The Adenovirus E1B 19-Kilodalton Protein Stimulates Gene Expression by Increasing DNA Levels

CHRISTINE H. HERRMANN AND MICHAEL B. MATHEWS*

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

Received 23 May 1989/Accepted 24 August 1989

In transient expression assays, the adenovirus E1B 19-kilodalton (19K) tumor antigen increases expression from viral promoters and the promoter for the cellular 70-kilodalton heat shock protein (hsp70). To study the mechanism of this effect, we constructed HeLa cell lines that contain stably integrated copies of the 19K gene. Compared with a 19K⁻ control cell line, 19K⁺ cells produced a significantly higher level of expression from every promoter introduced into the cells by transfection. The 19K protein also increased expression of an RNA polymerase III-transcribed gene but did not affect the level of expression of the endogenous hsp70 gene. The rate of transcription from transfected promoters, as measured by a nuclear run-on assay, was higher in the 19K⁺ cells than in the 19K⁻ control cells. Furthermore, the level of plasmid DNA remained higher in the 19K⁺ cell line, suggesting that the 19K protein stabilizes transfected plasmid DNA. The elevated DNA levels seemed to account in full for the increased transcription. The role of the 19K protein in increasing gene expression during viral infection was found to be due to a replication-dependent increase in viral DNA levels. Thus, the 19K protein activates transcription indirectly by producing a higher level of viral or plasmid DNA. The DNA stabilization function of the 19K protein is probably related to the protective role of the 19K protein during viral infection and represents the first example of a viral oncogene product that modulates gene expression by regulating viral and plasmid DNA levels.

Adenovirus encodes two genes, E1A and E1B, located at the left end of the viral genome, which together are capable of transforming primary rodent cells fully. The E1A gene alone is sufficient to immortalize cells, but the E1B gene or another cooperating oncogene, such as *ras*, is necessary to produce a completely transformed phenotype (for a review, see reference 5). The E1A and E1B genes also have several important functions during lytic infection. The E1A gene is required for full transcriptional activation of the other early viral genes (reviewed in reference 5). In addition, the multifunctional E1A proteins stimulate expression of certain endogenous and exogenous cellular genes, including genes transcribed by RNA polymerase III, inhibit the expression of several viral and cellular enhancer-dependent genes, and induce cellular DNA synthesis (5).

The E1B gene also influences a variety of viral and host cell functions. The E1B region encodes two major protein products of 55 and 19 kilodaltons (55K [495R] and 19K [176R], respectively), which can be synthesized from a single mRNA species, the 22S mRNA (7, 12). Although their coding sequences overlap, the 19K and 55K polypeptides initiate at separate AUG codons (the 19K protein at nucleotide 1711 and the 55K protein at nucleotide 2016) in different reading frames; as a result, the proteins do not share any amino acid sequence homology. The 19K protein can also be synthesized from a 13S mRNA, whose relative abundance increases at late times in infection such that the level of the 19K protein is highest late in infection (29, 39, 55). In addition, the E1B region encodes several small proteins related to the 55K polypeptide, but they are not essential for lytic infection or transformation (2, 24-26, 49). The 55K protein, on the other hand, is important for transformation and functions during lytic infection to facilitate the transport and stabilization of viral transcripts in the cyto-

plasm while blocking these processes for cellular messages (for a review, see reference 41).

The 19K protein is essential for both viral and DNA-mediated transformation of rodent cells (3, 4, 6, 8, 34, 43, 44, 53). It has been detected in the nuclear envelope and cytoplasmic membranes of transformed cells and virus-infected cells at early times but has been found exclusively in the nuclear envelope at late times in infection (50). A posttranslational acylation of the 19K protein may play a role in its association with cellular membranes (27). Viruses containing lesions in the 19K coding region are unimpaired or only slightly defective for growth in HeLa cells (34, 52, 53), but the growth characteristics of 19K mutant viruses depend strikingly on the host cell. In human KB cells, 19K mutant viruses are extremely defective for growth (42, 46, 54), whereas in WI38 cells (a nontransformed human diploid secondary lung fibroblast cell line), the 19K mutant viruses exhibit a growth advantage over wild-type virus (54). In some human cell lines, e.g., KB, infection with a virus that does not express a functional 19K protein results in the appearance of a large-plaque morphology (the *lp* phenotype) (8, 9, 43, 45). These mutants also cause an enhanced and accelerated cytopathic effect on the host cell (*cyt*) (34, 43-45) and the extensive degradation of viral and chromosomal DNA (*deg*) in most cell lines (10, 13, 23, 34, 42, 53). Since these phenotypes occur in the absence of the 19K protein, this tumor antigen has been implicated in preserving DNA and cellular integrity during adenovirus infection.

The 19K protein also modulates viral gene expression. In virus infections, the 19K protein appears to have a negative effect on viral gene expression in the presence of the E1A 13S or 12S products (52, 54; unpublished results), but in the absence of the E1A 13S or 12S products, the 19K protein exerts a positive effect on the expression of viral genes (19, 51). The E1B gene has also been suggested to increase the level of E1A expression in transformed rodent cell lines (38, 48). The stimulation of viral gene expression by the 19K

* Corresponding author.

protein is apparent in plasmid cotransfection experiments regardless of the presence or absence of E1A. In addition to increasing expression from adenovirus early promoters (19) and a late promoter (47), the 19K protein elevates the level of expression from the promoter for the cellular 70-kilodalton heat shock protein (hsp70) (19) and several enhancer-linked promoters (31, 57). An increased level of cytoplasmic mRNA is observed in the presence of the 19K protein, indicating that the 19K protein influences gene expression at or before the level of accumulation of mRNA in the cytoplasm (19, 31, 38, 47, 48, 57).

It has been assumed that the 19K protein exerts its effects at the transcriptional level. To test this assumption and to elucidate the mechanism of action of the 19K protein, HeLa cells lines that contain a high copy number of stably integrated 19K coding sequences were constructed. We found that the 19K protein indirectly activates transcription from promoters newly introduced into cells by increasing the stability of transfected plasmid DNA. Similarly, the increased rate of transcription from viral promoters during infection in the presence of the 19K protein was due to increased levels of viral DNA. The enhanced stability of viral and plasmid DNA is probably related to a protective function of the 19K protein during productive infection and represents a novel mechanism whereby an oncogene can modulate gene expression.

MATERIALS AND METHODS

Plasmids and viruses. Construction of the adenovirus promoter-chloramphenicol acetyltransferase (CAT) plasmids and the VA RNA₁-encoding plasmid (pMHVA) has been described previously (11, 28). pSV₂CAT was obtained from C. Gorman, Genetech, Inc. (15), dpm126 was obtained from W. Herr, Cold Spring Harbor Laboratory (18), hsp70CAT was from R. Morimoto, Northwestern University (56), and HIVCAT (pU3R-III) was from W. Hazeltine, Harvard Medical School (36). The pMT19K and pMT19K1520 constructs were provided by E. White, Cold Spring Harbor Laboratory (E. White and R. Cipriani, Proc. Natl. Acad. Sci. USA, in press). pMT19K contains the 19K-coding region from nucleotides 1704 to 2256 inserted downstream of a 1.4-kilobase-pair mouse metallothionein I promoter fragment. pMT19K1520 is identical to pMT19K except that a fragment from pdl1520D (4) containing a C-to-T transition at nucleotide 2022 replaced the wild-type sequence in this region. This mutation results in termination of the 55K protein after formation of a dipeptide but does not alter the structure of the 19K protein. pON3 was obtained from D. Hanahan, Cold Spring Harbor Laboratory, and contains a 1.4-kilobase-pair fragment of Tn5 carrying the neomycin resistance gene in a vector that contains the *lac* UV5 promoter, the simian virus 40 (SV40) early promoter, the SV40 small t intron, and an SV40 polyadenylation site. The single-stranded CAT M13 clones, used as probes in the nuclear run-off reactions, were obtained from L. Shan Su and D. Knipe, Harvard Medical School, and contain 780 base of CAT sequences that detect sense or antisense CAT RNA. The adenovirus M13 clones were provided by P. Hearing, State University of New York at Stony Brook, and detect the sense mRNA from each region. The E2 clone contains adenovirus type 5 sequences from 59.5 to 70.0 map units, E4 contains sequences from 89.1 to 93.5 map units, L1 contains sequences from 31.5 to 37.3 map units, and L3 contains sequences from 53 to 58.5 map units. The probes used to detect sense or antisense actin transcription in

transfected cells contain the human β -actin cDNA cloned in M13 and were provided by M. Laspi, Cold Spring Harbor Laboratory. A denatured double-stranded human β -actin probe was used to detect actin transcription in infected cells.

Adenovirus type 5 *dl309* (21) was used as the wild-type control. The *dl337* mutant contains a deletion within the coding region of the 19K protein that removes sequences between nucleotides 1769 and 1912 but does not affect the 55K-coding region (34). The E1ACAT virus contains the E1ACAT chimeric gene in place of the E1 region (19). The 9S and 9S.R2 viruses contain the E1A 9S cDNA in place of the genomic E1A gene and differ only by a point mutation at nucleotide 1769, resulting in a single-amino-acid substitution in the 19K-coding region of the 9S.R2 virus (30, 54). The viruses were grown and titrated by plaque assay on 293 cells (16).

Construction of cell lines. Monolayers of HeLa cells (ATCC CCL 2) in 60-mm-diameter dishes were transfected with 0.1 μ g of pON3 or cotransfected with 0.1 μ g of pON3 and 1 μ g of pMT19K or pMT19K1520, supplemented with 10 μ g of salmon sperm DNA, by the method previously described (19). The cells were grown in Dulbecco modified Eagle medium plus 10% fetal calf serum, 100 μ g of streptomycin per ml, and 100 μ g of penicillin per ml (DMEM+) for 2 days, at which time the cells were trypsinized, diluted 1:5 into selective medium (DMEM+ containing 1 mg of G418 [Gibco Laboratories] per ml), and fed every 3 to 4 days. At 3 to 4 weeks post-transfection, single colonies were isolated and expanded. The cell lines were maintained in the presence of G418. The individual clones were initially screened for expression of the 19K protein by immunofluorescence; all of the clones that were positive by this assay, as well as some negative controls, were further characterized.

Transfections and CAT assays. Transfection experiments were performed by the calcium phosphate precipitation technique as previously described (19) except that after the glycerol shock, the cells were fed with DMEM+ containing 125 to 150 μ M ZnCl₂ and 1.25 to 1.5 μ M CdSO₄ unless otherwise stated in the figure legends. CAT activity was assayed as previously described (15, 19) and quantified by using an AMBIS Beta Scanner (Automated Microbiology Systems, Inc.). A unit of CAT activity is defined as 1% conversion of chloramphenicol to its acetylated forms by an extract prepared from half of the cells from a 6-cm-diameter dish during a 60-min incubation.

Immunoblot analysis. Monolayer cultures of cells were washed twice in phosphate-buffered saline and lysed with A buffer (10 mM Tris [pH 7.4], 140 mM NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40) or sample buffer (22). Polypeptides were separated in 20% sodium dodecyl sulfate (SDS)-polyacrylamide gels and electroblotted onto nitrocellulose filters for 40 min at 200 mA, using a PolyBlot transfer system (American Bionetics, Inc.). The nitrocellulose blots were reacted with a monoclonal antibody (517) against the 19K protein (kindly provided by E. Harlow, Cold Spring Harbor Laboratory), and immune complexes were detected by using ¹²⁵I-labeled rabbit anti-mouse immunoglobulin G (Dupont, NEN Research Products), followed by autoradiography.

RNA analysis. Total cytoplasmic RNA was isolated and analyzed by a riboprobe-RNase protection assay. A 5- μ g amount of sample RNA was hybridized with gel-purified ³²P-labeled antisense RNA generated by T7 polymerase (generously provided by J. Dunn, Brookhaven Laboratory). Hybridization was carried out at 45°C overnight, after a 5-min incubation at 85°C, in a buffer containing 80% formamide, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid)

(PIPES), 400 mM NaCl, and 1 mM EDTA. The resulting hybrids were then incubated for 60 min at 30°C in a buffer containing 10 µg of RNase A per ml, 10 µg of RNase T₁ per ml, 10 mM Tris (pH 7.6), 5 mM EDTA, and 300 mM NaCl. The reactions were incubated an additional 15 min at 37°C with 50 µg of proteinase K in 1% SDS, extracted once each with phenol and chloroform-isoamyl alcohol (24:1, vol/vol), and precipitated with ethanol. The protected RNA was denatured by heating for 5 min at 90°C, resolved by electrophoresis in an 8% polyacrylamide–7 M urea sequencing-type gel, and visualized by autoradiography in the presence of an intensifying screen.

Immunoprecipitation and gel electrophoresis. Cells were labeled with 200 µCi of [³⁵S]methionine (Translabel; ICN Pharmaceuticals Inc.) per 2 × 10⁶ cells in DMEM+ without methionine for 4 h. The cells were washed in phosphate-buffered saline, pelleted, and lysed in 100 µl of A buffer. A 10-µl sample of lysate was mixed with an equal volume of 2× sample buffer (22), and the polypeptides were separated by electrophoresis in a 15% SDS-polyacrylamide gel, followed by fluorography. For immunoprecipitation of the hsp70 protein, 2.5 µl of the precleared cell lysate was diluted 1:10 with A buffer and incubated with prewashed fixed *Staphylococcus aureus* (IgGSorb; The Enzyme Center, Inc.) for 15 min on ice. The supernatant was collected and incubated with a mixture of affinity-purified monoclonal antibodies that recognize the hsp70 protein (provided by K. Riabowol, Cold Spring Harbor Laboratory) (35) for 30 min on ice, followed by a 10-min incubation with *S. aureus*. The pellet was washed four times with 1 ml of NET2⁺ (150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.4], 0.5% deoxycholate, 0.5% Nonidet P-40, 0.1% SDS) and suspended in sample buffer (22). The proteins were separated by electrophoresis in a 15% SDS-polyacrylamide gel, followed by fluorography.

Analysis of transcription rates. Nuclei were isolated from transfected or infected cells, labeled RNA was prepared, and equal amounts of labeled RNA were hybridized to 5 µg of single-stranded or denatured DNA immobilized on nitrocellulose filters according to the method of Greenberg and Ziff (17). A total of 150 µCi of [³²P]UTP was used per reaction containing approximately 2 × 10⁷ nuclei. The filters were subjected to autoradiography in the presence of an intensifying screen.

Analysis of low-molecular-weight DNA. Plasmid or viral DNA was isolated from cells essentially as described by Hirt (20), linearized with *Hind*III, incubated in the presence of 20 µg of RNase A per ml, extracted with phenol and chloroform-isoamyl alcohol (24:1, vol/vol), and precipitated with ethanol. After denaturation in 0.2 N NaOH for 60 min at room temperature, the DNA was neutralized by the addition of 10 volumes of 6× SSC (0.9 M NaCl, 0.09 M sodium citrate). The DNA from transfected cells was spotted onto nitrocellulose filters in twofold dilutions, and the DNA from infected cells was spotted in fivefold dilutions. The filters were probed with ³²P-labeled RNA specific for CAT sequences and were subjected to autoradiography in the presence of an intensifying screen.

RESULTS

Construction and characterization of E1B 19K cell lines. The E1B 19K-coding sequence under the control of the heavy-metal-inducible mouse metallothionein I promoter in plasmid pMT19K was cotransfected into HeLa cells with pON3, a plasmid that encodes the neomycin resistance gene. After growth in selective medium, eight colonies were iso-

TABLE 1. Description of cell lines

Cell line	Plasmid transfected	19K copies/cell	Detection of 19K protein ^a	Complementation of <i>cyt</i> and <i>deg</i> phenotypes
HeLa			—	—
2A1	Neo ^r		—	—
2D3	Neo ^r		—	—
4A2	Neo ^r + MT19K	1	—	+
4A3	Neo ^r + MT19K	50–100	+	+
4B1	Neo ^r + MT19K	500–1,000	+	+
6E	Neo ^r + MT19K1520	50–100	+	+

^a By immunofluorescence and immunoblot analysis.

lated and expanded. We also obtained four colonies from cells transfected with pMT19K1520, which encodes wild-type 19K protein but contains a point mutation at nucleotide 2022, resulting in termination of the 55K protein after two amino acids. As a control, we picked several clones arising from cells transfected with the neomycin resistance gene alone. Two of the cell lines transfected with pMT19K and one transfected with pMT19K1520 produced detectable amounts of the 19K protein after metal induction by immunoblot analysis (Table 1; Fig. 1A). One cell line, 4B1, synthesized very high levels of the 19K protein, comparable to the level seen in infected cells at 36 hours postinfection (hpi). Two other 19K⁺ cell lines, 4A3 and 6E, produced lower levels of the 19K protein, somewhat less than the level found in 293 cells, a cell line that constitutively expresses the E1A and E1B proteins (16). Another clonal line, 4A2, did not produce enough 19K protein to be detected by this analysis or by immunofluorescence. The other three 19K⁺ cell lines (4A3, 4B1, and 6E) displayed faint staining of the nuclear envelope by immunofluorescence, but 4B1 cells also showed bright punctate cytoplasmic fluorescence (data not shown). Although the 4A3 and 4B1 cell lines can theoretically synthesize the N-terminal portion of the 55K protein, we failed to detect it by immunofluorescence or by immunoprecipitation using an antipeptide antibody (25), nor did we detect it in 6E cells that are unable to make the 55K protein (data not shown), but the 55K protein was clearly evident in *dl309*-infected HeLa cells.

The amount of 19K protein expressed by the cell lines was dependent on the concentration of zinc and cadmium salts in the medium. Figures 1B and C show immunoblots of protein extracted from 4A3 and 4B1 cells grown in the presence of different concentrations of these ions. For both cell lines, there was a low but detectable level of 19K protein in the absence of added heavy metals, but the level of 19K protein expression was greatly induced by the addition of heavy metals, especially in the case of 4B1 cells. Because high concentrations of zinc and cadmium produced a cytopathic effect on the cells, the remainder of the experiments reported here were performed at or below 150 µM Zn²⁺ and 1.5 µM Cd²⁺.

The cell lines were also examined for 19K mRNA expression by isolating cytoplasmic RNA from uninduced cells and analyzing it by a riboprobe-RNase protection assay (Fig. 1D). The 19K-specific RNA probe was protected by 4A3, 4B1, and 293 cell RNA but not by the control cell line RNA. We further analyzed the cell lines for 19K DNA content by Southern blot analysis (data not shown) and estimated the amount of 19K DNA per cell by comparison with reconstructions containing pMT19K plasmid DNA. The copy number of the 19K gene (summarized in Table 1) correlated

FIG. 1. (A to C) Detection of the 19K protein from infected and transformed cell lines by immunoblot analysis. (A) Plates (60-mm diameter) of confluent cells, incubated in the presence of 180 μ M ZnCl_2 and 1.8 μ M CdSO_4 , were lysed in A buffer. Equal amounts (30 μ g) of total protein from each cell line were used. 2A1 control cells were uninfected (lane 1) or infected with *dl309* at a multiplicity of infection of 10 and harvested at the times shown (lanes 2 to 4). The infected 2A1 cells were not metal induced. (B) 4A3 cells on 60-mm-diameter dishes were incubated in regular media or medium containing 1.5 $\times$, 2.0 $\times$, 2.5 $\times$, or 3.0 $\times$ Zn/Cd (1 $\times$ Zn/Cd is 100 μ M ZnCl_2 plus 1 μ M CdSO_4) for 68 h and then lysed in 400 μ l of SDS-gel sample buffer. The 2A1 control cells were treated with 1.5 $\times$ Zn/Cd. (C) 4B1 cells were treated with 1.0 $\times$ or 2.0 $\times$ Zn/Cd, as indicated, for the times shown. The 2A1 control cells were treated with 2.0 $\times$ Zn/Cd and harvested at 126 hpt. (D) Detection of 19K mRNA in the cell lines. Total cytoplasmic RNA (5 μ g) isolated from uninduced cells was hybridized to ^{32}P -labeled RNA complementary to 19K mRNA and digested with RNase A and RNase T₁. The RNA protected by 4A3 and 4B1 RNA migrated more slowly than did that protected by 293 cell RNA because of the presence of linker sequences present in the probe and 19K cell line RNA but not in 293 cells. ^{32}P -labeled *Hpa*II-digested pBR322 size markers are shown in lane 5; undigested probe is shown in lane 6. M, Marker.

with the amount of 19K protein produced by the cell lines (Fig. 1).

Complementation of viral mutants. As discussed above, 19K mutant viruses induce several phenotypes, including an accelerated and increased cytopathic effect on the host cell (*cyt*) and the degradation of viral and chromosomal DNA (*deg*). We therefore tested the 19K⁺ cell lines to determine whether the 19K protein expressed by them could complement mutant viruses for these phenotypes. Cells were mock infected or infected with the phenotypically wild-type *dl309* virus or with the 19K mutant virus *dl337*, which contains a large deletion of 19K-coding sequences. The cells were observed microscopically for the *cyt* phenotype and are shown at 38 hpi in Fig. 2. The control cells infected with the wild-type virus, *dl309*, looked similar to mock-infected cells, whereas control cells infected with the 19K mutant *dl337* showed a cytopathic effect. However, the 19K⁺ cell lines (4A3 and 4B1) infected with *dl337* did not display the *cyt*

phenotype. We also assayed for complementation of the *deg* phenotype and found that, in contrast to the control cell lines (HeLa, 2A1, and 2D3), the cell lines transformed by the 19K gene (4A2, 4A3, and 4B1) did not exhibit the *deg* phenotype when infected with the 19K mutant virus *dl337* (data not shown). We conclude that the 19K protein made by the cell lines functions like its virally expressed counterpart.

Increased activity of transfected RNA polymerase II and III promoters. The E1B 19K protein stimulates expression from all of the adenovirus early promoters and the cellular hsp70 promoter when cotransfected into HeLa cells and stimulates expression from the viral late promoters in the presence of the E1A gene (19). We have now studied the effect of the 19K protein produced in the cell lines on the expression of adenovirus promoters and several other promoters. A 19K⁻ (2A1 or 2D3) and a 19K⁺ (4A3) cell line were transfected with a plasmid containing a promoter fused to the CAT reporter gene and harvested at 70 h posttransfection (hpt) for

FIG. 2. Complementation of an E1B mutant for the *cyt* phenotype by the 19K cell lines. 2D3 (19K⁻), 4A3 (19K⁺), and 4B1 (19K⁺) cell lines were mock infected or infected with *dl309* or *dl337* at a multiplicity of infection of 10 and incubated in the presence of 125 μ M ZnCl₂ and 1.25 μ M CdSO₄. Cells were photographed at 38 hpi. Magnification, $\times 200$.

assay of CAT activity (Table 2). A large increase in the expression of the adenovirus early promoters (E1A, E1B, E2e, E3, and E4) was observed in the 19K⁺ cell line compared with the control line, ranging from a 7- to 22-fold-higher level of expression in the 19K⁺ cell line. There was also a 4- to 10-fold increase in the expression of the late promoters (IX, IVa₂, major late promoter, and E2L).

The stimulatory effect of the 19K protein was not restricted to adenovirus promoters. Two other viral promoters, the SV40 early promoter linked to its enhancer and the human immunodeficiency virus type 1 long terminal repeat, as well as the promoter for the cellular hsp70, produced a higher level of CAT activity in a 19K-expressing cell line than in a control cell line (Table 2). The stimulation of the SV40 early promoter by the 19K protein is consistent with previous observations (31, 57), but in contrast to a previous report (57), we found that a functional enhancer was not absolutely required for the effect of the 19K protein, since a construct containing a mutant SV40 enhancer (dpm126) showed an increased level of CAT activity in the 19K⁺ cell line.

Previous work using plasmid cotransfection assays indicated that the 19K protein acts at or before the level of accumulation of mRNA in the cytoplasm. To confirm this result with our 19K-expressing cell line, E1A-directed CAT mRNA levels were measured by a riboprobe-RNase assay (Fig. 3A). In agreement with results of earlier studies, there

TABLE 2. Effect of the 19K gene on CAT expression

Promoter ^a	CAT activity ^b (U)		Fold increase (19K ⁺ /19K ⁻)
	19K ^{-c}	19K ^{+d}	
Adenovirus			
E1A CAT ^e	13.0	283.0	22
E1B CAT	0.8	5.8	7
IX CAT	0.6	4.3	7
IVa ₂ CAT	0.4	1.6	4
MLP CAT	2.1	20.3	10
E2L CAT	0.4	1.6	4
E2e CAT ^e	0.4	4.8	12
E3 CAT	2.9	35.7	12
E4 CAT	10.7	153.0	14
Other ^f			
SV ₂ CAT (enh ⁺)	203.0	4,748.0	23
dpm126 (enh ⁻)	0.5	4.0	8
HIV-1 CAT	1.7	46.4	27
hsp70 CAT	2.0	26.4	13

^a MLP, Major late promoter; enh⁺, enhancer containing; enh⁻, enhancer lacking; HIV-1, human immunodeficiency virus type 1.

^b Averages from three experiments except as noted otherwise.

^c 2A1 or 2D3 cells.

^d 4A3 cells.

^e Average from four experiments.

^f Average from two experiments.

FIG. 3. CAT mRNA and VA RNA₁ levels in a 19K⁻ and 19K⁺ cell line. (A) A 4- μ g sample of the E1ACAT plasmid was transfected into 2D3 (19K⁻) or 4A3 (19K⁺) cells. The cells were incubated in the presence of 125 μ M ZnCl₂ and 1.25 μ M CdSO₄, and at 70 hpt cells were harvested and total cytoplasmic RNA was prepared. A 5- μ g sample of RNA was hybridized to ³²P-labeled RNA complementary to CAT mRNA and then digested with RNase A and RNase T₁. Lane 1 contained total RNA isolated from 2D3 cells that were not transfected with the E1ACAT plasmid. ³²P-labeled *Hpa*II-digested pBR322 size markers are shown in lane 4, and undigested probe is shown in lane 5. (B) RNA was isolated from 293 cells (lane 1), 2A1 cells (lanes 2 and 3), or 4A3 cells (lanes 4 and 5) at 96 hpt. Cells were uninduced (lanes 1, 2, and 4) or incubated with 150 μ M ZnCl₂ and 1.5 μ M CdSO₄ (lanes 3 and 5) before RNA extraction. Total cytoplasmic RNA (5 μ g) was hybridized to ³²P-labeled antisense VA RNA₁ (+73 to -28, relative to the VA RNA₁ start site at +1) and treated with RNases. VA RNA₁ purified from wild-type adenovirus-infected cells was used as a control in the hybridization and RNase reactions (lane 6).

was a higher level of CAT mRNA in the 19K⁺ cell line (4A3) than in the control cell line (2D3). To determine whether the 19K protein could also influence the expression of a polymerase III-transcribed gene, the adenovirus VA RNA₁ gene was transfected into untreated or induced cells, and VA RNA₁ levels were analyzed by a riboprobe-RNase protection assay. Human 293 cells were also transfected as a control, since the transfected VA RNA₁ gene has been shown to be expressed at a high level in these cells (14). More VA RNA₁ was synthesized in the 19K⁺ cell line than in

FIG. 4. Immunoprecipitation of hsp70 and analysis of total cell protein. 2A1 cells (lanes 1 and 2), 4A3 cells (lanes 3 and 4), or 293 cells (lane 5) were labeled with [³⁵S]methionine 24 h after induction with 150 μ M ZnCl₂ and 1.5 μ M CdSO₄ (lanes 2 and 4). (A) Hsp70 protein immunoprecipitated from the labeled cell lysate; (B) total cell lysate. Proteins in panels A and B were separated by electrophoresis in a 15% SDS-polyacrylamide gel. The arrow indicates hsp70.

the control cell line (Fig. 3B). The level of VA RNA₁ was further elevated by the addition of heavy metals, which correlated with the increase in 19K protein levels in the 4A3 cell line (Fig. 1B).

Expression of an endogenous cellular gene in a 19K⁺ cell line. The 19K protein elevated expression from all transfected polymerase II- and III-transcribed promoters tested, including the promoter for the cellular hsp70 gene. We therefore asked whether the 19K protein could enhance expression of the endogenous hsp70 gene. There was no difference in the amount of hsp70 protein present in the control and the 19K⁺ cell line as detected by immunoprecipitation even when 19K levels were induced by the addition of heavy metals (Fig. 4A). As a control, hsp70 was also immunoprecipitated from 293 cells, which express a high level of hsp70 (32). We conclude that although the 19K protein increases expression from the hsp70 promoter when it is located on a transfected plasmid, it does not increase expression of the endogenous hsp70 protein. We also examined total cell protein by one-dimensional gel electrophoresis and at this level of analysis did not find any significant increases in the levels of cellular proteins in the 19K⁺ cell line in comparison with a control cell line (Fig. 8B). Further examination of total cell protein by two-dimensional gel electrophoresis also failed to reveal any dramatic changes in protein levels between the control (2A1 and 2D3) and 19K⁺ (4A3 and 4B1) cell lines (data not shown).

Increase in transcription rates from transfected promoters in a 19K⁺ cell line. The results presented thus far indicate that the 19K protein acts as a general activator of expression of transfected genes but does not regulate the expression of endogenous genes. To learn about the level of control at which the 19K protein acts, nuclei were isolated from E1ACAT-transfected control or 19K⁺ cells at various times posttransfection, and the rate of transcription was measured

FIG. 5. Transcription rate and plasmid DNA measurements in $19K^-$ and $19K^+$ cell lines. (A) Nuclei were isolated from 2A1 ($19K^-$) or 4A3 ($19K^+$) cells transfected with E1ACAT and incubated in the presence of $150 \mu M$ $ZnCl_2$ and $1.5 \mu M$ $CdSO_4$ at the times indicated. Labeled RNA was prepared and hybridized to $5 \mu g$ of single-stranded DNA probes immobilized on nitrocellulose filters. The probes detect sense (+) or antisense (-) actin RNA, sense (+) or antisense (-) CAT RNA, or transcription through M13 sequences. CAT activity in extracts prepared from a parallel set of transfected cells is shown. (B) 2A1 ($19K^-$) or 4A3 ($19K^+$) cells transfected in parallel with those used in panel A were harvested at the times shown. Denatured Hirt DNA isolated from these cells was spotted in twofold dilutions onto nitrocellulose filters and probed with ^{32}P -labeled antisense CAT RNA.

by a nuclear run-on assay (Fig. 5A). Because the 19K protein did not affect the expression of endogenous cellular genes (see above), cellular actin gene transcription was used to normalize the rate of CAT mRNA synthesis. The rate of CAT-specific transcription, as indicated by the CAT sense probe, was greater in the $19K^+$ cell line (4A3) than in the control cell line (2A1) at all times measured, indicating that the 19K protein enhanced the rate of transcription from transfected promoters.

Increased plasmid DNA levels in a $19K^+$ cell line. Because of the generality of the effect of the 19K protein on transcription and because some mutants in the 19K protein are associated with DNA degradation during infection, we measured the level of plasmid DNA in cells transfected in parallel with those used to analyze transcription rates. Hirt DNA was isolated at various times posttransfection and spotted in twofold dilutions onto nitrocellulose filters, which were probed with ^{32}P -labeled antisense CAT RNA. The levels of plasmid DNA in the two cell lines at 24 hpt were

equivalent, (Fig. 5B), indicating that the uptake of DNA in these cell lines was similar. In the 2A1 control cell line, the vast majority of plasmid DNA had disappeared by 76 hpt, with only about 1% of the plasmid DNA present at 24 hpt still remaining at 76 hpt. At 120 hpt, plasmid DNA was barely detectable, with only about 0.1% of the DNA still evident. In contrast, although much of the plasmid DNA was degraded in 4A3 cells expressing the 19K protein, a higher level of plasmid DNA was retained in this cell line. Approximately 3% of the level of plasmid DNA present at 24 hpt still remained in the $19K^+$ cell line at 76 hpt and approximately 0.5% remained at 120 hpt, so that there was two- to threefold more DNA present at 76 hpt and four- to fivefold more plasmid DNA at 120 hpt in the $19K^+$ cell line than in the control cell line. In other experiments, we observed an 8- to 16-fold increase in DNA levels at the latest time point (Fig. 6A and data not shown). This result suggested that the 19K protein stabilizes transfected plasmid DNA and raised the possibility that the increased rate of transcription in the $19K^+$ cell line (Fig. 5A) resulted from increased levels of plasmid DNA. The decline in plasmid DNA levels from 24 to 120 hpt was reflected by a decrease in CAT mRNA synthesis at late times.

The plasmid (E1ACAT) used in the transfection experiments shown in Fig. 5 and 6A was closed circular, supercoiled DNA. To determine whether the 19K protein could stabilize linearized DNA (the state of viral DNA), the E1ACAT plasmid was linearized by digestion with *PvuI* and transfected. Although it is difficult to predict the structure of this DNA once it is inside the transfected cell, this DNA was nevertheless stabilized by the 19K protein (Fig. 6B). There was at least eightfold more DNA in the $19K^+$ cell line than in the control line at 124 hpt, with a corresponding increase in CAT activity (Fig. 6D). We also tested whether the plasmid DNA had to be transcribed in order to be stabilized by measuring the DNA levels of the promoterless plasmid, pBACAT, which is transcriptionally inert (Fig. 6D). There was at least eightfold more pBACAT in the $19K^+$ cell line at 120 hpt (Fig. 6C), indicating that the 19K protein increases the stability of transfected DNA regardless of its transcriptional activity. The slightly higher level of plasmid DNA in the $19K^+$ cell line at the earliest time point (approximately twofold; Fig. 6A to C) probably reflects the evidently rapid rate of degradation because when this DNA was examined by Southern blotting, a large smear of DNA was apparent in both cell lines (not shown).

To rule out the possibility that the stabilizing effect of the 19K protein was specific for CAT sequences, we measured the levels of the VA RNA₁-encoding plasmid and found that the level of this plasmid was approximately eightfold higher in the $19K^+$ cell line than in the $19K^-$ cell line at 96 hpt (data not shown), comparable to the degree of stabilization observed for the CAT-containing plasmids. Another control experiment demonstrated that >95% of DNA was recovered in the Hirt fraction (data not shown), excluding the possibility that a population of plasmid DNA was not recovered by the Hirt extraction. We also found that the majority of plasmid DNA (>90%) was present in the nuclear fraction, where the additional templates would be accessible to the transcriptional machinery (data not shown).

Increased DNA levels account for increased mRNA synthesis. These results indicate that the 19K protein stabilizes transfected plasmid DNA, but does the increase in DNA stability account entirely for the large increase in CAT enzyme activity or is there another effect of the 19K protein at a later stage, perhaps at the transcriptional level? We

FIG. 6. Stability of supercoiled, linearized, and nontranscribed plasmid DNA in transfected 19K⁻ and 19K⁺ cell lines. 2A1 (19K⁻) or 4A3 (19K⁺) cells were transfected with closed, supercoiled E1ACAT (A), linearized E1ACAT (E1ACAT/PvuI) (B), or the promoterless, closed, supercoiled pBACAT plasmid (C). The cells were incubated in the presence of 125 μ M ZnCl₂ and 1.25 μ M CdSO₄; at 23, 68, or 124 hpt; parallel plates of cells were harvested and assayed for CAT activity or levels of CAT DNA. (A to C) Assays in which denatured Hirt DNA was spotted onto nitrocellulose in twofold dilutions of DNA and probed with ³²P-labeled RNA specific for CAT sequences; (D) CAT activity of corresponding extracts.

reasoned that if the amounts of plasmid DNA in the two cell types were equal, the rates of transcription should be equivalent if the 19K protein acts at the level of DNA stability and not directly at the transcriptional level. The data in Fig. 5 suggest that, at least at 76 and 120 hpt, the increase in plasmid DNA levels in the 19K⁺ cell line was reflected by a corresponding increase in transcription rates. At 24 hpt, however, there appeared to be a distinctly higher rate of transcription in the 19K⁺ cell line, although the levels of DNA were equivalent in the two cell lines. It is possible,

however, that the difference in transcription rates at 24 hpt could be a result of the rapid rate of degradation seen at early times, as discussed above, so that although the DNA levels appeared fairly similar by dot blot analysis, the DNA in the 19K⁻ control cell may already have been partly degraded and therefore not able to be transcribed. To get around this potential problem, various amounts of the E1ACAT plasmid was transfected into the 19K⁺ cells and a constant amount of the E1ACAT plasmid was transfected into the control cells in an attempt to achieve equal levels of plasmid DNA in both

FIG. 7. Plasmid DNA and transcription rate measurements in 19K⁻ and 19K⁺ cell lines transfected with different amounts of DNA. (A) 2A1 (19K⁻) cells were transfected with 25 μ g of the E1ACAT plasmid, and 4A3 (19K⁺) cells were transfected with 1.6, 3.1, 6.3, 12.5, or 25 μ g of the E1ACAT plasmid and incubated in the presence of 150 μ M ZnCl₂ and 1.5 μ M CdSO₄. Cells were harvested at 114 hpt (A). Hirt DNA was prepared and spotted onto nitrocellulose filters in twofold dilutions. The filters were probed for CAT-specific sequences. (B) Labeled RNA was hybridized to 5 μ g of single-stranded DNA probes that detect sense (+) or antisense (-) actin, sense (+) or antisense (-) CAT RNA, and M13. CAT activity assayed in corresponding extracts is shown at the bottom.

cell lines at late times posttransfection, when the rate of degradation appeared to be less rapid. In the experiment shown in Fig. 7A, the control 2A1 and 19K⁺ 4A3 cells contained similar levels of DNA at 114 hpt when the 4A3 cells were transfected with fourfold less DNA than the 2A1 control cells. Analysis of transcription rates from a parallel set of transfected cells showed that the rates of transcription in the cells containing equal levels of plasmid DNA were comparable (Fig. 7B). This results argues that the 19K protein acts at the level of DNA stability and does not directly influence the rate of transcription.

Increased transcription and viral DNA levels by the 19K protein during viral infection. The 19K protein also positively regulates gene expression during viral infection in the absence of the E1A 13S or 12S products (19, 51), and since the 19K protein normally functions in the context of viral infection, it was important to determine whether the 19K protein acts at the transcriptional level during viral infection. HeLa cells were infected with a virus containing the E1ACAT chimeric gene in place of the E1 region or coinfecting with this E1ACAT virus together with either the 9S virus or the 9S.R2 virus. The 9S virus contains the E1A 9S cDNA in place of the genomic E1A gene and expresses a wild-type 19K protein, whereas the otherwise identical 9S.R2 virus contains an amino acid substitution in the 19K protein. The 19K protein increased the rate of transcription from the E2, E4, L1, and L3 regions and from the E1A promoter, as measured by CAT mRNA synthesis (Fig. 8A). We found that the level of viral E1ACAT DNA was higher in cells coinfecting with the 19K-expressing 9S virus than in cells coinfecting with the 19K mutant virus or infected with E1ACAT alone (Fig. 8C). Therefore, the 19K protein produced an increase in the level of viral DNA even though the efficiency of replication in the presence of the 9S virus was less than 1% that of the wild-type virus (data not shown). The 9S.R2 virus allowed even less replication to occur but, somewhat surprisingly, was not completely defective. In contrast, the E1ACAT virus on its own did not replicate at all, presumably because of the absence of all E1-region gene products.

These data suggest that the increased rate of transcription resulted from increased levels of viral DNA. To determine whether the 19K protein affected transcription rates directly, we held the level of viral DNA constant by inhibiting DNA replication with hydroxyurea. The levels of viral DNA in the presence and absence of the 19K protein were comparable (Fig. 8D). We found that the rates of transcription of adenovirus early and late genes under these conditions were quite similar, with possibly a small increase in E2 and E4 transcription at 34 hpi, which was no longer evident by 72 hpi (Fig. 8B). In agreement with this result, White et al. (51) detected little difference in the levels of the E2 72K DNA-binding protein in 9S- or 9S.R2-infected cells when viral DNA replication was inhibited. We conclude that in infections as in transfection, the 19K protein does not have a significant effect on transcription but acts primarily to increase the level of viral DNA.

DISCUSSION

Transformation of primary cells by adenovirus requires two genes, the E1A and E1B oncogenes. Products of both of these genes regulate the expression of other viral genes as well as of cellular genes. Although the mechanism of action of the E1A gene products is by no means fully understood, the E1A proteins act at the transcriptional level, and much

progress has been made in identifying cellular factors that interact with E1A-inducible promoters. The action of the E1B 19K protein is less well understood. Although it has been shown that the 19K protein regulates viral gene expression, the level at which it functions has not been firmly established. To allow us to study the mechanism of action of the 19K protein, we required a system in which the 19K protein is efficiently expressed and in which we could detect a large stimulation of gene expression. The cell lines we have constructed provide such a system. Stimulation of the adenovirus early promoters and the hsp70 promoter was substantially greater with use of the cell lines that was detected in our earlier cotransfection experiments (>20-fold versus approximately 5-fold for E1ACAT) (19). Furthermore, an effect on the late promoters was detectable in the cotransfection experiments only when the 19K protein was expressed in conjunction with the E1A 13S product, which boosts 19K expression. In the cell lines, however, there was a 4- to 10-fold increase in late promoter expression even in the absence of E1A gene products. This finding is in agreement with the results of Vales and Darnell (47), who observed a 19K-dependent increase in the levels of late polypeptide IX mRNA as well as in levels of other E1B transcripts. It is apparent that the cell lines offer a greater sensitivity, presumably because there is no lag in 19K expression in the cell lines as occurs when the 19K protein is expressed from a transfected plasmid.

We believe that the effects on DNA stability and gene expression reported here are due to an effect of the 19K protein rather than to clonal variations in the cell lines for several reasons. First, three different 19K⁺ cell lines (4A3, 4B1, and 6E) express higher levels of CAT activity than do two control cell lines (2A1 and 2D3) or the parental HeLa cell line (data not shown). Although the 19K⁺ cell line (4A3) used in most of the experiments reported here can theoretically synthesize the N-terminal portion of the 55K protein, a truncated 55K protein cannot be responsible for the effect on DNA stability and gene expression because 6E cells, which are unable to synthesize the 55K protein, retained increased levels of plasmid DNA and produced an increased level of CAT activity (not shown). Second, while the 19K⁺ cells express higher levels of CAT activity and VA RNA₁ levels than do the control cells, even in the absence of added zinc and cadmium ions, CAT activity and VA RNA₁ levels are further stimulated after induction with heavy metals in the 19K⁺ cells but not in the 19K⁻ cells, corresponding to an increased level of 19K protein in the 19K⁺ cell lines (Fig. 1B and 3B and data not shown).

The chief conclusion of this study is that the 19K protein acts as a general activator of genes newly introduced into cells by transfection or infection. (However, this effect is overridden during infection by products of the E1A gene [52].) The stabilization of plasmid DNA by the 19K protein accounts for the nonspecific increase in expression of polymerase II- and III-transcribed genes, whereas there is no effect on the expression of cellular genes in their normal chromosomal context.

Increasing the stability of plasmid DNA is a novel mechanism whereby an oncogene can increase the expression of foreign genes. As shown here and reported previously by Alwine (1), plasmid DNA is highly unstable in transfected cells. Therefore, increasing the persistence of plasmid DNA allows more DNA to be available for transcription, especially at late times posttransfection. This result is consistent with our observation that the largest stimulation of gene expression by the 19K⁺ protein occurs at late times (Fig. 5A

FIG. 8. Transcription rates and viral DNA levels in infected cells. Cells were infected with the E1ACAT virus, coinfecting with the E1ACAT and 9S (19K⁺) viruses, or coinfecting with the E1ACAT and 9S.R2 (19K⁻) viruses at a multiplicity of infection of 50 in the absence (A and C) or presence (B and D) of hydroxyurea, and cells were harvested at 34 or 72 hpi. (A and B) Nuclei were isolated, and labeled RNA was prepared and hybridized to single-stranded M13 clones that detect sense RNA from the E2, E4, L1, and L3 regions of adenovirus or E1A promoter-directed CAT sense (+) or antisense (-) RNA. Control probes were M13 and denatured pBR322 and actin plasmids. CAT activity from corresponding extracts is shown. (C and D) Denatured Hirt DNA was spotted onto nitrocellulose filters in fivefold dilutions and hybridized to ³²P-labeled antisense CAT RNA.

and 6D; 19), although the effect of the 19K protein as measured by CAT enzymatic activity is probably exaggerated because the stable CAT protein is allowed to accumulate over the period of several days. Alwine (1) has also observed an increase in gene expression as a result of increased plasmid stabilization, using 293 cells which constitutively express E1A and E1B proteins. Although it was not emphasized and did not separate out effects of different

E1B products, a similar, albeit smaller, effect was also apparent in KB cells that stably express the E1A and E1B genes or the E1B gene alone relative to KB cells that do not contain the E1B gene. The ability of the 19K protein to stabilize plasmid DNA is consistent with the fact that the 19K protein is known to protect viral and chromosomal DNA from degradation. It has been reported that the appearance of the *deg* phenotype

during productive infection is dependent on the expression of the E1A 13S or 12S products, but since the experiments reported here were performed in the absence of E1A products, the presence of E1A proteins cannot be required for the stabilization effect. It is possible that infection by adenovirus under conditions in which the E1A 13S or 12S products are expressed results in the induction of a cellular nuclease, so that the stabilizing function of the 19K protein becomes more readily apparent.

It seems likely that the mechanism by which the 19K protein is acting in transfected cells is similar to that in viral infection. One way the 19K protein could increase the level of viral DNA is by acting directly in the replication process, but this seems unlikely, since efficient DNA synthesis occurs in reconstituted *in vitro* systems in the absence of the 19K protein (40). Furthermore, plasmid DNA does not contain a known origin of replication, and an analysis of transfected plasmid DNA by digestion with restriction enzymes that distinguish between replicated and nonreplicated templates did not reveal any evidence for plasmid replication (not shown). Alternatively, the 19K protein could increase viral DNA copy number under replicating conditions by stabilizing the newly replicated DNA. It is possible that newly replicated DNA, like plasmid DNA, is susceptible to degradation by a cellular nuclease.

The increase in DNA stability suggests a role that the 19K tumor antigen may play in the transformation process. It is clear that the E1B gene alone is not sufficient to induce transformation and that whereas transformation normally requires the cooperation of the E1A and E1B genes, overexpression of the E1A gene, directed by the strong metallothionein promoter, allows E1A on its own to induce the phenotypic changes associated with transformation (38). It is possible that expression of the E1A gene in its normal context, especially in plasmid transfection experiments, may not be high enough to bring about the transformed phenotype. Perhaps the role of the 19K protein in DNA-mediated transformation is to increase the copy number of E1A templates so that a higher level of E1A proteins may be expressed. Interestingly, the *ras* gene, which can substitute for the E1B gene in a plasmid transformation assay (37), has also been reported to increase DNA stability (33), suggesting that these two oncogenes may operate by similar mechanisms in cooperating with E1A to transform cells.

How does the 19K protein stabilize DNA? We considered several possible mechanisms. First, the 19K protein could act directly by binding to DNA and physically protecting it from degradation, but this appears unlikely since there is no evidence that the 19K protein binds to DNA. Second, the 19K protein could interact with and block the action of a cellular nuclease. Although there is no evidence that the 19K protein forms a stable complex with a cellular protein (unpublished results), the 19K protein may interact transiently with a cellular nuclease to inactivate it. Third, the 19K protein could bring about a more general change in the cell that leads to increased DNA stability. Since nuclear envelope localization of the 19K protein is correlated with its function in blocking the appearance of the *deg* phenotype (50), we believe it likely that the localization of the 19K protein is important for the stabilization of DNA by virtue of its ability to alter the structure or distribution of plasmid and viral DNA. E. White and R. Cipriani (personal communication) have demonstrated that the 19K protein perturbs the structure of the nuclear lamina and the intermediate filament network. This change in nuclear lamina organization may compartmentalize or in some other manner protect DNA

from degradation. Elucidation of the mechanism of DNA stabilization by the 19K protein awaits further study.

Finally, since the 19K protein increases gene expression nonspecifically by increasing plasmid DNA levels, the cell lines described here may be useful in transient and stable transfection assays. For example, these cell lines may provide a more sensitive approach to the study of the regulation of weak promoters by boosting basal-level expression of these promoters or may aid in the establishment of permanent cell lines.

ACKNOWLEDGMENTS

We thank Ronnie Packer for excellent assistance with the cell work and Jim Duffy and Dave Greene for art work and photography.

This work was supported by Public Health Service program project grant CA13106 from the National Cancer Institute.

LITERATURE CITED

1. Alwine, J. C. 1985. Transient gene expression control: effects of transfected DNA stability and *trans*-activation by viral early proteins. *Mol. Cell. Biol.* 5:1034-1042.
2. Anderson, C. W., R. C. Schmitt, J. E. Smart, and J. B. Lewis. 1984. Early region 1B of adenovirus 2 encodes two coterminal proteins of 495 and 155 amino acid residues. *J. Virol.* 50:387-396.
3. Babiss, L. E., P. B. Fisher, and H. S. Ginsberg. 1984. Effect on transformation of mutations in the early region 1B-encoded 21- and 55-kilodalton proteins of adenovirus 5. *J. Virol.* 52:389-395.
4. Barker, D. B., and A. J. Berk. 1987. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. *Virology* 156:107-121.
5. Berk, A. J. 1986. Functions of adenovirus E1A. *Cancer Surv.* 5:367-387.
6. Bernards, R., M. G. W. de Leeuw, A. Houweling, and A. J. van der Eb. 1986. Role of the adenovirus early region 1B tumor antigens in transformation and lytic infection. *Virology* 150:126-139.
7. Bos, J. L., L. J. Polder, R. Bernards, P. I. Schrier, P. J. van den Elsen, A. J. van der Eb, and H. van Ormondt. 1981. The 2.2 kb E1B mRNA of human adenovirus 12 and adenovirus 5 codes for two tumor antigens starting at different AUG triplets. *Cell* 27:121-131.
8. Chinnadurai, G. 1983. Adenovirus 2 *lp*⁺ locus codes for a 19 kd tumor antigen that plays an essential role in cell transformation. *Cell* 33:759-766.
9. Chinnadurai, G., S. Chinnadurai, and J. Brusca. 1979. Physical mapping of a large plaque mutation of adenovirus type 2. *J. Virol.* 32:623-628.
10. D'Halluin, J. C., C. Allart, C. Cousin, P. A. Boulanger, and G. Martin. 1979. Adenovirus early function required for the protection of viral and cellular DNA. *J. Virol.* 32:61-71.
11. Déry, C. V., C. H. Herrmann, and M. B. Mathews. 1987. Response of individual adenovirus promoters to the products of the E1A gene. *Oncogene* 2:15-23.
12. Esche, H., M. B. Mathews, and J. B. Lewis. 1980. Proteins and messenger RNAs of the transforming region of wild-type and mutant adenoviruses. *J. Mol. Biol.* 142:399-417.
13. Ezoe, H., R. B. Lai Fatt, and S. Mak. 1981. Degradation of intracellular DNA in KB cells infected with *cyt* mutants of human adenovirus type 12. *J. Virol.* 40:20-27.
14. Gaynor, R. B., L. T. Feldman, and A. J. Berk. 1985. Transcription of class III genes activated by viral immediate early proteins. *Science* 230:447-450.
15. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
16. Graham, F. L., J. Smiley, W. C. Russell, and R. Nairu. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36:59-77.
17. Greenberg, M. E., and E. B. Ziff. 1984. Stimulation of 3T3 cells

- induces transcription of the c-fos proto-oncogene. *Nature* (London) **311**:433-438.
18. Herr, W., and J. Clarke. 1986. The SV40 enhancer is composed of multiple functional elements that can compensate for one another. *Cell* **45**:461-470.
 19. Herrmann, C. H., C. V. Déry, and M. B. Mathews. 1987. Transactivation of host and viral genes by the adenovirus E1B 19K tumor antigen. *Oncogene* **2**:25-35.
 20. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
 21. Jones, N., and T. Shenk. 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. *Proc. Natl. Acad. Sci. USA* **76**:3665-3669.
 22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **227**:680-685.
 23. Lai Fatt, R. B., and S. Mak. 1982. Mapping of an adenovirus function involved in the inhibition of DNA degradation. *J. Virol.* **42**:969-977.
 24. Lewis, J. B., and C. W. Anderson. 1987. Identification of adenovirus type 2 early region 1B proteins that share the same amino terminus as do the 495R and 155R proteins. *J. Virol.* **61**:3879-3888.
 25. Lucher, L. A., K. H. Brackmann, J. S. Symington, and M. Green. 1984. Antibody directed to a synthetic peptide encoding the NH₂-terminal 16 amino acids of the adenovirus type 2 E1B-53K tumor antigen recognizes the E1B-20K tumor antigen. *Virology* **132**:217-221.
 26. Matsuo, T., W. S. M. Wold, S. Hashimoto, A. Rankin, J. Symington, and M. Green. 1982. Polypeptides encoded by transforming region E1B of human adenovirus 2: immunoprecipitation from transformed and infected cells and cell-free translation of E1B-specific mRNA. *Virology* **118**:456-465.
 27. McGlade, C. J., M. L. Tremblay, S.-P. Yee, R. Ross, and P. E. Branton. 1987. Acylation of the 176R (19-kilodalton) early region 1B protein of human adenovirus type 5. *J. Virol.* **61**:3227-3234.
 28. Mellits, K. H., and M. B. Mathews. 1988. Effects of mutations in stem and loop regions on the structure and function of adenovirus VA RNA₁. *EMBO J.* **7**:2849-2859.
 29. Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk. 1984. Control of adenovirus E1B mRNA synthesis by a shift in the activation of RNA splice sites. *Mol. Cell. Biol.* **4**:966-972.
 30. Moran, E., T. Grodzicker, R. J. Roberts, M. B. Mathews, and B. Zerler. 1986. Lytic and transforming functions of individual products of the adenovirus E1A gene. *J. Virol.* **57**:765-775.
 31. Natarajan, V. 1986. Adenovirus-2 E1A and E1B gene products regulate enhancer mediated transcription. *Nucleic Acids Res.* **14**:9445-9456.
 32. Nevins, J. R. 1982. Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. *Cell* **29**:913-919.
 33. Pater, A., and M. M. Pater. 1988. Expression of the control elements of BK and SV40 viruses in human cells exhibiting different transformed phenotypes. *Virology* **163**:625-628.
 34. Pilder, S., J. Logan, and T. Shenk. 1984. Deletion of the gene encoding the adenovirus 5 early region 1B 21,000-molecular-weight polypeptide leads to degradation of viral and host cell DNA. *J. Virol.* **52**:664-671.
 35. Riabowol, K. T., L. A. Mizzen, and W. J. Welch. 1988. Heat shock is lethal to fibroblasts microinjected with antibodies against hsp70. *Science* **242**:433-436.
 36. Rosen, C. A., J. G. Sodroski, and W. A. Haseltine. 1985. The location of *cis*-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. *Cell* **41**:813-823.
 37. Ruley, H. E. 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature* (London) **304**:602-606.
 38. Senear, A. W., and J. B. Lewis. 1986. Morphological transformation of established rodent cell lines by high-level expression of adenovirus type 2 E1A gene. *Mol. Cell. Biol.* **6**:1253-1260.
 39. Spector, D. J., M. McGrogan, and H. J. Raskas. 1978. Regulation of the appearance of cytoplasmic RNAs from region 1 of the adenovirus 2 genome. *J. Mol. Biol.* **126**:395-414.
 40. Stillman, B. 1983. The replication of adenovirus DNA with purified proteins. *Cell* **35**:7-9.
 41. Stillman, B. 1986. Functions of the adenovirus E1B tumor antigens. *Cancer Surv.* **5**:389-404.
 42. Subramanian, T., M. Kuppuswamy, J. Gysbers, S. Mak, and G. Chinnadurai. 1984. 19-kd tumor antigen coded by early region 1B of adenovirus 2 is required for efficient synthesis and for protection of viral DNA. *J. Biol. Chem.* **259**:11777-11783.
 43. Subramanian, T., M. Kuppuswamy, S. Mak, and G. Chinnadurai. 1984. Adenovirus *cyt*⁺ locus, which controls cell transformation, is an allele of *lp*⁺ locus, which codes for a 19-kilodalton tumor antigen. *J. Virol.* **52**:336-343.
 44. Takemori, N., C. Cladaras, B. Bhat, A. J. Conley, and W. S. M. Wold. 1984. *cyt* gene of adenovirus 2 and 5 is an oncogene for transforming function in early region E1B and encodes the E1B 19,000-molecular-weight polypeptide. *J. Virol.* **52**:793-805.
 45. Takemori, N., J. L. Riggs, and C. Aldrich. 1968. Genetic studies with tumorigenic adenoviruses. I. Isolation of cytotoxic (cyt) mutants of adenovirus type 12. *Virology* **36**:575-586.
 46. Takemori, N., J. L. Riggs, and C. Aldrich. 1969. Genetic studies with tumorigenic adenoviruses. II. Heterogeneity of *cyt* mutants of adenovirus type 12. *Virology* **38**:8-15.
 47. Vales, L. D., and J. E. Darnell. 1989. Promoter occlusion prevents transcription of adenovirus polypeptide IX until after DNA replication. *Genes Dev.* **3**:49-59.
 48. van den Elsen, P. J., A. Houweling, A. J. van der Eb. 1983. Morphological transformation of human adenoviruses is determined to a large extent by gene products of region E1A. *Virology* **131**:242-246.
 49. Virtanen, A., and U. Pettersson. 1985. Organization of early region 1B of human adenovirus type 2: identification of four differently spliced mRNAs. *J. Virol.* **54**:383-391.
 50. White, E., S. H. Blose, and B. W. Stillman. 1984. Nuclear envelope localization of an adenovirus tumor antigen maintains the integrity of cellular DNA. *Mol. Cell. Biol.* **4**:2865-2875.
 51. White, E., A. Denton, and B. Stillman. 1988. Role of the adenovirus E1B 19,000-dalton tumor antigen in regulating early gene expression. *J. Virol.* **62**:3445-3454.
 52. White, E., B. Faha, and B. Stillman. 1986. Regulation of adenovirus gene expression in human WI38 cells by an E1B-encoded tumor antigen. *Mol. Cell. Biol.* **6**:3763-3773.
 53. White, E., T. Grodzicker, and B. W. Stillman. 1984. Mutations in the gene encoding the adenovirus early region 1B 19,000-molecular-weight tumor antigen cause degradation of chromosomal DNA. *J. Virol.* **52**:410-419.
 54. White, E., and B. Stillman. 1987. Expression of adenovirus E1B mutant phenotypes is dependent on the host cell and on synthesis of E1A proteins. *J. Virol.* **61**:426-435.
 55. Wilson, M. C., and J. E. Darnell, Jr. 1981. Control of messenger RNA concentration by differential cytoplasmic half-life. Adenovirus messenger RNAs from transcription units 1A and 1B. *J. Mol. Biol.* **148**:231-251.
 56. Wu, B., R. E. Kingston, and R. Morimoto. 1986. Human HSP70 promoter contains at least two distinct regulatory domains. *Proc. Natl. Acad. Sci. USA* **83**:629-633.
 57. Yoshida, K., L. Venkatesh, M. Kuppuswamy, and G. Chinnadurai. 1987. Adenovirus transforming 19-kd T antigen has an enhancer-dependent *trans*-activation function and relieves enhancer repression mediated by viral and cellular genes. *Genes Dev.* **1**:645-658.