Activation Transcription Factor 1 Involvement in the Regulation of Murine H-2D<sup>d</sup> Expression*

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Resistance to radiation leukemia virus-induced leukemia is correlated with an increase in H-2D expression on the thymocyte surface. Recently, it has been shown that elevated H-2D<sup>d</sup> expression on the infected thymocyte is a result of elevated mRNA transcription and that the transcriptional increase is correlated with elevated levels of a DNA binding activity, H-2 binding factor 1 (H-2 BF1), which recognizes the 5′-flanking sequences (5′-TGACGCG-3′) of the H-2D<sup>d</sup> gene. This target for transcription factor binding has been found to be identical in the 5′-regulatory region of 12 rodent class I genes, nine of which have been shown to be functional genes. Furthermore, this cis-element is found 5′ of 20 primate class I genes (15 human genes), seven of which are known to be functional. Here, we demonstrate that activation transcription factor 1 (ATF-1) is one component of H-2 BF1. In addition, the levels of ATF-1 mRNA in uninfected and radiation leukemia virus-infected thymocytes parallel those of H-2D<sup>d</sup> mRNA, and therefore, it is suggested that ATF-1 up-regulates the transcription of the H-2D<sup>d</sup> gene after radiation leukemia virus infection of thymocytes. Transfection experiments also demonstrate that ATF-1 activates a reporter plasmid that contains the H-2 BF1 motif, but not a reporter lacking this motif. This is the first demonstration of the interaction of ATF-1 with 5′-regulatory sequences of major histocompatibility complex class I genes.

The MHC<sup>1</sup> encodes highly polymorphic cell-surface antigens that play an important role in recognition and elimination of virus-infected or neoplastic cells by the immune system (1, 2). Murine H-2 class I gene expression has been shown to be significantly altered in thymic cells after both radiation leukemia virus (RadLV) infection and RadLV-induced transformation (3). Early studies using H-2 congenic mouse strains demonstrated that the H-2D<sup>d</sup> locus of the MHC is sufficient to result in RadLV-induced thymoma development (4). Following infection of resistant mouse strains, there is a rapid increase in the level of D<sup>d</sup> antigen on the surface of infected thymocytes. The immune system of resistant strains recognizes the infected cells, and a cell-mediated immune response is detectable in vivo; no such immune response is seen in susceptible hosts (5). Soon thereafter, the virus-infected cells are eliminated from the resistant mice, and tumors arise in only a very small percentage of these animals (5). In the susceptible mouse strains (mice carrying the H-2D gene of a haplotype other than D<sup>d</sup>), the levels of H-2 expression are variable, no cell-mediated immune response is generated, and a high percentage of infected mice go on to develop thymomas. These observations clearly indicate the importance of immunosurveillance in determining the phenotypic response (resistance versus susceptible) following RadLV infection and potential tumorigenesis.

More recently, we have examined the stimulation of H-2D<sup>d</sup> expression at the molecular level. It was shown that infection by RadLV results in the increased transcription of H-2 gene(s) and a concomitant increase in H-2D<sup>d</sup> antigen on the surface of the thymocytes (6). Also detectable in nuclear extracts prepared from infected thymocytes is an increased DNA binding activity that specifically recognizes the sequence 5′-TGACGCG-3′ in the 5′-flanking region of the H-2D<sup>d</sup> gene. This trans-acting factor has been termed H-2 binding factor 1 (H-2 BF1). The importance of this CRE-like cis-factor in class I regulation was first noted by Israel et al. (7) in 1989 and subsequently by Dey et al. (8), Saji et al. (9), and Nobunaga et al. (10) in 1992. Although, in these studies, the biological function of this cis-element in class I regulation was suggested, the trans-factor(s) that bind to this element were not identified. We have previously shown that proteins of 27 and 65 kDa bind to this cis-sequence as determined by UV photocross-linking studies (10).

After an exhaustive search of GenBank<sup>™</sup> sequences, we have shown that this target binding sequence is present in the 5′-regulatory region of many class I genes. Notable among the findings is that this cis-element is identical in 17 out of 22 class I genes that have been shown to be functional, i.e. not pseudo-genes, by transfection analysis. The extraordinary conservation of this sequence across evolutionary lines emphasizes its potential importance in the regulation of expression of the major histocompatibility antigens.

In the experiments described here, we have screened a plasmid cDNA expression library using the yeast one-hybrid system (11). Numerous cDNA clones have been isolated, and one group of these cDNA clones is shown to encode activation transcription factor 1 (ATF-1). Using gel supershift assays, ATF-1 is demonstrated to be one component of the in vivo H-2 BF1 complex. Several facts support this evidence. Both human and mouse ATF-1 are known to bind to the CRE binding motif (12, 13), which is similar to the H-2 BF1 binding sequence. Murine ATF-1 has a molecular mass of 29 kDa, comparable to the 27-kDa protein observed in UV cross-linking studies of H-2.
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BF1 (10). The expression level of mRNA encoding ATF-1 closely parallels that of H-2 mRNA in both RadLV-infected and normal thymuses. Transfection experiments also demonstrate that ATF-1 activates a reporter plasmid that contains the H-2 BF1 motif. These observations suggest that ATF-1 is involved both in the normal regulation of H-2 expression and in the stimulation of transcription from the H-2Dα gene after RadLV infection of mouse thymocytes.

MATERIALS AND METHODS

Mice, Virus, and Cells—B10.T(6R) mice resistant to RadLV were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at the New York University Medical Center. RadLV was prepared as cell-free extracts of thymomas induced by intrathymic injection of similarly passaged RadLV (14). Preparation of infected thymocytes for RNA preparation and nuclear extraction was as described (10). Cell lines were derived from RadLV-induced thymomas and maintained as described by Bach and Meruelo (15). F9 cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% calf serum on gelatin-coated dishes.

cDNA Library Preparation and Screening—cDNA plasmid screening using the one-hybrid system was performed by the method of Wang and Reed (11). Briefly, cDNA was synthesized from the mRNA isolated from a RadLV-induced, thymoma-derived cell line (15) using the Superscript plasmid system (Life Technologies, Inc.). The product was directionally cloned into the SalI-NotI sites of the yeast expression vector pC86. The ligation products were introduced by electroporation (Bio-Rad) into Escherichia coli strain DH10B (Life Technologies, Inc.). The cDNA library was composed of 2.6 × 10^8 independent clones with an average insert size of 1.5 kb. The reporter plasmid pRS315HIS#7×4, derived from pRS315HIS (11), contained four tandem copies of the H-2 BF1 binding site (10) inserted into the BamHI site upstream of the GAL1 minimal promoter linked to the HIS3 gene. The yeast strain yWAM2 was first transformed with the pRS315HIS#7×4 plasmid by the lithium acetate method (11) and selected on leucine-deficient medium. This yeast strain was then transformed with the RadLV-transformed thymoma cell line cDNA described above. The transformants were plated on synthetic dextrose medium (16) without histidine (but including leucine and tryptophan) and incubated for 4 days at 30 °C. Colonies on these plates were transferred by replica plating onto synthetic dextrose plates without histidine, leucine, and tryptophan and incubated for another 4 days. Viable yeast colonies were picked and grown in synthetic dextrose medium with histidine, leucine, and tryptophan. Plasmid DNAs were isolated (16) and analyzed.

Northern Blot Analysis—Total RNA was isolated according to the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (17). Northern blotting and hybridization were carried out as described previously (18). Hybridization probes used were cDNA-H2 D1, cDNA-H2 D2, and cDNA-H2 Dβ (19). Hybridization to a single strand, a broadly cross-reactive cDNA subclone of H-2 Ia lacking the repetitive sequences (19, 20); ATF-1, excised by BamHI and HindIII from the ATF-1A clone (see Fig. 1); and β-actin (21).

Gel Mobility Shift Assays—Nuclear extracts were prepared from thymocytes of both RadLV-infected and normal mice of the B10.T(6R) strain as well as from the RadLV-induced, thymoma-derived cell line according to Dignam et al. (22). Protein concentration of extracts was measured by the Bio-Rad protein assay. The following oligonucleotide and its complement containing the H-2 BF1 motif were synthesized: 5′-CAGCTATGCAGCCGGCTG-3′. Complementary oligonucleotides containing the mutant H-2 BF1 motif were also synthesized: 5′-CAGCTATGCGACACGTT-3′. Complementary oligonucleotides containing the CRE found in the T-cell receptor Vβ promoter were also synthesized (13): 5′-CAGCTATGCACATTTA-3′. Equimolar amounts of these oligonucleotides were annealed with their complement and 5′-end-labeled by T4 polynucleotide kinase (New England Biolabs Inc., Beverly, MA). The labeled oligonucleotides were incubated with either the nuclear extracts or the in vitro translation products and electrophoresed through nondenaturing 5% polyacrylamide gels with 0.5 × TBE (Tris borate/EDTA) at 150 V for 2 h (Fig. 2A) or for 7 h (Figs. 2B and 3–5) (10).

In Vitro Protein Synthesis—ATF-1 cDNA was subcloned into pSG5 (Stratagene, La Jolla, CA), and the ATP-1 protein was synthesized in vitro by the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer’s instructions. Unmodified pSG5 was also used for in vitro translation as a negative control.

Gel Mobility Supershift Assays—For gel supershift assays, specific monoclonal antibodies were mixed with binding reactions (as described above) of the labeled oligonucleotides and nuclear extracts, incubated for 1 h on ice, and loaded onto the gel. Specific monoclonal antibodies against ATF-1 (products sc-270x and sc-243x), ATF-2 (product sc-187x), and c-Jun (product sc-45x) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid Constructs and Transfection Assays—The plasmid pRc/RSV/ATF-1 expression vector was generated by subcloning ATF-1A cDNA into pRc/RSV (Invitrogen, Carlsbad, CA). The plasmid pRc/RSV-1-FTA contained the ATF-1A cDNA in the antisense orientation, but was otherwise identical to pRc/RSV/ATF-1. The plasmids Δ−65CAT and Δ−122CAT, containing sequences of the mouse H-2Dα gene from positions −65 to +20 and from positions −122 to +20, respectively, linked to the gene for chloramphenicol acetyltransferase, were obtained from Dr. I. Stroyanowski (23). The mammalian expression vector for the catalytic subunit of the eAMP-dependent protein kinase, RSV-CHO-PKA-Cα version 2, was obtained from Dr. R. A. Maurer (24). Cells (5 × 10^6) F9 cells/10-cm dish) were transfected by the LipofectAMINE method (Life Technologies, Inc.) according to the manufacturer’s instructions. The DNAs for each transfection contained 5 μg of reporter plasmid (Δ−65CAT or Δ−122CAT), 5 μg of RSV-CHO-PKA-Cα version 2 or pBluescript II SK+ (Stratagene), 5 μg of pRc/RSV/ATF-1 or pRc/RSV-1-FTA, and 1 μg of pSV-β-galactosidase control vector (Promega) as an internal reference. The total amount of plasmid DNA was 16 μg/transfection. After 40 h, extracts were prepared, and chloramphenicol acetyltransferase assays were performed as described (25). Acetylated chloramphenicol was measured using the 4-methylumbelliferyl program (Version 1.60) from autoradiographed film and corrected for β-galactosidase activity to normalize for transfection efficiency (26).

RESULTS

The H-2 BF1 Binding Site Is Present 5′ of MHC Class I Genes of Many Species—Class I sequences in GenBank™ were searched for the presence of the H-2 BF1 target element by using GCG Version 8 software (Genetics Computer Group, Madison, WI). Eighteen murine H-2 class I gene sequences were identified that contain at least 150 bp of 5′-flanking sequence data. Of these 18, 11 encode classical transplantation antigens, while seven are either of the TLα or Qa families. All 10 genes, in addition to Dα, that encode transplantation antigens contain their 5′-sequence the identical 7-bp sequence identified as the binding target for H-2 BF1 (Table I). Eight of these 11 transplantation genes have been found to be active following transfection into cells. The seven H-2 genes not containing this cis-element are in the TLα/Qa families (27–31). Furthermore, two of the genes have been shown to be pseudogenes, and none of the seven have been demonstrated to be functional after transfection. Four rat class I genes were located that contain significant sequence data 5′ of the gene. One of these, RT-1C, has been shown to be functional and does indeed contain the H-2 BF1 target sequence (32). Of the three remaining isolates, two are functional but are TLα-like (33, 34), and the third is a pseudogene (35). Two swine genes were also identified and found to contain the binding sequence; one of these has been shown to be functional, while the other has not (36, 37).

The data bank search resulted in 24 human class I genes containing sufficient sequences 5′ of the coding region for examination. Thirteen of these were found to contain the identical sequence that serves as a target for H-2 BF1 and activates transcription in the mouse; five of these have been shown to be functional (Table I). Of the 11 that do not contain the sequence, two are pseudogenes (38–40), two are HLA-A genes (41, 42), two are non-HLA-A/B/C genes that resemble the H-2 TLα/Qa genes, and the remainder (43, 44), and the final five have been shown to be functional class I genes (45–47). Two isolate of HLA-E and HLA-Au24 are apparently functional in the thymus (42, 43). All of the substitutions within the target sequence occur in either the first or last base of the septum, none in the “core” of 5 bases. Other primate sequences that contain the H-2 BF1 target are found in the gorilla, chimpanzee, and orangutan (Table I). These latter sequences, however, were not isolated as part of an intact class I gene, i.e. these promoter regions were
cloned following polymerase chain reactions on genomic DNA from these species. The genes to which they are linked have not been isolated and therefore not tested for functionality. Several other sequences from primates, including human, have been isolated in this manner and do not contain the binding sequence. Until the genes themselves are isolated and examined, the significance of this observation is difficult to assess. In conclusion, however, of the class I genes that have been shown to be functional, 17 out of 22 do contain the H-2 BF1 cis-target sequence.

Cloning of ATF-1 Using the One-hybrid System in Yeast—
The cDNA library was prepared from RNA isolated from a RadLV-induced B10.T(6R) thymoma cell line as described under “Materials and Methods.” More than 10 library equivalents (3.5 \times 10^7 yeast colonies) were screened, and 49 cDNA plasmids were recovered and analyzed. From the partial sequencing of clones and the digestion patterns using a number of restriction enzymes, 22 plasmids were found to have homology to the cDNA of mouse ATF-1 (13). Based on the insert size, these clones were divided into two groups: one group with \(-1.5\)-kb inserts and another group with \(-2.4\)-kb inserts. The longest insert of each group was subcloned into pBluescript II SK\(^+\) and SK\(^-\) (Stratagene) and sequenced by the single chain termination method of Sanger et al. (48) using Sequenase (U. S. Biochemical Corp.). The 1472-bp nucleotide cDNA insert of the first group, referred to as ATF-1A, was identical to mouse ATF-1 (13) except that ATF-1A had an additional 201 bp in the 5’-noncoding region and a truncation of \(-760\) bp in the 3’-noncoding region (Fig. 1B). Two single base pair insertions were also found in the 3’-noncoding region. The second group, referred to as ATF-1B, contained a 2372-bp nucleotide insert. The coding region was found to be identical to the mouse ATF-1 cDNA. The 3’-noncoding sequence differed in that in addition to the two single base pair insertions mentioned above, one more single base pair insertion was seen, and three single base pair deletions and one single base pair substitution were identified (Fig. 1C). ATF-1B had 258 bp in the 5’-noncoding region not seen in ATF-1; the 201-bp overlap between ATF-1A and ATF-1B is identical, however. These observations would indicate that it is most probable both ATF-1A and ATF-1B are not complete reverse transcripts. The difference in the size of ATF-1A and ATF-1B in the 3’-end could be explained by utilization of two different polyadenylation sites, thereby generating mRNAs of \(-1.5\) and \(-2.4\) kb (13, 49, 50). Differences in 1 or 2 bases in the 3’-noncoding region may represent divergence between BALB/c mice, the strain used in the generation of the hybridoma used by Lee et al. (13), and the B10.T(6R) mice used in these experiments. Both cDNAs, ATF-1A and ATF-1B, have

| Gene                  | Sequence                        | Source |
|-----------------------|---------------------------------|--------|
| Murine class I        |                                 |        |
| H-2 D\(^{a}\)         | GGGACACTGATGACGCGCTGGCAGGTC      | 65     |
| D\(^{a}\)             |                                 | 66     |
| D\(^{b}\)             |                                 | 67     |
| K\(^{a}\)             | C............A.T......C........   | 68     |
| L\(^{a}\)             |                                 | 69     |
| D\(^{a}\)             |                                 |        |
| K\(^{a}\)             | C............A.T......C........   |        |
| D\(^{a}\)             |                                 |        |
| K\(^{a}\)             | C............A.T......C........   |        |
| Rat class I           |                                 |        |
| RT 1.C\(^{a}\)        | C...T.............A...AT...      | 70     |
| Swine class I         |                                 |        |
| SLA                   |                                 |        |
| PD1\(^{a}\)           |                                 |        |
| d haplotype           |                                 |        |
| Human class I         |                                 |        |
| HLA                   |                                 |        |
| Cu\(2\)\(^{a}\)       | A...T...CG......TCCC...TT...     | 72     |
| B27\(^{a}\)           | T.A.T...C......TCCC...AT...      | 73     |
| Cu1B\(^{a}\)          | T.A.T...C......TCCC...AT...      | 74     |
| Cu2B\(^{a}\)          | T.A.T...C......TCCC...AT...      | 74     |
| B\(^{7}\)             | A...T...CG......TCCC...CT...     | 74     |
| B5\(^{1}\)            | A...T...CG......TCCC...TT...     | 46     |
| Bu57\(^{a}\)          | A...T...CG......TCCC...TT...     | 75     |
| B                    |                                 |        |
| C1                    | T.A.T...C......TCCC...AT...      | 76     |
| B51-cd3.3             | CA.GT...CG......TCCC...TT...     | 76     |
| B35                   | A...T...CG......TCCC...TT...     | 77     |
| B                    |                                 |        |
| B51a                  |                                 |        |
| B7a                   |                                 |        |
| CuBL18                |                                 |        |
| Cu5                   |                                 |        |
| JY328                 |                                 |        |
| Gorilla class I       |                                 |        |
| Gogo                   |                                 |        |
| M:B26                 |                                 |        |
| Sb16                  |                                 |        |
| Chimpanzee class I    |                                 |        |
| Pat                    |                                 |        |
| K1811                 | A...T...CG......TCCC...TT...     | 79     |
| T1725                 | A...T...CG......TCCC...TT...     | 79     |
| Orangutan class I     |                                 |        |
| Pogy C1524            | CA...T...CG......TCCC...TT...    | 79     |

\(^{a}\) These genes have been shown to be functional by transfection and expression.

\(^{b}\) G. D. Brown D. R. Morris, and D. Meruelo (1997) Eur. J. of Immunol., in press.
Fig. 1. A, schematic representation of the sequences for the previously published mouse ATF-1 cDNA (13) and ATF-1A and ATF-1B cDNAs. The solid bar of each cDNA indicates the open reading frame. Small cross-hatched bars indicate potential polyadenylation signals (AATAAA and TATAAA) (13). H and B identify HindIII and BamHI restriction sites, respectively. The hatched bar, excised by BamHI and HindIII, indicates the probe used for Northern analysis. The open box shows the structure of the ATF-1 protein; BASIC and ZIP indicate the basic region and leucine zipper of mouse ATF-1. B, sequence of the 5'-noncoding regions of ATF-1A and ATF-1B. The 5'-ends of ATF-1 and ATF-1A are indicated on the longer ATF-1B. The beginning of translation (ATG) is also indicated. C, sequence of the 3'-noncoding regions of both clones. Single base pair deletions are indicated by asterisks; insertions are boxed; and a single base pair substitution is indicated in parentheses above the ATF-1B sequence. The 3'-end of ATF-1A is indicated. Potential polyadenylation signals are indicated in boldface.
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FIG. 1—continued

Fig. 2. A, gel mobility and competition assays of in vitro translation products of ATF-1A. End-labeled, double-stranded H-2 BF1 binding motif (WT) oligonucleotide (1 × 10⁵ cpm) was incubated with in vitro translation products. Lane 1, in vitro products of the pSG5 vector alone; lane 2, in vitro products of ATF-1A cDNA subcloned into pSG5; lanes 3–5, unlabeled, double-stranded oligonucleotide (H-2 BF1 motif) at 50-, 100-, and 150-fold molar excess, respectively, used as a competitor prior to the addition of radiolabeled probes; lanes 6–8, unlabeled, double-stranded oligonucleotide (mutant H-2 BF1 motif (MUT)) at 50-, 100-, and 150-fold molar excess, respectively, used as a competitor. F indicates free radiolabeled oligonucleotide probes. Samples were electrophoresed through nondenaturating 5% polyacrylamide gels with 0.5 × TBE at 150 V for 2 h. B, gel mobility and competition assays of nuclear extracts. End-labeled, double-stranded wild-type oligonucleotide (1 × 10⁵ cpm) was incubated with nuclear extracts. Lane 1, 1 µg of nuclear extracts from the RadLV-induced, thymoma-derived cell line; lanes 2–4, unlabeled oligonucleotide (WT) at 50-, 100-, and 150-fold molar excess, respectively, used as a competitor prior to the addition of radiolabeled probes; lanes 5–7, unlabeled oligonucleotide (MUT) at 50-, 100-, and 150-fold molar excess, respectively, used as a competitor. Samples were electrophoresed through nondenaturating 5% polyacrylamide gels with 0.5 × TBE at 150 V for 7 h.

only one long open reading frame, which encodes activation transcription factor 1. This protein has a molecular mass of 29 kDa and has been grouped into the bZIP (basic leucine zipper) family of transcription factors (51) (Fig. 1).

ATF-1 Binds to the H-2 BF1 Binding Motif—Binding to the double-stranded H-2 BF1 oligonucleotide was detected in in vitro translated ATF-1A (Fig. 2A, lane 2), whereas no binding of the in vitro products of the pSG5 vector alone was seen (lane 1). This is further supported by the fact that these clones were isolated using the one-hybrid system in yeast, where recognition of the cis-element contained in the reporter plasmid by the expressed cDNA product is a prerequisite for isolation. Binding to the CRE consensus oligonucleotide was also detected in nuclear extracts from the cell line and in vitro translated ATF-1A (data not shown). Specificity of binding was demonstrated by the competition assays. The addition of unlabeled H-2 BF1 oligonucleotides (WT) at 50-, 100-, and 150-fold molar excess resulted in a reduction of binding activity (Fig. 2A, lanes 3–5, respectively). The addition of mutant oligonucleotide (MUT) at 50-, 100-, and 150-fold molar excess had little effect on binding (Fig. 2A, lanes 6–8, respectively).

Parallel experiments were performed using nuclear extracts from the RadLV-induced, thymoma-derived cell line (Fig. 2B). Four bands (bands A–D) were observed in the absence of competitor (Fig. 2B, lane 1). Specificity of binding was demonstrated by the competition assays. The addition of unlabeled H-2 BF1 oligonucleotide (WT) at 50-, 100-, and 150-fold molar excess resulted in a reduction of all four bands (Fig. 2B, lanes 2–4, respectively). On the other hand, the addition of mutant oligonucleotide (MUT) at 50-, 100-, and 150-fold molar excess had little effect on bands A–C and partial reduction of band D (Fig. 2B, lanes 5–7, respectively). The differences in migration patterns are a result of differing gel electrophoresis times (see figure legends).

The ATF-1 Homodimer Is One Component of H-2 BF1 in Vivo—To directly compare the migration of DNA-protein complexes seen in the gel shift analysis, the bound cell line extract and in vitro products were run on the same nondenaturing 5% polyacrylamide gel (Fig. 3). Four bands (bands A–D) were observed in the cell line nuclear extract as described above (Fig. 3, lane 1). The same four bands were subsequently observed in thymocyte extracts from both RadLV-infected and normal B10.T(6R) mice (see Fig. 5). The in vitro products of ATF-1A in lane 2 comigrated with band C in lane 1. No binding was observed in the in vitro products of the pSG5 vector only (Fig. 3, lane 3). Because it has been shown that ATF-1/CREB proteins usually form either homodimers or heterodimers (51), band C in lane 1 is probably the ATF-1 homodimer since only one cDNA was included in the translation reaction. When the [³⁵S]methionine-labeled in vitro translation products of ATF-1A were analyzed on denaturing SDS-polyacrylamide gels, some truncated (<27 kDa) and elongated (>27 kDa) forms

1789 ATCTTTATATTTACTAATGTGAGTTTTAATGATTATTTAAAGTT
1839 ATATAGTGGTGTAGCAAATATTTTGATCTCAAGATATCATTTTT
1889 TATATGATGAGGTAATTTCTAATTGCTGATAATGGAATTGTA
1939 TTGTATATAAAAAGTTATCTAACAGGCTGTGTCAGFAACCCAGCA
1989 TTTAATCTCTTGTATTTTGTCTGTTGTCACCTTGGAAC•TGCAAGACTGT
2038 TGGTGACTCCACAGAAGTAGTTTCTATATTGCTGTGAAATTAAAAACATT
2088 TGATAAAG
were observed (data not shown). Therefore, it is not surprising that the binding of in vitro translated ATF-1A (Fig. 3, lane 2) did not result in a single discrete band.

To demonstrate that ATF-1 is indeed a component of H-2 BF1 in the thymocyte, we examined the effect of two anti-ATF-1 antibodies on the mobility of the H-2 BF1-DNA complex. Antibodies to two other closely related, but distinct members of the leucine zipper family of transcriptional activators were used as negative controls (anti-c-Jun and anti-ATF-2). Supershift of bands A–C with one anti-ATF-1 antibody (Santa Cruz Biotechnology product sc-270x) was observed (Fig. 4, lane 2). With another anti-ATF-1 antibody (Santa Cruz Biotechnology product sc-243x), only band C was shifted up (Fig. 4, lane 3). On the other hand, anti-c-Jun and anti-ATF-2 antibodies had no effect on the migration of the complex (Fig. 4, lanes 4 and 5, respectively). As we have shown in Fig. 3, band C is most probably the ATF-1 homodimer. The resulting supershift of band C with both anti-ATF-1 antibodies confirmed this. These data demonstrate that the H-2 BF1 complex, present in normal thymocytes and at elevated levels in RadLV-infected and RadLV-induced thymoma cell lines, contains ATF-1 as one of the components.

Based on the gel shift data using the cell line nuclear extracts, the H-2 BF1 complex is composed of four forms (bands A–D; Figs. 2B and 3–5). As described above, band C represents the ATF-1 homodimer. The following results led to the conclusion that band A is the CREB homodimer and that band B is the ATF-1/CREB heterodimer. 1) With specific anti-CREB-1 antibody, band A was totally and band B was partially shifted up; 2) the migration of in vitro translated CREB cDNA was the same as that of band A; and 3) the migration of in vitro translated ATF-1 and CREB cDNAs, after mixing, was the same as that of band C (data not shown). Since one of the anti-ATF-1 antibodies (product sc-270x; Fig. 4, lane 2) cross-reacts somewhat with CREB-1 according to the manufacturer’s analysis, it is consistent that not only band C, but also bands A and B were shifted up. On the other hand, the other anti-ATF-1 antibody (product sc-243x) specifically affects the migration of ATF-1 homodimers, but not that of the ATF-1/CREB heterodimer in the gel mobility shift analysis (data not shown). It is therefore consistent that only band C was shifted up with this antibody. Band D was consistently faint as compared with the other bands. In addition, the antibodies used in these experiments

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had no effect on the migration of band D. At this time, the composition of band D has not been determined.

Binding Activity Comparison between Nuclear Extracts from Different Sources—To compare the binding activity among normal thymocytes, RadLV-infected thymocytes, and RadLV-induced thymoma cell lines, the protein concentration of each nuclear extract was carefully measured simultaneously and was repeated in triplicate. 1 μg of nuclear protein from each preparation was used for gel mobility shift assay (Fig. 5). In every preparation, bands A–D were observed as described above. In two preparations from RadLV-infected thymocyte tissue (Fig. 5, lanes 3 and 4) and two from the RadLV-induced thymoma cell line (lanes 5 and 6), H-2 BF1 binding activity was higher than that seen in two individual preparations from normal thymocytes (lanes 1 and 2). Bands A–C were especially prominent. One preparation from RadLV-infected thymocytes (Fig. 5, lane 3) showed the highest binding activity when compared with other preparations. These data were consistent with the Northern data (Fig. 6) described below.

Expression of ATF-1 in RadLV-infected Thymocytes and the RadLV-induced, Tumor-derived Cell Line—The highly conserved MHC class I probe hybridized to an mRNA of 1.6 kb and showed that the levels of H-2 mRNA are elevated in the RadLV-induced, thymoma-derived cell line RNA (Fig. 6A, lane 1) and in two out of three RNA samples extracted from RadLV-infected thymocytes (lanes 3–5). Two bands, corresponding to 2.4 and 1.5 kb, were detected by ATF-1A hybridization in the samples of RadLV-infected murine thymocytes (Fig. 6B, lanes 3–5) and the RadLV-induced, thymoma-derived cell line (lane 1); these sizes are in agreement with the two cDNA clones isolated representing differential use of polyadenylation signals (Fig. 6B, arrows). The levels of H-2 and ATF-1 mRNA were increased both in RadLV-infected B10.T(6R) thymocytes (two of three samples assayed) and in the cell line derived from a RadLV-induced thymoma as compared with uninfected thymocytes (Fig. 2, A, lanes 2; and B, lane 2). The level of β-actin cDNA hybridization was relatively consistent in all RNA preparations and demonstrates that the variances seen above are not a result of varying RNA concentrations (Fig. 6C). The quantity of H-2 mRNA in different preparations of RadLV-infected thymocytes very nearly parallels that of ATF-1 mRNA. The data therefore suggest that ATF-1 may be involved in the up-regulation of transcription from the H-2D′ gene after RadLV infection of thymocytes. One of three RNA preparations from RadLV-infected B10.T(6R) thymocytes did not show the increase in H-2 and ATF-1 mRNA levels (Fig. 2, A, lane 3; and B, lane 3). This is consistent with the fact that usually ~70% of thymocyte preparations from individual RadLV-infected B10.T(6R) mice show increases in H-2 expression on the surface as measured by fluorescence-activated cell sorting analysis, while the other 30% of the preparations do not (data not shown).

Cotransfection Assays—To determine whether ATF-1 could activate transcription of the H-2D′ gene in vivo, F9 cells were transfected with either Δ−65CAT or Δ−122CAT reporter plasmids with or without expression vectors encoding the catalytic subunit of the cAMP-dependent protein kinase and ATF-1 as described above. As shown in Fig. 7B, the Δ−65CAT and Δ−122CAT plasmids contain sequences found 5′ of the mouse H-2D′ gene from positions −65 to +20 and from positions −122 to +20, respectively, linked to the gene for chloramphenical acetyltransferase (CAT) (23). The hatched box represents the H-2 BF1 motif (TGACGCG). Stippled boxes represent CAAT and TATA boxes.
motif (TGACGCG, from positions –99 to –93), while the former does not. In Fig. 7A, the induction index was calculated as follows: the experimental chloramphenicol acetyltransferase value was divided by the value for the Δ−65CAT reporter plasmid and antisense ATF-1 expression plasmid without the cAMP-dependent protein kinase plasmid (the first bar in Fig. 7A) in each experiment. Data are shown as the means ± S.D. of four independent experiments. The Δ−65CAT reporter plasmid showed only small increases (<2.5-fold). The Δ−122CAT reporter plasmid plus pRe/RSV/ATF-1 with kinase showed an 8.5-fold increase on average, whereas the Δ−122CAT reporter plasmid plus pRe/RSV/ATF-1 without kinase and the Δ−122CAT reporter plasmid plus pRe/RSV/1-FTA (antisense) showed a <3.0-fold increase on average. The combination of ATF-1 and the cAMP-dependent protein kinase expression plasmids with the Δ−122CAT reporter plasmid produced a response that was significantly greater than that produced by other combinations. This is consistent with the report that ATF-1 activates transcription in response to cAMP-dependent protein kinase A (52).

**DISCUSSION**

Early experiments in the RadLV system indicated the importance of immunosurveillance in controlling tumorigenesis and the impact that altered MHC expression has on the effectiveness of the immune response of the host to virus-infected cells. Subsequently, investigations in several other systems including RNA (AKR murine leukemia virus, Moloney leukemia virus, and vesicular stomatitis virus) and DNA (herpes simplex virus, SV40, adenovirus, and polymya virus) tumor viruses also demonstrated alterations in class I gene expression resulting from viral infections and/or virus-mediated transformations (for review, see Ref. 53). In general, elevation of class I gene expression has been correlated with disease resistance. The picture that emerges from all of these systems is that viral infection often leads to enhanced transcription and MHC expression, while viral transformation leads to decreased expression. The net result of these changes is highly significant for the ability of the immune system to either eliminate or fail to respond to virus-infected/transformed cells. It has been established that viral antigens are presented to immune effector cells in the context of histocompatibility antigens. Studies in the systems mentioned above have led to the recognition that the quantitative expression of MHC molecules is as important to the effectiveness of the immune system as is the nature of the MHC molecule involved. One key goal is to define the mechanisms by which viruses alter expression of MHC genes.

Our laboratory first demonstrated that in RadLV-infected cells, increased cell-surface expression of H-2D^d antigen is a result of elevated levels of transcription (10). The cis-regulatory element was located through DNA footprinting analysis; the protein complex that recognized and interacted with this sequence was termed H-2 BF1 and was shown to be composed of at least two proteins, one of which had a molecular mass of ~27 kDa (6, 10). The cis-element is of interest in that it has been found to be perfectly conserved in the 5′-region of nine out of nine known functional genes encoding murine transplantation antigens. Likewise, the sequence is identical in the majority of primate MHC genes that have been shown to be functional.

In the experiments described here, we have used the yeast one-hybrid system to identify murine ATF-1 as being one component of H-2 BF1. H-2 BF1 is the thymus-specific transcriptional activator complex (54) that recognizes sequences (5′-TGACGCGC-3′) 5′ of the H-2D^d gene. The molecular mass of ATF-1 approximates that of one component of H-2 BF1 as estimated from previous UV photocross-linking studies (10). Originally, ATF-1 was cloned by screening cDNA libraries with double-stranded DNA probes containing the CRE (12, 13). The CRE binding motif is known to be essential for the basal transcriptional activity of many promoters (55, 56), and ATF-1 has been shown to be involved in the expression of numerous genes, including calcitonin, Na,K-ATPase α-subunit, and interleukin-1β (57–60). Our data show that ATF-1 can bind both to the CRE binding motif and to the similar H-2 BF1 motif. The observation that anti-ATF-1 antibody changes the mobility of the H-2 BF1-DNA complex in gel mobility assays is strongly supportive of the conclusion that ATF-1 is one component of the H-2 BF1 complex. Northern analysis data show that the levels of H-2 mRNA in various preparations of RadLV-infected thymocyte RNA very nearly parallel those of ATF-1 mRNA. Transfection experiments also demonstrate that ATF-1 activates the reporter plasmid that contains the H-2 BF1 motif, but not the plasmid without the H-2 BF1 motif. Thus, it is also suggested that ATF-1 up-regulates the transcription of the H-2D^d gene after RadLV infection of thymocytes. This is the first evidence implicating ATF-1 in the transcriptional regulation of class I MHC genes.

Previously, we demonstrated that the H-2 BF1 complex was composed of at least two proteins with approximate molecular masses of 27 and 65 kDa (6, 10). During the screening process for clones recognizing the H-2 BF1 binding motif, many clones were isolated that did not show homology to ATF-1. Sequence data show that these isolates are the p65 subunit of NF-κB and that, in fact, the *in vitro* translated protein product of these clones does associate with ATF-1 *in vitro*.^2^ It has been shown that NF-κB does, under some circumstances, associate with other transcription factors such as c-Jun and c-Fos (61), the Epstein-Barr virus bZIP transactivator (BZLF1) (62), and CCAAT/enhancer-binding protein family members (63). Although preliminary attempts to demonstrate the association between ATF-1 and NF-κB in *vitro* have not been successful, the possibility that, under certain circumstances, ATF-1 could associate with NF-κB in *vitro* is under investigation. While conducting UV photocross-linking analysis, it was noted that detection of the 27-kDa protein was very strong and consistent; detection of the 65-kDa protein required higher amounts of nuclear extracts and was more variable from extract to extract. In some nuclear preparations, a protein of ~35 kDa was also detected in addition to the 27-kDa protein (data not shown). As mentioned above, we have found that the H-2 BF1 complex is composed of at least three forms: the ATF-1 homodimer, CREB homodimer, and ATF-1/CREB heterodimer.^2^ Murine CREB has a molecular mass of 37 kDa (64), and therefore, the 35-kDa protein detected in UV photocross-linking analysis is most probably CREB.

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