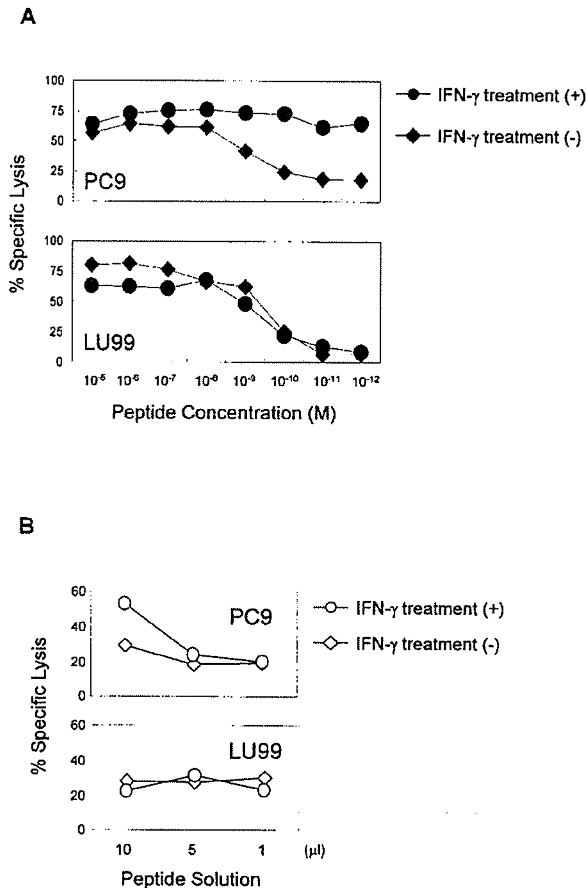

FIGURE 5 – The effects of IFN-γ on susceptibility of PC9 and LU99 cell lines to K3-1. (*a*) Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 against HLA-A24-positive lung cancer cells, PC9 or LU99, either treated (closed circle) or untreated (closed diamond) with 100 U/ml IFN-γ for 48 hr, as determined in ^{51}Cr release assay. The assay was performed in the presence of the indicated concentrations of the peptide $hTERT_{461}$ at an effector-target ratio of 5. (*b*) Naturally processed peptides were isolated from PC9 and LU99 cells, either treated (open circle) or untreated (open diamond) with IFN-γ, and concentrated. Indicated volumes of the peptide solution were pulsed on ^{51}Cr-labeled T2-A24 cells. K3-1-mediated target cell lysis was assessed at an effector-target ratio of 5.

Down-regulation of hTERT expression induced by IFN-γ

Very recently, Lee *et al.*[28] reported that telomerase activity and hTERT expression are attenuated by IFN-γ treatment, mediated by interferon regulatory factor-1 (IRF-1) in human cancer cell lines. As demonstrated in Figure 6, hTERT expression was decreased in LU99, LC99A and QG56-A24 cells after the IFN-γ treatment but not in PC9, 11-18 or MEG-01 cells. Taking into account the uniform up-regulation of immunoproteasome genes, TAPs and HLA-A*2402 molecules, the results strongly suggest that epitope supply to the surfaces of PC9 cells was increased and decreased to those of LU99 cells after IFN-γ treatment. Lee *et al.*[28] reported that induction of IRF-1 was closely correlated with attenuation of hTERT expression induced by IFN-γ treatment. However, IRF-1 induction was observed not only in cell lines such as LU99, LC99A and QG56-A24 where hTERT expression decreased but also in PC9 and MEG-01 where it did not (Fig. 6).

Attenuation of hTERT expression and K3-1-mediated cell lysis of primary lung cancer cells after IFN-γ treatment

Finally, we tested primary adenocarcinoma cells, obtained from a pulmonary fluid sample, to see the impact of IFN-γ treatment on hTERT expression and sensitivity to K3-1. As demonstrated in Figure 7, both hTERT expression and K3-1-medited cell lysis were attenuated after IFN-γ treatment. The results strongly suggest that IFN-γ impacts on hTERT expression and sensitivity to hTERT-specific CTLs *in vivo* as well as *in vitro*.

DISCUSSION

It was earlier reported that an HLA-A24-restricted $hTERT_{461}$-specific CTL could efficiently lyse hematological malignancies.[14] Thus, in our study, we addressed the question whether this epitope-specific CTL could similarly lyse lung cancer cells. An $hTERT_{461}$-specific CTL clone, K3-1, was generated from a healthy donor by repeated peptide stimulation and demonstrated to specifically lyse at least some lung cancer cell lines in an HLA-A24-restricted fashion. However, other examples of HLA-A24-positive lung cancer cell lines were not effectively lysed (Fig. 2*a*, center column), despite possessing telomerase activity. Sequence analysis of hTERT in these lung cancer cell lines revealed no mutation around the epitope (data not shown). Furthermore, K3-1 cytotoxicity against these cell lines was enhanced in the presence of cognate peptide, suggesting an insufficient epitope density. Pretreatment of the cell lines with IFN-γ did not, however, augment the CTL-mediated cytotoxicity. Ayyoub *et al.*[31] reported that an HLA-A2-restricted hTERT peptide 540–548-specific CD8+ T cells did not recognize tumor because of inefficient antigen processing, and we speculate that the epitope $hTERT_{461}$ is not processed and/or presented efficiently in some cell lines for unknown reasons.

IFN-γ plays important roles in the immune response not only to virus infection but also to tumors, up-regulating various genes including HLA class I,[32,33], ER peptide transporters (*e.g.* TAP1, 2),[34,35] proteasome β subunits (*e.g.* LMP2, 7, 10)[36–38] and proteasome regulators (*e.g.* PA28),[39], which contribute to antigen pro-

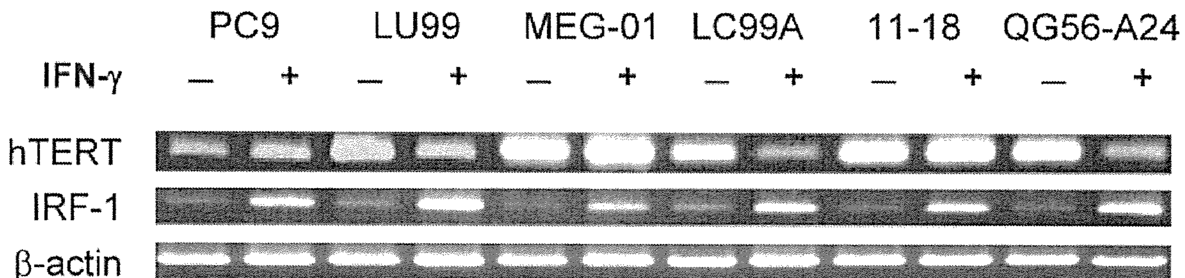

FIGURE 6 – RT-PCR analysis of hTERT and IRF-1 in cancer cell lines. Semi-quantitative RT-PCR analysis was performed using specific primers to hTERT, IRF-1 and β-actin. The mRNAs were isolated from cancer cells either treated or untreated with IFN-γ for 48 hr.

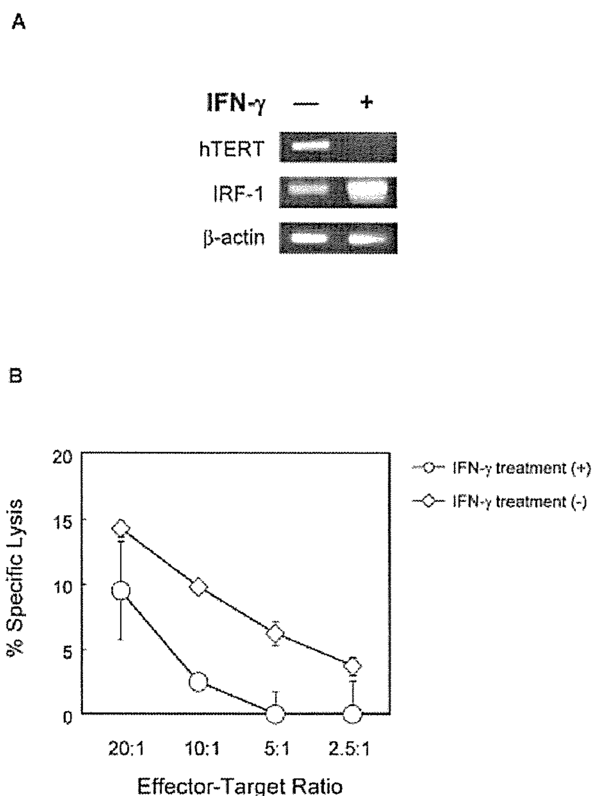

FIGURE 7 – Impact of IFN-γ treatment on primary lung cancer cells. (*a*) Freshly isolated adenocarcinoma cells from an HLA-A24-positive patient was treated or untreated with IFN-γ for 48 hr. Semi-quantitative RT-PCR analysis was performed using specific primers to hTERT, IRF-1 and β-actin. (*b*) Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 was determined with (open circle) or without (open diamond) IFN-γ pre-treatment (100 U/ml for 48 hr). Assays were performed at the indicated effector-target ratios. Bars indicate standard deviations.

cessing and presentation. In general, IFN-γ treatment is believed to enhance the presentation of peptides in the context of HLA class I molecules on the surface of target cells, leading to more efficient recognition by CTL. In our study, however, K3-1-mediated lysis of PC9, LU99, LC99A and MEG-01 cells was differentially affected by IFN-γ pre-treatment (Fig. 3). In addition, CTL assays using acid extracted peptides from cells indicated that the epitope was increased on PC9 but not LU99 cells after IFN-γ treatment (Fig 5*b*).

Paradoxical effects of IFN-γ on CTL recognition have in fact been reported. One example is induction of immunoproteasomes by IFN-γ treatment, with destruction of RU1-specific CTL epitopes and loss of CTL recognition.[40] In our study, immunoproteasomes did not appear to cleave the peptide within the $hTERT_{461}$ because the K3-1-mediated cytotoxicity against PC9 or MEG-01 cells was enhanced after IFN-γ treatment. In addition, LCL have been shown to express immunoproteasomes constitutively,[41] as here confirmed by Western blot analysis (Fig. 4*c*). Some telomerase-positive LCL were efficiently lysed by K3-1 in an HLA-A24-restricted fashion (data not shown), suggesting that immunoproteasomes do not destroy the $hTERT_{461}$ epitope. Another paradoxical effect of IFN-γ is mediated by inhibitory natural killer cell receptors expressed on the effector cells inducing inhibitory signaling.[42] Such receptors bind to several HLA-class I molecules, which could be upregulated by IFN-γ, thus executing the inhibitory effect of CTL-mediated target cell lysis after treatment with IFN-γ. Indeed, Malmberg *et al.*[43] reported that IFN-γ treatment inhibited lysis of ovarian cancer cells by specific CTL *via* a CD94/NKG2A-dependent mechanism. However, this could be excluded in the present case because LU99 cells treated with IFN-γ were efficiently lysed after being pulsed with cognate peptide (Fig. 3), and surface expression of CD94 on K3-1 was not detected by flow cytometric analysis (data not shown). In addition, inhibitory natural killer cell receptors, such as p58.1/KIR2DL1 or p58.2/KIR2DL2/3, were not found to be expressed on K3-1 (data not shown).

We demonstrated that hTERT expression itself was attenuated in the LU99 cells after IFN-γ treatment, resulting in inefficient recognition by the hTERT-specific CTL. The same attenuation was observed in primary lung cancer cells obtained from a lung cancer patient (Fig.7). A few reports revealed that IFN-γ reduces the expression of tumor antigens, such as MART-1/Melan A[44] or murine gp70.[45] With regard to these antigens, IFN-γ may promote immune-escape of tumors because these are not necessary for tumor proliferation. However, it is of particular interest to consider the effects of IFN-γ on telomerase activity in cancer immunity. IFN-γ may exert an anti-tumor influence primarily by suppressing hTERT transcription, resulting in limited proliferative potential. If such hTERT suppression is no longer occurring by whatever mechanism, such as IRF-1 gene inactivation as observed in some cancer cells,[46] IFN-γ might increase hTERT epitope processing and presentation leading to augmented susceptibility to specific CTL, as shown in PC9 cells (Figs. 3 and 5). Thus, the effects of IFN-γ on tumor cells through modulation of hTERT expression can be considered to feature a "fail safe" mechanism for efficient anti-tumor activity due to its impact on innate and adaptive immunity. With regard to clinical application, immunotherapy for malignant tumors using hTERT-specific CTL has unique advantages. hTERT-specific CTL not only kill tumor cells through the recognition of epitopes expressed on their surfaces but also produce and release IFN-γ *in situ*. Indeed, Le Poole *et al.*[44] reported that examination of melanoma lesions by quantitative reverse transcriptase-polymerase chain reaction revealed up to 188-fold more abundant IFN-γ transcripts produced by T cells when compared to control skin. In such circumstances, hTERT expression of tumor cells could be downregulated, resulting in suppressed tumor growth. However, some HLA-A24 positive lung cancer cells with hTERT expression were not efficiently recognized by hTERT-specific CTL, probably because of low epitope density on the cell surface. The effects of hTERT-specific CTL against such tumor cells might thus be limited. Downregulation of K3-1-mediated lysis was less pronounced with LC99A cells, although there was clear attenuation of hTERT transcription after IFN-γ treatment (Figs. 3 and 6). The reason is unclear but it could be speculated that more efficient processing and/or presentation might compensate for any shortage of hTERT proteins.

A previous study revealed that hTERT transcription may be decreased after IFN-γ treatment through induction of IRF-1.[28] Our study also confirmed downregulation of hTERT expression after IFN-γ treatment in 3 of 6 cell lines examined and primary lung cancer cells from a patient, in parallel with IRF-1 induction. However, in PC9, MEG-01 and 11–18 cells where hTERT expression did not decrease, IRF-1 was also induced. These equivocal findings for IRF-1 might be related with functional inactivation of the IRF-1 gene[46,47] or deletion or mutation of putative IRF-1 binding sites in the hTERT promoter. Alternatively, other yet-to-be identified third party molecules that cooperate with IRF-1 might be inactivated. Further studies are required to clarify the

mechanisms underlying the effect of IFN-γ upon down-regulation of hTERT expression.

In conclusion, we propose here a mechanism of attenuated CTL-mediated lysis of tumor cells through hTERT down-regulation induced by IFN-γ. Our study indicates that hTERT-specific CTL-based immunotherapy could be effective in patients with lung cancers which present relevant epitopes.

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Identification of an epitope from the epithelial cell adhesion molecule eliciting HLA-A*2402-restricted cytotoxic T-lymphocyte responses

Key words:
autoimmunity; CTL; dendritic cells; epithelial cell adhesion molecule; HLA-A24; immunotherapy

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Abstract: Because the epithelial cell adhesion molecule (Ep-CAM) is expressed in almost all carcinomas and human leucocyte antigen (HLA)-A*2402 is the most common allele in many ethnic groups, including Japanese, the identification of peptide sequences, which elicit HLA-A*2402-restricted Ep-CAM-specific cytotoxic T-lymphocyte (CTL) responses, would facilitate specific immunotherapy for various histological types of carcinomas. An epitope was identified through the following steps: (i) computer-based epitope prediction from the amino acid sequence of Ep-CAM, (ii) major histocompatibility complex (MHC) stabilization assay to determine the affinity of the predicted peptide with HLA-A*2402 molecules, (iii) stimulation of $CD8^+$ T cells with peptide-pulsed dendritic cells and (iv) testing the CTL specificity by means of enzyme-linked immunospot (ELISPOT) assays, CTL assays and MHC/peptide-tetramer staining. Peripheral $CD8^+$ T cells of four of five healthy donors after three rounds of stimulation with the peptide Ep-$CAM_{173-181}$ (RYQLDPKFI) secreted interferon-γ in ELISPOT assays when exposed to the peptide. A CTL clone specific to the peptide efficiently lysed Ep-CAM-expressing cancer cell lines in an HLA-A*2402-restricted fashion. Endogenous processing and presentation of the peptide in a lung cancer cell line were confirmed by means of cold target inhibition assays. The CTL clone was also lytic to normal bronchial epithelial cells but to a lesser extent at low effector: target ratios. All these data suggest that the peptide-specific CTL responses may play some roles both in anti-cancer and autoimmune reactions. The peptide should prove useful to study anti-Ep-CAM CTL responses among population possessing HLA-A*2402.

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Cytotoxic T-lymphocytes (CTLs) have become widely accepted as important players in resistance to cancer. Although various CTL epitopes of tumour-associated antigens have been identified so far (1, 2), the search for additional epitopes continues, because the expression of tumour antigens is heterogeneous among tumours of various histological origins, various patients and between individual lesions. From the clinical point of view, molecular characterization of

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additional tumour antigens is crucial for successful immunotherapy, because immunoselection of antigen-negative tumour cell variants has been observed during peptide vaccination (3–5).

Epithelial cell adhesion molecule (Ep-CAM), also referred to as EGP-2, 17-1A, GA733-2, KSA or PE-35 (6–10), was originally reported as a serologically defined surface antigen, highly expressed on many carcinomas of diverse histological origins, such as colon (11), lung (12), head and neck (13) and breast tumours (14), but with limited expression by normal epithelial cells (15, 16). Its function is to mediate Ca^{2+}-independent homotypic cell–cell adhesion. Because of its intensive and uniform expression in a variety of human tumours, Ep-CAM has become one of the most attractive targets for immunotherapy with monoclonal antibodies, or even for gene therapy (17). Treatment of a series of patients suffering from Dukes' C colorectal carcinoma with a monoclonal antibody against Ep-CAM, namely 17-1A, has been found to reduce mortality and recurrence (18, 19). Recently, it was reported that HLA-A*0201-restricted Ep-CAM-derived peptide-specific CTLs can lyse epithelial tumour cells but not normal cells (20, 21). Immunotherapy using such epitope peptides has potential efficacy.

Using a bioinformatic approach, in the present study, we first predicted seven peptide sequences in Ep-CAM, which might bind to HLA-A*2402 molecules, the most common allele in Japanese (more than 60%) and also present in persons of European descent (nearly 20%). Specific CTL was successfully induced in four of five healthy donors by using $Ep\text{-}CAM_{173-181}$ (RYQLDPKFI) and a $CD8^+$ CTL clone specific to this peptide showed cytotoxicity against $HLA\text{-}A24^+$ $Ep\text{-}CAM^+$ but not HLA-A24 cancer cells. Cold target inhibition assays suggested that the peptide was naturally processed and was presented on the surfaces of $HLA\text{-}A24^+$ $Ep\text{-}CAM^+$ cancer cells. The fine specificity of the peptide-specific CTL was extensively studied and the results were discussed in the light of anti-cancer and anti-self cellular immunity.

Materials and methods

Donors and cell lines

The study design and purpose, which had been approved by the Institutional Review Board of Aichi Cancer Center, Nagoya, Japan, were explained fully to all donors. Peripheral blood was obtained from five HLA-A24-positive healthy donors and peripheral blood mononuclear cells (PBMCs) were isolated by means of centrifugation on a Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient.

Human cancer cell lines – LU99, HSC-2, MKN28, MKN45 and COLO320DM cells – were purchased from the Japanese Collection of Research Bioresources (Tokyo, Japan) and LC-1/sq from RIKEN Cell Bank (Tsukuba, Japan). LC-1/sq cells were maintained in 45% RPMI 1640 medium (Sigma, St Louis, MO) and 45% Ham's F12 medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Life Technologies Limited, Auckland, New Zealand), L-glutamine, penicillin and streptomycin. COLO320DM and MKN28 were maintained in Dulbecco's modified Eagle medium (Sigma) with the same supplements. The other cancer cell lines were cultured in RPMI1640 medium with the same supplements (referred to as complete medium). HLA-A24-positive, normal human bronchial epithelial cells, designated as NHBE, were cultured according to the manufacturer's recommendations (CC2540, Clonetics Corp, BioWhittaker, Walkersville, MD). The HLA-A*2402 transfectants – T2-A24, QG56-A24 and A549-A24 – were established and were cultured as previously described (22, 23).

Reverse transcription polymerase chain reaction

Using a GenElute mRNA Miniprep kit (Sigma Chemical Co., St Louis, MO), total RNA was extracted from cultured cell lines. Gene-specific oligonucleotide primers were synthesized at Proligo (Kyoto, Japan) and were used in order to evaluate the mRNA expression of Ep-CAM. Forward and reverse primers used were as follows: ATG GCG CCC CCG CAG GTC CT and TTA TGC ATT GAG TTC CCT ATG CAT CTC ACC. Reverse transcription polymerase chain reaction (RT-PCR) was performed by using a thermal cycler (Perkin-Elmer, Wellesley, MA) and products were analysed by means of 1.5% agarose gel electrophoresis with ethidium bromide visualization.

Western blot analysis

Western blot analysis was performed as described previously (24) with slight modifications. Briefly, aliquots of 130-μg protein from the post-nuclear supernatant of the cell lysate were applied to 12% SDS-PAGE and were blotted onto Immobilon-P membranes (Millipore Corporation, Bedford, MA). After probing with a monoclonal antibody specific to Ep-CAM (clone 323/A3, Laboratory Vision, Fremont, CA), followed by peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Zymed, San Francisco, CA), proteins were visualized with the help of an ECL Western blot detection system (Amersham Biosciences, Buckinghamshire, UK).

Synthetic peptides

In order to identify potential HLA-A24-binding peptides within Ep-CAM (accession number M33011), we employed a computer-based program accessed through the World Wide Web site BioInformatics & Molecular Analysis Section (BIMAS) HLA peptide-binding predictions (available at http://bimas.dcrt.nih.gov/molbio/hla_bind/). Most peptides were synthesized with a Cleaved PepSet from Mimotope

(Melbourne, Australia), dissolved in 100 µl dimethyl sulfoxide and further diluted in 40% acetonitrile, 0.1 M HEPES (pH 7.4), where necessary. Characteristics of the seven synthetic peptides, designated as Ep_{31}, Ep_{173}, Ep_{185}, Ep_{250}, Ep_{225}, Ep_{296} and Ep_{304}, have been listed in Table 1. A human immunodeficiency virus-1 (HIV-1) envelope peptide RYLRDQQLL (25) (residues 584–592, designated as ENV_{584}) and an EBV (Epstein-Barr virus) latent membrane protein 2 peptide TYGPVFMCL (26) (residues 419–427, EBV-LMP2419) were synthesized by Toray Research Center (Kamakura, Japan).

Cell staining and flow cytometric analysis

Surface expression of HLA-A24 and Ep-CAM molecules was examined with the help of indirect immunofluorescence by using an anti-HLA-A24 monoclonal antibody (One Lambda, Inc., Canoga Park, CA), the anti-Ep-CAM monoclonal antibody and FITC-labelled (fluorescein isothiocyanate) anti-mouse IgG $F(ab')_2$ fragments (IMMUNOTECH, Marseille, France). MHC/peptide tetramers were produced as previously described (22, 27). The Ep-CAM-specific $CD8^+$ T cells were stained with PE-labelled HLA-A*2402 tetramers incorporating the Ep-CAM peptide, Ep_{173} (designated as the HLA-A24/Ep_{173} tetramer) or the HIV-1 peptide, ENV_{584} (HLA-A24/ENV_{584} tetramer). Flow cytometric analysis of the stained cells was performed by means of a FACSCalibur (Becton Dickinson, San Jose, CA) and the data were analysed with the help of CellQuest software (Becton Dickinson).

MHC stabilization assay

The seven synthesized peptides were used in an MHC stabilization assay by using T2-A24 cells as described earlier (22). Briefly, T2-A24 cells (2×10^5) were incubated with 200 µl of RPMI1640 containing 0.1% FCS and 5×10^{-5} M β-mercaptoethanol and each of the peptides at a concentration of 10 µM at 26°C for 16 h, followed by incubation at 37°C for 3 h. Surface HLA-A24 molecules were then stained with the anti-A24 monoclonal antibody and FITC-labelled anti-mouse IgG. Expression was measured in the FACSCalibur, and mean fluorescence intensity (MFI) was recorded. The percentage of MFI increase was calculated as follows: percentage of MFI increase = 100 × (MFI with the given peptide − MFI without the peptide)/(MFI without the peptide).

Characteristics of epithelial cell dhesion molecule (Ep-CAM) candidate peptides

Peptide designation	Amino acid sequence	Position	Sequence length	Score[a]	Percentage of MFI increase[b]
Ep_{31}	NYKLAVNCF	31–39	9	120	85
Ep_{173}	RYQLDPKFI	173–181	9	150	102
Ep_{185}	LYENNVITI	185–193	9	75	79
Ep_{225}	LFHSKKMDL	225–233	9	20	29
Ep_{250}	YYVDEKAPEF	250–259	10	198	57
Ep_{296}	KYEKAEIKEM	296–305	10	83	24
Ep_{304}	EMGEMHREL	304–312	9	5	16

[a]Estimated half-time of dissociation from HLA-A24 molecules (min), obtained with a computer program (World Wide Web site Bioinformatics & Molecular Analysis Section (BIMAS) HLA peptide-binding predictions).
[b]Synthetic peptides were tested for binding to human HLA-A*2402 molecules in MHC stabilization assays as described in the section entitled 'Materials and methods.' MFI, mean fluorescence intensity.

Table 1

Generation of Ep-CAM peptide-specific CTL lines and clones

Peripheral blood monocyte-derived dendritic cells (DCs) were generated as described previously (28). Briefly, plastic adherent cells were isolated from PBMCs and were cultured in RPMI1640 medium supplemented with 5% heat-inactivated human serum, 10 ng/ml of recombinant human interleukin-4 (IL-4) (R&D Systems, Minneapolis, MN) and 50 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (R&D Systems). On day 1 of incubation, 10 ng/ml of IL-1β PeproTech, Rocky Hill, NJ), 50 ng/ml of recombinant human tumour necrosis factor-α (TNF-α) (PeproTech) and 1 µM prostaglandin E_2 (Cayman Chemical Company, Ann Arbor, MI) was added for maturation. On days 2 or 3, the cells were harvested and were confirmed to express mature DC-associated antigens, such as CD1a, CD80, CD83, CD86 and HLA class-II molecules (data not shown). The DCs were pulsed with each of the synthetic peptides at a concentration of 10 µM in AIM-V medium (Gibco, Grand Island, NY) supplemented with 5×10^{-5} M β-mercaptoethanol for 2–4 h at room temperature and were irradiated (33 Gy). Thereafter, the DCs (1×10^5) were co-cultured with autologous $CD8^+$ T lymphocytes (1×10^6) purified with the aid of CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in RPMI1640 medium supplemented with 10% pooled human serum, 25 ng/ml of recombinant human IL-7 (R&D Systems) and 5 ng/ml of recombinant human IL-12 (R&D Systems) in a culture tube. After culture for 7 days, the cells were stimulated again by adding 1×10^5 peptide-pulsed autologous DCs prepared as described above. After culture for 7 additional days, the cells were stimulated a third time in the same manner. One day after each restimulation, recombinant human IL-2 (Takeda Chemical Industries, Osaka, Japan) was added to a final concentration of 20 U/ml. If necessary, rapidly growing cells were split into two to three tubes and were fed with fresh culture medium containing 20 U/ml of IL-2.

In order to establish T-cell clones, limiting dilution of the polyclonal CTLs was performed (22). After 2-week culture in 96-well plates, the specificity of growing cells was examined with CTL–CTL killing assays as previously described (29). Clones that were

killed only when pulsed with the Ep-CAM peptide were transferred into flasks and were expanded as detailed above.

Enzyme-linked immunospot assay

Enzyme-linked immunospot (ELISPOT) assays were performed as previously described (22). A total of 1×10^3 polyclonal $CD8^+$ T cells were co-cultured with peptide-pulsed T2-A24 cells (5×10^4) in wells of the MultiScreen-HA plates (Millipore) coated with 10 μg/ml of anti-interferon-γ (IFN-γ) monoclonal antibody (R&D Systems). All assays were performed in duplicate. After probing with a polyclonal rabbit anti-IFN-γ antibody (Genzyme, Cambridge, MA), followed by exposure to peroxidase-conjugated goat anti-rabbit IgG (Genzyme), IFN-γ spots were visualized and were counted under a dissecting microscope.

CTL assay

Target cells were labelled with ^{51}Cr as previously described (22). In some experiments, pre-determined amounts of blocking antibodies, W6/32 (anti-HLA class-I), MA2.1 (anti-HLA-A2) and A11.1 (anti-HLA-A24) were added to the wells 30 min before adding effector cells in order to determine the HLA restriction. The plates were incubated for 4 h at 37°C, and the supernatants were counted in a γ-counter. The percentage of specific ^{51}Cr release was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release −spontaneous release).

Cold target inhibition assays

Cold target inhibition assays were performed as described previously (30). Briefly, T2-A24 cells were incubated with the peptide Ep_{173} or EBV-$LMP2_{419}$ at a concentration of 10 μM for 1 h. After extensive washing, the indicated numbers of peptide-loaded cells were incubated with 2×10^4 cytotoxic effector cells for 1 h, and then 2×10^3 ^{51}Cr-labelled PC9 cells were added to each well. Cytotoxicity was assessed as described above.

Results

Selection of potential HLA-A24-binding peptides within Ep-CAM proteins

The computer program was applied in order to identify potential HLA-A*2402-binding peptides within the amino acid sequence of Ep-CAM and to predict HLA-binding peptides, on the basis of estimation of the half-time dissociation of the HLA/peptide complex. The peptide sequences were reviewed according to peptide motifs for HLA-A24 molecules (31–33), and seven peptides were synthesized (Table 1). MHC stabilization assays were performed in order to test their HLA-A*2402-binding efficiency by using T2-A24 cells. Most peptides increased the HLA-A24 expression on the cells, indicating that they bound and stabilized the HLA complexes on the cell surface, but peptide Ep_{304} gave a low value for the percentage of MFI (Table 1) and was excluded from further studies.

Screening of peptides antigenic for anti-Ep-CAM polyclonal CTL lines by means of ELISPOT assay

In order to identify peptides recognized by Ep-CAM-specific CTLs in the context of HLA-A*2402 molecules, $CD8^+$ T cells of five HLA-$A24^+$ healthy donors were stimulated with autologous DCs pulsed with each of the six peptides. After three rounds of stimulation, T cell lines from four donors produced significant numbers of IFN-γ spots when incubated with T2-A24 cells pulsed with peptide Ep_{173} (Fig. 1). Almost no spots were produced with control peptide ENV_{584} (data not shown). After four-time stimulation, the CTL line established from donor 4 specifically stained with the HLA-A24/Ep_{173} tetramer but not the HLA-A24/ENV_{584} tetramer (37.2 *vs* 0.06% of the total $CD8^+$ T cells, Fig. 2A). The intensity of the tetramer-positive cells was homogeneous and stronger than that of tetramer-negative cells by two- to three-fold on a logarithmic scale.

We established a T-cell clone, designated as C27, from limiting dilution culture of the Ep_{173}-specific polyclonal CTL line of donor 4. The study with tetramers indicated that both polyclonal and monoclonal Ep_{173}-specific $CD8^+$ T cells had high-affinity antigen receptors directed to HLA-A*2402/Ep_{173} complexes (Fig. 2B).

While an Ep_{250}-stimulated CTL line from donor 3 also specifically produced IFN-γ spots when incubated with Ep_{250} (Fig. 1), the establishment of Ep_{250}-specific CTL clones was unsuccessful. We, thus, further evaluated the Ep_{173}-specific CTL clone.

Characterization of the peptide Ep_{173}-specific $CD8^+$ CTL clone

Ep-CAM expression of cancer cell lines was examined by means of RT-PCR, Western blot analysis (Fig. 3) and indirect immunofluorescence (Table 2). The data showed concordance of results obtained with the three approaches. Twelve of 15 (80%) cancer cell lines appeared to express Ep-CAM. When HLA-A24 expression was examined with the help of indirect immunofluorescence by using an HLA-A24 monoclonal antibody, 10 were positive (Table 2).

As shown in Fig. 4(A), the Ep_{173}-specific CTL clone, C27, showed cytotoxicity to T2-A24 cells pulsed with Ep_{173} at a peptide

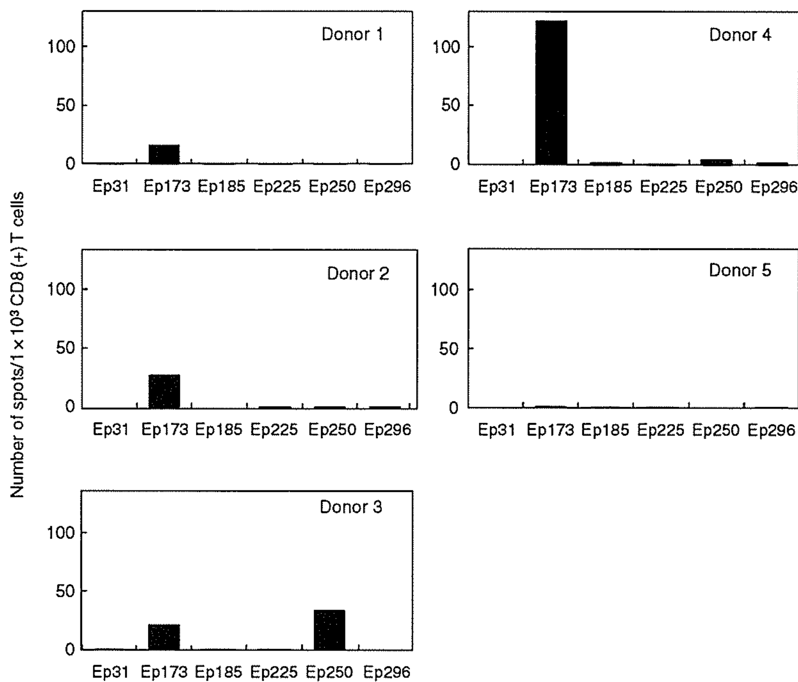

Fig. 1. **Evaluation of CD8$^+$ T cell lines by means of enzyme-linked immunospot (ELISPOT) assay.** Aliquots of CD8$^+$ T cells (1×10^3) were incubated with T2-A24 cells pulsed with the indicated peptide (10 μM) in wells of the ELISPOT plate. All assays were performed in duplicate.

concentration as low as 100 pM, but not with control peptide EBV-LMP2$_{419}$. Data for cytotoxicity of C27 against various cancer cell lines have been shown in Fig. 5. C27 efficiently lysed lung cancer cell lines PC9, LU99, LC99A and LC-1/sq, an oral squamous cell carcinoma cell line HSC-2 and a gastric cancer cell line MKN45 that expressed both HLA-A24 and Ep-CAM. However, no killing was observed with HLA-A24$^+$ Ep-CAM (11–18, COLO320 DM and A549-A24) or HLA-A24 (either Ep-CAM$^+$ or Ep-CAM) cell lines (QG56, A549 and MNK28). After the transfection of the HLA-A*2402 cDNA into HLA-A24 QG56 cells (QG56-A24), they were killed by C27. K562 cells were included in order to assess the degree of NK-like cytotoxicity of C27, which turned out to be negligible.

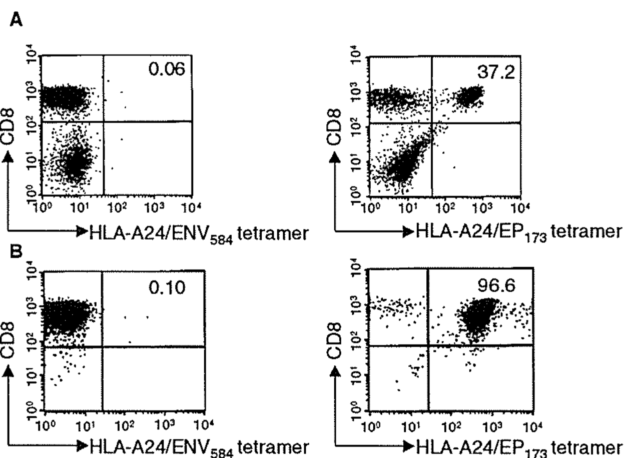

Fig. 2. **Tetramer staining of Ep$_{173}$ peptide-specific CD8$^+$ T cells.** Polyclonal CD8$^+$ T cells (A) and an Ep$_{173}$-specific cytotoxic T-lymphocyte (CTL) clone – C27 – (B) were stained with HLA-A24 tetramers incorporating Ep$_{173}$ or a control peptide, ENV$_{584}$. The percentages of tetramer-positive cells in CD8$^+$ T cells have been shown. HLA, human leucocyte antigen.

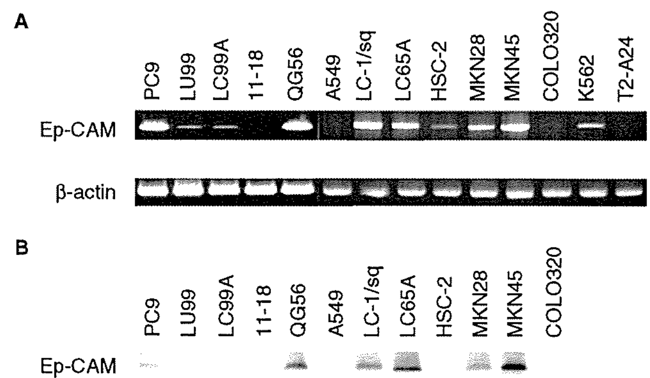

Fig. 3. **Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis of epithelial cell adhesion molecule (Ep-CAM) in cancer cell lines.** Semi-quantitative RT-PCR analysis was performed by using specific primers for Ep-CAM and β-actin (A). Western blot analysis of Ep-CAM was performed with a monoclonal antibody specific to Ep-CAM (B).

Characteristics of the cell lines used

Cell line	Origin	Surface expression (MFI[a]) of Ep-CAM	HLA-A24
Lung cancer			
LU99	Giant cell carcinoma	+ (22.55)	+ (87.16)
PC9	Adenocarcinoma	+ (383.67)	+ (43.53)
11–18	Adenocarcinoma	– (4.00)	+ (73.67)
LC99A	Large cell carcinoma	+ (15.32)	+ (96.71)
LC65A	Small cell carcinoma	+ (307.14)	– (3.67)
LC-1/sq	Squamous cell carcinoma	+ (198.94)	+ (70.56)
A549	Adenocarcinoma	– (6.40)	– (3.21)
A549-A24	Adenocarcinoma	Not done	+ (109.73)
QG56	Squamous cell carcinoma	+ (229.13)	– (2.65)
QG56-A24	Squamous cell carcinoma	Not done	+ (84.12)
Gastric cancer			
MKN28	Adenocarcinoma	+ (722.59)	– (7.67)
MNK45	Adenocarcinoma	+ (823.25)	+ (47.99)
Colon cancer			
COLO320DM	Adenocarcinoma	– (3.07)	+ (35.35)
Others			
HSC-2	Oral squamous cell carcinoma	+ (57.15)	+ (34.40)
K562	Chronic myelogeneous leukaemia	+ (41.45)	– (5.23)
T2-A24	B × T hybrid cells	– (3.05)	+ (197.75)
NHBE	Normal bronchial epithelial cells	+ (152.56)	+ (33.52)

[a]Mean fluorescence intensity (MFI) was examined with the help of flow cytometric analysis after cell staining by using anti-Ep-CAM or anti-HLA-A24 monoclonal antibodies and FITC-labelled anti-mouse IgG $F(ab')_2$ fragments.

Table 2

These data demonstrated that Ep_{173}-specific CTLs kill tumour cells expressing both HLA-A24 and Ep-CAM.

We further examined whether C27 might recognize naturally processed peptides presented on the surfaces of tumour cells in the context of HLA-A24. Cytotoxicity of C27 against PC9 cells ($HLA-A24^+$ $Ep-CAM^+$ lung cancer cell line) was blocked by monoclonal antibodies specific to HLA-A24 or pan-class-I molecules, but not an anti-HLA-A2 monoclonal antibody (Fig. 4B), confirming the HLA-A24 restriction. Cold target inhibition assays demonstrated that C27-mediated cytotoxicity against PC9 cells was specifically inhibited in the presence of T2-A24 cells pre-pulsed with the cognate but not an irrelevant peptide (Fig. 4C), indicating that C27 recognized peptides that were naturally processed and presented.

Because Ep-CAM is expressed in some sites of normal epithelial cells, there has been concern about potential autoimmune reactions after *in vivo* activation of T cells specific to the molecule. We, therefore, tested an HLA-A*2402-positive, normal human bronchial epithelial cell line, designated as NHBE, with clone C27. As demonstrated in Fig. 6(A), C27 exerted toxicity, especially at higher effector : target ratios (40 : 1 and 20 : 1), which was specifically blocked by T2-A24 cells pre-pulsed with the cognate peptide (Fig. 6B). At lower effector : target ratios (10 : 1 and 5 : 1), however, C27-mediated lysis of NHBE was apparently lower than that of cancer cell lines, such as PC9, LU99, LC99A and HSC-2 (Fig. 5).

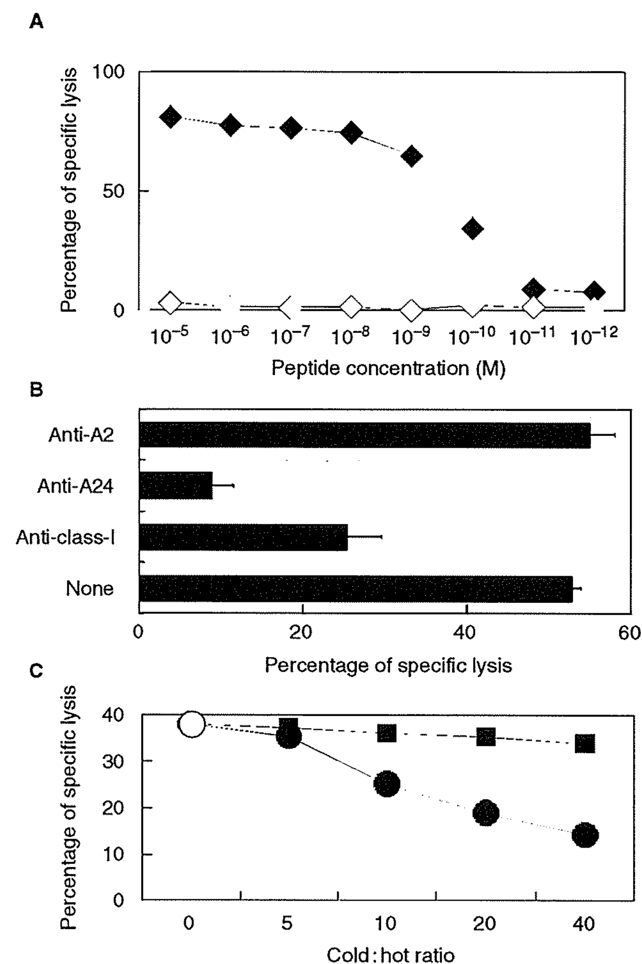

***Fig. 4.* Characterization of an Ep_{173}-specific cytotoxic T-lymphocyte (CTL) clone, C27.** An Ep_{173}-specific CTL clone, C27-mediated target cell (T2-A24) lysis in the presence of Ep_{173} (◆) and control peptide EBV-$LMP2_{419}$ (◇) has been shown (A). C27-mediated target cell (PC9) lysis was blocked with monoclonal antibodies specific to HLA-A24 and pan-class-I molecules (B). C27-mediated target cell (PC9) lysis was blocked with T2-A24 cells that had been loaded with Ep_{173} (●) but not with a control peptide EBV-$LMP2_{419}$ (■) (C). The cytotoxic assays were performed at effector to ^{51}Cr-labelled target ratios of 1, 10 and 5, in experiments shown in A, B and C, respectively. HLA, human leucocyte antigen.

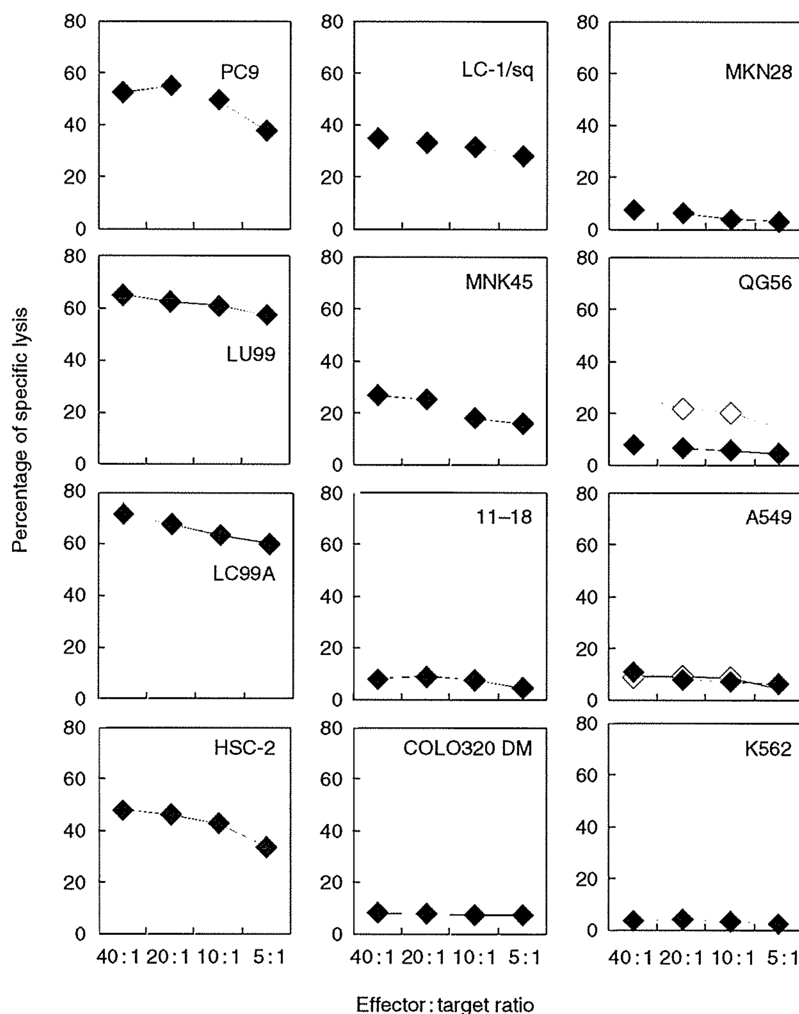

Fig. 5. **Cytotoxicity of the clone C27 against cancer cell lines.** An Ep_{173}-specific cytotoxic T-lymphocyte (CTL) clone, C27-mediated lysis of various cancer cell lines. Cell lines in the left and the centre columns express HLA-A24 on their surface, whereas those in the right do not. All the target cell lines except for 11–18, COLO320DM and A549 express the Ep-CAM. HLA-A*2402 transfectants of QG56 and A549 cells were also used as target cells (◇). K562 was included in order to assess natural killer-like cytotoxicity. HLA, human leucocyte antigen.

Discussion

According to recent findings, overexpression of Ep-CAM, a transmembrane protein mediating Ca^{2+}-independent cell–cell adhesion, is correlated with tumour grade (14, 34, 35), providing a useful marker for diagnosis of micrometastases (36) and a predictor of survival (14). The promoter region that regulates the Ep-CAM transcription has been cloned and it has shown to be negatively regulated by TNF-α (37).

Ep-CAM has become one of the major targets for immunotherapy with monoclonal antibody, because it is expressed in the vast majority of epithelial cell-derived cancers. The administration of Ep-CAM-specific murine monoclonal antibody (17-1A) into Duke's C colorectal cancer patients after surgery, for example, led to the prevention of distant metastasis and to prolonged survival after 7 years of follow-up evaluation (19). Recently, bispecific antibodies against Ep-CAM and CD3 (38, 39) or B7 (40) have been engineered in order to improve cytotoxicity with synergistic effects of antibodies and T lymphocytes. Considering the expression pattern, immunotherapy by using CTL directed against Ep-CAM appears attractive. Indeed, Ep-CAM peptides that elicited cellular immune responses restricted by HLA-A*0201 have recently been identified (4, 20). Of note, a natural T-cell response against Ep-CAM has been observed in colorectal cancer patients (41, 42). The available evidence, thus, suggests the possibility of therapeutic approaches using Ep-CAM as a target of CTLs.

We, in this study, explored immunogenic peptides derived from Ep-CAM that can elicit cellular immune responses against Ep-CAM$^+$ tumour cells in the context of HLA-A24, which is positive in nearly 20% of persons of European descent and more than 60% of Japanese. For that purpose, seven candidate peptides were first predicted from the Ep-CAM protein with a bioinformatic approach. Secondly, MHC

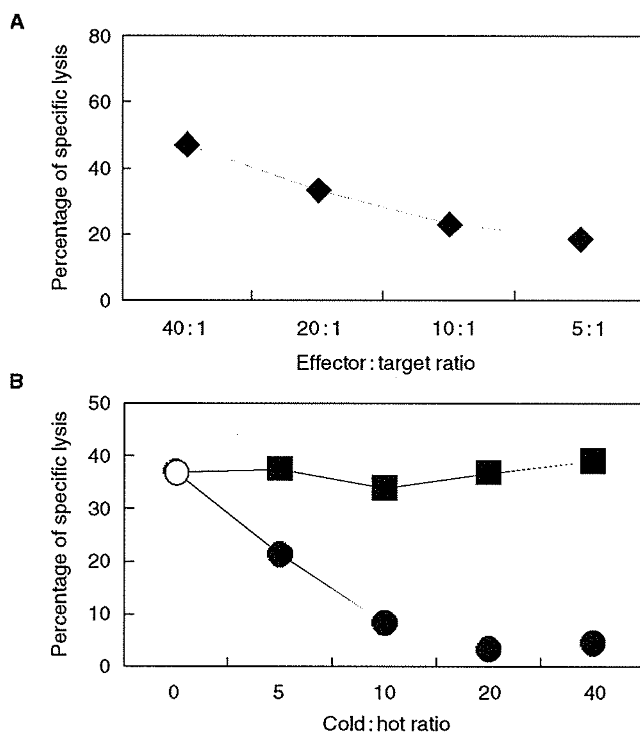

***Fig. 6.* Cytotoxicity of the clone C27 against normal bronchial epithelial cells.** C27-mediated lysis of normal bronchial epithelial cells (NHBE) expressing HLA-A24 and Ep-CAM (A) and C27-mediated NHBE cell lysis were blocked with T2-A24 cells pulsed with Ep_{173} (●) but not with a control peptide EBV-LMP2_{419} (■) (B). The effector to hot target ratio was 20. HLA, human leucocyte antigen.

stabilization assays were used in order to determine the binding affinity of the peptides with HLA-A*2402 molecules, revealing one peptide, Ep_{173} (RYQLDPKFI), to have the highest affinity and Ep_{304} (EMGEMHREL) the lowest. Thirdly, we successfully established Ep_{173}-specific polyclonal CTL lines from four of five healthy donors. A CTL clone, C27, demonstrated fine specificity for Ep_{173}, which was naturally presented on the surfaces of tumour cells, because C27-mediated PC9 cell lysis was blocked by both anti-HLA-A24 monoclonal antibody and Ep_{173}-pulsed cold target cells. An Ep_{250}-specific polyclonal CTL line was generated from donor 3, raising the possibility that Ep_{250} is another CTL epitope presented by HLA-A*2402. We, so far, could not establish CTL clones specific to Ep_{250}, which are required for further evaluation of the peptide.

It is of note that there is no correlation between the level of expression of Ep-CAM and the degree of CTL-mediated lysis (Figs. 3 and 5; Table 2). The level of HLA expression does not seem to explain the issue. We speculate that the Ep-CAM may be more resistant to proteasome degradation in cells with high Ep-CAM expression and low or moderate sensitivity to CTL-mediated lysis (LC-1/sq, MKN45 and QG56). On the contrary, there may be a higher turnover rate of the Ep-CAM in cells with low expression and high sensitivity to CTL-mediated lysis (LU99, LC99A and HSC-2).

Although Ep-CAM is a candidate target for both humoral and cellular immunotherapy, a persistent difficulty arises because of its presentation on normal epithelial tissues. Indeed, the CTL clone, C27, lysed HLA-A24-positive normal bronchial epithelial cells in an epitope-specific fashion, especially at high effector : target ratios (Fig. 6). However, at lower effector : target ratios, such as 10 : 1 and 5 : 1, C27-mediated lysis of NHBE was apparently lower than that of C27-sensitive cancer cell lines (Fig. 5). In addition, C27-mediated lysis of NHBE was more efficiently inhibited by T2-A24 cells pulsed with the cognate peptide (Fig. 6B) than that of the PC9 lung cancer cell line (Fig. 4C), implicating a lower density of HLA-A*2402/Ep_{173} complexes on the surface of NHBE cells.

It has been reported that an HLA-A*0201-restricted Ep-CAM-specific CTL line could not lyse normal bronchial epithelial cells under conditions, whereby epithelial tumour cells were efficiently killed (20). So far, immunization of colorectal carcinoma patients with a recombinant canarypox virus expressing Ep-CAM has been well tolerated and has been shown to induce anti-Ep-CAM CTL responses without causing autoimmune reactions (43). Interestingly, a monoclonal antibody specific to Ep-CAM did not localize to Ep-CAM-positive normal tissues of human Ep-CAM transgenic mice (6), indicating limited *in vivo* accessibility, which might explain any absence of autoimmunity. Another example of vaccination of tissue-specific self-antigen is provided by means of immunotherapy targeting CEA (carcino embryonic antigen). Greiner et al. (44) reported that vaccination of CEA-transgenic mice with a recombinant canarypox virus expressing CEA can generate substantial anti-tumour immunity with little or no autoimmunity. They proposed possible explanations including (i) differential susceptibility of tumour and normal tissues to the immune effector arms and (ii) blockage of the autoreactive T-cell activity by tolerizing antigen-presenting cells or the presence of regulatory T cells in order to terminate the response in the normal epithelia. Further studies are required in order to evaluate potential autoimmune reactions with Ep_{173} immunization. Littermates of human Ep-CAM transgenic mice (6) bred with HLA-A*2402 transgenic mice would provide a suitable model to study this issue.

In conclusion, we present, in this study, a novel HLA-A*2402-restricted epitope, Ep_{173} (RYQLDPKFI), which has the ability to induce $CD8^+$ T cells with high-affinity antigen receptors directed to HLA-A*2402/Ep_{173} complexes. All the data suggest that the epitope-specific CTL responses may play some roles in both anti-cancer and autoimmune reactions. The peptide should prove useful to study anti-Ep-CAM CTL responses among populations possessing HLA-A*2402.

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A Novel HLA-A*3303-Restricted Minor Histocompatibility Antigen Encoded by an Unconventional Open Reading Frame of Human TMSB4Y Gene[1]

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Female-to-male hemopoietic stem cell transplantation (HSCT) elicits T cell responses against male-specific minor histocompatibility (H-Y) Ags encoded by the Y chromosome. All previously identified H-Y Ags are encoded by conventional open reading frames, but we report in this study the identification of a novel H-Y Ag encoded in the 5′-untranslated region of the *TMSB4Y* gene. An HLA-A*3303-restricted CD8$^+$ CTL clone was isolated from a male patient after an HSCT from his HLA-identical sister. Using a panel of cell lines carrying Y chromosome terminal deletions, a narrow region controlling the susceptibility of these target cells to CTL recognition was localized. Minigene transfection and epitope reconstitution assays identified an 11-mer peptide, EVLLRPGLHFR, designated TMSB4Y/A33, whose first amino acid was located 405 bp upstream of the *TMSB4Y* initiation codon. Analysis of the precursor frequency of CTL specific for recipient minor histocompatibility Ags in post-HSCT peripheral blood T cells revealed that a significant fraction of the total donor CTL response in this patient was directed against the TMSB4Y epitope. Tetramer analysis continued to detect TMSB4Y/A33-specific CD8$^+$ T cells at least up to 700 days post-HSCT. This finding underscores the in vivo immunological relevance of minor histocompatibility Ags derived from unconventional open reading frame products. ***The Journal of Immunology*, 2004, 173: 7046–7054.**

Minor histocompatibility (minor H)[4] Ags are MHC-bound peptides derived from cellular proteins and are encoded by polymorphic genes, including Y chromosome-specific genes (1–3). Disparities in some minor H Ags in allogeneic hemopoietic stem cell transplantation (HSCT) have been shown to be associated with graft-vs-host disease (GVHD), graft rejection, or graft-vs-leukemia/lymphoma (GVL) effect (4–11). In the case of female to male HSCT, T cell clones specific for Y chromosome-encoded (H-Y) Ags were generated from the peripheral blood of recipients during GVHD or graft rejection, and their HLA class I or II epitopes have been identified, including *SMCY* (12, 13), *DFFRY* (14, 15), *UTY* (16, 17), *RPS4Y* (18), and *DBY* (19, 20). These five genes are among eight genes that have been reported to lie in the nonrecombining region of the human Y chromosome and have functional X homologues (21). Because all eight genes are sufficiently polymorphic with their X chromosome homologues to induce H-Y-specific T cell responses, it should be possible that more H-Y epitopes can be encoded either by the five genes that have proved to be immunogenic or by other Y chromosome genes (i.e., *ZFY*, *AMELY*, and *TMSB4Y*) for which H-Y epitopes have not yet been described.

In this study we report the identification of a novel human H-Y Ag, recognized by an HLA-A*3303-resricted CTL clone isolated from a male patient who developed chronic, but not acute, GVHD. The identified H-Y Ag is an 11-mer peptide, EVLLRPGLHFR, derived from *TMSB4Y*, a gene encoding thymosin β-4, Y isoform (22). Interestingly, the epitope identified in the *TMSB4Y* gene was encoded by the polymorphic region located 405 bp upstream of the initiation codon of the conventional open reading frame (ORF), whereas all minor H Ags identified to date are encoded by conventional ORF of the individual gene. There have been several reports describing CTL epitopes encoded by unconventional ORFs, such as untranslated regions (UTR) or alternative reading frames, most of which have been identified in tumor cells (reviewed in Ref. 23). To our knowledge, this is the first demonstration of a minor H Ag encoded in a region other than conventional coding region. Furthermore, we demonstrated, by CTL precursor (CTLp) frequency analysis, that a significant fraction of the total donor CTL responses in this patient was directed against the TMSB4Y epitope, and that the precursor remained detectable up to

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[4] Abbreviations used in this paper: minor H Ag, minor histocompatibility Ag; CI, confidence interval; CTLp, CTL precursor; DRiP, defective ribosomal product; GVHD, graft-vs-host disease; GVL, graft vs leukemia/lymphoma; HSCT, hemopoietic stem cell transplantation; H-Y Ag, Y chromosome-encoded Ag; LCL, B-lymphoblastoid cell line; ORF, open reading frame; UTR, untranslated region; STS, sequence-tagged site.

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700 days after HSCT. These findings underscore the in vivo immunological relevance of such a cryptic minor H Ag derived from unconventional ORF products.

Materials and Methods

Cell cultures and Abs

The HLA-A*3303-restricted $CD8^+$ CTL clone, 1B6, was isolated by limiting dilution from a cytotoxic T cell line generated from a PBMC sample obtained on day 50 post-HSCT from a 54-year-old man (HLA-A*2402/*3303, B*4403/*5401, Cw*0803/*1403) who had received his HLA-identical sister's marrow for treatment of chronic myelocytic leukemia. He did not develop acute GVHD, but did develop mild chronic GVHD of the skin and liver. The CTL clone was expanded as previously described (24) and frozen until use. B-lymphoblastoid cell lines (LCLs) were established from the donor and recipient and from normal volunteers. All blood or tissue samples were collected after obtaining written informed consent, and the study was approved by the institutional review board of Aichi Cancer Center.

The LCLs derived from individuals with Y chromosome deletions were provided by Dr. D. C. Page (Howard Hughes Medical Institute, Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA), and a detailed analysis of these lines has been previously reported (25). LCLs selected according to their deletion pattern and other cell lines including Raji were retrovirally transduced with HLA-A*3303 cDNA as described previously (26). LCLs were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS (Immuno-Biological Laboratory, Gunma, Japan), 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin/streptomycin. Primary dermal fibroblast lines from skin and oral mucosa, bone marrow stromal cell lines, and 293T cells were grown in IMDM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% FCS, 2 mM L-glutamine, and penicillin/streptomycin. mAbs, W6/32 (anti-pan HLA class I), HDR-1 (anti-HLA-DR), and A11.1M (anti-HLA-A24) were provided by Dr. K. Ito (Kurume University, Fukuoka, Japan).

Cytotoxicity assays

Target cells were labeled with 0.1 mCi of ^{51}Cr for 2 h, and 1×10^3 target cells/well were mixed with CTL at various E:T cell ratios in a standard 4-h cytotoxicity assay using 96-well, round-bottom plates. All assays were performed at least in duplicate. Cells were treated with IFN-γ (100 U/ml; Endogen, Woburn, MA) and TNF-α (10 ng/ml; Endogen) for 48 h where indicated. The percent specific lysis was calculated as follows: ((experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)) × 100. When necessary, allo-HLA-A24-specific CTL clones were used to confirm the susceptibility of the target cells.

Mapping of Y chromosome deletion mutant LCLs

Oligonucleotide primer pairs specific for sequence-tagged sites (STSs) previously mapped to the Y chromosome (25) were used to PCR-amplify the corresponding Y chromosomal target sequences from genomic DNA of each LCL. Amplification of STSs was performed as reported previously (16). Aliquots of each PCR were separated in 2% agarose or 5% acrylamide gels, and cell lines were scored as positive or negative for the presence of each STS. DNA extracted from LCLs derived from normal male and female donors served as positive and negative controls, respectively.

Detection of expression of the candidate genes

An RT-PCR assay was used to examine the expression of the candidate genes with cDNA synthesized from LCLs. PCR was performed in a total volume of 20 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each gene-specific primer, and 1 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) on a GeneAmp PCR system 9700 (Applied Biosystems). The PCR products were separated in 2% agarose gels and visualized with ethidium bromide staining.

PCR cloning of TMSB4Y gene

The conventional ORF sequence and full-length sequence of the *TMSB4Y* (GenBank accession no. NM_004202) were amplified from cDNA prepared from the recipient LCL and subcloned into a mammalian expression plasmid. The primer sequences used were as follows (*Hin*dIII and *Not*I sites are underlined, respectively): conventional ORF sense, 5′-TTAAGCTTCGCAGCCATGTCTGACAAACC-3′; conventional ORF antisense, 5′-ATGCGGCCGCCATGCCTGTTTAAGATTCGC-3′; full-length sense, 5′-TTAAGCTTTGGGAACAGACAGATCCTTTG-3′; and full-length antisense, 5′-ATGCGGCCGCTAGATTTCACTGCCCTCCCA-3′. PCR amplification was conducted in a total volume of 25 μl of 1× buffer containing 200 μM of each dNTP, 1.0 mM $MgSO_4$, 0.3 μM of each primer, and 1 U of KOD-Plus-DNA polymerase (TOYOBO, Osaka, Japan).

All products were digested with the restriction enzymes and ligated into *Hin*dIII-*Not*I-cut pEAK10 vector (Edge Biosystems, Gaithersburg, MD). The sequences of the cloned genes were verified by direct sequencing with BigDye Terminator kit (version 3.0, Applied Biosystems) on an ABI PRISM 3100 (Applied Biosystems).

Construction of truncated genes and minigenes for TMSB4Y

Expression plasmids encoding truncated forms of the TMSB4Y cDNA were constructed by RT-PCR using antisense primers that produced 345, 552, 754, and 955 bp DNA fragments. All products were ligated into the pEAK10 vector as described above. Minigene expression plasmids encoding the minimal, N or C terminus-extended polypeptides of the epitope predicted by BIMAS software (http://bimas.dcrt.nih.gov/molbio/hla_bind/) (27) and SYFPEITHI software (http://syfpeithi.de) (28) were constructed as previously described (29). The constructs all encoded a Kozak sequence and initiator methionine (CCACC-ATG) and a stop codon (TAG). Pairs of sense and antisense oligonucleotides were designed to form cohesive ends for *Hin*dIII and *Not*I sites at the 5′ and 3′ ends after hybridization, respectively, and all products were ligated into the pEAK10 vector and verified by sequencing.

Electroporation of LCL

The constructed vectors were introduced either into the donor LCL or into 293T cells. One million LCL were resuspended in 40 μl of OPTI-MEM I buffer (Invitrogen Life Technologies) and 4 μg of each plasmid in a 2-mm gap cuvette, and electroporated in an ECM 830 BTX Electro Square Porator (BTX, San Diego, CA) at 350 V and a pulse length of 1 ms. Then, cells were cultured in 4 ml of culture medium for 2 d, followed by selection with puromycin (0.7 μg/ml) for 3 d before use.

Transfection of 293T cells and cytokine release assays

293T cells were retrovirally transduced with HLA-A*3303 cDNA and selected in the presence of 1 μg/ml puromycin (referred to as 293T-A33). Aliquots of the 293T-A33 cells were transiently cotransfected with pEAK10 vectors encoding full-length TMSB4Y, a C-terminal deletion mutant cDNA, or minigenes of TMSB4Y. 293T-A33 cells were plated the day before transfection at 4×10^4 cells/100 μl/well into 96-well, flat-bottom microtiter plates and transfected with 6 μl of RPMI 1640 containing 90 ng of plasmid DNA and 0.27 μl of FuGENE 6 (Roche, Indianapolis, IN). After 24 h at 37°C, 100 μl of a cell suspension containing 1×10^4 CTL clone 1B6 in IMDM containing 20 U/ml IL-2 was added. Supernatants from the cocultures were harvested after 24 h and assayed for the presence of IFN-γ by ELISA.

Epitope reconstitution assay

The candidate peptide epitope identified by the minigene experiments and the homologous TMSB4X-encoded peptide were synthesized by standard methods. ^{51}Cr-labeled donor LCL were incubated for 30 min in medium containing 10-fold serial dilutions of the peptides and then used as target cells in standard cytotoxicity assays.

Real-time PCR assay for TMSB4Y expression

cDNA from a panel of different human adult and fetal tissues were purchased from BD Clontech (MTC panels human I and II; Palo Alto, CA) or synthesized from total RNA of human lung (BD Clontech) or various cultured cells. PCR amplification and real-time quantification analysis were performed using the TaqMan assay according to the manufacturer's instructions. The following sequences were used as primers and TaqMan probe to detect the mRNA region encoding the epitope: 5′-GACTAGAAAGCGGGCGCAG-3′ (sense; nt 302–320), 5′-ACTTCCGCGTTCAAGTGGTT-3′ (antisense; nt 415–434), 5′-(FAM)-TCCCTTCTCGACACGGAGTCTATGTGTAGT-(MGB)-3′ (TMSB4Y probe; antisense; nt 366–382). For the internal control, a primer and probe set for human GAPDH (Applied Biosystems) was used. PCR was performed in a 1× TaqMan Universal PCR master mix containing 10 pmol of each sense and antisense primer and 2 pmol of probe in a total volume of 25 μl in the ABI PRISM 7700HT Sequence Detector System (Applied Biosystems). The temperature profile was 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 62°C for 1 min for 40 cycles. Samples were quantified using relative standard curves for each amplification. All results are normalized with

respect to the internal control and are expressed relative to the levels found in a pool of male PBMC.

Limiting dilution-based CTLp frequency assay

The proportion of CTLp specific for the TMSB4Y peptide among the total CTLp against the recipient minor H Ags was quantitated using a standard limiting dilution assay. Purified CD8$^+$ T cells from the PBMC obtained at days 50 and 146 post-HSCT were cultured at 2-fold serial dilutions with 33 Gy-irradiated 3×10^4 CD40-activated B (CD40-B) cells generated from pre-HSCT recipient PBMC in 96-well, round-bottom plates in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% pooled human serum. IL-2 (50 U/ml) was added on days 2 and 5 after each restimulation with CD40-B cells. For each dilution, there were at least 12 replicates. After three rounds of stimulation, a split-well analysis was performed for peptide-specific cytotoxicity against ^{51}Cr-radiolabeled recipient PHA blasts or donor PHA blasts pulsed with TMSB4Y peptide or unpulsed. The supernatants were measured in a gamma counter after 4-h incubation. The wells were considered to be positive for lytic activity if the total cpm released by effector cells was $>2.5 \times$ SD above control wells (mean cpm released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated by L-Calc software (StemCell Technologies, Vancouver, Canada).

Tetramer construction and flow cytometric analysis

MHC-peptide tetramers were produced as described previously (30). In brief, HLA-A*3303 H chain and β_2-microglobulin (cloned in pHN1$^+$ vector; provided by the late Dr. D. C. Wiley, Howard Hughes Medical Institute, Harvard University, Cambridge, MA) were produced in XA90. The C terminus of the H chain was modified by the addition of a substrate sequence for the biotinylating enzyme BirA. Monomeric HLA/β_2-microglobulin/peptide complexes were folded in vitro in the presence of the peptide. The MHC complex was biotinylated and then converted into tetramers with PE-labeled streptavidin. For staining, PBMC or T cell lines were incubated with the tetramer at a concentration of 20 μg/ml at room temperature for 15 min, followed by FITC-conjugated anti-CD3 (BD Biosciences, San Diego, CA) and Tricolor anti-CD8 mAb (Caltag Laboratories, Burlingame, CA) on ice for 15 min. Cells were analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Results

*A CD8$^+$ CTL clone shows cytotoxicity against an H-Y Ag presented on HLA-A*3303$^+$ LCL*

CD8$^+$ CTL clone 1B6 efficiently lysed recipient LCL and PHA blasts, but not donor LCL (Fig. 1*A*). Addition of anti-pan HLA class I mAb, but not anti-HLA-A24 or anti-HLA-DR mAbs, significantly inhibited lysis of recipient LCL by 1B6. Transduction of HLA-A*3303 cDNA into a male LCL conferred susceptibility to 1B6, indicating that the clone was restricted by HLA-A*3303 (Fig. 1*B*). 1B6 showed very weak cytotoxicity against dermal or oral fibroblasts or against keratinocytes generated from HLA-A*3303-positive male individuals, whereas these targets were lysed moderately (i.e., 25–35%) by CTL specific for HLA-A24 alloantigen, which is shared by these targets. Even when they were treated with cytokines (IFN-γ and TNF-α), 1B6 still demonstrated relatively weak cytotoxicity, although HLA-A24-allospecific CTL induced robust cytotoxicity (Fig. 1*C*). Finally, 1B6 showed lytic activity only against male, but not female, LCLs transfected with HLA-A*3303 cDNA, indicating that the clone was specific for a H-Y Ag (data not shown).

The gene encoding the minor H Ag maps to deletion interval 5D on the Y chromosome

Cytotoxicity assay-based mapping was conducted to determine the location on the Y chromosome of the minor H gene encoding the epitope for 1B6. First, various LCLs were typed for terminal deletions of the Y chromosomes using the technique of STS content mapping (16, 21). Of these, a panel of LCLs with distinct terminal deletions was selected for transfection with HLA-A*3303 cDNA and assayed for susceptibility to 1B6.

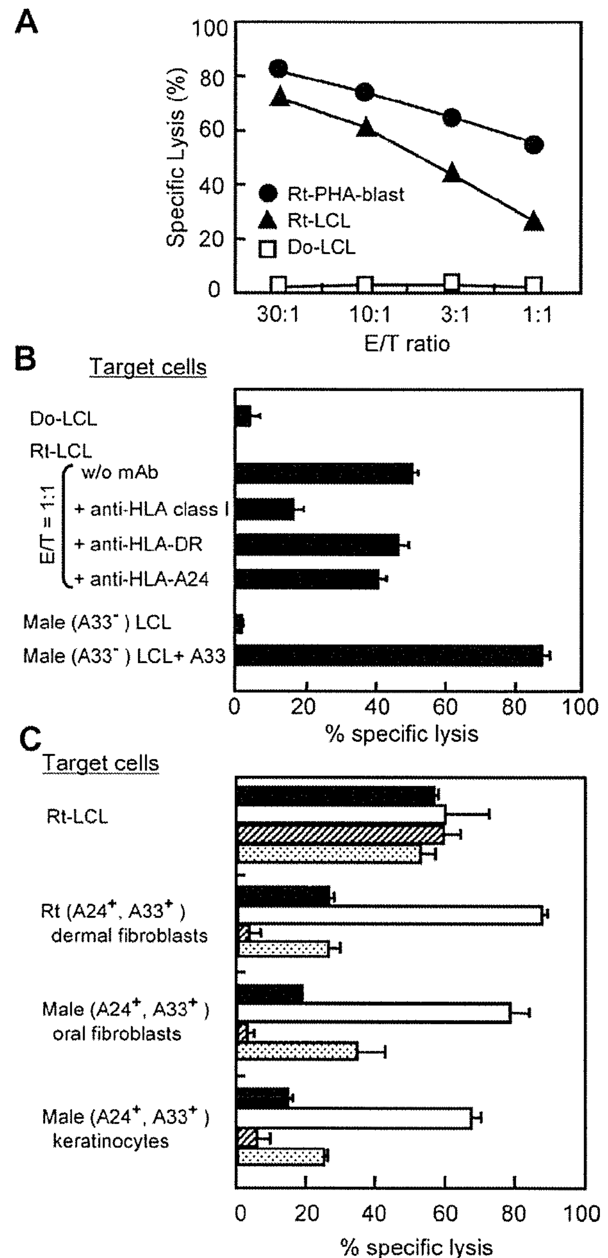

FIGURE 1. Specificity of the HLA-A*3303-restricted CTL clone, 1B6, analyzed in standard ^{51}Cr release assays. *A*, 1B6 recognition of target cells derived from recipient (Rt) LCL, PHA blasts, or donor (Do) LCL at the E:T cell ratios indicated. *B*, Ab blocking of the cytolysis was performed with anti-HLA mAbs (E:T cell ratio, 1:1). HLA-A*3303-negative male LCL with or without HLA-A*3303 transduction were tested at an E:T cell ratio of 10:1. *C*, Cytolytic activity of 1B6 or allo-HLA-A*2402-specific CTL against cytokine-treated or untreated HLA-A*3303 and A*2402-positive male B-LCL, dermal fibroblasts, oral fibroblasts, and keratinocytes was tested at an E:T cell ratio of 10:1. The cytokine treatment used was incubation of targets cells with 100 U/ml IFN-γ and 10 ng/ml TNF-α for 48 h before ^{51}Cr labeling. The lysis of cytokine-treated cells by 1B6 (■) or allo-specific CTL (□) and of cytokine untreated cells by 1B6 (▨) or allo-specific CTL (▩) is shown.

Fig. 2*A* shows the 43-interval deletion map of the 7 LCLs and their susceptibility to 1B6. LCL WHY10 and WHY12 that were lysed by 1B6 share only deletion intervals 5C and 5D, indicating that the region controlling the expression of this minor H Ag is

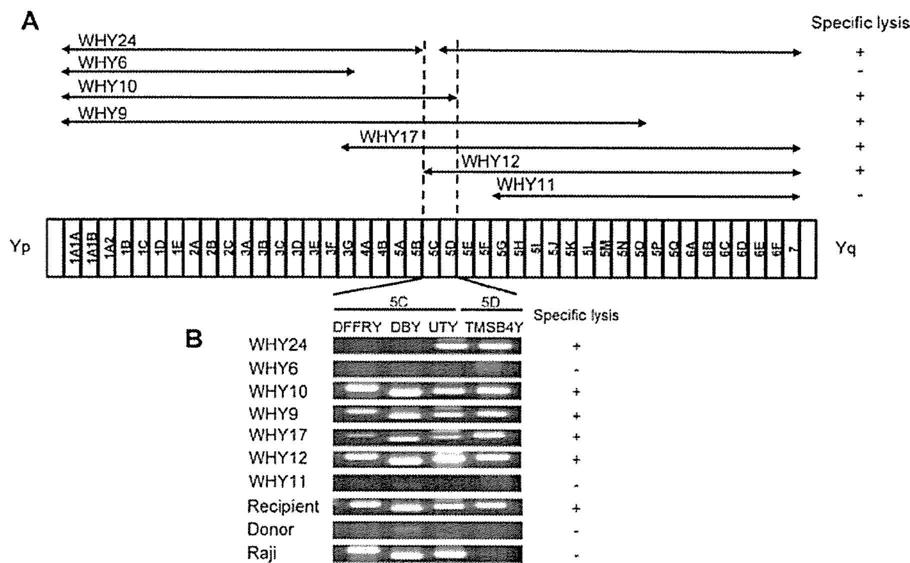

FIGURE 2. Mapping of the gene on the Y chromosome that encodes the minor H Ag recognized by 1B6. *A*, Genetic map of the LCLs carrying various terminal deletions of Y chromosome and their susceptibility to 1B6. Appropriate LCLs were selected based on their pattern of terminal deletions (16, 21, 25), transduced with HLA-A*3303 cDNA, and tested in standard ^{51}Cr release assays. The presence of the region encoding the minor H Ag in each LCL line is determined by its susceptibility to 1B6 (indicated in the *right column*). Bidirectional arrows indicate the conserved region(s) from deletion and are related to the 43-interval deletion map of the Y chromosome (21, 25). Vertical dotted bars indicate the region predicted to encode the minor H Ag. *B*, mRNA expression of four genes encoded in the deletion intervals 5C and 5D in selected cell lines and their recognition by 1B6. Female donor LCL served as a negative control. HLA-A*3303 transfected Raji cells, a Burkitt lymphoma cell line derived from a male patient, were also analyzed.

located within these two deletion intervals. The region was further narrowed down using the results from LCL WHY24, which was found to lack deletion interval 5C, but was nevertheless lysed by 1B6. Collectively, these results indicate that the gene encoding the minor H Ag maps to deletion interval 5D. Four genes, *DFFRY*, *DBY*, *UTY*, and *TMSB4Y*, all of which have X homologues, are encoded within deletion intervals 5C and 5D (21, 25). We examined mRNA expression of these four genes among the seven LCLs by RT-PCR (Fig. 2*B*). As expected from the results of deletion mapping, WHY6 and WHY11 were negative for the expression of all four genes; WHY10, WHY9, WHY17, and WHY12 were all positive. Because WHY24 was positive for the expression of TMSB4Y and UTY and was lysed by 1B6, the minor H Ag was encoded by either *UTY* or *TMSB4Y*. In addition, during the course of specificity analysis, we found that HLA-A*3303-transduced Raji cells were not killed by 1B6, although Raji cells are of male origin. RT-PCR analysis showed that they were negative for expression of TMSB4Y as shown in Fig. 2*B*. Moreover, female LCLs from the patient's HSCT donor transfected with any of three isoforms of *UTY* cDNA were not lysed by 1B6 (data not shown). These results indicated that *TMSB4Y* most likely encoded the minor H Ag.

The 5′ untranslated region of the TMSB4Y gene encodes the minor H Ag

To determine whether *TMSB4Y* indeed encodes the minor H epitope recognized by 1B6, we first tested CTL recognition of the female donor LCL transduced with the reported TMSB4Y ORF comprising 43 aa. However, 1B6 did not lyse the transfectant (Fig. 3*A*), suggesting either that the epitope is encoded not by *TMSB4Y* but by another gene located in deletion interval 5D, or that it is encoded elsewhere in the ~1.7-kb TMSB4Y cDNA. Recently, cryptic CTL epitopes encoded by alternative sources such as noncoding regions and nonconventional ORF have been described in both murine and human tumor cells (23). Thus, we cloned the full-length TMSB4Y cDNA (GenBank accession no. NM_004202) and then transduced donor LCL with it. As shown in Fig. 3*B*, female LCL expressing full-length TMSB4Y cDNA were lysed efficiently. Because the alternative ORF that is able to encode the antigenic peptide was unknown, a series of 3′ terminal deletion mutants of the TMSB4Y cDNA were prepared and tested for recognition by 1B6 by IFN-γ ELISA. Although cells transfected with TMSB4Y cDNA fragments extending from nt 1–552 were recognized when expressed in HLA-A*3303-transduced 293T cells, transfection of the fragment encoding nt 1–345 was not (Fig. 3*C*). These results indicated that the epitope was encoded in the 5′UTR between nt 346 and 552, which is at least 240 nt upstream of the reported ORF for the TMSB4Y protein (Fig. 4*A*).

Among three reading frames in this region, only one initiator methionine (nt 362–364) was found in the same reading frame encoding the TMSB4Y protein, followed by a polypeptide consisting of 19 aa, EVLLRPGLHFRNSCPILTT. This 19-mer contains a nonamer, LLRPGLHFR, which has the reported peptide-binding motif for HLA-A*3303 (i.e., Ala, Ile, Leu, Phe, Tyr, or Val at position 2, and Arg at C terminus) (31), with a predicted dissociation score of 9.0 by BIMAS software (27). However, a minigene construct encoding LLRPGLHFR failed to stimulate 1B6. Additional experiments using minigene constructs with N or C extensions finally identified the minimal epitope as EVLLRPGLHFR (Figs. 3*D* and 4*A*). Both Arg at the C terminus and Glu at the N terminus were essential for recognition by 1B6, indicating that Val and Arg are the likely N- and C-terminal anchors, respectively. The X homologue of TMSB4Y, TMSB4X cDNA (GenBank accession no. NM_021109), encoding thymosin β4, has much shorter 5′- and 3′UTR; thus, no corresponding region was found (Fig. 4*B*). However, a recently reported splice variant of *TMSB4X*, which includes 1076 bp of *TMSB4X* intron 1 (GenBank accession no. AK055976), has an initiator methionine and a following 32 aa in its 5′UTR upstream *TMSB4X* conventional ORF, and potentially encodes ETLFLPGLHFR, which differs from the 1B6

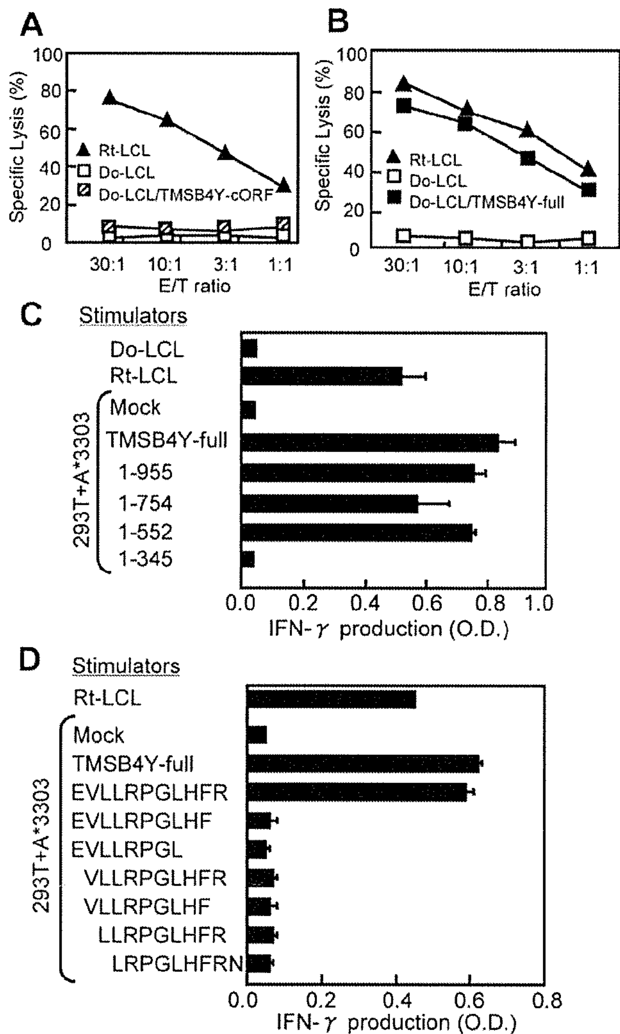

FIGURE 3. Localization of the minor H Ag epitope defined by 1B6 in TMSB4Y. *A*, Mammalian expression plasmid encoding the conventional ORF (cORF) of TMSB4Y, as identified in the deletion mapping (Fig. 2), was transfected into donor (Do) LCL, and recognition of transfected LCL by 1B6 was determined in a standard ^{51}Cr release assay. Recipient (Rt) LCL were used as a positive control. *B*, Plasmid encoding the full-length TMSB4Y cDNA containing the 5′- and 3′UTR (TMSB4Y-full; GenBank accession no. NM_004202) was transfected into donor LCL, and their susceptibility to 1B6 was tested as described above. *C*, Localization of the region encoding the minor H Ag by 3′ deletion mutants of the TMSB4Y cDNA. HLA-A*3303-transduced 293 T cells were transfected with plasmids encoding various 3′-deleted TMSB4Y cDNAs terminating at 345, 552, 754, or 955 nt and cocultured with 1B6. Supernatants were harvested and assayed for the presence of IFN-γ by ELISA. The OD of each supernatant is shown. *D*, Identification of the 1B6 epitope. The susceptibilities of HLA-A*3303-transduced 293 T cells transfected with minigene constructs encoding nonamer peptide (LLRPGLHFR) predicted by BIMAS software (27) and selected N- or C-terminally extended peptides were tested by ELISA.

epitope, EVLLRPGLHFR, by three amino acids (underlined; Fig. 4, *B* and *C*).

We next synthesized these two 11-mer peptides, EVLLRPGLHFR and ETLFLPGLHFR, and tested the cytotoxicity of 1B6 against donor LCL pulsed with serial dilutions of each peptide. The titration of peptide EVLLRPGLHFR recognized by 1B6 gave half-maximal lysis at a concentration of 20 nM, whereas peptide ETLFLPGLHFR failed to sensitize the donor LCL at any concentration tested (Fig. 5). Thus, EVLLRPGLHFR defines the HLA-A*3303-restricted 1B6 epitope, and we designated it TMSB4Y/A33.

mRNA expression of the TMSB4Y gene is found in various tissues and cell types

The X homologue of *TMSB4Y*, *TMSB4X*, has been shown to be expressed in a broad range of tissue types in rodents, with very high levels in spleen, thymus, and lung (32). To determine the distribution of *TMSB4Y* expression in different tissues, quantitative PCR analysis targeted to the 5′UTR of the mRNA was performed using a large panel of test samples derived from different tissues. PCR analysis demonstrated that the expression of *TMSB4Y* mRNA assessed by its 5′UTR was indeed observed in a wide range of normal tissues; from the highest expression in testis, prostate, pancreas, and hemopoietic cells to the lowest expression in dermal fibroblasts and skeletal muscles (~50-fold less than that in hemopoietic cells; data not shown). Expression of the mRNA in a panel of primary leukemic cells ranged from undetectable to levels similar to those seen in normal hemopoietic cells (data not shown).

TMSB4Y/A33-specific CD8$^+$ T cells are detectable in recipient post-transplant PBMC

A split-well assay was used to estimate the relative frequencies in the post-HSCT PBMC of CTLp specific for the TMSB4Y/A33 minor H Ag and those specific for other minor H Ags expressed on the recipient's hemopoietic cells. As shown in Fig. 6*A*, the frequencies of CTLp reactive with recipient PHA blasts and TMSB4Y/A33 peptide-pulsed donor PHA blasts in peripheral blood obtained on day 50 post-HSCT from which the 1B6 was derived were 324 (95% confidence interval (CI), 213–493) and 96 (95% CI, 47–175) per 10^6 peripheral blood CD8$^+$ cells, respectively, indicating that nearly a quarter of the CTL responses to recipient minor H Ags in this donor/recipient pair were indeed directed at the TMSB4Y/A33 minor H Ag. On day 146, the frequency of CTLp recognizing TMSB4Y peptide-pulsed donor PHA-blasts was 316 (95% CI, 216–464), and that for CTLp recognizing recipient PHA blasts was 3215 (95% CI, 2150–4808) per 10^6 peripheral blood CD8$^+$ cells, demonstrating that even at the later time point the CTL responses against TMSB4Y/A33 continued to account for a significant fraction (10%) of the total donor CTL responses against recipient minor H Ags in this donor/recipient pair (Fig. 6*B*).

In additional experiments, an HLA/peptide tetramer was used to confirm the presence of TMSB4Y/A33-specific CTL in unstimulated post-HSCT PBMC (Fig. *C*, *left column*) as well as in T cell lines prepared by stimulating these PBMC with the same stimulators used in the CTLp assay (Fig. *C*, *right column*). The assays clearly detected TMSB4Y/A33-specific CD8$^+$ T cells in PBMC obtained on day 696 (0.35%), but for PBMCs obtained on day 50 and 146, the presence of TMSB4Y/A33-specific T cells was not clear because of the low number of PBMC available. After in vitro stimulation, tetramer-positive cells became detectable for the latter two samples, although direct comparison with the CTLp results was not possible due to the use of different culture conditions in the two assays.

Discussion

In this study we have identified a gene, *TMSB4Y*, encoding a novel HLA-A*3303-restricted, H-Y Ag by testing HLA-A*3303-transfected cell lines carrying terminal deletions of the Y chromosome in cytotoxicity assays. This approach has previously been used to identify the HLA-B8-restricted H-Y Ag encoded by *UTY* (16).

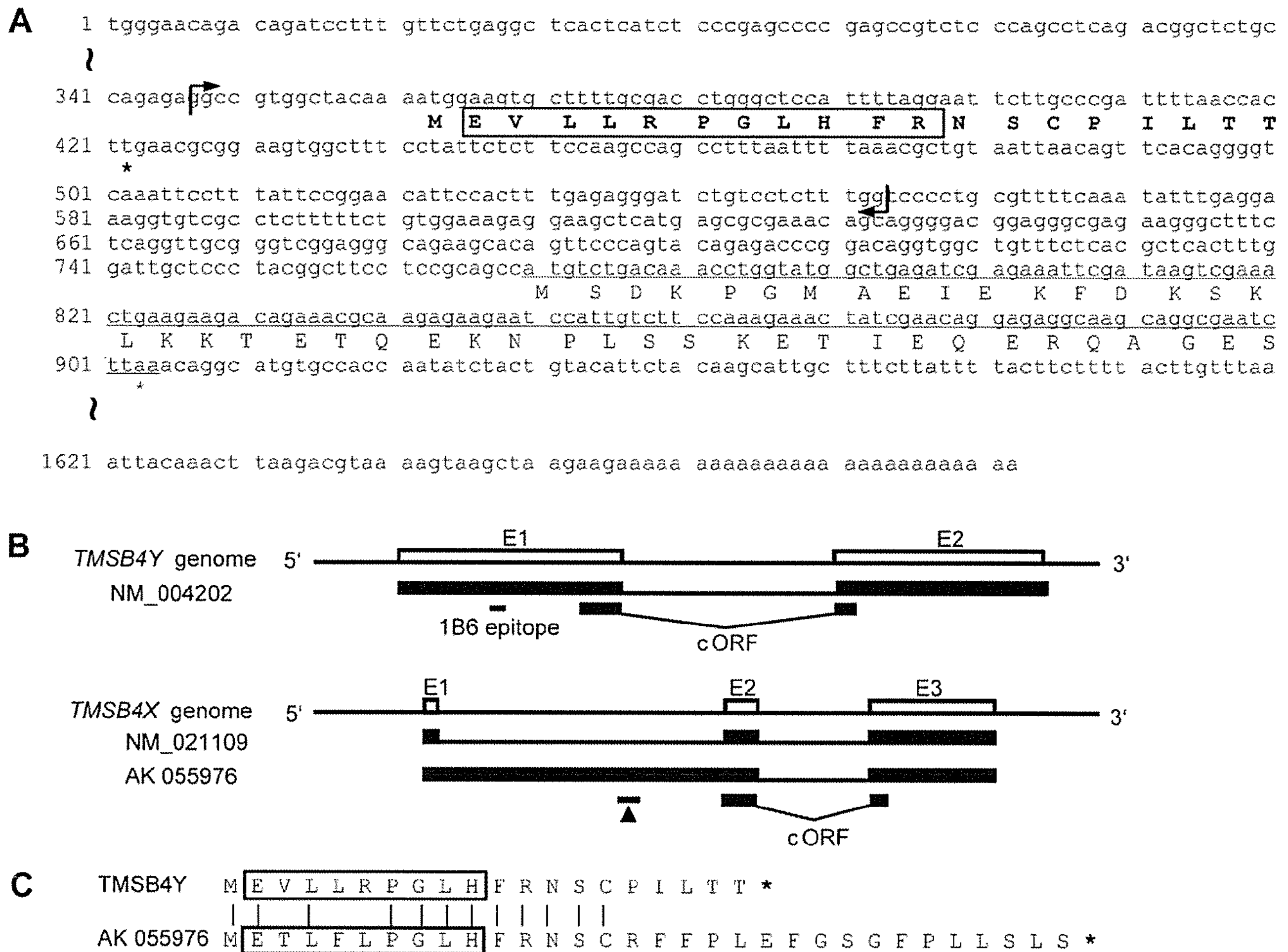

FIGURE 4. Location of the identified 1B6 epitope in the nucleotide and deduced amino acid sequences of the TMSB4Y cDNA (1702 bp; GenBank accession no. NM_004202). *A*, The deduced amino acid sequence is shown in one-letter designation below the nucleotide sequence for conventional ORF (the middle of the nucleotide sequence) and 5′UTR encoding the epitope recognized by 1B6. Asterisks indicate stop codons, and arrows indicate the region initially mapped by TMSB4Y cDNAs with nested 3′ deletions. The sequence corresponding to the identified peptide is boxed. *B*, Genomic organization of *TMSB4Y* and its X homologue, *TMSB4X*, and the relationship with their mRNAs. E1, E2, and E3 indicate exon 1, exon 2, and exon 3, respectively. The conventional ORF is indicated below the mRNA as cORF. The location of identified 1B6 epitope is shown below the 5′UTR of TMSB4Y cDNA. The putative X homologue peptide in a reported splice variant of *TMSB4X* containing its intron 1 (GenBank accession no. AK055976) is indicated by the arrowhead. *C*, Comparison of the deduced amino acid sequence in the 5′UTR encoding the 1B6 epitope in TMSB4Y and the putative X homologue (TMSB4X) peptide.

With the current discovery, all four identified genes that are encoded in deletion intervals 5C and 5D of the nonrecombining region of the human Y chromosome have been shown to encode at least one minor H Ag presented by class I or II HLA (14–17, 19, 20). Although the peptide sequence of the TMSB4Y/A33 minor H Ag identified in this study was 11 residues in length, and half-maximal lysis of peptide-pulsed female target cells was observed at a relatively high peptide concentration (~20 nM), it is likely that the 11-mer peptide is the minimal epitope, because it has a consensus Arg at the C terminus and a Val at the auxiliary anchor (position 2) (31). In addition, two computer algorithms predict that cleavage after the C-terminal Arg would be correctly performed by proteasomes (33, 34). Although all previously identified human H-Y Ags have homologue peptide on the ORF of their X homologous gene, it is not yet clear whether TMSB4Y/A33 minor H Ag has its homologue, because the longest cDNA clone (GenBank accession no. AK055976) assigned to be one of the splice variants of the *TMSB4X* gene containing the first intronic sequence was not detected by RT-PCR, whereas the full-length cDNA encoding thymosin β4 was readily detectable (data not shown). Thus, it is conceivable that the splice variant, AK055976, might be very rare or derived from a precursor mRNA.

Recently, evidence has been accumulating that cryptic polypeptides derived from noncoding regions, such as UTRs or introns, or encoded in alternative ORFs occasionally encode CTL epitopes for tumor or viral Ags in humans or mice (reviewed in Ref. 23). Of these, only one epitope is found in the 5′UTR of a cellular oncogene, c-*akt*, in the murine RL♂1 leukemia system (35). This unusual epitope is generated by insertion of the murine leukemia virus long terminal repeat into the exon of c-*akt*, resulting in transcription initiated at the cap site of the long terminal repeat. To the best of our knowledge, this is the first demonstration of a minor H Ag encoded outside a conventional ORF of a nonmutated gene. Although it is possible that the 19-residue ORF in the 5′UTR that encodes the epitope is an as yet unrecognized functional coding region, a search of the protein database, including the Protein-Protein Blast (http://www.ncbi.nlm.nih.gov/blast/), for amino acid sequence homology to this region did not identify any known functional domains.