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Analysis of Second-Site Revertants of a Murine Coronavirus Nucleocapsid Protein Deletion Mutant and Construction of Nucleocapsid Protein Mutants by Targeted RNA Recombination

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The Alb4 mutant of the coronavirus mouse hepatitis virus (MHV) is both temperature sensitive and thermolabile owing to a deletion in the gene encoding its nucleocapsid (N) protein. The deletion removes 29 amino acids that constitute a putative spacer region preceding the carboxyl-terminal domain of the protein. As a step toward understanding the structure and function of the MHV N protein, we isolated multiple independent revertants of Alb4 that totally or partially regained the ability to form large (wild-type-sized) plaques at the nonpermissive temperature. The N proteins of these revertant viruses concomitantly regained the ability to bind to RNA in vitro at a temperature that was restrictive for RNA binding by Alb4 N protein. Sequence analysis of the N genes of the revertants revealed that each contained a single second-site point mutation that compensated for the effects of the deletion. All reverting mutations were clustered within a stretch of 40 amino acids centered some 80 residues on the amino side of the Alb4 deletion, within a domain to which the RNA-binding activity of N had been previously mapped. By means of a targeted RNA recombination method that we have recently developed, two of the reverting mutations were introduced into a wild-type MHV genomic background. The resulting recombinants were stable and showed no gross phenotypic differences from the wild type. A detailed analysis of one, however, revealed that it was at a selective disadvantage with respect to the wild type.

Coronaviruses are a family of enveloped, positive-sense RNA viruses that infect a variety of mammals and birds, generally in a species-specific manner (11, 20, 22). The murine member of this family, mouse hepatitis virus (MHV), has been well studied because it is amenable to biochemical and genetic analysis and because it provides models for viral pathogenesis (3). Although its genome is the largest mature RNA molecule known (31 kb) (2, 13), MHV has a limited complement of structural proteins. The virion envelope, derived from an internal cellular membrane, exhibits two major glycoproteins, the spike (S) and the membrane (M) proteins, as well as a recently recognized minor component, the small membrane protein that is the product of gene 5b (26). A third major glycoprotein, the hemagglutinin-esterase (HE), is also found in some strains. Packaged within the MHV membrane envelope is a helically symmetric nucleocapsid, which is composed of the RNA genome coated with multiple molecules of the nucleocapsid protein (N), a highly basic phosphoprotein. In addition to protecting and structuring the virus genome, the N protein may play roles in viral RNA synthesis (1, 4) and in translation of viral mRNAs (23). Little structural information about N is available. On the basis of an amino acid sequence comparison of the N proteins of a number of MHV strains, we have proposed that this molecule consists of three highly conserved domains separated by spacer regions of variable composition (17). The most fundamental property of N, its ability to bind RNA, has been mapped to the central domain (domain II) (14, 16).

At present, only two coronavirus N protein mutants have been described, both in MHV-A59. One of these, Alb1, has a point mutation in the carboxy-terminal portion of domain I, a region that is highly conserved among the N proteins of all species of coronaviruses, which otherwise do not show a great degree of sequence homology (15). The other mutant, Alb4, contains a 29-amino-acid deletion encompassing the putative spacer region between domains II and III (9). To gain more insight into N protein structure and function, we have characterized 12 independent revertants of Alb4. Part of this analysis entailed the introduction into wild-type genomic backgrounds of two of the mutations responsible for reversion. This is the first report of the engineering of site-specific amino acid coding changes into the genome of a coronavirus.

MATERIALS AND METHODS

Virus and cells. MHV-A59 wild-type, mutant, revertant, and recombinant virus stocks were grown in mouse 17 clone 1 (17Cl1) cells. Plaque titer determinations and plaque purifications were performed in mouse L2 cells. Thermal inactivations to select revertant or recombinant viruses were carried out at 39 or 40°C and pH 6.5 as previously described (9).

Isolation of Alb4 revertants. The initial set of eight independent revertants of Alb4 (designated Alb4RevA through Alb4RevH) was obtained from virus stocks originating from individual plaques of Alb4 isolated at the permissive temperature, 33°C. Each virus stock was serially passaged twice at 33°C and then eight times at the nonpermissive temperature, 39°C. A portion of the 10th passage stock was thermally inactivated for 24 h at 40°C, and revertants were isolated as survivors able to form large (wild-type-size) or intermediate (between Alb4-size and wild-type-size) plaques at 39°C.

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A second set of four independent revertants (designated Alb4RevI through Alb4RevL) arose from various attempts to construct targeted recombinants in experiments similar to those described previously (9). These revertants, which were also isolated after thermal inactivation following multiple passages, all formed intermediate-size plaques at 39°C.

RNA-binding assay. The RNA-binding activity of N protein from wild-type, Alb4, revertant, or recombinant virus was assayed as described in detail previously (14). In brief, N protein was synthesized in vitro in a rabbit reticulocyte lysate programmed either with total cytoplasmic RNA from infected 17Cl1 cells or with N mRNA synthesized from constructed transcription vectors. Complex formation between [^{35}S]methionine-labeled translated N protein and an endogenous RNA in the reticulocyte lysate was determined by electrophoresis in a nondenaturing polyacrylamide gel system. As a control, translated samples were also analyzed by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10).

Cloning and sequencing of the N genes of revertants of Alb4. cDNA clones containing the entire N gene and part of the M gene of AlbRevH were generated from purified viral genomic RNA. First- and second-strand cDNAs were synthesized by standard techniques (6, 18), and the *Kpn*I-*Sac*I fragment running from the middle of the M gene to the distal portion of the 3′ untranslated region (UTR) was cloned into the *Kpn*I and *Sac*I sites of the vector pGEM-7Zf(+) (Promega). The same *Kpn*I-*Sac*I fragment from each of the other Alb4 revertants was cloned by using total RNA from infected cells, either by the same technique or by reverse transcription followed by PCR. In all cases, the primer for first-strand cDNA synthesis was complementary to nucleotides (nt) 18 to 35 from the end of the 3′ UTR. PCRs, carried out as described previously (9), used this primer plus a primer corresponding to nt 175 to 192 of the M gene. For each revertant, the sequence of the N protein-coding region was determined at least once on both strands. Nucleotide changes were verified on at least two additional independent clones and were finally confirmed by direct sequencing of PCR product obtained from infected-cell RNA. DNA sequencing was carried out by the method of Sanger et al. (19) with modified T7 DNA polymerase (Sequenase; U.S. Biochemical) and primers described elsewhere (17).

Plasmid constructs. Chimeric N genes composed of portions of the Alb4 N gene and the Alb4RevH N gene were constructed from plasmid pCR114, a T7 transcription vector containing most of the MHV leader sequence, the entire Alb4 N gene, and most of the MHV 3′ UTR (14). Derivative plasmids containing either the distal two-thirds, the middle one-third, or the distal one-third of the Alb4RevH N gene were generated by exchange of the *Apa*I-*Bst*EII, the *Apa*I-*Spe*I, or the *Spe*I-*Bst*EII fragments, respectively, of pCK1, a full-length clone of the Alb4RevH gene. The resulting plasmids used in further analysis were pCK7, which encoded a protein denoted 4/H/H, with residues 135 to 454 from Alb4RevH; pCK21, which encoded a protein denoted 4/H/4, with residues 135 to 306 from Alb4RevH; and pCK23, which encoded a protein denoted 4/4/H, with residues 307 to 454 from Alb4RevH (see Fig. 2B).

Two transcription vectors, used for the incorporation of site-specific mutations into the MHV N gene, were prepared from pCK70, a T7 transcription vector expressing the smallest subgenomic RNA (RNA7) of wild-type MHV-A59 (9). Plasmid pDP51 carries the Alb4RevH point mutation without the Alb4 deletion; and plasmid pDP80 carries the Alb4RevD point mutation without the Alb4 deletion (see Fig. 4). Each was constructed by exchange of the smaller *Nhe*I-*Eco*RI fragment of pCK70 with the corresponding fragment from a cDNA clone of the N gene of the respective mutant. Because the *Eco*RI site of pCK70 is not unique, this was carried out either by construction of an intermediate plasmid with subsequent transfer of the *Nhe*I-*Acc*I fragment or by a three-way ligation that included the smaller *Eco*RI-*Hin*dIII and the larger *Nhe*I-*Hin*dIII fragments of pCK70. A third vector, used for transduction of the Alb4RevH mutation into Alb4, was prepared from pB36, a T7 transcription vector expressing a synthetic MHV defective interfering RNA that contains the entire N gene (15). This plasmid, pCK160, was generated by exchange of the *Nhe*I-*Sac*I fragment of pB36 with the corresponding fragment from the Alb4RevH clone, pCK1 (see Fig. 8A). All constructs were verified by restriction analysis, and all newly generated junctions were verified by DNA sequencing.

RNA purification, synthesis, and sequencing. Viral genomic RNA was purified exactly as described previously (9). Total cytoplasmic RNA from MHV-infected 17Cl1 cell monolayers was purified by a Nonidet P-40 gentle-lysis procedure (8). Synthetic RNAs were transcribed with bacteriophage T7 RNA polymerase as described previously (9, 14), except that RNA products were purified with RNaid (Bio 101, Inc.). Direct RNA sequencing was carried out by a dideoxy-chain termination method (5), except that the labeling-reaction mixture contained 1 to 5 μg of RNA template, 10 pmol of primer, 39 mM Tris-HCl (pH 8.6), 48 mM NaCl, 4.8 mM $MgCl_2$, 3.2 mM dithiothreitol, 0.5 μM dGTP, 0.5 μM dCTP, 0.5 μM dTTP, 10 μCi of [α-^{32}P]dATP (3,000 Ci/mmol; Amersham), and 12 U of avian myeloblastosis virus reverse transcriptase (Life Sciences) in a final volume of 15.5 μl.

Targeted recombination. The second-site point mutations from Alb4RevH and Alb4RevD, in a background not containing the Alb4 deletion, were each transduced into MHV by the targeted RNA recombination method described previously (9), with Alb4 as the recipient virus and in vitro transcripts from *Nsi*I-truncated pDP51 or pDP80 as donor RNAs. The construction of recombinant viruses was confirmed by reverse transcription PCR (RT-PCR) analysis of infected cellular RNA by using a pair of primers flanking the Alb4 deletion, corresponding to nt 1077 to 1094 of the N gene and complementary to nt 18 to 35 from the end of the 3′ UTR (9). The relevant region of genomic RNA from purified recombinant virus was then directly sequenced with a primer complementary to nt 1011 to 1030 of the N gene.

The second-site point mutation from Alb4RevH, in a background that did contain the Alb4 deletion, was transduced into Alb4 with in vitro transcripts from *Nsi*I-truncated pCK160, as described previously (15). The construction of recombinant viruses was confirmed by sequencing genomic RNA from purified virus with primers complementary to nt 1011 to 1030 and nt 1267 to 1284 of the N gene for the Alb4RevH point mutation and the Alb4 deletion, respectively.

Growth competition between wild-type and recombinant viruses. Wild-type and recombinant Alb81 viruses mixed to give infectious titer ratios of 1:1 or 1:10 or Alb81 virus alone was used to inoculate 25-cm^2 17Cl1 cell monolayers at a total multiplicity of infection of 5 PFU/cell. Infections were carried out at both 33 and 39°C. Progeny released viruses (45 μl of 5 ml [total volume]) were used as the inoculum for a subsequent round of infection. Following eight such serial passages, the titer of the final round of released virus was determined on L2 cells, and 10 plaques of each sample were picked for analysis. Each plaque was used to infect a monolayer of 17Cl1 cells, and total infected-cell RNA was purified and sequenced with a primer complementary to nt 1011 to 1030 of the N gene.

TABLE 1. Characteristics of revertants of Alb4

Virus	Plaque size (mm)[a]	Earliest passage level of appearance	Fraction surviving heat treatment[b]
Alb4RevA	2.5–3.0	6	6.4×10^{-2}
Alb4RevB	2.5–3.0	6	8.9×10^{-2}
Alb4RevC	3.0	6	4.9×10^{-2}
Alb4RevD	2.0–2.5	8	8.4×10^{-3}
Alb4RevE	3.0–3.5	4	6.5×10^{-2}
Alb4RevF	2.5–3.0	6	7.9×10^{-2}
Alb4RevG	3.0	6	5.3×10^{-2}
Alb4RevH	3.0	4	5.8×10^{-2}
Alb4	1.0–1.5		3.2×10^{-4}
Wild type	3.5–4.0		9.4×10^{-2}

[a] The diameter was measured after incubation for 48 h at 39°C.
[b] Viruses (0.4×10^8 to 6.8×10^8 PFU/ml) were treated for 24 h at 40°C, as described in Materials and Methods.

RESULTS

Isolation of Alb4 revertants. Alb4 is a mutant of MHV-A59 that is both temperature sensitive and thermolabile (9). It forms very small plaques at the nonpermissive temperature, 39°C, in contrast to plaques formed by wild-type MHV. In addition, incubation of Alb4 virions at the nonpermissive temperature results in a substantial loss of infectious titer, typically 10^2- to 10^3-fold greater than the loss experienced by wild-type virus under identical conditions. Both aspects of the phenotype of this mutant have been shown to be caused by an internal deletion of 29 amino acids in a region near the carboxy terminus of the nucleocapsid protein (9). To obtain information about N protein structure and function, we undertook a search for revertants of Alb4. Virus stocks were begun from individual purified plaques of Alb4 and were serially passaged 10 times. Released viruses from passage 10 were thermally inactivated, and survivors were selected by plaque titer determination at 39°C and purified for further analysis. A multiple-passage protocol was used because in preliminary experiments, it had not been possible to detect revertants in single-passage stocks of Alb4. We considered that this indicated that reverting mutations were very rare or, alternatively, that multiple mutations might have to accumulate to compensate for the effects of the deletion.

The initial search yielded a set of eight revertants, designated Alb4RevA through Alb4RevH. All formed large (wild-type-size) plaques at the nonpermissive temperature, with the exception of Alb4RevD, which formed plaques intermediate in size between those of the wild type and of Alb4 (Table 1).

Additionally, the virions of all revertants exhibited thermal stabilities that were comparable to those of the wild type at the nonpermissive temperature, except for Alb4RevD, which had a thermal stability intermediate between that of the wild type and that of Alb4 (Table 1). Thus, the plaque size and the thermolability phenotypes were absolutely concordant. Because Alb4 is still viable at the nonpermissive temperature and because multiple passages were required to obtain revertants, we could not determine the frequency of reversion. Retrospective examination of earlier (even-numbered) passages of the stocks that gave rise to each revertant showed that at least four passages, and in one case as many as eight passages, had been required before revertants could be detected (Table 1). Notably, we did not observe intermediate-size plaques in early passages of stocks that ultimately yielded large-plaque revertants. This suggested that reversion of Alb4 did not occur by accumulation of multiple mutations, each producing an incremental increase in plaque size.

Functional analysis of revertant N proteins. In prior work, we developed an in vitro assay to localize the RNA-binding domain of the MHV nucleocapsid protein (14). In this assay, N protein translated in a reticulocyte lysate was found to bind to an endogenous RNA in the lysate, and as a consequence, it migrated toward the positive pole in a nondenaturing gel. Construction of a series of deletion mutants of N allowed the mapping of the RNA-binding function of this molecule to the central of three domains that had previously been postulated on the basis of interstrain sequence comparison (17). It was subsequently found that the RNA-binding ability of Alb4 N protein showed a marked temperature sensitivity in this assay. Although it exhibited no defect at ambient temperature (20°C), at 30°C in vitro translated Alb4 N protein failed to enter a nondenaturing gel whereas wild-type N protein was unimpaired in RNA binding at this temperature. By contrast, the N proteins of each of the eight revertants regained the ability to bind to RNA at 30°C, although quantitatively to a lesser extent than for wild-type N protein (Fig. 1). Thus, the plaque size and thermolability phenotypes of Alb4 and its revertants correlated with the RNA-binding ability of the N proteins of these viruses at an elevated temperature. Analysis of the same translation products by SDS-PAGE showed that all revertant N proteins had the same mobility as the Alb4 N protein (Fig. 1), indicating that each very probably had the same deletion as Alb4 and that reversion was due to one or more second-site mutations.

Mapping of the reverting mutation in Alb4RevH. We next focused on one of the revertants, Alb4RevH. To map the mutation(s) in this virus, its N gene was cloned and three chimeric transcription vectors were constructed, each of which contained portions of the Alb4 and the Alb4RevH N genes (Fig. 2). This approach was taken because we originally expected that a number of mutations might have arisen in the N gene during the multiple passages used to obtain the revertants and that only a subset of these would be relevant to the phenotypes under examination. Transcripts synthesized in vitro from the chimeric N vectors were used to program a reticulocyte lysate. When the resulting translated N proteins were assayed for RNA binding at 30°C, it was found that two of the chimeric constructs, 4/H/H and 4/4/H, retained the ability to bind to RNA to the same extent as had Alb4RevH (Fig. 2A). The remaining chimeric N protein, 4/H/4, like Alb4, was unable to bind to RNA. Since this assay depended only on the properties of N protein expressed in isolation from any other viral components, these results suggested that the reverting mutation(s) in Alb4RevH must be located in the N protein. Further, these results mapped the reverting mutation(s) to the

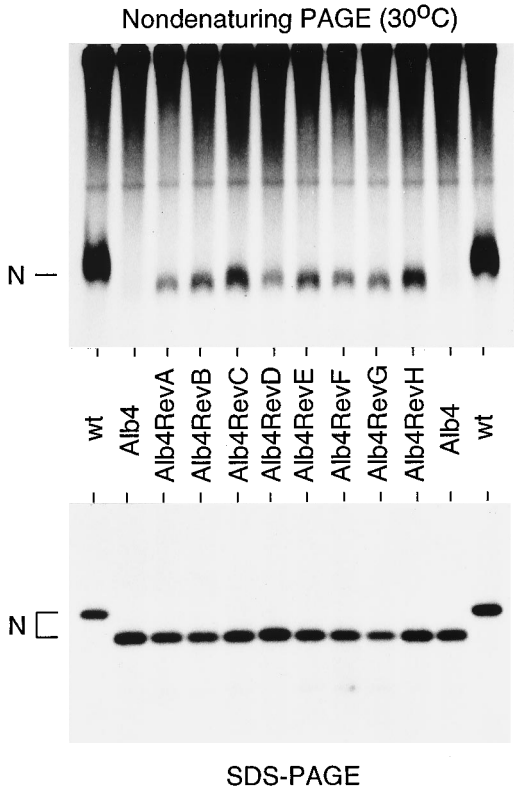

FIG. 1. Binding of RNA by translated N proteins. Total cytoplasmic RNA was purified from 17Cl1 cells infected with wild-type (wt) MHV, the mutant Alb4, or the revertants Alb4RevA through Alb4RevH. RNA was translated in a reticulocyte lysate, and [^{35}S]methionine-labeled N protein was analyzed by nondenaturing PAGE performed at 30°C (upper panel) or by SDS-PAGE (lower panel). In the upper panel, the narrow [^{35}S]methionine-labeled band migrating more slowly than N protein is due to an endogenous component of the reticulocyte lysate, which appears even in reactions not programmed with RNA (14).

distal one-third of the N protein (residues 307 to 454), since all constructs containing the carboxyl-terminal portion of Alb4 RevH N were able to bind to RNA, whereas all constructs containing the carboxyl-terminal portion of Alb4 N had lost the ability to bind to RNA (Fig. 2B). Sequence determination of the corresponding segment of the Alb4RevH N gene confirmed the presence of the Alb4 deletion and revealed only a single mutation, a change of nt 946 of the coding region from G to C, creating a change of amino acid 316 from glutamate to glutamine (Fig. 2B), within the domain of the N protein previously shown to be responsible for RNA binding (14). Moreover, contrary to our expectations, sequencing of the remainder of the Alb4RevH N gene, as well as half of the adjacent M gene, revealed no additional mutations. Thus, it was likely that a single mutation could compensate for the effects of the Alb4 deletion on plaque size, thermolability, and RNA binding.

Sequence analysis of other Alb4 revertants. The N genes of the remainder of the revertants were then cloned and sequenced. In addition, we cloned and sequenced the N genes of four other independent revertants, Alb4RevI through Alb4RevL. Members of this set had been obtained as byproducts from a number of targeted recombination experiments (9), and all of these formed intermediate-size plaques at the nonpermissive temperature (Table 2). As expected, all revertants had the identical deletion to that of Alb4. In accord with the result obtained for Alb4RevH, each revertant had only a single-nucleotide change in the entire region sequenced,

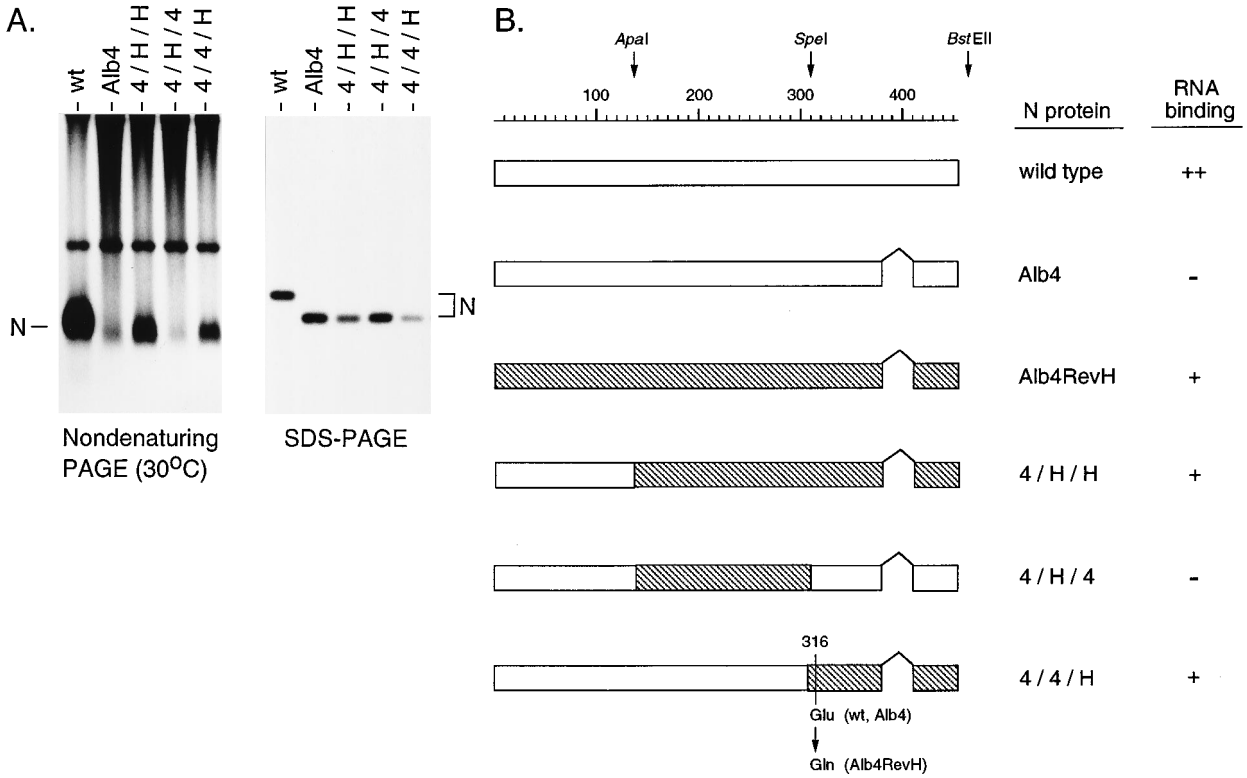

FIG. 2. RNA-binding analysis of Alb4 and Alb4RevH hybrid N proteins. RNA was synthesized in vitro from transcription vectors containing the wild-type N gene, the Alb4 N gene, or hybrid constructs comprising portions of the Alb4 and Alb4RevH N genes (4/H/H, 4/H/4, and 4/4/H) as detailed in Materials and Methods. (A) Nondenaturing PAGE performed at 30°C and SDS-PAGE of in vitro translated, [^{35}S]methionine-labeled N proteins. (B) Summary of the RNA-binding ability of wild-type, Alb4, Alb4RevH, and hybrid N proteins. The result for Alb4RevH is taken from Fig. 1. The number line at the top denotes amino acid residues in the wild-type N protein. The positions of restriction sites used in the construction of transcription vectors are indicated above. Open rectangles represent wild-type N protein sequence; hatched rectangles represent Alb4RevH N protein sequence.

which encompassed half the M gene, the entire N gene, and almost the entire 3′ UTR of the viral genome. Remarkably, all of the remaining large-plaque revertants, Alb4RevA through Alb4RevC and Alb4RevE through Alb4RevG, had the same mutation as Alb4RevH. By contrast, each of the intermediate-size plaque revertants had a different mutation: C to A at nt 844 (Alb4RevK), A to T at nt 854 (Alb4RevJ), A to G at nt 929 (Alb4RevD), A to C at nt 947 (Alb4RevL), and T to C at nt 962 (Alb4RevI). The resulting single-amino-acid changes in the revertants are summarized in Fig. 3. Although the types of changes observed do not fall into an obvious pattern, they are highly localized. All are clustered within a stretch of 40 amino acids centered some 80 residues to the amino side of the Alb4 deletion and within the RNA-binding domain of the N protein.

TABLE 2. Plaque sizes of Alb4 revertants and recombinants at different temperatures

Virus	Mutation(s)	Plaque size (mm) at[a]:		
		33°C	37°C	39°C
Alb4	Δ380–408	2.6	2.8	0.4
Alb4RevD	Q310R, Δ380–408	2.4	3.3	2.0
Alb4RevH	E316Q, Δ380–408	2.8	3.2	2.9
Alb4RevI	V321A, Δ380–408	2.5	3.1	2.0
Alb4RevJ	Q285L, Δ380–408	2.8	3.2	1.9
Alb4RevK	P282T, Δ380–408	2.5	3.0	1.6
Alb4RevL	E316A, Δ380–408	2.6	3.0	1.6
Alb81	E316Q	2.7	3.5	3.6
Alb83	Q310R	2.6	3.5	3.7
Wild type	none	2.8	3.3	3.4

[a] Plaque diameters were measured at 48 h postinfection for each L2 cell monolayer. Each value is the average for 10 or 11 plaques.

Construction of N protein mutants containing a reverting mutation in the absence of the Alb4 deletion. To gain insight into the nature of the mutations able to compensate for the Alb4 deletion, we sought to construct MHV mutants that contained a reverting mutation in the context of an otherwise wild-type N gene. Toward this end, the mutations from Alb4RevH and Alb4RevD were chosen to represent, respectively, large and intermediate-size plaque revertants. Mutants were constructed by means of a targeted RNA recombination technique that we have recently described (9). This method relies on recombination between Alb4 genomic RNA and synthetic subgenomic RNA7 (lacking the Alb4 deletion) to transduce a linked site-specific mutation into the MHV genome. For this purpose, two transcription vectors, pDP51 and pDP80, were made (Fig. 4). Each was derived from pCK70, a plasmid template for wild-type RNA7 (9), and contained, respectively, the second-site mutation from Alb4RevH or Alb4RevD. Candidate recombinant viruses were selected as thermally stable progeny from a cotransfection of Alb4 genomic RNA and synthetic RNA7 obtained by run-off T7 transcription of pDP51 or pDP80. Two plaques from each cotransfection were chosen for purification and further analysis: Alb81 and Alb82, which originated from pDP51 (Alb4RevH mutation), and Alb83 and Alb84, which originated from pDP80 (Alb4RevD mutation).

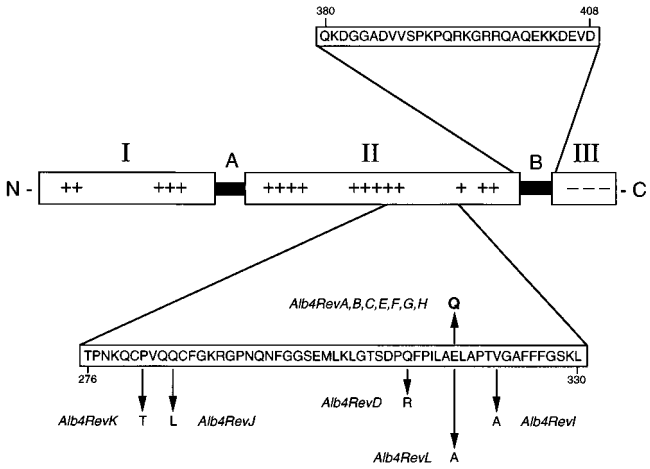

FIG. 3. Alb4 revertant N protein sequences. The single-amino-acid change in each of 12 independent revertants of Alb4 is given in the context of a three-domain model of the MHV N protein proposed previously (17). Indicated schematically are regions of N containing major clusters of net positive or net negative charge. Shown above the model are the residues that are deleted in the N proteins of Alb4 and its revertants.

RNA isolated from cells infected with Alb81 through Alb84 was analyzed by RT-PCR, using two primers flanking the region of the Alb4 deletion (Fig. 5A). This confirmed that each was, indeed, a recombinant, having regained the material that is deleted in Alb4. Alb81 through Alb84 each yielded a PCR product of the same size as that of wild-type MHV (573 bp), in contrast to the product obtained with Alb4 (486 bp). As shown in Fig. 5B, direct sequencing of genomic RNA isolated from purified virions of Alb81 and Alb83 revealed that they had received, respectively, the mutation from Alb4RevH or Alb4RevD. Thus, in isolation, these mutations were not grossly deleterious to the virus, unlike the Alb4 deletion for which they could compensate.

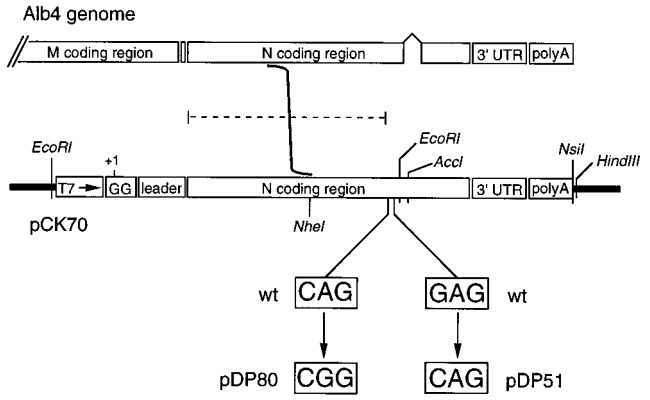

FIG. 4. Targeted recombination to construct mutants containing a reverting mutation in the absence of the Alb4 deletion. Transcription vectors pDP51 and pDP80 were derived from pCK70, a template for in vitro transcription of wild-type (wt) subgenomic RNA7 (9). Plasmid pDP51 has a single-base change at nt 946 of the N gene coding region, corresponding to the point mutation in Alb4RevH, but is otherwise identical to pCK70. Plasmid pDP80 has a single-base change at nt 929 of the N gene coding region, corresponding to the point mutation in Alb4RevD, but is otherwise identical to pCK70. Shown are restriction sites relevant to the construction of the vectors, as detailed in Materials and Methods. Indicated above the vector schematic is the region in which a crossover event between vector-derived synthetic RNA and Alb4 genomic RNA could generate the selected recombinants.

Characterization of constructed N protein mutants. As an initial step to characterize Alb81 and Alb83, plaque titers were determined at different temperatures. Table 2 compares the plaque sizes of the recombinants with those of wild type, Alb4, and each unique revertant. We consistently observed slightly larger plaques for Alb81 and Alb83 than for the wild type at 39 and 37°C, but it is not clear whether the magnitude of this was significant. Heat inactivation assays were also carried out to test the thermal stability of the recombinants. However, no difference was seen in the survival rate of the wild type, Alb81, and Alb83 after 24, 48, and 72 h of incubation at 40°C (data not shown).

To attempt to detect more subtle differences between wild-type virus and one of the recombinants, a coinfection experiment was carried out to assess whether one had a growth advantage over the other at either 33 or 39°C. Mixtures of wild-type virus and Alb81, at input ratios of 1:1 or 1:10, were serially passaged for eight rounds on monolayers of 17Cl1 cells. The identities of individual plaques obtained from released virus from the final passage were then determined by direct sequencing of RNA from infected cells (Table 3). We were surprised to find that at an input multiplicity ratio of 1:1 and at either 39 or 33°C, wild-type virus predominated by the eighth passage. Moreover, even if the input virus at the first passage contained only 1/10 as many PFU of wild-type virus as of Alb81, wild-type virus overwhelmingly outnumbered Alb81 by the eighth passage at 39°C and was at least as numerous as Alb81 by the eighth passage at 33°C. Thus, wild-type virus had a measurable competitive advantage over Alb81 for growth in tissue culture. In parallel control samples, Alb81 alone was passaged eight times at either temperature, and all plaques examined from the final passage still contained the expected mutation at codon 316 of the N gene (Table 3). The final-passage stocks of Alb81 also had titers comparable to those of all the mixed infections. This showed that Alb81 was stable and that the above results were not due to a high selective pressure for reversion of the point mutation that had been engineered into this recombinant.

To see whether there was a molecular correlate for these observations, we examined the temperature sensitivity of RNA binding by the N proteins of Alb81 and Alb83 translated in vitro from transcripts of pDP51 and pDP80 (Fig. 6). As noted above, at 30°C RNA binding by wild-type N protein was stable, whereas Alb4 N protein failed to enter the nondenaturing gel. At the same temperature, the N proteins of Alb81 and Alb83 were at least as able as the wild-type N protein to bind RNA. Significantly, when the assay was carried out at a still higher temperature, 37°C, wild-type N protein lost the ability to bind RNA. By contrast, at 37°C the N proteins of Alb81 and Alb83 retained most of the RNA binding that they had exhibited at the lower temperature. This indicated that the reverting mutations derived from Alb4RevH and Alb4RevD imparted a measure of thermal stability to the N molecule and that in the absence of the Alb4 deletion, these mutations actually caused N protein to bind more tightly to RNA than the wild-type N protein did.

The enhancement of RNA binding by the recombinant N proteins raised the possibility that the competitive advantage of wild-type virus over Alb81 was due to a delay in the uncoating of the viral genome in the earliest stages of infection. However, no difference was observed between the two viruses when we examined single-step growth carried out under the same conditions as those used for the initial passage of the competition experiment (Fig. 7). Thus, if removal of N protein from the genome of Alb81 produced a lag in the kinetics of infection relative to the wild type, this was too small to detect

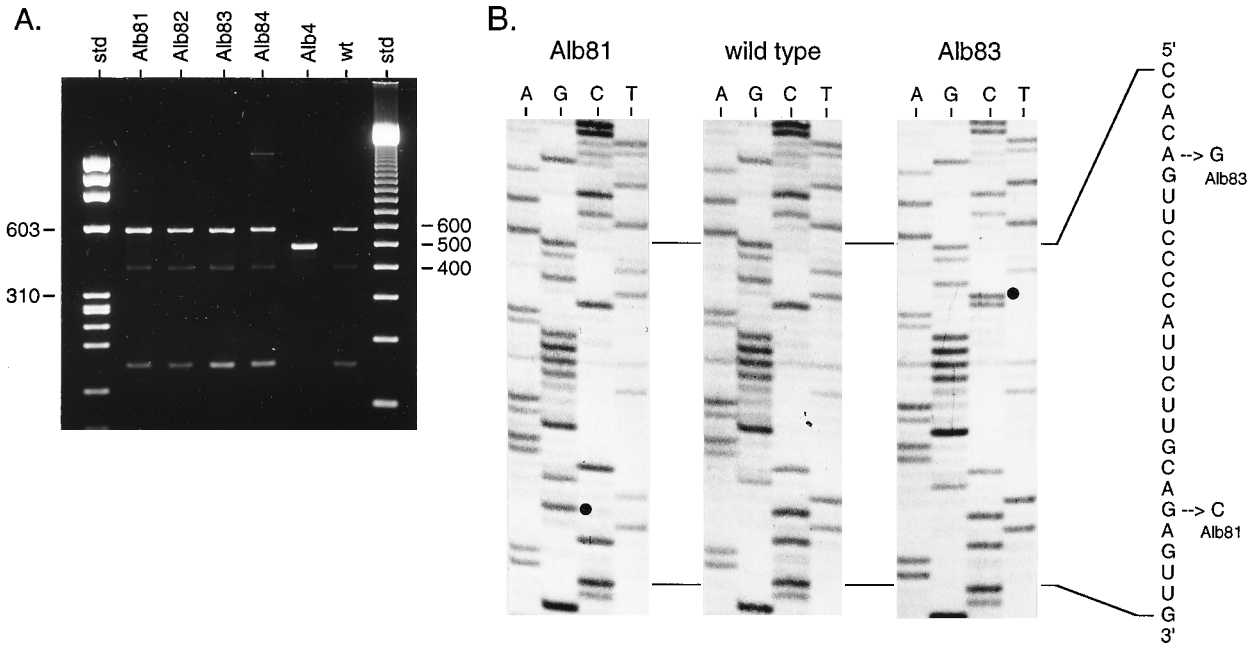

FIG. 5. Confirmation of the construction of recombinant viruses. (A) RT-PCR analysis of RNA isolated from cells infected with recombinant virus candidates Alb81 through Alb84 or wild type (wt) or Alb4 as controls. Sibling recombinants Alb81 and Alb82 originated from vector pDP51; sibling recombinants Alb83 and Alb84 originated from vector pDP80. RT-PCR products obtained with a pair of primers flanking the Alb4 deletion were electrophoresed on a 1.5% agarose gel. DNA molecular weight standards (std) were *Hae*III-digested ϕX174 replicative form (left) and a 100-bp ladder (right); fragment sizes are given in base pairs. (B) Sequencing of genomic RNA isolated from purified wild-type and recombinant viruses Alb81 and Alb83. A portion of the N coding region (nt 913 to 953) is shown. The indicated segment of the sequences is given as positive-sense RNA. Solid circles highlight the mutations in Alb81 and Alb83.

in a single passage and became measurable only following multiple passages.

Construction of an N protein mutant containing a reverting mutation in the presence of the Alb4 deletion. Since the Alb4 revertants had been obtained following multiple passages at the nonpermissive temperature, it remained possible that additional mutations outside the N gene were required for reversion of the Alb4 phenotype. To establish whether the E316Q mutation of Alb4RevH (and the other six large-plaque revertants) was sufficient for total reversion, this mutation, within an N gene containing the Alb4 deletion, was transduced into Alb4. In this case, this was accomplished by targeted recombination between Alb4 and a synthetic defective interfering RNA (15) into which we had inserted the N gene of Alb4RevH (Fig. 8A). Candidate recombinant viruses resulting from transfection of Alb4-infected cells with this defective interfering RNA were selected as progeny that were able to form large plaques at the nonpermissive temperature. These arose at the same frequency as those obtained with defective interfering RNA having a wild-type copy of the N gene. Two recombinants, designated Alb122 and Alb123, isolated from independent transfections, were chosen for further analysis. Direct sequencing of genomic RNA from purified virions of Alb122 and Alb123 confirmed that each had the Alb4RevH second-site point mutation in addition to the Alb4 deletion (Fig. 8B). Both the plaque sizes of these recombinants at the nonpermissive temperature and their thermal stability were indistinguishable from those of Alb4RevH (Table 4). These results (together with those shown in Fig. 2) demonstrated that a single mutation within the N gene was sufficient for reversion of the plaque size, thermolability, and RNA-binding phenotypes caused by the Alb4 deletion.

DISCUSSION

In this study, we have shown that the phenotypic effects of the large deletion in the N protein of an MHV mutant could be reversed by a single-amino-acid change that increases the affinity of N for RNA. The revertant analysis of Alb4 revealed five amino acid positions that could be altered to either totally or partially compensate for the temperature-sensitive growth and thermolability caused by the deletion of 29 residues separating putative domains II and III of the N molecule (Fig. 3). Among these, only one type of change was found that resulted in total reversion of the Alb4 phenotype. Because this muta-

TABLE 3. Growth competition between wild-type and recombinant Alb81 viruses[a]

Temp (°C)	Ratio (wt/Alb81) at:	
	First passage	Eighth passage[b]
39	1:1	10:0
	1:10	9:1
	0:1	0:10
33	1:1	9:1
	1:10	5:4
	0:1	0:10

[a] Mixtures of wild-type (wt) and Alb81, or Alb81 alone, were used to infect 17Cl1 cell monolayers at an initial total input multiplicity of infection of 5 PFU per cell. The cells were incubated at either 33 or 39°C, and released virus was passaged serially on 17Cl1 cells at the same temperature.

[b] Released virus from the eighth passage was subjected to titer determination by plaque assay. Ten (or, in one case, nine) plaques picked from each sample were used to infect 17Cl1 cells, from which cytoplasmic RNA was then purified and sequenced.

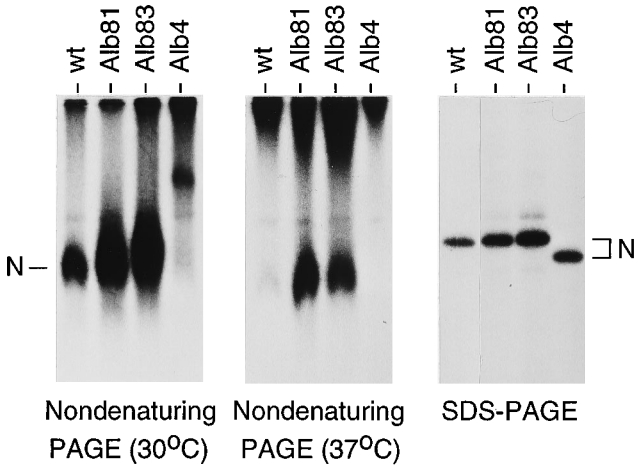

FIG. 6. RNA binding analysis of recombinant N proteins. RNA was synthesized in vitro from transcription vectors containing cloned copies of the N genes from wild-type (wt) MHV, the Alb4 mutant, or the recombinants Alb81 and Alb83. In vitro translated, [^{35}S]methionine-labeled N proteins were analyzed by nondenaturing PAGE performed at 30 and 37°C and by SDS-PAGE.

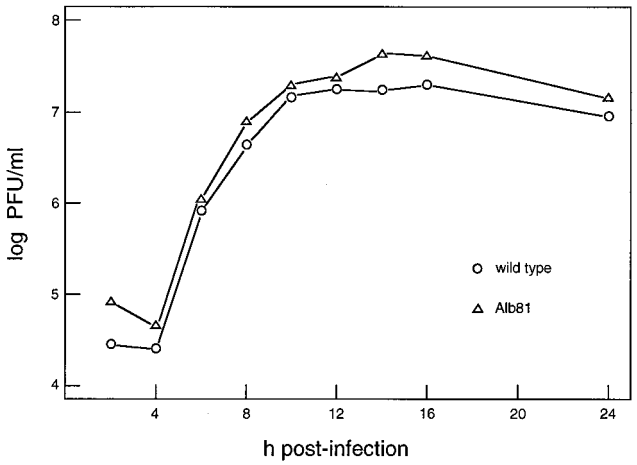

FIG. 7. One-step growth of wild-type and Alb81 viruses. 17Cl1 cells were infected with either wild-type MHV or recombinant Alb81 at a multiplicity of infection of 5 PFU per cell. The cells were incubated at 39°C, and the titers of progeny released virus, harvested from the medium at various times postinfection, were determined on L2 cells.

tion (E316Q) was independently isolated seven times (in Alb4RevA through Alb4RevC and Alb4RevE through Alb4 RevH), it is likely that it is the only possible mutation, or one of very few possible mutations, allowing total reversion. The transduction of this mutation back into the Alb4 genome established that it, alone, was sufficient for reversion of all aspects of the Alb4 phenotype (Fig. 8; Table 4).

The remaining five revertants, including one with an alternative change at position 316 (Alb4RevL), were different from each other, and each resulted in only an intermediate level of restoration of the wild-type phenotype. The common characteristic shared by both the total and partial reverting mutations was that all mapped to a relatively small locus in the second half of domain II of the N protein, a region to which we previously mapped the RNA-binding activity of N (14). Taken together, the reverting mutations do not fall into an obvious pattern. The single totally reverting mutation results in a net increase of positive charge, and it occurs at one of the very few positions in domain II that are highly conserved among all coronavirus N proteins. Most of the partially reverting mutations, however, cannot be similarly categorized. Indeed, for two of these, Alb4RevI and Alb4RevK, the reverting mutation results in an amino acid that is identical to that found at the same position in the N proteins of bovine coronavirus (12), turkey coronavirus (25), and human coronavirus OC43 (7). Thus, a subset of the reverting mutations of Alb4 are actually the residues selected through evolution in the different sequence contexts of these heterologous N proteins.

What effect does a reverting mutation have in the absence of the Alb4 deletion? We addressed this question by constructing MHV mutants containing either a totally or partially reverting point mutation within an otherwise wild-type N gene. Thus, an important outcome of this study was the demonstration that the technique of targeted RNA recombination can be used to stably introduce site-specifically engineered protein-coding changes into the MHV genome. Previous applications of this method had made silent mutations in open reading frame 1a and the N gene (24) or noncoding changes in the 3′ UTR (14). It was conceivable that a reverting mutation, by itself, would be as harmful to N protein function as the deletion it counteracted. If this were the case, we might not have been able to select such a mutant, since it might not have been distinguishable from its Alb4 parent. Alternatively, it was possible that a reverting mutation could act only in concert with the deletion and would be phenotypically silent in a wild-type background. The constructed mutants, Alb81 and Alb83, clearly fell closer to the latter possibility but were not completely as fit as the wild type. At least for the mutant examined in more detail, Alb81, the reverting mutation placed the virus at a distinct selective disadvantage with respect to wild-type virus (Table 3). However, this effect was apparent only after multiple passages and was too subtle to produce an observable difference in the single-step kinetics of growth of this mutant (Fig. 7).

How does a reverting mutation compensate for the Alb4 deletion? Since the Alb4 mutant is normal at the permissive temperature, the region that is deleted in its N protein must be dispensable for the functioning of this molecule. Thus, we believe that the net effect of the deletion is to generate a too close juxtaposition of domains II and III of the N protein, and this appears to create some sort of structural hindrance that severely impairs N function at the nonpermissive temperature. We have shown a clear correlation between the in vivo temperature sensitivity or stability of wild-type, Alb4, and revertant viruses and the relative affinities of their N proteins for RNA. This argues that the RNA-binding assay we have used measures a biologically relevant characteristic of N. Remarkably, the N proteins of the constructed mutants, Alb81 and Alb83, have a higher affinity for RNA than does wild-type N protein. RNA binding by the revertant N proteins can be seen, then, as resulting from the additive effects of a deletion mutation that reduces the affinity of N for RNA and a reverting mutation that enhances it. This may also explain the selective disadvantage observed for Alb81 with respect to wild-type virus. Since RNA binding is a dynamic process during the coronavirus replicative cycle, N protein that binds to RNA too tightly could be just as deleterious as N protein that does not bind tightly enough.

It must be noted that although the data show that the temperature dependences of the in vivo phenotypes of the viruses in this study and the in vitro RNA-binding affinities of their N proteins vary coordinately, there is not an exact correspondence between these in vivo and in vitro measured character-

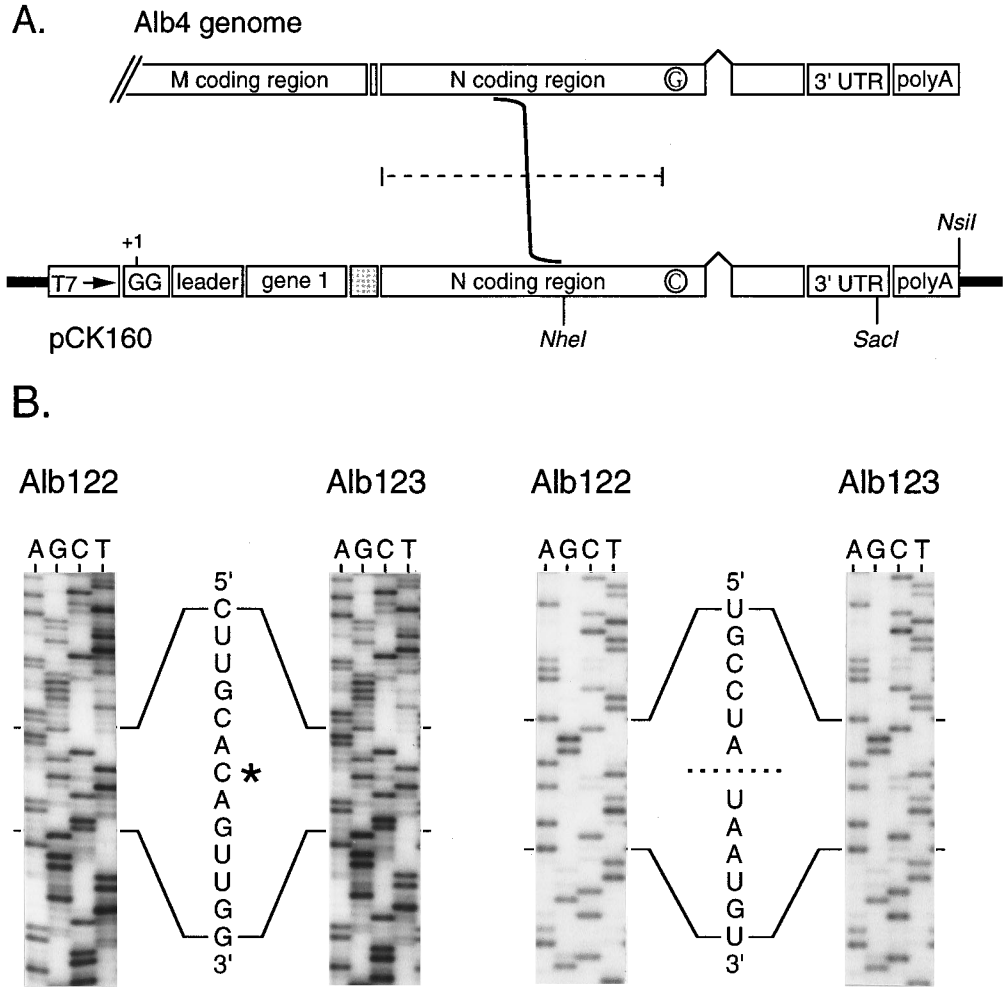

FIG. 8. Targeted recombination to construct a mutant containing a reverting mutation in the presence of the Alb4 deletion. (A) Transcription vector pCK160 was derived from pB36, a template for in vitro transcription of a synthetic MHV DI RNA (15), and contains both the Alb4 deletion and the Alb4RevH second-site point mutation. Indicated above the vector schematic is the region in which a crossover event between vector-derived synthetic RNA and Alb4 genomic RNA could generate the selected recombinant. (B) Sequencing of genomic RNA isolated from purified independent recombinant viruses Alb122 and Alb123. The left panels show the portion of the N coding region containing the Alb4RevH point mutation (asterisk). The right panels show the portion of the N coding region containing the Alb4 deletion, whose boundary is denoted by a broken line. In each, the indicated segment of the sequences is given as positive-sense RNA.

istics. For example, the transition temperature for the in vivo temperature-sensitive phenotype of the Alb4 virus is between 33 and 39°C, whereas the transition temperature for loss of in vitro RNA binding by the Alb4 N protein falls between 20 and 30°C. Thus, it is apparent that in vivo RNA binding must be stabilized in ways that are not fully mimicked by the in vitro assay. This is not very surprising, since RNA binding by N protein must be influenced by a number of variables, including conditions of ionic strength, pH, protein concentration, the nature of the RNA ligand, the presence of other viral or cellular components, and the state of N protein phosphorylation.

TABLE 4. Characteristics of reconstructed revertants of Alb4

Virus	Plaque size (mm)[a]	Fraction surviving heat treatment[b]
Wild type	2.9	1.3×10^{-1}
Alb4RevH	2.4	6.4×10^{-2}
Alb122	2.8	7.0×10^{-2}
Alb123	2.6	5.5×10^{-2}

[a] The plaque diameter was measured after incubation for 48 h at 39°C. Each value is the average for 24 to 26 plaques.

[b] Viruses (2.7×10^7 to 8.5×10^7 PFU/ml) were treated for 24 h at 40°C, as described in Materials and Methods.

Two recent studies have localized RNA binding in the MHV N protein. Our laboratory has used the nondenaturing gel assay described above to map the RNA-binding activity of this molecule to a region encompassing amino acids 136 through 397 (14). Nelson and Stohlman have further delimited RNA binding to amino acids 169 through 308 by using fragments of N protein produced by chemical cleavage (16). In their work, a Northwestern (RNA-protein) blotting assay was used, in which sequence-specific binding to the MHV leader RNA was carried out with nitrocellulose-immobilized N protein (21). While the conclusions of the two studies accord well, they are discrepant with respect to the carboxyl-terminal boundary of the RNA-binding domain, precisely in the region where the Alb4 reverting mutations occur. The smaller RNA-binding domain defined by Nelson and Stohlman (16) would not include three of the five Alb4 reversion sites found in the present study (Fig.

3), nor would it account for the abolition of RNA binding we previously observed with carboxyl-terminal deletions of N that ended at amino acid 336, 360, or 377 (14). A possible explanation for this disparity could be that the N protein has two RNA-binding domains, one for sequence-specific binding to leader RNA and another for non-sequence-specific RNA binding along the entire length of the MHV nucleocapsid. However, we think it is more plausible that the different boundaries obtained for the RNA-binding domains reflect differences between the two assays. It is likely that the nondenaturing gel electrophoresis assay that we have used requires a more stable N-RNA complex than the Northwestern assay owing to the duration, temperature, and ionic strength of electrophoresis conditions. Therefore, we favor the hypothesis that the region of the Alb4 reverting mutations participates in RNA binding, either through extension of the minimal binding site forming direct contacts with RNA or by promoting cooperative interactions between multiple monomers of N. This latter possibility would explain why the Northwestern assay was able to detect sequence-specific RNA binding whereas the nondenaturing gel assay failed to do so. In the Northwestern assay, individually immobilized N molecules would be more likely to act independently and not form cooperative multimers along an RNA molecule. By contrast, in the nondenaturing gel assay, sequence-specific binding would be masked by non-sequence-specific binding stabilized by N-N cooperative interactions. Further site-specific mutagenesis and biochemical studies will be necessary to more clearly elucidate the RNA-binding activity of the MHV N protein. The revertant analysis of the Alb4 mutant has defined one important region of N upon which such studies should focus.

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