1$ oligonucleotides were missing from $Ad2^+ND_3$.

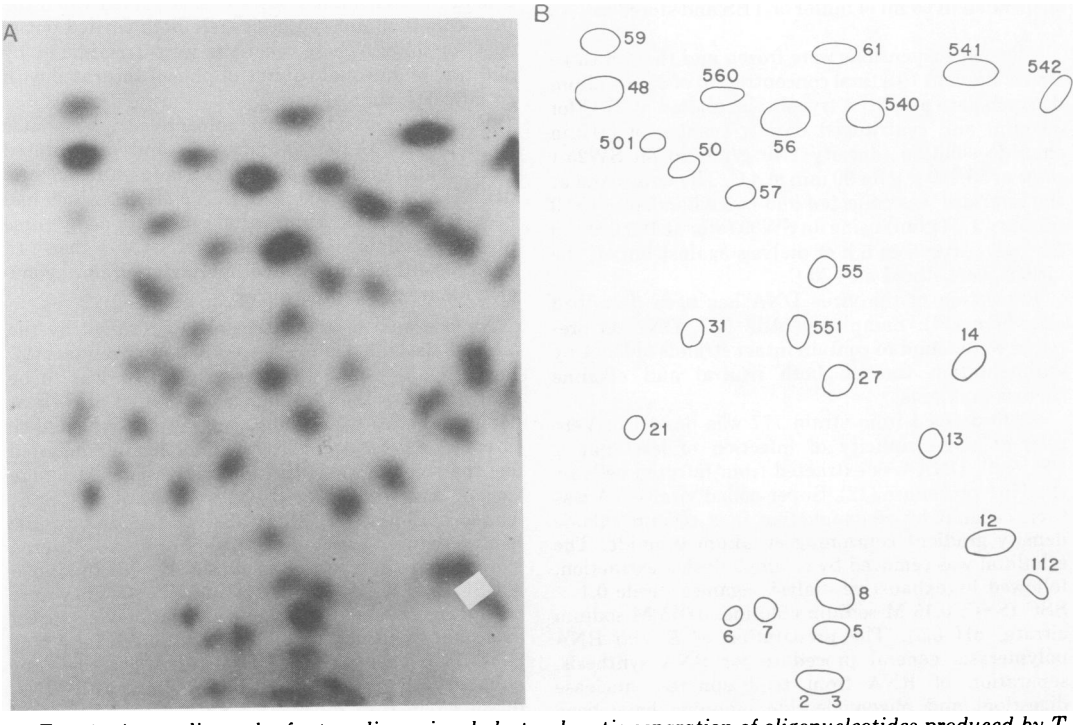


FIG. 1. *Autoradiograph of a two-dimensional electrophoretic separation of oligonucleotides produced by T_1 RNase digestion of SV40 complementary RNA annealed to $Ad2^+ND_1$ DNA. Electrophoresis was from left to right on Cellogel in 7 M urea at pH 3.5, and from bottom upwards on a DEAE cellulose-thin-layer chromatography plate using the "homo B" solution of Sanger et al (4). The RNA was synthesized on an SV40 DNA template, in the presence of [α-32P]UTP (sp. act. 36.6 Ci/mmol) for 30 min at 37 C, in the absence of EDTA, and annealed to $Ad2^+ND_1$ DNA as described in Materials and Methods. A, Autoradiograph. The blank spot indicates the position of marker dyes. B, Schematic sketch of autoradiograph, with numbers assigned to those oligonucleotides common to $Ad2^+ND_1$, $Ad2^+ND_3$ and SV40 transcripts.*

Experiments were performed to determine initial RNA sequences by use of short incubations at reduced temperatures. *E. coli* RNA polymerase was incubated with SV40 DNA at 18 or 24 C in the presence of EDTA, and RNA synthesis was initiated by the addition of $MgCl_2$ and then terminated after 5 to 20 s at 18 or 24 C. The resulting RNA was annealed to either $Ad2^+ND_3$ (Fig. 3) or $Ad2^+ND_1$ DNA (Fig. 4), eluted, and analyzed by T1 or pancreatic RNase digestion. Maps of RNase digests of the RNA from these two sources were similar and showed a limited number of prominent oligonucleotides (Fig. 3). Entirely similar results were obtained whether or not rifamycin (27) was added at the time transcription was initiated with $MgCl_2$. Table 1 summarizes the results of pancreatic RNase digestion of the principal oligonucleotides produced by complete T_1 RNase digestion of the RNA transcripts from brief syntheses after hybridization to either $Ad2^+ND_1$ or $Ad2^+ND_3$ DNA. Comparison of the analyses of the principal oligonucleotides from maps of transcripts prepared in 30-min syntheses and annealed to $Ad2^+ND_1$ or $Ad2^+ND_3$ DNA (Table 1a, b) with the analyses of the oligonucleotides from maps of transcripts prepared in brief syntheses and similarly annealed (Table 1d, e) showed that all the principal products obtained in the brief syntheses were derived from a segment of the DNA common to both $Ad2^+ND_1$ and $Ad2^+ND_3$. In addition to the results summarized in Table 1, the proposed sequences are based on results of digestion of the RNA with U2 RNase and spleen acid ribonuclease. The results of the complete sequence analysis of this segment of SV40 will be presented elsewhere. In control experiments, the oligonucleotides present in largest amounts in limited syntheses were the same when the RNA was purified by hybridization to whole SV40 rather than by hybridization to $Ad2^+ND_1$ or $Ad2^+ND_3$ (Fig. 4). Even when the RNA was digested and mapped without prior hybridization, this same set of oligonucleotides constituted the most prominent products (Fig. 5) although such maps were often less clean than those obtained from RNA previously annealed to $Ad2^+ND_1$ DNA. Nucleotide sequence analysis of RNA transcribed from SV40 DNA and annealed to $Ad2^+ND_1$ or $Ad2^+ND_3$ DNA has shown that this set of prominent oligonucleotides are ordered in a single continuous sequence. These results clearly indicate that under our conditions of restricted RNA transcription there is a preferred site for initiation of RNA synthesis on SV40 DNA which lies within the segment of SV40 DNA common to both $Ad2^+ND_1$ and $Ad2^+ND_3$.

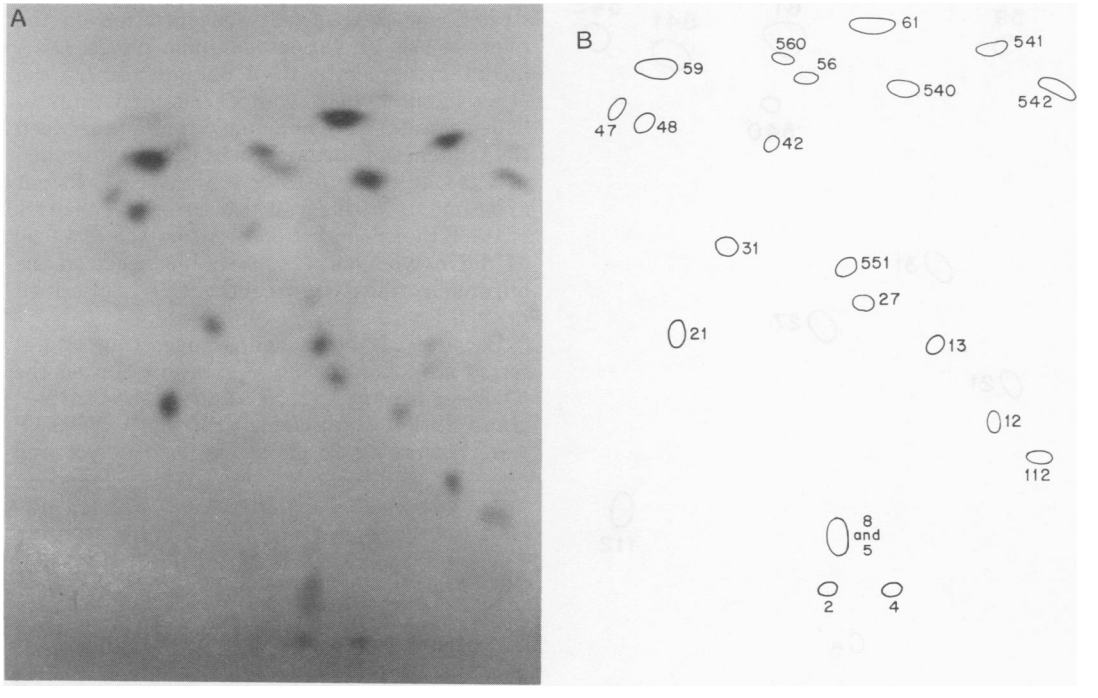
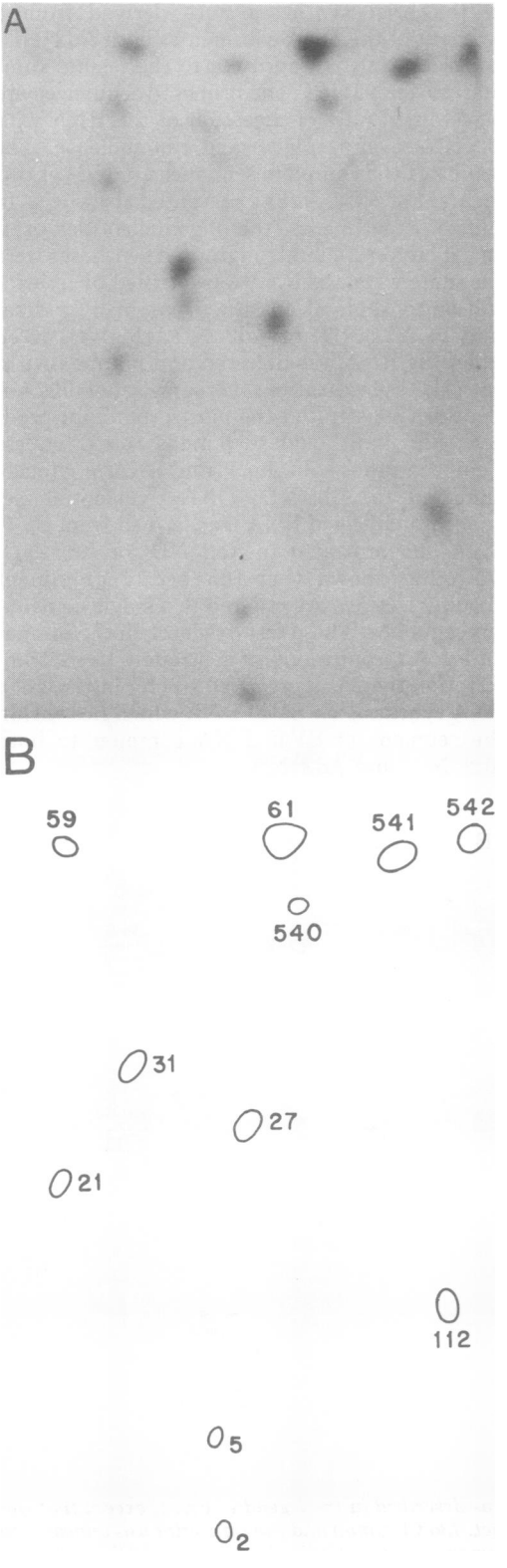


FIG. 2. *Autoradiograph of RNA prepared and analyzed as described in the legend to Fig. 1, except that the RNA was synthesized in the presence of [α-^{32}P] GTP (sp. act. 136 Ci/mmol) and the transcript was annealed to $Ad2^+ND_3$ DNA before digestion with RNase. A, Autoradiograph; B, Schematic sketch of autoradiograph with numbers assigned to the principal oligonucleotides.*



By prolongation of the time of incubation, we have been able to obtain a set of oligonucleotides approaching a total chain length of 100 nucleotides (Fig. 6). However, incubations for still longer periods of time do not result in any further lengthening of the RNA transcript as judged by the absence of any additional T_1 RNase digestion products in amounts stoichiometrically comparable to those found in shorter syntheses when the RNA was annealed to $Ad2^+ND_1$ DNA and eluted before analysis. Thus the initiation site for transcription under limiting conditions lies at least 100 nucleotides from the 5′ phosphorylated end of the transcribed strand of the SV40 region of $Ad2^+ND_1$ and $Ad2^+ND_3$ DNA. Our inability to extend the chain beyond 100 nucleotides may be due to a block in progression of the RNA polymerase molecules along the SV40 DNA rather than movement of the enzyme out of the region of SV40 DNA contained in $Ad2^+ND_3$. The other alternative requires that transcription proceeds on SV40 DNA in a direction away from the remainder of the $Ad2^+ND_1$ segment. Further experiments to test this model are in progress.

Several groups (1, 2, 20, 21, 32) have reported an influence of salt concentration on the kinetics of termination, release, and reinitiation of RNA transcribed on DNA templates, and Lebowitz (unpublished results) has recently demonstrated an effect of salt concentration on the relative use of various lambda phage DNA initiation signals by RNA polymerase. On the other hand, Lebowitz and Weissman (unpublished results) have noted no difference between the sequence of the lambda 6s RNA transcribed in vitro at either 0.18 or 0.27 M KCl. In our experiments with SV40 transcription, changes of the KCl concentration between 0.05 and 0.20 M did not have any appreciable effect on the oligonucleotide patterns of the RNA transcribed at 24 C for 10 s.

Because of the evidence suggesting a preferred site of initiation of transcription on the SV40 segment of $Ad2^+ND_1$ and $Ad2^+ND_3$ DNA, transcription experiments utilizing [γ-^{32}P]ATP were performed in an attempt to detect and

FIG. 3. *Autoradiograph of RNA prepared and analyzed as described in the legend to Fig. 1, except that the RNA was synthesized for 10 s at 25 C in the presence of [α-^{32}P] GTP (sp. act. 136 Ci/mmol) and annealed to $Ad2^+ND_3$ DNA as described in Materials and Methods. The oligonucleotide containing a 5′ terminal triphosphate streaked during electrophoresis on Cellogel and is not shown clearly in this and the following autoradiographs. A, Autoradiograph; B, schematic sketch of autoradiograph with numbers assigned to the oligonucleotides.*

determine the homogeneity of gamma-labeled initiation sequences within this DNA segment. Transcriptions were performed on the AD2 and AD2$^+$ND$_1$ DNA templates, and the RNA product was separated from substrate, digested with T$_1$ RNase, and analysed by one-dimensional chromatography. Figure 7a demonstrates the presence of about nine separate initiation fragments on Ad2 complementary RNA; in striking contrast, when Ad2$^+$ND$_1$ DNA was used as primer (Fig. 7b), the same fragments were seen plus one additional prominent and one or two less abundant large initiation fragments.

To determine whether these additional frag-

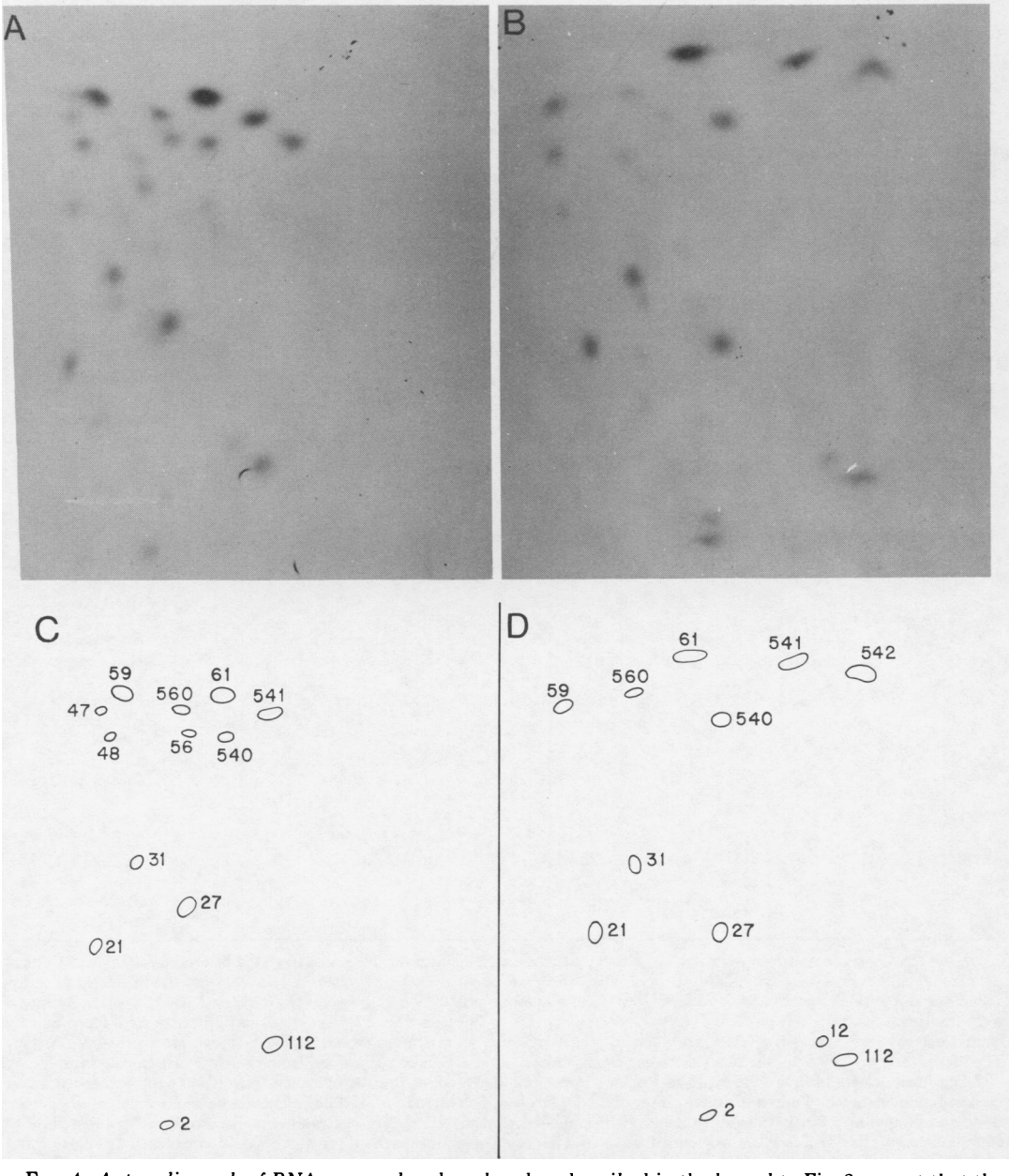


FIG. 4. *Autoradiograph of RNA prepared and analyzed as described in the legend to Fig. 2, except that the synthesis was for 20 s. A, Autoradiograph of RNA annealed to SV40 DNA. B, Autoradiograph of RNA annealed to Ad2$^+$ND$_1$ DNA. C and D, Schematic sketches of A and B, respectively, with numbers assigned to oligonucleotides.*

ments represented initiation points on the SV40 DNA included within the Ad2+ND$_1$ and Ad2+ND$_3$ hybrids, transcriptions were carried out using the DNA from these hybrids as templates and the radioactive product was annealed to SV40 DNA prior to T$_1$ RNase digestion and chromatographic analysis. The chromatogram of RNA processed by this method (Fig. 7c) demonstrates the presence of only one initiation spot having a mobility identical to that of the major additional spot in the Ad2+ND$_1$ transcript. Therefore, this product represents the principal initiation sequence in the SV40 segment of Ad2+ND$_1$ and Ad2+ND$_3$ DNA.

RNA was then synthesized on the SV40 DNA template, annealed to Ad2+ND$_1$ DNA, digested with T$_1$ RNase, and analyzed. Only one principal spot was seen, again moving with slow mobility and comigrating with the principal

TABLE 1. *Comparison of pancreatic RNase digestion products of oligonucleotides derived by T$_1$ RNase digestion of limited and extensive transcripts of SV40 DNA*[a]

Oligonucleotide no.	Pancreatic RNase digestion products[b]								Sequences derived[c]
	Radioactive precursor α-^{32}P-ATP	Source of RNA	Radioactive precursor α-^{32}P-CTP	Source of RNA	Radioactive precursor α-^{32}P-GTP	Source of RNA	Radioactive precursor α-^{32}P-UTP	Source of RNA	
2	ApApApUp, Cp, ApCp, ApApApGp	*abc*	ApCp, ApUp, Up, Cp, ApApApGp	*abcd*	ApApApGp	*abcde*	ApApApUp, ApUp, Up, Cp	*abcde*	. . .ApApApGp [Cp]
5	ApApUp, ApApApCp, Cp	*abcd*	ApApApCp, ApUp, Up	*abcd*	ApApUp	*abcd*	ApApUp, ApUp, ApApApCp, Gp	*abcd*	Up(CpCpApApAp CpUpCpApUp) CpApApUpGp [Up]
12	Cp, Up	*ab*	ApUp	*ab*	ApUp	*abcde*	ApUp, Gp, Cp, Up	*abcd*	UpApUpCpUpUp ApUpCpApUp Gp [Up]
21	Cp	*abc*	Cp, ApUp	*ab*	ApGp	*abcde*	ApUp	*abcd*	ApUpCpCpCpCp ApGp [Gp]
27	Cp, Up	*ab*	Up	*ab*	ApGp	*abcde*	ApGp, ApUp, Cp	*abcd*	CpApUpUpCpUp ApGp [Up]
31	ApApUp, Cp	*abc*	ApGp	*abcd*	ApGp	*abcde*	ApApUp	*abcd*	CpApApUpApGp [Cp]
112	Cp	*abc*	ApCp, Gp, Up	*abc*	Up	*abcde*	ApUp, ApCp, Up	*abcde*	CpApUpUpUp Up UpUpUpCpAp CpUpGp [Cp]
540	—	*ab*	Up	*ab*	Up, Gp	*abcde*	Cp	*abcd*	UpCpUpGp [Gp]
541	—	*ab*	—	*ab*	Up	*abcde*	Gp, Up	*abcde*	UpUpGp [Up]
542	—	*ab*	—	*ab*	Up		Gp, Up	*abcde*	UpUpUpGp [Up]
551	—	*ab*	Cp, Up	*ab*	Up	*abc*	Cp, Gp	*abcd*	CpUpCp(Cp, Up) CpUpGp [Up]

[a] Each RNA sample was digested with T$_1$ RNase. The resulting oligonucleotides were separated by electrophoresis on Celogel at pH 3.5 and homochromatography on thin-layer chromatography plates as described in Materials and Methods. Individual products were located by autoradiography, eluted, and digested with pancreatic RNase. These digestion products were fractionated on DEAE paper by electrophoresis at pH 3.5. The pancreatic RNase digestion products were identified by their electrophoretic mobility. They are all consistent with the sequences proposed in the last column of this table. This provides strong evidence for the identity of corresponding T$_1$ RNase products obtained from the different RNase samples.

[b] The italic letters indicate the types of RNA preparations analyzed. *a*, Results obtained from SV40 complementary RNA transcribed in vitro for 30 min and annealed to Ad2+ND$_3$ DNA as described in Materials and Methods. *b*, Results obtained from SV40 complementary RNA transcribed as in *a* but annealed to Ad2+ND$_1$ DNA. *c*, Results obtained from pulse-labeled SV40 complementary RNA, transcribed in vitro for 5 to 50 s. The total transcript (without purification by hybridization) was digested with T$_1$ RNase and mapped. *d*, Results obtained from RNA as mentioned in *c* but annealed to Ad2+ND$_3$ DNA. *e*, Results obtained from RNA as mentioned in *c* but annealed to Ad2+ND$_1$ DNA.

[c] The sequences derived are based principally on the analysis of the products of U$_2$ RNase as well as pancreatic RNase digestion of these oligonucleotides. Brackets indicate the nearest neighbor base and parentheses enclose nucleotides whose position is uncertain.

fragment from Ad2+ND$_1$ not present in transcripts of Ad2. When the SV40 transcript was digested and chromatographed without prior hybridization, the most prominent large initiation fragment comigrated with the fragment present in the transcripts of Ad2+ND$_1$ but absent in the transcript of Ad2. Furthermore, pancreatic RNase digestion of this initiation fragment derived from SV40 DNA and the comigrating fragment from Ad2+ND$_1$ DNA produced smaller radioactive fragments of identical mobility on electrophoresis on DEAE paper at pH 1.7. Thus the initiation signal on the SV40 segment of Ad2+ND$_1$ and Ad2+ND$_3$ appears to be identical with an initiation signal present in the intact SV40 DNA.

DISCUSSION

Several workers have shown that RNA may be annealed to and eluted from DNA and still retain relatively long intact nucleotide sequences. Blattner and Dahlberg (1) have annealed pulse-labeled lambda DNA transcripts to separate strands of mutant phage DNA to isolate single initiation sequences, and Lebowitz and Weissman (unpublished observations) have annealed lambda transcripts to the "r" strand of lambda DNA to isolate highly purified 6s RNA for sequence analysis. The present results demonstrate that it is possible to purify discrete segments of SV40 complementary RNA by annealing total SV40 transcripts to DNA from adenovirus-SV40 hybrid viruses. This makes it possible to analyze RNA containing genetic information that appears to code for specific antigens and which is of sufficient purity to permit detailed sequence determination.

We have confirmed that *E. coli* RNA polymerase transcribes SV40 DNA asymmetrically by demonstrating that oligonucleotides obtained by digestion of RNA hybridized to either Ad2+ND$_1$ or Ad2+ND$_3$DNA cannot be arranged into two complementary nucleotide sequences. For example, no pancreatic RNase product GpGpGpGpApUp (complementary to oligonucleotide 21 in Table 1) nor T$_1$ RNase product ApApApApApApApUpGp (complementary to oligonucleotide 112 in Table 1) was detected in our analyses.

Initiation of transcription at a preferred site on SV40 DNA indicates that there is an additional specificity beyond strand selection in the interaction of *E. coli* RNA polymerase and this DNA. Under our conditions of synthesis, it is clear that many of the starts occur at a single site on the SV40 DNA that corresponds to a segment of the genome included in the

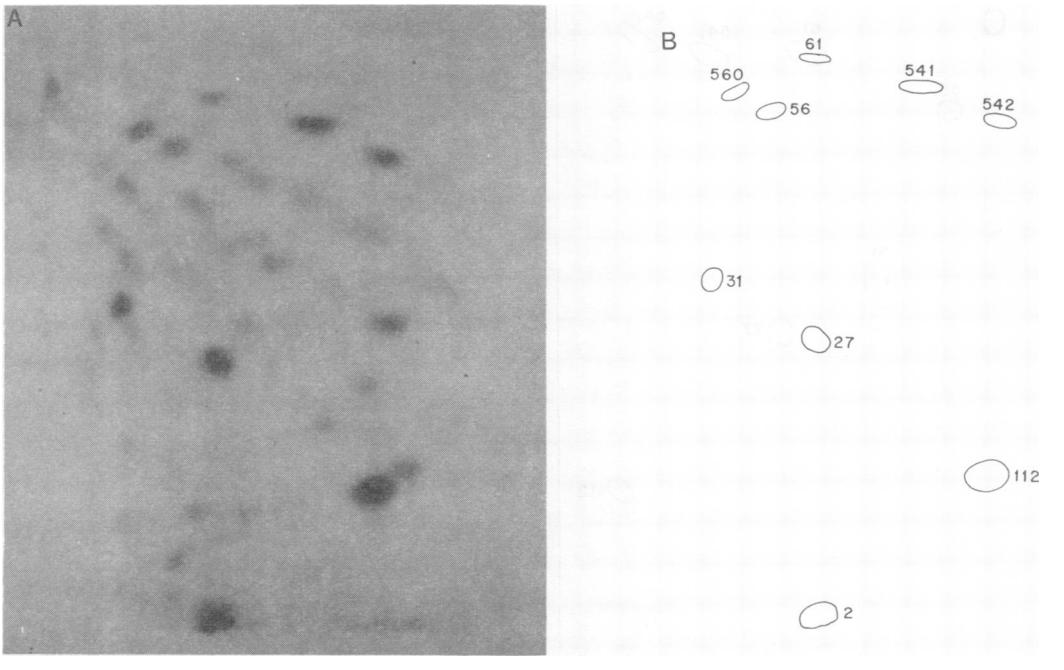


FIG. 5. *Autoradiograph of T$_1$ RNase digest of RNA synthesized as described in the legend to Fig. 1, except that the synthesis was carried out in the presence of [α-^{32}P]UTP (sp. act. 36.6 Ci/mMol) for 10 s. The total RNA synthesized was digested and mapped without purification by annealing to hybrid virus DNA. A, Autoradiograph, B, schematic sketch of autoradiograph with numbers assigned to oligonucleotides.*

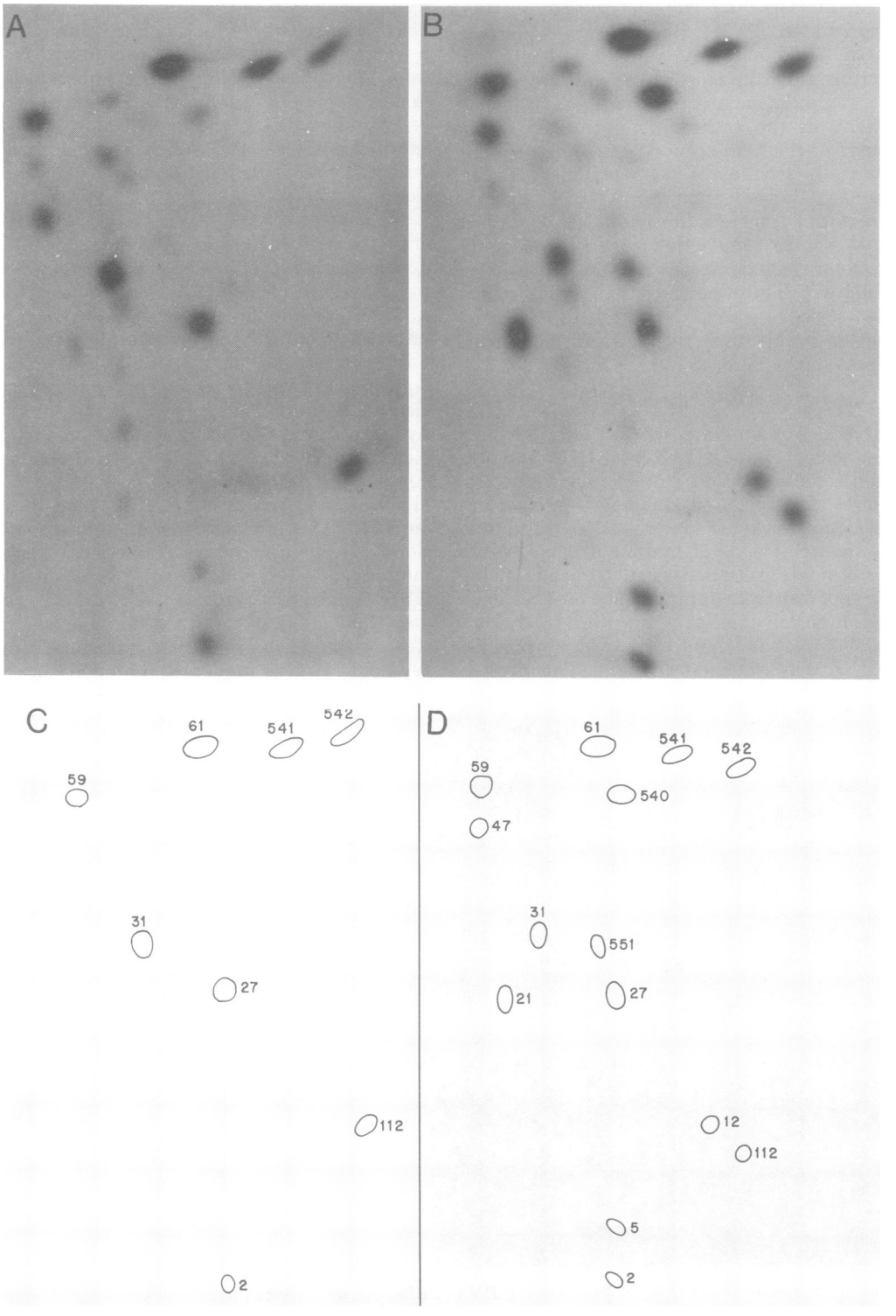


FIG. 6. *Comparison of oligonucleotide maps of T_1 RNase digests of RNA synthesized for 10 s (A and C) or 40 s (B and D). The RNA was synthesized in the presence of [α-^{32}P]GTP (sp. act. 136 Ci/mmol) and was annealed to Ad2$^+$ND$_1$ DNA. Analyses were performed as described in the legend to Fig. 1. A and B, Autoradiographs; C and D, schematic sketches of autoradiographs with numbers assigned to oligonucleotides.*

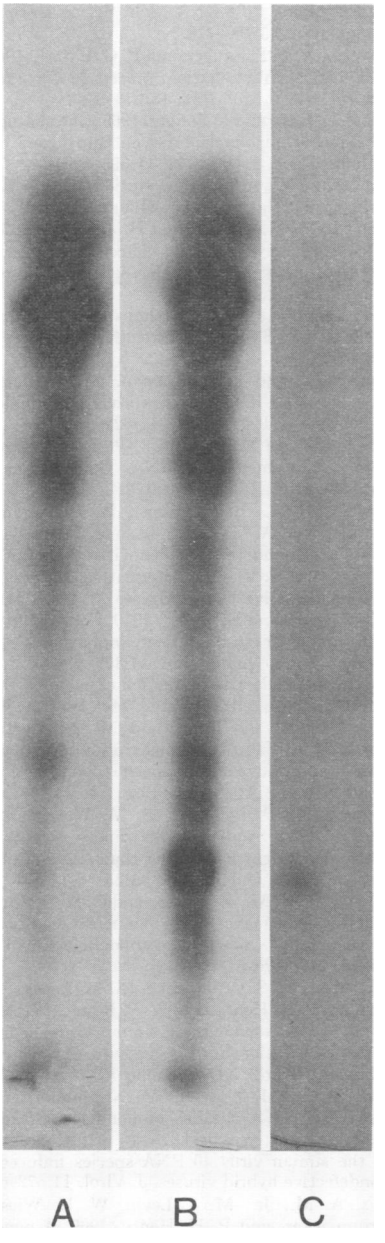


FIG. 7. *T_1 RNase digests of [γ-^{32}P]ATP-labeled RNA chromatographed on DEAE cellulose-thin-layer chromatography plates using "Homo B" (3) for development. A, RNA transcribed on Ad2 DNA as described in text; B, RNA transcribed on Ad2$^+$ND$_1$ DNA as described in text; C, RNA transcribed on Ad2$^+$ND$_1$ DNA and annealed to SV40 DNA.*

Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ hybrid viruses. This conclusion is based on our finding a limited number of prominent oligonucleotides in RNA synthesized on the SV40 DNA template at reduced temperatures for short periods of time which are identical to oligonucleotides in the RNA complementary to the SV40 DNA segments of Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$. In fact, when the SV40 complementary pulse-labeled RNA was annealed to Ad2$^+$ND$_1$ DNA prior to analysis, most oligonucleotides present in low yield in maps of unannealed RNA were lost and the prominence of the limited number of oligonucleotides found in the SV40 segments of the hybrid viruses was enhanced. Recent results indicate that the oligonucleotides found in the pulse-labeled RNA are also identical to products found in the transcript of the "*G*" fragment of SV40 DNA produced by cleavage with *Haemophilus influenza* restriction endonuclease (Dhar, Zain, Lebowitz, Weissman, Danna, Nathans, unpublished results). It is a curious fact that this is also the region of the SV40 genome in which DNA replication terminates (7).

Although our experiments establish a preferred site for initiation of transcription, it remains possible that under other conditions, additional sites might be detected. Delius, Westphal, and Axelrod (manuscript submitted for publication) have not detected a preferred initiation site on SV40 DNA, using a new technique for electron microscope investigation of the length of growing RNA chains transcribed from SV40 DNA by *E. coli* RNA polymerase. One possible reason for difference between their results and ours is that, under our conditions, most RNA chains are less than 100 nucleotides in length and fail to lengthen appreciably on extended incubation, whereas the RNA chains investigated by Delius and Westphal were, in general, of chain length of 100 or more.

We have not only shown a preferred site for initiation of transcription on the intact DNA of SV40 within the segment of genome carried by the Ad2$^+$ND$_1$ and Ad2$^+$ND$_3$ hybrids, but we have also demonstrated a specific initiation sequence labeled by [γ-^{32}P]ATP within the SV40 segment of the hybrid viruses. Furthermore, this initiation sequence appears not to be an artifact associated with the integration of SV40 genetic material into Ad2 DNA, since an identical [γ-^{32}P]ATP-labeled initiation fragment was identified in transcripts of intact SV40 DNA.

Specificity has also been found in strand selection, site of initiation (5, 9, 30) and even termination (14, 15, 21, 22, 23, 26, 27) in the transcription of several bacteriophage DNAs with purified *E. coli* RNA polymerase. For example, the enzyme preferentially transcribes one of the two strands of phage fd replicative form DNA and the transcripts contain relatively discrete RNA products with specific 5′ terminal sequences (31). In addition, transcription of λ^{b2b5} DNA gives RNA species with

unique initiation and termination points, complementary to only limited portions of the DNA coding for early functions of the phage (6, 22) and in one instance there is evidence that the starting sequences of the in vitro and in vivo transcripts of lambda are the same (1).

Detailed sequence analysis of the initiation site for *E. coli* RNA polymerase in the SV40 segment in $Ad2^{+}ND_1$ may provide new information about the way in which initiation of transcription is signaled in DNA sequences and the extent to which this signal is universal among cells. Experiments are in progress to establish the direction of transcription of SV40 DNA and to correlate the location of the in vitro transcription initiation site reported here with the sites of initiation of early and late gene transcription in vivo and with the site of termination of SV40 DNA replication.

Oxman et al. (24) have suggested that SV40 genes may be transcribed into RNA covalently linked to adenovirus messages in cells infected with an adenovirus-SV40 hybrid virus. Control of SV40 transcription by an adenovirus promotor would also be consistent with the delayed appearance of peak U antigen levels in cells infected with $Ad2^{+}ND_1$ (20). Even if the *E. coli* RNA polymerase start signal of the SV40 segment of $Ad2^{+}ND_1$ did represent an in vivo promoter, it is still possible that additional transcription could be controlled by an "upstream" adenovirus promotor.

ACKNOWLEDGMENTS

The authors acknowledge the excellent technical assistance of Su-Fang-Su and Graciela Z. Hebert, and are indebted to M. N. Oxman for providing the stock SV40 virus, and to J. Pan for growing the virus. We also are grateful to Joan Steitz for reading this manuscript and making many valuable suggestions.

The research was supported by American Cancer Society Grants #VC-ID 56444 and E-630 and Public Health Service Grants #CA-10, 13706 51669, and #CA-12, 145-02, both from the National Cancer Institute.

ADDENDUM IN PROOF

Recently H. Westphal and H. Delius (Le Petit Symposium, in press) studied the transcription of linear forms of SV40 DNA prepared by cleavage with R_1 restriction endonuclease. They found a preferred initiation site for *E. coli* RNA polymerase situated about 0.15 map units from one end of the linear DNA. This probably corresponds to the initiation site described in the present report.

LITERATURE CITED

1. Blattner, F. R., and J. E. Dahlberg. 1972. RNA synthesis startpoints in bacteriophage λ: are the promotor and operator transcribed? Nature N. Biol. **237**:227-232.
2. Bremer, H. 1970. Influence of KCl on the *in vitro* transcription of T4 DNA. Cold Spring Harbor Symp. Quant. Biol. **35**:109-119.
3. Brownlee, G. G., F. Sanger, and B. G. Barrell. 1965. A two dimensional fractionation procedure for radioactive nucleotides. J. Mol. Biol. **13**:373-398.
4. Brownlee, G. G., and F. Sanger. 1969. Chromatography of ^{32}P labeled oligonucleotides on thin layers of DEAE cellulose. Eur. J. Biochem. **11**:395-399.
5. Chamberlin, M. 1970. Transcription 1970: a summary. Cold Spring Harbor Symp. Quant. Biol. **35**:851-873.
6. Cohen, S. N., N. Maitra, and H. Jurwitz. 1967. Role of DNA in RNA synthesis. XI. Selective transcription of λ DNA segments *in vitro* by RNA polymerase of *E. coli*. J. Mol. Biol. **26**:19-38.
7. Danna, K. J., and D. Nathans. 1972. Bidirectional replication of SV40 DNA. Proc. Nat. Acad. Sci. U.S.A. **69**:3097.
8. Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. **12**:829-842.
9. Hayashi, Y., and M. Hayashi. 1970. Fractionation of φX 174 specific messenger RNA. Cold Spring Harbor Symp. Quant. Biol. **35**:171-177.
10. Henry, P. H., L. E. Schnipper, R. J. Samaha, C. S. Crumpacker, A. M. Lewis, Jr., and A. S. Levine. 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. VI. Characterization of the DNA from five nondefective hybrid viruses. J. Virol. **11**:665-671.
11. Herskowitz, I., and E. Signer. 1970. Control of transcription from the r strand of bacteriophage γ. Cold Spring Harbor Symp. Quant. Biol. **35**:355-368.
12. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365-369.
13. Khoury, G., J. C. Byrne, and M. A. Martin. 1972. Patterns of SV40 DNA transcription after acute infection of permissive and nonpermissive cells. Proc. Nat. Acad. Sci. U.S.A. **69**:1925-1928.
14. Larsen, C. J., P. Lebowitz, S. M. Weissman, and B. DuBuy. 1970. Studies of the primary structure of low molecular weight RNA other than tRNA. Cold Spring Harbor Symp. Quant. Biol. **35**:35-46.
15. Lebowitz, P., S. M. Weissman, and C. M. Radding. 1971. Nucleotide sequence of a ribonucleic acid transcribed *in vitro* from λ phage deoxyribonucleic acid. J. Biol. Chem. **246**:5120-5139.
16. Levin, M. J., C. S. Crumpacker, A. M. Lewis, Jr., M. N. Oxman, P. H. Henry, and W. P. Rowe. 1971. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. II. Relationship of adenovirus 2 DNA and SV40 DNA in the $Ad2^{+}ND_1$ genome. J. Virol. **7**:343-351.
17. Levine, A. S., M. J. Levin, M. N. Oxman, and A. M, Lewis, Jr. 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. VII. Characterization of the simian virus 40 RNA species induced by five nondefective hybrid viruses. J. Virol. **11**:672-681.
18. Lewis, A. M., Jr., M. J. Levin, W. H. Wiese, C. S. Crumpacker, and P. H. Henry. 1969. A nondefective (competent) adenovirus-SV40 hybrid isolated from the Ad2-SV40 hybrid population. Proc. Nat. Acad. Sci. U.S.A. **63**:1128-1135.
19. Lewis, A. M., Jr., A. S. Levine, C. S. Crumpacker, M. J. Levine, R. J. Samaha, and P. H. Henry. 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. V. Isolation of additional hybrids which differ in their simian virus 40-specific biological properties. J. Virol. **11**:655-664.
20. Lewis, A. M., Jr., and W. P. Rowe. 1971. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. I. A newly characterized simian virus 40 antigen induced by the $Ad2^{+}ND_1$ virus. J. Virol. **7**:189-197.
21. Maitra, U., and F. Barash. 1969. DNA dependent synthe-

sis of RNA by *E. coli* RNA polymerase. Release and reinitiation of RNA chains from DNA templates. Proc. Nat. Acad. Sci. U.S.A. **64**:779-786.
22. Maitra, U., A. H. Lockwood, J. S. Dubnoff, and A. Guha. 1970. Termination, release, and reinitiation of RNA chains from DNA templates by *E. coli* RNA polymerase. Cold Spring Harbor Symp. Quant. Biol. **35**:143-156.
23. Millete, R. L., C. D. Trotter, P. Herrlich, and M. Schweiger. 1970. *In vitro* synthesis, termination and release of active messenger RNA. Cold Spring Harbor Symp. Quant. Biol. **35**:135-142.
24. Oxman, M. N., W. P. Rowe, and P. H. Black. 1967. Studies of adenovirus SV40 hybrid viruses. VI. Differential effects of interferon on SV40 and adenovirus t antigen formation in cells infected with SV40 virus, adenovirus and adenovirus-SV40 hybrid viruses. Proc. Nat. Acad. Sci. U.S.A. **57**:941-948.
25. Richardson, J. 1969. RNA polymerase and the control of RNA synthesis. Prog. Nucl. Acid. Res. Mol. Biol. **9**:75-116.
26. Roberts, J. W. 1969. Termination factor for RNA synthesis. Nature (London) **224**:1168-1174.
27. Roberts, J. W. 1970. The P factor: termination and anti-termination in λ. Cold Spring Harbor Symp. Quant. Biol. **35**:121-126.
28. Sipple, A., and G. Hartman. 1968. Mode of action of rifamycin on the RNA polymerase reaction. Biochem. Biophys. Acta. **157**:218-219.
29. Sugiura, M., T. Okamoto, and M. Takanami. 1970. RNA polymerase sigma factor and the selection of initiation site. Nature (London) **225**:598-600.
30. Summers, W. C., and R. B. Siegel. 1970. Regulation of coliphage T_7 RNA metabolism *in vivo* and *in vitro*. Cold Spring Harbor Symp. Quant Biol. **35**:253-258.
31. Takanami, M., T. Okamoto, and M. Sugiura. 1970. The starting nucleotide sequences and size of RNA transcribed *in vitro* on phage DNA template. Cold Spring Harbor Symp. Quant. Biol. **35**:179-187.
32. Westphal, H. 1970. SV40 DNA strand selection by *E. coli* RNA polymerase. J. Mol Biol. **50**:407-420.
33. Witmer, H. J. 1971. Effect of ionic strength and temperature on the *in vitro* transcription of T4 DNA. Biochem. Biophys. Acta. **246**:29-43.