JOURNAL OF VIROLOGY, Dec. 1992, p. 6979–6988
0022-538X/92/126979-10$02.00/0

Vol. 66, No. 12

Negative Regulation of the Major Histocompatibility Complex Class I Enhancer in Adenovirus Type 12-Transformed Cells via a Retinoic Acid Response Element

ANASTASIA KRALLI,† RUOWEN GE,‡ ULLRICH GRAEVEN, ROBERT P. RICCIARDI, AND ROBERTO WEINMANN*

The Wistar Institute of Anatomy and Biology, 3601 Spruce Street, Philadelphia, Pennsylvania 19104

Received 10 July 1992/Accepted 27 August 1992

In cells transformed by the highly oncogenic adenovirus type 12 (Ad12), the viral E1A proteins mediate transcriptional repression of the major histocompatibility class I genes. In contrast, class I transcription is not reduced in cells transformed by the nononcogenic Ad5. The decreased rate of class I transcription is, at least in part, the result of a reduced major histocompatibility complex class I enhancer activity in Ad12-transformed cells and correlates with an increase in the levels of a DNA-binding activity to the R2 element of the enhancer (R. Ge, A. Kralli, R. Weinmann, and R. P. Ricciardi, J. Virol. 66:6969–6978, 1992). Employing transient transfection assays, we now provide direct evidence that the R2 element can confer repression in Ad12- but not Ad5-transformed cells. Repression by R2 was observed only in the presence of the positive enhancer element R1 and was dependent on (i) the number of the R2 elements and (ii) the relative arrangement of R2 and R1 elements. The putative R2-binding repressor protein, R2BF, was similar in molecular weight and binding specificity to members of the thyroid hormone/retinoic acid (RA) receptor family. RA treatment abrogated the R2-mediated repression in Ad12-transformed cells and had no effect on the activity of R2/R1-containing promoters in Ad5-transformed cells. These results are consistent with the presence of an R2-binding repressor in Ad12-transformed cells. In the absence of RA, the repressor compromises enhancer activity by interfering with the activity of the positive *cis* element R1. RA treatment of Ad12-transformed cells may render the repressor inactive.

Adenoviruses of all serotypes can transform primary cells in vitro, but only some can form tumors in syngeneic immunocompetent animals. The ability of the highly oncogenic adenovirus type 12 (Ad12) to form tumors in animals correlates with its ability to reduce the levels of expression of major histocompatibility complex (MHC) class I mRNAs and proteins (11, 43). The decrease in surface levels of MHC class I antigens is reflected by the lower susceptibility of the Ad12-transformed cells to allogeneic cytotoxic T lymphocytes, suggesting a means by which Ad12-transformed cells escape immune surveillance and develop into proliferating and evasive tumors (6, 11, 43, 47). In contrast, cells transformed by the nononcogenic Ad5 display high levels of class I RNAs and antigens, are readily recognized by allogeneic cytotoxic T lymphocytes, and form tumors only in immunodeficient mice (6, 11, 43). Two viral immediate-early genes, E1A and E1B, cooperate in transforming primary cells. Primary cells transformed with Ad12 E1A and Ad5 E1B have low levels of MHC class I expression and are highly oncogenic, while cells transformed with Ad5 E1A and Ad12 E1B have normal levels of class I antigens and are nononcogenic (6). Thus, E1A, a potent transcriptional regulator, can alter the levels of expression of the MHC class I genes and thereby the oncogenic potential of adenovirus-transformed cells (6, 49).

The reduced levels of MHC class I mRNA in Ad12-transformed cells reflect decreased rates of transcription of the endogenous class I genes (2, 13). Ad12-mediated transformation of cells derived from transgenic mice that carry the growth hormone gene under the control of the class I *H-2K*^{b} regulatory elements shows that sequences between −2015 and +12, relative to the transcription initiation site, are sufficient to mediate down-regulation of the transgene (36). Furthermore, a transiently transfected chloramphenicol acetyltransferase (CAT) gene driven by the *H-2K*^{b} regulatory sequences (−1049 to +12) is expressed at much lower levels in Ad12-transformed cells than in Ad5-transformed cells (14). Most significantly, the *H-2K*^{b} enhancer (−215 to −156), in the context of either the homologous *H-2K*^{b} minimal promoter or heterologous minimal promoters, displays much lower activity in Ad12-transformed cells than in Ad5-transformed cells (14).

The MHC class I enhancer (Fig. 1A) is composed of multiple *cis*-acting elements; the best characterized among them is R1 (−170 to −160), a positive regulator of transcription (5, 7, 8, 21). Mutations in the R1 element abolish basal enhancer activity as well as the ability to respond to stimuli such as tumor necrosis factor and tetradecanoyl phorbol acetate (22). R1, similar in DNA sequence to the κB element of the immunoglobulin enhancer, can be bound in vitro by several proteins, such as H2TF1, PRDII-BF1/MBP-1, and members of the c-Rel family (NFκB and p50) (4, 5, 12, 25, 52). Binding to the R1 element has been reported to be higher in extracts from Ad5-transformed cells than in extracts from Ad12-transformed cells (37, 40). However, the functional

* Corresponding author.
† Present address: Department of Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, CA 94143-0448.
‡ Present address: Department of Cardiovascular and Molecular Biology, Medical Research Division, Lederle Laboratories, Pearl River, NY 10965.

significance of the increased binding to R1 in Ad5-transformed cells and the role of R1 in class I transcription in Ad12-transformed cells have not been studied. The element R1′ (−186 to −177), an imperfect repeat of R1, appears to be dispensable for basal enhancer activity (7). However, either an R1′-R1 dimer or an R1-R1 dimer can confer regulation by signals such as tumor necrosis factor, whereas a monomer alone cannot. Thus, R1′ may increase the range of stimuli that the enhancer can respond to or the sensitivity of the enhancer to such stimuli (22). The R2 element (−205 to −184) is conserved among the various class I genes but does not contribute significantly to basal enhancer activity, at least in the cells in which it has been tested (7). Its DNA sequence is reminiscent of both the cyclic AMP response element (CREB) and DNA recognition sites of members of the nuclear hormone receptor superfamily (20). Indeed, the R2 site can confer cyclic AMP responsiveness upon a heterologous promoter (22) and is recognized by H-2RIIBP/retinoid X receptor beta (RXRβ), a member of the RXR family (20, 34). R2-binding activity is significantly higher in extracts from Ad12-transformed cells than in extracts from Ad5-transformed or non-E1A-containing cell lines (3, 14, 37).

In the accompanying report, we have shown that the MHC class I enhancer is significantly less active in Ad12- than in Ad5-transformed cells (14). The poor enhancer activity in Ad12-transformed cells correlates with the increased binding of nuclear factors to the R2 element, suggesting the presence of a repressor that may mediate its effects via the R2 site (14).

In this study, we examined the transcriptional activities of the individual enhancer elements R1 and R2 in Ad5- and Ad12-transformed cells to determine their relative contributions to enhancer activity in these cells. We showed that R2 can indeed repress transcription specifically in Ad12- but not in Ad5-transformed cells. Both the sequence of the R2 site and its position relative to the R1 site critically influence the repression obtained in Ad12-transformed cells. The binding specificity of the putative repressor is similar to that of members of the thyroid hormone receptor (TR)/retinoic acid (RA) receptor (RAR) family. Accordingly, R2-containing promoters introduced into Ad12-transformed cells can respond to RA. In contrast, R2-containing promoters in Ad5-transformed cells do not respond to RA. Our results support a model whereby in Ad12-transformed cells, a repressor binds to the R2 element and impairs the ability of the positive element R1 to stimulate transcription, thereby compromising enhancer activity. RA treatment of the Ad12-transformed cells may modify or replace the repressor, so that R2 can no longer function as a negative DNA element.

MATERIALS AND METHODS

Cell lines. Adenovirus-transformed cell lines were derived from primary cultures of tissues from embryos or newborn BALB/c mice. The two Ad5-transformed cell lines, KAd5-3 (BK5-3) and Wt5a (BK5-A), and the two Ad12-transformed cell lines, F10-12 (BK10-3F) and 12A1 (BBR12-1), have been previously described (11, 14).

Transfections. Cells were transfected by the calcium phosphate coprecipitation method essentially as described by Graham and van der Eb (19). Cells were grown in 100-mm plates to about 30 to 40% confluency. Twenty micrograms of DNA (5 μg of reporter plasmid, 4 μg of plasmid pRSV-β-gal, and 11 μg of pBluescript KS$^-$ vector [pBS; Stratagene, La Jolla, Calif.]) was mixed with 62.5 μl of 2 M $CaCl_2$ in 0.5 ml of sterile H_2O and added dropwise to 0.5 ml of 2× HBS (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 275 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 10 mM glucose, pH 7.05). DNA-calcium precipitates were allowed to form for 30 min at room temperature and then added to the cells. Six hours after the DNA was added, the cells were washed twice with phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) and refed fresh medium containing serum. Cells were harvested 46 to 50 h after the DNA was added.

Cells treated with RA and thyroid hormone (T3) were grown in minimal essential medium supplemented with fetal bovine serum that had been stripped of thyroid hormones and glucocorticoids (10). RA (all-*trans*; Sigma, St. Louis, Mo.) and T3 (kindly provided by Mitch Lazar) were added at final concentrations of 1 μM and 10 nM, respectively, at the same time as the DNA-calcium phosphate precipitate. Both RA and T3 were replenished after the cells were washed.

β-Gal and CAT assays. Transfected cells were harvested and lysed by three freeze-thaw cycles. For the β-galactosidase (β-Gal) assay, 1 to 10 μl of cell lysate was incubated with 5 mM chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim, Indianapolis, Ind.) in a final volume of 200 μl of 50 mM potassium phosphate (pH 7.5)–1 mM $MgCl_2$ at 37°C. After a 5- to 30-min incubation, the A_{578} was measured. One unit of β-Gal was arbitrarily defined as the amount of enzyme that upon 30 min of incubation with 5 mM chlorophenol red-β-D-galactopyranoside at 37°C gave an A_{578} reading of 1.0. The cell lines under study did not contain endogenous enzymatic activities that could use chlorophenol red-β-D-galactopyranoside as a substrate.

For the CAT assay, 1 to 100 μl of cell lysate was incubated with 2 μl of [^{14}C]chloramphenicol (50 μCi/ml, 0.83 μmol/ml) and 5 μl of 20 mM acetyl coenzyme A in a final volume of 150 μl of 0.25 M Tris-HCl (pH 7.5) at 37°C. After a 60-min incubation, the mixtures were extracted with 750 μl of ethyl acetate, lyophilized, resuspended in 25 μl of ethyl acetate, and analyzed by thin-layer chromatography. Conversion to acetylated [^{14}C]chloramphenicol was quantitated by a computerized radioanalytic imaging system (AMBIS, San Diego, Calif.). In transfections with the weakest-expressing constructs (e.g., −37H2), amounts of extract containing the equivalent of 100 to 300 U of β-Gal were assayed, while in those with strongly expressed promoters (e.g., $R1_4$H2), amounts of extract containing the equivalent of 0.5 to 50 U of β-Gal were assayed. CAT values in the bar graphs were normalized to the β-Gal activity of the cell extract.

Plasmids. All constructs were cloned in pBS. For −37H2, the *Sac*II-*Bam*HI fragment from pH2-37 (14) was cloned between the *Sma*I and *Bam*HI sites of the polylinker of pBS. The plasmid contains the −37 to +12 (relative to the transcription initiation site) fragment of the *H-2K^b* promoter fused to the reporter CAT gene.

Oligonucleotides R1, R2, and R2m (mutated R2) were subcloned, as monomers, dimers, or tetramers, in the *Xho*I site of the polylinker of −37H2, upstream of the *H-2K^b* minimal promoter. The enhancer-containing, double-stranded DNA oligonucleotide H2E was subcloned in the *Hin*dIII site of the polylinker of plasmid −37H2. The orientation and sequence of each oligonucleotide were determined by DNA sequencing. To introduce the same sequences upstream of the thymidine kinase (TK) promoter, the *Xba*I-*Sst*I fragment or the *Hin*dIII-*Sst*I fragment that contains the TK promoter (up to bp −105) and the CAT gene from plasmid pBLCAT2 (33) was used to replace the *H-2K^b* minimal promoter and CAT gene.

TABLE 1. Sequences of oligonucleotides used in this study

Oligonucleotide	Sequence
H2E (*H-2K*^{b} promoter)	tcgAGGCAGTGAGGTCAGGGGTGGGGAAGCCCAGGGCTGGGGATTCCCCATCTgtcga agcTCCGTCACTCCAGTCCCCACCCCTTCGGGTCCCGACCCCTAAGGGGTAGAcagct
R1 (*H-2K*^{b} promoter)	tcgAGGGCTGGGGATTCCCCATCTg CCCGACCCCTAAGGGGTAGAcagct
R2 (*H-2K*^{b} promoter)	tcgAGGCAGTGAGGTCAGGGGTGGG CCGTCACTCCAGTCCCCACCCagct
R2m (the underlined base pairs differ from these in the site in the *H-2k*^{b} promoter)	tcgAGGCAGTGAAATCAGGGGTGGG CCGTCACTTTAGTCCCCACCCagct
CREB (fibronectin promoter)	ACAGTCCCCGTGACGTCACCCGGGAGCCC TGTCAGGGGCACTGCAGTGGGCCCTCGGG
EREvit (estrogen response element [ERE] from the vitellogenin promoter)	AAGTCAGGTCACAGTGACCTGCTCA TTCAGTCCAGTGTCACTGGACGAGT
AP-1 (human metallothionein II promoter)	tcgaCGTGACTCAGCGCGC GCACTGAGTCGCGCGagct

The thyroid response element (TRE) palindrome (TREp) construct TREpMTVCAT (referred to as ΔMTV-TREp-CAT) was a kind gift of R. Evans (48). It contains a TREp in place of the glucocorticoid response element in the mouse mammary tumor virus long terminal repeat fused to the CAT gene.

Oligonucleotides. The DNA sequences of the oligonucleotides cloned upstream of the *H-2K*^{b} minimal promoter or used in DNA band shift assays are shown in Table 1 (small letters indicate base pairs not present in the native context of the elements).

The TREp element was excised with *Xba*I and *Eco*RI from pTREp/BS (a kind gift of M. Lazar) in which the TREp is subcloned at the *Bam*HI site of pBS. The sequence of TREp is as follows:

ggatcTCAGGTCATGACCTGAGAggatcc
cctagAGTCCAGTACTGGACTCTcctagg

Nuclear extract and DNA band shift assays. Nuclear extracts were prepared according to Shapiro et al. (45). Final protein concentrations were 8 to 10 mg/ml. In vitro binding of nuclear proteins to labeled DNA was carried out essentially as described by Sen and Baltimore (44). Briefly, nuclear extract (2 μg) was incubated for 30 min at 30°C with 50 fmol of ^{32}P-labeled probe and 1.0 μg of poly(dI-dC) in 10 mM HEPES (pH 7.9)–60 mM KCl–1 mM $MgCl_2$–0.5 mM EDTA–1 mM dithiothreitol–10% glycerol. DNA-protein complexes were resolved in a 5% polyacrylamide gel containing 45 mM Tris-HCl (pH 8.0), 45 mM boric acid, and 0.5 mM EDTA. The gels were dried, and the DNA was visualized by autoradiography. Where indicated, a 20-fold molar excess of unlabeled double-stranded competitor DNA was added to the binding reaction.

UV cross-linking. The R2 oligonucleotide was nick translated by a mix of DNA polymerase I (2.5 U) and DNase I (20 pg) in the presence of 50 μCi of [^{32}P]dCTP, 5-bromo-2′-dUTP (Sigma), 1 mM dATP, and 1 mM dGTP for 1 h at 15°C. The binding reactions were carried out similarly to band shift analysis except that 6 μg of nuclear extract and 150 fmol of the R2 oligonucleotide were used and all binding reactions were in the presence of 40-fold molar excesses of the R2m and CREB oligonucleotides. R2m and CREB had no effect on the levels or the migration of complex A or the cross-linked protein. Following a 30-min binding reaction, the samples were exposed to UV light (300 nm; Fotodyne, New Berlin, Wis.) for 45 min. DNA-protein complexes were resolved in a 5% native polyacrylamide gel; the band containing complex A was cut from the gel, impregnated with 2× Laemmli buffer (30) for 5 min, and loaded on an 8% denaturing gel. Electrophoresis was followed by autoradiography of the dried gel.

RESULTS

The enhancer element R1 activates transcription in both Ad5- and Ad12-transformed cells. The *H-2K*^{b} enhancer is significantly less active in Ad12- than in Ad5-transformed cells (14). Reduced enhancer activity correlated with the increased binding of a factor to the R2 enhancer element, suggesting that elevated levels of a repressor protein in Ad12-transformed cells may be responsible for the low enhancer activity. We sought to determine the relative contribution of each of the enhancer elements R1 and R2 (Fig. 1A) to *H-2K*^{b} transcription in adenovirus-transformed cells as well as to test the hypothesis that R2 could repress enhancer activity in Ad12-transformed cells. Thus, oligonucleotides containing the enhancer elements R1 and R2 were subcloned into eukaryotic expression vectors, and their effects on transcription from a minimal promoter were analyzed; a mutant R2 element with a 2-bp substitution, inactive in binding (see Fig. 4C), was used as a reference for wild-type R2 activity.

Specifically, either the entire *H-2* class I enhancer or a tetramer of R1 or of R2 (wild type or mutated) was cloned upstream of the *H-2K*^{b} minimal promoter (−37H2) directing the expression of the bacterial CAT gene. The constructs were transfected into independently derived Ad5 (Wt5a and KAd5-3)- and Ad12 (F10-12 and 12A1)-transformed cells, and extracts were prepared after 48 h. Transfection efficiency was controlled for by the cotransfection of a reporter plasmid (pRSV-β-gal) that contained the Rous sarcoma virus long terminal repeat driving the β-Gal gene. In agreement with the data presented in the accompanying report, the enhancer H2E was able to stimulate transcription 5- to 10-fold better in Ad5- than in Ad12-transformed cells (Fig. 1B and data not shown). In contrast to the entire enhancer, a tetramer of R1 activated transcription with comparable efficiency in Ad5 (Wt5a and KAd5-3)- and Ad12 (F10-12 and 12A1)-transformed cells (Fig. 1B and data not shown) (29). Tetramers of R2 (wild type or mutant) had no effect on promoter activity in all cell lines tested.

These results confirmed our previous finding that the activity of the class I enhancer is low in Ad12-transformed cells. The reduced activity was not simply due to the lack of

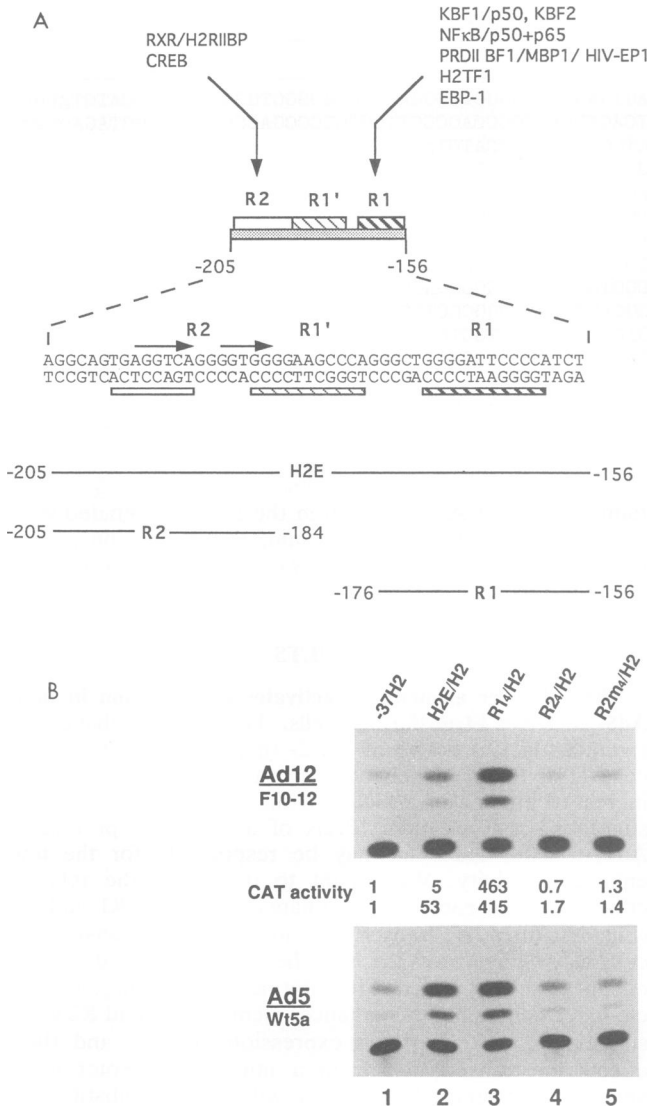


FIG. 1. Evidence that enhancer element R1 activates transcription efficiently in Ad5- and Ad12-transformed cells. (A) The MHC class I enhancer. Depicted are the *H-2K*^{b} enhancer, elements R1, R1′, and R2, and the proteins that have been reported to bind to these regions (4, 5, 21, 22, 25, 46). Below the diagram is the DNA sequence of the entire enhancer. The R1 and R1′ elements are underlined by striped boxes. The horizontal arrows indicate the sequences within R2 which resemble the GGTCA motif of TR/RAR binding sites. Underlined by the white box is the consensus CREB sequence. The three oligonucleotides that were cloned upstream of the *H-2K*^{b} minimal promoter in the reporter plasmids or used as probes in the binding studies are represented below. (B) Transfection assay. Five micrograms of each of the indicated constructs was cotransfected with 4 μg of pRSV-β-gal and 11 μg of pBS vector into Ad12- or Ad5-transformed cells. After 48 h, extracts were prepared and assayed for β-Gal and CAT activities. −37H2, the *H-2K*^{b} minimal promoter; H2E/H2, the entire enhancer upstream of the minimal promoter; $R1_4$/H2, $R2_4$/H2, and $R2m_4$/H2, tetramers of R1, wild-type R2, and mutated R2, respectively, upstream of the *H-2K*^{b} minimal promoter. The amounts of cell extract assayed in each CAT reaction, expressed in units of β-Gal, were as follows: for Ad12-transformed cells, 400 U in lanes 1, 2, 4, and 5 and 40 U in lane 3; for Ad5-transformed cells, 300 U in lanes 1, 4, and 5, 100 U in lane 2, and 20 U in lane 3. Indicated between the chromatograms are the normalized relative CAT activities (the CAT activity of −37H2 in each cell line was defined as 1.0).

positive factors acting via the R1 element, since an R1 tetramer could activate transcription in both Ad12- and Ad5-transformed cells. The fact that the R2 element had no effect on transcription suggested either that R2 was not a regulatory element in the cell lines under study or that its role in transcription was not detectable in the assay system used. A possible explanation was that the levels of transcription from the −37H2 construct, although high enough to give CAT activity well above background, were too low to give a sensitive assay for a repressor function. Alternatively, an R2 function could not be detected because R2 might not be able to act alone but only in the context of the enhancer, i.e., in the presence of the positive element R1.

To circumvent the problem of low basal expression, similar recombinant plasmids that contained either the H2E, the tetramer of R1, or the tetramer of R2 (wild type or mutant) were subcloned upstream of the TK promoter driving the CAT gene and were transfected into the Ad5- and Ad12-transformed cells. These constructs retained some of the TK upstream elements (up to bp −105 relative to the transcription initiation site), gave readily detectable levels of transcription in all cell lines tested, and provided a more sensitive system for the detection of a possible repressor function of R2. Still, the R2 tetramer had only small negative effects (two- to threefold; data not shown) on transcription in Ad12-transformed cells, suggesting that R2 could not efficiently repress transcription from the TK promoter. The R1 tetramer in the context of the TK promoter activated transcription in both Ad5- and Ad12-transformed cells (data not shown), confirming that R1-binding factors can activate transcription in Ad12-transformed cells.

R2 can repress R1-dependent transcription from the *H-2K*^{b} promoter. The R2 element, separated from its natural context, exerted little effect on the transcriptional activity of the *H-2K*^{b} minimal or TK promoter. Still, R2-binding factors may repress transcription in the context of the intact enhancer, e.g., by interfering with the function of positive factors which bind to neighboring enhancer elements, such as R1. To test such a possibility, different combinations of the R2 and R1 elements were multimerized and subcloned upstream of the −37H2 minimal promoter. The constructs included (i) a monomer of R2 upstream of a monomer of R1, tetramerized ($[R2_1R1_1]\times4$); (ii) a monomer of R2 upstream of a dimer of R1, as a monomer, a dimer, and a tetramer ($[R2_1R1_2]\times1$, -×2, and -×4); (iii) a dimer of R2 upstream of a dimer of R1, as a monomer, a dimer, and a tetramer ($[R2_2R1_2]\times1$, -×2, and -×4); and (iv) a tetramer of R2 upstream of a tetramer of R1 ($[R2_4R1_4]\times1$). To control for the function of R2-binding proteins, a similar series of constructs was generated with a mutant R2 element (R2m) deficient for R2 factor binding. The R2/R1-containing constructs were transfected into Ad5- and Ad12-transformed cells, and their transcriptional activities were determined by assaying the levels of CAT activity. Figure 2 shows the results from transfections of $[R2_2R1_2]\times1$, -×2, and -×4 into Wt5a and F10-12 cells. R2, proximal to the R1 element, could repress transcription in the Ad12- but not Ad5-transformed cells. The 2-bp substitution that prevented binding to the R2 element (R2m) had no effect on *H-2K*^{b} transcription in Wt5a (Ad5)-transformed cells but allowed increased transcription in F10-12 (Ad12)-transformed cells. The R2 mutation was actually releasing repression rather than activating transcription, since CAT activity was restored to the level expected for the number of copies of the R1 element. Furthermore, the R2m tetramer was not able to activate transcription from the *H-2K*^{b} minimal promoter (Fig. 1B).

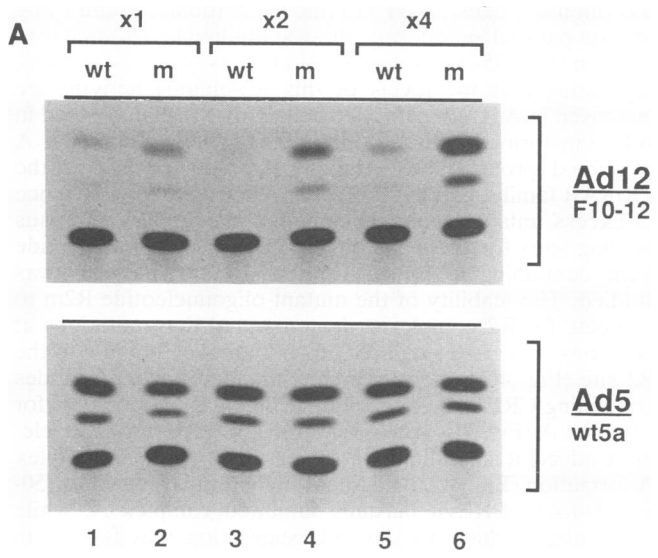
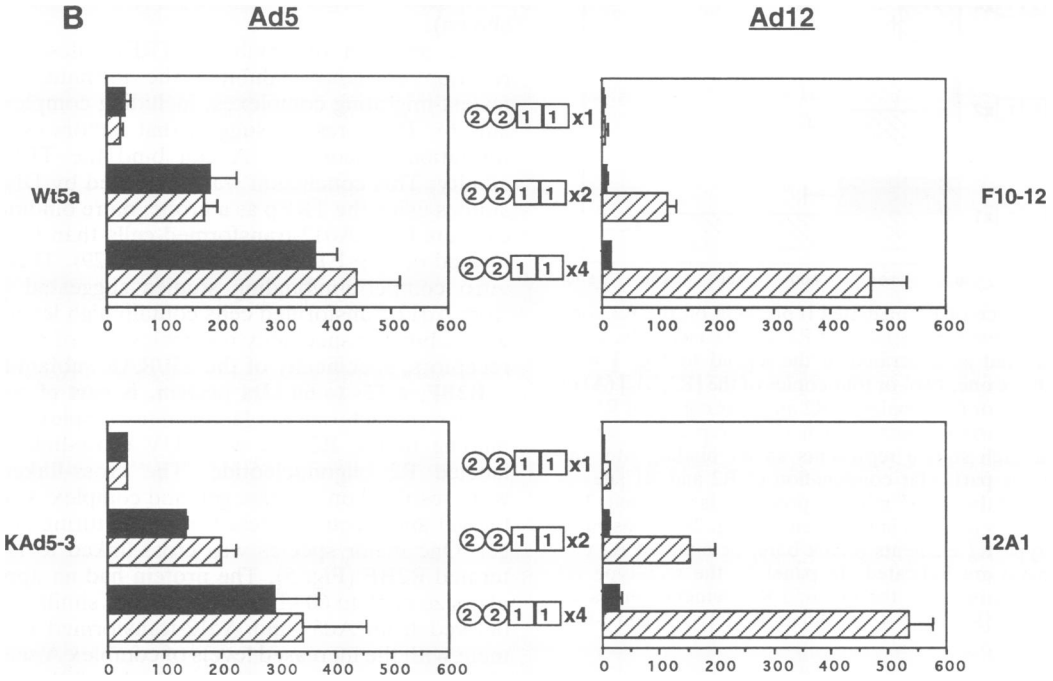


FIG. 2. Repression by R2 of R1-dependent transcription from the *H-2K*^{b} promoter in Ad12- but not Ad5-transformed cells. The [$R2_2R1_2$] cassette, present in one, two, or four copies and containing wild-type or mutated R2, was cloned upstream of the *H-2K*^{b} minimal promoter. Five micrograms of each construct was cotransfected with 4 μg of pRSV-β-gal and 11 μg of pBS vector into Ad5- or Ad12-transformed cells, and extracts were assayed for CAT activity as described in the legend to Fig. 1. (A) CAT activities from a representative experiment. wt, wild-type R2 site; m, mutated R2 site (R2m). The amounts of cell extract assayed in each CAT reaction, expressed in units of β-Gal, were as follows: for Ad12-transformed cells, 300 U in lanes 1 and 2 and 30 U in lanes 3 to 6; for Ad5-transformed cells, 100 U in lanes 1 and 2, 20 U in lanes 3 and 4, and 10 U in lanes 5 and 6. (B) Summary of CAT activities in two Ad5- and two Ad12-transformed cell lines. The values indicated are relative, i.e., the ratio of CAT activity of each construct (normalized with respect to β-Gal activity) divided by the CAT activity of the minimal promoter construct (−37H2). In this way, minimal promoter activity was arbitrarily defined as 1.0 in each cell line. Each circle represents an R2 binding site, and each square represents an R1 binding site; the number of times the [$R2_2R1_2$] cassette is repeated in the transfected construct is shown. The activities of the transfected constructs containing wild-type R2 elements (black bars) or mutated R2 elements (striped bars) are indicated.



Therefore, the wild-type R2 element could repress R1-dependent transcription in the Ad12-transformed F10-12 cells. Similarly, R2 conferred repression in 12A1 (Ad12-transformed) cells but had no effect in KAd5-3 (Ad5-transformed) cells (Fig. 2B).

Transfection of multiple different constructs containing R2 and R1 elements corroborated that R2 had no effect on transcription in Ad5-transformed cells but repressed transcription in Ad12-transformed cells (Fig. 3). The efficiency of R2-mediated repression varied in the different constructs. First, repression was dependent on the number of R2 copies. Constructs containing the [$R2_1R1_2$] instead of the [$R2_2R1_2$] cassette demonstrated that two copies of R2 reduced expression more efficiently than did one copy (Fig. 3A and B). Second, the relative positioning of the R1 and R2 elements was important for efficient repression; R2 sites interspersed between R1 sites could repress transcription better than could a series of R2 sites upstream of a series of R1 sites. The repression activity of four R2 sites in the presence of four R1 sites was higher in construct [$R2_1R1_1$]×4 (9-fold) or [$R2_2R1_2$]×2 (16-fold) than in [$R2_4R1_4$]×1 (3-fold) (Fig. 3C). The importance of the relative positioning of the elements supports the notion that R2 interferes with R1-dependent transcriptional activation and suggests that the R2 function may be dependent on interactions between proteins bound, directly or indirectly, to the R1 and R2 elements. Furthermore, a comparison of the activity of the R2m-containing constructs in Fig. 3C (striped bars) suggests that the spacing between the R1 sites per se is important in determining the efficiency with which the four R1 sites enhance transcription, possibly reflecting cooperativity at the contiguous R1 sites.

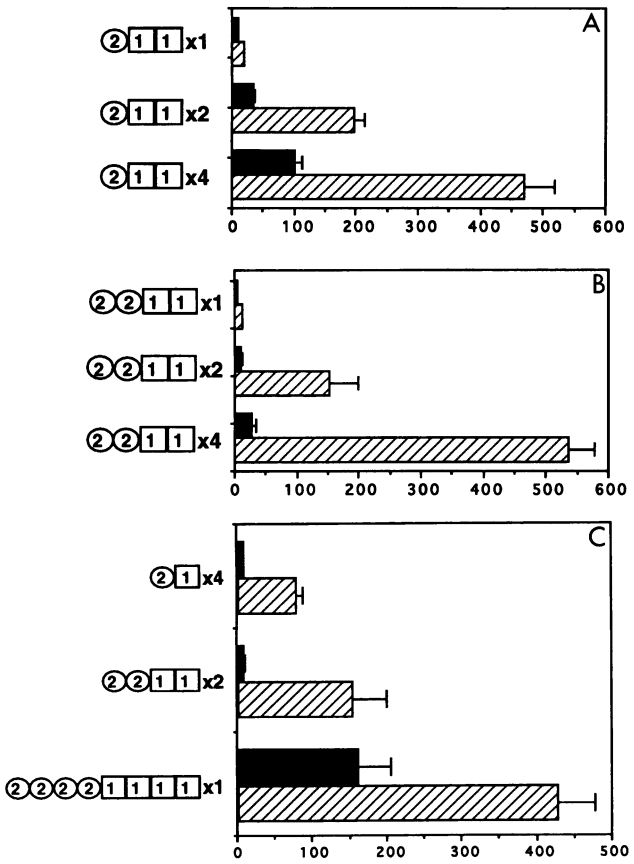


FIG. 3. Dependence of R2-mediated repression on the R2 copy number and the relative arrangement of R2 and R1 elements. 12A1 cells were transfected as described in the legend to Fig. 1 with constructs containing one, two, or four copies of the $[R2_1R1_2]$ (A) or $[R2_2R1_2]$ (B) cassette or four copies of R2 and four copies of R1 (C) in different relative arrangements. Each circle represents an R2 binding site, and each square represents an R1 binding site; the number of times the particular combination of R2 and R1 sites is repeated upstream of the *H-2K*^{b} minimal promoter is indicated. The relative CAT activities (defined in the legend to Fig. 2) of constructs containing wild-type R2 elements (black bars) or mutated R2 elements (striped bars) are indicated. In panel C, the wild-type R2 element, when compared with the mutated R2 element, repressed transcription 9-fold in $[R2_1R1_1]\times4$, 16-fold in $[R2_2R1_2]\times2$, and 3-fold in $[R2_4R1_4]\times1$.

The Ad12-transformed cell-specific R2-binding factors can also recognize the TREp. Analysis of the R2 DNA sequence reveals similarities to several other known DNA binding sites, such as a consensus CREB site and a consensus AP-1 binding site (Fig. 4A). In addition, the R2 element contains two direct repeats (a perfect and a degenerate one) of the AGGTCA sequence that is found in several TREs and RA response elements (RAREs) (Fig. 4A). Indeed, one of the RXRs, H-2RIIBP/RXRβ, has been cloned on the basis of its binding to the R2 site (20).

Four specific DNA-protein complexes were resolved in a DNA band shift assay performed with nuclear extracts from Ad5- and Ad12-transformed cells and an R2-containing double-stranded oligonucleotide. The level of one of these complexes, termed complex A, was significantly higher in Ad12-transformed cell extracts (Fig. 4B) (14). Nuclear extracts from other cell lines, such as HeLa (human placental), S49 (mouse T cells), NIH 3T3 (mouse fibroblasts), and Pys-2 (mouse parietal endoderm), showed binding levels similar to those in the Ad5-transformed cell extracts (data not shown), suggesting that the levels of this R2-binding activity are increased in Ad12-transformed cells rather than decreased in Ad5-transformed cells. To determine whether complex A contained proteins belonging to the AP-1/CREB or the TR/RAR family, binding to R2 was analyzed in the presence of excess unlabeled oligonucleotides containing consensus binding sites for these factors. In addition, oligonucleotide R2m, containing a 2-bp substitution in the R2 element, was studied. The inability of the mutant oligonucleotide R2m to compete for R2 complexes demonstrated that mutations at positions −195 and −196 (GG to AA) abolish binding to the R2 site (Fig. 4C, lane 4). Double-stranded oligonucleotides containing CREB or AP-1 binding sites did not compete for complex A (Fig. 4C, lanes 7 and 8). The EREvit oligonucleotide added at a 20-fold molar excess did not inhibit complex A formation (Fig. 4C, lane 5). At higher molar excesses (50- to 100-fold), EREvit partially inhibited complex A, while other oligonucleotides did not, suggesting that factors in complex A have a low affinity for the ERE site (data not shown).

The addition of unlabeled TREp sites in the binding reaction efficiently inhibited the formation of the three fastest-migrating complexes, including complex A (Fig. 4C, lane 6). These results suggest that factors essential for the formation of complex A can bind the TREp with high affinity. This conclusion was confirmed by DNA band shift studies using the TREp as a probe; more binding was seen in extracts from Ad12-transformed cells than from Ad5-transformed or non-E1A-containing cells (29). Therefore, our in vitro competition/binding studies suggested that extracts from Ad12-transformed cells contain high levels of a protein whose binding specificity resembles that of nuclear hormone receptors, specifically of the TR/RAR subfamily.

R2BF, a 55- to 60-kDa protein, is part of complex A. To further characterize the factor(s) in complex A, the proteins binding to the R2 site were UV cross-linked to the ^{32}P-labeled R2 oligonucleotide. The cross-linked complexes were resolved on a native gel, and complex A was cut out of the gel and electrophoresed in a denaturing polyacrylamide gel. One major species was cross-linked to DNA and was termed R2BF (Fig. 5). The protein had an apparent molecular size of 55 to 60 kDa and displayed similar mobility when derived from Ad5- and Ad12-transformed cells. In agreement with the increased levels of complex A seen in the band shift assay, we found higher levels of the R2BF protein cross-linked to the R2 oligonucleotide when extracts from Ad12-transformed cells were used.

The R2 element responds to RA treatment of the Ad12-transformed cells. The R2-binding factors could recognize and bind to the TREp, suggesting that they could be members of the RA or TR/RAR family. If so, R2 might be able to respond to RA or to T3. To test this possibility, constructs containing the $[R2_2R1_2]\times4$ or $[R2m_2R1_2]\times4$ element upstream of the *H-2K*^{b} minimal promoter were transfected into Ad5- and Ad12-transformed cells. The cells were treated with RA, T3, or both. To control for the presence of the appropriate cellular receptors, we also tested the TREpMTV CAT (ΔMTV-TREp-CAT) construct, which can respond to both RA and T3 (48).

T3 had no effect on expression of the control plasmid TREpMTVCAT or the R2-containing constructs in the Ad5- and Ad12-transformed cells tested (Fig. 6). The lack of response to T3 was likely due to the absence of the proper

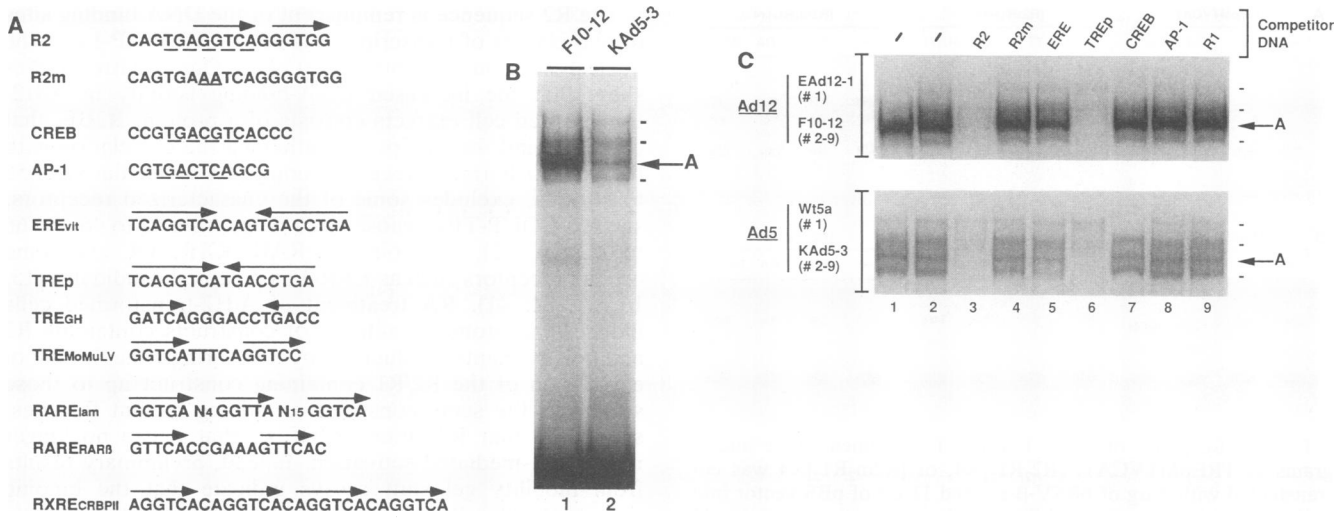


FIG. 4. Binding and competition analysis of R2-binding activities. (A) The R2 DNA sequence and other related DNA binding sites. The sequences shown are as follows: R2, the class I enhancer element R2 from the *H-2K*^{b} promoter; R2m, the R2 mutant used in this study (the 2-bp substitution is underlined); CREB, the CREB site from the fibronectin promoter (38); AP-1, the AP-1 binding site from the human metallothionein promoter (31); EREvit, the ERE from the vitellogenin promoter (26); TREp (48); TRE_{GH}, the TRE from the growth hormone promoter (17); TRE_{MoMuLV}, the TRE from the Moloney murine leukemia virus long terminal repeat (42); $RARE_{lam}$ the RARE from the B1 laminin promoter (50); $RARE_{RAR\beta}$, the RARE from the RARβ promoter (9); and RXRE, the retinoid X response element from the CRBPII promoter (35). The arrows indicate the repeats of the motif (A)GGTCA that TRs and RARs are thought to recognize; the dashed underline indicates the consensus CREB/AP-1 binding site. (B) Binding to the R2 element. DNA band shift analysis using extracts (2 μg) from Ad12 (F10-12) (lane 1)- or Ad5 (KAd5-3) (lane 2)-transformed cells and 50 fmol of ^{32}P-labeled R2 oligonucleotide. (C) Competition analysis. Extracts from Ad12- or Ad5-transformed cells were assayed for binding in the absence of competitor DNA or in the presence of a 20-fold molar excess of the following oligonucleotides: R2 (lane 3), R2m (lane 4), EREvit (lane 5), TREp (lane 6), CREB (lane 7), AP-1 (lane 8), and R1 (lane 9). Complex A is indicated by an arrow; the other complexes are indicated by bars.

TRs and was not investigated further. In contrast, RA activated transcription via the R2 element in Ad12-transformed cells but not in Ad5-transformed cells (Fig. 6). RA treatment of Ad12-transformed cells caused a 15-fold activation of constructs containing wild-type R2 sites and a 2-fold activation of constructs containing mutant R2 sites. A two-fold activation was also observed with wild-type and mutant R2 constructs in Ad5-transformed cells and with the minimal promoter −37H2 construct in both Ad5- and Ad12-transformed cells (not shown), suggesting that the effect was not significant. In summary, the control TREpMTVCAT responded to RA treatment of both Ad5- and Ad12-transformed cells (although the response in Ad12-transformed cells was more efficient), while the wild-type R2 element responded to RA only in Ad12-transformed cells.

DISCUSSION

MHC class I genes are expressed at lower levels in cells transformed by Ad12 than in cells transformed by Ad5 (6, 11, 43). In the accompanying report, we show that the low levels of MHC class I expression in Ad12-transformed cells are at least partly due to a reduced class I enhancer activity that correlates with an increase in factor binding to the R2 element of the enhancer (14). In this study, we show that the factor(s) binding to R2 can indeed repress transcription when R2 is in an appropriate promoter context. R2 is a poor repressor of transcription from a minimal promoter or the TK promoter but can efficiently repress R1-dependent transcription when juxtaposed to this positive enhancer element. Our results suggest that proteins that recognize the R2 element can repress the activity of factors binding to R1, thereby compromising enhancer activity. Furthermore, we

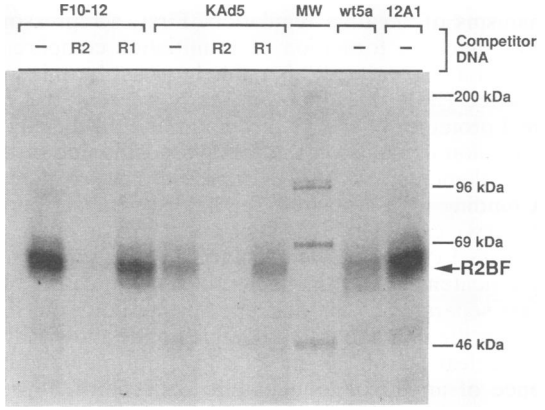


FIG. 5. UV cross-linking of complex A to R2. Oligonucleotide R2, nick translated in the presence of [^{32}P]dCTP and bromodeoxyuridine triphosphate, was incubated with extracts from Ad12 (F10-12 and 12A1)- or Ad5 (KAd5-3 and Wt5a)-transformed cells in the presence of unlabeled R2m and CREB oligonucleotides (−) or in the presence of unlabeled R2m, CREB, and R2 or R1, as indicated (at 40-fold molar excess of each unlabeled oligonucleotide). R2m and CREB had been previously shown to have no effect on complex A or on R2BF cross-linking to DNA (data not shown). The complexes were UV cross-linked for 45 min and resolved on a 5% native polyacrylamide gel. The band representing complex A was excised from the gel, incubated with 2× Laemmli buffer, and run on a sodium dodecyl sulfate–8% polyacrylamide gel, ^{14}C-labeled protein molecular standards were coelectrophoresed, and DNA-protein complexes were visualized by autoradiography. Molecular sizes of the standards are shown at the right. R2BF indicates the predominant band cross-linked to the R2 element.

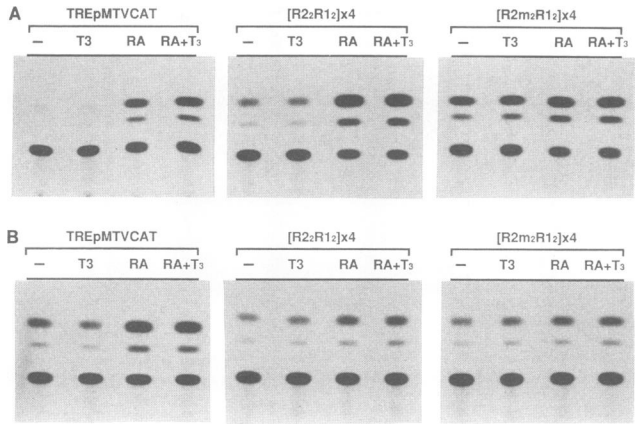


FIG. 6. Response of R2 to RA and T3 treatment. Five micrograms of TREpMTVCAT, $[R2_2R1_2]\times4$, or $[R2m_2R1_2]\times4$ was cotransfected with 4 μg of pRSV-β-gal and 11 μg of pBS vector into Ad12- (A) or Ad5-transformed (B) cells that were treated with 10 nM T3, 1 μM RA, or 10 nM T3 and 1 μM RA (RA + T_3), as indicated. After 48 h, cells were harvested and extracts were assayed for β-Gal and CAT activities. The amounts of cell extract assayed in each CAT reaction, expressed in units of β-gal, were as follows: in Ad12-transformed cells, 100 U for TREpMTVCAT, 30 U for $[R2_2R1_2]\times4$, and 14 U for $[R2m_2R1_2]\times4$; in Ad5-transformed cells, 150 U for TREpMTVCAT, 0.5 U for $[R2_2R1_2]\times4$, and 0.5 U for $[R2m_2R1_2]\times4$.

have characterized the R2-binding activity and found that it consists of at least one 55- to 60-kDa protein, R2BF, whose size and DNA-binding specificity are reminiscent of those of TRs and RARs.

Repression of transcription may occur via several distinct mechanisms of negative regulation. First, a repressor may directly affect the formation of an initiation complex at the transcription start site. R2 is unlikely to act by this mechanism since (i) it does not efficiently repress the *H-2K*^{b} minimal promoter or the TK promoter and (ii) the efficiency of repression depends on the relative positioning of the R2 and R1 elements. Second, a repressor may compete for DNA binding with a positive regulator of transcription (1). Although we cannot exclude such a mechanism in the native MHC class I enhancer, we consider it unlikely in this study using concatemerized oligonucleotides in which the binding sites are separated by 20 to 25 bp. Furthermore, our in vitro studies on the individual R1 oligonucleotide show that binding of nuclear factors to the R1 site is not inhibited by the presence of an R2 oligonucleotide (29). Third, repression may result from the masking of activation domains on positive regulators by proteins that bind on adjacent sites, as in the case of the yeast **a**2 repressor (24). Such protein interactions may explain not only the requirement for R1 in R2-mediated repression but also the correlation between the efficiency of repression and the relative positioning of R1 and R2 elements. For example, stronger repression was seen when R2 and R1 sites were interspersed, as in $[R2_2R1_2]\times2$, in which three out of four R1 sites are adjacent to an R2 site, than in $[R2_4R1_4]\times1$, in which only one of four R1 sites is close to an R2 site. Indeed, if interactions between R2- and R1-binding proteins do occur, we predict that changing the spacing between R1 and R2 may also affect the efficiency of repression. Furthermore, it is possible that R2-mediated repression is specific for a certain class of transcriptional activators.

The R2 sequence is reminiscent of the DNA binding sites of two classes of transcription factors, CREB/AP-1 and the nuclear hormone receptors (Fig. 4A). Our in vitro studies show that the increased DNA-binding activity in Ad12-transformed cell extracts consists of a protein, R2BF, that can also bind the TREp, strengthening the correlation with the nuclear hormone receptor family. Its molecular size, 55 to 60 kDa, excludes some of the characterized receptors, such as COUP-TFs, whose sizes range from 42 to 46 and 66 to 72 kDa (51), and points to RAR, RXR, TR, and some orphan receptors such as ERR-1 as potential candidates (15, 16, 32, 34, 41). RA treatment of Ad12-transformed cells induces the promoter activity of constructs containing R2 and R1 elements. Induction by RA brings the levels of expression of the R2/R1 containing construct up to those seen with the same construct containing mutant R2 sites, suggesting that RA alters R2BF so that it can no longer repress R1-mediated activation. Indeed, preliminary results from mobility gel shift assays indicate that the binding activity to the R2 site is not changed in extracts from Ad12-transformed cells grown in the presence of RA, suggesting that RA induces changes that affect transcription and not binding. Recent results of others (55) suggest that RA, rather than affecting DNA binding, may alter the selection of dimerization partners and thus affect transcription. For example, RA may influence the formation of distinct RXR homo- or heterodimers which are transcriptionally active but which do not differ from the transcriptionally inactive dimers in size and charge (55). Alternatively, RA may induce a novel activity that can compete with or substitute for the repressor and possibly activate transcription. In summary, the ability of RA to activate transcription via the R2 site in Ad12-transformed cells and the DNA binding specificity of R2BF strongly suggest that RARs can bind to R2 and regulate class I enhancer activity. This suggestion is further supported by the ability of H-2RIIBP/RXRβ to recognize the R2 element (20) and to confer RA inducibility to the MHC class I regulatory sequence from −213 to −123 (which include the enhancer) when transiently transfected into NT-2 cells (39).

Several nuclear hormone receptors have been reported to form heterodimers between themselves or with unidentified nuclear proteins that were termed coregulators (18). Recently, RXRs were found to heterodimerize with TRs, RARs, and COUP-TFs and were recognized as a subgroup of these coregulators (27, 28, 32, 53, 54); the heterodimers display enhanced DNA binding to the respective TREs, RAREs, and COUP sites and confer strong activation of transcription from promoters that contain these elements. DNA-protein cross-linking of complex A revealed a single protein (Fig. 5). It is possible that R2BF binds as a monomer or a homodimer. Alternatively, R2BF may bind as a heterodimer, but either the other protein does not efficiently cross-link to DNA or it is very similar in molecular weight to R2BF. Cloning of R2BF will help us address this question.

We have also addressed the role of the enhancer element R1 in the down-regulation of the MHC class I enhancer activity in Ad12-transformed cells. Experiments with the R1 tetramer-containing constructs show that the R1-binding factors can efficiently activate transcription in both Ad5- and Ad2-transformed cells. Constructs with two copies of R1 do reveal somewhat lower levels of R1 activity in Ad12-transformed cells than in Ad5-transformed cells; still, dimers of R1 are considerably active in Ad12-transformed cells, which suggests that the reduced enhancer activity cannot be explained solely by changes in the activity of R1. In support of

the transfection results, we have not been able to see significant and reproducible differences between Ad5- and Ad12-transformed cells in the levels of R1-binding proteins (29). Thus, although R1 may contribute to the down-regulation of enhancer activity in Ad12-transformed cells, other elements of the enhancer can also determine enhancer activity.

The ability of the R2-binding factors to repress R1-dependent transcription can explain the low levels of class I enhancer activity that we observe in Ad12-transformed cells. In contrast to our study, experiments by Katoh et al. suggest that the class I enhancer is activated and not repressed by Ad12 E1 (23). A possible explanation of the discrepancy in these results may lie in the transformation state of the different cells under study. Katoh et al. introduced Ad12 E1A into established 3Y1 cells, whereas in this study, Ad12 E1A and E1B were used to transform primary cells. The Ad12 E1A- and Ad5 E1A-expressing 3Y1 cells displayed similar levels of DNA-binding activity to the R2 enhancer element. The Ad12-specific increased binding to R2 has been independently documented by three different groups using human, mouse, and rat cell lines that have been derived from primary cells transformed by Ad12 DNA (3, 14, 37). From this point of view, the results of Katoh et al. are consistent with our findings in that the lack of an increase in R2-binding activity correlates with lack of repression of enhancer activity.

Our studies reveal a novel function for the MHC class I enhancer R2 element, i.e., the ability to negatively regulate enhancer activity. The R2-binding proteins that are activated by Ad12 E1A transformation are cellular proteins that are likely to modulate enhancer activity in a developmental stage- or tissue-specific manner. Interestingly, extracts from developing and adult murine tissues reveal high levels of R2 binding in the brain and early in development, i.e., cell types or stages at which MHC class I genes are not expressed (7).

ACKNOWLEDGMENTS

We thank Nobuo Horikoshi, Uli Mueller, and Mitch Lazar for helpful discussions during this study, Mitch Lazar for kindly providing T3 hormone and plasmid pTREp/BS, and R. Evans for providing TREpMTVCAT. We also thank Peter Curtis, Uli Mueller, and Keith Yamamoto for helpful comments on the manuscript.

This work was supported by National Cancer Institute grants CA 44466 and CA 10815 to R.W. and grant CA 29797 to R.P.R. A.K. was supported by a Monica Shander fellowship, and U.G. was supported by grant GR984/2-1 from the Deutsche Forschungsgemeinshaft.

REFERENCES

1. **Ackerblom, I. E., E. P. Slater, M. Beato, J. D. Baxter, and P. Mellon.** 1988. Negative regulation by glucocorticoids through interference with a cAMP responsive element. Science **241**:350–353.
2. **Ackrill, A. M., and G. E. Blair.** 1988. Regulation of major histocompatibility class I gene expression at the level of transcription in highly oncogenic adenovirus transformed cells. Oncogene **3**:483–487.
3. **Ackrill, A. M., and G. E. Blair.** 1989. Nuclear proteins binding to an enhancer element of the major histocompatibility class I promoter: differences between highly oncogenic and nononcogenic adenovirus-transformed rat cells. Virology **172**:643–646.
4. **Baldwin, A. S., Jr., L. P. LeClair, H. Singh, and P. A. Sharp.** 1990. A large protein containing zinc finger domains binds to related sequence elements in the enhancers of the class I major histocompatibility complex and kappa immunoglobulin genes. Mol. Cell. Biol. **10**:1406–1414.
5. **Baldwin, A. S., Jr., and P. A. Sharp.** 1987. Binding of a nuclear factor to a regulatory sequence in the promoter of the mouse *H-2K*^{b} class I major histocompatibility gene. Mol. Cell. Biol. **7**:305–313.
6. **Bernards, R., P. I. Schrier, A. Houweling, J. L. Bos, and A. J. van der Eb.** 1983. Tumorigenicity of cells transformed by adenovirus type 12 by evasion of T-cell immunity. Nature (London) **305**:776–779.
7. **Burke, P. A., S. Hirschfeld, Y. Shirayoshi, J. W. Kasik, K. Hamada, E. Appella, and K. Ozato.** 1989. Developmental and tissue-specific expression of nuclear proteins that bind the regulatory element of the major histocompatibility complex class I gene. J. Exp. Med. **169**:1309–1321.
8. **David-Watine, B., A. Israël, and P. Kourilsky.** 1990. The regulation and expression of MHC class I genes. Immunol. Today **11**:286–292.
9. **de Thé, H., M. D. M. Vivanco-Ruiz, P. Tiollais, H. Stunnenberg, and A. Dejean.** 1990. Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. Nature (London) **343**:177–180.
10. **Dobner, P. R., E. S. Kawasaki, L.-Y. Yu, and F. C. Bancroft.** 1981. Thyroid or glucocorticoid hormone induces pre-growth-hormone mRNA and its probable nuclear precursors in rat pituitary cells. Proc. Natl. Acad. Sci. USA **78**:2230–2234.
11. **Eager, K. B., J. Williams, D. Breiding, S. Pan, B. Knowles, E. Appella, and R. P. Ricciardi.** 1985. Expression of histocompatibility antigens H-2K, -D, and -L is reduced in adenovirus-12-transformed mouse cells and is restored by interferon γ. Proc. Natl. Acad. Sci. USA **82**:5525–5529.
12. **Fan, C.-M., and T. Maniatis.** 1990. A DNA-binding protein containing two widely separated zinc finger motifs that recognize the same DNA sequence. Genes Dev. **4**:29–42.
13. **Friedman, D. J., and R. P. Ricciardi.** 1988. Adenovirus type 12 E1A gene represses accumulation of MHC class I mRNA at the level of transcription. Virology **165**:303–305.
14. **Ge, R., A. Kralli, R. Weinmann, and R. P. Ricciardi.** 1992. Down-regulation of the major histocompatibility complex class I enhancer in adenovirus type 12-transformed cells is accompanied by an increase in factor binding. J. Virol. **66**:6969–6978.
15. **Giguere, V., E. S. Ong, P. Segul, and R. M. Evans.** 1987. Identification of a receptor for the morphogen retinoic acid. Nature (London) **330**:624–629.
16. **Giguere, V., Yang, N., Segui, P., and R. M. Evans.** 1988. Identification of a new class of steroid hormone receptors. Nature (London) **331**:91–94.
17. **Glass, C. K., A. Franco, C. Weinberger, V. A. Albert, R. M. Evans, and M. G. Rosenfeld.** 1987. A c-erb A binding site in rat growth hormone gene mediates trans-activation by thyroid hormone. Nature (London) **329**:738–741.
18. **Glass, C. K., S. M. Lipkin, O. V. Devary, and M. G. Rosenfeld.** 1989. Positive and negative regulation of gene transcription by a retinoic acid-thyroid receptor heterodimer. Cell **59**:697–708.
19. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus DNA. Virology **52**:456–467.
20. **Hamada, K., S. L. Gleason, B.-Z. Levi, S. Hirschfeld, E. Appella, and K. Ozato.** 1989. H-2RIIBP, a member of the nuclear hormone receptor superfamily that binds to both the regulatory element of major histocompatibility class I genes and the estrogen response element. Proc. Natl. Acad. Sci. USA **86**:8289–8293.
21. **Israël, A., A. Kimura, M. Kieran, O. Yano, J. Kanellopoulos, O. Le Bail, and P. Kourilsky.** 1987. A common positive trans-acting factor binds to enhancer sequences in the promoters of mouse H-2 and β_2-microglobulin genes. Proc. Natl. Acad. Sci. USA **84**:2653–2657.
22. **Israël, A., O. Le Bail, D. Hatat, J. Piette, M. Kieran, F. Logeat, D. Wallach, M. Fellous, and P. Kourilsky.** 1989. TNF stimulates expression of mouse MHC class I genes by inducing an NFκB-like enhancer binding activity which displaces constitutive factors. EMBO J. **8**:3793–3800.
23. **Katoh, S., K. Ozawa, S. Kondoh, E. Soeda, A. Israël, K. Shiroki, K. Fujinaga, K. Itakura, G. Gachelin, and K. Yokoyama.** 1990. Identification of sequences responsible for positive and negative

regulation by E1A in the promoter of H-2K^{bm1} class I MHC gene. EMBO J. **9**:127–135.
24. **Keleher, C. A., C. Goutte, and A. D. Johnson.** 1988. The yeast cell-type-specific repressor a2 acts cooperatively with a non-cell-type-specific protein. Cell **53**:927–936.
25. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerie, and A. Israël.** 1990. The DNA binding subunit of NFκB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62**:1007–1018.
26. **Klein-Hitpaß, L., M. Schorpp, U. Wagner, and G. U. Ryffel.** 1986. An estrogen-responsive element derived from the 5′ flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells. Cell **46**:1053–1061.
27. **Kliewer, S. A., K. Umesono, R. A. Heyman, D. J. Mangelsdorf, J. A. Dyck, and R. M. Evans.** 1992. Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. Proc. Natl. Acad. Sci. USA **89**:1448–1452.
28. **Kliewer, S. A., K. Umesono, D. J. Mangelsdorf, and R. M. Evans.** 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signalling. Nature (London) **35**:446–449.
29. **Kralli, A.** 1991. Ph.D. thesis. University of Pennsylvania, Philadelphia.
30. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
31. **Lee, W., A. Haslinger, M. Karin, and R. Tjian.** 1987. Two factors that bind and activate the human metallothionein IIA gene in vitro also interact with the SV40 promoter and enhancer regions. Nature (London) **325**:368–372.
32. **Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader, and P. Chambon.** 1992. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell **68**:377–395.
33. **Luckow, B., and G. Schütz.** 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids Res. **15**:5490.
34. **Mangelsdorf, D. J., E. S. Ong, J. A. Dyck, and R. M. Evans.** 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature (London) **345**:224–229.
35. **Mangelsdorf, D. J., K. Umesono, S. A. Kliewer, U. Borgmeyer, E. S. Ong, and R. M. Evans.** 1991. A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. Cell **66**:555–561.
36. **Meijer, I., A. G. Jochemsen, C. M. de Wit, J. L. Bos, D. Morello, and A. J. van der Eb.** 1989. Adenovirus type 12 E1A down regulates expression of a transgene under control of a major histocompatibility complex class I promoter: evidence for transcriptional control. J. Virol. **63**:4039–4042.
37. **Meijer, I., H. van Dam, A. J. M. Boot, J. L. Bos, A. Zantema, and A. J. van der Eb.** 1991. Co-regulated expression of junB and MHC class I genes in adenovirus-transformed cells. Oncogene **6**:911–916.
38. **Montminy, M. R., and L. M. Bilezikjian.** 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature (London) **328**:175–178.
39. **Nagata, T., J. H. Segars, B.-Z. Levi, and K. Ozato.** 1992. Retinoic acid-dependent transactivation of major histocompatibility complex class I promoters by the nuclear hormone receptor H-2RIIBP in undifferentiated embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA **89**:937–941.
40. **Nielsch, U., S. G. Zimmer, and L. E. Babiss.** 1991. Changes in NF-κB and ISGF3 DNA binding activities are responsible for differences in MHC and β-IFN gene expression in Ad5- versus Ad12-transformed cells. EMBO J. **10**:4169–4175.
41. **Petkovich, M., N. J. Brand, A. Krust, and P. Chambon.** 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature (London) **330**:444–450.
42. **Sap, J., L. de Magistris, H. Stunneberg, and B. Vennström.** 1990. A major thyroid hormone response element in the third intron of the rat growth hormone gene. EMBO J. **9**:887–896.
43. **Schrier, P. I., R. Bernards, R. T. M. J. Vaessen, A. Houweling, and A. J. van der Eb.** 1983. Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells. Nature (London) **305**:771–775.
44. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46**:705–716.
45. **Shapiro, D. J., P. A. Sharp, W. W. Wahli, and M. J. Keller.** 1988. A high efficiency HeLa cell nuclear transcription extract. DNA **7**:47–55.
46. **Shirayoshi, Y., J.-I. Miyazaki, P. A. Burke, K. Hamada, E. Appella, and K. Ozato.** 1987. Binding of multiple nuclear factors to the 5′ upstream regulatory element of the murine major histocompatibility class I gene. Mol. Cell. Biol. **7**:4542–4548.
47. **Tanaka, K., T. Yoshioka, C. Bieberich, and G. Jay.** 1988. Role of the major histocompatibility complex class I antigens in tumor growth and metastasis. Annu. Rev. Immunol. **6**:359–380.
48. **Umesono, K., V. Giguere, C. K. Glass, M. G. Rosenfeld, and R. M. Evans.** 1988. Retinoic acid and thyroid hormone induce gene expression through a common responsive element. Nature (London) **336**:262–265.
49. **Vaessen, R. T. M. J., A. Houweling, A. Israël, P. Kourilsky, and A. J. van der Eb.** 1986. Adenovirus E1A-mediated regulation of class I MHC expression. EMBO J. **5**:335–341.
50. **Vasios, G. W., J. D. Gold, M. Petcovich, P. Chambon, and L. J. Gudas.** 1989. A retinoic acid-responsive element is present in the 5′ flanking region of the laminin B1 gene. Proc. Natl. Acad. Sci. USA **86**:9099–9103.
51. **Wang, L.-H., N. H. Ing, S. Y. Tsai, B. W. O'Malley, and M.-J. Tsai.** 1991. The COUP-TFs compose a family of functionally related transcription factors. Gene Expression **1**:207–216.
52. **Yano, O., J. Kanellopoulos, M. Kieran, O. Le Bail, A. Israël, and P. Kourilsky.** 1987. Purification of KBF1, a common factor binding to both H-2 and β_2-microglobulin enhancers. EMBO J. **6**:3317–3324.
53. **Yu, V. C., C. Delsert, B. Andersen, J. M. Holloway, O. V. Devary, A. M. Näär, S. Y. Kim, J.-M. Boutin, C. K. Glass, and M. G. Rosenfeld.** 1991. RXRβ: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell **67**:1251–1266.
54. **Zhang, X.-K., B. Hoffman, P. B.-V. Tran, G. Graupner, and M. Pfahl.** 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature (London) **355**:441–446.
55. **Zhang, X.-K., J. Lehmann, B. Hoffmann, M. I. Dawson, J. Cameron, G. Graupner, T. Hermann, P. Tran, and M. Pfahl.** 1992. Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. Nature (London) **358**:587–590.