Ribosomal Scanning Past the Primary Initiation Codon as a Mechanism for Expression of CTL Epitopes Encoded in Alternative Reading Frames

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Summary

An increasing amount of evidence has shown that epitopes restricted to MHC class I molecules and recognized by CTL need not be encoded in a primary open reading frame (ORF). Such epitopes have been demonstrated after stop codons, in alternative reading frames (RF) and within introns. We have used a series of frameshifts (FS) introduced into the Influenza A/PR/8/34 nucleoprotein (NP) gene to confirm the previous in vitro observations of cryptic epitope expression, and show that they are sufficiently expressed to prime immune responses in vivo. This presentation is not due to sub-dominant epitopes, transcription from cryptic promoters beyond the point of the FS, or internal initiation of translation. By introducing additional mutations to the construct exhibiting the most potent presentation, we have identified initiation codon readthrough (termed scanthrough here, where the scanning ribosome bypasses the conventional initiation codon, initiating translation further downstream) as the likely mechanism of epitope production. Further mutational analysis demonstrated that, while it should operate during the expression of wild-type (WT) protein, scanthrough does not provide a major source of processing substrate in our system. These findings suggest (*i*) that the full array of self- and pathogen-derived epitopes available during thymic selection and infection has not been fully appreciated and (*ii*) that cryptic epitope expression should be considered when the specificity of a CTL response cannot be identified or in therapeutic situations when conventional CTL targets are limited, as may be the case with latent viral infections and transformed cells. Finally, initiation codon readthrough provides a plausible explanation for the presentation of exocytic proteins by MHC class I molecules.

CD8$^+$cytotoxic lymphocytes (CTL) recognize and respond to the products of protein degradation (epitopes) associated with MHC class I molecules at the cell surface (1–5). It has been generally assumed that degradation of full-length protein supplies the majority of epitope for antigen presentation. However, there are many instances reported in the literature describing presentation from gene fragments and mini-genes that encode only the epitope (6–8), implying that full-length protein is not a requirement for efficient antigen presentation to CTL. More surprisingly, evidence exists that questions the basic assumption that epitopes must to be encoded in primary open reading frames. Examples include the presentation of epitopes that follow stop codons (9), are encoded in alternate translation reading frames (10–13), are presented in the absence of any obvious initiation codons (14) or are even processed and presented in the apparent absence of a related promoter (15). Explanations that have been proposed to account for these unusual circumstances include ribosomal frameshifting (11, 13), alternative translation initiation codons (14) and the possibility of subgenic transcription events (the pepton hypothesis; 16). However, in most cases the underlying mechanism(s) have not been elucidated.

Until recently, cryptic epitope expression had remained an in vitro phenomenon. The immunological significance of unconventionally produced epitopes has now been demonstrated. An immunogenic human melanoma epitope has been mapped to a region of a novel gene encoded partially by exon 2 and the following intron (17), implying that the epitope is expressed only when imprecise splicing events occur. The mutation that elicits the CTL response is encoded in the intron, outside of the primary open reading frame (ORF)[1]. Wang et al. have mapped responses of a melanoma-specific CTL line to a peptide encoded within an alternate reading frame of the gp75 gene (12). An earlier study by Uenaka et al. (18), in which a murine leukemia-specific epitope was mapped to the 5′ untranslated region of the gene, suggests that alternative translation mechanism may also provide cryptic epitopes in vivo. In a more con-

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[1] *Abbreviations used in this paper:* BFA, brefeldin A; FS, frameshift; NP, nucleoprotein; ORF, open reading frame; RF, reading frame; UTR, untranslated region; vac, vaccinia virus; WT, wild-type.

trolled system, Elliott et al. have demonstrated the ability to prime mice to an epitope shifted to an alternate reading frame by a single base deletion (13).

The potential for unconventional epitopes to be encoded in alternative reading frames (RF) and untranslated regions (UTR) requires that the whole mRNA, not just the primary RF, be considered for examination when determining possible substrates for efficient antigen processing and presentation. In addition to providing a new array of epitopes, these mechanisms could provide a source of conventional RF substrate that is, by virtue of its abbreviated form, more efficiently processed and presented than full-length proteins. For both in-frame and out-of-frame epitopes this may be more common than generally imagined. Additionally, epitopes in alternate reading frames may be important contributors to immune responses in vivo, involved in the activation of autoimmune disorders and of benefit in targeting CTL responses in cancer immunotherapy.

Several potential explanations for the production of cryptic epitopes are available for consideration, including many at the level of translation. The inherent flexibility of the scanning and translating ribosome has produced variants of conventional translation (19) that could provide a basis for cryptic epitope expression, including: (*i*) initiation codon scanthrough (where the scanning ribosome bypasses the 5′ AUG and initiates at an AUG further downstream; references 20–22); (*ii*) reinitiation of translation (where a ribosome that has terminated translation does not dissociate from the message and begins translation at a downstream AUG codon (22–24); (*iii*) ribosomal frameshifting (the scanning ribosome changes RFs in mid-translation, a process greatly enhanced by the presence of a "slippery site", upon which the ribosome shifts and a "pseudo-knot", which causes the ribosome to pause over the slippery site (25, 26); (*iv*) translation termination readthrough (the context of the stop codon is insufficient to cause 100% of translating ribosomes to terminate; references 27, 28); and (*v*) internal initiation of translation (an internal ribosome entry site provides a "landing pad" for ribosomes to initiate translation from internal AUGs; references 29–31).

To elucidate the mechanism and gauge the potential of cryptic epitope production, we have imposed a series of controlled genetic changes upon the influenza A PR/8/34 nucleoprotein (NP) gene, predicted to prevent the translation of some or all of three well-defined and broadly spaced MHC class I–restricted epitopes. These manipulations not only confirmed the possibility of cryptic epitope expression, but further demonstrated in vitro and in vivo that cryptic translation is a physiologically relevant and an immunologically important mechanism for supplying substrate to the MHC class I antigen processing and presentation pathway.

Materials and Methods

Chemicals. General chemical supplies were obtained from Sigma Chem. Co. (St. Louis, MO). Molecular biology reagents were obtained from New England Biolabs (Beverly, MA), except where noted.

Animals. 6-8-wk-old female inbred CBA (H2-k), BALB/c (H2-d) and C57Bl/6 (H2-b) strain mice were obtained from Taconic (Albany, NY) or Jackson Labs (Bar Harbor, ME) and maintained in Thomas Jefferson University Animal Facilities.

Tissue Culture. L929 (H-2K^{k}), L-Kd (H2-K^{k}, H2-K^{d}; L929 transfected with H2-K^{d}; reference 32), and MC57G (H2-D^{b}) cells were grown in DMEM supplemented with 5% FCS (Sigma), 37°C/ 9% CO$_2$.

Molecular Manipulations. All enzymes used for the manipulation of the NP gene were used according to manufacturers directions. PCR primers and oligonucleotide linkers were synthesized by Kimmel Cancer Institute Nucleic Acid Facility. NP containing a silent ApaI site in the H2-K^{d} (bp457-463) epitope has been described elsewhere (8). PCR directed mutagenesis was used to incorporate a silent AatII site in the H2-K^{k} epitope (bp165-171): GAG GGA CGT CTG ATC. These altered sites were then used in additional cloning of the NP gene. Most manipulations were done using a Bluescript II SK +/− vector (Stratagene Inc., La Jolla) with NP inserted in the EcoRI site into the multiple cloning site. A similar strategy was used to insert an NheI site into NP$_{366-374}$.

Frameshifts. Frameshift A (FS-A) was created by encoding a dropped base 5(C) and a SalI site in a 5′ primer annealing to the 5′ end of NP (TCG CAG TCG ACA TCA AAA TCA TGG GTC CCA A). A 3′ reverse primer encompassing the AatII site at bp165-171 allowed the production of a fragment for ligation, after digesting with SalI and AatII, into NP-Bluescript cut with SalI and AatII. This was then shuttled into pSC11 (8) via the SalI and NotI restriction sites for vaccinia (vac) recombination. FSs B through E were created by cutting NP with AatII, NarI, ApaI, or AvrII restriction enzymes, respectively. The resulting digests were then incubated with DNA polymerase Large Fragment (Klenow fragment) and then religated (T4 DNA ligase; GIBCO BRL, Gaithersburg, MD). Ligation reactions were used to transform competent DH5α *E. coli*. Transformants were screened for loss of the restriction site and then sequenced to confirm the mutation. Mutant NP fragments were then cloned into pSC11.

Substitutions. Due to the location of the ATG codons that we wished to ablate, two-step primer directed PCR mutagenesis was used. A PCR primer pair encoding an ATG to ATC substitution and the appropriate surrounding sequence were used in independent reactions with the 5′ primer pairing with an NP-specific 3′ primer, and the 3′ primer pairing with an NP-specific 5′ primer. FS-A DNA was used as the template. The products of the independent PCRs were gel purified to remove any surplus primers. The PCR products were then mixed and allowed to anneal and extend in the absence of primers for three rounds of a PCR. The flanking distal primers used in the first reaction were then added for the remaining cycles. The resultant extended PCR, encoding the mutations, was ethanol precipitated and subject to restriction digestion for ligation into FS-A.

Epitope Knockouts. PCR primers encoding substitutions of the anchor residues were used to direct elimination of the H2-K^{k}, H2-K^{d}, and H2-D^{b} epitopes. TTG GAT CAG ACG TCC CTC ATA AGT ACT encodes a D51T substitution and a silent ScaI reporter site. AAC AAG GCC CCT CGT ACG CTG TGC AGT TGC encodes a Y148A and a silent BsiW1 reporter site. These two oligos were used for 3′ priming PCRs with 5′ primers spanning the SalI site at the front of the gene. GTT CAA ATT GCT AGC AAT GAA GCT ATG encodes a 5′ primer with a N370A substitution and a silent NheI site. This was used for a PCR reaction, paired with a vector derived 3′ primer, to create a fragment from the NheI site to the NotI site at the end of the gene.

NP/M321 Mutant. Complementary oligonucleotides directing an ATG to ATC mutation (GTC GGA AAA ATC ATT GGT and ACC AAT GAT TTT TCC GAC) were used in a two-step PCR reaction, in combination with primers annealing to the region encompasing the SalI site preceding the gene and the engineered ApaI site within the NP/147-155 epitope. The PCR product was trimmed with SalI and ApaI and then used to replace the wild-type sequence between SalI and ApaI.

Thermostable Duplex Barrier. The first stage of the barrier was created using a 5′ PCR primer encoding the left hand side of the barrier, the KpnI loop and an XbaI cloning site. This was used in conjunction with a SalI containing 3′ primer, that anneals to the front of the NP gene, to produce a fragment that inserted between the XbaI and SalI sites in NP-Bluescript. The second stage 5′ primer encoded the KpnI loop at the 5′ end, the right hand side of the barrier and a 3′ SalI site. In conjunction with a 3′ primer that anneals to internal NP sequence, a PCR fragment was produced that formed the duplex when inserted at the KpnI site of the first fragment (using an internal NP AatII site as the other restriction point). The completed duplex barrier-NP fragment was cloned into pSC11 by digestion at the XhoI and NotI restriction sites, taking advantage of the cohesive end compatibility between SalI and XhoI. Primer sequences were (*i*) first stage: ACG CTC GAG GGG GCG CGT GGT GGC GGGGTA CCA CGC GTC GAC GGT ATC GCG ATA AG and (*ii*) second stage: CGG GGT ACCCCG CCA CCA CGC GCC CCG CTC GAC CAC CAT GGT GTC.

Immunoprecipitations. 1 × 10^6 L-Kd cells were infected at 10 PFU/cell. After 6 h incubation at 37°C in DMEM with 40 μM LLnL proteasome inhibitor (a kind gift from Dr. Ken Rock, Dana-Farber Cancer Institute), the cells were starved for 30 min with Met/Cys-free DMEM (Biofluids Inc., Rockville, MD), followed by a 45-min pulse with 60 μCi ^{35}S Met-Cys/10^6 cells (Amersham Corp., Arlington Heights, IL). Cells were lysed in the presence of 2 mM PMSF (Sigma) and an inhibitor cocktail consisting of 0.2 mM AEBSF, 1 mM EDTA, 20 μM Leupeptin and 1 mM Pepstatin (Calbiochem-Novabiochem, San Diego, CA) and nuclei removed by pelleting at 12,000 g. The resulting supernatants were precleared for 12 h at 4°C with protein A–Sepharose beads, then incubated with rotation for 3 h at 4°C with protein A–Sepharose beads to which the NP-specific monoclonal antibodies H19S24 (a gift from Dr. W. Gerhard, Wistar Institute, Philadelphia, PA) and HB65 (American Type Culture Collection [ATCC], Rockville, MA) had been bound. The beads were then washed four times and boiled in reducing buffer before separation on a 10% SDS-PAGE.

Viruses. Vaccinia recombinants were made as previously described (8). Briefly all altered genes were ligated between the SalI and NotI sites in modified pSC11 for expression from the $P_{7.5}$ promoter. These plasmids were then introduced into the vaccinia genome via homologous recombination in CV-1 cells (CCL 70; ATTC) and plaque purified in 143B HuTK$^-$ (CRL 8303; ATTC) cells in the presence of BrdU (Boehringer Mannheim, Indianapolis, IN). In all cases the integrity of the recombinant was determined by isolating vac DNA and sequencing after PCR amplification of the mutant gene. Additionally, viral expression products were analyzed by northern blotting to confirm mRNA size. Control vac used in the experiments were generated by recombining the pSC11 plasmid with no insert into the vac genome.

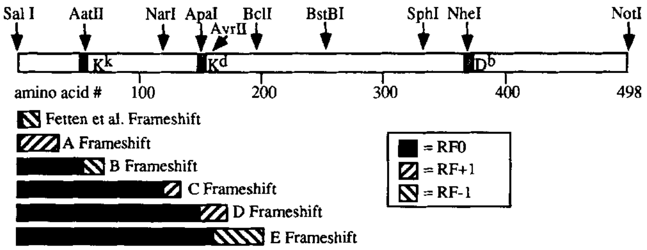
CTL Assay. APC were infected for 1 h at 37°C with vaccinia recombinants at 10 PFU/cell at a concentration of 10^7 cells/ml in balanced salt solution containing 0.1% BSA. After 1 h, 10 ml of preconditioned (37°C, 9% CO_2) DMEM + 5% FCS was added and the cells incubated a further 3 h with rotation. Cells were pelleted and resuspended with 50 μl/ 10^6 cells of IMDM with 7.5% FCS containing 100 μCi of $Na^{51}CrO_4$ (Amersham Corp.) and incubated for 1 h at 37°C. APC were then washed three times with DPBS and resuspended in IMDM and combined with CTL populations in round-bottom plates at 10^4 cells/well. APCs and CTL were coincubated for 4 h at 37°C before 100 μl of supernatants were collected and counted in a gamma detector (Wallac, Turku, Finland). The data are presented as percent specific ^{51}Cr, defined as 100 × ([experimental cpm − spontaneous cpm]/[total cpm − spontaneous cpm]). In some assays Brefeldin A (BFA) was added to a final concentration of 5 μg/ml and maintained at that concentration until harvest of supernatants.

Generation of CTL. CTL restricted to H2-K^k, H2-K^d, or H2-D^b were derived from NP immunized CBA, BALB/c or C57BL/6 mice, respectively, as described elsewhere (33). Mice were immunized by intraperitoneal injection of 10^7 PFU of NP-vac. After at least 2 wk, spleens from appropriate mice were harvested and one-third of cells infected with PR8 for restimulation. Secondary cultures were incubated at 37°C/9% CO_2 for 6–7 d before harvesting for effector populations. For in vivo priming assays, duplicate mice were injected intraperitoneal with 4 × 10^6 PFU of each recombinant vac and left for 2 wk before splenocytes were restimulated in vitro with PR8 or wild-type vaccinia virus.

Results

Construction of Out-of-Frame Epitopes in Influenza Nucleoprotein. To pursue studies on the contribution of epitopes encoded in alternate reading frames to MHC class I–mediated immune responses, we introduced a series of FSs (depicted in Fig. 1) into the NP gene. NP contains three well-defined MHC class I epitopes, which encompass amino acids 50-57 (NP_{50-57}: H2-K^k restricted), 147-155 ($NP_{147-155}$: H2-K^d restricted), and 366-374 ($NP_{366-374}$: H2-D^b restricted) (34–37). The FS series is predicted to have diverse effects on the production of the three epitopes. For example, frameshift A (FS-A) is a shift into the +1 RF (where the primary ORF is defined as RF0) at the second codon. This is predicted to cause termination at codon 16, which would lead to no epitope production by conventional translation. Even if all termination codons in the +1 RF were to be ignored by the translating ribosome, translation of the regions encoding the three epitopes will produce nothing resembling NP_{50-57}, $NP_{147-155}$, or $NP_{366-374}$, as shown in Fig. 1. The frameshifted mutant NP genes were recombined into the vaccinia virus (vac) genome and the resultant viruses were checked for fidelity by PCR amplification and sequencing of the altered gene. Immunofluoresence staining of cells infected with the vac recombinants, using an antibody that precipitates NP_{1-158} (HB-65), yielded no specific signal and initial NP-immunoprecipitations from cells infected with the vac recombinants, using monoclonal antibodies having complementary specificities, did not yield any specific bands (data not shown).

Presentation of Out-of-frame Epitopes In Vitro. Several studies have demonstrated expression of epitopes despite their



	RF0	+1 RF	-1 RF
NP 50-57	SDYEGRLI	*L*GTVDP	VIMRDG*S
NP 147-155	TYQRTRALV	LSEDKSSCS	LIRGQELLF
NP 366-374	ASNENMETM	FQ*KYGDYG	LPMKIWRLW

Figure 1. Positioning of the NP frameshift mutations and the predicted impact upon NP translation with respect to three MHC class I epitopes. These frameshifts have the effect of moving the NP_{50-57}, $NP_{147-155}$, and $NP_{366-374}$ epitopes into alternate reading frames. The nature of the frameshift, their position and their predicted translation termination points are shown. The predicted effect on translation in the region where each epitope sequence is encoded (if the upstream termination codon is ignored) is depicted.

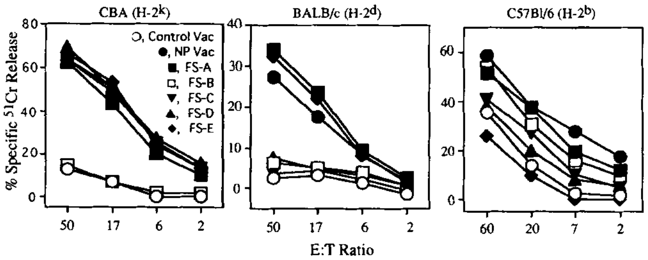


Figure 2. In vitro presentation of NP frameshift recombinants. APC expressing $H2-K^k$ (L-Kd), $H2-K^d$ (L-Kd), or $H2-D^b$ (MC57G) were infected for 4 h with vaccinia recombinants before ^{51}Cr labeling and incubation with NP-specific CTL generated by priming with NP vac and restimulating in vitro with PR8 as described in Materials and Methods. NP-Vac and FS-A are presented to all haplotypes, WT control vac is presented on none and FS-B, FS-C , FS-D, and FS-E are presented at different degrees to each haplotype. Vac restimulated splenocytes were used to confirm equal infection of target APCs (data not shown).

having been shifted out of the conventional RF (10, 11, 13, 14). To determine whether this was true for our constructs using the vac expression system, each was tested for presentation of all three NP epitopes. Cells expressing the $H2-K^k$, $H2-K^d$, or $H2-D^b$ MHC class I molecules were infected with the vaccinia recombinants and incubated with secondary culture CTL derived from CBA ($H-2^k$), BALB/c ($H-2^d$), and C57Bl/6 ($H-2^b$) mice immunized to NP. Because the location of the FSs and the consequential effect on NP translation, presentation would be predicted to differ for each construct. FS-A: (a shift into the +1 RF at codon 2, predicted to terminate at codon 33) is not predicted to present any of the epitopes, although a previous study with a similar mutant (10) showed retained presentation of NP_{50-57} and $NP_{147-155}$. Our results confirm and extend those findings, in that all three epitopes are presented in the face of FS-A. Frameshift B (FS-B: a shift to the −1 frame at codon 55 (actually within the NP_{50-57} epitope), predicted to terminate at codon 70) is also predicted to prevent presentation of all three epitopes. When assayed, presentation of NP_{50-57} and $NP_{147-155}$ are indeed ablated, while $NP_{366-374}$ is surprisingly still capable of sensitizing target cells to CTL-mediated lysis. Frameshift C (FS-C: a shift to the +1 frame at codon 121, terminating at codon 151) maintains presentation of NP_{50-57} (as expected since the FS occurs after this epitope) but is unable to present $NP_{147-155}$. In contrast, $NP_{366-374}$ is presented when expressed from this mutant, though at much reduced levels in comparison to FS-A and FS-B. Frameshift D (FS-D: a shift to the +1 frame at codon 151, terminating at codon 164) presents partially as predicted, with NP_{50-57} retaining presentation, $NP_{147-155}$ not presented (as the FS is within this epitope) and $NP_{366-374}$ presented slightly above control vac. This very low level of presentation was consistently observed in many independent assays. Frameshift E (FS-E: directs a shift to the −1 frame at codon 175, terminating at codon 201) is the only construct that performs completely as predicted, maintaining presentation of NP_{50-57} and $NP_{147-155}$, while losing any presentation of $NP_{366-374}$ (Fig. 2). It is especially noteworthy that presentation of the $H2-D^b$ epitope ($NP_{366-374}$) is proportional to the distance of the FS event from the 5′ end of the gene. This conforms to Kozak's predictions for ribosomal reinitiation potential, in that the further the termination event is from the 5′ cap, the less likely reinitiation is to occur (38).

In Vivo Presentation of Cryptic Epitopes. Aside from a recent publication (13), demonstration of alternative RF cryptic epitope presentation has been restricted to in vitro observations. Having established in our system that mechanisms exist that can overcome the effect of the FSs and enable translation at levels that are sufficient to sensitize the infected presenting cells in vitro, we wished to assess the ability of these epitopes to be presented in vivo. C57Bl/6 ($H-2^b$) mice were inoculated with recombinant vac expressing FS-A, FS-B, FS-C, FS-E, NP-vac, or control vac. After 2 wk, their spleens were used to make in vitro secondary cultures by restimulation with PR8. To confirm equal priming, a fraction of the splenocytes was restimulated with wild-type (WT) vac (Fig. 3, sub-graphs). Each of the CTL populations was tested in standard chromium release assays for specific lysis of cells infected with WT NP, presenting the $NP_{366-374}$ epitope. The data in Fig. 3 show that, in addition to CTL from NP-primed mice, CTL derived from mice primed with FS-A and FS-B were capable of specifically lysing cells expressing WT NP. In contrast, FS-C and -E did not produce any significant priming. Therefore it is likely that the same mechanisms that are responsible for the production of cryptic epitopes in this scenario are available to the immune system during natural immune responses. It should be noted, however, that cryptic epitope expression in our system appears to be stronger when analyzed in vitro, compared to in vivo. Only FS-A and FS-B constructs were capable of priming responses to NP in vivo (with FS-B priming much poorer), while FS-A to FS-D were all detected in vitro.

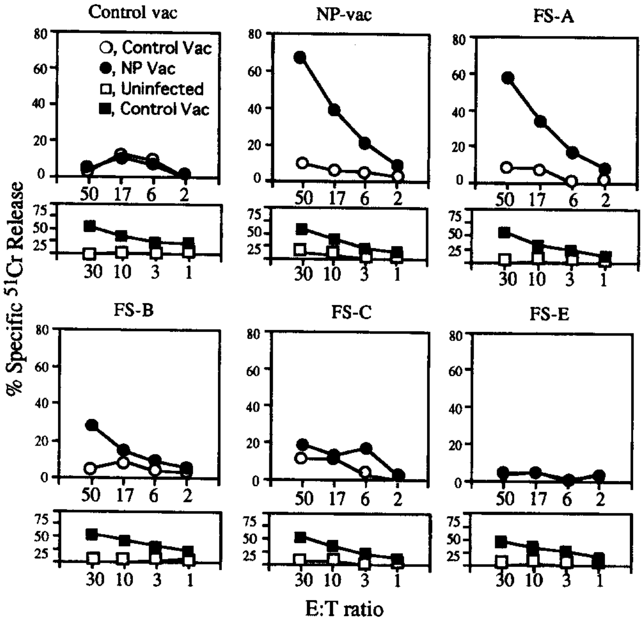


Figure 3. In vivo immunogenicity of alternative RF epitopes. Duplicate C57Bl/6 mice were inoculated with recombinant vaccinia expressing FS-A, FS-B, FS-C, or FS-E, control vac or NP-vac. Splenocytes derived from these mice were restimulated with PR8 (for NP$_{366-374}$-specific CTL) or WT vac (for vac-specific controls). After 6 d cultures were tested for the ability to lyse APCs infected with NP-vac, control vac or left uninfected. All constructs were capable of eliciting vac-specific responses but only FS-A and FS-B elicited significant NP-specific responsed.

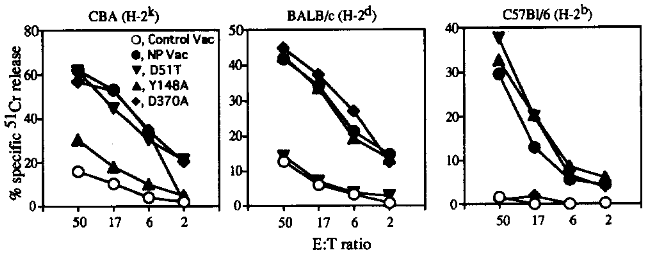


Figure 4. Subdominant epitopes do not account for the vast majority of presentation from the frameshifted constructs. Anchor residues from each of the three epitopes, NP$_{50-57}$ (D51T), NP$_{147-155}$ (Y148A), and NP$_{366-374}$ (D370A) were mutated to ablate MHC class I binding, thus allowing any subdominant epitopes to be presented. Vac recombinants were assayed in standard ^{51}Cr release assays with respect to control-vac and NP-Vac.

In Vitro Cryptic Epitope Presentation Is Not Due to Subdominant Epitopes. To address the argument that antigen presentation observed from the expression of these constructs is due to subdominant epitopes, we created a series of constructs that should eliminate presentation of the described epitopes. This is of particular concern for the NP$_{366-374}$ epitope as an observation by Oukka et al. (39) showed that an additional H2-D^{b} epitope exists at NP$_{55-63}$. In their system this epitope was shown to be produced at levels that are toleragenic, although this might not be true in a recombinant vac expression system. Three separate NP mutants were constructed in which one of the dominant MHC class I binding anchor residues was substituted with a non-anchor amino acid for each of three epitopes: the substitutions were $D_{51}\rightarrow T_{51}$ (D51T, H2-K^{k}), $Y_{148}\rightarrow A_{148}$ (Y148A, H2-K^{d}) and $D_{370}\rightarrow A_{370}$ (D370A, H2-D^{b}), respectively, changes that are predicted to abrogate the binding of the epitope to the restriction element. (40). Fig. 4 shows that eliminating the respective anchor residue of NP$_{147-155}$ and NP$_{366-374}$ ablates presentation, while presentation of NP$_{50-57}$ is severely curtailed. In each case, expression and presentation of the unmutated epitopes were not affected. The in vivo response by H-2b mice to FS-A could also be ascribed to recognition of NP$_{366-374}$; spleen cells from C57Bl/6 mice primed with FS-A and restimulated with PR8 did not respond to the D370A mutant (data not shown). These data imply that the presentation that is observed with the frameshifted constructs is due to the translation of the dominant epitopes 50-57 (H2-K^{k}), 147-155 (H2-K^{d}) and 366-374 (H2-D^{b}), although some NP-specific presentation from the D51T mutant is occurring. This may be due either to the expression of a very minor sub-dominant epitope or some persistent binding and CTL antigenicity on the part of the mutated epitope. Pertaining to this, we have noticed in many of our studies that NP$_{50-57}$ is a highly immunogenic epitope, capable of eliciting very strong immune responses (Bullock, T.N.J., and L.C. Eisenlohr, unpublished observations).

In Vitro Presentation of Out-of-Frame Epitopes Is Not Due to Cryptic Promoter Activity or Internal Initiation of Translation. Several alternative explanations, aside from cryptic translation, could account for the presentation of the out-of-frame epitopes from cells infected with the vac recombinants. The expression of out-of-frame epitopes that we have ob-

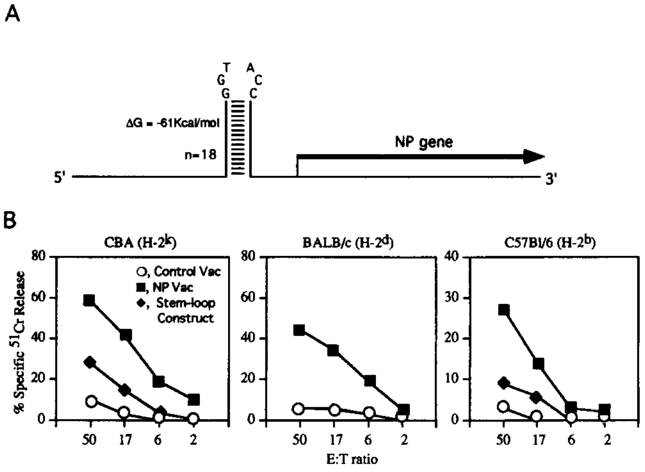


Figure 5. Limited influence of cryptic vac promoters and sites for internal initiation of translation in expression of the NP gene. A thermostable duplex barrier was inserted in front of the NP gene to block the progress of scanning ribosomes (*A*). Such a barrier would be circumvented by vac promoters that exist in the NP gene. When assayed to test for the ability to sensitize APCs for CTL activity (*B*), presentation of all three epitopes from the stem-loop containing gene was severely reduced in comparison to NP-vac, though not quite to the level of control vac for NP$_{50-57}$ and NP$_{366-374}$. Vac-specific killing was unaffected (data not shown).

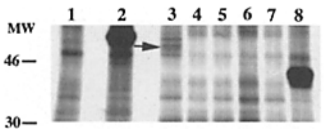


Figure 6. Immunoprecipitation of frameshifted mutants of NP expressed from vaccinia recombinants. Cells were infected with the indicated vac recombinants for 6 h at 37°C in the presence of the proteasome inhibitor LLnL and were pulse labeled for 45 min with ^{35}S Met/Cys. Cells were lysed, pre-cleared and immunoprecipitations were carried out using NP-specific antibodies in the presence of protease inhibitors. Lane *1*, control vac; lane *2*, NP-vac; lanes *3*–*7*, FS-A to -E; lane *8*, $NP_{147-498}$. Arrow denotes the NP-specific FS-A product.

served could have been due to cryptic vac promoters within the NP gene. These could be located downstream of the FS mutations and allow conventional translation of the primary ORF from RF 0. This is a potential explanation for the behavior of FS-B and FS-C (lack of presentation of $NP_{147-155}$ but presentation of $NP_{366-374}$). To address this possibility, we inserted a thermostable duplex barrier between the P7.5 vac promoter and codon 1 of NP (Fig. 5 *a*). Such a barrier is predicted to block the progression of a ribosome scanning from the 5′ end of the NP gene, as previously demonstrated by Kozak using a similar structure (20). Indeed we were not able to immunoprecipitate any specific product expressed from this construct (data not shown). If presentation is dependent upon the ribosome scanning unimpeded from the 5′-cap to AUG_1, then expression of the three epitopes in NP would be ablated. However, if cryptic vac promoters exist within the NP gene, the barrier would not be incorporated into the mRNA and translation of the epitopes should be unhindered. The data shown in Fig. 5 *b* demonstrate that there is a drastic reduction of presentation of all three epitopes. The remaining presentation seen for NP_{50-57} and $NP_{366-374}$ could be due to some cryptic vac promoter activity at a level that can be detected by CTL. However, the graded presentation of $NP_{366-374}$ with respect to the FS series argues against this possibility. The retained presentation may in fact be due to a biochemically undetectable amount of leakiness in the duplex barrier. Certainly, the level of epitope expressed from this construct cannot account for the majority of NP_{50-57} and $NP_{366-374}$ presentation seen with FS-A and FS-B. As more fully discussed below, we believe that $NP_{147-155}$ is not an efficiently presented epitope. This is a feasible explanation for these results and others (Figs. 2 and 7) in which presentation of NP_{50-57} and $NP_{366-374}$ persists while that of $NP_{147-155}$ is lost. This result also makes it very unlikely that internal initiation of translation is responsible for significant levels of cryptic epitope production, as this mechanism would also allow bypass of the thermostable duplex and translation from an internal entry site.

Immunoprecipitation of Frameshifted Constructs. Results described thus far suggested that bypass of the frameshift mutations occurs in our system at the level of translation. Initial screens using a cocktail of NP-specific mAbs only immunoprecipitated NP from NP-vac-infected cells (data not shown). However, the products from cryptic translation are likely to be produced at low levels and may be inherently unstable. Therefore we repeated the screens in the presence of the proteasome inhibitor LLnL and the infected, pulsed cells were lysed in the presence of a protease inhibitor cocktail consisting of AEBSF (serine proteases), EDTA (metalloproteases), leupeptin (broad range), and pepstatin (acid proteases). As can be seen from Fig. 6, the antibody cocktail was capable of immunoprecipitating NP (lane *2*) and a fragment consisting of $NP_{147-498}$ (lane *8*). Under these conditions a band with molecular weight slightly smaller than NP was precipitated from FS-A-infected cells (lane *3*). No specific bands were precipitated from lysates of cells infected with FS-B, FS-C, FS-D, or FS-E.

Ribosomal Scanthrough to the AUG_{32} Codon Appears to Be the Mechanism Responsible for Presentation of FS-A. The immunoprecipitable smaller band from FS-A suggested initiation at an internal AUG, either via reinitiation (after encountering a termination codon, in our case codon 33, the ribosome then proceeds to translate from a downstream AUG) or scanthrough (the ribosome bypasses AUG_1 in preference of an alternate downstream AUG, e.g., codon 13, 32, or 43). The shorter band also argues against an early natural frameshifting event by the ribosome as the product of this mechanism should be full-length. To discern between these possibilities, we eliminated two AUG start codons from FS-A in separate constructs (Fig. 7 *a*). This was accomplished by primer directed mutagenesis, substituting ATG to ATC at either codon 13 or codon 32 within FS-A, affecting a methionine to isoleucine substitution (termed

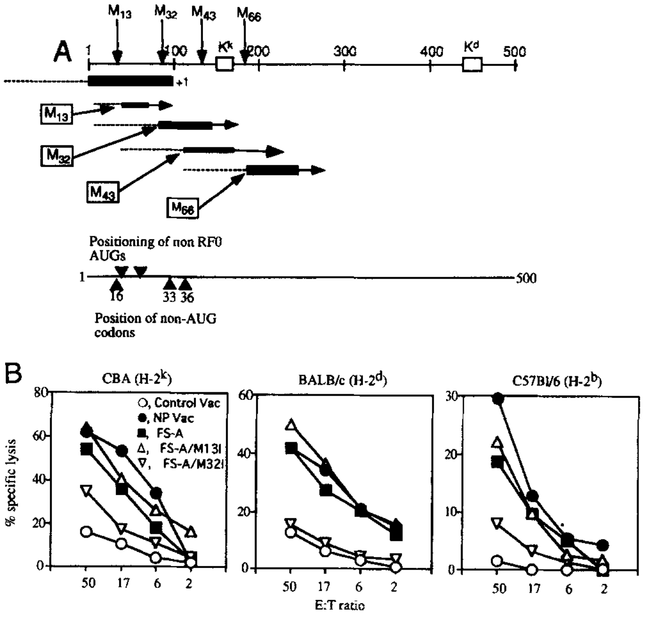


Figure 7. Effect of AUG to AUC substitutions on presentation of FS-A. (*A*) Positioning of potential conventional initiation (AUG) codons at the 5′ end of FS-A. To account for the method of out of frame epitope production from FS-A, AUG_{13} and AUG_{32} were serially disrupted by substitution, thus making them unavailable for translation initiation. (*B*) M131 and M321 mutations were introduced into FS-A and recombined into vaccinia. The recombinants were assayed in standard ^{51}Cr release assays with respect to WT-vac, NP-vac and FS-A, with NP-specific bulk splenocyte secondary cultures.

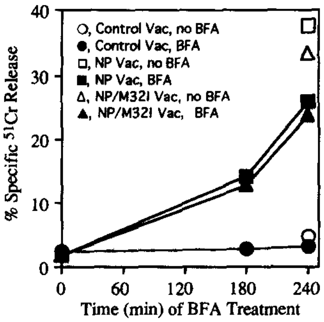


Figure 8. Scanthrough to AUG$_{32}$ does not provide a major source of processing substrate during the expression of WT NP. The M$_{32}$ codon in the WT NP gene was mutated from ATG to ATC. P815 target cells were infected with a vac recombinant expressing the NP/M32I mutant, control vac, or NP vac. Target cells were left untreated or exposed to BFA at various times post infection and then maintained on BFA for the remainder of the assay. 5 h post-infection target cells were co-incubated with NP-specific CTL. Values shown are for an effector/target ratio of 65.

FS-A/M13I, or FS-A/M32I, respectively). When assayed in standard chromium release assays, presentation of FS-A was little affected by the M$_{13}$I mutation (Fig. 7 *b*). However, the FS-A/M32I recombinant had virtually no ability to sensitize cells for CTL lysis. The severity of this single mutation on presentation was quite surprising given the persistence of presentation in the face of the FS mutations which might be considered to be much more severe manipulations of the gene. As with our other recombinants in this work, we have sequenced the relevant region of the vac genome and can find no changes other than those intended. This implies that AUG$_{32}$ is intrinsically important in the translation of epitope for antigen presentation of FS-A. As there is no translation termination codon in the −1 reading frame (adopted by the ribosome after the FS) until codon 33, this also suggests that ribosomal scanthrough, and not reinitiation, is responsible for the production of out-of-frame epitopes from FS-A.

Initiation at AUG$_{32}$ Does Not Provide a Major Source of Substrate in the Processing of Wild-type NP. An attractive hypothesis proposed in several different forms is that products of aberrant gene expression are processed with particular efficiency due to their immediate targeting for degradation. With this concept in mind we tested the possibility that initiation at M$_{32}$ is an important event even in the processing of wild-type NP. We employed site-directed mutagenesis to alter the thirty second codon of wild-type NP from ATG to ATC, effecting a M→I change. The mutant appeared to be identical to WT NP in terms of stability and nuclear localization (data not shown). NP/M32I was also indistinguishable from wild-type NP in the presentation of NP$_{147-155}$, the epitope that should be most sensitive to any compromise in the level of processing substrate (Fig. 8). In this assay, target cells were pulsed with control virus, NP vac, and NP/M32I vac and then exposed to brefeldin A (BFA) at various points post infection for the remainder of the assay. BFA blocks transport of proteins to the cell surface and can therefore be used as an indirect measure of the relative efficiency with which class I–peptide complexes are formed (41). Under these conditions, in which time points associated with sub-maximal presentation were clearly employed, the kinetics with which the epitope was formed from the wild-type and mutant proteins are essentially identical. Thus, scan-through to AUG$_{32}$ does not appear to provide a significant source of processing substrate during the expression of wild-type NP. This result was supported by performing of a similarly structured assay in which the kinetics of NP$_{366-374}$ expression from wild-type and FS-A were compared (not shown). The amount of NP$_{366-374}$ produced by infection with FS-A was significantly lower than that produced as a result of infection with WT NP vac.

Discussion

In this manuscript we provide evidence confirming and extending previous observations by others concerning the processing and presentation of cryptic epitopes (9–14, 42). Our in vitro presentation data concurs with the results of Fetten et al. and provides the additional support of a third nucleoprotein epitope, NP$_{366-374}$. In comparison to all but one preceding study (13), we have used the recombinant vac expression system. For purposes of this study, this strategy offers several advantages over other expression systems. First, vac has a cytoplasmic replication cycle and therefore uncontrolled genomic integration and NP mRNA splicing are not an issue. Second, vac infects a wide range of cells which allows us to study the presentation of multiple, differentially restricted epitopes. Vac is also efficient at priming immune responses, permitting us to assess the antigenicity of cryptic epitopes in vivo (43, 44).

The study of cryptic epitope expression has provided a forum for the debate as to whether CTL are sensitive to very low levels of antigen production. It has been proposed that antigen needs to be well expressed (detectable by conventional biochemistry) to elicit a CTL response (10), while others have suggested that CTL respond to vanishingly small amounts of epitope (14). Our results are more in agreement with the latter position. Immunoprecipitable quantities of FS-A were only achieved in the presence of proteasomal and protease inhibitors and we were not able to immunoprecipitate any product from FS-B infected cells (Fig. 6). Despite this, both FS-A and FS-B were well presented at NP$_{366-374}$. The low levels of antigen produced from these mutant constructs (FS-A and FS-B) were also sufficient to prime NP-specific immune responses in vivo (Fig. 3), confirming our in vitro results, and providing evidence that cryptic epitopes encoded in alternative RFs can play a significant role in natural immune responses. An important observation from these data is that not all of the FSs that could elicit CTL responses in vitro were capable of priming in vivo. This suggests that there is a difference in sensitivity levels between the two assay techniques and care should be taken when assessing the true activity of cryptic epitopes in immune responses.

Two strategies that we adopted were very helpful in eliminating potential mechanisms underlying the initial results. First, point mutations at anchor residues in each of the conventional epitopes allowed us to ascribe virtually all of the bypass of the FS mutations to expression of NP$_{50-57}$, NP$_{147-155}$, and NP$_{366-374}$ and not emergent subdominant epitopes. Second, the insertion of a thermostable duplex within the 5′ UTR of the NP gene indicated that the majority of epitope production from the NP gene is due to translation proceeding from the 5′ end of the NP gene, as opposed to either translation from abbreviated transcripts resultant from vac promoters within the NP gene, or from sites of internal initiation (Fig. 4). The graded diminution in $NP_{366-374}$ presentation as the FS became more distal (Fig. 2) also argues against the influence of cryptic vac promoter with NP. If a cryptic promoter within the NP gene was responsible for this low level of $NP_{366-374}$ expression, one would expect an abrupt loss of presentation with the first FS downstream of the promoter. The low level of H-2^b reactivity associated with the duplex construct seems likely to be due to biochemically undetectable leaking of scanning ribosomes through the thermostable duplex. This level of leakiness is probably not sufficient to elicit $NP_{147-155}$ responses, however, as we believe $NP_{147-155}$ is inefficiently processed relative to NP_{50-57} and $NP_{366-374}$. This is perhaps due to a major proteasomal cleavage site within the epitope itself (Yellen-Shaw, A., and L.C. Eisenlohr, manuscript in preparation), which would explain why its expression is absent with the duplex barrier in place. None of our manipulations completely ablated H-2^k reactivity and we believe it quite possible that there is a minor H-2^k -restricted epitope elsewhere in the NP gene. However, we do not think that this persistent low level of reactivity compromises our conclusions. In those cases where we observed H-2^k-restricted epitope expression relevant to the central theme of this work (FS-A and FS-A/M13I), presentation levels were maximal and therefore almost entirely attributable to expression of NP_{50-57}. These findings, therefore, permitted us to focus our attention on mechanisms at the level of translation.

Examination of our results with respect to the positioning of the FSs suggest that at least two variants of conventional translation account for cryptic epitope presentation. It is apparent from the introduction of ATG→ATC substitutions superimposed upon FS-A, that AUG_{32} is nearly requisite for CTL responses to the products of FS-A. This mutation determined that scanthrough was the probable mechanism at work for FS-A, as there are no termination codons between the FS event and codon 32. By definition, ribosomal reinitiation is not responsible for epitope expression from FS-A. The effect of the single point mutation on AUG_{32} initiated translation was startling in its severity considering that the substitution at AUG_{13} had no effect on the presentation of any of the epitopes under study (Fig. 7 *b*). Scanthrough is not a vac-specific mechanism. It has been directly observed in transfected yeast and mammalian cells (22–24) and therefore should be possible under many conditions of gene expression.

Although we have shown that ribosomal scanthrough can successfully produce cryptic epitopes when initiating near the 5′-cap, a second mechanism seems likely to operate for FS-B expression as the distance from the 5′-cap that scanthrough can successfully operate appears limited. This is seen in the failure of AUG_{66} to compensate for the loss of AUG_{32} in producing substrate for epitope excision, despite the excellent context surrounding this codon (CCG-GAAUGG; Kozak has defined good initiation context as $CC(A/G)^{-3}CCA^{+1}UGG^{+4}$, with the purine at −3 being in place 97% of the time and the guanine at +4 important in the absence of the −3 purine; reference 45). With this in mind, it is most likely that FS-B-expressed epitopes are produced by an alternate method. Reinitiation of translation seems a strong possibility. There are limitations to this mechanism with respect to both the length of the upstream ORF (23) and the intercistronic distance (24, 46, 47), but many possible FS-B reinitiation events are still permitted by these criteria. The fact that $NP_{366-374}$ is presented from FS-B while $NP_{147-155}$ is not suggests that reinitiation may occur downstream of $NP_{147-155}$. However, there are several other influencing factors with $NP_{147-155}$, principally its inefficient expression even from WT NP as mentioned above. A remaining question is why the FS-B expression mechanism is not available to FS-A/M_{32}I. Theoretically, the AUG codon that is used for reinitiation in the expression of the three epitopes from FS-B should still be available for FS-A/M_{32}I translation, unless the positioning of the two conventional translation termination codons (33 and 70, respectively) is significant. Experiments designed to elucidate the mechanism underlying the bypass of FS-B are ongoing and may shed light on this question.

Another demonstrated means of cryptic epitope expression is the initiation of translation at non-AUG codons (48). These alternative codons include AUU, UGG, GAU, ACG, CUG, and GCG, of which the latter three are the more potent at initiation. Recent studies have shown that such codons are capable of driving the translation of an epitope in the absence of a conventional initiation codon (14). There is one in-frame alternative initiation codon (GAU_{16}) preceding AUG_{32} in NP and 2 (AUU_{33}, AUU_{36}) immediately following, all of which are in optimal initiation context. However, it is apparent from the effect of the AUG_{32} substitution that alternative translation initiation codons do not contribute significantly to epitope production from FS-A in our system, although this might have been different if the stronger alternative initiation codons had been available.

As a natural extension of our in vivo results, demonstrating the strong priming potential of FS-A, it seems likely that ribosomal scanthrough of the primary initiation codon (AUG_1) could be occurring during the production of proteins in vivo. Therefore, it is quite possible that this translation mechanism is providing processing substrate from alternative RFs for many genes in the natural situation. This is dependent upon the portion of the gene that scanthrough can use as a template, and whether this region of the gene encodes an immunogenic epitope. Although our data suggest that scanthrough is limited to the 5′ end of mRNA, the potency of this mechanism is demonstrated by the ribosome bypassing four AUG codons and one alternative initiation codon before initiating translation at AUG_{32} (Fig. 7 *a*). The scanthrough initiation is at a strength and frequency that enabled us to immunoprecipitate a polypeptide smaller than NP from recombinant vac infected cells, using NP-specific antibodies under the appropriate conditions. Furthermore, in a more controlled setting, we have confirmed the ability of ribosomes to ignore the 5′ initiation codon (even those with excellent context) and utilize a downstream initiation codon in an alternative RF to produce sufficient epitope for CTL activation (T.N.J. Bullock and L.C. Eisenlohr, manuscript submitted). Encouraged by this result, we have tested the possibility of identifying naturally expressed epitopes encoded in alternative RFs with a computer based search of the viral protein database, targeting sequences from the alternative translation RFs conforming to the HLA-A2.1 motif (38). Numerous alternative ORFs at the 5′ end of the conventional ORF conformed to our parameters and we are currently determining the influence of such natural cryptic epitopes in immune responses. Why have such epitopes not been previously identified? The most reasonable answer probably lies in the fact that there has not been a pressing need to identify them. Standard techniques for epitope determination focus on the primary ORFs, with T cell clones that do not map to these regions being discarded. The extra effort required to investigate all three RFs would only be used in such prominent cases as tumor antigens or alloantigens. Recently, Malarkannan et al. (42) reported on an alloreactive epitope encoded in an alternative translation RF. Overlapping peptides failed to identify the epitope and its nature eluded the authors until they expressed the appropriate region in the different reading frames. This general sequence of events was also experienced with two epitopes expressed by human melanoma lines. One was shown to be partially encoded in an intron, for which expression is dependent upon aberrant splicing (17). The second lies in an alternate reading frame of the gp75 gene (12).

The recent report of Elliott et al. provides the first direct evidence for cryptic epitope expression in vivo (13). This study and ours are similar in that both employ expression of NP via a recombinant vac system although in their case the NP gene product is preceded by a leader sequence. However, results of the two studies are strikingly different. Elliott et al. observed strong presentation of $NP_{366-374}$ in vitro and clear presentation in vivo even though the product was shifted to the +1 frame within codon 160. This frameshift is in close proximity to our FS-D (also a shift into the +1 frame, at codon 151) which had very limited in vitro antigenicity. We did not test the FS-D construct in vivo but given results with FS-B, FS-C, and FS-E we expect it would not stimulate a detectable response. The discrepancy may be due to the differing conditions of the in vitro assays used to detect expression of the epitope and to assess priming in vivo. A more intriguing possibility is that translation coupled with translocation (as would occur with a leader-encoded sequence) is more susceptible to frameshifting. Indeed, the structure of the *E. coli* ribosome, as recently deduced by low-dose cryoelectron microscopy (49, 50), suggests that there are two channels through which the polypeptide can exit the ribosome. The exit of one channel is in the membrane associated domain of the 50S subunit and may therefore be reserved for translocated (secreted) proteins. It has been demonstrated that interactions of the nascent peptide with the exit channel can influence decoding by inducing transient mRNA/peptidyl tRNA dissociation and stalling (51, 52). Assuming a similar arrangement exists for eukaryotic ribosomes, perhaps exit through the exocytic channel induces a higher incidence of frameshifting as compared with exit through the cytosolic channel. The mechanism underlying expression of an epitope in an alternate reading frame of the melanoma-associated gp75 gene (12) has not yet been reported. Its position within the primary open reading frame (beginning 294 bases from the primary ATG) and the number of preceding ATG triplets (5) do not eliminate scanthrough as a possibility. However, this protein is exocytic (53, 54) and the same mechanism operating in conjunction with the exocytic version of NP may also be at work here.

Scanthrough translation may also have consequences for in-frame epitopes as well. We hypothesized that instability of the NP/32-498 product underlies presentation of FS-A and, further, that this product is a significant source of processing substrate in the expression of WT NP. However, presentation levels of the $NP_{147-155}$ epitope from the NP/M32I mutant were indistinguishable from those associated with WT NP (Fig. 8). Additionally, in experiments not shown, a BFA-mediated analysis demonstrated presentation of epitope from FS-A to be significantly lower than that from wild-type NP. Thus, we still do not understand the significance of the M_{32} codon and do not yet have evidence that anything other than full-length NP provides the bulk of the starting processing substrate for generation of the epitopes in our study. However, for other proteins it remains possible that truncated products generated by scanthrough account for significant quantities of epitope. Further, other mechanisms that would generate truncated products, such as premature termination, remain to be investigated.

A second extension of our findings involves the presentation of MHC class I epitopes derived from exocytic proteins. It has been generally assumed that the MHC class I–restricted presentation of these proteins is the result of mis-translation, resulting in the cytosolic proteolysis and processing of a fragment of these proteins that is not translocated into the endoplasmic reticulum (ER) (3, 37, 55). More recently, it has been proposed that a mammalian equivalent of the US11 gene of cytomegalovirus (CMV) may actively expel misformed membrane proteins from the ER into the cytosol, whereupon they can be rapidly degraded (56). In addition to these mechanisms, we wish to propose that ribosomes scanning through primary initiation codons and initiating from downstream codons, thereby bypassing the leader sequence required for ER translocation, may make a significant contribution to the substrate for processing and presentation of exocytic proteins.

The expression of cryptic epitopes could have an important immunological impact and physiological relevance in areas such as thymic education, CTL mediated autoimmunity, vaccine design and cancer immunotherapy. Further investigations in this area should help elucidate the potential influence of the inherent flexibility of translation in the development of immune responses.

We thank Mark R.M. Sermarini, Carol Laughlin, and Laura L. Franlin for technical assistance and John F. Atkins for helpful discussions.

These studies were supported, in part, by grants from the American Cancer Society (IM-726), the National Institutes of Health (AI 36331) and the Dean's Overage Research Program of Jefferson Medical College.

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Received for publication 19 April 1996 and in revised form 17 July 1996.

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