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Protein Coding Assignment of Avian Reovirus Strain S1133

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Avian reovirus S1133 encodes 10 primary translation products, 8 of which are structural components of the viral particle and 2 of which are nonstructural proteins. The identity of the gene that codes for each of these polypeptides was determined by in vitro translation of denatured individual genome segments.

Avian reoviruses are an important cause of disease conditions in poultry. In particular, reovirus-induced arthritis provokes considerable economic losses in poultry farming (4). Although the pathological effects of avian reoviruses in poultry have been extensively investigated, relatively little is known about their biochemistry and molecular biology. Previous studies have shown that avian reoviruses share many characteristics with their mammalian counterparts (13, 20). Both types of virus have a genome consisting of 10 segments of double-stranded RNA (dsRNA), separable into three size classes, which are enclosed within an outer capsid 70 to 80 nm in diameter (7). Extensive biochemical analyses of mammalian reoviruses have led to the identification of all virus-encoded proteins and to a comprehensive understanding of polypeptide composition and distribution within the viral particle (17). Much less is known about the proteins encoded by avian reoviruses. There have been previous studies of strain S1133 (13, 19, 20), but the protein coding assignment for this strain has yet to be completed. Ni et al. (12) recently identified 12 proteins, 10 structural and 2 nonstructural, by electrophoretic analysis of purified avian reovirus strain 176 virions and of infected cell lysates; however, whether the intracellularly synthesized proteins were of viral origin was not confirmed. Wickramasinghe et al. (21) detected 10 viral proteins in avian reovirus strain RAM-1 preparations by precipitation with mouse anti-RAM-1 serum but did not investigate which proteins were structural and which were nonstructural.

The first goal of the present study was to identify the primary translation products of avian reovirus S1133 mRNAs. S1133 avian reovirus was plaque cloned three times, and virus from a large plaque was grown at low multiplicity in chicken embryo fibroblasts (CEF) (20). Virus was purified as described previously (19), and viral mRNA was transcribed in vitro from heat-shocked reovirions (60°C for 30 s). Viral transcripts were translated in rabbit reticulocyte lysates as described elsewhere (1), and samples were analyzed on a discontinuous sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) gel (10) in which acrylamide was replaced by Hydrolink (AT Biochem, Malvern, Pa.) to achieve good resolution of all reoviral proteins. As can be observed in lane 2 of Fig. 1, viral mRNAs programmed the synthesis of at least 10 polypeptides: 3 of the λ class, 3 of the μ class, and 4 of the σ class. These proteins were also synthesized in reticulocyte extracts programmed with RNA isolated from S1133-infected CEF (Fig. 1, lane 3) and in cytoplasmic extracts of infected cells (Fig. 1, lane 5) but not in extracts of mock-infected cells (Fig. 1, lane 9) or in reticulocyte extracts programmed with cellular mRNAs (Fig. 1, lane 8). The fact that these proteins were recognized by antibodies to purified reovirions confirms their viral origin (Fig. 1, lanes 4 and 6). Comparison of the primary translation product profiles (Fig. 1, lanes 2 and 3) with the purified reovirion profile (Fig. 1, lane 7) indicates that eight of the primary products are structural components of the virus while two are nonstructural. The latter two proteins were synthesized in the cytoplasm of infected but not of mock-infected CEF (Fig. 1, lanes 5 and 9) and were thus identified as nonstructural viral proteins and designated μNS and σNS. Both polypeptides have been previously identified by Ni et al. (12) as nonstructural proteins of avian reoviruses S1133 and 176. However, those researchers did not investigate whether these polypeptides are of viral origin or whether they are cellular proteins whose synthesis is induced by viral infection; our results demonstrate that μNS and σNS are primary translation products of S1133 mRNAs.

The data shown in Fig. 1 thus indicate that avian reovirus S1133 encodes 10 primary translation products, of which 8 (λ1, λ2, λ3, μ1, μ2, σ1, σ2, and σ3) are structural and two (μNS and σNS) are nonstructural components. This is the expected result for a 10-gene virus. However, we cannot rule out the possibility that one or more minor polypeptides encoded by bicistronic genes were not detected by our experimental procedures (see below).

Comparison of the polypeptide profiles in Fig. 1 indicates that a μ class polypeptide which is present in cytoplasmic extracts of infected cells (Fig. 1, lane 5) and in purified virions (Fig. 1, lane 7) and is recognized by antibodies to avian reovirus (Fig. 1, lane 6) is not a primary translation product (Fig. 1, lanes 2 and 3). This polypeptide, which is probably a posttranslational cleavage product, was provisionally designated μ2C in view of the precursor-product relationship recently reported for μ2 and μ2C of avian reovirus 176 (12).

The second goal of this study was to identify the gene that codes for each viral polypeptide. The coding assignments for the mammalian reoviruses have been known for some time (11), but for avian reovirus, only the assignment of the S class genes of strain S1133 has been reported (18). To complete the avian reovirus assignment, all 10 genomic segments of avian reovirus S1133 were individually isolated and their in vitro translation products were analyzed by SDS-PAGE as follows. First, approximately 200 μg of dsRNA obtained by phenol extraction of 2 mg of purified reovirions was loaded onto a 3-mm-thick SDS–10% PAGE discontinuous gel (10) which was run at 140 V for 17 h. The gel was stained for 30 min with 0.5 μg of ethidium bromide per ml, and bands were visualized under UV light and then excised from the gel. RNA was extracted from crushed gel pieces by diffusion in accordance

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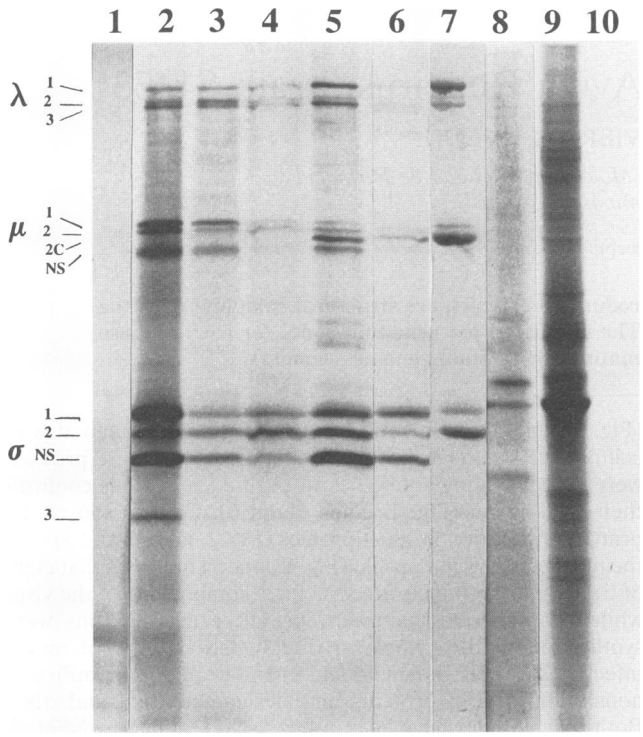

FIG. 1. SDS-PAGE analysis of [^{35}S]methionine-labeled avian viral polypeptides. Reticulocyte lysates programmed with endogenous mRNA (lane 1), with mRNA transcribed in vitro by heat-shocked S1133 reovirions (lane 2), or with cytoplasmic RNA extracted from S1133-infected (lane 3) or mock-infected (lane 8) CEF were analyzed by SDS-PAGE on a 10% Hydrolink gel. Cytoplasmic extracts of S1133-infected (lane 5) or mock-infected (lane 9) CEF, in both cases previously labeled at 24 h postinfection with 25 μCi of [^{35}S]methionine per ml for 1 h, were analyzed on the same gel, as well as purified reovirions (lane 7) that had been grown in CEF containing 10 μCi of [^{35}S]methionine per ml. Immunoprecipitates of the samples in lanes 3, 5, and 9 with antibodies against purified S1133 are shown in lanes 4, 6, and 10, respectively. Each viral protein is labeled at the left in accordance with the conventions adopted for mammalian reoviruses.

with the procedure of Sambrook et al. (15). About 4 to 6 μg of RNA from each segment was obtained (i.e., the yield was about 25%). As can be seen from lanes C of Fig. 2, the S1133 genome comprises 10 segments which were resolved as discrete bands in SDS-PAGE, with a migration profile similar to that previously reported by Gouvea and Schnitzer (5). No additional dsRNA bands were revealed by ethidium bromide staining, and densitometric analysis of the bands in lanes C of Fig. 2 showed that all 10 segments were present in equimolar amounts. These data argue strongly against the possibility that the S1133 genome undergoes rearrangement. All 10 S1133 dsRNAs were isolated as individual species (Fig. 2, lanes L1 to S4).

The dsRNAs were denatured with hydroxymethyl mercury (2) and translated in reticulocyte lysates. The final concentrations of the components of the reaction mixture were as follows: reticulocyte lysate (Boehringer Mannheim), 60%; dsRNA, 80 μg/ml (when translating individual segments) or 160 μg/ml (when translating unfractionated dsRNA); hydroxymethyl mercury, 0.3 mM; EDTA (pH 8.0), 6 μM; potassium acetate, 100 mM; magnesium acetate, 1.5 mM; and [^{35}S] methionine (>1,000 Ci/mmol; Amersham), 1 mCi/ml. After

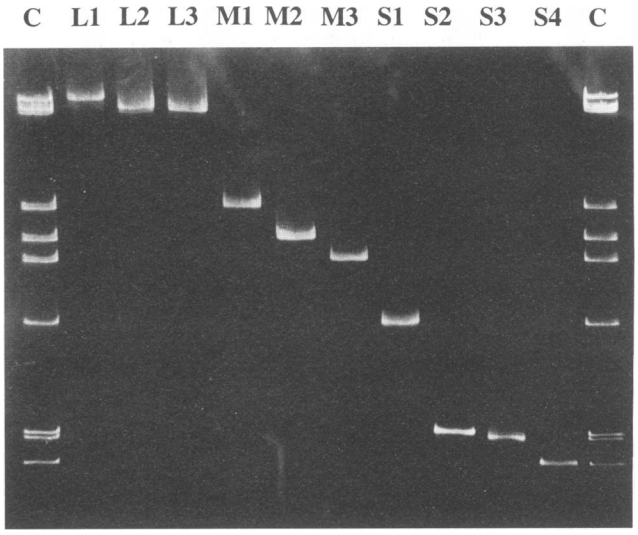

FIG. 2. SDS-PAGE analysis of individual S1133 genomic segments. Total dsRNA (lanes C) or individual genomic segments (lanes L1 to S4) were analyzed by SDS-PAGE, and the gel was stained with ethidium bromide before being photographed under UV light.

incubation for 1 h at 30°C, samples were analyzed by SDS-PAGE as for Fig. 1. The in vitro translation products of each of the 10 S1133 genes are shown in Fig. 3. Of the S class genes, segment S1 codes for σ3, S2 codes for σ1, S3 codes for σ2, and S4 codes for σNS; these results are in complete agreement with those of Schnitzer (18). In the L and M classes, there was complete correspondence between ranking of segment size and the corresponding ranking of protein size. L1 codes for λ1, L2 codes for λ2, and L3 codes for λ3, while M1 codes for μ1, M2 codes for μ2, and M3 codes for μNS. This is contrast to the

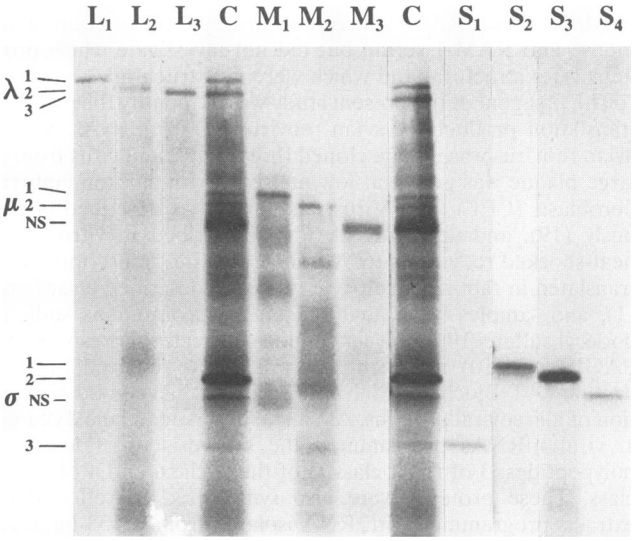

FIG. 3. Autoradiogram of in vitro translation products of denatured individual genomic segments of avian reovirus S1133. Total dsRNA (lanes C) or individual genomic segments (lanes L1 to S4) were denatured with hydroxymethyl mercury prior to translation in reticulocyte lysates in the presence of [^{35}S]methionine. Samples were analyzed by SDS-PAGE as described in the legend to Fig. 1.

findings for the S class genes, since the largest S segment (S1) codes for the smallest polypeptide, σ3. Assuming that the molecular weights of both S1 and σ3 are reliably reflected by their mobilities in SDS-PAGE, this suggests that only about 43% of the ≈1,800 nucleotides of the s1 mRNA code for σ3. This situation is unlike that of all reovirus genes sequenced to date, most of which use more than 95% of their length for coding (17). It is possible that σ3 is the cleavage product of an undetected precursor; however, this seems unlikely, since reticulocyte lysates do not normally cleave proteins in the absence of added microsomes (6) and since we were unable to detect any candidate precursor by pulse-chase analysis either in infected cells or in reticulocyte extracts programmed with viral mRNAs (data not shown). Furthermore, equimolar amounts of more than one protein would be produced from the precursor if proteolysis occurred: we did not detect any polypeptides other than σ3, even when using electrophoretic gels specially designed to resolve small peptides (16; data not shown).

Another possibility is that the S1 gene codes for both σ3 and another polypeptide which either is translated very inefficiently or has insufficient methionines to be detected by our experimental procedures. Interestingly, the S1 gene of mammalian reoviruses, which specifies cell attachment protein σ1, has been shown to be functionally bicistronic (8). The s1 transcript of mammalian reovirus type 3 contains two out-of-phase overlapping reading frames, allowing synthesis of both σ1 and σ1s, a minor nonstructural polypeptide with an unknown function (3). The initiation codon for σ1, located at positions 13 to 15, contains a C at −3 and therefore has a weak context for initiation of translation (8). According to the leaky-scanning model proposed by Kozak (8), this means that some of the ribosomal subunits coming from the cap do not stop at this codon but resume scanning until reaching the second AUG. This second AUG at positions 71 to 73, has a strong context, with an A at −3 and a G at +4, and serves as the initiation codon for σ1s (8). The picture seems to be different for the S1 gene of the avian reovirus. We sequenced more than 100 nucleotides from the 5′ end of the S1133 s1 mRNA (data not shown) and found that the first AUG, located at positions 33 to 35, has a strong context for initiation, with an A at −3 and a G at +4. Furthermore, no stop codons in phase with this AUG codon were found within the 28 downstream codons sequenced. These results suggest that all of the ribosomal subunits recruited by the cap must start translation at this codon. Since the only in vitro translation product of the avian reovirus S1 gene detected in reticulocyte extracts was σ3 (Fig. 3), it seems probable that this AUG serves as the initiation codon for this polypeptide. Synthesis of another protein from this mRNA is very unlikely, unless reinitiation occurs (9, 14). To determine whether the S1 gene of avian reovirus S1133 is bicistronic and, if so, to elucidate how this is achieved, we are currently sequencing the entire S1 segment and the amino-terminal end of σ3.

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REFERENCES

1. **Benavente, J., and A. J. Shatkin.** 1988. Avian reovirus mRNAs are nonfunctional in infected mouse cells: translational basis of virus host-range restriction. Proc. Natl. Acad. Sci. USA **85**:4257–4261.
2. **Cleveland, D. R., H. Zarbl, and S. Millward.** 1986. Reovirus guanylyltransferase is L2 gene product lambda 2. J. Virol. **60**:307–311.
3. **Fajardo, J., and A. J. Shatkin.** 1990. Translation of bicistronic viral mRNA in transfected cells: regulation at the level of elongation. Proc. Natl. Acad. Sci. USA **87**:328–332.
4. **Glass, S. E., S. A. Naqi, C. F. Hall, and K. M. Kerr.** 1973. Isolation and characterization of a virus associated with arthritis of chickens. Avian Dis. **17**:415–424.
5. **Gouvea, V. S., and T. J. Schnitzer.** 1982. Polymorphism of the genomic RNAs among the avian reoviruses. J. Gen. Virol. **61**:87–91.
6. **Jackson, R. C., and G. Globel.** 1977. Post-translational cleavage of presecretory proteins with an extract of microsomes from dog pancreas containing signal peptidase activity. Proc. Natl. Acad. Sci. USA **74**:5598–5602.
7. **Joklik, W. K. (ed.).** 1983. The Reoviridae. Plenum Publishing Corp., New York.
8. **Kozak, M.** 1982. Analysis of ribosome binding sites from the S1 message of reovirus. Initiation at the first and second AUG codons. J. Mol. Biol. **156**:807–820.
9. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108**:229–241.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **McCrae, M. A., and W. K. Joklik.** 1978. The nature of the polypeptide encoded by each of the 10 double-stranded RNA segments of reovirus type 3. Virology **89**:578–593.
12. **Ni, Y., R. F. Ramig, and M. C. Kemp.** 1993. Identification of proteins encoded by avian reoviruses and evidence for post-translational modification. Virology **193**:466–469.
13. **Nick, H., D. Curriefen, and H. Becht.** 1975. Structural and growth characteristics of two avian reoviruses. Arch. Virol. **48**:261–269.
14. **Peabody, D. S., and P. Berg.** 1986. Termination-reinitiation occurs in the translation of mammalian cell mRNAs. Mol. Cell. Biol. **6**:2695–2703.
15. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
16. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 KDa. Anal. Biochem. **166**:368–379.
17. **Schiff, L. A., and B. N. Fields.** 1990. Reoviruses and their replication, p. 1275–1306. *In* B. N. Fields, D. M. Knipe, et al. (ed.), Virology. Raven Press, New York.
18. **Schnitzer, T. J.** 1985. Protein coding assignment of the S genes of the avian reovirus S1133. Virology **141**:167–170.
19. **Schnitzer, T. J., T. Ramos, and V. Gouvea.** 1982. Avian reovirus polypeptides: analysis of intracellular virus-specified products, virions, top component, and cores. J. Virol. **43**:1006–1014.
20. **Spandidos, D. A., and A. F. Graham.** 1976. Physical and chemical characterization of an avian reovirus. J. Virol. **19**:968–976.
21. **Wickramasinghe, R., J. Meanger, C. E. Enriquez, and G. E. Wilcox.** 1993. Avian reovirus proteins associated with neutralization of virus infectivity. Virology **194**:688–696.