A non-AUG-defined alternative open reading frame of the intestinal carboxyl esterase mRNA generates an epitope recognized by renal cell carcinoma-reactive tumor-infiltrating lympho...

Article *in* The Journal of Immunology · August 1999

Source: PubMed

CITATIONS
88

READS
43

6 authors, including:

Christophe Ronsin
Biomnis
20 PUBLICATIONS **1,144** CITATIONS
SEE PROFILE

Nicolas Aknouche
Centre Hospitalier Universitaire de Nice
4 PUBLICATIONS **157** CITATIONS
SEE PROFILE

Catherine Gaudin
French Institute of Health and Medical Research
35 PUBLICATIONS **989** CITATIONS
SEE PROFILE

A Non-AUG-Defined Alternative Open Reading Frame of the Intestinal Carboxyl Esterase mRNA Generates an Epitope Recognized by Renal Cell Carcinoma-Reactive Tumor-Infiltrating Lymphocytes In Situ[1]

Christophe Ronsin, Véronique Chung-Scott, Isabelle Poullion, Nicolas Aknouche, Catherine Gaudin, and Frédéric Triebel[2]

A number of Ags recognized by tumor-reactive T cells have been characterized, including nonmutated gene products and a variety of epitopes shown to arise from either mutated or alternatively processed transcripts. Here, we report that the screening of a cDNA library with an HLA-B7-restricted renal cell carcinoma-reactive T cell clone derived from tumor-infiltrating lymphocytes (TILs) that were clonally amplified in vivo (as assessed by *TCRBV* complementarity determining region-3 length distribution analysis) resulted in the isolation of a nonamer encoded by an alternative open reading frame (ORF) (a +1 frameshift) of the intestinal carboxyl esterase gene. This peptide binds HLA-B*0702-presenting molecules as assessed in an immunofluorescence-based peptide binding assay using transfected T2 cells. Constitutive expression of this alternative ORF protein was observed in all transformed HLA-B7$^+$ renal cell lines that were recognized in cytotoxicity assays by the TILs. The intestinal carboxyl esterase gene is transcribed in renal cell carcinoma tumors as well as in normal liver, intestinal, or renal tissues. Mutation of the natural ATG translation initiation site did not alter recognition, indicating that frameshifting (i.e., slippage of the ribosome forward) and recoding are not involved. In addition, a point mutation of the three AUG codons that may be used as alternative translation initiation sites in the +1 ORF did not abolish recognition, whereas mutation of an upstream ACG codon did, indicating that the latter codon initiates the translation of the alternative ORF. These results further extend the types of Ags that can be recognized by tumor-reactive TILs in situ (i.e., leading to clonal T cell expansion). *The Journal of Immunology,* **1999, 163: 483–490.**

A variety of products have been shown to be recognized by tumor-reactive T cells, with most of them being isolated from melanoma patients. Some of these Ags represent nonmutated gene products, the expression of which is restricted to the testis in the tissues of normal adults (MAGE-1, MAGE-3, BAGE, and GAGE) (1–4). Other nonmutated genes are differentiation Ags that are also expressed in normal melanocytes but not in other normal tissues. These include the MART-1/MelanA (5, 6), gp100 (6), tyrosinase (7, 8), and gp75 (9) melanocyte lineage gene products. Melanoma-reactive T cells have also been shown to recognize mutated products of the β-catenin (10), MUM1 (11) and cyclin-dependent kinase-4 (12) genes. Renal cell carcinoma (RCC)[3]-reactive T cells have also been shown to recognize point-mutated gene products such as HLA-A2 (13) or hsp70-2 (14).

Also, some Ags recognized by tumor-reactive T cells may be generated by alternatively processed transcripts including intronic sequences, as in the case of MUM1 (11), *N*-acetylglucosaminyltransferase-V (15), or gp100 (16). The T cell-mediated surveillance of the integrity of the cell may extend beyond the genome and its intronic regions and focus also on peptides coded by an alternative open reading frame (ORF) located within the primary ORF, as in the case of gp75/tyrosinase-related protein-1 (TRP-1) (17) and NY-E50-1 (18). There are only a few examples of the usage of alternative ORFs in eukaryotes reported in the literature, and the biologic significance of the corresponding gene products is unknown. Nonetheless, it may be speculated that these products may serve as antigenic targets of the Ag-processing machinery to increase the efficiency of immune surveillance. There is an increasingly apparent relationship between abnormal translational control of gene expression (as for c-*myc* or fibroblast growth factor-2) (19–21) and cancer; thus, the identity of immunogenic peptides in tumors may very well extend beyond the ones derived from the primary ORF. Understanding the mechanisms by which alternative ORFs are translated in tumor cells may have important implications in tumor immunology.

This study demonstrates that a peptide derived from an alternative ORF of the intestinal carboxyl esterase (iCE) gene is recognized by an HLA-B7-restricted RCC-reactive T cell clone. The reactive tumor-infiltrating lymphocytes (TILs) are amplified in situ at the tumor site. Unexpectedly, this alternative ORF is initiated from a cryptic non-AUG (ACG) codon.

Materials and Methods

Cell lines

K562 cells and the EBV-transformed B cell line from patient 1 were cultured in medium consisting of RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 1% L-glutamine (200 mM,) 1% sodium pyruvate

Laboratoire d'Immunologie Cellulaire, Institut Gustave-Roussy, Villejuif, France

Received for publication December 14, 1998. Accepted for publication April 8, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was funded by a research grant from Ligue Nationale contre le Cancer and Comité des Hauts de Seine and by the Association pour la Recherche sur le Cancer.

[2] Address correspondence and reprint requests to Dr. Frédéric Triebel, Laboratoire d'Immunologie Cellulaire, Institut Gustave Roussy, 39, rue Camille Desmoulins, 94805 Villejuif Cedex, France. E-mail address: ftriebel@igr.fr

[3] Abbreviations used in this paper: RCC, renal cell carcinoma; ORF, open reading frame; TIL, tumor-infiltrating lymphocyte; CDR3, complementarity determining region-3; iCE, intestinal carboxyl esterase; MLTC, mixed lymphocyte-tumor cell culture; TCGF, T cell growth factor; nt, nucleotide; IU, international units; TRP-1, tyrosinase-related protein-1.

0022-1767/99/$02.00

(200 mM), 1% HEPES, 5% FCS, and penicillin (50 international units (IU)/ml) (Life Technologies). WEHI-164 clone 13 (W13) was kindly provided by Dr. Benoît J. Van den Eynde Bruxelles, Belgium; COS-7 cells were cultured in RPMI 1640 (Seromed, Biochrom KG, Berlin, Germany) supplemented with 1% L-glutamine (200 mM), 1% sodium pyruvate (200 mM), 1% HEPES, 5% FCS, and penicillin (50 IU/ml).

Patients and establishment of RCC cell lines

RCC cell lines were established as described previously (22). Primary tumors were obtained from untreated patients who had undergone radical nephrectomy. The RCC-1 cell line was established from patient 1 (HLA A1, A32, B7, B12–44, Cw5, Cw7), a 56-year-old male with a clear and granular renal cell carcinoma without metastasis. After surgery, fragments were processed by enzymatic digestion, and tumor cell suspensions were plated in complete RCC medium (22). The tumor cell lines RCC-2 (HLA A1, A3, B7, B8, Cw7, Cw7), RCC-3 (HLA A1, A29, B22, B15–62/63, Cw1, Cw7–17), RCC-4 (HLA A3, A19–29, B7, B12–44, Cw7, Cw16), RCC-5 (HLA A1, A3, B7, B22–56, Cw1, Cw7), RCC-6 (HLA A9–24, A32, B12–44, B18, Cw5, Cw5), RCC-7 (HLA A1, A28–68, B8, B40–60, Cw3, Cw7), and RCC-8 (HLA A2, A10–25, B18, B13, Cw8, Cw6), which were derived from the primary tumor of patients 2, 3, 4, 5, 6, 7, and 8, respectively, were maintained in complete RCC medium.

Generation of CTLs from TILs of patient 1

Autologous TILs were generated from a thawed suspension of dissociated tumor cells. An autologous mixed lymphocyte-tumor cell culture (MLTC) was performed as follows: on day 1, dissociated tumor cells were seeded at 2×10^6 TILs in 6-well flat-bottom plates (Falcon, Becton Dickinson, NJ) in RPMI 1640 (Life Technologies) containing 1% L-glutamine (200 mM), 1% sodium pyruvate (200 mM), 8% human AB serum (Institut Jacques Boy, S.A, Reims, France), penicillin (50 IU/ml) supplemented with 5% T cell growth factor (TCGF), and 50 IU/ml of human IL-2 (rIL-2) (Roussel Uclaf, Romainville, France), hereafter referred to as MLTC complete medium. MLTC complete medium was removed every 3 days as needed and replaced with new MLTC complete medium. On days 7, 15, and 21, 2×10^6 TILs were restimulated with 2×10^5 irradiated (100 Gy) autologous tumor cells seeded in 6-well flat-bottom plates with MLTC complete medium. On day 15, TILs were tested for cytotoxic activity against the autologous RCC-1 and K562 cell lines, characterized for surface phenotype by direct immunofluorescence, and cloned by limiting dilution. TILs were seeded to 0.6–600 cells/well in 96 V-shaped microwell plates (Nunc, Roskilde, Denmark) preseeded with irradiated autologous tumor cells (1×10^4/well) as stimulators and irradiated allogeneic PBLs (8×10^4/well) and irradiated EBV-transformed B cells (2×10^4/well) as feeder cells in a total volume of 200 μl of MLTC complete medium. Every 3 days, 60 μl of supernatant were removed from each well and replaced by 60 μl of fresh medium. Clones were screened for cytotoxicity in a standard 4-h chromium release assay. Every 7–10 days, CTL clones were restimulated with the allogeneic feeder cell line and the autologous tumor cell line as described above.

Cytotoxicity assay

The cytolytic activity of the CTLs was assessed in a standard ^{51}Cr release assay as described previously (22). Target cells (RCC cell lines, K562) were labeled for 1 h with 50–100 μCi of ^{51}Cr (DuPont New England Nuclear, Boston, MA) at 37°C. A total of 2×10^3 cells were seeded into 96-microwell plates in 100 μl of RPMI 1640 supplemented with 5% FCS. Effector cells were added to the wells at different E:T ratios ranging from 40:1 to 0.1:1. For an inhibition of lysis by mAbs, target cells were preincubated for 2 h in the presence of a saturating mAb concentration before the addition of effector cells. The 96-microwell plates were incubated at 37°C for 4 h, and supernatants were collected and counted for the release of ^{51}Cr. For blocking cytotoxicity or TNF production, the following mAbs were used: W6/32, a pan-MHC class I mAb, and B1.23.2 (ME1), an HLA-B/C-specific mAb.

Transfection of COS-7 cells and screening of transfectants

Transfection experiments were performed with COS-7 cells using the DEAE-dextran-chloroquine method (5, 7, 23). At 3 days before transfection, COS-7 cells were seeded in 96-microwell, flat-bottom plates at 5×10^3 cells/well in 150 μl of RPMI 1640 containing 20% FCS. Transfection experiments were performed in duplicate in two different microwell plates. For transfection, medium was discarded and replaced by 30 μl of transfection mixture containing 35 μg of DEAE-dextran (Sigma) and 0.1 mM chloroquine (Sigma), with 100 ng of plasmid DNA representing a pool of ~200 recombinant clones from the cDNA library and 100 ng of the autologous *HLA-B*0702* plasmid. COS-7 cells were incubated for 4 h at 37°C, medium was removed, and cells were incubated for 2 min in 1× PBS buffer containing 10% of DMSO solution. Cells were washed once in 1× PBS buffer and incubated with RPMI 1640/10% FCS for 2 days. After 2 days, transfected COS-7 cells were tested for their ability to stimulate the production of TNF by the 3B8 clone, as assessed with the WEHI assay.

Transfected COS-7 cells were tested for their ability to stimulate the production of TNF (24). A total of 2×10^3 CTLs (clone 3B8) were added to 96-microwell, flat-bottom plates containing transiently transfected COS-7 cells in 100 μl of RPMI 1640/10% FCS. Each supernatant was collected after 18 h, and its TNF content was determined by testing its cytotoxic effect on WEHI-164 clone 13 cells (25) in an MTT colorimetric assay.

Complementarity determining region-3 (CDR3) size analysis

CDR3 size analyses of *TCRBV* gene segments expressed by the CTL clone 3B8 or found in blood or tumor fragments were performed as described previously (22). The procedure used for CDR3 size analysis includes independent RT-PCR amplifications of *TCRBV-BC* fragments (26) followed by a runoff of the PCR products using nested fluorescent *TCRBC* or *TCRBJ* primers (27) and size determination of fluorescent runoff products by electrophoresis on an automated DNA sequencer ABI 373 (Perkin-Elmer Applied Biosystems, Foster City, CA) using Immunoscope software (28). Because the 5′ and 3′ primer positions are fixed, variations in the size of the runoff products are only due to differences in the length of CDR3 regions. Each peak is characterized by its position (CDR3 size) and an intensity of fluorescence (arbitrary fluorescence unit or fluorescence unit). The graphs representing CDR3 size patterns were standardized at 100% for the highest peaks. In blood from healthy donors, most profiles reflecting CDR3 size diversity in a given V_β subfamily displayed five to eight peaks at 3-nucleotide (nt) intervals with a nearly Gaussian distribution (21). Dominant peaks were defined as high-intensity signals with a dramatic decrease in other CDR3 signals.

Construction of the cDNA library

Poly(A)$^+$ RNA was extracted from the RCC-1 cell line using a maxi Message Marker kit (R&D Systems, Abingdon, U.K.) according to the manufacturer's instructions. First-strand cDNA was synthesized with the Superscript Choice System (Life Technologies, Gaithersburg, MD) using an oligo(dT) primer containing a *Not*I site at its 5′ end followed by second-strand cDNA synthesis. Blunt-end cDNAs were ligated to semi*Bst*XI adapters (Invitrogen, San Diego, CA), digested with *Not*I, and subsequently fractioned by chromatography on Sephacryl S-500 HR columns. cDNA size fractions were subcloned into the *Bst*XI and *Not*I sites of the pcDNAI expression vector. Recombinant plasmids were electroporated into *Escherichia coli* MC1061/P3, and bacteria were selected on Luria-Bertani-agar plates with ampicillin (50 μg/ml) and tetracycline (10 μg/ml). In screening experiments, the RCC-1 cDNA library was divided into 400 pools of 200 cDNA clones. Each pool of bacteria was amplified, and plasmid DNA was extracted using the alkaline lysis method (29).

Isolation of full-length iCE cDNA and of truncated or point-mutated iCE cDNA

Total RNA was extracted from an RCC cell line according to the guanidine-isothiocyanate/cesium chloride centrifugation procedure (30). Reverse transcription was performed on 5 μg of total RNA in a reaction volume of 20 μl using a cDNA Cycle Kit (Invitrogen) according to the manufacturer's instructions. A total of 1 μl of the cDNA reaction was used in a PCR using *Taq* DNA polymerase (Perkin-Elmer). For the amplification of human iCE cDNA (31), the following primers were used: primer P1, 5′-CCCAAGCTTGGTGAATAGCAGCGTGTCCGC-3′ (nt −28 to −48, sense) and primer P2, 5′-TGCTCTAGAAGGGAGCTACAGCTCTGT GTG-3′ (nt 1666–1687, antisense). PCR conditions were 10 min at 95°C followed by 30 cycles of amplification (94°C for 1 min, 60°C for 2 min, and 72°C for 3 min with a final extension for 10 min at 72°C). The PCR product obtained was digested by *Hin*dIII and *Xba*I and subsequently subcloned into the *Hin*dIII and *Xba*I sites of the pcDNAI expression vector for sequencing. The numbers in parentheses represent the nucleotide numbers complementary to the iCE cDNA published sequence (GenBank accession no. Y09616). iCE site-directed mutants were prepared by encoding the desired point mutation in overlapping oligonucleotide primers and generating the mutants by PCR (32). The sequencing of PCR products was performed with a DNA sequencing kit (ABI Prism, Perkin-Elmer Applied Biosystems).

Northern blot analysis

Total RNA was extracted from various primary tumors using the guanidinium isothiocyanate/cesium chloride centrifugation technique (30). RNA poly(A)$^+$ was prepared as described above from RCC cell lines and untransformed renal cell lines. A total of 5 μg of poly(A)$^+$ RNA or 10 μg of total RNA were subjected to electrophoresis in a 1.2% agarose formaldehyde gel and transferred to Hybond-N$^+$ (Amersham, Little Chalfont, U.K.) nylon membranes. The RNA blot was hybridized both with a 2C2 cDNA fragment corresponding to nt 1033–2009 of the published human iCE cDNA sequence (31) and with GAPDH cDNA as probes. All probes were labeled with [α-^{32}P]dCTP (3000 Ci mmol^{-1}) using the Prime-IT II Random Primer labeling kit (Stratagene, La Jolla, CA). Hybridization was performed at 48°C for 16 h.

Membranes were washed twice with 2× SSC at 52°C, washed once for 15 min with 0.2 SSC/0.1% SDS, and subsequently autoradiographed or analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

An RCC-specific CTL clone isolated from TILs

We first stimulated TILs from patient 1 with irradiated autologous tumor cells in the presence of low-dose IL-2 and TCGF (22). After 15 days of MLTC, a specific cytolytic activity against the autologous tumor cells was detected (31% of lysis at a 40:1 E:T ratio); TILs were cloned by limiting dilution in the presence of autologous tumor cells, EBV-transformed B cells, and allogeneic PBLs, with addition of IL-2 and TCGF. We isolated a TCR$\alpha\beta^+$ CD8$^+$ clone, termed 3B8, which lysed the autologous RCC cell line but not the NK-sensitive K562 target cells. The cytotoxicity of clone 3B8 against the autologous RCC-1 cell line was blocked with W6/32 mAb (Fig. 1*A*). In both cytotoxicity (Fig. 1*B*) and TNF production (data not shown) assays, all allogeneic HLA-B7$^+$ RCC cell lines (RCC-2, RCC-4, and RCC-5 in Fig. 1*B*) and none of the HLA-B7$^-$ RCC cell lines (RCC-3, RCC-6, RCC-7, and RCC-8) were recognized by CTL clone 3B8. The autologous EBV-transformed B cell line or PHA blasts were not recognized by 3B8 (data not shown). Therefore, it was concluded that the Ag recognized by 3B8 is presented by the HLA-B7 molecule and appears to be commonly expressed in RCC cell lines. The six HLA class I molecules from RCC-1 were isolated by RT-PCR (33), cloned in pcDNAI, and sequenced. The nucleotide sequence of autologous HLA-B7 cDNA led us to identify the allele involved as being *HLA-B*0702*. Transfection of this HLA allele in two HLA-B7$^-$ allogeneic RCC cell lines was sufficient to induce recognition (TNF secretion) by CTL clone 3B8, confirming that this clone led us to identify a shared Ag that is expressed by all RCC (data not shown).

In situ clonal expansion of a TIL subpopulation with similar TCRVB-BC *and* TCRVB-BJ *CDR3 length as the RCC-specific CTL clone 3B8*

For the 3B8 clone, a signal was obtained with only one of the 24 V_β subfamily primers (*TCRVB5*) and only one of the 13 *TCRBJ* primers (*TCRBJ1S2*) tested. CDR3 size distribution analysis showed that the *TCRBV5J1S2* clonotype of 3B8 was dominant in the tumor (as shown with *TCRBV5-BC* primers in Fig. 2*A* and with *TCRBV5-BJ1S2* primers for a more refined analysis in Fig. 2*B*), whereas such a clonotype was not found in PBMCs (a nearly Gaussian CDR3 length distribution with *TCRBV-BC* primers, see Fig. 2*A*). This result strongly suggests that the 3B8 clone was expanded specifically at the tumor site as shown previously by cDNA sequencing in several cases (14, 34–36).

Identification of a cDNA coding for the Ag

A cDNA library was constructed in the pcDNAI expression vector from RNA extracted from the RCC-1 cell line. The cDNA library was divided into 400 pools of 200 recombinant plasmids; each pool was cotransfected in duplicate in COS-7 cells with the expression vector pcDNAI containing the cDNA encoding for autologous *HLA-B*0702*. COS-7 cells were then tested for their ability to stimulate the production of TNF by 3B8. After 48 h, cotransfected COS-7 cells were incubated for 24 h with 3B8; the concentration of TNF in the culture supernatants was measured by its cytotoxic effect on WEHI cells. The amounts of TNF found in the supernatants ranged from 8 to 11 pg/ml except for two pairs of higher duplicates (14 and 15 pg/ml). For each pool of bacteria corresponding to these candidate wells, plasmid DNA was extracted and subcloned. A second screening was performed by transfecting COS-7 cells with 50 pools of 50 recombinant plasmids extracted from positive duplicates. Finally, a third screening in COS-7 cells led us to the isolation of two identical cDNA clones (cDNA clones 2C2 and 3G7) that transferred the expression of the Ag in HLA-B7$^+$ COS-7 cells. The results obtained with these cDNA clones are shown in Fig. 3*A*.

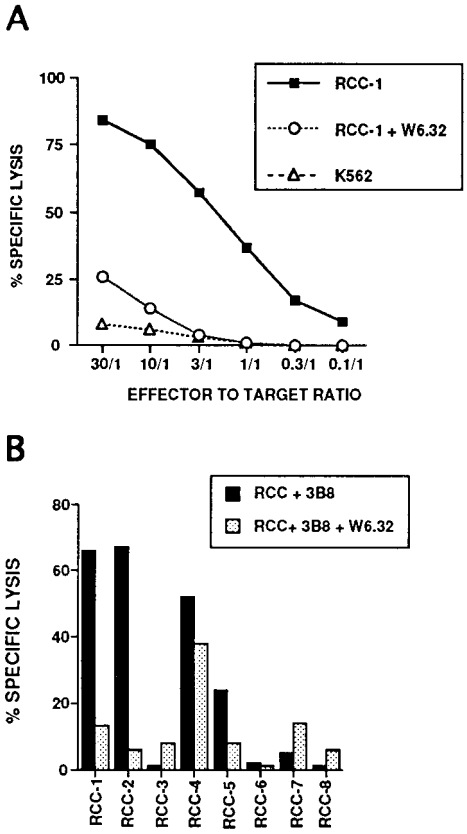

FIGURE 1. *A*, Specific lysis of the autologous RCC-1 cell line by CTL clone 3B8. The cytotoxicity of clone 3B8 toward the autologous RCC-1 cell line and K562 cells was tested in a standard chromium release assay at different E:T ratios. Blocking of lysis by mAb W6.32 is also shown. *B*, Cytotoxicity of 3B8 against various allogeneic cell lines. 3B8 was tested with the autologous RCC-1 cell line and with various allogeneic RCC cell lines in a standard chromium assay at an E:T ratio of 18:1. As a control, mAb W6.32 was used to block the HLA class I molecules involved in Ag presentation.

The sequence of cDNA 2C2 was 1250 nt long, with a 100% homology to nt 763-2009 (nucleotides are numbered from the start codon) of a recently identified cDNA coding for a putative iCE (31). To identify the full-length iCE cDNA corresponding to the published sequence, RT-PCR was performed from total RNA extracted from an RCC cell line; the corresponding 1.6-kb PCR product was subcloned in pcDNAI vector and sequenced. The nucleotide sequence was identical with the iCE published sequence.

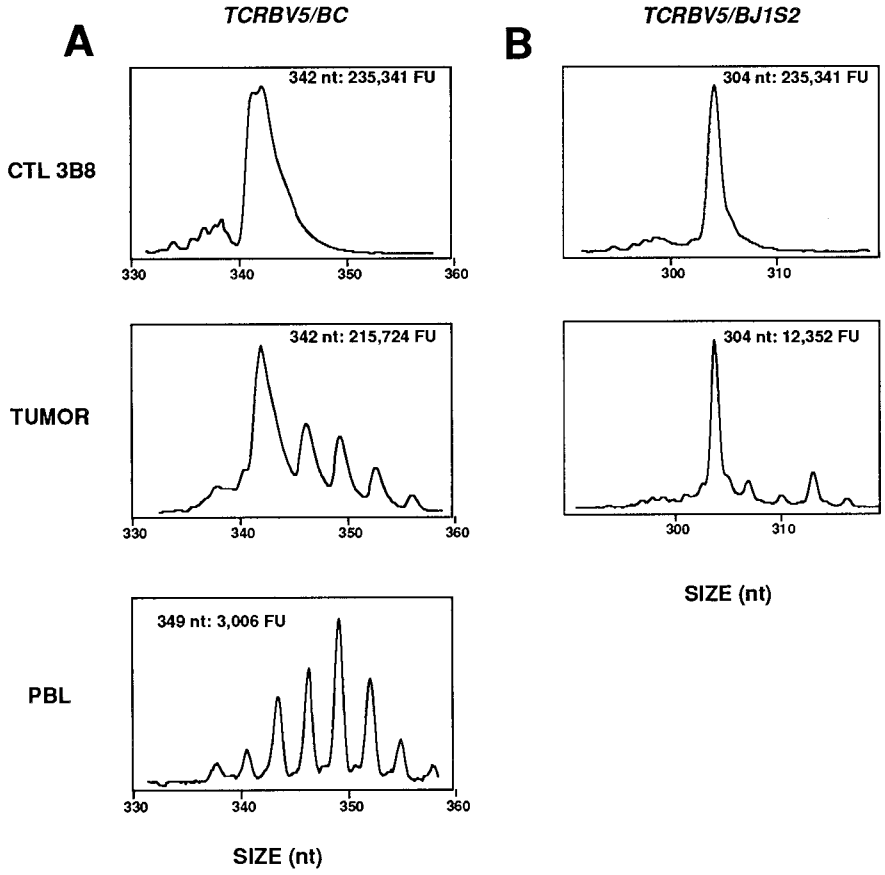

FIGURE 2. CDR3 size analysis in TILs and in clone 3B8 using selected *TCRBV* (*A*) and *TCRBJ* (*B*) primers. RNA was reversed transcribed and amplified for 40 cycles using *TCRBV5* and *BC* primers. Amplified DNA was copied for five cycles in a runoff reaction using either a nested fluorescent *TCRBC* (*A*) or a *TCRBJ152* (*B*) primer (13 *BJ* primers tested: *BJ1S1-BJ1S7*, *BJ2S1-BJ2S6*). The amplified products were analyzed on an automated sequencer. The profiles obtained show the sizes in nucleotides (*x*-axis) and the fluorescence intensity (*y*-axis) of the amplified products. The absolute fluorescence unit values obtained for dominant peaks are shown.

Cotransfection experiments in COS-7 cells showed that the full-length iCE cDNA was able to confer recognition by 3B8.

Identification of the antigenic peptide

To delimit the minimal nucleotidic region coding for the antigenic peptide, various truncated cDNAs corresponding to the iCE coding region were obtained from cDNA clone 2C2 (Fig. 4). These cDNA fragments subcloned into the pcDNAI expression vector were transfected into COS-7 cells together with pcDNAI containing the autologous *HLA-B*0702* cDNA. A minimal nucleotidic coding region was located between nt 763-1033. To reduce the nucleotidic sequence coding for the Ag, several truncated cDNAs were obtained by PCR amplification; these truncated cDNAs were cotransfected with the *HLA-B*0702* allele in COS-7 cells. COS-7 cells transfected with a fragment ranging from nt 763 to 855 were recognized by CTL 3B8 but not with a fragment ranging from nt 763 to 834 (Fig. 4), indicating that the peptide coding region was located between nt 763 and 855. After examination of the corresponding amino acid sequence, all possible nonamers and decamers were synthesized and tested for their ability to render autologous EBV-transformed B cells sensitive to lysis by 3B8. None of them were found to be positive at 10^{-4} or 10^{-5} M.

Finally, we found an alternative ORF (a +1 translational reading frame shift leading to a 453-nt ORF) with three ATGs at nt positions 476, 479, and 803 that encodes a nonamer (SPRWW PTCL) in the 763- to 855-nt minimal region. This nonameric sequence includes HLA-B7 anchoring residues at positions 2, 3, and 9. Half-maximal lysis of autologous EBV-transformed B cells was obtained with $<10^{-6}$ M of this nonapeptide (Fig. 5).

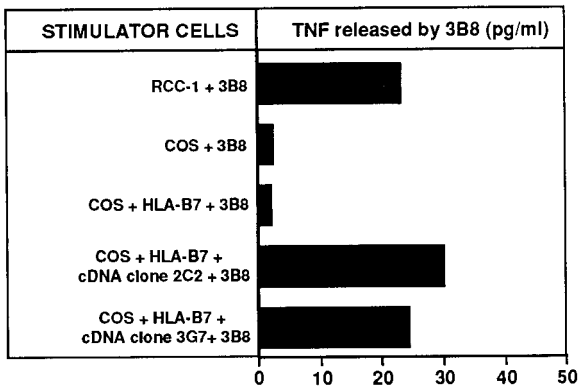

FIGURE 3. Stimulation of CTL 3B8 by COS-7 cells transiently cotransfected with expression vector pcDNAI containing cDNA clone 2C2 or 3G7 and the autologous *HLA-B*0702* cDNA. Control stimulator cells included the RCC-1 cell line as a positive control and COS-7 cells transfected with *HLA-B*0702* cDNA alone as negative control. The iCE cDNA was transiently cotransfected into COS-7 cells with the *HLA-B*0702* cDNA, and 3B8 was added after 48 h. The production of TNF was assessed by its cytotoxic effect on WEHI cells after 18 h. Control stimulator cells included the RCC-1 cell line as a positive control and COS-7 cells transfected with *HLA-B*0702* alone as a negative control.

Binding of iCE peptide to HLA-B7

HLA-A2 binding peptide Ags are known to up-regulate the expression of HLA-A2 molecules on T2 cells (37). Similarly, we used *HLA-B*0702*-transfected T2 cells (38) to analyze the binding ability and stability of the iCE peptide (Fig. 6). The binding of the iCE peptide was stable over time at 50 mM for ≥4 h, in contrast to the control HLA-A2-restricted hsp 70 peptide (14).

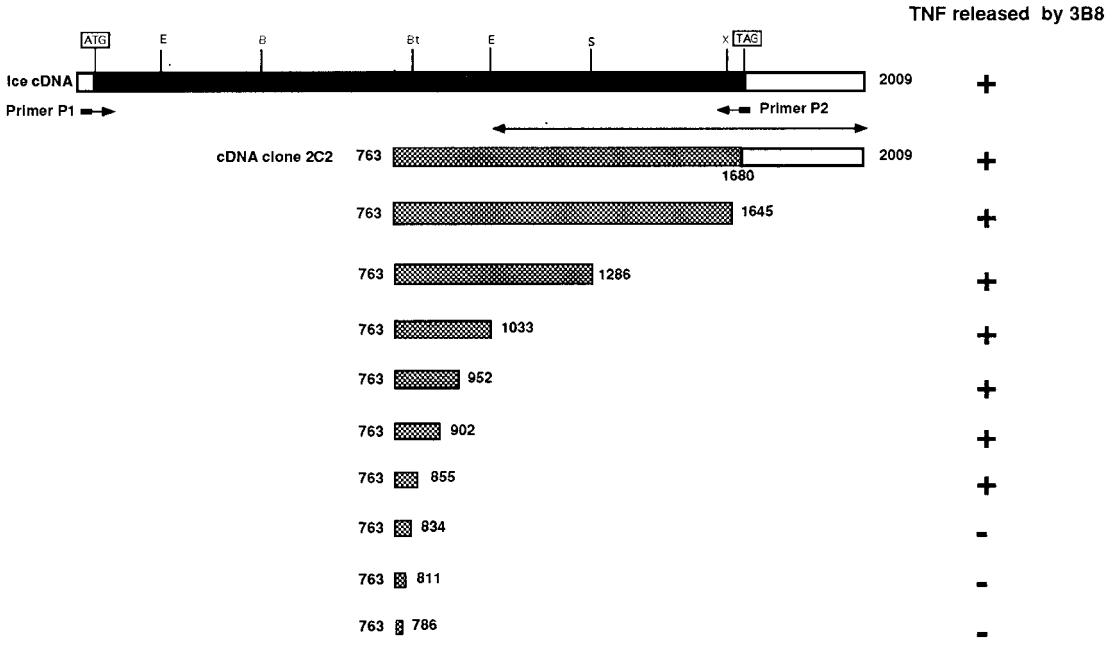

FIGURE 4. Location of iCE cDNA sequence coding for the antigenic peptide recognized by 3B8. A schematic representation of the full-length iCE cDNA sequence, cDNA clone 2C2, and various truncated 2C2 cDNAs is shown. Untranslated 3′ and 5′ regions are represented by open boxes. The human iCE translated sequence is represented by a filled box, the cDNA clone 2C2 and truncated 2C2 cDNAs are indicated by cross-hatched boxes. Nucleotides are numbered from the natural ATG start codon. Small black boxes with an arrow indicate the location of primer P1 and primer P2. The cDNA used as probe for hybridizing the RNA blot is indicated by a double-headed arrow. Restriction sites are as follows: B, *Bam*HI; Bt, *Bst*XI; E, *Eco*RI; S, *Sma*I; X, *Xba*I. The recognition by CTL 3B8 of COS-7 cells transiently cotransfected with the autologous *HLA-B*0702* cDNA and various truncated cDNAs is indicated. Transfected cells were incubated for 24 h with 5000 3B8 cells, and the amount of TNF in the supernatants was measured by its cytotoxic effect on WEHI-13 cells.

Tissue distribution of iCE mRNA

To determine the tissue distribution of iCE mRNAs, a human RNA Master blot (Clontech, Palo Alto, CA) consisting of a nylon membrane to which poly(A)$^+$ RNAs from 50 human tissues have been immobilized in separate dots was hybridized with a ^{32}P-labeled cDNA clone, 2C2, as a probe. iCE mRNA was detected in the liver, kidney, small intestine, colon, and heart and was weakly expressed in the pituitary gland, adrenal gland, prostate, and stomach (data not shown). No signal was found in fetal tissues, bone marrow, peripheral leukocytes, lung, and brain. To identify the mRNA species, a Northern blot was prepared with RNA poly(A)$^+$ from various RCC cell lines and untransformed renal cell lines (Fig. 7*A*) as well as total RNA extracted from various primary tumors (i.e., renal tumors, melanoma, bladder tumors, neuroblastoma, and colon tumors) (Fig. 7*B*). The RNA blot was hybridized with a cDNA probe corresponding to nt 1033–2009 of the 2C2 sequence. As shown in Fig. 7*A*, two mRNA species (4.5 kb and 3.5

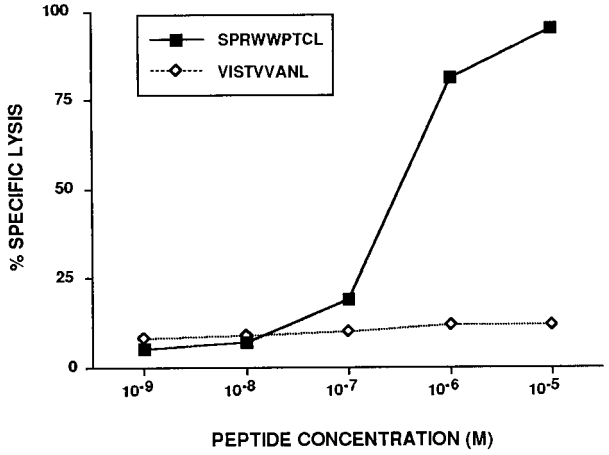

FIGURE 5. Lysis of autologous EBV-transformed cell lines incubated with iCE-encoded peptide by CTL 3B8. A total of 2000 ^{51}Cr-labeled, EBV-transformed cells were incubated for 1 h in the presence of the HLA-B7-restricted iCE peptide or another HLA-B7-restricted control peptide. CTL 3B8 were then added at an E:T ratio of 30:1. Chromium release was measured after 4 h.

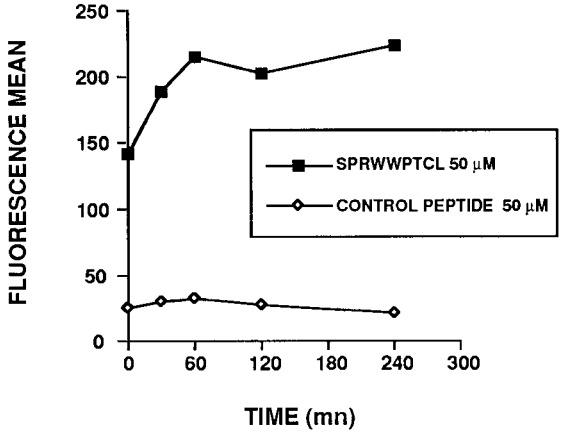

FIGURE 6. Induction of HLA-B7 expression on T2 cells by iCE peptide. T2 cells were incubated at 26°C for 16 h in serum-free medium with peptides at a concentration of 50 μM. Next, peptides were added again and cells were incubated at 37°C. At intervals of 30 min or 1 h, aliquots of cells were collected; changes in HLA-B7 expression were monitored by flow cytometry with an anti-HLA-B7 mAb (HB59). An HLA-A2-restricted hsp70 peptide was used as a control.

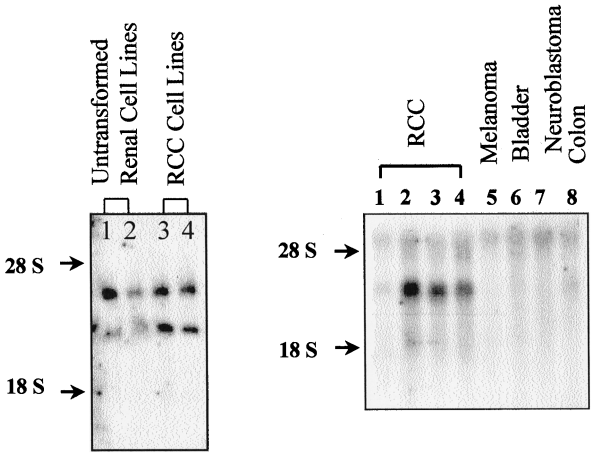

FIGURE 7. Analysis of iCE RNA transcripts in various cell lines (*A*) and tumor fragments (*B*). A total of 5 μg of poly(A)$^+$ RNA (*A*) and 10 μg of total RNA (*B*) were loaded on a denaturing 1% agarose-formaldehyde gel. RNA was transferred to a membrane, and the RNA blot was hybridized with ^{32}P-labeled cDNA clone 2C2 fragment as a probe. Hybridization with a GAPDH probe was used as an internal control for the loading of even amounts of RNA for analysis (data not shown).

kb) described previously by Schwer et al. (31) were detected in RCC carcinoma cell lines as well as in untransformed renal cells. In renal primary tumors a single mRNA transcript (3.5 kb) was detectable, whereas no iCE transcript was detected in primary tumors of different histotypes (Fig. 7*B*). Although an additional-2.2 kb transcript has been reported (31) in the small intestine and liver, no such transcript was detected in the various cell lines or primary tumors tested. Thus, the iCE protein is encoded in RCC tumors by a predominantly expressed single mRNA species (3.5 kb).

A cryptic non-AUG codon initiates an alternative ORF

A stop codon was first introduced at position 807 of the full-length iCE cDNA (Fig. 8*A*) just before the nonamer encoding sequence to confirm that the peptide recognized in the cytotoxicity assays is indeed encoded by the corresponding sequence in COS-7 transfection assays. This point mutation (mutant A) abolishes CTL 3B8 recognition following cotransfection with *HLA-B*0702* into COS cells (Fig. 8*B*). We subsequently mutated the natural AUG translation initiation site at position 3; this point mutant (mutant B) was still recognized (Fig. 8*B*), indicating that neither the natural iCE amino acid sequence nor a chimeric sequence resulting from programmed translational frameshifting (that is, for iCE slippage of the ribosome one codon forward) and recoding of the downstream sequence (39, 40) is coding for the recognized peptide.

In addition to ribosomal frameshifting (41), a ribosomal scanning mechanism that initiates translation at a downstream ATG has been shown to lead to the production of alternative reading frames recognized by T cells (42). Point mutations were then introduced on the full-length iCE cDNA at each of the three ATG sites found in the +1 ORF upstream from the nonameric peptide (mutant C for positions 476 and 479 and mutant D at position 803) to test whether the corresponding mutated iCE pcDNAI constructs were still able to confer recognition by CTL 3B8 in TNF release assays when cotransfected with *HLA-B*0702* into COS cells. As shown in Fig. 8*B*, none of these mutations abolishes recognition by CTL 3B8. These results strongly suggest that a cryptic non-AUG codon is used in iCE cDNA as an alternative translation initiation site.

To delimit the minimal nucleotidic region coding for this cryptic non-ATG codon, we introduced stop codons that would interrupt

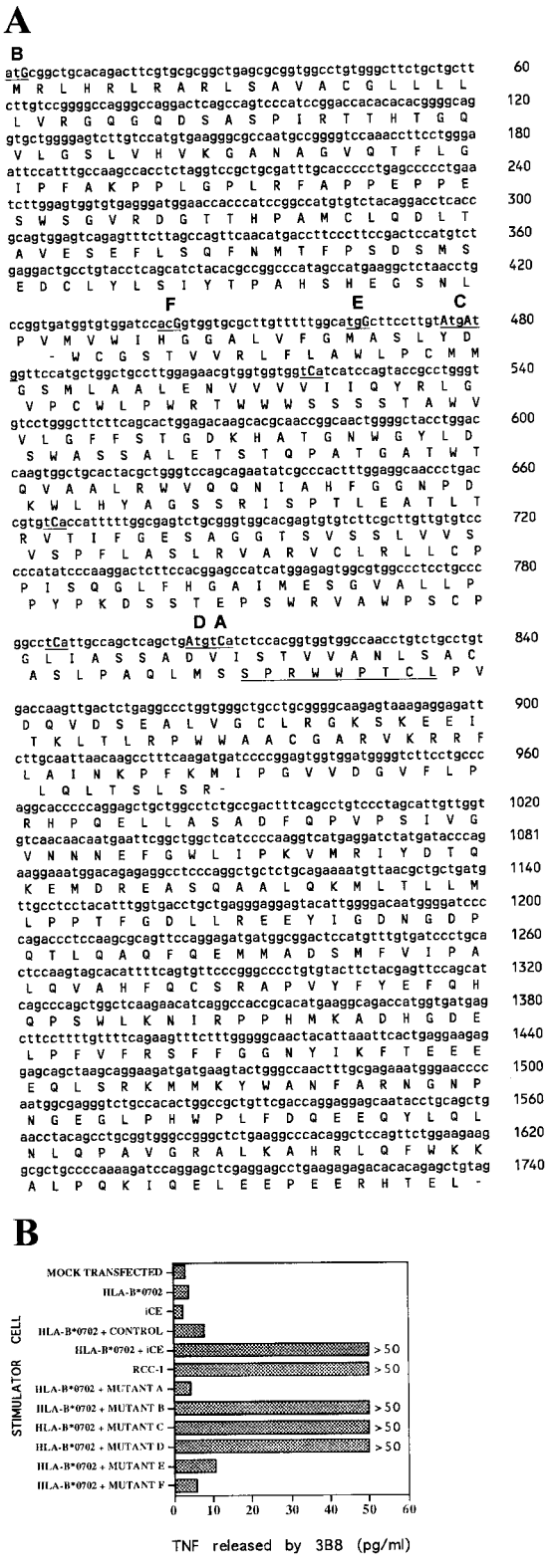

FIGURE 8. A non-ATG defined ORF of iCE is recognized by CTL 3B8. *A*, Sequence of the iCE cDNA coding region with the primary and the alternative (a +1 shift) ORFs. Positions of mutated nucleotides are shown in bold capital letters, the corresponding codons are underlined, and the locations of mutants A-F (tested in *B*) are shown above the mutated codons. The sequence of the antigenic peptide encoded by the +1 ORF is underlined. *B*, Point mutants (A-F) were tested for their ability to stimulate TNF release from clone 3B8 following cotransfection with *HLA-B*0702* into COS cells. Negative controls include mock transfection with *HLA-B*0702* or iCE cDNA alone or cotransfection with *HLA-B*0702* and a control pcDNAI plasmid.

the +1 ORF at different positions upstream from the antigenic peptide (between positions 428 and 809), with point mutations at positions 466 (mutant E), 519, 666, and 786 of the full-length iCE cDNA (Fig. 8*A*). All four of these mutants abolished CTL 3B8 recognition following cotransfection (see the result of mutant E for position 466 in Fig. 8*B*). A minimal nucleotidic region was then located between nt 428 and 466. We subsequently searched for possible non-ATG codons (CTG, ACG) in this short sequence and found only one, an ACG codon at position 440. Mutation of this codon to ACT (mutant F) abolished CTL 3B8 recognition (Fig. 8*B*). Thus, the first non-AUG codon in the +1 ORF was used to initiate the translation process.

Discussion

iCE cDNA was originally isolated from a human small intestine cDNA library (31). It has 65% homology to other carboxyl esterases of different mammalian species. It is expressed in human intestine, liver, or kidney and is supposed to be important for xenobiotic control and detoxification of the intestinal mucosa (31). We tested a large series of T cell epitopes encoded in the minimal nucleotidic region of the regular iCE ORF and found that none of them were recognized in the context of the *HLA-B*0702* class I restriction element. In contrast, a 453-nt ORF encoded in this region following a +1 frameshifting was found to encode a nonamer with HLA-B7 anchoring residues at positions 2, 3, and 9 (SPRWWPTCL). Half-maximal lysis was obtained with $<10^{-6}$ M of nonapeptide in target sensitization assays. The binding of this nonapeptide to *HLA-B*0702*-transfected T2 cells was stable over time, suggesting that low amounts of this alternative ORF expression may be sufficient to induce T cell recognition in vitro as well as T cell proliferation in vivo, as shown in the latter case by the in situ amplification at the tumor site of the corresponding TIL subpopulation.

Our results reveal that a novel mechanism may be involved in generating T cell epitopes. An alternative ORF induced by a cryptic non-AUG codon leading to a +1 translational reading frame was shown here to encode a tumor Ag recognized by TILs. In two other examples, gp75/TRP-1 (17) and NY-E50-1 (18), peptides recognized by TILs are coded by an alternative ORF located within the primary ORF. A mechanism by which the alternative ORF is translated has been suggested for gp75/TRP-1 (17), where recognition was affected by the presence of an internal AUG preceding the epitope. In addition to this ribosomal scanning mechanism, ribosomal frameshifting (39, 40) has been suggested for the production of T cell epitopes (41); however, in iCE, this possibility was excluded by the mutation of the natural ATG translation initiation site, which did not affect peptide recognition. In fact, the presence of the first cryptic internal translation initiation site (an ACG codon at position 440) in the +1 alternative ORF of iCE was enough to direct the expression of sufficient amounts of iCE peptide for T cell activation in vitro as well as in vivo (i.e., leading to in situ T cell clonal expansion). The leaky scanning model, in which ribosomes occasionally bypass the first AUG with a poor Kozak consensus sequence and initiate translation at a downstream translation initiation site may apply to iCE because of the presence of a pyrimidine at position +4 in place of a purine.

To our knowledge, this is the first example of an epitope coded by a non-ATG-defined alternative ORF and recognized by tissue-reactive T cells in human disease. It is not known whether low levels of expression of this alternative ORF in vivo may result in a failure to induce T cell tolerance to these products, leading to recognition in normal adult tissues. In the present study, untransformed HLA-B7$^+$ renal cell lines established in vitro were in fact recognized in cytotoxicity assays by the TIL-derived 3B8 clone (data not shown). It has been shown that non-ATG-defined alternative initiations of translation of the fibroblast growth factor-2 molecule are induced in stressed or transformed cells compared with ATG-defined ones (20). Similarly, the expression of non-ATG-initiated forms of iCE may be up-regulated in tumors, leading to the in situ clonal expansion of the corresponding TILs, as observed in the present study. This alternative iCE ORF would in the latter case represent an interesting tumor Ag for use in the immunotherapy of patients with hepatocarcinoma or colon or renal adenocarcinoma (tumors that may express iCE mRNA). More generally, these findings also raise the possibility that alternative ORFs induced by non-AUG codons in the three translational reading frames may encode T cell epitopes in some human diseases, such as cancer or autoimmune disorders. Short ORFs could be found in large numbers, and this may greatly increase the repertoire of nonmutated T cell epitopes recognized in adult tissues.

Acknowledgments

We thank Dr. C. Lutz for providing the *HLA-B*0702*-transfected T cells, Dr. J-P. Rousset and A.-C. Prats for helpful discussions, W. Mar for correcting the manuscript, and Drs. E. Angevin and B. Escudier for help in providing tumor samples.

References

1. Van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. D. Plaen, B. Van den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science 254:1643.*
2. Van den Eynde, B., and V. G. Brichard. 1995. New tumor antigens recognized by T cells. *Curr. Opin. Immunol. 7:674.*
3. Gaugler, B., B. Van den Eynde, P. Van den Bruggen, P. Romero, J. J. Gaforio, E. D. Plaen, B. Lethe, F. Brasseur, and T. Boon. 1994. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J. Exp. Med. 179:921.*
4. Boel, P., C. Wildmann, M. L. Sensi, R. Brasseur, J. C. Renauld, P. Coulie, T. Boon, and P. Van den Bruggen. 1995. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. *Immunity 2:167.*
5. Coulie, P. G., V. Brichard, A. V. Pel, T. Wölfel, J. Schneider, C. Traversari, S. Mattei, E. D. Plaen, C. Lurquin, J. P. Szikora, J. C. Renauld, and T. Boon. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med. 180:35.*
6. Kawakami, Y., S. Eliyahu, K. Sakaguchi, P. F. Robbins, L. Rivoltini, J. R. Yannelli, E. Appella, and S. A. Rosenberg. 1994. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor-infiltrating lymphocytes. *J. Exp. Med. 180:347.*
7. Brichard, V., A. V. Pel, T. Wölfel, C. Wölfel, E. D. Plaen, B. Lethé, P. Coulie, and T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med. 178:489.*
8. Robbins, P. F., M. El-Gamil, Y. F. Li, S. L. Topalian, L. Rivoltini, K. Sakaguchi, E. Appella, Y. Kawakami, and S. A. Rosenberg. 1995. Cloning of a new gene encoding an antigen recognized by melanoma-specific HLA-A24-restricted tumor-infiltrating lymphocytes. *J. Immunol. 154:5944.*
9. Wang, R. F., P. F. Robbins, Y. Kawakami, X. Q. Kang, and S. A. Rosenberg. 1995. Identification of a gene encoding a melanoma tumor antigen recognized by HLA-A31-restricted tumor-infiltrating lymphocytes. *J. Exp. Med. 181:799.*
10. Robbins, P. F., M. El-Gamil, Y. F. Li, Y. Kawakami, D. Loftus, E. Appella, and S. A. Rosenberg. 1996. A mutated β-catenin gene encodes a melanoma-specific antigen recognized by tumor-infiltrating lymphocytes. *J. Exp. Med. 183:1185.*
11. Coulie, P. G., F. Lehmann, B. Lethe, J. Herman, C. Lurquin, M. Andrawiss, and T. Boon. 1995. A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proc. Natl. Acad. Sci. USA 92:7976.*
12. Wölfel, T., M. Hauer, J. Schneider, M. Serrano, C. Wolfel, E. Klehmann-Hieb, E. D. Plaen, T. Hankeln, K. H. Meyer zum Buschenfelde, and D. Beach. 1995. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science 269:1281.*
13. Brandle, D., F. Brasseur, P. Weynants, T. Boon, and B. J. Van den Eynde. 1996. A mutated HLA-A2 molecule recognized by autologous cytotoxic T lymphocytes on a human renal cell carcinoma. *J. Exp. Med. 183:2501.*
14. Gaudin, C., F. Kremer, E. Angevin, V. Scott, and F. Triebel. 1999. A hsp70-2 mutation recognized by cytolytic T lymphocytes on a human renal cell carcinoma. *J. Immunol. 162:1730.*
15. Guilloux, Y., S. Lucas, V. G. Brichard, A. Van Pel, C. Viret, E. D. Plaen, F. Brasseur, B. Lethe, F. Jotereau, and T. Boon. 1996. A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanoma is encoded by an intron sequence of the *N*-acetylglucosaminyltransferase V gene. *J. Exp. Med. 183:1173.*

16. Robbins, P. F., M. El-Gamil, Y. F. Li, E. B. Fitzgerald, Y. Kawakami, and S. A. Rosenberg. 1997. The intronic region of an incompletely spliced gp100 gene transcript encodes an epitope recognized by melanoma-reactive tumor-infiltrating lymphocytes. *J. Immunol. 159:303*.
17. Wang, R. F., E. Appella, Y. Kawakami, X. Kang, and S. A. Rosenberg. 1996. Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes. *J. Exp. Med. 184:2207*.
18. Wang, R. F., S. L. Johnston, G. Zeng, S. L. Topalian, D. J. Schwartzentruber, and S. A. Rosenberg. 1998. A breast and melanoma-shared tumor antigen: T cell responses to antigenic peptides translated from different open reading frames. *J. Immunol. 161:3598*.
19. Nanbru, C., I. Lafon, S. Audigier, M. C. Gensac, S. Vagner, G. Huez, and A. C. Prats. 1997. Alternative translation of the proto-oncogene c-*myc* by an internal ribosome entry site. *J. Biol. Chem. 272:32061*.
20. Vagner, S., C. Touriol, B. Galy, S. Audigier, M. C. Gensac, F. Almaric, F. Bayard, H. Prats, and A. C. Prats. 1996. Translation of CUG- but not AUG-initiated forms of human fibroblast growth factor 2 is activated in transformed and stressed cells. *J. Cell Biol. 135:1391*.
21. Galy, B., A. Maret, A. C. Prats, and H. Prats. 1999. Cell transformation results in the loss of the density-dependent translational regulation of the expression of fibroblast growth factor 2 isoforms. *Cancer Res. 59:165*.
22. Angevin, E., F. Kremer, C. Gaudin, T. Hercend, and F. Triebel. 1997. Analysis of T-cell immune response in renal cell carcinoma: polarization to type 1-like differentiation pattern, clonal T cell expansion, and tumor-specific cytotoxicity. *Int. J. Cancer 72:431*.
23. Seed, B. 1987. An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. *Nature 329:84O*.
24. Traversari, C., P. Van der Bruggen, I. F. Luescher, C. Lurquin, P. Chomez, A. Van Pel, E. De Plaen, A. Amar-Costesec, and T. Boon. 1992. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J. Exp. Med. 176:1453*.
25. Espevik, T., and J. Nissen-Meyer. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J. Immunol. Methods 95:99*.
26. Genevee, C., A. Diu, J. Nierat, A. Caignard, P. Y. Dietrich, L. Ferradini, S. Roman-Roman, F. Triebel, and T. Hercend. 1992. An experimentally validated panel of subfamily-specific oligonucleotide primers (Vα1-w29/Vβ1-w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. *Eur. J. Immunol. 22:1261*.
27. Even, J., A. Lim, I. Puisieux, L. Ferradini, P. Y. Dietrich, A. Toubert, T. Hercend, F. Triebel, C. Pannetier, and P. Kourilsky. 1995. T-cell repertoires in healthy and diseased human tissues analysed by T-cell receptor β-chain CDR3 size determination: evidence for oligoclonal expansions in tumours and inflammatory diseases. *Res. Immunol. 146:65*.
28. Pannetier, C., M. Cochet, S. Darche, A. Casrouge, M. Zoller, and P. Kourilsky. 1993. The sizes of the CDR3 hypervariable regions of the murine T-cell receptor β chains vary as a function of the recombined germ-line segments. *Proc. Natl. Acad. Sci. USA 90:2472*.
29. Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res. 7:1513*.
30. Sambrook, J., E. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31. Schwer, H., T. Langmann, R. Daig, A. Becker, C. Aslanidis, and G. Schmitz. 1997. Molecular cloning and characterization of a novel putative carboxylesterase, present in human intestine and liver. *Biochem. Biophys. Res. Commun. 233:117*.
32. Mikaelian, I., and A. Sergeant. 1992. A general and fast method to generate multiple site directed mutations. *Nucleic Acids Res. 20:376*.
33. Ennis, P. D., J. Zemmour, R. D. Salter, and P. Parham. 1990. Rapid cloning of HLA-A,B cDNA by using the polymerase chain reaction: frequency and nature of errors produced in amplification. *Proc. Natl. Acad. Sci. USA 87:2833*.
34. Farace, F., F. Orlanducci, P. Y. Dietrich, C. Gaudin, E. Angevin, M. H. Courtier, C. Bayle, T. Hercend, and F. Triebel. 1994. T cell repertoire in patients with B-chronic lymphocytic leukemia: evidence for multiple in vivo T cell clonal expansions. *J. Immunol. 153:4281*.
35. Farace, F., E. Angevin, I. Poullion, C. Leboullaire, G. Ferir, D. Elias, B. Escudier, and F. Triebel. 1997. T-cell receptor CDR3 size distribution analysis to evaluate specific T-cell response to cancer vaccines. *Int. J. Cancer 71:972*.
36. Gaudin, C., P. Y. Dietrich, S. Robache, M. Guillard, B. Escudier, M. J. Terrier-Lacombe, A. Kumar, F. Triebel, and A. Caignard. 1995. In vivo local expansion of clonal T cell subpopulations in renal cell carcinoma. *Cancer Res. 55:685*.
37. Nijman, H. W., J. G. Houbiers, M. P. Vierboom, S. H. van der Burg, J. W. Drijfhout, J. D'Amaro, P. Kenemans, C. J. Melief, and W. M. Kast. 1993. Identification of peptide sequences that potentially trigger HLA-A2.1-restricted cytotoxic T lymphocytes. *Eur. J. Immunol. 23:1215*.
38. Smith, K. D., and C. T. Lutz. 1996. Peptide-dependent expression of HLA-B7 on antigen processing-deficient T2 cells. *J. Immunol. 156:3755*.
39. Farabaugh, P. J. 1996. Programmed translational frameshifting. *Annu. Rev. Genet. 30:507*.
40. Matsufuji, S., T. Matsufuji, Y. Miyazaki, Y. Murakami, J. F. Atkins, R. F. Gesteland, and S. Hayashi. 1995. Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme. *Cell 80:51*.
41. Elliott, T., H. Bodmer, and A. Townsend. 1996. Recognition of out-of-frame major histocompatibility complex class I-restricted epitopes in vivo. *Eur. J. Immunol. 26:1175*.
42. Bullock, T. N. J., and L. C. Eisenlohr. 1996. Ribosomal scanning past the primary initiation codon as a mechanism for expression of CTL epitopes encoded in alternative reading frames. *J. Exp. Med. 184:1319*.