The Journal of Immunology

Alternative Translational Products and Cryptic T Cell Epitopes: Expecting the Unexpected

On Ho and William R. Green

J Immunol 2006; 177:8283-8289; ;
doi: 10.4049/jimmunol.177.12.8283
http://www.jimmunol.org/content/177/12/8283

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

THE JOURNAL OF IMMUNOLOGY

BRIEF REVIEWS

Alternative Translational Products and Cryptic T Cell Epitopes: Expecting the Unexpected

On Ho and William R. Green[1]*†*

Although CD8 T cell epitopes have been studied extensively, often overlooked are unconventional cryptic epitopes generated from nontraditional sources of peptides/proteins and/or mechanisms of translation. In this review, we discuss alternative reading frame epitopes, both mechanistically and also in terms of their physiologic importance in the induction of antiviral and antitumor CTL responses. Issues of the influence of cryptic translational products on foreign and self-Ag diversity, thymic selection, and the T cell repertoire; disease pathogenesis; and approaches to vaccine design are discussed in context of the potentially large impact of unconventional epitopes on T cell immunity. *The Journal of Immunology,* **2006, 177: 8283–8289.**

Surveillance of the immune system by CD8 T cells is critical for the detection and elimination of aberrant cells, either infected by viruses or bacteria, arising from tumor development or expressing heterologous histocompatibility Ags. To detect foreign or abnormal self-gene products, the repertoire of peptide/MHC class I complexes on the target cell surface is sampled for peptides that may serve as ligands for the highly sensitive and uniquely specific AgRs of class I-restricted CD8 T cells. For effective immune surveillance, it is therefore crucial that the peptide repertoire presented by MHC class I molecules be as broadly inclusive as possible.

CD8 T cells and unconventional epitopes

MHC class I-presented peptides are 8–11 aa in length and generally adhere to a characteristic allele-specific motif, typically defined by two anchor residues critical for binding specifically to the class I groove. An early but comprehensive report compiled by Rammensee et al. (1) provided the first listing of MHC binding peptides, the size of which now appears very modest compared with the most recent database with 17,129 distinct sequences for >400 MHC molecules (2). Thus, the binding requirements of many different MHC molecules are fulfilled by generation of a broad pool of CD8 T cell ligands.

The expansiveness of the peptide repertoire suggests many sources for supplying the Ag-processing pathway, particularly via the degradation of newly synthesized proteins. Thus, defective ribosomal products (DRiPs)[2] consisting of polypeptides resulting from premature termination and/or improper folding lack biological function and are degraded (3–5). Indeed, an almost immediate proteasomal degradation may await perhaps ~30% of synthesized polypeptides defined as rapidly degraded polypeptides (6) that may provide a source of antigenic peptides. Based on this concept that synthesis of functional proteins is significantly <100% efficient, a current hypothesis is that aberrant DRiP and rapidly degraded polypeptide translation products may be the major source of class I-associated peptides. This view can be expanded to embrace cryptic translation products, or polypeptides synthesized by unconventional cellular translational mechanisms (7), as another source of class I epitopes. Presumably, these sources of peptides would be difficult to identify because unconventional translation would suggest a low abundance of these epitopes. On the contrary, when T cells were used to probe a cDNA expression library, an unusual cryptic translation product encoded at the vector/cDNA insert junction was detected despite an estimated expression frequency of this antigenic peptide at <10 copies/cell (8). Furthermore, in vivo studies have demonstrated priming for a specific CTL response against a cryptic translational epitope, generated in the early stages of a natural retroviral infection (9). Thus, efficient Ag processing, along with the extremely high sensitivity of CD8 T cells for recognition of peptide/MHC complexes (10), may overcome likely low levels of expression by cryptic proteins.

Cryptic epitopes may derive potentially from all levels of gene and protein expression (11). The pepton hypothesis suggesting that epitopes could result from transcription of short genetic regions by a novel polymerase, yielding antigenic pepton-RNA that associated directly with the class I molecule (12), was intriguing, but no/little evidence emerged to support this idea (13). Atypical epitopes may also arise from posttranscriptional regulatory events, e.g., human melanoma peptides generated by either incompletely spliced message (14), mutation of a normally noncoding intronic sequence (15), or exon extension as shown with a cryptic peptide derived from a murine sarcoma (16). At the translational level, mechanisms such as ribosomal frameshifting (17), internal initiation using internal ribosome

*Department of Microbiology and Immunology, Dartmouth Medical School, Lebanon, NH 03756; and †Norris Cotton Cancer Center, Dartmouth Hitchcock Medical Center, Lebanon, NH 03756

Received for publication June 22, 2006. Accepted for publication September 28, 2006.

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] Address correspondence and reprint requests to Dr. William R. Green, Dartmouth Medical School, Department of Microbiology and Immunology, 603W Borwell Building, One Medical Center Drive, Lebanon, NH 03756. E-mail address: william.r.green@dartmouth.edu

[2] Abbreviations used in this paper: DRiP, defective ribosomal product; ARF, alternative reading frame; HCV, hepatitis C virus; MAIDS, mouse AIDS; ORF, open reading frame; TIL, tumor-infiltrating lymphocyte; TK, thymidine kinase.

0022-1767/06/$02.00

entry sites (18, 19), initiation codon scanthrough (20, 21), doublet decoding (22), or initiation from non-AUG codons (23, 24) may contribute to the biosynthesis of nontraditional peptides. Recent evidence implicates posttranslational processing as yet another possibility: that is, epitopes generated by removal of an internal segment of a larger polypeptide, followed by ligation of the remaining carboxy- and amino-terminal residues (25, 26).

Alternative reading frame (ARF)-encoded epitopes: a translational mechanism affecting antiviral responses

Of the increasing number of class I peptides identified from cryptic translation, most are of viral or tumor cell origin, suggesting that viral infection or transformation may induce mechanisms prone to unconventional translation (7). Our lab defined a cryptic retroviral epitope while assessing resistance in the LP-BM5 retrovirus-induced mouse AIDS (MAIDS) model. Strongly lytic $CD8^+$ CTL were generated to the highly homologous *gag* polyprotein of both the replication-defective BM5def and replication-competent BM5eco viruses, in a MHC-dependent manner: only H-2^d-restricted CTL could be elicited, even in F_1 mice (27, 28). Characterization of MAIDS-resistant BALB/c-derived CTL raised against viral *gag* showed their specificity for an immunodominant K^d-presented epitope (SYNT GRFPPL) encoded by a novel and previously unrecognized +1 alternative translational open reading frame (ORF2) of the LP-BM5 genome (Fig. 1) (9). By adoptive transfer studies using disease-susceptible CD8-deficient BALB/c recipient mice, we have reported recently a physiologically relevant role for this ARF-encoded ORF2 epitope by demonstrating CTL-mediated full protection against LP-BM5-induced disease in vivo (29).

ARF epitopes have been identified in other viral systems, notably for HIV-1 (30). These cryptic peptides were processed and presented during the natural course of HIV-1 infection, priming CTL responses in infected individuals. Some of these epitopes are highly conserved in the sequences of circulating HIV-1 strains. However, some variation of a *gag* ARF-derived peptide resulted in a cross-reactive epitope found at a high frequency in specifically clade B viruses. While the need to preserve the function of the viral protein encoded by the primary ORF no doubt impacts the conservation of a corresponding overlapping ARF epitope, this comprehensive clade analysis suggests that ARF epitope expression may also be affected by immunological, virological, and drug-selective pressures (30). Although the in vivo protective role of these recognized ARF epitopes against HIV infection or AIDS cannot be directly addressed, their presence supports a role for ARF proteins as a source of antigenic peptides.

ARF epitopes and antitumor responses

Identification of several ARF antigenic peptides encoded by genes of nonvirally induced tumors emphasizes that ARF epitopes can also derive from cryptic translation of self proteins (Table I). Following studies that demonstrated metastatic tumor regression in a melanoma patient infused with tumor-infiltrating lymphocytes (TILs) together with a regimen of IL-2 and cyclophosphamide (31, 32), the TIL-defined CTL epitope was found to originate from the self *TRP-1*/*gp75* gene by translation of the ORF3 register (33, 34). Subsequently, several ARF epitopes have been documented in other tumor systems. Using therapy with recombinant adenovirus, 1 of 16 patients demonstrated complete regression of mediastinal metastases and was tumor-free at >5 years. T cell clones derived from this patient were directed against a novel melanoma Ag generated from a short ARF of the *BING-4* gene (35). Relatively high levels of BING-4 mRNA expression were found in many melanoma cell lines but not in other tumor types. However, ARF epitopes are not restricted to melanomas but have been identified, for example, from prostate- and breast tumor-associated protein (36) and in microsatellite DNA of an O-linked *N*-acetylglucosamine transferase gene found in unstable colorectal cancers, the latter resulting from a −1 frameshift mutation (37). An ARF protein encoded by an intestinal carboxyl esterase gene, and initiated by a non-AUG codon, is the source of an epitope recognized by

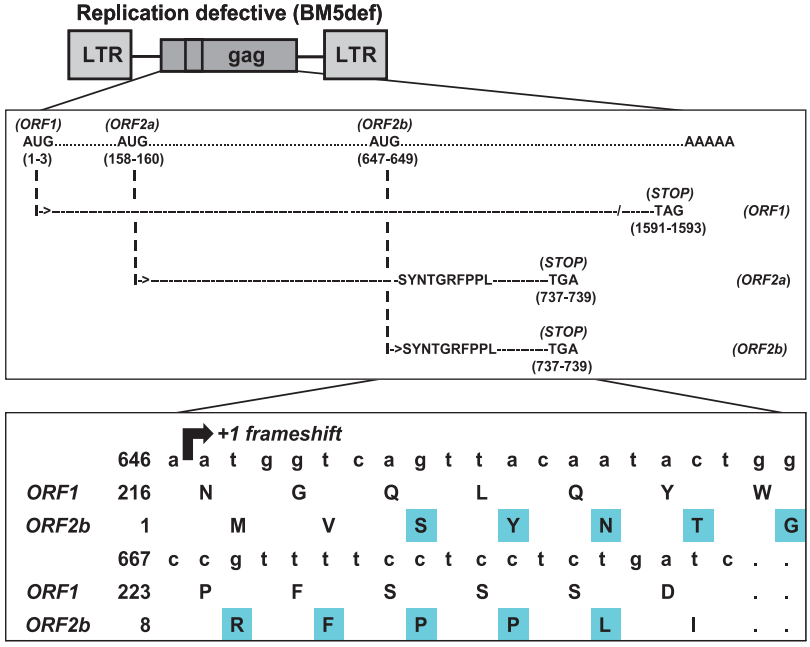

FIGURE 1. Generation of a cryptic CTL epitope derived from pathogenic ARF proteins in a retrovirus system. Putative mRNA transcripts and corresponding protein products of the pathogenic BM5def *gag* gene are shown. Translation of the ARF (ORF2) may initiate at either of two start sites, ORF2a and ORF2b, via a +1 frameshift of the primary ORF (ORF1). The cryptic K^d-restricted immunodominant CTL peptide SYNTGRFPPL (highlighted), processed from the larger precursor proteins, induces a protective CTL response against MAIDS disease (29). Conversely, the ORF2a and ORF2b protein products, individually or in combination, appear to be necessary for MAIDS pathogenesis (59).

Table I. *Cryptic T cell epitopes derived from extended ARF tumor proteins*

Disease System	Gene of Origin	Location	Epitopes (MHC restriction)	Extended ARF Protein(s)	References
Melanoma	*TRP-1/gp75*	ORF3	MSLQRQFLR (HLA-A31)	24-aa product	34
Melanoma	*BING-4*	ORF2	CQWGRLWQL MCQWGRLWOL[a] (HLA-A2)	10-aa product	35
Breast/prostate cancer	*TCRγ*	exon 2	FVFLRNFSL FLRNFSLML[b] (HLA-A2)	TCRγ alternate reading frame protein (TARP)	36
Colorectal cancer	*O-linked N-acetylglucosamine transferase (OGT)*	exon 5	SLYKFSPFPL (HLA-A2)	OGT(FS)[c]	37
Renal cell carcinoma	*Intestinal carboxyl esterase*	ORF2	SPRWWPTCL (HLA-B7)	151-aa product[d]	38
Melanoma	*CDKN2A*	exon 2	AVCPWTWLR[e] (HLA-A11)	p14ARF p16INK4a	39

[a] Minimal 9-mer and overlapping 10-mer peptides demonstrated similar stimulation of IFN-γ production by an antitumor lymphocyte clone (35).
[b] FVFLRNFSL, compared with FLRNFSLML, demonstrated higher binding affinity and better induction of peptide-specific responses by CD8 T cells (36).
[c] Mutational frameshifting giving rise to the OGT(FS) protein occurs in >40% of colorectal cancers (37).
[d] Translation initiation site of extended ARF protein is characterized as a non-AUG/ACG codon (38).
[e] Frameshift mutations in the shared exon 2 sequence produced transcripts from the p14ARF, and p16INK4a promoters, both of which encode the same T cell epitope (39).

TILs from a renal cell carcinoma patient (38). Also adding additional layers of complexity to consider in the cryptic translation process is the generation of two distinct products from the *CDKN2A* tumor suppressor gene. Coding in part from different exon 1 sequences, the proteins also derive from a common exon 2 sequence that is, however, translated from two alternative ORFs (39). Recognition of epitopes derived from both ARF proteins has been demonstrated with TILs that mediated complete disease regression in a melanoma patient (39).

Molecular mechanisms involved in translational frameshifting

Ribosomal frameshifting occurs when the ribosome slips to an alternative, overlapping reading frame in midtranslation. This event is determined by short homopolymeric nucleotide sequences (slippery sites) or stimulatory sequences that can cause the ribosome to stall during translation, especially, for example, while slowly decoding a rare tRNA (reviewed in Ref. 40). Additional secondary structures may also cause interruption of normal translation, including stem loops and pseudoknots (reviewed in Ref. 41).

Programmed translational frameshifting, or recoding, occurs frequently, and most of these events involve −1 frameshifting at particular signal-specific sites located between two overlapping reading frames (reviewed in Ref. 42). For most retroviruses, recoding is necessary at the 3′ end of the *gag* gene to synthesize the replication proteins coded by the *pol* gene, forming a GagPol polyprotein at 5–10% of *gag* translation (reviewed in Ref. 43). In other retroviruses, such as HIV, which possess the additional *pro* gene, expression of viral protease may require up to two frameshifts. Although recoding is commonly associated with viruses, programmed −1 and +1 frameshifting has been documented in both bacteria (44, 45) and at least two human cellular genes: *Edr*, providing the first evidence of −1 translational recoding in a eukaryote (46), and the gene for ornithine decarboxylase antizyme, in which +1 frameshifting occurs via a downstream mRNA pseudoknot (47).

In contrast, incidental translational frameshifting is very rare, due to the chance occurrences of similar signals as those orchestrating programmed recoding. In a unique example discussed by Zook et al. (48), frameshifting in a HSV mutant allowed for low-level production of wild-type thymidine kinase (TK) protein, despite interruption of the coding sequence with the insertion of an additional guanine nucleotide (49). In fact, the site of insertion was a guanine-rich sequence in the mRNA suitable as a frameshifting signal, resulting in realignment of the reading frame (50) and production of normal TK protein at 1–2% of wild-type translation (49). These levels were sufficient for HSV reactivation from latency but not high enough for virus susceptibility to acyclovir treatment (51, 52). Taking advantage of this system, a model conventional influenza nucleoprotein epitope in tandem with the OVA SIINFEKL peptide was engineered downstream of the TK element (48). Cryptic nucleoprotein and SIINFEKL expression, although dependent on the low level of incidental frameshifting, was sufficient to generate CTL responses, with the anti-SIINFEKL CTL capable of protection against tumor cells stably transfected to express OVA.

Because the sequences that stimulate frameshifting may be found in theoretically any region of the reading frame, it appears that ribosomal frameshifting is a more effective mechanism for generating cryptic epitopes than other methods of alternative translation (48). Thus, understanding the frequency of frameshifting events may provide information on cryptic epitope expression and the threshold levels necessary for induction of T cell responses. Using a bioinformatics approach and applying the algorithms for predicting ribosomal frameshifting sites to the genomes of all organisms for which large DNA databases were available, a significant number of heptanucleotide consensus signals for inducing −1 programmed recoding events was identified (53). This and other studies suggest that ribosomal frameshifting may be more common than previously thought, and furthermore, the list of known mechanisms may be incomplete. Thus, rather than ribosomal pausing, the ribosome may slide over a "hungry" codon waiting for an aminoacyl-tRNA in short supply and resume several codons downstream without translation of bypassed nucleotides (54). In contrast to the standard heptanucleotide signals for frameshifting, a sequence of only four adenosine nucleotides may also serve as a slippery site (55). Alternatively, an aminoacyl tRNA may be out-of-frame while binding the ribosome, thus mediating frameshifting without ribosomal slippage (56).

Impact of cryptic epitopes on T cell selection and immunity

T cell maturation occurs through a highly sophisticated system involving two separate selections, with the majority of thymocytes defaulting to a programmed cell death upon failure to bind self MHC/peptide complexes with sufficient avidity during positive selection. Of the remaining thymocytes, those that are highly self-reactive are further eliminated during negative selection, thereby establishing central tolerance and limiting the possibility of autoreactive T cell populations. In considering how cryptic translational epitopes may impact T cell development and ability to respond to Ag, Schwab et al. (57) generated transgenic mice expressing in parallel two model conventional epitopes, one placed in a conventional translation context, and the other engineered into a nontranslated region downstream of a termination signal and directed by non-AUG-induced initiation. This study demonstrated the induction of thymic self-tolerance by a cryptic peptide, suggesting that beyond their role in eliciting a peripheral T cell response, cryptic epitopes may also help to shape the TCR repertoire.

Although cryptic self-peptides can thus direct central tolerance in some cases, they may generally be preferentially involved in positive selection. In particular, while low-avidity recognition by the TCR appears sufficient for positive selection, high-avidity interactions are thought to be necessary for thymocyte deletion during negative selection. Although generated at presumably low concentrations, often from unconventional translational mechanisms, cryptic epitopes would seem to satisfy the threshold for low-avidity binding and allow their participation in positive selection. Conversely, their low abundance would ostensibly render them less functional in high avidity-requiring negative selection events. Thus, cryptic epitopes may contribute to expansion of the TCR repertoire without mediating comparable compensating negative selection. The likely low density of cryptic epitopes may be less detrimental to the magnitude of peripheral T cell responses. In fact, in the study by Schwab et al. (57), despite expression of cryptic peptide levels a 100-fold lower, the cryptic epitope-induced T cell response was only 3-fold smaller in comparison to the response elicited by the conventional epitope.

Cryptic translational products and disease pathogenesis

CTL recognition of a peptide derived from an alternative ORF of the M-CSF expressed by a renal cell carcinoma provides an example of an apparent disconnect between ORF1 and ORF2 translation (58). Thus, the ARF peptide was in fact not tumor specific, but was expressed in normal tissues that did not produce the ORF1-generated M-CSF protein. However, more commonly, both primary and ARF sequences have been conserved, clearly not for coding of an antigenic epitope, but rather because viruses and tumors appear to use extended ARF proteins for their own advantage (Tables I and II).

A unique feature of the LP-BM5 retrovirus complex responsible for murine AIDS is a largely conserved ORF2 encoding a protective CTL epitope in both the pathogenic BM5def and nonpathogenic BM5eco viruses (Fig. 1) (9). We discovered that BM5def, but not BM5eco helper, virus encoded an extended, uninterrupted ORF2 containing two possible initiation sites, ORF2a and ORF2b (Table II). Mutated $ORF2a_{AUG}$ or $ORF2b_{AUG}$, BM5def virus, rescued with wild-type BM5eco, successfully infected susceptible C57BL/6 mice but was unable to induce disease, in contrast to pathogenic wild-type rescued BM5def (59). Because the mutation strategy allowed ORF1 to remain intact, the putative 193 and 30 aa, protein products translated from the ORF2a and ORF2b start sites, respectively, may each be required for disease. Alternatively, only the ORF2a product may be needed in addition to ORF1 for MAIDS pathogenesis: the engineered mutation at the ORF2b AUG site may act not to inhibit translation initiation but rather to substitute threonine for methionine, inadvertently introducing a deleterious missense mutation affecting the function of the ORF2a protein.

Examples of ARF proteins with potential roles in disease pathogenesis have been identified in other viral systems (Table II). Chen et al. (60, 61) identified the ARF location of a CTL epitope and used this information to demonstrate a novel ARF-encoded influenza A protein localized to the mitochondria that can induce apoptosis of monocytes, a feature characteristic of influenza virus infection. Similarly, an alternative ORF in hepatitis C virus (HCV) shown to encode the targets of both T and

Table II. *Cryptic translational T cell epitopes derived from extended ARF viral proteins likely involved in pathogenesis*

Disease System	Gene of Origin	Location	Epitopes (MHC restriction)	Extended ARF Protein(s)	Role in/Mechanism of Pathogenesis	References
LP-BM5 retrovirus	*gag*[a]	ORF2	SYNTGRFPPL (H-2K[d])	gag_{ORF2a} gag_{ORF2b}	Yes/to be determined[b]	9, 11, 27–29, 59
Influenza A virus	*PB1*[c]	ORF2	LSLRNPILV (H-2D[b])	PB1-F2	Potentially/apoptosis of monocytes	60, 61
HCV	*HCV*[d]	ORF2	APGWVCARL[e] (HLA-B7) WVCARLGRL (HLA-A2, HLA-B7) RLPSGRNLV (HLA-A2) GPGLSPGTL (HLA-B7)	F protein (also called ARFP or core+1 protein)	Potentially/repression of p21 negative regulation of cell cycle	62, 63

[a] *gag* is the gene encoding the core structural proteins of the retrovirus.
[b] Mutation of the ORF2b AUG initiation site did not affect translation of the ORF2a product, suggesting gag_{ORF2a} may be solely responsible for disease, perhaps by targeting a cellular protein (59).
[c] *PB1* is the gene segment encoding the PB1 protein, one of three critical subunits of the viral polymerase.
[d] HCV genome encodes a single polyprotein, which is proteolytically processed to generate 10 distinct proteins, including the core.
[e] Epitopes were predicted from the sequence of the HCV-JA isolate. IFN-γ and IL-10 responses were observed from PBMC in 21% of HCV-seropositive patients to these predicted/"consensus" epitopes, demonstrating priming to the identical or cross-reactive epitopes of the patient virus (62).

B cell-mediated responses (62) also codes for an ARF protein thought to function as a repressor of p21 function, similar to its ORF1 counterpart (63). Because p21 as a negative regulator of cell cycle progression inhibits tumor induction, this ARF product may contribute to HCV oncogenesis.

Additional identified viral ARFs are less well characterized immunologically but may also be novel sources of cryptic epitopes. In a subgroup of Theiler's murine encephalomyelitis virus, translation of an ARF produces a protein that appears to increase demyelinating activity (64) and may also play a role in cell tropism, thus restricting infection to cell populations that serve as reservoirs for the virus (65). Variations in the hepatitis B virus genome have revealed cryptic ORFs encoding long ARF sequences associated predominantly with patients demonstrating severe disease, suggesting a role of these sequences in viral pathogenesis (66).

Nonviral ARF proteins can also be critical to pathogenesis. Specifically, ARF self-proteins may supply autoantigens or function as inheritable disease gene products. In autoimmune Reiter's syndrome, an autoreactive CTL clone was stimulated by a cryptic transframe epitope, partially derived from both ORF1 and ORF2 by +1 frameshifting. The cryptic epitope was shown subsequently to be encoded by the cellular IL-10 gene but in a form cross-reactive with an epitope from an IL-10 homolog of EBV (67). The ARF product encoded by the alternative testis transcripts gene in *Drosophila melanogaster* demonstrated sequence homology with the bovine version of a predominant autoantigen in Graves' disease (68), warranting further studies into the translational source of the protein and epitopes responsible for this human disease.

Immunotherapeutic approaches may also engage ARF and other cryptic epitopes, sometimes in unexpected ways. With regard to transplantation of allogeneic immunocompetent tissue, disparity for the minor histocompatibility male-specific (H-Y) Ag can be a significant inducer of graft-vs-host disease. T cell responses specific for a novel H-Y Ag were detected in a male patient with chronic graft-vs-host disease after transplantation of stem cells from a female donor. Notably, the target epitope was translated from an upstream region of the primary ORF of the *TMSB4Y* gene encoding the Y homolog of the thymosin β-4 protein (69).

On the other side of the pathogenesis equation, at least one host mechanism employs an ARF protein to counter disease (70). Beyond a role in stabilizing and stimulating the p53 tumor suppressor (71), p14ARF (p19ARF murine), derived from the *INK4a* gene, may also inhibit tumor cell growth by targeting members of the E2F transcription factor family (72). Mutations affecting p14ARF expression have been associated with several human cancers.

A final note on ARF products and their roles in pathogenesis relates to the meaning of the word "alternative." Particularly when ARF translation is initiated by an AUG (CUG) normal start codon and produces an extended protein shown (or inferred) to (likely) have a biological function, is it meaningful to term this an "alternative" reading frame? Beyond the fact that ARFs are usually described after the original ORF is defined, should the amount of coding capacity, level or efficiency of translation, and/or documentation of function (and if so, as based on in vitro or in vivo activities?) determine whether this is indeed an ARF, and therefore an ARF epitope? These and other specifics emphasize the difficulty in formulating a yes/no definition for ARFs. In this review, we have adopted the "standard," or at least chronologically based, definition—i.e., "alternative" refers to the ORF(s) other than the generally accepted ORF (ORF1) originally defined for a given known gene. While the differences between this and other possible definitions may not be simply semantic, we suggest that the preceding and following discussion is not affected by adoption of this convention. Moreover, the present definition of "ARF" serves to underscore the concepts that such ARFs generally derive from previously unrecognized sequences of coding potential by various cryptic translation mechanisms, as described above, and correspondingly that these reading frames, unexpected at the time of their discovery, may provide new epitopes as targets for immunological intervention.

Vaccine design involving ARF epitopes

Based on the early identification of a *TRP-1/gp75*-encoded ARF epitope as a target of TILs mediating partial regression of lung metastases in a melanoma patient (31–34), alternative ORFs were considered a novel source of additional tumor Ags with potential clinical applications. In another melanoma, the CAMEL protein was defined as the translation product of an alternative ORF in the LAGE-1 gene. Although the first 11 aa of CAMEL appeared to constitute the immunodominant CTL epitope, simultaneous and efficient generation of both regular ORF1 and cryptic ARF products of LAGE-1 were capable of inducing CD8 T cell responses against melanoma tumors examined in vitro (73, 74). Whereas here the ARF epitope was thus a constituent, not the exclusive, target epitope, potent antitumor protection can be achieved in the absence of ORF1 targets, as demonstrated in mice immunized with a mixture of only ARF tumor peptides (75).

The design of an effective cancer vaccine may need to involve several arms of the immune system, including helper T cell function and Ab responses. CD4 T cell clones have been identified with specificity for ARF-derived tumor Ags from the previously described CAMEL protein (76, 77) and from the TARP protein associated with prostate and breast cancers (78). In addition, proteins generated from ARF translation have been implicated in the induction of Ab responses against HCV infection (79) and in lung, prostate, breast, and ovarian cancer patients (80).

Perhaps the most critical feature of a vaccine is the ability to generate memory responses. Memory CD8 T cell responses specific for predicted epitopes derived from the previously described HCV ARF protein were detected in seropositive patients (62), suggesting the possibility that efficient recall responses may generally be a subsequent feature of the induction of primary CTL directed against cryptic reading frame peptides. We have obtained potentially similar findings in the retrovirus-induced MAIDS model, in which adoptively transferred ARF-specific CD8 T cells were able to recall an Ag-specific IFN-γ response beyond 2 mo after virus infection (O. Ho and W. R. Green, unpublished observations).

Concluding remarks

The presence of cryptic translational products across a broad range of disease systems suggests a rethinking of the role these proteins may play in host immunity and disease pathogenesis. Alternative ORF translation as a source of novel peptide epitopes should increase the receptor repertoire available for T

cell recognition of pathogens and tumors. Yet, as with proteins derived from conventional translation, cryptic products may be encoded from inheritable disease genes, generate self-Ags targeted by autoimmune mechanisms, and, more likely than not, as extended proteins, add to the arsenal of mechanisms used by viruses and tumor cells to promote pathogenesis, including potentially immune evasion. Associated with the propensity for viruses and tumors to use alternative translation and multiple, overlapping reading frames to efficiently encode proteins required for their replication and pathogenic function, this approach toward maximizing the informational content of the genome may unwittingly lead to a greater degree of sequence conservation of associated epitopes, both cryptic and conventional (Tables I and II). Thus, the selective pressure to maintain the amino acid coding sequences simultaneously for, say, overlapping viral ORF1 and ORF2 registers to preserve the function of both the ORF1- and ORF2-extended viral proteins would put severe constraints on the ability to tolerate variations in an embedded cryptic ORF2 (or conventional/ORF1) epitope. Such epitopes positioned in an area of overlapping reading frames might make excellent candidates for conserved targets that the virus cannot vary to escape from immune detection. Thus, despite their presumably low abundance, ARF epitopes can be expressed at functional levels in vivo at the priming stage and at the target level without the use of overexpression systems (9), and can provide in vivo targets for protective immune responses (29, 48).

In light of the remarkable impact these proteins and their epitopes may have on immunological and/or virological and tumor functions, better understanding of the mechanisms underlying cryptic translation is needed to elucidate the frequency and efficiency of generating unconventional but biologically relevant gene products. The ability to predict those products of unconventional translation that may ultimately benefit vs hinder the host can facilitate a more rational design of antiviral and tumor vaccines and immunotherapeutic approaches. In this regard, production of epitopes and extended proteins derived from cryptic translation should no longer be viewed as unexpected phenomena but rather normal biological realities, albeit ones which require further study.

References

1. Rammensee, H.-G., T. Friede, and S. Stevanovic. 1995. MHC ligands and peptide motifs: first listing. *Immunogenetics* 41: 178–228.
2. Bhasin, M., H. Singhn, and G. P. S. Raghava. 2003. MHCBN: a comprehensive database of MHC binding and non-binding peptides. *Bioinformatics* 19: 665–666.
3. Yewdell, J. W., L. C. Anton, and J. R. Bennink. 1996. Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules? *J. Immunol.* 157: 1823–1826.
4. Schubert, U., L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell, and J. R. Bennink. 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404: 770–774.
5. Yewdell, J. W. 2005. Serendipity strikes twice: the discovery and rediscovery of defective ribosomal products (DRiPS). *Cell. Mol. Biol.* 51: 635–641.
6. Qian, S.-B., M. F. Princiotta, J. R. Bennink, and J. W. Yewdell. 2006. Characterization of rapidly degraded polypeptides in mammalian cells reveals a novel layer of nascent protein quality control. *J. Biol. Chem.* 281: 392–400.
7. Shastri, N., S. Cardinaud, S. R. Schwab, T. Serwold, and J. Kunisawa. 2005. All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. *Immunol. Rev.* 207: 31–41.
8. Malarkannan, S., M. Afkarian, and N. Shastri. 1995. A rare cryptic translation product is presented by Kb major histocompatibility complex class I molecule to alloreactive T cells. *J. Exp. Med.* 182: 1739–1750.
9. Mayrand, S.-M., D. A. Schwarz, and W. R. Green. 1998. An alternative translational reading frame encodes an immunodominant retroviral CTL determinant expressed by an immunodeficiency-causing retrovirus. *J. Immunol.* 160: 39–50.
10. Sykulev, Y., M. Joo, I. Vturina, T. J. Tsomides, and H. N. Eisen. 1996. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity* 4: 565–571.
11. Mayrand, S.-M., and W. R. Green. 1998. Non-traditionally derived CTL epitopes: exceptions that prove the rules? *Immunol. Today* 19: 551–556.
12. Boon, T., and A. V. Pel. 1989. T cell-recognized antigenic peptides derived from the cellular genome are not protein degradation products but can be generated directly by transcription and translation of short subgenic regions: a hypothesis. *Immunogenetics* 29: 75–79.
13. Lindahl, K. F. 1991. Do we need a pepton hypothesis? *Immunogenetics* 34: 1–4.
14. 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: 303–308.
15. 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: 7976–7980.
16. Uenaka, A., Y. Hirano, H. Hata, S. Win, T. Aji, M. Tanaka, T. Ono, J. C. A. Skipper, K, Shimizu, and E. Nakayama. 2003. Cryptic CTL epitope on a murine sarcoma Meth A generated by exon extension as a novel mechanism. *J. Immunol.* 170: 4862–4868.
17. Weiss, R. B., D. M. Dunn, J. F. Atkins, and R. F. Gesteland. 1987. Slippery runs, shifty stops, backward steps, and forward hops: −2, −1, +1, +2, +5, and +6 ribosomal frameshifting. *Cold Spring Harbor Symp. Quant. Biol.* 52: 687–693.
18. McBratney, S., C. Y. Chen, and P. Sarnow. 1993. Internal initiation of translation. *Curr. Opin. Cell Biol.* 5: 961–965.
19. Macejak, D. G., and P. Sarnow. 1991. Internal initiation of translation mediated by the 5′ leader of a cellular mRNA. *Nature* 353: 90–94.
20. Bullock, T. N., A. E. Patterson, L. L. Franlin, E. Notidis, and L. C. Eisenlohr. 1997. Initiation codon scanthrough versus termination codon readthrough demonstrates strong potential for major histocompatibility complex class I-restricted cryptic epitope expression. *J. Exp. Med.* 186: 1051–1058.
21. Bullock, T. N., 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: 1319–1329.
22. Bruce, A. G., J. F. Atkins, and R. F. Gesteland. 1986. tRNA anticodon replacement experiments show that ribosomal frameshifting can be caused by doublet decoding. *Proc. Natl. Acad. Sci. USA* 83: 5062–5066.
23. Malarkannan, S., T. Horng, P. P. Shih, S. Schwab, and N. Shastri. 1999. Presentation of out-of-frame peptide/MHC class I complexes by a novel translation initiation mechanism. *Immunity* 10: 681–690.
24. Shastri, N., V. Nguyen, and F. Gonzales. 1995. Major histocompatibility class I molecules can present cryptic translation products to T cells. *J. Biol. Chem.* 270: 1088–1091.
25. Hanada, K., J. W. Yewdell, and J. C. Yang. 2004. Immune recognition of a human renal cancer antigen through post-transcriptional protein splicing. *Nature* 427: 252–256.
26. Vigneron, N., V. Stroobant, J. Chapiro, A. Ooms, G. Deviovanni, S. Morel, P. van der Bruggen, T. Boon, and B. J. van den Eynde. 2004. An antigenic peptide produced by peptide splicing in the proteasome. *Science* 304: 587–590.
27. Schwarz, D. A., and W. R. Green. 1994. CTL responses to the gag polyprotein encoded by murine AIDS defective retrovirus are strain dependent. *J. Immunol.* 153: 436–441.
28. Mayrand, S.-M., P. A. Healy, B. E. Torbett, and W. R. Green. 2000. Anti-gag cytolytic T lymphocytes specific for an alternative translational reading frame-derived epitope and resistance versus susceptibility to retrovirus-induced murine AIDS in F_1 mice. *Virology* 272: 438–449.
29. Ho, O., and W. R. Green. 2006. Cytolytic $CD8^+$ T cells directed against a cryptic epitope derived from a retroviral alternative reading frame confer disease protection. *J. Immunol.* 176: 2470–2475.
30. Cardinaud, S., A. Moris, M. Fevrier, P. S. Rohrlich, L. Weiss, P. Langlade-Demoyen, F. A. Lemonnier, O. Schwartz, and A. Habel. 2004. Identification of cryptic MHC I-restricted epitopes encoded by HIV-1 alternative reading frames. *J. Exp. Med.* 199: 1053–1063.
31. Topalian, S. L., D. Solomon, F. P. Avis, A. E. Chang, D. L. Freerksen, W. M. Linehan, M. T. Lotze, C. N. Robertson, C. A. Seipp, P. Simon, et al. 1988. Immunotherapy of patients with advanced cancer using tumor-infiltrating lymphocytes and recombinant interleukin-2: a pilot study. *J. Clin. Oncol.* 6: 839–853.
32. Rosenberg, S. A. 1997. Cancer vaccines based on the identification of genes encoding cancer regression antigens. *Immunol. Today* 18: 175–182.
33. Wang, R. F., and S. A. Rosenberg. 1996. Human tumor antigens recognized by T lymphocytes: implications for cancer therapy. *J. Leukocyte Biol.* 60: 296–309.
34. Wang, R. F., M. R. Parkhurst, Y. Kawakami, P. F. Robbins, and S. A. Rosenberg. 1996. Utilization of an alternative open reading frame of a normal gene in generating a novel cancer antigen. *J. Exp. Med.* 183: 1131–1140.
35. Rosenberg, S. A., P. Tong-On, Y. Li, P. Riley, M. El-Gamil, M. R. Parkhurst, and P. F. Robbins. 2002. Identification of BING-4 cancer antigen translated from an alternative open reading frame of a gene in the extended MHC class II region using lymphocytes from a patient with a durable complete regression following immunotherapy. *J. Immunol.* 168: 2402–2407.
36. Oh, S., M. Terabe, C. D. Pendleton, S. Bhattacharyya, T. K. Bera, M. Epel, Y. Reiter, J. Phillips, W. M. Linehan, C. Kasten-Sportes, et al. 2004. Human CTLs to wild-type and enhanced epitopes of a novel prostate and breast tumor-associated protein, TARP, lyse human breast cancer cells. *Cancer Res.* 64: 2610–2618.
37. Ripberger, E., M. Linnebacher, Y. Schwitalle, J. Gebert, and M. von Knebel Doeberitz. 2003. Identification of an HLA-A0201-restricted CTL epitope generated by a tumor-specific frameshift mutation in a coding microsatellite of the OGT gene. *J. Clin. Immunol.* 23: 415–423.

38. Ronsin, C., V. Chung-Scott, I. Poullion, N. Aknouche, C. Gaudin, and F. Triebel. 1999. 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. *J. Immunol.* 163: 483–490.
39. Huang, J., M. El-Gamil, M. E. Dudley, Y. F. Li, S. A. Rosenberg, and P. F. Robbins. 2004. T cells associated with tumor regression recognize frameshifted products of the CDKN2A tumor suppressor gene locus and a mutated HLA class I gene product. *J. Immunol.* 172: 6057–6064.
40. Namy, O., J. P. Rousset, S. Napthine, and I. Brierly. 2004. Reprogrammed genetic decoding in cellular gene expression. *Mol. Cell* 13: 157–168.
41. Alam, S. L., J. F. Atkins, and R. F. Gesteland. 1999. Programmed ribosomal frameshifting: much ado about knotting! *Proc. Natl. Acad. Sci. USA* 96: 14177–14179.
42. Baranov, P. V., R. F. Gesteland, and J. F. Atkins. 2002. Recoding: translational bifurcations in gene expression. *Gene* 286: 187–201.
43. Hatfield, D., and S. Oroszian. 1990. The where, what, and how of ribosomal frameshifting in retroviral protein synthesis. *Trends Biochem. Sci.* 15: 186–190.
44. Rodriguez, I., P. Garcia, and J. E. Suarez. 2005. A second case of −1 ribosomal frameshifting affecting a major virion protein of the *Lactobacillus* bacteriophage A2. *J. Bacteriol.* 187: 8201–8204.
45. Baranov, P. V., R. F. Gesteland, and J. F. Atkins. 2002. Release factor 2 frameshifting sites in different bacteria. *EMBO J.* 3: 373–377.
46. Shigemoto, K., J. Brennan, E. Walls, C. J. Watson, D. Stott, P. W. J. Rigby, and A. D. Reith. 2001. Identification and characterization of a developmentally regulated mammalian gene that utilizes −1 programmed ribosomal frameshifting. *Nucleic Acids Res.* 29: 4079–4088.
47. 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: 51–60.
48. Zook, M. B., M. T. Howard, G. Sinnathamby, J. F. Atkins, and L. C. Eisenlohr. 2006. Epitopes derived by incidental translational frameshifting give rise to a protective CTL response. *J. Immunol.* 176: 6928–6934.
49. Hwang, C. B., B. Horsburgh, E. Pelosi, S. Roberts, P. Digard, and D. M. Coen. 1994. A net +1 frameshift permits synthesis of thymidine kinase from a drug-resistant herpes simplex virus mutant. *Proc. Natl. Acad. Sci. USA* 91: 5461–5465.
50. Horsburgh, B. C., H. Kollmus, H. Hauser, and D. M. Coen. 1996. Translational recoding induced by G-rich mRNA sequences that form unusual structures. *Cell* 86: 949–959.
51. Griffiths, A., S. H. Chen, B. C. Horsburgh, and D. M. Coen. 2003. Translational compensation of a frameshift mutation affecting herpes simplex virus thymidine kinase is sufficient to permit reactivation from latency. *J. Virol.* 77: 4703–4709.
52. Griffiths, A., M. A. Link, C. L. Furness, and D. M. Coen. 2006. Low-level expression and reversion both contribute to reactivation of herpes simplex virus drug-resistant mutants with mutations on homopolymeric sequences in thymidine kinase. *J. Virol.* 80: 6568–6574.
53. Hammell, A. B., R. C. Taylor, S. W. Peltz, and J. D. Dinman. 1999. Identification of putative programmed −1 ribosomal frameshift signals in large DNA databases. *Genome Res.* 9: 417–427.
54. Gallant, J. A., and D. Lindsley. 1998. Ribosomes can slide over and beyond "hungry" codons, resuming protein chain elongation many nucleotides downstream. *Proc. Natl. Acad. Sci. USA* 95: 13771–13776.
55. Gramstat, A., D. Prufer, and W. Rohde. 1994. The nucleic acid-binding zinc finger protein of potato virus M is translated by internal initiation as well as by ribosomal frameshifting involving a shifty stop codon and a novel mechanism of P-site slippage. *Nucleic Acids Res.* 22: 3911–3917.
56. Farabaugh, P. J., H. Zhao, and A. Vimaladithan. 1993. A novel programmed frameshift expresses the POL3 gene of retrotransposon Ty3 of yeast: frameshifting without tRNA slippage. *Cell* 74: 93–103.
57. Schwab, S. R., K. C. Li, C. Nang, and N. Shastri. 2003. Constitutive display of cryptic translation products by MHC class I molecules. *Science* 301: 1367–1371.
58. Probst-Kepper, M., V. Stroobant, R. Kridel, B. Gaugler, C. Landry, F. Brasseur, J. P. Cosyns, B. Weynand, T. Boon, and B. J. van deyn Eynde. 2001. An alternative open reading frame of the human macrophage colony-stimulating factor gene is independently translated and codes for an antigenic peptide of 14 amino acids recognized by tumor-infiltrating CD8 T lymphocytes. *J. Exp. Med.* 193: 1189–1198.
59. Gaur, A., and W. R. Green. 2005. Role of a cytotoxic T lymphocyte epitope-defined, alternative gag open reading frame in the pathogenesis of a murine retrovirus-induced immunodeficiency syndrome. *J. Virol.* 79: 4308–4315.
60. Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, et al. 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nat. Med.* 7: 1306–1312.
61. Chen, W., J. R. Bennink, and J. W. Yewdell. 2003. Systematic search fails to detect immunogenic MHC class I-restricted determinants encoded by influenza A virus noncoding sequences. *Virology* 305: 50–54.
62. Bain, C., P. Parroche, J. P. Lavergne, B. Duverger, C. Vieux, V. Dubois, F. Komurian-Pradel, C. Trepo, L. Gebuhrer, G. Paranhos-Baccala, et al. 2004. Memory T cell-mediated immune responses specific to an alternative core protein in hepatitis C virus protein. *J. Virol.* 78: 10460–10469.
63. Basu, A., R. Steele, R. Ray, and R. B. Ray. 2004. Functional properties of a 16 kDa protein translated from an alternative open reading frame of the core-encoding genomic region of hepatitis C virus. *J. Gen. Virol.* 85: 2299–2306.
64. Chen, H. H., W. P. Kong, L. Zhang, P. L. Ward, and R. P. Roos. 1995. A picornaviral protein synthesized out of frame with the polyprotein plays a key role in a virus-induced immune-mediated demyelinating disease. *Nat. Med.* 1: 927–931.
65. Yamasaki, K., C. C. Weihl, and R. P. Roos. 1999. Alternative translation initiation of Theiler's murine encephalomyelitis virus. *J. Virol.* 73: 8519–8526.
66. Faure, E. 2006. Alternative peptide-fusion proteins generated by out-of-frame mutations, just upstream ORFs or elongations in mutants of human hepatitis B viruses. *Virus Res.* 117: 185–201.
67. Saulquin, X., E. Scotet, L. Trautmann, M. A. Peyrat, F. Halary, M. Bonneville, and E. Houssaint. 2002. +1 Frameshifting as a novel mechanism to generate a cryptic cytotoxic T lymphocyte epitope derived from human interleukin 10. *J. Exp. Med.* 195: 353–358.
68. Madigan, S. J., P. Edeen, J. Esnayra, and M. McKeown. 1996. *att*, a target for regulation by *tra2* in the testes of *Drosophila melanogaster*, encodes alternative RNAs and alternative proteins. *Mol. Cell. Biol.* 16: 4222–4230.
69. Torikai, H., Y. Akatsuka, M. Miyazaki, E. H. Warren III, T. Oba, K. Tsujimura, K. Motoyoski, Y. Morishima, Y. Kodera, K. Kuzushima, and T. Takahashi. 2004. A novel HLA-A*3303-restricted minor histocompatibility antigen encoded by an unconventional open reading frame of human TMSB4Y gene. *J. Immunol.* 173: 7046–7054.
70. Quelle, D. E., F. Zindy, R. A. Ashmun, and C. J. Scherr. 1995. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 83: 993–1000.
71. Llanos, S., P. A. Clark, J. Rowe, and G. Peters. 2001. Stabilization of p53 by $p14^{ARF}$ without relocation of MDM2 to the nucleolus. *Nat. Cell Biol.* 3: 445–452.
72. Martelli, F., T. Hamilton, D. P. Silver, N. E. Sharpless, N. Bardeesy, M. Rokas, R. A. DePinho, D. M. Livingston, and S. R. Grossman. 2001. p19ARF targets certain E2F species for degradation. *Proc. Natl. Acad. Sci. USA* 98: 4455–4460.
73. Aarnoudse, C. A., P. B. van den Doel, B. Heemskerk, and P. I. Schrier. 1999. Interleukin-2-induced, melanoma-specific T cells recognize CAMEL, and unexpected translation product of *LAGE-1*. *Int. J. Cancer* 82: 442–448.
74. Rimoldi, D., V. Rubio-Godoy, V. Dutoit, D. Lienard, S. Salvi, P. Guillaume, D. Speiser, E. Stocker, G. Spagnoli, C. Servis, et al. 2000. Efficient simultaneous presentation of NY-ESO-1/LAGE-1 primary and nonprimary open reading frame-derived CTL epitopes in melanoma. *J. Immunol.* 165: 7253–7261.
75. Graddis, T. J., M. L. Diegel, C. J. McMahan, L. Tsavler, R. Laus, and D. Vidovic. 2004. Tumor immunotherapy with alternative reading frame peptide antigens. *Immunobiology* 209: 535–544.
76. Slager, E. H., C. E. van der Minne, M. Kruse, D. D. Krueger, M. Griffioen, and S. Osanto. 2004. Identification of multiple HLA-DR-restricted epitopes of the tumor-associated antigen CAMEL by $CD4^+$ Th1/Th2 lymphocytes. *J. Immunol.* 172: 5095–5102.
77. Slager, E. H., M. Borghi, C. E. van der Minne, C. A. Aarnoudse, M. J. E. Havenga, P. I. Schrier, S. Osanto, and M. Griffioen. 2003. $CD4^+$ Th2 cell recognition of HLA-DR-restricted epitopes derived from CAMEL: a tumor antigen translated in an alternative open reading frame. *J. Immunol.* 170: 1490–1497.
78. Kobayashi, H., T. Nagato, K. Oikawa, K. Sato, S. Kimura, N. Aoki, R. Omiya, M. Tateno, and E. Celis. 2005. Recognition of prostate and breast tumor cells by helper T lymphocytes specific for a prostate and breast tumor-associated antigen, TARP. *Clin. Cancer Res.* 11: 3869–3878.
79. Walewski, J. L., T. R. Keller, D. D. Stump, and A. D. Branch. 2001. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. *RNA* 7: 710–721.
80. Mollick, J. A., F. S. Hodi, R. J. Soiffer, L. M. Nadler, and G. Dranoff. 2003. MUC1-like tandem repeat proteins are broadly immunogenic in cancer patients. *Cancer Immun.* 3: 3–17.