The immunoprotective MHC II epitope of a chemically induced tumor harbors a unique mutation in a ribosomal protein

Toyoshi Matsutake and Pramod K. Srivastava*

Center for Immunotherapy of Cancer and Infectious Diseases, University of Connecticut School of Medicine, Farmington, CT 06030-1601

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CD4$^+$ T lymphocyte clones, generated from mice immunized with the methylcholanthrene-induced fibrosarcoma Meth A (H-2^d), are restricted by I-E^d and recognize a unique antigen on Meth A. The antigen has been purified and characterized as the ribosomal protein L11. The antigenic epitope is contained within the sequence EYELRK*H*NFSDTG and is generated by substitution of Asn by His (italic) caused by a single point mutation. The tumor contains the wild-type and the mutated alleles. Immunization of BALB/cJ mice with the mutated epitope but not with the wild-type epitope protects mice against a subsequent challenge with the Meth A sarcoma. Adoptive transfer of CD4$^+$ clones into BALB/c mice renders the mice specifically resistant to Meth A sarcoma. The mutated L11 epitope is thus shown to be an immunoprotective epitope *in vivo* by several criteria.

T cell response to tumors is critical for successful protective immunity to cancers. Depletion of T lymphocytes *in vivo* renders mice unable to be immunized (1), and adoptive transfer of T lymphocytes from tumor-immune mice can successfully transfer tumor immunity (2–9). These observations have led to efforts to identify the antigenic epitopes of cancers recognized by T lymphocytes. Most such efforts have been directed to identification of MHC I-presented cancer epitopes recognized by CD8$^+$ T lymphocytes (10–18), with a considerably smaller number of MHC II-presented epitopes recognized by CD4$^+$ T lymphocytes having been identified and characterized.

MHC II-presented human cancer epitopes, like their MHC I counterparts, have turned out to be a mixture of unmutated cancer testis antigens (19–21), differentiation antigens (22, 23), and mutated unique antigens restricted to an individual cancer (24–26). Only a single MHC II-presented cancer epitope of a murine cancer has been reported thus far (27). This is a mutated allele of a ribosomal protein L9. Although the human cancers are the obvious targets of immunotherapy, identification of antigenic epitopes of murine cancers permits their use in experimental models that allow far more experimental flexibility in a far shorter time than do clinical trials with humans (28). Indeed, the entire edifice of immunotherapy with the MHC I-presented epitopes of human cancers is built on the pioneering corresponding studies in a murine system (12).

We describe here the identification and characterization of the dominant MHC II-presented epitope of the chemically induced mouse sarcoma Meth A. This is the first identified MHC II-presented epitope of a chemically induced murine cancer; the only other identified MHC II-presented epitope of a murine cancer came from an UV-induced squamous cell carcinoma (27). The results show surprising and interesting similarities and differences between the two epitopes and their activities *in vivo*.

Materials and Methods

Generation and Maintenance of CD4$^+$ Lines and Clones. BALB/c mice were immunized s.c. twice 10 days apart with cell lysate or cytosol emulsified with complete Freund's adjuvant. Two weeks after the immunization, spleen cells (5×10^6 cells per well) were cultured in the presence of cell lysate or cytosol. Restimulation was performed in the presence of irradiated BALB/c antigen-presenting cells (APCs) and lysate and cytosol every 2 weeks. Sometimes CD4$^+$ T cells were purified by MACS column (Miltenyi Biotec, Auburn, CA). Specificity was checked by [^{3}H]thymidine uptake. T cell clones were obtained by limiting dilution (0.5–0.1 cell per well). During expansion, specificity was checked by [^{3}H]thymidine uptake or cytokine release in the supernatant by ELISA (Endogen, Cambridge, MA). Typically, the activity of the bulk CD4$^+$ T cell line is shown by thymidine incorporation, and the activity of clones is measured by IL-5 release.

Purification of L11. Cells (6×10^7 cells per ml) were lysed by five cycles of freeze and thaw (27). Briefly, a 50 ml pellet of Meth A ascites was washed with PBS three times and incubated in freshly made hypotonic buffer (20 mM $NaHCO_3$, pH 7/1 mM PMSF) for 1 h and homogenized by Dounce homogenizer. After dead cells were removed, the lysates were centrifuged at 100,000 $\times$ *g* for 90 min. Supernatant was treated as a cytosol preparation. The cytosol fraction was diluted 1:1 into calcium- and magnesium-containing PBS (150 mM NaCl/2 mM $MgCl_2$/2 mM $CaCl_2$) and was applied to the Con A-agarose column. The Con A-unbound fraction was precipitated by 30–40% or 20–50% saturation of ammonium sulfate, and the precipitant was solubilized in 20 mM sodium phosphate (pH 7) and applied to a DEAE-agarose column (25 ml) that was eluted with an 0–1 M NaCl gradient on a BioCAD system (PerSeptive Biosystems, Framingham, MA). Pooled fractions containing the most active antigenic component recognized by CD4$^+$ T cell clones were concentrated by Centricon 10 (Amicon) and applied to the Mini Prep Cell (Bio-Rad) with 12% SDS/PAGE running at 200 V for 6 h. Elution was performed at 500 μl for 3 min per fraction. Active fractions were pooled and concentrated by Centricon 10, resolved on a 12% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was cut into 1-mm slices, and the individual slices were put into culture with a CD4$^+$ T cell clone with irradiated BALB/c splenic APCs for determination of antigenic content. Coomassie blue-stained bands corresponding to the most active slices were sent for the protein sequencing.

Reverse Transcription–PCR. Reverse transcription–PCR was performed with a Gene Amp EZ rTth RNA PCR kit (Perkin–Elmer) with 0.3 μg of total RNA and an Rneasy mini kit (Qiagen, Chatsworth, CA) and 22.5 pmol of primers for human (29) or rat (30) ribosomal protein L11 synthesized by GIBCO/BRL.

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Abbreviation: APC, antigen-presenting cell.

*To whom reprint requests should be sent at: University of Connecticut School of Medicine, MC1601, Farmington, CT 06030-1920. E-mail: srivastava@nso2.uchc.edu.

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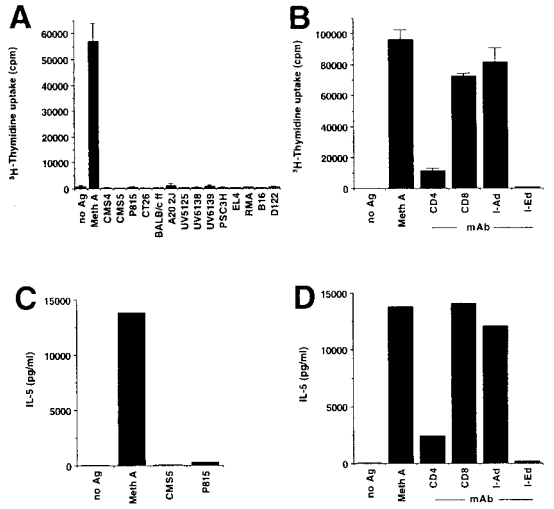


Fig. 1. Characterization of the Meth A-specific CD4$^+$ line and a representative clone 24D3 derived from it. (*A*) TML-01 proliferates in response to lysates of Meth A but not other tumors and normal tissues indicated. TML-01 cells (2×10^4 cells) were cultured in the presence of 5×10^5 irradiated BALB/c splenic APCs with or without lysate of tumors or normal fetal fibroblast (ff) (5×10^3 cell equivalents per milliliter) for 4 days. [^{3}H]Thymidine (0.5 μCi per well) was added 16–18 h before harvesting and counting. (*B*) Antigen-specific proliferation of TML-01 is blocked by anti-CD4 (PharMingen) but not anti-CD8 monoclonal antibody (PharMingen) and by anti-I-E^d (Accurate Chemicals) but not anti-I-A^d monoclonal antibody (PharMingen). (*C*) CD4$^+$ T cell clone 24D3, derived from TML-01, is stimulated in response to lysates of Meth A but not other tumors indicated. IL-5 secretion was measured in the supernatant of day 2 culture by ELISA (Endogen). (*D*) Stimulation of clone 24D3 is blocked by anti-CD4 but not anti-CD8 monoclonal antibody and by anti-I-E^d but not anti-I-A^d monoclonal antibody.

Human L11 Primers. The human L11 primers were 5′-2:ATCCGCAAACTCTGTCTCAAC, 5′-3:CTGACGCGAGCAGCCAAGGTG, 3′-2:CTCTTTGCTGATTCTGTGTTT, 3′-3:CTTGTCTGCGATGCTGAAACC.

Results

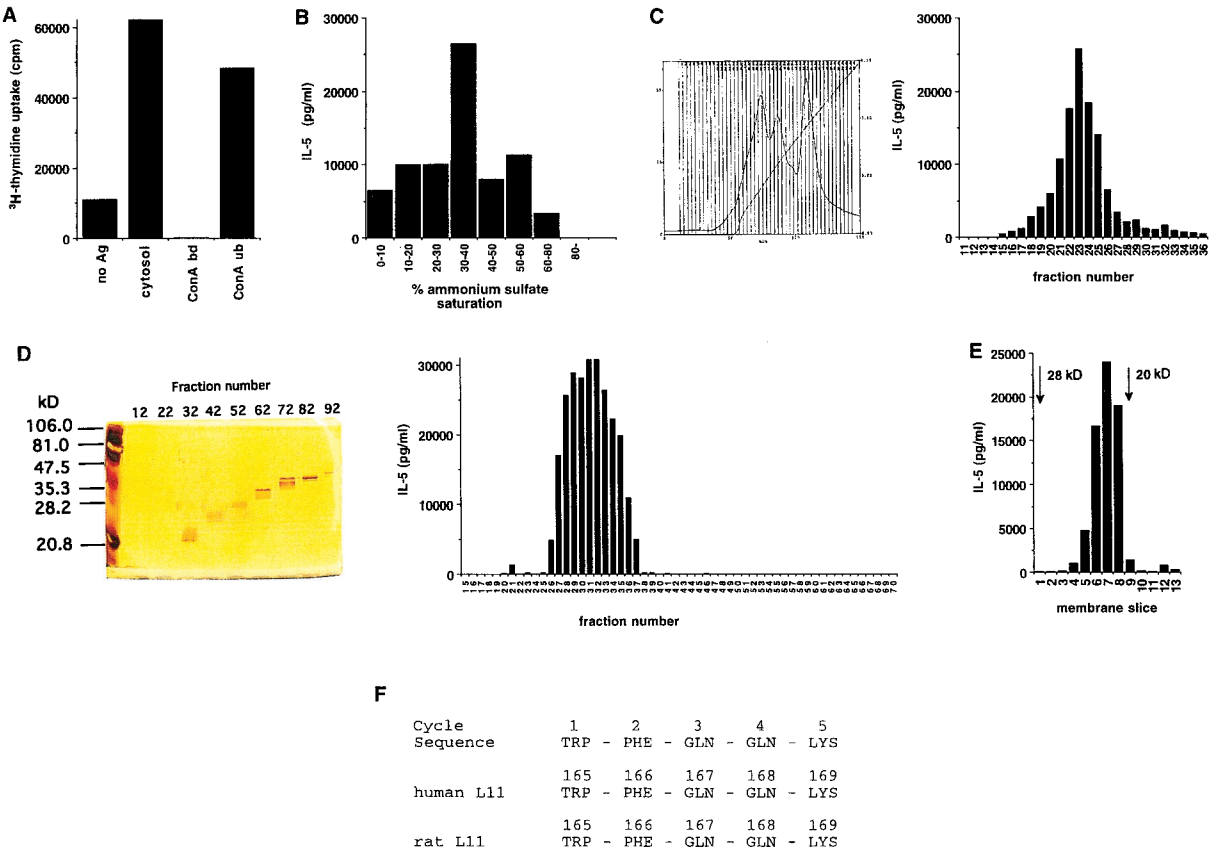
Generation of a CD4$^+$ Line and CD4$^+$ T Lymphocyte Clones Against the Meth A Fibrosarcoma. BALB/cJ (H-2^d) mice were immunized with a lysate of the methylcholanthrene-induced Meth A fibrosarcoma as described in *Materials and Methods*. A bulk line was established by stimulation of the spleen cells of the immunized mouse with APCs pulsed with the Meth A lysate. This line, designated TML-01, proliferated in response to lysates of Meth A but not to lysates of 14 other cell types tested, including normal fibroblasts, other methylcholanthrene-induced fibrosarcomas, UV-induced squamous cell carcinomas, lymphomas, a melanoma, and a lung carcinoma (Fig. 1*A*). This line therefore demonstrated the individual specificity characteristic of the immunogenicity of tumors (34, 35). The antigen-specific proliferation of TML-01 was abrogated by anti-CD4 but not by anti-CD8 antibody, and by anti-I-E^d but not by anti-I-A^d antibody (Fig. 1*B*). Several CD4$^+$ clones were derived from TML-01, and each showed the precise characteristics observed in the bulk line (Fig. 1 *C* and *D*).

Purification and Characterization of the Antigen Recognized by TML-01. Supernatant ($100{,}000 \times g$) of Meth A lysate was applied to a Con A-Sepharose column, and the unbound and bound fractions were tested for their ability to stimulate proliferation of TML-01, as described in *Materials and Methods*. The unbound fraction was found to be positive (Fig. 2*A*) and was further fractionated by increasing saturation with ammonium sulfate. The proteins precipitating between 30% and 40% saturation were found to be the most active (Fig. 2*B*) and were applied to a DEAE column eluted by a sodium chloride gradient. Fractions eluting at a NaCl concentration of ≈550 mM were the most antigen-positive (Fig. 2*C*) and were further resolved by preparative SDS/PAGE (Fig. 2*D*). The fractions eluting at molecular masses between 20 kDa and 28 kDa were found to be the most antigen-positive (Fig. 2*D*) and were pooled and resolved once again by SDS/PAGE. The proteins were transferred to a membrane that was sliced into ≈1-mm slices, and each slice was tested for its antigen content as described in *Materials and Methods*. The slices containing a band of 21–22 kDa were found to be antigen-positive (Fig. 2*E*), were subjected to digestion with trypsin, and were sequenced by Edman degradation. An amino-terminal sequence Trp-Phe-Gln-Gln-Lys, which was identical to the ribosomal protein L11 (amino acid positions 165–169 in humans and 165–169 in rats) was obtained (Fig. 2*F*).

Characterization of the Antigenic Epitope. The cDNA encoding L11 was amplified by a PCR, from the total RNA preparation of the Meth A sarcoma and from a mouse CD4$^+$ clone as a normal tissue control. The amplified products were sequenced and compared with each other and the human and rat L11 sequences (Fig. 3*A*). The deduced amino acid mouse and rat sequences were identical, and the human sequence was also highly homologous. The cDNA amplified from the Meth A showed two sequences: one was identical to the normal mouse L11 sequence, and the other differed from it by a single base pair substitution at position 289, from A to C (Fig. 3*B*, Meth A). This difference was confirmed by cloning the amplified Meth A cDNA into a pCR-4-TOPO vector and sequencing individual clones. Meth A was observed to contain both the mutated sequence and a normal sequence identical to that present in the normal lymphocytes of mice (Fig. 3*B*, Meth A mutated, wild type). Sequencing of the corresponding region of amplified cDNA from a number of other tumors, including the CMS4 and CMS5 sarcoma, did not reveal the existence of the mutated sequence (Fig. 3*B*).

Examination of the ORF of L11 showed that the single base pair substitution at bp 289 in the 97th codon at the first position resulted in translation of a His in place of an Asn (Fig. 4*A*). Peptides (19-mer) spanning the wild type and the mutant sequence were synthesized (Fig. 4*A*), and the wild type and the mutant peptides were tested in a proliferation assay with the bulk line TML-01 and nine CD4$^+$ clones derived from it (Fig. 4*B*). It was observed that the TML-01 and each of the clones proliferated in response to the mutant but not the wild-type peptide. The wild type and the bulk peptide were titrated for their ability to stimulate the CD4$^+$ clones; the mutated peptide was able to stimulate the clones at concentrations as low as 10 nM, whereas the wild-type peptide did not stimulate them, even at 10 μM. Truncated versions of the 19-mer mutant peptide were synthesized and tested for their ability to stimulate the clone 24D3: the 19-mer, 17-mer, 15-mer, and 13-mer peptides (Fig. 4*A*) were each able to stimulate the clone equally at concentrations between 10 μM and 10 nM (Fig. 4*B*), indicating that the antigenic epitope was contained within the 13-mer sequence.

Immunological Activity of the Antigenic Epitope *in Vivo*. BALB/cJ mice were immunized with ribosome preparations (polysome) from Meth A or normal liver of BALB/cJ mice and were challenged with 10^5 Meth A cells. Mice immunized with Meth A-derived polysomes were observed to be resistant to tumor challenge, whereas those immunized with liver-derived polysomes or with PBS succumbed to it ($P < 0.005$) (Fig. 5*A*). Mice were also immunized with the wild type or the mutant peptide in Freund's incomplete adjuvant as described in *Materials and Methods* and were challenged with 10^5 Meth A cells. The tumors



F

Cycle	1	2	3	4	5
Sequence	TRP	PHE	GLN	GLN	LYS
	165	166	167	168	169
human L11	TRP	PHE	GLN	GLN	LYS
	165	166	167	168	169
rat L11	TRP	PHE	GLN	GLN	LYS

Fig. 2. Purification and identification of the antigen recognized by TML-01 and 24D3. (*A*) Meth A lysates (30 ml cytosol derived from a ≈15-ml cell pellet) were fractionated by Con A-agarose affinity chromatography, and the bound and unbound fractions were assayed for antigenic activity, as described in *Materials and Methods*. (*B*) Con A-unbound proteins were fractionated by increasing saturation with ammonium sulfate, and each fraction was tested for antigenic activity. The proteins precipitating between 30% and 40% saturation were found to be the most active. This fraction was solubilized in 20 mM of sodium phosphate buffer and was resolved by DEAE-agarose chromatography by elution through a 0–1 M NaCl gradient on BIOCAD (*C*). The curved line shows absorbance at a wavelength of 280 nm, and the straight line depicts the shape of the salt gradient as conductivity. Each fraction (10 μl of 3 ml) was tested for antigenic activity. Fraction 24 was found to be the most active. (*D*) Fractions 22–24 of *C* were pooled and concentrated to 500 μl by Centricon 10, loaded onto a Mini Prep Cell, and separated on a 12% SDS gel at 200 V for 6 h. Proteins were eluted at 500 μl for 3 min per fraction. Each fraction (10 μl) of the preparative gel was tested for antigenic activity. Activity was present in fractions 27–36, which were smaller than 28 kDa. (*E*) Fractions 28–33 of the preparative gel in *D* were pooled, concentrated, and loaded onto a 12% SDS/PAGE gel. The proteins were transferred to a nitrocellulose membrane, which was cut into 1-mm slices. The slices were placed in culture with CD4$^+$ T cells and splenic APCs. Activity was measured by IL-5 release in the supernatant. Activity was present in the fractions corresponding to a ≈21- to 22-kDa band. (*F*) The nitrocellulose slice corresponding to the active fraction in *E* was subjected to digestion with trypsin and Edman degradation (Keck Facility of Yale University). One of the fragments showed a signal through the first five cycles (*F*). This sequence was identical to the sequence of ribosomal protein L11 of rats and humans.

grew progressively in mice immunized with PBS or the wild-type peptide, but mice immunized with the mutant peptide were relatively albeit not completely resistant to Meth A tumor challenge ($P < 0.005$) (Fig. 5*B*). The biological activity of the peptides was also tested by adoptive transfer of the antigen-specific CD4$^+$ T clones to BALB/cJ mice, which were then challenged with Meth A or the antigenically distinct CMS5 sarcoma cells immediately after the adoptive transfer. Mice that received the mutated L11-specific CD4$^+$ cells were completely resistant to challenge with the Meth A but not CMS 5 sarcoma ($P < 0.001$) (Fig. 5*C*). Mice that received CD4$^+$ cells from normal spleens were equally sensitive to Meth A and CMS5 sarcomas (data not shown).

Discussion

We have identified the dominant MHC II-presented epitope of the Meth A fibrosarcoma. Each of the nine clones isolated from the bulk line TML-01 shows identical characteristics as shown here and shares an identical Vβ chain composition (unpublished observations). This degree of dominance of a single CD4$^+$ clone is surprising in light of the fact that the line TML-01 was cloned soon after the spleen cells were isolated from the immunized mouse. Analysis of the CD4$^+$ clones isolated from other mice immunized with the Meth A sarcoma have also confirmed the dominance of this clone (unpublished observations). While this paper was under review, additional evidence for dominance of the immune response to an L11 epitope came to light through an independent approach: Ono *et al.* (36) report that the dominant antibody response in the sera of Meth A-bearing mice is directed against the L11 ribosomal protein. The sera do not discriminate, however, between the mutant and the wild-type L11. The antigen recognized by the CD4$^+$ cells clearly contains a unique mutation in the ribosomal protein L11. The mutation is not detected in any normal cells, tissues, or a wide range of other tumors tested structurally and immunologically, including tumors induced by the same carcinogen and of the same histological type as Meth A. The Meth A cells contain the wild type as well as the mutated alleles of L11. Because the Meth A sarcoma was induced in 1962 (34) and has been in culture for many passages, we have obtained an early passage of this tumor and found it to contain the

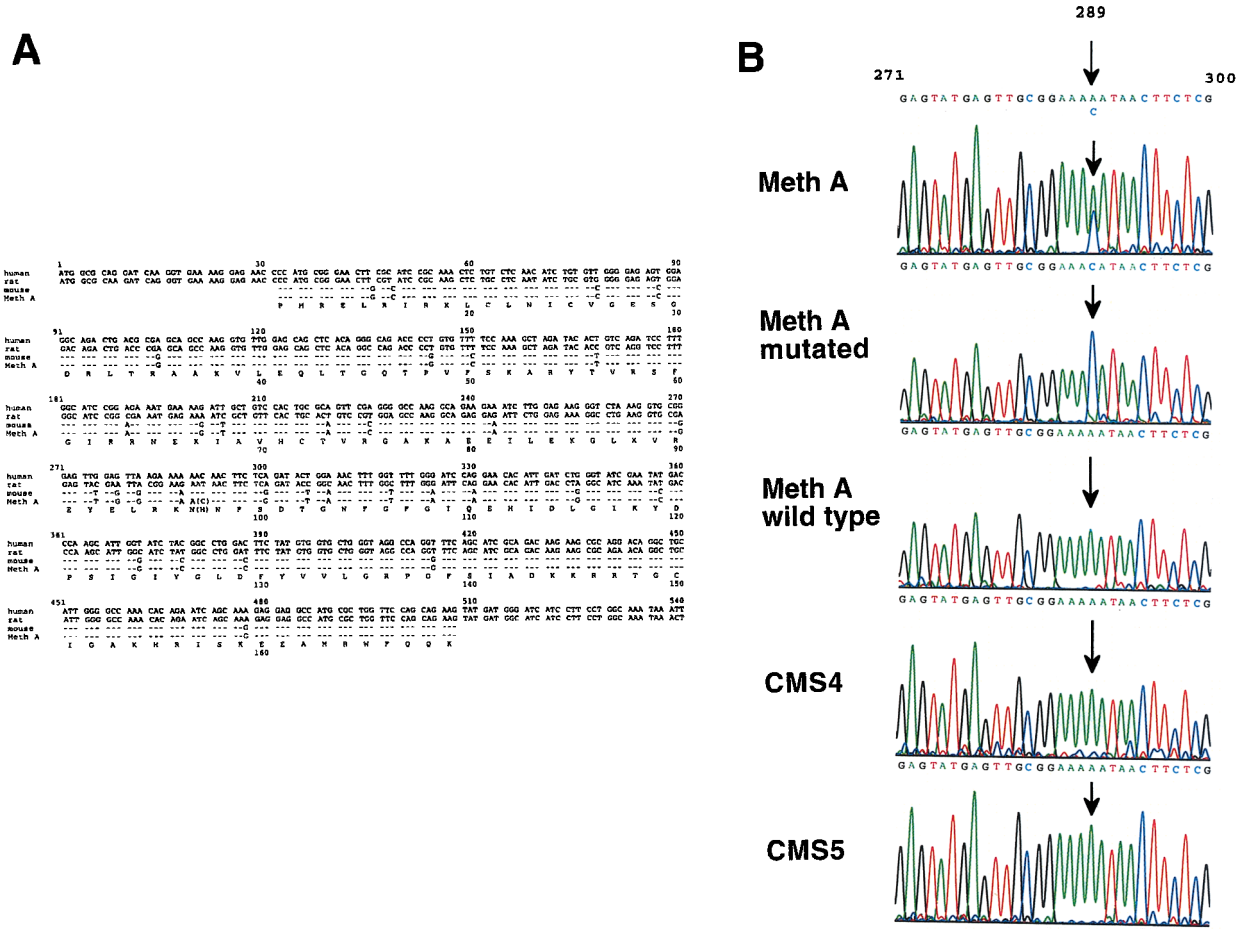


Fig. 3. Molecular characterization of mouse L11 and of a point mutation in L11 in Meth A. (*A*) Sequence of cDNA encoding L11 from a $CD4^+$ T cell clone (as normal control) and Meth A compared with human (29) and rat (30) L11 cDNA. The sequences in the normal control and Meth A were identical except for an A-to-C substitution at base pair 289 and a corresponding amino acid substitution of N by H change at amino acid 97. The Meth A contained both the normal and mutated sequences. (*B*) Base pairs 271–300 of L11 cDNA from Meth A, CMS4, and CMS5 were amplified by PCR. Cloned normal or mutated PCR products derived from the Meth A L11 sequence were also sequenced. Direct sequencing of amplified product derived from Meth A shows double sequences (A and C) at position 289. Direct sequencing of amplified products of CMS4 and CMS5 tumors shows only a single sequence A at that position. Sequences of cloned L11 PCR products derived from Meth A show normal or mutated sequence A or C at position 289.

mutation in L11 (data not shown), thus indicating that the mutation is not a result of antigenic drift *in vitro*. The L11 mutation described here is therefore a true tumor-specific and unique antigen. The uniqueness of the antigen corresponds to the individuality of antigenicity seen in all tumors tested (34, 35).

Although we designate this antigen as a tumor-specific antigen, we are aware of the inevitable and unavoidable caveats that must be borne in mind in so naming it. Discussion of two levels of caveats is in order. First, we would have liked access to the normal tissues of the mouse in which the Meth A fibrosarcoma was originally induced in 1962, to formally demonstrate that the mutation was not present, by an odd coincidence, in the original mouse itself. However, the normal tissues are unavailable. Second, even the availability of the normal tissue would not have addressed the issue, as one must also entertain the possibility that the mutation may have existed not in all normal tissues of the original mouse but only among a group of cells (descendants of a given mutated stem cell), one of whose members happened to have been transformed. For all practical purposes, therefore, it is impossible to prove that this antigen is a tumor-specific antigen. The L11 mutation is termed a tumor-specific antigen in recognition of these reservations.

Monach *et al.* (27) have described the only other MHC II-presented epitope of a murine cancer. This epitope of the UV-induced tumor 6132A is also a result of an amino acid substitution in a ribosomal protein, although a different one. Both mutations are individually tumor-specific and unique. There are interesting differences between the two systems. The L9 gene is mutated on both chromosomes in the 6132A tumor and is an example of loss of heterozygosity seen in many tumors. The L11 mutation described here, on the other hand, coexists with its unmutated counterpart, thus indicating that the mutation does not confer any growth advantage on the tumor and that its wild-type allele is not a candidate for a tumor suppressor gene. Whether or not the observation that the MHC II-presented tumor epitopes in two entirely different tumors (chemically versus UV-induced, fibrosarcoma versus squamous cell carcinoma, *k* versus *d* haplotype) are derived from mutations in ribosomal proteins is simply a coincidence or is reflective of some deeper meaning can for now only be a matter of speculation. Ribosomes play a key role in cell division, and it is conceivable that alterations in their structure can have powerful effects on cellular physiology, including, perhaps, malignant transformation. However, this argument can be made equally well for genes encoding proteins involved in signaling, cell division, cytoskeleton, transcriptional and translational control, DNA replication, cell surface receptors for external ligands, and

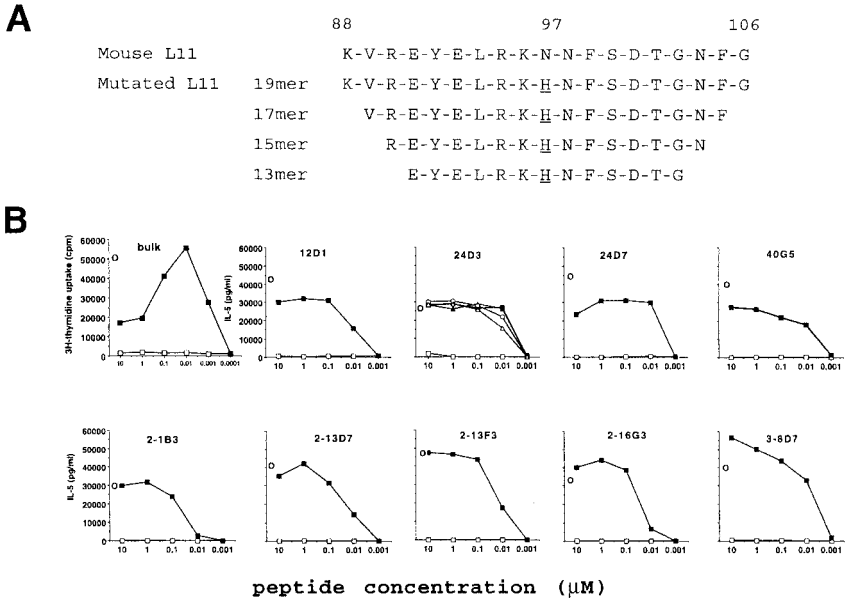


Fig. 4. Characterization of the antigenic moiety recognized by TML-01 and the clones derived from it. (*A*) Sequence of synthetic peptides containing the mutation at amino acid 97 and the normal counterpart, and of the various truncated derivatives of the mutated peptide, as indicated. (*B*) The line TML-01 and nine clones derived from it were tested against the titrated quantities of the wild type (□) or mutated peptides (■) in the presence of splenic APCs. The symbol (○) in the upper left corner of each panel denotes the activity against Meth A lysate, which was used as a positive control. The full-length normal and mutated peptides and the various derivatives of the mutated peptide (17mer, ▲; 15mer, ○; 13mer, △) were tested for activity against the clone 24D3. Activity was measured by [^{3}H]thymidine incorporation (TML-01) or IL-5 secretion (all clones).

mitochondrial structure and function among others, i.e., for an enormously large segment of the transcriptome. This multiplicity of ways in which transformation may be achieved does not negate a role for ribosomal changes in transformation, but it does highlight the dangers of seeking a deeper link between the two events. It may be argued additionally that ribosomes are extremely abundant, and hence alterations in their structure have a much higher probability of competing for presentation by

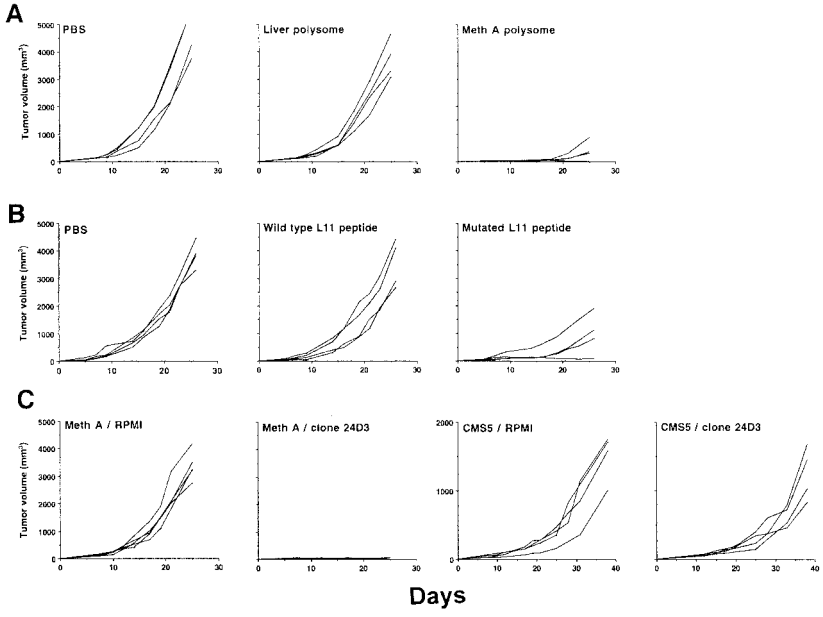


Fig. 5. Immunogenicity of the mutated L11 peptide *in vivo*. (*A*) BALB/cJ mice were immunized s.c. twice one week apart with PBS or 10 μg of polysome (prepared as described in ref. 31) derived from normal liver or from Meth A. Mice were challenged intradermally with 1×10^5 Meth A cells 1 week after the last immunization. The kinetics of tumor growth in individual mice is shown. (*B*) Mice were immunized with PBS, or the wild type or mutant 19-mer peptide (100 μg peptide/PBS emulsified with an equal volume of incomplete Freund's adjuvant per mouse) and were challenged as in *A*. The kinetics of tumor growth in individual mice is shown. (*C*) Adoptive transfer of clone 24D3 into BALB/cJ mice protects them against Meth A but not antigenically distinct CMS5 sarcoma. Mice received medium alone or 1×10^7 cells of CD4$^+$ T cell clone 24D3 by adoptive transfer (32). Mice were challenged with 1×10^5 Meth A or 2×10^5 CMS5 cells intradermally, 3 h after adoptive transfer. The kinetics of tumor growth in individual mice is shown. Tumor volume (mm^3) = $0.4 \times$ (longest diameter) $\times$ (shortest diameter)^2 (33). *P* values were calculated by Student's *t* test.

MHC molecules and for recognition by T cells. This line of thinking too has its perils, particularly lack of precedent. Among the many antigenic epitopes of cancers and viruses defined thus far, there is little evidence that the abundance of an antigen makes it particularly presentable. It may simply be premature at this point to read a deeper meaning into the fact that two of two MHC II epitopes of murine cancers identified thus far are derived from ribosomal proteins.

The ability of the L11 mutated peptide to mediate tumor immunity as tested in prophylactic assays *in vivo* makes it possible to identify it as a MHC II-presented tumor-specific antigen. The mutated L9 epitope of the UV-induced squamous cell carcinoma was not tested in this manner; its activity was shown not by immunization but by adoptive transfer of the peptide-specific $CD4^+$ T lymphocytes. The only other tumor antigens shown to immunize protectively against tumors include the MHC I-presented mutated allele of ERK-2 of the CMS5 fibrosarcoma (17) and the mutated p53 allele of the Meth A sarcoma (37). It is interesting to note that although a number of other antigens of murine tumors, such as P1A of the P815 mastocytoma and the AH1 of the CT26 colon carcinoma, have been identified and have been tested for their tumor-protective ability, it is only the mutated (unique) antigens that have been shown to be tumor protective *in vivo* (14, 38). The tumor-protective activity of any of the several unmutated human tumor antigens identified has also not been demonstrated, and mutated unique human tumor antigens remain untested clinically. It must be borne in mind that the data available thus far are too sketchy to draw any conclusions with respect to the efficacy of mutated versus shared tumor antigens. The lack of immunizing activity of P1A or AH1 epitopes may derive from many other factors, among them the lack of $CD4^+$ help. Observations reported here highlight the powerful role that such help can play in tumor rejection *in vivo*.

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