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# Specificity of Initiation of Transcription of Simian Virus 40 DNA I by *Escherichia coli* RNA Polymerase: Identification and Localization of Five Sites for Initiation with [$\gamma$-$^{32}$P]ATP

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Simian virus 40 (SV40) DNA I was transcribed with *Escherichia coli* RNA polymerase in the presence of $\gamma$-$^{32}$P-labeled ribonucleoside triphosphates in order to investigate the specificity of initiation of in vitro transcription. ATP and GTP served as predominant initiating nucleotides, the former being incorporated about twice as much as the latter. Cleavage of [$\gamma$-$^{32}$P]ATP-labeled SV40 complementary RNA (cRNA) with T1 RNase followed by homochromatographic analysis of the resultant 5′ initiation fragments revealed the presence of four specific initiation fragments 6 to 9 nucleotides in length, designated $A_I$, $A_{II}$, $A_{IIIa}$, and $A_{IIIb}$. By means of hybridization of [$\gamma$-$^{32}$P]ATP-labeled SV40 cRNA to DNA from specific adenovirus 2-SV40 hybrids and specific restriction endonuclease fragments of SV40 DNA before chromatographic analysis, it was possible to identify and determine approximate localizations of five [$\gamma$-$^{32}$P]ATP initiation sites on the SV40 genome: one in *Hin*-G close to the *Hin*-G-B junction, giving rise to the $A_{II}$ fragment, two in the overlapping fragment *Hin*-A–*Hae*-A, giving rise to $A_I$ and $A_{III}$ fragments, and two in the fragment *Hin*-A–*Hae*-E, also giving rise to $A_I$ and $A_{III}$ fragments. All five sites either fall within or lie near regions of the genome that are cleaved by S1 nuclease and subject to partial alkaline denaturation. These five sites lie on the minus strand of SV40 DNA and initiate RNAs that are copied in a leftward direction. Cleavage of [$\gamma$-$^{32}$P]GTP-labeled cRNA with pancreatic RNase liberated three major 5′ initiation fragments of short length, $G_I$, $G_{II}$, and $G_{III}$, suggesting the presence of three principal GTP initiation sites.

Whereas controversy exists concerning the specificity of initiation and strand selection during in vitro transcription of simian virus 40 (SV40) DNA by mammalian RNA polymerase (16, 17, 29, 30), a substantial body of evidence points to considerable specificity in the transcription of SV40 DNA by *Escherichia coli* RNA polymerase. At the level of strand selection, Westphal (41) initially demonstrated by means of nucleic acid hybridization methods asymmetry in SV40 transcription by the *E. coli* enzyme, a finding subsequently confirmed by direct nucleic acid sequence analysis of the transcript (12). Studies from several laboratories (19, 28, 37) have revealed that the in vitro transcript is copied from the same DNA strand (minus or E strand) as that copied in early lytic infection.

At the level of chain initiation, two lines of evidence point to retention of specificity in the interaction between SV40 DNA and *E. coli* RNA polymerase. First, under conditions of reduced temperature, transcription is initiated at a preferred site on the SV40 genome (44); this site has been localized to a small segment of SV40 DNA contained in the adenovirus-SV40 hybrid, $Ad2^+ND_3$, 0.17 map units from the unique *E. coli* R1 restriction endonuclease cleavage site. Second, under standard transcription conditions, a limited number of discrete sites on SV40 DNA from which RNA transcripts are initiated have been visualized by electron microscopy (10, 15, 43), one of which appears to correspond in localization to the preferred site for initiation of in vitro transcription (44).

Termination of in vitro SV40 transcription, on the other hand, appears to be nonspecific or even lacking under standard transcription conditions, for discrete RNAs cannot be detected in such transcriptions (22) and the circular genome may be transcribed multiple times without successful termination (10). Partially specific termination of transcription, however, has

been observed at reduced temperatures (44) in which a specific transcript of 100 to 110 nucleotides in length is synthesized from the preferred initiation site. And recently, a number of discrete RNAs have been transcribed from the SV40 genome under conditions of restricted substrate concentration (22), indicating specificity at the levels of both initiation and termination of transcription.

The present study was motivated by the desire to further explore the specificity of initiation of transcription of SV40 by *E. coli* RNA polymerase, to attempt localization of sites of initiation of in vitro transcription on the SV40 genome, and to compare these sites with genomic sites corresponding to the 5′ termini of early and late lytic (11, 20, 38) and transformed-cell (5, 21) mRNA's and with sites that are susceptible to cleavage with single-strand nuclease (3) or most readily denaturable under alkaline conditions (33). For these purposes, we have utilized [$\gamma$-$^{32}$P]ATP and -GTP to specifically label the initiation sites of in vitro transcripts followed by RNase digestion and homochromatography to isolate individual 5′-terminal initiation oligonucleotides. This methodology has permitted identification of five principal ATP initiation sites and suggests the presence of three principal GTP initiation sites. All five ATP initiation sites have been mapped on the SV40 genome; two, and possibly three, of these initiation sites fall in regions of the genome that are attacked by single-strand nuclease, and the remaining two sites lie near one of these regions. All five sites lie within regions of the genome that are most susceptible to alkaline denaturation. None of the ATP sites appear to correspond to 5′ termini of in vivo virus-specific mRNA's.

## MATERIALS AND METHODS

Propagation of SV40, from strain 776 stock, and the adenovirus 2-SV40 hybrids, Ad2$^+$ND$_{1-5}$, and purification of their DNAs have been described previously (14, 34, 44). SV40 DNA I, purified in CsCl-ethidium bromide equilibrium gradients, was used in all transcription experiments. Purified hybrid viruses were a generous gift from A. Lewis, Jr. The separated strands of Ad2$^+$ND$_4$, prepared as described elsewhere (35), were generously provided by C. Patch and A. S. Levine.

Restriction endonucleases from *Haemophilus influenzae* and *H. aegyptius* were purified by established methodology (24, 39). SV40 DNA I cleaved with the former yielded 11 major fragments, and with the latter 16 fragments, as reported elsewhere (9, 24). These are designated the *Hin*-A-K and *Hae*-A-M fragments, respectively.

RNA polymerase was purified from *E. coli* 1113 as previously described (25). Transcriptions were generally carried out in reactions of 125 $\mu$l containing 0.18 M KCl, 0.033 M Tris-hydrochloride, pH 7.9, 6 mM mercaptoethanol, 0.165 mM CTP, UTP, ATP, and GTP, 10 to 20 $\mu$g of SV40 DNA I, and 20 $\mu$g of RNA polymerase. Quantitative incorporation studies were carried out in reactions of 30 $\mu$l containing 5 $\mu$g of SV40 DNA and 0.5 or 5 $\mu$g of RNA polymerase. Either [$\gamma$-$^{32}$P]ATP or -GTP (New England Nuclear Corp.; specific activity, 18 to 34 Ci/mmol), CTP or UTP (Amersham-Searle Corp.; specific activity, 3 Ci/mmol), or [$\alpha$-$^{32}$P]ATP (New England Nuclear Corp.; specific activity, 70 Ci/mmol) served as the radioactive substrate in each reaction. For quantitative incorporation studies, the $\gamma$-$^{32}$P-labeled nucleotide triphosphates were desalted by passage through columns of Sephadex G-10 in water, since the commercial preparations often contained inhibitory salt concentrations. After incubation at 37°C for 20 to 30 min, 10 $\mu$g of pancreatic DNase (Worthington; RNase-free) was added to each 125-$\mu$l reaction and the incubation was continued for 10 min more. Reactions were stopped by extraction with 0.25% sodium dodecyl sulfate and an equal volume of water-saturated phenol, and labeled RNA was separated from radioactive substrate on Sephadex G-100 columns. Radiolabeled RNA was precipitated in the presence of 100 $\mu$g of carrier *E. coli* tRNA by addition of 0.1 volume of 20% potassium acetate and 2 volumes of ethanol before nucleic acid hybridization.

Nucleic acid hybridizations between [$\gamma$-$^{32}$P]ATP or -GTP-labeled RNAs and the DNAs of SV40 and the adenovirus-SV40 hybrids or fragments of SV40 DNA were carried out on nitrocellulose filters by a slight modification of the method of Gillespie and Spiegelman (13), as described elsewhere (44). After annealing at 65°C for 14 to 16 h, filters were washed on each side with 50 ml of 2× SSC (SSC, standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Filters containing [$\gamma$-$^{32}$P]ATP-labeled RNA were then treated with 25 IU of T1 RNase, and filters containing [$\gamma$-$^{32}$P]GTP-labeled RNA were treated with 0.8 $\mu$g of pancreatic RNase in 2 ml of 2× SSC for 45 min at room temperature. Filters were then rewashed as above with 2× SSC, incubated in 2 ml of 0.15 M sodium iodoacetate, 0.1 M sodium acetate, pH 5.2, for 40 min at 54°C, rewashed in 2× SSC, and then brought to 100°C for 5 min in 1.5 ml of 0.01× SSC. Eluted radiolabeled RNA was precipitated as noted above before RNase digestion and two-dimensional mapping.

Oligonucleotides containing 5′-[$\gamma$-$^{32}$P]ATP and [$\gamma$-$^{32}$P]GTP labels were obtained, respectively, by digestion of eluted RNA with T1 RNase (25 IU/100 $\mu$g of carrier tRNA) and pancreatic RNase (4 $\mu$g/100 $\mu$g of tRNA) in 10 $\mu$l of 0.01 M Tris-hydrochloride, pH 7.4, 1 mM EDTA for 50 min at 37°C. Digests were spotted on Cellogel approximately 2 cm apart and electrophoresed in 7 M urea at pH 3.5 for 5 min at 4,000 V. They were transferred to DEAE thin-layer plates and chromatographed in the second dimension with the homochromatography B mixture of Brownlee and Sanger (7). Autoradiograms were obtained with Kodak RP-Xomat film, using exposure times of 1 to 3 weeks.

For secondary enzymatic digestion, 5′-terminal

oligonucleotides were scraped from thin-layer plates, suctioned into plugged syringes, and washed extensively with ethanol to remove urea. Oligonucleotides were eluted with 30% triethylamine bicarbonate, dried, and digested for 1 h at 37°C in 15 μl of either pancreatic RNase (1 mg/ml) or T1 RNase (2,500 IU/ml) in 0.01 M Tris-hydrochloride, pH 7.4, 1 mM EDTA or U2 RNase (20 IU/ml) in 0.05 M sodium acetate, pH 4.5, 2 mM EDTA. Digests were electrophoresed on DEAE paper at pH 1.7 at 150 mA for 24 to 40 h, and autoradiograms were obtained as above.

## RESULTS

**Incorporation of $\gamma$-$^{32}$P-labeled ribonucleoside triphosphates into SV40 cRNA.** To determine which of the four ribonucleoside triphosphates serve as initiator nucleotides in transcription of SV40 DNA by *E. coli* RNA polymerase, small-scale identical transcriptions were conducted, using 5 μg of SV40 DNA, two different concentrations of RNA polymerase, and desalted [$\gamma$-$^{32}$P]ATP, -CTP, -GTP, and -UTP as substrates. Transcriptions were linear over the 20-min incubation period. Only ATP and GTP were incorporated significantly into complementary RNA (cRNA) (Fig. 1), the former about twice as well as the latter. Thus only ATP and GTP served as initiators of in vitro transcription of SV40 DNA, and ATP appeared to initiate twice the number of RNA chains as GTP.

**Demonstration of specific [$\gamma$-$^{32}$P]ATP initiation fragments.** An assay dependent upon nucleotide sequence was used to examine the specificity of initiation of transcription of SV40 DNA by *E. coli* RNA polymerase. SV40 cRNA's labeled in the 5′ position with [$\gamma$-$^{32}$P]ATP and -GTP were digested with T1 and pancreatic RNases, respectively, and then subjected to homochromatographic analysis. Only oligonucleotides derived from the 5′ end of cRNA were visualized by autoradiography.

Figure 2 provides chromatographic patterns of T1 RNase digests of ATP-labeled RNA. Most commonly, three spots with slow mobility and one or more with rapid mobility were visualized (Fig. 2a). The mobilities of the three slowly migrating spots have been compared with mobilities of fully or partially sequenced oligonucleotides similarly separated on DEAE thin-layer chromatography plates developed with homochromatography B solution (45; S. Weissman, unpublished data). From these comparisons, the three slowly migrating 5′ oligonucleotides, designated $A_I$, $A_{II}$, and $A_{III}$, appear to carry 11 to 14 negative charges and are therefore probably 6 to 9 nucleotides in length. In many transcriptions, the amounts of these three initiation fragments relative to one another have been somewhat variable. However, the amounts of $A_I$ and $A_{III}$ have always been either less than or approximately equal to the amount of $A_{II}$. Because these slowly migrating oligonucleotides are transcribed from a limited number of specific loci on the SV40 genome (see below), they are thought to be specific 5′ transcription initiation fragments. On one occasion, in which a strong oligonucleotide solution was used for homochromatography (Fig. 2b), a pattern showing a single major rapidly migrating spot and four rather than three slowly migrating spots was obtained. From the appearance of the spots, it appears most likely that the $A_{III}$ spot was resolved into two components, designated $A_{IIIa}$ and $A_{IIIb}$. Additional evidence derived from genomic localization of the [$\gamma$-$^{32}$P]ATP initiation fragments (see below) is also consistent with the presence of two components of the $A_{III}$ spot.

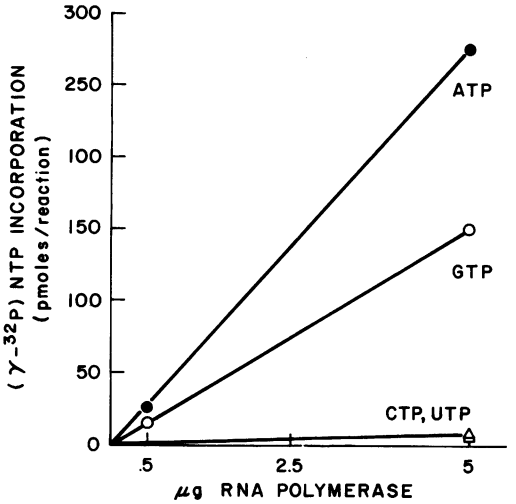


FIG. 1. *Incorporation of [$\gamma$-$^{32}$P]ATP (●), -GTP (○), -CTP (△), and -UTP (□) into SV40 cRNA. Transcriptions were performed as described in Materials and Methods. Deproteinized reaction mixtures were passed through Sephadex G-100 columns, and incorporation was determined from the amounts of radioactivity in the excluded RNA peak and the included substrate peak.*

The rapidly migrating spots in Fig. 2 moved slightly more slowly than marker ATP. They released labeled inorganic phosphate upon treatment with alkaline phosphatase, indicating that their 5′ phosphate is not blocked in any way, and they were not degraded by vigorous pancreatic or U2 RNase digestion, indicating that they do not contain additional nucleotides on the 3′ side of ATP. The more rapidly migrating spot in Fig. 2b (marked with an open arrow) moved more rapidly than marker pppApUp on

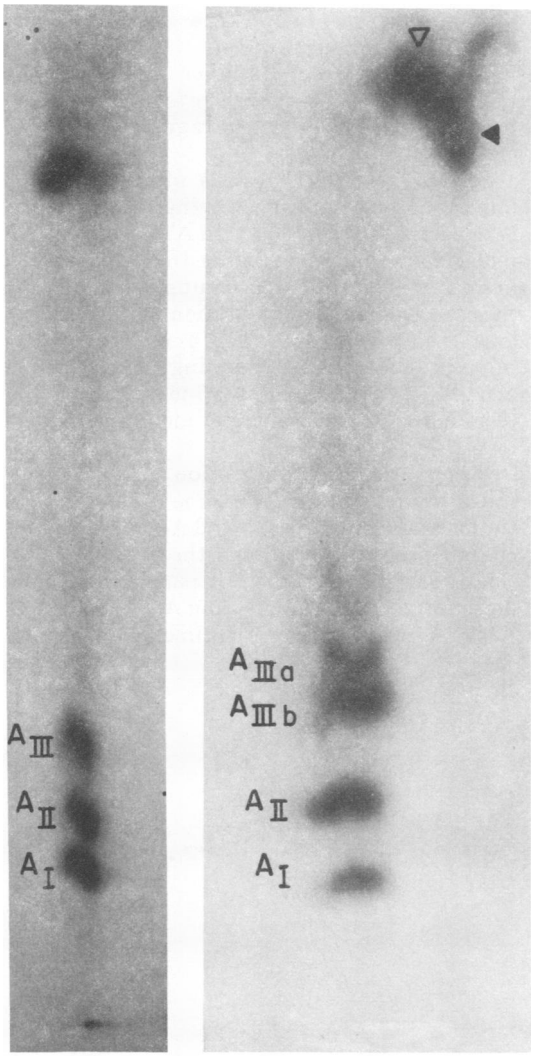


FIG. 2. *Homochromatograms of the [γ-32P]ATP-labeled 5′ initiation fragments of SV40 cRNA after hybridization to and elution from SV40 DNA. Transcriptions and transcript isolation were performed as described in Materials and Methods. Transcript RNA was hybridized to 5 μg of SV40 DNA fixed to nitrocellulose filters for 16 h at 65°C in 2× SSC plus 0.1% sodium dodecyl sulfate, and the hybrid was treated with T1 RNase and extensively washed before elution of bound RNA. Eluted RNA was digested with T1 RNase (25 IU per 100 μg of carrier tRNA) at 37°C for 50 min, after which the digests were electrophoresed in tandem in a rightward direction on Cellogel strips in 8 M urea at pH 3.5 and chromatographed in an upward direction in the homochromatography B mixture of Brownlee and Sanger (7) at 65°C. The three principal 5′ oligonucleotides in (a) have been designated $A_I$, $A_{II}$ and $A_{III}$. In (b), the $A_{III}$ oligonucleotide has been resolved into two components, $A_{IIIa}$ and $A_{IIIb}$.*

electrophoresis at pH 1.7 on DEAE paper, whereas the second spot (marked with a closed arrow) co-migrated with marker pppApUp under these conditions. Unlike the three 5′ oligonucleotides with slow mobility, these spots were present in extremely variable amounts; in most transcriptions they accounted for no more than 25% of the total radioactivity of the homochromatogram. Their relative presence was reduced by hybridization of the transcript RNA to SV40 DNA, and they were inconsistently visualized in the digests of SV40 cRNA annealed to and eluted from a wide variety of SV40 DNA fragments. This information leads us to feel that these spots could not be additional [γ-32P]ATP initiation fragments; the more rapidly migrating of these spots is likely pppAp derived nonspecifically during the T1 RNase digestion, whereas the slower spot most likely represents an unidentified derivative of ATP that is of little significance in considering the specific transcription initiation points on SV40 DNA.

In certain transcripts, additional 5′-labeled oligonucleotides, with mobility more rapid than $A_{III}$ but less rapid than the uppermost spots, were noted; however, their yield was also very variable and they never accounted for more than 10 to 15% of the total yield of terminal oligonucleotides. In summary, these data reveal four specific [γ-32P]ATP initiation fragments in SV40 cRNA, indicating four principal nucleotide sequences on SV40 DNA from which transcription with ATP is initiated with specificity by *E. coli* RNA polymerase.

It has been suggested that removal of superhelical turns from a closed circular supercoiled DNA leads to a restriction in the number of sites utilized by bacterial RNA polymerases for initiation of in vitro transcription (6). We therefore transcribed SV40 DNA II under our standard transcription conditions and homochromatographed the cRNA after digestion with T1 RNase. However, the chromatographic pattern of [γ-32P]ATP initiation fragments was virtually identical to that obtained for cRNA transcribed from SV40 DNA I.

Preliminary experiments were also conducted with [γ-32P]GTP-labeled RNA in an attempt to find specific initiation oligonucleotides. When this RNA was digested with pancreatic RNase, three major spots with mobilities only slightly slower than marker GTP, designated $G_I$, $G_{II}$, and $G_{III}$, and several relatively minor spots were visualized (Fig. 3). The most prominent of these minor spots, $G_{IV}$, carried only about one-third the radioactivity of each major spot. The three major spots were all digestible with T1 RNase, with release of labeled

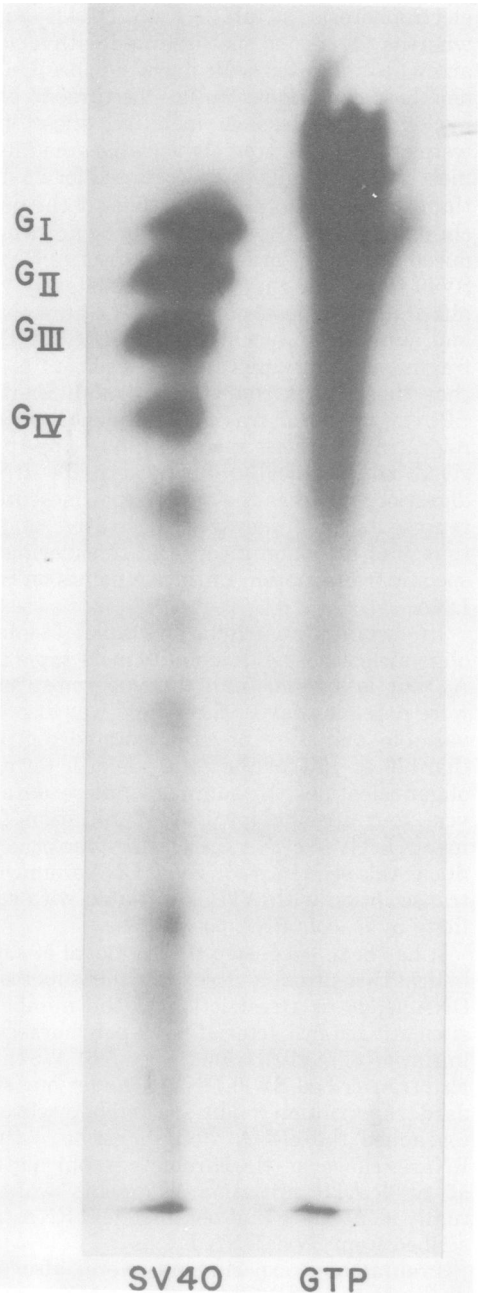


FIG. 3. *Homochromatograms of the [γ-32P]GTP-labeled 5′ initiation fragments of SV40 cRNA after hybridization to and elution from SV40 DNA. Conditions for transcription, transcript isolation hybridization, and homochromatography are described in the Fig. 2 legend except that the filter-bound DNA-RNA hybrid was treated with pancreatic RNase, extensively washed, eluted and then digested with pancreatic RNase (4 μg per 100 μg of carrier tRNA) at 37°C for 50 min. The three principal 5′ oligonucleotides have been designated $G_I$, $G_{II}$, and $G_{III}$, and a quantitatively less significant oligonucleotide has been designated $G_{IV}$. [γ-32P]GTP was cochromatographed with the GTP initiation oligonucleotides.*

pppGp, and are hence true initiation fragments. Further experiments with [γ-32P]GTP-labeled cRNA are now in progress, and the remainder of the results reported here concern the [γ-32P]GTP initiation fragments of SV40 cRNA.

**Size of [γ-32P]ATP cRNA of SV40.** It was important to determine whether RNA chains labeled in the 5′ position with ATP were of high molecular weight or whether they represented prematurely terminated chains of small size. Sucrose gradient sedimentation of SV40 cRNA labeled in this manner (Fig. 4) revealed 5′ terminally labeled RNA spanning the size range of 5 to 6S to approximately 26 to 28S. Thus, the terminally labeled transcript included RNAs of long chain length.

**Pancreatic RNase digestion of [γ-32P]ATP initiation fragments.** The three [γ-32P]ATP initiation fragments, $A_I$, $A_{II}$, and $A_{III}$, were further characterized by examining the 5′ oligonucleotides liberated from each by pancreatic RNase digestion (Fig. 5). From $A_I$ and $A_{III}$, oligonucleotides migrating 1.50 to 1.60 times more rapidly

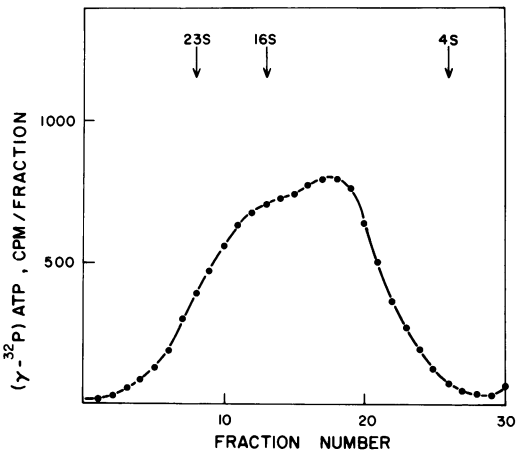


FIG. 4. *Sucrose gradient sedimentation analysis of a [γ-32P]ATP-labeled transcript of SV40 DNA. Transcription was carried out as described in Materials and Methods. After purification of the transcript by passage through a Sephadex G-100 column, RNA was precipitated and sedimented on a 5 to 20% sucrose gradient in 0.01 M Tris-hydrochloride, pH 7.5, 0.2 M LiCl, 0.5% sodium dodecyl sulfate, and 1.0 mM EDTA for 2.5 h at 50,000 rpm in the Spinco SW50.1 rotor. Nonlabeled E. coli 4S and ribosomal 16 and 23S RNAs were cosedimented as size markers.*

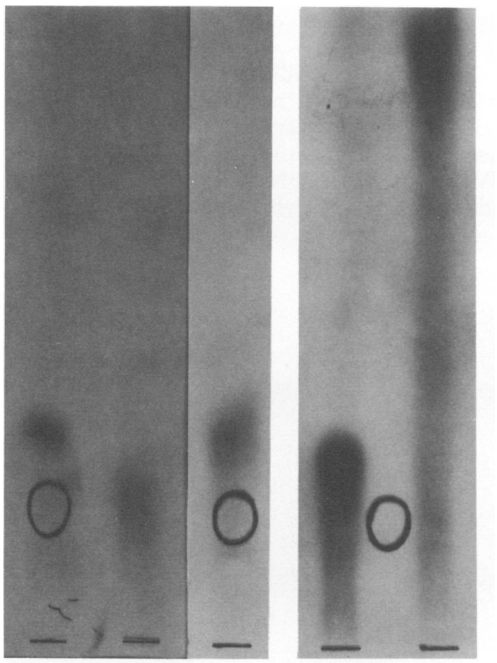


FIG. 5. *One-dimensional electrophoretic migration of the 5′ oligonucleotides liberated from the [γ-$^{32}$P]ATP-labeled 5′ initiation fragments of SV40 cRNA ($A_I$, $A_{II}$, and $A_{III}$) by pancreatic RNase digestion. The $A_{I-III}$ spots were scraped from a homochromatogram similar to that in Fig. 2a, freed of urea, and digested with pancreatic RNase (1.0 mg/ml) for 1 h at 37°C. The digests were electrophoresed in an upward direction on DEAE-cellulose paper at pH 1.7 at 150 mA for 40 h. The yellow dye, orange G, outlined in black circles, served as an electrophoretic marker. The migration of the 5′ pancreatic RNase oligonucleotides pppApUp and pppApCp, liberated from the 5′ termini of in vitro transcribed 6 and 11S RNAs of λb$_2$b$_5$c DNA, are shown on a separate electropherogram in the right panel for comparison. The small spot on the electropherogram of $A_I$ to the right of the leading edge of the marker dye is an artifact of autoradiography.*

than marker orange G dye on electrophoresis at pH 1.7 on DEAE paper were liberated. pppApUp, liberated from [γ-$^{32}$P]ATP-labeled 6S RNA synthesized in vitro on the λb$_2$b$_5$c template (25), migrated 1.50 times more rapidly than orange G under identical conditions (Fig. 5). The only other oligonucleotide with the structure ppp(Ap)$_n$Pyp that could migrate in this region was ppp(Ap)$_{4-5}$Cp, and this sequence is not present in total digests of SV40 cRNA (S. M. Weissman, unpublished observations). These data thus suggest that both $A_I$ and $A_{III}$ initiation fragments terminate in the sequence pppApUp. The 5′ oligonucleotide derived from $A_{II}$ migrated more slowly, falling on the leading edge of the marker dye. pppApApApUp, isolated from the 5′ terminus of an RNA copied from SV40 DNA by *E. coli* RNA polymerase under restrictive transcription conditions (44), migrated similarly under these electrophoretic conditions (12; S. M. Weissman and R. Dhar, unpublished observations). The only other oligonucleotide that could migrate in this region under these conditions was ppp(Ap)$_{6-8}$Cp, and this sequence was also excluded in digests of SV40 cRNA. We therefore believe that the $A_{II}$ initiation fragment likely terminates with the 5′ sequence pppApApApUp. As pointed out subsequently, the $A_{II}$ initiation fragment does in fact map in the same region of the SV40 genome as the pppApApApUp terminal RNA synthesized under restrictive transcriptional conditions.

The uniqueness of the pancreatic products of $A_I$, $A_{II}$, and $A_{III}$, taken together with the failure of the $A_I$ and $A_{II}$ oligonucleotides to resolve into additional components and the resolution of $A_{III}$ into only two components when subjected to a long first-dimensional electrophoresis as well as second dimensions under varying homochromatography conditions, suggests that the four major [γ-$^{32}$P]ATP initiation fragments are unique oligonucleotides.

**Localization of [γ-$^{32}$P]ATP initiation sites on the SV40 genome.** To localize the sites of initiation of transcription by [γ-$^{32}$P]ATP on the SV40 genome, we used initially a series of five adenovirus 2-SV40 hybrids (26, 27) containing overlapping segments of SV40 DNA covalently inserted at a unique point (18, 23, 32) into the adenovirus 2 genome (see Fig. 11). In the first localization experiment, a [γ-$^{32}$P]ATP transcript of SV40 DNA was hybridized to and eluted from the DNA of Ad2$^+$ND$_4$, the hybrid containing the longest SV40 segment (48% of the genome) (14, 18, 23, 32), before T1 RNase digestion and chromatographic analysis of the resultant 5′ initiation fragments. Figure 6b reveals the presence of all three principal 5′ initiation fragments, $A_I$, $A_{II}$, and $A_{III}$, in the eluted RNA. Thus at least one copy of each of these initiation fragments is derived from the area of the SV40 genome represented in Ad2$^+$ND$_4$.

It has previously been reported that a prominent [γ-$^{32}$P]ATP initiation site exists within the segment of SV40 DNA included within the hybrid virus Ad2$^+$ND$_3$ (44). The SV40 segment of this virus represents only 6.4% of the SV40 genome (18, 23, 32). To determine which of the three [γ-$^{32}$P]ATP initiation fragments is transcribed from this region, a [γ-$^{32}$P]ATP-labeled transcript of SV40 DNA was first annealed to

and eluted from this hybrid and then T1 RNase treated and cochromatographed with the same transcript annealed to SV40 DNA. Only the $A_{II}$ 5′ oligonucleotide was visualized in the RNA annealed to $Ad2^+ND_3$ (Fig. 6e); although faint, it was identifiable in four independent experiments. Neither the $A_I$ nor $A_{III}$ initiation fragment was visualized in any of these experiments. Thus, it is the $A_{II}$ fragment that is derived from a site included in the SV40 segment of the $Ad2^+ND_3$.

Approximate localizations of the $A_I$ and $A_{III}$ initiation sites on the SV40 DNA segment of $Ad2^+ND_4$ were determined in a similar manner: by hybridization of [$\gamma$-$^{32}$P]ATP-labeled SV40 cRNA to the DNAs of the three remaining adenovirus-SV40 hybrids, $Ad2^+ND_1$, $_{-2}$ and $_{-5}$, containing, respectively, 18, 36, and 29% of the

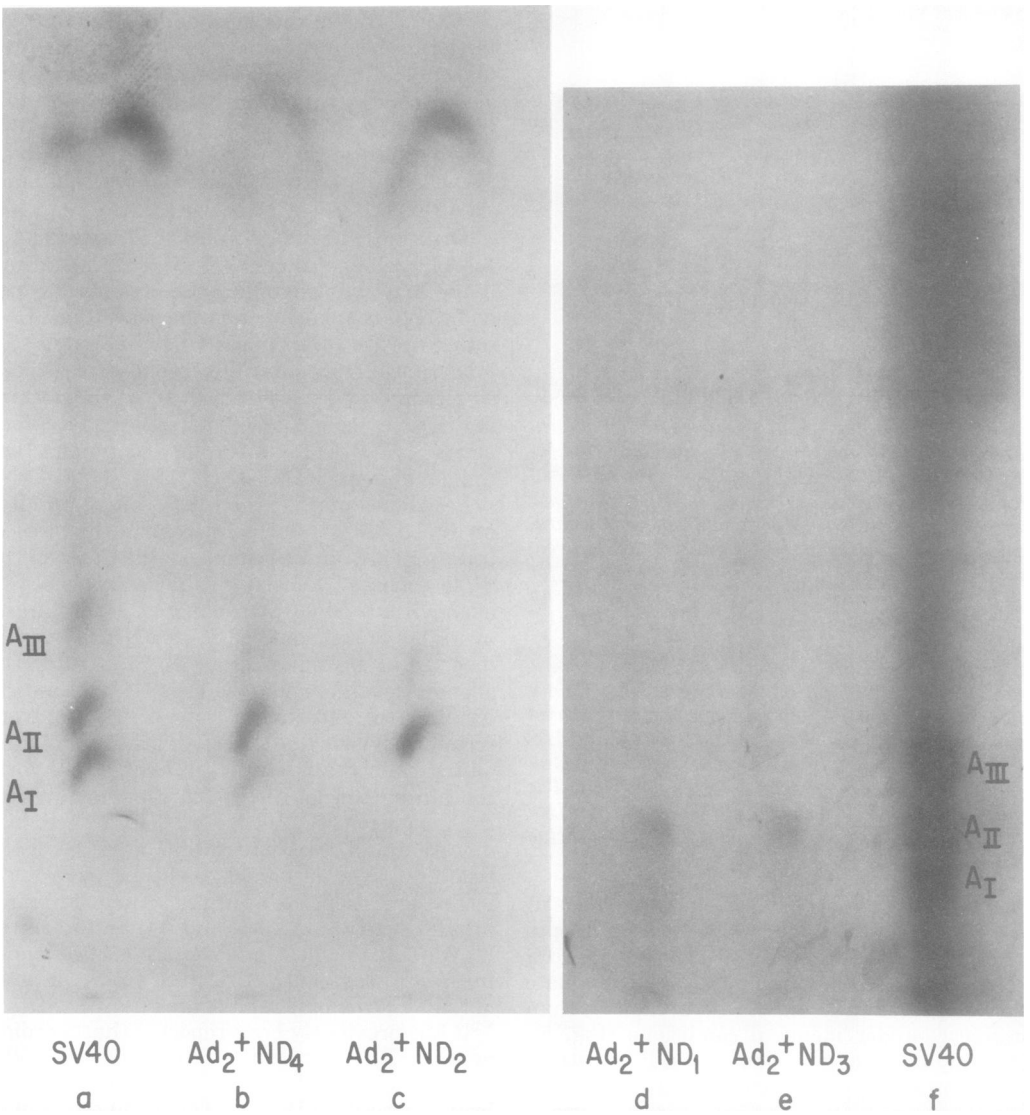


FIG. 6. *Homochromatograms of the [$\gamma$-$^{32}$P]ATP-labeled 5′ initiation fragments of SV40 cRNA after hybridization to and elution from the DNAs (2 $\mu$g) of SV40 (a), $Ad2^+ND_4$ (b), $Ad2^+ND_2$ (c), $Ad2^+ND_1$ (d), and $Ad2^+ND_3$ (e) and without any prior hybridization (f). The conditions for transcription, transcript isolation, hybridization, T1 RNase digestion, and homochromatography are described in the Fig. 2 legend and in Materials and Methods. The SV40 transcript hybridized to $Ad2^+ND_5$ gave a homochromatographic pattern similar to those for cRNA hybridized to $Ad2^+ND_1$ and $Ad2^+ND_3$.*

SV40 genome (18, 23, 32), before nuclease digestion and chromatography. Like labeled RNA hybridized to and eluted from $Ad2^+ND_3$, RNA annealed to $Ad2^+ND_1$ (Fig. 6d), and $Ad2^+ND_5$ DNA contained only one 5′ oligonucleotide, $A_{II}$. RNA bound to $Ad2^+ND_2$, however, contained not only the $A_{II}$ fragment but also the $A_{III}$ spot (Fig. 6c). These results were reproduced in two to four independent experiments for each of the hybrid DNAs. Thus, an $A_{III}$ site may be localized to the 7% of the SV40 genome present in $Ad2^+ND_2$ but absent in $Ad2^+ND_5$. Furthermore, since the $A_I$ fragment was not visualized in the digest of RNA annealed to $Ad2^+ND_2$, an $A_I$ initiation site must be included in the 12% of the SV40 genome present in $Ad2^+ND_4$ but absent in $Ad2^+ND_2$.

The map of the five adenovirus-SV40 hybrids used in these localization studies has been colinearly related to the map of the fragments of SV40 DNA derived by cleavage with restriction endonucleases from *H. influenzae* (22), *H. aegyptius* (23), and *E. coli* containing RII factor (40) (see Fig. 11). By looking for the presence of the characteristic initiation fragments after binding of [$\gamma$-$^{32}$P]ATP-labeled SV40 cRNA to various restriction endonuclease-derived fragments, it was thus possible to confirm and also to more precisely determine the positions of the $A_I$, $A_{II}$, and $A_{III}$ initiation sites already identified on the SV40 DNA segments of the hybrid viruses and also to search for additional initiation sites beyond the confines of the SV40 segment of $Ad2^+ND_4$. Table 1 reveals the extent of binding of the SV40 transcript to the *Hin*-G, -B, -I, -H, and -A, *Hae*-A, -C, and -D, and *Eco*RII-F fragments, all of which are included totally or almost totally in the SV40 segment of $Ad2^+ND_4$; to *Hae*-E, half of which is included in the SV40 segment of $Ad2^+ND_4$ and half without; and also to the unresolved mixture of *Hin*-C and -D, *Hin*-E, and *Hae*-B, which together with a portion of *Hae*-E span the area of the SV40 genome not included within $Ad2^+ND_4$. The efficiency of hybridization to these fragments was somewhat low, probably owing to lability of the terminal $^{32}$P at 65°C for 14 to 16 h and possibly also to susceptibility to cleavage with traces of phosphatase during T1 RNase treatment of filters before RNA elution. Nevertheless, it is apparent that [$\gamma$-$^{32}$P]RNA bound significantly only to *Hin*-G and -A and *Hae*-A, -C, and -E, and *Eco*RII-F, essentially not at all to *Hin*-B and -I and *Hae*-B, and not at all to the remaining fragments. In control experiments, identically prepared filters containing nearly identical quantities of the *Hin* fragments all bound SV40 [$\alpha$-$^{32}$P]cRNA. The fact that *Hin*-C-D, *Hin*-E, and *Hae*-B did not bind appreciable amounts of [$\gamma$-$^{32}$P]RNA indicates the absence of [$\gamma$-$^{32}$P]ATP initiation sites in the contiguous 45% of the genome between the left terminus of *Hin*-C and the right terminus of *Hae*-B, most of the genome beyond the confines of the SV40 segment of $Ad2^+ND_4$ (see Fig. 11).

TABLE 1. *Hybridization of [$\gamma$-$^{32}$P]ATP-labeled transcripts of SV40 DNA to restriction endonuclease fragments of SV40 DNA*[a]

| Fragment | Input cpm | Cpm bound |
|---|---|---|
| *Hin*-A | 25,000 | 1,006 |
| -B | 25,000 | 12 |
| -C-D | 25,000 | 0 |
| -E | 25,000 | 0 |
| -G | 25,000 | 214 |
| -H | 25,000 | 0 |
| -I | 25,000 | 5 |
| *Hae*-A | 19,500 | 1,104 |
| -B | 19,500 | 8 |
| -C | 19,500 | 428 |
| -D | 19,500 | 0 |
| -E | 16,800 | 680 |
| *Eco*RII-F | 15,400 | 168 |

[a] Hybridizations were performed as described in Materials and Methods between approximately 0.2 $\mu$g of each restriction endonuclease fragment fixed to a nitrocellulose filter and the indicated amount of input transcript RNA. All hybrids were treated with T1 RNase before washing and counting by the method of Cerenkov.

Chromatographic analysis of the initiation fragments derived from RNA hybridized to and eluted from *Hin*-A, -B, -G, and -I, *Hae*-A, -C, and -E, and *Eco*RII-F is demonstrated in Fig. 7 and 8. The $A_{II}$ initiation fragment was visualized only in the chromatograms of RNA annealed to the overlapping fragments *Hin*-G, *Hae*-C, and *Eco*RII-F; thus the significance of the single light $A_{II}$ spot in the chromatograms of RNA annealed to $Ad2^+ND_1$ and $Ad2^+ND_3$ (Fig. 6d, e) is confirmed, and only one $A_{II}$ initiation site can be localized on the SV40 genome. Of importance as negatives, $A_I$ and $A_{III}$ spots were not visualized in the *Hae*-C chromatogram and only very weakly in the *Hin*-G chromatogram, both of which demonstrated very prominently the $A_{II}$ spot. The $A_I$ and $A_{III}$ 5′ oligonucleotides were visualized prominently in the chromatograms of RNA hybridizing to *Hin*-A, *Hae*-A, and *Hae*-E. Thus *Hae*-A and *Hae*-E, which together overlap all of *Hin*-A, each possess an $A_I$ and $A_{III}$ initiation site; the initiation sites within *Hae*-A must lie within the confines of the SV40 segment of $Ad2^+ND_4$, whereas it is not clear whether the initiation sites within *Hae*-E lie within or beyond the confines of the SV40 segment in $Ad2^+ND_4$. From these chromatograms, it is not possible to discern which of the $A_{III}$ components lie in *Hae*-

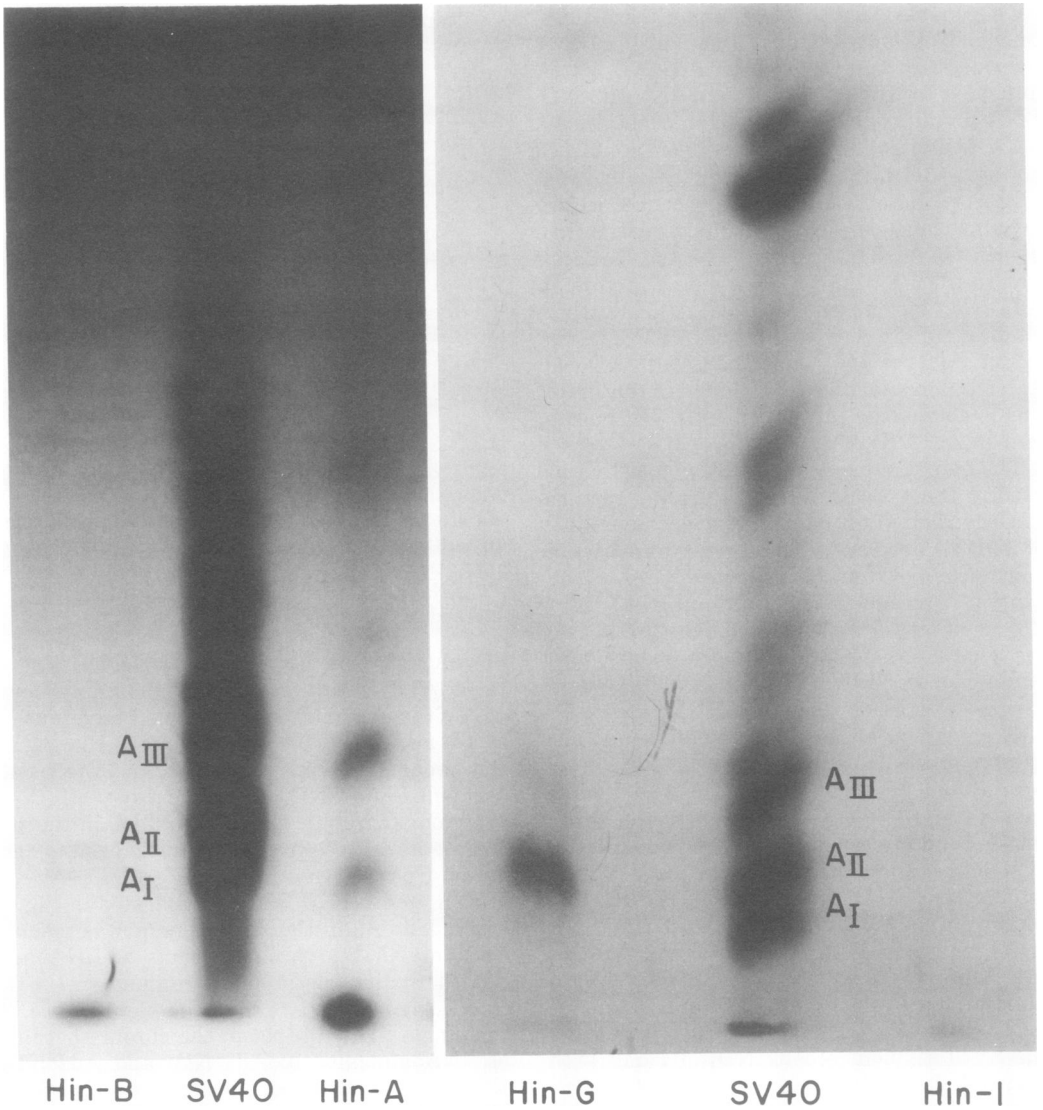


FIG. 7. *Homochromatograms of the [γ-$^{32}$P]ATP-labeled 5′ initiation fragments of SV40 cRNA after hybridization to and elution from the A, B, G, and I fragments (0.2 μg) derived from digestion of SV40 DNA with Haemophilus influenzae restriction endonuclease. The 5′ initiation fragments from unhybridized SV40 cRNA served as markers. The conditions for transcription, transcript isolation, hybridization, T1 RNase digestion, and homochromatography are described in the Fig. 2 legend and in Materials and Methods.*

A and -E, respectively. The chromatogram of RNA annealed to *Hae*-E also showed a relatively faint spot above the $A_{III}$ spot. From its migration and the fact that this chromatogram was developed with a relatively weak homochromatography B solution, it is clear that this spot is not one of the major 5′ oligonucleotides and that the $A_{III}$ spot is not resolved into its two components. The chromatogram of the small amount of RNA eluting from the filters containing *Hin*-B and *Hin*-I gave no detectable spots.

From the combined data on initiation fragment localizations, using hybrid viruses and DNA fragments, it may be concluded that a total of five principal [γ-$^{32}$P]ATP initiation sites exist on the SV40 genome: two $A_I$ initiation sites, the first on the span of DNA common to *Hin*-A, *Hae*-A, and Ad2$^+$ND$_4$ but not Ad2$^+$ND$_2$ and the second on a span of DNA common to *Hin*-A and *Hae*-E; a single $A_{II}$ initiation site on the span of DNA common to *Hin*-G, *Hae*-C, *Eco*RII-F, and Ad2$^+$ND$_3$; and two $A_{III}$ initiation sites, the first on the span of DNA common to

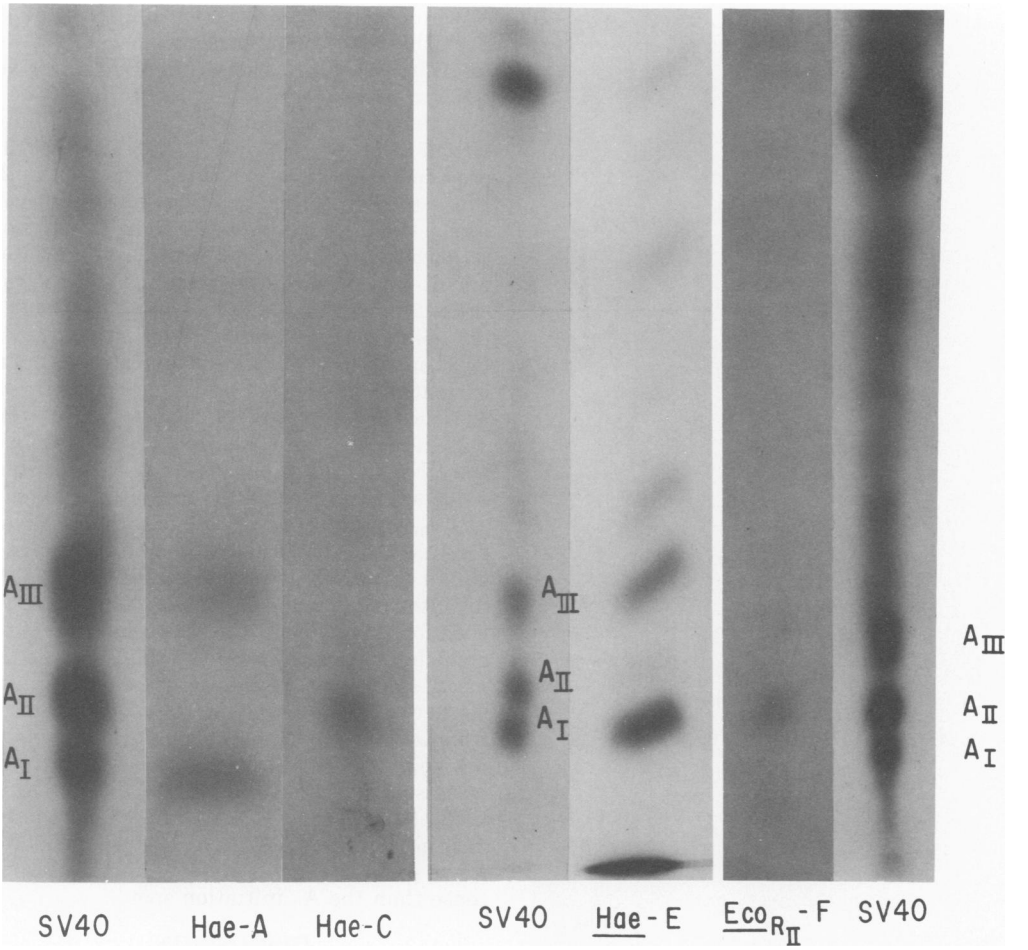


FIG. 8. *Homochromatograms of the [γ-32P]ATP-labeled 5' initiation fragments of SV40 cRNA after hybridization to and elution from the A, C, and E fragments (0.2 μg) derived from cleavage of SV40 DNA with H. aegyptius restriction endonuclease and the F fragment (0.2 μg) derived by cleavage with restriction endonuclease from E. coli carrying RII factor. The 5' initiation fragments from unhybridized SV40 cRNA served as markers. The conditions for transcription, transcript isolation, hybridization, T1 RNase digestion, and homochromatography are described in the Fig. 2 legend and in Materials and Methods.*

*Hin*-A, *Hae*-A, and Ad2$^+$ND$_2$ and the second on a span of DNA common to *Hin*-A and *Hae*-E (see Fig. 11). The order of the $A_I$ and $A_{III}$ sites on *Hae*-E has not been determined.

**Localization of ATP initiation sites to the minus (early) DNA strand.** *E. coli* RNA polymerase transcribes only the minus strand of SV40 DNA provided there are no nicks in the DNA (19, 28, 37, 41). To be certain that the [γ-$^{32}$P]ATP initiation fragments described were copied from the minus strand, a [γ-$^{32}$P]ATP transcript of DNA I was annealed to the separated strands of Ad2$^+$ND$_4$. Almost 95% of the total counts annealed hybridized to the minus strand, and about 5% annealed to the plus (late) strand. Furthermore, the three characteristic initiation fragments were present in the chromatogram of the T1 RNase digest of RNA eluted from the minus strand (Fig. 9). Thus, the five [γ-$^{32}$P]ATP initiation sites we have described may be localized on the minus strand of SV40 DNA. Of interest, an additional minor initiation oligonucleotide migrating just ahead of the $A_{III}$ fragment was visualized in the chromatogram of cRNA annealing to the minus strand. The small amount of hybridization of the radiolabeled transcript to the plus strand of Ad2$^+$ND$_4$ is most likely due to slight contamination with minus strands.

**Direction of transcription from three ATP initiation sites.** It has been demonstrated that early in lytic infection of permissive cells, RNA is transcribed over an approximately 50% span of the minus strand of SV40 DNA in a leftward

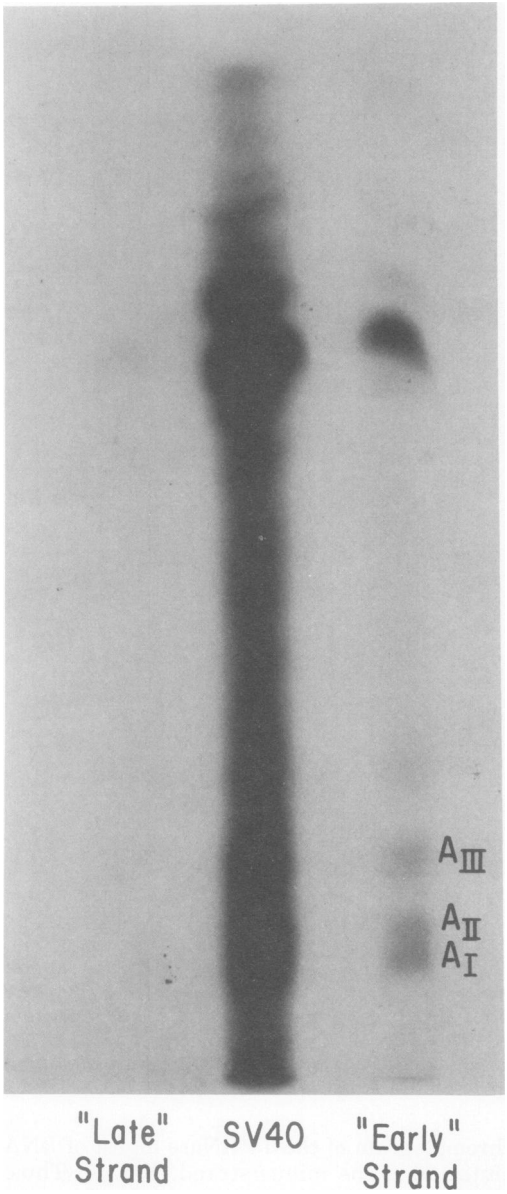


FIG. 9. *Homochromatograms of the [γ-$^{32}$P]ATP-labeled 5′ initiation fragments of SV40 cRNA after hybridization to and elution from the separated strands (approximately 2 μg each) of Ad2$^{+}$ND$_4$ DNA. The early strand of hybrid virus DNA contains the template for SV40 RNAs synthesized early in lytic infection, and the late strand contains the template for the additional RNAs synthesized late in lytic infection. The 5′ initiation fragments from unhybridized SV40 cRNA served as markers. The conditions for transcription, transcript isolation, hybridization, T1 RNase digestion, and homochromatography are provided in the legend of Fig. 2 and in Materials and Methods. The amount of rapidly migrating material, probably pppAp (see text), in the cRNA binding to early strand is exceptionally large in this experiment.*

or counterclockwise direction as the SV40 genome is conventionally drawn (11, 12, 20, 38) (see Fig. 11). Since transcription on any given DNA strand must always proceed in a 3′ to 5′ direction, in vitro transcription on the minus strand from the ATP initiation sites must also proceed in this direction. It is pointed out in the Discussion that the $A_{II}$ initiation site is very likely the predominant site from which transcription at reduced temperature is initiated, and it has been shown previously that transcription from this site does indeed proceed to the left (12). Leftward transcription from at least one each of the $A_I$ and $A_{III}$ sites was demonstrated by performing a transcription in the presence of [γ-$^{32}$P]ATP on the SV40 DNA template, annealing the terminally labeled RNA to the DNA of an adenovirus-SV40 hybrid whose SV40 segment terminates to the left of the $A_I$ and $A_{III}$ initiation sites, omitting the T1 RNase digestion of the hybrid, and then examining the eluted RNA for the presence of the $A_I$ and $A_{III}$ initiation fragments. When Ad2$^{+}$ND$_5$ DNA was used for this hybridization, not only the 5′ oligonucleotide from the $A_{II}$ site but also the terminal fragments originating at $A_I$ and $A_{III}$ sites were visualized (Fig. 10). Thus transcription from at least one each of the $A_I$ and $A_{III}$ sites proceeds in a leftward direction. It is probably because of the failure of all RNA chains initiated at the $A_I$ and $A_{III}$ sites to extend the full distance into the Ad2$^{+}$ND$_5$ SV40 segment that these spots in Fig. 10 are considerably less intense than the $A_{II}$ initiation spot.

## DISCUSSION

The contention that *E. coli* RNA polymerase transcribes SV40 DNA with specificity has rested on the asymmetrical nature of transcription under standard (41) and restricted (22) polymerization conditions, demonstration by electron microscopy of a limited number of DNA sites from which RNA chains arise under standard conditions (15, 43), and the presence of discrete RNA species (22) and transcription from a preferred initiation site (44) under conditions that greatly restrict total RNA synthesis. The findings reported here are concerned with the identification and localization of transcription initiation sites on SV40 DNA and offer additional support for specificity of the interaction between SV40 DNA and *E. coli* RNA polymerase.

Our experiments demonstrate incorporation of [γ-$^{32}$P]ATP and -GTP, but not [γ-$^{32}$P]CTP and

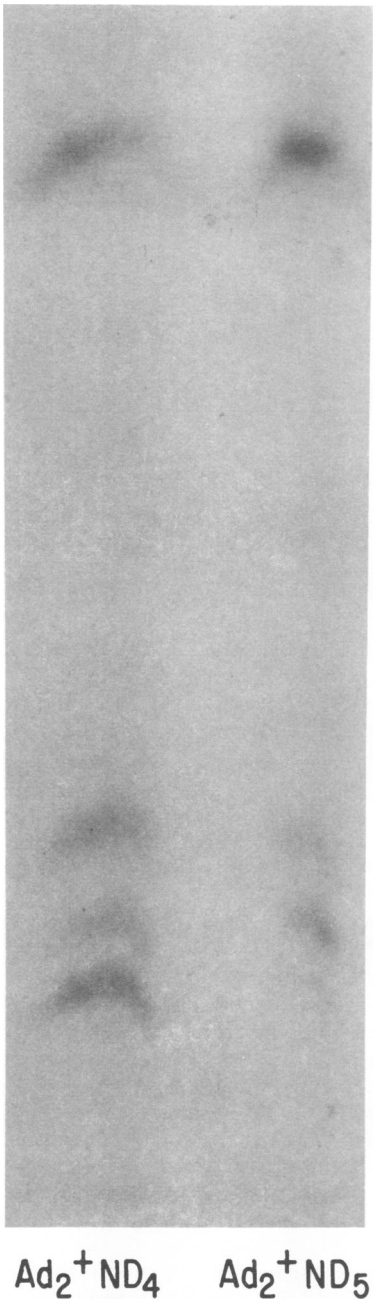


FIG. 10. *Homochromatograms of the [γ-32P]ATP-labeled 5' initiation fragments of SV40 cRNA after hybridization to and elution from the DNAs of Ad2+ND4 and Ad2+ND5 (2 μg each). The conditions for this experiment were identical to those in Fig. 2, except for the omission of T1 RNase digestion of the nitrocellulose filter-fixed DNA-RNA hybrids before elution of RNA for further nuclease digestion and homochromatography.*

-UTP, into SV40 cRNA by *E. coli* RNA polymerase. Furthermore, we have found four principal specific [γ-32P]ATP initiation fragments and three principal specific [γ-32P]GTP initiation fragments in the RNA transcribed from SV40 DNA I by the bacterial RNA polymerase. From these data we conclude that in vitro initiation is confined to the purine ribonucleoside triphosphates and that specific initiation is restricted principally to four nucleotide sequences on SV40 DNA for ATP and three for GTP. Additional transcription initiation sites for GTP and ATP have been noted, but quantitatively these appear to be of minimal significance, and qualitatively some may be nonspecific. With regard to ATP initiations, our experiments further show that one of the initiation fragments, $A_{II}$, is copied from only one site on the SV40 genome, whereas the $A_I$ fragment and the $A_{III}$ complex (composed of $A_{IIIa}$ and $A_{IIIb}$ initiation fragments) are each copied from two separate sites on the SV40 genome. Thus a total of five principal [γ-32P]ATP initiation sites have been identified on the SV40 genome. Total incorporation of [γ-32P]ATP is approximately twice that of [γ-32P]GTP into SV40 cRNA. Given five principal [γ-32P]ATP initiation sites, it appears likely to us that there are three principal [γ-32P]GTP initiation sites on the SV40 genome or, stated otherwise, that each of the three principal [γ-32P]GTP initiation fragments is derived from only a single site on the SV40 genome. Alternatively, the three principal [γ-32P]GTP initiation fragments could be derived in a rather nonspecific manner from a multiplicity of initiation sites; however, we have no direct evidence bearing on this point since we have not yet mapped the [γ-32P]GTP initiation fragments on the viral genome. In contrast to our results for SV40, transcription of the DNA of the related virus, polyoma, is initiated largely, if not exclusively, with GTP and specifically with the dinucleotide sequence pppGpUp (A. A. Travers, Ph.D. thesis, University of Cambridge, Cambridge, England, 1968).

It has been estimated from direct electron microscopic visualization in two separate studies that superhelical SV40 DNA binds in nonrandom fashion three (15) and up to six (43) molecules of *E. coli* RNA polymerase. For polyoma DNA I, of approximately equal molecular weight, from four to nine enzyme-binding sites have been visualized in the electron microscope (8, 36). Our finding of five specific [γ-32P]ATP initiation sites and possibly three additional [γ-32P]GTP sites on SV40 DNA is compatible with or somewhat higher than the most reliable electron microscopic estimate for the number of

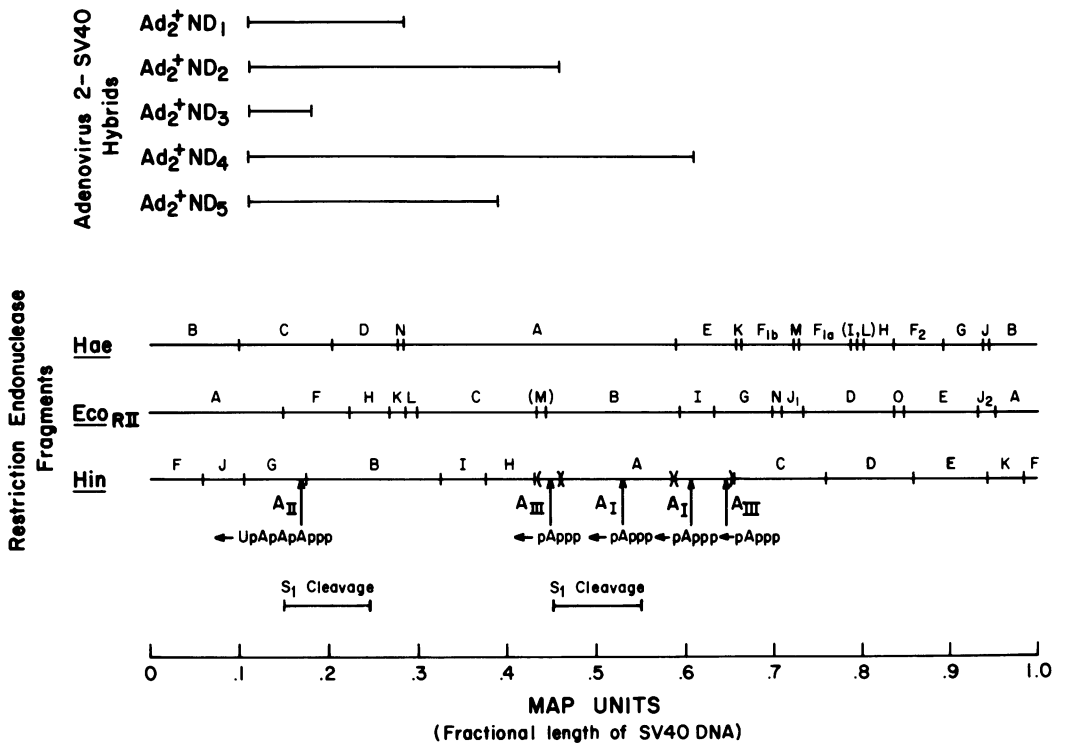


FIG. 11. *Cleavage map of the SV40 genome and the SV40 segments of five adenovirus 2-SV40 hybrids indicating the localizations on SV40 DNA of five sites from which transcription is initiated in the presence of [γ-$^{32}$P]ATP. Whereas the precise localization of the $A_{II}$ site has been determined (see text), the approximate localizations of the $A_I$ and $A_{III}$ sites are indicated by parentheses. The order of the $A_I$ and $A_{III}$ sites within Hae-E has not been determined. Transcription from these sites is in a leftward direction as the map has been drawn.*

transcription initiation sites on SV40 DNA (43). It should be noted that these electron microscopic studies used transcriptions in the presence of rifampin after RNA polymerase binding to DNA on the basis of studies suggesting that rifampin restricts initiations specifically to promotor sites (2). It has unfortunately not been possible for us to use rifampin restriction in our experiments, for reinitiation is essential to obtain adequate incorporation of terminal gamma label into cRNA for the type of analytic methods we have used. However, we have attempted to restrict initiations by means of drastically reducing substrate concentrations (22) and by transcribing SV40 DNA II, but in both experiments [γ-$^{32}$P]ATP and -GTP are both incorporated into cRNA in a ratio of approximately 2:1, and the three characteristic [γ-$^{32}$P]ATP initiation fragments are visualized on homochromatography.

Figure 11 summarizes our findings on the approximate localizations on the SV40 genome of all five sites from which in vitro transcription with ATP is initiated. These localizations have now been confirmed by direct transcriptions of SV40 DNA fragments in the presence of [γ-$^{32}$P]ATP (P. Lebowitz and P. K. Ghosh, unpublished observations). Furthermore, nucleic acid sequence analysis of these genomic regions (Dhar and Weissman, unpublished observations) has revealed nucleotide sequences consistent with the terminal sequences and length of the [γ-$^{32}$P]ATP initiation oligonucleotides.

The single $A_{II}$ site lies in the segment of the genome common to *Hin*-G, *Hae*-C, *Eco*RII-F, and Ad2$^+$ND$_3$. This DNA segment, adjacent to the *Hin*-G-B junction, spans 125 nucleotides (40), the sequence of which has been determined (11, 12, 45). From the facts that the $A_{II}$ initiation fragment is copied from the SV40 minus strand in a leftward direction, is approximately eight nucleotides in length, and starts with the sequence pppApApApUp, there are only two locations on the minus strand within this stretch of 125 nucleotides from which it can arise, both with the sequence pTpTpTpApTpTpTpCp. One of these sites, 30 nucleotides from the *Hin*-G-B junction (45), at 0.17 map units,

serves as the site for preferred initiation of transcription at reduced temperatures. It thus seems most probable that the $A_{II}$ initiation site is indeed identical to the site for preferred initiation of transcription at reduced temperatures. Furthermore, examination of the homochromatograms of [$\gamma$-$^{32}$P]ATP-labeled SV40 initiation fragments in the present paper has revealed either approximately equal quantities of the three initiation fragments or relatively more of the $A_{II}$ fragment. Given a single $A_{II}$ initiation site but two $A_I$ and $A_{III}$ initiation sites, we conclude that under standard conditions, too, the $A_{II}$ site is a preferred site for initiation of transcription.

In their electron microscopic analysis of transcription initiation sites on SV40 DNA, Delius et al. (10) were also able to identify a strong site for initiation at 0.17 map units on the SV40 genome. Although growing RNA chains were detected in their study in the 0.40 to 0.60 map unit region of the genome, it was not possible by this method to localize specific initiation sites or specify their number. Our localizations of the $A_I$ and $A_{III}$ sites correlate well with the electron microscopic localizations of these growing transcripts and also provides more precise information on the specific number and localizations of initiation sites in this area of the genome.

Allet et al. (1) have defined a class of promoters on the basis of their proximity to cleavage sites, of *H. influenzae* d-II and *H. parainfluenzae* I restriction endonucleases, both of which share a cleavage specificity, and the fact that their binding of *E. coli* RNA polymerase blocks cleavage by these two enzymes at such sites. The site for preferred initiation of transcription, the $A_{II}$ site, is one such promotor, for binding of RNA polymerase at this locus blocks cleavage by these two enzymes at the *Hin*-G-B junction. In contrast, the $A_I$ and $A_{III}$ initiation sites cannot be of this class of promotors for they do not lie near any cleavage sites of these enzymes.

It has been shown that the single-strand-specific nuclease S1 is capable of cleaving SV40 DNA I in two specific regions of the genome, from 0.15 to 0.25 and 0.45 to 0.55 map units (3). T4 phage gene 32 binds to SV40 DNA within the latter region, at 0.46 map units (31). Furthermore, the latter region lies within the broad segment of the genome, from 0.40 to 0.65 map units, which is most susceptible to partial alkaline denaturation (33); the former region coincides with a segment of the genome that is somewhat less susceptible to alkaline denaturation. The alkaline denaturation data suggest that these two regions of the SV40 genome, probably because of a specific structural feature, e.g., a high adenine-thymine content, are inherently unstable and susceptible to denaturation by a variety of chemical or physical factors. Imposition of superhelicity on the closed circular DNA molecule is one such factor favoring strand denaturation. Since SV40 DNA II is not cleaved by S1 nuclease (3), the combined S1 nuclease and alkaline denaturation data therefore suggest that as a result of its supercoiled configuration, the SV40 DNA I helix exists in a denaturable state in these two regions. It is thus of great interest that the $A_{II}$ initiation site lies within the former S1-susceptible region and that the remaining four sites lie either within or close to the second S1-susceptible region and within the broad region most susceptible to alkaline denaturation. Since *E. coli* RNA polymerase initiates transcription from the $A_I$, $_{-II}$, and $_{-III}$ initiation sites on SV40 DNA II, it is clear that "single strandedness" or susceptibility to denaturation is not essential for initiation of transcription at these specific sites. However, our preliminary experiments suggest that SV40 DNA II is not as efficient a template as supercoiled SV40 DNA for initiation of transcription from these sites. It thus appears quite likely that "single strandedness" or susceptibility to denaturation facilitates the recognition, binding, and initiation of transcription from these specific sites on the SV40 genome by *E. coli* RNA polymerase. This notion agrees with the findings that SV40 DNA II, a tightly hydrogen-bonded structure, is a relatively poor template for transcription with *E. coli* RNA polymerase (42) and that the priming ability of phage lambda DNA with *E. coli* RNA polymerase increases as negative superhelical turns are imposed upon the closed circular form of this DNA (6).

For bacteriophage lambda, it has been shown that three sites of initiation of in vitro transcription by *E. coli* RNA polymerase correspond to sites of initiation of in vivo transcription (H. A. Lozeron, M. L. Funderburgh, J. E. Dahlberg, B. P. Stark, and W. Szybalski, Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, V312, p. 237; citation in reference 4). For SV40, the precise termini of early and late lytic RNA (12; R. Dhar, S. M. Weissman, K. Subramanian, and J. Pan, manuscript in preparation) have recently been identified by nucleic acid sequencing methodology; in addition, the approximate termini of SV40-specific mRNA in transformed cells have been determined by nucleic acid hybridizations (5, 21). It is not yet entirely clear whether these termini always

arise during post-transcriptional processing of larger viral or covalently linked virus-host RNAs or whether they might correspond to precise sites of initiation and termination of transcription on the SV40 genome. Nevertheless, from the information presently available on the 5′ terminus of early mRNA in the *Hin*-C fragment and transformed-cell RNA in the *Hin*-C and -D fragments, it may be deduced that neither of the $A_I$ nor $A_{III}$ sites corresponds to the principal in vivo 5′ terminus for early lytic and transformed-cell mRNA transcription. Rather, all four $A_I$ and $A_{III}$ sites lie within the segment of SV40 DNA transcribed early in lytic infection. We do not yet have any information on whether one or more of the [$\gamma$-$^{32}$P]GTP initiation sites may lie in the region of initiation of early gene transcription.

## ACKNOWLEDGMENTS

We thank A. Lewis, Jr., for generously providing the purified adenovirus-SV40 hybrid viruses and A. S. Levine and C. Patch for generously providing the separated strands of $Ad2^+ND_4$ DNA. We are also very grateful to R. Dhar for purification of certain samples of hybrid virus DNA.

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## ADDENDUM IN PROOF

After submitting this manuscript, we learned that in vitro transcription of SV40 DNA I by *E. coli* RNA polymerase is inhibited by pretreatment of the DNA with *N*-cyclohexyl-*N*′-$\beta$-(4-methyl morpholinium)-ethyl carbodiimide, a reagent binding only to single-stranded DNA (P. Hale and J. Lebowitz, *In Molecular Mechanisms in the Control of Gene Expression,* Academic Press Inc., 1976, p. 135–142). The mechanism of inhibition is due primarily to the inability of RNA polymerase to bind to supercoiled DNA. This result is consistent with our finding that the five [$\gamma$-$^{32}$P]ATP transcription initiation sites are located in the "single-stranded" regions of SV40 DNA I.

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