Requirement for Distal Upstream Sequences for Maximal Transcription In Vitro of Early Region IV of Adenovirus

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A series of deletion mutants spanning the adenovirus early region IV (EIV) promoter were tested for transcription activity in vitro. At least three elements were found to be important for maximal transcription in HeLa whole-cell extracts. Deletion of the TATA box drastically reduced the transcription activity from the EIV promoter. Sequences between nucleotides -58 and -44 are also important for efficient transcription since deletion of this region reduced activity by 50%. More importantly, sequences residing upstream from -140 critically influence the level of EIV transcription. Deletion of sequences between nucleotides -325 (the right terminus of adenovirus genome) and -140 reduced the level of transcription more than 10-fold. It is possible that a specific cellular factor stimulates EIV transcription by recognition of these upstream sequences. The dependence of transcription from the EIV promoter on a distal upstream element may explain some aspects of the regulation of this promoter.

To understand the molecular mechanisms for the regulation of transcription mediated by eucaryotic RNA polymerase II, sequences and factors required for accurate and efficient initiation must be identified. Recently, cell-free systems capable of initiating transcription accurately from a variety of promoters have been developed (28, 44). Such systems facilitate the definition of sequence signals responsible for the regulation of transcription. It has been suggested that sequences which function to potentiate initiation of transcription in vivo and in vitro are comprised of at least three elements. The first element, the "TATA box," is found between 20 and 30 nucleotides upstream from the initiation site on most eucaryotic promoters (7). There are, however, examples of promoters which either lack a discernable TATA box or are fully active when this element has been deleted (3, 13, 16, 29, 35, 40). Typically, the second set of elements required for efficient transcription of eucaryotic genes lies between 50 and 150 base pairs (bp) upstream of the initiation site (10, 17, 18, 31-33, 38). The third type of element, called an enhancer sequence, stimulates transcription in vivo when placed either 5' or 3' of a promoter segment (2, 9, 14, 34).

Transcription of the early region IV (EIV) promoter of adenovirus in vivo (6, 37) and in vitro (20) is specifically inhibited by a viral DNA binding protein. Transcription in vivo of the same promoter is also stimulated by adenovirus (EIA) gene products (4, 25). Thus, the EIV promoter provides an interesting system for studying both positive and negative regulation of transcription by various factors.

To resolve the identity and functional role of the EIV promoter sequences that regulate transcription, we constructed a series of mutants with deletions of sequences close to the initiation site. Individual mutants were tested for transcriptional activity in vitro. These experiments identify at least three distinct signals upstream from the initiation site of EIV.

MATERIALS AND METHODS

Construction of deletion mutants. Plasmid pSmaII of adenovirus type 5 was constructed as follows. Digestion of plasmid pRIB5 (84 to 100 map units; 5) with *EcoRI* and *SmaI* yields the *SmaII* fragment (98.3 to 100 map units), with an *EcoRI* linker attached to the right terminus. This fragment was inserted between the *EcoRI* and *SmaI* sites of pBR313. To construct 5'- or 3'-deletion mutants, pSmaII DNA (10 µg) was linearized with *EcoRI* or *SmaI*, respectively, and incubated at 25°C with 5 U of *Bal31* (Bethesda Research Laboratories) in 100 µl of 20 mM Tris-hydrochloride (pH 8.1)-100 mM NaCl-12 mM CaCl₂-1 mM EDTA. Portions (25 µl) were removed at 90, 180, and 260 s and put into tubes containing 175 µl of 25 mM EDTA, extracted twice with phenol-chloroform, and precipitated with ethanol. Samples were incubated in 100 µl of 50 mM Tris-hydrochloride (pH 7.2)-10 mM MgSO₄-2 mM dithiothreitol-50 µg of bovine serum albumin per ml-0.1 mmol of each deoxyribonucleotide triphosphate-10 U of DNA polymerase Klenow fragment for 30 min at room temperature. The reaction was heated for 10 min at 65°C to inactivate the enzyme. *BamHI* linkers (decamer; Collaborative Research, Inc.) were phosphorylated with T4 polynucleotide kinase (P-L Biochemicals) and added to the blunt-end termini with 20 U of T4 DNA ligase (P-L Biochemicals). The molar ratio of linkers to mutant DNAs was 40:1. Incubations were at 4°C for at least 12 h in a 150-µl reaction mixture containing 50 mM Tris-hydrochloride (pH 7.5)-7 mM MgCl₂-10 mM dithiothreitol-0.1 mM ATP. The DNA fragments obtained after *BamHI* and either *SmaI* or *EcoRI* digestions were eluted from 1.4% agarose gels and inserted into *BamHI*- and *SmaI*-digested pBR313 or *BamHI*- and *EcoRI*-digested pBR322 for 5'- or 3'-deletion mutants, respectively. Recombinants were transfected into competent *Escherichia coli* HB101. Sequence analysis to determine the exact endpoints of the deletions was performed by the Maxam-Gilbert technique (30). The 5'- and 3'-deletion mutants were named as E- and S-series mutants, respectively.

To construct the sequential internal deletion mutants,

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recombination of matching 5'- and 3'-deletion mutants was performed. As the endpoints of all 5'- and 3'-deletion mutants terminate with the same synthetic *Bam*HI restriction site, the fragments of the S-series mutant DNAs were digested with *Eco*RI and *Bam*HI and purified by electrophoresis in agarose gels. These were subsequently inserted into *Eco*RI- and *Bam*HI-digested E-series mutant DNAs.

In vitro transcription. A whole-cell extract was prepared from HeLa cells as described previously (28). Recombinant plasmids were grown in *E. coli* HB101. Plasmid DNAs were extracted by established procedures (26, 39) and digested with various restriction endonucleases to distinguish the runoff transcripts by their sizes. Plasmid DNAs of the E-series, internal deletion (ID)-series, and pSmaII were digested with *Tth*111I; plasmid DNAs of the S-series and mutants pRIB5 (EIV wild type [WT]) and pFLBH (major late [ML]) were digested with *Ava*I, *Bgl*II, and *Pvu*I, respectively. The mixture of these linearized plasmid DNAs was used as the template DNA for transcription in vitro. When a whole-cell extract was used in the transcription reaction, the standard reaction mixture (25 μ l) contained the following: 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9), 40 mM KCl, 5 mM MgCl₂, 0.05 mM EDTA, 0.8 mM dithiothreitol, 7% glycerol, 5 mM creatine phosphate, 300 μ M ATP, 100 μ M GTP, 100 μ M CTP, 5 μ M UTP, 10 μ Ci of [α -³²P]UTP (New England Nuclear Corp.), 18 μ g of poly(deoxyinosinic acid-deoxycytidylic acid) · poly(deoxyinosinic acid-deoxycytidylic acid) per ml, 5 to 10 μ g of template DNA per ml, and 9 μ l of HeLa cell extract.

When transcription in a reconstituted system was compared with that in a whole-cell extract, the preincubation pulse-chase procedure was used (A. Fire, M. Samuels, and P. A. Sharp, *J. Biol. Chem.* in press). The preincubation mixture (20 μ l) contained 12 mM HEPES (pH 7.9), 60 mM KCl, 5 mM MgCl₂, 0.6 mM dithiothreitol, 1 mM EDTA, and 12% glycerol. The preincubation in a reconstituted system contained template DNA (5 to 10 μ g), RNA polymerase II (20 U), RNasin (15 U), and partially purified HeLa transcriptional factors: 1 μ l of [AB], 0.6 μ l of [CB], 2 μ l of [DB], and 4 μ l of [CD] (41). The preincubation in a whole-cell extract contained template DNA (5 to 10 μ g/ml), poly(deoxyinosinic acid-deoxycytidylic acid) (18 μ g/ml), and 9 μ l of a whole-cell extract. After a 1-h preincubation at 30°C, transcription was initiated by the addition of nucleotide (3 μ l) to concentrations of 30 μ M ATP, CTP, and GTP and 1 μ M [α -³²P]UTP (760 Ci/mmol). After a 10-min incubation at 30°C, 2 μ l of a chase nucleotide mixture was added to give a final concentration of 1 mM. An additional 15 min of incubation at 30°C allowed the elongation of the nascent RNA chains.

Analysis of in vitro RNA transcripts. Extraction of RNA products, denaturation with glyoxal, and agarose gel electrophoresis were performed as previously described (19). RNA products were also analyzed with M13 selection by the method of Hansen and Sharp (20a) with slight modifications. RNA products were resuspended in 15 μ l of 50 mM HEPES (pH 6.95)–0.75 M NaCl–1 mM EDTA with a mixture of recombinant M13 phage DNAs: 0.2 μ g of M13 XH11 (an M13-adenovirus recombinant containing –261 to +197 of the ML promoter and bounded by *Xho*I and *Hind*III restriction sites) and 0.2 μ g of M13 XE11 (an M13-adenovirus recombinant containing –331 to +251 of the EIV promoter and bounded by *Eco*RI and *Sma*I restriction sites) as described previously (M. Samuels, A. Fire, and P. A. Sharp, *J. Biol. Chem.*, in press). Hybridization mixtures were heated to 70°C for 5 min and incubated at 50°C for 1 to 2 h. Hybridizations were stopped with 200 μ l of cold buffer

containing 10 mM HEPES (pH 7.5), 1 mM EDTA, and 0.2 M NaCl and treated with 5 U of RNase T₁, a single-strand RNA-specific endonuclease, for 30 min at 30°C. The T₁ was subsequently destroyed by the addition of 50 μ g of proteinase K to the reaction for 60 min at 30°C. The hybrids were precipitated with ethanol, resuspended in 80% formamide–0.05 M Tris-borate (pH 8.3)–1.25 mM EDTA, heated in boiling water for 5 min to dissociate them, and analyzed by acrylamide gel electrophoresis. Autoradiograms obtained with preflashed film were traced by an ATAGO densitometer to quantitate the amount of radioactivity in each band.

RESULTS

Construction of deletion mutants and their transcriptional activity. A series of mutants with deletions upstream from the 5' end of the EIV transcription unit were constructed. The 5'-deletion mutants, E-series, were derived from pSmaII (Fig. 1A), and the exact endpoints of the deletions were determined by DNA sequencing (Fig. 1B). EIV transcripts have a heterogeneous set of capped 5' termini, and the position of the furthest-upstream cap site (a uridine residue) is numbered as +1 (1, 12, 22). Whole-cell extracts prepared from uninfected HeLa cells are capable of initiating transcription in vitro at the EIV promoter of adenovirus (12, 27). To compare the transcriptional efficiencies of mutant DNAs to those of the WT EIV promoter, two template segments were added to a standard reaction mix. The mutant and WT templates have been cleaved to generate 1,200- and 1,000-nucleotide runoff RNAs, respectively (21). After a 60-min incubation in the presence of [α -³²P]UTP, the RNA products were extracted and resolved by electrophoresis in denaturing glyoxal gels. All 5'-deletion mutants with endpoints between –140 and –58 exhibited ca. 10-fold reduction in transcriptional efficiency as compared with that of WT EIV

FIG. 1. 5'-Deletion mutants at the EIV promoter region. (A) Structure of plasmid pSmaII. The sequence of the EIV gene is indicated by a box. The pBR313 sequence is represented by a line. The straight arrow indicates the region of progressive deletion. Only relevant restriction sites are displayed. (B) Endpoints of 5'-deletion mutants. The number of nucleotide residues from the initiation site (+1) of EIV transcription is indicated for each E-series mutant. The sequence TATATATA is the TATA box of EIV. The endpoint of deletion for each mutant is shown as double lines for the EIV gene sequence and dashed lines for the pBR313 sequence. The EIV WT extends 325 bp upstream from the transcription initiation site.

FIG. 2. Effect of progressive 5' deletion on transcription in vitro of the EIV promoter. Transcription was done in the standard reaction mixture containing a HeLa whole-cell extract. Both the mixtures of each E-series plasmid DNA (4.2 μ g/ml) cleaved with *Tth*III and the *Bgl*II-cleaved pRIB5 (2.1 μ g/ml) internal control were used as template DNAs. Transcription reactions were incubated for 60 min at 30°C. RNA was extracted from the reaction mixture and analyzed by agarose gel electrophoresis. The marker (lane M) was 5'-end-labeled fragments of *Hinf*I-cleaved SV40 DNA, the sizes of which are given in nucleotides. The arrow indicates the position of the transcripts from E-series mutant DNA. The percentage of mutant transcription as compared with that of WT transcription is listed in parentheses at the bottom of each lane.

(Fig. 2, lanes 1 through 7). Transcriptional activity of the mutant retaining 44 bp upstream of the cap site (pE-044) was further depressed approximately twofold (Fig. 2, lane 8); no accurate transcription was detected with the mutant retaining only 5 bp of upstream sequences (pE-005, lane 9). These results indicate that sequences extending between -44 and -5 are essential for accurate initiation of EIV transcription, whereas sequences further upstream than -140 greatly influence the efficiency of the transcription. It is unlikely that the newly joined pBR322 sequences are suppressing transcription from EIV since the equivalent constructions with the ML promoter of adenovirus were not influenced by these sequences (24).

The 3'-deletion mutants, S-series, were also constructed from pSmaII (Fig. 3A) with exact boundaries determined as above by DNA sequencing (Fig. 3B). The in vitro transcriptional activities of these templates were also compared with that of the WT EIV promoter (Fig. 4). The activities of both pS-004 and pS-016 DNAs, which had deletions of sequences to -4 and -16, respectively, were similar to that of EIV WT (Fig. 4, lanes 7 and 8). The efficiency of transcription from pS-021 was drastically reduced. Similarly, transcripts from pS-027, pS-029, pS-059, pS-086, and pS-138 were only detected at a very low level. These results indicate that sequences lying between -21 and -16 were critically important in determining the efficiency of the transcription of the EIV gene.

ID-series mutants were constructed from two opposing libraries of deletion mutants, the E- and S-series mutants.

FIG. 3. 3'-Deletion mutants at the EIV promoter region. (A) Procedure of constructing 3'-deletion mutants. EIV DNA and pBR313 sequences are indicated by a box and a line, respectively. The straight arrow represents the region of progressive deletion. (B) Endpoints of 3'-deletion mutants. The endpoint deletion for each mutant is shown as double lines for the EIV gene sequence and dashed lines for the pBR322 sequence. The number of nucleotides from the initiation site (+1) of EIV transcription is indicated for each mutant (S-series). The sequence TATATATA comprises the TATA box of EIV.

Combinations of adjacent 5'- and 3'-deletion mutants were used to create deletions in the promoter region of the EIV gene (Fig. 5). The in vitro transcription activities of these mutants were also compared to that of the WT EIV promoter (Fig. 6). Plasmids pID-2, -3, -4, and -8 yielded levels of transcripts similar to that of pSmaII (Fig. 6, lanes 1, 2, 3, 4,

FIG. 4. Effect of progressive 3' deletion on transcription in vitro of the EIV promoter. Transcription in vitro was done under standard conditions containing a HeLa whole-cell extract. Both the mixtures of each S-series DNA (3.2 μ g/ml) cleaved with *Ava*I and the *Bgl*II-cleaved pRIB5 (3.2 μ g/ml) were used as template DNAs. RNA was extracted for the reaction mixture and analyzed by electrophoresis in denaturing glyoxal gels. The arrow indicates the position of the transcript from the S-series mutant DNA.

FIG. 5. Internal deletion mutants at the EIV promoter region. The sequential internal deletion mutants, ID-series, were constructed by recombination of matching E- and S-series mutants. The nucleotide number of each deletion site is indicated. EIV and pBR313 sequences are displayed by double and dashed lines, respectively. The EIV WT extends to 325 bp upstream from the transcription initiation site.

and 8). However, plasmids pID-5 and -7, missing sequences from -61 to -44 and from -21 to -5, respectively, gave three- and twofold lower levels of transcription, respectively. No accurate transcription was detected when plasmid pID-6, missing TATA sequences, was used as the template (Fig. 6, lane 6). These results again indicate that the sequences extending from -61 to -44 influence transcription efficiency and that the sequences between -29 and -21, i.e., the TATA box, are essential for accurate and efficient transcription from the EIV promoter.

Efficiency of transcription with changes in template concentration. The low level of transcription of the E-series suggested that a sequence beyond -140 was important for activity (Fig. 2). To test whether deletions missing this element would compete with WT template, various concentrations of E-series DNAs were examined in a whole-cell extract system. Mixtures of WT template pRIB5 and either pE-103 or pID-2 were used as template DNAs. The amounts of pRIB5 DNA were kept constant in each reaction, and the amounts of either pE-102 or pID-2 DNA were varied over a 10-fold range of DNA concentrations (Fig. 7). An increase in transcripts from pID-2 and a corresponding decrease of transcripts from WT template were observed with the addition of higher concentrations of pID-2 DNA. At the highest concentration of total DNA, transcription from both templates was nonspecifically inhibited (Fig. 7, lanes 1 and 6). Mutant pE-103 gave increased levels of transcription with the addition of higher concentrations of DNA, but the level of WT template transcription remained almost constant until activity was nonspecifically inhibited (Fig. 7, lane 1). Even at a maximum level of pE-103 DNA (Fig. 7, lane 2), this template yielded ca. 10-fold lower levels of transcripts than did pID-2 (Fig. 7, lane 7). The specific suppressive effect of the addition of high concentrations of pID-2 DNA on the transcription of WT template probably reflects a competition for a limiting factor. The lack of specific suppression by pE-103 DNA suggests that sequences between the terminus of adenovirus DNA and -140 were necessary for this specific competition.

Transcription of EIV promoter in reconstituted system. The unusual dependence of the EIV promoter on distal upstream sequences for in vitro transcription suggests that a specific factor(s) might be required for efficient transcription. It has been demonstrated that a reconstituted system which includes RNA polymerase II and three separate but not purified factors initiates transcription precisely at the adenovirus ML and EIV promoters (41). To test whether the same

set of factors recognizes the upstream signal sequences in the EIV promoter region, the efficiency of transcription of pE-103 and pID-2 templates was examined in both the whole-cell extract and the reconstituted system. In both cases, the activities of pE-103 and pID-2 were compared with those of the ML template. The mixture of pFLBH (ML) with either pE-103 or pID-2 DNA was transcribed, and the resulting transcripts were separated either by electrophoresis in denaturing glyoxal gels or by hybridization with an excess of a mixture of recombinant M13 DNAs (Fig. 8A and B, respectively); as described in the legend, different ratios of template DNAs were used in various reactions. In particular, the ratio of EIV to ML was 1:1 in lanes 3 and 4 and 7:1 in lanes 7 and 8 (Fig. 8). In the latter case, the hybridized RNA was analyzed by the procedure of Hansen and Sharp (20a) with omission of the filter-binding step. The hybrids were digested with T_1 nuclease and analyzed by gel electrophoresis (see above).

The efficiency of transcription from pE-103 and pID-2 in a whole-cell extract system was clearly different (Fig. 2 and 8). pE-103 DNA yielded a ca. 13-fold lower level of transcription than did pID-2 DNA. However, pE-103 and pID-2 templates gave the same level of runoff transcription in a reconstituted system (Fig. 8, lanes 7 and 8). These results

FIG. 6. Effect of internal deletion on transcription in vitro of the EIV promoter. Transcription in vitro was done in the standard reaction mixture containing a HeLa whole-cell extract and both the mixture of each ID-series plasmid DNA (3.2 μ g/ml) cleaved with *Tth*1111 and the *Bgl*II-cleaved pRIB5 (3.2 μ g/ml). RNA was extracted after incubation for 60 min at 30°C and analyzed by electrophoresis in denaturing glyoxal gels. The lengths of the markers, the same as those in Fig. 2, are indicated. The arrow indicates the position of the transcript from ID-series mutant DNAs. The percentage of WT transcription is listed in parentheses for each mutant.

FIG. 7. Comparison of transcriptional activity of pE-103 and pID-2 DNA with increasing concentrations of DNA. (A) Dose response of transcriptional activity of pE-103 and pID-2 DNAs. Transcription in vitro was done in the standard reaction mixture containing a HeLa whole-cell extract and poly(deoxyinosinic acid-deoxycytidylic acid). Two kinds of DNA templates were used in each reaction: a constant amount of the *Bgl*II-cleaved pRIB5 (4.3 μ g/ml) and various amounts of the *Tth*111I-cleaved pE-103 DNA (lane 1, 24 μ g/ml; lane 2, 12 μ g/ml; lane 3, 6 μ g/ml; lane 4, 3 μ g/ml; lane 5, 1.5 μ g/ml) or pID-2 DNA (lane 6, 24 μ g/ml; lane 7, 12 μ g/ml; lane 8, 6 μ g/ml; lane 9, 3 μ g/ml; lane 10, 1.5 μ g/ml). Transcription reactions were incubated for 60 min at 30°C. RNA was extracted from the reaction mixture and analyzed by electrophoresis in denaturing glyoxal gels. The lengths of the markers, the same as in those in Fig. 2, are shown. The arrow indicates the position of the transcription from pE-103 or ID-2 DNA. (B) Analysis of the amount of template DNAs. The amount of template DNA was examined by electrophoresis in neutral agarose gels. The numbers of the reactions correspond with those in (A).

were more apparent when assayed by using recombinant M13 single-stranded DNAs to quantitatively hybridize radioactive RNA transcribed from EIV and ML promoters (Fig. 8B). EIV and ML templates yielded runoff transcripts of 1,050 and 840 bases, respectively, whereas only 250 and 200 bases, respectively, of these RNAs were complementary to sequences in the M13 recombinant DNAs. These hybridization assays also indicated that pID-2 and pE-103 templates

had comparable activities in the reconstituted system, whereas pID-2 templates were 11-fold more active in the whole-cell extract system.

DISCUSSION

Transcription of the EIV gene of adenovirus is probably regulated by a variety of viral and cellular factors. Previous experiments concerning EIV transcription in vivo have indicated that the viral EIA (4, 25) and EIIA (6, 37) gene products regulate EIV transcription in the positive- and negative-acting fashion, respectively. Suppression of transcription from the EIV promoter by the EIIA DNA binding protein was also demonstrated in vitro (20). General cellular factors are required in addition to RNA polymerase II for EIV transcription in vitro. To understand the molecular mechanisms of the regulation of EIV transcription, we have characterized the in vitro transcription of a series of mutants with deletions in the region of the promoter.

At least three separate sequence signals upstream from the 5' terminus of EIV mRNAs were required for optimal transcription. The first signal is characterized by deletion of sequences containing the TATA box, between -29 to -21 nucleotides. The internal deletion mutant missing these DNA sequences (pID-6) lacked transcriptional activity. The second set of signals for the EIV promoter is located from -61 to -44 nucleotides upstream from the transcription start site. Deletion of these sequences reduces transcription efficiency two- to threefold in vitro. The third signal is located in an upstream control region which lies between -140 and -325 (-325 is the right terminus of the adenovirus genome). Deletion mutants of this region have 10-fold less transcriptional activity in vitro.

The EIV promoter activity in vitro is unusual in its apparent complete dependence on TATA sequences for an initiation reaction that is stimulated 10-fold by other sequences far upstream. Previous studies have shown that intact TATA sequences are essential for the accurate transcription in vitro of many promoters (8, 18, 24, 42, 43). For these promoter sites, recognition of the TATA sequences is probably the rate-limiting step for initiation in vitro and, thus, alterations in these sequences reduce the efficiency of transcription. In contrast, several in vivo (3, 15, 33) and in vitro (20a, 35) transcriptional studies suggest that the TATA sequences are not always of primary importance for transcription of some promoters. In the case of these initiation sites, particularly the early promoter of simian virus 40 (SV40), the frequency of initiation is primarily determined by the efficiency of processes directed by sequences other than TATA. The EIV promoter is somewhat unique in that upstream sequences stimulate initiation by increasing the rate of the TATA-specified reaction.

The only well-documented example, other than this study of EIV, of a set of sequences stimulating transcription in vitro over lengths greater than 100 nucleotides is the dependence of the early and late promoters of SV40 on the guanosine-cytosine-rich repeats (PyPyCCGCCC region; 11, 20a). McKnight and Kingsbury (32) have shown that the -100 to -50 region of the herpes thymidine kinase gene containing sequences of CCCGCCC and GGGCGGCG is important for transcription in oocytes. Sequences similar to the repeats in SV40 and the guanosine-cytosine-rich elements in the *tk* gene are present in the regions around -254 (CGCGGG), -236 (GGCGGG), and -215 (GGCGGA) bp upstream from the EIV transcription initiation site. The last two regions are part of the inverted terminal repetition

FIG. 8. Transcriptional activity of pE-103 and pID-2 templates in a whole-cell extract or a reconstituted system. Transcription from the promoters of pE-103 and pID-2 was compared by transcribing both templates in either a whole-cell extract (lanes 1 through 4) or a reconstituted system containing a mixture of RNA polymerase II and three transcription factors (lanes 5 through 8). The EIV DNA template was pE-103 or pID-2 DNA digested with *Tth1111* endonuclease, and the ML template was pFLBH DNA digested with *PvuI* endonuclease. The preincubation pulse-chase procedure as described in the text was used. The RNA product was extracted from the reaction mixture and analyzed either (A) by electrophoresis in denaturing glyoxal gels or (B) by hybridization with an excess of M13 cloned DNAs as described in the text. The number of each lane in (A) is analogous with that shown in (B). The template DNA was: lane 1, pFLBH (10 μ g/ml); lane 2, pID-2 (10 μ g/ml); lane 3, pFLBH (5 μ g/ml) and pID-2 (5 μ g/ml); lane 4, pFLBH (5 μ g/ml) and pE-103 (5 μ g/ml); lane 5, pFLBH (2.5 μ g/ml); lane 6, pID-2 (10 μ g/ml); lane 7, pFLBH (1.25 μ g/ml) and pID-2 (8.75 μ g/ml); lane 8, pFLBH (1.25 μ g/ml) and pE-103 (8.75 μ g/ml). 5'-End-labeled fragments of *HinfI*-cleaved SV40 DNA were used as the size marker.

sequences of adenovirus. Studies in vivo of deletions of the promoter region of EIA have shown that synthesis of mRNAs initiated at the normal EIA site increases as it is positioned closer to the inverted terminal repeat (23). Perhaps this stimulation is due to the same transcription elements found upstream of the EIV promoter.

Comparison of transcription in vitro from the EIV promoter and ML promoter suggests the former may be dependent on a specific cellular factor. Accurate transcription of the EIV promoter can be obtained with three fractions of a HeLa whole-cell extract and purified RNA polymerase II. These fractions were prepared by using an assay containing the ML promoter of adenovirus as template, and these fractions probably solely recognize sequences adjacent to the TATA box for initiation (24). All deletions of the EIV promoter retaining the TATA element had comparable activity in the reconstituted system. However, in the whole-cell extract, templates which retained the upstream -140 to -325 sequences were preferentially transcribed to templates deleted in these sequences. This suggests that the reconsti-

tuted reaction is missing a factor(s) important for efficient transcription in vitro of the EIV promoter and that the effect of such factors is mediated by distal upstream sequences. This situation is reminiscent of the SV40-specific transcription factor which probably recognizes the above-mentioned guanosine-cytosine repeat region (11). Whether the same factor is involved in the stimulation of EIV and SV40 promoters remains to be determined.

Transcription from the EIV promoter of adenovirus is suppressed during the intermediate stage of infection (37). Studies of temperature-sensitive mutants of adenovirus suggest that the 72,000-molecular-weight DNA binding protein encoded by early region II is responsible for this suppression (6). The addition of purified 72,000-molecular-weight DNA binding protein to an in vitro reaction specifically suppresses EIV transcription (20). Adenovirus DNA replication is initiated at both termini of the genome by formation of a complex containing the 72,000-molecular-weight DNA binding protein (36). It is possible that suppression of EIV transcription results from the binding of the 72,000-molecular-weight

DNA binding protein and other cellular proteins that are part of the replication complex to sequences overlapping the transcriptional element in the -140 to -325 region.

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