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E1a Transactivation of Human HSP70 Gene Promoter Substitution Mutants Is Independent of the Composition of Upstream and TATA Elements

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We have analyzed 41 deletion, linker scan, and substitution mutants of the human HSP70 gene promoter for activation by the adenovirus E1a region. No natural element of the HSP70 gene promoter was required for activation. To investigate specific interactions between E1a and transcription factors, a set of 24 promoters containing all possible combinations of eight different upstream or TATA motifs was investigated for E1a stimulation. E1a transactivated the promoter regardless of the particular TATA motif present. Furthermore, there was no dramatic correlation between any upstream motif and activation by E1a. These data suggest that E1a does not stimulate transcription via an interaction with any specific transcription factor but instead suggest that E1a interacts via the general transcription machinery.

The adenovirus E1a proteins are potent activators of transcription, stimulating expression from both viral and cellular promoters (3, 15). Despite extensive experimentation, the mechanism of E1a transactivation remains poorly understood. E1a has shown no sequence-specific DNA-binding activity (8), and detailed promoter mutagenesis has not identified an element important for induction by E1a that is not also required for full basal levels of transcription (11, 30, 42; reviewed in references 2 and 18). Therefore, attention has turned to the host-cell DNA-binding transcription factors for potential targets. For example, studies focused on viral promoters have suggested that E1a increases the transcriptional activity of the cellular factors E2F and E4F by increasing their DNA-binding activities (19, 32). Others have proposed that the factor which binds the TATA element (e.g., transcription factor TFIID) is the target that mediates induction by E1a (22, 35, 51).

E1a may not modify a specific factor. It has been proposed that E1a possesses both promoter-directing and transcriptional activation domains analogous to those of cellular transcriptional activators (23). In this model, E1a utilizes its promoter-directing domain to recognize and bind to a DNA-bound protein on the promoter, after which the activation domain functions to stimulate transcription. It was proposed that a limited number of DNA-bound factors may be recognized by E1a to mediate transcriptional activation; for example, activation transcription factor (ATF), a factor common to many E1a-induced promoters, might be one target (7, 14, 20, 21, 25). An explicit prediction of this model is that E1a physically interacts with a domain present on the promoter complex. If this domain is formed by one or more specific DNA-bound factors, one would predict that certain factors or combinations of factors would produce an inducible promoter, whereas other combinations would produce a promoter refractory to stimulation. The numerous reports of specific factors being necessary for full induction of a promoter may reflect the ability of those factors to create a domain that interacts effectively with E1a.

Alternatively, E1a might not recognize any specific domain formed by DNA-bound factors but instead may replace or augment the activity of one of the non-DNA-binding factors needed for promoter function (9, 18). In this hypothesis, E1a would still be physically present on the promoter, but its presence would not be determined by any specific DNA-binding factor. Further, the ability of E1a to stimulate would be determined not only by its presence but also by whether its mechanism of action complemented the ability of the preexisting complex to function. These different mechanisms of E1a action can be distinquished. If E1a either modifies a factor or uses a factor to bind to the promoter, then one would expect promoters that contain such a factor to be preferentially stimulated by E1a. If instead E1a generally alters the transcription machinery, strong correlations between the level of induction and the presence of any individual factor would not be expected. We therefore have quantitatively assessed the ability of E1a to stimulate a number of nonsense and substitution mutants of the human HSP70 gene promoter (hereafter referred to as the HSP70 promoter).

The endogenous HSP70 promoter is stimulated by the E1a proteins after infection (16, 31), and the mechanism of this stimulation has been extensively studied (35, 47, 48). Whereas one study reported that a mutation in the TATA region eliminates E1a inducibility of this promoter (35), a second study failed to locate any specific factor required for stimulation (46). We report here an extensive analysis of linker scan and deletion mutations in this promoter that also fails to identify a factor required for stimulation. We have extended these observations by systematically replacing the HSP70 TATA region and upstream CCAAT region with various TATA and upstream motifs (41). Analysis of the E1a inducibility of these chimeric promoters shows no clear correlation of E1a stimulation with any individual factor or with any specific combination of factors. Rather, we note an inverse correlation between the basal activity of a promoter and its level of induction: weak promoters tend to be stimulated more than strong promoters. These data are most consistent with a model in which E1a functions by replacing or augmenting the activity of one of the non-DNA-binding

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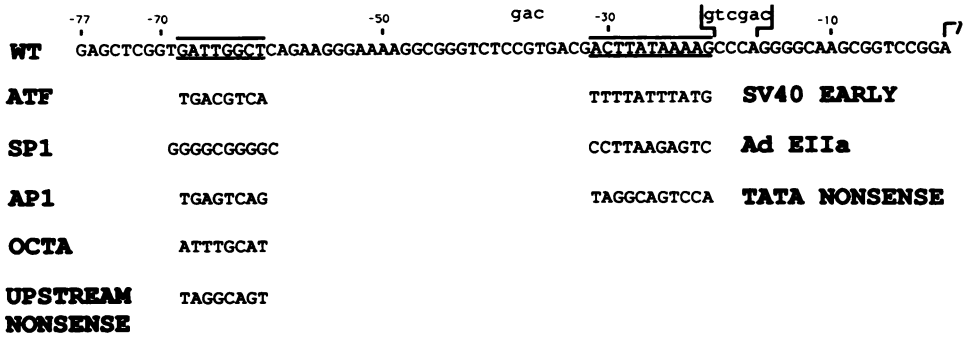

FIG. 1. Sequence of the human HSP70 promoter. The CCAAT and TATA sequences are highlighted, as these are the sites that were varied in the substitution mutants. The sequence motifs used to create the substitution mutants are shown. Base changes resulting from the cloning procedures are shown in small letters above the wild-type (WT) sequence (41).

factors at this promoter, not by specifically interacting with any one sequence specific DNA-binding factor.

MATERIALS AND METHODS

Plasmids. All plasmids were purified by banding twice with ethidium bromide-CsCl centrifugation. DNA concentrations were determined spectrophotometrically and verified by agarose gel electrophoresis. Construction of hsp70 linker scan mutants (13) and substitution mutants (41) has been described previously. Deletion mutants (generous gift of Holly Prentice) were created by cutting with the restriction endonuclease *Nhe*I (+7) and digesting with *Bal* 31 exonuclease, after which ends were blunt-end ligated. Plasmid pΔ-18/+23 deletes between bases −18 and +23 inclusive, whereas plasmid pΔ+1/+65 deletes bases +1 to +65 inclusive (Fig. 1).

The pseudo-wild-type promoter is encoded by plasmid pIR17-84, which contains wild-type HSP70 promoter sequences to −84 and a deletion of bases +65 to +98. This deletion results in a truncated signal (130 bases) on S1 analysis.

Virus. Wild-type adenovirus type 5 (Ad5) and mutant *dl*312 were grown on HeLa and 293 cell monolayer cultures, respectively. Mutant *dl*312 is deleted for most of the E1a region and part of the E1b region (15). Titers to determine PFU were performed on the same cell lines (12).

Infection and transfection. The infection-transfection experiments were performed essentially as described previously (35). Briefly, HeLa cells were grown in Dulbecco modified Eagle medium (DMEM) and split 1:5 16 to 24 h before the addition of the calcium phosphate-DNA precipitate. Infections with Ad5 or *dl*312 were done in serum-free DMEM at 20 PFU per cell (1 ml of virus dilution per 100-mm-diameter plate) for 1 h at 37°C. Cytosine arabinoside (40 μg/ml of culture medium) was added after infection (and every 8 h after the first addition) to inhibit viral replication and maintain high levels of E1a. In some cases (chloramphenicol acetyltransferase [CAT] experiments), mock infection (with serum-free DMEM) was substituted for *dl*312 infection. Control experiments in which mock infection, *dl*312 infection, and wild-type Ad5 infection were compared revealed that CAT expression levels were identical after mock or *dl*312 infection (data not shown). DNA precipitates were added 8 h after infection (13). Cells were washed and fed 12 h later and harvested 12 h after the wash. For CAT experiments, transfection mixtures contained 20 μg of the test plasmid and 2 μg of pXGH5, which expresses human growth hormone (34) and is used to control for transfection efficiency. For RNA analysis, 10 μg of the test plasmid, 10 μg of the internal reference plasmid pIR17-84, and 2 μg of pXGH5 were added. Human growth hormone and CAT assays were as described previously (1, 34).

RNA preparation and analysis. Total cellular RNA was obtained by the guanidinium-CsCl method (1). Preparation of the single-stranded human HSP70 probe (containing bases +229 [5′ end labeled] to −133 of the HSP70-CAT fusion gene) and S1 nuclease digestion analysis have been described elsewhere (13). Gels were quantitated by film densitometry with a Kratos model SD3000 (Schoeffel Instrument Co.) spectrodensitometer.

Data analysis. For S1 gels (RNA data) quantitated by densitometry, the induced strength of a promoter (Table 1) is (SM/IR), where SM is the signal (appropriate and downstream initiation site present with the nonsense and simian virus 40 [SV40] early TATA motifs) from a given substitution mutant and IR is the signal from the internal reference promoter cotransfected with that promoter. The 24 chimeric promoters were all analyzed by S1 analysis in three separate experiments, two of which were quantitated and averaged to give the values presented. Because of the low level of basal expression from these promoters that is a consequence of the infection-transfection protocol (see above), the basal strength (i.e., in the absence of E1a) of each promoter was calculated in an identical manner from data obtained by transfections in which RNA was harvested 48 h after introduction of DNA. These data are presented in Fig. 3 of the accompanying paper (41). Note that in the accompanying paper the numerical analysis of these data was processed by arbitrarily setting the strength of the wild-type HSP70 promoter (CCAAT/hsp70) to 1.0 in order to ease comparison, whereas the raw values are presented here (Table 1).

The induction ratio (Table 1) is determined by dividing the induced strength by the basal strength. This value therefore relates the strength of the promoter in the absence of E1a to the strength of the promoter in the presence of E1a. It thus gives a quantitative value for the ability of E1a to induce the promoter.

To better relate this calculation to the values from the CAT experiments, fold induction for the wild-type HSP70 promoter (CCAAT/hsp70) was determined by averaging its stimulation in eight separate infection-transfection experiments assayed for CAT activity (see below); this value is shown in parentheses in Table 1. The fold induction value for the remaining substitution mutants was determined by relating their induction ratios (i.e., from two RNA experiments) relative to the CCAAT/hsp70 induction ratio value by the

TABLE 1. Stimulation of all 24 chimeric promoters by E1a expression[a]

Promoter	Induced strength	Basal strength	Induction ratio	Fold induction	CAT induction
CCAAT/hsp70	1.06	0.68	1.6	(56)	82
ATF/hsp70	0.73	0.66	1.1	38	33
SP1/hsp70	0.45	0.60	0.7	24	56
AP1/hsp70	0.29	0.11	2.6	91	21
OCTA/hsp70	0.13	0.10	1.3	45	62
Up. Nons./hsp70	0.08	0.12	0.7	24	110
CCAAT/SV40E	0.62	0.73	0.8	28	13
ATF/SV40E	0.21	0.08	2.6	91	75
SP1/SV40E	0.19	0.08	2.4	84	40
AP1/SV40E	0.07	0.04	1.7	59	17
OCTA/SV40E	0.10	0.01	10	350	67
Up. Nons./SV40E	0.04	0.03	1.3	45	30
CCAAT/EIIa	0.59	0.68	0.8	28	10
ATF/EIIa	0.59	0.51	1.2	42	22
SP1/EIIa	0.39	0.37	1.0	35	35
AP1/EIIa	0.22	0.11	2.0	70	21
OCTA/EIIa	0.20	0.11	1.8	63	34
Up. Nons./EIIa	0.08	0.03	2.7	94	39
CCAAT/TATA Nons.	0.36	1.05	0.3	10	17
ATF/TATA Nons.	0.21	0.03	7.0	240	44
SP1/TATA Nons.	0.33	0.36	0.9	31	29
AP1/TATA Nons.	0.17	0.10	1.7	59	13
OCTA/TATA Nons.	0.19	0.20	0.9	31	72
Up. Nons./TATA Nons.	0.12	0.07	1.7	59	14

[a] For description of column headings and relevant calculations, see Materials and Methods.

equation (IR/1.6) × 56. Here, IR is the induction ratio for a given substitution mutant, 1.6 is the induction ratio for CCAAT/hsp70, and 56 is the fold induction for CCAAT/hsp70.

CAT induction is $(CAT_I/GH_I)/(CAT_B/GH_B)$, where CAT_I and CAT_B represent the percent conversion of [^{14}C]chloramphenicol to the acetylated form under E1a-induced and basal conditions, respectively, and GH_I and GH_B are the expression levels (in ^{125}I counts) of human growth hormone under E1a-induced and basal conditions, respectively. Values are averages from two separate experiments in which all promoters were analyzed.

RESULTS

No sequence element is required for stimulation of the human HSP70 promoter by E1a. The human HSP70 promoter (Fig. 1) is regulated by a variety of external stimuli, including expression of the 289-amino-acid E1a protein (31, 48). A series of linker scan mutants has been constructed that alters nearly every base pair of the proximal region of this promoter and has identified three critical promoter elements to which transcription factor proteins have been shown to bind. These include a factor that binds to the CCAAT site at −65 (e.g., CP1); Sp1, which binds the GC box at −45; and TFIID, the TATA-box-binding factor (13, 29, 47, 49, 50). The proximal HSP70 promoter also contains putative ATF- and AP2-binding sites, although the contribution of these sites to the basal level of transcription from this promoter is unclear (13, 47; H. Prentice, unpublished data). In addition, a number of deletion mutants of this promoter have been constructed, including one that removes sequences from −84 to −1250 as well as others that delete sequences downstream (to +65) of the initiation site (Fig. 1). To determine whether any of these promoter sequences are required for stimulation by E1a, we analyzed these mutants with an adenovirus infection-transfection protocol in HeLa cells.

Infections were performed in parallel with wild-type Ad5 and the E1a region deletion mutant *dl*312. The promoter constructs were introduced by calcium phosphate precipitation into cells 8 h after infection (at 20 PFU per cell), and the cells were harvested 24 h later. An HSP70 pseudo-wild-type construct (13) was included in each transfection as a control for stimulation. Total cellular RNA was isolated, and transcript levels and initiation sites were assayed by S1 nuclease digestion. Each mutant promoter tested was stimulated upon expression of the E1a region, including promoters that had a mutated TATA element (Fig. 2, lanes 21 to 22) and that contained two and even three mutations simultaneously in the three important HSP70 proximal promoter elements (lanes 25 to 32). In addition, promoters that contained gross deletions of sequences downstream of the initiation site were stimulated (lanes 33 to 36).

Similar results were seen with a protocol in which E1a-expressing plasmids were cotransfected with the mutant promoters, although the degree of stimulation was much less dramatic (averaging 3.5-fold; data not shown). Two recent studies analyzing E1a inducibility of linker scan mutations in this promoter have been published. The data reported here are in general agreement with those of Williams et al. (47), whereas they differ somewhat from those of Simon et al. (35), who observed a dramatic decrease in E1a inducibility of a promoter with a mutant TATA region. Stimulation of the 22–26 (i.e., altered in bases 22 to 26) TATA linker scan mutation by E1a (11-fold stimulation by CAT analysis) was consistently less than that seen with the wild-type promoter (53-fold) in experiments in which stimulation of these two constructs was directly compared. A more extensive TATA linker scan mutation showed similar behavior (Table 1; see below). Other mutants in the promoter showed little or no effect on the degree of E1a stimulation, in agreement with results of both prior studies (35, 47). These data extend prior studies by analyzing the effects of multiple mutations in the basal elements and by analyzing downstream deletions (Fig. 2). Stimulation of each of these mutants was within threefold of the stimulation of the wild-type promoter, as determined by CAT analysis (data not shown).

E1a inducibility of substitution mutants of the HSP70 promoter. In the analysis described above, we were unable to identify a specific sequence element required for stimulation of the human HSP70 promoter by E1a. This observation suggests that E1a may not specifically interact with any factor bound to the promoter but instead may function via the transcription complex as a whole (47). To test this possibility more thoroughly, we sought to alter some of the components of the basal transcription complex that normally act at the HSP70 promoter. Previous studies have suggested that E1a may function via specific transcription factors, such as ATF or TFIID (14, 20, 21, 25, 35, 51). We therefore decided to analyze E1a inducibility of a set of substitution mutants in which we had replaced the two most active components of the HSP70 basal promoter, CCAAT at −65 and TATA, with other motifs. Mutation of these two components had the most dramatic effect on E1a inducibility of

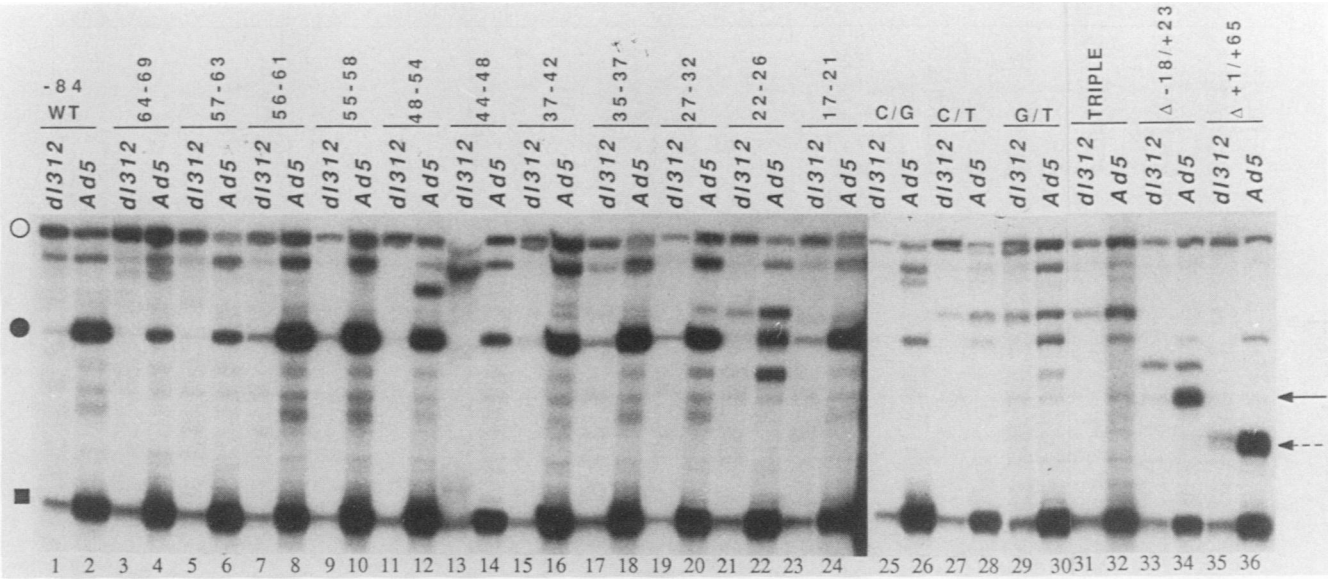

FIG. 2. Demonstration that the human HSP70 promoter does not have any element needed for activation by E1a. RNA from cells that were infected with either *dl*312 (odd-numbered lanes) or Ad5 (even-numbered lanes) was analyzed by S1 nuclease digestion, and the resulting fragments were separated on an 8% polyacrylamide–urea denaturing gel. Cells were transfected with 10 μg each of pIR17-84 and the indicated wild type (WT) or linker scan mutants (13). Linker scan mutants are indicated by the bases altered (i.e., 64–69 denotes that bases 64 through 69 are altered by the linker). Double mutants (lanes 25 through 30) are indicated by the sequence elements affected (C, CCAAT element; G, GC box; T, TATA). The triple mutant (lanes 31 and 32) is mutated in all three of these elements. The endpoints of the deletion mutants are also shown (lanes 33 through 36). Marked are appropriate initiation from the test promoter (●) and internal reference promoter (■) and discontinuity between probe and RNA with each of the two deletion mutants (◀—, ◀- - -). Bands above the signals for the test promoters resulted from fortuitous upstream transcription and discontinuities between the probe and the linker scan or sequences in the plasmid beyond −84. ○, Undigested S1 probe.

this promoter in both our studies and previous work (Table 1; 35, 47). We speculated that if any individual factor conferred an increased ability to interact with E1a, then we would see a consistent increase in the ability of promoters containing that factor to be stimulated by E1a. Similarly, if a factor was refractory to E1a stimulation, then promoters dependent on that factor for activity might be refractory to stimulation.

The substitution mutants contained five different upstream motifs (ATF, Sp1, AP1, octamer [OCTA], and a nonsense control) and three different TATA motifs (SV40 early, adenovirus EIIA, and a nonsense control) in place of the wild-type CCAAT and TATA elements (Fig. 1; 41). The six resultant upstream motifs (including CCAAT) and four resultant TATA motifs (including HSP70 TATA) were juxtaposed in all possible ways to create a set of 24 promoters. These mutants were created in the context of the wild-type HSP70 promoter and therefore also contained the several binding sites for known factors in this promoter. Mutation of these other sites does not alter E1a inducibility of the promoter (see above; 35, 47) and has either a weak or nonexistent effect on basal strength (13). Expression from these substitution mutants is therefore primarily dependent on the substituted sites, and indeed the level of expression from these constructs varied by over 50-fold (Table 1). Thus, by analyzing E1a inducibility of these constructs, it should be possible to determine whether any of these factors is preferentially able to interact with E1a.

Inducibility of each promoter was determined by using the infection-transfection protocol described above. Each promoter drives expression of the bacterial CAT gene. Assaying the product of this gene showed that each of the 24 promoters was stimulated by E1a (Table 1). In agreement with our previous results, each of the promoters that contained a mutated TATA element (TATA nonsense [TATA Nons.]) was transactivated, as was the promoter with a mutation in two elements (Upstream nonsense [Up. Nons.]/TATA Nons.). Separate experiments were harvested for total cellular RNA to show that E1a activated transcription from the same start sites used by these promoters under basal conditions (in Fig. 3). Note that the presence of E1a did not correct the start site error in the TATA mutants (Fig. 3).

All experiments harvested for RNA included a pseudo-wild-type HSP70 promoter as an internal reference. This promoter contained a deletion in the 5′ untranslated region and thus produced a shorter signal on S1 analysis (Fig. 3). By comparing the signal from the test promoter with the signal from the internal reference promoter in the same lane, it was possible to determine the relative strength of the test promoter under E1a-induced conditions (i.e., induced strength). The basal strength of each of the 24 promoters has been determined previously in the same way, using a transfection protocol that leaves the DNA in the cell for 48 h before harvest (41). Dividing the induced strength by the basal strength for each of the promoters gives the induction ratio, that is, the extent of stimulation of the test promoter by E1a relative to the stimulation of the pseudo-wild-type HSP70 promoter. It is extremely difficult to determine this value by directly comparing RNA levels from *dl*312 and wild-type Ad5-infected cells. The protocol allows for only 12 h between washing of the $CaPO_4$ precipitate from the cells and harvesting of RNA, and therefore the transcript levels in the absence of E1a are extremely difficult to observe (the data of Fig. 2, where we were barely able to observe these levels, represent a 2-week exposure from an unusually efficient transfection). RNA levels from the *dl*312-infected samples

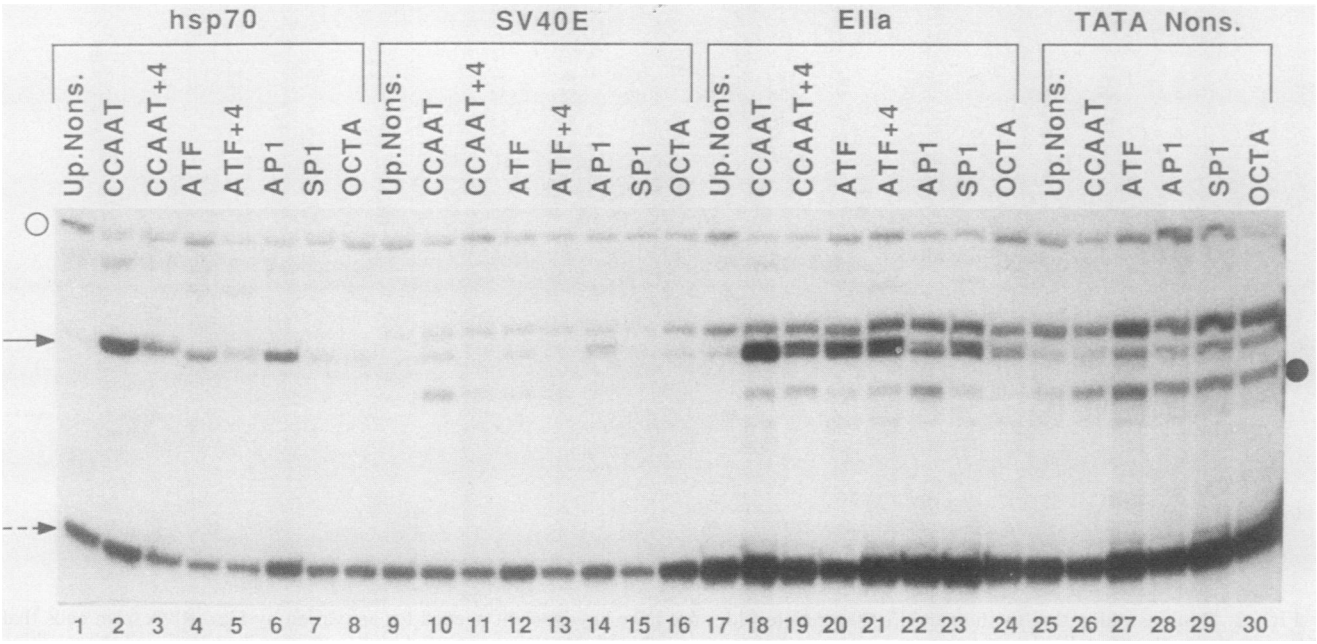

FIG. 3. E1a-induced expression levels of chimeric promoters. RNA from cells infected with Ad5 was analyzed by S1 nuclease digestion as for Fig. 2. Cells were transfected with 10 μg each of the test promoter and pIR17-84 (pseudo-wild-type promoter; contains wild-type sequences to −84 and a deletion of nucleotides +65 to +98). The solid and broken arrows represent appropriate initiation from the test promoter and internal reference promoter, respectively. Promoters are grouped according to the TATA element that they contain. Symbols: ●, message that initiated approximately 25 bases downstream in the constructs containing the SV40 early, adenovirus EIIa, and TATA nonsense TATA sequences; ○, undigested S1 probe. Also included in this experiment were various spacing mutants (ATF+4/hsp70, etc.) that inserted 4 base pairs between the upstream site and the TATA sequence (41).

from the same experiment as the wild-type infected samples shown in Fig. 3 and from repeat experiments were not detectable (data not shown).

The values for induction of each promoter as determined by CAT levels and by RNA levels are compared in Table 1. Whereas among the stronger promoters there was good correlation between these two measurements, with the weaker promoters the correlation broke down. Two types of errors led to this lack of correlation. With these weaker promoters, the CAT level obtained under basal conditions was higher than the level of appropriately initiated transcripts (data not shown; 41). We interpret this to imply that the basal CAT level from these weaker promoter constructs is erroneously high due to background translation of RNA(s) with an inappropriate start site(s) and therefore does not accurately reflect appropriately initiated transcription from the promoter. A second, less serious source of error is that the level of transcripts from these weaker promoters was very low (e.g., OCTA/SV40 early TATA [SV40E]) and therefore difficult to measure, leading to values that are less precise than with stronger promoters. We base the following analysis of the data primarily on the values obtained from the RNA data, as we believe these data to be most accurate.

Plotting the induction ratio versus the basal strength of the 24 promoters showed an interesting correlation (Fig. 4). Although the relationship was not linear, there was an inverse relationship between the basal strength of a promoter and the extent to which that promoter was stimulated by E1a. In general, those promoters that were weakest under basal conditions were stimulated most by E1a; conversely, those promoters that were strongest were stimulated least.

Our 24 promoter constructs consist of combinations of eight different sequence elements (five upstream elements and three TATA elements). In light of recent speculation that certain sequence elements are necessary to confer E1a inducibility on a promoter, we were interested in whether any of the elements tested here was preferentially E1a inducible. The average fold stimulation for all of the promoters containing a given sequence element can be calculated from Table 1. Since each promoter was stimulated significantly by E1a (Table 1), each specific promoter element also showed stimulation. However, there was no strong correlation between the degree of induction and the presence of a specific upstream sequence element. Promoters containing the binding site for ATF were among the most dramatically stimulated. As mentioned previously, the binding site for this factor is present in a number of E1a-inducible promoters and as such has been proposed as a candidate target for E1a action.

In summary, each of the 41 promoter constructs that we tested was induced at least 10-fold by E1a introduced by infection. That these effects were due to some viral protein other than E1a is not likely, since similar results were seen when E1a was introduced on a plasmid (data not shown). In addition, we were concerned that the extent of the observed stimulation of our promoters by E1a was due to saturation of a rate-limiting factor and therefore not due to a linear response to E1a activity. If this were the case, the strength of the promoter observed under induced conditions would not represent the intrinsic E1a-inducible strength of that promoter but rather an artifactual maximum level. To address this issue, various amounts of selected promoters were tested in the infection-transfection assay. The results showed that the response to E1a was not affected by increasing amounts of the promoter construct from 5 to 20 μg (data not shown).

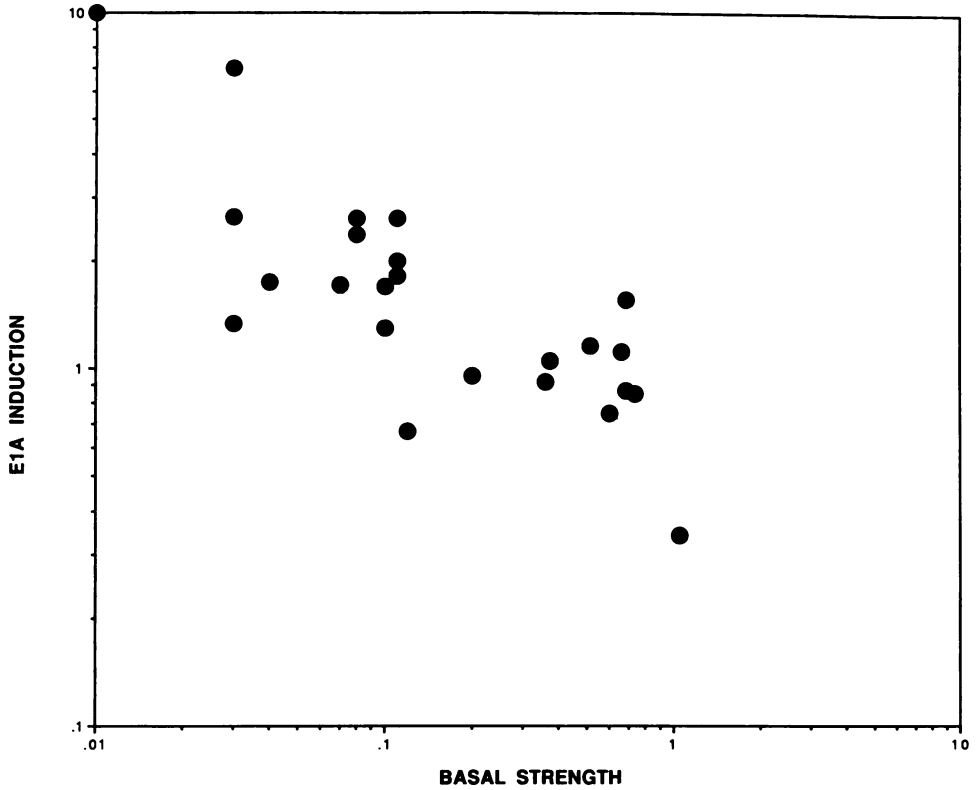

FIG. 4. Inverse relationship between the basal strength of a promoter and its extent of E1a induction. The gel of Fig. 3 and that from a similar experiment were quantitated by densitometry, and the induction ratio was calculated as described in Materials and Methods. This value is defined as E1a induction for simplicity. E1a induction is plotted versus basal strength on a full log scale. A computer fitted a regression line to these points with an equation $y = 2.97 - 3.22x$ and a correlation coefficient of simple determination (R) = 0.20. When the three most extreme values (OCTA/SV40E, ATF/TATA Nons., and CCAAT/TATA Nons.) were eliminated, a regression line with a negative slope ($y = 1.93 - 1.50x$) was retained and R increased to 0.35.

DISCUSSION

Stimulation of transcription by the adenovirus E1a region is a distinctive form of eucaryotic gene regulation. Whereas many mammalian regulatory factors function by binding to a specific DNA sequence (5, 26) and thereby stimulate only promoters that contain that sequence, E1a does not function through any one sequence, does not specifically bind to DNA, and therefore is not limited in the promoters that it stimulates (1, 8, 18). Rather, E1a promiscuously stimulates transcription: it increases expression from a broad variety of promoters in transfection experiments and stimulates a smaller but still substantial number of viral and cellular promoters during the natural course of infection (1, 6, 11, 16, 31, 33, 39, 40, 42).

One of the endogenous cellular promoters stimulated by E1a during infection is the human HSP70 promoter (31). We analyzed a large group of linker scan and deletion mutants of this promoter, covering almost every base from upstream of the promoter to +65, in hopes of finding an element important for stimulation by E1a. Every mutant was stimulated. Most notable was that a linker scan mutation disrupting the TATA box (mutation 22–26) was induced by E1a. The contribution of this element to stimulation of the HSP70 promoter by E1a has been controversial. Simon et al. (35) reported that altering this element rendered the promoter uninducible by E1a in an infection-transfection assay similar to that used here. Recently, however, Williams et al. (47) used both transfection and infection protocols to show that a TATA nonsense mutant remained inducible by E1a. Our data are in general agreement with those of Williams et al. both in a lack of requirement for the TATA box and in the lack of requirement for any individual element.

We report an analysis of E1a transactivation of 22 mutated HSP70 promoters containing an alteration of the TATA box (Fig. 2 and 3; Table 1). Two different TATA nonsense mutants were used (22–26 and an 11-base-pair nonsense sequence used in the chimeric promoters) as well as substitution mutants in which the HSP70 TATA was replaced with the SV40 early or EIIa TATA motif (Fig. 1). We found that the three mutants similar to those reported by Simon et al. (35) to be noninducible by E1a (CCAAT/SV40E, CCAAT/EIIa, and CCAAT/TATA Nons.; Table 1) were the least stimulated of any of the analyzed constructs. However, this effect was obtained only in the context of the CCAAT upstream element; when the upstream motif was changed (e.g., to ATF or Sp1), alterations in the TATA motif no longer reduced the ability of E1a to transactivate the promoter. These observations point out the complexity of E1a regulation and argue that the ability of E1a to regulate a promoter depends not only on the individual elements present but also on promoter context.

This point is strengthened by the analysis of all of the substitution mutants of the HSP70 promoter. These promoters were created by replacing the TATA and upstream CCAAT motifs with a variety of upstream binding motifs and TATA motifs from both E1a-induced and E1a-repressed

promoters (4, 7, 14, 20, 21, 25, 36, 37, 43, 45, 46, 51). The basal level of expression from these promoters varies more than 50-fold, and thus the substituted elements play a predominant role in determining the functions of these chimeric promoters. We reasoned that if E1a worked preferentially with certain transcription factors, then certain of these chimeric promoters would be more inducible than others and the pattern of activation would point to factors that interact with E1a. We found that all of the chimeric promoters were stimulated by E1a, but the degree of stimulation varied by over an order of magnitude.

There was no dramatic correlation between the degree of stimulation and the presence of any individual element (Table 1). Previous studies have implicated upstream factor ATF in stimulation of numerous viral promoters by E1a (7, 14, 21), and the TATA motif found in the wild-type HSP70 promoter has been implicated in stimulation of the E1b and major late promoters of adenovirus (22, 51). Stimulation of chimeric promoters containing the ATF-binding site was somewhat more pronounced than stimulation of other promoters, but promoters containing the OCTA motif, not heretofore considered an E1a-inducible element, were similarly elevated. Promoters containing the HSP70 TATA element were somewhat less stimulated as a group than promoters containing other TATA elements. The only correlation that emerged from analyzing the degree of stimulation of the chimeric promoters was between basal strength of the promoter and inducibility. Weak promoters tended to be more strongly stimulated than strong promoters (Fig. 4).

Mechanism of E1a stimulation. We have analyzed 41 deletion, linker scan, and substitution mutants of the human HSP70 promoter for stimulation by the E1a region, and all are stimulated. What do these data tell us about the mechanism by which E1a stimulates transcription? Present theories concerning E1a function place E1a at the promoter and have it stimulating expression by an unknown interaction. The data that E1a functions at the promoter come from two sources. First, fusion of E1a to the transactivating domain of VP16 makes E1a a better transactivator (23). Because VP16 is believed to function at the promoter, these data are interpreted to argue that E1a can bring the transactivating domain of VP16 to the promoter. Second, intact E1a and a peptide from the transactivating domain of E1a (17, 24, 27, 28) can both stimulate transcription in vitro (10, 38). These data are most simply interpreted as meaning that E1a is functioning directly, since it seems less likely that a complicated cascade could be recapitulated in vitro.

We believe that the data presented here argue that E1a comes to the promoter independent of specific DNA-binding proteins and acts via the general transcription machinery. We base this conclusion on the observation that there is no motif common to the large number of promoters that we have analyzed, yet all are stimulated. Furthermore, there is no compelling correlation between the presence or absence of any factor-binding site and the degree of stimulation. Although these data leave open the possibility that there exist multiple factors that can specifically interact with the E1a protein, we argue that these data are more consistent with a model in which E1a replaces or facilitates the activity of one or more of the non-DNA-binding factors needed for transcription. Several factors are believed to be involved in most if not all initiation of transcription by RNA polymerase II. These include, besides polymerase II, TFIIA, TFIIB, and TFIIE (44). TFIID, which does specifically bind to the TATA box, may also function at promoters that lack this sequence element and therefore could also be considered a general factor in this discussion.

A model in which E1a replaces or enhances the activity of one or more of these factors is consistent with all available data on E1a activation. To stimulate transcription, E1a must alter a rate-limiting step in the initiation process. The varying degrees of stimulation we observe here is most consistent with E1a acting at a step which varies (with promoter) in the extent to which it is rate limiting. Previous observations that certain elements of certain promoters are needed for activation by E1a would be explained by arguing that removal of those elements changes the rate-limiting step for transcription to one not affected by E1a. Certain factors, such as ATF, may be common to many promoters stimulated by E1a because they function via a mechanism that is consistent with a maximal ability of E1a to activate transcription (i.e., ATF does not act at the same step as does E1a). Specificity of E1a activation during the infection process may well not be determined by which promoters E1a is interacting with but instead by whether that interaction is productive. A model in which E1a does not interact specifically with any particular DNA-binding factor is therefore more consistent with available data then a model invoking specific interactions. Proof of such a model will require detailed kinetic experiments with purified factors.

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