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cis Requirement for N-Specific Protein Sequence in Bovine Coronavirus Defective Interfering RNA Replication

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A naturally occurring 2.2-kb defective interfering (DI) RNA of the bovine coronavirus, structurally a simple fusion of the genomic termini, contains a single contiguous open reading frame (ORF) of 1.7 kb composed of the 5′-terminal 288 nucleotides of polymerase gene 1a and all 1,344 nucleotides of the nucleocapsid protein (N) gene. The ORF must remain open throughout most of its sequence for replication to occur. To determine the qualitative importance of the N portion of the chimeric ORF in DI RNA replication, transcripts of mutated reporter-containing constructs were tested for replication in helper virus-infected cells. It was determined that the N ORF could not be replaced by the naturally occurring internal I protein ORF, accomplished by deleting the first base in the N start codon which leads to a +1 frameshift, nor could it be replaced by the chloramphenicol acetyltransferase ORF. Furthermore, 3′-terminal truncations of the N gene leaving less than 85% of its total length were likewise not tolerated. Small in-frame deletions and in-frame foreign sequence insertions of up to 99 nucleotides within certain regions of the N ORF were tolerated, however, but the rate of DI RNA accumulation in these cases was lower. These results indicate that there is a requirement for translation of most if not all of the N protein in *cis* for optimal replication of the bovine coronavirus DI RNA and suggest that a similar requirement may exist for viral genome replication.

Defective interfering (DI) RNAs contain portions of the parental virus RNA genome but are incapable of self-replication (reviewed in references 12 and 39). Often the DI genome is a truncated version of the parental genome, having arisen from extensive internal deletions of one or more regions. For DI RNA replication, virus-specific proteins, usually including the RNA-dependent RNA polymerase, must be supplied in *trans* by the helper (usually parental) virus. However, DI RNAs contain the *cis*-acting signals required for RNA replication and sometimes encapsidation and can be useful, especially for RNA viruses such as coronaviruses for which synthetic infectious genomic transcripts are not yet available, for identifying the viral genomic replication and packaging signals.

One kind of *cis*-acting requirement identified for defective RNAs of some (2, 5, 8, 18, 20, 49, 50) but not all (1, 22, 23) plus-strand RNA viruses is an open reading frame (ORF) that must remain open for replication. Interestingly, for poliovirus (32) and turnip yellow mosaic virus (49), the linkage between translation and replication is also found for the viral genome from which the DI RNA was derived, suggesting the same linkage mechanism might function for both the viral genome and DI RNA.

All of the naturally occurring coronavirus DI RNAs that have been cDNA cloned and analyzed as infectious transcripts contain an ORF throughout most of their sequences. The ORF is composed of in-frame fusions of one to four discontiguous regions of the polymerase (*pol*) gene and some fraction (ranging from 21% for mouse hepatitis virus [MHV] to 100% for bovine coronavirus [BCV]) of the N gene (4, 5, 17, 18, 26, 28, 46) (Fig. 1). The general feature of the continuous ORF in the coronavirus DI RNAs suggests that it may play an important role in DI RNA replication. This view is supported by studies in which the MHV DI RNA ORF, experimentally truncated by frameshift mutations, became restored after one passage in cell culture (5, 18). Reversion was thought to have resulted from a recombination event or point mutation. These results indicated a strong preference but not a rigid requirement for translation of the MHV DI RNA ORF for replication. Paradoxically, in other experiments with MHV DI RNA, engineered mutants with little or no ORF accumulated to high levels (17, 18), and the chloramphenicol acetyltransferase (CAT) gene in both open and closed (i.e., inverted) forms reportedly could replace the MHV DI RNA ORF in replicating constructs (24, 26). Furthermore, very large DI RNAs of infectious bronchitis virus (35) and MHV (16) that break the pattern of a continuous ORF requirement have been recently described. These contain a genome with interrupted ORFs, possibly derived from natural intergenic regions, and most or all of the N gene. They may be replicating variants of the virus genome, however, since for MHV DI RNA at least, no helper virus was required for replication (16).

Our preliminary studies with the cloned, reporter-containing BCV DI RNA pDrep1 (4) had indicated that there was a strict ORF requirement for replication of the DI RNA. In our studies, no revertants to an ORF from a closed one could be found after three serial passages of progeny virus that would have contained reverted DI genomes (4) or after one passage of transfected cells. Thus, differences between the behavior of BCV DI RNA and MHV DI RNA became apparent. To identify what properties of the fused ORF are important for BCV DI RNA replication, we performed a series of mutational analyses of pDrep1 and examined the effects on transfected transcripts in helper virus-infected cells. From these studies, we conclude that some N-specific protein must be synthesized in *cis* for BCV DI RNA replication. This requirement, therefore, is in many ways similar to that described for DI RNAs of poliovirus (32), turnip yellow mosaic virus (49), cowpea mosaic virus (2), and clover yellow mosaic virus (50). The apparent rigidity of the requirement for the N ORF in BCV DI RNA replication and the lower recombination rate in BCV than in MHV may reflect functional differences in the polymerases between these two otherwise closely related coronaviruses.

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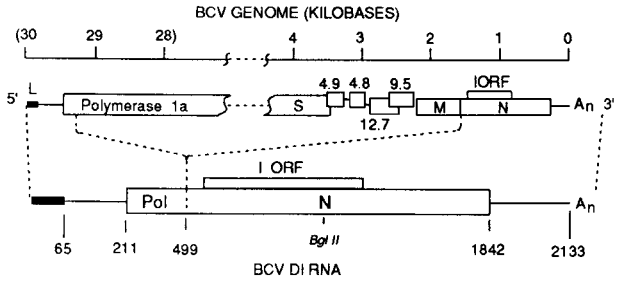

FIG. 1. Structure of the BCV DI RNA relative to viral genome. L, indicated by a filled small box, represents the BCV 65-nt leader. Large open boxes identify ORFs. The beginning of the start codon for the *pol*-N ORF in the DI RNA is at base 211, and the beginning of that for the N gene is at base 499. The position of the internal ORF (IORF) within the N gene and the *Bgl*II restriction endonuclease site used for insertion of the 30-nt reporter sequence to create pDrep1 (4) are shown.

The requirement for N synthesis in *cis*, furthermore, may reflect a similar requirement for virus genome replication.

MATERIALS AND METHODS

Virus and cells. A stock of the Mebus strain of BCV, derived from virus plaque purified three times, had an infectious titer of 2.6×10^7 PFU/ml and was used as the helper virus in all experiments. This stock contained the natural DI RNA described previously (4, 11). Virus was grown on HRT-18 cells as described previously (4, 11).

Synthetic oligonucleotides. Oligonucleotides used in this study are described in Table 1.

Construction of plasmids. Plasmid constructs (Fig. 2) were all modifications of pDrep1 (4); for each, mutated regions were confirmed by sequencing, and the predicted ORFs were confirmed by in vitro translation of T7-generated transcripts. pDM1 was generated by a PCR overlap mutagenesis procedure (13). For this, the gel-purified 219-nucleotide (nt) PCR product from a pDrep1-templated reaction with primers leader(−) and DM(+) and the gel-purified 920-nt product from a reaction with primers DM(−) and TGEV#8(+), primer leader(−), and primer TGEV#8(+) were used together in an overlap PCR to form a product of 1,117 nt from which the 443-nt *Hpa*I-*Xba*I fragment was used to replace the analogous region in pDrep1.

pNM1 was similarly generated except that the overlap PCR used the 503-nt fragment from a reaction with the primers leader(−) and NM(+) and the 508-nt fragment from a reaction with primers NM(−) and TGEV#8(+), primer leader(−), and primer TGEV#8(+). From the resulting 1,117-nt fragment, the 443-nt *Hpa*I-*Xba*I fragment was used to replace the analogous region in pDrep1. pNMTG was serendipitously generated during the preparation of pNM1.

pDIN was generated by a modification of pNMTG. (i) pD92, a construct identical to pDrep1 except that its 5′-terminal 92 nt were missing (not shown), was cut at base 1093 with *Bgl*II, made blunt by a 4-nt fill-in with Klenow enzyme, and religated to form pD92B (not shown). (ii) The *Xba*I-*Hin*dIII fragment from pD92B containing the 4-nt insert was used to replace the analogous fragment in pNMTG.

To introduce premature stop codons into the pDrep1 ORF, pDrep1 was linearized with one of six restriction endonucleases, made blunt, and religated. For pN5d (a 5-nt deletion at the *Nsi*I site), pN4d (a 4-nt deletion at the *Nsi*I site), pB4d (a 4-nt deletion at the *Bst*XI site), and pNd2d (a 2-nt deletion at the *Nde*I site), linearized pDrep1 DNA was made blunt with mung bean nuclease and religated. For pS4i (a 4-nt insertion at the *Spe*I site), pT1i (a 1-nt insertion at the *Tth*111I site), pX4i (a 4-nt insertion at the *Xba*I site), linearized pDrep1 DNA was made blunt by fill-in with T4 DNA polymerase and religated.

Constructs pΔ930-1046, pΔ930-1047, and pΔ930-1067, containing the indicated internal deletions, were generated by sequentially linearizing pDrep1 with *Tth*111I and blunt ending by fill-in with T4 DNA polymerase, cutting with *Bsu*36I and blunt ending with mung bean nuclease, and religation of the gel-purified large fragment. pΔ930-1046 had the predicted 117-nt in-frame deletion, whereas pΔ930-1047 and pΔ930-1067 arose as an apparent result of overdigestion with mung bean nuclease.

pDrep-CAT was generated by splicing the *Bam*HI fragment of the CAT-containing plasmid pCM4 (Pharmacia) into *Xba*I-linearized pDrep1 after both had been filled in with Klenow enzyme.

pDrep-gpD4 was generated by subcloning a 95-nt T4 DNA polymerase blunt-ended fragment (5′ggatcccAAATATGCCTTGGTGGATGCCTCTCTCAAGA TGGCCGACCCCAATCGCTTTCGCGGCAAAGACCTTCCGgtcctggacgggaa ttatc3′; lowercase letters refer to primer sequences used in the initial cloning) encoding the neutralizing epitope of herpes simplex virus gD (amino acids [aa] 1 to 23) (6) into the T4 DNA polymerase blunt-ended *Spe*I site of pDrep1 (4).

RNA (Northern) blot hybridization. (i) Assay for DI RNA replication. Replication of transcripts from each mutant plasmid was assayed in a transfection experiment, and the products were analyzed by RNA blot hybridization as previously described (4). For this, ^{32}P-end-labeled oligonucleotide TGEV#8(+) was used as the probe. Relative amounts of accumulation were determined with a Bio-Rad Imaging Densitometer. For measurement of virus replication, virus genome and mRNAs were identified by probing with ^{32}P-labeled N(+) oligonucleotide (Table 1) after removal of the TGEV#8(+) probe by stripping.

(ii) Measurement of RNA stability. RNA transcripts of pDrep1, pDIN, and pT1i were transfected into uninfected cells at near 80% confluency for 10 min, and cytoplasmic RNA was extracted at the times indicated and analyzed by quantitative RNA blotting (4). The number of molecules per cell was calculated from an N(+) probe with a specific activity of 5.4×10^5 cpm per pmol, determined as previously described (11), and from our measured yield of 12 μg of RNA per 7.2×10^5 cells. The amount of radioactivity in the transfected RNA species was normalized to the amount of 18S rRNA in each sample. For quantitation of 18S rRNA, end-labeled probe 18S(+) was used on the same blot after removal of the TGEV#8(+) probe by stripping. Quantitation was done with the Ambis Photoanalytic Imaging System (AMBIS, Inc., San Diego, Calif.).

In vitro translation and immunoprecipitation. In vitro translation of transcripts was done in wheat germ lysate, using [^{35}S]methionine as specified by the manufacturer (Promega Biotec). Immunoprecipitation with a BCV N-specific rabbit polyclonal antiserum was performed as described previously (40). Products were analyzed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel as described previously (40).

TABLE 1. Synthetic oligonucleotides used in this study

Oligonucleotide[a]	Sequence[b]	Binding region[c]
Leader(−)	5′GAGCGATTTGCGTGCGTGCATCCCGC3′	7–32
DM(+)	5′GTTGATCTTCGACAATGTGACC3′	204–225
DM(−)	5′GGTCACATTGTCGAAGATCAAC3′	204–225
NM(+)	5′GTAAAAGACAACCTTTCTTGAAGT3′	486–509
NM(−)	5′ACTTCAAGAAAGGTTGTCTTTTAC3′	486–509
N(+)	5′CCAGAACGATTTCCAAAGGACGCTCT3′	532–557
TGEV#8(+)	5′CATGGCACCATCCTTGGCAACCCAGA3′	1099–1128
TGEV#7(−)	5′TCTGGGTTGCCAAGGATGGTGCCATG3′	1099–1128
18S(+)	5′CTGCTGGCACCAGACTTGCCCTCCAA3′	595–620

[a] Oligonucleotide binds to either plus-sense RNA, as indicated by (+), or to minus-sense RNA, as indicated by (−).

[b] Underlined bases indicate differences from genomic sequence.

[c] Numbers correspond to pDrep1 plus-strand sequence, except for oligonucleotides TGEV#8(+) and TGEV#7(−), in which cases numbers refer to the TGEV N gene plus-strand sequence (15), and oligonucleotide 18S(+), in which case numbers refer to the 18S RNA sequence (45).

RESULTS

A single fused *pol*-N ORF within the BCV DI RNA is required in *cis* for RNA replication: the N ORF region cannot be supplanted by the I or CAT ORF or be severely truncated. The BCV DI RNA from which the reporter-containing construct pDrep1 was derived (4) is a simple fusion between the N-terminal portion of the RNA-dependent RNA *pol* gene 1a and the entire N ORF, resulting in a single fused ORF (Fig. 1). This region of gene 1a shows a sequence nearly identical to that of the same region of the MHV genome (4) and therefore probably represents the first 96 aa of a homolog of MHV p28 (14). To test whether the BCV DI ORF plays a functional role in RNA replication, site-specific point mutations were introduced into pDrep1 such that the character of the ORF was changed (Fig. 2), and each mutant was tested under conditions known to support pDrep1 RNA replication (4) (Fig. 2). Replication was judged to have occurred if a reporter-containing product appeared in new cells following infection with the progeny virus from transfected cells. For strongly replicating molecules, such as pDrep1, an increase in intracellular amounts by 48 or 96 h posttransfection following an initial decrease could also at times be observed. The replication of pDrep1 was assayed in parallel in all experiments, and the

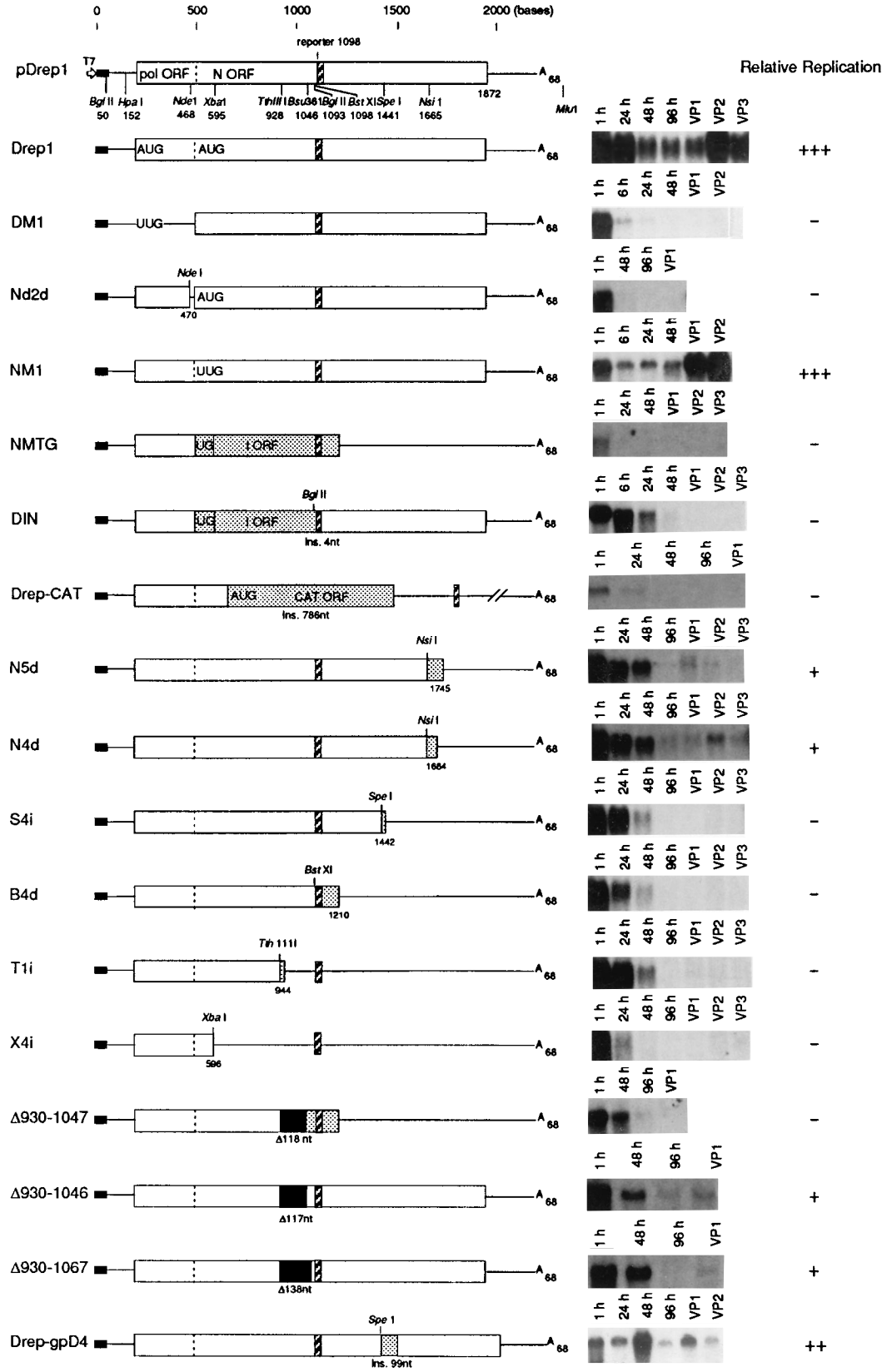

FIG. 2. Replication assay specific for transcripts of pDrep1 and its mutant derivatives. The structure of pDrep1, and of RNAs derived from pDrep1 and its mutant derivatives, are shown. Restriction endonuclease sites are described in reference to pDrep1 DNA. In-frame *pol* and N ORF sequences are indicated by large open boxes, and other ORF sequences are indicated by stippled boxes and are otherwise identified. The 30-nt reporter sequence derived from the TGEV N gene is indicated by a hatched box, and deleted regions of pDrep1 sequence are indicated by filled boxes. Northern blot analyses specific for reporter sequence were done on cytoplasmic RNA extracts prepared at the indicated times posttransfection or on RNA extracted from cells at 48 h after infection with progeny virus. VP1, VP2, and VP3 indicate virus passage numbers. Blots were probed with the ^{32}P-end-labeled oligonucleotide TGEV#8(+), which binds to the plus-strand reporter sequence (Table 1). Relative replication identifies the accumulated amount of DI RNA at 48 h postinfection from passage 1 virus compared with the amount of pDrep1 RNA measured in parallel. +, approximately 30% of pDrep1 RNA accumulation (+++); −, no accumulation at any of the times assayed. Ins., insertion.

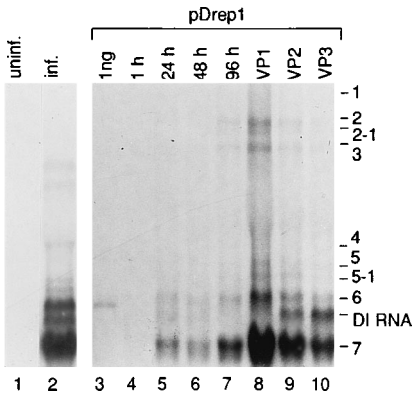

FIG. 3. Evidence of viral RNA and wt DI RNA accumulation in helper virus-infected cells. Uninf. and inf. (lanes 1 and 2) indicate cytoplasmic RNA from mock- and helper virus-infected cells, respectively, extracted at 48 h postinfection and probed with radiolabeled N(+) oligonucleotide that binds to N gene sequence (see Table 1). A marker identifies wt DI RNA. Lanes 3 to 10, the blot described in Fig. 2 for pDrep1 RNA was stripped and probed with radiolabeled N(+) oligonucleotide. Both wt DI RNA and pDrep1 are identified by the probe.

replication rates of mutants relative to pDrep1 were determined by examining cytoplasmic RNA at 48 h after the first passage of progeny virus. Helper virus was also shown to have replicated in all experiments by probing the same Northern blot for viral RNA (not shown, but data were essentially identical to those shown in Fig. 3 for pDrep1).

To determine whether the *pol* region of the DI RNA ORF is required for replication, two mutants of pDrep1 with a disrupted *pol* region were tested. In the first, the *pol*-N ORF start codon in pDrep1 was converted to a leucine codon (UUG) by changing the single base A-211 to U, thus forming pDM1 (Fig. 2). No accumulation of pDM1 RNA was observed in a replication assay (Fig. 2), nor, as expected, was a full-length *pol*-N ORF product made by in vitro translation (Fig. 4A, lane 3). Proteins the size of N and larger, however, were synthesized from pDM1 RNA, apparently initiated from the N start codon at base 499 and from other upstream potential start codons at bases 454, 385, and 268 (4, 19), as indicated by immunoprecipitation with an N-specific polyclonal antiserum (Fig. 4B, lanes 3, 6, and 7). These results suggest that translation of the Pol-N ORF fusion protein is required for replication. They also indicate that the requirement must be in *cis* since the natural DI RNA present in helper virus-infected cells (4) (Fig. 3, lane 2) was not providing the function in *trans*. The second mutant, pNd2d, was prepared by making a two-base deletion in pDrep1 at the *Nde*I site (base 469) which creates a truncation of the *pol* ORF at base 470 (Fig. 2). pNd2d RNA, like pDM1 RNA, did not replicate (Fig. 2), nor was a Pol-N product made by in vitro translation (data not shown). Furthermore, it was demonstrated with mutant pNM1, a construct in which the N start codon of pDrep1 had been converted to a leucine codon by changing A499 to U (Fig. 2), that independent synthesis of N from its initiator codon in the *pol*-N ORF was not required for replication (Fig. 2) and that the *cis* function for replication was being fulfilled by the full-length fusion protein (Fig. 4B, lane 4). The results of experiments with mutants pDM1, pNd2d, and pNM1, therefore, together indicate that the *pol* region of the DI ORF is important for DI RNA replication, possibly because of a property of the Pol product expressed in *cis*, but this was not examined further.

To determine whether the N portion of the fusion ORF is required for DI RNA replication, the N portion of the *pol*-N sequence was replaced by ORFs of two separate proteins and tested. In the first, the N ORF was replaced by the naturally occurring I protein ORF encoded within the N gene sequence but in the +1 reading frame (40) (Fig. 1). For this, pNMTG was created by deleting base A-499 in pDrep1, the first base in the N translation start codon. This caused a total replacement of the N ORF in the fusion sequence with the I ORF and added 20 amino acids of non-N, non-I sequence upstream of the I initiation codon (Fig. 2). pNMTG RNA, from which no part of the N protein should be made, did support translation of a protein of the predicted size (Fig. 4A, lane 5) that, as expected, was not immunoprecipitable with an N-specific antibody (Fig. 4B, lane 5). pNMTG RNA did not replicate in transfected cells or after three passages of progeny virus (Fig. 2). Furthermore, replication could not be rescued by restoring the continuity of the C-terminal region of the N protein through a four-base insertion at the *Bgl*II site in construct pDIN (Fig. 2). This RNA, as illustrated in Fig. 4A, lane 6, supported synthesis of a protein indistinguishable in size from the full-length Pol-N ORF product of pDrep1. These results indicate that some *cis*-acting quality of the N-terminal region of the N protein, rather than only the process of translation through the C-terminal region of the N ORF, is required for replication of the DI RNA molecule.

To test for the N ORF requirement in a second construct, the N ORF in pDrep1 was replaced in large part, though not totally, by an in-frame insertion of the CAT gene. For this, pDrep-CAT was created by inserting a 786-nt fragment containing the CAT gene into the *Xba*I site of pDrep1 just downstream of the N start codon (Fig. 2). Transcripts of this likewise failed to replicate (Fig. 2), although a protein of the predicted

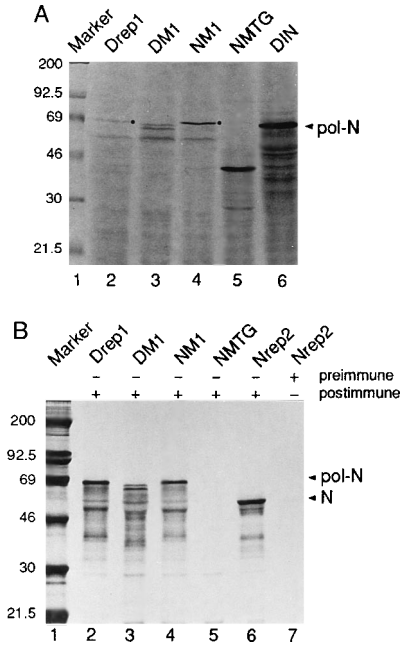

FIG. 4. Proteins synthesized in vitro from pDrep1 RNA and its mutant derivatives. (A) SDS-polyacrylamide gel electrophoresis of in vitro-translated products of mutant transcripts. Proteins were radiolabeled with [^{35}S]methionine during synthesis and identified by autoradiography. Filled circles identify the predicted full-length Pol-N fusion ORF product. (B) SDS-polyacrylamide gel electrophoresis of immunoprecipitated products of in vitro translation. [^{35}S]methionine-labeled products of in vitro synthesis were immunoprecipitated with rabbit polyclonal antiserum specific for BCV N protein (lanes 2 to 6). Preimmune serum was used in lane 7. Nrep2 is RNA synthesized from pNrep2, a construct encoding BCV N mRNA (mRNA 7) but containing the same in-frame reporter sequence as pDrep1 (4). Sizes are indicated in kilodaltons.

size was synthesized in vitro (data not shown), indicating again the importance of the N protein in *cis* for replication.

To determine what properties of the N ORF are required in *cis* for DI RNA replication, frameshift mutations giving rise to a series of truncated N ORFs were tested. For this, stop codons were introduced into the N ORF region of pDrep1 by separately cutting at six unique restriction endonuclease sites, blunt ending, and religating. These manipulations resulted in frameshifts of +1 or −1 and consequent ORF truncations near the restriction endonuclease sites. Truncations of the N ORF near the *Spe*I, *Bst*XI, *Tth*111I, and *Xba*I sites created mutants pS4i, pB4d, pT1i, and pX4i, respectively, which produced nonreplicating transcripts (Fig. 2). However, *Nsi*I site mutants pN5d and pN4d, which gave carboxy-terminal N ORF truncations of 41 and 62 aa, respectively, did replicate, but not as well as transcripts of pDrep1 (Fig. 2). Truncated proteins of the predicted sizes were obtained by in vitro translation of the truncated constructs (data not shown). These results indicate that nearly the entire N ORF may be required in *cis* for BCV DI RNA replication.

Some in-frame deletions and insertions of foreign sequence within the N ORF are tolerated for replication. In-frame deletions of 117 nt (39 aa) and 138 nt (46 aa) from within the N-terminal half of the N ORF beginning at the *Tth*111I site to form pΔ930-1046 and pΔ930-1067, respectively, resulted in truncations of the N ORF in mutants that yielded truncated polypeptides when translated in vitro (data not shown). Although these mutants replicated, their rates of accumulation were slower than that of pDrep1 (Fig. 2). These results suggest, as did the results for pDIN, that N-specific protein sequence within its N-terminal half is important in *cis* for replication. A deletion of 118 nt at the *Tth*111I site resulted in a frameshift such that only the N-terminal 143 aa of N protein were made, followed by 55 aa of the I protein, upon translation in vitro (Fig. 2). The resulting construct, pΔ930-1047, yielding a truncated protein of the predicted size (data not shown), did not replicate (Fig. 2), which is consistent with the results for the truncated pDIN mutant showing that the N-terminal region of N alone is insufficient to provide the *cis*-acting function.

Although the degree of potential inhibition could not be measured, the in-frame insertion of the 30-nt transmissible gastroenteritis virus (TGEV)-specific sequence into the N ORF of the natural DI RNA to form pDrep1 had shown that the added sequence does not prevent replication (4). In the study presented here, an in-frame insertion of 99 nt at the *Spe*I site appeared to inhibit replication only moderately (Fig. 2). These results indicate that some changes in the quality of the N ORF product, at least in terms of spacing, can be tolerated when the protein is fulfilling its *cis*-acting function.

Repair of DI RNA mutations was not observed upon virus passaging. The BCV DI RNA as well as the replicating pDrep1 RNA has been shown to be packaged as evidenced by at least four serial passages in virus and by the presence of the DI RNA in purified virions (4, 11), although the packaging signal for this DI RNA has not yet been characterized. In contrast to the rapid repair of MHV DI RNA mutants (5, 18), no replicating revertants of BCV DI RNAs from nonreplicating constructs with lesions within the *pol*-N ORF were found after a single passage of infected cells (i.e., 96 h posttransfection) or after one to three serial passages of virus, nor was there reversion of the replicating mutant pNM1, as evidenced by sequencing of PCR-amplified cDNA after two passages of virus (data not shown).

Differences in RNA replicability cannot be explained by differences in RNA stability. One possible mechanism for the observed dependence of viral RNA replication on translation in *cis* is that translatable molecules might have been more stable and therefore more likely to replicate (36). To test whether the nonreplicating molecules are less stable, decay rates for pDrep1, pDIN, and pT1i RNA transcripts were compared by quantitative RNA blot analysis after transfection into uninfected cells (Fig. 5A). For each construct, approximately 90 copies of RNA per cell entered after 10 min of transfection, but all decayed with similar rates through 10 h posttransfection (Fig. 5B), indicating that differences in replicability cannot be explained by differences in stability.

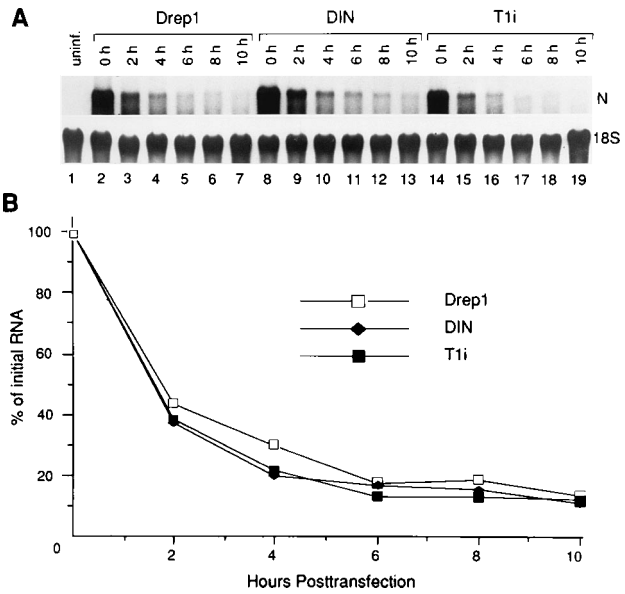

FIG. 5. Decay of pDrep1, pDIN, and pT1i RNA molecules in uninfected cells. (A) RNA was transfected into uninfected (uninf.) cells and cytoplasmic RNA was extracted at the indicated times and probed with reporter-specific ^{32}P-end-labeled probe [TGEV8(+)] in an RNA blot analysis (Table 1). After quantitation, the blot was stripped and probed with 18S RNA-specific probe (Table 1). Timing began with the termination of the transfection period. (B) Radioactivity in the DI RNA bands was normalized to that in the 18S rRNA, and the relative amounts were plotted. From the specific activity of the probe, it was determined that for all three RNA species, 100% at 0 h posttransfection represented approximately 90 molecules per cell.

DISCUSSION

In experiments presented here, we have shown that both the polymerase-specific and N protein-specific sequences encoded within the BCV DI RNA as a fused ORF are required in *cis* for replication of the DI RNA. From a mutational analysis of the N portion of the DI ORF, we further conclude that the quality of the N protein throughout large regions must be preserved in order to maintain the replication function of DI RNA and that other protein sequences, including those for the I or CAT protein, will not substitute. A limited number of small in-frame insertions and deletions within the N ORF had a small inhibiting effect on replication. The results of our experiments therefore lead us to conclude that the rigid ORF requirement for BCV DI RNA replication rests not only on the process of translation but also on a quality of the N protein that is somehow utilized in *cis* for replication.

Our results for BCV DI RNA were in surprising contrast to those for MHV DI RNA, the only coronavirus DI RNA for which replicational requirements have been characterized (5, 17, 18, 25, 26, 28, 48). Two reports specifically addressing the N ORF requirement of MHV DI RNA appeared in print

during the writing of this discussion (25, 48). The surprise in our results stems from the fact that BCV and MHV are structurally closely related viruses and would therefore be expected to be fundamentally similar in their replicational requirements, as reflected in DI RNA replication (4, 10, 21). The differences in DI RNA behavior, however, are clear in three basic respects. (i) Whereas the entire N gene sequence is present in the naturally occurring BCV DI RNA ORF (4) and in a replicating synthetic MHV DI RNA patterned after pDrep1 (29), only the 3′-terminal 21% (28) to 26% (46) of the N ORF is present in naturally occurring MHV DI RNAs, and as little as 10% (17) to no (25, 48) N sequence is required for replication of MHV DI RNA mutants. One possible explanation for this difference is that a potential *cis*-acting N function is mimicked by the fusion protein synthesized from the mosaic MHV DI ORF. Inspection of the internal polymerase regions contained within the ORFs of MHV DI RNAs DIssE (28) and MIDI (46) interestingly reveal stretches of basic amino acids, one property of N protein that is probably important for its function (30, 33). Other unknown properties, however, might be mimicked and provide a function important in replication. (ii) Whereas maintenance of the naturally occurring large ORF in BCV DI RNA is a strict requirement for replication, it is not so, per se, for MHV DI RNA, although DI RNAs with large ORFs that have been examined were found to have a competitive advantage (5, 18). Many MHV DI RNA mutants with no or very truncated ORFs replicated (5, 17, 18, 25, 48). This difference from BCV might reflect a difference in polymerase behavior. It might be, for example, that N is needed as a component of the replication complex and MHV polymerase is better able to utilize N in *trans*. (iii) Whereas no recombination was observed for BCV DI RNA leading to restoration of the ORF, even in the presence of a natural wild-type (wt) DI RNA that should have enhanced chances for recombinational repair (29, 34, 47), it was frequently found for MHV DI RNAs (5, 18, 29, 47). These results may again reflect differences in the polymerases or possibly helicases of BCV and MHV. Small mutations in brome mosaic virus helicase, for example, can affect the pattern of recombination in this virus (31). Conceivably, BCV and MHV helicases could differ enough that one would effect a higher rate of recombination.

One puzzling question regarding pDrep1 replication is why mutations blocking *pol* ORF synthesis but allowing N (or N-like) protein to be synthesized, e.g., mutant pDM1, did not replicate since N would be available to provide the *cis*-acting function. One possibility consistent with our results showing that transfected synthetic molecules of reporter-containing N mRNA did not replicate (4) is that the (partial) Pol 1a protein product serves to target the pDrep1 RNA into a replication complex. Further study is needed to determine the function of the *pol* ORF portion of pDrep1.

The rigid *cis* ORF requirement for replication is a property of BCV DI RNA that is shared with the DI RNAs of poliovirus (8, 20), turnip yellow mosaic virus (49), clover yellow mosaic virus (50), and cowpea mosaic virus (2). In the case of poliovirus and turnip yellow mosaic virus, for which infectious synthetic genomic transcripts have been studied, the *cis*-acting ORF requirement for viral genome replication exists as well (32, 49). In these cases, however, the products of the *cis*-acting ORF are nonstructural proteins thought to be a part of the RNA-synthesizing machinery (7, 32, 49). For clover yellow mosaic virus, capsid protein, analogous to the coronavirus nucleocapsid protein, is a product of the required DI RNA ORF (50). Although not demonstrated, it is conceivable that the *cis*-acting ORF requirement in DI RNA replication reflects the need for local placement of the ORF product in the assembly of the minus-strand (antigenome) RNA-synthesizing complex (32, 49). A similar complex might also be used for the synthesis of coronavirus subgenomic mRNA minus strands (anti-mRNAs) shown to be involved in mRNA amplification (3, 9, 10, 11, 38, 41, 42). If the ORF product is used stoichiometrically, furthermore, this function could also explain the requirement for continued protein synthesis during coronavirus minus-strand synthesis (37).

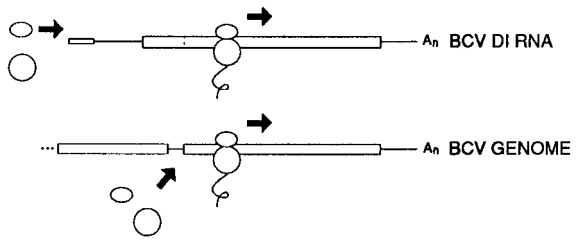

FIG. 6. Possible origins of a *cis*-acting N protein for the replication of DI RNA and coronavirus genome. For the DI RNA genome, ribosomes presumably enter at the 5′ terminus and scan to encounter the *pol*-N start codon. The model predicts that for replication of the viral genome, ribosomes would enter the polycistronic genome internally near the N start codon to translate N and thus provide the *cis*-acting function.

The results with BCV DI RNA, therefore, fit a model proposed earlier for the plus-strand turnip yellow mosaic virus (49) and poliovirus (32), in which the nascent protein is channeled into formation of a complex involved in initiating minus-strand synthesis. How can the *cis* requirement for N in the replication of DI RNA be reconciled with viral genome replication since in the viral genome N is synthesized from the last in a linear series of discontinuous ORFs? Assuming that DI RNA behavior reflects that of the genome, then a requirement for N in *cis* predicts a mechanism of internal ribosomal entry for synthesis of the N protein. Such a mechanism might be similar to those demonstrated for synthesis of the infectious bronchitis virus 12.4-kDa protein from gene 3c in the tricistronic mRNA3 (27) or to the MHV 36-kDa protein from gene 5b in the bicistronic mRNA5 (44). It could be that the intergenic region serves in part as a ribosomal entry site (Fig. 6). The intergenic sequence contains the consensus UCUAAAC thought to possibly function in transactivation of translation of coronaviral mRNAs in infected cells (43). It might be that a similar mechanism acts in *trans* to activate translation from an internal site on the coronavirus genome.

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