Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 2846–2849, May 1986
Biochemistry

The immunoglobulin heavy chain enhancer is stimulated by the adenovirus type 2 E1A products in mouse fibroblasts

[RNA polymerase B (II) promoter/*trans*-acting factor/cell specificity/repression/transcription]

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Contributed by P. Chambon, December 23, 1985

ABSTRACT **In contrast with our previous results (Hen, R., Borrelli, E. & Chambon, P. (1985) *Science* 230, 1391–1394), which demonstrated that the mouse immunoglobulin heavy chain transcriptional enhancer is repressed in lymphoid cells by the products of the adenovirus type 2 E1A transcription unit, we show here that these products activate the same enhancer in mouse fibroblast L cell lines that contain stably integrated copies of a recombinant in which the enhancer is inserted upstream from the chicken conalbumin promoter. In addition, competition experiments suggest that the activity of the heavy chain enhancer may be repressed by a *trans*-acting factor in mouse L cells. We speculate that the E1A products may prevent the action of this cellular repressor in these cells.**

The adenovirus type 2 (Ad2) E1A proteins have a pleiotropic effect on the regulation of eukaryotic gene transcription. They both activate the promoters of the other early adenovirus genes (ref. 1 and refs. therein) and of a number of cellular genes (2, 3) and repress the activity of some transcriptional enhancers (4–6). We have shown that the E1A proteins negatively regulate not only enhancers that are active in a wide range of cells, like those of simian virus 40 (SV40) and polyoma virus, but also the cell-specific enhancer of the mouse immunoglobulin heavy chain gene that is fully active only in lymphoid B cells (see ref. 7 for a review). This cell specificity could be either due to the presence of a specific positive transcriptional factor(s) in lymphoid cells or to the presence of a repressor in nonlymphoid cells or to a variable ratio of these regulatory factors in lymphoid versus nonlymphoid cells. *In vivo* footprint experiments have suggested the existence of lymphoid cell-specific positive factor(s) interacting with the heavy chain gene enhancer (8). *In vitro* (ref. 9; J. Dougherty, P. Augereau and P.C., unpublished results) and *in vivo* (ref. 10; C. Lemaire, J. L. Imler, C.W. and B.W., unpublished results) experiments have revealed the presence of sequences within the enhancer region that negatively regulate transcription, suggesting the existence of *trans*-acting repressor(s). In addition, the heavy chain enhancer may also interact with positive transcriptional factor(s) present in nonlymphoid cells, since it has been reported that the heavy chain enhancer has some transcriptional stimulatory activity in L cells and other nonlymphoid cell lines (10). *In vitro* transcription competition experiments between SV40 and heavy chain enhancers have also suggested that nonlymphoid cells contain a positive factor(s) that can interact with the heavy chain enhancer (11).

We have now investigated the effects of the Ad2 E1A proteins on the activity of the mouse heavy chain enhancer present in a chimeric promoter recombinant which has been integrated into the genome of nonlymphoid cells (mouse L cells). To our surprise, the heavy chain enhancer that is not fully active in these cells was activated by the E1A products. Our results also suggest that the heavy chain enhancer can be negatively regulated in L cells, and we speculate that the E1A proteins may relieve this repression.

MATERIALS AND METHODS

All recombinants are described in Fig. 1 with the exception of IGE and pβ2X which are described in refs. 11 and 14, respectively. L-C, L-C1, L-CMI, L-CMI1 cell lines were derived from mouse LMTK$^-$ cells (15) that were cotransfected with either the plasmid pTCT (L-C and L-C1) or pTCTMI (L-CMI and L-CMI1) and the plasmid pTK (15) that carries the herpes simplex virus thymidine kinase (*tk*) gene used for selecting the transformed cells. The L-CMIE1A cell line was obtained by cotransfecting the plasmids pE1ASV (Fig. 1) and pY3 [a plasmid carrying the gene coding for the resistance to hygromycin (16)] in L-CMI cells.

All transfections were carried out with the calcium phosphate precipitation technique. Cytoplasmic RNA was isolated from the transfected cells and analyzed by quantitative S1 nuclease mapping as described (17) using the following single-stranded probes labeled at the 5′ end with ^{32}P. The conalbumin probe was a *Bam*HI fragment isolated from plasmid pTCT (Fig. 1), which yields an S1 nuclease-protected fragment of 67 nucleotides when hybridized to RNA initiated at the conalbumin cap site. The E1A probe was an *Eco*RI–*Sau*3A fragment of plasmid pE1ASV (Fig. 1), which yields an S1 nuclease-protected fragment of 130 nucleotides when hybridized to RNA initiated at the E1A cap site. The E2A probe was a *Sma* I–*Sau*3A fragment of plasmid pBX (1), which gives a protected fragment of 47 nucleotides when hybridized to RNA initiated at the Ad2 E2A cap site. The tk probe was a *Bam*HI–*Bgl* II fragment of plasmid pTK, which yields a protected fragment of 49 nucleotides when hybridized to RNA initiated at the herpes simplex virus *tk* cap site. The rabbit β-globin probe was a *Bst*NI fragment of plasmid pβ2X (14), which gives a protected fragment of 134 nucleotides when hybridized to RNA initiated at the rabbit β-globin cap site.

Nuclear run-on transcription assays using L-C, L-CMI, and L-CMIE1A cells were performed as described (1, 6).

RESULTS

Activation of the Heavy Chain Enhancer in Mouse L Cells by the Ad2 E1A Products. In recombinant pTCTMI the heavy chain enhancer is inserted upstream from the conalbumin promoter present in recombinant pTCT (Fig. 1). Both pTCT and pTCTMI were stably integrated in mouse LMTK$^-$ cells using the herpes simplex virus *tk* gene as a selective marker. L-C and L-C1 cell lines contain pTCT, and L-CMI and

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Abbreviations: Ad2, adenovirus type 2; SV40, simian virus 40; tk, thymidine kinase.

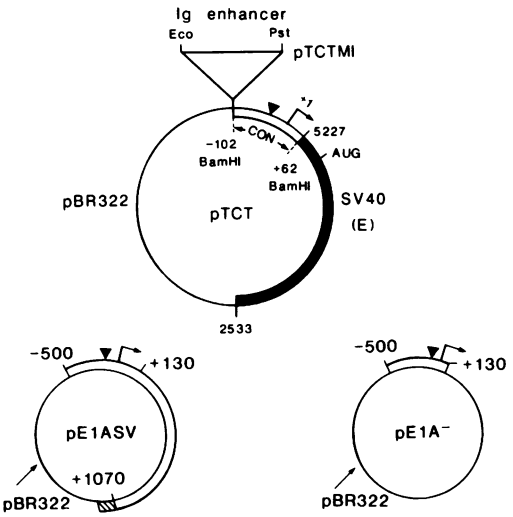

FIG. 1. Recombinant pTCT (12) contains the chicken conalbumin promoter linked to the SV40 large tumor (T) antigen coding sequence. Recombinant pTCTMI (10) was derived from pTCT by inserting the *Eco*RI–*Pst* I fragment containing most of the heavy chain enhancer (13) into the *Bam*HI site located 102 nucleotides upstream from the conalbumin start site. pE1ASV contains the entire Ad2 E1A transcription unit from position −500 to +1070, while pE1A⁻ contains only the E1A promoter region from position −500 to +130 (4) (see Fig. 4).

L-CMI1 cell lines contain pTCTMI (each cell line contains approximately five copies of either pTCT or pTCTMI). Cytoplasmic RNA was extracted from these cell lines and analyzed by quantitative S1 nuclease mapping. As shown (10), the presence of the heavy chain enhancer increased by 5- to 7-fold the level of RNA transcribed from the conalbumin promoter (Con+1, in Fig. 2), which was very low in its absence (compare lanes 1 and 2 with lanes 3 and 4 in Fig. 2). The four L-C and L-CMI cell lines were infected with Ad2 at 100 plaque-forming units, and RNA was analyzed after 15 hr. The level of mRNA transcribed from the conalbumin promoter was not affected in the L-C cell lines (Fig. 2, compare Con+1 in lanes 1 and 5 and in lanes 2 and 6); in contrast infection of the two L-CMI cell lines resulted in a dramatic increase (20- to 50-fold) of this level (compare the Con+1 signal in lanes 3 and 7 and in lanes 4 and 8). The level of RNA transcribed from the *tk* promoter was not significantly affected by Ad2 infection under the same conditions (compare in Fig. 2 the *tk*+1 signal in lanes 1 to 4 with that in lanes 5 to 8). Judging from the level of E1A mRNAs, the time course of infection was the same in all cell lines (data not shown). Note also that transcription initiated upstream from the conalbumin promoter in pBR322 sequences is not affected by Ad2 infection (EP in Fig. 2).

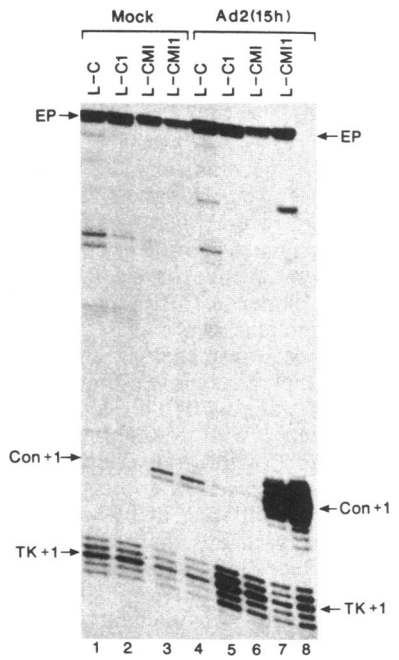

FIG. 2. S1 nuclease analysis of cytoplasmic RNA (10 μg) extracted from L-C, L-C1, L-CMI, and L-CMI1 cells either mock-infected or infected for 15 hr with Ad2 at high multiplicity (100 plaque-forming units) as indicated. Con+1 and tk+1 correspond to RNA initiating at the conalbumin and tk cap site, respectively. EP corresponds to RNA initiating upstream from the end point of homology of the conalbumin probe. Similar results were obtained in several independent experiments.

To demonstrate that the E1A products are involved in the stimulatory effect, the L-CMI cell line was infected with the adenovirus type 5 mutant dl312 (18), which lacks the E1A transcription unit (Fig. 3*B*, the E1A+1 signal is absent in lane 4). As expected in a dl312 infection, the level of the Ad2 early E2A mRNA 36 hr after infection was similar to that present 5 hr after infection with the Ad2 wild-type virus (compare E2+1 in Fig. 3*C*, lanes 2 and 4). It is clear that no stimulation of transcription from the conalbumin promoter could be observed following infection with dl312 (Fig. 3*A*, lane 4), while transcription was stimulated approximately 5-fold 5 hr after infection with the wild-type virus (Fig. 3*A*, lanes 1 and 2).

The above results indicate that the E1A products are required for the observed stimulation of the heavy chain enhancer activity. To demonstrate that the E1A proteins alone can activate the heavy chain enhancer in the L-CMI cell line, the isolated E1A transcription unit, rather than the entire virus, was used. In the first set of experiments, the L-CMI cell line was transfected with the recombinant pE1ASV (Fig. 1), which contains the entire E1A transcription unit, and the level of RNA transcribed from the conalbumin promoter was analyzed 36 hr after transfection. A 4-fold increase in transcription was obtained when 20 μg of pE1ASV was transfected, while transcription remained un-

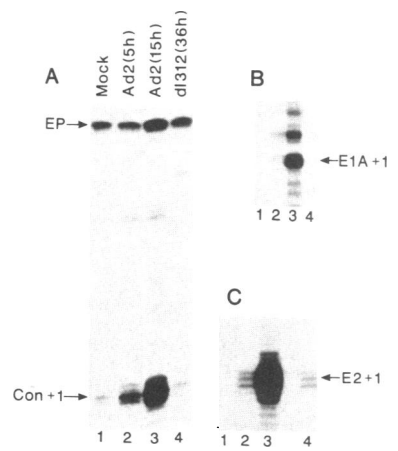

FIG. 3. S1 nuclease analysis of cytoplasmic RNA (10 μg) extracted from L-CMI cells infected with Ad2 or the adenovirus type 5 mutant dl312 (18). Lanes 1, mock-infected cells; lanes 2, Ad2-infected cells harvested after 5 hr; lanes 3, Ad2-infected cells harvested after 15 hr; lanes 4, dl312-infected cells harvested after 36 hr. (*A*, *B*, and *C*) RNA was analyzed with the conalbumin probe, the Ad2 E1A probe, and the Ad2 E2A probe, respectively. E1A+1 and E2+1 correspond to RNA that initiates at the E1A and E2A main cap sites, respectively. Con+1 and EP are as in Fig. 2. These results are representative of several independent experiments.

changed when the recombinant pE1A$^-$ (Fig. 1), which lacks the E1A coding region, was used (Fig. 4*A*, compare the Con+1 signal in lanes 1–3). This stimulation is weaker than that obtained after 15 hr of Ad2 infection. This is probably due to the poor efficiency of transfection of the L-CMI cell line, since immunofluorescence studies have shown that only 10% of the L cells transfected with a recombinant containing the SV40 early transcription unit express large tumor (T) antigen (not shown). However, we cannot exclude that other Ad2 products may also play a role in the heavy chain enhancer stimulation. In a second set of experiments the E1A transcription unit was stably integrated in the genome of the L-CMI cell line. Only one clone (L-CMIE1A) was obtained that constitutively expressed the E1A products, most probably due to the toxicity of these proteins. The level of E1A transcription was low in these cells (Fig. 5, see dot E1A in lane 5 and results not shown). Nevertheless, the level of RNA transcribed from the conalbumin promoter in the transformed cell line L-CMIE1A was 5-fold higher than in the control cell line L-CMI (compare the Con+1 signal in lanes 4 and 5 of Fig. 4*B*). In contrast, the level of tk mRNA was not affected (tk+1 signal in lanes 4 and 5). The low level of E1A expression in these cells probably explains why the stimulation was not more efficient than that observed after 5 hr of Ad2 infection (see above).

The Activation by the E1A Products Is at the Transcriptional Level. The observation that the stimulation by the Ad2 E1A products occurred only with recombinants containing the heavy chain enhancer suggests that it is the rate of transcription rather than the RNA stability that is affected. This hypothesis was supported by nuclear run-on transcription analysis performed on nuclei from L-C and L-CMI cells, either mock-infected or infected with Ad2 (Fig. 5, lanes 1–4). In the L-C cells that contain the recombinant pTCT (Fig. 1), the low level of transcription initiating at the conalbumin promoter and, therefore, hybridizing to SV40 DNA was not affected by Ad2 infection (Fig. 5, see dots SV40 in lanes 1 and 2). In contrast, in the L-CMI cells that contain recombinant pTCTMI (Fig. 1), the level of transcription hybridizing to the SV40 DNA was higher in the infected cells (Fig. 5, see dots SV40 in lanes 3 and 4). The magnitude of the stimulation does not appear to be as high as in the analysis of cytoplasmic RNA by S1 nuclease mapping (Fig. 2), most likely because transcripts that initiate in pBR322 sequences (see above) also hybridize with the SV40 DNA and are, therefore, responsible for a background level of transcription that is not affected by Ad2 infection (see dots pBR in Fig. 5). Nuclear run-on transcription analysis was also performed on nuclei from

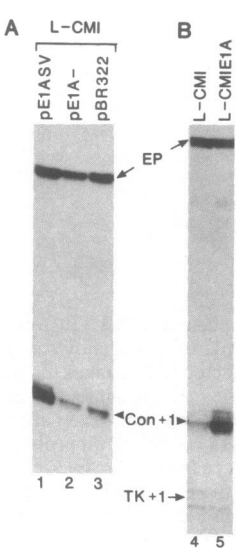

FIG. 4. (*A*) S1 nuclease analysis of cytoplasmic RNA (10 μg) extracted from L-CMI cells transfected with 20 μg of pE1ASV (lane 1), 20 μg of pE1A$^-$ (lane 2), or 20 μg of pBR322 (lane 3). RNA was analyzed with a conalbumin probe as in Fig. 2. Similar results were obtained in several independent transfection experiments with different plasmid preparations. (*B*) S1 nuclease analysis of RNA (10 μg) extracted from L-CMI cells (lane 4) and L-CMIE1A cells (lane 5). RNA was analyzed with conalbumin and tk probes (see Fig. 2).

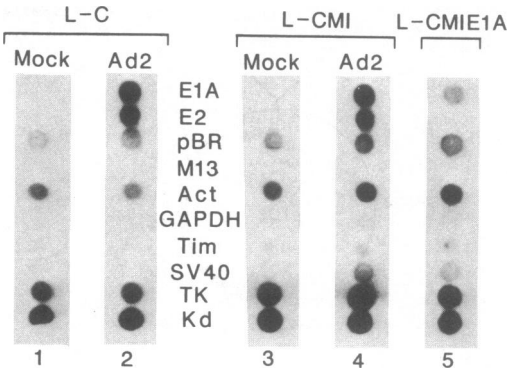

FIG. 5. Nuclear run-on transcription experiments with RNA transcribed from 10^7 nuclei of L-C, L-CMI, and L-CMIE1A cells as indicated. Lane 1, mock-infected L-C cells; lane 2, Ad2-infected L-C cells, harvested after 15 hr; lane 3, mock-infected L-CMI cells; lane 4, Ad2-infected L-CMI cells harvested after 15 hr; lane 5, L-CMIE1A cells. The following DNAs (1 μg) were dotted on nitrocellulose filters and hybridized with the *in vitro* synthesized RNA: E1A, *Eco*RI–*Bam*HI fragment from recombinant pE1ASV (extending from position −500 to +1070); E2, Ad2 *Sma* I–*Hin*dIII fragment corresponding to the E2A early transcription unit; pBR, pBR322; M13, Mp7 phage; Act, cDNA fragment corresponding to a rat actin gene (19); GAPDH, cDNA fragment corresponding to the rat glyceraldehyde phosphate dehydrogenase gene (19); Tim, cDNA fragment corresponding to the rat triose phosphate isomerase gene (19); tk, plasmid pTK (15); Kd, H2-K^d mouse genomic clone containing a repetitive sequence (20).

L-CMIE1A cells (L-CMI cells in which the E1A transcription unit has been stably integrated, see above). As expected the level of transcription hybridizing to SV40 DNA was higher than in the noninfected L-CMI cells (Fig. 5, compare lanes 3 and 5). In all these nuclear run-on transcription experiments the level of RNA corresponding to a number of genes and to repetitive sequences present in a genomic clone corresponding to the *H2-K*^{d} gene was not significantly affected by Ad2 infection nor by the presence of a stably integrated copy of the E1A gene (Fig. 5, lanes 1–5).

The Heavy Chain Enhancer May Be Negatively Regulated in L Cells. The previous results could be explained by a derepression of the heavy chain enhancer by the Ad2 E1A products. To test whether this enhancer is negatively regulated in mouse L cells, we performed a series of cotransfection experiments with recombinant pTCTMI and increasing amounts of the recombinant IGE that contains the isolated heavy chain enhancer sequence (the *Eco*RI–*Pst* I heavy chain enhancer fragment cloned in pBR322) (11). Recombinant pβ2X (14), which contains two copies of the entire rabbit β-globin gene, was cotransfected as an internal control. Transcription from the conalbumin promoter was increased by more than 8-fold when a 10-fold excess of recombinant IGE was cotransfected with pTCTMI (Fig. 6, compare Con+1 in lanes 1 and 3), while transcription from the globin gene promoters was increased by at most 2-fold (Glob+1 in Fig. 6). Transcription initiating in pBR322 sequences upstream from the heavy chain enhancer was also increased (Fig. 6, EP signal in lanes 1–3), indicating that it may also be activated by the heavy chain enhancer. These results suggest that the activity of the heavy chain enhancer may be negatively regulated in L cells by a *trans*-acting factor(s) that interacts with the enhancer sequence. Other titration experiments (C.W. and B.W., unpublished results) support this possibility.

DISCUSSION

We demonstrate here that the Ad2 E1A products activate the heavy chain enhancer in mouse L cells. This result is

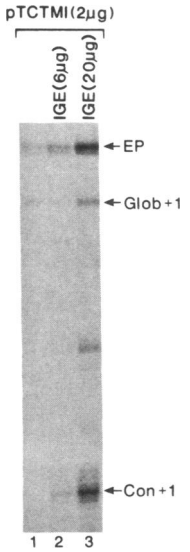

FIG. 6. S1 nuclease analysis of cytoplasmic RNA (10 μg) extracted from mouse LMTK⁻ cells transfected with recombinant pTCTMI (2 μg), pβ2X (0.5 μg) and either 6 μg or 20 μg of recombinant IGE. pBR322 DNA was added to adjust to 22.5 μg the total amount of transfected DNA. Glob+1 corresponds to RNA initiated at the globin cap sites of pβ2X. Con+1 and EP are as in Fig. 2.

unexpected since the activity of this enhancer is repressed by the E1A products in lymphoid cells (6). These opposite effects were obtained under the same experimental conditions—i.e., Ad2 infection of either MPC11 lymphoid cells or mouse L cells containing integrated copies of the recombinant pTCTMI in which the heavy chain enhancer is located upstream from the chicken conalbumin promoter. The differential effects of the E1A proteins on the activity of the heavy chain enhancer might be related to the mechanism underlying its marked cell specificity. The heavy chain enhancer is fully efficient at activating transcription from heterologous promoters in lymphoid cells only, which has suggested the existence of positively acting lymphoid-specific factors (see ref. 7 for a review). *In vivo* footprint experiments (8) as well as *in vitro* studies (ref. 9, and P. Augereau, J. Dougherty, and P.C., unpublished results) have supported this hypothesis by revealing specific DNA–protein interactions in lymphoid cells or lymphoid cell extracts. In nonlymphoid cells the heavy chain enhancer has a weaker stimulatory activity, and it has been shown that the sequences responsible for this activity correspond to a subset of those involved in lymphoid cells (10). This suggests that at least some of the factors responsible for the enhancer activity in lymphoid cells are different from those acting in nonlymphoid cells. Thus, one could explain why the E1A proteins do not repress the weak activity of the heavy chain enhancer in L cells by assuming that these proteins specifically prevent the action of the lymphoid cell-specific factors. However, this does not explain why the E1A proteins activate the heavy chain enhancer in L cells. They may activate the enhancer either directly or by relieving a negative regulation. In the first hypothesis the E1A proteins would replace, at least to some extent the lymphoid cell-specific factor(s), which are missing in L cells, and the E1A-mediated repression observed in lymphoid cells would be explained by a competition between the E1A proteins and the lymphoid cell-specific factor(s). There are, however, a number of observations that suggest that the heavy chain enhancer is repressed in nonlymphoid cells. The use of cycloheximide to block protein synthesis in L cells containing an integrated copy of an heavy chain gene has resulted in a stimulation of transcription of this gene, suggesting the presence of a labile repressor molecule (21). Furthermore, sequences acting negatively both *in vivo* and *in vitro* have been detected in the heavy chain enhancer region (see refs. 9, 10, and P. Augereau, J. Dougherty, and P. C., unpublished results). Our present experiments, which show that the activity of the heavy chain enhancer in L cells is stimulated by cotransfection with increasing amounts of isolated enhancer sequences, support the possibility that a repressor is operating in these cells. How the E1A products could prevent such a repression is unknown. However, since the E1A products are known to repress the activity of various enhancers (see above), it is tempting to speculate that they could compete with a cellular repressor present in L cells. Assuming that they would be less efficient at repressing, this competition may lead in fact to a derepression.

We thank C. Kédinger, P. Augereau, W. Schaffner, L. Matrisian, and R. Breathnach for gifts of recombinants and viruses; M. Acker and the members of the cell culture service for their technical assistance; and B. Boulay, C. Werlé, and the members of the secretariat for preparing the manuscript. This work was supported by Centre National de la Recherche Scientifique (6984), Institut National de la Santé et de la Recherche Médicale, the Ministère de l'Industrie et de la Recherche (84V0803), the Fondation pour la Recherche Médicale, and the Association pour le Développement de la Recherche sur le Cancer. E.B. was supported by a fellowship from the Université Louis Pasteur.

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