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Immunology

Immunogenic (tum$^-$) variants of mouse tumor P815: Cloning of the gene of tum$^-$ antigen P91A and identification of the tum$^-$ mutation*

(tumor immunology/cosmid/mastocytoma P815)

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ABSTRACT **Mutagen treatment of mouse P815 tumor cells produces tum$^-$ variants that are rejected by syngeneic mice because these variants express new surface antigens. These "tum$^-$ antigens" are recognized by cytolytic T lymphocytes but induce no detectable antibody response. Transfection of P815 cell line P1.HTR with DNA of tum$^-$ variant P91 yielded transfectants expressing tum$^-$ antigen P91A. They were detected by their ability to stimulate proliferation of cytolytic T lymphocytes [Wölfel, T., Van Pel, A., De Plaen, E., Lurquin, C., Maryanski, J. L. & Boon, T. (1987) *Immunogenetics* 26, 178–187]. A cosmid library of a cell line expressing antigen P91A was transfected into P1.HTR. Transfectants expressing the antigen were obtained. By packaging directly the DNA of a transfectant with λ phage extracts, we obtained a small cosmid population containing as major component a cosmid that transferred the expression of P91A. The assay of various restriction fragments of this cosmid led to the isolation of an 800-base-pair fragment containing the P91A sequence required for transfection. Comparison with a homologous cDNA showed that this fragment contained only one of the several exons of the P91A gene. The normal and the tum$^-$ forms of the gene differ by one nucleotide located in this 137-base-pair exon. The essential role of this mutation, which produces an amino acid change, was confirmed by site-directed mutagenesis. No significant sequence similarity was found between the 800-base-pair fragment and any recorded gene.**

In vitro mutagen treatment of mouse tumor cell lines generates at high frequency immunogenic variants that are rejected by syngeneic mice (1). Since they fail to form tumors, these stable variants have been named "tum$^-$" as opposed to the original "tum$^+$" cell, which forms progressive tumors. Most tum$^-$ variants express transplantation antigens not found on the tum$^+$ cell. The existence of these "tum$^-$ antigens" can be demonstrated *in vivo* by cross-immunization experiments or *in vitro* with cytolytic T lymphocytes (CTLs) (2).

We have studied a series of tum$^-$ variants derived from mastocytoma P815, a tumor induced in a DBA/2 mouse with methylcholanthrene (3). 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 (4). Despite numerous attempts, we failed to obtain antibodies against these variant-specific antigens.

The diversity of tum$^-$ antigens is considerable: the analysis of 15 P815 tum$^-$ variants demonstrated the existence of a different antigen on each of them (5). To understand the underlying genetic mechanism, we undertook the cloning of the "tum$^-$ genes" that determine the expression of tum$^-$ antigens. As a first step, we attempted DNA-mediated transfer of antigen P91A, one of the two tum$^-$ antigens of P815 variant P91. Other workers have described the production of transfectants for surface antigens (6, 7), but in these instances, the antigen-expressing transfectants were detected with antibodies. For tum$^-$ antigens, we had to develop procedures ensuring detection with CTLs. This required a DNA-recipient cell line that was of *H-2^d* haplotype like P815 and that showed good sensitivity to CTL lysis. The P815 tum$^+$ clonal line P1 fulfilled those requirements but proved unsuitable because of its very low transfection efficiency. Fortunately, a highly transfectable P815 cell, P1.HTR, could be selected by repeated transfection cycles (8). We then developed a detection test based on the ability of some anti-tum$^-$ CTL clones to be stimulated by P815 cell populations containing only 3% of the relevant tum$^-$ variant. This test ensured reliable screening of several thousand transfectants.

By cotransfecting P1.HTR cells with DNA of tum$^-$ variant P91 and plasmid pSVtk-neoβ, we obtained transfectants expressing tum$^-$ antigen P91A at a frequency of 1/13,000 among the transfectants expressing the neomycin-resistance (Neo^r) phenotype (9). We found one secondary transfectant, P91.T6.T1, that had received both the *neo^r* and the P91A sequence from the primary transfectant (Fig. 1). But in a λ phage library prepared from this transfectant, none of the phages that carried the *neo^r* marker had the ability to transfer the expression of P91A. As we had observed that the expression of P91A could be transferred by fragments of P91 DNA that were smaller than 30 kilobase pairs (kb) (9), we decided to transfect P1.HTR with the DNA of a cosmid library obtained from a transfectant expressing antigen P91A. Our aim was to obtain transfectants from which the P91A sequence could be recovered with the help of the cosmid sequences.†

MATERIALS AND METHODS

Cell Lines. Fig. 1 summarizes the tumor-cell lines and transfectants. The derivation from mastocytoma P815.X2 of clonal line P1 (tum$^+$) and of tum$^-$ variant P91 by treatment of P1 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was described earlier (3). The highly transfectable line P1.HTR.tk$^-$

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Abbreviations: CTL, cytolytic T lymphocyte; Neo^r, neomycin resistance; Amp^r, ampicillin resistance; Tet^r, tetracycline resistance; HmB^r, hygromycin B resistance.

*This is paper IX of a series; paper VIII is ref. 9.

†The P91A sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Lab., Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03652).

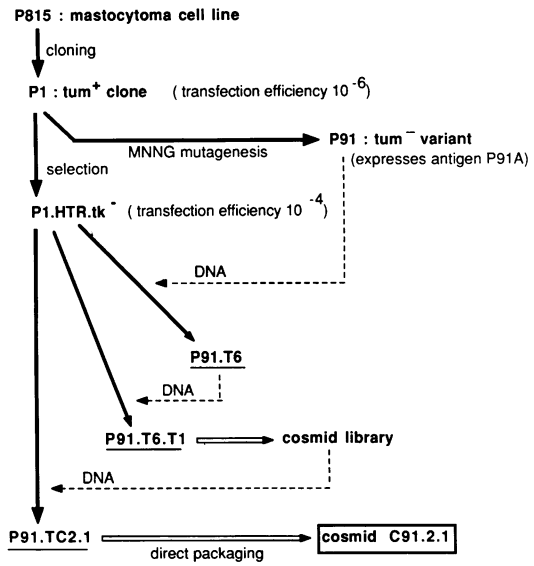

FIG. 1. Tumor cell lines and transfectants expressing antigen P91A (underlined). MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

(referred to below as P1.HTR) was derived from P1 by repeated cycles of transfection (8).

Construction and Amplification of Cosmid Libraries. Cosmid arms of vector c2RB (Fig. 2 and ref. 10) were obtained by cleavage with restriction endonuclease *Sma* I and treatment with calf intestinal phosphatase followed by digestion with *Bam*HI. Genomic DNA (100 μg/ml) was digested with *Mbo* I (0.05 unit/μg) for three time points as described by Grosveld *et al.* (11). The preparations were pooled and centrifuged on a NaCl gradient (11). Fractions containing DNA of 35–50 kb (1.5 μg) were added to cosmid arms (1.5 μg), coprecipitated, resuspended in 10 μl of ligation buffer (130 mM Tris Cl, pH 7.5/10 mM $MgCl_2$/10 mM dithiothreitol/2 mM ATP), and incubated at 14°C for 15 hr with 4 units of bacteriophage T4 DNA ligase (Amersham). The ligated DNA was packaged with Gigapack extracts (Stratagene). The product was titrated on *Escherichia coli* ED8767 as described (11). We obtained 2 × 10^5 to 1 × 10^6 Amp^r colonies per μg of insert DNA.

To amplify the cosmid groups, packaging mixtures producing ≈50,000 cosmid colonies 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 without shaking for 1 hr. The suspension was then titrated, diluted with 40 ml of LB medium containing ampicillin (25 μg/ml), and incubated under strong aeration for 8–15 hr. One-third of the culture was used to inoculate 2 liters of LB medium in the presence of ampicillin (25 μg/ml). When the bacterial concentration reached 2 × 10^8 cells per ml (OD_{600} = 0.8), chloramphenicol (170 μg/ml) was added. Total cosmid DNA was then isolated by the alkaline lysis procedure and purified on CsCl gradients (12).

Transfection. Transfections were performed on P1.HTR by the calcium phosphate precipitation method, as described (9). For the transfection of the c2RB cosmid library, groups of 5 × 10^6 P1.HTR cells were treated with 60 μg of DNA of the amplified library and 2 μg of cosmid pHMR272 (13). Transfectants were selected with hygromycin B (350 μg/ml). For transfection with isolated plasmids, cells were treated with 10 μg of DNA and 2 μg of pSVtk-neoβ (14) and selected with neomycin analog G418 (1.5 mg/ml).

The detection of transfectants expressing antigen P91A was described before (9). In brief, antibiotic-resistant transfectants were divided in microcultures of 30 cells. When they had grown to 6 × 10^4 cells, they were duplicated and 500 cells from CTL clone CTL-P91:6 were added. Stimulation was ascertained 5 days later by visual observation of CTL proliferation and by measuring the specific lytic activity of the CTL content of every microculture. The duplicates of positive microcultures were subcloned and cells expressing the P91A antigen were identified by visual observation of their lysis by CTL-P91:6 (9). Positive clones were confirmed by assay of chromium release from ^{51}Cr-loaded cells.

Cosmid Rescue. For direct packaging, high molecular weight DNA of transfectants was packaged in Gigapack extracts. Infection of ED8767 and selection of Amp^r colonies were as above.

For rescue with cosmid arms of pTL6, the two types of arms of pTL6 (15) were prepared by digestion with either *Pvu* II or *Bst*EII, treatment with phosphatase, and digestion with *Bgl* II. DNA of transfectants was partially digested with *Mbo* I and fragments of 50–100 kb were prepared by centrifugation (11). Cosmid arms (1.5 μg) were mixed with 1.5 μg of insert DNA. After ligation, packaging, and infection of ED8767, Tet^r or Amp^r colonies were selected to recover the cosmids obtained with *Bst*EII arms and *Pvu* II arms, respectively (see Fig. 2).

cDNA Libraries. Total cellular RNA was purified by the LiCl/urea method (16). cDNA libraries in bacteriophage λgt10 were prepared as described (17).

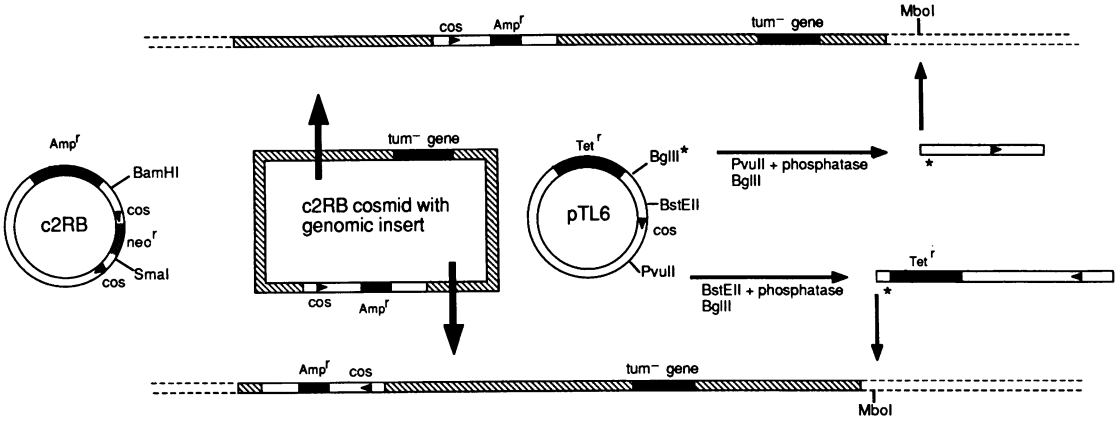

FIG. 2. Relative orientation of the tum⁻ gene, cohesive-end (*cos*) site, and ampicillin-resistance (Amp^r) marker in the transfectant according to where the circular cosmid is opened during transfection (large arrows). Each configuration can be rescued only by one pTL6 arm as indicated. The orientation of the *cos* site of pTL6 relative to those of c2RB was determined by examining the yield of cosmids obtained by ligating DNA partially digested with *Mbo* I with each of the four possible combinations involving one c2RB and one pTL6 arm. Tet^r, tetracyline-resistance marker.

Site-Directed Mutagenesis. The mutagenic oligonucleotide d(CTCAGAACCACCGAGCCC), synthesized by Eurogentec (Liège, Belgium), was used to modify the sequence cloned in bacteriophage M13 by the method of Nakamaye and Eckstein (Amersham kit) (18).

RESULTS

Transfection of Cosmids in P1.HTR Cells. To obtain transfectants representative of a complete cosmid library (700,000 cosmids), it appeared necessary to produce ≈35,000 independent transfectants, assuming an average integration of 20 cosmids. Since P1.HTR has a transfection efficiency of 10^{-4}, this implied that $>3.5 \times 10^8$ cells must be submitted to transfection (8). Previous experience had shown that groups of 5×10^6 cells are optimally transfected with 60 μg of DNA. It was therefore necessary to produce more than 4 mg of DNA from the cosmid library. This enormous amplification (10^8) led us to choose the double cohesive end (*cos*)-site cosmid vector c2RB, which can be conveniently produced in large amounts (10). High molecular weight DNA of P91.T6.T1, a secondary transfectant that expresses antigen P91A, was partially digested with *Mbo* I and 40- to 50-kb fragments were ligated to the *Bam*HI site of c2RB cosmid arms. The library was divided into 19 groups of 35,000 independent cosmids. Each group was considerably amplified and DNA was extracted. In the amplified population, 60% of the cosmids had retained inserts of more than 35 kb, whereas the others had lost them.

DNA from each cosmid group was cotransfected into P1.HTR with pHMR272, which confers resistance to hygromycin B (HmB^r) (13). Approximately 3000 HmB^r transfectants per group were tested for their ability to stimulate proliferation of an anti-P91A CTL clone, and the positive transfectants were confirmed by a lytic assay as described (9). Two of the 19 cosmid groups produced transfectants expressing P91A. Group 7 produced one P91A transfectant (P91.TC7.1) out of 3000 HmB^r transfectants. When group 2 proved positive, additional cells were transfected, so that three P91A transfectants P91.TC2.1, -2, and -3 were obtained from a total of 10,000 HmB^r cells. A Southern blot (19) performed with the DNA of the four transfectants expressing P91A showed that they had integrated a number of cosmids ranging from 6 to more than 30.

Rescue of a Cosmid Transferring the Expression of Antigen P91A. Lau and Kan (20) reported that a cosmid containing a globin gene, which had been used to obtain transfectants, could be recovered by packaging directly the DNA of these transfectants into λ phage heads. Presumably, this was made possible by occasional tandem integration of two cosmids producing a pair of *cos* sites that are separated by 39–52 kb of DNA, the size that can be packaged in λ phage heads. By packaging 1 μg of DNA of transfectant P91.TC2.1, we obtained about 240 cosmid colonies selected with the Amp^r marker of c2RB (Table 1). Cosmid DNA was prepared from 16 colonies. Nine of these produced the same 37-kb cosmid, C91.2.1. When it was cotransfected into P1.HTR cells with pSVtk-neoβ, 80% of the Neo^r transfectants were found to express antigen P91A (Table 2). Direct packaging of the DNA of P91.TC2.3 and P91.TC7.1 produced several thousand cosmids per microgram of DNA, a number in line with the higher number of cosmids integrated in these transfectants (Table 1). However, no transfectant expressing antigen P91A was obtained with these cosmids. In agreement with this negative result, no positive colony was identified when these cosmids were hybridized later with the *Bam*HI–*Pvu* II fragment of cosmid C91.2.1 (Fig. 3).

The success of the "direct packaging" with the DNA of some but not all transfectants was anticipated, since one does not expect every transfected tum^- gene to be integrated between two adequately spaced *cos* sites. But we wanted a procedure whereby we could recover tum^- sequences integrated near a *cos* site irrespective of the nearby presence of a second *cos* site and of their position relative to the first *cos* and the drug-resistance marker of the cosmid. Lund *et al.* (15) have described such a method, which involves the ligation of new cosmid arms to partially digested DNA of the transfectant. As shown in Fig. 2, two types of cosmid arms can be produced from cosmid pTL6, and each of these arms should be able to rescue the tum^- sequence integrated in one of the two main possible configurations. The results obtained with cosmid transfectants P91.TC2.1, -TC2.3, and -TC7.1 are shown in Table 1. Cosmids containing the P91A tum^- sequence could be rescued from each transfectant, with either one or the other arm of pTL6.

Table 1. Recovery of cosmids from transfectants

Transfectant	No. of cosmid copies	No. of cosmids obtained per μg of transfectant DNA		
		Direct packaging	pTL6 rescue	
			*Bst*EII arm	*Pvu* II arm
P91.TC2.1	6	240*	45*	5
P91.TC2.3	>30	28000	1700	650*
P91.TC7.1	30	5900	2060	380*

*Group where cosmids carrying P91A sequences were observed. Cosmids obtained by direct packaging and those rescued from P91.TC2.1 with the *Bst*EII arm of pTL6 were tested by transfection. All the groups obtained from P91.TC2.3 and P91.TC7.1, either by direct packaging or by pTL6 rescue, were tested by hybridization with the *Bam*HI–*Pvu* II fragment of C91.2.1 (Fig. 3).

Isolation of a 0.8-kb Fragment Transferring the Expression of P91A. The restriction map of cosmid C91.2.1 (Fig. 3) showed that the structure was more complex than anticipated: it contains two noncontiguous cosmidic regions and confers the HmB^r phenotype. Presumably, one of the two *cos* sites that allowed packaging was provided by cotransfected cosmid pHMR272.

The ability of C91.2.1 to transfect the expression of the tum^- antigen was not abolished by digestion with restriction enzymes *Bam*HI, *Hin*dIII, and *Bgl* II. A number of restriction fragments of C91.2.1 were transfected into P1.HTR, either directly or after cloning in suitable plasmids. A large *Bam*HI fragment (13.8 kb) and a large *Hin*dIII fragment (17.5 kb) produced transfectants expressing antigen P91A (Fig. 3). These two fragments overlap by only 3.9 kb; the essential

Table 2. Transfection of P91A fragments and homologous fragments of the normal gene

DNA*	Clones expressing P91A,† no./no. of Neo^r clones
Cosmid	
C91.2.1 (P91)	40/48
C1.91A.1 (P1)	0/41
3.9-kb *Bam*HI–*Hin*dIII fragment	
C91.2.1	35/38
C1.91A.1	0/31
0.8-kb *Pst* I–*Pvu* II fragment	
C91.2.1	26/48
C1.91A.1	0/48
Site-specific mutant	
M13.mut.2 (adenine at 274)	33/96
M13.mut.3 (guanine at 274)	0/96

*The 3.9-kb and 0.8-kb fragments of cosmids C91.2.1 and C1.91A.1 were cloned in vectors c2RB and M13tg130, respectively. M13.mut.2 and -3 were products of site-directed mutagenesis of the 0.8-kb fragment of C1.91A.1.

†Clones expressing the P91A antigen were detected by a visual lysis test with CTL-P91:6.

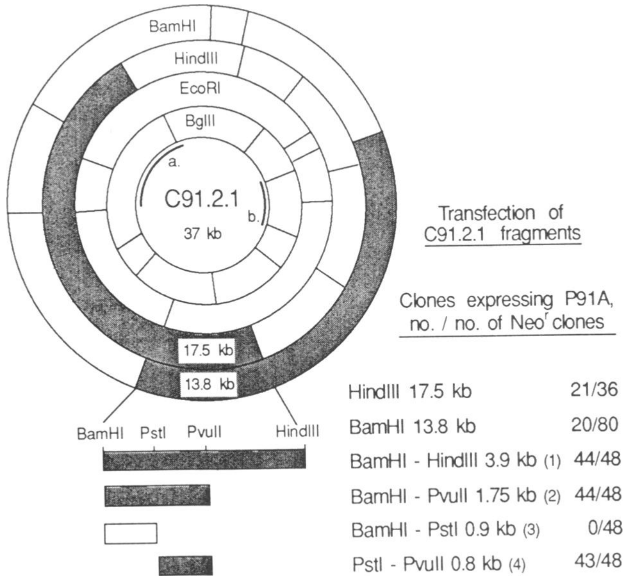

FIG. 3. Restriction map of C91.2.1. Solid lines indicate the location of the cosmid sequences (a and b). The HmB^r gene is located in region a. Fragments of C91.2.1, not cloned or cloned in c2RB, pBR322, pUC8, or pUC9 (as indicated by 1, 2, 3, and 4 in parentheses, respectively), were cotransfected with pSVtk-neoβ. The drug-resistant cells were cloned, and clones were tested for lysis by CTL-P91:6.

tum$^-$ sequence must be located in this overlap region. Expression of P91A could indeed be transfected by the 3.9-kb *Bam*HI–*Hin*dIII fragment. This fragment could be reduced further to a 1.75-kb and even to a 0.8-kb fragment that transferred the expression of the antigen (Fig. 3).

Structure of the 0.8-kb P91A Fragment. The sequence of the 0.8-kb *Pst* I–*Pvu* II fragment that transfers the expression of P91A is shown in Fig. 4. The exon/intron structure of the fragment was determined by comparing its sequence to that of homologous cDNA regions. cDNA libraries were derived with the λgt10 system (17) from P1 and from P91. They were screened by hybridization with the 1.75-kb *Bam*HI–*Pvu* II fragment of C91.2.1. A few positive P1 and P91 cDNA clones were obtained. By comparison with their sequence, we located on the genomic 0.8-kb fragment a single exon of 137 base pairs, bounded by consensus splice acceptor and donor sequences. Only one reading frame can be read through the entire exon. The corresponding amino acid sequence is shown in Fig. 4.

The hybridization pattern of several cDNA clones with various restriction fragments of cosmid C91.2.1 indicated that gene P91A is longer than 8 kb and contains several exons located both before and after the exon of the 0.8-kb fragment.

A single band of intensity corresponding to one copy was seen on Southern blots of *Eco*RI-digested P815 DNA hybridized with the 0.8-kb P91A fragment, even in low-stringency salt and temperature conditions. A computer search† failed to reveal sequence similarity between this fragment and recorded mouse or human gene sequences. The search was done using the FASTN program, with a *K*-tuple parameter of 3 and of 6 (21).

Presence of a Mutation in the P91A tum$^-$ Gene. To compare the sequence of the 0.8-kb transfecting fragment of the P91A gene with that of its normal homolog, we prepared a cosmid library from tum$^+$ cell line P1, which does not express antigen P91A. Approximately 700,000 cosmids were screened by hybridization with the 1.75-kb *Bam*HI–*Pvu* II fragment of cosmid C91.2.1. Four positive cosmids were identified. From one of those (C1.91A.1), we isolated a 3.9-kb *Bam*HI–*Hin*dIII fragment and a 0.8-kb *Pst* I–*Pvu* II subfragment that were clearly homologous to the transfecting fragments of C91.2.1. As expected, transfection with the fragments of P1 origin did not produce cells expressing antigen P91A (Table 2).

The sequence of the normal 0.8-kb fragment was found to differ from the P91A tum$^-$ fragment by only a single nucleotide located in the exon: a guanine residue in the normal gene is replaced by an adenine in the tum$^-$ sequence at position 274 (Fig. 4). This transition corresponds to an amino acid change from arginine to histidine. To confirm that this mutation was sufficient to cause the expression of antigen P91A, we carried out site-directed mutagenesis on the 0.8-kb fragment of P1 origin cloned in M13 with an oligonucleotide representing the mutant sequence centered around position 274 (18). Phages were obtained that had acquired the tum$^-$ mutation and others that had kept the normal sequence, as seen by differential hybridization with oligonucleotides carrying either the normal or the mutant sequence. M13.mut.2, which was sequenced to verify that it carried the tum$^-$ mutation (adenine in position 274), transferred the expression of antigen P91A (Table 2). M13.mut.3, whose sequence had remained normal (guanine in position 274), did not.

†EMBL/GenBank Genetic Sequence Database (1987) EMBL Nucleotide Sequence Data Library (Eur. Mol. Biol. Lab., Heidelberg), Tape Release 12.

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PstI                                           50                                                100
CTGCAGCGCCACACTTGGTTACTTATGCATTCTTCAGCTACCAAGAAATACTGGGCTTATTTTAAATGGTGCAGGTCCCAGCGTTTGTCCATAGGCTTTG

                                               150                                               200
TGTGCAACTCTTTGCAGTTCTCTTGGATATGCACCTAGGAACAGAATTGCACGCTCATGATATTTCCTCTGTGACTTTTGGAGGACTTACACTTGCTTCT

                                                        250                                 ▼
CTTTTGCCTCCCGACCCCTGCCAG GCA CAG AAA ATC TCT GAT GAC CTG ATG CAG AAG ATC AGC ACT CAG AAC CAC CGA GCC
                         Ala Gln Lys Ile Ser Asp Asp Leu Met Gln Lys Ile Ser Thr Gln Asn His Arg Ala

                     300                                                             350
CTG GAC CTC GTA GCT GCC AAG TGT TAC TAT TAT CAC GCC CGG GTC TAT GAG TTC CTG GAC AAG CTG GAT GTG GTG
Leu Asp Leu Val Ala Ala Lys Cys Tyr Tyr Tyr His Ala Arg Val Tyr Glu Phe Leu Asp Lys Leu Asp Val Val

                                               400                                               450
CGC AGGTATGGGGAGTCAAGGTCCCCGTGGATCAGAGCCCTGGTCATCACTAGTGCAGAGGTGTGGGTCTGGAGAGAGTGAGGAACAAGTGGGTGTGA
Arg
                                               500                                               550
GGGTCTGTGTTAGTCACTGTTCTGCTGCTGAGAGGGGACACCATCACCCGGGTGCTGTATGAAGGAAAACATTTCACTGGGGGCTTACCTACAGTTGTGG

                                               600                                               650
AGGTTTAGTCCATGATCGAGACAACTTGTTCAGGCTGGAGAATAGCTAGGAGCTGCGTCCTTAGCTGAGGGGCTGGGGACTGGCGTGGGCTTCTGAAATC

                                               700                                               750
TCAGTGACATGCTTCCTCCAACAAGCCACGCCTTCTAGTCCTTCTACGTCTGTGACCTCGTGCCCTCCTGGTGACTGAGCGCTCAGGTATCCAAGCCTGA

                                               800                          PvuII
TGGACAGTCTGTTCAGTCCACCCTGGGGGGCCCAGCAGGGAGCAAGCCCTGTGCTGAGGGAGTGGCAGCTG
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FIG. 4. Sequence of the 0.8-kb *Pst* I–*Pvu* II fragment of cosmid C91.2.1 that transfers the expression of tum$^-$ antigen P91A. The splice donor and acceptor sequences at the boundaries of the exon are underlined. The position of the tum$^-$ mutation (nucleotide 274) is indicated by ▼; it is occupied by a guanine in the normal form of the P91A gene. This fragment and the homologous fragment of cosmid C1.91A.1 were cloned in M13tg130 and M13tg131 and sequenced by the dideoxynucleotide chain-termination method.

Blot hybridization was performed with mRNA from both P1 and P91 cells. Hybridization with the 1.75-kb *Bam*HI–*Pvu* II fragment of gene P91A revealed a unique 2.2-kb band of similar intensity on the blots of both P1 and P91 origin (data not shown). The expression of tum$^-$ antigen P91A results therefore from a point mutation occurring on a protein that is already expressed in the original tum$^+$ cell.

DISCUSSION

Cosmid transfection and rescue combined with detection of antigen-expressing transfectants by CTL stimulation enabled us to clone the gene that determines the expression of tum$^-$ antigen P91A. Preliminary results suggest that these procedures will ensure the cloning of other tum$^-$ genes. We hope that they will also lead to the isolation of the genes of other transplantation antigens that are recognized by T cells but do not induce an antibody response. Most tumor-associated transplantation antigens, male-specific antigen H-Y, and minor histocompatibility antigens belong to this category (22).

Our approach was facilitated by the ability of small fragments of the P91A gene to transfer the expression of the antigen, as this lifted the requirement that the rescued cosmid must carry the entire gene. How this comes about is presently unclear. The 0.8-kb fragment appears to lack the transcriptional control sequences that could ensure autonomous expression. Possibly, some fragments integrate during transfection close to a promoter, so that a peptide encoded by the exon is produced. The recent work of Townsend *et al.* (23) on influenza antigens suggests that such a small peptide or part of it could associate with a class I histocompatibility molecule so as to be recognized by CTLs. An alternative possibility is that some of the transfected fragments engage in homologous recombination with the normal gene present in the recipient cell. This has been reported, but with much lower frequencies than those we observe (24). We cannot explain our high frequency by the presence of a large number of homologous genes, since the 0.8-kb P91A fragment hybridizes with a single band on Southern blots.

Our results begin to clarify the genetic nature of tum$^-$ variants. One paradox concerning these stable variants was their exceptionally high frequency: 1–20% in tumor cell populations reduced to 1% survival by mutagen treatment (5). This led us to consider the possibility that tum$^-$ variants arise through the expression of a gene that is normally silent. For tum$^-$ antigen P91A, this hypothesis is clearly ruled out by our results. The P91A gene of tum$^-$ variant P91 has undergone a point mutation, and the single mRNA species that hybridizes with the transfecting P91A sequence is also produced by the original P1 tum$^+$ cell. We therefore conclude that a protein that is already expressed in the tum$^+$ cell undergoes a structural change that results in the expression of a strong antigen recognized by CTLs. The simplest interpretation of our results is that we have isolated the structural gene for the P91A antigen. The recent work with influenza peptides (23) suggests a way of obtaining direct proof of the role of the P91A exon that contains the tum$^-$ mutation: evaluation of the ability of synthetic peptides corresponding to the region surrounding the mutation to render P1 cells sensitive to lytic attack by anti-P91A CTLs.

The relevance of the tum$^-$ antigens defined with CTLs to the rejection of tum$^-$ variants is amply demonstrated by two sets of observations. First, when variants that had lost their tum$^-$ antigen were selected *in vitro* with the appropriate CTLs, we observed that these antigen-loss variants had regained their ability to form tumors (25). Second, when we analyzed *in vitro* the cells of the rare progressive tumors that were obtained after injection of tum$^-$ variants, we found that they had lost tum$^-$ antigens. This was observed with antigen P91A (26). Moreover, we observed recently that P1 cells expressing antigen P91A after transfection with the P91A sequence produce tumors after considerably longer delay than that observed with the original P1 cells, even though the transfected cells expressed only one of the two tum$^-$ antigens of P91 (unpublished data).

The relation between P91A and genes of the major histocompatibility complex (H-2, in the mouse) deserved careful examination, because tumor-specific antigens of a UV-induced tumor were found to be H-2 class I variant molecules (27). It appears unlikely that P91A is related to H-2 because, in addition to the lack of sequence homology, the length of the mutated P91A exon is very different from that of the exons coding for external domains of class I and class II H-2 molecules. Structural data reported for tumor-specific antigens of tumor Meth A (28, 29) show no sequence homology with our P91A fragment.

It is of course impossible to predict that the mutational origin of tum$^-$ antigen P91A will also apply to other tum$^-$ antigens. However, on the basis of the present evidence, it appears reasonable to adopt the term tum$^-$ mutants rather than tum$^-$ variants.

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