Stimulation of in vitro transcription by the upstream element of the adenovirus-2 major late promoter involves a specific factor

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ABSTRACT

We have previously reported that sequences located upstream from the TATA box of the Adenovirus-2 major late promoter (Ad2MLP), between -34 and -97, are necessary for efficient transcription in vivo and in vitro (1). We have utilized an in vitro competition assay to demonstrate that the upstream element requirement involves the binding of a specific factor(s), which results in stimulation of in vitro transcription from the Ad2MLP. DNA fragments prepared from Ad2MLP upstream sequence mutants which are transcribed much less efficiently than the wild-type promoter both in vivo and in vitro were shown to be unable to bind this factor. We have also constructed chimeric promoter recombinants containing the 21bp repeat upstream element of the SV40 early promoter inserted upstream from the Ad2MLP TATA box. The SV40 upstream element stimulates in vitro transcription from the heterologous Ad2MLP TATA box element; competition experiments show that the Ad2MLP upstream element-specific factor is different from the SV40-specific factor Sp1.

INTRODUCTION

The development of in vitro transcription systems (cell-free extract) (see 2, 3 for reviews) has led to a greater understanding of both the DNA sequence and protein factor requirements for accurate and efficient transcription in eucaryotes ("accurate" refers to transcription initiating at the in vivo start (cap) sites; "efficient" refers to the amount of accurate transcription). Multiple complementary fractions prepared from either S-100 (4), whole cell (5) or nuclear (6) extracts of cells such as HeLa are necessary for accurate and efficient transcription by RNA polymerase B (II)(7-13).

In vivo studies have shown that RNA polymerase B promoters (see 1-3, 14-18 for reviews and references) consist of several promoter elements located upstream from the mRNA startsite. For a number of promoters, the in vivo effect of these elements has been reproduced in vitro. The TATA box or proximal upstream element located 25-30 bp upstream from the startsite appears to be required for both accurate and efficient in vitro transcrip-
tion (14, 19-24). The distal or upstream elements, usually located 40-110 bp upstream from the start site, have been shown to be required for maximal-ly efficient in vitro transcription from a number of viral and cellular genes, such as the Adenovirus-2 major late (Ad2ML) (1, 25), SV40 early (26-28), silkworm fibroin (29) and sea urchin histone H2a (30) transcription units. The in vivo stimulatory effect of the SV40 enhancer has been repro-duced, in part, in vitro using both the homologous SV40 early (28, 31) and heterologous conalbumin and Ad2ML (32, 33) promoter elements. Recent competition and footprinting experiments have shown that these promoter elements may be specifically recognized by transcription factors in vitro: (i) the TATA box element appears to bind a general transcription factor (10, 34); (ii) the upstream elements of both the SV40 early promoter (21bp repeat region) (35) and the Drosophila heat shock hsp70 gene (36) bind specific factors required for efficient transcription; and (iii) the SV40 enhancer element appears to bind a specific trans-acting factor (31, 33).

We have previously demonstrated that efficient in vivo and in vitro transcription from the Adenovirus-2 major late promoter (Ad2MLP) requires the presence of an upstream sequence (1). Moreover, we suggested that a transcription factor may interact with this upstream sequence. In this study we present evidence which strongly supports the existence of such a factor which appears to be distinct from those which recognize other promoter elements. In addition, using a chimeric promoter, we show that the SV40 21bp repeat upstream element can functionally replace the Ad2MLP upstream sequence for stimulation of transcription from the Ad2MLP TATA box element. However, the factor which specifically interacts with the SV40 upstream element, but not the Ad2MLP upstream factor, appears to be required for efficient in vitro transcription from this chimeric promoter.

MATERIALS AND METHODS

Construction of Recombinants. The pM series (Fig. 1 and 2A) was constructed using standard cloning techniques (37). pM677 (Fig. 1) contains the wild-type Ad2MLP region from -677 (EcoRI site) to +33 (BamHI site) of the pSVA series previously described (1) inserted between the EcoRI and BamHI sites of pBR322. pM97 and pM34 (Fig. 1) were similarly constructed from the pSVA series and contain deletions upstream of positions -97 and -34, respectively (1). pM1 (Fig. 2A) contains two point mutations at positions
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-60 and -62 which were created by oligonucleotide-directed mutagenesis (for details of this construction and also those of pM2 and pM677-X, see ref. 38). pM2 (Fig. 2A) contains two point mutations at positions -54 and -125 (also created by oligonucleotide-directed mutagenesis), generating two SmaI sites at positions -52 and -124, respectively; pM3 and pM4 (Fig. 2A) were obtained by inversion and deletion, respectively, of the SmaI fragment from pM2. pM5 and pM6 (Fig. 2A) were constructed by insertion of the SV40 21bp repeat region [from the repaired NcoI site at SV40 position 38 (coordinates in the BB8 system) to a repaired BamHI site created at SV40 position 101 (39)] into the SmaI site of pM4 in its natural (pM5) and reverse (pM6) orientation with respect to the SV40 early promoter capsites. pM677-X (Fig. 2A) corresponds to the recombinant pM677 described above into which a XhoI site was created at position -31 in the Ad2MLP TATA box by oligonucleotide-directed mutagenesis; pM1-X, pM2-X, pM3-X and pM4-X, all with a XhoI site in the TATA box, were similarly constructed from pM1, pM2, pM3 and pM4, respectively. A series of pM plasmids containing two Ad2MLP regions (see Fig. 1) was constructed by insertion of the repaired BamHI Ad2MLP-containing fragment from pSVA97 and pSVA34 (see ref.1) into the PvuII site of pM34, resulting in recombinants pM34.97 and pM34.34, respectively (gifts of P. Sassone-Corsi). The nomenclature adopted for these double promoter-containing plasmids is such that the first and second numbers correspond to the insertions of the Ad2MLP into the EcoRI-BamHI and PvuII sites, respectively. pM97.97, pM97.34, pM6.34 and pM6.97 (Fig. 1) were then constructed by recombination of appropriate PstI-Sall fragments between pM34.97 and pM97, pM34.34 and pM97, pM34.34 and pM97, pM34.34 and pM6, and pM34.97 and pM6, respectively.

In vitro transcription. The preparation of HeLa whole cell extract (WCE) was described previously (5). The standard in vitro transcription run-off assay consisted of a 15 min preincubation at 24° in a 16 μl reaction volume containing 6-8 μl WCE (the optimum amount was determined for each extract), 25 mM Tris-HCl (pH 7.9), 5 mM MgCl2, 20 mM (NH4)2SO4, 0.1 mM EDTA, 0.5 mM DTT, 7.5% glycerol, 10 mM KCl and 200 ng of the specific DNA template. After the preincubation period, 4 μl of a mixture containing 5 mM MgCl2, 10 mM KCl, 1.25 mM each of ATP, GTP and UTP, and 6.25 μM CTP containing 2-5 μCi [32P]α-CTP was added to start RNA synthesis. The standard competition assay was as described above, except that variable amounts of competitor DNA fragment (40-160 ng) were added in the preincuba-
Fig. 1. Structures of the pM series of Ad2MLP-containing recombinants. The construction of the pM series is described in Materials and Methods. pM677 contains the wild-type Ad2MLP region (solid heavy line) from -677 to +33 from pSVA677 (1) inserted between the EcoRI and BamHI sites of pBR322 (solid light line). pM97 and pM34 contain deletions (blank space) from positions -97 to -363 and positions -34 to -372 of the Ad2MLP sequence, respectively. pM6 (see Fig. 2A) contains the SV40 sequence (coordinates in the BBM system, ref. 50) from position 38 to 105 (hatched line) inserted into the Smal site between position -124 to -52 of pM4 (see Fig. 2A). The pM series of recombinants having two Ad2MLP regions contain either the pSVA97 or pSVA34 (1) Ad2MLP region (solid heavy line), including pBR322 EcoRI-BamHI flanking sequences (solid light line), inserted into the PvuII site of pM97, pM34 and pM6. Lost restriction enzyme sites are indicated in parentheses. DNA templates from the series of pM plasmids containing a single Ad2MLP region were prepared for in vitro transcription analysis either by linearizing plasmids at the SalI site (309 nucleotide run-off transcript) or by purifying EcoRI-SalI and EcoRI-Sphi fragments (309 and 218 nucleotide run-off transcripts, respectively). DNA templates from the series of pM plasmids containing two Ad2MLP regions were prepared for in vitro transcription analysis by digestion with AccI, resulting in 212 and 309 nucleotide run-off transcripts from the insertions of the Ad2MLP into the PvuII and EcoRI-BamHI sites, respectively.

A reaction mixture and 200 ng of the specific DNA template was added with the nucleotides to start RNA synthesis. Reactions were stopped after 45 min at 24°C, and the RNA run-off transcripts were purified and analyzed on 5% acrylamide-8.3 M urea gels as described in (10).
RESULTS

Requirement for upstream sequences of the Adenovirus-2 major late promoter for efficient transcription in vitro.

The wild-type Ad2MLP region from -677 to +33 and a series of Ad2MLP mutants were cloned into pBR322 between the EcoRI and BamHI sites (Materials and Methods and Figs. 1 and 2A). Transcription of these recombinants, linearized at the Sall site, was analyzed in vitro by run-off transcription using a WCE prepared from HeLa cells (Fig. 2B-D). As previously observed for point mutants in the conalbumin TATA box element (20, 40), the Ad2MLP TATA box mutant pM677-X (Fig. 2A) is transcribed at an almost undetectable level in vitro (Fig. 2B, lane 3). In agreement with our previous report (1), the Ad2MLP deletion mutant pM97, which lacks sequences upstream of position -97, is transcribed in vitro with the same efficiency as the wild-type plasmid pM677 (Fig. 2C, compare lanes 5 and 7); in contrast, pM34, which lacks sequences upstream of position -34, is transcribed less efficiently than pM97 (Fig. 2C, compare lanes 5 and 6).

To ensure that the upstream sequences which are required for efficient transcription in vitro are the same as those responsible for the stimulation of transcription in vivo, additional upstream sequence mutants were transcribed in vitro. The results of the in vivo transcription of those mutants will be published elsewhere (38). The double point mutant pM1, which contains two base changes at positions -60 and -62 (Fig. 2A) and is poorly transcribed in vivo [20% relative to pM97 (38)], is transcribed at approximately the same level as pM34 in vitro [Fig. 2C, compare lanes 6 and 8; the in vivo transcription of pM34, relative to pM97, is 3-5% (1)]. The double point mutant pM2, which contains two base changes at positions -54 and -125 (Fig. 2A), is transcribed at a slightly reduced rate when compared to pM97 both in vivo (70-80%, ref. 38) and in vitro (Fig. 2C, compare lane 5 with lane 9). Conversely, pM3 and pM4, in which the sequence from position -52 to -124 has been inverted or deleted, respectively (Fig. 2A), are transcribed at almost the same level as pM34 both in vivo [5-10% and 5% relative to pM97, for pM3 and pM4, respectively (38)] and in vitro (Fig. 2C, compare lane 6 with lanes 10 and 11). Thus, we have qualitatively, but not quantitatively, reproduced the in vivo effect (1, 38) of the various Ad2MLP mutants in vitro. The inability to faithfully reproduce in vitro the Ad2MLP upstream sequence requirement observed in vivo is likely due to the more prominent role of the TATA box sequence, as compared to the upstream sequence, in the efficiency of in vitro trans-
Fig. 2. Sequence and in vitro transcription efficiency in a whole cell extract of the pM series of Ad2MLP-containing recombinants. (A) The nucleotide sequence of the non-coding strand of the Ad2MLP between -130 and +1 is shown (5) for the wild-type Ad2MLP recombinant pM677 and mutants thereof, described in Materials and Methods and in Fig. 1. Point mutations are indicated by the underlining of the altered base. In the case of pM97 and pM34, the dotted lines refer to sequences of the Ad2MLP upstream of positions -370 and -379, respectively. In the case of pM5 and pM6, the
nucleotide sequence of the non-coding and coding (with respect to the SV40 early cap sites) strand of the SV40 21bp repeat upstream element from position 38 to 105 is shown, respectively (50). (B, C, D) In vitro transcription efficiency of the wild-type Ad2MLP recombinant pM97/ and mutants thereof was determined by run-off transcription in a WCE as described in Materials and Methods (see also legend to Fig. 1 and text). The specific DNA templates (200 ng plasmid, linearized at the SalI site), indicated at the top of each lane, were added in the preincubation step. Only the relevant portion of the gel autoradiogram is shown (309 nucleotide run-off, see legend to Fig. 1). The amount of α-amanitin added to inhibit RNA polymerase B transcription was 0.5 μg/ml (lane 2). Lane M: size markers, [32P] 5' end-labelled MspI fragments of pBR322.

cription. Recently, Jove and Manley (25) reported that the sequences of the Ad2MLP between -51 and -66 are required for efficient in vitro transcription. Taking these results together with our previous in vivo and in vitro data (1, 38), and those presented here (Fig. 2C), we conclude that sequences located between -52 and -66, and centered around position -60/-62, are required for efficient transcription from the Ad2MLP both in vivo and in vitro.

Upstream sequence requirement using recombinants containing two Adenovirus-2 major late promoter regions.

The results obtained from in vitro transcription of different DNA templates in separate reaction mixtures are often subject to a great deal of variability. For instance, it is difficult to ensure that in any particular experiment the amount and/or the physical state (extent of deproteinization and double helical integrity) of the various DNA templates are equivalent. Furthermore, the recovery of the in vitro synthesized RNA transcripts may not be the same from each reaction mixture. In fact, we have encountered some variability in our results. In the experiment shown in Fig. 2C, the amount of transcription from pM97 is approximately 3-4 fold greater than from pM34, whereas in Fig. 3A, however, the amount of transcription from pM97 is 8-10 fold and 6-8 fold greater than pM34 using as template either the purified EcoRI-SalI (309 nucleotide run-off transcript, compare lane 2 with lane 3) or the EcoRI-SphI (218 nucleotide run-off transcript, compare lane 4 with lane 5) DNA fragments, respectively. In order to avoid these variabilities, we have constructed recombinant plasmids having two Ad2MLP regions, both of which contain the same TATA box element and initiation site, but differ in their upstream sequence (Materials and Methods and Fig. 1), to examine further the requirement of upstream sequences for efficient in vitro transcription.
Fig. 3. Comparison of in vitro transcription efficiencies of the "97" and "34" templates in a WCE using either single (A) or double promoter-containing plasmids (B). (A) pM97 and pM34 were transcribed in a WCE as described in Materials and Methods. 40 ng of either EcoRI-SalI (designated Sal) or EcoRI-SphI (designated Sph) fragments prepared from pM97 or pM34, as indicated, plus 160 ng of pBR322, linearized at the BamHI site, were added in the preincubation step. (B) pM34.97 (lanes 6-8), pM97.34 (lanes 9-11), pM97.97 (lanes 12-14) and pM34.34 (lanes 15-17) were transcribed in a WCE as described in Materials and Methods. Varying amounts (as indicated in ng at the top of each lane) of AccI-digested DNA (designated Acc) were added per reaction mixture during the preincubation step. 309, 218 and 212 are the expected sizes of run-off transcripts (see legend to Fig. 1). M: size markers as in Fig. 2B.

The DNA templates were prepared for in vitro transcription by digestion with AccI; the expected sizes of the run-off transcripts initiated at the Ad2MLP inserted between the EcoRI and BamHI sites and into the PvuII site are 309 and 212 nucleotides, respectively (Fig. 1). Based on the expected number of cytosine residues incorporated into the transcripts, the theoretical ratio of the intensity of the 309 and 212 nucleotide run-off bands originating from identical promoters such as those present in either pM97.97 or pM34.34 is 1.64. In fact, the observed ratio is greater than 1.64 for both pM97.97 (Fig. 3B, lanes 12-14) and pM34.34 (Fig. 3B, lanes 15-17). As quantitated by densitometry of autoradiograms from at least 8 different experiments, using 200 ng of either pM97.97 or pM34.34 as DNA template, the amount of RNA transcript initiating from the promoter inserted between the EcoRI and BamHI sites is 2-2.5 fold greater than that
from the promoter inserted into the PvuII site. Therefore, when in vitro transcription efficiencies are compared using plasmids containing the Ad2MLP region from both pM97 (here called the "97" template) and pM34 (here called the "34" template), the amount of transcription from the promoter inserted into the PvuII site should be multiplied by 2-2.5.

As shown in Fig. 3B, the "97" template is clearly more efficiently transcribed than the "34" template, using as DNA either pM34.97 (lanes 6-8, the 309 and 212 nucleotide run-off transcripts are from the "34" and "97" templates, respectively) or pM97.34 (lanes 9-11, the 309 and 212 nucleotide run-off transcripts are from the "97" and "34" templates, respectively; see also Fig. 7, lanes 1 and 4). Taking into consideration the above difference in transcription efficiency observed between the two Ad2MLP insertions, densitometry of autoradiograms of several experiments similar to that shown in Fig. 3B (lanes 6-11) have indicated that at the DNA concentration used for all subsequent experiments (200 ng, see below), the "97" template was consistently 6-8 fold more efficiently transcribed than the "34" template in the double Ad2MLP-containing plasmids. Furthermore, the relative transcription efficiency of the "97" as compared to the "34" template was significantly greater at lower DNA template concentrations (Fig. 3B, compare lane 6 with lane 7 and lane 9 with lane 10), in agreement with the recent report of Jove and Manley (25). Therefore, it appears that using recombinants containing two Ad2MLP regions the in vivo effect of the upstream sequence can be reproducibly mimicked in vitro. Such recombinants have been used in the experiments reported below.

The in vitro effect of the Adenovirus-2 major late promoter upstream element is specifically competed out by preincubation with a DNA fragment which contains only the upstream sequence.

To test the hypothesis that the stimulatory effect of the Ad2MLP upstream sequence requires interaction with a specific factor, we performed a competition assay in which the WCE was preincubated, prior to addition of the DNA template, with a DNA fragment containing the upstream sequence, but no other functional promoter element of the Ad2MLP. Similar competition experiments have been used to show that a transcription factor stably binds to the TATA box region of the conalbumin and Ad2ML promoters (10). As a control, we first analyzed the effect of preincubating the WCE with a DNA fragment from pBR322 which does not appear to contain TATA box-like sequences (41). Preincubation of the WCE with increasing amounts of the pBR322 EcoRI-EcoRV fragment [here called pBR322(RI/RV)] has only a small...
Fig. 4. Competition of in vitro transcription from the "97" and "34" templates. pM34.97 (200 ng, digested with AccI) was transcribed in a WCE as described in Materials and Methods after a 15 min preincubation of the WCE with various amounts of DNA fragments, pBR322(RI/RV), pM677-X(Xho) and SV40(Nco), as indicated. pBR322(RI/RV) is the EcoRI-EcoRV fragment (coordinates 0 to 187) of pBR322 (52); pM677-X(Xho) is the XhoI fragment (coordinates -260 to -31) of pM677-X (see Fig. 2A); and SV40(Nco) is the NcoI fragment (SV40 coordinates 333 to 38), with an SphI deletion (200 to 128), from pHB3 (see ref. 28). "97" and "34" are the 212 nucleotide and the 309 nucleotide run-off transcripts from the "97" and "34" templates, respectively.

As quantitated by densitometry of the autoradiograms, the uncorrected ratio of "97" to "34" template transcription (here called "97/34" ratio) varied from 2.2 to 2.6 in several experiments similar to that shown in Fig. 4. However, amounts of pBR322(RI/RV) fragment greater than 80 ng significantly decrease transcription from both the "97" and "34" templates (Fig. 4, lanes 3 and 4). To maximize the sensitivity of the competition assay, a constant amount of pBR322(RI/RV) fragment (40 or 80 ng) was introduced into all subsequent preincubation mixtures. In contrast to the effect observed with the pBR322(RI/RV) fragment alone, preincubation of the WCE with 40 or 80 ng of a DNA fragment containing the upstream sequence of the Ad2MLP between positions -260 and -31 (Fig. 2A) [here called pM677-X(Xho)] inhibited run-off transcription from the "97" to a far greater extent than from the "34" template (Fig. 4, compare lanes 5 and 6 with lanes 3 and 4, respectively). In this particular experiment, 80 ng of the pM677-X(Xho) fragment, which corresponds to a 9 fold molar excess of upstream sequence competitor, appears to completely prevent the effect of the upstream sequence present in the "97" template (compare the "34" to "97" ratio in Fig. 4, lane 6 with...
Fig. 5. Competition of in vitro transcription from the "97" and "34" templates with Ad2MLP (A) and heterologous promoter (B) upstream element DNA fragments. pM34.97 (200 ng, digested with Accl) was transcribed in a WCE as described in Materials and Methods after a 15 min preincubation of the WCE with 40 ng of pBR322(RI/RV) plus 80 ng of the competitor "upstream" DNA fragments, as indicated. (A): pBR322(RI/RV) and pM677-X(Xho) are as in Fig. 4; pM1-X(Xho), pM2-X(Xho), pM3-X(Xho), and pM4-X(Xho) are the XhoI fragments from position -260 to -31 of the Ad2MLP from pM1-X, pM2-X, pM3-X and pM4-X, respectively (Fig. 2A). (B): pBR322(RI/RV), pM677-X(Xho) and SV40(Nco) are as in Fig. 4. SV40 "21bp" is the EcoRV-SaiI fragment from BR21 (31), which contains the 21bp repeat region of SV40 from position 101 to 122 [BamHI and Sall sites at positions 101 and 32, respectively, were created by oligonucleotide-directed mutagenesis (39)] inserted between the BamHI and Sall sites of pBR322; SV40 "72bp" is the BamHI-PvuII fragment from DB14 (31), which contains the 72bp enhancer region of SV40 from position 113 to 270 (BamHI site at position 113 and PvuII site at 270) inserted between the BamHI and PvuII sites of pBR322; CON(-104/-44) is the EcoRI-EcoRV fragment from Con60C, which contains the repaired AluI-BstNI fragment from position -102 to -44 of the conalbumin promoter (10) inserted into the Clal site of pBR322; CON(-274/-29) is the PstI-EcoRI fragment from ConXR, which contains the PstI-Xbal fragment from position -274 to -29 of the conalbumin promoter TATA mutant (40) inserted between the PstI and EcoRI sites of pBR322; E3(-236/-37) is the EcoRI-BamHI fragment of E3XB which contains the Adenovirus-5 EIII promoter EcoRI-Xmal fragment from position -236 to -37 (53) inserted between the EcoRI and BamHI sites of pBR322; E2(-250/-64) is the SmaI-Xbal fragment from position -250 to -64 of LN-64 (54), which contains an Xbal linker insertion at position -64 of the Adenovirus-2 El1a promoter; and TK(-200/-40) is the Clal-HindIII fragment of pTK1, which contains the Herpesvirus thymidine kinase promoter (55) repaired PvuII-BstNI fragment from position -200 to -40 inserted into the HindIII site of pBR322. M: size markers as in Fig. 2B. "97" and "34" as in Fig. 4. The numbers below each lane (R:"97/34") refer to the average uncorrected ratio of "97/34" template run-off transcript formed, as determined by quantitation of autoradiogram scans of at least three separate competition prebinding experiments (see text).
the "34" to "34" ratio in Fig. 3B, lanes 15-16).

To test whether the above competitive effect involves the sequences which are required for stimulation of transcription by the upstream element, we next studied the ability of DNA fragments prepared from Ad2MLP mutants (see above) to compete out transcription from the "97" template (Fig. 5A). As previously observed (Fig. 4), preincubation of the WCE with the wild-type Ad2MLP upstream sequence DNA fragment, pM677-X(Xho), considerably reduces the transcription of the "97" template relative to the "34" template (Fig. 5A, compare lane 2 with lane 3). As expected, the DNA fragment prepared from the mutant pM4 [here called pM4-X(Xho)], which contains a deletion of the upstream sequence required for efficient in vivo and in vitro transcription (see above), was not effective in the competition assay (Fig. 5A, lane 7). The result obtained with the DNA fragment [here called pM1-X(Xho)] prepared from the mutant pM1 is particularly interesting. Transcription from this mutant which has only two base changes, at -60 and -62 (Fig. 2A), is severely impaired both in vivo and in vitro (see above); as shown in Fig. 5A, lane 4, the pM1-X(Xho) fragment does not compete out transcription from the "97" template. Since the pM1-X(Xho) and pM677-X(Xho) fragments have identical DNA sequences except for the two point mutations, we can eliminate the possibility that the competitive ability of the latter was due to some structural feature of the fragment itself, such as the XhoI ends. We conclude from the competition assays described above that there exists a factor(s) in the WCE which specifically interacts with sequences in the upstream element of the Ad2MLP.

Whereas a good correlation exists between the lack of competitiveness of the pM1-X(Xho) and pM4-X(Xho) fragments (Fig. 5A) and the low in vitro transcription efficiency of pM1 and pM4 (Fig. 2C), the results obtained from competition experiments with DNA fragments prepared from pM2 and pM3 (here called pM2-X(Xho) and pM3-X(Xho), respectively) are apparently inconsistent with their transcription efficiencies (Fig. 5A, lanes 5 and 6). The pM2-X(Xho) fragment does not appear to compete efficiently, whereas pM2 transcription efficiency is only slightly reduced both in vivo and in vitro (see above). This apparent discrepancy may reflect a situation in which the decreased binding of the upstream factor to its specific recognition sequence would be sufficient to prevent efficient competition, but not to strongly affect the efficiency of transcription. On the other hand, the pM3-X(Xho) fragment may compete to some extent, although the in vivo and in vitro transcription efficiency of pM3 is drastically reduced (see above).
In this case, it is possible that inversion of the upstream sequence does not fully prevent the binding of the upstream factor, but that this binding is totally unproductive for stimulation of in vivo and in vitro transcription. Studies directly analyzing the binding capacity of the upstream factor are in progress to test the validity of these hypotheses.

We next asked whether the factor(s) which recognizes the upstream element of the Ad2MLP also interacts with other upstream or enhancer promoter elements. We first tested the SV40 early promoter 21bp repeat upstream element and the 72bp repeat enhancer element, which have been well-characterized in vivo and in vitro, and have been shown to bind specific trans-acting factors present in both a WCE and a nuclear extract (31, 33, 35). Preincubation of the WCE with an SV40 fragment containing both the 21bp upstream repeat and the 72bp enhancer elements [here called SV40 (Nco), see legend to Fig. 4] did not inhibit transcription from the "97" template (compare lanes 7 and 8 with lanes 3 and 4 in Fig. 4, and lane 8 with lane 10 in Fig. 5B); in contrast, under identical conditions, the Ad2MLP upstream sequence fragment pM677-X(Xho) was effective in the competition assay (Fig. 4, lanes 5 and 6 and Fig. 5B, lane 9). Similarly, neither the isolated SV40 21bp repeat upstream element (here called SV40 "21bp") nor the isolated SV40 72bp repeat enhancer element (here called SV40 "72bp") inhibited transcription from the "97" template (Fig. 5B, lanes 11 and 12). The amounts of competitor fragments used in these and all subsequent experiments (80 ng), corresponds to an 8-12 fold molar excess of upstream sequence DNA. We then tested (Fig. 5B) promoter upstream elements which, although they have not been shown to bind transcription factors, are required for efficient in vivo and/or in vitro transcription: (i) conalbumin [CON(-102/-44) or CON(-274/-29)], whose promoter upstream sequence is required for efficient in vitro transcription in a HeLa WCE (a 2-3 fold stimulating effect is seen with sequences between -44 and -102; B. Wasylyk, personal communication); (ii) Adenovirus-2 EIIa [E2(-250/-64)], whose promoter upstream sequence is required for efficient expression in vivo and in vitro (42) and displays an homology to a consensus upstream sequence (43) and to the upstream sequences required for efficient in vivo transcription from both the Ad2ML (1) and rabbit β-globin (17, 44) promoters; (iii) Adenovirus-5 EIII [E3(-236/-37)], whose promoter upstream sequence is required for efficient in vivo and in vitro transcription (45); and (iv) Herpes virus thymidine kinase [TK(-200/-40)], whose promoter upstream sequence is required for efficient in vivo transcription (46) and
Fig. 6. Stability of interaction between the Ad2MLP upstream promoter element and its specific factor.

The strategy for the experiment is shown at the top of the Figure. The template (pM34.97; 200 ng, digested with AccI) or competitor (120 ng of pBR322(RI/RV) or 40 ng of pBR322(RI/RV) plus 80 ng of pM677-X(Xho)) was first preincubated for 15 min at 24°C with the WCE, as indicated. A second preincubation for the indicated lengths of time, with either the competitor or template (as above) was performed to complete the preincubation period. pBR322 (RI/RV) and pM677-X(Xho) are as in Fig. 4. RNA synthesis was started by the addition of nucleotides. The uncorrected "97/34" ratios were determined as in Fig. 5, from the data of 2 separate experiments.

displays a strong sequence homology to the SV40 21bp repeat upstream element, yet does not appear to bind the SV40 upstream-specific factor Spl (18). A comparison of the uncorrected "97/34" ratios for these different upstream competitor fragments indicates that none of these upstream sequences appear to interact with the factor(s) that interacts with the Ad2MLP upstream sequence (Fig. 5B, compare lane 9 with lanes 13-17). The interaction between the Adenovirus-2 major late promoter upstream element and its factor is stable.

The strategy used to test the stability of the interaction between the Ad2MLP upstream element and its specific factor is outlined in the top panel of Fig. 6. The decrease of run-off transcription from the "97" template (as determined by a lowered uncorrected "97/34" ratio), which results from a preincubation of the pM677-X(Xho) fragment with the WCE, appears to be stable for at least 60 min when subsequently challenged with the specific pM34.97 DNA template (Fig. 6, lower panel, open squares). The
interaction of the Ad2MLP upstream factor with the specific DNA template is similarly stable when challenged for up to 60 min with the specific competitor fragment (Fig. 6, lower panel, closed squares); under similar conditions, the presence of the pBR322(RI/RV) fragment has no effect (Fig. 6, lower panel, triangles). Since the specific competitor fragment pM677-X(Xho) does not contain the Ad2MLP TATA box, we infer that the recognition and stable binding of the Ad2MLP upstream factor to its specific DNA sequence is independent of the presence of the TATA box element.

Stimulation of in vitro transcription controlled by the Ad2MLP TATA box element by a heterologous upstream element is not competed out by the Ad2MLP upstream sequence.

It was of interest to determine whether stimulation of transcription by an upstream element requires its homologous TATA box element, or whether it could also stimulate transcription when placed upstream from a heterologous TATA box element, as has been shown for the SV40 72bp repeat enhancer element (32, 33). To answer this question, we constructed chimeric SV40-Ad2ML promoter recombinants containing the SV40 21bp repeat upstream element inserted upstream from the Ad2MLP TATA box (see Fig. 1 and Materials and Methods for details of the constructions). The plasmid pM5, which has the SV40 21bp repeat region inserted in its natural orientation (with respect to the SV40 early capsites), is transcribed in a WCE at approximately the same level as pM677 (Fig. 2D, compare lanes 12 and 13). Interestingly, pM6, which differs from pM5 by having the 21bp repeat region inserted in the reverse orientation, is transcribed even more efficiently than pM677 (Fig. 2D, compare lanes 12 and 14). Thus, the SV40 21bp repeat upstream element can functionally replace the Ad2MLP upstream element in vitro. The ability of the SV40 early promoter upstream element to stimulate in vitro transcription bidirectionally has also been observed with the homologous SV40 early promoter (28).

We next asked which factor is required for the stimulation of transcription brought about by the SV40 21bp repeat region in these chimeric promoter recombinants. We constructed double promoter-containing plasmids with the pM6 promoter region (here called the SVAd2 template) inserted between the EcoRI and BamH1 sites, and either the "97" (pM6.97) or "34" (pM6.34) template promoter region inserted into the PvuII site of pBR322 (Fig. 1). The DNA fragment containing the SV40 21bp repeat upstream element sequence from SV40 position 101 to 32 (SV40 "21bp"), which is known to bind the SV40-specific factor Spl (35) was tested for its ability to inhibit
Fig. 7. Competition of in vitro transcription from the Ad2ML and SV40-Ad2ML promoters by the SV40 and Ad2ML promoter upstream elements. pHJ4.97 (lanes 1-3), pM97.34 (lanes 4-6), pM6.97 (lanes 7-9) and pM6.34 (lanes 10-12) (200 ng each, digested with AccI, see legend to Fig. 1) were transcribed in a WCE as described in Materials and Methods after a 15 min preincubation of the WCE with 120 ng of pBR322(RI/RV) (lanes 1, 4, 7, 10) or 40 ng of pBR322(RI/RV) plus 80 ng of either pM677-X(Xho) or SV40 "21bp" (see legend to Fig. 5), as indicated. M : size markers as in Fig. 2B. 309 and 212 : run-off transcripts as described in legend to Fig. 1.

transcription from the SVAd2 template. As a first control, we repeated the competitions using the pM677-X(Xho) competitor fragment and either the pM34.97 or pM97.34 DNA templates. The results shown in Fig. 7 demonstrate that the ability of the pM677-X(Xho) fragment to compete out transcription from the 97 template is clearly independent of the construction chosen to study the competition [compare lane 1 with 2 (212 nucleotide run-off) and 4 with 5 (309 nucleotide run-off)]. As previously shown (Fig. 5B, lane 11), the SV40 "21bp" fragment does not compete out run-off transcription from the "97" template [Fig. 7, compare lane 1 with 3 (212 nucleotide run-off) and 4 with 6 (309 nucleotide run-off)]. The SVAd2 template (309 nucleotide run-off in lanes 7-12) is also transcribed in vitro from these double promoter-containing recombinants more efficiently than either the "97" or "34" templates [Fig. 7, lanes 7 and 10, respectively (212 nucleotide run-off)]. As expected, the pM677-X(Xho) fragment, but not the SV40 "21bp" fragment, inhibits transcription from the "97" template [Fig. 7, lanes 8 and 9 (212 nucleotide run-off)]. In contrast, the SV40 "21bp" fragment, but not the pM677-X(Xho) fragment, inhibits transcription from the SVAd2 template [Fig. 7, lanes 8 and 9 (309 nucleotide run-off)]. We have also obser-
ved that the SV40 "21bp" fragment, but not the pM677-X(Xho) fragment, inhibits in vitro run-off transcription from the major capsites of the SV40 early promoter (results not shown and see ref. 31). Under the assay conditions used here, we also show that the "97" and SVAd2 promoter regions are apparently in competition with each other for some limiting general transcription factor(s), since inhibiting by competition one promoter region results in increased transcription from the other promoter region (Fig. 7, compare lanes 8 and 9 with lane 7). It is, however, remarkable that transcription of the "34" promoter region was barely detectable using the pM6.34 template (Fig. 7, lanes 10-12). The nature of the limiting factor is unknown at present, as well as why the SVAd2 promoter region is much more efficient than the "97" promoter region at competing for such a factor.

From all the above observations, we conclude that the binding of factor Spl is required for efficient in vitro transcription from a chimeric promoter containing the SV40 21bp repeat upstream element and the Ad2MLP TATA box element. Furthermore, in support of previous results (11), we find that factor Spl is not involved in the stimulation of in vitro transcription from the Ad2MLP.

DISCUSSION

We have previously shown that upstream sequences between positions -34 and -97 are necessary for efficient transcription from the Ad2MLP both in vivo and in vitro (1). Our results have been recently confirmed by Jove and Manley (25), who have reported that sequences between -51 and -66 are necessary for efficient in vitro transcription with a HeLa WCE. We have analyzed further the in vitro stimulatory effect of sequences upstream from the Ad2MLP TATA box. We show here that the double point mutant pM1, which contains base changes at positions -60 and -62, is transcribed in vitro approximately 3-4 fold less efficiently than the wild-type promoter (Fig. 2C), in agreement with the in vivo effect of the mutation (38). To avoid the problem of variability inherent to in vitro transcription analyses (see above), we constructed recombinants containing two promoter regions, one containing ("97" template) and the other lacking ("34" template) the Ad2MLP upstream sequence (Fig. 1); the in vitro effect of the Ad2MLP upstream element was readily apparent using these double promoter-containing plasmids (Fig. 3B).

A competition assay was utilized to provide three lines of evidence
demonstrating that the upstream sequence element of the Ad2MLP forms a stable complex with a specific trans-acting factor(s), which is involved in the stimulation of \textit{in vitro} transcription. (a) Transcription from the "97" template, but not the "34" template, was competed out by preincubation of a HeLa WCE with a DNA fragment containing the upstream sequence element (Fig. 4). Furthermore, the interaction between the upstream sequence and its specific factor was stable (Fig. 6). (b) DNA fragments containing the mutated Ad2MLP upstream elements, which are transcribed less efficiently than the wild-type promoter \textit{in vivo} (38) and \textit{in vitro} (Fig. 2C), did not effectively compete out transcription from the "97" template (Fig. 5A). (c) DNA fragments containing upstream sequence elements prepared from heterologous promoters, which have been shown to be required for efficient transcription \textit{in vivo} and/or \textit{in vitro}, did not compete out transcription from the "97" template (Fig. 5B).

The inability of both the SV40 21bp and 72bp repeat sequences to compete out transcription from the Ad2MLP (Figs. 4 and 5B) is in agreement with the findings of Dynan and Tjian (11) that the factor Spl which binds to the 21bp repeat upstream element of the SV40 early promoter does not stimulate \textit{in vitro} transcription from the Ad2MLP, and with recent results (31, 33) which show that the Ad2MLP upstream sequence does not compete out transcription from promoters containing either the SV40 upstream or enhancer elements. The fact that the Ad2EIIaE upstream sequence did not compete for transcription from the Ad2MLP is noteworthy, since a sequence homology exists between the upstream sequences of the Ad2EIIaE, β-globin and Ad2ML promoters in the upstream regions which have been shown to be required for efficient \textit{in vivo} and/or \textit{in vitro} transcription (1, 17, 18, 38, 42, 44, this paper) and correspond to the upstream consensus sequence noticed previously (43). Recent studies have also shown that the SV40 upstream element-specific factor Spl does not stimulate transcription from the thymidine kinase promoter, although a strong upstream sequence homology exists (18). Thus, it is apparent that the specificity of interaction between factors and upstream elements requires more than the presence of a consensus sequence.

We have also shown that the SV40 21bp repeat upstream element inserted upstream from the Ad2MLP TATA box, in its natural or reverse orientation, can functionally replace the Ad2MLP upstream element (Fig. 2D); in these recombinants, factor Spl, but not the Ad2MLP upstream factor, is involved in the stimulation of transcription (Fig. 7). Taken together
with the fact that the Ad2MLP DNA fragments used in competition experiments do not contain the Ad2MLP TATA box sequence, we conclude that the stable binding of an upstream factor to its specific recognition sequence does not require the presence of the TATA box element.

The ability of the SV40 21bp repeat upstream element to functionally replace the upstream element of the Ad2MLP is reminiscent of the ability of the SV40 72bp repeat enhancer to stimulate in vitro transcription from heterologous promoters, in particular the conalbumin and Ad2ML (32, 33) promoters. Since competition studies suggest that the same general "TATA box" factor recognizes both the conalbumin and Ad2ML promoter TATA box region (10), we may suppose that the different factors recognizing the SV40 21bp repeat upstream element, the SV40 72bp repeat enhancer and the Ad2MLP upstream element operate through similar mechanisms in order to stimulate initiation of transcription in vitro, possibly by facilitating the binding of the TATA box factor. Furthermore, these factors appear to form, independently, stable complexes in vitro and do not require the homologous TATA box element to stimulate transcription. In contrast with procaryotic promoters (for reviews and refs. see 47-49), promoter elements for eucaryotic genes coding for proteins appear to be interchangeable and flexible in their relative positions; that is, efficient promoter regions can be assembled from heterologous elements and a fixed arrangement of these elements is not absolutely required. How this is achieved remains a challenge for future in vitro studies.

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REFERENCES

38. Hen, R., Wintzerith, M., Miyamoto, N.G. and Chambon, P. (manuscript in preparation).