The Gene Coding for a Major Tumor Rejection Antigen of Tumor P815 Is Identical to the Normal Gene of Syngeneic DBA/2 Mice

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Summary

We showed previously that mouse mastocytoma P815 expresses several distinct antigens that are recognized by cytolytic T lymphocytes (CTL) of syngeneic DBA/2 mice. Antigens P815A and P815B are usually lost jointly and are targets for immune rejection responses in vivo. We used a cosmid library and a CTL stimulation assay to obtain transfectants expressing tumor rejection antigen P815A. From these transfectants we retrieved gene P1A which transferred the expression of both P815A and B. This gene is unrelated to three previously isolated genes coding for tum$^-$ antigens. It encodes a putative protein of 224 amino acids which contains two highly acidic domains showing homology with similar regions of nuclear proteins. The P1A gene expressed by tumor P815 is completely identical to the gene present in normal DBA/2 cells. Expression of the gene was tested by Northern blots. Cells from liver, spleen, and a number of mast cell lines were negative, but mast cell line L138.8A produced a high level of P1A message and was lysed by CTL directed against antigens P815A and B. We conclude that major tumor rejection antigens of P815 are encoded by a gene showing little or no expression in most normal cells of adult mice.

Mouse tumors express antigens that are targets of T cell mediated rejection responses in the syngeneic animals. The first evidence for the existence of these "tumor rejection antigens" was obtained with experimental tumors induced by chemical carcinogens such as methylcholanthrene (1, 2, 3). The antigens expressed by these tumors elicited immune rejection responses and were different for every tumor. They were named tumor-specific transplantation antigens (TSTA)[1] (4). Later, similar antigens were observed on tumors induced by ultraviolet radiation (5). Spontaneous tumors, on the other hand, are usually nonimmunogenic and were therefore thought to be devoid of tumor rejection antigens (6). However, immunogenic variants of spontaneous tumors were obtained by mutagenesis and these variants were shown to induce an immune protection against the original tumor (7). This indicated that spontaneous mouse tumors express tumor rejection antigens which can constitute targets for syngeneic rejection responses even though they are not immunogenic on their own. Similar results have been obtained recently by transfecting foreign genes into spontaneous tumors (8).

A number of tumor rejection antigens have been characterized in vitro with cytolytic T lymphocytes (CTL) derived from mice immunized with tumor cells and highly specific CTL clones have been obtained (9, 10, 11, 12, 13). However, the nature of these antigens has remained elusive because they do not elicit any antibody response, and could therefore not be isolated by immunoprecipitation. The same situation applies to other antigens recognized by CTL, such as minor histocompatibility antigens, male-specific H-Y antigens (14), and also tum$^-$ antigens, which are present on immunogenic variants obtained by mutagen treatment of mouse tumor cells (10).

We have recently cloned the genes coding for three tum$^-$ antigens expressed by tum$^-$ variants of mouse tumor P815 (15, 16, 17). The cloning procedure involved gene transfection and the detection of antigen-expressing transfectants by their ability to stimulate the appropriate CTL (18). The sequencing of the three genes revealed that they were completely unrelated and showed no homology with any gene previously recorded in the data banks. For these three genes, the allele borne by the tum$^-$ variants was found to differ from the normal allele by a point mutation located in a coding region (16, 17, 19). These mutations create new antigenic peptides that are presented to the syngeneic CTL by a class I molecule of the MHC.

[1] *Abbreviations used in this paper:* BrdU-bromodeoxyuridine; CTL, cytolytic T lymphocytes; LB, Luria-Bertani; tk, thymidine kinase; TSTA, tumor-specific transplantation antigens.

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We have extended our approach to tumor rejection antigens of mastocytoma P815, a tumor obtained in a DBA/2 mouse treated with methylcholanthrene. These antigens are recognized by CTL and are involved in the immune rejection response occurring in vivo (20). We report here the cloning and analysis of a gene coding for a tumor rejection antigen of P815.

Materials and Methods

Cell Lines. Mastocytoma P815 was obtained in a DBA/2 mouse that had been treated with methylcholanthrene. P1 is a clonal line isolated from a permanent cell line derived from the transplantable P815-X2 subline (21). The highly transfectable line P1.HTR.tk$^-$ (referred to as P1.HTR) was derived from P1 after repeated cycles of transfection and selection for thymidine kinase expression (tk$^+$) followed by reverse selection with bromodeoxyuridine (BrdU) (22). PO.HTR cells were derived from the P1.HTR variant by immunoselection with CTL clones as described (12). L fibroblastic cell line DAP-3 (H-2k) and transfected derivatives expressing H-2L^d and H2-D^d were a gift from K. Ozato (National Institutes of Health, Bethesda, MD) (23). We obtained the transfectants expressing K^d by transfecting DAP L cells with plasmid p191.1 (24).

The culture medium was DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% (v/v) FCS (Gibco Laboratories). P815 cells were cultured in tissue culture plasticware, and incubated in water-saturated air containing 8% CO_2 at 37°C. Medium for untransfected PO.HTR tk$^-$ was supplemented with BrdU (100 μg/ml) (Janssen Chemica, Beerse, Belgium) until 4 d before transfection to prevent appearance of tk$^+$ revertants. The L138.8A mast cell line was a gift from L. Hültner (Institut für Experimentelle Hämatologie, München, FRG) (25, 26), and was cultivated in RPMI medium supplemented with L-arginine (5.5×10^{-4} M), L-asparagine (2.4×10^{-4} M), L-glutamine (1.5×10^{-3} M), 2-ME (5×10^{-5} M), 10% FCS, and 10% IL-3–containing culture supernatant of WEHI-3B cells. Mast cell line MC/9 was obtained from the American Type Culture Collection (Rockville, MD) and cultivated in Iscove's modified DME supplemented with 500 U/ml of rIL-9 (a gift of J. Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium) and with the same additives as in the medium used for L138.8A.

CTL Clones. The derivation from DBA/2 mice of CTL clones specific for P815 tumor antigens A, B, C, and D was described previously (20). We maintained CTL clones in long-term culture by transferring every 3–4 d 5×10^4 CTL to 1 ml cultures containing 5×10^6 irradiated (30 Gy) DBA/2 feeder spleen cells and 10^5 tk$^-$ P1.HTR cells as stimulators in 24-well tissue culture plates. The culture medium was DME supplemented with L-arginine (5.5×10^{-4} M), L-asparagine (2.4×10^{-4} M), L-glutamine (1.5×10^{-3} M), glucose (3.5 g/l), HEPES (10^{-2} M), 2-ME (5×10^{-5} M), and 10% FCS and HAT (10^{-4} M hypoxanthine, 3.8×10^{-7} M aminopterin, and 1.6×10^{-5} M 2-deoxythymidine). In addition, cultures contained 50% of supernatant from secondary MLC (27) as a source of growth factor. For the stimulation assays, CTL cultures initiated 4 d earlier were divided into two parts and supplemented with an equal volume of fresh CTL culture medium with 50% MLC supernatant. No fresh feeder cells or stimulator cells were added. 2 d later, the CTL were harvested and added to the microcultures of transfected cells.

DNA-mediated Gene Transfer and Selection of Transfected Cells. Plasmid and genomic DNA were prepared as described (18). We used a transfection procedure similar to the Corsaro and Pearson (28) modification of the method of Wigler et al. (29). Cellular DNA (60 μg) and 3 μg of pHMR272 (30) were mixed in 940 μl of 1 mM Tris-Cl, pH 7.5, 0.1 mM EDTA; 310 μl of CaCl2 1 M were then added. This solution was slowly added under constant agitation to 1.25 ml of 50 mM Hepes, 280 mM NaCl and 1.5 mM Na_2HPO_4, adjusted to pH 7.1 with NaOH. The calcium phosphate-DNA precipitates were allowed to form for 30–45 min at room temperature. PO.HTR cells (5×10^6) were centrifuged for 10 min at 400 *g*. After removal of the supernatant, the pellets were resuspended directly in the medium containing the DNA precipitates. The mixture was incubated for 20 min at 37°C and then added to a 80 cm^2 tissue culture flask containing 22.5 ml DMEM supplemented with 10% FCS. After 24 h, the medium was replaced. 48 h after transfection, the cells were collected and counted. Selection of transfected cells in mass cultures was then carried out in culture medium supplemented with hygromycin B (350 μg/ml) (Calbiochem-Behring Corp., San Diego, CA). For each group two flasks were prepared with 8×10^6 cells in 40 ml medium per flask. To estimate the number of independent transfectants, 10^6 cells from each group were plated in 5 ml DMEM with 10% FCS, 0.4% bactoagar (Difco Laboratories), and 300 μg/ml hygromycin B. Colonies were counted 12 d later. The average of two independent determinations was multiplied by 5 to estimate the number of independent transfectants in the corresponding group. A correction was made for the cloning efficiency of P815 cells in agar, which was observed to be ~0.3.

Chromium Release Assay for CTL Activity. Chromium release assay was previously described (10). Briefly, CTL were incubated with 2,000 ^{51}Cr-labeled cells at various effector/target ratios in 96-well conical microplates in a final volume of 200 μl. The chromium released in the supernatant was measured after 4 h of incubation at 37°C.

Screening of Transfectants with CTL. 8 d after the transfection, the antibiotic-resistant transfectants were separated from the dead cells by density centrifugation with Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and were maintained in nonselective medium for 1 or 2 d. These cells were then plated in 96-well microplates (round bottom) at 30 cells/well in 200 μl of culture medium. After 5 d, the wells contained ~6×10^4 cells and replica plates were prepared by transferring 1/10 of the wells to microplates which were incubated at 30°C. 1 d later, the master plates were centrifuged, the medium was removed and 750 CTL-P1:5 (anti-P815A) were added to each well together with 10^6 irradiated syngeneic feeder spleen cells in CTL culture medium containing 40 U/ml of recombinant human IL-2. The medium also contained HAT to kill the stimulator cells. 6 d later, the plates were examined visually to identify the wells where the CTL had proliferated. For the plates where proliferating microcultures were observed, aliquots of 100 μl of all the wells were transferred to another plate containing ^{51}Cr-labeled P1.HTR target cells (2,000–4,000/well). Chromium release was measured after 4 h (10). The replica microcultures corresponding to the microcultures showing high CTL activity were expanded and cloned by limiting dilution in DME with 10% FCS. After 5 d, ~200 clones were collected and screened with CTL-P1:5 in a visual lysis assay.

Visual Lysis Assay for CTL Activity. Aliquots (~10^3/cells) from individual clones were incubated either alone or together with 2×10^4 CTL in DME with 10% FCS on 96-well round-bottom microplates (12). Before use, the CTL were separated from dead feeder and stimulator cells by density centrifugation on Ficoll-Paque. 1 d later, the plates were examined microscopically to compare the number of surviving cells in the wells containing CTL to that in the control wells.

Genomic Libraries and Cosmid Transfection. Total genomic DNA of transfectant P1A.T2 was partially digested with Sau3A1 and fractionated by NaCl density gradient ultracentrifugation to enrich for 35–50 kb DNA fragments (31). These fragments were ligated to cosmid arms of c2R.B (32) that were obtained by cleavage with SmaI and treatment with calf intestinal phosphatase followed by digestion with BamHI. The ligated DNA was packaged into λ phage components (Gigapack Gold; Stratagene Cloning Systems, La Jolla, CA). The product was titrated on *Escherichia coli* ED8767 as described (31). We obtained about 9×10^5 $Ampi^r$ colonies per microgram of DNA insert. To amplify the cosmid groups, packaging mixtures representing 30,000 independent cosmids were mixed with 2 ml of ED8767 bacteria in 10 mM $MgCl_2$, incubated 20 min at 37°C, diluted with 20 ml of Luria-Bertani (LB) medium, and incubated for 1 h. The suspension was titrated, then used to inoculate 1 L of LB medium in the presence of ampicillin (50 μg/ml). When the bacterial concentration reached 2×10^8 cells/ml (OD_{600} = 0.8), a 10 ml aliquot was frozen and chloramphenicol (200 μg/ml) was added to the rest of the culture for overnight incubation. Total cosmid DNA was isolated by the alkaline lysis procedure and purified on CsCl gradient. Groups of 5×10^6 PO.HTR cells were treated with 60 μg of DNA of each amplified cosmid group and 4 μg of pHMR272.

The genomic library of normal DBA/2 kidney cells was prepared in λ ZapII (Stratagene). High mol wt DNA was digested with EcoRI and fractionated by agarose gel electrophoresis. Fragments from 6 to 8 kb were recovered from the gel and ligated to EcoRI-digested λ ZapII. The recombinant DNA was packaged in λ phage components and titrated on *E. coli* strains XL1-Blue and PLK-A (Stratagene). The screening of this library was performed with the P1A probe a (Fig. 6). The 8-kb insert of a positive clone was transferred to plasmid vector pBluescript by in vivo excision according to Stratagene's protocol.

Cosmid Recovery by Direct Packaging. High mol wt DNA of the cosmid transfectants expressing P815A was directly packaged in λ phage components with Gigapack Gold extracts (Stratagene). The product was titrated on *E. coli* ED8767 with ampicillin selection. For transfection, these groups of cosmids were amplified like the cosmid library.

mRNA Analysis and cDNA Library. Total cellular RNA was prepared by the guanidine-isothiocyanate procedure (33). Poly-A^+ mRNA was purified by chromatography on oligo-dT-cellulose column (Pharmacia Fine Chemicals) as described (33).

For the Northern blot analysis, the RNA samples were fractionated on 1% agarose gels containing 0.66 M formaldehyde. Gels were treated with 10× SSC (SSC:0.15 M sodium chloride, 0.015 M sodium citrate [pH 7.0]) for 30 min before overnight blotting on nitrocellulose membranes (BA85; Schleicher and Schuell, Inc., Keene, NH). After 2 h baking at 80°C, the membrane was prehybridized for 15 min at 60°C in a solution containing 10% dextran sulfate (Pharmacia Fine Chemicals), 1% SDS, and 1 M NaCl. Hybridization was then performed by adding to the prehybridization solution the denatured labeled specific probe together with 100 μg/ml denatured salmon sperm DNA. The human β-actin cDNA probe was kindly provided by A-M. Lebacq. The hybridization was carried out overnight at 60°C. The blots were washed twice at room temperature with 2× SSC and twice at 60°C with 2× SSC supplemented with 1% SDS. The P1 cDNA library was prepared in λ gt10 as described (34). The DBA/2 mouse spleen cDNA library was prepared in λ gt10 using adaptors (cDNA cloning system, Amersham kit; Amersham Corp., Arlington Heights, IL).

DNA Sequencing and Homology Search. DNA sequence analysis was performed on clones generated by a variety of restriction digests and by specific priming with synthetic oligonucleotides. All sequencing reactions were performed by the dideoxy chain-termination method (United States Biochemicals Corp., Cleveland, OH; or Pharmacia Kit; Pharmacia Fine Chemicals). The computer search for sequence homology was done with program FASTA, with K-tuple parameters of 3 and 6 (35). Genbank database release 65 (October 1990) was used.

Amplification of the 5' cDNA End by Polymerase Chain Reaction. The amplification was performed as described (36). The primer for the synthesis of the cDNA corresponded to positions 320–303. For the amplification, we used a 3' primer corresponding to positions 286–266 and the 5' primers described by Frohman et al. (36). We obtained a band of the expected size (270 bases) which hybridized with P1A probe a (Fig. 6) on a Southern blot. This band was cloned in M13 tg130 and tg131 and was sequenced.

Results

We have shown previously that clonal cell line P1 derived from tumor P815 expresses four distinct antigens recognized by CTL of syngeneic DBA/2 mice immunized with P815 cells (20). Stable CTL clones, which showed strict specificity for P815, were used to obtain antigen-loss variants of P815 by selecting in vitro tumor cells that were resistant to lysis. Other antigen-loss variants that were also resistant to some anti-P815 CTL clones were found in tumor cell populations that had escaped immune rejection in vivo. Examples of antigen-loss variants selected in vitro (isc) or in vivo (ist) are shown in Fig. 1. The analysis of these variants with a panel of anti-P815 CTL clones revealed a total of four distinct antigens, which were named P815A, B, C, and D. Antigens P815A and B showed linkage: immune selection with anti-A CTL repeatedly resulted in concurrent resistance to anti-B CTL. However, one antigen-loss variant was resistant to anti-A and not to anti-B CTL, suggesting that antigens A and B might be distinct (Fig. 1). The loss of antigens A and B on several variants that had escaped immune rejection in DBA/2 mice, provided strong evidence for an essential role of these antigens in the immune rejection response occurring in vivo against P815 (20).

Transfection with Genomic DNA. To clone the gene coding for antigen P815A, we resorted to a gene transfection approach. The highly transfectable cell line P1.HTR, which had been used successfully for the transfection of tum^- genes, was not a suitable DNA recipient, because it expressed antigen A (22). We therefore submitted P1.HTR to selection with anti-A CTL clone P1:5 and obtained a variant that expressed neither antigen A nor B. An additional selection with anti-C CTL clone P89:15 yielded clone PO.HTR which had also lost C (Fig. 3). This clone was used for all the transfection experiments.

To screen the transfectants for expression of antigen A, we relied on a test based on the ability of antigen-expressing transfectants to stimulate anti-A CTL clone P1:5 (18). In reconstruction experiments, microwells were seeded with 750 CTL and with a mixture of 1,500 P1.HTR cells (which express A) and 44,000 PO.HTR cells (which do not). A significant proliferation of the CTL was observed visually after 5 d, and the stimulation of the CTL was confirmed by testing the

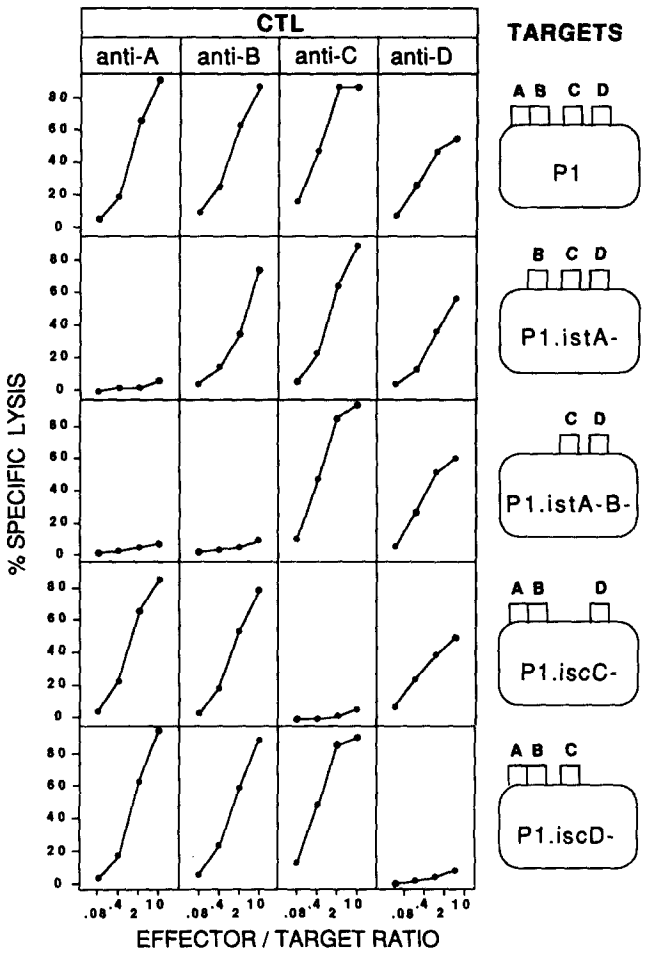


Figure 1. Antigens recognized by DBA/2 CTL on P815 clone P1 and on immunoselected variants. Antigen-loss variants P1.iscC$^-$ and P1.iscD$^-$ were isolated by in vitro selection with anti-P815 CTL clones P89:15 (anti-C) and P89:20 (anti-D), respectively. Antigen-loss variants P1.istA$^-$ and P1.istA$^-$B$^-$ were found in P1 cell populations that had escaped tumor rejection in vivo. These variants were resistant to anti-P815 CTL clone P1:3 and CTL clone P35:10 which were defined as anti-A and anti-B, respectively. The cytolytic activity was measured in a 4-h ^{51}Cr release assay.

lytic activity of aliquots of the microcultures against P1.HTR (Fig. 2 *A*). This indicated that identification of transfectants expressing antigen A should be feasible by testing pools of 30 transfected cells.

Fifteen groups of 5 × 10^6 PO.HTR cells were transfected with genomic DNA of P1.HTR and with selective plasmid pHMR272, which confers hygromycin resistance. After selection by hygromycin, the transfected populations were amplified and distributed by pools of 30 cells into 100–400 microwells according to the number of independent hygromycin-resistant transfectants obtained in each group. This number, which was estimated by an agar colony test, varied from 500 to 3,000 and our aim was to have each independent transfectant represented in at least four microwells. The microcultures were incubated until they contained approximately 6 × 10^4 cells. At this stage an aliquot was transferred to a duplicate well and 750 anti-A CTL were added to each microculture. 5 d later the proliferation of the CTL was evaluated visually. Three of the fifteen groups of transfectants gave a few positive microcultures. The lytic activity against P1.HTR of all the microcultures of these groups was assessed by a chromium release test and most microcultures where proliferation had been observed displayed a lytic activity well above background (the results obtained with two positive groups of transfected cells are shown in Fig. 2 *B*). For each group, the duplicate microcultures corresponding to several positive wells were subcloned and more than 1% of the clones were found to be lysed by the anti-A CTL. Thus, 3 independent transfectants expressing antigen P815A were obtained from a total of 33,000 hygromycin-resistant transfectants.

The transfectants were tested with CTL directed against the P815 antigens. All three were lysed by the anti-A CTL as effectively as P1.HTR. They were also lysed by anti-B CTL, confirming the linkage between antigens A and B and suggesting that these antigens were encoded by the same gene. Results obtained with transfectant P1A.T2 are shown in Fig. 3.

Transfection with a Cosmid Library and Recovery of the Gene Transferring the Expression of Antigens P815A and B. We

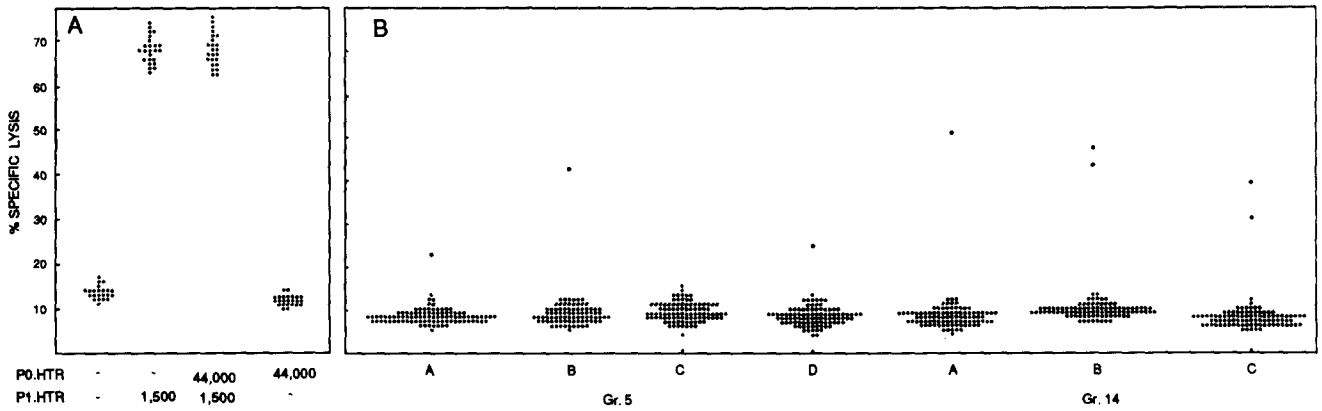


Figure 2. Detection of transfectants expressing antigen P815A. (*A*) Reconstruction experiments with microcultures seeded with 750 anti-A CTL (P1:5) and either no stimulating cells, 1,500 P1.HTR cells, 1,500 P1.HTR cells plus 44,000 PO.HTR cells, or 44,000 PO.HTR cells. After 6 d, the lytic activity of the microcultures was tested against chromium-labeled P1.HTR cells. Each point represents the lytic activity of a single microculture. (*B*) Detection of transfectants expressing antigen P815A in two groups of PO.HTR cells transfected with genomic DNA of P1.HTR. For groups 5 and 14, 400 and 300 wells, respectively, were seeded with 30 hygromycin-resistant transfected cells. After amplification and duplication of these microcultures, anti-A CTL clone P1:5 was added. 6 d later, the lytic activity of the microcultures was tested on P1.HTR. Each point represents the lytic activity of a single microculture.

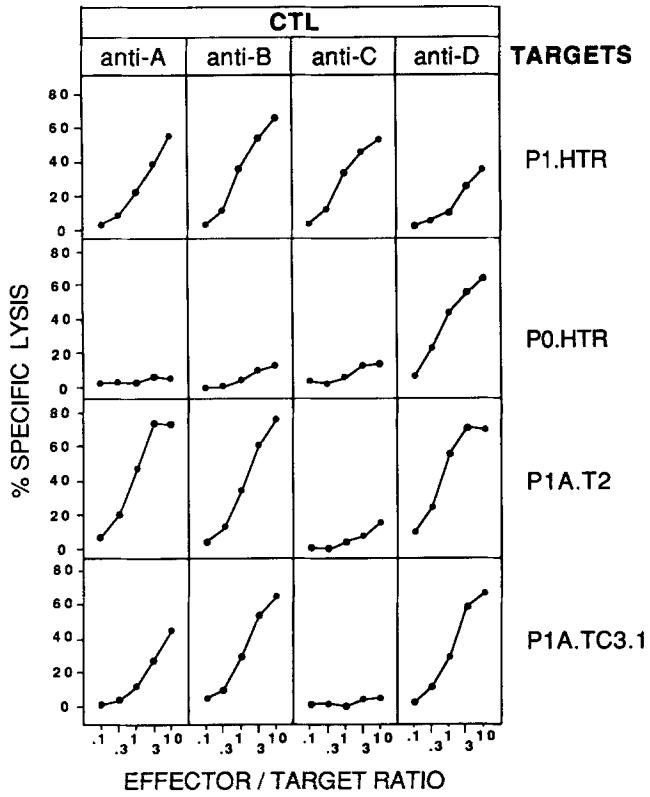
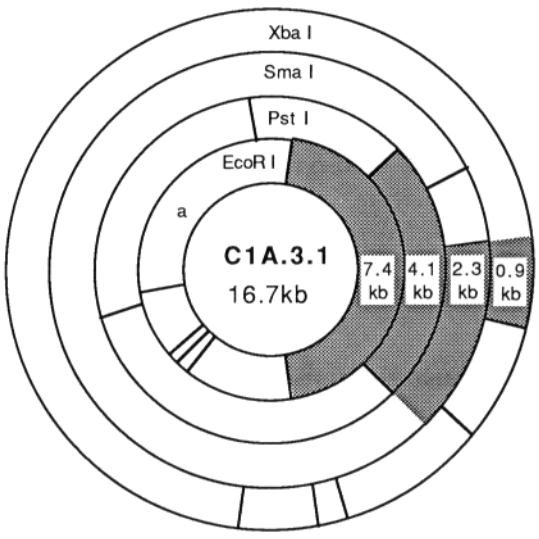


Figure 3. Sensitivity of P1.HTR, PO.HTR, genomic transfectant P1A.T2, and cosmid transfectant P1A.TC3.1 to lysis by anti-A CTL-P1:5, anti-B CTL-P35:10, anti-C CTL-P89:15, and anti-D CTL-P1.204:8. Chromium release was measured after 4 h.



Transfection of restriction fragments

	No. of clones expressing P815A / no. of HmB^r clones
4.1 kb Pst I - Pst I	2/16
2.3 kb Sma I -Pst I	16/96
0.9 kb Sma I - Xba I	22/96

Figure 4. Restriction map of cosmid C1A.3.1. Restriction fragments, cloned in vector pUC18 or obtained by electroelution were transfected into PO.HTR. 60 μg of DNA were cotransfected with 4 μg of pHMR272 into 5 × 10^6 PO.HTR cells. After 2 d, dilutions of the cells were seeded in agar plates containing hygromycin. 7 d later, the hygromycin resistant (HmB^r) colonies were transferred into microwells and tested individually with anti-A CTL (P1:5) in a visual assay. The fragments shown in grey were able to transfect the antigen. The 5.1-kb EcoRI fragment marked "*a*" corresponds to the sequence of vector c2RB.

showed previously that genes coding for tum$^-$ antigens could be recovered directly from transfectants obtained with a cosmid library (15). This approach was therefore applied to P815A. A library of 650,000 cosmids was prepared with cosmid vector c2RB and DNA of genomic transfectant P1A.T2. Twenty one groups of ~30,000 cosmids were amplified, the DNA of each group was transfected into PO.HTR with plasmid pHMR272 and an average of 3,000 transfectants per group were tested for antigen expression. The transfectants were screened for their ability to stimulate anti-A CTL clone P1:5 as described for the genomic transfections. One group of cosmids repeatedly produced positive transfectants at a frequency of about 1/5,000 drug-resistant transfectants. Here again these transfectants expressed both A and B. The sensitivity to anti-P815 CTL of cosmid transfectant P1A.TC3.1 is shown in Fig. 3.

The DNA of three independent cosmid transfectants was isolated and packaged directly with λ phage extracts (15). Cosmids were obtained for each transfectant. They were pooled, amplified, and transfected into PO.HTR. The three pools produced a high frequency of transfectants expressing antigen A (Table 1). By analysis with restriction enzymes, we identified seven different cosmids in the pool of 32 cosmids derived from transfectant P1A.TC3.1. These cosmids were transfected individually and four of them proved capable of transferring the expression of antigens A and B. We selected for further analysis cosmid C1A.3.1, which was only 16.7 kb long, presumably because it had undergone partial deletion during amplification.

The restriction map of C1A.3.1 is shown on Fig. 4. All the EcoRI restriction fragments were transfected, and only the 7.4-kb EcoRI fragment produced transfectants that were

Table 1. *Transfer of the Expression of Antigen P815A by Cosmids Obtained by Direct Packaging*

Transfectant obtained with the cosmid library	No. of cosmids obtained by direct packaging of 0.5 μg of DNA*	No. of transfectants expressing P815A/no. of HmB^r transfectants‡
TC3.1	32	87/192
TC3.2	32,000	49/384
TC3.3	44	25/72

* The DNA of the transfectants indicated in the first row was packaged in a λ phage extract and used to infect *E. coli* ED8767. The number of ampicillin-resistant bacterial colonies is indicated.

‡ The DNA of the cosmids derived by direct packaging was cotransfected in PO.HTR with pHMR272. Independent drug-resistant transfectants were cloned by limiting dilution and were tested for lysis by anti-A CTL P1:5 in a visual assay.

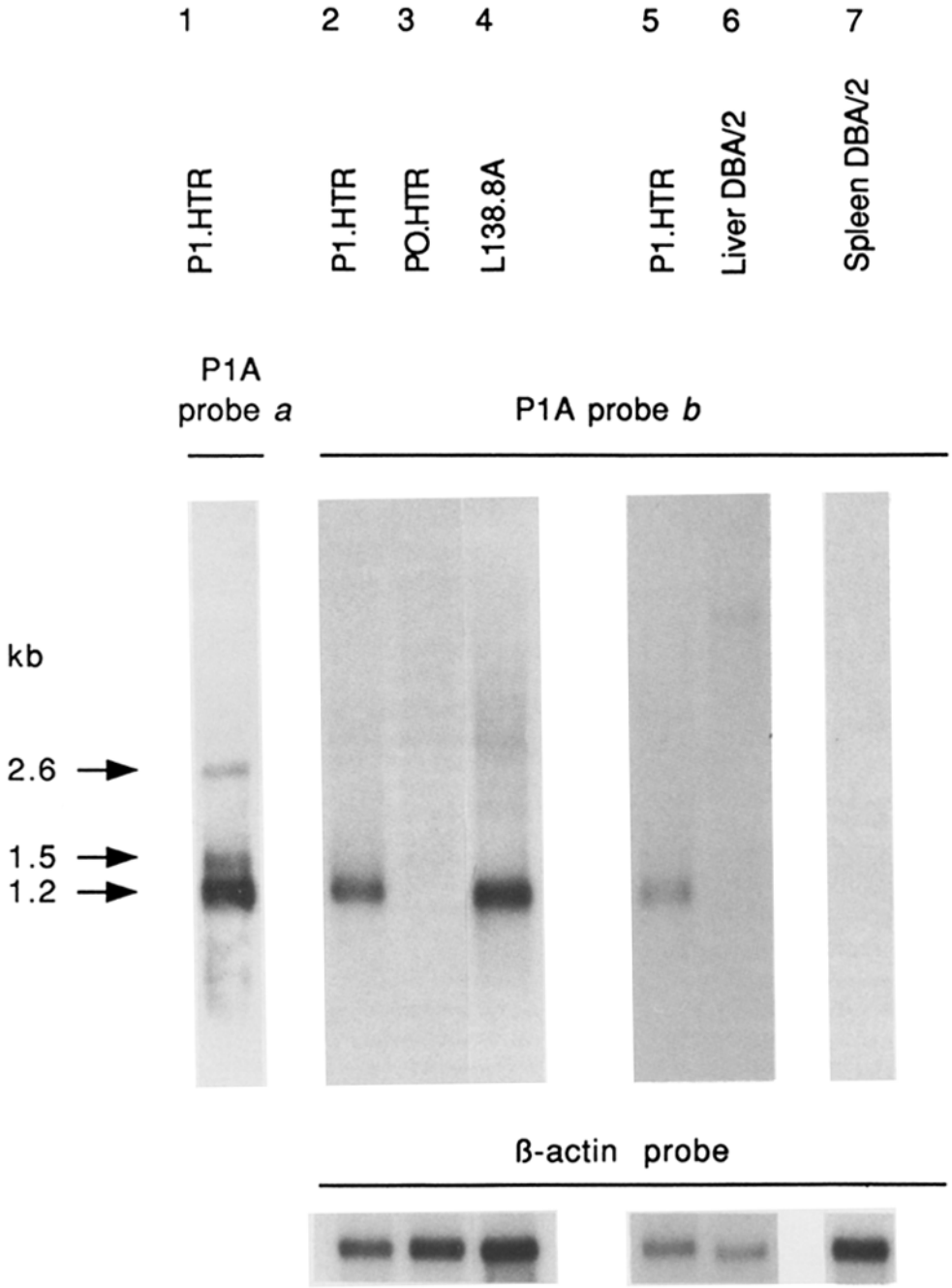


Figure 5. Northern blot analysis of the expression of gene P1A. Lanes *1*–*7* contained, respectively, 6, 2, 2, 2, 1, 5, and 2 μg of polyA$^+$ RNA of the cells indicated. Lane *1* was hybridized with probe a (Fig. 6) of gene P1A which crossreacted with the mRNA of nucleolin (2.6 kb) and nucleolar protein N038/B23 (1.5 kb). Lanes *2*–*7* were hybridized with probe b (Fig. 6) of gene P1A and also with a β-actin probe. Hybridization with both probes were performed either successively on the same blot (lanes *2*, *3*, *4*, and *7*) or simultaneously on two identical blots (lanes *5* and *6*). Washing and exposure conditions were the same for the three probes.

lysed with the anti-A CTL clone. Of the PstI fragments, only a 4.1-kb fragment, which was included in the 7.4-kb EcoRI fragment, gave a positive result (Fig. 4). Further digestion of this fragment with SmaI also produced a positive 2.3-kb fragment. Finally, we found that a 900 bases SmaI/XbaI fragment transferred efficiently the expression of antigen A. All these fragments transferred the expression of antigen B also (data not shown).

Presentation of Antigens P815A and P815B by Ld. To identify the class I molecules responsible for presentation of antigens A and B, cosmid C1A.3.1 was transfected into fibroblast line DAP (H-2^k), that had previously been transfected with either K^d, D^d, or L^d. Both A and B were presented to the appropriate CTL only by cells transfected with the L^d gene (Table 2).

Structure of Gene P1A. The fragment of 900 bases of cosmid C1A.3.1 that transferred the expression of antigen A was used as a probe on a Northern blot prepared with poly-A$^+$ RNA of P1.HTR. It revealed a band of 1.2 kb and two fainter bands of larger size (Fig. 5, lane *1*). Using the same probe to screen a cDNA library prepared with poly-A$^+$ RNA of P1, we identified a cDNA clone. It had only a 1-kb insert, suggesting that the 5′ end was missing. Using oligonucleotides corresponding to sequences of this cDNA, we obtained by PCR

Table 2. *H-2-Restriction of Antigens P815A and P815B*

Recipient cell*	No. of clones lysed by the CTL/no. of HmB^r clones‡	
	CTL anti-A	CTL anti-B
DAP ($H\text{-}2^k$)	0/208	0/194
DAP + K^d	0/165	0/162
DAP + D^d	0/157	0/129
DAP + L^d	25/33	15/20

* Cosmid C1A.3.1 containing the entire P1A gene was transfected in DAP cells previously transfected with $H\text{-}2^d$ class I genes as indicated.
‡ Independent drug-resistant colonies were tested for lysis by anti-A or anti-B CTL in a visual assay.

performed on P1.HTR cDNA a perfectly overlapping 5′ end sequence adding 200 bases (36). This provided a plausible 1.2-kb sequence for the mRNA (Fig. 7).

A 5.7-kb region of cosmid C1A.3.1, that included the SmaI-XbaI transfecting fragment, was sequenced. The comparison with the cDNA sequence delineated the exons indicated in Fig. 6 and Fig. 7. We concluded from these data that gene P1A has a length of approximately 5 kb and comprises 3 exons. An open reading frame coding for a putative protein of 224 amino acids starts in exon 1 and ends in exon 2 (Fig. 7). The fragment of 900 bases which transfers the expression of antigens A and B contains only exon 1. The promoter region contains a CAAT box and an enhancer sequence which has been observed in the promoter of most MHC class I genes (37, 38).

A computer search revealed no significant similarity between gene P1A and sequences previously recorded in data banks, with the exception of a stretch of 95 bases corresponding to part of an acidic region encoded by exon 1 (positions 524 to 618). This sequence is highly similar to sequences coding for an acidic region in two nucleolar proteins, namely mouse nucleolin (61 identical bases out of 95) and mouse nucleolar protein NO38/B23 (56 identical bases out of 95) (39, 40). To test whether these homologies provided the explanation of the crosshybridizing bands found in the Northern blots, we screened a mouse spleen cDNA library with the 900 bases P1A fragment and we obtained cDNA clones corresponding closely to the sizes of the crosshybridizing bands. They were partially sequenced and the 2.6-kb cDNA was found to correspond exactly to the reported cDNA sequence of mouse nucleolin whereas the 1.5-kb cDNA corresponded to the mouse nucleolar protein NO38/B23.

The putative product of gene P1A has a Mr of 25 kD. There is a potential nuclear targeting signal (Fig. 7, residues 5–9) (41). A striking feature of protein P1A is the presence of a large acidic domain located at positions 83–132, which includes the region of homology between P1A and the two nucleolar proteins. This region contains a putative phosphorylation site (Ser 125). Close to the C-terminus, there is a second acidic domain, an uninterrupted stretch of 14 glutamate residues. A similar C-terminal region was found in a murine homeodomain protein, which was shown to have a nuclear localization (42).

The P1A Sequence Is Identical in Tumor P815 and in Kidney Cells. A λ phage library was prepared with the DNA of

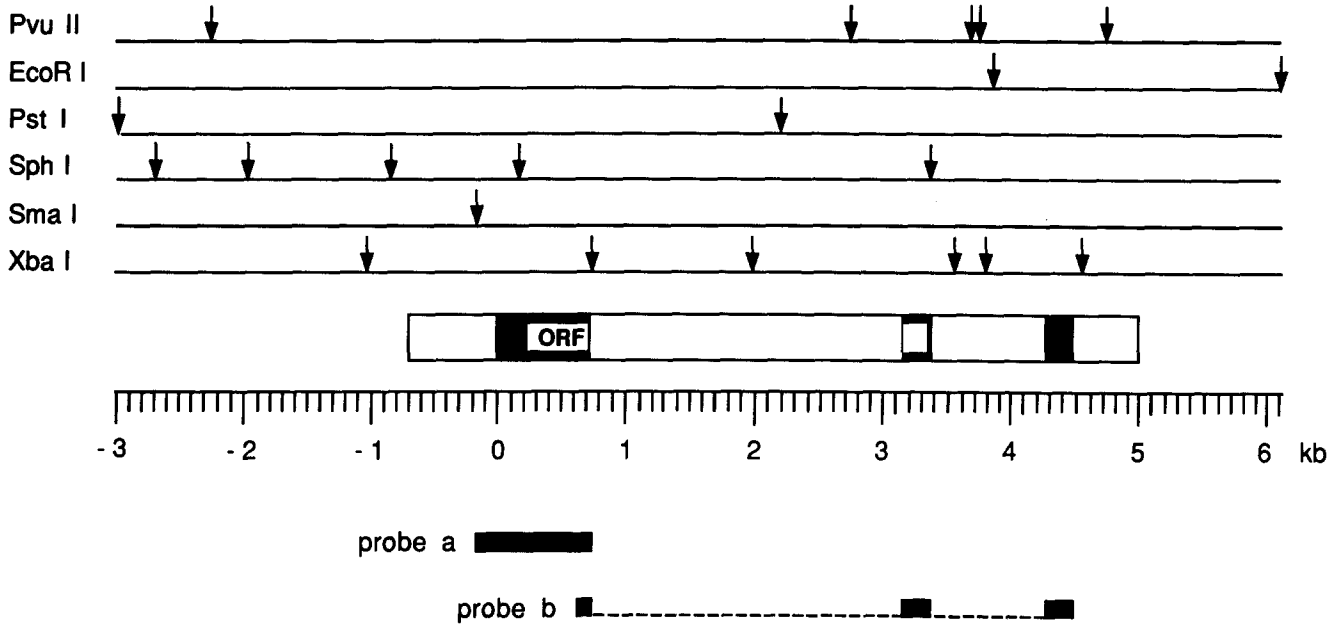


Figure 6. Structure of gene P1A and restriction sites. The box represents the part that was sequenced with the exons in black and the open reading frame (ORF) of exons 1 and 2. The 0.9-kb SmaI/XbaI fragment, which is able to transfer the expression of the antigen is delineated as probe a. Probe b is an incomplete cDNA of 0.6 kb. The region preceding the SphI site at −0.8 is rearranged in cosmid C1A.3.1. The gene was retrieved from another library where it was not rearranged as seen by comparison with genomic Southern blots. The restriction map indicated here is the genomic map.

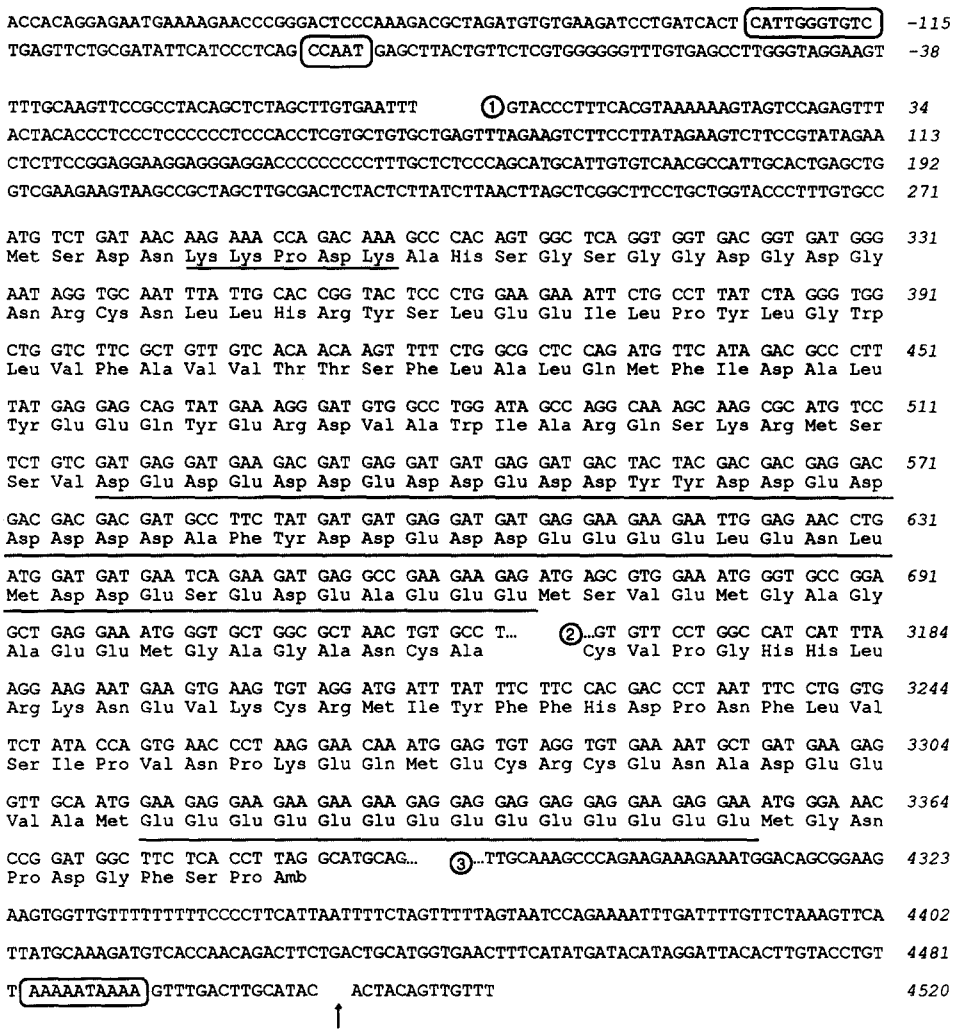


Figure 7. Sequence of the three exons and of the 5′ region of gene P1A and sequence of the protein encoded by the longest open reading frame. The numbering corresponds to the scale of Fig. 6. The nucleotide indicated as the first in exon 1 corresponds to the 5′ cDNA end obtained by the PCR. Boxes show an enhancer sequence reported to be present in the promoter of most MHC class I genes (37, 38), a CAAT box, and a consensus polyadenylation site. The start of the poly(A) tail is indicated by an arrow. In the amino acid sequence, a putative nuclear targeting signal is underlined, as well as two regions rich in acidic residues. The sequence between position −0.7 to 5 (Fig. 6) is available from EMBL/GenBank under accession number M36386. The cDNA sequence is also recorded in EMBL/GenBank under accession number M36387.

DBA/2 kidney cells and a clone hybridizing with a P1A probe was identified. From this clone, which contained exon 1 and 2 of gene P1A, the sequence corresponding to positions −0.7 to 3.8 of Fig. 6 was obtained. The sequence from 3.8 to 4.5 was obtained by PCR amplication of kidney DNA. We did not find a single difference between these sequences and that of the gene carried by the P815 tumor cells. Moreover, when the gene isolated from kidney cells was transfected into PO.HTR, it transferred the expression of antigens A and B as efficiently as the gene isolated from P815 (Fig. 8). We conclude that the expression of these antigens by tumor P815 does not result from a mutation affecting the protein produced by gene P1A.

No evidence was found that a rearrangement had occurred in the near vicinity of the gene carried by P815 cells. Southern blot analysis with a probe containing exon 1 revealed an EcoRI band and a PstI band of the same size, 8 kb and 5 kb, respectively, in P815 cells and in kidney cells. An exon 3 probe revealed an EcoRI band of 2.3 kb in both cells (data not shown).

Expression of Gene P1A. The 1.2-kb mRNA of gene P1A was not observed on Northern blots prepared with RNA of liver or spleen cells of DBA/2 mice (Fig. 5), suggesting that most normal cells do not express gene P1A.

Because P815 was originally described as a mastocytoma, we also tested a number of mast cell lines for expression of the gene. Mast cell line MC/9 (43) and short term cultures of mast cells isolated from mouse bone marrow were negative on Northern blots (data not shown). In contrast, a strong signal was obtained with L138.8A (Fig. 5), a mast cell line derived from BALB/c bone marrow by culture in conditioned medium containing IL-3 (25). We took advantage of the fact that BALB/c and DBA/2 mice share the H-2^d haplotype to test the sensitivity of cultured L138.8A to lysis by anti-P815 CTL clones. A high level of lysis was observed with anti-A and anti-B CTL, but not with anti-C and anti-D CTL (Fig. 9).

Mouse tumor cell lines of other histology were also tested for expression of gene P1A: Northern blots of teratocarcinoma cell line PCC4 (44) and of leukemias LEC (7) and WEHI-3B were all negative.

Discussion

We have isolated a gene coding for a tumor rejection an-

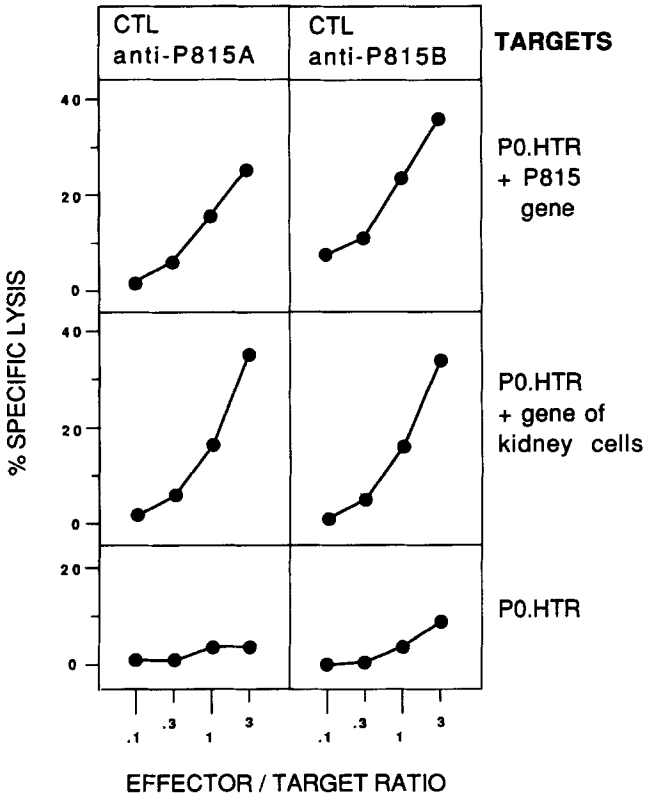


Figure 8. Transfections with gene P1A isolated from tumor P815 or from normal cells. Restriction fragments containing the two first exons of gene P1A isolated from P815 or from normal DBA/2 kidney cells and cloned in pUC18 or in pBluescript respectively were transfected into PO.HTR. 60 μg of DNA were cotransfected with 4 μg of pHMR272 into 5 × 10^6 PO.HTR cells. After 6 d of selection in hygromycin, the population of HmB^r transfectants was chromium-labeled and tested with CTL-P1:5 (anti-A) and CTL-P35:10 (anti-B) in a 4-h chromium release assay. Lysis of PO.HTR is shown as control.

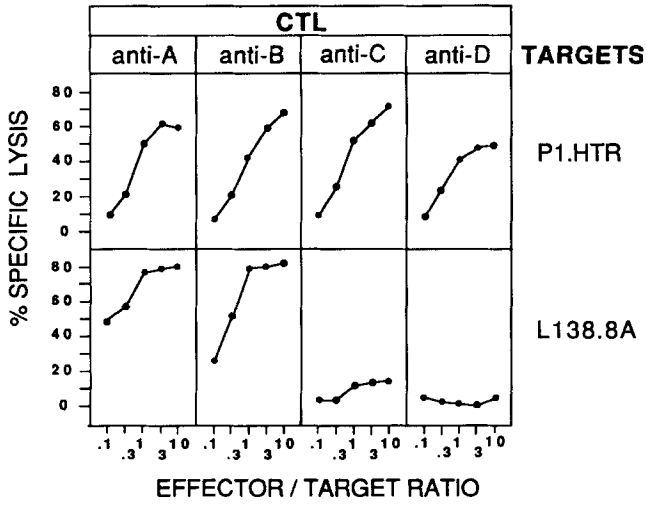


Figure 9. Sensitivity of L138.8A mast cell line to lysis by anti-A CTL-P1:5, anti-B CTL-P35:10, anti-C CTL-P89:15, and anti-D CTL-P1.204:8. Lysis of P1.HTR is shown as control. Chromium release was measured after 4 h.

tigen by an approach involving the transfection of a cosmid library. This approach had been used previously to clone three genes coding for tum^- antigens (15, 16, 17, 18) and our results provide additional evidence for its wide applicability. It is nevertheless worth mentioning that a library of 1,400,000 cosmids derived from P815 cells failed to provide any transfectants expressing antigen P815A, even though a total of 100,000 independent transfectants were tested. We then derived another library from a genomic transfectant expressing antigen P815A in the expectation that the DNA surrounding the transfected gene might be different and perhaps less inhibitory to cosmid integration and multiplication. We were successful, but it would be premature to conclude that the use of transfectant DNA to build the cosmid library was a decisive factor.

No significant similarity with the sequence of P1A was found in gene data banks except for the sequences of two genes coding for nucleolar proteins that share an acidic domain (39, 40, 45). Proteins of the *myc* family of oncogenes also belong to this class of nuclear proteins (40, 46), but no significant homology was found between the sequences of *myc* and P1A.

Both anti-A and anti-B CTL recognize a product of gene P1A in association with L^d. To establish the exact relation between the epitopes recognized by these two CTL, it will be necessary to identify the relevant antigenic peptides. P815A and B may correspond to two different peptides encoded by P1A. Alternatively, the two CTL may recognize the same peptide, but with different affinities, so that a decrease in the amount of peptide bound to L^d may affect recognition by anti-A CTL more deeply than that by anti-B.

Unlike the three genes coding for tum^- antigens, the antigenicity of gene P1A does not result from a point mutation that modifies its protein product. There is no sequence difference between the gene expressed in tumor P815 and that present in normal cells of DBA/2 mice. Moreover, transfection of the gene isolated from normal DBA/2 kidney cells confers sensitivity to anti-A and anti-B CTL. We conclude that tumor rejection antigens P815A and B result from the expression by tumor P815 of a gene that is silent or expressed at a very low level in most if not all normal cells of adult mice. This suggests a correlation between expression of gene P1A and antigenicity, in contradiction with an hypothesis that we considered (47).

To the extent that tumor rejection antigens would be generated by point mutations like the tum^- antigens, one would expect that these antigens would be strictly specific for individual tumors. On the other hand, the identity of gene P1A carried by tumor P815 with the gene carried by normal cells suggests that some tumor rejection antigens may be shared by independent tumors of the same histology. Antigens P815A and B are shared by P815 and by mast cell line L138.8A, which incidentally is tumorigenic in DBA/2 mice. This differs from the conclusions drawn from the study of methylcholanthrene-induced sarcomas which express a large variety of tumor rejection antigens (4). It is difficult at the present time to compare our results with those obtained for antigens of sarcoma

MethA because these antigens were isolated on the basis of their ability to stimulate immune responses in vivo and not as targets of CTL (48, 49). Clearly more genes need to be analyzed to decide whether the situation observed with P1A is the rule or the exception.

Mice that reject P815 cells do not present obvious health impairment. How can antigens, that are encoded by normal genes, elicit an immune rejection response against the tumor without severe autoimmune consequences? A first explanation may be provided by the long-standing notion of "oncofetal" antigens, implying that tumors reexpress fetal antigens which have disappeared from all normal cells before the establishment of immunological tolerance. Hence the absence of tolerance for these antigens and the absence of autoimmune consequences of the response, since no normal cell of the adult animal would express these antigens. An alternative possibility is that gene P1A is expressed by mast cell precursors located in the bone marrow during a brief stage of their differentiation. These cells would not by themselves induce an immune response either because of tolerance or because of their small number and dispersion. The antitumoral response might eliminate some of these precursor cells, but after the rejection of the tumor, the active effector T cells would disappear and mast cell differentiation could resume without damage. To clarify these issues, we intend to carry out a systematic analysis of the expression of P1A messenger RNA in adult and fetal tissues.

We consider that the product of gene P1A represents a major tumor rejection antigen of tumor P815 for the following reasons. First, more than 10 independent occurrences of P815 tumor-escape after nearly complete rejection produced P815 cells that had lost the expression of antigens P1A-B. Moreover, when these antigen-loss variants were injected into mice, we never observed the partial rejection phase (20). Second, in many mice that mount an immune response against P815, the major part of the CTL activity is directed against antigens P1A-B. However, it is worth pointing out that there is as yet no evidence that antigens P815A and B can elicit a T cell response on their own. P815 expresses at least two other antigens recognized by CTL. One cannot exclude that a response against these antigens creates conditions that strongly facilitate the response against A and B. This would be in line with the phenomenon observed with tum$^-$ variants derived from spontaneous mouse tumors which were devoid of any immunogenicity. The mice that rejected these tum$^-$ variants, which expressed new antigens, also acquired an immune memory against an antigen present on the parental tumor cells (7).

We hope that the identification of genes coding for tumor rejection antigens will soon be extended to several other mouse tumors. It will be important to find out whether these antigens always result from the expression of a gene which is silent in most or all normal cells or whether mutations also contribute. It is also essential to realize that, in the absence of a mutation specific for the allele expressed by the tumor, the only evidence that an antigen defined as a CTL target plays a role in the rejection of the tumor cells is the correlation between the resistance to this CTL and tumor escape in vivo (20). It is only because such evidence is clearly provided by antigen-loss variants for P815A and B that we can exclude that they are merely the targets of an artefactual autoimmune response favored by the conditions of mixed lymphocyte-tumor cell cultures.

Our approach may also be applicable to human tumor rejection antigens, because it is commonly observed that the blood lymphocytes of many cancer patients can be restimulated in vitro with their tumor cells to produce CTL that lyse these tumor cells with specificity (50, 51). Because stable CTL clones can be obtained, it should be possible to use the transfection approach to identify the genes coding for the antigens recognized by these CTL.

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