Adenovirus transforming 19-kD T antigen has an enhancer-dependent *trans*-activation function and relieves enhancer repression mediated by viral and cellular genes

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The adenovirus E1b region codes for two major tumor antigens of 19 kD and 55 kD, which are important for cell transformation. Our results indicate that the 19-kD T antigen possesses two enhancer-regulatory functions. It can *trans*-activate enhancer-linked promoters and relieve enhancer repression mediated by viral and cellular repressors. The 19-kD activation function enhances expression from different promoters linked to SV40, Py, E1a, and immunoglobulin heavy-chain enhancers. Enhancer activation by the 19-kD protein appears to be cell type-specific, since the heavy-chain and SV40 enhancers were not *trans*-activated in myeloma cells whereas the same enhancers were *trans*-activated in fibroblasts. The 19-kD enhancer activation function appears to be dominant over the enhancer repression function of E1a, since in cells expressing the 19-kD protein there is no significant repression despite a large increase in E1a expression. The 19-kD T antigen activates the Py enhancer in undifferentiated F9 cells indicating that the activation function of E1b masks enhancer repression by an "E1a-like" cellular gene product. The enhancer activation function of the 19-kD T antigen may be important for cell transformation and cell differentiation.

[Key Words: Enhancer activation; repression; derepression; transformation; differentiation]

Received May 13, 1987; revised version accepted July 8, 1987.

The adenovirus E1b region, which maps between map positions 4.5 and 11.5 on the viral genome, codes for two major tumor antigens of 19 kD and 55 kD (for review, see Sussenbach 1984). In addition to these two major proteins, other polypeptides as well as mRNAs that could potentially code for polypeptides related to the 55-kD T antigen have also been identified (Green et al. 1982; Anderson et al. 1984; Virtanen and Pettersson 1955). The 19-kD T antigen has been shown to play an important role in cell transformation in cooperation with the E1a region (Chinnadurai 1983; Mak and Mak 1983; Babiss et al. 1984; Logan et al. 1984; Subramanian et al. 1984; Takemori et al. 1984; Barker and Berk 1987). The 55-kD T antigen has also been shown to be important for transformation (Bernard et al. 1983; Logan et al. 1984; Barker and Berk 1987). However, the relative importance of these two E1b-coded proteins in the conversion of E1a-transformed cells to the fully transformed phenotype has not been fully elucidated.

To delineate the roles of the 19-kD and 55-kD T antigens in cell transformation in cooperation with E1a, we have been performing a two-stage transformation

assay, using baby rat kidney cells immortalized by E1a (E1a-BRK). For second-stage transformation, DNA segments coding for either the 19-kD or the 55-kD T antigens were transfected, using the *neo*^r marker coded from plasmid pSV₂*neo* as the dominant selection marker. During these studies, we observed that E1a-BRK cells were relatively refractile to *neo*⁺ transformation by transfection of pSV₂*neo* alone. However, cotransfection of DNA fragments encoding the E1b region substantially increased the formation of *neo*⁺-resistant colonies in E1a-immortalized BRK cells. This observation, in light of the known role of E1a in repression of the activity of SV40 (Velcich and Ziff 1985), polyoma (Borelli et al. 1984), and immunoglobulin heavy-chain (Hen et al. 1985) enhancers, prompted us to examine the role of the E1b region in enhancing *neo*⁺ transformation by pSV₂*neo*. These studies have revealed that the 19-kD protein coded by the E1b region possesses novel enhancer-regulatory functions. The 19-kD regulatory activities may be important for cell transformation.

Relatively little is known about enhancer regulation. The two adenovirus transforming genes E1a and E1b 19-kD T antigen appear to have opposite effects on enhancer-driven gene expression. The 19-kD gene is rela-

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tively well characterized, and a large number of mutants are available in this region. This gene product will be an important tool to investigate enhancer regulation.

Results

Effect of Ad7 E1b T antigens on pSV₂neo transformation of E1a-BRK cells

To study the roles of the E1b 19-kD and 55-kD tumor antigens of adenoviruses in transformation, we established several BRK cell lines immortalized and partially transformed by the left 4.5% of the Ad2 (group C) genome, which encodes the E1a proteins. We assayed conversion of E1a-immortalized cells to the fully transformed phenotype by transfection of E1b coding regions from different adenovirus serotypes and found that the Ad7 (group B) E1b region had the best transformation complementing activity (K. Yoshida and G. Chinnadurai, in prep.). Therefore, some of the initial experiments described here were carried out using plasmids that express the Ad7 E1b. In the two-step transformation assays, the E1b plasmids were cotransfected on E1a-immortalized cells using the *neo* resistance marker (Colbere-Garapin 1981; Southern and Berg 1982) expressed from pSV₂neo (Southern and Berg 1982), and the transfected cells were maintained under antibiotic G418 for positive selection of cells harboring the E1b sequences. During these studies, it was observed that E1a-BRK cells consistently yielded fewer *neo*⁺ colonies (i.e., without E1b) than two spontaneously immortalized rat cell lines, CREF (Fisher et al. 1982) and 3Y1 (Kimura et al. 1975), that do not contain E1a (Table 1). We reasoned that this relative inhibition of *neo*⁺ transformation of E1a-BRK cells may be due to the enhancer-dependent repression (see Borelli et al. 1984; Velcich and Ziff 1985) of the SV40 early promoter-enhancer elements contained in pSV₂neo. In accordance with this expectation, we also observed that when *neo*⁺ expression was under the control of the HSV-tk promoter (which lacks elements analogous to other enhancers), consistently greater numbers of *neo*⁺ transformants were obtained in E1a-BRK cells than with pSV₂neo (data not shown). However, it was

surprising that cotransfection of pRA (*Rsa*I-A fragment, N1431–4024), coding for the Ad7 E1b 55-kD and 19-kD T antigens, with pSV₂neo caused about 30-fold stimulation in *neo*⁺ colony formation in E1a-BRK cells and no such dramatic effects were observed with CREF and 3Y1 cells (Table 1). Similarly, cotransfection of a mutant plasmid, pRA-A (for description of various mutants, see Fig. 1 and Materials and methods), capable of coding the entire 19-kD T antigen and only a severely truncated form of the 55-kD T antigen (amino-terminal 90 amino acids), also caused a large increase in *neo*⁺ transformation of E1a-BRK cells. On the other hand, mutant pRA-N, capable of coding for the 55-kD T antigen but containing a mutation that abolishes the initiation codon for the 19-kD T antigen, caused only a relatively modest increase in the frequency of *neo*⁺ transformation. In contrast to the effect of E1b on *neo*⁺ expression in E1a-BRK cells, cotransfection of the various E1b plasmids with pSV₂neo did not have any significant effect on the frequency of induction of *neo*⁺ transformants in the spontaneously transformed rat cell lines, CREF and 3Y1. It would appear that the level of *neo* expression directed by pSV₂neo alone in the cell lines is sufficient to ensure maximal frequency of *neo*⁺ colony induction; further increases in *neo* expression, such as that evidenced upon cotransfection of the 19-kD T antigen (see below), do not result in increased *neo*⁺ transformation rates in CREF and 3Y1 cells. These results suggest that the 19-kD T antigen coded by the E1b region of Ad7 has a stimulatory role in the *neo*⁺ transformation of Ad2 E1a-expressing cells when *neo* expression is under the transcriptional control of SV40 promoter–enhancer elements.

Effect of Ad7 E1b on SV40 early promoter activity in E1a-BRK cells

The observation that the Ad7 E1b 19-kD T antigen caused a substantial increase in the frequency of *neo*⁺ transformation of E1a-BRK cells prompted us to examine the effect of Ad7 E1b on the expression of pSV₂neo in these cells. To study the effect of E1b T antigens, pSV₂neo was cotransfected with different E1b plasmids on two different E1a-BRK cell lines (E1a-BRK-1 and E1a-BRK-2); total cytoplasmic RNA was extracted 48 hr after transfection and subjected to S1 nuclease analysis. Results obtained with the E1a-BRK-1 cell line are shown in Figure 2A. In E1a-BRK-1 cells transfected with pSV₂neo and pRA-NA defective in both the 19-kD and 55-kD T antigens, there was a very low level of *neo* expression. However, in cells cotransfected with pSV₂neo and pRA (wt E1b) or pRA-A (defective in the 55-kD T antigen), a significant amount of the 390-nucleotide S1 nuclease-protected DNA fragment specific for the *neo* probe was observed. In contrast, cells cotransfected with pRA-N, expressing only the 55-kD T antigen, contained very low amounts of *neo*-specific RNA, as in the case of pRA-NA. Semiquantitative estimation of the intensity of the S1-protected DNA fragments revealed that the levels of pSV₂neo expression induced by pRA or pRA-A were about 10–15 times greater

Table 1. Effect of Ad7 E1b on *neo*⁺ transformation of E1a-BRK and spontaneously immortalized rat cells.

Cotransfected plasmid	Number of colonies/dish ^a		
	E1a-BRK	CREF	3Y1
pRA	32	30	14
pRA-N	5	27	9
pRA-A	42	23	19
pUC13	1	30	14

^a Average of two independent experiments. 60-mm dishes containing about 5×10^5 cells were transfected with 2 μ g of pSV₂neo and 8 μ g of either E1b plasmid or pUC13 vector DNA. About 24 hr after transfection, cells in each dish were split into five dishes and subjected to G418 selection from 24 hr after plating.

Figure 1. Organization of the group C (Ad2 and Ad5) and group B (Ad7) adenovirus E1b regions. This figure is based on Bos et al. (1981) and Dijkema et al. (1982) as summarized by Sussenbauch (1984). Only the major mRNAs and proteins are indicated. The mutations at various restriction sites are indicated as follows: (H) *HindIII*; (S) *SstI*; (K) *KpnI*; (X) *XmnI*; (B) *BglII*; (N) *NcoI*; and (A) *AccI*. (pm2022) Point mutation at position 2022 (Barker and Berk 1987) on the Ad2 genome. The Ad2 and Ad5 coordinates are given on the top and the Ad7 coordinates are given at the bottom of each diagram. The Ad2 coordinates are given within parentheses.

than the levels induced by pRA-NA or pRA-N in four separate experiments. Similar results were also obtained with the E1a-BRK-2 cell line (results not shown). These results suggest that the very low levels of *neo*⁺ transformation of E1a-BRK cells may be due to the poor expression of pSV₂*neo* in these cells. The level of pSV₂*neo* expression and *neo*⁺ transformation of E1a-BRK cells can be increased by cotransfection of Ad7 E1b plasmids coding for functional 19-kD T antigen.

The effect of Ad7 E1b T antigens on the expression of another test gene coding for chloramphenicol acetyl transferase (CAT) under the control of the SV40 early

promoter–enhancer elements in the plasmid pSV₂CAT (Gorman et al. 1982) was also tested on E1a-BRK-1 cells (Fig. 2B). Cotransfection of pSV₂CAT with pRA or pRA-A caused about five- to eightfold increase in CAT activity compared to cells cotransfected with pSV₂CAT and pRA-N or pRA-NA. Similar results were also obtained with comparable Ad5 *wt* or mutant E1b plasmids (not shown). These results on CAT expression are in very good agreement with the results on *neo* expression and indicate that the Ad7 E1b 19-kD T antigen induces substantial stimulation of expression from the SV40 early promoter in E1a-expressing BRK cells.

Figure 2. Effect of Ad7 E1b T antigens on SV40 early promoter expression in E1a-BRK cells. (A) Expression of pSV₂*neo*. E1a-BRK-1 cells were cotransfected with 5 μ g of pSV₂*neo* and 5 μ g of the various E1b plasmids (Wigler et al. 1979). Total cytoplasmic RNA was extracted 48 hr after transfection, treated with 50 U/ml RQ1 DNase (Promega Biotec), and analyzed by quantitative S1 nuclease method as described (Hurwitz and Chinnadurai 1985). A 1883-bp fragment (*BglII*–*PstI*) 5'-end-labeled at the *BglII* site, located within the *neo* sequences of pSV₂*neo*, was used as the probe. (B) Expression of pSV₂CAT. E1a-BRK-1 cells were transfected with pSV₂CAT and the various E1b plasmids as in A. CAT activity was determined from the extracts prepared 48 hr after transfection as described by Gorman et al. (1982). (AcCm) Acetyl chloramphenicol; (Cm) chloramphenicol.

Lack of E1a-mediated repression in the presence of E1b

The initial observations with Ad7 E1b T antigens were further extended using the E1a and E1b genes of Ad5 in transient assays in rat or human cells that do not contain E1a. This system also facilitates a more direct assay of the E1b effect on E1a-mediated repression of pSV₂neo. Plasmids containing the E1a and wt E1b coding sequences of Ad5 (pXC, mp 0–15.0, *Xho*I-C fragment) or E1a and various E1b mutations (illustrated in Fig. 1 and described under Materials and methods) were cotransfected with pSV₂neo on the rat fibroblast cell line CREC or human HeLa cells, and *neo*-specific RNA was analyzed 48 hr after transfection. The results on CREC cells are shown in Figure 3A; similar results were also observed with HeLa cells. In cells cotransfected with pSV₂neo and pUC13 vector DNA, there was significant synthesis of *neo* RNA. However, in cells cotransfected with pSV₂neo and pXC-K (mutated at the *Kpn*I site at position 2048 and expressing functional E1a but defective in both E1b T antigens), expression of *neo* was greatly reduced compared to the cells that did not receive E1a (i.e., transfected with pUC13). This is expected

since it has been known that E1a represses the SV40 promoter activity (Velcich and Ziff 1985). In cells cotransfected with pXC-S (mutated at the *Sst*I site at position 1770), which expresses E1a and the E1b 55-kD T antigen but not the 19-kD T antigen, the level of *neo* expression was also very low and similar to that in cells transfected with pSV₂neo and pXC-K. However, in cells cotransfected with pSV₂neo and wt pXC or pXC-H, which contains a mutation in the 55-kD T antigen coding region (at the *Hind*III site at position 2805), there was a substantial increase in *neo* expression, indicating that E1a-mediated repression of SV40 early promoter activity is not observed in the presence of the amino-terminal half of E1b. An enhancerless β -globin plasmid, pG2, was cotransfected in these experiments as a control for normalizing transfection efficiencies, which did not vary by more than 10%. Since the pXC-S mutation specifically affecting the 19-kD T antigen did not cause stimulation of *neo* expression compared with cells transfected with pXC-K, expressing only E1a, the E1b function that relieves E1a-mediated repression appears to be specific for the 19-kD T antigen. All these E1b mutants were observed to express comparable levels of E1b (see Fig. 4); therefore, the observed effect of pXC-S mutation may not be due to unusually unstable E1b RNA. It is also noteworthy that a comparable Ad2 19-kD mutation (*dl250*) with similar effects as pXC-S has been transferred to the viral genome and has been shown to induce E1b 55-kD and 55-kD-related proteins at levels comparable to that observed in wt infected cells (see Subramanian et al. 1984).

Enhanced E1a expression in the presence of E1b

The RNA samples from cells transfected with pSV₂neo and the pXC-derived plasmids were also analyzed for expression of E1a (Fig. 3B). Cells transfected with wt pXC, expressing both 19-kD and 55-kD T antigens, or pXC-H, expressing the amino-terminal half of E1b, contained substantially larger amounts of E1a-specific RNA than cells transfected with pXC-S (defective in the 19-kD T antigen) or pXC-K (defective in both 19-kD and 55-kD T antigens). These results indicate that the E1b 19-kD T antigen also has a pronounced effect on the expression of E1a. It is unlikely that the observed effect of E1b is due to any *cis* effects of the E1b region since we have also observed similar activation of E1a expression when the E1a and E1b sequences are present on two different plasmids (see Fig. 7).

Transactivation of pSV₂neo by E1b

To determine whether the effect of the E1b 19-kD T antigen that we observed was due to any possible *trans*-activation function of its own, rather than due to the removal of repression by E1a, we constructed plasmids containing only E1b (p Δ XC) by removal of most of the E1a sequences including the promoter and enhancer regions. Since E1a has been shown to be required for efficient expression of other early genes (Berk et al. 1979; Jones and Shenk 1979), some of the earlier experiments were carried out using plasmids expressing E1b under

Figure 3. Effect of Ad5 E1b T antigens on E1a-mediated repression of SV40 promoter. CREC cells were transfected with 5 μ g of pSV₂neo and 10 μ g of pUC13 or pXC or its mutant derivatives. (A) Total cytoplasmic RNA was analyzed for *neo* expression as described under Fig. 2. (B) E1a expression was analyzed in the same RNA samples by including an E1a probe 5'-end-labeled at nucleotide position 1343 (*Xba*I site). The E1a probe (*Xba*I–*Eco*RI) was prepared from plasmid pLA1 Δ b.

Figure 4. *Trans*-activation of pSV₂neo by Ad5 E1b. (A) Expression of E1b from pΔXC-derived plasmids was analyzed using a probe (*Xba*I–*Nco*I) 5′-end-labeled at position 2210 of the Ad5 genome (*Nco*I site). This probe was prepared from plasmid pΔXC. (B) *Trans*-activation of pSV₂neo by various pΔXC-derived plasmids was analyzed as described under A.

the transcriptional control of MSV-LTR. However, in a number of subsequent experiments using both Ad5 and Ad7 E1b plasmids, we have observed that these plasmids can express significant amounts of E1b without *trans*-activation by E1a. It is noteworthy that some of the earlier studies have also indicated that among the various adenovirus early genes, E1b is the least dependent on E1a (see Solnick and Anderson 1982). CREF or HeLa cells were cotransfected with pSV₂neo and *wt* pΔXC, expressing E1b but not E1a, or the various E1b mutant derivatives of pΔXC. The RNA from transfected cells was analyzed for E1b and *neo* expression. As seen in Figure 4A, the E1b-specific RNA (of expected sizes depending on the mutations) was efficiently expressed in cells transfected with the various pΔXC-derived plasmids, which do not contain E1a. In a number of different experiments, the levels of E1b expression from pΔXC-derived plasmids were slightly lower than the levels of expression from pXC-derived plasmids (results not shown). When these RNA samples were analyzed for *neo*-specific RNA (Fig. 4B), cells transfected with pSV₂neo and pUC13 or pΔXC-K (defective in both E1b proteins) expressed reduced levels of *neo* RNA. In contrast, cells cotransfected with plasmids expressing *wt* E1b (pΔXC) sequences or the amino-terminal half of E1b (pΔXC-H) expressed about 15- to 20-fold more *neo* RNA (as determined from three independent experiments), suggesting that the E1b region has a *trans*-activating function. Similar activation of *neo* expression was not observed in cells transfected with pΔXC-S (defective in 19-kD T antigen), indicating that the 19-kD T antigen is essential for this *trans*-activation function.

Effect on E1a-mediated repression of polyoma enhancer

Since repression of the SV40 promoter-enhancer elements of pSV₂neo was not observed in the presence of 19-kD T antigen, we tested whether repression of the polyoma (Py) enhancer by E1a occurs in the presence of this protein. For this purpose, a plasmid construct,

pG2Py (Hen et al. 1986), expressing the rabbit β-globin gene under the control of β-globin promoter and Py enhancer, was cotransfected with pUC13 or the various pXC-derived plasmids on CREF cells, and β-globin-specific cytoplasmic RNA was quantitated (Fig. 5A).

As expected, expression of β-globin was repressed in cells transfected with pG2Py and pXC-K (which expresses E1a and no functional E1b) by about three- to fivefold compared with cells cotransfected with pUC13. Similar low levels of globin were also expressed in cells cotransfected with pG2Py and pXC-S. However, cotransfection of pG2Py with *wt* pXC or pXC-H increased globin expression to levels comparable to those observed in cells that do not express any E1a (lane pUC13), indicating that in the presence of the amino-terminal portion of the E1b region, E1a-mediated repression of Py enhancer is not evident. This release of repression was evident in four independent experiments. The fact that the mutation in the 19-kD T antigen coding region does not significantly alter the level of repression is indicative again of the 19-kD T antigen being essential in overcoming E1a-mediated repression of the Py enhancer.

Trans-activation of Py enhancer-driven β-globin expression

To determine whether E1a by itself can *trans*-activate the Py enhancer–β-globin promoter elements of pG2Py as in the case of pSV₂neo, we cotransfected pG2Py and the various E1b plasmids, and β-globin RNA expression was assayed (Fig. 5B). These results more or less paralleled the results obtained with pSV₂neo (see Fig. 4) again, revealing *trans*-activation of globin expression (by about four- to sixfold in four different experiments) by plasmids pΔXC or pΔXC-H, whereas plasmids pΔXC-S or pΔXC-K did not have any significant activation effect compared with pUC13. In some of these experiments, two additional E1b mutant plasmids were also tested. pΔXC-XB, an Ad2-Ad5 E1b plasmid that retains the en-

Figure 5. Effect of Ad5 E1b T antigens on pG2Py expression. CREF cells were cotransfected as described under Fig. 2 with pG2Py and pXC (A) or pΔXC (B) derived plasmids, and globin expression was determined S1 nuclease analysis of total cytoplasmic RNA. A 5'-end-labeled (at *Bam*HI site) probe specific for the second exon of rabbit β -globin was prepared from plasmid pG2 (Hen et al. 1986) and used for the S1 analysis. The autoradiograms in the bottom panel are longer exposures of the top panels.

tire 19-kD T antigen coding region and deletes most of the 55-kD T antigen coding region from N2256 (Ad2, *Xmn*I site) to N3322 (Ad2, *Bgl*II site), and plasmid p19Kpm2022-X, which contains the 19-kD T antigen coding region (*Xba*I–*Xmn*I site) and a point mutation that converts the third codon in the 55-kD T antigen reading frame into a nonsense codon (Barker and Berk 1987), were found to *trans*-activate pG2Py to a similar extent as pΔXC-H (data not shown). Mutant pΔXC-XB can only code for the amino-terminal 80 amino acids of the 55-kD T antigen, whereas plasmid p19Kpm2022-X can only code for two amino-terminal amino acids of the 55-kD protein and is therefore almost totally defective in all the 55-kD-related proteins (see Virtanen and Pettersen 1985). The use of these mutants further reinforces the results indicating that the 19-kD T antigen is essential for the *trans*-activation and derepression functions and suggests that the 55-kD T antigen may not have a significant role in these functions.

Effect on immunoglobulin enhancer-driven CAT expression

Since the experiments described in the preceding sections revealed that gene expression driven by SV40 (Fig. 4), Py (Fig. 5), and possibly E1a (Figs. 3 and 7) enhancers was activated by the 19-kD T antigen, we also tested whether the E1b region could *trans*-activate gene expression mediated by the immunoglobulin heavy-chain enhancer. For this purpose we used a test plasmid construct, pAcatH (Garcia et al. 1986) expressing the CAT gene under the minimal SV40 promoter (Laimins et al. 1982) and the heavy-chain enhancer. Plasmid pAcatH and the various E1b plasmids were cotransfected on the

mouse myeloma cell line S194 (ATCC T1B-19), and CAT activity was determined.

As seen in Figure 6A, cotransfection of pAcatH with the wt E1b region (pΔXC) or plasmids expressing functional 19-kD T antigen (pΔXC-H) or 55-kD T antigen (pΔXC-S) had no significant effect on pAcatH expression compared with pΔXC-K, which is defective in both E1b proteins. To determine whether E1a-mediated repression of the heavy-chain enhancer (Hen et al. 1985) could be overcome by E1b, pAcatH was cotransfected with pXC-derived plasmids, and CAT activity was determined (Fig. 6B). As expected, plasmid pXC-K expressing only E1a almost totally repressed the expression of pAcatH (compared with pΔXC-K lane). When pAcatH was cotransfected with pXC or pXC-H, there was a very small amount of CAT expression. However, this level of CAT expression, in three independent assays, ranged within 5–10% of the level seen in cells that did not express any E1a (lane pΔXC-K) and, therefore, suggests that the E1b 19-kD T antigen does not have a prominent effect (such as observed for the SV40 and Py enhancers) on the release of E1a-mediated repression of the heavy-chain enhancer in myeloma cells.

Trans-activation is cell type-specific

Although we have observed *trans*-activation of gene expression driven by SV40 and Py enhancers in rat fibroblasts (CREF) and in human epithelial cells (HeLa), we did not observe *trans*-activation of gene expression driven by immunoglobulin heavy-chain enhancer in myeloma cells.

The lack of 19-kD T antigen-mediated *trans*-activation in the latter case may be due to the cell type or due

Figure 6. Effect of E1b (A) and E1a plus E1b (B) on pAcatH expression. Mouse myeloma cells (S194, ATCC TIB-19) were transfected with pAcatH (1 μ g) and the various Ad5 plasmids (3 μ g) by the DEAE dextran method as described by Garcia et al. (1986), and the CAT activity was determined from extracts prepared 48 or 60 hr after transfection. (AcCm) Acetyl chloramphenicol; (Cm) chloramphenicol.

to the nature of the heavy-chain enhancer sequences. To determine whether the E1b *trans*-activation property is functional in myeloma cells, we examined the effect of E1b on pSV₂CAT expression. It is known that SV40 promoter-enhancer elements are functional in myeloma cells (Banerji et al. 1983). In cells transfected with pSV₂CAT and pΔXC-K there was significant CAT expression (Fig. 7A). In cells transfected with pSV₂CAT and pΔXC-H, the level of CAT expression did not increase further, suggesting that the E1b *trans*-activation function is not apparent in myeloma cells. This conclusion was further substantiated by examining the expression of pAcatH in rat fibroblasts (Fig. 7B). It has been reported that the immunoglobulin enhancer is functional (albeit at reduced levels in the natural orientation) in fibroblasts (Wasylyk and Wasylyk 1986). CREF cells were cotransfected with pAcatH and pΔXC-H, and the CAT expression was quantitated (Fig. 7B). Plasmid pΔXC-H reproducibly *trans*-activated pAcat-H expression about fivefold, suggesting that 19-kD-mediated *trans*-activation of the IgH enhancer may be regulated by the cell type.

Trans-activation is specific for enhancer

To determine whether *trans*-activation by E1b is specific for the SV40 or Py enhancers, we cotransfected a derivative of pSV₂*neo* lacking the enhancer, pSV₂*neo*ΔE, or pG2 (Hen et al. 1986), containing only the rabbit β -globin promoter, along with the various E1b plasmids, and the expression of *neo* or β -globin RNA was analyzed.

As seen in Figure 8A, cells cotransfected with pSV₂*neo*ΔE and pUC13 expressed very low levels of *neo* RNA. A similar low level of expression was also observed when pSV₂*neo*ΔE was cotransfected with the E1b-defective plasmid pΔXC-K or the 19-kD T antigen–

defective plasmid pΔXC-S. Cotransfection of pSV₂*neo*ΔE with pΔXC (wt E1b) or pΔXC-H (19-kD positive) did not enhance the expression of *neo* RNA. Similarly, the level of β -globin expression observed in cells cotransfected with pG2 and pUC13 did not increase when pG2 was cotransfected with the various E1b plasmids. These results reveal that the effect of E1b on pSV₂*neo* and pG2Py observed in the preceding experiments (Fig. 4B and Fig. 5B) is predominantly due to its effect on the SV40 and Py

Figure 7. Effect of cell type on *trans*-activation by E1b. Mouse myeloma (S194) cells (A) or rat CREF cells (B) were transfected with pSV₂CAT and E1b plasmids (A) or pAcatH and E1b plasmids (B) as in Fig. 6, and CAT activity was determined.

Figure 8. Effect of E1b on enhancerless SV40 early and β -globin promoters. CREF cells were transfected with pSV₂neoΔE or pG2 with various E1b plasmids and *neo* or β -globin specific RNA was analyzed as described in Figs. 2 and 3.

enhancer elements, respectively. A marginal *trans*-activation effect for 19-kD on pG2-driven globin expression (less than twofold; Fig. 5B, lane ΔXC-H) was not apparent in most other experiments, including those where pG2 was included as an internal control for monitoring transfection efficiencies.

Activation of E1a expression is dependent on the E1a upstream sequences

In the case of E1a, the upstream noncoding sequences have been reported to contain functional enhancer elements (Hearing and Shenk 1983; Hen et al. 1983; Imper-

Figure 9. Effect of E1a upstream sequences on E1b *trans*-activation. (A) Organization of various enhancer elements of E1a. (B) Effect of E1b T antigens on E1a expression from pGC212Δb or pLA1Δb. E1a (5 μ g) and E1b (15 μ g) plasmids were transfected on HeLa cells and E1a expression was analyzed as described under Fig. 3.

iale et al. 1983). However, the elements identified by these three groups are located at different regions as illustrated in Figure 9A. One of the Ad2 plasmids (pGC212) constructed in our laboratory (Chinnadurai 1983) contains a deletion of the left-most 309 bp of the viral genome (see Imperiale et al. 1983). Therefore, the plasmid lacks the enhancer elements identified by Hen et al. (1983) in their entirety and most of the sequences identified by Hearing and Shenk (1983) but retains the element identified by Imperiale et al. (1983). To determine whether the upstream sequences deleted in this plasmid could modulate the effect of E1b on E1a expression observed in our studies (Fig. 3B), a derivative of pGC212 lacking all the E1b coding sequences (pGC212Δb) was cotransfected with various Ad5 E1b plasmids. As seen in Figure 9B, pΔXC-K and pΔXC-S induced comparable levels of E1a expression from pGC212Δb. In cells transfected with pGC212Δb and pΔXC or pΔXC-H there was a modest increase in E1a expression of about twofold. In these experiments, the E1a expression from an E1a plasmid pLA1Δb that contains the entire upstream noncoding sequences was also compared. As in the case of pGC212Δb, the E1b plasmids pΔXC-K and pΔXC-S induced more or less similar levels of E1a expression from pLA1Δb. However, the E1b plasmids pΔXC and pΔXC-H had a more pronounced effect on E1a expression, resulting in a greater than 15-fold increase (as measured in three independent experiments) in E1a expression, compared to cells that do not express E1b (pΔXC-K) or E1b 19-kD T antigen (pΔXC-S). These results suggest that the effect of E1b on E1a expression is modulated by the upstream enhancer-containing sequences. The modest twofold increase in E1a expression from pGC212Δb is probably regulated by the upstream enhancerlike element contained in this plasmid and identified by Imperiale et al. (1983).

Activation of Py enhancer in undifferentiated F9 cells by E1b

It has been reported that undifferentiated F9 embryonal carcinoma cells contain an "E1a-like" activity (Imperiale et al. 1984). Consistent with this notion, it has been shown that the Py enhancer is repressed in these cells, whereas a mutant enhancer that is also not repressed by E1a is active (Hen et al. 1986). To determine whether E1b could activate Py enhancer in F9 cells, we cotransfected with pG2Py and various E1b plasmids and analyzed the expression of β-globin (Fig. 10).

F9 cells transfected with plasmid pG2Py* containing the enhancer mutation (Py ECF 9.1) and pUC13 carrier DNA expressed significant levels of globin RNA, whereas in cells transfected with pG2Py containing wt Py enhancer and carrier pUC13 vector DNA or E1b-defective plasmid pΔXC-K, the expression of globin was repressed. However, cells cotransfected with pΔXC, containing wt E1b coding sequences, or pΔXC-H, containing the mutation within the 55-kD T antigen, expressed globin RNA at levels comparable to that in cells transfected with pG2Py*, indicating that in the presence of the amino-terminal half of E1b, the wt Py enhancer is

Figure 10. Effect of E1b T antigens on Py enhancer expression in F9 cells. F9 undifferentiated cells were cotransfected with pG2Py or pG2Py* and the various E1b plasmids by the calcium phosphate method. Total cytoplasmic RNA was extracted 24 hr after transfection and analyzed for β-globin expression.

not repressed. This release of repression was reproducible in three separate experiments. In cells transfected with pG2Py and the E1b plasmid defective in the 19-kD T antigen, there was no significant alteration in the extent of repression of pG2Py, suggesting that the 19-kD T antigen is essential for activation of the Py enhancer in undifferentiated F9 cells.

Discussion

Trans-activation by E1b

Our results demonstrate that the early region E1b of Ad5 and Ad2 (group C) and Ad7 (group B) has a *trans*-activation function, which is especially pronounced on viral and cellular enhancer element (SV40, Py, E1a, and immunoglobulin heavy-chain)-driven promoters. We have localized this *trans*-activation function within the 19-kD T antigen coding region since mutations that exclusively map in this region abolish the enhancer activation function whereas plasmids that can only code for the 19-kD T antigen retain this activity. The 19-kD *trans*-activation function differs from the well-known E1a function (Berk et al. 1979; Jones and Shenk 1979) and also from a recently identified function encoded by the E4 region (Goding et al. 1985). The *trans*-activation functions encoded by E1a and E4 gene products activate the adenovirus early region E2 efficiently, whereas the E1b 19-kD T antigen is much less efficient in its action on the E2 promoter (K. Yoshida and G. Chinnadurai, unpubl.). Our results indicate that significant amounts of E1b can be expressed without E1a and that this level of E1b expression is sufficient to induce a large effect on the expression of the test genes CAT, *neo*, β-globin, and E1a linked to three different promoter elements (SV40, β-globin, and E1a) driven by the four enhancer elements tested.

We have shown that the *trans*-activation function me-

diated by the 19-kD protein is specific for enhancers, since removal of the 72-bp repeats from the SV40 early promoter and of the upstream sequences from the E1a promoter greatly reduces the effect of the 19-kD protein. Similarly, expression of rabbit β -globin from its own promoter is not altered by 19-kD T antigen but the β -globin promoter linked to the Py enhancer is *trans*-activated. The E1a DNA sequences modulating the 19-kD-mediated *trans*-activation of E1a expression also appear to be located primarily within the two E1a enhancer elements (Hearing and Shenk 1983; Hen et al. 1983). Since E1a expression is also stimulated from plasmid pGC212 Δ b, although to a much lesser extent, it is possible that the 19-kD protein may be exerting this limited effect via the enhancer element identified by Imperiale et al. (1983), which is present in pGC212 Δ b. The various enhancer–promoter test gene combinations that we have utilized appear to respond to 19-kD T antigen in rat fibroblasts and human HeLa cells. The immunoglobulin heavy-chain enhancer does not seem to be *trans*-activated by 19-kD protein in mouse myeloma cells. Since the E1b effect was also not observed with SV40 early promoter–enhancer in myeloma cells, although the heavy-chain enhancer appeared to respond to E1b *trans*-activation in CREF fibroblasts, it seems that this 19-kD protein function may be modulated in a cell type–specific manner. This observation suggests that the 19-kD T antigen may function directly or indirectly by interacting with some cellular factors. It has been demonstrated in a number of instances that enhancer elements display cell type specificity (for review, see Voss et al. 1986), indicating that the enhancer elements interact with some cellular factors.

The 19-kD T antigen is predominantly localized on the nuclear envelope and in cytoplasmic membrane components (Persson et al. 1982; White et al. 1984) of cells infected or transformed by Ad2. In addition, biochemical fractionation of Ad2-infected cells has indicated that a significant fraction of this protein is also present in the nucleoplasm (Green et al. 1982). Therefore, it is possible that the 19-kD function may be mediated by direct association with the target DNA or by other, indirect means such as facilitating formation of transcription complexes mediated by enhancers. It is noteworthy that enhancers have been shown to stimulate formation of active transcription complexes (Treisman and Maniatis 1985; Weber and Shaffner 1985). A direct DNA interaction role would be analogous to the E2 *trans*-activator of BPV1, which has been shown recently to bind directly to enhancers (Androphy et al. 1987; Moskaluk and Bastika 1987). All the enhancer elements [including the E1a enhancer element identified by Hen et al. (1983)] activated by the 19-kD protein contain the so-called enhancer core element GTGG A/T A/T A/T (Welher et al. 1983) and may therefore constitute a candidate region for DNA–protein interactions.

Effect on enhancer repression

Our results show that in the presence of the 19-kD T

antigen coded by group C (Ad2 or Ad5) and group B (Ad7) adenoviruses, the repression of SV40 and Py and possibly E1a enhancers mediated by Ad2 or Ad5 is not evident. Recently, it has been reported that in cells transfected with plasmid pGC212 (which contains Ad2 E1a and the amino-terminal half of E1b, i.e., the *Hind*III–G fragment) and an Ad2 major late promoter (MLP) construct linked with the SV40 enhancer, transcription from the MLP is not repressed (Natarajan 1986). However, repression of MLP was observed when the E1b region of pGC212 was mutated, suggesting that in the presence of the 19-kD T antigen, the SV40 enhancer may not be repressed. Our results suggest that the antirepression effect may be related directly to the *trans*-activation function of 19-kD protein, since in myeloma cells where there was no significant *trans*-activation of the immunoglobulin enhancer, E1a-mediated repression was not released efficiently. The 19-kD *trans*-activation function appears to be dominant over E1a-mediated repression. Despite a large increase in E1a expression in the presence of E1b, there is no significant E1a-mediated repression (see Fig. 3). It has been shown earlier that repression by E1a is manifested in a dose-dependent manner (Borelli et al. 1984). The mechanism of E1a-mediated enhancer repression is not known. The anti-E1a effect of the 19-kD protein may be useful to probe the E1a repression of enhancers.

Our results indicate that E1b has a large effect on the expression of E1a. This effect typically ranged from 15- to 25-fold compared with cells transfected with E1a alone. It has been proposed that E1a autoregulates its own expression by enhancer repression (Borelli et al. 1984; Smith et al. 1985; Tibbetts et al. 1986). However, data inconsistent with this model have also been reported (Hearing and Shenk 1985). If E1a indeed autoregulates by enhancer repression, the observed effect of 19-kD protein on E1a expression may be due to the perturbation of the enhancer repression effect. Alternatively, since we have observed *trans*-activation of enhancer-driven gene expression by 19-kD T antigen, the observed effect of E1b on E1a expression could simply be due to the *trans*-activation by 19-kD T antigen.

We note that a 19-kD role opposite to the one we have observed on E1a expression has been proposed by White et al. (1986). They observed that in the human diploid fibroblast cell line WI-38 (in which adenovirus growth is relatively restricted) infected with Ad2 or Ad5 mutants defective in the 19-kD protein, the early region E2a is overexpressed. They have proposed that the enhanced expression of E2a is due to elevated levels of E1a expression, suggesting that E1a expression is under negative control by the 19-kD protein. Here again, it is possible that during viral infection, several viral gene products may interact among themselves in a complex manner; alternatively, the effect could be due to the nature of WI-38 cells. We would like to point out that we have observed the *trans*-activation function not only during transfection of isolated E1a and E1b genes, but also in cell lines that stably express the 19-kD T antigen; therefore, it appears to be an intrinsic property of the protein.

Enhancer activation in F9 cells

Our results indicate that the Py enhancer could be activated in undifferentiated F9 cells by the 19-kD T antigen, suggesting that E1b can also efficiently overcome the repression mediated by a cellular gene product. In addition, we have observed *trans*-activation of Py mutant enhancer (Py*, Py ECF 9.1) reported not to be repressed by E1a (Hen et al. 1986). It has recently been reported that the activated cellular oncogene T24-c-Ha-ras also activates Py and Py* enhancers in F9 cells (Wasylyk et al. 1987). These results would imply that activation of the Py enhancer by the 19-kD protein and by T24 ras need not necessarily involve reversal of the repression effect mediated by E1a or the "E1a-like" cellular repressor but may reflect an E1a effect-independent *trans*-dominant mechanism. However, repression of Py mutant enhancers that are active in F9 cells by E1a has also been observed (Velcich et al. 1986). Therefore, it may be difficult at present to draw definitive conclusions as to whether enhancer activation by 19-kD protein and T24 ras leads to a true reversal of E1a or "E1a-like" repression. T24 ras has been reported to activate enhancers in myeloma cells (Wasylyk et al. 1987). However, the 19-kD protein does not *trans*-activate the SV40 and immunoglobulin heavy-chain enhancer in these cells. In addition, we have also observed in several experiments that T24 ras does not activate the SV40 early promoter in E1a-BRK cells (results not shown), whereas E1b efficiently activates under the same conditions. Although we have not examined the Py enhancer per se in myeloma cells, it would appear from the above two observations that *trans*-activation by these two transforming genes may involve somewhat different mechanisms. However, it is interesting that these two oncogenes have similar enhancer activation function in F9 cells and also cooperate with E1a in oncogenic conversion of primary rodent cells (Ruley 1983). Protein p21, coded by the ras oncogene, resides on the inner surface of the plasma membrane (Shih and Weeks 1984), and since enhancer activation by T24 ras parallels activation by the tumor promoter TPA, it has been postulated that T24 ras activates previously inactive enhancer factors by phosphorylation via protein kinase C (Wasylyk et al. 1987). In contrast, the 19-kD T antigen has a different subcellular localization (i.e., in the nuclear membrane, cytoplasmic membrane, and nucleoplasm), and enhancer activation by this protein may occur by an alternate mechanism.

It should be pointed out that it is also possible that the 19-kD protein may activate the Py enhancer via induction of differentiation-related cellular changes instead of overcoming the effect of the cellular "E1a-like" activity. It has been reported that Py enhancer activity increases during differentiation (Katinka et al. 1981; Sekikawa and Levine 1981; Fujimura and Linney 1982). It is possible that the 19-kD protein may induce some cellular changes associated with the differentiation of F9 cells, thereby resulting in the activation of the Py enhancer. It is noteworthy that the introduction of cellular oncogenes *c-fos* (Müller and Wagner 1984) and *ras* (Weinberg

1984) into F9 cells has been shown to induce differentiation. If the 19-kD T antigen induces some changes associated with differentiation, it is possible that differentiated F9 cells may contain a "19-kD-like" activity.

Enhancer activation and cell transformation

A number of studies have indicated that the 19-kD T antigen is important for adenovirus-mediated cell transformation. All the Ad5 and Ad7 plasmid constructs that we have used in the present study have also been tested for transformation activity on BRK cells. These studies have also clearly indicated that the 19-kD T antigen is essential for transformation (K. Yoshida and G. Chinnadurai, unpubl.). The biochemical function of 19-kD T antigen in cell transformation remains to be elucidated. Since several viral and cellular oncogenes have been implicated in transcriptional regulation (Kingston et al. 1985), the enhancer activation function of 19-kD T antigen identified in the present study may also be important for cell transformation. It is possible that the 19-kD T antigen may activate some dormant cellular genes by enhancer *trans*-activation and that these E1b-activated cellular genes may directly or indirectly lead to cell transformation. It is interesting that the E1a and E1b genes required for immortalization and transformation have opposite effects on enhancers. The E1a gene represses enhancer-linked promoters and *trans*-activates enhancerless promoters. The 19-kD T antigen, on the other hand, *trans*-activates enhancer-linked promoters and relieves E1a-mediated repression. It is possible that the immortalization step by E1a may require repression of a subset of cellular genes and that the transformation process by 19-kD T antigen may activate a second set of cellular genes or deactivate the E1a-repressed genes.

An alternate mechanism would be that the E1b-mediated enhancer activation may lead to cell transformation by regulating the expression of E1a. It has been reported earlier that E1a-immortalized cells express unusually low amounts of E1a RNA compared to cells transformed by E1a and E1b (van den Elsen et al. 1983). It has also been reported that a high level of E1a expression from a strong mouse metallothionein promoter could lead to full transformation of NIH-3T3 cells (Senear and Lewis 1986). Although it is not known whether high levels of E1a expression could lead to full transformation of BRK and other primary cells, it is possible that the highly pronounced effect of 19-kD T antigen on E1a expression that we have observed could lead to full transformation mediated essentially by E1a. Although both alternatives could be true, more work is needed to elucidate fully the possible relationship between the 19-kD regulatory function and cell transformation.

Materials and methods*Cells*

Rat embryo fibroblast cell line CREF (Fisher et al. 1982) was originally obtained from P. Fisher and was grown in Dulbecco modified MEM (DMEM) containing 5% fetal calf serum.

Human HeLa cells were grown in monolayer culture using DMEM containing 10% fetal calf serum. Baby rat kidney (BRK) cells were prepared from 6-day-old Fisher rats by treatment with collagenase-dispase (Boehringer Mannheim, Biochemicals, Indianapolis, Indiana) and grown in α -MEM containing 10% fetal calf serum. BRK cells were immortalized (Ela-BRK) by transfection of a plasmid, pHpaI-E, containing the Ela coding sequences of Ad2. F9 embryonal carcinoma cells were obtained from C. Carlin and maintained in DMEM containing 10% fetal calf serum. S194 myeloma cells were obtained from American Type Culture Collection and were grown in suspension cultures using DMEM and 10% horse serum.

Plasmids

The Ad2 Ela-containing plasmid, pHpaI-E, which contains the left 1569 bp (*HpaI*-E fragment) of the Ad2 genome cloned at the *PstI* site of pBR322, was originally obtained from J. Darnell. pXC was constructed by recloning the left-most 5788 bp (*XhoI*-C fragment) of Ad5 from plasmid pBL209 (Babiss et al. 1983) in pUC13. Mutant derivatives of pXC were constructed by linearizing pXC with various restriction endonucleases, followed by blunt-ending with T4 DNA polymerase or the Klenow fragment of DNA PolI (Maniatis et al. 1982) and recloning. Mutant plasmid pXC-S contains a 4-bp deletion at the *SstI* site (N1770), pXC-K contains a 4-bp deletion at the *KpnI* site (N2048), and pXC-H contains a 4-bp insertion at the *HindIII* site (N2805). Plasmid p Δ XC and the mutants p Δ XC-S p Δ XC-K, and p Δ XC-H were derived from the pXC, pXC-S, pXC-K, and pXC-H plasmids, respectively, by deleting the Ela region up to the *Xba-I* site (N1340). The Ad7 E1b-containing plasmid pRA was constructed by cloning the *RsaI*-A fragment (N1431–4024) in pUC13. Mutant plasmids pRA-N and pRA-A contain a 4-bp deletion at the *NcoI* site (N1600) and a 2-bp insertion at the *AccI* site (N2178), respectively. The deletion in plasmid pRA-N was introduced by S1 nuclease treatment. The double-mutant plasmid pRA-NA was constructed by ligating mutated segments from pRA-N and pRA-A. The various Ad5 and Ad7 E1b mutations are illustrated in Figure 1. Plasmids pSV₂*neo* (Southern and Berg 1982) and pSV₂CAT (Gorman et al. 1982) were obtained from P. Southern and B. Howard, respectively. Plasmids pG2Py, pG2Py*, and pG2 (Hen et al. 1986) were obtained from P. Chambon. Plasmid pSV₂*neo* Δ E was constructed by deleting the enhancer sequences between N126 (*NsiI*) and N270 (*PvuII*) of the SV40 genome and by recloning the *neo* transcription unit in pUC13. Plasmid pGC212 Δ b was constructed by deleting the E1b sequences (*SstI*–*HindIII*) from pGC212 (Chinnadurai 1983). Plasmid pLA1 Δ b was similarly constructed by deleting the E1b sequences from pLA1 (Tamanai and Stillman 1982). Plasmid p19Kpm2022-X was constructed by cloning the Ad2 19-kD coding region (*XbaI* to *XmnI* site) from mutant plasmid pBEpm2022 (Barker and Berk 1987) into a pUC13 vector carrying the SV40 early poly(A) addition signals.

Transfections

Plasmid DNAs were introduced into cells for transient expression assays either by the CaPO₄ or the DEAE dextran methods. Where appropriate, the enhancerless β -globin plasmid pG2 was cotransfected as an internal control for normalizing transfection efficiencies. Levels of E1b expression also served as additional controls in most experiments.

Acknowledgments

This work was supported by research grants CA-33616 and CA-31719 from the National Cancer Institute. We thank A.

Berk, C. Carlin, P. Chambon, B. Howard, P. Jat, J. Nevins, P. Sharp, C. Queen, and C.S.H. Young for various plasmids and cells. K.Y. was supported in part by a Postdoctoral Fellowship from the Japan Society for Promoting Science.

Note added in proof

Trans-activation of viral and cellular promoters by the 19-kD T antigen has also been observed by C.H. Herrmann, C.V. Déry, and M.B. Mathews (*Oncogene*, in press).

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Genes Dev. 1987, **1**:

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