Structure of the Gene of tum- Transplantation Antigen P198: A Point Mutation Generates a New Antigenic Peptide

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Summary

Mutagen treatment of mouse tumor cell line P815 produces tum- variants that are rejected by syngeneic mice because they express new transplantation antigens. These tum- antigens are recognized by cytotoxic T lymphocytes (CTL) but induce no detectable antibody response. By transfecting P815 cell line P1.HTR with DNA of tum- variant P198, we obtained transfectants expressing tum- antigen P198 that could be identified on the basis of their ability to stimulate anti-P198 CTL. This was repeated with DNA of a cosmid library derived from variant P198, and a cosmid carrying the sequence encoding antigen P198 was recovered from a transfectant. Gene P198 is 3 kb long and contains eight exons. It shows no homology with previously identified tum- gene P91A, nor with any gene presently recorded in the data banks. The long open reading frame codes for a 23.5-kD protein. The antigenic allele of gene P198 differs from the normal allele by a point mutation located in exon 7. This mutation causes an Ala to Thr change, and was shown by site-directed mutagenesis to be responsible for the expression of the antigen. An 11-amino acid synthetic peptide covering the sequence surrounding the tum- mutation rendered P815 cells sensitive to lysis by anti-P198 CTL. The homologous peptide corresponding to the normal sequence of the gene did not, but it was able to compete for binding to major histocompatibility complex molecule Kd. We conclude that tum- mutation P198 generates a new epitope recognized by syngeneic T cells. As observed with gene P91A, we found that a fragment of gene P198 that contained only exons 3–7, cloned in nonexpression vectors, transferred efficiently the expression of the antigen.

Transplantation antigens, such as minor histocompatibility antigens, tumor-specific transplantation antigens (TSTA)1, or male-specific antigen H-Y often induce cytolytic T cell responses, but do not elicit antibody responses (1). These antigens can therefore not be isolated by immunoprecipitation like the major histocompatibility antigens, and their structure and genetic origin have remained largely unknown. We have developed a gene transfection approach aimed at identifying directly the genes that code for this type of antigen, and we have applied it to the tum- transplantation antigens that arise on mouse tumor cells when they are treated with mutagenic agents.

In vitro mutagen treatment of mouse tumor cell lines generates at very high frequency stable immunogenic variants that are rejected by syngeneic mice (2–4). Since they fail to form tumors, these variants have been named tum-, as opposed to the original tum+ cell, which forms progressive tumors. Most tum- variants express new transplantation antigens not found on the tum+ cell. The existence of these tum- antigens has been demonstrated in vivo by crossimmunization experiments and in vitro with CTL (5, 6).

A large number of tum- variants have been derived from mastocytoma P815, a tumor induced in a DBA/2 mouse with methylcholanthrene (7). For most P815 tum- variants, stable CTL clones have been obtained that show a strict specificity for the immunizing variant and thus define one or several tum- antigens (8). The tum- antigens defined by CTL are relevant to the rejection of the variants, as shown by the correlation between the loss of these antigens and the reversal of the tum- phenotype (9, 10). The diversity of tum- antigens is considerable: the analysis of 15 P815 tum-
variants revealed the existence of a different antigen on each of them with no evidence of crossreactivity (11). This large diversity is reminiscent of that observed with TSTA expressed by tumors induced with chemical carcinogens (12).

To clone the genes that determine the expression of tum- antigens, we have developed an approach based on gene transfection and detection of antigen-expressing transfectants with CTL, since no antibodies are available (12). By transfecting P815 tum+ cells with a cosmid library prepared with the DNA of a cell expressing tum- antigen P91A, we have obtained transfectants expressing this antigen, and from these transfectants, we have recovered a cosmid carrying the encoding gene (14). This enabled us to obtain the sequence of the gene coding for tum- antigen P91A (15). This gene shows no significant homology with any of those presently reported in data bases. It codes for a 60-kD protein that does not appear to be localized at the cell surface, an observation in line with the demonstration that antiinfluenza CTL recognize MHC-bound peptides corresponding to viral proteins that remain inside the cell. A mutation that causes the expression of the antigen is located in an exon and appears to be the only difference between the normal and the antigenic allele. A short synthetic peptide corresponding to the coding region located around the tum- mutation makes P815 cells sensitive to lysis by anti-P91A CTL. A major consequence of the tum- mutation is the creation of a strong agglutetope enabling this peptide to bind to H-2 molecule Ld (15).

Whereas the results obtained with tum- antigen P91A provided a first clarification about the nature of this type of antigen, there remained several questions. Would our cloning approach prove generally applicable? Would the direct generation of new antigenic peptides by point mutations prove to be a major immunogenic mechanism? And finally, would the genes coding for other tum- antigens resemble P91A or would they be completely unrelated? To answer these questions we set out to clone other tum- antigens. We present here the results obtained with one of them.

Materials and Methods

Cells. The derivation from mastocytoma P815.X2 of clonal line P1 (tum+) and of tum- variant P198 by treatment of P1 with N-methyl-N' nitro-N-nitrosoguanidine was described earlier (7). Repeated cycles of transfection of P1 ensured the selection of the highly transfectable cell line P1.HTR.tk-, which has a transfection efficiency of 10-4 with the calcium phosphate precipitation method (16). DAP L cells (H-2b) and transfected derivatives T111 (expressing H-2Le) and T483 (expressing H-2Dk) were a gift from K. Ozato (17). DAPT191 clone (transfected with the K1 gene) was described previously (15). The cultures were maintained in DME (Gibco Laboratories, Grand Island, NY) containing 10% FCS (Gibco Laboratories) and incubated in tissue culture flasks at 37°C in air containing 8% CO2.

CTL Clones. CTL clones CTL-P198:6 and CTL-P91:6 show strict specificity for tum- variants P198 and P91, respectively. Their derivation and their long-term culture conditions were described previously (8).

Transfection. For the transfection of genomic DNA, we used the method described by Wölfel et al. (13). Briefly, groups of 5 × 104 Pl.HTR tk- cells were incubated for 20 min at 37°C in preformed calcium phosphate DNA precipitate containing genomic DNA (60 μg) mixed with plasmid pSVtkneoβ (6 μg) as selective marker (18). The following day, the medium containing the precipitate was replaced by fresh medium. Selection of the transfectants was carried out 1 d later by incubating 1.6 × 105 cells of each group in 80 ml of medium containing 1.5 mg/ml of the neomycin analog G418 (geneticin; Gibco Laboratories). To estimate the number of independent transfectants, 106 cells were plated for a colony test in 5 ml medium containing 0.4% bactoagar (Difco Laboratories, Inc., Detroit, MI) and 1.2 mg/ml of G418. A correction was made for the cloning efficiency of P815 cells in agar, which is ~0.3.

The same procedure was applied for the transfection of the c2RB cosmid library. Groups of 5 × 106 Pl.HTR cells were transfected with 60 μg of DNA of the amplified library and 2 μg of cosmid pHMR272 coding for the resistance to hygromycin B (19). The transfectants were selected after 48 h in a medium containing 350 μg/ml of hygromycin B.

Transfections with isolated plasmids were performed by using the cells with 10 μg of plasmid DNA and 2 μg of pSVtkneoβ.

Screening of Transfected Cells. For the detection of transfectants expressing antigen P198, we used the method described in Wölfel et al. (13). 8 d after selection, the drug-resistant transfectants were separated from the dead cells by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscata) before being plated on 96-well round-bottomed microplates at 30 cells per microculture of 200 μl. Depending on the number of independent transfectants, 1–5 plates were prepared for each group. After 5 d at 37°C, each well contained ~3 × 104 cells: aliquots of 20 μl were transferred to duplicate wells that were incubated at 30°C. The following day, the master plates were centrifuged, and the cells were resuspended in 200 μl of a medium containing 1,000 CTL-P198:6, 106 irradiated DBA/2 feeder splenocytes, 10 U/ml rIL2 (a kind gift of Walter Fiers, State University of Ghent, Belgium), and 3.8 × 10-9 M mycophenolic acid, 3.8 × 10-6 M mimosine, 1.6 × 10-3 M 2-deoxythymidine) to prevent the proliferation of the Pl.HTR tk- stimulatory cells. After 5 d, the proliferation of the CTL was evaluated. For the plates where proliferating microcultures were observed, aliquots of 100 μl of all the wells were transferred to a separate plate containing 2,000 51Cr-labeled P198 cells per well. Chromium release was measured after 4 h. The duplicates of the positive wells were subcloned by limiting dilution so as to obtain 150–300 clones. After 5 or 6 d, these clones were screened for lysis by CTL-P198:6 in a visual assay followed by a chromium release test for final confirmation.

Visual Lysis Assay. Aliquots (105 cells) from individual clones were incubated either alone or together with 2 × 106 CTL-P198:6 in DME with 10% FCS in 96-well round-bottomed microplates. 1 d later, the plates were examined for the presence of surviving tumor cells (9).

Assay for Cytolytic Activity. The protocol used has been previously described (6). CTL and 2,000 51Cr-labeled targets were incubated at various ratios in 96-well conical microplates in a final volume of 200 μl. Chromium release was measured after 4 h. The duplicates of the positive wells were subcloned by limiting dilution so as to obtain 150–300 clones. After 5 or 6 d, these clones were screened for lysis by CTL-P198:6 in a visual assay followed by a chromium release test for final confirmation.

Cosmid Libraries. Cosmid arms of c2RB were prepared by cleavage with Smal and treatment with calf intestinal phosphatase, followed by digestion with BamHI (20). Genomic DNA was partially digested with Sau3A1 (0.05 U/μg) for three time points and centrifuged on a NaCl gradient (21). Fractions containing DNA fragments of 35–50 kb were ligated to c2RB arms overnight at 14°C with T4 DNA ligase (Amersham Corp., Arlington Heights,
by the Sanger's dideoxy chain termination method using the en-
wereamplified. DNA was extracted and purified on a CsCl gradient.

Cosmid Rescue. 1 μg of high molecular weight DNA of trans-
fectants expressing P198 antigen was directly packaged into λ phage
components (Gigapack Gold, Stratagene). The product was titrated
on E. coli ED8767 with ampicillin selection. Groups of cosmids
were amplified and purified on a CsCl gradient.

DNA Sequencing and Homology Search. Restriction fragments
of cosmids C198.3.1 and C1.198.1 were subcloned into M13 vectors
as described (15, 22–24). Nucleotide sequences were determined
by the Sanger’s dideoxy chain termination method using the en-
gineered form of the T7 DNA polymerase (Sequenase Kit; United
States Biochemical Corp., Cleveland, OH). The computer search
for sequence homology was done with program FASTN, with K-
tuple parameters of three and six (25). EMBL database release 18
(March 1989) and Genbank release 60 (June 1989) were used.

mRNA Analysis. cDNA libraries were constructed in Agt10
(14, 26; Amersham cDNA synthesis system). Northern blots were
prepared and hybridized as described (15). SI nuclease mapping and
RNAase protection assay were performed following the protocol
described by Ausubel et al. (27).

The rapid amplification of the 5’ end of P198 mRNA was per-
formed as described by Frohman et al. (28). The primer used for
the synthesis of the cDNA was 5’-CACCTTGAGGCGCTCC-3’
(position 3557–3541). For the amplification, we used 5’-AGCCT-
GGCCTCTTGG-3’ (position 3535–3519) as 3’ primer, and the
primers described by Frohman et al. as 5’ primers. The amplified
product was cloned in M13mp18 using the Sall site of the 5’ primer
and a SphI site located in the P198 sequence at position 3501;
resulting clones were sequenced.

Site-directed Mutagenesis. The mutagenic oligonucleotide 5’-CCA-
GAGTGGTTGTCACTGCC-3’, synthesized by Eurogentec (Liège,
Belgium), was used to modify the sequence of the BglII-BglII frag-
ment of cosmid C1.198.1, which was cloned in bacteriophage
M13tg130 following the method of Nakamaye and Eckstein (29;
Amersham site-directed mutagenesis system).

Peptide Production and Assay. Peptides were synthesized by G.
Corradin (Ludwig Institute for Cancer Research, Lausanne Branch,
Institute of Biochemistry, University of Lausanne), using Merrifield’s
solid-phase method (30).

For the assay on P815, microwells received various concentrations
of the peptide diluted in 50 μl of DMEM and 2,000 51Cr-
labeled target cells in 50 μl. After 15 min at 37°C, 20,000 CTL
were added in 100 μl of medium. The cells were incubated for 4 h at 37°C before the chromium release was measured.

Results

P198 is a tum− variant obtained after mutagen treatment of P1, a clonal cell line derived from mouse tumor P815 (7). By restimulating in vitro spleen lymphocytes of DBA/2 mice that had rejected tum− variants, stable CTL clones were produced that recognized either all P815 cells or only the immunizing variant (8). Thus, CTL-P198:6 was obtained that lysed P198 but neither P1 nor other tum− variants derived from P1 (Fig. 1). This CTL defined tum− antigen P198.

Transfection of tum− Antigen P198. We demonstrated earlier
that the expression of tum− antigen P198 is dominant in
(P1 × P198) somatic hybrids (31). Therefore, we used gene
transfection as a first step in the cloning of the gene encoding
this antigen. Following the approach that had ensured the
isolation of transfectants expressing tum− gene P91A (13),
we transfected with DNA of tum− variant P198 the highly
transfectable clone P1.HTR, which had been derived from P1 (16). For the detection of transfectants expressing antigen P198, we used a test relying on stimulation of CTL-P198:6.

In reconstruction experiments, microwells were seeded with
1,000 of these anti-P198 CTL and with a mixture of 2 × 103 P198 cells and 6 × 104 P1.HTR cells, which do not express antigen P198. A significant proliferation of the CTL was observed visually after 5 d, and it could be measured
by testing the lytic activity of the microcultures against P198
(Fig. 2A). This indicated that identification of transfectants
expressing antigen P198 should be feasible by testing the trans-
fected cells by pools of 30.

A total of 42 groups of 5 × 106 P1.HTR cells were
cotransfected with DNA of variant P198 and plasmid
pSVtkneoβ, which confers resistance to geneticin. Each group
produced 300–1,500 independent geneticin-resistant transfec-
tants. After selection in geneticin, the transfected populations
were amplified and distributed by pools of 30 cells into 100–500
wells, according to the estimated number of independent trans-
fectants present in the population. These microcultures were allowed to grow to \(3 \times 10^4\) cells and, at this point, aliquots were transferred to duplicate wells. 1,000 anti-P198 CTL were then added to each well and, 5 d later, the proliferation of CTL in the microcultures was evaluated visually. For those plates where proliferation was observed in some wells, the lytic activity of all the microcultures was tested on \(^{51}Cr\)-labeled P198 cells. Positive microcultures were observed in 2 of the 42 groups of transfectants (Fig. 2 B). This corresponded to a frequency of one antigen-expressing transfectant per 13,000 drug-resistant transfectants. The duplicates of the positive microcultures were subcloned, and the clones were tested for their sensitivity to the anti-P198 CTL with a visual assay. Antigen-expressing clones were found, and their lysis by CTL-P198:6 was observed to be comparable with that of P198 (Fig. 3).

**Isolation of a Cosmid Transferring Expression of Antigen P198.** Because we had been unable to recover \(\text{tum}^-\) gene P91A on the basis of its linkage to the cotransfected plasmid, and because a cosmid retrieval approach had worked readily for this gene, we applied the cosmid approach to P198. A library of \(4 \times 10^5\) cosmids was constructed with DNA of variant P198, which was partially digested with Sau3A1 and ligated to vector c2RB (14). The library was divided into four groups of \(10^5\) independent cosmids that were amplified by a factor of \(10^8\) to obtain enough DNA for transfection. We applied the transfection and detection procedure that had been used with genomic DNA. Each cosmid group was cotransfected with selective plasmid pHMR272, which codes for hygromycin resistance. Transfectants expressing antigen P198 were obtained with three of the four cosmids groups. A total of five independent transfectants were obtained.

The DNA of each transfectant was extracted and packaged directly with \(\lambda\) phage extracts; all produced cosmid populations, which were amplified and transfected. Only those cosmids derived from transfectant P198.TC3 produced transfectants expressing the antigen, and the frequency was very low (1/4,500). A Southern blot analysis revealed that the DNA of one of these secondary transfectants contained only a small number of integrated cosmids. We therefore used this DNA for a second direct packaging experiment, and this time, we obtained a cosmid population that transferred the P198 phenotype at high frequency; >50% of the drug-resistant transfectants expressed the antigen. This cosmid population consisted of only three different cosmids. Two of them transferred the expression of antigen P198, and a 43-kb cosmid C198.3.1 was selected for further analysis.

**A 1.2-kb Fragment Transfers the Expression of Antigen P198.** A restriction map of cosmid C198.3.1 was obtained (Fig. 4). A 14-kb XbaI restriction fragment produced transfectants that were lysed by anti-P198 CTL. Further digestion of this fragment produced a 5-kb HindIII fragment and eventually a 1.2-kb BglII-HindIII fragment that transferred the expression of the antigen (Fig. 4).

**Expression of Gene P198.** The 5-kb HindIII fragment that transferred the expression of antigen P198 was used to probe a Northern blot prepared with poly(A)\(^+\) RNA isolated from variant P198. A single band of 0.7 kb was observed. A band of similar size and intensity was revealed on a parallel lane containing RNA of P1, which does not express the antigen. The expression of the antigen by variant P198 is therefore not due to the activation of a silent gene.

**Structure of Gene P198.** Using the 1.2-kb BglII/HindIII fragment as a probe, we screened \(3 \times 10^8\) phages of a \(\lambda\)gt10 cDNA library derived from P1. 15 positive clones were found. We sequenced the longest completely processed cDNA, which comprised 649 bases before the polyadenylation site. To ensure that we had the complete 5' end sequence of the message, we applied a modification of the polymerase chain reaction developed by Frohman et al. (28). This indicated that
the full-length processed mRNA contains 653 bases. Comparison of the cDNA sequence with that of cosmid C198.3.1 recovered from the transfectant indicated that this cosmid lacked the 3' end of gene P198. A cosmid that appeared to carry the complete gene was identified in a library derived from P1 by using the 1.2-kb BgIII/HindIII probe. The complete sequence of the gene was obtained by sequencing overlapping fragments of the two cosmids.

Gene P198 is ~3 kb long and comprises eight exons bordered by consensus splicing sites (Fig. 5). The longest open reading frame present on the P198 cDNA sequence codes for a 23.5-kD protein of 203 amino acids. The complete sequence of the gene is shown in Fig. 6. The cap site indicated there corresponds to the 5' end of the cDNA clones expanded by polymerase chain reaction. It was confirmed by S1 nuclease and ribonuclease protection assays. A GATAA sequence showing some homology with the consensus TATA box and two GC-rich regions are located, respectively, 33, 204, and 263 nucleotides upstream of this site. The open reading frame starts 18 nucleotides after the cap site at an ATG codon surrounded by a consensus initiation sequence (32). It terminates at a TGA codon located in exon 8. A short 3' untranslated region (27 bases) contains a polyadenylation signal located 20 bases before the poly(A) addition site.

The sequence of gene P198 shows no homology with previously cloned tum- gene P91A, nor with any gene presently recorded in data banks.

Identification of a Point Mutation in the Antigenic Allele of P198. Southern blot analysis was performed on P1 and P198 DNA with two probes derived from gene P198. Probe a, which comprised exon 1 (see Fig. 5), hybridized to a single homologous fragment on P1 and P198 DNA. Probe b, which comprised only exon 7 (see Fig. 5), hybridized to several different bands, suggesting that several genes contain homologous sequences. For both probes, no additional band was observed in the P198 lanes, and all the bands of P1 and P198 coincided. This makes it unlikely that a rearrangement of the gene was responsible for the expression of the antigen.

The 1.2-kb BgIII/HindIII transfecting fragment of cosmid C198.3.1 comprised exons 3–7. The sequencing of the homologous fragment from the cosmid identified in the P1 library revealed a point mutation in exon 7: the guanine residue located in position 4114 in the normal allele was replaced by an adenine in the antigenic allele. This transition directs a change from alanine to threonine in the protein encoded by the long open reading frame.

By site-directed mutagenesis, we demonstrated that this mutation was responsible for the expression of the antigen.
An M13 construct containing the 1.2-kb fragment of the normal allele was mutated with an oligonucleotide carrying the mutation. A resulting clone, which carried the mutation, was reached at a concentration ~10 nM (Fig. 8 A). We verified that the effect of this peptide was specific: it did not induce considerable lysis when they were incubated in the presence of other peptides.

**Identification of an Antigenic Peptide Recognized by Anti-P198 CTL.** Because of the compelling evidence that most CTL recognize short peptides bound to class I MHC molecules, we examined whether synthetic peptides corresponding to various parts of the putative P198 protein could render P815 cells susceptible to lysis by anti-P198 CTL. We were guided by the location of the turn mutation. Six different peptides containing the mutated amino acid were tested (Fig. 7). Peptide P198-14-24, which comprised amino acids 9–24 of exon 7, and also various shorter peptides, induced considerable lysis when they were incubated in the presence of P1.HTR cells. The most active was P198-14-24: a concentration of 0.7 nM produced half of the maximal lysis, which was reached at a concentration ~10 nM (Fig. 8 A). We verified that the effect of this peptide was specific: it did not induce the lysis of P1.HTR by anti-P198 CTL (Fig. 8 A).

**Figure 6.** Sequence of gene P198. The numbering of the nucleotides corresponds to the scale of Fig. 5. The exons are numbered from 1 to 8. A possible TATA box and two GC-rich regions located before the first exon are underlined. The location of the point mutation (A in the antigenic allele instead of G in the normal allele in position 4114) is indicated by an asterisk. The sequence of antigenic peptide P198-14-24 is boxed. The polyadenylation signal and the start of the poly-A tail are indicated. The amino acid sequence corresponds to the amino acid sequence of an M13 construct containing the 1.2-kb fragment of the normal allele was mutated with an oligonucleotide carrying the mutation. A resulting clone, which carried the mutation, was transferred the expression of an antigenic peptide recognized by anti-P198 CTL. Because of the compelling evidence that most CTL recognize short peptides bound to class I MHC molecules, we examined whether synthetic peptides corresponding to various parts of the putative P198 protein could render P815 cells susceptible to lysis by anti-P198 CTL. In this search, we were guided by the location of the turn mutation. Six different peptides containing the mutated amino acid were tested (Fig. 7). Peptide P198-9–24, which comprised amino acids 9–24 of exon 7, and also various shorter peptides, induced considerable lysis when they were incubated in the presence of P1.HTR cells. The most active was P198-14–24: a concentration of 0.7 nM produced half of the maximal lysis, which was reached at a concentration ~10 nM (Fig. 8 A). We verified that the effect of this peptide was specific: it did not induce the lysis of P1.HTR cells by a CTL clone directed against another turn antigen (data not shown). The polyadenylation signal and the start of the poly-A tail are indicated. The amino acid sequence corresponds to the open reading frame. This sequence has been submitted to the EMBL/GenBank Data Library and has the accession number X51528.
Table 1. Expression of Antigen P198 after Site-directed Mutagenesis

| Transfected fragments* | Lysis of neo'-transfected cells with anti-P198 CTL† | Clones expressing P198 neo' clones (per/§) |
|------------------------|-----------------------------------------------|------------------------------------------|
|                        | E/T = 3                                      | E/T = 1                                  |
| Fragment of C198.3.1 (origin P198): A at position 4114 | 33                                          | 17                                       | 11/17                                    |
| Fragment of C1.198.1 (origin P1): G at position 4114 | 0                                           | 0                                        | 0/17                                     |
| Fragment of C1.198.1 with G4114 replaced by A | 38                                          | 19                                       | 7/17                                     |

* Fragments corresponding to position 2983-4172 of the gene (Fig. 6) were isolated from cosmids C198.3.1 and C1.198.1 and subcloned in M13tg130. The fragment isolated from C1.198.1 was submitted to site-directed mutagenesis to replace the G in position 4114 by A. The constructs were cotransfected with pSVtkneoβ into P1.HTR.

† The geneticin-resistant transfectants were submitted to a 4-h 51Cr release assay with CTL-P198:6 at various E/T ratios.

§ The geneticin-resistant transfectants were subcloned, and 17 clones were then tested for their lysis by CTL-P198:6 with a visual assay.

The ability to bind to the MHC molecule. Peptide P198-16-24 did not compete with peptide P198-14-24, indicating that the tyrosine in position 15 is required for MHC binding (Fig. 8 B).

In agreement with antibody inhibition experiments that indicated that Kd was the restricting element of antigen P198 (33), we found that DAP cells (H-2d) that had been transfected with Kd were lysed by anti-P198 CTL in the presence of the peptide, whereas DAP cells transfected with Dd or Ld were not lysed (Fig. 9).

**Effect of the tum- Mutation.** Synthetic peptide P198+.14-24, corresponding to the normal sequence of the gene, did not induce the lysis of target cells by anti-P198 CTL, even at very high concentrations (Fig. 8 A). This could be due either to the inability of the peptide to bind to the class I-presenting molecule, or to the absence of the epitope recognized by the anti-P198 CTL. To distinguish between these two possibilities, we performed competition experiments (Fig. 8 B): peptide P198+.14-24 was clearly able to inhibit competitively the lysis induced by homologous peptide P198-14-24, indicating that the P198 tum- mutation does not create the aggretope for Kd binding, but rather a new epitope. As a specificity control, we verified that peptide P198+.14-24 had no inhibitory effect on the lysis of tum- variant P91 by the relevant CTL clone CW3/701.1 and peptide

![Figure 7](image-url)
Lysis of P1.HTR cells

![Graph A]

Figure 8. Induction of lysis and competition by synthetic P198 peptides. (A) Lysis of P1.HTR cells by CTL-P198:6 in the presence of increasing concentrations of peptides encoded by the mutated or normal allele of gene P198. 3Cr-labeled cells were incubated with CTL-P198:6 for 4 h at an E/T ratio of 10:1 in the presence of various concentrations of the peptides. (B) Comparison of peptides P198-14-24, P198-15-24, and P198-16-24 as competitors for peptide P198-14-24. 3Cr-labeled P1.HTR cells were preincubated for 15 min at 37°C with increasing concentrations of the competitor peptides before adding peptide P198-14-24 at a concentration of 6 nM. The cells were then incubated for 4 h with CTL-P198:6 at a 10:1 E/T ratio. (C) Comparison of peptides P198-14-24 and P198-14-24 as competitors for peptide HLA-CW3 170-182 inducing lysis by Kd-restricted CTL-CW3/701.1. The experiment was performed as in B, except that the CW3 peptide was added at 100 nM and that the CTL to target ratio was 3:1.

Discussion

We have isolated the gene that codes for tum- antigen P198. This antigen was defined as the target of CTL clones specific for tum- variant P198. It is also relevant to the rejection of HLA-CW3 170-182 (Fig. 8 C) [34]. In agreement with the results obtained for anti-P198 lysis, both were shown to be effective competitors.

Figure 9. Identification of Kd as presenting molecule for antigen P198. Lines T483, T191, and T111 were derived from H2k fibroblast line DAP by transfection with plasmids containing the genes coding for Dd, Kd, or Ld. 2,000 3Cr-labeled target cells were preincubated for 45 min with increasing concentrations of peptide P198-14-24 before a 4-h Cr release assay with CTL-P198:6 at an E/T ratio of 10:1. All the target cells, except P1.HTR, were incubated in the presence of 25% of secondary allogeneic MLC supernatant during the 3 d preceding the test.

Figure 8. Induction of lysis and competition by synthetic P198 peptides. (A) Lysis of P1.HTR cells by CTL-P198:6 in the presence of increasing concentrations of peptides encoded by the mutated or normal allele of gene P198. 3Cr-labeled cells were incubated with CTL-P198:6 for 4 h at an E/T ratio of 10:1 in the presence of various concentrations of the peptides. (B) Comparison of peptides P198-14-24, P198-15-24, and P198-16-24 as competitors for peptide P198-14-24. 3Cr-labeled P1.HTR cells were preincubated for 15 min at 37°C with increasing concentrations of the competitor peptides before adding peptide P198-14-24 at a concentration of 6 nM. The cells were then incubated for 4 h with CTL-P198:6 at a 10:1 E/T ratio. (C) Comparison of peptides P198-14-24 and P198-14-24 as competitors for peptide HLA-CW3 170-182 inducing lysis by Kd-restricted CTL-CW3/701.1. The experiment was performed as in B, except that the CW3 peptide was added at 100 nM and that the CTL to target ratio was 3:1.

The transfection approach, which previously ensured the cloning of the gene of tum- antigen P91A, proved to be applicable to antigen P198. Among the P1.HTR cells that had integrated genomic DNA from the tum- variant, transfec-
tants expressing antigen P198 were found at a frequency of 1/13,000. A similar frequency was observed with gene P91A (13). Considering that the-mammalian genome comprises ~6 x 10^6 kb, that tum- mutants are heterozygous for the mutation, and that transfec-
tants are estimated to incorporate ~1,000 kb of DNA (35), the theoretical frequency is 1/6,000. It appears, therefore, that P1.HTR transfec-
tants express tum- genes efficiently and that detection by CTL stimulation is also efficient. In the library of 400,000 cosmids prepared with DNA of variant P198, three were found to transfer the expression of the antigen. This compares with 2/700,000 for the P91A cosmid library (14). These values are also close to the theoretical frequency, which is ~1/150,000. The direct recovery of a cosmid carrying the tum- gene proved to be more arduous for P198 than for P91A. Direct packaging of one of three cosmid transfec-
tants produced a major cosmid species carrying gene P91A. For P198, none of five cosmid transfec-
tants did, and we had to use a secondary transfec-
tant to obtain a cosmid carrying the gene. Because direct packaging of a transfected gene requires insertion of the gene between two cos sites in the same orien-
tation, and at a distance of 40–50 kb, it is not surprising that only a minority of the cosmid transfectants lend themselves to direct cosmid recovery. Nevertheless, the results obtained in the cloning of gene \(P198\) are on the whole very similar to those obtained with \(P91A\). These and similar results obtained with a third tum\(^-\) gene (36) show that our approach should be applicable to many transplantation antigens.

In the course of the cloning of gene \(P198\), we repeated a surprising observation made with gene \(P91A\). Promoterless gene fragments that were cloned in vectors that are not eucaryotic expression vectors were found to transfer efficiently the expression of the antigen. This may apply only to transfected DNA, due for instance to preferential insertion into the actively transcribed regions. On the other hand, it may reflect a new mechanism leading to the production of antigenic peptides by a translation process that starts shortly before the region encoding the antigenic peptide (37, 38).

The antigenic allele of gene \(P198\) differs from the normal allele by a point mutation in exon 7. This mutation is responsible for the antigenicity, as shown by site-directed mutagenesis, and it appears to be the only difference between the two alleles. A short synthetic peptide corresponding to the sequence surrounding the Thr residue introduced by the tum\(^-\) mutation was found to render P1 cells sensitive to lysis by anti-\(P198\) CTL. This demonstrates that gene \(P198\) is the structural gene of the antigen. A similar situation was reported for tum\(^-\) gene \(P91A\), there also, an antigenic peptide was encoded by the region surrounding the tum\(^-\) mutation (15). However, the role of the \(P198\) tum\(^-\) mutation appears to be quite different from that of the \(P91A\) mutation. For \(P91A\), the homologous peptide corresponding to the normal sequence of the gene does not induce lysis of P1 by the anti-\(P91A\) CTL and does not compete with the antigenic peptide. We concluded that this tum\(^-\) mutation makes the peptide capable of binding to the presenting molecule, which is L\(^\alpha\). For antigen \(P198\), the peptide encoded by the normal allele does not render P1 cells sensitive to lysis by the CTL, but it competes with the antigenic peptide for binding to K\(^\alpha\). It appears, therefore, that the \(P198\) tum\(^-\) mutation generates a new epitope on a peptide that is already capable of binding to the K\(^\alpha\) molecule. The normal peptide is presumably not recognized by the T lymphocytes because of natural tolerance.

The structure of gene \(P198\) is completely different from that of MHC or Ig genes. It is also unrelated to the gp96 antigenic protein found on methylcholanthrene-induced tumor methA (39). Finally, it bears no homology with gene \(P91A\) nor with the gene that encodes tum\(^-\) antigen P35B (36). These results suggest that a large number of genes are potential sources of tum\(^-\) antigens. Thus, we now have an explanation of the combination of stability, very high frequency, and diversity of tum\(^-\) variants. These variants are stable because they are mutants. They are very diverse, because their antigens are derived from many, possibly all, the genes of the mammalian genome, and they are extremely frequent, presumably because new antigenic peptides are generated by a significant proportion of the mutations affecting all these genes.

It is tempting to believe that tumor-specific transplantation antigens also arise through genetic mechanisms acting on a large variety of genes. Like tum\(^-\) antigens, tumor-specific transplantation antigens are very diverse (39), and carcinogens have mutagenic effects. But to prove this, it will be necessary to clone the genes coding for TSTA. This may be possible with the approach used for tum\(^-\) antigens. Minor histocompatibility antigens may also result from random mutations throughout the genome. The first gene coding for a minor histocompatibility antigen has recently been isolated. It codes for a mitochondrial protein and it is located in the mitochondrial genome, resulting in maternal inheritance (40). Here also, the different alleles differ by point mutations (41). Remarkably, this antigen is presented by a class I molecule that is not located in the MHC complex.

The demonstration that CTL can recognize peptides encoded by genes producing viral proteins that remain intracellular (42, 43) opened the possibility that T cells could exert surveillance over all cellular proteins, whether or not they are located on the cell surface. Our results demonstrate that this immunosurveillance effectively occurs: mutations occurring throughout the mammalian genome appear to generate very frequently new antigenic peptides recognized by T cells. T cell immunosurveillance of the integrity of the mammalian genome is therefore not an hypothesis anymore but a demonstrated process, whose mechanism is beginning to be understood.

This is paper XII of a series; paper XI is Szikora et al. (36).

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