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Translation from Cryptic Reading Frames of DNA Vaccines Generates an Extended Repertoire of Immunogenic, MHC Class I-Restricted Epitopes

Reinhold Schirmbeck,2* Petra Riedl,* Nicolas Fissolo,* Francois A. Lemonnier,† Antonio Bertoletti,‡ and Jörg Reimann*  

To test whether simple expression units used in DNA vaccines can generate immunogenic, MHC class I-binding epitopes by translating other than the primary open reading frame (ORF), we constructed a vector (pCI/SX) that encodes the smallest hepatitis B surface Ag in the primary ORF, and a C-terminal fragment (residue 344–832) of the polymerase (Pol) in an alternative (out-of-frame) reading frame. pCI/SX efficiently primed multispecific, HLA-A2-restricted CD8+ T cell responses to epitopes of hepatitis B surface Ag and of Pol (Pol3, Pol3–832). Pol3-containing products generated from pCI/SX were detected only by T cell assays, but not by biochemical assays. Priming Pol-specific T cell responses to epitopes generated from alternative ORFs depended on promoter sequences that drive transcription in the DNA vaccine (human CMV-derived promoter sequences being more efficient than SV40-derived promoter sequences). Human CMV promoter-driven Pol constructs encoding different Pol fragments in primary or alternative reading frames elicited comparable levels of Pol3-specific T cell responses. We confirmed efficient T cell priming to epitopes from alternative ORFs by constructing DNA vaccines that encode an SV40-derived cT-A2–272 protein fused either in frame or out of frame with an immunogenic OVA fragment (OVA18–385). Similar OVA-specific CD8+ T cell responses were primed by both alternative vaccine constructs. Hence, DNA vaccine-stimulated T cell responses to epitopes generated from alternative ORFs seem to be a regular event, although its biological role and risks are largely unexplored. The Journal of Immunology, 2005, 174: 4647–4656.

The CD8+ T cell is an important specific effector cell against many intracellular pathogens and tumors that specifically recognize antigenic, MHC class I-binding peptides. The consensus is that the primary source of antigenic peptides presented by class I molecules are normal or defective nascent proteins that arise by translating either conventional (primary) or alternative (cryptic) reading frames (1–3). The latter can result from the use of alternative reading frames of a coding sequence, or of untranslated regions of DNA, mRNAs, introns, or intron/exon junctions. Aberrant initiation of mRNA or protein synthesis may result from, e.g., initiation codons in translated or untranslated sequences, frame shifting during translation, defective splicing of primary transcripts, or overriding of stop signals (3). These events seem to be errors intrinsic to the complex transcription/translation machinery. T cell-stimulating epitopes generated from cryptic products translated from alternative open reading frames (ORFs)1 have been found in (human and mouse) tumors and in virus infections (4–10). It is unknown whether delivering Ag by DNA vaccines (11–13) primes T cell responses to immunogenic epitopes generated from alternative ORFs.

Genetic (nucleic acid or DNA) vaccination is a potent tool to elicit CD8+ T cell responses (11, 12). Expression vectors used in DNA vaccines contain a transcription unit composed of a heterologous promoter/enhancer sequence, the Ag-encoding sequence, and termination sequences. The constructs are designed such that the Ag is expressed from the primary open reading frame with the first ATG motif of the Ag-encoding sequence located downstream of the promoter. We have reported different strategies to construct vectors that can deliver an extended spectrum of immunogenic information. These involved the following approaches: 1) different Ags were coexpressed from a single transcription unit by incorporating internal ribosomal entry sequences between two (or even more) genes cloned into a bi-(multicistronic) vector to allow their coexpression under control of a single promoter (14, 15). 2) Bidirectional human CMV (HCMV)-derived promoter elements allow coexpression of two Ags by generating two separate expression units up- and downstream of the promoter sequence (16). 3) Two overlapping reading frames of a single sequence that encode two defined viral Ags (under a single heterologous promoter control) allow the delivery of more antigenic information in a compact form by an expression plasmid of limited size (17). In the present study, we analyzed whether CD8+ T cell responses can be generated from cryptic products translated from other than the primary ORF encoded by DNA vaccines (3).

The 3.2-kb genome of the hepatitis B virus (HBV), one of the smallest known viral genomes, contains four partially overlapping ORFs encoding the core, polymerase (Pol), surface (HBsAg), and Ls, large HBsAg; MS, middle HBsAg; Pol, HBV polymerase; S, small HBsAg; T-Ag, large SV40 tumor Ag; tg, transgenic.

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1 Abbreviations used in this paper: ORF, open reading frame; HBsAg, hepatitis B surface Ag; HBV, hepatitis B virus; HCMV, human CMV; hsp, heat shock protein; D-89081 Ulm, Germany. E-mail address: reinhold.schirmbeck@medizin.uni-ulm.de
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trans activator (X) proteins (Fig. 1A) (18, 19). The HBsAg-encoding sequence overlaps the sequence encoding Pol (Fig. 1B) (18). In the present study, we analyzed an HCMV-driven DNA vaccine that encodes the small HBsAg in the primary ORF and an overlapping C-terminal Pol_{344–832} fragment in an alternative (out-of-frame) ORF. No endogenous HBV-specific promoter sequences (19, 20) are located in this HBV sequence. Multispecific MHC class I-restricted epitopes to Pol and HBsAg have been characterized (21–27). The Pol_{344–832} fragment encoded in the alternative ORF contained at least three different A2-restricted epitopes (Figs. 1 and 2) and, thus, it can be used as a defined model Ag for cryptic transcription/translation products expressed from alternative reading frames. Immunization experiments showed that the pCI/SX vaccine efficiently coprimed CD8+ T cells specific for A2-restricted epitopes processed from HBsAg and from cryptic Pol-specific translation products. We showed that: 1) the HCMV promoter plays a critical role in generating cryptic translational products from alternative Pol ORF that give rise to immunogenic Pol_{303–811} epitope, and 2) HCMV promoter-driven Pol constructs, encoding different Pol fragments in the alternative or primary reading frame, elicited comparable levels of Pol3-specific T cell responses.

In the second part of the study, we generated different constructs producing an immunogenic epitope of OVA. The OVA_{18–385} fragment (containing the well-characterized K\^1-restricted SIINFEKL epitope) was cloned C terminally (in frame or out of frame) behind the SV40 cT_{1–272} protein (28, 29). The OVA-specific CD8+ T cell responses primed by constructs producing the epitope from either an out-of-frame or an in-frame translation product were compared. B6 (H-2d) mice vaccinated with these constructs developed similar OVA-specific CD8+ T cell responses. Hence, CD8+ T cell responses can be primed equally well by immunogen products generated from either the primary or an alternative ORF of a DNA vaccine.

Materials and Methods

Mice

C57Bl/6d (B6) mice (H-2d) and HLA-A2 transgenic (A2-tg) HHH mice were bred and kept under standard pathogen-free conditions in the animal colony of Ulm University. Male and female mice were used at 12–16 wk of age.

Vector constructs

HBV Pol-encoding plasmids: pCI/Pol. The HBV Pol was amplified by PCR from the pTKTHBV2a generous gift of A. Meyer, Martin-Luther-König-Karl University of Halle-Wittenberg. The HBV Pol encoding sequence overlaps the sequence encoding Pol_{303–811} epitope and a reverse primer with a SflI site (AAGTGCGACTCAGGTGTTCTCTCAGGAC) and a reverse primer with a SflI site (AAAGATCGATCCGTTTACAATTCCGAC) (18). In generating pCI/Pol, the HBV Pol_{344–832} fragment was amplified by PCR from the plasmid pTKTHBV2 using a forward primer with a SflI site (AAAGATCGATCCGTTTACAATTCCGAC) and a reverse primer with a SflI site (AAAGATCGATCCGTTTACAATTCCGAC) and a reverse primer with a SflI site (AAAGATCGATCCGTTTACAATTCCGAC). The product was cloned in frame behind the cT_{1–272} encoding sequence of pCI/cT_{1–272} vector (28) using KpnI/SallI.

cP/CX and its derivatives. The XhoI/BglII fragment of HBV encoding the small HBsAg up to the HBV poly(A) sequence was cloned into the XhoI/ BamHI-digested pCI vector to generate the pCI/SX plasmid. pCI/SX_{Amin}: the intron sequence was deleted from the pCI/SX vector by BglII restriction and religation to create pCI/SX_{Amin}, the pCI/SX vector was digested with BglII/XhoI, blunted with Klenow, and religated to create the promoterless pCI/SX_{Amin}. pCI/SX: pCI/SX plasmid was digested with XhoI/StuI, and the HBsAg-encoding fragment was cloned into XhoI/StuI-digested pCI. pSf/SX: the HCMV promoter of pCI/SX vector was exchanged with the SV40 early promoter sequences derived from pSf vector (catologue number E1712; Promega) to generate the plasmid pSf/SX. pCI/SX_{non sense}: the pCI/SX vector was digested using NheI/ XhoI and religated. This vector encodes the S42–226 and the Pol_{344–832} in an overlapping sequence. pCI/SX_{non sense}': A HBV Pol fragment was amplified by PCR from the pCI/SX, using a forward primer with a NhI site (AAAGCTTGGAGCAAGATTGCATGTAT) and a reverse primer (CGAGAGGATCCAAAAGCGA). The product was cloned into NhI/BamHI cut pCI/SX vector. This vector encodes the S42–226 and the Pol_{344–832} in an overlapping sequence. pCI/SX_{non sense} c': An HBV Pol fragment was amplified by PCR from the pCI/SX, using a forward primer with a NhI site (AAAGCTTGGAGCAAGATTGCATGTAT) and a reverse primer (CGAGAGGATCCAAAAGCGA). The product was cloned into NhI/BamHI cut pCI/SX vector using NhI/BamHI. This vector encodes the Pol_{344–832} sequence.

OVA-encoding plasmids: pCI/OVA. An OVA construct was generated that started at the methionine at OVA position 8 for detail, see Swiss-Prot: OVAL.CHICK. The OVA_{8–385} encoding sequence was amplified using a forward primer with a XhoI site (GGGTCCGAAGGCAAGATCTTTTT) and a reverse primer with a NsiI site (GGGGCGGCGGCTTCAATTCACTAT) (18). The OVA construct was cloned into pCI/Pol using XhoI/NsiI to generate the OVA constructs. pCI/cT-OVA1: An OVA_{8–385} encoding fragment was amplified by PCR from pCI/OVA plasmid, using a forward primer with a KpnI site (AAAGTGATCCATCAAGAATGCATGACG) and a reverse primer with a NsiI site (GGGGCGGCGGCTTCAATTCACTAT) (18). The wild-type OVA sequence, the forward primer contained one additional A nucleotide. The product was cloned into pCI/cT-OVA2 vector to generate the pCI/cT-OVA1 construct. This construct encodes a fusion protein consisting of the cT_{1–272} fragment, and, mediated by the frame shift of the additional A nucleotide in the forward primer, 10 residues not related to OVA or large SV40 tumor Ag (T-Ag) (IQGAQSPCCPQ), followed by a stop signal (cT + 10). Due to a start signal in the alternative reading frame, this construct additionally encodes a N-terminal 40-residue product not related to OVA or T-Ag. (MILKORKRLKLKCPGSLVELKEKLKH HANENIFYCVPIA), followed by the OVA_{8–385} fragment (OVA*). pCI/cT-OVA2: The OVA_{8–385} coding fragment was amplified by PCR from the pCI/OVA plasmid using a forward primer with a KpnI site (AAAGTGATCCATCAAGAATGCATGACG) and a reverse primer with a NsiI site (GGGGCGGCGGCTTCAATTCACTAT) (18). The product was cloned into pCI/cT_{1–272} vector to generate the pCI/cT-OVA2 DNA construct expressing the cT_{1–272}/OVA fusion protein plus 3-amino acid (IQG) not related to T-Ag or OVA (cT-OVA).

Hepatitis B core Ag-encoding plasmid. The pCIC DNA vaccine has been described (30).

Transient expression assays

Chicken hepatoma LMH cells (CRL-2117; American Type Culture Collection; Promocochem) or human epithelial kidney HEK293 cells (CRL-1573; American Type Culture Collection; Promocochem) were transiently transfected with plasmid DNA using the CaPO_4 method, as described previously (28, 29). Where indicated, cells were labeled with [35S]methionine, extracted with lysis buffer, and immunoprecipitated using the anti-Pol mAb 9F9, polyclonal rabbit anti-SV40 T-Ag and protein A-Sepharose. The precipitates were processed for SDS-PAGE, followed by fluorography. Alternatively, cellular extracts from unlabelled cells were directly processed for SDS-PAGE and analyzed by Western blotting using the anti-Pol mAb 9F9, polyclonal rabbit anti-SV40 T-Ag serum, or polyclonal anti-OVA serum (C-6534; Sigma-Aldrich), followed by a 35S-labeled protein plus 3-amino acid (IQG) not related to T-Ag or OVA (cT-OVA).

DNA vaccination

We injected 100 μg of DNA (50 μl of PBS containing 1 μg/μl plasmid DNA) into each tibialis anterior muscle, as described (32).

Peptides

Synthetic, HPLC-purified peptides used in the described experiments were obtained from Jerini BioTools.

Splenic specific CD8+ T cell frequencies

Spleen cells (1.5 × 10^6/ml) were incubated for 1 h in serum-free BioWhitaker-Ultra Culture medium (catologue number BE12-725F; Cambrex Bio Science) with the indicated amounts of the specific or the control peptides. Thereafter, 5 μg/ml brefeldin A was added, and the cultures were incubated for further 4 h. Cells were harvested and surface stained with PE-conjugated anti-CD8 mAb (catologue number 01055B; BD Pharmingen). Surface-stained cells were fixed with 2% paraformaldehyde in PBS before further staining for intracellular cytokines. Cytokine-generated cultures were incubated in 200 μl of phorbol ester (1 nM) and anti-CD3 antibodies (1 μg/ml) in 200 μl of activation buffer (HBSS, 0.5% BSA, 0.5% saponin, and 0.05% sodium azide) and incubated with FITC-conjugated anti-IFN-γ mAb (catologue number 55441; BD Pharmingen) for 30 min at room temperature and

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washed in permeabilization buffer. Stained cells were resuspended in PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide. We determined the frequencies of IFN-γ* CD8*T cells by flow cytometry analyses. The mean numbers of IFN-γ* CD8*T cells/10⁵ CD8*T cells are shown.

Results
Expression of viral Ags from alternative reading frames of a DNA vaccine

The pCI/SX vector contains sequences encoding the small HBsAg (S) and its 3’ untranslated sequence up to the HBV poly(A) sequence. It also contains in an overlapping, alternative ORF the sequence encoding the C-terminal Pol344–832 fragment (Fig. 1, B and C). The nucleic acid sequence as well as the amino acid sequences of the alternative HBsAg- and Pol-specific ORFs for the pCI/SX DNA vaccine as well as the A2-restricted epitopes are shown in Fig. 2. The pCI/Pol vector encodes the 832 residue Pol protein, and the large HBsAg (LS) in an alternative reading frame (Fig. 1, B and C) (17). Cells transfected with pCI/SX plasmid DNA efficiently expressed (glycosylated and nonglycosylated) small HBsAg (S) (Fig. 1D). Cells transfected with the pCI/Pol vector expressed lower levels of the (glycosylated and nonglycosylated) large pre-S1/pre-S2/S (LS), middle pre-S2/S (MS), and small (S) HBsAg (Fig. 1D) (17). We did not detect expression of Pol or its fragments in cells transfected with either pCI/SX or pCI/Pol plasmid DNA using anti-Pol mAb 9F9 that binds an extreme C-terminal linear epitope with high affinity (Fig. 1D) (31).

An immunogenic Pol epitope is generated from a product translated from the alternative reading frame of the HBsAg-encoding expression plasmid pCI/SX

A2-tg mice were injected with the pCI/SX DNA vaccine. Spleen cells obtained from these mice 12–14 days postvaccination were restimulated ex vivo with titrated amounts of the Pol- or HBsAg-derived peptides (Fig. 2B). The numbers of CD8*T cells specifically inducible to IFN-γ expression by each of the peptides were determined by flow cytometry. The pCI/SX DNA vaccine efficiently primed CD8*T cell responses to the A2-restricted epitopes S1 and S2 of HBsAg and also to the Pol3 epitope of Pol (Fig. 3). This vaccine did not prime CD8*T cell responses to the Pol1 and Pol2 epitope of Pol (Fig. 3). The pCI/Pol DNA vaccine (which coexpresses HBsAg and Pol from alternative reading frames) efficiently primed CD8*T cell responses to the Pol1 and Pol3, but prime responses inefficiently to the Pol2 epitope and the HBsAg epitopes S1 and S2 (Fig. 3). CD8*T cell responses to Pol3 were always higher than those to Pol1 (Fig. 3). Interestingly, the magnitude of the Pol3-specific T cell responses primed by either pCI/Pol (Pol3 epitope processed from the primary ORF) or pCI/SX (Pol3 epitope processed from the alternative ORF) was comparable (Fig. 3). Stimulation of splenic T cells ex vivo with titrated amounts of the different MHC-I-binding peptides of Pol reached saturating conditions when >2.5 × 10⁻⁷ M respective peptides were used (Fig. 3). Under these saturating conditions, CD8⁺
IFN-γ T cell frequencies were comparable between the different groups. Control experiments confirmed the specificity of the responses to Pol and HBsAg. These responses did not cross-react to the C1 epitope of the control core Ag of HBV (Fig. 3). Similarly, vaccination with pCI/C (encoding the HBV core protein) induced a C1-specific CD8 T cell response that did not cross-react with the Pol or HBsAg epitopes (Fig. 3). Thus, our data demonstrate that the Pol3 epitope can be efficiently processed from peptides generated from translation products of an alternative ORF. Stable and unstable expression of Pol (fragments) gives rise to Pol3-specific T cell responses generated from products translated from alternative ORFs have been identified in different experimental systems (4–10), but we report in this work the first example that it also operates in DNA vaccination. Sensitive CD8 T cell-priming/presentation assays have been used to detect expression of such cryptic protein products. Confirming this finding, we found strong T cell responses to the Pol3 epitope generated from products of alternative reading frames. But, it proved difficult to detect cryptic translation products in biochemical assays. We could not detect Pol fragments in lysates of metabolically labeled, pCI/SX-transfected cells using immunoprecipitation and/or Western blotting with the Pol-specific mAb 9F9 (Fig. 1D). Also, Pol expression was not detected in pCI/Pol-transfected cells using the same protocols (Fig. 1D) (17). These data show that Pol protein expression is difficult to detect. We cannot distinguish between low Pol expression and a high turnover rate of Pol. Hence, specific T cell priming by DNA vaccination is more a sensitive readout than most currently available biochemical tools to detect products from in-frame or out-of-frame transcription/translation.

We next asked whether stable overexpression of a Pol fragment enhances Pol3-specific T cell priming. The Pol585–832 fragment encodes only the Pol3 epitope (but not the Pol1 or Pol2 epitope) (Figs. 1, B and C, and 2A). The heat shock protein 73 (hsp73)-facilitated expression system (28) was used to overexpress this Pol fragment. Its sequence was fused in frame behind the hsp73-capping, DnaJ-homologous cT1–272 protein domain derived from a cytosolic SV40 T-Ag variant. This generated the pCI/cT-Pol585–832 DNA vaccine (Fig. 1C). The hsp73-bound cT-Pol585–832 fusion protein was efficiently expressed in transfected cells and accumulated to high levels (Fig. 1D). A2-tg mice vaccinated with this DNA vaccine developed a Pol3-specific CD8 T cell response. The magnitude of this response was comparable to that primed by DNA vaccines expressing Pol either in frame (pCI/Pol) or out of frame (pCI/SX) (Fig. 3). Thus, DNA vaccines with strikingly different levels of Pol expression prime Pol3-specific T cell responses with similar efficacy. The magnitude of Pol expression thus does not correlate with efficient generation of immunogenic epitopes. Rapid Pol degradation may make its detection of expression from pCI/Pol or pCI/SX difficult, but may facilitate the generation of immunogenic peptides.

Different HBsAg-encoding expression constructs support CD8 T cell priming to the Pol3 epitope

We constructed variants of plasmid pCI/SX, tested their HBsAg expression, and measured their relative efficacy to prime A2-restricted T cell responses. We introduced the following deletions into the pCI/SX construct (Fig. 4A): deletion of C-terminal sequences encoding the Pol570–832 sequence (construct 2: pCI/S); elimination of the intron between the promoter and the start codon of HBsAg (construct 3: pCI/SX\_P); or elimination of the promoter (construct 5: pCI/SX\_P). Furthermore, we exchanged HCMV-derived promoter sequences with SV40 promoter sequences (construct 4: pSI/SX). These constructs were tested for HBsAg expression (Fig. 4B). Efficient HBsAg expression was apparent from pCI/SX (lane 1) and pCI/S (lane 2). Expression of HBsAg was lower when the intron was deleted (pCI/SX\_P; lane 3).
3), or when the promoter sequences were changed (pSI/SX; lane 4), confirming previous results (32). No HBsAg expression was detected from the pCI/SX\_\text{AP} plasmid (lane 5). Pol-specific translation products were not detected in transfected cells (data not shown).

In A2-tg mice vaccinated with these constructs, CD8\(^+\) T cell priming to the S2 and Pol3 epitopes was determined (Fig. 4C). S2-specific CD8\(^+\) T cell responses were efficiently elicited in mice vaccinated with the pCI/SX, pCI/S, pCI/SX\_\text{AP}, or pSI/SX DNA vaccines. The pCI/SX\_\text{AP} did not elicit S2-specific CD8\(^+\) T cell responses. CD8\(^+\) T cell responses to Pol3 were efficiently induced in mice by vaccination with pCI/SX or pCI/SX\_\text{AP}, but not with pCI/S, pCI/SX\_\text{AP}, or pSI/SX. HBsAg expression from HCMV-derived, but not SV40-derived promoter sequences thus supported generation of the immunogenic Pol3 epitope from a translation product of an alternative ORF. Intron sequences were not necessary for Pol3 priming.

Expression constructs encoding Pol fragments in alternative and primary reading frames support Pol3-specific CD8\(^+\) T cell priming

Vaccinating mice with the pCI/SX DNA vaccine (encoding the Pol\(_{144–832}\) fragment in an alternative ORF) primed CD8\(^+\) T cells specific for the Pol3, but not the Pol1 epitope (Fig. 3). A switch to the alternative ORF that generates the immunogenic Pol products may thus operate downstream of the position of the Pol1 epitope (Fig. 2A). To test whether immunogenic Pol3 peptides are translated from different cryptic or primary reading frames dependent on specific Pol sequences, we constructed pCI/SX-based nonsense vectors. The pCI/SX\_\text{nonsense} \_A vector was created by deleting the NheI/XhoI fragment (encoding the S-specific start codon) from the pCI/SX plasmid. The remaining sequence encodes the Pol\(_{136–832}\) and S\(_{42–226}\) fragments (Fig. 5A). Assuming that the first ATG motif is used to start translation, this construct may produce a S\(_{75–226}\) fragment from the primary ORF and a Pol\(_{133–832}\) fragment from an alternative ORF (Fig. 5A). Transient transfection followed by Pol-specific immunoprecipitation and/or Western blot analyses did not detect Pol or S fragments (data not shown), but a similar 1–74 deletion construct of HBsAg has been detected (33).

A2-tg mice were vaccinated with the pCI/SX or pCI/SX\_\text{nonsense} \_A constructs. CD8\(^+\) T cell priming to the S2, Pol1, and Pol3 epitopes (present on both constructs) was determined (Fig. 5). Pol3-specific (but not Pol1-specific) CD8\(^+\) T cell responses were efficiently elicited in mice vaccinated with both constructs. The pCI/SX\_\text{nonsense} \_B DNA coinduced S2-specific CD8\(^+\) T cell responses (Fig. 5B). Thus, truncated coding sequences support generation of immunogenic epitopes from both reading frames.

We further constructed the pCI/SX\_\text{nonsense} \_B vector containing the overlapping coding regions Pol\(_{447–832}\) and S\(_{144–226}\), and the pCI/SX\_\text{nonsense} \_C vector containing only the Pol\(_{685–832}\)-coding regions (Fig. 5A). These constructs did not express detectable levels of Pol or S (data not shown). Assuming that the first ATG motif is used to start translation, these constructs may produce the Pol\(_{699–832}\) or Pol\(_{499–832}\) proteins from the primary ORF (Fig. 5A). A2-tg mice vaccinated with these constructs generated high CD8\(^+\) T cell responses to Pol3 (Fig. 5B). Priming of Pol3-specific CD8\(^+\) T cells is comparable when translation products were generated from Pol fragments expressed from alternative ORFs (pCI/SX; pCI/SX\_\text{nonsense} \_A) or primary ORFs (pCI/SX\_\text{nonsense} \_B; pCI/SX\_\text{nonsense} \_C) (Fig. 5). The vector pCI/SX\_\text{nonsense} \_B encodes the S\(_{144–226}\) sequence, but there is no ATG in this ORF preceding the S2 epitope (Fig. 5A). Hence, no S2-specific responses were primed with this construct.

Induction of OVA-specific CD8\(^+\) T cell responses

We extended the study to an unrelated Ag system using OVA. We compared generation of an immunogenic K\(^\alpha\)-restricted OVA
epitope from primary and an alternative translation product. We designed a pCI/cT-OVA1 expression plasmid that encodes the hsp73-binding cT1–272 fragment and a 10-residue spacer IQGAQSPPCQ in the primary ORF (cT/H1100110; Fig. 6, A and B). This construct also encodes (in an alternative open reading frame) a fusion protein containing N terminally a 40-residue fragment MILIQKRRKLNKCPGSLVPFKELKVHHANENIFYCPIAI initiated from an upstream ATG start signal and the OVA35–385 (OVA*; Fig. 6, A and B). In the control pCI/cT-OVA2 construct, we inserted the OVA18 –385 sequence C terminally in frame to the cT1–272 fragment to generate the cT-OVA18 –385 fusion protein (cT-OVA; Fig. 6, A and B). We further cloned a OVA8–385 sequence into the pCI vector to generate the pCI/OVA construct (OVA; Fig. 6). These constructs allowed the comparative evaluation of the immunogenicity of the Kb-restricted OVA257–264 epitope SIINFEKL expressed from either the alternative (pCI/cT-OVA1) or primary (pCI/cT-OVA2, pCI/OVA) OVA-encoding ORFs. OVA-specific Western blotting revealed efficient expression of the OVA protein or the cT-OVA fusion protein by cells transfected with pCI/OVA or pCI/cT-OVA2 plasmid DNA (Fig. 6C). Cells transiently transfected with pCI/cT-OVA1 plasmid DNA efficiently expressed the N-terminal cT + 10 protein, but not the OVA* fragment, as expected (Fig. 6C).

B6 mice were vaccinated with pCI/cT-OVA1, pCI/cT-OVA2, pCI/OVA, or control pCI plasmid DNA (100 μg/mouse i.m.). The OVA- and T-Ag-specific CD8+ T cell responses were read out 12 days postvaccination. CD8+ T cell responses of B6 mice specific for the Kb-restricted OVA257–264 epitope were comparable when the epitope was generated by translation products generated from primary ORFs (pCI/cT-OVA2, pCI/OVA) or an alternative ORF (pCI/cT-OVA1) (Fig. 6D). pCI/cT-OVA1 and pCI/cT-OVA2 DNA vaccines primed similar CD8+ T cell responses to the SV40 T-Ag, i.e., the Db-restricted T206–215 epitope SAINNYAQKL (Fig. 6D) (34). These data confirm the efficient generation of CD8+ T cell-stimulating epitopes by HCMV-driven translation products of alternative reading frames.

**Discussion**

We used in vivo priming by a single injection of a DNA vaccine to assess (in a quantitative and specific CD8+ T cell readout) the relative efficacy with which immunogenic epitopes are generated from in-frame (primary ORF) vs out-of-frame (alternative ORF) translation products using different Ag systems. We demonstrate that CD8+ T cell responses specific for unrelated epitopes processed from cryptic translation products are efficiently primed by simple DNA vaccines. These data support the notion that expressing Ag in the primary ORF of a DNA vaccine may lead to copriming of immunogenic, MHC class I-binding epitopes generated from cryptic translation products of alternative reading frames.

The HBsAg-encoding pCI/SX DNA vaccine efficiently primed CD8+ T cell responses to the A2-restricted epitopes S1 and S2 of HBsAg, and to the Pol3 epitope of Pol. Thus, T cell responses were
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Characterization of CD8+ T cell responses to Pol3 (2)

**A** Constructs encoding different HBsAg/Pol sequences in the primary (p) or alternative (a) ORF

![Diagram](https://via.placeholder.com/150)

**B** Vaccination of A2-tg mice

![Graphs](https://via.placeholder.com/150)

efficiently primed by immunogenic translation products of in-frame and out-of-frame reading frames of a DNA vaccine. The Pol1 and Pol2 epitopes also encoded in this alternative reading frame (defined by the first ATG start signal downstream of the promoter) are indicated. Furthermore, the primary (p) and alternative (a) reading frames are indicated.

Conventional expression vectors used in DNA vaccines contain a transcription unit encoding the Ag sequence cloned downstream from a strong promoter/enhancer sequence that drives expression of the immunogenic protein from a primary reading frame. Promoter sequences used in DNA vaccines are often from viruses, e.g., CMV, SV40, or retroviruses. Our data suggest that the type of promoter used to express an Ag in a DNA vaccine has an influence on the efficacy of generating epitopes from alternative reading frames. HCMV-driven pcIX/SX, but not SV40-driven pSI/SX DNA vaccines induced Pol3-specific T cell responses. Hence, HCMV-derived promoter sequences are apparently more efficient than SV40-derived promoter sequences in supporting generation of cryptic translation products from which immunogenic epitopes can be processed, but the molecular events underlying this finding are unclear. We have shown that DNA vaccines with SV40-derived promoter sequences produce lower levels of Ag from the primary reading frame than vaccines in which Ag expression is driven by HCMV-derived promoter sequences (Fig. 3B) (32). Thus, the efficacy of a promoter may play a role in the decreased generation of cryptic Pol-specific translation products. Alternatively, different promoter sequences may have different fidelities with which they use a primary reading frame.

We used different approaches to detect Pol-derived translation products from alternative reading frames using the anti-Pol mAb 9F9 directed against the extreme C terminus of Pol close to the location of the Pol3 epitope. We tried to detect newly synthesized Pol or Pol fragments in lysates of 35S-labeled transfectants or their steady state levels by Western blotting. We were only successful in detecting hsp73-associated cT-Pol585-832 fusion Ag, but not in detecting the Pol protein nor any cryptic Pol fragments (Fig. 1D). Thus, Pol expression does not seem to correlate with efficient generation of immunogenic epitopes because apparently very different levels of Pol expression from DNA vaccines primed T cell responses in mice of comparable efficacy (Fig. 3).
FIGURE 6. CD8$^+$ T cell responses to OVA expressed by DNA vaccines in a primary or an alternative reading frame. A and B, Maps and sequences of the pCI/cT-OVA1, pCI/cT-OVA2, and pCI/OVA constructs. The pCI/cT-OVA1 construct encodes two proteins: the cT + 10 protein that encodes the cT1–272 protein plus 10 (C-terminal) residues not related to OVA or T-Ag (IQGAQSPPCQ) and, in an alternative ORF, the OVA$^*$ protein that encodes 40 N-terminal residues (MILIQKQKRLNKCPGSLYFPFELKHSVHANE NIFYCPIAI) and the OVA15–385. The pCI/cT-OVA2 construct encodes the cT1–272-OVA18–385 (cT-OVA) fusion protein in the primary reading frame. pCI/OVA encodes a OVA8–385 (OVA) protein in the primary ORF. C, Expression of OVA-encoding constructs. Human epithelial kidney HEK293 cells were transiently transfected with DNA from the pCI/cT-OVA1, pCI/cT-OVA2, pCI/OVA, or (control) pCI constructs. Nonlabeled extracts of transfected cells were processed for OVA- or T-Ag-specific Western blotting (using polyclonal anti-OVA or anti-T-Ag sera and $^{35}$S-labeled protein A). The position of OVA, cT-OVA, and cT1–272 is indicated. D, B6 mice were vaccinated i.m. with the pCI/cT-OVA1, pCI/cT-OVA2, pCI/OVA, or (control) HBsAg vaccine. Spleen cells obtained 12–14 days postvaccination were restimulated for 4 h ex vivo with the antigenic, Kb-binding OVA257–264, Db-binding T206–215, or (control) HBsAg peptide. Cells were surface stained for CD8 and intracellularly stained for IFN-$\gamma$. Mean numbers of IFN-$\gamma^+$ CD8$^+$ T cells/10$^5$ splenic T cells (±SD) of three mice from a representative (of three independent) experiment(s) are shown.
nascence protein (but not the stability of mature proteins) is critical in generating MHC class I-binding epitopes (3).

We tested different expression constructs encoding HBsAg and Pol fragments from alternative reading frames of the same sequence. In the pCI/SX<sub>nonsense</sub> A construct, two reading frames apparently generated protein fragments that could not be detected by biochemical assays (Fig. 5). However, this nonsense construct induced CD<sup>8</sup><sup>+</sup> T cell responses to Pol and HBsAg. HCMV-derived promoter sequences can apparently initiate expression from different reading frames of the same sequence and generate products that can elicit CD<sup>8</sup><sup>+</sup> T cell responses in vivo (Fig. 5).

We assume that the first ATG motif is used to start translation and hence defines the primary reading frame. Different Pol constructs, encoding different N-terminal sequences of Pol (Pol<sub>144–832</sub>, Pol<sub>417–832</sub> or Pol<sub>685–832</sub>) in the primary or alternative reading frame, induced comparable Pol3 (Pol<sub>103–811</sub>)-specific T cell responses (Fig. 5). Thus, the HCMV promoter is able to drive translation of immunogenic products from primary as well as alternative reading frames. We further confirmed efficient HCMV-driven generation of cryptic expression units with a DNA construct (pCI/T-OWA1) encoding an SV40 T-Ag-derived cT + 10 protein in the primary reading frame and an OVA<sub>188–358</sub>-encoding OVA<sup>+</sup> protein in an alternative ORF (Fig. 6). This construct elicited in B6 mice OVA-specific CD<sup>8</sup><sup>+</sup> T cell responses with an efficacy comparable to that of DNA constructs that encode OVA or the cT-OWA fusion protein (i.e., pCI/OWA or pCI/T-OWA2). Thus, the HCMV promoter is able to drive translation of immunogenic products from an alternative OVA-encoding reading frame. The molecular mechanism underlying this observation is unknown. The phenomenon seems to have general importance. We demonstrated efficient use of alternative reading frames for the generation of immunogenic, T cell-stimulating epitopes in two unrelated and independent systems (HBV-Pol/S and OVA). It operated efficiently in two different cryptic ORFs, and was independent of intron sequences and of the level of protein translation from primary or alternative reading frames.

Generation of immunogenic epitopes from alternative reading frames is not restricted to viral Ag systems, but may be a feature intrinsic to the transcription or protein synthesis machinery (3). Experiments with OVA-encoding constructs confirmed the validity of the concept that out-of-frame translation products are a rich source of immunogenic epitopes, despite the fact that these translation products are not found by sensitive protein detection tools. This offers the chance to build an extended repertoire of antigenic exceptions that prove the rules?

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Disclosures

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