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Down-Regulation of the Major Histocompatibility Complex Class I Enhancer in Adenovirus Type 12-Transformed Cells Is Accompanied by an Increase in Factor Binding

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In transformed cells, the E1A gene of adenovirus type 12 (Ad12) represses transcription of class I genes of the major histocompatibility complex. The tumorigenic potential of Ad12-transformed cells correlates with this diminished class I expression. In contrast, the E1A gene of the nontumorigenic Ad5 does not affect class I expression. We show here that a transfected reporter chloramphenicol acetyltransferase plasmid driven by an *H-2*K promoter (−1049 bp) was expressed at much lower levels in Ad12- than in Ad5-transformed mouse cells. Analysis of mutant constructs revealed that only 83 bp of *H-2* DNA, consisting of the enhancer juxtaposed to the basal promoter, was sufficient for this differential expression. Whereas the *H-2* basal promoter alone was somewhat less active in Ad12-transformed cells, the *H-2* TATA box itself did not appear to be important. The *H-2* enhancer proved to be the principal element in Ad12 E1A-mediated repression, since (i) substitution of the *H-2* enhancer by simian virus 40 enhancers overcame the repression, and (ii) when juxtaposed to either its native or heterologous basal promoters, the *H-2* enhancer was functional in Ad5- but not Ad12-transformed cells. Mobility shift assays showed that there is a DNA-binding activity to the 5′ site (R2 element) of the enhancer that is significantly higher in Ad12- than in Ad5-transformed cells. These results suggest that decreased class I enhancer activity in Ad12-transformed cells may, at least in part, be due to the higher levels of an enhancer-specific factor, possibly acting as a repressor.

All adenovirus serotypes can transform rodent cells in vitro, but only some are capable of generating tumors in rodents. This difference has been used to group adenoviruses as highly oncogenic, weakly oncogenic, or nononcogenic (15). Expression of the major histocompatibility complex (MHC) class I genes is diminished in cells transformed by the highly oncogenic adenovirus type 12 (Ad12) but not by the nononcogenic Ad5 (13, 39). Class I genes encode surface antigens (H-2K, H-2D, and H-2L in mice) that are expressed by most cells and play an important role in immune recognition (reviewed in references 11 and 44). Since foreign and class I antigens must be corecognized by cytotoxic T lymphocytes for lysis of target cells to occur (53), the reduced levels of class I antigens on Ad12-transformed cells may favor their escape from immunosurveillance and act as an important step in tumorigenesis (7, 13, 43, 52).

In both human and mouse cells transformed by Ad12, the levels of each of the class I antigens and mRNAs are greatly reduced (13, 39, 47). Nuclear run-on experiments demonstrated that the low class I mRNA levels result from a decrease in the rate of transcription (1, 16). Reduced transcription is not due to structural perturbation of the class I genes, since they remain responsive to interferon stimulation (12, 13). The Ad12 E1A gene, which encodes two overlapping proteins of 266 and 235 residues, is capable of repressing class I transcription in the absence of other adenovirus genes (47).

E1A is multifunctional. It transforms primary cells in cooperation with other oncogenes such as the adenovirus E1B, polyomavirus middle T antigen, and *ras* genes (reviewed in references 8 and 37); for this process to occur, the binding of cellular proteins to E1A protein appears to be essential (51). E1A also transactivates certain viral and cellular genes, likely by interacting with cellular transcription factors (6, 24, 32, 50). In addition, E1A prevents some enhancers, e.g., the simian virus 40 (SV40) and immunoglobulin G enhancers, from stimulating transcription (9, 22, 48). Since most of these functions appear to be common to E1A genes of both Ad5 and Ad12, it is intriguing to consider how only Ad12 E1A represses class I transcription.

To determine what class I DNA regulatory elements are involved in transcriptional down-regulation by Ad12 E1A, we transfected Ad5- and Ad12-transformed mouse cell lines with *H-2* promoter-driven reporter chloramphenicol acetyltransferase (CAT) plasmids. These cell lines are genetically identical ($H\text{-}2^d$) and have been extensively characterized by our laboratory with respect to reduced class I expression (12, 13, 16, 52). We show here that expression of an exogenous $H\text{-}2K^b$ promoter was significantly lower in Ad12- than in Ad5-transformed cells. Specifically, the class I enhancer (−205 to −159) was found to be much less active in Ad12-transformed cells. This finding, together with an observed higher DNA factor-binding activity to the 5′ region of the enhancer (R2 element) in extracts from Ad12-transformed cells, leads us to speculate the presence of an enhancer repressor that down-regulates MHC class I transcription. In the accompanying report (30), we show that R2 does function as a negative element in Ad12-transformed

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cells and that its ability to repress transcription is dependent on factor binding.

MATERIALS AND METHODS

Cell lines. Cell lines transformed with Ad5 (Wt5a, KAd5-3, and BrAd5) and Ad12 (F10-12, EAd12-1, and 12A1) were derived from primary mouse BALB/c (*H-2*^{d}) cells and have been described previously (13).

Plasmid constructions. *H-2* constructs were made in either plasmid pML or pBluescript. The parental *H-2* promoter plasmid was constructed by subcloning the *H-2K*^{b} fragment, *Pvu*II-*Nru*I (−1049 to +12 relative to the transcription initiation site), upstream of the coding sequences of the reporter CAT gene (34, 49). *H-2* promoter deletion plasmids (pH2-364, pH2-118, and pH2-37) were constructed by cutting pH2-1049 with restriction enzymes *Xba*I, *Ppu*MI, and *Sac*II, respectively, and then subjecting the fragments to mung bean nuclease digestion and religation. For plasmids pH2-215, pH2-H2(E+IRS), pH2-H2E, and pH2-SVE, a polymerase chain reaction (PCR) was used to generate inserts with modified *Sac*II ends, which were cloned into the *Sac*II site of pH2-37. The inserts were −38 to −215, −123 to −215, and −159 to −205 of the *H-2* promoter in pH2-215, pH2-H2(E+IRS), and pH2-H2E, respectively, and two copies of the SV40 72-bp enhancer (19) in pH2-SVE. pH2-H2(E+IRS)s is the same as pH2-H2(E+IRS) except for the second *Sac*II site at position −215, which was destroyed by site-directed mutagenesis. pH2-s-H2(E+IRS) was made by inserting a PCR-amplified 80-bp fragment of pBR322 (bp 4210 to 4290) into the *Sac*II site of pH2-H2(E+IRS)s. The sources of SV40 and herpes simplex virus type 1 thymidine kinase (TK) sequences were plasmids pSV2-CAT (19) and pHSV106 (33), respectively. In pSV-H2(E+IRS) and pTK-H2(E+IRS), the *H-2* enhancer is joined to the basal promoters of the early SV40 and herpes simplex type 1 TK genes, respectively. To construct pSV-H2(E+IRS), the SV40 early promoter of pSV2CAT was amplified by PCR from position −35 relative to the transcription start site (a *Sac*II site was included in the oligonucleotide primer) to the *Nco*I site within the CAT coding region. The amplified DNA was used to replace the *Sac*II-to-*Nco*I fragment of pH2-H2(E+IRS)s. To construct pTK-H2(E+IRS), the TK promoter from pTKCAT was amplified by PCR from position −40 (a *Sac*II site was included in the primer) to the *Nco*I site within the CAT gene, and the amplified DNA was used to replace the *Sac*II-to-*Nco*I fragment of pH2-H2(E+IRS)s. Every construct was verified by DNA sequencing (38).

Site-directed mutagenesis. The *H-2* TATA box (TATAA AGT) mutations were made from single-stranded templates (pH2-37 and pH2-364), using Amersham in vitro mutagenesis reagents. Oligonucleotides with the sequences CGGACGC TGGATATTTAGTCCACGCAGCCC and CGGACGCTGG ATATTTATTCCACGCAGCCC were used to generate double- and triple-point mutations where indicated by the underlines. All mutations were verified by DNA sequencing (38).

Transfection and CAT assay. Cells were plated in 10-cm dishes and the next day (when at 30 to 50% confluency) cotransfected with reporter CAT plasmid (15 μg) and pRSV-β-gal plasmid (5 μg) by the calcium phosphate method (20). After 48 h, 100 μl of extract was prepared. To determine the β-galactosidase activity, 20 μl of the extract was added to 380 μl of 5 mM chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and incubated at 37°C for 30 min, and the optical density at 578 nm was measured. The same extract was used to determine CAT activity (19). To correct for transfection efficiency, the volume of extract used for each CAT assay was adjusted to an equivalent β-galactosidase activity. Conversion to acetylated [^{14}C]chloramphenicol was determined by thin-layer chromatography and quantitated by using a radioanalytic scanner (AMBIS Microbiology Systems, San Diego, Calif.). For each construct, the CAT values represent the average of at least four transfections.

Nuclear extracts and binding reactions. Nuclear extracts were prepared according to Shapiro et al. (40). Final protein concentrations were 8 to 10 mg/ml. For mobility shift assays, 2 to 6 μg of nuclear extract was incubated for 30 min at 30°C with 25 to 50 fmol of ^{32}P-end-labeled double-stranded oligonucleotides and 1.0 μg of poly(dI-dC) in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–60 mM KCl–1 mM $MgCl_2$–1 mM EDTA–1 mM dithiothreitol–10% glycerol. DNA-protein complexes were resolved on 5% polyacrylamide gels containing 45 mM Tris-borate and 1 mM EDTA and were visualized by autoradiography of the dried gel. For competition experiments, nuclear extracts were incubated with unlabeled double-stranded DNA for 5 min at room temperature prior to addition of labeled probe. The oligonucleotides used are shown in Table 1.

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence
H2E (−205 to −156)	5′-TCGAGGCAGTGAGGTCAGGGGTGGGGAAGCCCAGGGCTGGGGATTCCCCATCTG-3′ 3′-CCGTCACTCCAGTCCCCACCCCTTCGGGTCCCGACCCCTAAGGGGTAGACAGCT-5′
R2 (−205 to −184)	5′-TCGAGGCAGTGAGGTCAGGGGTGGG-3′ 3′-CCGTCACTCCAGTCCCCACCCAGCT-5′
R1 (−176 to −156)	5′-TCGAGGGCTGGGGATTCCCCATCTG-3′ 3′-CCCGACCCCTAAGGGGTAGACAGCT-5′
SP1	5′-CTAGTCCCGCCCCTAACTCCGCCCAT-3′ 3′-AGGGCGGGGATTGAGGCGGGTAGACT-5′
CREB	5′-ACAGTCCCCGTGACGTCACCCGGGAGCCC-3′ 3′-TGTCAGGGGCACTGCAGTGGGCCCTCGGG-5′
H2 TATA (−35 to −13)	5′-TCGACGGACGCTGGATATAAAGTCCAC GCCTGCGACCTATATTTCAGGTGAGCT-3′

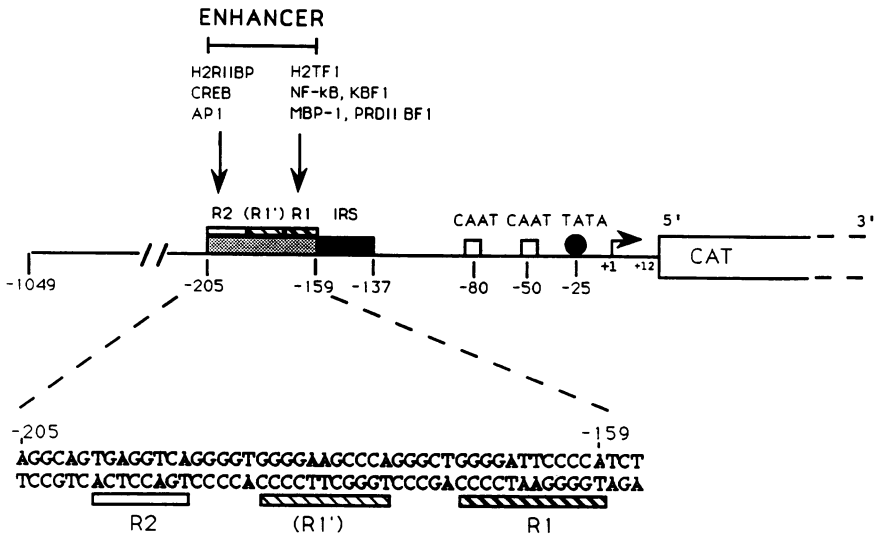

FIG. 1. Regulatory elements of the *H-2* promoter. Depicted is the reporter CAT gene linked to 1,061 bp of the *H-2* promoter. Indicated is a TATA box located 25 bp upstream of the transcription start site, two CAAT boxes (at −50 and −80), an IRS located between −137 and −154, and an enhancer located between −205 and −159. Indicated above the arrows are the cellular factors that bind to the enhancer sites R1 and R2; R1′ is an imperfect repeat of R1. The sequence of the complete enhancer is shown. The R2 site is recognized by a member of the steroid receptor family, H2-RIIBP (retinoid X receptor beta) as well as having a consensus binding sequence for CREB and AP-1 (21, 29). The R1 site has a 13-bp recognition sequence for H2TF1, NF-κB, KBF1, PRDII-BF1, and MBP-1 (3–5, 14, 25, 26, 41). Both R1 and R2 sites have been implicated in the control of MHC class I transcription (4, 10, 25, 28).

RESULTS

Expression of *H-2* promoter plasmids is down-regulated in Ad12-transformed cells. In Ad12-transformed cells, the diminished level of class I expression results from a block in transcription (1, 16). To determine whether the promoters of the MHC class I genes are regulated by Ad12 E1A, plasmids containing a CAT reporter gene, driven by the class I *H-2K*^{b} promoter, were transfected into Ad5- and Ad12-transformed mouse cell lines. The *H-2* CAT plasmids (pH2-1049 and pH2-364) used in this experiment contained 1,049- and 364-bp sequences upstream of the transcriptional start site of the *H-2K*^{b} promoter, respectively, and included known transcriptional regulatory elements (11, 28, 29, 35, 49) (Fig. 1). CAT activity from both plasmids was much lower in Ad12- than in Ad5-transformed cells (Fig. 2). In contrast, both Ad5- and Ad12-transformed cells expressed nearly equivalent levels of CAT activity when transfected with pSV2CAT, in which the SV40 early promoter drives the CAT gene (Fig. 2). These results reflect the disparity in transcription of endogenous class I genes between Ad5- and Ad12-transformed cells and indicate that there is not a general difference in the ability of these cells to express exogenous genes. Furthermore, they demonstrate that sequences contained within 364 bp upstream of the *H-2* promoter transcription start site are sufficient for down-regulation by Ad12 E1A. Consistent with these results was the low activity obtained in Ad12- but not Ad5-transformed cells with use of the human class I HLA-A2 promoter (data not shown).

Promoter deletion suggests elements involved in repression. To locate sequences within the *H-2* promoter which might be down-regulated by Ad12 E1A, a series of 5′ deletions was constructed from the parental *H-2* CAT plasmid, pH2-1049 (Fig. 3). If Ad12-specific negative transcription elements reside in the upstream regulatory region of the class I gene, their removal is expected to result in equivalent CAT activity in Ad5- and Ad12-transformed cells. However, the levels of CAT activity remained consistently low when each *H-2* promoter deletion construct was transfected into Ad12-transformed cells (Fig. 3). In contrast, a gradual decrease in promoter activity in Ad5-transformed cells accompanied the deletion of regulatory elements (constructs pH2-118 and pH2-37). In both transformed cells, the level of CAT activity obtained with pH2-364 or pH2-215 was always higher (about 1.5 to 2 times) than that of the longest construct, pH2-1049,

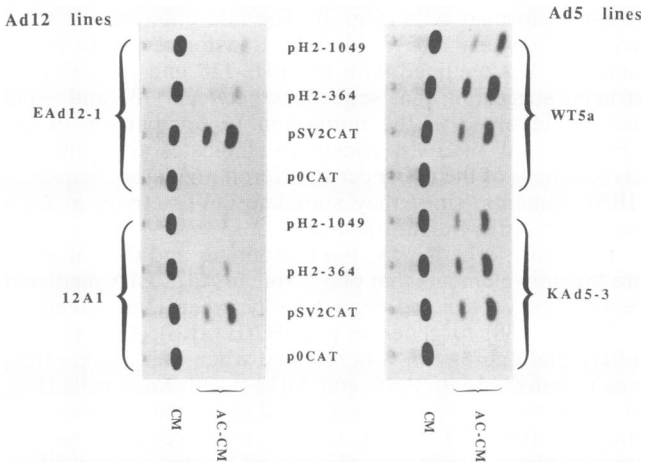

FIG. 2. Repression of exogenous *H-2* promoter activity in Ad12-transformed cells. CAT plasmids were cotransfected into Ad5 (Wt5a and KAd5-3)- and Ad12 (EAd12-1 and 12A1)-transformed mouse cells lines, with plasmid pRSV-β-gal used as an internal standard. All CAT activities were normalized to equivalent β-galactosidase levels. The *H-2* plasmids contain up to bp −1049 and −364 of the *H-2* promoter sequences relative to the start site of transcription. Control plasmid pSV2CAT contains the SV40 enhancers and early promoter, and p0CAT is promoterless. AC-CM represents the acetylated forms of [^{14}C]chloramphenicol (CM).

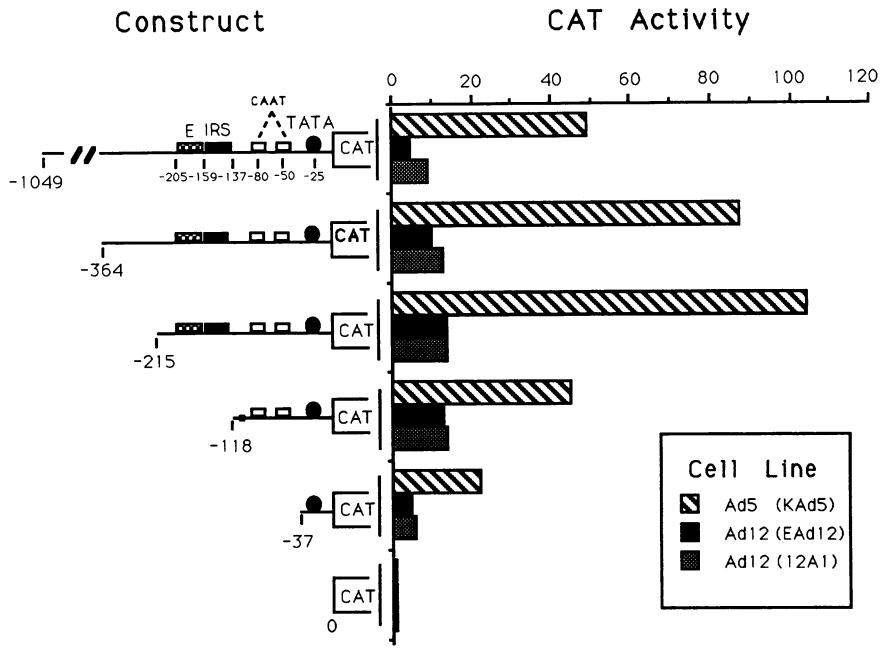

FIG. 3. Analysis of the *H-2* promoter deletion constructs in adenovirus-transformed cells. In the *H-2* deletion constructs, the length of the *H-2* promoter remaining after deletion is indicated, as are the locations of the enhancer (dotted box), the adjacent IRS (filled box), the CAAT boxes (open boxes), the TATA box (filled circle), and the reporter CAT gene. Each deletion construct was cotransfected with plasmid pRSV-β-gal into transformed cell lines, and CAT activity was determined after normalization of values to equivalent β-galactosidase levels. Shown in the histogram are the CAT activities from the Ad5-transformed cell line KAd5 and from the Ad12-transformed cell lines EAd12 and 12A1. All CAT values are the averages of at least four different experiments.

suggesting the presence of a negative (*cis*-regulatory) element in the *H-2* promoter that is not related to E1A repression.

The basal promoter could be a target for Ad12 E1A-mediated repression, since the level of CAT activity of construct pH2-37 was invariably higher (four- to fivefold) in Ad5-transformed cells (Fig. 3). The fold difference in CAT activity between Ad5- and Ad12-transformed cells is the same as that obtained with the pH2-118 and pH2-37 constructs, suggesting that sequences between −37 and −118 are not involved in the repression. A comparison of the pH2-215 and pH2-118 constructs indicates the potential involvement of the enhancer-interferon regulatory sequence (IRS) elements, since they stimulate CAT activity in Ad5- but not in Ad12-transformed cells.

To address whether the basal promoter and the enhancer are the only elements that play a role in Ad12 E1A-mediated repression, we deleted sequences between these elements (−118 to −38) to generate pH2-H2(E+IRS) (Fig. 4). Full differential expression was retained when pH2-H2(E+IRS) was transfected into Ad5- and Ad12-transformed cells (Fig. 4). Moreover, when the normal *H-2* sequences between the enhancer-IRS and TATA regions were replaced with a random plasmid sequence of identical length, a major difference in CAT activity between Ad5- and Ad12-transformed cells was still observed [pH2-s-H2(E+IRS); Fig. 4].

These studies demonstrate that only 130 bp, which include the enhancer-IRS and basal promoter elements, are sufficient to recapitulate the negative effect of Ad12 E1A. Deletion of sequences upstream of the enhancer (−1049 to −215), as well as those between the enhancer-IRS and basal promoter (−118 to −38), had no effect on the differential class I expression between the Ad5- and Ad12-transformed cells.

Alteration of the TATA box of the *H-2* basal promoter does not restore class I transcription in Ad12-transformed cells. The *H-2* TATA element has a sequence that is known to be responsive to E1A transactivation (42). When the *H-2* TATA sequence (TATAAAGT) was converted by site-directed mutagenesis into an SV40 TATA sequence (TATTTATT) known to be nonresponsive to E1A transactivation (42) or to an SV40-like TATA sequence (TATTTAGT) and placed into the background of pH2-364 or pH2-37, the CAT activity in either Ad5- or Ad12-transformed cells was not changed (data not shown). These results imply that the differential expression of the *H-2* basal promoter does not result from the *H-2* TATA box sequence being repressed by Ad12 E1A or activated by Ad5 E1A. Although the TATA box is the only apparent regulatory element in the −37 region, we cannot rule out an effect due to other sequences.

The *H-2* enhancer fails to function in Ad12-transformed cells. From the deletion analysis (Fig. 3 and 4), the shortest sequence of the *H-2* promoter sufficient for retaining full differential expression in Ad5- and Ad12-transformed cells was a 130-bp fragment containing the enhancer-IRS region joined to the basal promoter. To evaluate the contribution of the enhancer alone in repression, we tested pH2-H2E, in which the 46-bp *H-2* enhancer was placed directly upstream of the 37-bp basal promoter (Fig. 4). pH2-H2E was still expressed at much lower levels in Ad12-transformed cells [compare pH2-H2(E+IRS) with pH2-H2E; Fig. 4]. Thus, the IRS sequence is not essential for Ad12 E1A-mediated repression. Most importantly, the *H-2* enhancer fails to stimulate transcription from its basal promoter in Ad12-transformed cells but does so efficiently in Ad5-transformed cells. When the SV40 72-bp enhancer repeats were juxtaposed to the *H-2* basal promoter (pH2-SVE), CAT activity was ex-

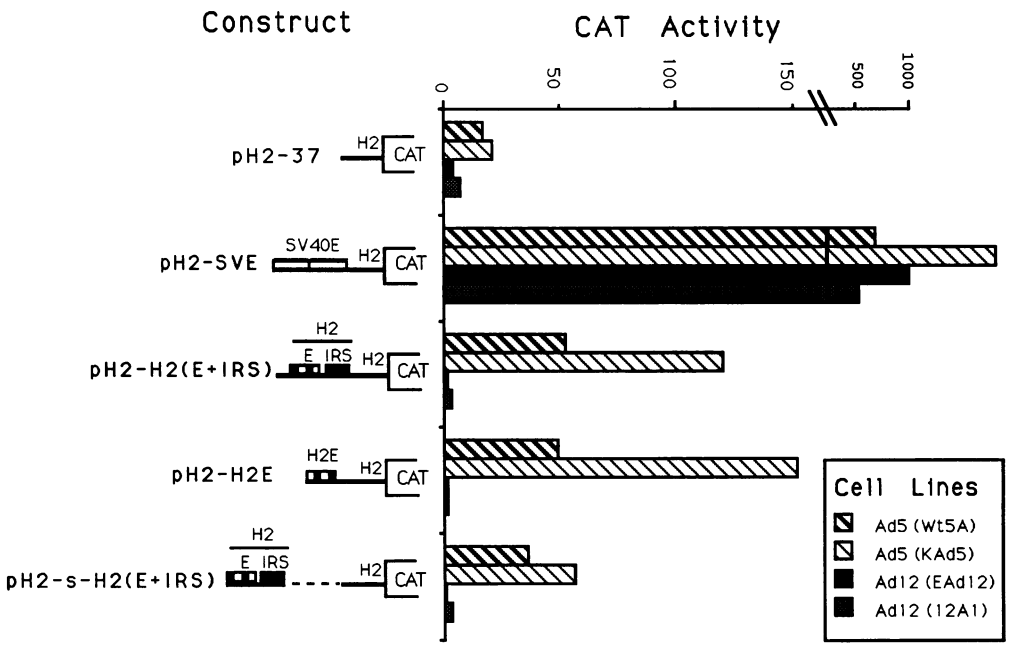

FIG. 4. Failure of the *H-2* enhancer to stimulate transcription in Ad12-transformed cells. Represented are the *H-2* basal promoter constructs linked to homologous or heterologous enhancers. The *H-2* basal promoter (pH2-37), containing bp −37 to +12 of the *H-2K*^{b} gene, was juxtaposed to SV40 enhancers (pH2-SVE), the *H-2* enhancer and IRS [pH2-H2(E+IRS)], or the *H-2* enhancer alone (pH2-H2E). In pH2-s-H2(E+IRS), the *H-2* enhancer and basal promoter are separated by plasmid DNA which is of the same length as in the native *H-2* promoter. Indicated are the SV40 enhancers (open boxes), the *H-2* enhancer (dotted box), and the IRS (filled box). Transfection was carried out as described in the legend to Fig. 3. Shown in the histogram are the CAT activities from the Ad5-transformed cell lines Wt5A and KAd5 and from the Ad12-transformed cell lines EAd12 and 12A1. A two- to threefold difference in CAT activity was typically observed among the different Ad5-transformed cell lines. All CAT values are the averages of at least four transfections.

pressed at very high levels in both Ad5- and Ad12-transformed cells (Fig. 4). Furthermore, a monomer or a dimer of an SP1 binding site activated *H-2* promoter transcription with comparable efficiency in Ad5- and Ad12-transformed cells (not shown). Therefore, the basal promoter is capable of being stimulated in Ad12-transformed cells. Still, in the context of its own enhancer, the *H-2* basal promoter may contribute to the low levels of class I transcription in these cells.

To determine how the class I enhancer functions in the context of heterologous basal promoters, a fragment containing the *H-2* enhancer was linked to the early SV40 or herpes simplex virus type 1 TK basal promoter [pSV-H2(E+IRS) or pTK-H2(E+IRS), respectively; Fig. 5]. In Ad5-transformed cells, the enhancer stimulated CAT activity four- to fivefold relative to the SV40 and TK basal promoters, while in Ad12-transformed cells, the *H-2* enhancer had no stimulatory effect. Thus, in the Ad12-transformed cells, the class I enhancer is incapable of functioning as a transcription element.

In summary, these results demonstrate that the *H-2* enhancer is a principal element in Ad12 E1A-mediated repression. When juxtaposed either to its native basal promoter or to heterologous basal promoters, the *H-2* enhancer stimulates transcription in Ad5- but not Ad12-transformed cells. Maximum differential expression of class I genes between Ad5- and Ad12-transformed cells requires the *H-2* enhancer. The *H-2* basal promoter is not sufficient to confer repression when linked to a heterologous enhancer, e.g., the SV40 enhancer, or to an upstream activator sequence such as the SP1 site.

Increased factor binding to the *H-2* enhancer in Ad12- versus Ad5-transformed cells. Differential regulation of class I transcription in adenovirus-transformed cells could be due to the absence of a positive factor or the presence of a negative factor that binds to the *H-2* promoter. First, we compared Ad5- and Ad12-transformed cells for differences in binding activity throughout the 5′ regulatory region of the class I promoter. Extracts were prepared from two Ad5- and three Ad12-transformed cell lines and analyzed for their DNA-binding activities by mobility shift assays, using DNA fragments that scanned the promoter from −474 to +12.

A major difference was detected with a fragment (−215 to −122) that included the enhancer and the IRS. Extracts from Ad12-transformed cells showed a higher binding activity to this fragment. Separation of the enhancer and the IRS at −165 indicated that this differential binding was to the enhancer and not to the IRS (data not shown).

Mobility shift assays with an oligonucleotide (H2E) that represents the complete *H-2* enhancer (−205 to −156) confirmed that Ad12-transformed cell extracts contain higher levels of an enhancer-binding activity designated complex A (Fig. 6). We emphasize that the sequences contained within the enhancer oligonucleotide accounted for all of the differential binding found with the larger fragments. By comparison, several other DNA-protein complexes were observed at similar levels in extracts from both cell types with the exception of a minor low-mobility complex that was at slightly higher levels in Ad12-transformed cells. All of the complexes were specific, since their formation was inhibited by an excess of unlabeled *H-2* enhancer oligonucleotide (Fig. 6b, lanes 2 and 9) but not by an unrelated oligonucleotide (lanes 7 and 14).

Since the *H-2* enhancer contains at least two known

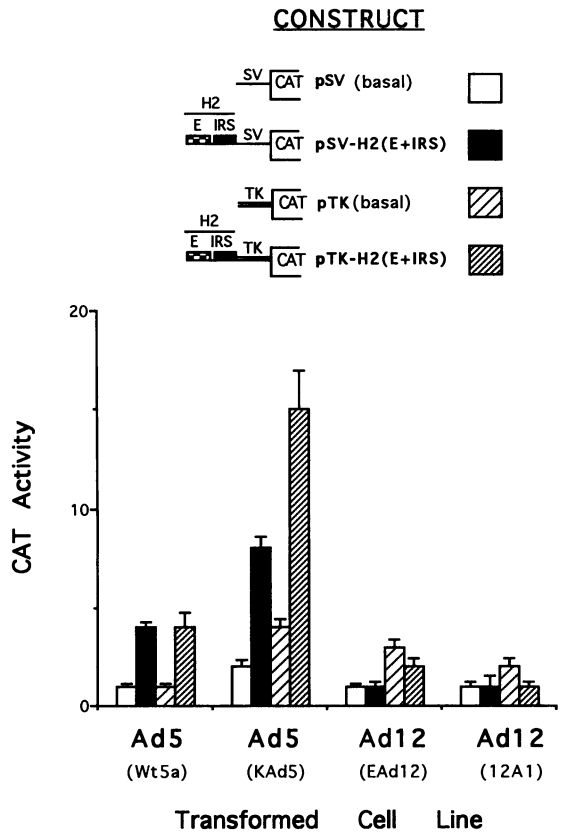

FIG. 5. Inactivity of the *H-2* enhancer linked to heterologous basal promoters in Ad12-transformed cells. Shown are constructs of the *H-2* enhancer joined to heterologous basal promoters. The *H-2* enhancer and IRS elements were joined to the SV40 early and TK basal promoters [pSV-H2(E+IRS) and pTK-H2(E+IRS)]. The basal promoter constructs, pSV and pTK, contained 35 and 40 bp of upstream sequences, respectively. Indicated are the basal promoters of SV40 (single line), TK (double line), the *H-2* enhancer (dotted box), and the IRS (filled box). The histogram represents the CAT activities of the *H-2* enhancer and heterologous promoter constructs in transformed cells. Transfection was carried out as described in the legend to Fig. 3. Shown for each cell line is the CAT activity of the heterologous basal promoter, either alone or juxtaposed to the *H-2* enhancer. All CAT values are the averages of at least four transfections.

factor-binding sites (R1 and R2; Fig. 1), it was important to determine which of them was responsible for complex A. Therefore, we analyzed binding in the presence of excess cold oligonucleotides that contained site R1 (−176 to −156) or site R2 (−205 to −184). Complex A formation was effectively inhibited by the R2 oligonucleotide (Fig. 6b, lanes 4 and 11) but not by the R1 oligonucleotide (lanes 3 and 10), suggesting that its binding depends on the R2 site. Formation of all enhancer-binding complexes was inhibited by a combination of oligonucleotides R1 and R2 (Fig. 6b, lanes 5 and 12). This finding suggested that no other *H-2* enhancer sequences were recognized by these nuclear extracts. Thus, Ad12-transformed cells have significantly higher levels of a DNA-binding activity to the R2 site of the *H-2* enhancer, represented by complex A, than do Ad5-transformed cells.

Mobility shift assays with oligonucleotide R2 confirmed the increased DNA-binding activity to the R2 site (Fig. 7; compare lanes 2 and 6 with lanes 10, 14, and 18). R2, which contains a putative CREB site, has been reported to be recognized by AP-1 (29) and by H-2RIIBP (21). Formation of complex A was inhibited by an oligonucleotide excess that contained the CREB site of the fibronectin gene (Fig. 7, lanes 4, 8, 12, 16, and 20). Similarly, competition with CREB sites of the somatostatin gene or of the CREB-related ATF site of the adenovirus E3 promoter failed to inhibit complex A formation indicating that this activity is distinct from CREB. Moreover, an oligonucleotide that contained an AP-1 site failed to compete (data not shown). As expected, the R1 oligonucleotide did not inhibit complex A formation (Fig. 7, lanes 5, 9, 13, 17, and 21).

Binding assays with other regions of the *H-2* promoter indicated no major differences in DNA-binding activities of nuclear extracts from Ad5- and Ad12-transformed cells (not shown). We also tested binding to a DNA recognition site, SP1, which is not present in the pH2-1049 promoter construct. As shown in Fig. 8, all Ad5 and Ad12 nuclear extracts exhibited similar levels of SP1 binding, suggesting that the difference observed with the *H-2* enhancer is specific and does not reflect a general difference in binding activity between the two types of transformed cells.

DISCUSSION

The E1A gene of Ad12 is known to repress class I transcription in transformed cells (1, 16). We show here that this phenomenon is also observed with exogenous class I promoters. Specifically, the class I *H-2K*^{b} promoter driving the reporter CAT gene is expressed at reduced levels in Ad12- but not in Ad5-transformed cells. Significantly, a construct containing just the basal promoter (−37 to +12) and the enhancer (−205 to −159) retained the same differential expression between Ad5- and Ad12-transformed cells as was observed with the full-length pH2-1049 promoter construct. The importance of the *H-2* enhancer in Ad12 E1A-mediated repression was demonstrated by showing that it can stimulate transcription of heterologous basal promoters in Ad5- but not in Ad12-transformed cells. Thus, the *H-2* enhancer alone is sufficient for down-regulation of transcription in Ad12-transformed cells. In correlation with the genetic analysis, we find a higher binding activity to the R2 site of the enhancer (−205 to −185) with nuclear extracts from Ad12- compared with Ad5-transformed cells, suggesting the presence of an R2-binding repressor in Ad12-transformed cells. This binding activity (referred to as complex A) recognizes the same site for the recently described factor H-2RIIBP, a member of the steroid hormone receptor superfamily (21). The levels of H-2RIIBP mRNA are the same in Ad5- and Ad12-transformed cells (unpublished data), indicating either that the protein levels or binding activity of H-2RIIBP may differ in the two cell types or that other distinct factors are involved. Indeed, tissue-specific and developmental differences in factor binding to the R2 site (and the R1 site) have been observed (10).

In the class I enhancer there is an AP-1/CREB consensus binding site that overlaps with the H-2RIIBP binding site (28, 29). Recently, both Ad5 and Ad12 E1A were shown to be capable of mediating repression of the collagenase promoter through an AP-1 complex binding site (17, 36, 46). Notably, the surrounding DNA context of the AP-1 binding site appears to be influential, since an AP-1 site of the c-*jun* promoter is activated by both Ad5 and Ad12 E1A (46). Our data suggest that class I repression by Ad12 E1A is not through an AP-1 complex, since an AP-1 oligonucleotide could not compete for R2-binding factors. In accord with our

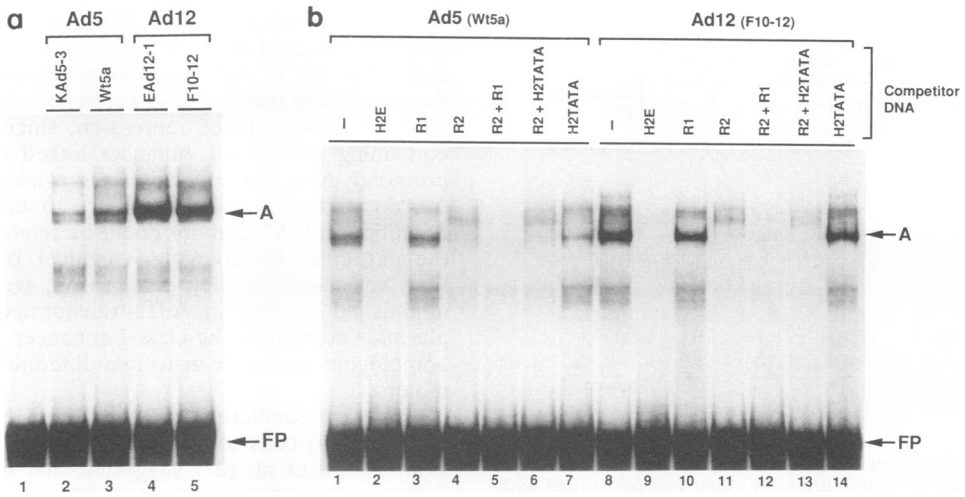

FIG. 6. Evidence that Ad12-transformed cells contain a higher binding activity to the *H-2* enhancer than do Ad5-transformed cells. (a) DNA mobility shift analysis of the *H-2* enhancer, using extracts from Ad5- and Ad12-transformed cells. ^{32}P-labeled oligonucleotide H2E containing the *H-2* enhancer was incubated with either no extract (lane 1) or 6 μg of extract from Ad5 (lanes 2 and 3)- or Ad12 (lanes 4 and 5)-transformed cell lines. DNA-protein complexes were resolved on a 5% polyacrylamide gel. A, the complex that is more abundant in extracts from Ad12-transformed cells; FP, free probe. (b) Competition analysis of binding activities to the *H-2* enhancer. Extracts from Ad5 (Wt5a)- or Ad12 (F10-12)-transformed cells (lanes 1 to 7 and 8 to 14, respectively) were incubated with labeled *H-2* enhancer oligonucleotide (H2E) in the absence of competitor oligonucleotide (lanes 1 and 8) or in the presence of a 20-fold excess of unlabeled oligonucleotide (lanes 2 to 7 and 9 to 14), as indicated. The positions of complex A (A) and free probe (FP) are indicated.

observations, Ackrill and Blair (2) showed that in Ad12- but not Ad5-transformed rat cells, there was a binding activity to the 5′ region of the class I enhancer which was distinct from AP-1, CREB, and ATF.

Interestingly, N-*myc* expressed in neuroblastoma cells was shown to repress class I transcription by reducing factor binding to the R1 enhancer site (31). The R1-binding factors are also greatly reduced in AKR tumor cells, in which class I mRNA levels are low (23). Using the R1 site probe in mobility gel shift assays, we observed a slowly migrating complex present at slightly higher levels in extracts of Ad5- compared with Ad12-transformed cells (data not shown). Thus, while the role of the R1 site in negative regulation by Ad12 E1A cannot be excluded, functional studies, described in the accompanying report (30), clearly demonstrate that the R2 element can interfere with the ability of the R1 site to stimulate transcription.

The role of the *H-2* basal promoter in repression is not clear. The activity of the pH2-37 construct was consistently four- to fivefold higher in each of the Ad5-transformed cell lines than in Ad12-transformed lines. In contrast, SV40 and TK basal promoters did not show significant differences between Ad5- and Ad12-transformed cell lines. The *H-2* TATA box sequence per se is not important, since convert-

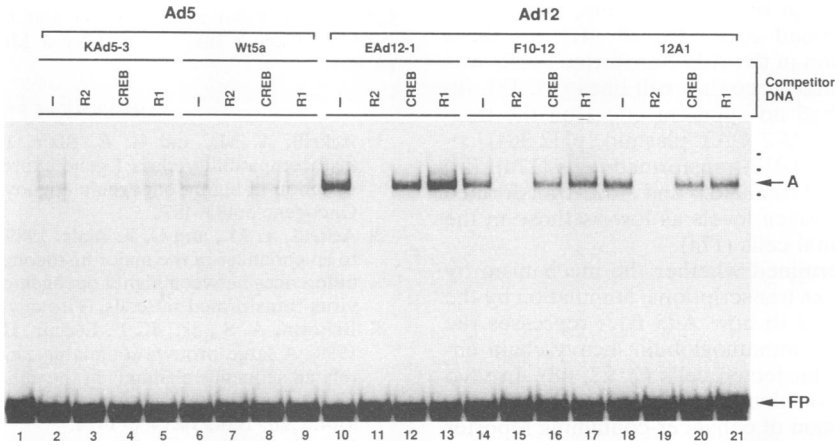

FIG. 7. Recognition by complex A of the R2 site of the *H-2* enhancer. Shown is the mobility shift analysis of ^{32}P-labeled oligonucleotide 205/185, representing the R2 site of the *H-2* enhancer. The labeled oligonucleotide was incubated without extract (lane 1) or with 2 μg of nuclear extract from either Ad5 (lanes 2 to 9)- or Ad12 (lanes 10-21)-transformed cells in the absence or presence of a 20-fold excess of unlabeled competitor DNA. Indicated are samples with no competitor (lanes 2, 6, 10, 14, and 18), self-competitor R2 oligonucleotide (lanes 3, 7, 11, 15, and 19), an oligonucleotide containing the CREB site of the fibronectin gene (lanes 4, 8, 12, 16, and 20), and the R1 oligonucleotide (lanes 5, 9, 13, 17, and 21). DNA-protein complexes were resolved on a 5% polyacrylamide gel. The positions of complex A (A) and free probe (FP) are indicated.

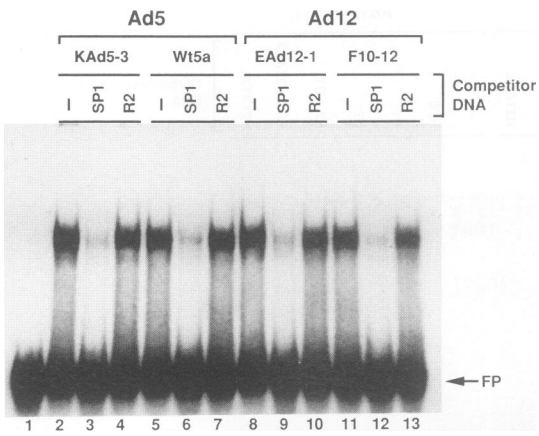

FIG. 8. Similar levels of binding activity of SP1 in Ad5- and Ad12-transformed cells. A mobility shift assay was performed with no extract (lane 1) or 6 μg of nuclear extract from Ad5- or Ad12-transformed cells and a ^{32}P-labeled oligonucleotide containing the SP1 site, without competitor DNA (lanes 2, 5, 8, and 11) or with a 20-fold excess of unlabeled oligonucleotide SP1 (lanes 3, 6, 9, and 12) or R2 (lanes 4, 7, 10, and 13). DNA-protein complexes were resolved on a 5% polyacrylamide gel. FP, free probe.

ing it to other TATA box sequences failed to restore *H-2* transcription in Ad12-transformed cells. Significantly, the SV40 enhancer stimulated the *H-2* basal promoter activity to similarly high levels in both Ad5- and Ad12-transformed cells, as did insertion of SP1 binding sites upstream of the *H-2* basal promoter. These results suggest that an element other than the TATA box may be responsible for the low basal promoter activity in the Ad12-transformed cells and that its effect can be overcome by a strong heterologous enhancer.

In our experiments, the possibility that the transfected *H-2* promoter is not down-regulated by Ad12 E1A but rather is transactivated by Ad5 E1A is unlikely for the following reasons. (i) In transformed mouse, human, and rat cells expressing Ad12 E1A, the levels of class I antigens and their mRNAs were lower than in the parental cells, control cell lines, and Ad5-transformed cells (13, 18, 47). In these studies, class I expression in the Ad5-transformed cells was not higher than in parental or control cell lines (13, 18). (ii) An Ad5 E1A plasmid had no apparent effect on the transcriptional activity of an *H-2* CAT plasmid (pH2-364) cotransfected into HeLa or Ad12-transformed cells (17a). (iii) Somatic fusions generated from Ad5- and Ad12-transformed cells displayed class I antigen levels as low as those in the Ad12-transformed parental cells (17a).

It remains to be determined whether the mechanism by which Ad12 E1A represses transcriptional stimulation by the class I enhancer is related to how Ad5 E1A represses the SV40, polyomavirus, and immunoglobulin heavy-chain enhancers in infected or transfected cells (9, 22, 48). In vivo competition with excess enhancer fragments was able to relieve Ad5 E1A repression of enhancer-containing reporter plasmids, suggesting that a negative effect is mediated through E1A alone or in combination with a negative cellular factor (9). It is important to note that the SV40 enhancer constructs used in this study were expressed at similar levels in all adenovirus-transformed cell lines, suggesting that if any E1A-mediated repression did occur, it was to the same extent in both Ad5- and Ad12-transformed cells.

In accord with our results, Kimura et al. (28) also showed that a transfected *H-2* CAT plasmid was down-regulated in Ad12- but not in Ad5-transformed mouse cells. However, they suggested that the class I enhancer is not the sole target of Ad12 E1A-mediated repression, since a CAT construct containing the class I enhancer linked to the conalbumin promoter was not extensively down-regulated in Ad12-transformed cells. Their inability to detect a substantial reduction in CAT activity could be related to the nature of the particular heterologous enhancer promoter construct that was used (28). By comparison, we achieved greatly diminished activity in Ad12-transformed cells with CAT plasmids containing the class I enhancer linked either to its homologous promoter or to two different heterologous promoters.

There are conflicting results of studies using established (nonprimary) cells to analyze Ad12 E1A-mediated repression. Katoh et al. (27) suggested that in established 3Y1 cells, an element upstream of the class I enhancer (−1836 to −1521) is a target for repression, and that the enhancer is actually stimulated by E1A. In contrast, Vaessen et al. (45) failed to see any difference in class I expression following transfection of Ad5 or Ad12 E1A into 3Y1 cells. Similarly, we have never been able to produce any difference in class I synthesis in established cell lines in which we introduced E1A of Ad5 or Ad12 (18).

Our study clearly shows that the class I enhancer is involved in Ad12 E1A-mediated repression in transformed mouse cells. This repression correlates with an increase in factor binding to the R2 element of the class I enhancer. Since transcriptional regulation of class I genes is potentially controlled by the binding of both positive and negative regulatory factors, the increased binding of a negative factor in Ad12-transformed cells could result in decreased transcription. Indeed, in the accompanying report (30), we show that the R2 element can repress R1-dependent transcription from the class I promoter in Ad12-transformed cells.

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