In Vitro Transcription of Adenovirus

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A series of recombinants of adenovirus DNA fragments and pBR322 was used
to test the transcriptional activity of the nine known adenovirus promoters in a
cell-free extract. Specific initiation was seen at all five early promoters as well as
at the major late promoter and at the intermediate promoter for polypeptide IX.
The system failed to recognize the two other adenovirus promoters, which were
prominent in vivo only at intermediate and late stages in infection. Microhetero-
geneity of 5' termini at several adenovirus promoters, previously shown in vivo,
was reproduced in the in vitro reaction and indeed appeared to result from
heterogeneous initiation rather than 5' processing. To test for the presence of
soluble factors involved in regulation of mRNA synthesis, the activity of extracts
prepared from early and late stages of infection was compared on an assortment
of viral promoter sites. Although mock and early extracts showed identical
transcription patterns, extracts prepared from late stages gave 5- to 10-fold
relative enhancement of the late and polypeptide IX promoters as compared with
early promoters.

Initiation of transcription by RNA polymerase
II is the first step in synthesis of mRNA in
eucaryotic cells. Weil et al. (40) have demon-
strated that this process can be duplicated in a
soluble in vitro reaction by mixing purified RNA
polymerase II and an S100 cytoplasmic extract.
Manley et al. (24) found that an extract of whole
HeLa cells will also specifically initiate tran-
scription by endogenous RNA polymerase II at
promoter sites on both viral and cellular DNAs.
In fact, these in vitro systems produce RNA
with 5' termini identical to those of mature
mRNA's both in nucleotide sequence and 5'
modification by capping and methylation. The
exogenously added template DNA can then be
manipulated by deletion and substitution meth-
ods to define sequences directing the in vitro
reaction. In general, sequences in the vicinity of
the TATA consensus sequence or Goldberg-
Hogness box seem essential for in vitro initiation
(9, 20, 26, 36, 38, 39). In addition, alterations in
sequences as far as 30 nucleotides on either side
of the TATA sequence can affect the level of in
vitro initiation at a promoter site.

The availability of cell-free transcription sys-
tems recognizing eucaryotic promoters allows
direct access to questions of transcriptional reg-
ulation. It has already been shown, for instance,
that the binding of a purified analog of simian
virus 40 T antigen is capable of specifically re-
pressing in vitro transcription from the simian

virus 40 early region (29; U. Hansen and P. A.
Sharp, personal communication). In most eucar-
yotic systems one is not so fortunate as to have
purified regulatory factors whose activities have
been well characterized in vivo. In vitro systems
should provide a tool for identifying such factors
as well as in characterizing their modes of action.

One situation where such factors must exist is
during productive infection of human cells by
adenovirus. The course of synthesis of different
adenovirus genes during the lytic cycle follows
a pattern: "early" functions begin expression at
1 to 4 h postinfection, and their expression is
seen to decrease during the latter part of the
early phase (4 to 7 h postinfection) (33). At 6 to
12 h postinfection DNA replication begins, thus
increasing template copy number and poten-
tially shifting the configuration of the template.
The intermediate mRNA's for polypeptides IX
and IVa$_2$ and the late form of 72K DNA-binding
protein mRNA, all of which are undetectable at
early times, are made in copious amounts during
the first part of the late stage (see references 35
and 42 for review). The initiation of transcription
occurs at the late promoter during the early and
intermediate stages, but these transcripts are
processed to yield only two mRNA's (8, 21, 28,
33). During the late stage of infection at least 13
different RNAs are processed from the late tran-
scription unit and account for 50% of total
mRNA synthesis (35, 42). This high level of

transcription from the late promoter may be due to a shift in the specific activity (initiations per template per minute) during the course of infection. However, positive regulation of the late promoter is hard to assess given the tremendous expansion in the pool of functional DNA template at late times.

MATERIALS AND METHODS

Cells and virus. HeLa S-3 cells were grown in suspension in RPMI or minimal essential medium with 5% horse serum. For virus infection, cells were concentrated 10-fold in the absence of serum, and 50 PFU of an adenovirus type 2 (Ad2) lysate stock per cell was added. Adsorption was carried out for 60 min, after which medium containing 2% horse serum was added to original volume. This was taken as the zero point of infection. To insure that infection had indeed occurred, a sample of each infection was carried to 32 h postinfection, fixed in phosphate-buffered saline with 10% formaldehyde, and then stained with fluorescent antibodies to adenovirus late protein; 96 to 98% of the cells were infected according to this assay.

Preparation of extract. Extracts were prepared as described by Manley et al. (24). Briefly, cells were washed in phosphate-buffered saline and then swelled and homogenized in a Dounce homogenizer in 4 volumes of hypotonic buffer. After addition of sucrose and glycerol the nuclei were lysed with the addition of ammonium sulfate to 10% saturation. The chromatin and other debris were removed by ultracentrifugation at $175,000 \times g$ for 3 h, and the supernatant was concentrated by ammonium sulfate precipitation. This precipitate was resuspended and dialyzed in 100 mM KCl–12.5 mM $MgCl_2$–2 mM dithiothreitol–17% glycerol–0.5 mM EDTA, and samples were stored at −70°C after freezing in liquid N_2. Protein concentrations varied from 10 to 20 mg/ml, and extracts contained 0.1 to 0.5 mg of RNA per ml. Repeated freezing in dry ice and thawing for 1 min at 30°C produced no change either in levels or specificity of transcription. All transcriptions described in this paper were performed with extract frozen and thawed only once.

Transcription conditions. Except where noted, conditions were as described by Handa et al. (18). Preparative reactions were done at somewhat lower salt concentrations (40 mM KCl, 5 mM $MgCl_2$, 0.8 mM dithiothreitol, 7% glycerol), which enhanced transcription severalfold without changing specificity.

DNA and extract concentrations. Titrations of both DNA and extract yield nonlinear responses. Fixing extract and measuring runoff transcripts as a function of DNA concentration yields (i) a threshold DNA concentration below which no transcription occurs, and (ii) an inhibitory effect of high DNA concentration (24, 40). The requirement for a minimal DNA concentration is nonspecific—i.e., by using a concentration of a promoter-specific DNA which is below the threshold, "carrier" DNA, such as pBR322 or *E. coli* DNA, can be added to stimulate specific transcription. U. Hansen (personal communication) has shown that the duplex alternating copolymers polydeoxyinosinic-deoxycytidylic acid [poly(dIC:dIC)] and polydeoxyadenylic-deoxythymidylic acid [poly(dAT:dAT)] will act as carrier DNA, thereby demonstrating a total lack of sequence specificity in the bulk DNA requirement. A further advantage of these copolymers as carrier DNA is that the transcribed RNA products of the carrier poly(dIC:dIC) and poly(dAT:dAT) contain only two nucleotides. Thus, poly(dIC:dIC) carrier in a reaction containing $[\alpha\text{-}^{32}P]UTP$ yields no radioactive background. The key aspect of bulk DNA dependence is that at a fixed total DNA concentration, the molar yield of transcripts per promoter site is constant and independent of the source of carrier DNA. An exception to this generality is that at high carrier DNA concentrations, synthetic copolymers tend to more severely inhibit transcription than natural DNA.

A critical dependence of transcription upon extract concentration has been previously reported (24). In fact, DNA concentration dependence and extract protein concentration dependence are related. Specific transcription can be obtained in a range of 4 to 18 mg of extract protein per ml. At low extract concentration the DNA optima tend to be lower (in the range of 10 μg/ml). There is still a bulk DNA dependence, but it is less steep and the threshold concentrations are lower. At a high extract concentration the DNA titration becomes much sharper, and the threshold becomes much higher. At higher protein concentrations, often it is necessary to use 60 μg of DNA per ml for any transcription. Thus, for each new extract it is necessary to do careful DNA and extract titrations to determine optimal conditions.

For a given promoter, very short runoff transcripts (<300 nucleotides [n]) have a higher DNA optimum than longer runoff transcripts. This effect has been taken into account in the regulation and DNA titration experiments described here by measuring the synthesis of different-length runoff products from a promoter. No length dependence for transcription was observed with runoff products between 400 and 4,000 n.

Analysis of RNA products. Analytical reactions were done in 20 μl. After 90 min at 30°C, 150 μl of stop buffer (7 M urea, 100 mM LiCl, 0.5% sodium dodecyl sulfate, 10 mM EDTA, 350 μg of tRNA per ml, 10 mM Tris [pH 7.9]) and 300 μl of phenol-chloroform-isoamyl alcohol buffered by 20 mM Tris (pH 7.5) were added, and the tubes were blended in a Vortex mixer and centrifuged at $12,000 \times g$ for 15 min. The aqueous phase (without interface) was extracted once more with phenol-chloroform-isoamyl alcohol and once with chloroform and then pooled with 200 μl of 1.0 M NH_4 acetate and 900 μl of ethanol. The RNA precipitate was collected after 60 min at −70°C and washed with 1 ml of ethanol. The pellet was air dried in an inverted tube for 5 min and resuspended in 20 μl of 10 mM $Na_2H(PO_4)$ (pH 6.8)–1 mM EDTA; to this was added 50 μl of 1.4 M deionized glyoxal–70% dimethyl sulfoxide–10 mM $Na_2H(PO_4)$ (pH 6.8)–1 mM EDTA–0.04% bromophenol blue. After 1 h at 50°C, 25 μl of the sample was loaded on 1.4% agarose gels in 10 mM $Na_2H(PO_4)$–1 mM EDTA (27). DNA restriction fragments were denatured with glyoxal and run as markers; 0.1% sodium dodecyl sulfate was added to the running buffer, and the gel was prerun for 10 min at 100 V to prevent the appearance of a sodium dodecyl sulfate front.

Preparative reactions for RNA fingerprinting. Large-scale reactions were as above, except that a second precipitation with 0.5 M NH_4 acetate and 3

volumes of ethanol was done. Autoradiography of 7 M urea–8% acrylamide gels was done at room temperature. Bands of interest were cut out, electroeluted, and subjected to fingerprinting as described previously (6, 31). End labeling of RNA by decapping and kinasing and selection on filters has been described previously (2, 3a, 43). Alkali breakage before selection and RNase A treatment on filters has been omitted.

RESULTS

In vitro transcription of Ad5: runoff mapping. Sizing of transcription runoff RNAs provides a sensitive means of identifying promoter sites. For this purpose, cloned restriction endonuclease fragments encompassing each of the nine known promoter regions of adenovirus have been constructed (K. Berkner et al., manuscript in preparation; see Table 2 for list of promoter sites). These recombinant pBR322-viral DNAs are cleaved with a variety of restriction enzymes and then used as template in a whole cell extract (WCE) reaction mix. The RNA products were specifically labeled by incorporation of $[\alpha\text{-}^{32}P]$ UTP and detected by autoradiography after electrophoresis in denaturing glyoxal gels (27). Detection of a particular length of α-amanitin-sensitive RNA product positions the promoter site relative to the terminus of the viral DNA fragment. Transcription of fragments generated by cleavage with other endonucleases confirms uniquely the position of the promoter site(s).

Some of the bands resolved after electrophoresis of $[\alpha\text{-}^{32}P]$UTP-labeled RNA are not due to the initiation of transcription at promoter sites on viral DNA. First, some faint products are insensitive to α-amanitin (1 μg/ml) and are not RNA polymerase II products. These products are primarily due to various amounts of end labeling of ribosomal RNA present in the extracts (24). A second source of labeled bands is the inefficient but specific RNA polymerase II transcription of the pBR322 DNA present in the template (R. Kaufman and F. Laski, personal communication). High-molecular-weight RNA products are also generated by ligation of DNA segments containing active promoters to other fragments (unpublished results). Finally, it is possible that some of the faint bands generated during transcription are the consequence of RNA processing. In several cases, detailed analysis has failed to confirm this, and, where tested, transcripts were found to be stable on 2-h chases in both uninfected and infected extracts. For the above reasons, it is essential that a variety of templates be used to map a particular transcriptional region.

The HindIII G (0.0 to 7.9-map-unit [m.u.]) fragment of Ad5 DNA contains sites for initiation of EIa and EIb early mRNA's. This fragment was cloned into pBR322 through EcoRI linkers attached at 0.0 m.u. and through the HindIII site at 7.9 m.u. and was a kind gift of Kathleen Berkner. When cleaved with KpnI, this plasmid generated 1,550- and 345-n RNAs. These sizes agree with the predicted size runoff RNAs for initiation at the EIa (1,550 n) and EIb (350 n) promoter sites (Fig. 1A). Similarly, cleavage of the plasmid with XbaI or HpaI also yielded the correct size runoff RNAs (Fig. 1A). Additionally, RNA polymerase II and factors in the WCE recognize a site for initiation some 300 n upstream from the EIa promoter (PEIa; denoted with a dashed arrow in Fig. 1A). Initiation at this site generated α-amanitin-sensitive runoff transcripts of 1,850, 1,400, and 1,165 n after cleavage with KpnI, HpaI, and XbaI, respectively. mRNA's with 5' termini mapping upstream from the EIa promoter have been reported (5, 12, 30, 41); thus, this may be an active promoter in vivo.

A comparison of in vitro transcription from the EIb and polypeptide IX promoter sites can be obtained by use of the Ad5 SmaI F fragment (2.8 to 11.1 m.u.). This fragment was cloned into pBR322 by attachment of EcoRI linkers (Fig. 1B). In vitro transcription of this plasmid after cleavage with EcoRI generated products of 2,250 and 380 n from the EIb and polypeptide IX promoter regions, respectively (Fig. 1B). The products predicted from DNA sequence are 2,240 and 360 n. Similarly, transcription of the KpnI- or HindIII-cleaved SmaI F-pBR322 plasmid also yielded the expected runoff products (Fig. 1B). Thus, both the EIb and polypeptide IX promoter sites are recognized and are about equally active. No other major in vitro RNA polymerase II promoter site was observed in the SmaI F fragment.

The HindIII B fragment (72.8 to 89.1 m.u.) of Ad5 encompasses initiation sites for both early regions II (EII) and III (EIII). This fragment was cloned into pBR322 by ligation to the HindIII cleavage sites. Somewhat surprisingly, in vitro transcription of this plasmid after cleavage with a variety of different restriction endonucleases yielded strong runoff transcripts only from the EIII promoter. For example, transcription of plasmid DNA after cleavage with either EcoRI, XhoI, KpnI, or XbaI generated RNAs of lengths 2,380, 2,200, 1,175, and 980 n, respectively, from the EIII promoter site (Fig. 2A). However, transcription of the same plasmid after cleavage with SalI or BamI produced only very faint bands of lengths 1,320 or 1,080 n, consistent with initiation at the EII promoter site. Thus the EII promoter is relatively inactive in the WCE system; however, other experiments show that the levels of EII transcription seen in vitro are indeed specific (see below).
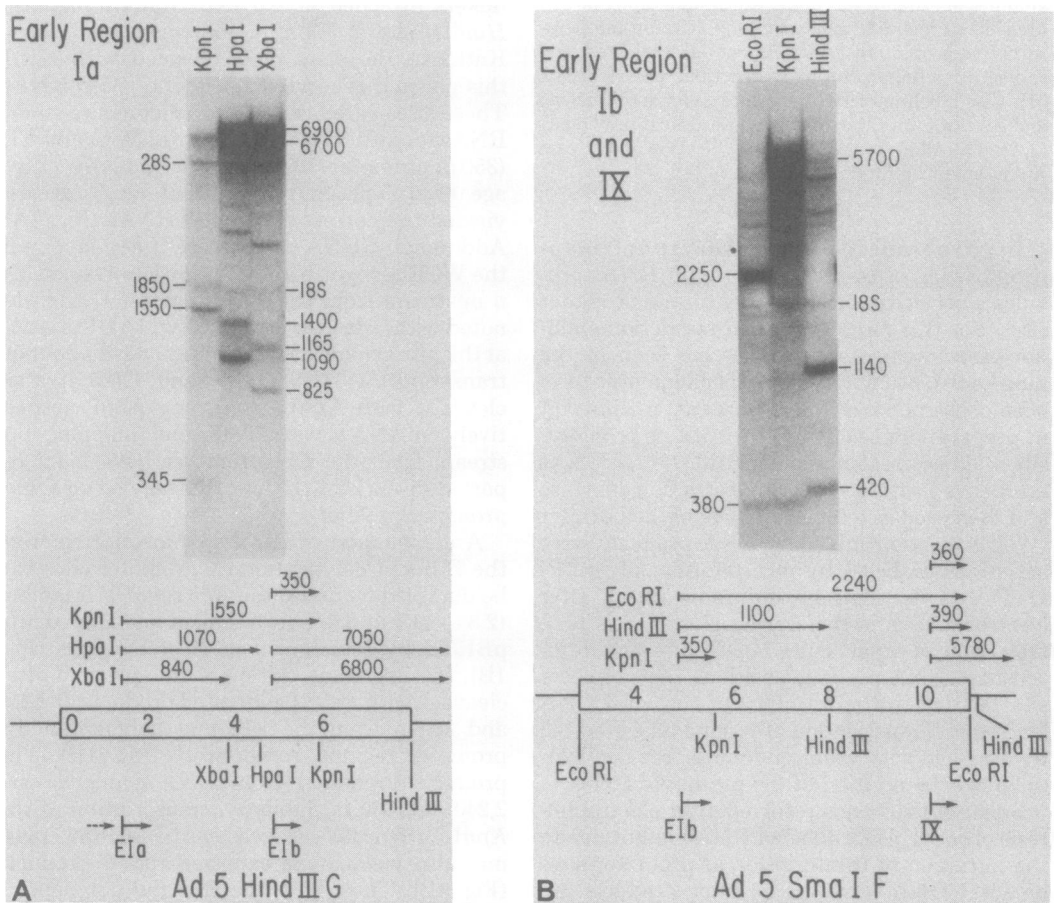
FIG. 1. *Transcription of EIa and EIb regions. Clones of HindIII G and SmaI F fragments of Ad5 were cleaved as shown, and unfractionated cut DNA was transcribed in an uninfected extract. Each 20-μl reaction mix contained 160 μg of WCE protein; 1 μg of specified DNA; 500 μM ATP, CTP, and GTP; 50 μM UTP at 2 Ci/mmol; 60 mM KCl; 10% glycerol; 7.5 mM MgCl₂; 5 mM EDTA; 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid–NaOH (pH 7.9); 8 mM creatine phosphate; and 0.5 mM dithiothreitol. Transcription was for 75 min at 30°C. The autoradiograms shown are 40-h exposures with intensifying screen. A, Digests of Ad5 HindIII G recombinant show EIa and an upstream initiation site. Nucleotide lengths alongside gel denote sizes calculated from DNA and RNA markers. Diagram shows localization of in vivo promoters and expected lengths of runoff transcripts (22, 23, 37). The bands of 1,550, 1,090, and 825 n are generated by EIa runoffs. Bands of 1,850, 1,400, and 1,165 n result from initiation at 0.7 m.u. Bands of 345, 6,900, and 6,700 n correspond to the EIb promoter. Bands between 18S and 28S are produced by initiation in the pBR322 vector. B, Digests of Ad5 SmaI F fragment show initiation at EIb and IX promoter sites. The runoff transcripts expected from both the EIb and IX promoter sites are indicated by their respective lengths. End-labeled 18S RNA is also indicated.*

Runoff transcription of early region IV of Ad5 was tested by using the EcoRI B fragment (84 to 100 m.u.). This fragment was cloned into pBR322 by attachment of EcoRI linkers to the right terminus of the genome and then insertion through the EcoRI site at 84 m.u. The addition of this plasmid after cleavage with either SmaI, HindIII, or KpnI to an in vitro reaction mix generated RNAs of 235, 660, and 2,020 n, respectively—the sizes expected for products initiated

at the EIV promoter site. There was another specific initiation site for RNA polymerase II weakly detected in the EcoRI B fragment. This site maps at 96.3 m.u., and its transcripts are in the same direction as the EIV promoter. Initiation at this site generated the 980- and 1,485-n products seen from templates cleaved with KpnI and SmaI, respectively (Fig. 2B). There is no known in vivo Ad5 mRNA with 5' termini mapping at 96.3 m.u. It is difficult to decide whether
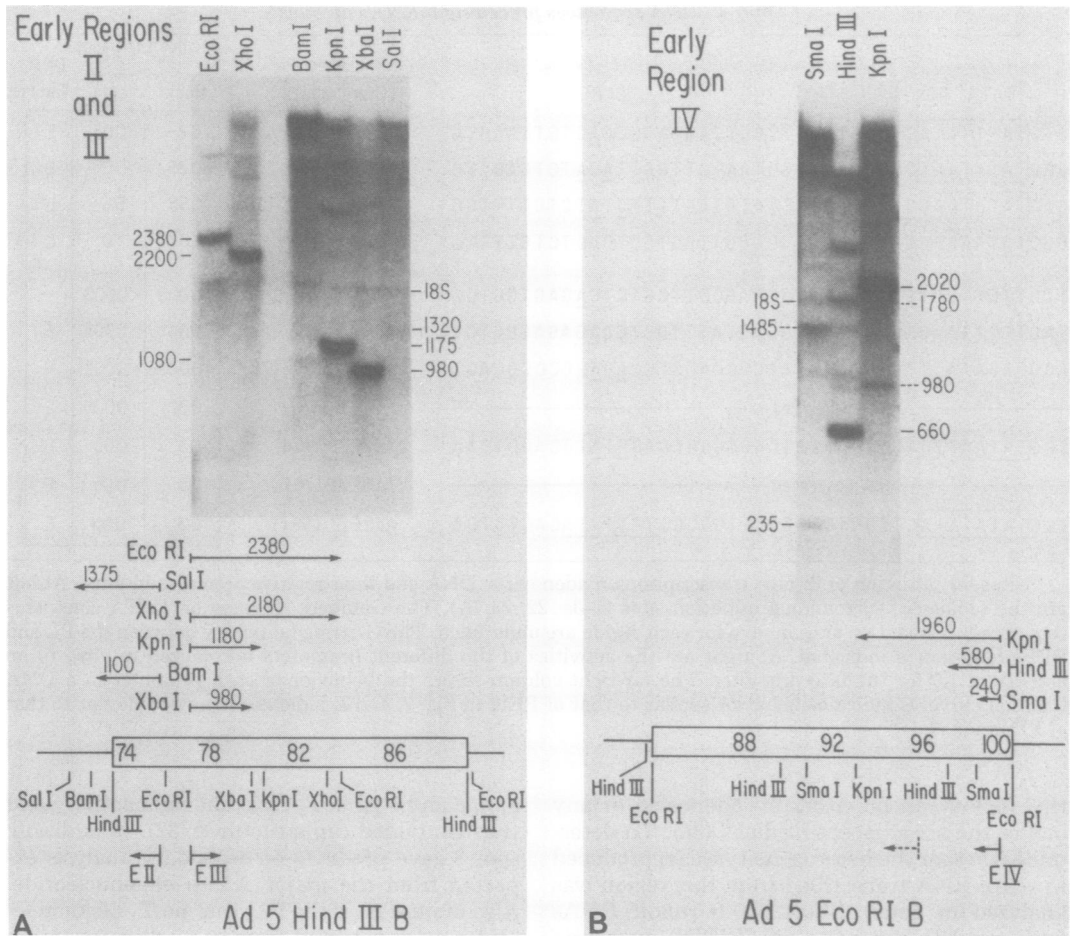
FIG. 2. *Transcription of EII and EIII region. Conditions of transcription were as in Fig. 1. A, Digests of Ad5 HindIII B recombinant show EII and EIII transcription. Bands of 2,380, 2,200, 1,175, and 980 n in the lanes marked EcoRI, XhoI, KpnI, and XbaI, respectively, correspond to EIII. Faint bands of 1,320 n and 1,080 n in lanes marked SalI and BamHI correspond to EII. B, Digests of Ad5 EcoRI B fragment show transcription of EIV and a minor initiation site (dotted lines). Expected runoff products from EIV are given in the diagram (34, 35). The lower molar yield of the 235-n runoff transcript in the first lane is due to a higher DNA optimum for very short transcripts (see text). Initiation at site near 96.3 m.u. generates bands of 1,485, 1,780, and 980 n on SmaI-, HindIII-, and KpnI-cleaved templates, respectively. Bands of 1,760 n in the HindIII lane and those migrating between 18S and 28S in all other lanes are pBR322 transcripts.*

this is artifactual recognition by the in vitro system or an undetected in vivo promoter.

Two intermediate stage promoters, those for IVa_2 and the late form of EII mRNA (EIIL) are not utilized in the in vitro system. Transcription of a fragment containing the latter promoter will be described below. Transcription of the IVa_2 promoter was assessed by using the *Bal* I E fragment of Ad2 (14.7 to 21.5 m.u.) cloned with *Bam*I linkers into the *Bam*I site of pBR322. The fragment was cut either with *Bam*I or with *Bam*I and *Hin*dIII. In both cases, a 460-n runoff would be expected from the IVa_2 promoter

(Baker and Ziff, in press), in addition to late promoter transcripts of 1,750 and 196 n from the two templates, respectively. Though the late promoter bands were very strong, the expected comigrating IVa_2 runoffs were not seen (0.5% of the late promoter level would have been detected; data not shown).

Fidelity of in vitro initiation at the EIV promoter. Baker and Ziff (3, 3a) and Hashimoto and Green (19) have shown that the EIV promoter site generates microheterogeneous 5' termini. As indicated in Table 1, the capped nucleotide forming the 5' end of mRNA's from

TABLE 1. *DNA sequences preceding mRNA cap sites*[a]

"TATA" BOX	CAPS	Promoter (Coordinate)	Relative Efficiency		DNA Titration
			Mock	Late	
GTG TATTTATA CCCGGTGAGTTCCTCAAGAGGCC<u>A</u>C TCTTGAGTG		Ad5 EIa (1.4)	0.2	0.03	E
GGG TATATAAT GCGCCGTGGGCTAATCTTGGTT<u>ACA</u>TCTGACCTC		Ad5 EIb (4.7)	0.35	0.05	E
GAA TATATAA GGTGGGGGTCTTATGTAGTTTT<u>GTA</u>TCTGTTTTGC		Ad5 Protein IX (9.8)	0.35	0.6	L
GGC TATAAAA GGGGGTGGGGGCGCGTTCGTCCTC<u>A</u>C TCTCTTCCG		Ad2 Major Late (16.4)	1.0	1.0	L
TCCTTCGTGCTGGCCTGGACGCGAGCCTTCGTC<u>TCA</u>GAGTGGTCC		Ad2 IVa$_2$ (15.9)	<0.005	<0.005	—
TAGTCCTTAAGAGTCAGCGCGCAGTATTTGCTG<u>AA</u>GAGAGCCTCC		Ad2 EII (75)	0.04	0.005	E
AGGTACAAATTTGCGAAGGTAAGCCGACGTCC<u>ACA</u>GCCCCGGAGT		Ad2 EIIa Late (72)	<0.005	<0.005	—
———————— Not Sequenced ————————		Ad5 EII (75)	0.02	0.005	E
GGG TATAA CTCACCTGAAAATCAGAGGGCGAGG<u>TA</u>TTCAGCTCAA		Ad2 EIII (76.6)	0.3	0.05	E
———————— Not Sequenced ————————		Ad5 EIII (76.6)	0.3	0.05	E
TCC TATATATA CTCGCTCTGCACTTGGCCC<u>TTTTTT</u>ACACTGTGA		Ad5 EIV (99.1)	0.3	0.05	E

[a] Sites for initiation of in vivo transcription on adenovirus DNA and their relative activities in vitro. At left are the sequences surrounding initiation sites (1–3a, 22, 23, 37). The Goldberg-Hogness or TATA consensus sequence is boxed. Cap sites in vivo for each region are underlined. The G-string homology between the PL and PIX promoters is indicated. At right are the activities of the different promoters normalized in vitro to an activity of 1.0 for the late promoter. The far-right column shows the behavior of each promoter in a DNA titration curve. E indicates behavior similar to that of PEIa in Fig. 7, and L indicates behavior similar to that of PL in Fig. 7.

this region can be either an adenosine or any one of the six adjacent uridines (3a). To determine whether the heterogeneity was reproduced in vitro, RNA transcribed from this region was analyzed in detail. Short 250-n runoff RNAs from the EIV promoter site (PEIV) were prepared by transcription of the *Sma*I-cleaved *Eco*RI B fragment of Ad5 (Fig. 2B). These RNAs were labeled in vitro by incorporation of either α-^{32}P-labeled UTP, ATP, or GTP, and the products were resolved by electrophoresis in urea-acrylamide gels. The runoff bands were identified by autoradiography and electroeluted. After digestion with RNase T$_1$, the oligonucleotides were analyzed by two-dimensional fingerprinting (3a).

Figure 3A is an autoradiograph of a T$_1$ fingerprint of ^{32}P-labeled in vivo Ad2 nuclear RNA selected by hybridization to the Ad2 *Sma*K fragment (98.3 to 100 m.u.) (3a). Most of the T$_1$ oligonucleotides predicted from the EIV sequence were identifiable in the two-dimensional pattern (Fig. 3). Panels B, C, and D of Fig. 3 show the equivalent T$_1$ fingerprints of 250-n runoff transcripts labeled in vitro by [α-^{32}P] UTP, -ATP, and -GTP, respectively. Secondary analyses of these T$_1$ oligonucleotides with RNases T$_2$ and A are presented in Table 2. Spots

a, b, c and 8 gave T$_2$-resistant radioactivity and thus contained cap structures (32). In addition, spot 8 gave products on secondary analysis expected from the major A cap oligonucleotide. Also of interest is the fact that no T$_1$ oligonucleotides were detected from sequences upstream of or spanning the in vivo initiation site. At this limit of analysis the in vitro and in vivo 5′ termini are identical.

A more precise means of examining the 5′ termini of in vitro-transcribed RNA is to uniquely label the 5′-terminal phosphate. This can be accomplished by removal of the 7-methyl guanosine by periodate oxidation and β elimination. The 5′ end is exposed by treatment with alkaline phosphatase, allowing end labeling with [γ-^{32}P]ATP and polynucleotide kinase (3a). Since the in vitro RNA products were diluted into a mixture of endogenous RNA, the 5′-labeled RNA was selected on filters containing the *Sma*I K fragment. Figure 4 shows such a comparison of the T$_1$ fingerprints of in vivo and in vitro Ad5 EIV RNA labeled at their 5′ termini. The series of labeled T$_1$ oligonucleotides is identical in the two cases. Some 60% of the initiations are at the adenine position, whereas the remaining 40% are distributed between the six adjacent uridine positions. The identification of the la-

beled 5' termini as cap sites was further confirmed by the presence of labeled dinucleotides protected from T_2 digestion by 2'-O-methylation. A small fraction of in vitro-capped termini was unmodified by 2'-O-methylation as is shown by the presence of type O caps in the T_2 digest of the major A cap oligonucleotide in Fig. 3 (spot 8, Table 2). About 20% of the in vitro-synthesized RNA from PEIV did not have any cap structure at all, since about this fraction of the in vitro 5' termini (and none of the in vivo termini) can be labeled with [γ-^{32}P]ATP by simple treatment with phosphatase and polynucleotide kinase. This subset of in vitro RNA chains also lack 2'-O-methylation on their terminal nucleotides. Whether this suggests that only a fraction of the newly initiated chains is modified by capping, and thus that cap formation is not a necessary component of transcription, or that only a fraction of the newly in vitro-synthesized caps is methylated and thus stable, cannot be determined with this methodology. The addition of (2 mM) S-adenosylmethionine to the WCE did not affect methylation levels or the fraction of capped 5' termini (data not shown).

Heterogeneously capped 5' termini could be generated either by endonuclease cleavage or by initiation of transcription at multiple sites. The frequency of initiation with a particular nucleotide would be expected to be dependent on the concentration of its triphosphate precursor. Conversely, the frequency of endonuclease cleavage should be independent of specific nucleotide concentrations. Panels C, D, and E of Fig. 4 show a comparison of 5' termini synthesized in the presence of normal UTP and ATP concentrations, limiting UTP concentrations, and limiting ATP concentrations, respectively. The visible demonstration that changes in the ratio of ATP to UTP concentrations are specifically reflected in the ratio of adenine to uridine caps indicates that the microheterogeneous EIV termini are derived from initiation at each cap site.

In vitro transcription of early region II. Early region II is unique among early regions of adenovirus for several reasons: (i) it is transcribed at the early and late stages of infection by initiation at different promoter sites (7, 8); (ii) the early promoter site PEII (75 m.u.) does not have a TATAAA consensus sequence positioned 25 to 31 n upstream from the cap site (Table 1); (iii) the late promoter site for early region II (PEIIL) at 72 m.u. has a TACAAA sequence at the expected consensus sequence positions (3a); and (iv) both EII and EIIL promoter sites are efficient in vivo. It is therefore somewhat surprising that neither of these pro-

moters is efficiently recognized in the WCE. The Ad2 EcoRI F (70.7 to 75.9 m.u.) fragment encompasses both EII and EIIL promoter sites (Fig. 5). In vitro transcription of this fragment after cleavage with EcoRI, KpnI, and SstI generated low levels of runoff transcripts of 1,460, 1,240, and 880 n, respectively, those expected from initiation at PEII. The PEII activity detected is about 0.04 of that observed from the late promoter of Ad2 (Fig. 5; a myriad of other minor bands appear on this overexposure, some of which correspond to the initiation sites within pBR322). Even this low level of runoff transcription was not observed for initiation at PEIIL (72 m.u.). An RNA of 320 n would be predicted for an EcoRI-cleaved template (see Fig. 5), and an RNA of 1,200 n would be predicted from BglI cleavage (data not shown).

To show that the in vitro system actually initiates correctly at PEII, RNA transcribed in the WCE was subjected to 5' terminus analysis as described previously for PEIV. In this case, RNA was extracted from a very large reaction mix (2 ml) and 5' labeled as described above. The 5'-labeled RNA was selected by hybridization to a HindIII-EcoRI fragment spanning from 72.8 to 75.9 m.u., digested with RNase A, and fingerprinted. The two large oligonucleotides (spots 1 and 2 of Fig. 6) were shown to carry the 2'-O-methylation and behaved identically to spots derived from the in vivo termini on secondary analysis shown in Fig. 5B and C of reference 2. Thus, the faint bands from PEII are initiated at the same sites as in vivo mRNA's. Interestingly, many of the smaller oligonucleotides in Fig. 6 also have 2'-O-methylated bases. Since the in vitro reaction mix was scaled up 10-fold over that used in Fig. 4, these capped oligonucleotides probably represent other in vitro initiation sites within the 1,100-n DNA fragment used to select hybrids for fingerprinting. This suggests that RNAs initiated at nonpromoter sites are also modified by cap synthesis.

A similar labeled 5' terminus analysis with a hybridization probe spanning the EIIL promoter site selected a number of 5'-terminal 2'-O-methylated oligonucleotides, none of which corresponds to the in vivo PEIIL initiation sequence. This demonstrates that PEIIL is very infrequently utilized as a promoter site in vitro.

Relative transcriptional activities of the different promoters. To determine the relative rates of transcription of different viral promoters under a variety of conditions, an equimolar mixture of template fragments (see Fig. 8A) was constructed. Each fragment generated runoff transcripts of unique length. Since the in vitro transcripts are labeled with [α-^{32}P]UTP,
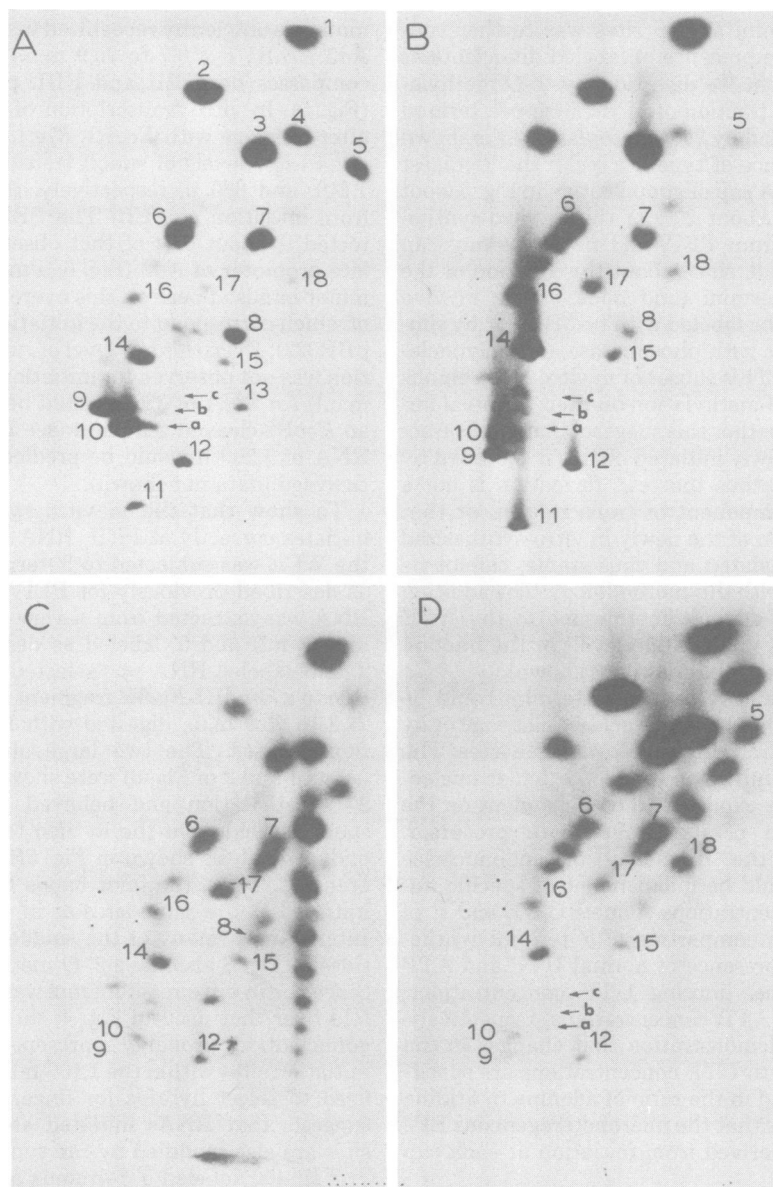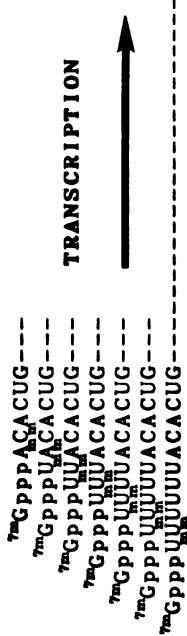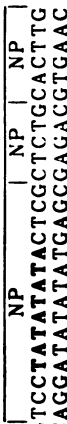
FIG. 3. *Fingerprints of 250-n runoff transcripts from PEIV. A, Ad2 early nuclear RNA was selected on filters by hybridization to Ad2 SmaI K fragment (98.3 to 100 m.u.). RNA was labeled with $^{32}PO_4$ in vivo as described previously (2). Selected RNA was treated with RNase T_1 on filters, the RNA was eluted, and an RNase T_1 digest was resolved by the two-dimensional fingerprint analysis of Brownlee et al. (6, 31). B, Ad5 EcoRI B recombinant was cut with SmaI and transcribed in a reaction mix as noted in the text. Nucleotide concentrations were 50 μM each ATP, CTP, and GTP and 100 μM [α-^{32}P]UTP at 450 Ci/mmol. The 250-n runoff band was electroeluted from urea acrylamide gel and fingerprinted with RNase T_1 as above. Spot 8 is the major A cap, and spots a, b, and c are three of the minor U caps. C, The same template as in B was transcribed with 75 μM [α-^{32}P]ATP (specific activity, 304 Ci/mmol), 50 μM CTP and GTP, and 5 μM UTP. The series of spots along the right side appears to be oligoadenylic acid. D, The same template as in B was transcribed with [α-^{32}P]GTP (specific activity, 600 Ci/mmol); 50 μM ATP, CTP, and UTP; and 29 μM GTP. Diagram, Sequence flanking EIV promoter site of Ad5 (34) shows aligments for T_1 spots of panels A through D on the basis of secondary analyses presented in Table 2. Oligonucleotides upstream or traversing the cap and not found in in vitro runoff products are marked NP. Spot 13, found in nuclear RNA, is absent from the in vitro runoff products since the template is truncated in this oligonucleotide. Note the conformation of the major A cap (spot 8) by identification of RNase A products. Similar analysis of the in vivo spots has been shown (3a).*

r 5′ CGTAGGTTCGCGTGCGGTTTTCTGGGGTGTTTTTGTGGACTTTAACCGTTACGTCATTTTTAGTCCTATATACTCGCTCTGCACTTG
l 3′ GCATCCAAGCGCACGCCAAAAGAGACCACAAAAACACCTGAAATTGGCAATGCAGTAAAAATCAGGATATATGACGAGACGTGAAC

-100   -80   -60   -40   -20

7mGpppApCpACUG----
7mGpppUpACUG----
7mGpppUpUpACACUG----
7mGpppUpUpUUACACUG----
7mGpppUpUpUUACACUG----
7mGpppUpUpUUUACACUG----
7mGpppUpUpUUUUACACUG----

TRANSCRIPTION

GCCCTTTTTTACACTGTGACTGATTGAGCTCGGTCCGTGTCGAGTGGTGTTTTCTTTTTTACTGGTAAGGCTGACTG
CGGGAAAAATGTGACACTGACTAACTCGACGCCAGGCACAGCTCACCACAAAAAAAATTATCCAAAGAAAAATGACCATTCCGACTGAC

+1   +20   +40   +60   +80

TTAGGCTGCCGCTGTGAAGCGCTGTATGTTGTTCTGGAGCGGAGGGTGCTATTTTGCCTAGGCAGGAGGGTTTTCAGGTGTTTATGTG
AATCCGACGGCGACACTTCGCGACATACAACAAGACCTCGCGCCTCGCCACGATAAACGGATCCGTCCTCCCAAAAGTCCACAAATACAC

+100   +120   +140   +160

TTTTTCTCTCTATTAATTTGTTATACCTCCTATGGGGGCTGTGTAATGTGTCTCTACGCCTGCGGGTATGTATTCCCCGGG 3′ r
AAAAAGAGAGATAATTAAAACAATATGGAGGATACCCCCGACATTACAACAGAGATGCGGACGCCCATACATAAGGGGGCCC 5′ l

+180   +200   +220   +240

Sma I

TABLE 2. *Analysis of RNase T_1 oligonucleotides from Ad5 EIV RNA transcribed in vitro*

Spot no.[a]	RNase A products[b]		
	[α-^{32}]UTP label[c]	[α-^{32}P]ATP label[c]	[α-^{32}P]GTP label[c]
1	NA	NA	NA
2	NA	NA	NA
3	NA	NA	NA
4	NA	NA	NA
5	Gp	NA	Cp
6	AUp, Gp	Up, Gp	AUp, Up
7	ACp, Gp	Gp	[AAGp, AGp, Gp, (AUp, Cp, Up)][d]
8	ACp, Gp	[Type O and I cap cores][e]	NP
9	ACp, Cp, Up	Up	Up, Gp
10	AAUp, Up, (ACp, Cp)[f]	AAUp, Up	AGp
11	AAUp, AUp, Cp, Up, (Gp)	NA	NA
12	AUp, Cp, (Up)	AUp, Up	AUp, Gp
13	NP	NP	NP
14	AUp, Up, Cp	AAUp,[d] Cp, Up	Up, AGp
15	Cp	Up	ACp
16	AUp, Gp, Up	Up	AUp, (Up)[d]
17	AAUp, Gp	AAUp, Up	AAUp
18	Cp	NA	AGp
a	[Cp, Up, cap I core][e]	NP	[cap I core, Up][e]
b	[Cp, Up, cap I core][e]	NP	[cap I core, Up][e]
c	[Cp, Up, cap I core][e]	NP	[cap I core, Up][e]

[a] Numbers correspond to the spot numbers of Fig. 3.

[b] For secondary analysis, RNase T_1 oligonucleotides were redigested with RNase A. Products from the secondary digests were fractionated by electrophoresis on DEAE paper at pH 3.5.

[c] These are the products resulting from RNase A redigestion of the RNase T_1 oligonucleotides from the fingerprints, shown in Fig. 3B, C, and D, of RNA labeled in vitro with [α-^{32}P]UTP, [α-^{32}P]ATP, or [α-^{32}P]GTP. NA indicates that the corresponding spot was not analyzed; NP indicates that the spot was not present in the fingerprint. Products in parentheses were barely detectable. Abbreviations: A, adenosine; C, cytidine; U, uridine; G, guanosine; P, phosphate.

[d] This product(s) was not predicted by the Ad5 sequence in Fig. 3.

[e] These products are the result of redigestion with RNase T_2.

[f] Spot 10 was contaminated with spot 9.

the amount of radioactivity incorporated per mole of product is proportional to length. The late promoter site (PL) was the most active template in the mix, but only generated a faint band of 225 n under these conditions. In addition to the adenovirus promoters, transcripts from the various vector sequences were observed, one migrating slightly below 18S and others migrating well above the EIb band at 2,575 n. The relative strengths of various promoter sites can be approximated by densitometry of gels similar to that in Fig. 8A or of gels containing a single runoff transcript such as those in Fig. 1 and 2 (Table 1). The two methods yield comparable results under similar conditions.

It is well known that the in vitro transcription activity of different promoters can vary markedly with changes in total DNA concentration. It has been reported (29; U. Hansen and P. A. Sharp, personal communication) that relative transcriptional activities of different simian virus 40 promoters vary with DNA concentration. A similar observation with adenovirus promoters

is shown in Fig. 7. An equimolar mixture of template DNAs for PEIa (1,220-n runoff) and PL (924-n runoff) was prepared, and a DNA-titration curve was performed. As shown in Fig. 7A, both early and late promoters gave qualitatively similar titration curves, with a threshold value below which no transcription was seen, and inhibition by high DNA concentration (see above). A graph of the molar ratio of early and late transcripts is shown in Fig. 7B. There was a 20-fold decrease in the ratio over a DNA range of 7 to 56 μg/ml. That this is a function of bulk DNA concentration is shown by a similar curve when total promoter DNA was held constant and DNA concentration was titrated up with increasing concentrations of duplex copolymer poly(dIC:dIC). Adenovirus promoters fall into two general classes in the DNA titration curve: all early promoters respond like pEIa, whereas the polypeptide IX promoter behaves like the late promoter (data not shown).

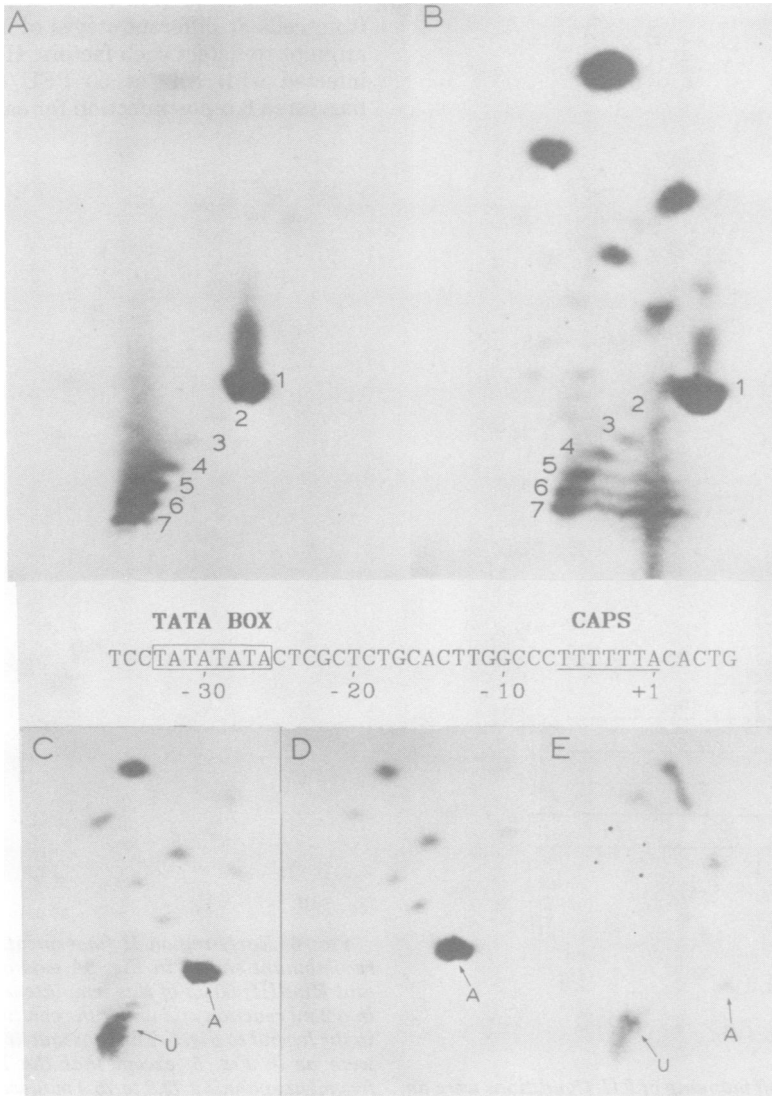A comparison of the relative efficiencies of the different promoters each at their optimal DNA

FIG. 4. *Localization of 5' termini from the EIV region. RNAs were 5' labeled by decapping and kinasing using previously described procedures (3a). The RNA was selected and fingerprinted as in Fig. 3A. In both A and B, spot 1 and spots 2 through 7 derive from the major A terminus and minor U termini, respectively. In each case, identity of the spots was confirmed by redigestion with nuclease P1, RNases T_2 and A, and chromatography on 540 or DEAE paper (3a). A, Analysis of cytoplasmic RNA prepared from cycloheximide-treated, Ad5-infected HeLa cells at 5 h postinfection. B, Analysis of in vitro-transcribed RNA; 6.4 µg of BglII-cleaved EcoRI B recombinant was transcribed in a 180-µl reaction mix under preparative conditions (see text). The prominent spots above the A terminus are not 2'-O-methylated and thus probably do not correspond to capped 5' termini. A number of the very minor spots were shown to have 2'-O-methylation and probably represent minor initiation events. The sequence of EIV showing seven capped termini and the TATA box is shown in the diagram. C, D, and E, Variability of relative levels of A and U caps with nucleotide concentration. Transcriptions in 0.2 ml with 4 µg of HindIII-cleaved EcoRI B were analyzed in A. Spots derived from A caps and U caps are indicated. C, Nucleotide concentrations were 50 µM ATP, GTP, CTP, and UTP. D, Nucleotide concentrations were 50 µM GTP and CTP, 500 µM ATP, and 1 µM UTP (endogenous pool). E, Nucleotide concentrations were 50 µM GTP and CTP, 500 µM UTP, and 10 µM ATP. Incorporation was much lower in this sample, due to limiting ATP.*

concentration is shown in Table 1. It should be noted that whereas the trailing and leading peak shapes differ, the actual optimal DNA concen-

trations for the different promoters under the conditions used were quite similar.

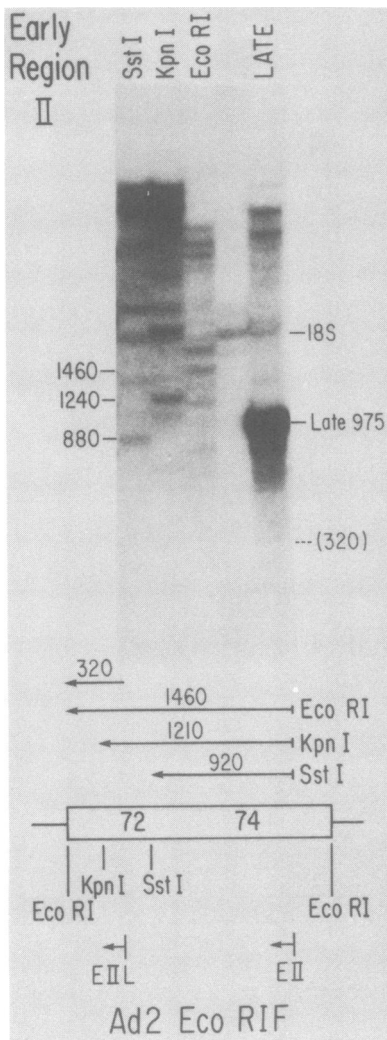Regulation of transcription is the major level

FIG. 5. Runoff mapping of EII. Conditions were as in Fig. 1, except that autoradiography was for 150 h. Bands of 880, 1,240, and 1,460 n in the SstI, KpnI, and EcoRI lanes, respectively, correspond to EII transcription (2, 13). Bands of 2,300, 1,960, and 1,740 n correspond to transcripts from pBR322. The other minor bands have not been characterized. The lack of a band migrating at 320 n in the EcoRI-digested lane indicates that transcription from the EIIL promoter at 72 m.u. does not occur under these conditions. In addition, transcription of a BglI-cleaved template which would generate EIIL runoff of 1,200 n failed to yield such a band (data not shown). A major late promoter incorporation done under the same conditions is shown.

of gene control during the adenovirus lytic cycle. Factors responsible for this regulation must appear or disappear (or both) during various phases of the lytic cycle. Extracts were prepared

from cells at different stages of infection in an attempt to detect such factors. HeLa cells were infected with Ad2 at 50 PFU/cell and were harvested 6 h postinfection for early extracts or
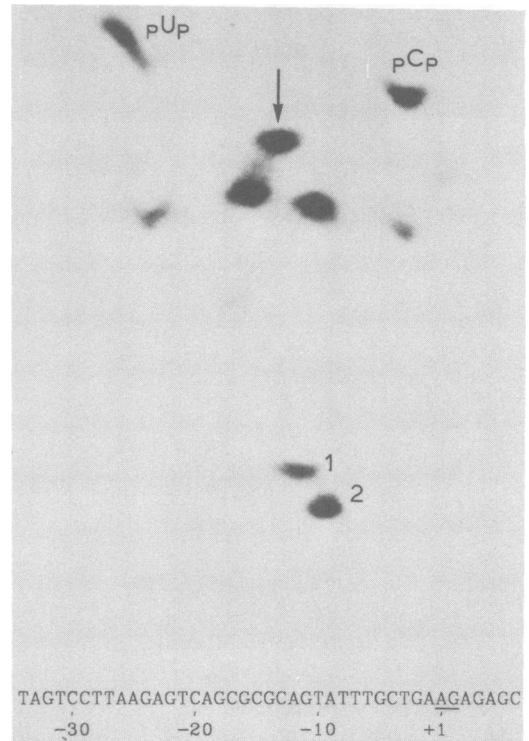

TAGTCCTTAAGAGTCAGCGCGCAGTATTTGCTGAAGAGAGC

−30   −20   −10   +1

FIG. 6. Early region II fingerprint. The EcoRI F recombinant shown in Fig. 3A was cut with EcoRI and HindIII; 50 µg of this template was transcribed in a 2-ml reaction mix under the conditions described in the legend to Fig. 5. The subsequent manipulations were as in Fig. 5, except that the HindIII-EcoRI fragment spanning 72.8 to 75.9 m.u. was immobilized on filters to select RNA. The selected RNA was digested with ribonuclease A (which cuts on the 3' side of pyrimidines). The lower doublets of spots 1 and 2 were identified as GAGAGC and AGAGAGC, respectively, which correspond to the in vivo termini for EII as reported in Baker et al. (2). This was confirmed by secondary analysis with RNases T$_2$ and T$_1$ and nuclease P1. These spots in addition carry the 2'-O-methylation indicative of capped termini. Because the reaction is scaled up 10-fold from that in Fig. 5, 5'-labeled termini from background initiation have been proportionally enhanced. The CU spot indicated by an arrow and a number of the other oligonucleotide spots carry 2'-O-methylation, indicating that they were derived from capped 5' termini encoded within the fragment. These other termini were not seen in vivo (Fig. 3). The diagram below shows the sequence around the promoter site of EII (75.0 m.u.) with A and G 5' termini at positions +1 and +2, respectively.
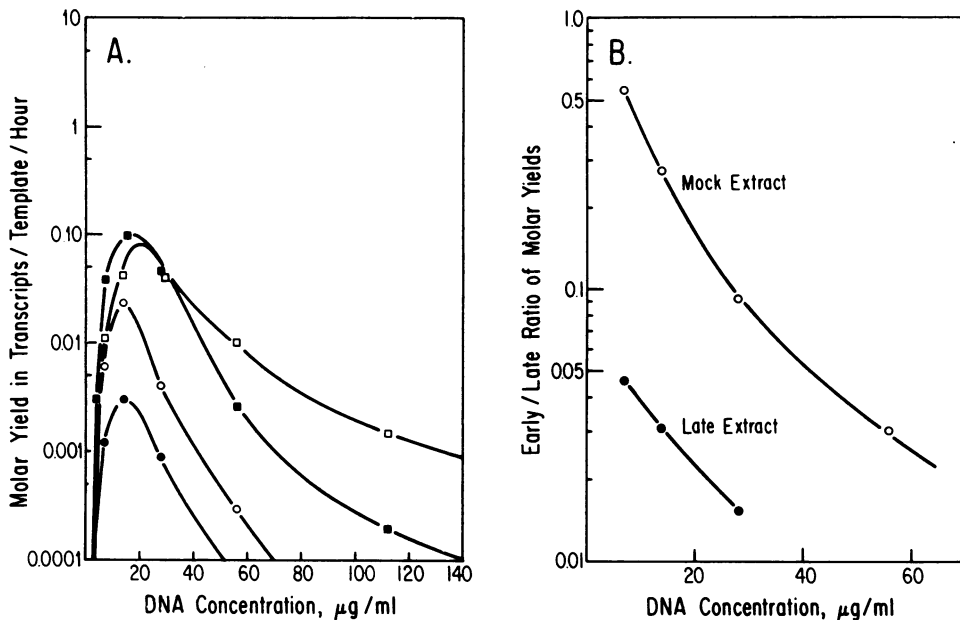
FIG. 7. DNA titration curves for EIa and the late promoter in uninfected and infected extracts. Cleaved plasmids containing PEIa and the late promoter were mixed in an equimolar ratio for template. The EIa promoter generated a runoff of 1,220 n, and the Ad2 late promoter generated a runoff of 974 n. Similar results were obtained with a 4,000-base-pair runoff of the late promoter. Transcription conditions were as in Fig. 1, except that nucleotide concentrations were 1/10 of those given. Quantitation was by scanning with a laser densitometer. A, Specific activity of promoters expressed in moles of transcript per mole of template in a 1-h reaction. Symbols: □, PL promoter in mock extract; ○, PEIa in mock extract; ■, PL in late extract; ●, PEIa in late extract. B, Early/late ratio as a function of DNA concentration.

21 h postinfection for late extracts. In parallel, a mock-infected extract was prepared from the same batch of HeLa cells. These extracts had roughly similar total transcriptional activities.

In light of variation in relative activity of different promoters with changes in DNA concentration it is important to ensure that any effects seen in infected extracts are not simply the result of endogenous viral DNA contaminating those extracts. Extracts were assayed for viral DNA by two methods. (i) Total nucleic acid corresponding to several reaction mixes was electrophoresed on neutral agarose gels with and without HindIII digestion and stained with acridine orange (27). No viral DNA was observed. (ii) The level of endogenous VA RNA synthesis in infected cell extracts was measured and compared with a standard of added viral DNA. Less than 0.02 µg of template viral DNA was observed per 20 µl of reaction mix. The addition of three times this concentration of viral DNA sequences to the transcription shown in Fig. 8A did not change the ratio of the runoff transcripts (data not shown). All of the extracts also gave similar bell-shaped DNA titration curves for the various promoters (Fig. 7A), indicating that a certain

bulk of exogenous DNA was still needed for transcription to occur.

Since the strong in vivo promoter for EII was only marginally active in extracts from uninfected cells, it was interesting to test its activity in extracts from early and late-infected cells. The Ad5 HindIII B fragment cloned into pBR322 was cleaved with XhoI and SalI, which would generate runoffs of 2,200 and 1,375 n from the EIII and EII promoter sites, respectively. Only low levels of the 1,375-n EII runoff were generated with mock and early extracts; this runoff was undetectable in a late extract (data not shown). The EIII promoter site was fully active in both mock and early extracts, but had less activity in late extracts. Similar experiments have been done with DNA fragments spanning the EIIL or the IVa2 promoter sites. In neither case was transcription detected from early or late extracts. Thus, infected extracts seem to lack components which allow transcription from these promoters.

When transcription from the equimolar mixture of efficient promoters was compared in extracts from mock, early, and late stages in infection, a shift was observed (Fig. 8B). Although
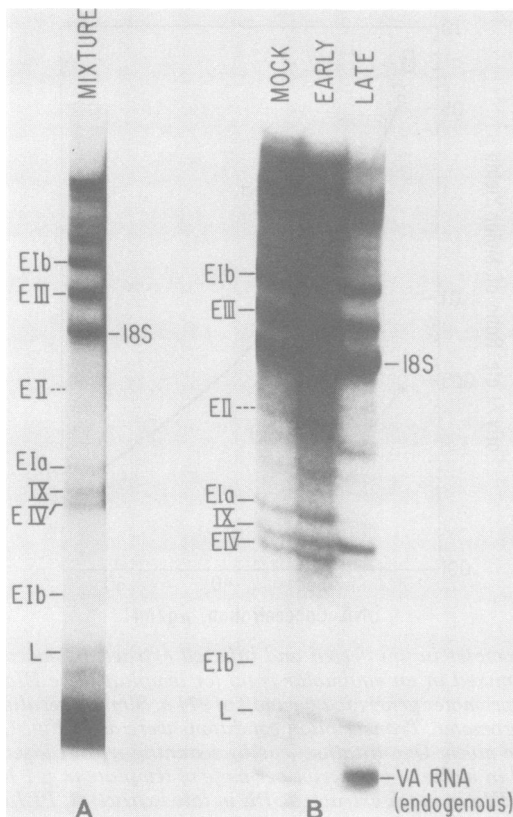
FIG. 8. *Mixture of templates from different regions. An equimolar mixture of the following cleaved plasmids was prepared: Ad5 HindIII G recombinant cut with XbaI and KpnI, which generates runoffs of 340 n from PEIb, 840 n from PEIa, and 1,165 n from the initiation site at 0.7 m.u. (Fig. 1A); Ad5 SmaI F recombinant cut with BamHI, which generates runoffs of 2,575 n from PEIb and 715 n from PIX (Fig. 1B); Ad2 BalI (14.7)-HindIII (17.0) cloned from BamHI to the HindIII site of pBR322—this recombinant cut with EcoRI, which generates a runoff of 225 n from the major late promoter; Ad5 HindIII B recombinant cut with XhoI and SalI, which generates runoffs of 1,375 n from PEII and 2,180 n from PEIII (Fig. 2A); Ad5 EcoRI B recombinant cut with HindIII, which generates a 680-n runoff from PEIV (Fig. 2B). The equimolar mixture (10 μg/ml) was supplemented with 15 μg of poly(dIC:dIC) per ml of carrier as noted in the text. Omission of any of the above DNAs removed the corresponding runoff bands from the labeled RNA pattern. A, Mixture transcribed in an uninfected extract. Due to differences in runoff length and promoter strength, it was often necessary to examine several exposures to accurately compare activity. B, Comparison of extracts from mock-, early-, and late-infected cells. Cells were harvested, and extracts were prepared from mock-infected cells and cells at 6 and 21 h postinfection with Ad2. Activities were determined for a variety of extract concentrations, and the reactions giving peak transcription are shown. The relative ratios of bands*

mock and early extracts produced essentially identical responses, the late extract showed preferential transcription of the intermediate PIX and the late (PL) promoters relative to the early promoter sites. In Fig. 8B, it is particularly striking to compare PIX with the flanking PEIa and PEIV runoffs. This shift in transcription specificity has been observed repeatedly with different mock and late extracts. The observation of relative enhancement of transcription of the PIX and PL promoter sites in late extracts did not change with variations in time of incubation, ionic strength, or nucleotide concentration, suggesting that simple differences in preparation do not account for the shift. Figure 7 shows a comparison of the DNA titration curves for the two extracts. Note that the DNA concentrations for optimal transcription of the early and late promoters in the two extracts are similar, but that the PEIa/PL ratio of the late extract remains 10-fold lower than that in an uninfected extract throughout the course of the DNA titration curve. A more complete description of the comparison of the transcription capacity in mock and late extracts is presented by Fire et al. (11).

DISCUSSION

Adenovirus mRNA synthesis results from initiation of transcription at nine sites on viral DNA (35, 42). Baker and Ziff (3a) have defined the set of capped nucleotides at each of these positions (Table 1). Six of these nine sequences are efficiently recognized by RNA polymerase II and factors for initiation of transcription in the WCE system. Each of these six sites has an obvious TATA or Goldberg-Hogness consensus sequences between −25 to −31 n. Two previous studies have shown that the EIa, EIb, and IX promoter sites are active in in vitro systems (24, 39). Three sites which encode RNA 5' ends, the promoter sites for IVa₂ (15.9 m.u.) and the early and late promoter sites for region II (PEII [75 m.u.] and PEIIL [72.0 m.u.]), do not have consensus TATA sequences and are poorly recog-

from an extract did not change over the range of the titrations. Protein concentrations were: mock, 6.3 mg/ml; early, 6.1 mg/ml; late, 5.8 mg/ml. A 150-n band in the late extract resulted from endogenous VA RNA (virus-associated small RNA) synthesis. The level of VA transcription is comparable to that obtained with 1.0 μg of exogenously added Ad2 DNA per ml. Two runoff bands of 2,575 and 340 n from EIb have been included. Lighter and darker exposures allow comparisons of these two bands in the different extracts. The ratio of radioactivity in these two EIb runoffs did not vary. The band of 1,140 n, which was strongly enhanced in the late extract, corresponds to the rightward promoter at 0.7 m.u.

nized in the WCE reaction mix. Very sensitive analysis of capped termini shows that transcription is specifically initiated at the PEII position, but at 1/25 the frequency observed from the Ad2 late promoter. With the same assay, transcription from the late promoter site for region II (PEIIL) is not detected (less than 0.5% of PL [16.4 m.u.]). This site has TACAAA at the position of the consensus sequence. The promoter site for the rabbit α-uteroglobin has an identical consensus sequence and has been reported to be efficiently transcribed in this system (S. Woo, personal communication). Thus, factors in the WCE must be discriminating between these promoters on the basis of sequences other than the TACAAA.

Several other results also suggest that RNA polymerase II and factors in the extract interact with sequences in addition to the TATA. Many perfect consensus sequences in viral or vector DNAs are not recognized for transcription initiation. In fact, in all of the adenovirus DNA sequences surveyed in this study, only two sites were detectably utilized for initiation that were not previously known to be in vivo initiation sites; these sites mapped on the r strand at approximately 0.7 m.u. and on the l strand at 96.3 m.u. The former may account for minor mRNA's detected from Ad2-, Ad5-, Ad7-, and Ad12-infected and -transformed cells (5, 12, 30, 41). No Ad2 mRNA has been mapped with a 5' terminus at 96.3 m.u. (5).

A direct test of the importance of the TATA sequences for in vitro transcriptional activity emerges from studies in which these sequences have been modified by deletion or mutation. Hu and Manley (20) concluded that the TATAAA sequence in the late Ad2 promoter was essential for in vitro transcriptional activity. Studies of similar deletions in the conalbumin (9), ovalbumin (36), early simian virus 40 (26), and late Ad2 (9) promoters have given similar results. In fact, Wasylyk et al. (38) showed that conversion of the third base, T, to a G in the conalbumin promoter sequence of TATAAA almost abolishes the in vitro reaction. Additionally, Hu and Manley's results indicated that deletion of sequences from either −51 n upstream or +5 n downstream of the late cap site also affected the efficiency of transcription, suggesting the in vitro reaction senses 60 n of sequence (20). Thus, the picture emerges that the TATA consensus sequence is of central importance for in vitro recognition, and that sequences lying 25 n to either side of this site can affect the efficiency of initiation.

There are reasons to suspect that the TATA consensus sequence is of secondary importance for in vivo transcription initiation. Where it has been studied, deletion of the TATA sequence does not significantly reduce in vivo initiation of transcription from a region, but does generally render an RNA product with widely dispersed 5' termini (4, 10, 14, 15). Hence, the TATA consensus sequence seems to have a positioning role for RNA polymerase II. In addition, there seems to be a marked in vivo dependence on sequences beyond −60 n (4, 10, 15–17), whereas deletion of these sequences has no effect in vitro. The in vitro reaction as it is now constituted is only a shadow of the in vivo process; it probably is only responsive to the higher affinity of RNA polymerase II and factors for sequences involved in positioning the complex for initiation.

One of the more remarkable features of the in vitro system is the fidelity of its reproduction of the nucleotide specificity of the in vivo initiation. This was shown previously for the late promoter of Ad2 (24, 40) and is vividly seen here in the microheterogeneity of in vitro initiation at the EIV promoter site. Both in vivo (3a) and in vitro RNA initiation at the Ad5 PEIV occur at any one of seven adjacent nucleotides, a string of six thymidine bases, or the adjacent adenosine (Table 1). This suggests that the biochemical complex specifying the in vitro initiation is identical to that specifying the actual initiation event in vivo. Although the relative ratios of PEIV caps under standard conditions in vitro are the same as those in vivo, the distribution of sites of in vitro initiation is sensitive to the concentration of free nucleotide triphosphates. Reduction of relative UDP or ATP concentrations can drastically reduce the fraction of chains initiated with uridine or adenine, respectively. Thus, initiation at multiple sites rather than RNA processing by 5' cleavage must account for the heterogeneous termini.

The lytic cycle of adenovirus evolves through a series of temporal stages where subsets of the nine promoters listed in Table 1 are optimally active. Either factors, RNA polymerase II or the viral template, must be modified during the course of the cycle to enhance transcription of various sites. The finding that WCEs from uninfected cells initiate at either early, intermediate, or late promoter sites with roughly comparable efficiency suggests that the in vivo transcription regulation is not fully reproduced in the in vitro system. This is further suggested by the lack of observed differences between extracts from mock- and early-infected cells as well as the lack of stimulation of the early PEII or intermediate PEIIL and PIVa$_2$ promoters in any of the infected extracts. Thus, some of the factors that play a role in adenovirus transcription in vivo are either missing or nonfunctional in WCEs.

One striking feature of the lytic cycle of adenovirus is the shift in rates of transcription between early and late promoters at the time of viral DNA replication. A shift in the ratio of transcription of late versus early promoters was also observed in vitro. An increase in the total DNA concentration enhanced transcription of both PL and PIX promoters relative to any early promoter in extracts prepared from either uninfected or late-infected cells. PL and PIX were also distinguished from the early promoters in their relatively enhanced activity in extracts prepared from late-infected cells. The ratio of transcription from either PL or PIX relative to an early promoter was 10-fold higher in late extracts than in mock extracts (Table 1). Thus, soluble factors in uninfected extracts distinguish late promoters from early promoters. Shifts in the level of these factors in late extracts could account for the enhanced transcription of late promoters. It should be possible in the future to identify these factors. Similarly, it is likely that some common sequence feature of PL and PIX promoters mediates their common enhanced recognition. Perhaps this feature is the string of guanine bases immediately 3' to their TATA consensus sequences. In any case, manipulation of DNA sequences around early and late promoter sites should permit identification of important features.

ACKNOWLEDGMENTS

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