MOLECULAR AND CELLULAR BIOLOGY, Aug. 1986, p. 2994–2999
0270-7306/86/082994-06$02.00/0

Vol. 6, No. 8

The E1A 13S Product of Adenovirus 5 Activates Transcription of the Cellular Human HSP70 Gene

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Received 18 March 1986/Accepted 13 May 1986

Expression of the human gene encoding the major heat shock protein, HSP70, was induced during cell growth by serum stimulation and after infection with adenovirus 5. In this study we showed that HSP70 gene expression could be induced by adenovirus 5 infection, even in the absence of exogenous serum factors. Whereas serum stimulation induced the expression of the endogenous HSP70 gene, it had no effect on early adenovirus promoters. However, expression of both the cellular HSP70 gene and the adenovirus E3 promoter were activated during adenovirus infection. By using a collection of reconstructed mutant viruses, we identified the 13S product of the E1A region as the specific transcriptional *trans*-activator of the HSP70 gene.

Although the sequence of events during productive adenovirus infection of human cells has been well documented (36), little is known about the events leading to adenovirus-mediated cellular transformation. Genetic studies have revealed that the viral E1A gene is necessary for cellular transformation (6, 14, 18, 22, 30, 32). E1A has complex effects on transcription: it stimulates transcription of early adenoviral (1, 17, 23, 26–28) and cellular (9, 12, 19, 25, 34, 35) promoters but represses the effects of viral enhancers (4, 37). A particularly attractive hypothesis for the mechanism by which viral transforming gene products mediate cellular transformation predicts that this *trans*-regulatory activity may alter cellular gene expression. Specifically, those genes promoting cell growth would be stimulated, and genes restricting cell growth would be repressed. To date, four endogenous cellular genes have been shown to be responsive to E1A: a rat class I MHC gene is repressed (31), whereas a human β-tubulin gene (34), a human HSP70 gene (19, 25), and a mouse MHC H-2K gene (29) are stimulated. The induced synthesis of human HSP70 during adenovirus 5 (Ad5) infection is dependent on E1A gene expression and is due to an increase in both the rate of transcription and the accumulation of HSP70 mRNA (19, 25).

The human HSP70 gene is a good candidate for a cellular gene that is growth regulated and induced after virus infection. Expression of human HSP70 has been shown to be stimulated during Ad5 infection (19, 25), and HSP70 is expressed at high levels in Ad5-transformed human embryonic kidney cells (cell line 293) (11, 25, 40). The expression of the HSP70 gene is induced by serum stimulation in parallel with an increase in cellular DNA synthesis in two human cell lines, HeLa and 293 (41). Hence, the human HSP70 gene serves as an example of a gene whose expression is regulated during cell growth and is also responsive to E1A *trans*-regulation. Here we show that HSP70 gene expression can be induced by Ad5 infection, even in the absence of exogenously supplied serum and the growth factors therein. Furthermore, this induction is dependent on the Ad5 E1A 13S product, which confers both transcriptional activation and cellular immortalization functions.

To distinguish induction of HSP70 gene expression by Ad5 infection from that elicited by serum stimulation, we used three culture conditions for Ad5 infection of HeLa cells: (i) conditioned medium, (ii) fresh medium, and (iii) serum-free medium. HeLa cells were grown to 80% confluence, and the medium was saved (conditioned medium). The cells were either mock treated or infected with Ad5 at a multiplicity of infection of 10 PFU per cell for 30 min in serum-feee medium. After mock or viral infection, the cells were fed either conditioned medium, fresh medium containing 10% serum, or serum-free medium. To facilitate our analysis, we used a HeLa cell line, 27-T, derived by cotransfection with the pSV2neo selectable marker and pKCAT23, in which expression of chloramphenicol acetyltransferase (CAT) is dependent on the E3 promoter (38). This allowed us to simultaneously examine expression of the endogenous HSP70 gene and E1A-dependent activation of the E3 promoter. At various times postinfection (p.i.), the cells were harvested, total cytoplasmic RNA was prepared, and the levels of HSP70 mRNA were determined by the S1 nuclease protection method (2) by using a cloned probe to protect a 150-nucleotide 5′ terminal fragment (40). The addition of fresh serum to the mock-infected cells stimulated the level of HSP70 mRNA (Fig. 1A, lanes 7 through 10), thus masking the effect of adenovirus infection (Fig. 1B, lanes 7 through 10). An 8.5-fold increase in the level of HSP70 mRNA was observed by 16 h p.i. with Ad5 relative to the level at 0 h p.i. However, in mock-infected cells at 16 h p.i., HSP70 mRNA levels were induced sixfold as a result of serum stimulation alone. Therefore, the level of induction of HSP70 by Ad5 infection above that induced by serum stimulation in mock-treated cells was only 1.4-fold. These results are quantitated and presented in Fig. 1D.

To examine the effect of adenovirus infection without serum stimulation of the HSP70 gene, we used two culture conditions: saved (conditioned) or serum-free medium. Relative to the level in mock-infected cells, the level of HSP70 induction in adenovirus-infected cells cultured in saved medium was fourfold greater (Fig. 1A and B, lanes 3 through 6) and was even more pronounced (5.5-fold) in cells cultured in serum-free medium (Fig. 1A and B, lanes 11 and 12) Thus, the different culture conditions modulated the basal level of HSP70 gene expression but not its Ad5 responsiveness (Fig. 1C and D). The effects of serum stimulation and adenovirus infection on HSP70 gene expression do not appear to be additive (Fig. 1C through E). We conclude from these

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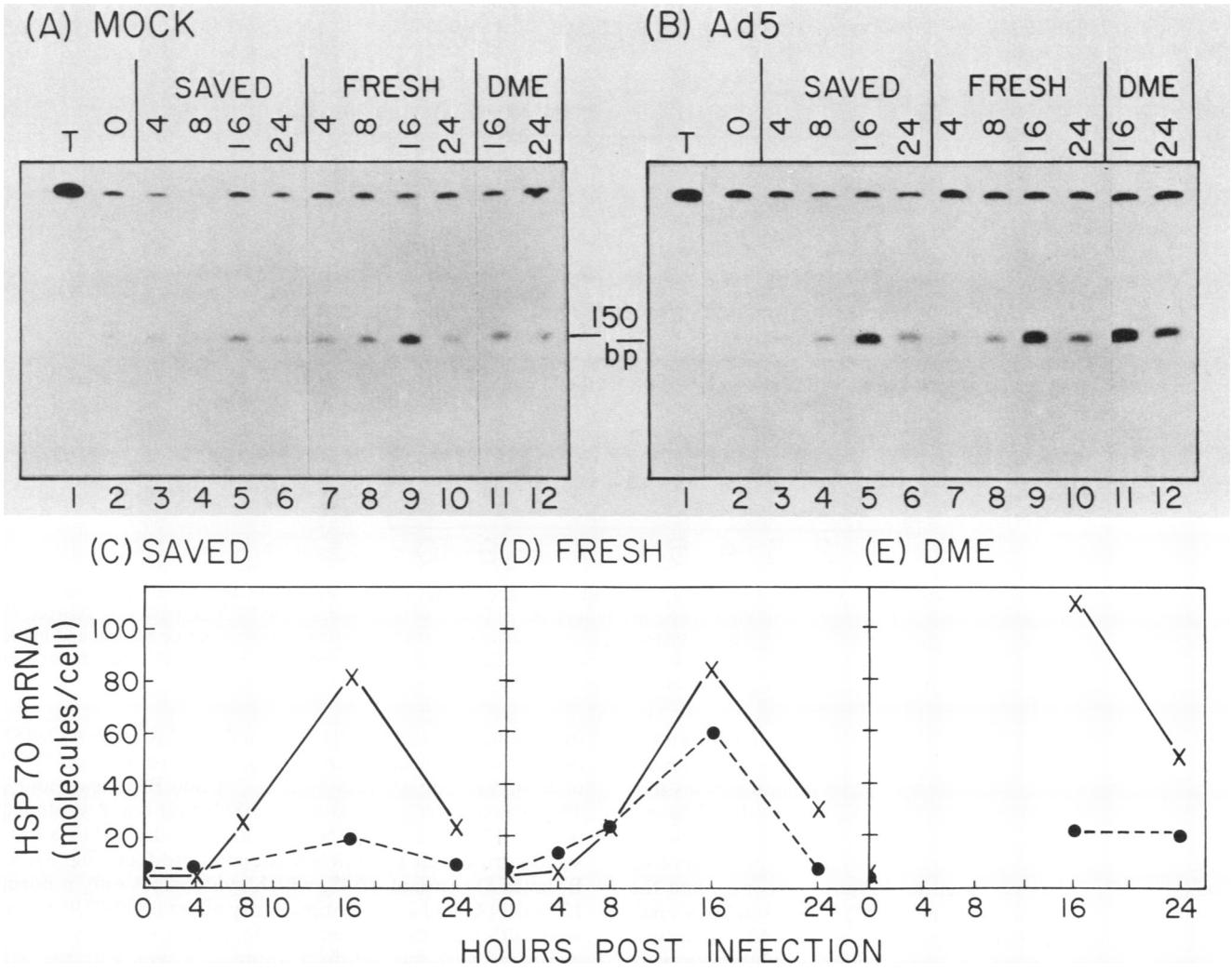

FIG. 1. Induction of human HSP70 gene during mock (A) or Ad5 (B) infection of HeLa 27-T cells (containing integrated E3-CAT genes; 13) under three culture conditions: saved serum-supplemented medium (SAVED), fresh serum-supplemented medium (FRESH), or serum-free medium (DME). HeLa cells were grown in serum-supplemented medium, Dulbecco modified Eagle medium (DME) plus 10% fetal calf serum, to 80% confluence. The medium was removed and saved. Ad5-infected cells were washed with Dulbecco modified Eagle medium and incubated with virus at a multiplicity of infection of 10 PFU per cell in Dulbecco modified Eagle medium for 30 min. Mock-infected cells were similarly treated in the absence of virus. The cells were then incubated with the indicated culture medium. At the indicated hours p.i. cytoplasmic RNA was prepared, and 10 μg was used to measure the level of HSP70 mRNA by the S1 nuclease protection method.(2). The ^{32}P-labeled DNA probe (T) used for S1 nuclease analysis was a 450-base-pair (bp) *Bam*HI-*Hin*dIII fragment, 5′ end labeled at the *Bam*HI site, which contains 300 base pairs of upstream flanking and 150 base pairs of nontranslated HSP70 sequences. (This fragment was obtained from plasmid pH-17, a deletion construct of the HSP70-CAT fusion gene [42].) The probe results in a 150-base-pair S1-nuclease-resistant product. HSP70 mRNA levels were determined by scanning densitometric measurements (C through E). Symbols: ×, Ad5-infected cells; ●, mock-infected cells. Autoradiograms of varying exposure times were scanned, and the approximate number of HSP70 mRNA molecules per cell was calculated from the relative intensity of marker equivalents. For example, the sample (T) applied in lanes 1 of panels A and B contained 2×10^8 molecules. Ten micrograms of cytoplasmic RNA corresponds to approximately 10^6 cells. Therefore, this marker corresponded to 200 molecules per cell in 10^6 cells.

experiments that the adenovirus-induced expression of the HSP70 gene is masked by serum-stimulated expression under standard conditions of viral infection. Fortunately, we were able to identify conditions in which adenovirus induction of HSP70 gene expression can be distinguished from serum-stimulated expression. This reveals that the induction of HSP70 mRNA can occur independently of serum-stimulated activation of the cellular HSP70 promoter.

We monitored the process of adenovirus infection by two means: the expression of E3-CAT activity and the synthesis of late viral proteins. Samples of the cells analyzed in the experiments depicted in Fig. 1 were prepared for enzymatic CAT assays (10) or were labeled in vivo with [^{35}S]-methionine. Under all conditions of adenovirus infection, activation of the E3 promoter was detected (Fig. 2B) and similar levels of late viral proteins were synthesized (data not shown). The E3 promoter was not activated in mock-infected cells. Therefore, serum stimulation alone does not provide a cellular E1A-like activity. The effects of serum stimulation and adenovirus infection on E3-CAT activity and HSP70 gene expression are shown in Table 1.

Previous studies have suggested that activation of the HSP70 gene requires expression of the adenovirus E1A region (19, 25). The E1A transcription unit encodes three

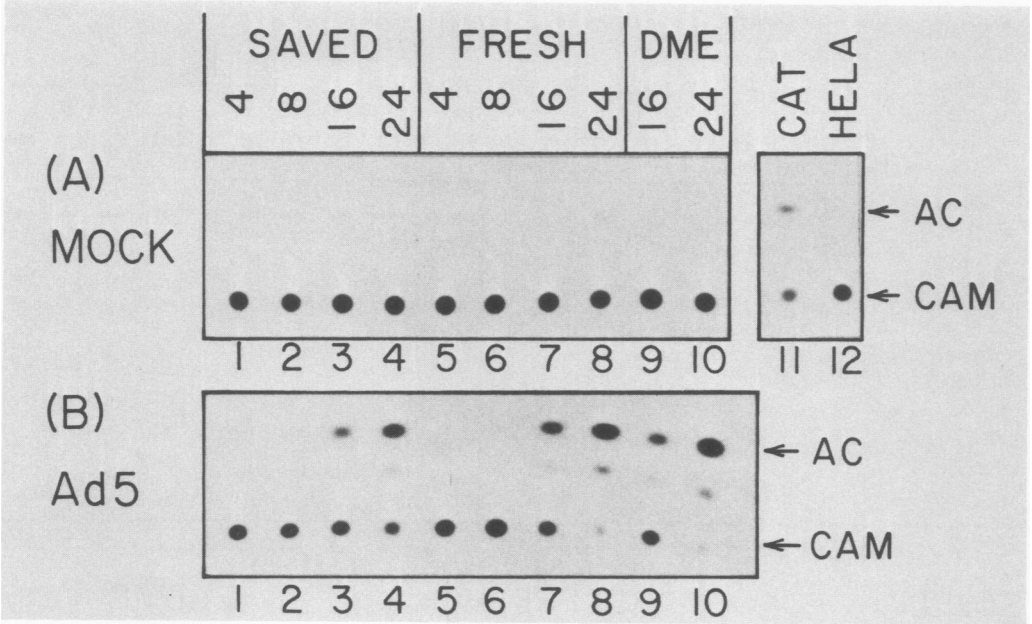

FIG. 2. Expression of integrated E3-CAT genes in mock (A)- or Ad5 (B)-infected HeLa 27-T cells under the three culture conditions described in the legend to Fig. 1. Extracts of cells from the experiment depicted in Fig. 1 were prepared, and CAT activity was assayed (10) by incubating 2 nmol of [^{14}C]chloramphenicol, 520 μM acetyl coenzyme A, and 50 μg of extract for 1 h at 37°C. A positive control and marker was provided by the bacterial enzyme (CAT), and a negative control was provided by the HeLa cell extract (HELA). The unacetylated substrate (CAM) and diacetylated (AC) forms of chloramphenicol are indicated by arrows.

mRNA species of 13S, 12S, and 9S (3, 7, 33). To determine which of these products is required for *trans*-activation of the HSP70 gene, we used a collection of mutant viruses that differentially express the E1A mRNA species. In all cases, the cells were infected with virus stocks at a multiplicity of infection of 10 PFU per cell and cultured in serum-free medium. RNA was prepared at various times p.i., and the level of HSP70 mRNA was measured by S1 nuclease analysis. In addition, expression of the E3-CAT gene was monitored for CAT activity. No temporal differences were observed through 24 h p.i. The data for 16 h p.i. are presented in Fig. 3. As expected, infection of 27-T cells with the virus *dl*312 (18), which lacks a functional E1A gene, did not result in activation of either the HSP70 or E3-CAT gene (Fig. 3A and B, lanes 1 through 3). Similarly, mutant viruses that could only express the 12S and 9S E1A mRNAs (*dl*520) or the 9S mRNA alone (*dl*526) also failed to activate HSP70 or E3-CAT gene expression (Fig. 3A and B, lanes 5 and 9). This was also true of a mutant (*dl*522) that could synthesize normal 12S- and 9S-mRNA-encoded products but synthesized a truncated 13S-mRNA-encoded product (Fig. 3A and B, lanes 8). Mutant *dl*347, which synthesizes only a normal 12S mRNA, had a slight stimulatory effect on HSP70 expression, although the maximum effect seen was ony twofold compared with the eightfold stimulation seen with the wild-type virus. In contrast, mutant *pm*975, which can synthesize normal 13S and 9S mRNAs, induced both HSP70 and E3-CAT gene expression to the same extent as did the wild-type virus (Fig. 3A and B, lanes 7). The results for the mutant viruses were quantitated and are presented in Table 2. Clearly, a functional 13S-mRNA-encoded product is required for the activation of adenovirus early promoters (1, 17, 21, 23, 26–28), such as the E3 promoter, and the human HSP70 promoter.

TABLE 1. Effects of serum stimulation and adenovirus infection on E3-CAT activity and HSP70 gene expression[a]

Infection and culture condition (medium)	E3-CAT activity (pmol/μg per h)[b]	Amt of HSP70 mRNA (molecules/cell)[c]
Mock infection		
Saved	0	20
Fresh	0	60
Serum free	0	20
Ad5 infection		
Saved	27	80
Fresh	33	85
Serum free	36	110

[a] Level of HSP70 mRNA and E3-CAT activity for cells harvested at 16 h p.i., as depicted in Fig. 1 and 2.

[b] CAT activity is expressed as picomoles of [^{14}C]chloramphenicol converted to acetylated forms per microgram of cell protein extract per hour of incubation at 37°C and was calculated from percent converted material, derived by scanning densitometric measurements. CAT activity was not detectable in mock-infected cells.

[c] Derived as described in the legend to Fig. 1.

The induction of the HSP70 promoter could have been in response to events occurring during productive infection and may not have been directly attributable to the E1A gene. This possibility was ruled out by a series of cotransfection experiments. A plasmid containing 2.4 kilobases of 5′ flanking sequences and 150 nucleotides of 5′ noncoding sequences of the HSP70 gene fused to the bacterial CAT gene (pHB-CAT; 40) was transfected into HeLa cells in the presence or absence of a second plasmid containing the E1A gene. Expression of the pHB-CAT plasmid was readily detected in transfected cells; expression was found to be at least 10-fold higher in the presence of the E1A gene (Fig. 4). Consistent with the viral infection results presented above, the 13S-mRNA-encoded product was essential; cotransfection with a plasmid containing the E1A 13S cDNA sequences gave stimulation similar to that of the plasmid containing the wild-type sequences (Fig. 4, lane 3), whereas the 12S cDNA sequences failed to stimulate at all (Fig. 4, lane 4).

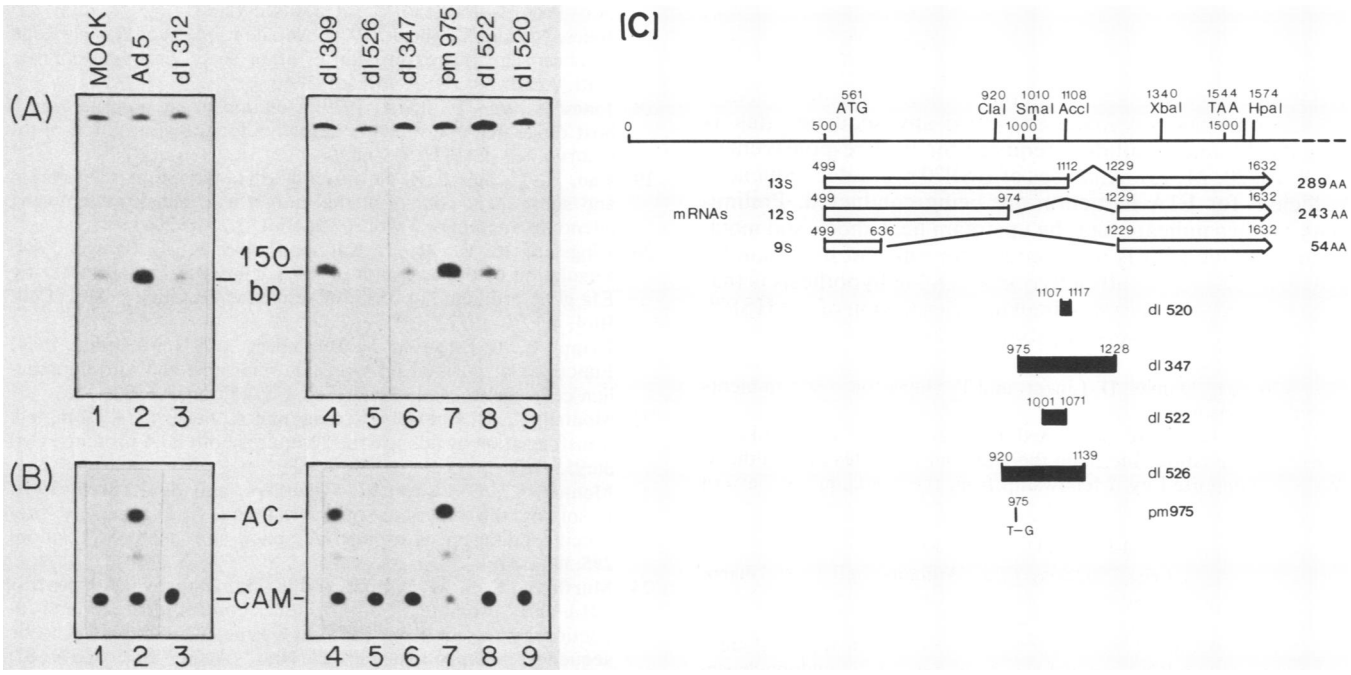

FIG. 3. Induction of human HSP70 (A) and integrated E3-CAT (B) genes in HeLa 27-T cells upon infection by mutant viruses under serum-free medium culture conditions. At 16 h p.i., HSP70 mRNA was measured by the S1 nuclease assay, as described in the legend to Fig. 1, and CAT activity was measured as described in the legend to Fig 2. bp, Base pairs; AC and CAM, diacetylated and unacetylated forms, respectively, of chloramphenicol. (C) Physical maps of the adenovirus E1A region and various adenovirus mutants used in this study. The left-hand end of the Ad5 genome is represented by the top line; the positions of key restriction endonuclease cleavage sites are indicated together with the translation initiation and termination codons. The structures of the three mRNAs synthesized from this region during productive infection are shown together with the predicted size of the polypeptide that can be translated from each message. At the bottom are shown the alterations present in each of the viral mutants. The construction and phenotypes of these mutants have been described in detail elsewhere (for mutants *dl*520, *dl*522, and *dl*526, see reference 13; for *dl*1347, see reference 39; for *pm*975, see reference 23). The nature of the E1A products that can be synthesized by each mutant is shown in Table 2. AA, Amino acids.

We propose that the human HSP70 gene is an example of a cellular gene whose expression is growth regulated (41) and is *trans*-activated by a viral transforming gene product, specifically the Ad5 E1A 13S product. The human HSP70 promoter contains at least two regulatory elements: one which is required for heat shock and metal induction and a separate element which is required for serum stimulation (42). The E1A 13S product could therefore *trans*-regulate HSP70 gene expression via either of these promoter ele-

TABLE 2. HSP70 mRNA and E3-CAT activity in virus-infected cells[a]

Infecting virus	Expression of mRNA:			Amt of HSP70 mRNA (molecules/cell)	E3-CAT activity (pmol/μg per h)
	13S	12S	9S		
Mock infected	−	−	−	24	1.8
*dl*309	+	+	+	176	39.3
*dl*312	−	−	−	22	1.8
*pm*975	+	−	+	130	53.1
*dl*520	−	+	+	10	1.2
*dl*526	−	−	+	24	1.2
*dl*347	−	cDNA	−	50	1.5
*dl*522	Truncated	+	+	30	1.2

[a] Cells were harvested at 16 h p.i. Results are depicted in Fig. 3. CAT activity was calculated as described in Table 1, footnote *b*, and HSP70 mRNA levels were determined as described in the legend to Fig. 1. Equivalent amounts of cytoplasmic RNAs did not correspond to cell equivalents among the different infections. Relative to other samples, two- and threefold more cells from *dl*347 and *dl*522 infections were needed to obtain equivalent amounts of cytoplasmic RNAs.

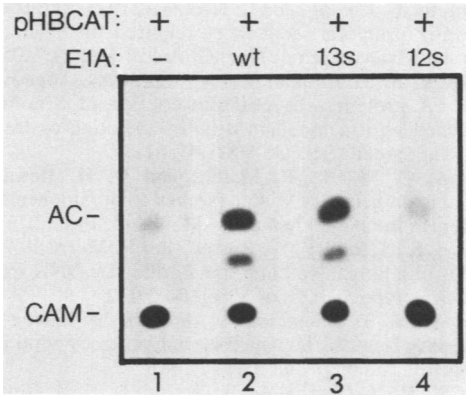

FIG. 4. Induction of HSP70 promoter by cotransfection with E1A-containing plasmids. HeLa cells were transfected with the plasmids containing the indicated sequences, and 48 to 72 h later extracts were prepared and tested for CAT activity (10). Plates (100 mm) of cells were transfected with 5 μg of the plasmid pHB-CAT (40) and, where indicated, with 7.5 μg of the E1A-containing plasmid. The E1A-containing plasmids were previously described (13): wild-type (wt) sequences in pCE, 13S cDNA sequences in pJN20, and 12S cDNA sequences in pJF12. The cells were subsequently cultured in serum-free medium. A portion of each extract was incubated with [^{14}C]chloramphenicol for 15 min at 37°C. AC and CAM, diacetylated and unacetylated forms, respectively, of chloramphenicol.

ments or perhaps other promoter elements. There is no obvious homology between any of the early adenovirus promoters and the HSP70 promoter (15). Furthermore, mutational analysis of a number of E1A-responsive early viral promoters has failed to reveal any sequence that is specifically and absolutely required for E1A responsiveness (5, 8, 16, 20, 24, 43). Analyses of HSP70 promoter sequences sufficient for E1A induction are being conducted. Preliminary studies indicate that the upstream heat shock and metal regulatory element is not required for efficient induction by E1A. A particularly attractive and testable hypothesis is that E1A-induced and serum-stimulated expression of the HSP70 gene use the same sets of promoter elements.

We thank L. Dumas, D. Linzer, and T. Shenk for their comments on this manuscript.

These studies were supported by grants from the Leukemia Society Foundation, Inc., and the National Institutes of Health. B. Wu was supported by a fellowship from the Leukemia Society of America.

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