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Neither the RNA nor the Proteins of Open Reading Frames 3a and 3b of the Coronavirus Infectious Bronchitis Virus Are Essential for Replication

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Gene 3 of infectious bronchitis virus is tricistronic; open reading frames (ORFs) 3a and 3b encode two small nonstructural (ns) proteins, 3a and 3b, of unknown function, and a third, structural protein E, is encoded by ORF 3c. To determine if either the 3a or the 3b protein is required for replication, we first modified their translation initiation codons to prevent translation of the 3a and 3b proteins from recombinant infectious bronchitis viruses (rIBVs). Replication in primary chick kidney (CK) cells and in chicken embryos was not affected. In chicken tracheal organ cultures (TOCs), the recombinant rIBVs reached titers similar to those of the wild-type virus, but in the case of viruses lacking the 3a protein, the titer declined reproducibly earlier. Translation of the IBV E protein is believed to be initiated by internal entry of ribosomes at a structure formed by the sequences corresponding to ORFs 3a and 3b. To assess the necessity of this mechanism, we deleted most of the sequence representing 3a and 3b to produce a gene in which ORF 3c (E) was adjacent to the gene 3 transcription-associated sequence. Western blot analysis revealed that the recombinant IBV produced fivefold less E protein. Nevertheless, titers produced in CK cells, embryos, and TOCs were similar to those of the wild-type virus, although they declined earlier in TOCs, probably due to the absence of the 3a protein. Thus, neither the tricistronic arrangement of gene 3, the internal initiation of translation of E protein, nor the 3a and 3b proteins are essential for replication per se, suggesting that these proteins are accessory proteins that may have roles in vivo.

Avian *Infectious bronchitis virus* (IBV) is in the genus *Coronavirus*, the family *Coronaviridae*, and the order *Nidovirales* (22, 23, 28). Together with the genetically closely related *Turkey coronavirus* (13, 29), *Pheasant coronavirus* (14), and viruses recently detected in three species of wild birds (34), it forms the group 3 coronaviruses. IBV primarily causes respiratory disease in domestic fowl, though it also replicates at many epithelial surfaces of the alimentary tract, oviduct, and kidney (12, 15).

The virus has a 27.6-kb single-stranded RNA genome of positive polarity associated with a nucleocapsid (N) protein, surrounded by an envelope in which are present the large spike (S) glycoprotein, a smaller integral membrane (M) protein, and a few copies of a much smaller envelope (E) protein, which is targeted to Golgi membranes (18, 19, 47), where it interacts with the M protein (39). The E protein is required for virus particle formation (3, 26, 56). A recombinant murine hepatitis virus (MHV) that did not produce E protein was able to produce infectious virus, but the titers were at least 3 orders of magnitude less than that of wild-type virus (36). A mutant transmissible gastroenteritis virus with a deleted E gene was able to replicate only when the E protein was provided in *trans* (43).

Nonstructural (ns) proteins associated with viral-RNA replication and transcription are encoded by gene 1. Interspersed among the structural-protein genes are small ns-protein genes that vary in number, position, and sequence among the coronaviruses (11, 23, 37). IBV has two such genes, 3 and 5 (Fig. 1A) (5). Gene 5 is functionally bicistronic and encodes two open reading frames (ORFs), 5a and 5b, which are expressed in IBV-infected cells (41). Proteins 5a (8, 57) and 5b (8) are accessory proteins that are not essential for replication. Gene 3 is functionally tricistronic (40), having three ORFs, 3a, 3b, and 3c. It is the third ORF, 3c, which encodes the E protein of IBV (51).

Plasmid expression studies suggest that translation of ORF 3c to produce the E protein is initiated when ribosomes bind to a structure formed by the preceding 3a and 3b sequences (38, 42). The 3a and 3b ORFs are strongly conserved, not only among serotypes of IBV (40), but also in the other group 3 coronaviruses of turkeys and pheasants (13, 14), indicative of a role not only for the 3a and 3b nucleotide sequences, but also for the encoded proteins. The 3a and 3b proteins are indeed synthesized in IBV-infected cells (40). Recently, it has been demonstrated that some 3a is associated with a novel domain of the smooth endoplasmic reticulum (45). However, a mutant isolate of the Beaudette strain of IBV, following multiple passage in Vero cells, was found to contain a single nucleotide insertion in the 3b sequence, resulting in a C-terminally truncated 3b protein that no longer localized to the nucleus, indicating that 3b is not essential for replication (50).

To investigate the requirement for the 3a and 3b proteins for replication, and the requirement for the internal initiation of translation of the 3c ORF, we used our reverse genetic system (6, 8–10, 33) to produce isogenic recombinant IBVs (rIBVs)

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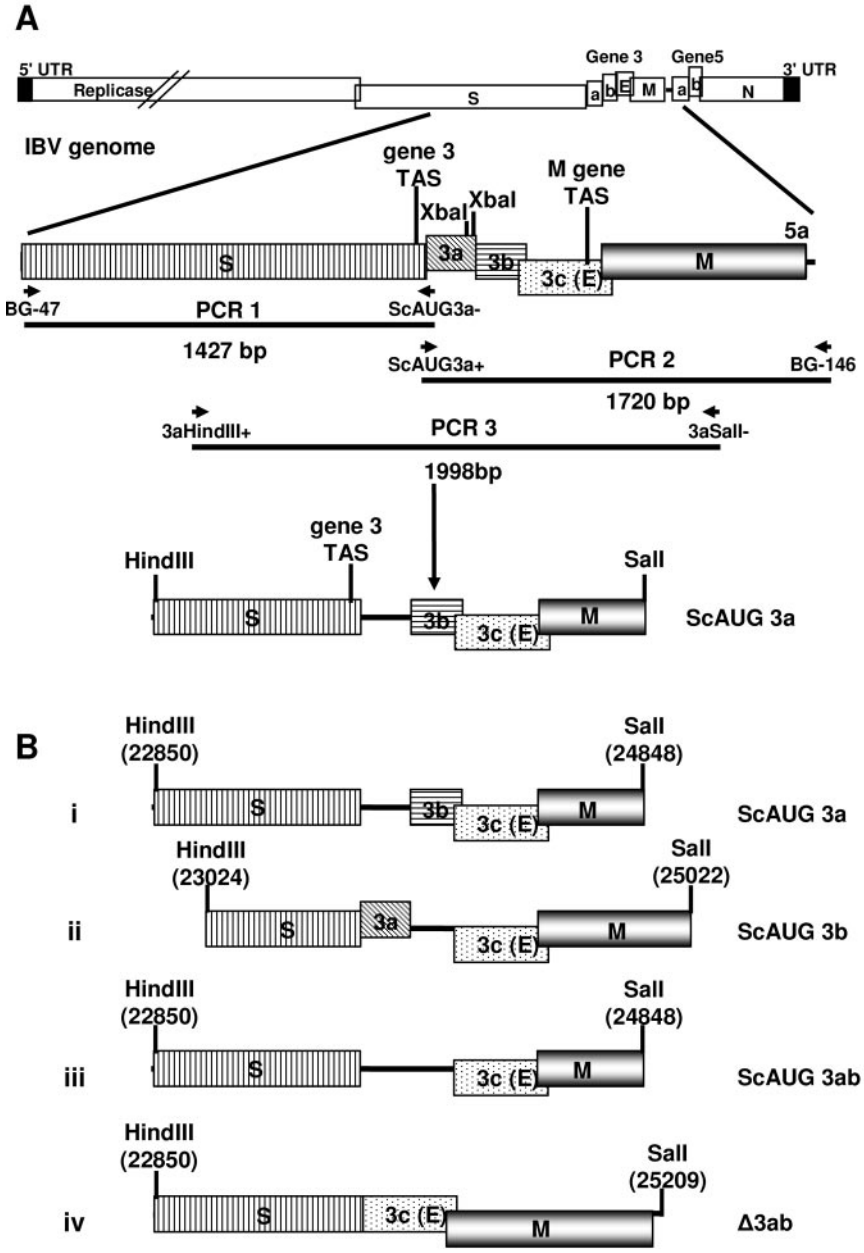

FIG. 1. Construction of the modified IBV gene 3 cDNAs. (A) Modifications to the Beaudette gene 3 were carried out using overlapping PCR mutagenesis. Shown is the method used to generate the modified cDNA with a modified 3a translation initiation codon. In the first two PCR amplifications, two complementary oligonucleotides, ScAUG3a− and SCAUG3a+, were used to introduce the two A^{23857}TG-to-ACC nucleotide substitutions to retain the S gene termination codon and modify the 3a translation initiation codon. PCR 3 was done to join the two initial PCR products, resulting in a contiguous cDNA with the introduced modifications. Two restriction endonuclease sites, HindIII and SalI, were introduced at the ends of the PCR 3 products for cloning the modified cDNAs into pGPTNEB193rev. The HindIII and SalI restriction endonuclease sites were introduced at different positions relative to the IBV genome, depending on the modified cDNA being generated. (B) Four modified gene 3 cDNAs that were initially cloned into pGPTNEB193rev and then used to produce the rIBVs. The positions of the ORF 3a and 3b sequences are shown as black lines following modification of the translation initiation codon to indicate that the sequences are retained but that translation of the gene product is lost. The numbers associated with the HindIII and SalI restriction endonuclease sites represent the IBV genomic positions at the ends of the modified cDNAs. The oligonucleotides used to modify the 3a or 3b translation initiation codons or resulting in the deletion of the coding sequences are shown in Table 1. The ScAUG3ab cDNA was produced as described in Materials and Methods using sequences from pGPT-ScAUG3a and pGPT-ScAUG3b, pGPTNEB193rev-derived plasmids containing the modified 3a and 3b translation initiation codons.

with specific modifications in gene 3. At the heart of our system is a full-length clone (27.6 kb) of the Beaudette strain of IBV cloned at the thymidine kinase locus of the genome of vaccinia virus. Modifications to the IBV gene 3 sequence were introduced into the full-length IBV cDNA sequence in the vaccinia virus genome by a transient dominant selection (TDS) procedure (25), and rIBVs were rescued from the modified IBV cDNA sequences.

TABLE 1. Oligonucleotides used for PCR mutagenesis

PCR[a]	Oligonucleotide	Sequence[b]	Polarity	Position[c]
For recombinant BeauR-ScAUG3a				
1	BG-47	CTTATTGAAGACCTTCTA	+	22441–22459
1	Sc3aAUG−	GACTTTGGAT**GT**TCAAACAGAC	−	23847–23868
2	Sc3aAUG+	GTCTGTTTGA**AC**ATCCAAAGTC	+	23847–23868
2	BG-145	CTGTTGCTACACTTTCTAGC	−	25140–25159
3	3a+HindIII	CGTAAGCTTCTATTCTTACTGAGACTATGG	+	22856–22876
3	3a−SalI	GCAGTCGACGATTCTGGATTAAATGACCAC	−	24834–24854
For recombinant BeauR-ScAUG3b				
1	BG-47	CTTATTGAAGACCTTCTA	+	22441–22459
1	Sc3bAUG−	CTAAGTTTAA**GT**TTAGTCTAGAC	−	24019–24041
2	Sc3bAUG+	GTCTAGACTAA**AC**TTAAACTTAG	+	24019–24041
2	BG-145	CTGTTGCTACACTTTCTAGC	−	25140–25159
3	3b+HindIII	CGTAAGCTTATATTAGAGTGTCACAACAGCG	+	23030–23051
3	3b−SalI	GCTGTCGACGGTGTACAAACAAATATATC	−	25009–25028
For recombinant BeauR-Δ3ab				
1	BG-47ex	CTTATTGAAGACCTTCTATTTACAA	+	22441–22465
1	Δ3ab−	GACTTATTCAATAAATTCAT<>CATCAAACAGACTTTTTAG	−	23840–24226
2	Δ3ab+	GACCTAAAAAGTCTGTTTGATG<>ATGAATTTATTG	+	23836–24218
2	BG-146ex	TCTACCACTCTAAGTTGAGTTAATA	−	25542–25567
3	3a+HindIII	CGTAAGCTTCTATTCTTACTGAGACTATGG	+	22856–22876
3	IGR-SalI	GCAGTCGACGAATAATTTTAAATACTCTCTAC	−	25215–25192

[a] PCRs 1 and 2 produced ~1-kb products encoding the modified sequences at their 3′ and 5′ ends, respectively. The products from PCRs 1 and 2 formed the templates for PCR 3, which resulted in a 2-kb product encoding a centrally located modified sequence.

[b] Nucleotides with single underline in boldface indicate substituted nucleotides to modify the translation initiation codons. Double lines indicate introduced restriction endonuclease sites (with three non-IBV-encoded nucleotides to the left of the restriction site), and < > indicates a deletion of part of gene 3.

[c] The nucleotide positions relate to the genome of IBV Beau-R (10), accession number AJ311317.

MATERIALS AND METHODS

Viruses and cells. Vaccinia virus vNotI/IBV$_{FL}$ is a recombinant vaccinia virus (rVV), derived from rVV vNotI/TK, into which the full-length cDNA of IBV strain Beaudette-CK had been inserted (10). IBV Beaudette-R (Beau-R) was recovered from this full-length cDNA of Beaudette-CK; they are isogenic except for two nucleotide substitutions, C^{19666}→U (a serine-to-leucine substitution in the replicase gene) and A^{27087}→G (a silent mutation in the N gene). Beau-R was used in the experiments described here after five passages in chick kidney (CK) cells, which were prepared as previously described (46). Recombinant vaccinia viruses were propagated and titrated in monkey kidney fibroblasts (CV-1 cells) or monkey kidney epithelial cells (Vero cells) grown in Dulbecco's modified Eagle medium supplemented with 0.37% (wt/vol) sodium bicarbonate, L-glutamine, 10% fetal calf serum, and antibiotics (6, 8–10). Fowlpox virus rFPV-T7 was a recombinant fowlpox virus expressing the bacteriophage T7 RNA polymerase under the control of the vaccinia virus P$_{7.5}$ early-late promoter and was propagated and titrated in chicken embryo fibroblast cells in 199 medium supplemented with 2% newborn calf serum (7). Tracheal-organ cultures (TOCs) were approximately 1-mm transverse sections of trachea taken from 19-day-old, specific-pathogen-free (SPF) Rhode Island Red chicken embryos and incubated in glass test tubes rotated once every 7 min (17, 33).

Recombinant DNA techniques. Recombinant DNA techniques followed standard procedures (1, 48) or were carried out according to the manufacturer's instructions. All IBV-related nucleotide and amino acid residue numbers refer to the positions in the IBV Beau-R genome (10), accession number AJ311317. All plasmids were amplified in *Escherichia coli* DH5α (Invitrogen).

PCR mutagenesis. Overlapping PCR mutagenesis (Fig. 1A) was used to introduce a number of modifications into gene 3 of IBV (an example is shown in Fig. 1A). The ATG translation initiation codons of ORF 3a and ORF 3b were each mutated to AAC (Table 1) to make the modified cDNAs ScAUG3a (Fig. 1B, i) and ScAUG3b (Fig. 1B, ii), respectively ("ScAUG" stands for scrambled [modified] AUG codon). A third modified cDNA had ORF 3a and all except the final 17 nucleotides of ORF 3b deleted (from the first codon of ORF 3a up to and including codon GT^{24203}G of ORF 3b, which is immediately upstream of the first potential translation start codon of ORF 3c), to produce the modified cDNA Δ3ab (Fig. 1B, iv). All gene 3 modifications were produced by overlapping PCR mutagenesis during amplification of 2-kb sections (1 kb on either side of the modification) of the Beaudette genome from pFRAG-3 (10), a template plasmid containing a cDNA copy of the 3′ end of the Beaudette genome from the NheI site in ORF 1b at position 13806 to the 3′ end of the genome. For each modification, two different PCR products approximately 1 kb in length were generated using modified oligonucleotides (Table 1), so that the modified sequence was near the 5′ and 3′ ends. The two PCR products were used as the templates in a third PCR, resulting in three 2-kb products with a centrally located gene 3 modification. The oligonucleotides used in the construction of each modified sequence are described in Table 1.

Plasmid constructions. The amplified sequences were inserted into SalI- and HindIII-digested pGPTNEB193rev (a gift from M. A. Skinner, Institute for Animal Health) (4, 6, 8), a plasmid encoding the *E. coli* guanine xanthine phosphoribosyl transferase (*Eco gpt*) gene under the control of the vaccinia virus P$_{7.5}$ early-late promoter (Fig. 2). Plasmid pCI-N (a gift from Julian Hiscox, University of Leeds) (32) comprised the Beau-CK nucleoprotein gene inserted into pCI-Neo (Promega) so that it was under the control of both the cytomegalovirus RNA polymerase II promoter and the T7 RNA polymerase promoter. A modified gene 3 cDNA containing both modified ORF 3a and 3b AUG translation initiation codons (ScAUG3ab) (Fig. 1B, iii) was produced using sequences from the pGPT-derived plasmids containing the singly modified ATGs. Essentially, an Eco47III fragment, containing the 3b AUG modification, was used to replace the corresponding sequence in pGPT-ScAUG3a, resulting in pGPT-ScAUG3ab, a plasmid containing a cDNA with both 3a and 3b modified ATGs.

Generation of recombinant vaccinia viruses containing full-length IBV cDNAs

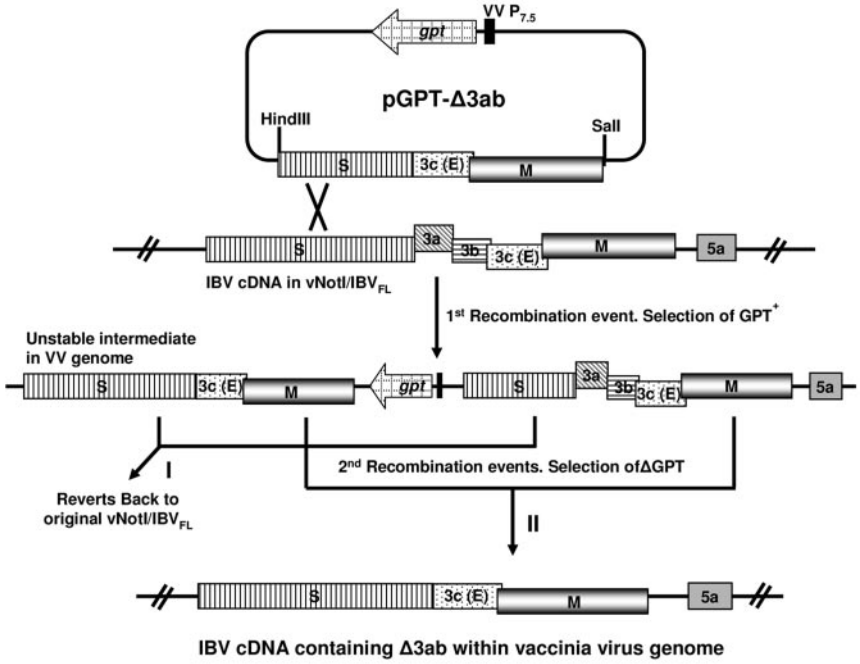

FIG. 2. Schematic diagram showing the transient dominant selection process for modifying the full-length cDNA within the genome of vaccinia virus vNotI/IBV$_{FL}$. The modified cDNAs shown in Fig. 1B were introduced as HindIII-SalI fragments into the TDS GPT transfer/recombination vector pGPTNEB193rev. The diagram outlines the two-step process for modifying the IBV cDNA within vNotI/IBV$_{FL}$ using the modified cDNA containing the 3ab deletion in pGPT-Δ3ab. In step 1, the complete plasmid DNA is integrated into the full-length IBV cDNA by a single-step homologous-recombination event; a potential recombination event is shown. The resultant rVVs have a GPT$^+$ phenotype, allowing selection in the presence of MPA. Removal of MPA results in two types of spontaneous intramolecular recombination events, I and II, due to the instability of the IBV cDNA with tandem repeats of similar sequences. I results in reversion to the Beaudette genotype (no introduced modifications), and II results in an rVV encoding the required modified gene 3 sequences. Both recombination events result in the loss of GPT. The example outlined shows the generation of an rIBV with the 3ab sequence deleted and the 3c (E) sequence directly under the control of the gene 3 TAS.

with modifications in gene 3. Recombinant vaccinia viruses were generated by TDS (25) using the *Eco gpt* gene as the transient selectable marker, as described previously (6, 8). Briefly, CV-1 cells were infected with vNotI/IBV$_{FL}$ (an rVV containing the full-length IBV Beau-R cDNA) (10) and then transfected with a pGPT-based plasmid carrying a modified gene 3 sequence. Homologous recombination between the plasmid-encoded IBV cDNA and the vNotI/IBV$_{FL}$ IBV cDNA led to insertion into the modified gene 3 sequence and the *Eco gpt* gene (Fig. 2). The rVVs were initially selected in the presence of mycophenolic acid (MPA; 25 μg/ml), xanthine (250 μg/ml), and hypoxanthine (15 μg/ml). Several *gpt*$^+$ rVV clones for each gene 3 modification were selected during three rounds of plaque purification in the presence of MPA and then plaque purified three times in the absence of MPA, resulting in a second single homologous recombination event. The second recombination event resulted in the loss of the *Eco gpt* gene, along with one of the duplicated IBV cDNA sequences, so that approximately 50% of the resulting rVVs contained the modified gene 3 sequence (6, 8). The rVVs lacking the *Eco gpt* gene and containing the required gene 3 modifications were identified by PCR amplification using oligonucleotides BG-69 (5′-GCTTTTGCCACTATTATCTTC-3′, corresponding to gene 2) and BG-143 (5′-CAAGAGTACAATTTGTCTCG-3′, corresponding to gene 4) and sequence analysis. Two rVVs representing each gene 3 modification were isolated following two independent TDS recombination attempts.

Recovery of recombinant IBVs. Recombinant vaccinia virus DNA representing each of the modified IBV full-length cDNAs was purified, and corresponding rIBVs were recovered, as previously described (6, 8–10). Recombinant IBVs were characterized and used for subsequent experiments after three passages in CK cells. Two independent clones of each rIBV were rescued (differentiated by the number "1" or "2" at the end of the name of the virus) from each of the two rVV DNAs, except for rIBVs ScAUG3b and ScAUG3ab, for which only one rIBV was recovered. Regions proximal and distal to the ends of the flanking modified gene 3 cDNAs from each of the rIBVs were sequenced; this confirmed that the intended modifications were present.

Multistep growth kinetics in CK cells. Confluent monolayers of CK cells in six-well plates were inoculated with 1 ml of CK medium containing 10 PFU of Beau-R and mutant ScAUG3a-1, ScAUG3a-2, ScAUG3b, ScAUG3ab, Δ3ab-1, or Δ3ab-2 in triplicate for each time point. This small inoculum was used to increase the chance that any reduction in the replication capacity of an rIBV, compared with Beau-R, would be observed. Following an attachment period of 1 h at 37°C, the cells were washed twice in phosphate-buffered saline (PBS) and then incubated at 37°C in 3 ml of CK medium. At selected time points, medium was removed, stored at −70°C, and subsequently analyzed by plaque assay on CK cells. Analysis of variance (Minitab v.12) was used to determine the statistical significance of the results, where P values of < 0.05 were considered to be significant.

Growth kinetics in TOCs. Groups of five TOCs in glass test tubes were inoculated with 10^4 PFU of each virus in 0.5 ml of medium. Following an attachment period of 1 h at 37°C, the TOCs were washed twice with PBS and then incubated at 37°C in 2 ml of medium with rotation (17). At selected time points, three tubes containing each mutant were removed, and the medium was stored at −70°C and subsequently analyzed by plaque assay on CK cells.

Growth kinetics in 10-day-old chicken embryos. Ten-day-old SPF Rhode Island Red chicken embryos were inoculated with 0.1 PFU of rIBV per embryo in 0.1 ml of PBS into the allantoic cavity. The embryos were incubated at 37°C in an egg incubator (Octagon, Brinsea, United Kingdom), and the allantoic fluid was harvested at specific time points after the embryos were chilled at 4°C. The allantoic fluid was stored at −70°C and subsequently analyzed by plaque assay on CK cells.

Assessment of pathogenicity in 10-day-old chicken embryos. Groups of five 10-day-old SPF Rhode Island Red chicken embryos were inoculated with 1 PFU (as determined by titration in CK cells) in 0.1 ml of PBS via the allantoic route and incubated at 37°C in an egg incubator. The embryos were candled three or four times daily for signs of IBV-induced pathology: no movement of the embryo and/or apparent disappearance of the large blood vessels was indicative that the embryo would die.

Confirmation of virus identities. Total cellular RNAs were harvested during each of the growth kinetics and pathogenicity experiments by the RNeasy method (QIAGEN) and amplified for the presence of IBV gene 3 sequences by

reverse transcription (RT)-PCR using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech) (8). The IBV gene 3-derived RT-PCR products were analyzed by sequence analysis for the presence of the gene 3 modifications. This revealed that in all experiments, the virus that was recovered was indeed the intended virus.

Serial passage of rIBVs. Confluent monolayers of CK cells in T25 flasks were infected with 1 ml of undiluted medium after the third passage (P_3) of rIBV in CK cells. Infected cells were incubated at 37°C for 2 days, and the medium was used undiluted to infect another flask. This was repeated to passage 30. Total cytoplasmic RNAs were extracted from the P_{30} cell sheets using the QIAGEN RNeasy kit, and the sequence of the IBV gene 3 was established.

Northern blot analysis of rIBVs. Poly(A)-containing RNAs were purified from rIBV-infected cell lysates using Oligo-dT-Dynabeads (Dynal) and separated in denaturing 1% agarose-2.2 M formaldehyde gels. The RNAs were transferred onto Hybond XL membranes (Amersham) and detected with a psoralen-biotin-labeled probe (Ambion), derived from the IBV 3′ untranslated region (positions 26941 to 27607), and streptavidin-alkaline phosphatase conjugate (Brightstar; Ambion) (8, 21, 24, 30).

Western blot analysis of the rIBVs. rIBV-infected CK cells were lysed 24 h postinfection with RIPA lysis buffer (Santa Cruz Biotechnology) (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing X1 broad-spectrum protease inhibitor cocktail (Complete protease inhibitor cocktail; Boehringer) at 4°C and centrifuged at 14,000 × *g* for 10 min at 4°C. The proteins in the supernatants were denatured with SDS lysis buffer containing dithiothreitol for 5 min at 100°C and separated by Tris-glycine-buffered SDS-polyacrylamide gel electrophoresis using 15% acrylamide gels. The proteins were electroblotted onto Hybond-C (Amersham) membranes and detected using the Advance enhanced-chemiluminescence (ECL) detection system (Amersham Bioscience) (8). The IBV 3a and M proteins were detected using rabbit antisera (gifts from C. E. Machamer, The Johns Hopkins School of Medicine), and the E protein was detected using mouse monoclonal antibodies. The rabbit antisera were diluted 1:100,000 and the monoclonal antibodies were diluted 1:1,000 in PBS, 0.1% Tween 20, and 2% (wt/vol) ECL blocking agent (PBS-Tween blocking agent), followed by goat anti-rabbit and/or mouse immunoglobulin G conjugated to horseradish peroxidase diluted 1:100,000 in PBS-Tween blocking agent, and exposed to film.

Sequence analysis. Nucleotide sequences were determined using either an ABI prism BigDye terminator cycle-sequencing ready-reaction kit (Applied Biosystems) or a CEQ DTCS quick start kit (Beckman-Coulter). Sequence reactions were analyzed on an Applied Biosystems 377 DNA sequencer or a CEQ 8000 capillary sequencer. PREGAP4 and GP4 of the Staden Sequence Software Programs (2) were used for sequence entry, assembly, and editing.

RESULTS

Recombinant IBVs with modified 3a and 3b translation initiation codons. Translation of ORF 3c to produce the E protein is governed by sequences corresponding to ORFs 3a and 3b. In order to minimize the possibility of interfering with the production of E protein, we initially made only minimal nucleotide substitutions to the ORF 3a and 3b translation initiation codons to prevent translation of the respective proteins.

Recombinant IBVs representative of each type of mutant virus were recovered, indicating that neither the 3a nor the 3b protein is essential for replication. This was further confirmed, following comparison of the growth kinetics of the rIBVs with Beau-R, in CK cells (inoculated with 10 PFU) and in 11-day-old embryos (inoculated with 0.1 PFU). These small inocula were used to increase the chance that any reduction in the replication capacity of an rIBV, compared with Beau-R, would be observed. The growth kinetics of mutant viruses unable to translate ORF 3a (Fig. 3A and 4A), ORF 3b (Fig. 3B and 4B), or both ORFs 3a and 3b (Fig. 3C and 4C) were very similar to those of the wild-type virus. Sequence analysis confirmed that the viruses isolated at the end of each experiment were the intended ones and that the gene 3 sequences had acquired no new mutations.

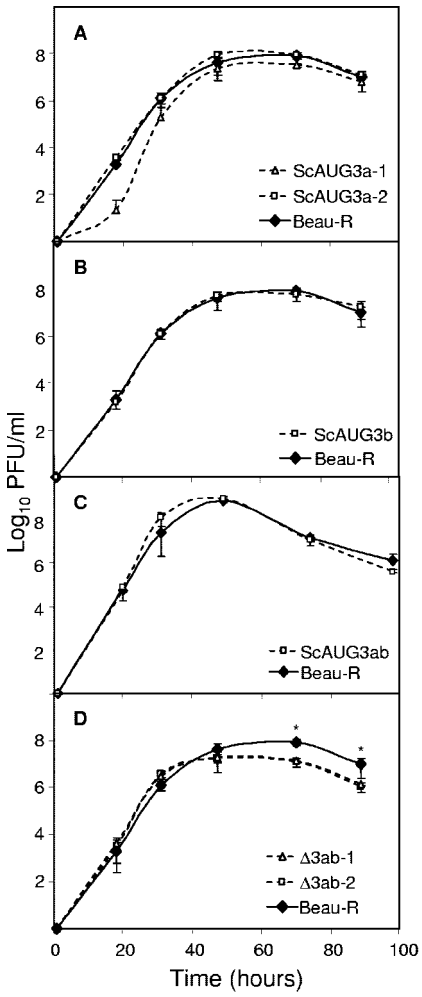

FIG. 3. Growth kinetics of the rIBVs in CK cells. Monolayers of CK cells were inoculated in triplicate with 10 PFU of virus. After 1 h at 37°C, the cells were washed and incubation was continued. Medium was harvested over a period of 100 h for analysis by plaque assay on CK cells. The growth kinetics of Beau-R are compared with those of the rIBVs: (A) ScAUG3a, (B) ScAUG3b, (C) ScAUG3ab, and (D) Δ3ab. Where two independent rIBVs encoding the same modification had been rescued (A and D), both data sets have been illustrated. The error bars represent the standard deviations from three replicates. The asterisks indicate the time points at which the titers of mutant viruses were significantly different from those of Beau-R ($P < 0.05$).

Western blot analysis, using a rabbit antiserum against 3a protein, confirmed that no 3a protein was produced by mutants ScAUG3a and ScAUG3ab, whereas the 3a protein was expressed, as expected, by wild-type Beau-R and by ScAUG3b (Fig. 5). We were unable to demonstrate the absence of the 3b protein, as attempts to make an antiserum to the protein had been unsuccessful.

To assess the stability of the nucleotide substitutions, ScAUG3a, ScAUG3b, and ScAUG3ab were passaged 30 times in CK cells. The sequences representing the modified sequences and flanking regions of 1 to 2 kb on each side of the modification were sequenced. No changes to the introduced nucleotide substitutions were identified in viruses isolated after 30 passages in CK cells.

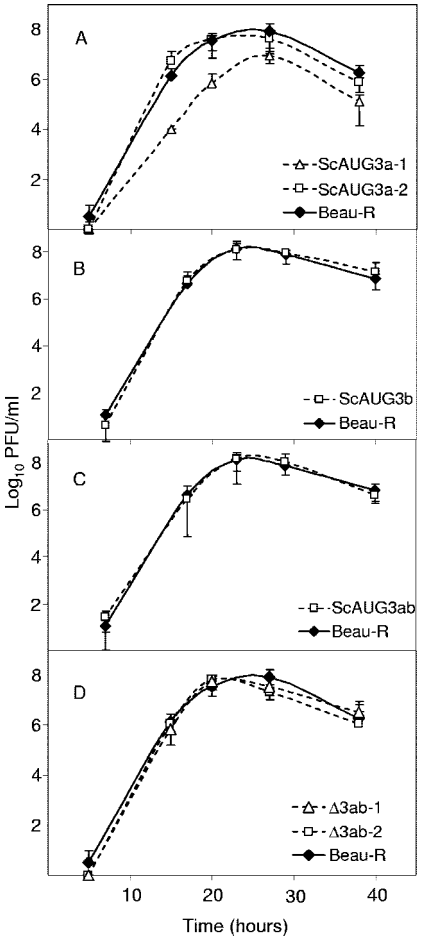

FIG. 4. Growth kinetics of the rIBVs in 10-day-old embryonated eggs. Embryos were inoculated in triplicate via the allantoic cavity with 0.1 PFU of each virus. At each time point, the embryos were chilled at 4°C before the allantoic fluids were analyzed for progeny virus by plaque assay on CK cells. The growth kinetics of Beau-R are compared with those of the rIBVs: (A) ScAUG3a, (B) ScAUG3b, (C) ScAUG3ab, and (D) Δ3ab. Where two independent rIBVs encoding the same modification had been rescued (A and D), both data sets have been illustrated. The error bars represent the standard deviations from three replicates.

Recombinant IBVs with most of ORFs 3a and 3b deleted. The results with viruses containing the modified translation initiation codons demonstrated that neither 3a nor 3b, nor both proteins together, is essential for replication cell culture or in embryonated eggs. To test this notion further, we deleted the coding region for ORF 3a and most of 3b (the 3′ end of ORF 3b could not be removed, as it overlaps the start of ORF 3c, encoding the E protein). In addition to removal of the coding regions of ORFs 3a and 3b, such a deletion resulted in the removal of the sequences believed to be involved in the internal initiation of translation of 3c (E). The deletion resulted in the 3c translation initiation codon replacing the codon responsible for initiation of 3a, resulting in ORF 3c being under the direct control of the gene 3 transcription-associated sequence (TAS). The deletion comprised all of the ORF 3a sequence (from nucleotide position 23856, i.e., from the first nucleotide of the ORF 3a AUG) and most of ORF 3b (up to nucleotide 24204; up to, but not including, the first

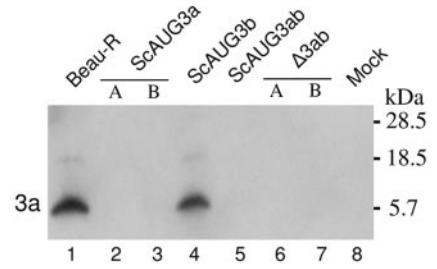

FIG. 5. Western blot analysis for the presence of the ORF 3a protein. Recombinant IBVs containing a modified 3a translation initiation codon should not express the 3a protein. Proteins present in infected (lanes 1 to 7) and mock-infected (lane 8) CK cell extracts were separated by SDS-PAGE, transferred to a Hybond-C membrane, and probed with a rabbit anti-3a serum, followed by ECL detection. The 3a protein was detected in cells infected with Beau-R (lane 1) and with rIBV ScAUG3b (lane 4) and not with viruses unable to express the 3a protein: ScAUG3a (lanes 2 and 3), ScAUG3ab, and Δ3ab (lanes 6 and 7). Where two independent rIBVs encoding the same modification had been rescued (lanes 2 and 3 and lanes 6 and 7), both mutants were analyzed.

nucleotide of the first ORF 3c AUG, 18 nucleotides upstream of the ORF 3b translation stop codon). Two independent rIBVs, Δ3ab-1 and Δ3ab-2, that contained the 3ab deletion in gene 3 were recovered; sequence analysis confirmed that 3c was under the control of the gene 3 TAS. Northern blot analysis confirmed that Δ3ab-1 and -2 synthesized a subgenomic (sg) mRNA 3 (3.4 kb) shorter than the sg mRNA (3.8 kb) synthesized by Beau-R or the other rIBVs. Likewise, both rIBVs synthesized an sg mRNA 2 (6.9 kb) shorter than the sg mRNA 2 (7.3 kb) from Beau-R and the other rIBVs (Fig. 6, lanes 6 and 7). The sizes of the other sg mRNAs (4 to 6) and the sg mRNA profiles of the other rIBVs (lanes 3 to 5) were similar to those of Beau-R (lane 1). Western blot analysis revealed that, as expected, no 3a protein was produced by mutants Δ3ab-1 or -2 (Fig. 5).

As with the other mutants, the titers of Δ3ab-1 and -2 produced in CK cells and embryonated eggs were very similar to those of Beau-R (Fig. 3D and 4D). Sequence analysis confirmed that the viruses isolated at the end of each experiment were the intended ones and that the gene 3 sequences had no other mutations. The replication of Δ3ab-1 and -2 not only confirmed that neither the 3a nor the 3b protein was essential for replication, but also showed that neither the 3a nor the 3b nucleotide sequence was essential for replication. This was particularly noteworthy, as the rIBVs with the deleted 3a-3b region now contained a gene 3 sequence, with 3c (E) protein translation no longer controlled by internal initiation within the 3a-3b nucleotide sequence. Rather, the E ORF was now directly under the control of the gene 3 TAS and was expressed by cap-dependent translation from sg mRNA 3. To see whether production of the E protein was affected by this change, Western blot analysis was performed with a mixture of anti-M and anti-E protein sera. This showed that whereas mutants ScAUG3a, ScAUG3b, and ScAUG3ab, with modified AUGs, had E/M signal ratios similar to each other and to Beau-R, rIBVs Δ3ab-1 and -2 produced less E protein (Fig. 7). Scanning analysis of the amounts of protein expressed, determined from several blots, indicated that production of the E protein by Δ3ab-1 and -2 was

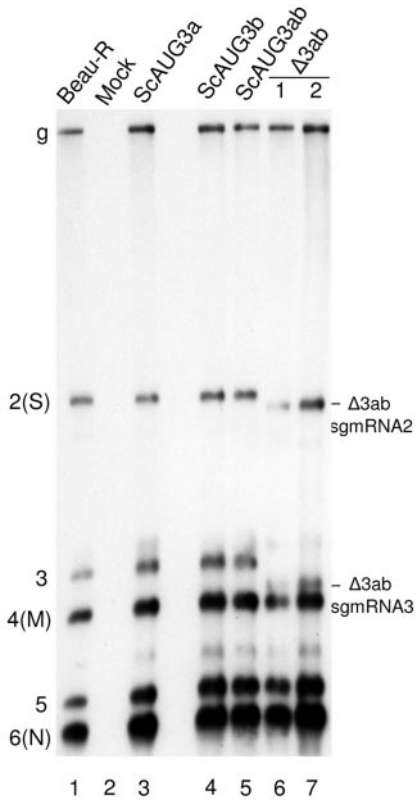

FIG. 6. Northern blot analysis of IBV-derived RNAs from CK cells infected with IBV. The IBV genomic (g) and sg mRNAs (numbered on the left) produced in CK cells infected by Beau-R (lane 1) and rIBVs ScAUG3a (lane 3), ScAUG3b (lane 4), ScAIG3ab (lane5), and Δ3ab (two independent mutants, 1 and 2 [lanes 6 and 7]) are shown. Mutants Δ3ab-1 and -2 produced a smaller gene 2 (sgmRNA2) and gene 3 (sgmRNA3) mRNA, as expected. The IBV-derived RNAS were detected using a 666-bp probe corresponding to the 3′ untranslated region of the IBV genome and present in all the sg mRNAs, as well as in gRNA.

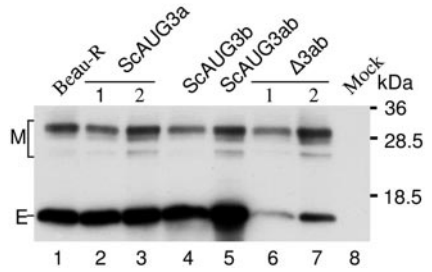

FIG. 7. Western blot analysis for the presence of the IBV E protein (3c protein) in CK cells infected with IBV. Analysis of E protein expression showed that mutants Δ3ab-1 and -2 (lanes 6 and 7) produced less E protein than the amounts expressed in CK cells infected with Beau-R (lane 1) and the other rIBVs: ScAUG3a (lanes 2 and 3), ScAUG3b (lane 4), and ScAUG3ab (lane 5). The proteins were separated by SDS-PAGE, transferred to a Hybond-C membrane, and probed with a mouse monoclonal antibody to the E protein and a rabbit polyclonal serum against the M protein, followed by ECL detection.

approximately fivefold less than that by the other viruses. This suggests that the cap-dependent translation of sg mRNA 3 from Δ3ab-1 and -2 was less efficient than when it was driven by internal initiation within the 3a-3b sequence.

Replication of rIBVs in tracheal-organ cultures. The reduced production of E protein by the Δ3ab mutants had not resulted in a decrease in production of infectious virus in CK cells and embryonated eggs. Similarly, mutant ScAUG3b, which was unable to produce the 3b protein, replicated to titers similar to those of wild-type Beau-R in TOCs (Fig. 8D). The viruses that were unable to produce the 3a protein initially replicated to titers similar to those observed for Beau-R (Fig. 8). However, the titers of the rIBVs declined after 25 h postinfection by 1.0 to 1.5 $\log_{10}$ units, whereas they remained higher for Beau-R and the mutant ScAUG3b. The common factor among the rIBVs whose titers declined early was the lack of expression of the 3a protein; the absence of the 3b protein did not result in an early decline in the titer of the virus. The earlier fall in titer for the viruses unable to produce the 3a protein was reproducible; the experiments illustrated in Fig. 8C and D were performed at different times from each other and from those of Fig. 8A and B. These ex vivo experiments confirmed that the 3a and 3b proteins are not essential for replication per se but suggested that they might have a role in vivo. Sequence analysis confirmed that the viruses isolated at the end of each experiment were the intended ones and that the gene 3 sequences had no other mutations.

Lethality of rIBVs for embryonated eggs. To see if the rIBVs reduced the lethality of Beau-R in 10-day-old embryonated eggs, mutants ScAUG3a-1 and -2, ScAUG3b, ScAUG3ab, and Δ3ab-1 and -2 were inoculated into 10-day-old chicken embryos at a dose of 1 PFU per egg. The time taken for Beau-R to kill 50% of the embryos was 35 h postinfection, and all the rIBVs were in the range of 34 to 37 h postinfection. Thus, inability to produce the 3a and 3b proteins had not attenuated the lethality of the virus for embryos. We did not assess the pathogenicity of the mutants in hatched chickens, as Beau-R already has a nonpathogenic phenotype (27, 33).

DISCUSSION

Our results show that neither the IBV 3a nor 3b protein is essential for replication per se; they can be considered accessory proteins. Shen et al. (50) reported that during multiple passages of IBV Beaudette in Vero cells, there was an insertion of a nucleotide within the 3b ORF. The resulting frame shift would have resulted in the production of a greatly truncated 3b protein. The mutant virus replicated at least as well as wild-type Beaudette in Vero cells and chicken embryos, demonstrating that 3b is not essential for replication. We have recently shown that the IBV 5a and 5b ns proteins are accessory proteins (8). The small ns-protein genes interspersed among the structural-protein genes of MHV (20), transmissible gastroenteritis coronavirus (44, 53), and feline coronavirus (31) have also been removed with little or no effect on the replication of the viruses in vitro, showing them to be accessory proteins. Deletion of the accessory proteins of MHV (20) and feline coronavirus (31) attenuated their pathogenicity in mice and cats, respectively, which strengthens the view that the accessory proteins have roles in vivo, i.e., are advantageous for survival in a host with innate and adaptive immune responses.

The earlier decline in titer of IBV mutants unable to produce the 3a protein in TOCs (not observed in CK cells or in

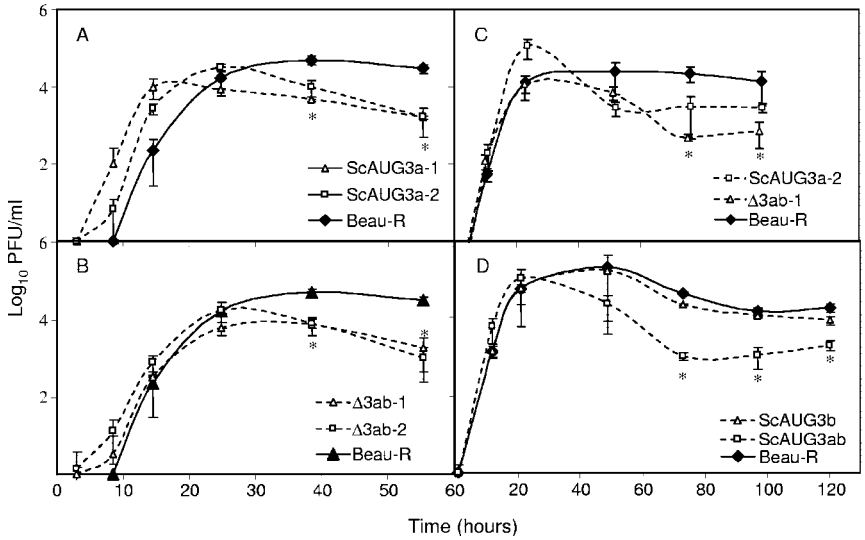

FIG. 8. Growth kinetics of the rIBVs in TOCs (ex vivo). Groups of five TOCs were inoculated in triplicate with 10^4 PFU of virus. After 1 h at 37°C, the TOCs were washed and incubation was continued. Medium was harvested over a period of up to 125 h for analysis of progeny virus by plaque assay on CK cells. The growth kinetics of Beau-R are compared with those of the rIBVs: (A) ScAUG3a, (B) Δ3ab, and (D) ScAUG3b and ScAUG3ab. (C) Repeat of the experiment in panels A and B, in which one of each pair of recombinant viruses was examined and the time course was extended to 100 h. Where two independent rIBVs (-1 and -2) encoding the same modification had been rescued, both data sets have been illustrated. The error bars represent the standard deviations from three replicates. The asterisks indicate the time points at which the titers of mutant viruses were significantly different from those of Beau-R ($P < 0.05$).

10-day-old embryos) is intriguing. The TOCs are transverse sections of the tracheas of 18-day-old embryos that are immunocompetent (49). IBV replicates in the ciliated cells at the lumenal surface of the trachea (16, 35). In our experiments, it took several days for the Beaudette strain of IBV to cause ciliostasis of all the cells, an indicator of cell death—time in which innate immune responses would be expected to be activated and operative. It is possible that one or more populations of underlying cells, including immune cells, might also have produced antiviral responses. Macrophages containing IBV proteins, detected by immunofluorescence, have been observed in the lamina propria beneath the ciliated cells (16). Our observation with the 3a-negative mutants further supports the view that the coronavirus accessory proteins function to enhance the survival of the virus in vivo.

Studies of the transient expression of the IBV E protein have demonstrated that translation of the E protein is initiated internally (40), enabled by folding of the ORF 3a and 3b sequences. Similarly, plasmid studies have shown that the MHV 5a ORF enables internal initiation of the 5b ORF, which encodes E protein (55). In further plasmid studies, Liu and Inglis (42) reported that when ORFs 3a and 3b were removed, translation of ORF 3c did not occur. Our results with rIBV Δ3ab do not agree with this. Although it is likely that the secondary structure of the IBV genomic RNA would have been affected by the deletion of the 3a and most of the 3b sequences, any such change was not deleterious to the virus. Moreover, removal specifically of the structure that controls ORF 3c expression in wild-type IBV, formed by the 3a and 3b sequences, was also not disadvantageous for replication. This is similar to the situation with MHV. Although plasmid studies have demonstrated that translation of E from ORF 5b involves internal initiation of translation mediated by ORF 5a, ORF 5a is not required in recombinant MHV. Removal of MHV gene 4 and the 5a sequence resulted in a virus that replicated to titers within 10 times those of wild-type virus, demonstrating that neither the gene 4 proteins nor the 5a protein is essential for replication in vitro (20). In this mutant, as in our Δ3ab mutant, the expression of the E protein was from a newly created monocistronic gene at the 5′ end of the sg mRNA. These results again raise the question as to why IBV and MHV have the E protein expressed from a third and second ORF, respectively, rather than from a monocistronic gene. Bovine coronavirus, another group 2 coronavirus, has a monocistronic E protein gene, as have the group 1 coronaviruses (23, 37) and severe acute respiratory syndrome coronavirus (52, 54). Our Δ3ab mutants replicated as well as wild-type virus, showing conclusively that translation of the E protein from an sg mRNA via an internal ribosome entry mechanism is not essential for optimal replication of IBV in vitro. Nevertheless, the production of the E protein by Δ3ab was fivefold lower than that by wild-type virus. It is conceivable that the coronavirus E protein has a role in addition to the already identified one of facilitating virus particle formation; it might also have an accessory function, requiring maximal amounts of expression facilitated by translation via an internal ribosome entry mechanism, which is beneficial for this hypothetical role. In any case, the other coronaviruses noted above clearly do not need to maximize their production of the E protein by such a mechanism.

We shall be using our gene 3 mutants to help understand the functions of the 3a and 3b proteins.

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