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A Breast and Melanoma-Shared Tumor Antigen: T Cell Responses to Antigenic Peptides Translated from Different Open Reading Frames

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Infusion of TIL586 along with IL-2 into the autologous patient with metastatic melanoma resulted in the objective regression of tumor. Here, we report that screening a cDNA library from the 586mel cell line using CTL clones derived from TIL586 resulted in the isolation of a gene, *CAG-3 (cancer Ag gene 3)*. Sequence analysis revealed that CAG-3 encodes an open reading frame identical to NY-ESO-1, which was recently reported to be recognized by autologous serum from a patient with esophageal cancer. Thus, NY-ESO-1 appears to be an immune target for both Ab- and T cell-mediated responses. Significantly, NY-ESO-1-specific CTL clones were capable of recognizing two HLA-A31-positive fresh and cultured breast tumors. To our knowledge, this represents the first direct demonstration that tumor-specific CTL clones can recognize both breast and melanoma tumor cells. A 10-mer antigenic peptide ESO10–53 (ASGPGGGAPR) was identified from the normal open reading frame of NY-ESO-1 based on its ability to sensitize HLA-A31-positive target cells for cytokine release and specific lysis. Interestingly, two additional CTL clones that were sensitized with NY-ESO-1 recognized two overlapping antigenic peptides derived from an alternative open reading frame of the same gene. These findings indicate that CTLs simultaneously responded to two different gene products translated from the normal and alternative reading frames of the same gene. Understanding of this mechanism by which the alternative reading frame is translated may have important implications in tumor immunology. *The Journal of Immunology,* **1998, 161: 3596–3606.**

T he adoptive transfer of CTL or tumor-infiltrating lymphocytes (TIL)[3] along with IL-2 into the autologous patient with melanoma can result in the objective regression of tumor (1, 2), suggesting that T cells play an important role in antitumor immune response. To understand the molecular basis of T cell-mediated antitumor immunity, several groups have identified a number of genes encoding tumor rejection Ags in human melanoma that may be responsible for tumor regression (3–8). Based on their expression patterns, these tumor Ags can be divided into several classes. One class of tumor Ags, which includes MAGE1, MAGE3, BAGE, and GAGE, is encoded by genes that are expressed only in the tumor and testis, but not in other normal human tissues (9–12). The second class of Ags, such as MART-1/Melan-A, gp100, tyrosinase, gp75/TRP-1, and TRP-2, are differentiation Ags encoded by genes that are expressed only in melanocytes, melanomas, and normal retinal tissue (13–19). These tumor Ags are nonmutated self proteins. The third class of Ags are mutated, tumor-specific Ags recognized by T cells, including CDK4 (20), β-catenin (21), MUM-1 (22), and caspase 8 (23).

We previously isolated a gene encoding gp75/TRP-1 as a tumor Ag recognized by TIL586, which was associated with antitumor activity in vivo (18). T cell clones were then established from the TIL586 cell line by the limiting dilution method. Some of the clones were capable of recognizing A31 + normal melanocytes, 586mel tumor cells, and 586EBV B cells pulsed with the peptide ORF3P (MSLQRQFLR), derived from the alternative open reading frame of the *TRP-1* gene (24). Several T cell clones, however, did not appear to recognize the *TRP-1* gene and its ORF3P peptide, although they were capable of recognizing 586mel tumor cells and HLA-A31[+] melanocytes. This led to the identification of TRP-2 as a tumor Ag recognized by CTL clones (19). Interestingly, murine TRP-2 has been identified as a tumor Ag for B16 melanoma recognized by CTL generated by immunization of murine splenocytes with B16 tumor cells (25). This represents the first example of both human and mouse homologous proteins of TRP-2 acting as tumor Ags that are recognized by their corresponding CTLs. Therefore, the murine counterpart may be an ideal Ag for evaluating the role of differentiation Ags in antitumor responses in mice.

While a number of tumor Ags have been shown to be recognized by melanoma-reactive T cells, there are only limited data demonstrating direct recognition of tumor-shared Ags expressed in different types of tumor by CTL. The *MAGE* family of gene has been shown to be expressed in many types of tumor, but data demonstrating recognition of these tumor types by CTL have not been presented. Several reports have indicated that it is possible to generate CTL using peptides from HER-2/neu which is overexpressed in 20 to 30% of breast tumors (26–28). However, HER-2/neu is expressed in normal tissues as well. In this report, we demonstrate that CTL clones, or cloids, derived from the tumor-reactive TIL586 line recognize a breast and melanoma-shared tumor Ag encoded by the *cancer ag gene 3 (CAG-3),* identical to

NY-ESO-1 independently isolated by using the serum derived from a patient with esophageal cancer (29). Strikingly, we found that CTL clones were capable of recognizing HLA-A31-positive fresh and cultured breast tumor cells. Antigenic peptides from the normal open reading frame as well as an alternative open reading frame of the *NY-ESO-1* gene were identified. This represents the first example that two different gene products translated from the same gene can be recognized by CTL clones derived from a TIL with antitumor activity in vivo. Therefore, NY-ESO-1 could be an important tumor Ag for use in the immunotherapy of patients with breast cancer, melanoma, and other types of cancer.

Materials and Methods
Chemicals and reagents

The following chemicals and reagents were purchased from the sources indicated: RPMI 1640, AIM-V medium, Lipofectamine, and G418 from Life Technologies (Gaithersburg, MD); the eukaryotic expression vector pcDNA3.1 from Invitrogen (San Diego, CA); anti-HLA-A31 mAb from One Lambda (Canoga Park, CA); and anti-IgM Ab conjugated with FITC from Vector Laboratories (Burlingame, CA).

Cell lines and T cell clones

Melanoma cell lines 397mel, 397mel/A31, 586mel, 624mel, 624mel/A31, and EBV-transformed B cell lines 586EBV and 1510EBV were established in our laboratory and cultured in RPMI 1640 medium containing 10% FCS. Normal cultured melanocytes derived from infant foreskin (NHEM680 purchased from Clonetics, San Diego, CA) were cultured in melanocyte growth medium (MGM; Clonetics). 1295Br and 1315Br, fresh cryopreserved breast tumor digests, were cleaned with Ficoll gradient before use in T cell assays; 1295 fibroblast were cultured cells from the autologous patient for 22 to 64 days. 1315Br, culture A, were breast tumor cells grown in immunodeficient mice and then cultured in keratinocyte-SFM/2% FCS medium (Life Technologies), and 1315Br, culture B, were grown in Hams F12/5% FCS for 77 to 80 passages; these cells were kindly provided by Dr. Stephen Ethier, University of Michigan (Ann Arbor, MI). 1398Br was a human papillomavirus (HPV) E6/E7-immortalized breast tumor line established in the Surgery Branch, National Cancer Institute. Prostate tumor lines 1535Pro, 1542Pro, and 1510 fibroblast were HPV E6/E7-immortalized cell lines.

TIL586 were isolated from the tumor specimen of a patient with metastatic melanoma and grown in medium containing IL-2 (6000 IU/ml) (Chiron, Emeryville, CA) for 32 to 60 days as previously described (30). TIL586 and TIL1244 were predominantly $CD8^+$ T cells. TIL1244 recognized the TRP-2 peptide in the context of HLA-A31 and -A33 (31). The T cell clones or cloids were generated by limiting dilution methods (at 1 cell/well) from the TIL586 cell line, using allogeneic PBL (1×10^3 cells/well) as feeder cells in RPMI 1640 containing 10% human AB sera and 500 IU IL-2. After 12 days, the T cell clones were expanded in AIM-V medium containing 6000 IU/ml IL-2. To obtain an optimal expansion, we used the OKT3 expansion method, described by S. Riddell (32). Briefly, on day 0, 5×10^4 to 5×10^5 T cells were cocultured with HLA-A31$^+$ PBL (500:1, PBL:T cell ratio) and 586EBV B cells (100:1, EBV:T cell ratio) in 25 ml of RPMI 1640 containing 11% human sera, 30 ng/ml OKT3 Ab, and antibiotics. On day 1, IL-2 was added at a final concentration of 180 IU/ml. The medium was changed with fresh medium containing 11% human sera and 180 IU/ml IL-2 on day 5. The medium was then changed every 3 days. On days 12 through 14, T cells were harvested, counted, and cryopreserved.

cDNA library construction

Total RNA was extracted from 586mel using Trizol reagent (Life Technologies). Poly(A) RNA was purified from total RNA by the polyATtract system (Promega, Madison, WI) and converted to a cDNA using a cDNA construction kit (Life Technologies) with an oligo(dT) primer containing a *Not*I site. The cDNA was ligated to *Bst*XI adaptors and digested with *Not*I, then ligated to the expression vector pcDNA3.1. The cDNA library was electroporated into DH10B cells (Life Technologies). Plasmid DNA pools, each consisting of ~100 cDNA clones, were prepared from bacteria.

cDNA library screening and granulocyte/macrophage-CSF (GM-CSF) secretion assay

DNA transfection and GM-CSF assays were performed as previously described (18). Briefly, 200 ng of cDNA pools and 50 ng of the *HLA-A31* DNA were mixed with 2 μl of Lipofectamine in 100 μl of serum-free

DMEM for 15 to 45 min. The DNA/Lipofectamine mixture was then added to the COS-7 (5×10^4) cells and incubated overnight. The following day, cells were washed twice with AIM-V medium. CTL clones 5 or 10 were added at a concentration of 5×10^4 cells/well in AIM-V medium containing 120 IU/ml of IL-2. After 18 to 24 h of incubation, 100 μl of supernatant was collected and GM-CSF concentration was measured in a standard ELISA assay (R&D Systems, Minneapolis, MN). For testing peptide recognition, 586EBV, 1510EBV, or T2 cells were incubated with peptides at 37°C for 90 min, and then washed three times with AIM-V medium containing 120 IU/ml of IL-2. T cells were added and incubated for an additional 18 to 24 h; 100 μl of supernatant was collected for the GM-CSF assay. In some experiments, IFN-γ release assays were done using a standard ELISA kit (Endogen, Woburn, MA).

Northern blot analysis

Total RNA was isolated by the guanidine isothiocyanate/cesium chloride centrifugation method. Total RNA from normal human tissue was purchased from Clontech (Palo Alto, CA). Twenty micrograms of total RNA was subjected to electrophoresis in a 1.2% formaldehyde agarose gel and transferred to a nylon membrane. An 0.8-kb DNA fragment of the *NY-ESO-1* gene was labeled with [α-^{32}P]CTP by the random priming method. Prehybridization and hybridization were performed according to the QuickHyb protocol (Stratagene, La Jolla, CA). Membranes were washed twice with 2× SSC/0.1% SDS at room temperature for 15 min and twice with 0.1× SSC/0.1% SDS at 60°C for 30 min. The autoradiography was performed at −70°C.

Reverse transcriptase-PCR

Total RNA was extracted from tumor cell lines as described above. Five hundred nanograms of total RNA was used for conversion of RNA to cDNA by avian myeloblastosis virus (AMV) reverse transcriptase. cDNA was then amplified by PCR using the ESO-P2 (5′-GCGGCTTCAGGGCT GAATGGATG) and ESO-P5 (5′-AAGCCGTCCTCCTCCAGCGACA) primers and One-Step RT-PCR system (Life Technologies). PCR products were amplified under denaturation conditions at 94° for 30 s, annealing at 55° for 30 s, extension at 72° for 3 min for 40 cycles, and final elongation at 72° for 10 min. PCR products were analyzed on a 3% agarose gel.

Cytotoxicity assays

Cytolytic assays were performed as previously described (24). Briefly, the target cells were labeled with ^{51}Cr for 90 min. After washing three times, the cells were incubated with peptides at a concentration of 1 μg/ml for 90 min. The cells were washed again, counted, and then mixed with CTL clone 5 at the indicated E:T ratio. Chromium release was measured after a 4-h incubation. For titration of the peptides recognized by CTL clone 5, 586EBV B cells were incubated with various concentrations of the purified peptides. Percentage of specific lysis was determined from the equation: $(A − B)/(C − B) \times 100$, where A is the lysis of 586EBV B cells by CTL clone 5 in the presence of a peptide, B is the spontaneous release from 586EBV B cells in the presence of the same peptide but in the absence of effector cells, and C is the maximum chromium release.

The peptides were synthesized by a solid phase method using a peptide synthesizer (Model AMS 422, Gilson, Worthington, OH). Some peptides were purified by HPLC and had >98% purity. The mass of some peptides was confirmed by mass spectrometry analysis.

Results
CTL clones recognize tumor cells, but not normal melanocytes

The TIL586 cell line with antitumor activity in vivo had previously been shown to recognize the tumor Ag gp75/TRP-1 (18). Tumor-reactive CTL clones isolated from TIL586 by limiting dilution (24) appeared to recognize several distinct Ags. The first group of CTL clones recognized the ORF3P peptide derived from a gene product translated from an alternative open reading frame of the *TRP-1/ gp75* gene (24). The second group of CTL clones recognized TRP-2 as a tumor Ag in the context of HLA-A31 (19). A third group of 14 CTL clones, recently isolated, recognized 586mel tumor cells, but did not recognize normal HLA-A31$^+$ melanocytes nor COS-7 cells transfected with either *TRP-1* or *TRP-2* (see below) (Fig. 1). These results suggested that these T cell clones recognized additional tumor Ags expressed in 586mel tumor cells. Four CTL clones (clones 2, 5, 10, and 14) were expanded, and CTL clones 5 and 10 were used to screen cDNA libraries.
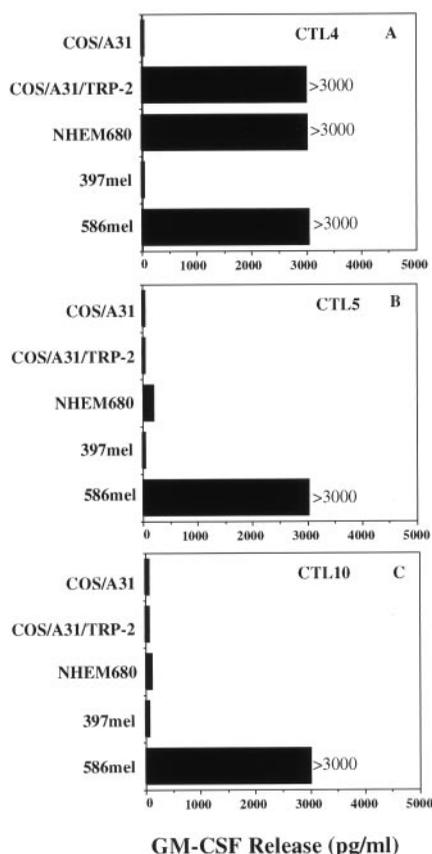
FIGURE 1. Recognition of various targets by CTL clones derived from TIL586. CTL clones were isolated by the limiting dilution method and were further expanded. *A*, GM-CSF release by CTL clone 4 was measured after coculturing with different target cells. *B* and *C*, In a separate experiment, GM-CSF release by CTL clones 5 and 10 was determined after coculturing with different stimulators. NHEM680 is a HLA-A31-positive normal melanocyte cell line, 397mel is an HLA-A31-negative melanoma cell line and 586mel is an HLA-A31-positive melanoma cell line.

FIGURE 2. Isolation of cDNA clones encoding a tumor Ag recognized by CTL clone 5. GM-CSF release by CTL clone 5 was measured after coculturing with 586mel, 397mel, COS-7 alone, COS-7 transfected with HLA-A31, or COS-7 transfected with HLA-A31 plus cDNA clones 1 and 2, respectively. 586mel and 397mel were used as the positive and negative controls.

We then tested whether these T cells recognized previously identified tumor Ags or melanocyte-lineage differentiation proteins. Therefore, the ability of CTL clones 5 and 10 to recognize COS-7 cells transfected with HLA-A31 cDNA along with genes encoding the known tumor Ags including MART-1, gp75/TRP-1, gp100, tyrosinase, and TRP-2 was examined. However, none of these Ags tested was recognized by CTL clones 5 and 10, indicating that these CTL clones recognized a new tumor Ag in the context of HLA-A31 (data not shown).

Isolation of a gene encoding a tumor Ag

To identify the new tumor Ag(s) recognized by CTL clones 5 and 10, we made a cDNA library derived from 586mel. Each cDNA pool consisted of ~100 cDNA clones. After screening a total of 2.5×10^5 cDNA clones, we identified 15 positive cDNA pools that conferred T cell recognition by CTL 5 or 10 when cotransfected into COS-7 along with *HLA-A31* cDNA. The positive clones were then tested for recognition by both CTL clones 5 and 10. It was found that both CTL clones recognized the same cDNA pools. Individual colonies were isolated from each positive pool and tested for T cell reactivity. Representative data are shown in Figure 2. CTL clone 5 recognized COS-7 cotransfected with cDNA clone 1 or 2 and *HLA-A31,* but not COS-7 alone, COS-7 transfected with cDNA clone 1 or 2, or transfected only with the *HLA-A31* cDNA (Fig. 2). DNA sequencing analysis indicated that all 10 of the cDNA-positive clones from different positive pools overlapped; the DNA and the amino acid sequence of these clones are shown in Figure 3. A search of all available databases revealed that the coding region of this gene, which we named cancer antigen gene 3 (*CAG-3;* GenBank accession no. AF038567), was identical to *NY-ESO-1,* which was recently reported to be an Ag recognized by a serum Ab derived from a patient with esophageal cancer (29). Our longest cDNA clone contained an additional 37 nucleotides upstream of the previously reported 5′-end untranslated region. Two other proteins in the databases were found to contain homologous sequences in a limited region. The gene product of NY-ESO-1 (we have used NY-ESO-1 for CAG-3 in the following text) has 52% similarity to the tegument protein (UL36) of herpes simplex virus type 1 in the 64-aa segment and 47% similarity to enterobactin synthetase component F (serine-activating enzyme) in the 48-amino acid region (Fig. 3*B*).

Recognition of new Ags by CTL clones in an HLA-A31-restricted fashion

To determine the HLA molecule responsible for presenting Ag to CTL clones 5 and 10, we transfected HLA-A31 cDNA into HLA-A31-negative tumor lines such as 397mel and 624mel and tested for T cell recognition. Transfectants of 397mel and 624mel expressing HLA-A31 were strongly recognized by CTL clones 5 and 10 (Table I), suggesting that T cell recognition was restricted by HLA-A31.

Table I. *Ag recognition by CTL clones 5 and 10 is HLA-A31-restricted*[a]

Cell Lines	Stimulators		GM-CSF Secretion (pg/ml)	
	Transfected gene	HLA-A31 expression	CTL clone 5	CTL clone 10
397mel	None	−	<50	<50
397mel	HLA-A31	+	>3000	>3000
624mel	None	−	<50	<50
624mel	HLA-A31	+	>3000	>3000
586mel	None	+	>3000	>3000

[a] GM-CSF in the supernatant was measured after a 24-h incubation of 5×10^4 CTL clone 5 or 10 cells with melanoma cell lines.
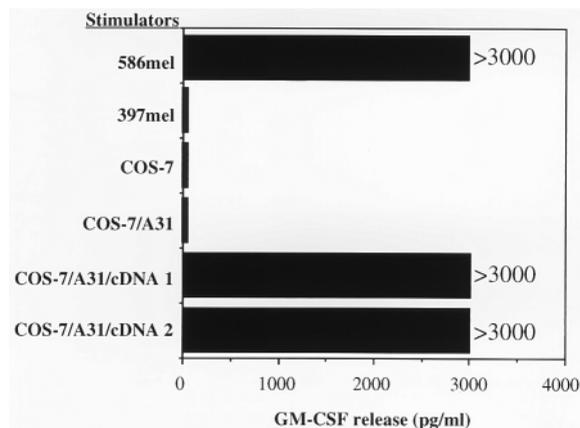
A

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AGCAGGGGGCGCTGTGTGTACCGAGAATACGAGAATACCTCGTGGGCCCTGACCTT
CTCTCTGAGAGCCGGGCAGAGGCTCCGGAGCCATGCAGGCCGAAGGCCGGGGCACA
                                ORF1 ▶ M  Q  A  E  G  R  G  T
         GGGGGTTCGACGGGCGATGCTGATGGCCCAGGAGGCCCTGGCATTCCTGATGGCCC
ORF1▶ G  G  S  T  G  D  A  D  G  P  G  G  P  G  I  P  D  G  P
            ORF2 ▶ M  L  M  A  Q  E  A  L  A  F  L  M  A
         AGGGGGCAATGCTGGCGGCCCAGGAGAGGCGGGTGCCACGGGCGGCAGAGGTCCCC
ORF1▶ G  G  N  A  G  G  P  G  E  A  G  A  T  G  G  R  G  P
ORF2▶Q  G  A  M ┌L  A  A  Q  E  R  R  V  P  R┐A  A  E  V  P
         GGGGCGCAGGGGCAGCAAGGGCCTCGGGGCCGGGAGGAGGCGCCCCGCGGGGTCCG
ORF1▶R  G  A  G  A  A  R ┌A  S  G  P  G  G  G  A  P  R┐G  P
ORF2▶ G  A  Q  G  Q  Q  G  P  R  G  R  E  E  A  P  R  G  V  R
         CATGGCGGCGCGGCTTCAGGGCTGAATGGATGCTGCAGATGCGGGGCCAGGGGGCC
ORF1▶ H  G  G  A  A  S  G  L  N  G  C  C  R  C  G  A  R  G  P
ORF2▶ M  A  A  R  L  Q  G
         GGAGAGCCGCCTGCTTGAGTTCTACCTCGCCATGCCTTTCGCGACACCCATGGAAG
ORF1▶ E  S  R  L  L  E  F  Y  L  A  M  P  F  A  T  P  M  E
         CAGAGCTGGCCCGCAGGAGCCTGGCCCAGGATGCCCCACCGCTTCCCGTGCCAGGG
ORF1▶A  E  L  A  R  R  S  L  A  Q  D  A  P  P  L  P  V  P  G
         GTGCTTCTGAAGGAGTTCACTGTGTCCGGCAACATACTGACTATCCGACTGACTGC
ORF1▶ V  L  L  K  E  F  T̲ ̲V̲ ̲S̲ ̲G̲ ̲N̲ ̲I̲ ̲L̲ ̲T̲ ̲I̲ R  L  T  A
         TGCAGACCACCGCCAACTGCAGCTCTCCATCAGCTCCTGTCTCCAGCAGCTTTCCC
ORF1▶ A  D  H  R  Q  L  Q  L  S  I  S  S  C  L  Q  Q  L  S
         TGTTGATGTGGATCACGCAGTGCTTTCTGCCCGTGTTTTTGGCTCAGCCTCCCTCA
ORF1▶L  L  M  W  I  T  Q  C  F  L  P  V  F  L  A  Q  P  P  S
         GGGCAGAGGCGCTAAGCCCAGCCTGGCGCCCCTTCCTAGGTCATGCCTCCTCCCCT
ORF1▶ G  Q  R  R
         AGGGAATGGTCCCAGCACGAGTGGCCAGTTCATTGTGGGGGCCTGATTGTTTGTCG
         CTGGAGGAGGACGGCTTACATGTTTGTTTCTGTAGAAAATAAAACTGAGCTACGAA
         AAAAAAAAAAAAAAAAAAAAAA
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B

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ENTF E. coli   894 QAAATGGDARQLVGYLVSQSGL-PLDTSAL--QAQLRETLPPHMVPVVLLQ 941
                   +  A G ++R L  YL       P++     +  L +  PP  VP VLL+
ESO-ORF1        77 RCGARGPESRLLEFYLAM-PFATPMEAELA--RRSLAQDAPPLPVPGVLLKEFTVSGNILTIRL 137
                   +  G  GP +  LLEF L M P+ T +   LA  R SLA  A  LP+    +L E ++ G +   +L
TEGU HSV11     604 QAGVAGPAAALLEFTLNMLPWKTAVGDFLASTRLSLADVAAHLPLVQHVLDENSLIGRLALAKL 667


ESO-ORF2         1 MLMAQEALAFLMAQGAMLAAQERRV 25
                   M    EAL FLM Q  M+ A + V
GDH C.symb     348 MPTTNEALRFLMQQPNMVVAPSKAV 372
```

FIGURE 3. Nucleotide and amino acid sequence of NY-ESO-1. *A*, Numbering of nucleotide sequence of *NY-ESO-1* starts from the first nucleotide in the 5′ untranslated region. ORF1 represents the gene product of NY-ESO-1, and ORF2 represents a 58-aa gene product translated from the ORF2. Antigenic peptides recognized by CTL clone 2 and 5 are boxed. In addition, a peptide poorly recognized by CTL clone 5 is underlined. *B*, Sequence alignment of the ESO-ORF1 protein with enterobactin synthetase component F (GenBank accession no. g250614) and tegument protein of herpes simplex virus type 1 (accession p10220). The residues are numbered with the first start site representing the first amino acid. Alignment of the ESO-ORF2 and glutamate dehydrogenase (accession g1942184) was also shown. The conserved amino acid substitution is indicated by the + symbol.

Breast cancer cells recognized by CTL clones

Northern blot analyses were performed using *NY-ESO-1* cDNA as a probe to evaluate the expression pattern in different tissues. Testis tissue was shown to be the only positive in the expression of *NY-ESO-1* among the normal human tissues tested. *NY-ESO-1* was found to be expressed in several types of cancers including melanoma and breast cancer (data not shown). These results were consistent with previously reported data (29). To determine whether the melanoma-reactive CTLs would also recognize other tumor cells, we tested T cell reactivity against HLA-A31-positive breast and prostate tumor cells by CTL clone 5. We used IFN-γ to monitor T cell recognition in these experiments, because prostate

tumor cells alone secret GM-CSF, but not IFN-γ. As shown in Table II, CTL clone 5 was capable of recognizing HLA-A31-positive 1295Br and 1315Br fresh breast tumor cells, but neither HLA-A31-negative 1405Br and 1411Br fresh breast tumor cells nor HLA-A31-positive 1295 fibroblast cells derived from the autologous patient 1295. In addition, CTL clone 5 recognized the cultured HLA-A31-positive 1315Br (culture A and B) cells (Table II), but did not respond to the cultured HLA-A31-negative cultured 1398Br breast cancer cells nor to the cultured HLA-A31-positive 1510 fibroblast. Although CTL clone 5 somehow did not respond to the cultured HLA-A31-positive 1315Br (culture A and B) cells in experiment 1, additional experiments showed T cell recognition

Table II. *Recognition of breast tumor cells by CTL clone 5[a]*

Stimulators		IF-γ Release (pg/ml) by CTL Clone 5	
Target cells	HLA-A31 expression	Expt. 1	Expt. 2
586mel	+	225	488
397mel	−	7	35
1315Br (fresh tumor 1)	+	171	520
1315Br (fresh tumor 2)	+	106	126
1295Br (fresh tumor)	+	265	358
1295 fibroblast	+	4	24
1405Br (fresh tumor)	−	15	28
1411Br (fresh tumor)	−	ND	74
1315Br (culture A)	+	23	224
1315Br (culture B)	+	0	155
1398Br	−	0	36
1510 fibroblast	+	8	ND
1535 prostate	+	24	ND
1542 prostate	−	17	ND

[a] IFN-γ in the supernatant was measured after an 18-h incubation of 1×10^5 CTL clone 5. In experiments 1 and 2, cytokine release from stimulators alone was <10 pg/ml.

Table III. *Screening of synthetic peptides with reactivity to CTL clone 5[a]*

Target Cells Pulsed with Peptides from NY-ESO-1		GM-CSF Release (pg/ml) by CTL Clone 5
1510EBV + ESO9-90	AQPPSGQRR	<50
1510EBV + ESO9-98	TPMEAELAR	<50
1510EBV + ESO9-99	PMEAELARR	<50
1510EBV + ESO9-38	GATGGRGPR	<50
1510EBV + ESO9-45	GPRGAGAAR	<50
1510EBV + ESO9-89	LAQPPSGQR	<50
1510EBV + ESO9-128	VSGNILTIR	<50
1510EBV + ESO9-35	IRLTAADHR	<50
1510EBV + ESO9-54	SGPGGGAPR	304
1510EBV + ESO10-127	TVSGNILTIR	293
1510EBV + ESO10-34	TIRLTAADHR	<50
1510EBV + ESO10-97	ATPMEAELAR	<50
1510EBV + ESO10-170	FLAQPPSGQR	<50
1510EBV + ESO10-98	TPMEAELARR	<50
1510EBV + ESO10-77	RCGARGPESR	<50
1510EBV + ESO10-68	AASGLNGCCR	<50
1510EBV + ESO10-171	LAQPPSGQRR	<50
1510EBV + ESO10-44	RGPRGAGAAR	<50
1510EBV + ESO10-72	LNGCCRCGAR	<50
1510EBV + ESO10-53	ASGPGGGAPR	>2000
1510EBV + None		<50

[a] 1510EBV B cells were incubated with individual peptides at a concentration of 0.1 μg/ml for 90 min. GM-CSF release was measured after coincubation of peptide-loaded 1510EBV cells with CTL clone 5. 1510EBV is an EBV-transformed B cell line expressing HLA-A31.

of the cultured HLA-A31-positive 1315BR (culture A and B) cells (data not shown). The expression of HLA-A31 and NY-ESO-1 in the fresh 1315Br and 1295Br tumor cells was confirmed by FACS analysis and RT-PCR analysis (Fig. 4). CTL clone 5 recognized neither the HLA-A31$^+$ 1535 prostate tumor cells, because of lack of expression of NY-ESO-1, nor the HLA-A31$^-$ 1542 prostate tumor cells (Table II). These results strongly suggest that an antigenic peptide of NY-ESO-1 was expressed at sufficient levels on the surface of breast tumor cells to be recognized by T cells. Therefore, NY-ESO-1 may serve as an immune target for the immunotherapy of patients with breast cancer.

Antigenic peptides derived from the normal open reading frames

To determine the antigenic epitopes recognized by NY-ESO-1-reactive CTL, we made a series of synthetic peptides based on the peptide-binding motif for HLA-A31, which consists of hydrophobic residues at position 2 and positively charged residues at position 9 (33). These peptides were pulsed onto HLA-A31-positive 1510 EBV B cells and tested for their ability to stimulate cytokine release by CTL clone 5. As shown in Table III, the 10-mer peptide ESO10–53 (ASGPGGGAPR), starting at position 53 of the NY-

ESO-1 protein, was strongly recognized by CTL clone 5, while the overlapping 9-mer peptides, ESO9-54 as well as ESO10–127, were weakly recognized when pulsed onto 1510EBV B cells. CTL clone 10 recognized the same peptide as CTL clone 5 (data not shown). Interestingly, CTL clone 2 did not recognize any of these peptides (Table III) even though it recognized 586mel and COS-7 transfected with NY-ESO-1 (see below). The reactivity of CTL clone 5 was undetectable when either the ESO9-54 or the ESO10–127 peptides were used at concentrations below 100 nM to sensitize EBV cells. To examine whether CTL clone 5 also recognized peptides that contained the core amino acid sequence with the extension of amino acid residues at either the N or C terminus, we made overlapping 11-mer, 12-mer, 13-mer, 14-mer, and 15-mer peptides, as well as several peptides containing substitutions at

FIGURE 4. FACS analysis of HLA-A31 expression and RT-PCR detection of *NY-ESO-1* in two fresh breast tumors. *A*, 1295 and 1315 fresh breast tumors were stained with anti-HLA-A31 Ab. After washes, the second goat anti-mouse IgM-FITC was used for FACS analysis. An isotype IgM control Ab was used for negative control. *B*, Expression of *NY-ESO-1* was detected by RT-PCR using primers ESO-P2 and ESO-P5 in both 1295 and 1315 fresh breast tumor cells. RNA from 586mel was used as a positive control, and MDA435 RNA and no RNA were used as negative controls. PCR products were separated on a 3% agarose gel and stained with ethidium bromide. One-kilobase DNA markers (Life Technologies) were loaded on both sides.
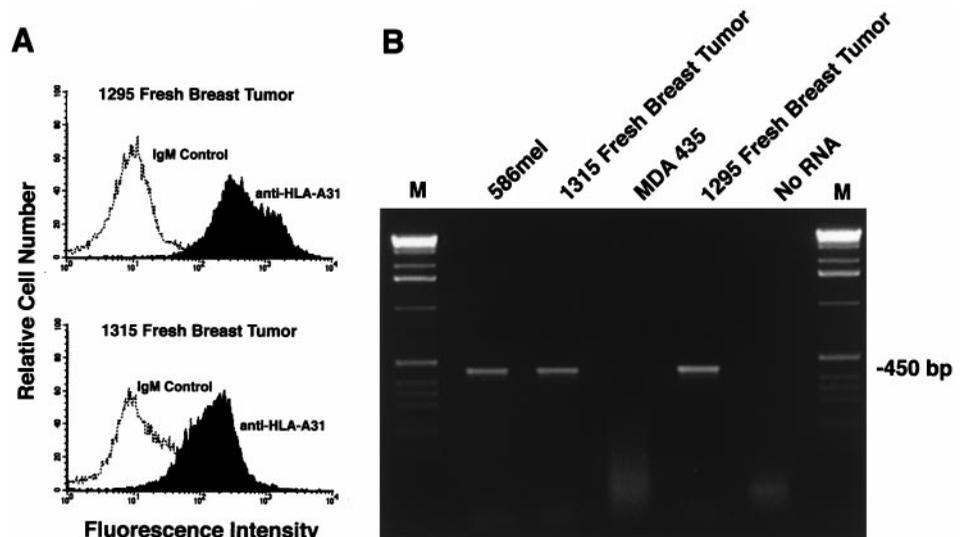
Table IV.   *Recognition of the modified peptides by CTL clone 5*[a]

| | GM-CSF Release (pg/ml) by CTL Clone 5 | |
Target Cells Pulsed with Peptides	Expt. 1 (1510EBV)	Expt. 2 (586EBV)
1510EBV + AGAARASGPGGGAPR	728	532
1510EBV + GAARASGPGGGAPR	659	678
1510EBV + AARASGPGGGAPR	844	738
1510EBV + ARASGPGGGAPR	840	738
1510EBV + RASGPGGGAPR	851	708
1510EBV + SGPGGGAPR	<50	<50
1510EBV + GPGGGAPR	<50	<50
1510EBV + ASGPGGGAPRG	<50	<50
1510EBV + SGPGGGAPRG	<50	<50
1510EBV + ASGPGGGAPR	1097	1044
1510EBV + A**A**GPGGGAPR	688	676
1510EBV + A**I**GPGGGAPR	428	550
1510EBV + A**L**GPGGGAPR	200	378
1510EBV + A**V**GPGGGAPR	556	512
1510EBV + A**T**GPGGGAPR	1630	1158
1510EBV + ASGPGGGAP**K**	<50	<50
1510EBV + ASGPGGGAP**H**	<50	<50
1510EBV + **T**SGPGGGAPR	320	346
1510EBV + **V**SGPGGGAPR	825	666
1510EBV + **L**SGPGGGAPR	<50	<50
1510EBV + **R**SGPGGGAPR	<50	<50
1510EBV + None	<50	<50

[a] 586EBV or 1510EBV B cells were incubated with individual peptides at a concentration of 0.1 μg/ml for 90 min. GM-CSF release was measured after coincubation of peptide-loaded EBV cells with 5×10^4 CTL clone 5. The wild-type 10-mer ESO10–53 peptide is underlined. Peptides with amino acid substitution are marked by bold face and underlines. 586EBV and 1510EBV are HLA-A31-positive EBV-transformed B cell lines.

either position 1, 2, or 10 of ESO10–53 (Table IV and Figure 3). CTL clone 5 was capable of recognizing 11-mer, 12-mer, 13-mer, 14-mer, and 15-mer peptides with amino acid extensions at the N terminus of the ESO-53 core peptide, although the longer peptides appeared to stimulate significantly less GM-CSF secretion than did the ESO10–53 10-mer peptide (Table IV). However, an extension of only a single amino acid residue at the C terminus of ESO10–53 abrogated its ability to stimulate T cells (Table IV). CTL clone 5 did not recognize the 8-mer peptide.

Titration experiments demonstrated that CTL reactivity was detectable at a 10-nM concentration of ESO10–53 peptide. GM-CSF release from CTL clone 5 increased with the increasing peptide concentrations, and no plateau was reached even at 10 μM peptide concentration (Fig. 5A). In addition to measuring cytokine release stimulated by ESO10–53, the ability of this peptide to sensitize target cells for lysis by CTL clone 5 was examined. CTL clone 5 was capable of lysing HLA-A31-positive tumor 586mel, but not the HLA-A31-negative and NY-ESO-1-positive tumor 397mel (Fig. 5B). CTL clone 5 lysed >60% of either 586EBV or 1510EBV B cells that had been incubated with the ESO10–53 peptide at an E:T ratio of 40:1, and ~5 to 10% lysis of target cells was observed at an E:T ratio of 2.5:1. CTL clone did not lyse either 586EBV or 1510EBV B cells alone or pulsed with an irrelevant peptide, nor did it lyse the HLA-A31-negative T2 cells pulsed with the ESO10–53 peptide (Fig. 5C).

Next, we tested whether T cell recognition of the 10-mer peptide could be improved by substituting amino acids at anchor residues. A number of synthetic peptides with modification at residues 1, 2, and 10 were made and tested for recognition by CTL clone 5 when pulsed onto 586EBV B cells (Table IV). The modified 10-mer peptides with a substitution at position 2 derived from the wild-type ASGPGGGAPR were still recognized by CTL clone 5 when pulsed on 586EBV B cells. The reactivity of peptides containing a substitution of either Ala, Ile, Leu, or Val at position 2 was lower than that of the wild-type peptide, while one peptide containing a

substitution of Thr for Ser at position 2 resulted in a slightly higher reactivity than the wild-type ESO10–53 peptide. In contrast, peptides containing substitutions of Arg with Lys or His completely lost their ability to stimulate T cells, suggesting that the Arg at the C terminus of the ESO10–53 peptide represents a critical anchor residue. Peptides with a substitution at position 1 were recognized poorly or not recognized at all by CTL clone 5 (Table IV). These results indicate that the ESO-53 peptide, ASGPGGGAPR, represents the best peptide for T cell recognition.

Antigenic peptides derived from an alternative open reading frame

Two additional CTL clones, clones 2 and 14, appeared to recognize 586mel as well as COS-7 cells transfected with NY-ESO-1 and HLA-A31 cDNA, but failed to recognize the ESO10–53 peptide (Fig. 6). CTL clone 5 and TIL1244 were used for the specificity controls. Additional experiments showed that CTL clone 2 did not respond to any of 19 other peptides containing the HLA-A31 binding motif derived from the normal open reading frame of NY-ESO-1 (Table III). To test the hypothesis that CTL may recognize a peptide from a gene product translated from an alternative open reading frame of the same gene, we made synthetic peptides with HLA-A31 binding motif on the basis of an amino acid sequence predicted from the second open reading frames (ORF2) (Fig. 3). Strikingly, CTL clone 2 recognized ESORF2-9–19 (AAQERRVPR) as well as the overlapping ESORF2-10–18 (LAAQERRVPR) peptides when pulsed onto 1510EBV B cells. Representative data for CTL clone 2 are shown in Figure 7A. CTL clone 14 recognized the same peptides as CTL clone 2 (data not shown). These results suggest that CTL clones 2 and 14 recognized an antigenic peptide derived from the ORF2 (Fig. 3). A protein database search revealed that the 58-aa protein of ORF2 has a 52% similarity to the chain A of glutamate dehydrogenase in a 25-aa region (34). Peptide titration experiments demonstrated that CTL
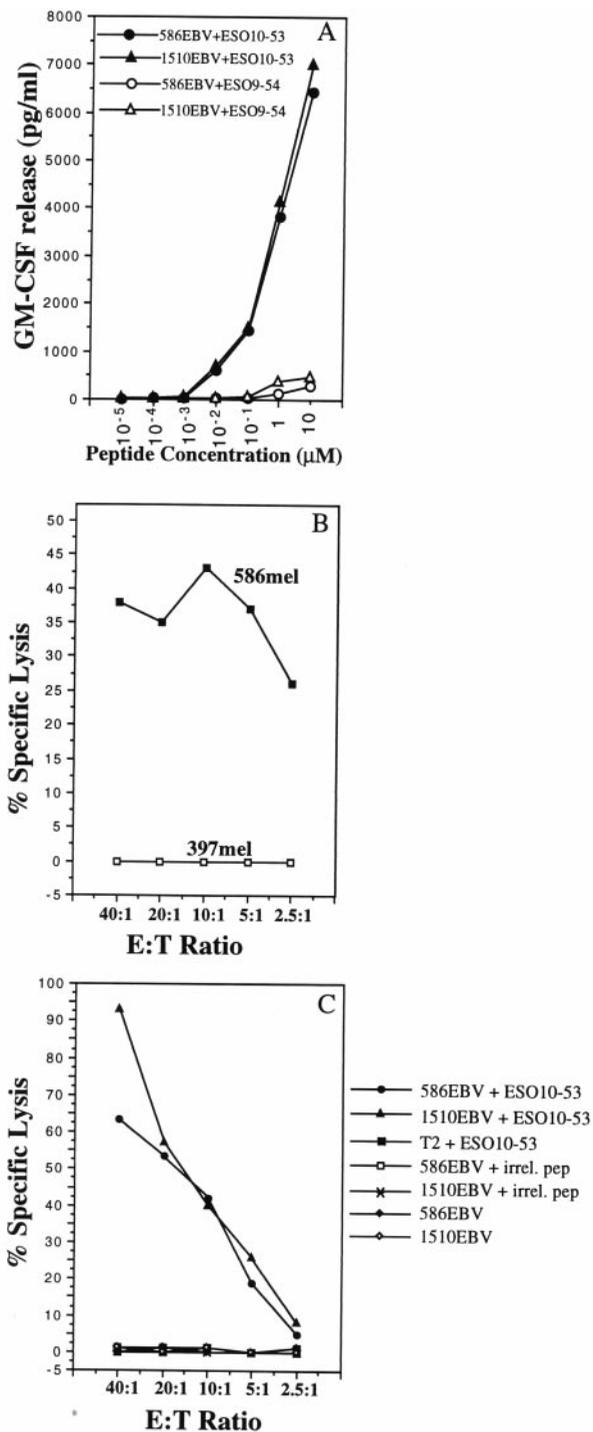
FIGURE 5. Characterization of the 9-mer ESO9-54 and 10-mer ESO10–53 derived from the normal open reading frame. *A*, 586EBV or 1510EBV B cells were pulsed with ESO10–53 and ESO9-54 peptides at different concentrations for 120 min. After two washes with AIM-V medium with 120 IU IL-2, CTL clone 5 (1 × 10⁵/well) was added and incubated for 18 to 24 h. GM-CSF release by CTL clone 5 was determined by ELISA. *B*, 586Mel cells were lysed by CTL clone 5, but 397mel cells were not lysed by CTL clone 5 at different E:T ratios. *C*, 586EBV and 1510EBV B cells were labeled with chromium overnight. The ESO10–53 peptide was then pulsed on the chromium-labeled 586EBV, 1510EBV, and T2 cells for 120 min. An irrelevant peptide containing an HLA-A31 peptide-binding motif was also pulsed on chromium-labeled 586EBV, 1510EBV B cells as negative controls. After peptide incubation and two washes, cytolysis of target cells by CTL clone 5 was determined in a 4-h chromium release assay. T2 pulsed with the ESO10–53 peptide was used for the specificity control.

clone 2 was capable of lysing 1510EBV pulsed with ESORF2-10–18 (LAAQERRVPR) at relatively low concentrations of peptide, but failed to lyse 1510EBV pulsed with ESO10–53 or HLA-A31-negative 1102EBV pulsed with ESORF2-10–18 (Fig. 7*B*). In addition, CTL clone 2 also recognized overlapping 11-mer, 12-mer, and 13-mer peptides with amino acid extensions at the N terminus of the ESORF2-10–18 peptide at relatively high concentrations (data not shown).

Additional experiments were conducted to determine whether CTL clones recognize the ORF2 gene product of the NY-ESO-1 in other tumor types. As shown in Figure 7*C*, the recognition pattern of CTL clone 2 was similar to that of CTL clone 5 on tumor cells. CTL clone 2 recognized the HLA-A31-positive fresh 1315Br and 1295Br breast tumor as well as 586mel and 1388mel, but did not recognize HLA-A31-negative fresh 1411Br breast tumor, 397mel, nor the HLA-A31-negative 1295 fibroblast. Although 1353mel expresses HLA-A31, neither CTL clone 2 nor clone 5 responded to 1353mel because 1353mel is an NY-ESO-1-negative tumor. As previously demonstrated, CTL clone 5 recognized the ESO10–53 ASGPGGGAPR peptide and CTL clone 2 recognized the ORF2-10–18 LAAQERRVPR peptide derived from the ORF2 following incubation with 1510EBV B cells (Fig. 7*C*). These results strongly suggest that the ORF2 gene product was translated, processed, and presented in melanoma as well as breast tumors. Therefore, we concluded that *NY-ESO-1* encode two different proteins: a protein with 180 aa (ORF1) recognized by CTL clones 5 and 10; and a protein with 58 aa (ORF2) recognized by CTL clones 2 and 14.

Discussion

NY-ESO-1 is a cellular immune target

Five differentiation Ags including tyrosinase, MART-1, gp100, TRP-1/gp75, and TRP-2 have been identified as melanoma Ags recognized by T cells derived from TILs (13–19), which had been shown to be associated with antitumor reactivity in vivo. Thus far, no TILs available in the Surgery Branch at National Cancer Institute recognize MAGE-1, BAGE, and GAGE, which are expressed only in the testis and in cancer cells. Here we show that NY-ESO-1, another cancer-shared Ag, is a tumor Ag recognized by HLA-A31-restricted T cells. NY-ESO-1 was independently isolated using the autologous serum from a patient with esophageal cancer (29), suggesting that the NY-ESO-1 gene product was an immune target for Ab-mediated immunity. Results from this study provide evidence that NY-ESO-1 is also an immune target recognized by T cells. This is further supported by a recent report that HLA-A2-restricted CTL established in a melanoma patient recognized NY-ESO-1 (35). Several tumor Ags, including MAGE-1, tyrosinase, and TRP-1, recognized by CTL have been found to be reactive with Ab as well (36, 37). Since NY-ESO-1 is not expressed in normal human tissues except the testis, which does not express MHC class I molecules and is considered as an immunologically privileged site, this gene product may constitute a safe immune target for the immunotherapy of patients with cancer.

NY-ESO-1 is a breast cancer Ag

Based on its gene expression pattern, NY-ESO-1 belongs to a member of an expanding family of Ags including MAGE-1, MAGE-3, BAGE, GAGE, and HOM-MEL-40 (9–12, 30). However, NY-ESO-1 is highly expressed in a significant proportion of breast, prostate, and bladder cancers (29) as compared with MAGE, BAGE, and GAGE. More importantly, CTL clones recognized two HLA-A31-positive fresh and cultured breast cancer cells (Table II and Fig. 7*C*). To our knowledge, this is the

FIGURE 6. CTL clones 2 and 14 recognize the *NY-ESO-1* gene, but not the ESO10–53 peptide derived from the NY-ESO-1 protein. CTL clones 2, 14, and 5 recognized *NY-ESO-1* when cotransfected with *HLA-A31* cDNA into COS-7 (*left panels*). TIL1244, which recognized *TRP-2* but not NY-ESO-1, was used as a specificity control. On the *right panels*, CTL clones 2, 5, 14, and TIL1244 recognized 586mel. However, CTL clones 2 and 14 did not recognize the ESO10–53 peptide, whereas CTL clone 5 strongly recognized the ESO10–53 peptide when pulsed onto HLA-A31-positive 586EBV B cells. TIL1244 recognized the TRP197–205 peptide derived from TRP-2. 586EBV B cells alone or pulsed with the ORF3P peptide from an alternative reading frame of TRP1 were negative controls. 397mel is an HLA-A31-negative, NY-ESO-1-positive tumor line.
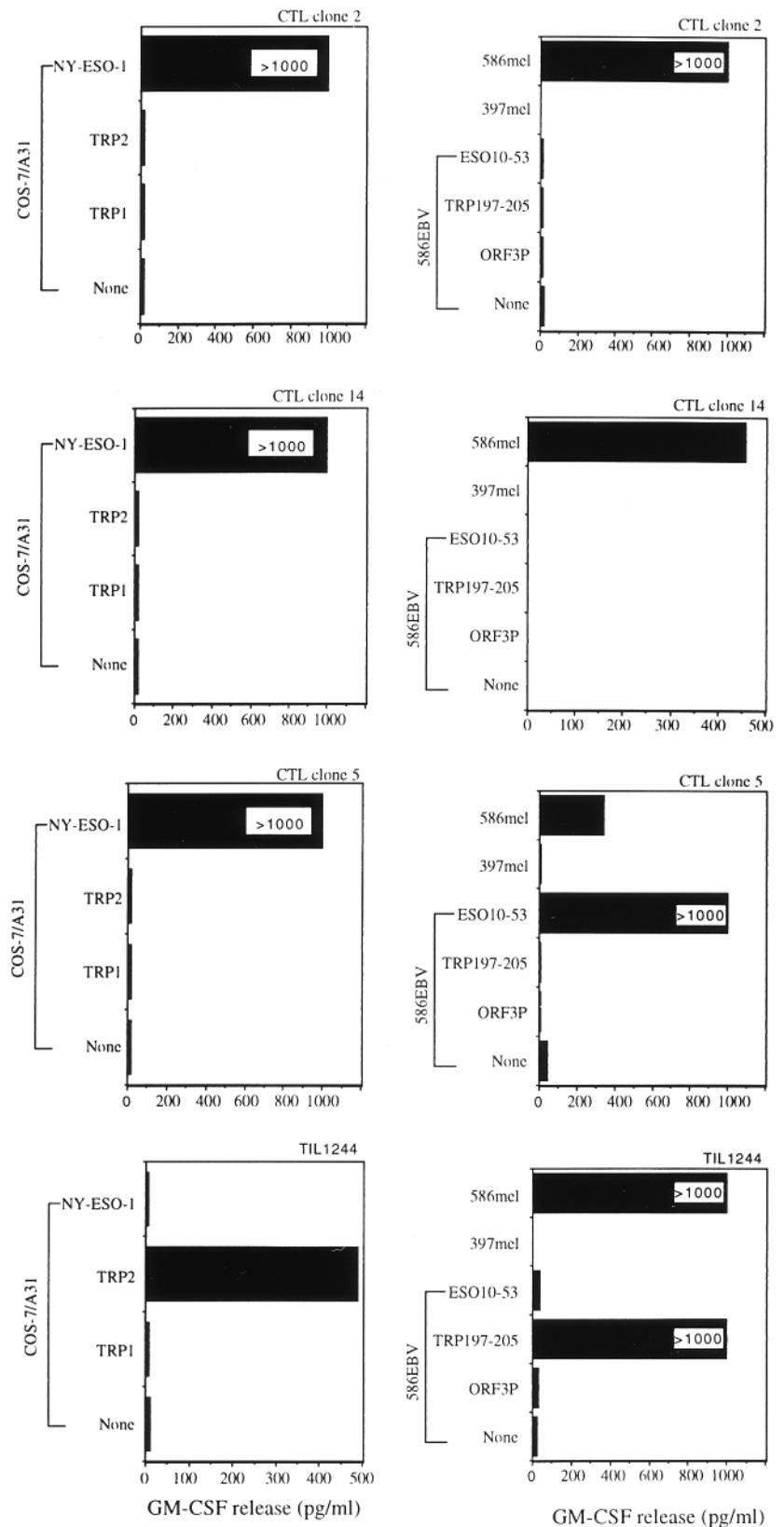
first demonstration of CTL recognition of NY-ESO-1 positive breast cancer cells. Although the expression of MAGE-1, MAGE-3, and others were reported to be detected by RT-PCR in breast tumors at a low frequency (<5–10%), CTL recognition of these breast tumors by the Ag-specific CTL has not been documented. It has been difficult to generate breast-reactive

CTLs from PBL in vitro, although MHC-restricted T cells that recognized HER-2/neu peptides on breast cancer cells have been reported (26–28). Identification of NY-ESO-1 peptides presented on the cell surface of breast cancers is important for the development of Ag-specific cancer vaccines for the treatment of patients with breast cancer. The CTL cloning approach

FIGURE 7. An alternative open reading frame of the *NY-ESO-1* gene and antigenic peptides recognized by CTL. *A*, Identification of antigenic peptides from the ORF2. Thirty peptides were synthesized based on all potential ORFs and screened. Representative data are shown here. Seven peptides derived from ORF2 (see Fig. 3) were pulsed on HLA-A31-positive 1510EBV B cells and tested for T cell recognition based on GM-CSF release. 1510EBV alone was used a negative control. *B*, 1510EBV B cells were labeled with chromium for 2 h. The ESORF2-10–18 peptide was then pulsed on the chromium-labeled 1500EBV (solid square) and HLA-A31-negative 1102EBV (open circle) at different concentrations. 1510EBV pulsed with ESO10–53, which was recognized by CTL clone 5, was used for the specificity control (solid triangle). After peptide incubation and three washes, cytolysis of target cells by CTL clone 2 was determined in a 4-h chromium release assay at an E:T ratio of 20:1. *C*, Several tumor lines and fresh breast tumors were tested for recognition by CTL clone 2 to determine whether the ORF2 is translated in different tumors. 1510EBV B cells pulsed with ESO10–53, ORF2-10–18, or alone were included to evaluate the reactivity and specificity of CTL clone 2 and 5. Expression of HLA-A31 on the tumor cells is indicated.

described here represents a strategy for the isolation of breast cancer Ags.

Translation of different ORFs as a mechanism for generating T cell epitopes

To define antigenic peptides, we found the 10-mer ASGPGG GAPR derived from the NY-ESO-1 protein as the best antigenic peptide recognized by CTL clone 5 although the 9-mer, 11-mer, 12-mer, 13-mer, 14-mer, and 15-mer peptides were also recognized. This reactivity may be due to the presence of two proline residues in these peptides. Proline residues in the core peptide sequence may allow the peptides to bulge out of the MHC binding pocket, and thus the anchored residues in the longer peptides can still fit into the HLA-A31 molecule. An alternative explanation is that the longer peptides may be processed to the shorter peptides by extracellular or serum proteases (38). However, when the longer peptides were pulsed onto 586EBV B cells in serum-free conditions, they were still recognized by CTL clone 5 (data not

shown). This experiment, however, does not exclude the possibility that these longer peptides were processed by extracellular proteases. The modified peptide, ATGPGGGAPR, with a substitution of Thr for Ser appeared to slightly improve T cell recognition. It has been shown that modified peptides could improve binding affinity to the MHC class I molecule, thus enhancing the immunogenicity of peptides (39). It is not clear why CTL clone 5 recognized the unrelated ESO10–127 peptide, but its recognition was weak and could only be detected at a relatively high peptide concentration.

Interestingly, two additional CTL clones recognized COS-7 transfected with NY-ESO-1 plus HLA-A31 cDNA, but not the ESO10–53 peptide, which was derived from ORF1 of the NY-ESO-1 gene (Fig. 6). Further analysis showed that CTL clone 2 recognized a peptide from the gene product translated from an alternative open reading frame (ORF2) of NY-ESO-1. Although there are several examples that two different proteins are translated from different ORFs from a single viral mRNA (40), very few cases have been reported in single eukaryotic mRNA. Two examples have been reported: *TRP-1/gp75,* which encodes two different proteins, gp75 recognized by sera from a patient with melanoma and a 24-aa gene product recognized by CTLs (24, 37); and 43 kDa-Ag recognized by squamous cell carcinoma-reactive CTL (41). Interestingly, the cyclin-dependent kinase inhibitor *p16* gene also encodes two gene products, p16 and p19^{ARF}. However, the alternative ORF gene product, p19^{ARF}, is translated from an alternatively spliced transcript that shares an identical sequence with the p16 transcript except the first exon (41). Recent studies showed that the alternative reading frame product p19^{ARF} plays a role in cell cycle regulation and tumor suppression (42). The *NY-ESO-1* gene reported here encodes two different proteins: a 180-aa protein encoded by ORF1 and a 58-aa protein encoded by ORF2. The ORF2 product is located inside ORF1. Strikingly, CTL clones 5 and 10 recognized antigenic peptides derived from ORF1, while CTL clones 2 and 14 recognized an antigenic peptide from ORF2. Both CTL clones 2 and 5 recognized several HLA-A31$^+$ melanomas as well as fresh breast tumors (Table II and Fig. 7*C*), suggesting that the translation of the alternative ORF2 may serve as a general mechanism in vivo for generating T cell epitopes.

The mechanism by which the alternative ORF is translated is currently not known. However, there are several potential explanations for the production of alternative reading frames. The leaky-scanning model is one of them: ribosomes occasionally bypass the first AUG with a poor KOZAK consensus sequence and initiates translation at a downstream AUG (43, 44). In the case of NY-ESO-1, the start codon for translation of ORF1 is not in the optimal context. There are four potential start codons that could be used to translate the ORF2. In our previous report, a CTL epitope was translated from an alternative reading frame of gp75/TRP-1 (24). Recognition of the epitope of ORF3 by CTL was affected by the presence of the first ATG codon used for the translation of the gp75 protein and was completely abolished when the internal ATG preceding the T cell epitope (ORF3P) was changed to ATC, suggesting that ribosomal scanning may be a possible mechanism. In the studies of the influenza nucleoprotein, CTL epitopes were produced via a ribosomal scanning mechanism (44). However, in a similar study, ribosomal frameshifting was suggested as a mechanism for the production of T cell epitopes (45). Other mechanisms have also been proposed for the production of T cell epitopes derived from different genes. For example, several T cell epitopes were identified from gene products translated from a cryptic initiation codon of the 5′ untranslated region (46, 47), from a non-ATG-defined open reading frame (48), and from introns of transcript (22, 49, 50). Since both the alternative open reading frames

of *gp75/TRP-1* and *NY-ESO-1* are located within the primary open reading frame, it is of particular interest to understand the underlying mechanism.

Although there are only a few examples of the usage of the alternative open reading frames in eukaryotes reported in the literature, we believe that more examples will be reported in the future when tumor-reactive CTL and autoantibodies are available and used to identify target proteins or peptides. Therefore, it is important to understand the biologic significance of the gene products translated from alternative open reading frames. One possibility is that these gene products serve as antigenic targets of the Ag processing machinery to increase the efficiency and capacity of the immune surveillance. Identification of T cell epitopes derived from different open reading frames of *NY-ESO-1* suggests that the identity of immunogenic peptides for cancer vaccines may not be limited only to peptides derived from the primary open reading frame. It is not clear at the present time whether the ORF2 gene product of *NY-ESO-1* and the ORF3 gene product of *gp75/TRP-1* have biologic functions in addition to immune responses of T cells. Further studies are needed to characterize the biologic function for both the ORF1 and ORF2 products of *NY-ESO-1*.

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