Murine Retroviruses Control Class I Major Histocompatibility Antigen Gene Expression via a *trans* Effect at the Transcriptional Level

LISE D. WILSON, DAVID C. FLYER,[†] AND DOUGLAS V. FALLER*

Division of Pediatric Oncology, Dana-Farber Cancer Institute, and Harvard Medical School, Boston, Massachusetts 02115

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Moloney murine leukemia virus (M-MuLV) and Moloney murine sarcoma virus (M-MSV) exert a regulatory effect on the class I genes of the murine major histocompatibility complex (MHC). We have previously shown that M-MuLV infection of mouse fibroblasts results in a substantial increase in cell surface expression of H-2K, H-2D, and H-2L proteins, whereas M-MSV, upon coinfection of the same cells, is apparently able to override the MuLV-induced increase in H-2 expression. As a result of this modulation, immune recognition of the infected cells is profoundly altered. Our efforts have been directed toward elucidating the molecular basis for this phenomenon. We report here that stimulation of interferon production as a result of infection with MuLV does not occur and, therefore, is not the cause of MuLV-induced enhancement of MHC expression. Control of H-2 class I and β_2 -microglobulin gene expression by M-MuLV, and probably by M-MSV, takes place at the transcriptional level as indicated by nuclear runoff studies and analysis of steady-state mRNA levels. Our demonstration that M-MuLV controls expression of widely separated endogenous cellular genes (those coding for H-2D, H-2K, H-2L, and β_2 -microglobulin), transfected class I MHC genes, and unintegrated chimeric genes consisting of fragments of class I MHC genes linked to sequences encoding a procaryotic enzyme, chloramphenicol acetyltransferase, suggests that M-MuLV exerts its effect in *trans* and not by proviral integration in the vicinity of the H-2 gene complex. Finally, we show that the sequences of at least one MHC gene, which are responsive to *trans* regulation by M-MuLV, lie within 1.2 kilobases upstream of the initiation codon for that gene.

Class I genes of the H-2 gene complex in mouse, and the HLA complex in humans, encode protein products which are expressed in association with β_2 -microglobulin on the surfaces of nucleated cells (23). In addition to serving as the target molecules in allogeneic graft rejection, these proteins are essential for the recognition and elimination of neoplastic and virus-infected cells by the cytotoxic T cells (CTLs) of the host immune system (37, 44). The level of expression of H-2 class I proteins on virus-infected cells has been correlated with the degree of effectiveness with which they are destroyed by the CTLs (18, 34). It is, therefore, reasonable to expect that viruses with the capability of altering H-2 expression in the cells they infect may be influencing their own survival.

Previously, this laboratory has reported that cells chronically express significantly increased surface levels of H-2K, H-2D, and H-2L proteins after infection with the Moloney murine leukemia virus (M-MuLV) (18). These infected cells are efficiently lysed by M-MuLV-specific CTLs and also by allospecific CTLs, a reflection of their increased level of H-2 class I antigens. Coinfection of cells with M-MuLV and the replication-defective, acutely transforming Moloney murine sarcoma virus (M-MSV) eliminates the H-2 enhancement, as well as profoundly decreasing the immunosensitivity of the infected cells. This report describes our efforts to define the mechanism by which these viruses control H-2 expression.

One mechanism by which a virus might affect class I murine major histocompatibility complex (MHC) antigen levels is through the stimulation of interferon production. The interferons have been shown to be potent enhancers of class I gene expression, in addition to their ability to stimulate the production of antiviral proteins and inhibit cell growth (16). However, in this report, we show that neither interferon nor any other soluble factor with direct class I-enhancing activity is produced by M-MuLV-infected cells; the virus must be physically present for the effect on class I expression to take place. Furthermore, our findings indicate that as a result of M-MuLV infection, cells increase their synthesis of H-2 class I mRNA, as well as β_2 -microglobulin mRNA. The increase is not evident upon coinfection of the same cells with M-MSV, suggesting that the inhibitory effect of the transforming virus is at the transcriptional level. Infection of cells transfected with H-2 class I genes indicate that the viral regulation of class I occurs in *trans*. We also show that sequences upstream of at least one H-2 class I gene are responsive to this *trans* regulator and will direct increased production of an attached bacterial gene in M-MuLV-infected cells.

MATERIALS AND METHODS

Viruses. M-MuLV clone 1 (12) was obtained from C. Tabin and R. Weinberg (Massachusetts Institute of Technology, Cambridge). Twenty-four-hour conditioned medium from the M-MSV-producer cell line, G8-124 (2), was used as the

* Corresponding author.

[†] Present address: Department of Microbiology, Milton S. Hershey Medical Center, Hershey, PA 17033.

source of the M-MSV/MuLV complex after passage through a 0.22- μ m-pore size Millex filter (Millipore Corp., Bedford, Mass.). Vesicular stomatitis virus (VSV), Indiana strain, was obtained from A. Huang. Viral infections of cultured cells were carried out as described (11).

Cells. BALB/c fibroblasts were obtained through the American Type Culture Collection, Rockville, Md., or as a gift from R. Weinberg. Infected and uninfected BALB/c cells were cultured in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine.

Monoclonal antibodies. Hybridoma cell lines producing monoclonal antibodies specific for H-2K^d (31.3.45), H-2D^d (34.2.12), and H-2K^b (B8-24-3) were kindly provided by David Sachs. Cell-free supernatants from hybridoma-conditioned medium were used undiluted for cell surface staining.

ConA supernatant. Spleen cells from Lewis rats (Charles River Laboratories, Wilmington, Mass.) were cultured in the presence of 5 μ g of concanavalin A (ConA; Pharmacia Fine Chemicals, Uppsala, Sweden) per ml for 24 h. The cell-free supernatant was partially purified by ammonium sulfate precipitation as described (33).

Immunofluorescent labeling and analysis. Live cells were labeled with hybridoma culture supernatants followed by fluorescein-conjugated, affinity-purified F(ab')₂ fragment of sheep anti-mouse immunoglobulin G (Cooper Biomedical, Malvern, Pa.), as described (18). After fixation in 2% paraformaldehyde, labeled cells were analyzed by an Epics V fluorescence-activated cell sorter (Coulter Electronics, Inc., Hialeah, Fla.).

VSV inhibition assay for interferon. BALB/c cells, uninfected or chronically infected with M-MuLV, were tested for interferon production by using a plaque inhibition assay. Cells were plated at 10³ per well in a 96-well tissue culture dish. As a positive control for interferon activity, BALB/c cells were also plated and cultured in medium containing 2.5% ConA supernatant. Forty-eight hours after plating, the medium was aspirated and replaced with serial dilutions of a VSV stock containing 10⁹ PFU/ml. Infection was allowed to proceed for 1 h at 37°C. Eight wells of each cell type were set up for each 10-fold dilution of virus tested. After infection, fresh medium (plus 2.5% ConA supernatant for the control wells) was added to the aspirated wells, and the cells were incubated at 37°C. The wells were scored visually for plaques 48 h after infection.

Northern blot analysis of total cellular RNA. Total cellular RNA was extracted from cells by using a modification of the guanidine hydrochloride extraction technique described by Strohman et al. (41). Briefly, after three serial ethanol precipitations in the presence of 6 M guanidine-100 mM potassium acetate, the nucleic acids were extracted with a phenol-chloroform mixture and ethanol precipitated. Twenty micrograms of RNA was loaded per lane on a 1.4% agarose gel containing formaldehyde and ethidium bromide (20). After electrophoresis, transfer to nitrocellulose was carried out using standard techniques. After baking in vacuo, prehybridization of the filters was performed in 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate; pH 7.0) and 50% formamide at 42°C. Hybridization was carried out under the same conditions, in the presence of 10% dextran sulfate and 2 $\times$ 10⁶ cpm of radiolabeled probe per ml of hybridization fluid. The blots were washed under stringent conditions (68°C, 0.2 $\times$ SSC), and autoradiography was carried out using intensifying screens at -70°C. A densitometric scanner (Helena Laboratories, Beaumont,

Tex.) was used to quantitate relative amounts of hybridization.

The probe used to detect *H-2* class I sequences was a 2.5-kilobase (kb) *Bam*HI fragment from a plasmid clone containing the *H-2D^b* genomic clone (kind gift of R. Flavell, Biogen). This fragment contains the strongly conserved exons 4 through 8 and the 3' untranslated sequences and hybridizes to all murine class I mRNA species. The probe for β_2 -microglobulin was previously described (18). Probes were labeled to a specific activity of >10⁸ cpm/ μ g, using the random oligo primer method (13). To normalize for the amount of RNA in each lane, blots were stripped of probe after autoradiography and rehybridized with a β -actin cDNA probe and a β -tubulin cDNA probe. The actin probe consisted of a 2 kb *Pst*I fragment derived from a chicken β -actin cDNA clone, and the tubulin probe consisted of a 1.7-kb *Pst*I fragment derived from a chicken β -tubulin cDNA clone (8).

Nuclear transcript runoff analysis. Nuclei were prepared from approximately 10⁸ actively growing fibroblasts (infected or uninfected), using a modification of the technique described by McKnight and Palmiter (30). Briefly, cells were lysed in a buffer containing 10 mM Tris (pH 7.5), 2 mM MgCl₂, 3 mM CaCl₂, 3 mM dithiothreitol, 0.3 M sucrose, and 0.5% Triton X-100, and nuclei were purified by centrifugation through a 2.3 M sucrose cushion. A sample of 6 $\times$ 10⁷ nuclei was incubated in 330 μ l of a reaction mixture containing 20 mM Tris (pH 8.0), 100 mM KCl, 4.5 mM MgCl₂, 0.4 mM each of ATP, CTP, and GTP, 2 mM dithiothreitol, 20% glycerol, 20 U of RNasin (Promega Biotech, Madison, Wis.) per 100 μ l, and 330 μ Ci of [³²P]UTP (New England Nuclear Corp., Boston, Mass.; specific activity, 3,000 mCi/mmol) for 30 min at 26°C. The nuclei were spun out of the reaction mixture, and the reaction was terminated by the addition of carrier RNA and DNase. After a 15-min incubation at 26°C, sodium dodecyl sulfate and proteinase K were added. After 30 min of digestion at 37°C, a hot phenol-chloroform extraction was performed, and the nucleic acids were precipitated in ethanol.

Two micrograms of DNA sequence specific for class I *H-2* molecules, β_2 -microglobulin, actin (see above), or pBR322 was denatured by heating to 100°C, adjusted to 1 M NaOH, neutralized, spotted onto nitrocellulose by using a dot-blot apparatus, and fixed by baking in vacuo. Prehybridization and hybridization were carried out in 40% formamide-4 $\times$ SSC-5 mM EDTA-0.4% sodium dodecyl sulfate, in the presence of 1 $\times$ Denhardt solution and 100 μ g of yeast tRNA per ml, at 40°C. For hybridization, 2.3 $\times$ 10⁷ cpm of labeled RNA was added to 750 μ l of hybridization buffer, and incubation at 40°C was carried out for 72 h. Washes of the filters were as described by Spindler et al. (40). After autoradiography, the filters were cut up, and the relative amounts of hybridization were quantitated by scintillation counting.

DNA-mediated *H-2* gene transfer. The *H-2K^b* gene, subcloned into the *Eco*RI site of pBR327, was kindly provided by R. Flavell and H. Allen (1). This DNA was coprecipitated with the selectable marker pMSVneo (17; see below) or pSV2neo (39) and using salmon sperm DNA as carrier. Transfection and isolation of clones were carried out as previously described (17). Clones were selected for cell surface expression of protein K^b by immunofluorescent staining and analyzed by fluorescence-activated cell sorting.

CAT vector constructions. The subclone of the genomic *H-2K^b* gene in pBR327 was digested with the restriction enzymes *Hind*III and *Nru*I to generate a 21-kb fragment. After isolation, the fragment was blunt-ended with the large

fragment of *Escherichia coli* DNA polymerase I and ligated to *Hind*III oligonucleotide linkers. The linked fragment was then ligated into the *Hind*III site of the chloramphenicol acetyltransferase (CAT) vector pSVOCAT (22). Transformation of competent *E. coli* HB101 resulted in Amp^r colonies which were screened by the minilysate technique (3) for insertion and correct orientation of the *H-2K^b* promoter sequences relative to the bacterial CAT gene (pK^bH3 NruCAT; see Fig. 5). The construction pK^bP2NruCAT was generated by isolating the 1.2-kb *Pvu*II-*Nru*I fragment of the *H-2K^b* gene, ligating it to *Hind*III linkers, and cloning it into pSVOCAT as described above (see Fig. 5). All enzymes and linkers were obtained from New England Biolabs (Beverly, Mass.) and were used according to the supplier's recommendations.

Assay for transient CAT expression. All CAT plasmids were isolated by lysozyme-sodium dodecyl sulfate lysis and cesium chloride-ethidium bromide equilibrium gradient centrifugation (28). Cells were plated at 10⁶ per 100-mm tissue culture dish approximately 18 h before transfection. Calcium phosphate precipitates containing 10 µg of the CAT vector and 20 µg of salmon sperm carrier DNA were prepared, and transfections were carried out as described previously (17). The CAT vector pSV2CAT (22), provided by R. Mulligan and J. Nye, was used as a positive control for CAT expression in each of the cell lines tested. Forty-eight hours after transfection, the cell monolayers were washed with, and scraped into, ice-cold phosphate-buffered saline. The pelleted cells were suspended in 100 µl of 0.25 M Tris hydrochloride (pH 7.8), and cell extracts were prepared by freezing and thawing the suspended cells three times. Cellular debris was removed by centrifugation in a microcentrifuge at 13,500 × *g* for 5 min at 4°C. The extracts were then measured for protein content and assayed for CAT activity as described by Gorman et al. (22). Controls including purified CAT enzyme (0.001 U per assay; Pharmacia, Piscataway, N.J.), instead of cell extract, were also run as standards for acetylation states of the chloramphenicol. In some experiments, to provide an internal control, the CAT plasmids were cotransfected with 2 µg of a plasmid, pTKGH, which contains the cDNA encoding the human growth hormone protein, under the transcriptional control of the thymidine kinase promoter (36). The plasmid was generously provided by D. Moore (Massachusetts General Hospital, Boston). Human growth hormone levels were measured in the culture medium from the transfected cells at 48 h, using a radioimmunoassay kit (Nichols Diagnostics Institute, San Juan Capistrano, Calif.).

Establishment of long-term CAT-expressing cell lines. Long-term, stable CAT-expressing lines were generated by transfecting BALB/c 3T3 cells with a calcium phosphate precipitate consisting of 10 µg of pK^bH3NruCAT, 0.5 µg of pMSVneo, and 20 µg of salmon sperm carrier DNA. The bacterial Tn5 phosphotransferase gene, termed *neo*, confers resistance to neomycin, kanamycin, and their analogs, including geneticin sulfate (G418; GIBCO). Its use as a selectable marker for eucaryotic gene expression has been reported (9, 39). In the pMSVneo construction, the *neo* gene has been placed under the control of the M-MSV 5' long terminal repeat sequences (17). Forty-eight hours after the cotransfection, the cells were replated in medium containing 0.5 mg of G418 per ml. Individual clones of G418-resistant cells were picked, using cloning cylinders, and subsequently expanded in G418-containing medium. Extracts of the G418-resistant clones were then prepared and assayed for CAT activity, as described above. Certain of the CAT-expressing

FIG. 1. H-2K^d antigen expression on the surface of BALB/c 3T3 cells exposed to M-MuLV-containing conditioned medium. BALB/c 3T3 cells were exposed for 48 h to: (A) medium conditioned by BALB/c 3T3 cells chronically infected with M-MuLV and producing infectious virions; (B) conditioned medium as in panel A, but which had been previously centrifuged (45,000 × *g* for 1 h) to pellet out virions; and (C) 2.5% of medium conditioned by ConA-stimulated splenocytes, as a source of gamma interferon. After exposure to these media, cells were labeled with monoclonal antibodies directed against H2-K^d. Cells were then stained with a fluorescein-conjugated anti-mouse immunoglobulin antiserum and analyzed on a flow cytometer as described. Solid lines, Mock-treated BALB/c 3T3 cells; broken lines, cells exposed to the conditioned media.

lines were transfected with 20 µg of the cloned, biologically active provirus, pMoV-9, a kind gift of R. Mulligan (7), or with carrier salmon sperm DNA alone. Cellular extracts were prepared and assayed 48 h later, as described above.

RESULTS

M-MuLV induces increased expression of H-2 class I antigens without the involvement of interferon. When BALB/c 3T3 cells were exposed for 48 h to conditioned medium from cells producing M-MuLV, an increase in cell surface expression of class I MHC antigens resulted (Fig. 1). All three BALB/c class I antigens (D^d, K^d, and L^d) were elevated. This effect could be due to infection of the BALB/c 3T3 cells by the M-MuLV virions contained in the conditioned medium or, alternatively, to soluble factors (e.g., interferon) elaborated into the conditioned medium by the M-MuLV producer cell line. Ultracentrifugation of the conditioned medium effectively removed infectious virions (data not shown), and medium treated in this way had no subsequent effect on class I MHC expression in BALB/c 3T3 cells. This suggests that it is the retrovirus itself which is influencing H-2 expression in the treated cells.

Other indirect evidence to support the role of the retrovirus in this effect comes from examination of the time course of MHC induction after exposure to the conditioned medium containing M-MuLV. Although Fig. 1 shows a 2-fold increase in class I antigen expression as early as 48 h after treatment with the virus-containing medium, the full 10-fold induction of antigen levels demonstrated previously (18) required 7 to 10 days. This length of time parallels the period required for spread of M-MuLV through the fibroblast culture (as monitored by the appearance of viral protein expression in the cells).

In theory, beta interferon could be elaborated by the BALB/c 3T3 cells after exposure to the M-MuLV in the

TABLE 1. Susceptibility of MuLV-infected cells to VSV infection^a

Infected cells	Virus titer (PFU/ml)	
	Expt 1	Expt 2
BALB/c 3T3	5×10^6	2×10^7
M-MuLV-BALB	5×10^6	2.5×10^7
ConA-BALB	$<10^2$	$<10^2$

^a Titer of the same virus stock as assayed on BALB/c 3T3 cells, M-MuLV-infected BALB/c 3T3 cells (M-MuLV-BALB), or BALB/c 3T3 cells treated for 48 h before infection with a source of gamma interferon (ConA-BALB). Results from two separate experiments, using different stocks of VSV, are shown.

conditioned medium and, thus, induce MHC expression. Three sets of experiments indicated that endogenous interferon production does not occur and therefore does not mediate the viral effect on H-2. First, conditioned medium from M-MuLV-infected BALB/c 3T3 cells did not contain any soluble (nonprecipitable) activity capable of inducing class I MHC on other BALB/c 3T3 cells (see Fig. 1). Analysis of mRNA species from newly infected or chronically infected cells, using a murine beta-interferon-specific cDNA probe, showed no detectable mRNA for interferon (data not shown). To rule out the possibility that very small quantities of interferon were being elaborated by the M-MuLV-infected cells and were acting on the cells in an autocrine manner, we compared M-MuLV-infected cells with uninfected cells for the presence of an interferon-induced antiviral state. MuLV-infected cells were as susceptible to infection with VSV as were non-M-MuLV-infected BALB/c 3T3 cells (Table 1). Taken together, these data argue strongly that endogenous interferon production does not mediate the M-MuLV induction of class I MHC antigens.

Levels of H-2 class I- and β_2 -microglobulin-specific mRNAs. The levels of H-2 class I and β_2 -microglobulin-specific mRNAs increased after cells were infected with M-MuLV; this was the result of increased transcription. The levels of MHC class I transcripts and β_2 -microglobulin transcripts were examined in uninfected BALB/c 3T3 cells and compared with the levels found in cells chronically infected with M-MuLV or MSV/M-MuLV. The probe used to detect MHC mRNA was selected to hybridize with all three types of BALB/c class I transcripts (D^d , K^d , and L^d). M-MuLV-infected cells expressed four- to sixfold more H-2 transcripts than did uninfected cells (Fig. 2a). Furthermore, levels of both β_2 -microglobulin mRNA species were also elevated in M-MuLV-infected cells, by a factor of approximately 5 (Fig. 2b). These quantitations were normalized to the amount of actin-specific and tubulin-specific mRNA per lane. Steady-state actin or tubulin mRNA levels appeared to be unchanged between infected and uninfected cells, suggesting that M-MuLV infection does not increase the level of all transcripts in the cell. Coinfection of cells with MSV and M-MuLV resulted in little or no increase in MHC-specific or β_2 -microglobulin-specific transcripts, compared to uninfected cells. Multiple hybridizations from three different MSV/M-MuLV-infected cell lines showed H-2 or β_2 -microglobulin mRNA levels ranging from 0.9 to 1.3 times the levels in uninfected cells.

To determine whether these differences in mRNA levels reflected differences in transcription rates for H-2 and β_2 -microglobulin genes in chronically infected cells, nuclear runoff experiments were performed. Nuclei were isolated from uninfected, M-MuLV-infected, and MSV/M-MuLV-

FIG. 2. Levels of MHC class I or β_2 -microglobulin mRNA transcripts in cells in the presence or absence of retrovirus. Equal amounts of total cellular RNA were extracted from uninfected BALB/c 3T3 cells (BALB), M-MuLV-infected cells (MuLV), or MSV/M-MuLV-infected cells (MSV/MuLV), separated on a 1.4% agarose gel, transferred to nitrocellulose, and hybridized to labeled probes specific for H-2 (a) and, after stripping of probe, for β_2 -microglobulin (b). The estimated molecular sizes of the hybridizing mRNA species are approximately 1.8 kb for panel a and 0.7 to 0.9 kb for panel b. Results of a 9-h autoradiographic exposure are shown here. (c and d) Autoradiograms of the same blot rehybridized sequentially to probes specific for β -actin (c) or β -tubulin (d), showing hybridizing transcripts of 2.0 and 1.8 kb, respectively.

infected cells. Transcription was then allowed to proceed in the presence of labeled triphosphates, and the H-2-specific, β_2 -microglobulin-specific, and actin-specific transcripts were then quantitated after exhaustive hybridization to homologous sequences. The results shown in Fig. 3 are representative of four separate nuclear runoff experiments. After quantitation of hybridized radioactivity and normalization relative to the actin transcripts, it was found that transcription of H-2 genes was increased 3.2-fold (range, 3.0 to 4.2) in M-MuLV-infected versus uninfected cells, and β_2 -microglobulin transcription was increased 1.5-fold (range, 1.4 to 2.0). In separate nuclear runoff experiments, we observed no

FIG. 3. Nuclear runoff analysis of class I MHC and β_2 -microglobulin transcription in retrovirus-infected cells. Strips of nitrocellulose, onto which DNA sequences specific for class I MHC (H-2), β_2 -microglobulin (β_2), actin, and plasmid (pBR) had been immobilized, were hybridized with 32 P-labeled nuclear transcripts from uninfected BALB/c 3T3 cells (BALB), M-MuLV-infected cells (MuLV), or MSV/M-MuLV-infected cells (MSV/MuLV). The results of a 24-h autoradiograph exposure from one such experiment are shown here.

FIG. 4. Effect of M-MuLV infection on cell surface expression of endogenous and transfected class I MHC antigens. BALB/c 3T3 cells transfected with, and expressing, the $H-2K^b$ gene were chronically infected with M-MuLV, and the levels of endogenous H-2 expression (H-2K^d, panel A) and transfected H-2 expression (H-2K^b, panel B) were examined by fluorescence-activated cell sorter analysis. Results are shown for one representative transfected clone (pK^b-2). Profiles marked Uninfected and Infected represent pK^b-2 and M-MuLV-infected pK^b-2, respectively. Profiles marked Treated were obtained from pK^b-2 exposed to 2.5% conditioned medium from ConA-stimulated rat splenocytes as a source of gamma interferon.

differences in the transcription of the *c-myc* or β -tubulin genes between uninfected or M-MuLV-infected cells, arguing against a general increase in gene transcription as a result of M-MuLV infection. In the cells coinfecting with MSV/M-MuLV, transcription of the class I MHC genes and β_2 -microglobulin genes was essentially identical to that found in uninfected cells (range, 1.0 to 1.2 and 1.0 to 1.3, respectively).

Thus, the increased levels of H-2 and β_2 -microglobulin mRNAs correlate roughly with the increases in transcription of the respective genes observed in M-MuLV-infected cells. The higher cell surface expression of class I MHC antigens after M-MuLV infection may be therefore chiefly attributable to effects at the transcriptional level.

H-2 class I genes introduced into cells by DNA-mediated gene transfer are inducible by M-MuLV. Retroviruses contain strong enhancer elements and can influence the transcription of genes in the vicinity of their integration (a *cis* effect). It is unlikely, however, that integration of M-MuLV in the vicinity of the murine MHC locus would up-regulate all three class I MHC genes (K^d , D^d , and L^d), which span a region of 100 kb on the genome, and, in addition, affect transcription of the β_2 -microglobulin gene, which is on a separate chromosome. To determine whether M-MuLV can affect transcription of exogenously introduced class I MHC genes, a K^b genomic clone, containing 4 kb of 5' upstream sequences, was transfected into BALB/c 3T3 cells. Clones expressing K^b antigen at the cell surface were identified. Treatment of such clones with a source of gamma interferon resulted in an increase in both endogenous (K^d) and exogenous (K^b) H-2 antigen expression (Fig. 4). Infection of these clones with M-MuLV similarly resulted in increased levels

of both the endogenous and transfected MHC antigens. Up-regulation of the newly introduced H-2 antigens was observed after M-MuLV infection in five of six independent K^b transfectants.

Integration of transfected genes occurs in an apparently random fashion, making it likely that the M-MuLV exerts control of MHC genes via a *trans* effect rather than due to proximity. It is, however, difficult to determine where in the genome of the clones the newly transfected K^b sequences have integrated, because of the large number of class I-related genomic restriction fragments which hybridize to a K^b-specific probe on a Southern blot. To eliminate this problem, we next demonstrated that M-MuLV can affect transcription of class I MHC genes in transient expression assays, during which genomic integration does not occur.

Upstream sequences of the $H-2K^b$ gene are susceptible to the M-MuLV *trans* effect. To determine whether sequences upstream from the H-2 class I protein-coding sequences contain a control region which responds to M-MuLV infection (or to a cellular product stimulated by M-MuLV), we subcloned two 5' fragments of the cloned $H-2K^b$ gene and inserted them into the plasmid vector pSVOCAT (22). The pSVOCAT vector (4.7 kb) contains the gene encoding the bacterial enzyme CAT as well as the simian virus 40 poly(A) addition site, the pBR322 origin of replication, and the pBR322 ampicillin resistance gene. It is a derivative of pSV2CAT, in which the simian virus 40 early promoter has been deleted for the accommodation of other promoter sequences. After transfection of the CAT vectors into cells, enzymatic activity can be measured as a quantitative assay of promoter function.

The K^b upstream fragments subcloned were the 2.1-kb *HindIII-NruI* fragment and the 1.2-kb *PvuII-NruI* fragment (Fig. 5). The *NruI* site was selected because *NruI* cleaves only once in the $H-2K^b$ 5' region, its recognition sequence lies 19 base pairs (bp) upstream from the ATG codon of the exon encoding the $H-2K^b$ leader peptide, and the essential sequences for effective promoter activity (i.e., TATA box, CCAAT box, cap site) are preserved by cutting at this site. After isolation by standard techniques, the *HindIII-NruI* fragment was filled in with the Klenow fragment of *E. coli* DNA polymerase I and ligated to *HindIII* oligonucleotide linkers. The *PvuII-NruI* fragment was directly ligated to *HindIII* linkers. The fragments were inserted into the pSVOCAT plasmid previously opened up with *HindIII*, which cleaves 29 bp 5' to the CAT-coding sequences.

The two chimeric K^b-CAT vectors, pK^bH3NruCAT and pK^bP2NruCAT, are shown in Fig. 5. These constructions were tested for activity by transfection into cell lines in which the $H-2K^b$ promoter should be active (BALB/c 3T3 and NIH 3T3). Extracts of these cells contained measurable CAT activity after transfection with the vectors containing the 5' K^b fragments in the correct orientation. When the $H-2K^b$ fragments were inserted into pSVOCAT in the opposite orientation, and transfected into the same cell lines, no CAT activity was detected (data not shown). Therefore, the $H-2K^b$ sequences that have been subcloned contain an active promoter capable of driving transcription of the CAT gene in mouse cells.

To determine whether the pH-2K^bCAT vectors contain sequences which are acted upon by M-MuLV, uninfected and M-MuLV-infected BALB/c 3T3 cells were transfected with the vectors. Forty-eight hours after transfection, cell extracts were prepared and assayed for CAT activity. The results of this transient assay for CAT expression are shown in Fig. 6 and Table 2. The level of CAT expression in

FIG. 5. Construction of pK^bH3NruCAT and pK^bP2NruCAT. The restriction map of the *H-2K^b* gene showing the location of the promoter, exons 1 through 8, and the 3' untranslated region has been adapted from Allen et al. (1). Restriction sites: E, *EcoRI*; B, *BamHI*; H3, *HindIII*; N, *NruI*; P2, *PvuII*; X, *XbaI*. The *NruI* site is located 19 bp upstream from the ATG of the *H-2K^b* exon 1 (leader sequence). The 2.1-kb *HindIII-NruI* fragment and the 1.2-kb *PvuII-NruI* fragment, both from the *H-2K^b* 5' region, were ligated to *HindIII* oligonucleotide linkers and cloned into the *HindIII* site of the pSVOCAT vector (4.7 kb). The positions of the pBR322 origin of replication (ori) and ampicillin resistance gene (Amp^r); simian virus 40 (SV40) addition site; and *E. coli* Tn9 CAT 5' untranslated region, coding sequence, and 3' untranslated region are depicted. The modified plasmids were banded in cesium chloride-ethidium bromide gradients before transfection.

M-MuLV-infected BALB/c 3T3 cells transfected with pK^bH3NruCAT is 4.5-fold higher than that in uninfected BALB/c 3T3 cells transfected with the same vector. Normalization of these data to those obtained when the control plasmid, pSV2CAT, was introduced into the cell lines did not alter the results. To provide an additional internal control, the transient expression assays using pK^bH3NruCAT were repeated with cotransfection of a plasmid containing the cDNA for human growth hormone under the control of a thymidine kinase promoter (plasmid pTKGH; 36). Conditioned media from the transfected cells were collected at the time that cell lysates were prepared for measurement of CAT activity, and the media were assayed for growth hormone by a radioimmunoassay. Again, normalization for expression of this cotransfected reporter gene still showed a relative four- to fivefold increase of expression from the *H-2*-driven CAT gene in M-MuLV-infected cells. Negligible CAT activity was observed when the promoterless pSVOCAT was used as the vector in either cell line. The relative increase in CAT expression in M-MuLV-infected cells compared with uninfected cells did not vary with time of incubation or with the amount of extract used in the incubation (data not shown). The magnitude of the increase in CAT expression driven from the *H-2K^b* promoter in M-MuLV-infected 3T3 cells is in the same range as the increase in steady-state class I mRNA levels shown in Fig. 2. When the second vector, pK^bP2NruCAT, was transfected

into the uninfected and infected cell lines, the difference in CAT expression was 2.3-fold (Fig. 6 and Table 2). These results suggest that the M-MuLV effect on *H-2* expression may be mediated through an interaction somewhere within the 2,100 bp upstream from the coding sequence for the class I proteins. Shortening that sequence to 1,200 bp decreases the magnitude of the observed effect, but does not eliminate it.

A comparison of CAT activity in extracts of MSV/M-MuLV-infected cells was also made (Fig. 6 and Table 2). These cells exhibited a 4.9-fold increase in CAT activity over 3T3 cells when transfected with pK^bH3NruCAT and a 1.8-fold increase over 3T3 cells when transfected with pK^bP2NruCAT. Therefore, the MSV/M-MuLV-infected cells, in which there is little or no increase in cell surface levels of *H-2* class I antigens and no increase in steady-state transcriptional rates of endogenous *H-2*-specific mRNA, are not inhibited in CAT expression driven from the 2.1 kb of *H-2K^b* 5' flanking sequences. Additional upstream sequences not included in the CAT constructions may be required to provide a target site for M-MSV to have its effect, or posttranscriptional mechanisms may also be involved in the control of class I MHC levels in the setting of a sarcoma virus infection.

To examine whether the CAT expression of cell lines in which the *H-2K^b*CAT vector was integrated into the genome could be affected by M-MuLV, BALB/c 3T3 cells were

FIG. 6. Effect of viral infection on the level of CAT expression directed by pK^bH3NruCAT and pK^bP2NruCAT. Uninfected (lane 1), M-MuLV-infected (lane 2), and MSV/M-MuLV-infected (lane 3) BALB/c 3T3 cells were transfected with calcium phosphate precipitates including 10 μ g of pK^bH3NruCAT (A) or pK^bP2NruCAT (B). Cell extracts were prepared 48 h posttransfection and assayed by incubating 500 μ g of extract with 1 μ Ci of [¹⁴C]chloramphenicol for 2 h at 37°C. The reaction products were separated by thin-layer chromatography. The autoradiograph of the thin-layer chromatography plate is shown with the positions of the unacetylated chloramphenicol (CAM) and acetylated reaction products (1-acetyl CAM and 3-acetyl CAM) indicated. Spots were cut from the plate and counted by liquid scintillation counting to determine the percent conversion of chloramphenicol to its acetylated derivatives (see text and Table 2).

cotransfected with pK^bH3NruCAT and pMSVneo (at a 20:1 ratio of pH-2K^bCAT to pMSVneo). Transfectants resistant to G418 were cloned, expanded, and assayed for CAT activity. Approximately 60% of clones assayed did express CAT, presumably driven by the *H-2K^b* promoter. We were never able to obtain CAT-expressing clones after transfection of BALB/c cells with the promoterless vector

TABLE 2. Levels of CAT enzyme activity during transient expression assays

Cell line ^a	Transfection with DNA species: ^b	% Chloramphenicol conversion ^c	Relative change ^d
3T3	pK ^b H3NruCAT	0.15	
M-MuLV-3T3		0.68	4.5
MSV-3T3		0.74	4.9
3T3	pK ^b P2NruCAT	0.16	
M-MuLV-3T3		0.37	2.3
MSV-3T3		0.29	1.8
K ^b 15.7B	Mock	0.32	
	pMoV9	2.2	6.9
	+IFN ^e	2.3	7.2
K ^b 15.10B	Mock	0.31	
	pMoV9	0.99	3.2
	+IFN ^e	0.62	2.0

^a 3T3 represents BALB/c 3T3 cells, either uninfected or infected with M-MuLV (M-MuLV-3T3) or MSV/M-MuLV (MSV-3T3). See text for description of cell lines K^b15.7B and K^b15.10B.

^b See text for description of plasmids pK^bH3NruCAT, pK^bP2NruCAT, and pMoV-9.

^c Relative amount of chloramphenicol converted to acetylated form, expressed as a percentage of total.

^d Ratio of chloramphenicol acetylated under various conditions, compared with the control for each group.

^e +IFN, A 48-h treatment with 2.5% ConA supernatant as a source of gamma interferon.

FIG. 7. A molecular clone of M-MuLV elevates CAT expression in pK^bH3NruCAT-transfected cell lines. The cloned cell lines, K^b15.7B and K^b15.10B, were established by cotransfecting BALB/c 3T3 cells with the plasmids pK^bH3NruCAT and pMSVneo, selecting for resistance to G418, and assaying for stable, uninduced CAT expression. Samples of 10⁶ CAT-expressing cells were plated on 100-mm dishes in Dulbecco modified Eagle medium–10% fetal bovine serum 18 h before treatment. O, Mock transfection; IFN, cells grown in 2.5% ConA supernatant as a source of gamma interferon; pMoV-9, transfection of cells with a calcium phosphate precipitate containing 20 μ g of cloned M-MuLV proviral DNA, followed by glycerol shock. Forty-eight hours after treatment, cell extracts were prepared and CAT activity was determined as described for Fig. 6.

pSVOCAT and pMSVneo, using the same ratio of the two (20:1) in the calcium phosphate precipitate.

The effect of M-MuLV on the level of CAT expression in these lines was determined by transfecting them with the cloned, biologically active provirus, pMoV-9 (7). The cell lines were also grown separately in the presence of a source of gamma interferon to determine whether the integrated *H-2K^b*-CAT sequences were responsive to a known activator of *H-2* class I gene expression. In each line tested, including the two representative CAT-expressing lines shown in Fig. 7 and Table 2, CAT activity increased after transfection with pMoV-9, as well as after exposure to supernatant containing gamma interferon. The difference ranged from two- to sevenfold with pMoV-9 or with interferon, depending on the cell line examined (Table 2). Control cell lines, which were G418 resistant but not CAT expressing, did not exhibit CAT activity after either pMoV-9 or gamma interferon treatment (data not shown).

These results show that the *H-2K^b* promoter in the CAT constructions is capable of similar function and similar response to modulators, whether present as an extrachromosomal element in the transient assays or stably integrated into genomic DNA of the transfected cells.

DISCUSSION

Regulatory effects on MHC genes have been observed in multiple cell types including chemically transformed cells, radiation-induced leukemia cells, and spontaneously occurring leukemia cells in high-incidence strains (reviewed in reference 21). Attempts have been made to correlate alterations in MHC antigen levels in tumors with changes in the capacity of the immune system to eliminate their metastatic potential (15). This report describes our efforts to understand the mechanisms by which the exogenous leukemia-inducing virus, M-MuLV, and the acute-transforming sarcoma virus, M-MSV, modulate the levels of class I *H-2* antigen expression in the cells they infect.

We have shown that soon after establishment of M-MuLV infection, cells in culture increase their surface levels of H-2 class I proteins (18). It is highly unlikely that the increase is due to de novo expression of previously unexpressed class I genes or to recombinational events leading to the formation of novel class I-like proteins, since increased levels of all three of the class I proteins (H-2K, H-2D, H-2L) are detected with specific monoclonal antibodies. Further evidence of the ability of M-MuLV to alter the expression of normal H-2 proteins is that the biological function of the proteins (the ability to serve as recognition structures for allospecific CTLs) is not only still intact, but strongly enhanced (18). This increase in class I expression in MuLV-infected is specific in that other MHC genes, including the class II (*Ia*) genes, are not induced (unpublished data).

There are increased steady-state levels of H-2 and β_2 -microglobulin mRNA transcripts in cells after infection with M-MuLV, and this is paralleled by an increase in specific gene transcription as assessed by nuclear runoff assay. (Various studies have indicated that relative levels of gene transcription measured by such assays correspond to transcriptional rates in vivo [10]). Therefore, the increased level of H-2 protein expression in M-MuLV-infected cells is most likely the result of an increase in the rate of transcription of the H-2 class I genes, as well as the gene encoding β_2 -microglobulin. The specificity of the control of H-2 and β_2 -microglobulin genes by MuLV is demonstrated by the lack of any observed effect by MuLV infection on the steady-state mRNA levels or transcriptional rates of other genes such as those for actin and tubulin and *c-myc*.

Having observed similar up-regulation of three class I genes, which are widely separated within the mouse MHC locus (1), and of the β_2 -microglobulin gene, which is located on a separate chromosome, we wished to test whether the M-MuLV transcriptional activator functions *in trans* and to rule out the possibility that the virus alters H-2 expression by placing viral promoters, enhancers, or both in proximity to the affected genes through proviral integration into appropriate areas of the host cell genome. While all cells in a M-MuLV-infected population displayed enhanced surface levels of H-2, an analysis of the genomes of these cells revealed no common M-MuLV integration sites within the population. Furthermore, we could detect no rearrangements or disruptions of the H-2 complex gene structure in M-MuLV-infected cells (unpublished data). Transfection studies with complete cloned H-2 genes showed that M-MuLV up-regulates both endogenous and transfected genes after infection. The incorporation of exogenous genes into the cellular genome after DNA-mediated gene transfer is known to be a random process (26). It is, therefore, improbable that the retrovirus is capable of integrating in the vicinity of all transfected H-2 genes in the cloned and bulk cell populations examined, modulating their expression *in cis*.

More compelling evidence for a *trans* activity of M-MuLV on class I H-2 genes, as well as some information relating to the H-2 sequences upon which it exerts its effect, were obtained in experiments utilizing H-2-CAT hybrid genes in transient expression assays of infected and uninfected cells. The time frame of these assays allowed for only minimal, if any, association of the transfected CAT vectors with the cellular genome. Therefore, most of the CAT mRNA was derived from transcription of the vectors as extrachromosomal elements. The expression of CAT directed by 1.2 kb of H-2K^b upstream sequences mimicked expression from the whole H-2K^b gene when transfected into M-MuLV-infected cells. Individual clones of CAT-expressing cell lines

generated through random integration of the H-2K^b-CAT vector also were stimulated to increase their basal level of CAT expression after exposure to the virus.

Other retroviruses may have different modes of action in affecting MHC gene expression. In their study of radiation leukemia virus (RadLV), another MuLV that modifies H-2 expression, Merulo et al. (31) obtained evidence for altered methylation and structural rearrangements of the H-2 complex in RadLV-induced tumors, presumably because of integration of viral genomes. Like M-MuLV, RadLV enhances H-2 expression in cells soon after infection (6, 32). However, transformed cells which arise after a long latent period in RadLV-infected animals show very little, if any, H-2 expression (31). Therefore, different retroviruses having the same initial effects on H-2 genes may operate through very distinct mechanisms. Merulo et al. also stated that certain other tumorigenic viruses tested did not lead to rearranged H-2 genes after infection.

Endogenous cellular beta interferon can be elaborated by cells in response to viral infection or to double-stranded polyribonucleotides. Interferons are capable of inducing class I MHC expression on mesenchymal cells (see Fig. 1). Although the response of cells to infection with M-MuLV is similar to the response of the same cells to interferon treatment, we have ruled out the involvement of interferon as a mediator in the augmentation of H-2 expression in this system. The infected cells do not produce detectable levels of interferon, or any other H-2-enhancing soluble factor, express no detectable mRNA for beta interferon, and demonstrate no interferon-induced antiviral state. We have previously shown that interferon treatment of M-MuLV-infected cells has an additive effect on the H-2 level, leading us to believe that the site of action of interferon differs from that of the virus (18). However, it is possible that very similar molecular mechanisms are employed by the virus and by interferon to achieve the same final effect.

Interferon treatment of fibroblasts and lymphoid cells leads to an increase in MHC mRNA levels (14). Friedman and Stark have identified a consensus sequence upstream of the protein-coding regions of certain interferon-regulated genes (19). Their analysis revealed a region of homology, indicating a possible target site for interferon (interferon response sequence) around 142 or 147 bp upstream of the TATA box of human MHC class I genes. An interferon response sequence has also been identified at positions -137 to -165 bp relative to the CAP site in H-2K^b, H-2L^d, and H-2K^k (25). Upon examination of the relationship between upstream sequences of H-2K^b and interferon induction in a transient assay, Israel et al. (24) concluded that an enhancer sequence, with which the interferon response sequence overlaps, is required. We have shown that the sequences included in the H-2K^b-CAT vectors enable our long-term transfected lines to increase their level of CAT expression after exposure to interferon. Thus, the interferon target sequences, as well as a presumptive distinct target sequence(s) for M-MuLV control, are within the 1.2 kb of upstream H-2K^b DNA. Fine mapping of the H-2K^b virus-control target sequence and an examination of sequences upstream of other class I genes and the β_2 -microglobulin gene are under way.

At this time it is not known whether M-MuLV itself, or a cellular product activated by the virus, is responsible for the changes in H-2 expression. Some retroviruses are known to carry the information for transcriptional activators within their genomes (35, 38). Broome and Gilbert (5) have determined that Rous sarcoma virus encodes a transactivator for

its own promoter, as well as for the nonviral preproinsulin II gene. The expression of a reading frame within the Rous sarcoma virus *gag* gene, other than the one responsible for the expression of the precursor protein for the viral group-specific antigens, most likely gives rise to the transactivator protein. Studies designed to map the location of the viral gene responsible for the activation of H-2 by M-MuLV are in progress.

The infection of cells with M-MuLV leads to increased levels of H-2 proteins at the cell surface, but infection with virus stocks which include both M-MuLV and MSV does not lead to the same effect (18). Because of this, MSV-infected cells are able to elude CTLs in vitro (18) and may be able to escape immune detection in vivo. Although the cell surface level of gp70, a product of the M-MuLV *env* gene, in the coinfecting cells is equivalent to that in the M-MuLV-infected cells, the induction of H-2 mRNA in the coinfecting and transformed cells has been inhibited. However, transfection of the *H-2K^b-CAT* vectors into MSV/MuLV-infected cells results in increased CAT expression as compared to transfection of the vector into uninfected cells. One explanation for this discrepancy may be that not enough of the *H-2K^b* upstream sequence is included in the CAT vectors for M-MSV to exert the inhibitory effect on transcription.

The whole-gene studies (steady-state mRNA analyses, nuclear runoffs) may reflect the true control by M-MSV because the *H-2K^b* genetic information is intact. This would predict a model whereby M-MSV would have a direct effect on *H-2* upstream sequences, rather than on an M-MuLV product. Evidence does exist for the regulation of eucaryotic gene expression by viral gene products through repression of enhancer-induced stimulation of transcription (4). It is possible that a target sequence for such a repressor encoded or induced by M-MSV might be located more than 2.1 kb upstream of the *H-2K^b* protein-coding sequences (although at this time we also cannot rule out the importance of the protein-coding sequences of the *H-2K^b* gene; these coding sequences are missing in the CAT construction, and may possibly represent other sites upon which M-MSV could have an effect.

Recently, Vogel et al. (43) have shown that the transient expression of a CAT gene directed by 1.9 kb of the 5' flanking region of the *H-2L^d* gene is not down-regulated in adenovirus type 12-transformed cells, even though the level of transcription of endogenous *H-2* genes in these cells is only 5% of the normal cell level. It is possible that the data from this system are analogous to our own data relating to the M-MSV effect on transient expression of a CAT gene directed by 2.1 kb of *H-2K^b* 5' flanking region. In each case, a viral *trans* regulation with target sequences over 2 kb away from the transcriptional start site may be involved in down-regulating gene expression. A second difference in the two types of gene control studies we describe is that the CAT assay is a measure of transient expression from DNA in an extrachromosomal state. M-MSV may only have the ability to repress the induction of gene expression by M-MuLV when the *H-2* sequences are located within the cellular genome. A third possible explanation for the discrepancy might be the existence of posttranscription events in the MSV-infected cells which act to decrease the half-life of H-2 and β_2 -microglobulin messenger species measured by both nuclear runoff and steady-state mRNA analysis. The hybrid *H-2-CAT* transcripts generated in the transfection experiments might be less susceptible to such posttranscriptional control.

It is logical that M-MSV has evolved a way to avoid

immune detection and elimination of the cells it infects by inhibiting the enhanced production of H-2 class I proteins to a level that is optimal for CTL recognition (18). However, why would M-MuLV encode an activity that is designed to enhance the level of these same proteins? M-MuLV and certain other murine retroviruses, such as RadLV, promote the induction of leukemia in infected mice after a long latent period through a complex multistep process involving the generation of novel recombinant retroviruses (42). After infection by these thymotropic retroviruses, and during the preleukemic phase, the major cell population in the thymus bears high levels of H-2 antigens and viral gene products (6, 32). It has been proposed that the increased levels of these cells surface proteins may help bring about the chronic immunostimulation which appears to be necessary during this phase, either to provide the recombinant leukemogenic viruses with a proliferating target cell population (27, 29) or, perhaps, to enhance autostimulation of MuLV-infected, MuLV-specific T lymphocytes, producing a preneoplastic lymphoid hyperplasia. Thus, the ability of M-MuLV to enhance H-2 expression in its target cells may be intrinsic to its ability to eventually generate lymphoid neoplasia, while the acutely transforming sarcoma viruses have evolved a mechanism to inhibit this immune antigen induction in the cells they transform, thereby evading immune surveillance and destruction.

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