Initiation Codon Scanthrough versus Termination Codon Readthrough Demonstrates Strong Potential for Major Histocompatibility Complex Class I–restricted Cryptic Epitope Expression

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Summary

Accumulating evidence shows that the repertoire of major histocompatibility complex class I–restricted epitopes extends beyond conventional translation reading frames. Previously, we reported that scanthrough translation, where the initiating AUG of a primary open reading frame is bypassed, is most likely to account for the presentation of cryptic epitopes from alternative reading frames within the influenza A PR/8/34 nucleoprotein gene. Here, we confirm and extend these findings using an epitope cassette construct that features two well-defined CD8$^+$ T cell (T_{CD8^+}) epitopes in alternative reading frames, each preceded by a single start codon. Expression of one epitope depends on scanning of the ribosome over the first AUG with translation initiation occurring at the second AUG. We find that scanthrough translation has great potency in our system, with its impact being modulated, as predicted, by the base composition surrounding the first initiation codon, the number of start codons preceding the point of alternate reading frame initiation, and the efficiency with which the epitope itself is generated. Additionally, we investigated the efficiency of eukaryotic translation termination codons, to assess codon readthrough as a mechanism for cryptic epitope expression from 3′ untranslated regions. In contrast with initiation codons, eukaryotic stop codons appear to be highly efficient at preventing expression of epitopes encoded in 3′ untranslated regions, suggesting that 3′ untranslated regions are not a common source of cryptic epitope substrate. We conclude that scanthrough is a powerful mechanism for the expression of epitopes encoded in upstream alternative open reading frames that may contribute significantly to T_{CD8^+} responses and to tolerance induction.

T_{CD8^+} activation depends upon interaction between the clonotypic TCR and a MHC class I molecule complexed with an 8–10 amino acid peptide epitope (1–5). Conventional models of antigen processing and presentation generally presume these epitopes to be derived from the cytosolic proteolysis of full-length, fully translated proteins. However, it has been demonstrated that T_{CD8^+} are also capable of recognizing either the products of gene fragments or mini genes expressing the minimal sequence of an epitope (6–8). In addition, there is sufficient evidence in the literature to suggest that strict adherence to conventional translation is not required for antigen presentation. Examples include the presentation of epitopes encoded in untranslated regions (UTRs)[1] (9, 10) and in alternative reading frames (RF) (11–15). This implies that regions of genes that are traditionally thought to be inaccessible to translation could be a potentially significant source of substrate for antigen processing and presentation.

The generally accepted model for translation initiation of most messages is the ribosomal scanning hypothesis (16–18), where a ribosome scans from the 5′-cap and begins translation from the first AUG in favorable context, as determined by surrounding nucleotide identity. This AUG defines the primary RF, or RF0. Extensive surveys of eukaryotic genes have shown that in most cases the first AUG encountered is in favorable context (19, 20) and biochemical studies have shown that a primary start codon is used by the great majority of scanning ribosomes (21). However, some ribosomes fail to initiate at the primary initiation codon, with factors such as proximity to the 5′-cap, and lo-

[1]*Abbreviations used in this paper:* NP, nucleoprotein; nt, nucleotide; ORF, open reading frame; RF, reading frame; T_{CD8^+}, CD8$^+$ T cell; UTR, untranslated region; vac, vaccinia virus.

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cal secondary structures as well as initiation codon context having influence (22, 23). We have recently demonstrated that initiation codon readthrough (scanthrough) is probably responsible for the production of epitopes encoded in alternative RFs from a mutated influenza A PR/8/34 nucleoprotein (NP) gene (24). In the system studied, it appeared that scanthrough was capable of utilizing an internal AUG codon to rescue the presentation of three NP epitopes (NP_{50-57}, $NP_{147-155}$, $NP_{366-374}$) that had been shifted out-of-frame by a base deletion in the second codon of the NP open reading frame (ORF). In addition to providing this alternative source of out-of-frame epitopes, scanthrough translation may simultaneously produce truncated, in-frame polypeptides that could be more efficiently processed than full-length protein by the class I processing machinery. This mechanism could also provide a plausible rationale for the presentation of exocytic proteins that do not normally access the cytosolic protein-degradation apparatus, by allowing initiation of translation after the signal sequence encoding region, resulting in the production of a nontranslocated substrate.

As in the case of start codons, not all translation stop codons cause termination of translation with equal efficiency. The sequence surrounding the termination codon dictates whether some readthrough and continued translation can occur. In *Saccharomyces cerevisiae*, termination codon readthrough has been shown to occur at levels up to 3.35% (25). This mechanism would allow for translation from the 3′ UTR, further expanding the available substrate for class I presentation.

In the current study, we have constructed a presentation cassette in order to assess the potential for ribosomal scanthrough in epitope expression under controlled conditions. This allowed us, first, to confirm our previous findings concerning initiation codon readthrough and, second, to assess directly the strength of initiation codon readthrough in the face of competitive AUG codons encoded in alternate RFs. In addition, we wished to discern whether epitopes encoded in 3′ UTR are also available for presentation. For this purpose, we tested the ability of ribosomes to read through strong and weak RF0 stop signals inserted in wild-type NP and translate a downstream epitope. These studies provide important insights into the translation mechanisms available for the production of substrate from unconventional coding regions.

Materials and Methods

Chemicals

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

Animals

6 to 8-wk-old female inbred CBA (H-2^k), BALB/c (H-2^d), and C57BL/6 (H-2^b) strain mice were obtained from Taconic (Albany, NY) or Jackson Labs (Bar Harbor, ME) and maintained in Kimmel Cancer Center Animal Facilities (Philadelphia, PA).

Tissue Culture

L929 (H-2^k), L-Kd (H-2^k, H-$2K^d$; L929 transfected with H-$2K^d$; reference 26) and MC57G (H-2^b) cells were maintained under standard conditions. Specifically, they were grown in DMEM supplemented with 5% FCS (Sigma) and incubated at 37°C and 9% CO_2.

Molecular Manipulations

All enzymes used for the manipulation of the NP gene were used according to the instructions of the manufacturer. PCR primers and oligonucleotide linkers were synthesized by the Kimmel Cancer Institute Nucleic Acid Facility (Philadelphia, PA). NP containing a silent Apa I site in the H-$2K^d$ (bp 457–463) epitope has been described elsewhere (8). Silent PCR directed mutagenesis was used to incorporate an Aat II site in the H-$2K^k$ epitope (bp 165–171): 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, La Jolla, CA) with NP inserted in the EcoRI site of the MCS.

Variable Initiation Cassettes. The region between the NP_{50-57} and $NP_{147-155}$ epitopes was removed by AatII to ApaI digestion of the NP gene in Bluescript II SK (+). The resulting vector was then ligated (T4 ligase; GIBCO BRL, Gaithersburg, MD) with an oligo pair encoding the reconstituted epitopes separated by 5 nucleotides (nt) and the initiation cassette consisting of an excellent context for ATG_{RF0} and an excellent context for ATG_{RF+1}. The two ATG codons were in alternate reading frames and separated by 13 nt, partially encoding a KpnI site for later manipulations. Oligonucleotide sequences (upper oligo only), TCG ACG GAT TAT GGC GGT ACC GGA TTA TGG CAA GCG ATT ACG AGG GAC GT and CTG ATC ACG CGA CTT ATC AGA GGA CAA GGG CC. After construction of the excellent–excellent cassette, we altered the context of the $ATG_{147-155}$ initiation codon using synthetic oligonucleotides. The initial construct was digested with SalI and KpnI to excise the ATG_{RF0} initiation region. Oligos encoding a similar initiation codon with different surrounding context were ligated into this site. Good context, TCG ACG GAT TAT GTC CGT AC. Fair context, TCG ACG GGT TAT GTC CGT AC. Poor context, TCG ACG GTT TAT GTC CGT AC. Null context, TCG ACG GAT TTT TGC CGT AC. The null construct for the downstream RF+1 was generated by KpnI to ApaI digestion of the excellent context of RF0 construct and replacement with an oligonucleotide pair designed to ablate the KpnI site and mutate the RF+1 start codon from AUG to ATC. The upper strand of the pair reads G̲GG ATT ATC̲ GCA AGC GAT TAC GAG GGA CGT (underline indicates the mutations). All the cassette variants were sequenced and then transferred to pSC11 via a SalI–NotI fragment containing the manipulated gene.

The reverse-frame cassette was made by digesting the excellent context of RF0 construct with KpnI and ApaI and replacing that fragment with a synthetic oligo pair that retained the KpnI site, inserted a single base (C) right after the KpnI site and a base (G) immediately following the ATG. The upper strand reads CC̲G GAT TAT GG̲G CAA GCG ATT ACG AGG GAC GT (inserted bases are indicated by underline). The null context of RF0 was created as described above for the variable initiation cassettes.

Variable Context Stop Codons. The NP gene in pSC11 was digested with BstBI and treated with alkaline phosphatase. Into

the BstBI site, self-annealing oligos encoding either a strong (CGT ATA AGC GCG CTT ATA) or weak (CGT ATG ACC GCG GTC ATA) stop signals were ligated. Correct ligations were analyzed by the loss of the BstBI site.

Viruses

Control vaccinia virus (vac), NP-expressing vaccinia virus, and the NP_{D51T} mutant designed to ablate the $NP_{50–57}$ epitope have been described elsewhere (24). Other recombinants described here were made as previously described (8). In brief, 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 (American Type Culture Collection [ATCC], Rockville, MD; CCL 70) and plaque purified in 143B $HuTK^-$ (ATCC; CRL 8303) 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.

CTL Assay

APC were infected for 1 h at 37°C with vac 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 were 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 mCi of $Na^{51}CrO_4$ (Amersham Corp., Arlington Heights, IL) 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 bottomed 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 γ detector (Pharmacia, Sweden). The data are presented as percent specific ^{51}Cr release, defined as 100 × ([experimental cpm − spontaneous cpm] / [total cpm − spontaneous cpm]).

Generation of CTL

T_{CD8^+} restricted to H-2^k, H-2^d, or H-2^b were derived from NP-immunized CBA, BALB/c, or C57BL/6 mice, respectively, as described elsewhere (27). Mice were immunized by intraperitoneal injection of 10^7 PFU of NP–vac in the case of C57BL/6, a vac expressing the isolated $NP_{50–57}$ epitope in the case of CBA, and a vac expressing the isolated $NP_{147–155}$ epitope in the case of BALB/c. 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.

Results and Discussion

Confirmation of Initiation Codon Scanthrough as a Viable Mechanism for MHC Class I Epitope Production. Our analysis of a series of frameshift mutations of the NP gene led us to suggest that ribosomal scanthrough of an initiation codon can provide a significant amount of substrate for epitope production from unconventional coding regions (24). A recombinant vac encoding the NP gene with a deletion in the second codon was able to express three NP epitopes, $NP_{50–57}$ (H-$2K^k$-restricted), $NP_{147–155}$ (H-$2K^d$-restricted), and $NP_{366–374}$ (H-$2D^b$-restricted), as assessed by sensitization of target cells in vitro. This mutant was also able to prime mice for anti-$NP_{366–374}$ responses at levels similar to those achieved by priming with wild-type NP-expressing vac. We were able to identify an internal AUG codon that played a prominent role in overcoming the frameshift and permitting the expression of the three NP epitopes. Owing to the location of the initiation and termination codons involved, scanthrough was determined to be the most likely mechanism operating in the expression of these epitopes.

We wished to confirm the potential of scanthrough in a controlled system that is not subject to alternative translation mechanisms such as reinitiation, in which the ribosome terminates translation and starts again at a downstream AUG (28–30). Kozak (23) has shown, using standard biochemical techniques, that the degree of scanthrough is dependent upon the context surrounding the first AUG (defined by the following optimal sequence: $CC(^A/_G)^{-3}CCAUGG^{+4}$, in which the −3 purine and the +4 guanine are most important) and that scanthrough initiation is limited to the upstream region of the message (31). We created a truncated NP gene in which the region between the $NP_{50–57}$ and $NP_{147–155}$ epitopes was eliminated, leaving them separated by 5 nt (Fig. 1 *a*). This had the effect of putting the two epitopes in alternate RFs: $NP_{50–57}$ in RF+1, $NP_{147–155}$ in RF0, with no alternative RF stop codons that could influence the expression of these epitopes. Mutation of the residues required for binding to the MHC class I molecule has confirmed that H-2^k and H-2^d responses are limited to $NP_{50–57}$ and $NP_{147–155}$, respectively (24). In front of the two epitopes we placed a translation initiation cassette consisting of tandem start codons in the same reading frames as the two epitopes. The second ATG (RF+1, excellent context) is in frame with $NP_{50–57}$. This start codon is preceded by an ATG in RF0 with varying contexts that are predicted to influence significantly the efficiency of translation initiation. Based upon established results (32), four contexts were provided that we designated excellent (A at −3, G at +4), good (A at −3, T at +4), fair (G at −3, T at +4), and poor (T at −3, T at +4). In this range, the level of initiation is predicted to vary over 10-fold. The AUG_{RF0} is in the same frame as the $NP_{147–155}$ epitope. To confirm that the epitopes depend upon the RF0 and RF+1 AUGs for expression we mutated either the RF0 or RF+1 ATG while keeping the remaining ATG in excellent context. A predicted outcome for the translation of this cassette is that a scanning ribosome should first encounter the AUG_{RF0}, responsible for translation of $NP_{147–155}$. However, a percentage of scanning ribosomes will bypass it, with some level of initiation occurring at the second AUG, resulting in the expression of $NP_{50–57}$. We anticipated that, as the initiation context surrounding $AUG_{147–155}$ was improved, more ribosome would be competed away from translating in the RF+1 and directed into RF0, resulting in decreased presentation of $NP_{50–57}$.

All of our constructs were recombined into the vac ge-

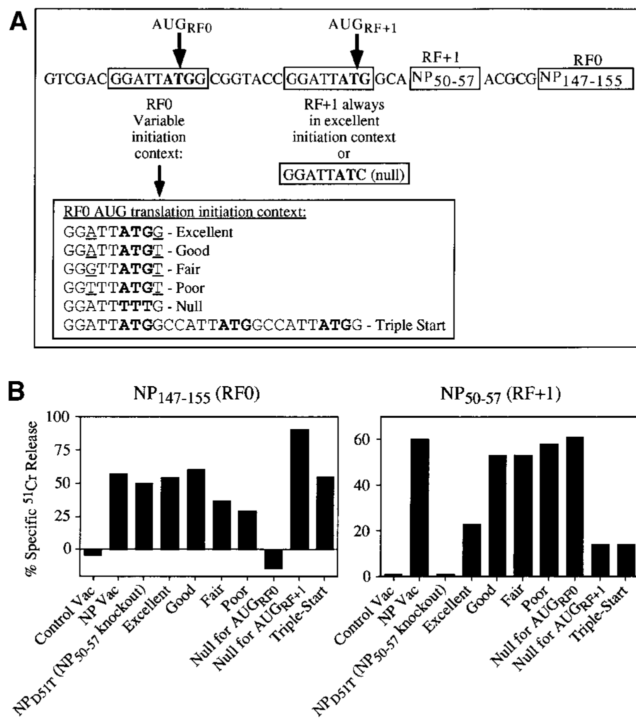

Figure 1. Presentation of an epitope in a downstream alternative reading frame depends upon the context and number of upstream initiation codons in the primary reading frame. (*a*) MHC class I presentation cassette. Two MHC class I–restricted epitopes, NP_{50-57} and $NP_{147-155}$, were placed in alternate RFs, each translated by different AUG initiation codons. The first AUG encountered by the scanning ribosome is in variable context and initiates translation of $NP_{147-155}$. The second AUG is in excellent context and initiates translation of NP_{50-57}. After $NP_{147-155}$, the sequence of NP is unaltered. Start codons or their null equivalents are indicated in bold. The −3 and +4 nucleotides involved in changing the initiation potential are underlined. (*b*) Scanthrough is an efficient mechanism of translation from alternative RFs. The MHC class I presentation cassette variants were recombined into the vac genome and tested for sensitization of L-K^d cells for lysis in a standard ^{51}Cr release assay. Specificity of the T_{CD8^+} effector population, generated as described in Materials and Methods, is indicated above each data set. The effector/target ratios shown are 30:1 for recognition of $NP_{147-155}$ and 13:1 for NP_{50-57}, respectively. Similar results were observed with three other ratios.

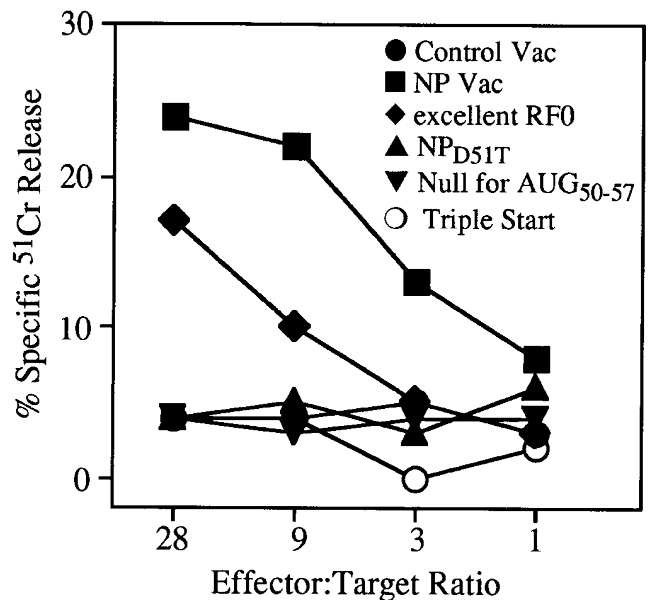

Figure 2. Presentation of NP_{50-57} from selected constructs. The indicated constructs were tested for target cell sensitization in a standard ^{51}Cr release assay.

nome to assay for the expression of these two epitopes by infected target cells in a standard ^{51}Cr release assay. To measure presentation of both epitopes in the same target cell, we utilized L929 (H-2^k) cells transfected with the gene encoding the K^d class I molecule (26). Presentation of the $NP_{147-155}$ epitope was quite strong when driven by initiation codons in either good or excellent contexts (Fig. 1 *b*). Levels of specific lysis were comparable to those associated with infection by a wild-type NP-expressing vac. Of note, presentation was diminished when the RF0 ATG was placed into fair and poor contexts. Mutation of the RF0 ATG resulted in complete loss of lysis above control levels, confirming its essential role in expression of the $NP_{147-155}$ epitope.

It is generally understood that T_{CD8^+} are exquisitely sensitive, requiring very little antigen expression and very few peptide–class I complexes at the cell surface to trigger effector function (13, 33–35). In this light, the diminution of $NP_{147-155}$ presentation resulting from downgrading of the RF0 ATG may be unexpected. However, we and others have noted the relative inefficiency with which $NP_{147-155}$ is presented (24, 36, 37), probably because of a major site for proteasome attack within the epitope (36). Antón et al. (37) have estimated that only 30 $NP_{147-155}$–K^d complexes result from the processing of wild-type NP. This compares with 1,800 NP_{50-57}–K^k complexes. Further supporting the notion that $NP_{147-155}$ expression is close to, if not below, levels associated with maximal T cell stimulation, we have recently noted that a 2- to 5 fold reduction in wild-type NP expression within target cells is sufficient to reduce $NP_{147-155}$-specific lysis appreciably (Yellen-Shaw, A.J., K.A. Puorro, and L.C. Eisenlohr, unpublished data). Using proinsulin synthesis as a readout, Kozak observed that a change from excellent to good context results in an ~2-fold decrease in expression, from good to fair, a 3.7-fold decrease, and from fair to poor, a decrease of at least 3.5-fold (32). Together, this information provides an explanation for the lower presentation of $NP_{147-155}$ in the fair and poor contexts.

Presentation of the NP_{50-57} epitope, which depends upon bypass of the RF0 AUG by a sufficient portion of ribosomes, followed a pattern predicted by the scanning hypothesis. When the RF0 AUG is in poor, fair, and good contexts, specific lysis was not significantly different from that associated with the $ATG_{147-155}$ null construct. When the RF0 ATG is in excellent context, presentation of NP_{50-57} was clearly compromised, though consistently present. This is reinforced by data from a separate assay shown in Fig. 2, which provides specific lysis figures over several effector/target ratios. The importance of the RF+1 AUG for this expression is confirmed by the low lysis associated with the

AUG_{RF+1} null construct. In this assay, levels were above those associated with control virus, but in other assays (see Fig. 2) the two were comparable. We also note the complete loss of NP_{50-57}-specific lysis with a single mutation within the NP_{50-57} epitope. In our previous publication, we observed only partial loss of lysis with this mutant (24). As we used NP_{50-57}-expressing vac to prime mice for effector populations in the studies reported here, and full-length NP vac for this purpose in the previous work, we strongly suspect the existence of a weaker H-2^k–restricted epitope within NP. Such an epitope has been suggested by Daly et al. (38) who generated a K^k-restricted T cell hybridoma with reactivity to NP that does not map to NP_{50-57}.

In accord with the scanning hypothesis, we reasoned that multiplying the number of RF0 start codons in excellent context would track a higher proportion of ribosomes to RF0, further reducing presentation of NP_{50-57}. Therefore, the single start codon module was replaced by a string of triple RF0 start codons, each in excellent context. This strategy significantly reduced levels of NP_{50-57} presentation. This construct is also shown in Fig. 2, where no lysis above that associated with negative control virus was observed.

These results demonstrate that even when the preceding start codon is in excellent context, there is a degree of scanthrough that permits presentation of an epitope in an alternative reading frame. As the context surrounding the primary AUG becomes more favorable, the potential for epitope expression in the alternative reading frame decreases. Epitope expression in the alternative reading frame was clearly compromised when the RF0 AUG was in excellent context. An extensive survey of vertebrate mRNAs revealed that only 25% have primary start codons in excellent context. Good and fair contexts are each present in 36% (72% total) of messages (20). Thus, the majority of messages are predicted to feature a level of scanthrough that would permit strong expression of an epitope in an alternative reading frame. The results further demonstrate that the level of scanthrough is also influenced by the number of upstream start codons in alternative RFs.

Reversal of the Cassette. As discussed above, the $NP_{147-155}$ epitope is inefficiently generated compared with NP_{50-57}. Therefore, conditions that permit activation of $NP_{147-155}$-specific T_{CD8^+} via scanthrough may be more limited. To test this, the pairing of the first and second start codons with $NP_{147-155}$ and NP_{50-57} was reversed as depicted in Fig. 3 *a* and described in Materials and Methods. This placed the first AUG and NP_{50-57} in RF0 and the second AUG and $NP_{147-155}$ (as well as the rest of NP, residues 156–498) in RF-1. Whereas NP_{50-57} in an alternative RF could be presented in the face of an upstream RF0 AUG in excellent context (see Fig. 2 *b*; Fig. 3), presentation of $NP_{147-155}$ in the same circumstance could not be detected (Fig. 3 *b*). Thus, in addition to the context and number of upstream AUGs, the epitope itself can determine the potential for T_{CD8^+} activation via scanthrough.

Altogether, our data indicate that scanthrough and reading of alternative ORFs that likely accompanies all translation to some degree is sufficient to activate T_{CD8^+}. The frequency with which epitopes are expressed in this manner, and the extent to which they play a role in the total response to foreign and self antigens, remains to be determined. However, we note that the cassette system employed may have underestimated the potential for this mechanism. The alternative ORFs in this study are much larger than those that naturally occur. For example, the average alternative ORF within NP encodes a peptide of 14 amino acids. Epitopes within such small products may be processed and loaded onto class I molecules hundreds- to thousands fold more efficiently than their equivalents that are part of larger proteins (37).

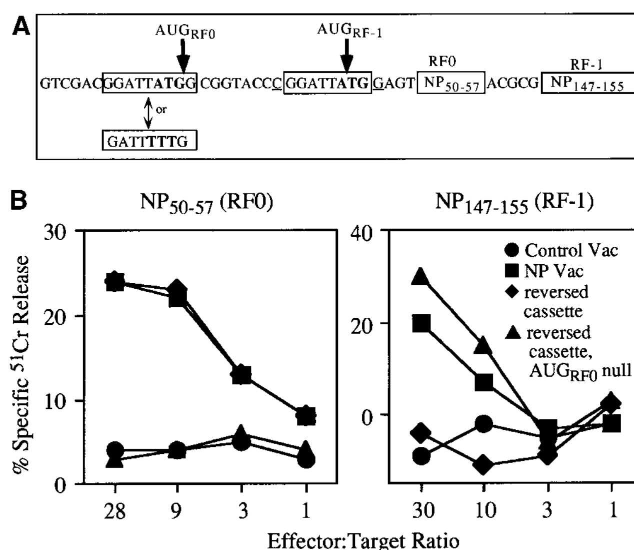

Figure 3. Reversal of the cassette. (*a*) Two base insertions, indicated by underline, were made to the original cassette resulting in pairing of RF0 ATG with NP_{50-57} and the RF-1 ATG with $NP_{147-155}$. (*b*) Scanning beyond an AUG in excellent context is not sufficient to allow presentation of $NP_{147-155}$. The indicated reverse and control constructs were tested for target cell sensitization in a standard ^{51}Cr release assay. Specificity of the effector populations is indicated above each data set.

Translation Termination Codons Are Efficient at Preventing the Expression of Downstream Epitopes. A genetic region that has great potential for supplying additional substrate for class I–restricted epitopes is the 3′ untranslated region. Part of this region could be available for translation if the translation termination codon is bypassed to a certain extent. In a manner similar to optimal initiation context, the sequence surrounding a stop codon can significantly influence translation termination (25, 39, 40), with certain sequences permitting a degree of leakthrough. Given the extreme sensitivity of T_{CD8^+}, even small amounts of stop codon readthrough could make the 3′ UTR of a gene available as a source of substrate for epitope production. To test this notion, we inserted a stop codon, with either weak or strong termination efficiency, in RF0 of the full-length, wild-type NP between $NP_{147-155}$ and $NP_{366-374}$ (Fig. 4 *a*). In *S. cerevisiae*, the weak context stop codon has been shown to allow 3.35% readthrough, whereas the strong context stop codon allows only 0.36% (25). We intentionally placed these stop codons far into the NP ORF to elim-

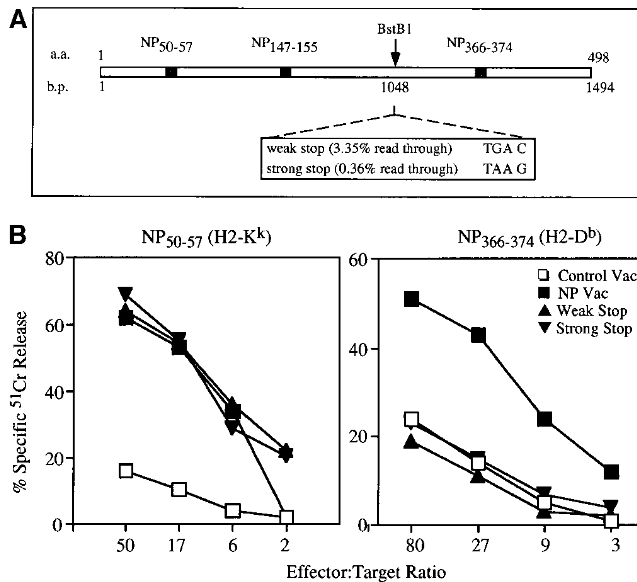

Figure 4. Testing stop codon readthrough as a means of cryptic epitope expression. (*a*) Positioning of in-frame stop codons in full-length NP. Either a weak or a strong termination codon (25) was inserted at the BstBI site in NP, between the $NP_{147-155}$ and $NP_{366-374}$ epitopes. The resulting constructs were recombined into vac. (*b*) In-frame epitopes encoded after stop codons are not presented. The constructs depicted in *a* and the indicated control recombinants were tested for target cell sensitization in a standard ^{51}Cr release assay. Specificity of the effector populations is indicated above each data set.

inate the confounding mechanisms of scanning and reinitiation that are possible in upstream regions of the message (19, 28, 30, 41). As seen in Fig. 4 *b*, both stop codons were very efficient at preventing $NP_{366-374}$ presentation. As expected, NP_{50-57} presentation was unaffected by these manipulations because this epitope lies upstream of the insertion site.

Therefore, it seems unlikely that translating ribosomes can productively penetrate stop codons in this region of the gene in this system. From our previous results showing presentation of epitopes from biochemically undetectable levels of substrate (24), we would have predicted that 3.35% readthrough of the termination codon would have produced sufficient amounts of $NP_{366-374}$ to sensitize target cells. In our hands, $NP_{366-374}$ is efficiently generated, resembling NP_{50-57} much more than $NP_{147-155}$ by several criteria (Yellen-Shaw, A.J., K.A. Puorro, and L.C. Eisenlohr, unpublished data). However, it may be that the vac expression system employed is less permissive in translation termination or that a more efficiently generated epitope would indicate a low level of readthrough.

The unavailability of $NP_{366-374}$ for presentation from these constructs is compatible with previous data in which frameshifts were introduced into the distal region of NP. These frameshifts were predicted to produce termination codons in regions of the mRNA that would not be available for reinitiation of translation (19, 41). This was confirmed by the loss of $NP_{366-374}$ presentation (24). This termination codon efficiency is also in concurrence with the work of Shastri and Gonzalez (13), who demonstrated that placing a termination codon directly in front of an epitope was highly efficient at preventing the expression of that epitope. Given these results, we speculate that cryptic epitope expression due to unconventional translation may be limited to the early regions of the open reading frame. Further investigation will be required to address this possibility, including an analysis of frameshifting, in which the actively translating ribosome shifts into a new reading frame. This is a mechanism that would not be limited to a particular region of the open reading frame and one that we have begun to investigate.

With these studies, we have demonstrated the potency of scanthrough in the 5′ region of a gene. In contrast, stop codon readthrough is apparently not available for epitope production. These studies have further elucidated the role of cryptic translation events in the production of substrate for immune responses. Such epitopes could play important roles in thymic education, T_{CD8^+}-mediated autoimmunity, cancer immunotherapy, and vaccine design.

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