Rubella virus (RUB) is an important pathogen of humans. RUB is a small, quasi-spherical, enveloped, nonsegmented, plus-strand RNA virus that is the sole member of the Rubivirus genus in the Togaviridae family (5). The RUB genome is roughly 10,000 nucleotides (nt) long and is capped and polyadenylated. In infected cells, three viral RNA species are synthesized: the genomic RNA, which also serves as the mRNA for translation of the nonstructural proteins (whose function is in viral RNA synthesis) from a long open reading frame (ORF) at the 5′ end of the genome; a complementary genome-length RNA of minus polarity which is the template for synthesis of plus-strand RNA species; and a subgenomic (SG) RNA which is initiated internally and contains the sequences of the 3′ terminal one-third of the genome and one of the nonstructural proteins involved in replication. In the nonstructural region, both endogenous promoters are found: a 25-nt promoter has been placed both between the ORFs and downstream from the ORFs (as a virus that synthesized two SG RNAs, one from which the structural protein ORF (SP-ORF) is translated and one from which the nonstructural proteins (NSP-ORF) and the entire 120-nt noncoding region consisting of the 3′-terminal 126 nt of the nonstructural protein ORF (NSP-ORF) and the entire 120-nt noncoding region are translated) and another 3′-proximal ORF. In the other togavirus genus, the alphaviruses, synthesis of the SG RNA is directed by a short (30 nt) promoter adjacent to an RNA polymerase promoter such that the mRNA for the translation of the structural proteins (C [capsid protein] and two envelope glycoproteins, E1 and E2) from a second, 3′-proximal ORF. In the other togaviruses, the alphaviruses, synthesis of the SG RNA is directed by a short (25-nt) sequence located immediately upstream from the SG start site known as the SG promoter (20).

Because RUB causes serious birth defects when infection occurs during the first trimester of pregnancy, live, attenuated vaccines were developed and have been used in vaccination programs in developed countries since 1970 (8). The standard vaccination strategy is universal vaccination of children at 15 to 18 months of age. The vaccine induces an immune response in over 95% of recipients and has been among the most successful live, attenuated vaccines developed. Because of their effective-
The genomic arrangements of the RUB constructs developed and used in this study. Robo302 (pCL1821 plasmid backbone [11]) and Robo402 (pBR322) contain the standard virus genome with its modular NSP- and SP-ORFs. Robo402 was additionally modified by the addition of a NolI site immediately following the NSP-ORF to produce Robo402/NolI. The region of the genome containing the putative SG promoter, nt 6260 to 6506, was duplicated by PCR; two amplicons were produced, the first using primers 106 and K1 (Table 1) and the second using primers K3 and 1. Following digestion of amplicon 1 with BglII and XbaI and amplicon 2 with XbaI and Ascl, a three-fragment ligation was performed with Robo302 digested with BglII and Ascl. GFP was PCR amplified from SINrep/GFP plasmid (obtained from I. Frolov) with primers that retained the IRES element. Following the NSP-ORF to produce Robo402/NsiI. The region of the genome containing the putative SG promoter, nt 6260 to 6506, was duplicated by PCR; two amplicons were produced, the first using primers 106 and K1 (Table 1) and the second using primers K3 and 1. Following digestion of amplicon 1 with BglII and XbaI and amplicon 2 with XbaI and Ascl, a three-fragment ligation was performed with Robo302 digested with BglII and Ascl. GFP was PCR amplified from SINrep/GFP plasmid (obtained from I. Frolov) with primers that retained the IRES element. Following the NSP-ORF to produce Robo402/NsiI. The region of the genome containing the putative SG promoter, nt 6260 to 6506, was duplicated by PCR; two amplicons were produced, the first using primers 106 and K1 (Table 1) and the second using primers K3 and 1. Following digestion of amplicon 1 with BglII and XbaI and amplicon 2 with XbaI and Ascl, a three-fragment ligation was performed with Robo302 digested with BglII and Ascl. GFP was PCR amplified from SINrep/GFP plasmid (obtained from I. Frolov) with primers that retained the IRES element.
of Robo302. Surprisingly, transcripts from this construct, Robo402/IRES, gave rise to viable virus which formed plaques on Vero cells. The average P0 titer of Robo402/IRES virus was $8.5 \times 10^6$ PFU/ml; the titer rose to 2.4 $\times 10^7$ PFU/ml at P3 and 6.0 $\times 10^7$ PFU/ml at P5. As shown in Fig. 5B, the predominant virus-specific RNA species in Robo402/IRES virus-infected cells was the genomic RNA. A faint band of with a size slightly larger than that of the standard SG RNA was present. The ratio of the intensity of this band relative to the genomic RNA was 0.08 in P1 and declined to 0.006 and 0.003 in P3 and P5, respectively (in comparison, the SG/genomic intensity ratio was 1.2 in Robo402/IRES-infected cells). Therefore, although the identity of this band was not determined (for example, it could have been due to adventitious use of the IRES as an SG promoter), it is doubtful that it plays a significant role in Robo402/IRES virus replication.

To complete construction of the vector, the SG promoter followed by the GFP gene was introduced into Robo402/IRES to produce siRobo402/GFP. Virus produced from this construct should synthesize a single SG RNA; in this SG RNA, the downstream primer (D) sequence is colinear with the genomic sequence. The downstream primer (D) sequence is at the 3' end of the amplicon with respect to the RUB genomic construct; the sequence of RUB nucleotides is thus complementary with the genomic sequence. The upstream primer (U) sequence is at the 5' end of the amplicon with respect to the RUB genomic construct; the sequence of RUB nucleotides is thus colinear or complementary are given in parentheses. NA, not applicable.

Restriction site(s) in the primers used for cloning and the corresponding name are in bold. In the case of primer K3, used to create the MCS in dsRobo302, several restriction sites are present; they are alternately shown in bold and all italics.

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Thus, we have successfully constructed RUB vectors which could be useful as both vaccine and expression vectors, and this is the first report of the use of RUB as a recombinant vector. The siRobo vector exhibited greater stability of foreign expression and the strategy of using an IRES to increase stability is also applicable to alphavirus vectors. Both dsRobo and siRobo vectors with MCSs have been developed. Since RUB replicates in a variety of vertebrate cell types and in most of these replication is to low titers and without accompanying cytopathogenicity (unlike the Vero cells used in this study), the niche for

![FIG. 2. Immunoprecipitation of CAT expressed by dsRobo and SIN vectors.](http://jvi.asm.org/)

Vero cells were mock transfected (MOCK) or transfected with dsRobo302/CAT transcripts or transcripts from a double-subgenomic SIN vector expressing CAT, pT52/CAT (dsSIN/CAT). The cells were metabolically radiolabeled with $[^35]$S)methionine (1,000 Ci/mm; Amersham) for 1.0 h at 25 (dsSIN/CAT) or 41 (MOCK and dsRobo/CAT) h posttransfection, followed by lysis with radioimmunoprecipitation buffer, immunoprecipitation using an anti-CAT monoclonal antibody (5' Prime-3' Prime, Inc.), and SDS-PAGE as described previously (4). The molecular weight standards (MW) (from top to bottom) are 200, 97, 68, 43, and 29 kDa; CAT is marked.

### TABLE 1. PCR primer pairs used in vector construction

<table>
<thead>
<tr>
<th>Sequencea</th>
<th>Restriction site(s)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>106 (U)</td>
<td>AGCTACCCGACCCGTAAC (5319–5335)</td>
</tr>
<tr>
<td></td>
<td>GCTCTGATGCACCTGGGCCCTTCTCGCTT (6488–6507)</td>
</tr>
<tr>
<td>107 (D)</td>
<td>XbaI</td>
</tr>
<tr>
<td>K1 (U)</td>
<td>GAAATCTAGGGCGCTGGAAACGCGGTAAAC74TGATCGCTATCGTGGCGAAG (6260–6280)</td>
</tr>
<tr>
<td></td>
<td>GAAGGGATGCAGCAAGG (7323–7340)</td>
</tr>
<tr>
<td>1 (D)</td>
<td>XbaI, StuI, BstI, MluI, HpaI, NsiI/Ppu10I</td>
</tr>
<tr>
<td>Amplicon I (EMCV IRES)</td>
<td>NaI</td>
</tr>
<tr>
<td>IR-5 (U)</td>
<td>CACATCATGTTATTGCTGCCCTTCACTCCTC (NA)</td>
</tr>
<tr>
<td>IR-3 (D)</td>
<td>CATGTTTGTTGGCAAAGCTTATC (NA)</td>
</tr>
<tr>
<td>Amplicon II</td>
<td>Eco47III</td>
</tr>
<tr>
<td>IR-R (U)</td>
<td>CGCGATGCCTTCTACTACCTCCCATCACCC (6433–6453)</td>
</tr>
<tr>
<td>1 (D)</td>
<td>GAAGGCGATGCCCAAGG (7323–7340)</td>
</tr>
</tbody>
</table>

a The sequences of oligonucleotide primers used in two manipulations, duplication of the SG promoter in Robo302, and substitution of the SG promoter in Robo402/NsiI with an IRES from EMCV are given. In both manipulations, two PCR amplicons were generated and the manipulation was done via a three-fragment ligation. The upstream primer (U) sequence is at the 5' end of the amplicon with respect to the RUB genomic construct; the sequence of RUB nucleotides is thus colinear with the genomic sequence. The downstream primer (D) sequence is at the 3' end of the amplicon with respect to the RUB genomic construct; the sequence of RUB nucleotides is thus complementary with the genomic sequence.

b Nucleotides in the primers containing RUB sequences are underlined; those in the genome (numbered from the 5' end) to which the nucleotides in the primer are colinear or complementary are given in parentheses. NA, not applicable.
a RUB expression vector would be for low-level expression without a drastic effect on the cell, which has been a problem with some of the highly cytopathic alphavirus vectors (17). While the expression experiments in this study used reporter genes, we have successfully expressed a truncated form of the immunogenic E proteins of Japanese encephalitis virus in both dsRobo and siRobo as prototype vaccine candidates (data not shown). Live, attenuated RUB vaccines have been universally accepted as effective and safe in childhood immunization programs. Thus, a RUB-based vaccine would be best used in a pediatric setting to target systemic pathogens against which universal immunization was desired, such as human immunodeficiency virus, respiratory syncytial virus, or one of the hepatitis agents; a cocktail of RUB-based vaccines targeting different pathogens could be used to induce immunity simultaneously against each pathogen targeted in the cocktail. To the end of developing a RUB vector acceptable as a vaccine vector, we recently constructed an infectious cDNA clone based on the RA27/3 vaccine strain (12), the vaccine used in the United States and Europe.

While the focus of this study was vector development, the results did add to our understanding of RUB replication strategy. First, the ability of dsRobo virus to synthesize two SG RNAs with equal efficiency demonstrates that the RUB SG promoter is somewhere within the duplicated region, i.e., 170 nt upstream from the SG RNA start site. The dsRobo302/GFP

FIG. 3. Immunoprecipitation of GFP expressed by dsRobo (A) and siRobo (B) vectors. Vero cells were mock infected (Mock), infected with Robo402 virus (R402), Robo402/IREs virus (402/IREs), or a passaged stock of dsRobo/GFP or siRobo/GFP virus. In these multiple passages, P0 is virus recovered from transfection which was subsequently passaged at a low MOI (~0.1 PFU/cell) to produce P1, P2, etc. For this experiment, the MOI for each virus stock was adjusted to ~1 PFU/cell. Three days postinfection, cells were metabolically radiolabeled with [35S]methionine (1,000 Ci/mmol; Amersham) for 1.5 h followed by lysis with radioimmunoprecipitation buffer, immunoprecipitation using an anti-GFP polyclonal immunoglobulin G (Clontech), and SDS-PAGE (4). In each panel, the three molecular weight standards (MW) are (from top to bottom) 68, 43, and 29 kDa; GFP is marked.

FIG. 4. Percentage of cells in cultures infected with dsRobo/GFP and siRobo/GFP viruses expressing GFP. Vero cells were infected at an MOI of 1 PFU/cell with dsRobo/GFP or siRobo/GFP stocks produced by multiple low-MOI passages (virus recovered from transfected cells, designated P0, was passaged in Vero cells to produce P1, P2, etc.). Three to four days postinfection, when 100% of the cells are infected with Robo302 virus under these conditions (10), to determine the percentage of cells expressing GFP, the infected cultures were trypsinized, and the cells were resuspended in medium and subjected to fluorescence-activated cell sorting analysis using a Becton Dickinson FACS Calibur flow cytometer (equipped with a 388-nm, 16-mW argon laser) with CellQuest software (Becton Dickinson); 20,000 events were used to determine each percentage.

FIG. 5. Virus-specific RNAs produced by Robo constructs. Vero cells were mock infected (Mock) or infected at an MOI of ~1 PFU/cell with Therien strain RUB (WT [wild type]), Robo302 or Robo402 virus (R302 or R402), or stocks of dsRobo, dsRobo/GFP, Robo402/IRES (402/IRES), or siRobo/GFP viruses passaged one (P1), three (P3), or five (P5) times in Vero cells (MOI of ~0.1 PFU/cell at each passage) (in panel B, the dsRobo/GFP virus [ds/GFP] was P1). Three days postinfection, total cell RNA was extracted and subjected to agarose gel electrophoresis and virus-specific RNA species were detected by Northern hybridization using a probe complementary to the RUB SP-ORF ([32P]CTP-la-beled negative-polarity RNA transcripts synthesized from pRUB-SP-ORF [7]). The amount of radioactivity present in RNA bands on autoradiographs was quantitated by densitometry with a Fujix BAS1000 Bio Imaging analyzer (Fuji Photo Film, Tokyo, Japan), using software provided by the manufacturer. G, genomic RNA; 28S, the 28S cell rRNA which causes a background blob; SG1, the standard SG RNA; SG2, SG RNAs engineered for expression of foreign genes. In the Robo402/IRES lanes, a faint band of unknown identity is marked with an arrowhead.
construct will be of use in mapping the precise boundaries of the SG promoter. Second, we unexpectedly discovered that RUB was viable with an IRES element in place of its SG promoter. A number of other virus families of vertebrates (the caliciviruses, astroviruses, and hepatitis E virus) and plants have evolved a modular expression strategy similar to that used by togaviruses in which the nonstructural and structural proteins are translated from two different ORFs. In all of these viruses, expression of the 3′-proximal structural protein ORF is driven by an SG promoter. Our results show that an IRES can function in this capacity as well in the absence of an SG RNA. Interestingly, it was recently shown that in the insect picorna-like virus family, which have a 3′-proximal structural protein ORF and synthesize no SG RNA, expression of this ORF is driven by an IRES element (16). However, while the IRES element was retained during multiple passaging of Robo402/IRE5 virus, passaging of the siRoboRUB/GFP virus which contained both the SG promoter and IRES resulted in deletions within the genome and reappearance of an SG RNA similar in size to the standard SG RNA, indicating that SG RNA synthesis was preferred.

Support for this study was provided by a grant from the World Health Organization and from PHS grant AI-21389 from NIAID.

We thank Birgit Neuhaus for help with image reproduction.

REFERENCES