MOLECULAR AND CELLULAR BIOLOGY, Apr. 1988, p. 1765–1774
0270-7306/88/041765-10$02.00/0

Vol. 8, No. 4

Factors Responsible for the Higher Transcriptional Activity of Extracts of Adenovirus-Infected Cells Fractionate with the TATA Box Transcription Factor

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Received 23 July 1987/Accepted 19 January 1988

Extracts of adenovirus-infected HeLa cells have 5- to 10-fold-higher activity for transcription from the major late promoter in vitro than do extracts of mock-infected or E1A mutant-infected cells (K. Leong and A. J. Berk, Proc. Natl. Acad. Sci. USA 83:5844–5848, 1986). In this study, we analyzed extracts from mock-infected cells and from cells infected with an E1A mutant, pm975, which expresses principally the large E1A protein responsible for the stimulation of transcription. These extracts were fractionated by phosphocellulose chromatography, a procedure which separates factors required for transcription from this promoter (J. D. Dignam, B. S. Shastry, and R. G. Roeder, Methods Enzymol. 101:582–589, 1983), allowing the quantitative assay of individual factors (M. Samuels, A. Fire, and P. A. Sharp, J. Biol. Chem. 257:14419–14427, 1982). Fractions eluted with 0.04, 0.35, and 0.6 M KCl, which contained RNA polymerase II, the upstream factor MLTF, and three general polymerase II transcription factors, had similar activities when prepared from virus-infected or from mock-infected cells. The sequence-specific DNA-binding activity of MLTF was also similar in the virus-infected- and mock-infected-cell extracts. In contrast, the 1.0 M KCl fraction prepared from virus-infected cells consistently exhibited activity severalfold higher than that of the equivalent fraction prepared in parallel from mock-infected cells. E1A protein eluted principally (>80%) in the 0.35 M KCl fraction. Results of others (M. Sawadogo and R. G. Roeder, Cell 43:165–175, 1985) have shown that the 1.0 M KCl fraction, containing 2 to 5% of the unfractionated protein extract, contains a factor which binds specifically to the major late promoter TATA box. These results, together with a recent genetic analysis of the E1B promoter which demonstrated that the TATA box was required for its efficient transcriptional activation (transactivation) by E1A (L. Wu, D. S. E. Rosser, M. Schmidt, and A. J. Berk, Nature (London) 326:512–515, 1987), are consistent with the model that E1A protein indirectly activates the TATA box transcription factor. Consistent with this model was the finding that mutants of the major late promoter containing only the TATA box and cap site region were transcribed at higher rates with extracts from virus-infected cells than with extracts from mock-infected cells. Other models consistent with the results are also discussed.

Adenovirus early region 1A (E1A) encodes products which stimulate transcription from all early viral promoters (5, 33), as well as the major late promoter (MLP) early in infection (50). E1A proteins are also required for the oncogenic transformation of rodent cells (4). Early in infection, two mRNAs of 12S and 13S are expressed from E1A. These mRNAs differ only in the size of the single intron removed by RNA splicing (51) and encode proteins of 289 and 243 amino acids, respectively. The 289-amino-acid E1A protein has much more transcription-stimulating activity than does the 243-amino-acid protein or the protein product from a 9S E1A mRNA expressed during the late phase of infection (46–48, 63). Microinjection of E1A protein synthesized in and isolated from *Escherichia coli* also stimulates transcription from adenovirus promoters (16), demonstrating that E1A protein, and not RNA, performs the transcriptional activation (transactivation) function. Transactivation by E1A protein is not limited to adenovirus genes. Transcription from a number of cellular genes, as well as herpesvirus genes, is stimulated when plasmid clones of the genes are cotransfected into HeLa cells with plasmids expressing E1A (15, 20, 24, 28, 34, 59–61). Furthermore, the E1A protein also stimulates transcription of genes transcribed by RNA polymerase III (2, 19, 30). Extracts from adenovirus-infected HeLa cells have severalfold-higher activity for transcription of these class III genes than do extracts of mock- or E1A mutant-infected cells (30, 67). This difference in activity does not seem to be due to the action of E1A protein in vitro, since E1A protein can be depleted from the extracts by immunoprecipitation without reducing the high activity of infected- or transformed-cell extracts (67). Rather, the higher activity of the infected-cell extracts appears to be due to the increased activity of the general polymerase III transcription factor TFIIIC (30, 67).

In general, systematic mutagenesis of early adenovirus promoters has not revealed promoter sequences required only for E1A transactivation (25, 28, 32, 35, 39, 41, 49, 64, 68). Promoter sequences required for the maximal level of E1A-transactivated transcription are also required for the maximal basal level of transcription in the absence of E1A protein. For the major late (8, 9, 11–13, 29, 41, 43, 45, 54, 55), E2 early (6, 32, 35, 49, 56, 65, 68), E4 (21, 22, 25, 38), and E1B promoters (64), the sequences required for maximal E1A-transactivated transcription appear to be binding sites for transcription factors found in the uninfected cell. Hence, it has been postulated that the E1A protein exerts its action on these promoters indirectly by increasing the activities of cellular transcription factors. Kovesdi et al. (36) have reported that the sequence-specific DNA-binding activity of a cellular factor, E2F, which interacts with the E2 early promoter, increases greatly after adenovirus infection and expression of E1A protein. While E2F appears to interact

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with the E2 early and E1A promoter regions, it does not bind to other adenovirus promoters (37) and therefore is probably not responsible for the transactivation of these other promoters by E1A.

In this study, we have further analyzed the mechanism of E1A transactivation by assaying the in vitro activities of transcription factors which interact with the MLP in extracts of adenovirus-infected cells. Extracts of adenovirus-infected HeLa cells have transcriptional activity severalfold higher than that of extracts of mock- or E1A mutant-infected cells for in vitro transcription from the MLP (18, 40). The higher activity was shown to be due to an increase in the number of templates transcribed in the infected-cell extracts (40). Extracts of mock- and adenovirus-infected cells were fractionated by chromatography on phosphocellulose, a procedure which partially separates HeLa cell transcription factors required for in vitro transcription from this promoter (13, 43, 54). By the nomenclature of Roeder and colleagues, four general transcription factors are fractionated such that TFIIA is found in the flowthrough fraction, fraction A. Most of the RNA polymerase II activity is eluted with a buffer containing 0.35 M KCl (fraction B). Factors TFIIB and TFIIE are eluted with a buffer containing 0.6 M KCl (fraction C). Factor TFIID, which interacts directly with the TATA box (55) is eluted with a buffer containing 1.0 M KCl (fraction D). Fraction D contains about 2 to 5% of the protein in the unfractionated extract. In addition to these general transcription factors, a transcription factor recently purified by Chodosh et al. (11), termed MLTF, stimulates transcription from the MLP severalfold in vitro (9, 45, 55) and in vivo (42). MLTF binds directly to a sequence 52 to 63 base pairs upstream from the transcription start site of the MLP and is found in both the A and B phosphocellulose fractions (9, 45, 55).

The transcriptional activities of these individual phosphocellulose fractions prepared from mock-infected and virus-infected HeLa cells were compared in in vitro transcription reactions reconstituted with phosphocellulose fractions from uninfected cells. We found that fractions A, B, and C had similar activities whether they were prepared from mock-infected or virus-infected cells. However, the D fraction prepared from virus-infected cells had several times the specific activity of the D fraction from mock-infected cells. This result is consistent with the model that E1A expression increases the activity of the TATA box transcription factor. Consistent with this model, we found that the higher transcriptional activity of infected-cell extracts was also observed with mutant templates containing only the TATA box and cap site regions.

MATERIALS AND METHODS

Preparation of cell extracts and phosphocellulose chromatography. Whole-cell extracts from mock-infected or mutant adenovirus pm975- (47) infected HeLa cells were prepared in parallel as described elsewhere (40). Extracts were prepared from 4 to 5 liters of mock- or virus-infected suspension cell cultures (see Table 1). The mock-infected- and virus-infected-cell extract protein (200 mg each) was loaded onto phosphocellulose (Whatman PC II) columns (30 ml of bed volume) and fractionated in parallel as described previously (54), except that 200 μg of bovine serum albumin per ml was added to the 1.0 M KCl buffer used to elute the D fraction. About 75% of the total protein (the center of the protein profile peak) in the flowthrough and in each of the step elutions were pooled. The pooled flowthrough fractions (fraction A) were quick frozen in small portions on dry ice. The pooled 0.35, 0.6, and 1.0 M KCl step elutions (fractions B, C, and D, respectively) were dialyzed two times for 1 h against 2 liters of buffer A plus 0.1 M KCl (54) and then divided into portions and quick frozen on dry ice. These fractions were stored at −70°C and thawed immediately before use. Samples were assayed for protein concentration (Table 1). Fractions stored for 6 months retained full activity and suffered no significant loss of activity after one cycle of thawing and freezing. Protein concentrations were determined by the method of Bradford (7).

In vitro transcription and analysis of RNA products by reverse S1 assay. Reconstituted transcription assays were performed by using 6 to 10 μg of the phosphocellulose A fraction, 3 to 6 μg of the B fraction, 4.8 to 5.6 μg of the C fraction, and 4.9 to 6.6 μg of the D fraction (including added bovine serum albumin). The 50-μl reaction volumes contained 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid)-NaOH (pH 7.9); 75 mM KCl; 5 mM $MgCl_2$; 2 mM creatine phosphate; 10% glycerol; 0.6 mM dithiothreitol; 0.6 mM EDTA; 250 μM each of ATP, CTP, and UTP; 25 μM GTP (P-L Biochemicals, Inc.); 10 μCi of [α-^{32}P]GTP (Amersham Corp., 3,000 Ci/mmol); and 0.4 μg of closed circular plasmid DNA as template at 30°C for 90 min. For the determination of transcriptional activity (see Fig. 1), the template was pBalIE, a pBR322 clone (54) of the adenovirus type 2 (Ad2) sequence (23) from nucleotides 5360 to 7751, −679 to +1713 with respect to the MLP transcription start site. For clarity, we refer to this template as pMLP −679 to +1713.

After extraction with phenol-chloroform, the RNA products were ethanol precipitated with 20 μg of carrier tRNA and 0.5 μg of single-stranded M13-MLP DNA. M13-MLP DNA contains the R strand of Ad2 from nucleotide −260 to +536 with respect to the MLP cap site at +1. The pellet was resuspended in 8 μl of 20 mM Tris (pH 7.0)-1 mM EDTA (TE). TE (2 μl) containing 2 M NaCl was added to give a final concentration of 0.4 M NaCl. The nucleic acid mixture was then heated to 80°C for 10 min and transferred to a 60°C water bath for 90 min. At the end of this hybridization reaction, the reaction tubes were subjected to a quick spin in

TABLE 1. Protein yields of extracts and phosphocellulose chromatography fractions from pm975- and mock-infected HeLa cells[a]

Extract	Amt of protein (mg)				
	Extracted	In column fractions			
		A	B	C	D
pm975 infected	248	97.5	86.4	5.49	4.27
Mock infected	216	75.6	89.1	6.39	5.46

[a] Whole-cell extracts were prepared in parallel from mock-infected and pm975-infected HeLa cells at 20 h postinfection as described elsewhere (40). Extracts were prepared from 2.2 × 10^9 cells each of both mock- and pm975-infected cells. Extract protein from the mock- and pm975-infected cells (200 mg each) was fractionated. Phosphocellulose columns were loaded and eluted as described in Materials and Methods. The columns were loaded in buffer containing 0.04 M KCl, with the flowthrough designated as fraction A and eluted in steps with 0.35 M KCl (fraction B), 0.6 M KCl (fraction C), and 1.0 M KCl (fraction D).

a microfuge to concentrate the reaction mixture to the bottom of the tubes. Then, 190 μl of a buffer containing 200 to 300 U of nuclease S1 (Bethesda Research Laboratories, Inc.), 31.5 mM sodium acetate (pH 4.6), 52.6 mM NaCl, 1 mM $ZnCl_2$, and 5% glycerol was added to each tube. After incubation at 30°C for 1 h, the S1-resistant RNA-DNA hybrid was ethanol precipitated with 20 μg of carrier tRNA after addition of ammonium acetate to 2 M and ethanol precipitated again from 0.3 M sodium acetate. The RNA-DNA hybrid was then denatured, glyoxylated, and analyzed on a 2% agarose gel (44). The gels were dried and exposed to XAR film (Eastman Kodak Co.) without an intensifying screen. RNA products were quantitated by densitometry, with the densest band normalized to a value of 100. The M13-MLP recombinant was constructed by ligating the Ad2 *Xho*I-*Sma*I fragment containing the MLP (nucleotides −260 to +536) to M13mp10 vector via the *Sma*I and *Sal*I sites.

Other templates containing sequences from the Ad2 MLP region used were pMLP −66 to +192, pMLP −66 to +33, and pMLP −51 to +33 (see Fig. 2, 3, and 7). For each of these, the indicated Ad2 sequence relative to the MLP initiation site was cloned between the *Eco*RI and *Hin*dIII sites of pBR322. RNA was analyzed as described above, by using a clone of the small *Eco*RI-*Sal*I fragment from these plasmids into the same sites of M13mp11 to protect specifically initiated RNA. Another template used was pHpstβ (obtained from T. Maniatis; see Fig. 4), the 4.4-kilobase-pair *Pst*I fragment including the promoter region of the human β-globin gene cloned into the *Pst*I site of pBR322. Specifically initiated RNA was protected from S1 digestion with a clone of the transcribed strand of the *Bam*HI fragment extending from nucleotides −1490 to +477 with respect to the start site of β-globin transcription into the *Bam*HI site of M13mp11.

Gel DNA-binding assay. The DNA binding reaction was performed at 30°C for 60 min in a 20-μl reaction mixture containing 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris hydrochloride (pH 7.9), 0.5 mM EDTA, 0.6 mM dithiothreitol, 12% glycerol, 0.5 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia, Inc.), 0.3 ng (~10^5 cpm) of a 5′-end-labeled DNA probe, and various amounts of mock- or Ad2-infected HeLa whole cell extracts (see legend to Fig. 5). The probe contained nucleotides −138 to −29 from the cap site at +1, including the MLTF binding site, and was made by 5′ end labeling the *Hin*dIII-*Ava*II fragment from pHB310 (31). DNA-protein complexes were resolved in a native polyacrylamide gel as described elsewhere (9).

RESULTS

Fractionation of factors responsible for the higher activity of infected-cell extracts with the TATA box transcription factor. Extracts (Table 1) were prepared in parallel from mock- and pm975-infected cells 20 h postinfection as previously described (40). Mutant pm975 does not express the 12S E1A mRNA but expresses the 13S mRNA encoding the E1A protein most active in stimulating transcription (47, 63). We observed earlier (40) that the maximal activity of extracts from Ad2-infected HeLa cells occurs at about 20 h after infection. Peak levels of E1A protein are reached between 14 and 20 h postinfection (57). Consequently, for the present experiments, we analyzed extracts prepared at the end of the period of peak expression of the large E1A protein, when one might expect to see the maximal accumulated effect of the action of E1A. These extracts were chromatographed, in parallel, on phosphocellulose columns and step eluted by the procedure of Samuels et al. (54; Table 1). Similar amounts of protein were recovered from whole-cell extracts and from each of the phosphocellulose column fractions of pm975- and mock-infected cells (Table 1). This result indicates that viral infection did not greatly alter the extraction of cell protein or the fractionation procedure.

Fraction A (the fraction which flows through the column in a buffer containing 0.04 M KCl) from uninfected HeLa cells contains TFIIA and MLTF activities (9, 13, 54, 55). The transcriptional activities of the A fractions from mock-infected and pm975-infected cells were assayed by adding 0 to 10 μg of A-fraction protein to transcription reactions containing fractions B, C, and D from the mock-infected cells and by using an Ad2 clone from −679 to +1713 with respect to the MLP initiation site as template (Fig. 1). Under these conditions, transcription largely depended on the addition of A-fraction protein. The very low but detectable level of RNA synthesized when no A-fraction protein was added was probably due to low levels of TFIIA present in the other fractions. However, as increasing amounts of the mock- or virus-infected-cell A fraction were added, the level of RNA synthesized increased in a linear fashion (Fig. 1). This linear dose response during titration of the A fraction indicates that none of the other transcription factors present in the remaining three phosphocellulose fractions was in limited quantity. Most importantly, there was no significant difference between the transcriptional activity of the A fraction prepared from mock-infected cells and that from virus-infected cells. This result indicates that adenovirus infection does not increase the activities of the TFIIA or MLTF factors present in the whole-cell extract.

The transcriptional activities of the remaining phosphocellulose fractions were similarly assayed by reconstituted in vitro transcription reactions in which increasing amounts of protein prepared from mock-infected or virus-infected cells were used to complement a fixed amount of the remaining protein fractions from mock-infected cells (Fig. 1). In the assay of fraction B, the reconstituted transcription system largely depended on the addition of B-fraction protein. There was a low level of RNA synthesis in the absence of B-fraction protein, but as the amount of either the mock- or virus-infected-cell B fraction was increased in the reconstituted system, the level of RNA transcribed increased linearly (Fig. 1). Again, there were no differences in the levels of RNA synthesized in the presence of mock- or virus-infected-cell B fractions at all concentrations of the proteins tested. Hence, the transcriptional activity of the B fraction, containing RNA polymerase II and MLTF (43, 54, 55), is not increased in adenovirus-infected cells. Earlier, it was shown that the total RNA polymerase II activities, assayed by nonspecific initiation of transcription from a synthetic homopolymer template or nicked salmon sperm DNA, in both mock- and adenovirus-infected-cell extracts are similar (40, 62). Our results (Fig. 1) indicate that the activity of any particular form of the polymerase required for specific initiation of transcription is also present at similar concentrations in extracts of mock-infected and pm975-infected cells.

The data shown in Fig. 1 were obtained by using a template which includes the MLTF binding site at −63 to −52, so that the transcriptional activity of MLTF was measured along with the activities of the general transcription factors in the A and B fractions. To be certain that these assays measured the transcriptional activity of MLTF, we tested the activities of the reconstituted transcription reac-

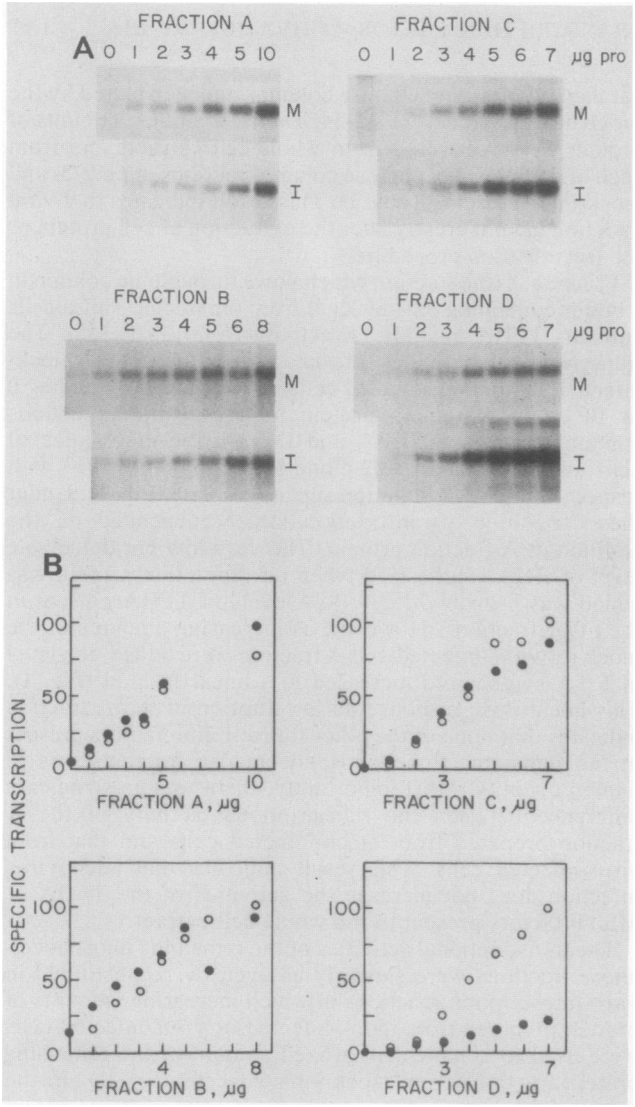

FIG. 1. Transcriptional activities of phosphocellulose fractions prepared from mock-infected and pm975-infected HeLa cells. (A) The individual phosphocellulose fractions prepared in parallel from mock-infected (M) and pm975-infected (I) HeLa cells were assayed by in vitro transcription as described in Materials and Methods using the Ad2 clone from −679 to +1713 with respect to the MLP initiation site as template. Autoradiograms show the regions of the gels showing the 536-nucleotide RNA generated by initiation at +1. (B) The intensity of the 536-nucleotide specifically initiated RNA band was measured by densitometry of direct autoradiograms obtained without intensifying screens. Intensities were normalized to a value of 100 for the darkest band in each titration and plotted versus the micrograms of protein added to the fraction being assayed. Phosphocellulose fractions were from pm975-infected cells (○) and mock-infected cells (●). For A-fraction titration, transcription reactions contained 3 μg of B-fraction protein, 4.8 μg of C-fraction protein, and 6.6 μg of D-fraction protein, all derived from mock-infected-cell extracts, and the indicated amount of the A-fraction protein from the mock- or pm975-infected cells. For the B-fraction assay, transcription reactions were reconstituted with 6 μg of A-fraction protein, 4.8 μg of C-fraction protein, 6.6 μg of D-fraction protein, all derived from mock-infected-cell extracts, and the indicated amount of B-fraction protein from mock- or pm975-infected cells. For the C-fraction assay, reactions had 10, 6.2, and 4.9 μg of the A, B, and D fractions, respectively, of mock-infected-cell extracts and the indicated amount of C fraction from mock- or pm975-infected cells. For the D-fraction assay, reactions were reconstituted with 10, 6.2, and 4.9 μg of the A, B, and C fractions, respectively, of mock-infected-cell extracts and the indicated amount of D fraction from mock- or pm975-infected cells.

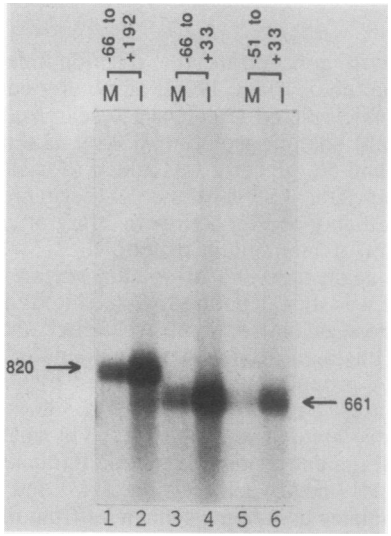

FIG. 2. Reconstituted in vitro transcription reactions with templates with or without the MLTF binding site. In vitro transcription reactions were performed and assayed as described in Materials and Methods. Transcription was performed with 10 μg of A-fraction protein, 6.2 μg of B-fraction protein, 4.9 μg of C-fraction protein, and 4.9 μg of D-fraction protein prepared from mock- (M) or pm975-infected (I) HeLa cells. The templates were pMLP −66 to +192 (lanes 1 and 2), pMLP −66 to +33 (lanes 3 and 4), and pMLP −51 to +33 (lanes 5 and 6). The S1-protected, specifically initiated RNAs were 820 nucleotides for the pMLP −66 to +192 template and 661 nucleotides for the pMLP −66 to +33 and pMLP −51 to +33 templates.

tions on templates with or without the MLTF binding site (Fig. 2). By using transcription reactions reconstituted with the A, B, C, and D fractions from mock- and pm975-infected cells, similar amounts of specifically initiated RNA were transcribed from two templates containing the MLTF binding site, pMLP −66 to +192 and pMLP −66 to +33 (Fig. 2, lanes 1 to 4; Table 2). Approximately one-fourth as much RNA was transcribed from a template lacking the MLTF binding site, pMLP −51 to +33 (Fig. 2, lanes 5 and 6; Table 2). Thus, the presence of the MLTF binding site resulted in an approximately fourfold increase in transcription under the conditions of these reconstituted transcription reactions. We conclude that the assays of the A and B fractions (Fig. 1) measured the transcriptional activity of MLTF, as well as of TFIIA and of RNA polymerase II. To measure the activities of TFIIA and RNA polymerase II in the absence of the effects of MLTF, we assayed the transcriptional activities of the A and B fractions using pMLP −51 to +33 as template. As with results shown in Fig. 1, we saw no significant difference in the activities of the A and B fractions prepared

TABLE 2. Quantitation of transcription reactions shown in Fig. 2[a]

Extract	Relative amt of RNA from template:		
	pMLP −66 to +192	pMLP −66 to +33	pMLP −51 to +33
pm975 infected	100	97	28
Mock infected	24	22	6.2

[a] The autoradiogram shown in Fig. 2 was analyzed by densitometry. The darkest band was assigned a value of 100, and the relative intensities of the other bands is shown.

from mock- and pm975-infected cells (data not shown). Therefore, we conclude that the transcriptional activities of MLTF, TFIIA, and RNA polymerase II were similar in the extracts of mock- and pm975-infected cells.

In the C-fraction assay (Fig. 1), no RNA product was synthesized in the absence of added C fraction. There was a linear response in the quantity of RNA product made in the presence of increasing amounts of both mock and infected C fractions, indicating that none of the other transcription factors required for specific initiation from the MLP was in limited supply in the reconstituted system under these conditions. As with the A and B fractions, the C fraction prepared from infected cells had transcriptional activity similar to that of the equivalent fraction from uninfected cells.

Results of the D-fraction assays (Fig. 1) showed that transcription depended on the addition of D-fraction protein. No RNA was detected in the absence of added D fraction, and transcription increased linearly with added D protein. Significantly, the activity of the D fraction prepared from infected cells was approximately fourfold greater than the activity of the equivalent fraction prepared in parallel from mock-infected cells. This observation was repeated in three successive fractionation experiments using three independent sets of virus-infected and mock-infected cell extracts. The ratios of D-fraction activity prepared from infected cells to that prepared from mock-infected cells were approximately 3, 4 (the results shown in Fig. 1), and 8 in the three experiments.

Reinberg et al. (52) recently reported the identification of a new cellular transcription factor in the phosphocellulose D fraction, termed TFIIX. TFIIX is not absolutely required for transcription from the MLP and has no effect on transcription from a template containing the promoter region from −400 to +33, but it stimulates RNA synthesis from a template containing sequences from −400 to +536 (52). Since the template used in the transcription reactions of Fig. 1 contained Ad2 sequences extending from −679 to +1713, we tested whether the higher activity of the D fraction from infected cells required Ad2 sequences from +33 to +536. We also tested whether the higher activity of the D fraction from infected cells required any Ad2 sequences upstream of the TATA box. To do this, the D fractions from mock and infected cells were assayed as described above, by using a template extending from −51 to +33 fused to pBR322 sequences, a template which lacks the sequences responsive to TFIIX (52) and sequences upstream of the TATA box region. The results (Fig. 3) were very similar to the results shown in Fig. 1. The D fraction from infected cells had four times the activity of the D fraction from uninfected cells with either template, indicating that the higher activity of the infected-cell D fraction was not due to an increase in the activity of TFIIX. Further, a template containing only the Ad2 sequence including the MLP TATA box and cap site regions from −51 to +33 was sufficient to obtain the higher transcriptional activity of the D fraction from infected cells.

It seemed possible that an as yet undiscovered factor induced during adenovirus infection might stimulate transcription from the MLP specifically and fractionate in the D fraction. To test whether the higher activity of the D fraction from virus-infected cells would be observed with a nonviral promoter, we titrated the D fractions with the human β-globin gene as template (Fig. 4). Transcription from the β-globin promoter is stimulated by E1A in vivo (24, 60) and appears to use the same TATA box transcription factor used by the MLP in vitro (53). The D fraction from virus-infected

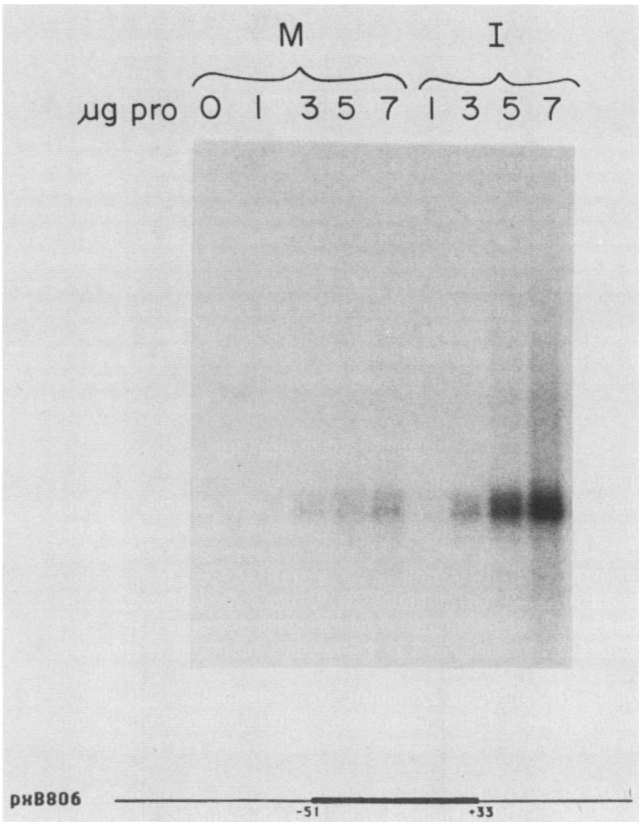

FIG. 3. In vitro transcriptional activity of phosphocellulose D fractions from mock-infected (M) and pm975-infected (I) cells with a template having an Ad2 sequence from −51 to +33 relative to the MLP start site. Ad2 sequence from −51 to +33 was cloned into pBR322 between the *Eco*RI and *Hin*dIII sites, resulting in pXB806 (31). This plasmid (diagrammed at the bottom of the figure) was used as the template for in vitro transcription as described in Materials and Methods. Reactions contained 10, 6.2, and 4.9 μg of the A, B, and C fractions, respectively, of mock-infected-cell extracts and the indicated amount of D-fraction protein (pro) from mock-infected or pm975-infected cells. An autoradiogram of the entire gel is shown.

cells had activity approximately four times higher than that of the fraction from mock-infected cells when the β-globin gene was used as template (Fig. 4), just as was observed during titrations with the MLP (Fig. 1 and 3). We conclude that the factor(s) responsible for the higher activity of the D fraction from virus-infected cells either stimulates the activity of a general transcription factor or is itself a general transcription factor.

Sequence-specific binding activity of MLTF unaffected by infection. From the results shown in Fig. 1, we concluded that extracts from mock- and pm975-infected cells had similar MLTF transcriptional activities. We also assayed the sequence-specific DNA-binding activity of MLTF by a gel retardation assay which can detect this activity in the unfractionated whole-cell extract (9).

By using the same labeled DNA fragment used by Carthew et al. (9) extending from −138 to −29 from the MLP start site and similar binding and gel electrophoresis conditions, we observed the same three major species of DNA-protein complexes they reported (designated I, II, and III in Fig. 5). As shown by Carthew et al. (9), we also observed that the formation of these species was inhibited by addition to the binding reaction of a 10-fold molar excess of the

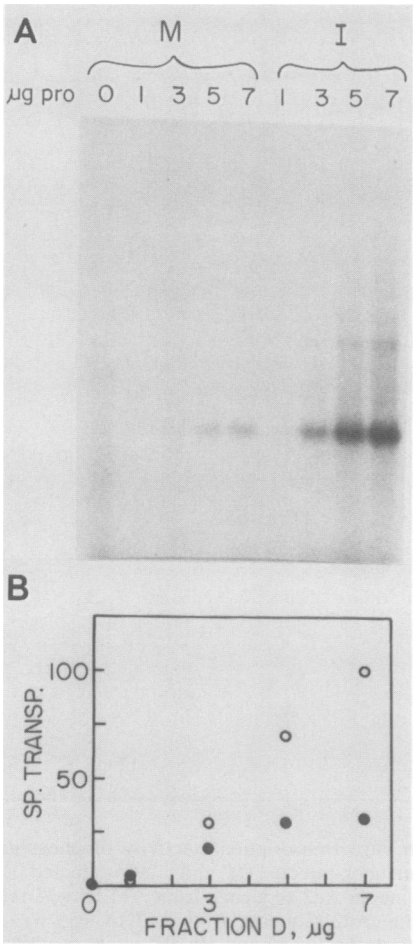

FIG. 4. (A) D-fraction titrations with the human β-globin promoter as template. Transcription reactions were performed as described in Materials and Methods, with 10, 6.2, and 4.9 μg, respectively, of the A, B, and C fractions derived from mock-infected cells plus the indicated amounts of D-fraction protein (pro) from mock-infected (M; ●) or pm975-infected (I; ○) cells. (B) Densitometric quantitation as described in the legend to Fig. 1. SP. TRANSP., Specific transcription.

unlabeled DNA fragment from −138 to −29, but not by a 50-fold molar excess of DNA fragments of similar size which did not contain sequences homologous to the MLTF-binding site from −63 to −52 (data now shown). Carthew et al. (9) showed that each of the three complexes generates a similar DNase I footprint centered over the binding site from nucleotide −63 to −52 and suggested that the more slowly migrating species II and III result from the interaction of DNA-bound MLTF protein with one or more additional proteins which do not make sequence-specific contacts with the DNA. The important point demonstrated in Fig. 5 is that the patterns of retarded specific DNA-MLTF complexes, I, II, and III formed at each protein concentration were not significantly different when extracts from mock-infected and Ad2-infected HeLa cells were compared, even though the extract from adenovirus-infected cells had transcriptional activity five times higher than that of the mock-infected-cell extract. These results indicate that there is no significant difference between the sequence-specific binding activity of MLTF in extracts from mock-infected cells and that in extracts of Ad2-infected HeLa cells.

Phosphocellulose fractionation of E1A protein in the whole-cell extract. The 289-amino-acid E1A protein which expresses most of the E1A transactivating activity can be detected in the whole-cell extract from infected cells by an immunoblot assay with a monospecific anti-E1A antiserum prepared against an E1A fusion protein expressed in *E. coli* (58). The immunoblot of the one-dimensional gel (Fig. 6) shows two predominant electrophoretic species which can be resolved into multiple distinct species by high-resolution two-dimensional gel electrophoresis (26). To determine how E1A protein fractionates during phosphocellulose chromatography, the phosphocellulose fractions were subjected to sodium dodecyl sulfate gel electrophoresis and immunoblot analysis (Fig. 6). Amounts of protein proportional to the total protein in each fraction were analyzed in lanes 7 to 10. More than 80% of the large E1A protein species eluted in the B fraction. However, E1A protein was detected in each of the phosphocellulose fractions, including the D fraction (lane 10), where it was more clearly observed when more protein was analyzed (lane 12). As expected, E1A protein was not detected in fractions from mock-infected cells.

TATA box region requirement for higher activity of unfractionated infected-cell extracts. The results presented above indicate that the component(s) responsible for the higher transcriptional activity of adenovirus-infected cell extracts fractionated principally in the D fraction during phosphocellulose chromatography. Since the TATA box binding transcription factor also elutes in the D fraction (55), the results are consistent with the model that the higher activity of the infected-cell extract is due to an increase in the activity of this factor. To test this model further, we analyzed the template activities of deletion mutants of the MLP in unfractionated extracts from mock-infected and pm975-infected HeLa cells. Transcription reactions were performed with templates including Ad2 sequences from −679 to +1713, −66 to +192, −66 to +33, and −51 to +33 with respect to the MLP cap site (Fig. 7). Transcription reactions with unfractionated extracts can be performed at much higher protein concentration than can reactions reconstituted with the phosphocellulose fractions. Consequently, several-times-more-specific RNA was produced, and it was possible to quantitate the amounts of transcribed RNA by cutting the specific bands from the gel (Fig. 7) and counting them directly (Table 3). These results demonstrate that the only MLP DNA sequence required to observe the higher activity of the infected-cell extract is the region from −51 to +33. This is approximately equivalent to the region protected from DNase I digestion by the partially purified TATA box transcription factor (55). Thus, these results are consistent with the model that the higher activity of the infected-cell extract is due to an increase in the activity of the TATA box transcription factor.

DISCUSSION

E1A protein stimulates transcription from nine adenovirus promoters which have no significant sequence homology in common (reviewed in reference 3). E1A functions also stimulate transcription from nonadenovirus genes when they are introduced into cells by transfection or by infection of a recombinant adenovirus (1, 10, 15, 20, 24, 28, 60, 61). Indeed, there are no promoters for RNA polymerase II which have been shown not to respond when they are introduced into adenovirus-infected cells expressing the large E1A protein. In the original work showing that transcription of the human β-globin gene is stimulated by E1A in transfection experiments, activation of the human α-globin

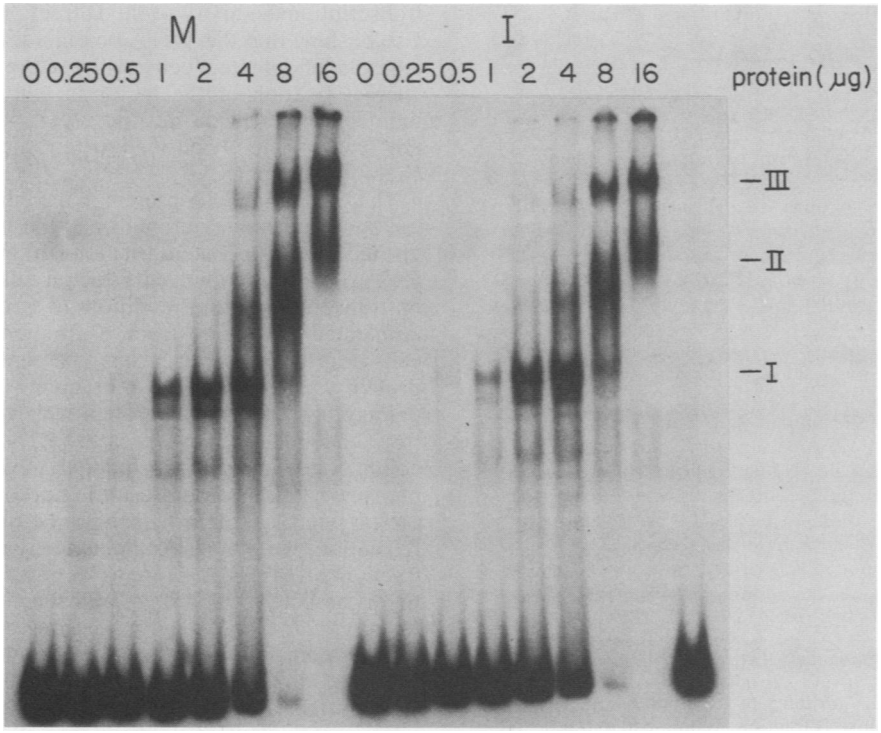

FIG. 5. MLTF sequence-specific DNA-binding activities in mock-infected (M) and Ad2-infected (I) HeLa cell extracts. Whole-cell extracts were prepared 20 h postinfection with Ad2. Labeled probe containing the MLTF binding site was incubated with the indicated amount of protein under the conditions of Carthew et al. (9) and subjected to electrophoresis on a 4% polyacrylamide gel, and the products were visualized by autoradiography. I, II, and III, Major species of DNA-protein complexes.

gene was not observed (24). However, later work showed that transcription of the α-globin gene is stimulated by E1A when the α-globin gene is infected into HeLa cells as part of an Ad5 recombinant (28). Clearly, there are no strict promoter sequence requirements for E1A transactivation.

In an analysis of the human β-globin promoter sequences required for transcription after transfection into an adenovirus-transformed cell line constitutively expressing E1A proteins, Green et al. (24) found that deletion of sequences upstream of the TATA box had little effect on expression, whereas TATA box mutants were expressed at a much lower level. Transcription from a mutant Ad2 E3 promoter deleted of all viral sequence upstream of the TATA box is also stimulated by E1A functions (39). The TATA box region is also crucial to E1A transactivation of the Ad2 E1B promoter. A systematic mutational analysis of the E1B promoter (64) indicated that it is an extremely simple promoter composed of a TATA box with a closely situated binding site for the transcription factor Sp1 (14). Mutations of the Sp1 site produce a weak promoter consisting essentially of a TATA box region only. Yet, transcription from the Sp1 site mutants is stimulated by E1A functions. In contrast, TATA box mutants are very weak promoters which are poorly transactivated by E1A (64).

The ability of E1A functions to stimulate transcription from these mutant promoters consisting of essentially only a TATA box region suggests that E1A protein stimulates the activity of general transcription factors which interact with this basic promoter element. To search for evidence that one of the basic transcription factors is in fact activated by the expression of E1A protein and to determine which of the factors are affected, we analyzed the transcriptional activity of extracts of adenovirus-infected HeLa cells. Several distinct host cell transcription factors are required for accurate initiation of transcription by RNA polymerase II (12, 14, 17, 43). The general transcription factors required for accurate initiation of transcription in vitro have been most thoroughly characterized in studies with the Ad2 MLP. In the nomenclature of the Roeder laboratory, transcription factors TFIIA, TFIIB, TFIIE, and TFIID are required for accurate in vitro initiation of transcription from the TATA box region of the MLP (13, 52, 53). Of these, only TFIID binds to promoter DNA (12, 17), making close contacts to the TATA box sequence (55).

In our initial studies, we found that extracts of adenovirus-infected HeLa cells have activity for in vitro transcription from the MLP 5- to 10-fold higher than that of extracts prepared in parallel from mock-infected or E1A mutant-infected cells (18, 40). This E1A-dependent increase in transcriptional activity was also observed in extracts of infected cells blocked in viral DNA replication and late gene expression (40). In the present study, we also found this

TABLE 3. Quantitation of transcription reactions shown in Fig. 7[a]

Extract	Amt of transcribed RNA (cpm)			
	pMLP −679 to +1713	pMLP −66 to +192	pMLP −66 to +33	pMLP −51 to +33
Mock infected	993	1,045	894	254
pm975 infected	8,054	22,417	9,653	2,578

[a] The bands containing specifically initiated RNA from the gel shown in Fig. 7 were cut out of the gel, added to a scintillation fluor cocktail, and counted in a scintillation counter. A background of 34 cpm was subtracted, and the net counts over background were shown.

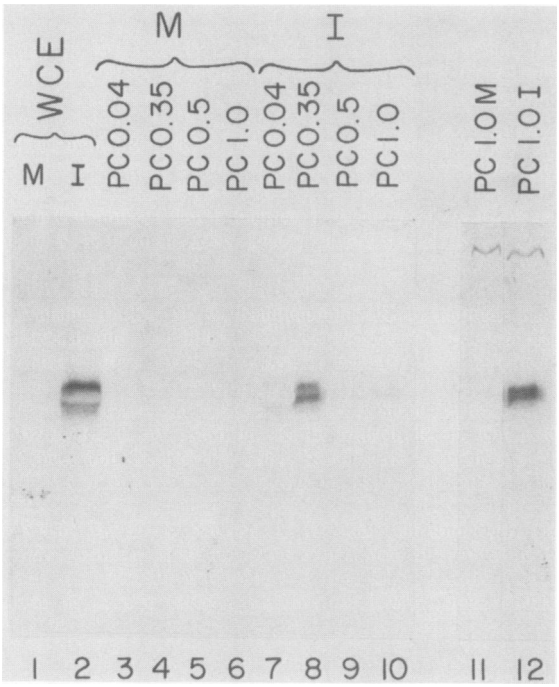

FIG. 6. Elution of E1A proteins of the pm975-infected-cell extract in the phosphocellulose B fraction. Whole-cell extracts (WCE) and the individual phosphocellulose fractions from mock-infected (M) or pm975-infected (I) cells were fractionated in a sodium dodecyl sulfate-10% polyacrylamide gel and transferred to a nitrocellulose membrane, and E1A proteins were visualized by using a polyclonal anti-E1A antiserum as described elsewhere (55). Lanes: 1 and 2, whole-cell extract protein (200 μg); 3 and 7, A-fraction protein (100 μg); 4 and 8, B-fraction protein (100 μg); 5 and 9, C-fraction protein (10 μg); 6 and 10, D-fraction protein (16 μg); 11 and 12, D-fraction protein (100 μg). The amounts of protein loaded in lanes 3 to 10 were approximately proportional to the total amount of protein recovered in the A, B, C, and D fractions in a typical phosphocellulose fractionation of the whole-cell extract (Table 1).

higher transcriptional activity with templates from which all known promoter elements upstream and downstream of the major late promoter TATA box have been deleted, a result consistent with the model that E1A expression results in an increase in the activity of one or more general transcription factors. Further, we fractionated the extracts from adenovirus- and mock-infected cells by phosphocellulose chromatography by following a procedure which separates the general transcription factors (13, 43, 54) and assayed the transcriptional activities of the individual fractions.

Fractions A, B, and C, containing RNA polymerase II, TFIIA, TFIIB, TFIIE, and the MLP upstream factor MLTF (9, 43, 45, 52–55) had similar activities in assays for their functions in the specific initiation of transcription (Fig. 1). MLTF sequence-specific binding activity was also similar in extracts of virus-infected and mock-infected cells (Fig. 4). The factor(s) principally responsible for the higher transcriptional activity of the infected-cell extract fractionated in the D fraction. This is the same fraction in which the TATA box transcription factor fractionates (55), and the obvious implication is that the higher activity of the infected-cell extract results from an increase in the activity of this factor. However, the D fraction is a crude protein fraction containing approximately 2 to 5% of the total protein in the whole-cell extract. Consequently, it is possible that another protein in the D fraction is responsible for the higher transcriptional activity of the D fraction from infected cells. Establishing that the TATA box factor is in fact activated in extracts of infected cells will require the direct physical characterization of the factor in extracts of infected and uninfected cells. Thus far, the TATA box factor activity has not been sufficiently purified to permit such a direct physical characterization (52).

The bulk of the E1A protein in the infected-cell extract did not stimulate transcription directly in vitro. More than 80% of the large E1A protein fractionated in the B fraction (Fig. 6). Yet, the B fraction from infected cells was no more active in transcription after addition to protein fractions from uninfected cells than was the B fraction from mock-infected cells (Fig. 1). However, a small percentage of the total E1A protein was found in the D fraction. This could include a unique form of the E1A protein which is physically distinct from the bulk of E1A protein. E1A proteins undergo a complex set of posttranslational modifications, including phosphorylation, which result in approximately 30 forms of the large E1A protein which can be distinguished by high-resolution two-dimensional gel electrophoresis (26). Also, a fraction of the E1A proteins in infected-cell extracts are tightly associated with specific host cell proteins of unknown function (27, 66). It may be that one of the minor forms of E1A protein fractionates in the D fraction and stimulates transcription directly in vitro. If a small fraction of the total E1A protein does act directly to stimulate transcription in vitro, we think it is unlikely that it acts catalytically (to activate transcription factors, for example), since mixtures of D fractions from infected and uninfected cells have activities which are close to the average of the two (K. Leong and A. J. Berk, unpublished observations).

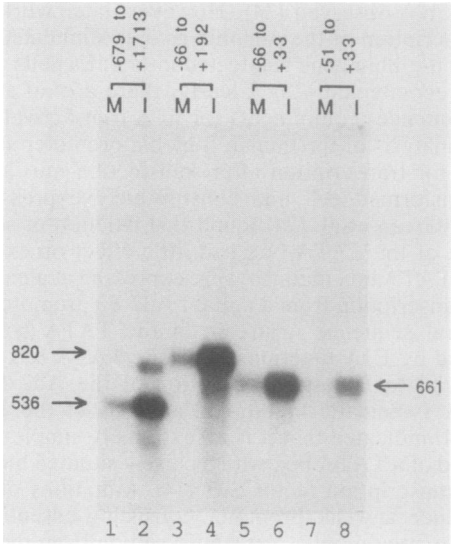

FIG. 7. Autoradiogram showing that only the TATA box and cap site regions of the MLP are required for the increased transcriptional activity of adenovirus-infected-cell extracts. Transcription reactions were performed with 300 μg of whole-cell extract prepared from mock- (M) or pm975-infected (I) HeLa cells as described elsewhere (40). The 50-μl reactions contained 2 μg of the following template DNAs: pMLP −679 to +1713 (lanes 1 and 2), pMLP −66 to +192 (lanes 3 and 4), pMLP −66 to +33 (lanes 5 and 6), and pMLP −51 to +33 (lanes 7 and 8). Specifically initiated RNAs protected from S1 digestion were 536 nucleotides for the pMLP −679 to +1713 template, 820 nucleotides for the pMLP −66 to +192 template, and 661 nucleotides for the pMLP −66 to +33 and pMLP −51 to +33 templates. A 1-h autoradiogram is shown.

There is evidence that E1A expression stimulates the activity of other host cell transcription factors. Kovesdi et al. (36) have identified a host cell factor which binds specifically to an element of the E2 early promoter region. The sequence-specific DNA-binding activity of this factor, E2F, increases after adenovirus infection, and this increase in activity requires the expression of E1A protein (36). E1A functions also stimulate the transcription of genes transcribed by RNA polymerase III (2, 19, 30). Analyses of RNA polymerase III transcription factor activities in extracts of virus-infected HeLa cells have led to the suggestion that E1A expression causes an increase in the activity of the general RNA polymerase III transcription factor TFIIIC (30, 67). Thus, evidence is accumulating that E1A protein acts, indirectly or directly, to increase the activities of a number of host cell transcription factors. Such a model would explain the ability of E1A protein to stimulate transcription from so many diverse promoters.

ACKNOWLEDGMENTS

We are grateful to Jim Manley for providing us with mutants of the MLP and to Carol Eng for excellent technical assistance.

This work was supported by Public Health Service grant CA 41062 and fellowships CA 07815 (to K.L.) and GM 10699 (to L.B.) from the National Institutes of Health.

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