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*J Immunol* 2006; 176:6928-6934; doi: 10.4049/jimmunol.176.11.6928
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Epitopes Derived by Incidental Translational Frameshifting Give Rise to a Protective CTL Response

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Aberrant gene expression can be caused by several different mechanisms at the transcriptional, RNA processing, and translational level. Although most of the resulting proteins may have no significant biological function, they can be meaningful for the immune system, which is sensitive to extremely low levels of Ag. We have tested this possibility by investigating the ability of CD8+ T cells (T_{CD8+}) to respond to an epitope whose expression results from incidental ribosomal frameshifting at a sequence element within the HSV thymidine kinase gene. This element, with no apparent functional significance, has been identified due to its ability to facilitate escape from the antiviral compound acyclovir. Using a recombinant vaccinia virus expression system, we find that in vitro and in vivo T_{CD8+} responses to the frameshift-dependent epitope are easily discernible. Furthermore, the in vivo response is at a sufficient level to mediate protection from a tumor challenge. Thus, the targets of immune responses to infectious agents can extend beyond the products of conventional open reading frames. On a per-cell basis, responses to such minimally expressed epitopes may be exceedingly effective due to the selective expansion of high avidity T_{CD8+}.


The CD8+ T lymphocyte (T_{CD8+}) is activated through recognition of Ag-derived peptides (epitopes), generally 8–11 aa in length, displayed at the surface of the Ag-bearing cell in complex with MHC class I molecules (1, 2). This population serves to limit the spread of intracellular parasites, either by outright killing of the infected cell, or by release of factors that halt or limit replication (3, 4). A striking property of T_{CD8+} is their exquisite sensitivity to very low levels of Ag. T_{CD8+} can be triggered by tens to hundreds of copies of epitope at the cell surface, which can be derived from quantities of precursor protein that are undetectable by standard biochemical techniques (5, 6).

Although some proteins are naturally expressed at very low levels, other trace species result from errors in gene expression during transcription, splicing, and translation. A number of variations on conventional translation are relevant in this regard. These variants include initiation at non-AUG codons (7–9), scanning over the primary AUG with initiation at an internal AUG (10, 11), and ribosomal frameshifting, in which the ribosome shifts into a new reading frame in midtranslation (12, 13). This last mechanism may have particularly rich potential for the generation of “cryptic” epitopes because, in contrast to the former two, it is not limited to early phases of translation.

Translational frameshifting can be separated into two general categories. Programmed alternative translation (recoding) is orchestrated by cis-acting signals in mRNA and occurs at high levels (14, 15). The most common form of recoding, programmed −1 frameshifting occurs at a specific location within the overlap of two open reading frames, the trans-frame products are generally functional proteins, and this mechanism is commonly associated with viruses that infect mammalian cells. A well-known example occurs during translation of the HIV gag-pol overlap. Frameshifting occurs at a shift prone site, a series of consecutive bases that allow for slippage of the A- and P-site tRNAs relative to the mRNA (16). High-level frameshifting is achieved by the presence of a downstream stimulatory RNA structure (17–19). Although the exact mechanism of frameshift stimulation is unknown (20, 21), it is generally believed that the RNA structure causes the translocating ribosome to pause, allowing for slippage of the tRNAs relative to the mRNA. Programmed ribosomal frameshifting at the HIV gag-pol shift site occurs at a frequency of ~5–10%, determining the ratio of the Gag to Gag-Pol polyprotein, which is critical for viral propagation (22–24).

The second category of ribosomal frameshifting is incidental, directed by sites in coding sequences that fortuitously bear base elements of highly evolved programmed recoding sites, and induce frameshifting at much lower frequencies. These include homopolymeric runs of nucleotides (slippery sites) and sequences that can stall the ribosome, due to slowly decoded codons in the A-site (25–28). A particularly interesting example provides the means for HSV escape from acyclovir treatment (29, 30). The thymidine kinase (TK) gene contains a run of seven consecutive guanines that allows for ~1% of translating ribosomes to shift into the +1 reading frame, negligibly reducing the yield of functional TK. In contrast, the escape mutant contains eight consecutive G’s (31) effectively disrupting the reading frame beyond the point of nucleotide insertion. Faithful translation of the mutant results in functionless TK.

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Received for publication December 8, 2004. Accepted for publication February 24, 2006.

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However, 1% of the time, the correct reading frame is regained during translation via the +1 frameshift event and functional TK is produced. This level of expression is sufficient for viral propagation but insufficient for developing toxic levels of acyclovir monophosphate, the conversion product (30).

In this study we asked whether incidental ribosomal frameshifting, as occurs during translation of the tk gene and ostensibly during the course of most, if not all, viral infections, can reveal “out-of-frame” epitopes at a level that is functionally meaningful. Using a recombinant vaccinia virus (vacc) expression system, several in vitro assays for epitope expression, as well as a tumor challenge model, we found that this is indeed the case.

Materials and Methods

Mice, cell lines, and chemicals

Six- to 8-wk-old female C3FeB6F1/J, B6C3F1, or C57BL/6 mice were purchased from Taconic Farms or The Jackson Laboratory and maintained in the Thomas Jefferson University Animal Facilities (Philadelphia, PA) according to Institutional Animal Care and Use Committee guidelines.

The following cell lines were maintained in DMEM (Cellgro; Fisher Scientific) supplemented with 5% FCS, 4% dialyzed fetal bovine serum (preserved in D2000; Amersham Biosciences) and 1% antibiotic-antimycotic (Gibco; Life Technologies). L1210 (HLA-1, DBA/2 strain) and P388 (HLA-2, C57BL/6 strain) were maintained in RPMI 1640 (Cellgro; Fisher Scientific) supplemented with 10% FCS and 50 µg/ml penicillin/streptomycin/ampicillin.

Construction of the NP/SIINFEKL gene has been described elsewhere (31). All recombinant viruses were made and plaque purified by the Department of Infectious Diseases, Washington University School of Medicine, St. Louis, MO.

Assays for epitope expression based upon use of the B3Z or DBFZ hybridomas have been previously described (32). Briefly, 5 × 10⁴ L-Kb or L-Db cells were infected in triplicate with 5 FFU/cell recombinant vac for 2 h in 250 µl of BSA supplemented PBS in the presence of 250 µg/ml arabinofuranosylcytosine. Cells were washed with PBS and cocultured in 96-well flat-bottom plates with 5 × 10⁴ B3Z or DBFZ cells in complete media supplemented with 250 µg/ml arabinofuranosylcytosine overnight. T cell activation was assessed by addition of the fluorogenic substrate 4-methylumbelliferyl β-d-galactoside (4-MUG) at 33 µg/ml. To compare results across several independent experiments, the values obtained with the frameshift constructs were normalized to those of the controls using the following formula for normalized value: (sample – Neg)/(Pos – Neg) × 100.

ELISPOT

Mice were immunized i.p. injection with 1 × 10⁶ PFU of recombinant vac diluted to 250 µl with BSA supplemented PBS. Fourteen days postinfection spleens were harvested, RBC lysed, and plated at 2.5 × 10⁶, 5 × 10⁶, or 1 × 10⁷ cells per well in 50 µl of RPMI 1640 containing 10% FCS and 50 µg/ml 2-ME in 96-well ELISPOT plates (Millipore) coated with 5 µg/ml anti IFN-γ Ab (51-2525KZ; BD Pharmingen). Specific activation was determined by addition of 2.5 × 10⁵ L-Kb or L-Db cells either untreated, pulsed with 10⁻⁶ M NP₅₀₋₅₇, SIINFEKL, or NP₃₆₆₋₃₇₄ synthetic peptide, or infected with nonrecombinant (wild-type, Cr-19) vac. Plates were incubated at 37°C for 18 h, washed two times with PBS and three times with wash buffer (PBS with 0.5% Nonidet P-40) and incubated with 5 µg/ml biotinylated anti IFN-γ Ab (51-1818KA; BD Pharmingen) for 2 h at room temperature. After washing, 10 µg/ml avidin-HRP (551950; BD Pharmingen) was added and incubated at room temperature for 1 h. Plates were washed first with wash buffer followed by PBS and spots visualized by addition of substrate solution (551951; BD Pharmingen). Spots were counted using Image-Pro software (Media Cybernetics), and values were obtained by subtracting background (i.e., unpulsed) from treated cells. Due to this manipulation, the SIINFEKL and 366–374 response in the negative control became negative because the values were actually less than the background.

To control for differences in priming between individual animals, individual SIINFEKL-specific responses were normalized to the NP₅₀₋₅₇ response as described earlier (36). The average NP₅₀₋₅₇ value for each experiment was determined. The fold difference of individual NP₅₀₋₅₇ responses from the average NP₅₀₋₅₇ response was calculated using the formula [(average NP₅₀₋₅₇ response – individual NP₅₀₋₅₇ response)/(individual NP₅₀₋₅₇ response)]. The fold difference was then multiplied by the appropriate SIINFEKL-specific response to obtain a normalized value.

Tumor challenge

Six-week-old female C57BL/6 mice were primed i.p. with 1 × 10⁷ PFU of recombinant vacs. Ten days later, they were anesthetized and shaved on the right flank. A total of 3 × 10⁵ E.G7 or EL-4 cells maintained at 5 × 10⁵/ml were washed three times with PBS and resuspended to 3 × 10⁵/ml. They were then injected s.c. in 100 µl of total volume PBS. Tumor volume was measured every other day beginning when tumor growth became visible by measuring the diameter in three dimensions using a vernier caliper.

Results

Generation of constructs

In broad outline, our strategy was to place a frameshifting element between sequences encoding two MHC class I-restricted epitopes. Translation of the upstream epitope is not impacted by the insertion and can be used to normalize expression of the downstream epitope, which requires ribosomal frameshifting at the element to be expressed. As indicated in Fig. 1, our base construct for these studies was the influenza virus NP, which contains three well-described epitopes at residues 50–57 (H2-Kk-restricted, NP₅₀₋₅₇ (37)), 147–155 (H2-Kk-restricted, NP₁₄₇₋₁₅₅ (38)), and 366–374 (H2-Dₑ-restricted, NP₃₆₆₋₃₇₄ (39)). A unique SplI site between the NP₁₄₇₋₁₅₅ and NP₃₆₆₋₃₇₄ epitopes served as an insertion point for
two different frameshifting elements, that of the HSV tk gene (both 7 and 8 consecutive G versions) and that at the HIV gag-pol interface. As mentioned, the gag-pol element directs recoding at a frequency of 5–10% and was included to contrast epitope production driven by the low level (≤1%) incidental frameshifting of the TK element. For the TK element, we used a version of NP (NP/SIINFEKL) that contains the widely used OVA-derived epitope \( \text{SIINFEKL} \) that contains the widely used OVA-derived epitope immediately upstream of the 366–374 epitope. Frameshift constructs were created by cloning sequence corresponding to the HSV TK frameshift site and surrounding sequence into the SpII site of the parent gene in such a way that a translational shift into the \(-1 \) or \(+1 \) reading frame, respectively, was necessary for production of the downstream epitopes. For HSV, the shift sites are underlined and the nucleotide additions are in bold. For HIV, the shift site is underlined.

FIGURE 1. Generation of viral frameshift (FS) constructs. All constructs were based upon the Influenza PR/8 NP containing endogenous MHC class I epitopes 50–57 (K\(^k\)-restricted), 147–155 (K\(^d\)-restricted), and 366–374 (D\(^d\)-restricted). For the HSV series of constructs, the 257–264 epitope from OVA (SIINFEKL, K\(^d\)-restricted) was inserted immediately upstream of the 366–374 epitope. Frameshift constructs were created by cloning sequence corresponding to the HSV TK frameshift site and surrounding sequence into the SpII site of the parent gene in such a way that a translational shift into the \(-1 \) or \(+1 \) reading frame, respectively, was necessary for production of the downstream epitopes. For HSV, the shift sites are underlined and the nucleotide additions are in bold. For HIV, the shift site is underlined.

For direct assessment of frameshifting mediated by the HIV and TK elements in the NP-based system, L929 cells expressing the transfected H2-K\(^d\) gene (L-K\(^d\)) were infected with the recombinant vaccinia panel, radiosabeled with \(^35\)S, and immunoprecipitated with mAbs specific for the N terminus of NP. As seen in Fig. 2A, bands corresponding to full-length 56-kD protein products are visible in lanes from both the HIV positive and HSV TK positive controls. Additionally, 38-kD bands are present in samples from frameshift constructs (HIV FS, HSV TK 7G, HSV TK 8G, Fig. 2A) as well as the negative controls (HIV Neg, HSV TK Neg, Fig. 2A), due to a termination codon in the \(-1 \) reading frame that is encountered by nonframeshifting ribosomes after traversing the point of insertion. A faint band with the same mobility as the full-length protein is present in the lane from the HIV construct (HIV FS, Fig. 2A) resulting from the well-characterized \(-1 \) frameshift event. Scanning densitometry of multiple samples established the predicted ratio for frameshifting at viral sequences.

Production of full-length protein as a result of ribosomal frameshifting at viral sequences. A, L-K\(^d\) cells infected with 5 PFU/cell of the indicated vac were labeled with \(^35\)S, and NP (HIV) or NP/SIINFEKL (NPS) TK protein immunoprecipitated. Protein species were resolved via PAGE and visualized by autoradiography with care being taken to avoid background bands migrating closely with the full-length protein product during analysis. B, L-K\(^d\) cells were infected with 5 PFU/cell of the indicated vac, and surface K\(^d\)-SIINFEKL expression quantified using mAb 26.D1.16. Quantification was achieved using calibration beads containing a known density of fluorescent probe. Background fluorescence obtained with the TK negative control sample was subtracted from the experimental samples, with absolute values representing from 1 to 22% increase over the negative control. Values represent the average of two independent experiments.
the frequency of frameshifting to be 5–16%, which is in good agreement with previous results (16). In the lanes corresponding to the HSV TK constructs (TK 8G and TK 7G, Fig. 2A), the level of full-length product resulting from +1 frameshifting was much lower, with a trace band being only sporadically appreciable. Thus, with the constructs supporting low-level frameshifting, the amount of protein available for production of the NP366–374 and OVA257–264 epitopes is at the limit of detection by immunoprecipitation.

One of the useful OVA257–264-specific reagents is a mAb, 26.D1.16, which is “TCR-like” in having specificity for the OVA257–264/Kb complex (42). L-Kb cells, capable of OVA257–264/Kb presentation, were infected with equal amounts of the TK series of recombinant vacs. MHC class I ligand cell surface density was determined by incubating samples in culture supernatant containing the 25.D1.16 Ab followed by anti-mouse, FITC-labeled IgG and analyzed by flow cytometry. Through the use of standard microbeads loaded with a known concentration of FITC, we were able to quantify the absolute number of complexes present. As seen in Fig. 2B (TK 8G and TK 7G), both HSV constructs produce a signal that is significantly above background. Importantly, this result suggests that frameshifting occurs during translation of wild-type TK, as has been reported previously (31). Thus, despite the full-length protein being barely detectable by immunoprecipitation, frameshifting occurs at a level that provides appreciable cell surface epitope as determined with this immune-based approach.

**Viral frameshift-derived epitopes stimulate T cell activation in vitro**

Detection of presented epitope by the 26.D1.16 Ab is highly sensitive and may, in fact, surpass the sensitivity of T cells in vitro and/or in vivo. As a first step in assessing the relevance of the TK frameshifting element for T cell activation, we determined the ability of the vac panel members to activate NP366–374/Dp– and OVA257–264/Kb–specific T cell hybridomas that produce β-galactosidase upon activation (43, 44). L929 cells transfected with the H2-Dp–encoding gene (L-Dp) were infected with the HIV recombinant vac panel and cocultured with the DBFZ T cell hybridoma (OVA257–264/Kb–specific), and activation was assessed using soluble 4-MUG. Results shown in Fig. 3 demonstrate that frameshifting at both the HIV and HSV sites produces sufficient epitope for hybridoma stimulation. For the HIV element, the level of activation resulting from the HIV frameshift construct ranged from 55 to 66% of that of the positive control over four independent experiments (HIV FS, Fig. 3). This finding was not especially surprising given the estimated frameshift frequency of 5–10%. For the HSV constructs (TK 8G and TK 7G, Fig. 3), the level of activation ranged from 10 to 26 and 13 to 22%, respectively, over four experiments. Thus, in an in vitro setting, even low-level frameshifting induced by the TK element results in clearly appreciable T cell responses as determined by a T cell hybridoma.

**In vivo response to frameshift-directed epitopes**

One of the main reasons for using the vac expression system is the documented ability of this virus to elicit significant TCD8+ responses both in vitro and in vivo. Thus, the same constructs used in the in vitro assays we have described can be used for assessing TCD8+ activation in vivo. H2-Kb/H2-Db F1 mice were primed with individual members of the HIV recombinant vac panel. Two weeks later, spleen cells were assessed by ELISPOT analysis for the presence of NP50–57 or NP366–374–specific population, which would be detectable only by expansion of the exceedingly low number of epitope-specific precursors. Because NP50–57 precedes the frameshift element, responses to this epitope should be roughly equivalent in all cases if the immunizations were equivalent, whereas responses to NP366–374 reflect frameshifting in vivo. As expected, the CD8+ T cell responses to target cells pulsed with NP50–57 peptide are relatively uniform among all constructs tested (Fig. 4A). In contrast, the NP366–374 response to peptide-pulsed targets is proportional to the expected frameshift frequency with negative values representing a response that was below the background for that experiment. Fig. 4B shows the normalized NP366–374–specific response, which corrects for the minor variations in priming as determined by the differences in the NP50–57 responses (36). The anti-NP366–374 response associated with the HIV frameshift construct remains significantly higher than the response to the negative control construct after this transposition. Again, this result is not surprising given the level with which the HIV element mediates frameshifting.

A similar approach was taken to assess the impact of the HSV TK element in vivo except that the resultant TCD8+ responses to the OVA257–264 epitope were measured instead of NP366–374. As with the HIV panel, responses to NP50–57 are essentially uniform among all constructs (Fig. 4C) with the normalized SIINFEKL–specific response shown for correction (Fig. 4D). Notably, both HSV constructs (TK 8G and TK 7G, Fig. 4C) are associated with a significant response to the OVA257–264 epitope. These results show that epitope production via incidental frameshifting during the translation of viral transcripts can induce a detectable TCD8+ response.

**Protective effect of TCD8+ stimulated by frameshift-derived epitopes**

Having demonstrated in vivo expansion of TCD8+ specific for an epitope expressed as a result of incidental frameshifting, we sought to determine whether this expanded population has any functional significance. Toward this end, we determined whether the frameshifting-dependent CD8+ population also protects against a tumor challenge. Mice were primed as described for the ELISPOT assay, and then challenged s.c. at 10 days post-vac injection with EL-4 tumor cells (naturally expressing the Kb class I molecule) or E.G7 tumor cells (EL-4 cells stably transfected to express OVA). As seen in Fig. 5A, tumor burden was significantly delayed in mice primed with either HSV construct (TK Pos or TK 8G) compared with growth in mice primed with the HSV negative construct (TK Neg, Fig. 5A). As expected, no difference was noted in mice challenged with the parental tumor line (Fig. 5B).
Discussion

We have demonstrated that the incidental ribosomal frameshifting directed by a fortuitous frameshift element within the tk gene is significant with respect to CD8+ T cell responses. Not only are such responses readily detectable both in vitro and in vivo, they also afford protection against a tumor challenge.

These findings should be distinguished from publications that have described the production of “neo-epitopes” as a result of frameshift mutations (45–47). Those cases involve nucleotide insertion/deletion, leading to an actual change in the reading frame. In our case, unconventional translation (i.e., ribosomal frameshifting) by the ribosome is responsible for the shift to an alternative reading frame. Ribosomal frameshifting within the human IL10 gene has been previously shown to produce a cryptic epitope as read out by T cells that had been extensively expanded in vitro following isolation from Reiters syndrome patients (12). Indeed, in vitro systems have elucidated many different mechanisms of aberrant gene expression that lead to cryptic epitope production. These include splicing errors (48, 49), translation initiation at a non-AUG (50), translation initiation at an internal AUG (10), and ribosomal frameshifting (12). Yet, in few sites has significance in vivo been demonstrated. In a report by Schwab et al. (51), a cryptic epitope produced from a non-AUG initiation site was expressed from a murine transgene. Spleen cells from the transgenic mouse were observed to elicit a T_{CD8+} response to the epitope upon transfer to nontransgenic counterparts, and the transgenic mice were shown to be tolerant to the epitope. Similarly, Wang et al. (52) showed that tumor infiltrating lymphocytes from patients with melanoma recognized an Ag produced from an alternative reading frame of the gp75 protein. In this case, the mechanism was felt to be initiated at an internal start codon. In a murine viral system, Mayrand and colleagues (53, 54) previously also showed that internal initiation was potentially capable of producing a cryptic epitope that was the target of a CD8 T cell response. Finally, a recent report by Cardinaud et al. (55) has shown that several cryptic epitopes are produced during the course of HIV replication and are capable of eliciting a T cell response. In these cases, insertion of a premature stop codon in the alternative reading frame abrogated epitope expression; however, the exact mechanism of epitope production remains to be elucidated. The work we have reported in this study demonstrates protection from tumor challenge as a result of ribosomal frameshifting, a process that we consider to have substantial potential for the production of cryptic epitopes.

Ribosomal frameshifting can ostensibly occur at any point during translation because sequences that induce frameshifting are not restricted to any region of an open reading frame. In contrast, aberrant initiation of translation is confined to upstream regions of the open reading frame because ribosomes lose the ability to initiate translation soon after engaging mRNA, presumably due to dissociation of initiation factors from the translation complex. Thus, ribosomal frameshifting would appear to be a more potent mechanism for the generation of cryptic epitopes. However, several other parameters, many poorly defined, need to be considered. First is the relative frequency with which these distinct events occur. Initiation at conventional start codons is influenced by surrounding sequence in predictable ways (11). The same rules appear to apply for internal initiation at conventional start codons (6). It is not yet known whether non-AUG initiation is similarly restricted and, therefore, how promiscuous this event is (8). Algorithms for predicting sites that stimulate ribosomal frameshifting are at early stages of development and so the general frequency of low-level frameshifting is also not known, especially in mammals. Interestingly, although some sequences, such as homopolymeric runs of nucleotides, appear straightforward, there are many sequences that induce frameshifting in ways that are not immediately obvious. Indeed, a recent search of the yeast genome for under-represented sequences that might undergo translational frameshifting identified a subset of sequences, some of which do not resemble known frameshift motifs, which are prone to nonprogrammed error frameshifting (56). Identified in this group were heptamers that may direct frameshift levels of between 0.5 and 8% with no obvious gene expression function. Likewise, an analysis of Escherichia coli coding sequences found known error prone sequences, which can promote higher than 1% frameshifting errors in coding sequences (57). Although these sites were under-represented in highly expressed genes, they were readily identified in many other genes. The unexpected conclusion is that error frameshifting, at least in some cases, is not sufficiently detrimental to trigger strong negative selection pressure. Consequently, error frameshifting at significant levels may be more widespread than is readily appreciated.

Also important to consider are the levels of expression that lead to meaningful T cell activation and how frequently they are exceeded for various aberrant species. The critical level will vary for each epitope due to factors such as affinity for the restricting class I molecule and efficiency of processing. With respect to this latter
it is worth a mention that some products of aberrant proteasomal degradation (58) a consequence that certainly de-
sequestered in aggregates that are resistant to the results reported suggest that simply counting by tetramer staining,
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INCIDENTAL RIBOSOME FRAMESHIFTING IN PROTECTIVE CD8 RESPONSE


