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Immunology

# Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation

(alphaviruses/RNA virus/gene expression/epitope mapping/mutagenesis)

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ABSTRACT **Sindbis virus (SIN) is a small positive-strand enveloped RNA virus that infects a broad range of vertebrate and insect cells. A SIN vector (called dsSIN), designed for transient expression of heterologous RNAs and proteins, was engineered by inserting a second subgenomic mRNA promoter sequence into a nonessential region of the SIN genome. By using this vector, dsSIN recombinants have been constructed that express either bacterial chloramphenicol acetyltransferase, a truncated form of the influenza hemagglutinin (HA), or minigenes encoding two distinct immunodominant cytotoxic T lymphocyte (CTL) HA epitopes. Infection of murine cell lines with these recombinants resulted in the expression of $\approx 10^6$–$10^7$ chloramphenicol acetyltransferase polypeptides per cell and efficient sensitization of target cells for lysis by appropriate major histocompatibility complex-restricted HA-specific CTL clones *in vitro*. In addition, priming of an influenza-specific T-cell response was observed after immunizing mice with dsSIN recombinants expressing either a truncated form of HA or the immunodominant influenza CTL epitopes. This SIN expression system allows the generation of high-titered recombinant virus stocks in a matter of days and should facilitate mapping and mutational analysis of class I major histocompatibility complex-restricted T-cell epitopes expressed via the endogenous pathway of antigen processing and presentation.**

Sindbis virus (SIN) is the prototype member of the *Alphavirus* genus of the family *Togaviridae* (for review, see ref. 1). Alphavirus virions mature by budding from infected host cells and consist of an icosahedral nucleocapsid surrounded by a lipid bilayer containing virus-encoded glycoproteins. The SIN genome consists of a single-stranded mRNA molecule of 11,703 bases with a 5′ cap structure and a 3′ poly(A) tract. Upon entry of the host cell, the 5′ two-thirds of the genomic RNA is translated to produce replicase proteins essential for cytoplasmic RNA synthesis. RNA amplification is initiated by the synthesis of full-length complementary minus-strand RNAs, which serve as templates for synthesis of additional genome-length plus strands. By initiation at an internal promoter sequence, genome-length minus strands are also used for synthesis of 3′-terminal subgenomic mRNAs that encode the SIN structural proteins. In certain vertebrate cell types, the SIN replication machinery can produce an estimated $5 \times 10^5$ molecules per cell of the subgenomic mRNA, $\approx 10^7$–$10^8$ molecules per cell of the virion structural proteins, and released virus titers in excess of $10^3$ infectious particles per cell. As a transient expression system for heterologous RNAs and proteins, SIN offers several potential advantages: (*i*) a broad range of susceptible host cells, including those of insect, avian, and mammalian origin; (*ii*) high levels of cytoplasmic RNA and protein expression without splicing; and (*iii*) the facile construction and manipulation of recombinant RNA molecules by using a full-length SIN cDNA clone from which infectious RNA transcripts can be generated by *in vitro* transcription (2).

Previous studies showed that replacement of the SIN structural protein coding region with the bacterial chloramphenicol acetyltransferase (CAT) gene resulted in a self-replicating RNA, transcription of a subgenomic CAT mRNA, and $\approx 10^8$ polypeptides per cell of active CAT (3). Since these RNA constructs do not encode structural proteins, their usefulness is limited by the efficiency of RNA transfection or alternatively requires the use of a helper system for packaging (refs. 3 and 4; B. Pragai and C.M.R., unpublished results). Here, we report the construction of SIN vectors that are both replication and packaging competent and that allow the rapid recovery of high-titered infectious recombinant virus stocks. Heterologous polypeptides are efficiently expressed via translation of a second subgenomic mRNA, which initiates from an additional subgenomic promoter located in the SIN 3′ noncoding region (NCR). Such SIN recombinants (called dsSIN), expressing either a truncated form of the influenza hemagglutinin (HA) protein (trHA) or minigenes encoding immunodominant cytotoxic T lymphocyte (CTL) epitopes, can infect murine tissue culture cells and sensitize them for lysis by appropriate class I major histocompatibility complex (MHC)-restricted CTLs. Hence, this system is immediately useful for rapid mapping and fine-structure analysis of T-cell epitopes expressed via the endogenous antigen-presentation pathway. In addition, immunization of mice with these SIN–influenza recombinants allows priming of influenza epitope-specific CTL responses *in vivo*.

## MATERIALS AND METHODS

**Shuttle Phagemids and dsSIN Vectors.** Standard methods were used for plasmid constructions and analysis (5). Plasmid structures were verified by restriction endonuclease analysis, and all regions derived by using PCR amplification (6), site-directed mutagenesis (7), or oligonucleotide adaptors were verified by sequence analysis.

Shuttle phagemid pH3′2J1 (Fig. 1) was constructed to facilitate mutagenesis and cloning of heterologous cDNAs into full-length SIN vectors designed for expression via a second subgenomic promoter located in the 3′ NCR. The second subgenomic promoter cassette, derived by PCR, contains an *Apa* I recognition site, a functional SIN promoter sequence from −109 to +49 (numbered relative to the nucleotide in the genome-length plus strand, which corresponds to the first nucleotide of the subgenomic mRNA; ref. 9), a

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Abbreviations: SIN, Sindbis virus; CAT, chloramphenicol acetyltransferase; NCR, noncoding region; HA, hemagglutinin; CTL, cytotoxic T lymphocyte; pfu, plaque-forming unit(s); FBS, fetal bovine serum; VVHA, recombinant vaccinia virus expressing influenza HA; MHC, major histocompatibility complex; CEF, chicken embryo fibroblasts; trHA, truncated form of the influenza HA protein.

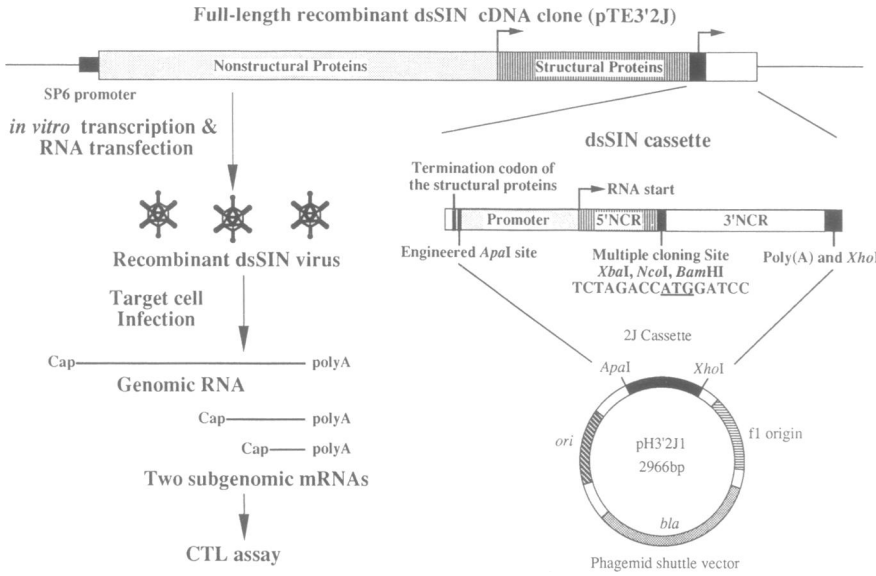


FIG. 1. Generation of recombinant dsSIN constructs. At the top is shown a schematic of a full-length dsSIN vector (plasmid pTE3'2J) containing an additional subgenomic mRNA promoter (marked by arrows) located immediately 3' to the open reading frame encoding the SIN structural proteins. The phagemid shuttle vector pH3'2J1, which contains several unique restriction sites (*Xba* I, *Nco* I, and *Bam*HI) to facilitate cloning heterologous sequences downstream from the second promoter and the 49 base 5' NCR derived from the SIN 26S subgenomic mRNA, is also shown. Surrounding the ATG of the *Nco* I recognition are nucleotides that facilitate eukaryotic translation initiation (8). After linearization of full-length plasmid templates with *Xho* I, 5' capped run-off transcripts are produced by using SP6 RNA polymerase. Recombinant dsSIN virus stocks are harvested after RNA transfection of BHK-21 cells and used for subsequent infection and transient expression assays.

sequence containing the recognition sites for *Xba* I, *Nco* I, and *Bam*HI (5'-TCTAGACCATGGATCCTAGA-3'), and the 3' NCR from pToto1101 from nucleotide 11,386 (2), which includes a 3' terminal poly(A) tract followed by an *Xho* I recognition site. The phagemid also contains the β-lactamase gene, the col E1 origin, and an f1 phage origin allowing rescue (10) of single-stranded SIN genome-sense DNA templates for mutagenesis.

An *Apa* I recognition site was inserted into the 3' NCR of full-length Sindbis cDNA clone pTE12 (11) after nucleotide 11,385. The dsSIN parent (called pTE3'2J) contains the second subgenomic promoter cassette inserted into the *Apa* I derivative of pTE12 using the *Apa* I and *Xho* I restriction sites (Fig. 1). dsSIN:CAT was constructed by inserting the *Xba* I–*Xho* I CAT module from pTRCAT (3) into the *Xba* I site of pTE3'2J after filling in the cohesive ends (5). dsSIN:trHA was constructed by subcloning the *Hin*dIII–*Eco*RI fragment from pMT21HA (ref. 12; Y.S.H., unpublished data) into the *Xba* I site of pTE3'2J after filling in the cohesive ends (5). dsSIN derivatives expressing short immunodominant influenza virus (A/Japan/57) polypeptides (see *Results*) were constructed by using the pH3'2J1 shuttle phagemid and synthetic double-stranded oligonucleotide adaptors flanked by 5' *Xba* I and 3' *Bam*HI restriction sites.

**Cell Lines.** Nonadherent P815 mastocytoma cells (*H-2*$^d$) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (wt/vol) glutamine. L929 monolayer cell cultures (*H-2*$^k$) were maintained in MEM supplemented with 7–10% FBS. The EL4 cell line (*H-2*$^b$) was maintained in RPMI 1640 and supplemented with 10% FBS and 1% glutamine. BHK-21 (American Type Culture Collection, clone 13) cultures were maintained in MEM containing 7% FBS. Secondary cultures of chicken embryo fibroblasts (CEF) were prepared as described (13).

**Virus Stocks.** Influenza virus strain A/Japan/305/57 (H2N2 subtype) was grown in the allantoic cavity of 10-day-old chicken embryos (14). dsSIN stocks were produced by lipofectin-mediated transfection of BHK-21 cells by using capped RNAs derived by SP6 RNA polymerase transcription of *Xho* I-linearized plasmid templates (2). Culture supernatants were harvested at 24 h postinfection, and aliquots were stored at −80°C until used. dsSIN virus titers were determined by plaque assay on CEF monolayers (15), and typical yields were over $10^{10}$ plaque-forming units (pfu) from ≈$10^7$ cells (≈$10^9$ pfu per ml).

**CTL-Mediated Cytolysis Assay.** Suspensions of P815 cells or trypsinized L929 cells were prepared and washed with MEM. Cells ($10^6$) were resuspended in 0.5 ml of MEM and infected for 1 h at 37°C with either dsSIN, the various dsSIN recombinants (at 50 pfu per cell as determined on CEF monolayers), or a recombinant vaccinia virus expressing influenza HA (VVHA). Following $^{51}$Cr labeling (150 μCi per 0.5 ml; 1 Ci = 37 GBq) for 2 h at 37°C, cells were counted and distributed in flat-bottom wells of 96-well plates ($10^4$ cells per well), appropriate CTLs were added, and incubation was continued for 6 h at 37°C. Effector cells (at an effector-to-target cell ratio of 5:1) included bulk CTL populations (16) or cloned CTL lines (17), which were passaged weekly in the presence of irradiated influenza A/Japan/305/57-infected splenocytes and a source of exogenous T-cell growth factor. The percentage of specific $^{51}$Cr release was determined by standard procedures (18). Each data point represents the mean of quadruplicate samples; standard deviations were typically <5%.

**Priming T-Cell Responses by Using dsSIN Recombinants.** Six- to 12-week-old BALB/cByJ (*H-2*$^d$) mice (The Jackson Laboratory) were infected by intraperitoneal injection of $10^6$ pfu of each dsSIN recombinant or parental SIN TE12 (11). Two weeks later, splenocytes from immunized mice were stimulated *in vitro* with influenza virus-infected syngeneic splenocytes and assayed for cytolytic activity on day 5 or 6.

## RESULTS

**Replication and Packaging-Competent SIN Vectors Expressing a Second Subgenomic mRNA.** Infectious SIN vectors were engineered to express foreign sequences under the control of an additional SIN subgenomic promoter, a strategy similar to that used for two positive-strand RNA plant viruses (19, 20). Several previous results are relevant to the design of these dsSIN vectors. First, the core promoter for SIN subgenomic mRNA transcription maps from position −19 to +5 relative to the subgenomic mRNA start (21); however, this sequence is ≈6-fold less active than the sequence from −98 to +14 (22). Second, the SIN promoter element (also called the "junction region"; ref. 21) allows precise transcription initiation when translocated into different SIN sequence contexts (21–23). Third, substantial alterations in the SIN 3' NCR are tolerated without affecting SIN replication in vertebrate cells (24), making this a likely insertion site for additional sequences. These observations suggested that foreign sequences, located downstream from a second subgenomic promoter ele-

ment in the 3′ NCR, might be efficiently expressed by the SIN transcription machinery via synthesis of a second subgenomic mRNA. pTE12 (11) was chosen as the parental full-length SIN cDNA clone for vector constructions since SIN TE12 exhibits enhanced expression levels in certain murine cell lines (unpublished results), which might allow efficient sensitization of target cells for CTL assays (see below). SIN TE12 is a SIN hybrid consisting of envelope proteins derived from a mouse-adapted neurovirulent SIN variant (25) and the remainder from the SIN lab strain Toto1101 (2, 23). The prototype vector, pTE3′2J (13,822 base pairs), contains a unique *Apa* I site positioned immediately downstream from the SIN structural protein open reading frame followed by the second promoter element (from −109 to +49), a short polycloning sequence, and the entire SIN 3′ NCR (see Fig. 1 and *Materials and Methods*). This subgenomic promoter element, which includes the sequences corresponding to the subgenomic mRNA 5′ NCR, may yield slightly higher transcription levels than the −98 to +14 promoter element (2) and may also enhance translation of the second subgenomic mRNA. Given the large size of pTE3′2J, a phagemid shuttle vector called pH3′2J1 was constructed to facilitate cloning of heterologous sequences into pTE3′2J, using the unique *Apa* I site and the unique *Xho* I site that immediately follows the 3′ poly(A) tract (Fig. 1).

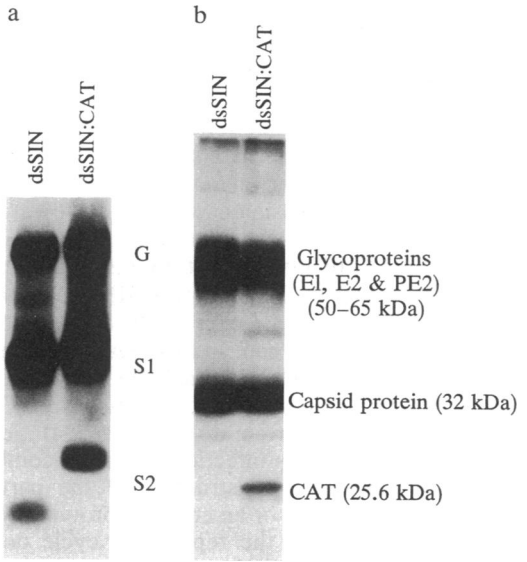


FIG. 2. Virus-specific RNAs and proteins in BHK cells infected with dsSIN recombinants. (*a*) BHK-21 monolayers were mock-infected or infected with either dsSIN or dsSIN:CAT at 50 pfu per cell and incubated at 37°C. Virus-specific RNAs were labeled with [$^3$H]uridine in the presence of actinomycin D from 3 to 6 h postinfection (13). Monolayers were lysed in SDS and proteinase K (26), and the RNAs were separated, without denaturation, by agarose gel electrophoresis in the presence of SDS (26). The positions of the genome-length RNAs (G), the subgenomic mRNAs encoding the SIN structural proteins (S1), and the second 3′ terminal subgenomic RNAs (S2) are indicated. Note that the expected ratio of [$^3$H]uridine label incorporated into S1 and S2 would be 11:1 (dsSIN) or 4:1 (dsSIN:CAT) if equimolar ratios of the two subgenomic RNAs were produced. Although other studies suggest that the second promoter is utilized at somewhat lower efficiency in full-length Sindbis constructs (22), the relative efficiency of the −109 to +49 promoter utilized in our studies has not been determined. (*b*) BHK-21 cells, infected as described above, were labeled with [$^{35}$S]methionine from 6 to 7 h postinfection. Washed monolayers were lysed with SDS, and the proteins were separated by SDS/PAGE using 15% polyacrylamide gels (27). Although not resolved in this gel, the SIN structural glycoproteins (E1 and E2) and precursor PE2 are indicated, as are the capsid protein and CAT and their predicted polypeptide molecular masses.

RNA transcripts of linearized pTE3′2J templates were found to be infectious when used to transfect BHK-21 cells, giving rise to virus with similar plaque morphology to TE12 but with slightly slower kinetics of virus release (data not shown). Culture supernatants with virus titers typically ranging from $10^8$–$10^9$ pfu per ml were obtained 24 h after transfection of BHK-21 cells. The CAT gene was inserted into pTE3′2J as a sensitive reporter molecule for gene expression in eukaryotic cells (see *Materials and Methods*), and the recovered virus (dsSIN:CAT) was used to study synthesis of virus-specific RNAs and proteins as well as expression levels of the heterologous CAT product by enzyme assay (Figs. 2 and 3). Infection of BHK-21 cells with dsSIN or dsSIN:CAT led to the accumulation of subgenomic mRNAs of the sizes appropriate for initiation at their respective second subgenomic mRNA promoters located in the 3′ NCR (Fig. 2*a*). [$^{35}$S]Methionine-labeled CAT protein could be readily detected in extracts from dsSIN:CAT-infected BHK-21 cells (Fig. 2) and, by 7 h postinfection, $\approx 10^7$ molecules of active CAT had accumulated per cell (Fig. 3).

Since one of our goals is to use the SIN expression system to study antigen processing and presentation in murine cells, dsSIN:CAT infectivity and the CAT expression levels were tested for three murine cell lines: L929 (*H-2$^k$*), P815 (*H-2$^d$*), and EL4 (*H-2$^b$*). All three murine cell lines could be infected by dsSIN:CAT, and by 7 h postinfection, $>10^6$ molecules of CAT per cell had accumulated (Fig. 3). Flow cytometry of dsSIN-infected L929 and P815 cells (stained with antiserum specific for the SIN structural glycoprotein E2; ref. 30) demonstrated that essentially 100% of the cells were infected under these conditions (data not shown).

**Sensitization of Target Cells by Using dsSIN Expression of Immunodominant Influenza Virus CTL Epitopes.** Several immunodominant CTL epitopes have been mapped for the influenza virus (strain A/Japan/305/57) HA protein. These include two K$^d$-restricted sites, HA residues 523–545 (called the "001" epitope) and residues 202–221 (called the "006" epitope), and one K$^k$-restricted site, HA residues 252–271 (called the "027" epitope) (31, 32). To examine the use of dsSIN recombinants for expression of CD8$^+$ T-cell epitopes and target cell sensitization, a dsSIN recombinant was constructed that should express a truncated version of the HA protein corresponding to the N-terminal 256 amino acid residues (dsSIN:trHA), including the signal sequence responsible for cotranslational translocation of the HA protein across the endoplasmic reticulum membrane. dsSIN recombinants expressing minigenes encoding 006-containing polypeptides with different flanking sequences (dsSIN:006-1, -2, or -3) or the 027 epitope (dsSIN:027) were also constructed (see *Materials and Methods* and the legend of Fig. 4). Analyses of the RNA patterns from these recombinant dsSIN virus stocks demonstrated the synthesis of second subge-

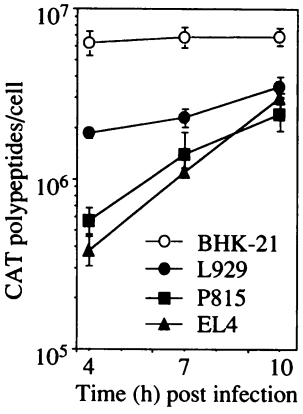


FIG. 3. CAT expression in murine tissue culture cell lines and BHK-21 cells. Murine L929 (*H-2$^k$*), P815 (*H-2$^d$*), EL4 (*H-2$^b$*), or BHK-21 hamster cells were infected with dsSIN:CAT at a multiplicity of infection of 50 pfu per cell (as determined on CEF monolayers) for 1 h at 37°C. Cells were incubated at 37°C for 4, 7, or 10 h and harvested, and CAT enzyme assays were performed as described (28, 29). The data and standard errors are averages from three independent experiments. Background values from uninfected cell extracts were <1000 molecules per cell.

nomic mRNAs of the sizes predicted for each heterologous insert (data not shown).

P815 target cells (*H-2*$^d$) were infected with dsSIN:trHA or the dsSIN:006 recombinants and tested for lysis by 006-specific CTL clones 11-1 and 40-2 (Fig. 4*a*). Previous cytotoxicity assays, using synthetic peptides, suggest that HA residues 202–212 are necessary for recognition and lysis by CTL clone 11-1, whereas residues 211–221 are necessary for lysis by CTL clone 40-2 (32). Target cells infected with either dsSIN:trHA or any of the dsSIN:006 recombinants were efficiently lysed by both CTL clones (Fig. 4*a*), indicating functional processing and presentation of HA epitopes when expressed either as part of a larger protein (trHA) or as small peptides (006-1, -2, and -3). Efficient specific lysis was also observed with either bulk T-cell populations from influenza-primed CBA mice (Fig. 4*b*) or 027-specific CTL clones (data not shown) and L929 (*H-2*$^k$) target cells infected with dsSIN:027. The level of specific lysis was comparable to that observed for influenza virus-infected target cells (Fig. 4*b* and data not shown).

**Generation of Anti-HA-Specific CTLs by Immunization with dsSIN Recombinants.** To examine the ability of dsSIN recombinants to stimulate an influenza HA-specific CTL response, BALB/c mice (*H-2*$^d$) were immunized with dsSIN:trHA or dsSIN:006 or with TE12, as a negative control. After 2 weeks, bulk CTL populations were prepared by stimulating splenocytes from immunized mice with influenza virus and were tested in a cytotoxicity assay. CTLs induced by immunization with dsSIN:trHA or dsSIN:006-1 and one or two *in vitro* stimulations with influenza virus lysed target cells infected with either influenza, VVHA (33), or dsSIN:006-1 (Table 1). Influenza virus-specific lysis did not occur with CTLs induced by SIN priming and SIN secondary stimulation (data not shown). Similar results were obtained when CBA mice (*H-2*$^k$) were immunized with dsSIN:027 (data not shown). These results indicate that dsSIN recombinants, expressing either trHA or short peptide epitopes, are able to prime influenza virus-specific CTL responses *in vivo*.

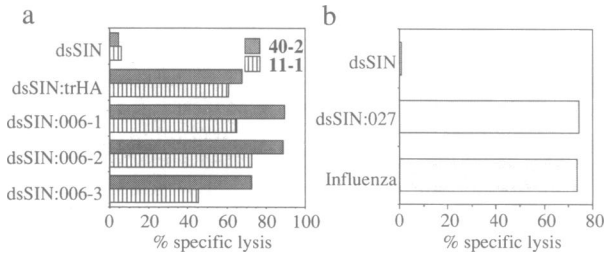


FIG. 4. Target cell sensitization by recombinant dsSIN vectors expressing trHA and immunodominant HA epitopes. (*a*) $^{51}$Cr release assay using P815 target cells (*H-2*$^d$) infected with the dsSIN parent, a recombinant dsSIN expressing the N-terminal 253 amino acids of HA (dsSIN:trHA), or three different dsSIN recombinants expressing the HA 006 epitope (HA residues 202–221; K$^d$-restricted) (32) in different sequence contexts. Cloned CTL lines 40-2 (darkly shaded bars) or 11-1 (hatched bars) (17) were used as effector cells. dsSIN:006-1, dsSIN:006-2, and dsSIN:006-3 express the polypeptides MRTLYQNVGTYVSVGTSTLNKPWILDATPQ, METEQRTLYQNVGTYVSVGTSSLNKRDPRRYAPMIRPAKLDVLPRN, and METEQRTLYQNVGTYVSVGTSSLNKRRS, respectively (where HA residues are underlined). (*b*) $^{51}$Cr release from L929 target cells (*H-2*$^k$) infected with parental dsSIN, influenza virus, or dsSIN:027. The effector cells (stippled bars) were splenic lymphocytes from CBA/J mice (*H-2*$^k$) that had been immunized with VVHA and restimulated *in vitro* with influenza virus (16). dsSIN:027 expresses the K$^k$-restricted HA 027 epitope (HA residues 251–271) (16), MDTINFESTGNLIAPEYGFKIS. The observed levels of spurious release from dsSIN-infected target cells were typically <10% for P815 target cells or <15% for L929 target cells. The higher level of release from dsSIN-infected L929 target cells may be due to virus-induced cytopathic effects because of the faster kinetics of SIN replication in this cell type (see Fig. 3). The % specific lysis = 100 × (specific lysis minus spontaneous lysis)/(total lysis minus spontaneous lysis).

Table 1. Priming of H-2 K$^d$-restricted HA-specific T cells *in vivo*

| Bulk CTLs | % specific lysis of target cells | | | | |
|---|---|---|---|---|---|
| | SIN | Flu | dsSIN:006 | VVHA | Uninfected |
| | Experiment 1 | | | | |
| SIN | 6/11 | 16/30 | 8/11 | ND | 8/13 |
| dsSIN:trHA | 6/10 | 42/68 | 21/37 | ND | 9/19 |
| dsSIN:006 | 9/15 | 55/78 | 25/40 | ND | 13/22 |
| | Experiment 2 | | | | |
| dsSIN:trHA | 12/22 | 75/89 | 41/57 | 39/48 | 14/24 |
| dsSIN:006 | 10/16 | 72/84 | 41/48 | 35/41 | 9/11 |

Splenocytes from mice immunized with the indicated virus were stimulated *in vitro* once (experiment 1) or twice (experiment 2) with influenza virus (Flu) and assayed for cytolytic activity by using either uninfected or infected (as indicated) P815 target cells. Pairs of numbers indicate the two different ratios of effector to target cells used: 5:1/10:1 (experiment 1) or 10:1/20:1 (experiment 2). The rather high background or cross-reactivity observed with influenza virus-infected target cells has sometimes been seen in these and other experiments; the basis for this observation is unknown.

## DISCUSSION

Several features of the dsSIN recombinant vectors described here make them appealing for transient expression of heterologous RNAs and proteins. First, recombinant virus constructs are readily assembled by using well-defined plasmid vectors and conventional methodology. Once plasmid templates for *in vitro* transcription of infectious RNA are made, high-titered infectious virus stocks can be generated in typically less than 24 h after transfection of appropriate host cells and collecting the culture supernatant. Such virus stocks can then be used, without further phenotypic selection or purification, for transient expression assays in a wide variety of different host cells. Some considerations for use of the present system are worth noting. First, for many applications it will be important that the biology of dsSIN replication be compatible with the functional expression of the heterologous product in a given host cell. In this regard, it should be noted that SIN infection can have varied effects on host DNA replication, RNA transcription, and translation of mRNA in different vertebrate and invertebrate cell types (34). Second, the successful recovery of an infectious dsSIN recombinant may depend on the length or structure of the particular heterologous RNA sequence or an encoded protein product. At essentially any stage in the replication cycle one can envision interference with SIN replication, which might preclude the recovery of the desired recombinant. In our limited experience thus far, packaging constraints on the length of dsSIN genomic RNAs appear to be an important determinant for rescue and stability of dsSIN recombinants. Recombinants with smaller inserts (e.g., <2 kilobases) tend to be more stable than those with larger inserts, such as a 3.4-kilobase cassette encoding β-galactosidase (C.S.H. and A. Grakoui, unpublished data). In the latter case, a substantial fraction of the virus recovered directly after transfection consists of deletion variants that no longer express functional β-galactosidase. Preliminary experiments suggest that dsSIN vectors with heterologous inserts positioned upstream from the structural protein open reading frame may be more stable; however, these constructs produce somewhat lower expression levels (C.S.H., A. Grakoui, C. Xiong, H. Huang, and C.M.R., unpublished data). In any case, the present dsSIN system should be readily applicable for expression of small proteins and peptides, as well as antisense RNAs (35), RNA decoys (36), or ribozymes (37). It is likely that future improvements will enable expression levels to be modulated by incorporating mutations in the SIN replicase (38, 39) or

subgenomic promoter (22, 23) or by making mutations that affect the stability (40) or translation efficiency of the heterologous mRNA.

In this report, we have shown the utility of dsSIN vectors for studying the endogenous pathway of antigen processing and presentation. The current view of the class I MHC presentation pathway is that peptides, produced by cytoplasmic proteolysis, are transported across the endoplasmic reticulum membrane and bind to immature class I MHC molecules. This formation of this complex and association with $\beta_2$-microglobulin facilitates transport to the cell surface, allowing recognition and lysis by antigen-specific CTL. dsSIN expression of trHA or of short peptides containing immunodominant CTL epitopes allows sensitization of target cells for efficient lysis by appropriate class I MHC-restricted influenza virus-specific CTLs. At least for murine cells, these results suggest that the dsSIN system should be readily applicable for mapping and fine-structure analysis of T-cell epitopes. In experiments to be reported elsewhere, we have defined the minimal peptide sequences required for endogenous presentation of the 006 epitope recognized by $K^d$-restricted CTL clones 11-1 and 40-2 and carried out an extensive mutagenesis study to determine the importance of residues in the core epitopes or flanking sequences on the efficiency of presentation (unpublished results). Given the wide vertebrate host range of SIN, it may also be possible to study T-cell responses in other animal systems including humans using these vectors. Compared to vaccinia virus recombinants, which have been extensively used to express heterologous genes and study T-cell immunity (41), the dsSIN system offers the advantages of rapid recombinant virus construction and isolation. Comparable specific lysis of L929 target cells has been observed after infection with either dsSIN:027 or by a vaccinia virus recombinant expressing the identical minigene construct (unpublished observations). Perhaps of greater importance for studying discrete steps in the antigen presentation pathway, SIN and vaccinia virus represent two alternative viral expression systems; if similar results are obtained with both systems, it is more likely that they reflect the normal presentation pathway as opposed to specific virus-induced effects on host cell biology.

In addition to the application of dsSIN expression vectors for *in vitro* studies, our results indicate that *in vivo* immunization with dsSIN recombinants can be used to prime specific T-cell responses. Both a larger polypeptide (trHA) as well as small peptides containing immunodominant influenza T-cell epitopes were effective as immunogens. Thus, like vaccinia (42), dsSIN vectors can be used as effective immunogens for generating $CD8^+$ and $CD4^+$ T-cell responses and can be used as a tool to dissect specific sites on viral polypeptides recognized by T lymphocytes. The ability to produce epitope-specific CTL populations or clones should also be useful for determining their role in viral pathogenesis or autoimmune disease. Other studies have shown that B-cell-dependent immunity can also be induced by immunization with infectious SIN recombinants carrying a discrete B-cell epitope from a heterologous virus and can lead to partial protection against challenge with the heterologous virus (43). Hence, it may be possible to use immunization with engineered animal RNA viruses to induce protective B- and T-cell-mediated immunity (see also refs. 44 and 45). Although SIN is not likely to be approved for use as a human vaccine, a parallel approach to the one used here for SIN may be applicable for developing live-attenuated vaccine strains using viruses with similar replication strategies, such as attenuated strains of Venezuelan equine encephalitis virus (46) or rubella virus.

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