JOURNAL OF VIROLOGY, May 1984, p. 666–669
0022-538X/84/050666-04$02.00/0

Vol. 50, No. 2

Characterization of Bovine Viral Diarrhea Virus Proteins

A. F. PURCHIO,†* RUBY LARSON, AND MARC S. COLLETT
Molecular Genetics, Inc., Minnetonka, Minnesota 55343

Received 14 November 1983/Accepted 19 January 1984

Virus-specific proteins were examined in cultured cells infected with bovine viral diarrhea virus. By using antisera obtained from virus-infected animals, three major virus-specific polypeptides with molecular weights of 115,000 (115K), 80K, and 55K were observed. Minor proteins of 45,000 and 38,000 daltons were also noted. Tryptic peptide mapping indicated that the 115K and the 80K polypeptides were structurally related. The 55K protein was glycosylated and appeared not to be related to the 115K and 80K proteins. Pulse-chase experiments failed to demonstrate any procursor-product relationship among any of these proteins, and all three polypeptides were found in purified virion preparations. The significance of these findings with respect to the replication of bovine viral diarrhea virus is discussed.

Bovine viral diarrhea (BVD) virus is classified as a member of the *Pestivirus* genus of the non-arthropod-borne togaviruses (5, 6) and has been identified as the causative agent of virus-induced diarrhea mucosal disease in cattle (5, 11, 14). Early studies identified four virus-specific proteins in infected bovine bone marrow cells, having molecular weights of 110,000 (110K), 93K, 70K, and 23K (12). Several years later, Matthaeus described three BVD viral structural polypeptides in purified virions with molecular weights of 57K, 44K, and 34K; the 57K and 44K proteins were found to be glycosylated (9). We have been studying the replication of BVD virus in cultured cells, and in this report we present our results on the synthesis of viral-specific proteins in bovine kidney cells.

Figure 1A shows a fluorogram of a sodium dodecyl sulfate-polyacrylamide gel analysis of BVD virus-specific proteins immunoprecipitated from infected Madin-Darby bovine kidney (MDBK) cells labeled with [^{35}S]methionine. Three major virus-specific proteins were noted (Fig. 1, track 6), having molecular weights of 115K, 80K, and 55K; minor proteins of 38K and 45K could also be seen. This same profile of virus-specific proteins was seen when infected cells were radiolabeled continuously for 24 h (data not shown). Note that no difference in the total radiolabeled protein profile of infected and uninfected cells can be seen (Fig. 1A, tracks 1 and 2). This pattern was not altered when labeling was carried out in the presence of actinomycin D (data not shown). When BVD-infected cells were labeled with [^{3}H]glucosamine and lysates were immunoprecipitated with BVD virus antiserum, only the 55K protein was labeled (Fig. 1B, track 2), indicating that this polypeptide was a glycoprotein.

To determine whether any structural relationship exists among these proteins, two-dimensional tryptic peptide mapping was performed on the [^{35}S]methionine-labeled proteins. The 80K protein is clearly related to the 115K protein, as shown by the mixing experiment in Fig. 2C. The 55K and

* Corresponding author.
† Present address: Oncogen, Seattle, WA 98121.

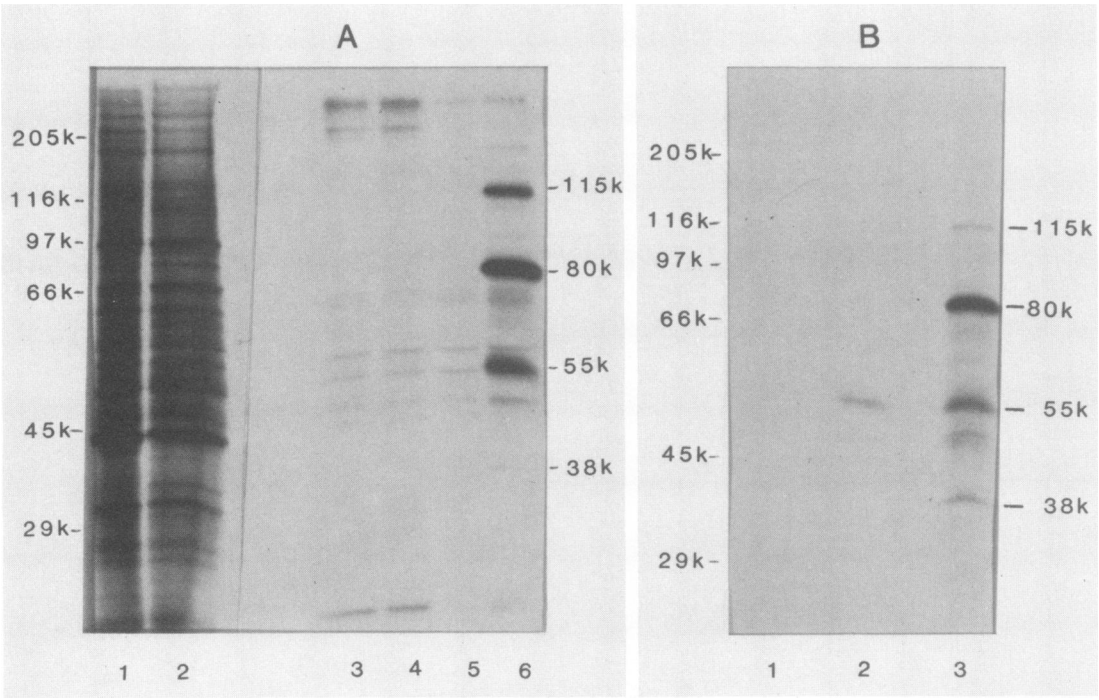

FIG. 1

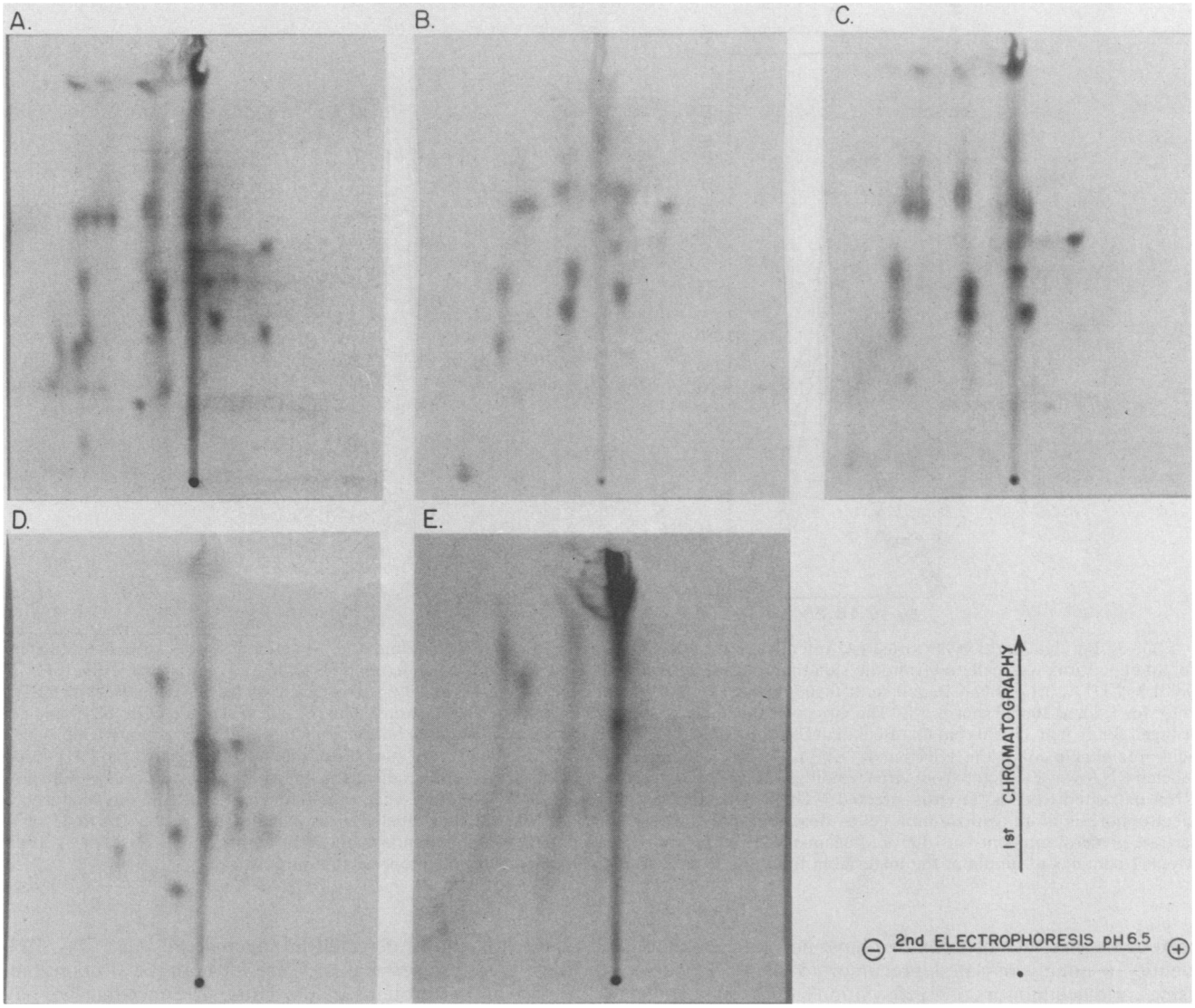

FIG. 2. Two-dimensional fractionation of [^{35}S]methionine-containing tryptic peptides of BVD virus-specific proteins. Two-dimensional tryptic peptide mapping was performed as previously described (4). Briefly, immunoprecipitated polypeptides to be analyzed were localized in polyacrylamide gels by autoradiography, excised, eluted, digested with tolylsulfonyl phenlalanyl chloromethyl ketone-trypsin (Millipore), and subjected to ascending chromatography in the first dimension followed by electrophoresis at pH 6.5 in the second. Proteins in panels: A, 11K, B, 80K; C, mix of 115K and 80K; D, 54K; E, 38K. Several independent preparations of the 38K protein had the identical tryptic map as that shown in panel E.

38K proteins appear to have peptide maps which differ from those of the 115K and 80K proteins (Fig. 2D and E). These results were substantiated by mixing experiments and comparative partial proteolysis mapping (3), using V8 protease, papain, and chymotrypsin (data not shown). Caution must be exercised with respect to the interpretation of the peptide maps of the 55K protein, as its glycosylation may alter the migration of tryptic peptides or inhibit cleavage by the

FIG. 1. Immunoprecipitation of BVD virus-specific polypeptides from [^{35}S]methionine-labeled lysates of infected bovine kidney cells. (A) BVD virus was grown in MDBK cells as described previously (13). At 20 to 24 h postinfection, cells were labeled with [^{35}S]methionine, harvested, lysed, and immunoprecipitated (1), and the immunoprecipitates were analyzed by electrophoresis on 10% polyacrylamide-sodium dodecyl sulfate gels (7) and fluorographed as described previously (2). For [^{3}H]glucosamine labeling, medium was removed from infected cells at 10 h postinfection and replaced with fresh medium containing 200 μCi of [^{3}H]glucosamine (Amersham Corp.) per ml. Labeling was for 15 h. BVD virus-specific antiserum was prepared by injecting a young calf, known to be free of BVD virus antibodies, with 10-ml samples of virus-containing supernatant fluid (10^{6} PFU/ml). Injections were made weekly for 7 weeks by injecting 5 ml of virus material intravenously and 5 ml intramuscularly. Tracks: 1, uninfected cell lysate; 2, infected cell lysate; 3, uninfected cell lysate immunoprecipitated with preimmune serum; 4, uninfected cell lysate immunoprecipitated with immune serum; 5, infected cell lysate immunoprecipitated with preimmune serum; 6, infected cell lysate immunoprecipitated with immune serum. The numbers on the left show the position of molecular weight standards in thousands. (B) Infected cells were labeled with [^{3}H]glucosamine and immunoprecipitated with: track 1, preimmune serum; track 2, immune serum. Track 3 shows the BVD virus-specific proteins immunoprecipitated from cells labeled with [^{35}S]methionine.

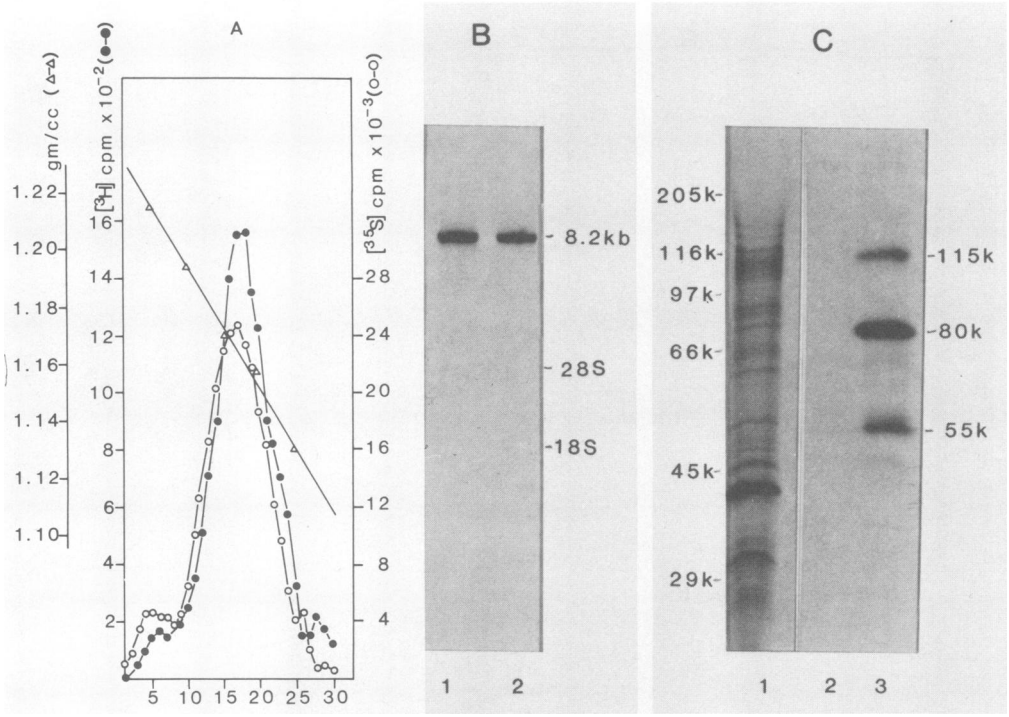

FIG. 3. Purification of BVD virus. (A) Infected culture fluids labeled with either [^{3}H]uridine or [^{35}S]methione were clarified at 8,200 × *g* for 10 min. Virus was pelleted from the supernatant as described previously (15), resuspended in STE (0.01 M Tris-hydrochloride [pH 7.2], 0.001 M EDTA, 0.15 M NaCl), and centrifuged through 5 ml of 20% sucrose onto a 1.5-ml 70% sucrose cushion (in STE) in a Beckman SW41 rotor for 1.5 h at 30,000 rpm at 5°C. The virus was then layered onto a 0 to 50% Na^+-K^+ tartrate–30 to 0% glycerol gradient (in STE) and centrifuged for 16 h at 35,000 rpm in a Beckman SW41 rotor at 5°C. Fractions were collected by bottom puncture and assayed for trichloroacetic acid-precipitable counts in ^{3}H (●) and ^{35}S (○). Density was determined by weighing 0.1-ml samples of individual fractions. (B) [^{3}H]uridine-labeled RNA was extracted from virus banding in fractions 16 to 21 of the tartrate-glycerol gradient shown in panel A (track 2). Track 1 shows RNA extracted from BVD virus-infected MDBK cells labeled with [^{3}H]uridine in the presence of actinomycin D. RNA was analyzed by electrophoresis in an agarose-urea gel as described previously (13). (C) [^{35}S]methionine-labeled virus banding in fractions 16 to 21 of the tartrate-glycerol gradient was dialyzed against STE and immunoprecipitated with preimmune (tract 2) or anti-BVD virus (track 3) serum. Track 1 contains a sample of the material in fractions 16 to 21 run directly without immunoprecipitation.

proteolytic enzymes or both. We have not yet been able to identify a nonglycosylated precursor to the 55K protein, using various inhibitors of glycosylation such as tunicamycin (data not shown). Furthermore, pulse-chase experiments failed to reveal a precursor-product relationship among any of the BVD proteins (data not shown).

To determine which of these proteins was associated with mature virions, BVD virus labeled with either [^{3}H]uridine or [^{35}S]methionine was purified as described in the legend to Fig. 3. A major peak of ^{3}H and ^{35}S radioactivity was detected in the tartrate-glycerol gradient (Fig. 3A). This peak corresponded to the peak of virus infectivity (data not shown). The [^{3}H]uridine-labeled RNA contained in this peak was the same 8.2-kilobase virus-specific RNA species previously found in infected cells (13). When the total [^{35}S]methionine protein profile of the virus peak was analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a complex pattern was observed (Fig. 3C, track 1). This has been observed by others (9) and is probably a consequence of the low titers of BVD virus obtained in cell culture (13). When the [^{35}S]methionine-labeled virus peak was immunoprecipitated with anti-BVD serum, the same major proteins seen in infected cell lysates (115K, 80K, and 55K) were detected (Fig. 3C, track 3).

The replication of BVD virus appears to differ from prototype togaviruses, such as Semliki Forest virus and Sindbis virus, in that only a single BVD virus-specific RNA species has been detected in infected cells (13). This RNA must therefore serve as message for both the structural and nonstructural proteins of the virus. The fact that we have been unable to detect a large polypeptide precursor to the virus-specific proteins seen in infected cells may mean that multiple functional initiation sites for protein synthesis exist on the 8.2-kilobase RNA, as has been described for other viral messages (8, 10). Alternatively, initiation of protein synthesis may take place at a unique site, and proteolytic cleavage of viral proteins may occur as the ribosome moves down the message. Synthesis and cleavage of viral proteins may take place too fast for a putative precursor polypeptide to be detected by the methods used in this study. Cell-free synthesis of viral polypeptides, using the 8.2-kilobase BVD RNA as message, should provide insight into the mechanism by which viral proteins are generated.

It is especially interesting that no precursor-product relationship could be demonstrated for the 115K and 80K proteins in view of their obvious structural relatedness (Fig. 2). This suggests that there may be functional differences between the two proteins.

The results described here are in general agreement with those of Pritchett and Zee (12), with respect to the molecular weights of BVD proteins. However, they differ from the results of Matthaeus (9), who described BVD virion proteins of 57K, 42K, and 34K, the two larger proteins being glycosylated. We have used the same procedure as that described by

Matthaeus for the purification of BVD virus and still observe the same polypeptide profile as that shown in Fig. 3. The discrepancy between the results reported here and those of Matthaeus may be due to differences in antisera, as the antisera used by Matthaeus may not recognize the 115K and 80K proteins described here. We cannot, however, rule out the possibility that the 115K and 80K proteins may be nonspecifically associated with virion particles throughout the virus purification steps used here.

A previous report (15) indicated that BVD-specific soluble proteins (those not incorporated into virions) were able to pass through a 10-nm membrane filter (Millipore Corp.), whereas intact virus was retained. When [^{35}S]methionine-labeled virions were passed through a 10-nm Millipore filter, we found that all of the BVD virus-specific immunoprecipitable protein was retained (data not shown), indicating that the proteins shown in Fig. 3 are virion associated and not free "soluble antigens."

We thank David Reed and Chuck Muscoplat for preparing antisera and Kit Wojcik for typing this manuscript.

LITERATURE CITED

1. **Bruggle, J. S., and R. L. Erikson.** 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–347.
2. **Chamberlain, J. P.** 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. **98**:132–136.
3. **Cleveland, D. W., S. G. Fischer, M. W. Kuschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
4. **Collett, M. S., E. Erikson, and R. L. Erikson.** 1979. Structural analysis of the avian sarcoma virus transforming protein: sites of phosphorylation. J. Virol. **29**:770–781.
5. **Fenner, F.** 1976. Classification and nomenclature of viruses. Second report of the International Committee on Taxonomy of Viruses. Intervirology **7**:44–47.
6. **Horzinek, M. C.** 1981. Non-arthropod-borne *Togaviruses*. Academic Press, Inc., New York.
7. **Laemmli, U. K.** 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
8. **Marsden, H. S., L. Haarr, and C. M. Preston.** 1983. Processing of herpes simplex virus proteins and evidence that translation of thymidine kinase mRNA is initiated at three separate AUG codons. J. Virol. **46**:434–445.
9. **Matthaeus, W.** 1979. Detection of three polypeptides in preparations of bovine viral diarrhea virus. Arch. Virol. **59**:299–305.
10. **Perez-Bercoff, R., and R. Kaempfer.** 1982. Genomic RNA of mengovirus. V. Recognition of common features by ribosomes and eucaryotic initiation factor 2. J. Virol. **41**:30–41.
11. **Peter, C. P., D. E. Tyler, and F. K. Ramsey.** 1967. Characteristics of a condition following vaccination with bovine virus diarrhea vaccine. J. Am. Vet. Med. Assoc. **150**:46–52.
12. **Pritchett, R. F., and Y. C. Zee.** 1975. Structural proteins of bovine viral diarrhea virus. Am. J. Vet. Res. **36**:1731–1734.
13. **Purchio, A. F., R. Larson, and M. S. Collett.** 1983. Characterization of virus-specific RNA synthesized in bovine cells infected with bovine viral diarrhea virus. J. Virol. **48**:320–324.
14. **Thomson, R. G., and M. Savan.** 1963. Studies on virus diarrhea and mucosal disease of cattle. Can. J. Comp. Med. Vet. Sci. **27**:217–224.
15. **Volenec, F. J., B. E. Sheffy, and J. A. Baker.** 1972. Immunobiologic heterotypic activity associated with viral and soluble components of bovine viral diarrhea virus. Arch. Gesamte Virusforsch. **36**:275–283.