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Immunology

Lack of class I H-2 antigens in cells transformed by radiation leukemia virus is associated with methylation and rearrangement of H-2 DNA

(virally induced loss of expression of major histocompatibility complex antigen/transformation)

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ABSTRACT **Transformation of murine thymocytes by radiation leukemia virus is associated with reduced expression of the class I antigens encoded in the major histocompatibility complex (MHC) and increased methylation and altered restriction enzyme patterns of MHC DNA. These changes may play a role in host susceptibility to virus-induced leukemogenesis and accord with the notion that viral genomes play a regulatory function when they integrate adjacent to histocompatibility genes.**

Associations between malignant disease and the murine major histocompatibility complex, MHC, have been extensively demonstrated (1), and various mechanisms have been proposed. In studies of MHC-linked resistance to neoplasia induced by radiation leukemia virus (RadLV) (2–5), we found dramatic alterations in the quantitative expression of some cell surface class I MHC antigens associated with infection and transformation of thymocytes. Several prior findings have suggested that these changes in H-2 antigen expression result from direct effects of RadLV on H-2 transcription (5, 6). In addition, as shown below, levels of class I mRNA are considerably reduced in cells transformed by RadLV, and transformation by RadLV is associated with methylation and rearrangements of MHC DNA.

MATERIALS AND METHODS

Mice. Mice used were bred at the New York University Medical Center, purchased from The Jackson Laboratory, or obtained from Chella David's colony at the Mayo Clinic.

Cell Cultures. *In vitro* adapted tumors of various inbred mouse strains were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution, and 0.1% Fungizone.

Hybridization Probes. Probes used in this study have been previously described by others: pH-2II (7), *Thy-1* (8), pγ2b (9), $p\beta_2$-microglobulin (10), and β-actin probe (11).

DNA Procedures. DNAs were isolated by the procedure of Blin and Stafford (12) and concentrations were determined spectrophotometrically by the diphenylamine method. Fifteen micrograms of DNA was digested with 60 units of enzyme in the buffer recommended by New England Biolabs, containing bovine serum albumin at 100 μg/ml. Incubations were carried out in 50 μl at 37°C for 16 hr to ensure completeness of digestion. This was monitored by withdrawing 10 μl of the 50-μl reaction mixture 2 min after starting and placing it in a tube containing 2 μl of λ phage DNA (0.75 ng/μl) or adenovirus DNA (0.75 ng/μl), ^{32}P-labeled by nick-translation to low specific activity (≈10^7 cpm/μg). At the end of digestion the reactions were stopped by addition of 12.5 μl of bromophenol blue loading buffer containing 20% Ficoll, 1 mM disodium ethylenediaminetetraacetate (EDTA), and 0.2% bromophenol blue. Results were confirmed in several independent experiments. DNAs were electrophoresed in 0.8% agarose gels (Bethesda Research Laboratories) overnight and transferred to nitrocellulose (Schleicher & Schuell BA85) by the method of Southern (13). The 12-μl samples (see above) were loaded into another agarose gel, electrophoresed, dried, and autoradiographed to monitor completeness of digestion.

mRNA Isolation. mRNA was isolated out by the procedure of Feramisco *et al.* (14) with a slight modification. About 1.0 × 10^8 cells were homogenized in 10 ml of 4 M guanidine isothiocyanate/140 mM 2-mercaptoethanol/2.0% Sarkosyl/50 mM Tris·HCl, pH 7.6/10 mM EDTA. Samples were extracted with phenol several times at 60°C, once with an equal volume of phenol/chloroform containing 4% (vol/vol) isoamyl alcohol, and finally with chloroform and 4% isoamyl alcohol; all were carried out in 0.1 M sodium acetate (pH 5.9). The nucleic acids were precipitated with 2.5 times the sample volume of ethanol (EtOH) overnight at −20°C. Nucleic acids were pelleted at 15,000 × *g* for 30 min, dried in a desiccator, and resuspended with 3 ml of 10 mM Tris·HCl, pH 7.5/2 mM EDTA/0.2% $NaDodSO_4$. Samples were incubated 5 min at 65°C to unfold secondary RNA structure. After 8 min of incubation on ice, NaCl was added to a 400 mM final concentration. The samples were placed over an oligo(dT)-cellulose (Calbiochem) column to bind mRNA (15, 16). mRNA was eluted from the column with 10 mM Tris·HCl/2 mM EDTA/0.2% $NaDodSO_4$ heated to 45°C. NaCl was added to the mRNA sample to 400 mM final concentration and then the mRNA was precipitated as above with EtOH and stored at −70°C until needed for electrophoresis.

mRNA Electrophoresis. Samples (10 μg) were resuspended with 4.5 μl of water/10 μl of 99% (vol/vol) formamide/3.5 μl of 37% (vol/vol) formaldehyde/2 μl of 10× buffer [10× buffer = 200 mM 3(*N*-morpholino)propanesulfonic acid (Mops)/50 mM sodium acetate/10 mM EDTA], heated at 65°C for 10 min, and placed on ice for 5 min. Five microliters of bromophenol blue loading buffer was added, and the mRNAs were loaded into a 1% agarose gel containing formaldehyde

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Abbreviations: MHC, major histocompatibility complex; RadLV, radiation leukemia virus; kb, kilobase(s); RFLP, restriction fragment length polymorphism.

and electrophoresed overnight at 15 mA. Gels were blotted onto nitrocellulose, rinsed in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate), and baked 5 hr at 80°C under reduced pressure.

Hybridization Procedures (for Both DNA and RNA). Only stringent conditions were used. After baking the filters were placed in 100 ml per filter of blocking solution consisting of 6× SSC and Denhardt's solution (0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone), prewarmed to 65°C. After incubation for 5 hr in a waterbath at 65°C with shaking, the filters were removed, air dried, placed in a heat-sealable plastic bag with hybridization solution consisting of 2× SSC, 0.01 M EDTA, ^{32}P-labeled denatured probe (labeled to at least 1.0×10^8 cpm/μg), and denatured calf thymus DNA at 5 μg/ml, and incubated overnight at 65°C. Nick-translations were performed with New England Nuclear Kit NEK 004B. The filters were then washed three times for 20 min in 2× SSC/0.5% $NaDodSO_4$/20 mM monobasic sodium phosphate/2.7 mM disodium pyrophosphate at 65°C, once in 1× SSC, then 0.4× SSC, and finally with 0.1× SSC until the Cerenkov radioactivity was below 100 cpm/5 ml. Filters containing mRNA were blocked with 0.1% Ficoll at 65°C for 1 hr and then incubated at 42°C in 50% (vol/vol) formamide/5× Denhardt's solution/50 mM sodium phosphate, pH 6.8/5 mM EDTA/0.1% $NaDodSO_4$/750 mM NaCl/5 mg of denatured calf thymus DNA per ml for 4 hr. Denatured probe, labeled to $\approx 10^8$ cpm/μg, was then added and incubated with the filter for ≈16 hr at 42°C. Filters were washed as above except that the first three 2× SSC washes were carried out at room temperature instead of 65°C.

RESULTS

RadLV Transformation Is Associated with a Decreased Expression of H-2 Class I mRNAs. Fig. 1 shows that class I mRNA is undetectable in RadLV tumors. This is consistent with our previous observations that transformation of thymocytes by RadLV leads to a loss of H-2 antigen expression as measured by two-dimensional gel electrophoresis, immunofluorescence, absorption analysis, and immunoprecipitation (2). This loss is particularly impressive in that the probe used in these experiments, pH-2II, is broadly cross-reactive, hybridizing with all *K, D, L, Qa*, and *TL* class I genes.

Changes in Methylation of MHC DNA Are Associated with RadLV-Induced Transformation of Murine Thymocytes. Fig. 2 *A* and *B* compares autoradiograms of DNAs from untreated mouse thymocytes and RadLV-induced, *in vitro* adapted tumors of various inbred strains after digestions with restriction enzymes *Msp* I and *Hpa* II followed by Southern gel electrophoresis and hybridization with a MHC DNA probe (7). *Msp* I and *Hpa* II both recognize the same sequence (CCGG), but digestion with *Hpa* II is inhibited when the second cytosine residue is methylated. The *Msp* I digests of RadLV tumor and normal thymic DNAs are identical, but the *Hpa* II patterns of DNA from RadLV-induced tumors are consistently different (in 100% of the more than 30 tumors examined) from those of normal thymus. For example, in the 3- to 10-kb region there are more restriction fragments visible in the lane that contains DNA from C57BL/6 RadLV-transformed cells than in the lane that contains DNA from normal cells (fragments 11–16 for RadLV and 7 and 8 for normal) (Fig. 2*A*). Several fragments (nos. 1–4) between 5 and 10 kb present in normal B10.G DNA are absent or altered in DNA from the RadLV-transformed counterparts (Fig. 2*A*). Since the only additional fragments seen in DNA from RadLV-transformed B10.G cells are 5 and 6, the missing fragments have probably not been cleaved from higher molecular weight fragments because hypermethylation inhibits digestion by *Hpa* II. In T7/T7 RadLV-transformed cells (Fig. 2*B*) numerous fragments present in the *Hpa* II pattern of normal cells (nos. 17–22) are not detectable, presumably for similar reasons. In B10.S transformed cells (Fig. 2*B*) fragments 24–31, present in the normal cells, are not detectable; new fragments (nos. 32–35) appear, having higher molecular weights than the normal fragments.

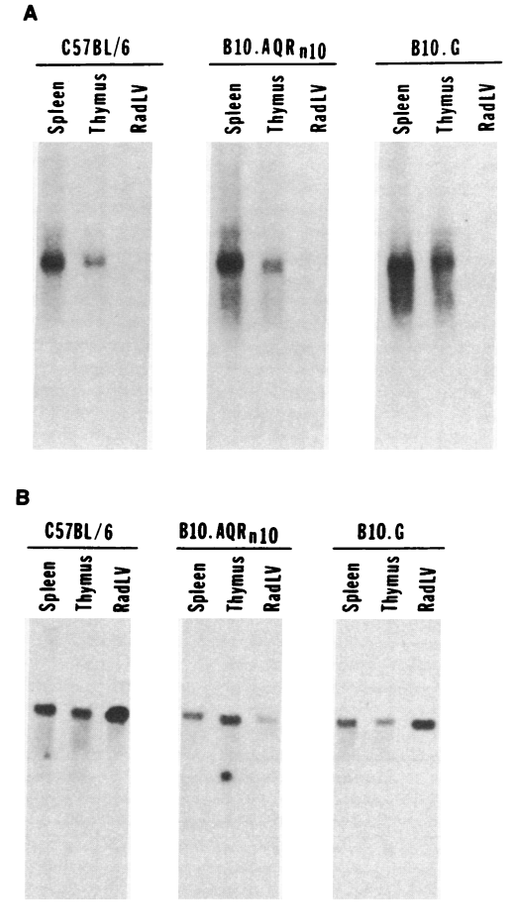

FIG. 1. Comparison of class I MHC mRNA levels in normal mouse thymocytes and splenocytes with those of RadLV-induced thymomas. (*A*) Expression of *H-2*, shown with the pH-2II probe, is observed in both normal cell types of each of the three mouse strains, while there is a considerable reduction of *H-2* in the corresponding thymoma. (*B*) Expression of β-actin mRNA is unaffected by RadLV transformation.

To confirm that the observed changes in *Hpa* II digestion patterns of *H-2* DNA seen in RadLV-transformed cells are related to transformation and not merely a result of adaptation to growth *in vitro*, several leukemic mice in which thymomas had been induced by RadLV were sacrificed, and their DNAs were prepared immediately. DNA from thymomas of these mice also revealed differences with their normal counterparts after *Hpa* II digestions (Fig. 2 *C* and *D*). For example, fragments 1–3 present in normal B10 cells are absent from tumor 50068 (Fig. 2*C*). In general the mean molecular weight of new fragments in the tumor cells (nos. 4–9) is higher than that of missing fragments in normal cells. In tumors 53101 and 56412 the pattern of *Hpa* II fragments differs significantly from that in the normal *Hpa* II digest (Fig. 2*D*). Again, the new *Hpa* II fragments in tumor DNA (nos. 12–16) are at higher molecular weights than those unique to normal DNA (nos. 10 and 11).

One result obtained in these studies is that every tumor appears to have unique alterations in methylation, even RadLV tumors derived from the same mouse strain (see Fig. 2 *C* and *D*). Since every tumor consistently gives the same

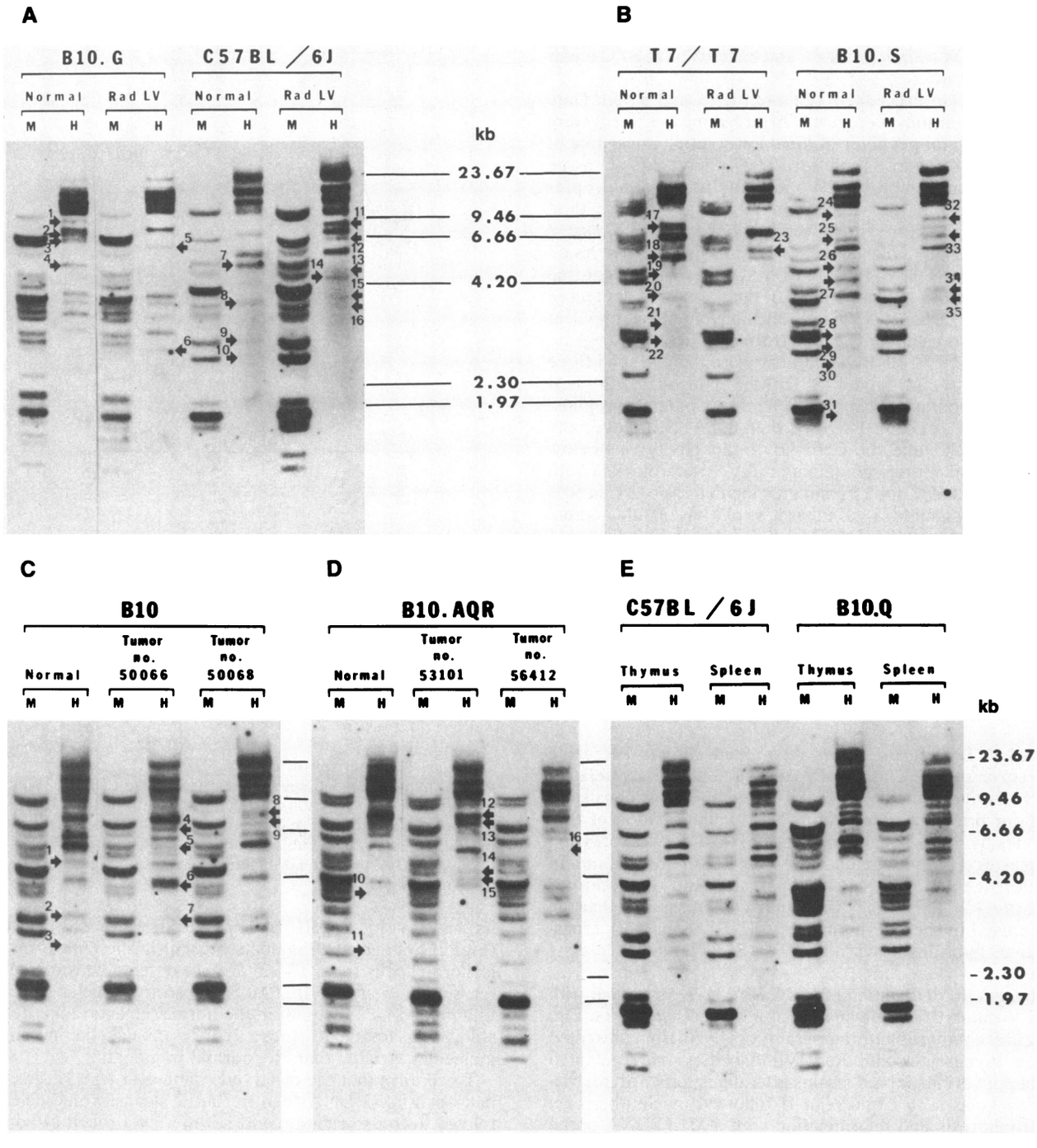

FIG. 2. (*A* and *B*) Comparison of *H-2* DNA fragments from normal thymocytes and RadLV-induced thymomas, adapted to *in vitro* growth (as discussed in ref. 4), after digestion with restriction endonucleases *Msp* I (M) or *Hpa* II (H). (*C* and *D*) Comparison of *H-2* DNA fragments from normal thymocytes and RadLV-induced thymomas removed immediately after sacrifice of moribund leukemic mice after digestion with restriction endonuclease *Msp* I and *Hpa* II. (*E*) Comparison of *H-2* DNA fragments from normal thymocytes and splenocytes of two inbred mouse strains after digestion with restriction endonucleases *Msp* I and *Hpa* II. Small numbers within the gel refer to individual restriction fragments. Numbers outside gels indicate molecular sizes of markers in kilobases (kb).

methylation pattern whenever it is assayed (in uncloned cell lines), this implies that alterations in methylation occur before the tumorigenic cells expand from a limited number of transformed cells. This notion would be consistent with a clonal origin for most RadLV tumors.

We sought to determine whether RadLV-associated changes in *H-2* DNA methylation are related to normal regulatory influences that affect *H-2* expression in various organs. On average, thymocytes express about one-fifth to one-tenth as much *H-2* antigens as splenocytes do (17), yet no differences between splenocytes and thymocytes are detected in the *Msp* I or *Hpa* II pattern of digestion (Fig. 2*E*). Thus methylation of *H-2* DNA is associated with RadLV transformation but not with normal regulatory influences affecting *H-2* expression in these cells.

An important question is whether the altered patterns of methylation associated with RadLV transformation are also found elsewhere (non-MHC) in the genome. We examined genes other than those coded by the MHC, such as *Thy-1*, *γ2b*, and β_2-microglobulin (*β2M*). DNA from at least 20

tumors derived *in vivo* and *in vitro* were analyzed with probes specific for these genes (8–10). No differences were observed between the *Msp* I or *Hpa* II digestion of *Thy-1* DNA from RadLV-transformed and uninfected/transformed thymocytes (data not shown). Similar results were obtained for β_2-microglobulin. In the case of *γ2b* no changes were observed except that DNA from one of several tumors examined appeared to be *less* methylated than normal DNA (data not shown).

Rearrangements of Class I DNA Regions Are Associated with RadLV Tumorigenesis. Rearrangements in MHC DNA also appear to accompany RadLV transformation as detected by digestion with restriction enzymes. Two examples of primary tumors in which restriction fragment length polymorphisms (RFLPs) are detected with class I probes are shown in Fig. 3 *A* and *B*. Novel fragments are not detected with probes for other genes such as *γ2b* and *β2M* (Fig. 3*A*). In the case of tumor 47589 (Fig. 3*B*) metastasis to the spleen had occurred and both thymus and spleen display the RFLPs (Fig. 3 *B* and *C*). While tumors with RFLPs are seen less frequently than tumors with changed DNA methylation patterns by using restriction enzymes (we have RFLPs in at least four additional *in vitro* adapted cell lines and one tumor from which DNA was made immediately after removal from the animal out of 25 tumors examined), it is difficult to ascertain the true incidence of tumors with novel RFLPs because of the multiplicity of fragments hybridizing with *H-2* probes. A displacement of a fragment to a different location in a gel may be masked by a fragment of the same size already present in normal DNA.

It was of interest to determine more precisely which class I genes were methylated and rearranged. While these studies are still preliminary and will be the subject of future publications, it is possible to conclude that alterations in methylation associated with RadLV appear to affect numerous class I genes, while RFLPs involve a more limited number of class I genes. We also find less extensive alterations in MHC DNA methylation, but no rearrangements, in some tumors induced by radiation and viruses other than RadLV (data not shown).

DISCUSSION

While the mechanisms of resistance to leukemogenesis are not known in many instances, host resistance to RadLV-induced neoplasia has been shown to rely on immune mechanisms responding to different quantitative levels of H-2 antigen expression (4). Since such expression might be markedly affected by both alterations in methylation and rearrangements of class I genes, these changes might play an important role in resistance to disease. Similar mechanisms

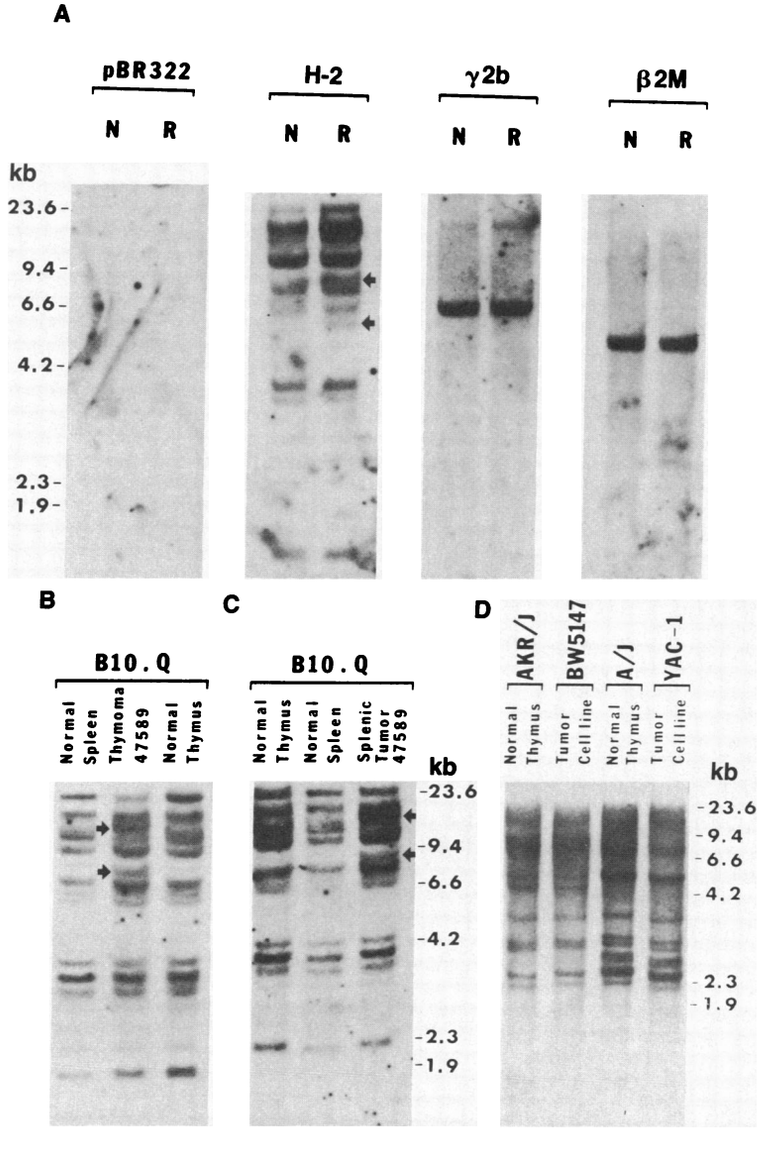

FIG. 3. Comparison of genomic DNA from normal thymocytes and RadLV-induced thymomas removed immediately after sacrifice of moribund leukemic mice. Thymoma DNA (tumor 50068) was digested with restriction endonuclease *Eco*RI. (*A*) Hybridization was done with ^{32}P-labeled pBR322, pH-2II (7), p*γ*2b (11), and a probe for *β2M* recognizing a segment of genomic DNA that encodes amino acids 3–95 of β_2-microglobulin and approximately 300 base pairs of intervening sequences (18). Novel RFLPs were observed only with the *H-2* probe and were confirmed in additional experiments. (Other novel RFLPs are shown in the next two panels.) N, normal thymocytes; R, RadLV-induced thymoma. (*B* and *C*). Comparison of *H-2* DNA fragments of normal thymocytes and splenocytes with those of thymocytes and splenocytes of RadLV-leukemic mouse 47589, in which thymoma cells had metastasized to the spleen. Digestion was with *Eco*RI. (*D*) Absence of class I-related, novel RFLPs in tumors induced by AKR virus (BW5147) or Moloney leukemia virus (YAC-1). DNA from these and other tumors (not shown) of non-RadLV origin were digested with a variety of restriction enzymes and hybridized with *H-2* probes. *Bam*HI digest is shown as an example.

may apply to other tumors. Taken together with our recent findings of integrated viral sequences adjacent to numerous histocompatibility genes (19, 20), the data in this paper are consistent with the possibility that type C RNA viruses may interact with histocompatibility genes to alter their expression in a physiologically important manner.

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