JOURNAL OF VIROLOGY, Mar. 1985, p. 908–919
0022-538X/85/030908-12$02.00/0
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Vol. 53, No. 3

Measles Virus *P* Gene Codes for Two Proteins

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Received 18 September 1984/Accepted 5 December 1984

The entirety of the phosphoprotein gene of measles virus has been sequenced. The gene is composed of 1,657 nucleotides and specifies a 507-amino-acid protein (P). A second overlapping reading frame was predicted from the sequence and specifies a 186-amino-acid protein (C). Through the use of antisynthetic peptide antibodies, we show that both proteins are expressed in virally infected cells. Both proteins are expressed from a functionally bicistronic mRNA through independent initiation of ribosomes at the respective AUG codons. Using immunofluorescent microscopy, we localized the C protein in the nucleus and in cytoplasmic inclusions within the infected cells.

The negative-strand RNA viruses are a family of viruses which share many structural and biochemical features (2, 27, 46). Their genomes consist of negative-strand (anti-message sense) RNA, which is associated with many molecules of a viral nucleocapsid protein to form a nucleocapsid structure. These nucleocapsids are surrounded by an envelope consisting of a lipid bilayer, one or two types of glycoproteins, and a nonglycosylated matrix protein. Because of these structural similarities, it was anticipated that the replicative strategies of these viruses would also be similar (2). Research over the intervening years has largely borne out these speculations. However, a number of variations on the basic negative-strand RNA virus replication strategy are now recognized. One of the most surprising is the employment of overlapping reading frames in some, but not all, of the viruses in this family. Although this phenomenon was originally described for influenza virus (25, 26), it has recently been found in Sendai virus (15) and is suspected to occur in the bunyaviruses (1, 7) and possibly the arenaviruses (1).

In a previous study, we identified two clones of measles virus which specified the carboxy-terminal region of the phosphoprotein (3, 4). In this communication, we report the molecular cloning and sequencing of the entire phosphoprotein gene. Sequence analysis indicates that this gene contains two open reading frames, one specifying a protein of 507 amino acids and the other a protein of 186 amino acids. We chemically synthesized peptides corresponding to different portions of the proteins encoded by the two reading frames and used these to raise antibodies specific for these proteins. Using these immune reagents, we demonstrated that both open reading frames are expressed in virus-infected cells and in cell-free translations of mRNA extracted from infected cells. The larger open reading frame codes for the phosphoprotein (P) and the smaller codes for a protein which we have designated C. Double-label indirect immunofluorescent microscopy reveals that the C protein colocalizes in the infected cell with the nucleocapsid protein, a location that suggests that this protein may function in either transcription or replication. The results of heteroduplex analysis, hybrid selection and translation, and Northern blot analysis demonstrate that a single mRNA codes for both proteins. These results indicate that the information stored in the second open reading frame of the phosphoprotein gene of measles virus is accessed differently than in the case of influenza virus. In the latter case, transcripts of the phosphoprotein gene are spliced in the nucleus of the infected cell to yield two different mRNAs, each of which is specific for one or the other gene product (25, 26). Our results indicate that in the measles virus system, the information in the two open reading frames are accessed by independent initiation of ribosomes at the first AUG codon of each open reading frame. Thus, it appears that the relative proportion of the two proteins specified by the single mRNA is controlled at the level of translation.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England Biolabs, Inc., Beverly, Mass., Boehringer Mannheim Biochemicals, Indianapolis, Ind., and Bethesda Research Laboratories, Gaithersburg, Md. Polynucleotide kinase was obtained from P-L Biochemicals, Milwaukee, Wis. Calf intestinal alkaline phosphatase and the Klenow fragment of DNA polymerase I were from Boehringer Mannheim. The cordycepin 3′-end-labeling kits, α-^{32}P-deoxyribonucleotide triphosphates (3,200 Ci/mmol), [γ-^{32}P]ATP (2,900 Ci/mmol), and [α^{32}P]GTP (760 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass.

Cells and virus. African green monkey kidney cell lines, CV-1 and Vero, were propagated as monolayers in Dulbecco modified Eagle medium, 10% fetal calf serum, penicillin, and streptomycin. MA 160 SSPE cells (MA 72046; M.A. Bioproducts, Walkersville, Md.), a human prostate cell line persistently infected with the Mantooth strain of measles virus (12), were grown as monolayers in the same medium. Plaque-purified Edmonston measles virus (obtained from E. Norrby) was passaged at a multiplicity of infection of 0.1 PFU per cell at 37°C. Virus was harvested at 30 to 35 h postinfection when 90% of the cells were involved in fusion events.

cDNA libraries. The cDNA library produced by oligodeoxythymidylic acid priming of measles mRNA has previously been described (16, 42). The measles genomic library was established by randomly priming 2 μg of intracellular nucleocapsid 50S (−) genomic RNA with salmon sperm DNA pentamers. The nucleocapsids from Edmonston virus-infected cells were purified essentially as described by

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Mountcastle and Choppin (33), except that the infected cells were scraped into distilled water and Dounce homogenized without the addition of detergents. Subsequent to cesium chloride banding, nucleocapsids were phenol extracted (two times) and applied to 15 to 30% sucrose gradients containing 0.2% sodium dodecyl sulfate (SDS) and centrifuged at 20,000 rpm for 15 h at 20°C with an SW41 rotor. The 50S RNA was recovered from the gradients and ethanol precipitated in preparation for cDNA cloning. Methods employed for first- and second-strand synthesis were as detailed by Rice and Strauss (38). Subsequently, the double-stranded DNA (200 ng) was S1 nuclease treated, oligodeoxycytidylate tailed, and inserted into the *Pst*I site of appropriately tailed pBR322. Transformation was performed by using RbCl-treated competent *E. coli* HB101 cells as described (28). Ampicillin-sensitive, tetracycline-resistant colonies were distributed into microtiter plates. The library was composed of ca. 2,000 clones.

End labeling and DNA sequencing. DNA fragments to be sequenced were 3′ end labeled by the addition of [^{32}P]cordycepin via terminal transferase or by the addition of the appropriate α-^{32}P-deoxyribonucleotide triphosphate by fill-in reactions catalyzed by the Klenow fragment of DNA polymerase I. End labeling at the 5′ end was performed by [γ-^{32}P]ATP by using polynucleotide kinase, subsequent to calf intestinal alkaline phosphatase treatment of the DNA. Labeled DNA fragments were subcut with restriction endonucleases to yield uniquely labeled fragments which were purified by gel electrophoresis. Sequence analysis of the labeled DNA was performed by the base-specific chemical cleavage method described by Maxam and Gilbert (29).

Preparation of mRNA. Vero cells infected with measles virus (multiplicity of infection, 1 PFU per cell) were harvested when 80 to 90% of the cells had fused. Monolayers of cells were washed with TNE buffer (10 mM Tris-hydrochloride [pH 8.8], 0.14 M NaCl, 2 mM EDTA) containing 0.5% Nonidet P-40, 0.1% sodium deoxycholate, 1% β-mercaptoethanol, and 100 U of RNasin per ml. Nuclei were removed (1,000 × *g*, 5 min), and the supernatant fluid was adjusted to 0.1% SDS and 0.3 M LiCl. Polyadenylated [poly(A)$^+$] mRNA was selected by passing the supernate over oligodeoxythymidylate-cellulose columns as described by Maniatis et al. (28).

In vitro translation and immunoprecipitation. The rabbit reticulocyte lysate translation system obtained from Promega Biotec, Madison, Wis., was used according to the recommendations of the supplier. Each reaction was performed with 2.0 μg of poly(A)$^+$ RNA and 50 μCi (1,000 Ci/mmol) of [^{35}S]methionine (New England Nuclear). The products of in vitro translation were immunoprecipitated as detailed (4) and separated by SDS-polyacrylamide gel electrophoresis (24). Subsequently, the gels were fluorographically enhanced, dried, and exposed to Kodak XR-5 X-ray film at −70°C.

Northern blot analysis. Poly(A)$^+$ RNA (1 to 2 μg) was electrophoretically separated in formaldehyde-agarose (1%) gels according to the method described by Derman et al. (10). The RNA was transblotted onto Zeta probe membrane filters (Bio-Rad Laboratories, Richmond, Calif.), and the filters were baked at 80°C under a vacuum for 2 h. Hybridization to end-labeled DNA fragments (5 × 10^6 dpm/μg) was carried out at 42°C for 12 to 15 h in 50% formamide–5× SSPE (0.90 M NaCl, 50 mM sodium phosphate [pH 7.0], 5 mM EDTA)–0.02% (wt/vol) bovine serum albumin-Ficoll 400-polyvinylpyrolidone–0.3% SDS–100 μg of sonicated, denatured, salmon sperm DNA per ml. After hybridization, the filters were washed at 45°C with 2× SSPE containing 0.2% SDS (three times for 20 min) and washed a final time at 55°C with 0.2× SSPE containing 0.2% SDS. Filters were exposed to Kodak XR-5 X-ray film using a screen and stored at −70°C until developed.

Peptide synthesis and antibody production. Synthetic oligopeptide synthesis, antibody production, affinity purification of antisynthetic peptide antisera, and enzyme-linked immunosorbent assays for the antisera have been in part previously described (4) and will be detailed elsewhere (C. Richardson, A. Berkovich, S. Rozenblatt, and W. Bellini, submitted for publication).

Immunofluorescence. Cells to be infected with measles virus or the MA 160 SSPE persistently infected cell line were grown on cover slips which were pretreated with detergent and 1N HCl. CV-1 or Vero cell monolayers were infected with Edmonston measles virus at a multiplicity of infection of 10 PFU per cell, incubated for various periods of time (6 to 36 h), fixed with 3% formaldehyde in phosphate-buffered saline (pH 7.4), and permeabilized with 0.05% Triton X-100. The MA 160 SSPE cells were fixed and permeabilized in the identical manner. Cells were incubated with a mouse monoclonal antibody to either the P or N protein of measles virus (4) and subsequently to either the C_1 or C_2 affinity-purified antipeptide antibodies (50 μg/ml). For blocking studies, a 1 M excess of either the C_1 or C_2 peptide was present during the incubations with the respective antisynthetic peptide antibody. Cells were then washed and incubated with an affinity-purified goat anti-rabbit immunoglobulin conjugated to tetramethylrhodamine isothiocyanate (Cappel Laboratories, Cochranville, Pa.). The cover slips were mounted on glass slides in 0.1 M Tris-hydrochloride (pH 8.8) and viewed with a Zeiss ICM 405 inverted microscope equipped with epifluorescence and differential interference contrast optics.

"Riboprobe" mapping of the P-C mRNA. The Riboprobe gene analysis system was purchased from Promega Biotec. Cloning of the P/C gene fragments into the Riboprobe vectors, pSP64 and pSP65, is detailed in the text. The synthesis of (−) and (+) genomic probes was performed according to the protocols provided with the system. Typical transcription reaction volumes were 50 μl and contained 1 μg of the linearized recombinant vector and 15 μM [α-^{32}P]GTP (760 Ci/mmol), 30 μM GTP, and 500 μM nucleoside triphosphates. Hybridization and RNase A and T1 trimming conditions were as described by Promega Biotec. Analysis of the single-stranded protected probe is described (see below and legend to Fig. 7).

RESULTS

Overlapping cDNA clones which span the phosphoprotein (*P*) gene. Libraries of cDNA clones of measles 50S genome or mRNA were generated by priming reverse transcriptase with mixed oligonucleotides generated from salmon sperm DNA or with synthetic oligodeoxythymidylic acid. These libraries, arranged in microtiter dishes, were screened by colony hybridization to identify a series of overlapping clones that span the *P* gene of measles. A clone previously derived from measles virus mRNA (Cl-G) (16) was recently determined to contain the last 400 nucleotides of the *P* gene (3, 4). This clone was used to probe the libraries, and the largest positive clone identified (pWB3A8) was used to rescreen the library. The overlapping clones identified in this manner extended into the nucleocapsid gene in one direction and into the matrix gene in the other (Fig. 1). The gene assignments for all of these clones were verified through the use of colony hybridization with a known phosphoprotein

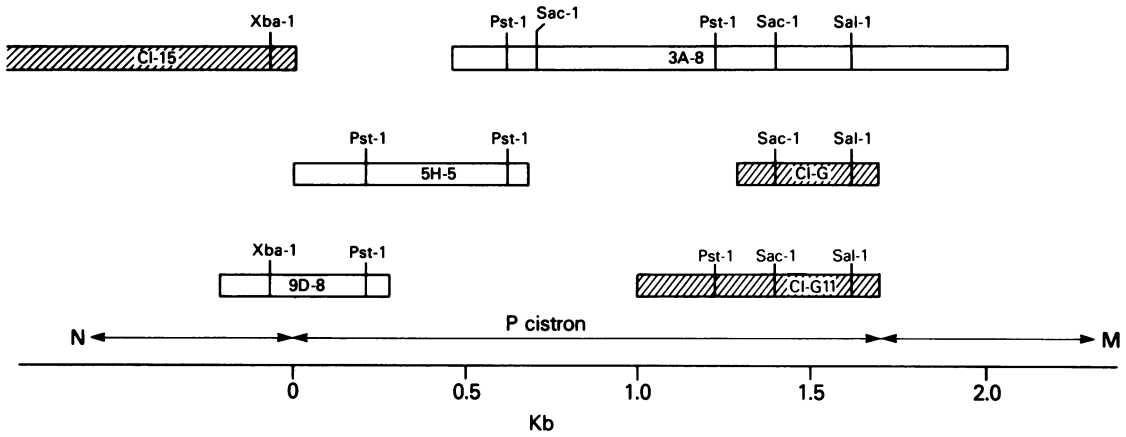


FIG. 1. Schematic representation of cDNA clones encompassing the phosphoprotein cistron and extending into the adjacent nucleocapsid and matrix genes of measles virus. Clones derived from the genomic library appear as open bars, and those derived from the mRNA library are shown as hatched bars.

clone, restriction endonuclease mapping, hybrid selection-mRNA translation, hybrid arrest of translation, and immunoprecipitation with antibodies which were directed against peptides constructed from the coding regions of the various cDNA clones (C. D. Richardson, A. Berkovich, S. Rozenblatt, and W. J. Bellini, J. Virol., in press). A schematic representation of these clones with respect to the measles genome is presented in Fig. 1.

Boundary region sequences define the *P* gene. Both strands of clones Cl-G, pWB-9D8, pGE-5H5, and pWB-3A8 were completely sequenced by the chemical method of Maxam and Gilbert (29). The overlapping sequences were collated, and the entire sequence of the *P* gene is presented in Fig. 2. We will describe elsewhere (Richardson et al., in press) that regular sequence homologies are observed at the intercistronic boundaries for the genes of measles virus (3). These sequences, 5′ . . . $TTATA_6CTTAGGA$ . . . 3′, show a high degree of homology with the boundary sequences of three other negative-strand RNA viruses: vesicular stomatitis virus, Sendai, and influenza. With these sequences as beginning and end points, the P cistron is 1657 nucleotides long and lies between the nucleocapsid (*NP*) gene and the matrix (*M*) gene.

***P* gene contains two overlapping reading frames.** The coding region for the phosphoprotein begins 60 nucleotides from the putative beginning of the mRNA (position 64 in Fig. 2) and specifies a 507-amino-acid protein (53.9 kilodaltons [kd]). The coding region terminates at the UAG codon lying 61 nucleotides from the end of the gene (Fig. 2). The size of this protein deviates somewhat from the estimates obtained from SDS-polyacrylamide gel electrophoresis, but this is not unexpected since the phosphoprotein of vesicular stomatitis virus behaves in a similar fashion (14). The primary structure of the protein indicates that it contains 32 proline residues which may yield a complex secondary and tertiary structure, which in turn may account for its slow electrophoretic mobility in acrylamide gels. It is interesting to note that despite the fact that the intercistronic boundaries of measles and Sendai viruses show good homology, comparison of the phosphoprotein sequence shown in Fig. 2 with that published for Sendai virus (15) reveals little homology.

The *P* gene of measles virus contains a second overlapping reading frame which could code for a second polypeptide. The initiation codon for this reading frame lies 22 nucleotides downstream from the first ATG triplet, and the second open reading frame is located at the 5′ end of the P phosphoprotein mRNA. The nucleotide sequence specifies a 186-amino-acid protein with a calculated molecular mass of 21,039 daltons. The protein is quite basic, with a pI of 8.7, and it contains eight proline residues. We will demonstrate in subsequent sections of this manuscript that this protein is synthesized in measles-infected cells, and we propose the name C for it, by analogy with the Sendai virus system (11, 13, 15). This measles protein may be identical to the ca. 20-kd proteins observed by Rima and Martin (40) and Vainionpää (47).

Identification of the C polypeptide as a unique viral protein through use of antibodies directed against specific peptides. To establish whether the second open reading frame in the phosphoprotein gene was actually expressed in measles-infected cells, two synthetic oligopeptides (C_1 and C_2) were prepared which corresponded to two regions of the amino acid sequence predicted for the C protein. These oligopeptides were coupled to a carrier protein and used to raise antibodies that would be specific for the C protein. There was no significant homology between the peptides synthesized and the phosphoprotein coded for in the first open reading frame. We recently reported that antibody directed against a synthetic peptide (P20) which consisted of the last 20 amino acids at the carboxy terminus of the P protein immunoprecipitated the phosphoprotein from cells infected with measles virus (4). To demonstrate that the 5′ portion of the *P* gene coded for two distinct proteins, another synthetic peptide (PN13) corresponding to the anticipated amino terminus of the phosphoprotein specified by the first open reading frame was also synthesized and used to prepare specific antisera. The sequence of these four oligopeptides and their relationship to the two proteins specified by the two open reading frames are illustrated in Fig. 2.

All antisera directed against synthetic peptides demonstrated exclusive reactivity with the particular peptide used to generate the antibody, as evidenced by specific enzyme-linked immunosorbent assays. The antisera were purified by affinity chromatography over columns prepared by coupling the specific peptides to a cross-linked agarose support. The affinity-purified antibodies were tested for immune reactivity with proteins labeled with [^{35}S]methionine synthesized in in vitro translations of poly(A)$^+$ mRNA. In parallel experiments, lysates of infected cells which had been labeled with [^{35}S]methionine were immunoprecipitated with these affini-

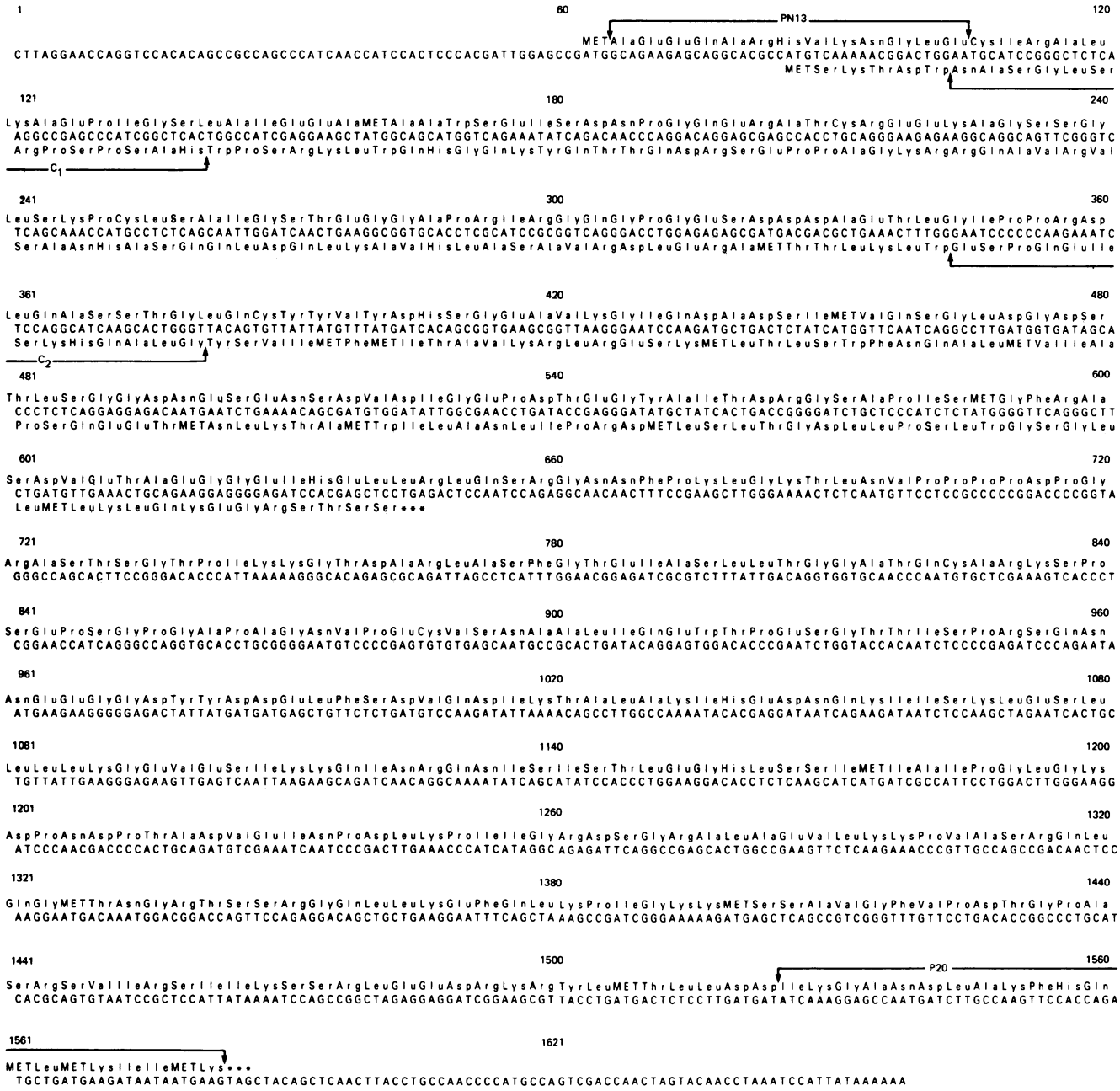


FIG. 2. Complete nucleotide sequence of the phosphoprotein gene of measles virus expressed as (+) sense DNA. The deduced amino acid sequence of the phosphoprotein (P) appears above the nucleotide sequence. The deduced amino acid sequence of the C protein appears below the nucleotide sequence. Bracketed regions designated PN13 and P20 denote the amino acid sequences employed for the construction of P-specific oligopeptides. Bracketed regions designated C_1 and C_2 denote the amino acid sequences of the oligopeptides constructed to the C protein. The dot appearing over the fourth nucleotide of the P cistron indicates the probable start of the mRNA.

ty-purified antibodies. Both sets of immune precipitates were analyzed by SDS-polyacrylamide gel electrophoresis together with appropriate controls.

Antibody to C_1 and C_2 synthetic oligopeptides specifically immunoprecipitated a 21-kd protein (C) from the products of an in vitro translation (Fig. 3a, lanes 2 and 3). This protein comigrates with a prominent protein present in the nonimmune precipitated products synthesized in the reticulocyte lysate system (Fig. 3a, lane 1). Anti-measles virus antisera from rabbits and humans do not precipitate the C protein (data not shown), nor does the control nonimmune rabbit serum (lane 7). These observations are consistent with the idea that the C protein is a nonstructural measles virus protein which is found only within the infected cell. Proteins of the same size as the C protein have been observed in measles-infected cells but not in uninfected cells (40, 47). However, it was not possible for those authors to distinguish whether the proteins were encoded by the measles genome or were simply host proteins whose synthesis was stimulated by the measles virus infection. Because of the similarity in

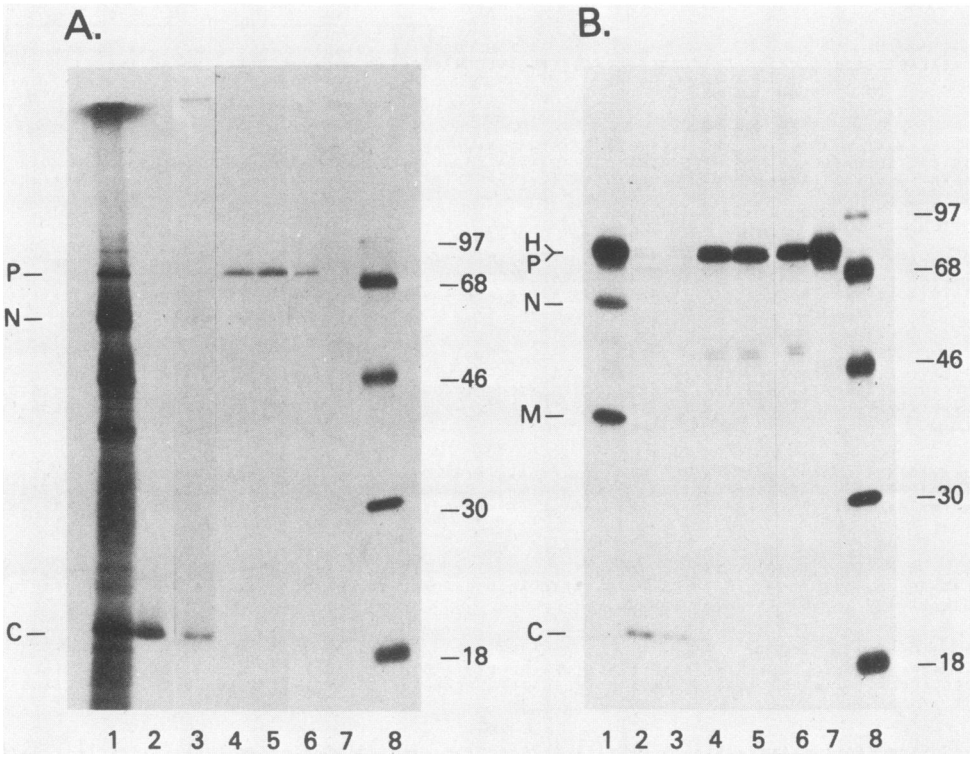


FIG. 3. Autoradiographs of SDS-polyacrylamide gels showing the specificities of the antisynthetic peptide antibodies. (A) Poly(A)$^+$ RNA (3 μg) from measles-infected cells was translated in vitro, and the [^{35}S]methionine-labeled products were used in immunoprecipitation studies. Lane 1, Total in vitro translated proteins; lane 2, [^{35}S]methionine-labeled translated proteins immunoprecipitated by C_1 antibody; lane 3, C_2 antibody; lane 4, PN13 antibody; lane 5, P20 antibody; lane 6, monoclonal anti-P antibody; lane 7, preimmune antibody; and lane 8, molecular weight standards. (B) Cells persistently infected with the Mantooth strain of measles virus were pusle-labeled for 4 h with 50 μCi of [^{35}S]methionine per ml. Cell lysates were immunoprecipitated with rabbit anti-measles serum (lane 1), C_1 antibody (lane 2), C_2 antibody (lane 3), PN13 antibody (lane 4), P20 antibody (lane 5), monoclonal anti-hemagglutinin (lane 7), and molecular weight standards (lane 8). The SDS-polyacrylamide (15%) gel did not resolve the hemagglutinin from the P protein.

size and association with the measles virus infection, it now seems likely that the protein observed in these early studies was the measles C protein.

The monoclonal anti-P antibody and the two antisera directed against synthetic peptides specified by the first open reading frame (P20 and PN13) all specifically precipitated the phosphoprotein from in vitro translation products (Fig. 3a, lanes 4, 5, and 6). These results indicate that both the 5′ and 3′ portions of the first open reading frame are translated into the phosphoprotein. Furthermore, since the PN13 peptide corresponds to the sequence immediately adjacent to the first methionine in this reading frame, these results are compatible with the notion that the first AUG is the translation initiation site for the phosphoprotein.

Immunoprecipitation studies with [^{35}S]methionine-labeled cells which were either acutely infected with the Edmonston strain of measles virus or persistently infected with the Mantooth SSPE strain (MA160 SSPE cells) were also performed. The results of the SDS-polyacrylamide gel analyses of the precipitates (Fig. 3b) are similar to those presented for the immunoprecipitates of the in vitro translation products. However, the cells which were acutely infected with the Edmonston strain of measles were found to produce less C protein than the cells persistently infected with the SSPE strain. This result was confirmed by immunofluorescence studies which are reported in a later section. The results obtained with the MA160 persistently infected cell line are reported in Fig. 3b. The C protein of the persistently infected cells migrated on SDS-polyacrylamide gels exactly as the in vitro synthesized counterpart (Fig. 3b, lanes 2 and 3). Again, neither the rabbit (data not shown) nor the human (lane 1) anti-measles serum precipitated the C protein from infected cells.

The monoclonal anti-P antibody and antisera directed against the peptides PN13 and P20 precipitated the phosphoprotein from persistently infected cells (Fig. 3b, lanes 4, 5, and 6). It is interesting to note that the phosphoprotein synthesized in infected cells often migrates on SDS gels as a doublet band, possibly reflecting varying degrees of phosphorylation of this protein.

The immune precipitation studies with proteins synthesized in infected cells establish that the predicted protein, C, is expressed during normal and persistent infections with measles virus. Since neither rabbit nor human antisera precipitated appreciable amounts of the C protein, it seems likely that it may not be incorporated into progeny measles virus particles.

Immunofluorescent localization of the P and C proteins in measles-infected cells. The availability of antipeptide antisera which specifically recognizes the C protein enabled us to investigate the localization of this protein by immunofluorescent microscopy. We used two different points of reference for the precise localization. The first is the Nomarski differential interference contrast image of the fixed and immunolabeled cells which reveals the major structures within the cell. The second is the cytoplasmic and intranu-

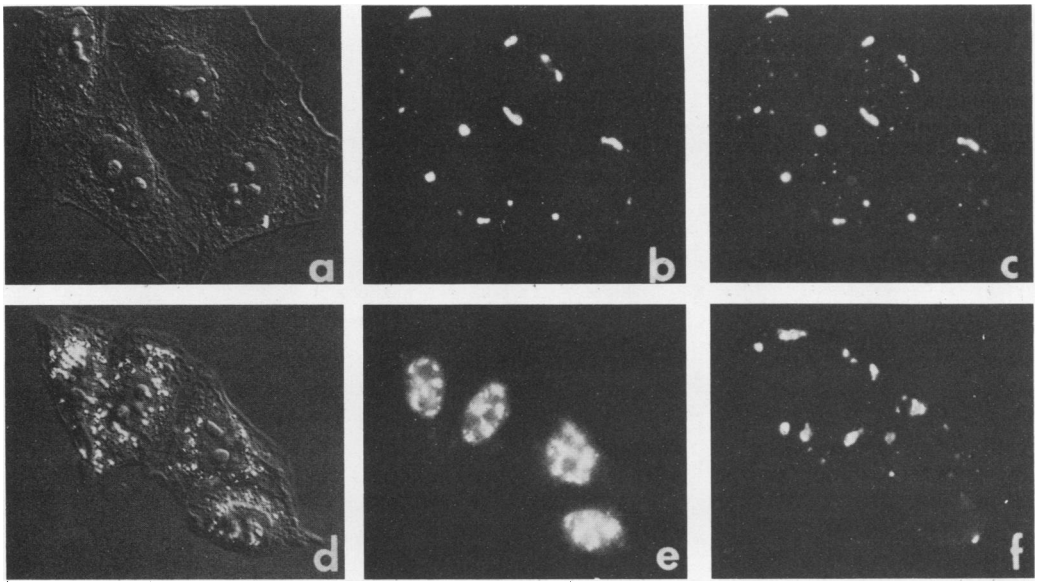


FIG. 4. Double indirect immunofluorescence of the persistently infected MA160 cells. (a) Nomarski differential interference contrast view of MA160 SSPE cells. (b) Immunofluorescent appearance of the same cells as in (a) labeled with the anti-C_1 antibody. (c) Immunofluorescent appearance of the same cells as in (a) labeled with a monoclonal antibody to the N protein. (d) Nomarksi differential interference contrast view of MA160 SSPE cells. (e) Immunofluorescent pattern of the same cells as in (d) labeled with the anti-C_2 antibody. (f) Immunofluorescent pattern of the same cells as in (d) labeled with the monoclonal anti-N protein.

clear inclusions of the measles nucleocapsid protein as visualized by using mouse monoclonal antibody directed against the nucleocapsid protein and a fluorescein tagged goat anti-mouse immunoglobulin G preparation. The typical experimental protocol called for fixing the persistently or acutely infected cells on cover slips, labeling the fixed cells with both sets of primary and fluorescent tagged secondary antibodies, washing them free of unbound antibodies, and photographing them with Nomarski differential interference optics or by fluorescent microscopy by using both the fluorescein and rhodamine channels. Examples of these localization experiments are shown in Fig. 4 and 5. Cells persistently infected with the Mantooth SSPE virus (MA160 SSPE) were investigated first because the immunoprecipitation experiments cited above demonstrated that they contained relatively large amounts of the C protein. Figure 4a shows a Nomarski image of a tetranucleated syncytium in relief. The four raised areas correspond to the location of the four nuclei, and the papillae on them correspond to the nucleoli. In Fig. 4b, the same field is viewed with fluorescent microscopy by using the rhodamine channel to reveal the location of the C protein. In this case, anti-C_1 peptide was used. Fig. 4c, the localization of the nucleocapsid protein is revealed by the fluorescein channel. The distribution of the nucleocapsid and the C protein as revealed by these antibodies appears very similar and, within the limits of resolution of this method, they colocalized perfectly. The major amounts of nucleocapsid and C protein are concentrated in inclusion bodies that lie near the nuclei. Much smaller amounts of nucleocapsid are found in intranuclear inclusions. When the anti-C_1 peptide was used as the detector system, the C protein was not apparent in the nuclei. Much to our surprise, when the same experiment was performed with the anti-C_2 peptide antisera, a different result was obtained (Fig. 4d through f). Here, the nucleocapsid is still seen localized in the perinuclear regions, but the C protein, as revealed with the anti-C_2 antisera, is localized in the intranuclear inclusions. This difference between the anti-C_1 and the anti-C_2 antisera is a qualitative, rather than a quantitative, difference; the anti-C_2 antisera weakly reacted with the cytoplasmic C protein (Fig. 4e). This difference may reflect different conformations of the C protein in the nucleus and cytoplasm. The epitopes for the anti-C_1 and anti-C_2 antibodies may not be equally available in all conformations of the C protein. The difference in the immunoreactivities of the anti-C_1 and anti-C_2 antisera are also reflected in the results obtained with cells acutely infected with the Edmonston strain of measles virus (Fig. 5). In Fig. 5a and d are shown two areas of syncytia formation in infected cells. In Fig. 5b and e, the C protein in these same fields was visualized with anti-C_1 and anti-C_2 antisera, respectively. In Fig. 5c and f, the location of the nucleocapsid protein is shown. In the acutely infected cells, the nucleocapsid is apparent as patches and inclusions in the cytoplasm and as punctate inclusions in the nuclei. The fluorescence observed with the anti-C_1 antisera colocalized with the cytoplasmic patches and inclusions, but little nuclear fluorescence was observed. The anti-C_2 antisera, on the other hand, recognized the C protein in the nucleus which colocalized with the nucleocapsid inclusions. The specificity of this fluorescence is indicated in Fig. 5g through j. Panels g and i are fluorescent micrographs showing nuclear and cytoplasmic nucleocapsid localizations in two fields of cells. Panels h and j show the same fields treated with the anti-C_1 and anti-C_2 antisera, respectively, but in the presence of the cognate synthetic peptides. The absence of any demonstrable fluorescence in these panels indicates that the binding of the antibodies to the cells is abrogated by the presence of the specific peptide used in raising the antibody.

The conclusion from the fluorescent immunomicroscopy experiments shown in Fig. 4 and 5 is that the C protein in both acute and persistent measles infections colocalizes with nucleocapsids in both the nucleus and cytoplasm. This colocalization suggests to us that the C protein may play a role in nucleocapsid assembly or function (RNA synthesis).

Single mRNA codes for both the P and the C proteins.

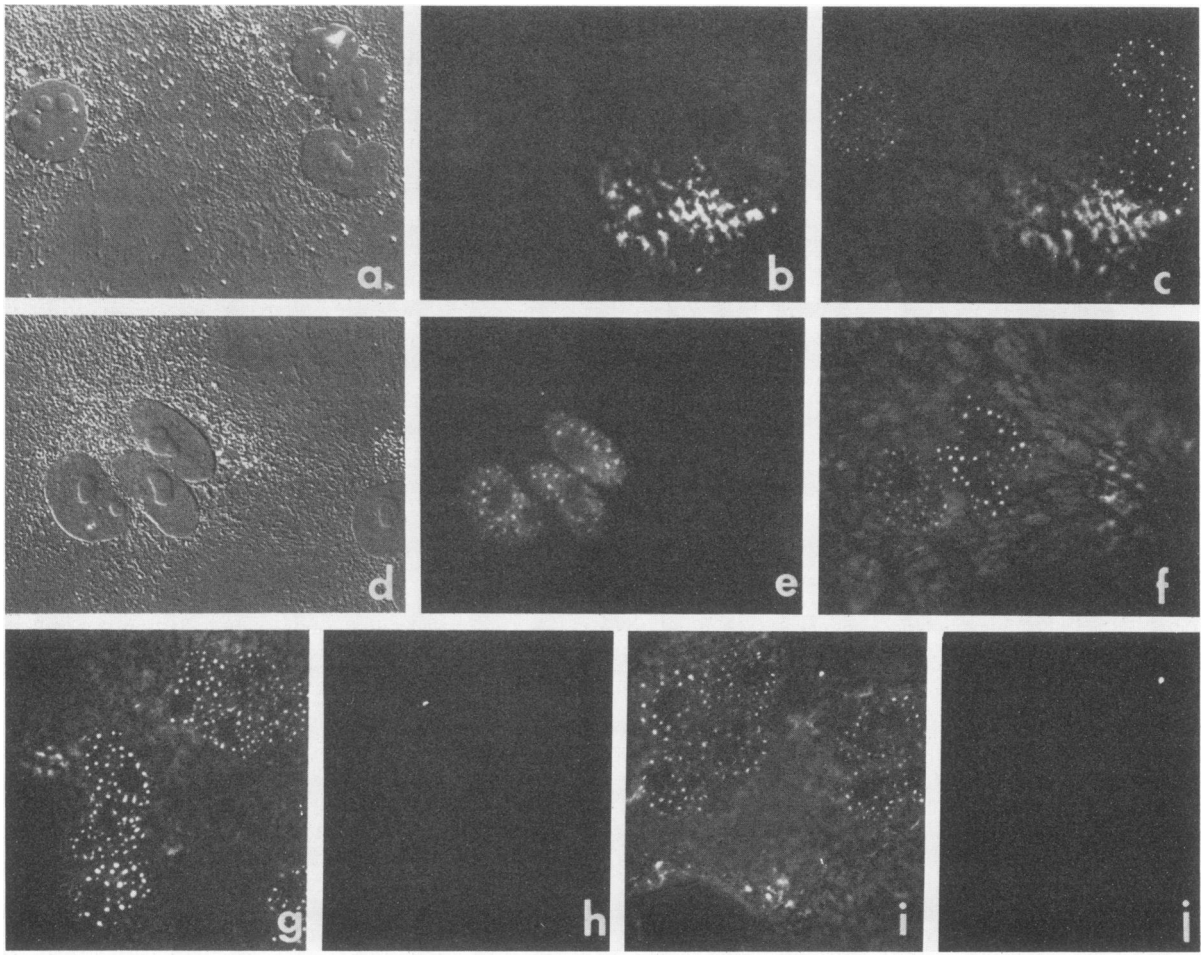


FIG. 5. Immunofluorescent labeling of acutely infected cells. CV-1 cells were infected with the Edmonston strain of measles virus and incubated for 24 h before fixation, permeabilization, and double indirect immunofluorescent labeling. (a and d) Nomarksi differential interference contrast views of giant cells appearing after measles infection. (b and c) The immunofluorescent staining patterns of the same cells as in (a), labeled with anti-C_1 antibody and monoclonal anti-N antibody, respectively. (e and f) The immunofluorescent localization patterns of the same cells shown in (d), labeled with the anti-C_2 antibody and anti-N antibody, respectively. (g) The immunofluorescent appearance of anti-N antibody-stained cells. (h) The same cells as in (g) stained with anti-C_1 antibody neutralized with a molar excess of C_1 peptide. (i) Immunofluorescent localization of the N protein. (j) The same cells as in (i) stained with anti-C_2 antibody after neutralization with the C_2 peptide.

Earlier work by others has demonstrated that each of two genes of influenza virus code for more than one protein through the use of overlapping reading frames (21, 25, 26). Analysis by these authors has revealed that each gene yielded multiple mRNAs which coded for these proteins and that these mRNAs were derived from a primary transcript by alternate splicing patterns. The replicative cycle of influenza virus involves an obligate nuclear phase during which the splicing can take place. The situation with measles virus is less clear, and an obligatory nuclear involvement or splicing of measles mRNA has not been demonstrated as yet. Furthermore, in measles virus both open reading frames begin near the very 5′ end of the P mRNA, and this region does not contain sequences homologous to the canonical splice acceptor or donor sites. Given this, it is unlikely that splicing and the generation of multiple mRNAs could account for the synthesis of the P and C proteins. We sought to distinguish between multiple mRNAs on the one hand and a single mRNA on the other which could code for both the P and C proteins. We pursued three lines of experimentation in answering this question. The first was an examination by Northern blot analysis of the mRNA from measles-infected cells. Measles virus mRNA and [^{14}C]rRNA were electrophoresed in separate lanes of an agarose-formaldehyde gel and transferred to nylon membrane by electroblotting. Strips of membrane containing both mRNA and rRNA standards were hybridized with ^{32}P probes from either the 5′ end of the P mRNA, which contained information for both open reading frames (Fig. 6, strip 2), or the 3′ end of the mRNA, which contained only information for the phosphoprotein (Fig. 6, strip 1). The autoradiograms show that both probes anneal to the same species of mRNA, an mRNA of ca. 1,750 bases and two mRNAs of 3,000 to 3,500 bases. The former RNA corresponds in size to a full-length transcript of the *P* gene with a poly(A) tail. The latter are probably readthrough dicistronic mRNAs of the type previously described for negative-strand RNA viruses (18, 51). For our present purposes, it is important to note that no mRNAs smaller than full length were found to contain information for the P or C proteins. These results indicate that if there are multiple mRNAs, they cannot be resolved on the basis of size by the techniques employed here. We next turned to hybrid selec-

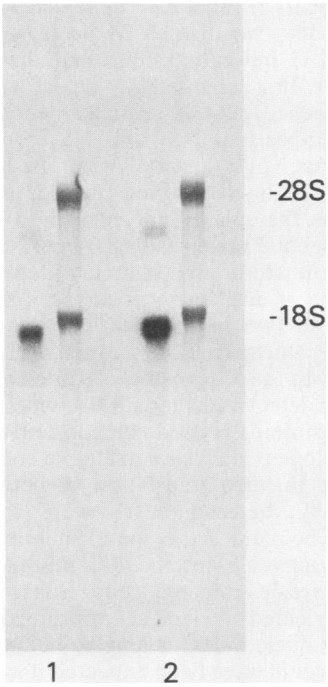


FIG. 6. Northern blot analysis of P/C mRNA. Measles mRNA (2 μg) and [^{14}C]rRNA markers were electrophoresed in formaldehyde-agarose gels and transblotted onto nylon membranes. Strip 1 was hybridized with an end-labeled probe (5×10^6 dpm/μg) derived from pWB3A8 by digesting this DNA with *Pst*I and *Sal*I. The resultant fragment was purified from acrylamide gels and 3′ end labeled as described in the text. Strip 2 was hybridized to *Pst*I digested and 3′-end-labeled pGE5H-5 (10^7 dpm/μg). [^{14}C]rRNA was run in the right-hand lanes of strips 1 and 2, and measles virus mRNA was run in the left-hand lanes.

tion and translation of measles mRNA in an effort to distinguish between the alternatives. mRNA selected by its hybridization to clone CLG-11 (Fig. 1) represents the very 3′ end of the gene and only contains information for the phosphoprotein. Translation of the selected mRNA yielded the C protein as well as the phosphoprotein (data not shown). This result suggests that if a separate mRNA encoding the C protein were present, it must contain the information at the very 3′ end of the gene, although this information is not expressed in the C protein.

These studies do not exclude the possibility that mRNAs of essentially identical size independently code for the P and C proteins. These messages would differ by a small spliced-out region in the mRNA for the C protein, which would effectively remove the initiator methionine employed for the start of the P protein. Thus, the AUG start position for the C protein would become the first 5′-proximal AUG in the mRNA encoding the C protein. The hypothetical splice should occur at or very near the AUG codon for the P protein start, i.e., ca. 70 nucleotides from the 5′ end of the mRNA.

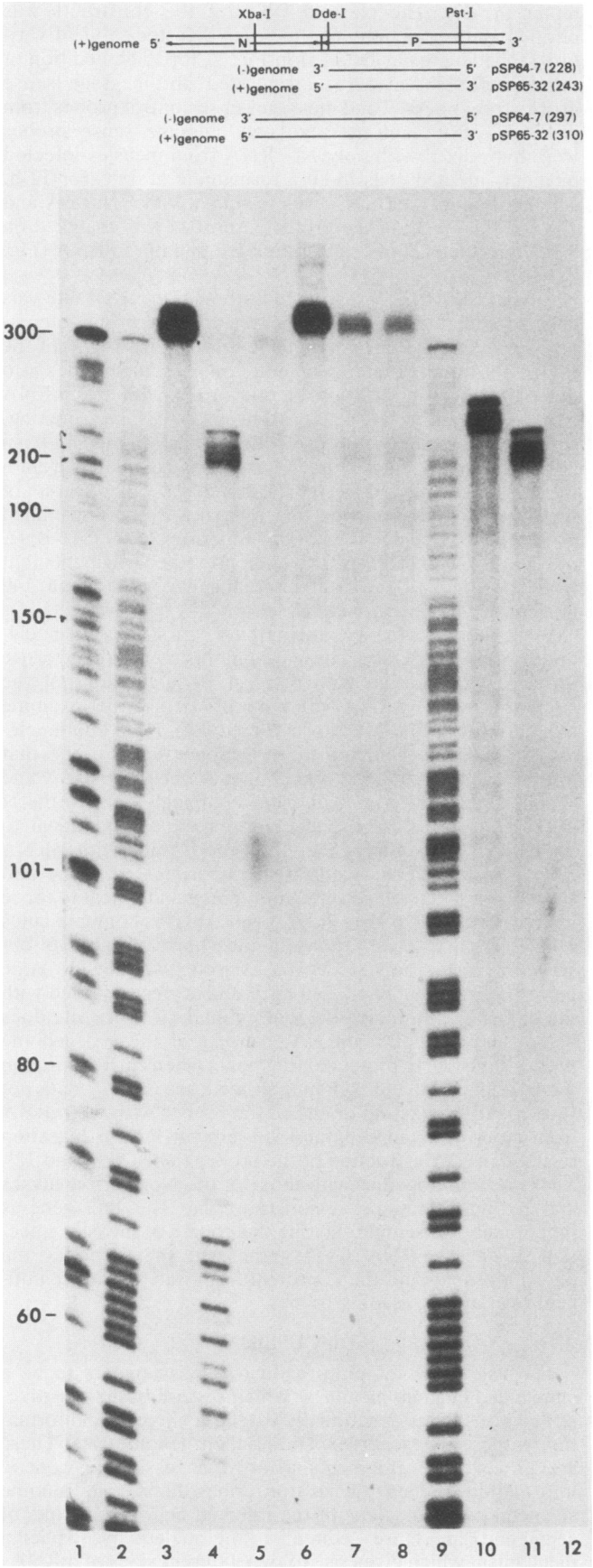
To examine this possibility, we prepared single-stranded RNA probes containing the complementary sequences of the first 211 nucleotides from the very 5′ end of the P/C mRNA. Clone pWB9D-8 was digested with *Pst*I and *Xba*I. The resultant 280-base-pair fragment was purified and inserted into the appropriately digested polylinker sites of the Riboprobe vectors, pSP64 and pSP65 (30), in opposite orientations, downstream from the SP6 bacteriophage promoter (6, 22). The plasmid DNA of the constructs was purified and restriction endonuclease digested to yield various probe lengths as detailed in Fig. 7, top. The addition of the SP6 RNA polymerase generated anti-message sense probes from pSP64-7 and message sense control probes from pSP65-32. Both anti-message and message sense probes were hybridized with poly(A)$^+$ RNA from measles-infected or mock-infected cells in 80% formamide at 45°C for 12 h. The heteroduplexed RNA was trimmed with RNase A and T1, and the length of the protected probes was analyzed on 8% sequencing gels as single-stranded denatured RNA (Fig. 7, bottom).

Clone pSP64-7(297) restricted at the unique *Xba*I site was used to generate a (−) genome sense RNA probe encompassing 211 nucleotides complementary to the 5′ end of the P-C mRNA and extending an additional 71 nucleotides into the *N* gene. Hybridization of this probe with the mRNA from measles-infected cells, followed by RNase trimming, resulted in a nested set of protected fragments ranging from 211 to 222 nucleotides (Fig. 7, lane 4) as calculated from a DNA sequencing ladder (lanes 1 and 2). The degree of ragged ends appears to be that reflected in the untrimmed probe and may be due to multiple initiations of the SP6 RNA polymerase. Nevertheless, no large protected fragments that would be compatible with an internal splice site, i.e., ca. 140 nucleotides in length, were observed.

Very short fragments ranging between 40 and 70 nucleotides were detected. These fragments very likely arose through hybridization with the last 70 nucleotides of the nucleocapsid (N) mRNA with the probe. To further examine this question, pSP64-7(228) restricted with *Dde*I endonuclease was used to generate a (−) genome sense probe that terminated at the intercistronic boundary between the *N* and *P* genes but carried no sequences complementary to the N mRNA. In all other respects, this probe was identical to pSP64-7(297) (see top of Fig. 7). Hybridization with mRNA from measles-infected cells with the truncated probe and subsequent trimming resulted in fragments identical to those observed with the pSP64-7(297) probe (Fig. 7, compare lanes 4 and 11). In contrast, none of the shorter fragments observed in lane 4 appeared with this probe. Again, no large protected fragment of ca. 140 nucleotides was observed with the pSP64-7(228), indicating that an internal splice site does not occur in the P/C mRNA. Neither of the (+) genome probes gave rise to protected fragments when carried through the hybridization and trimming procedures (lane 7; data not shown). Hybridization of any of the probes with the mRNA from mock-infected cells and subsequent RNase digestion resulted in the destruction of the probes (lanes 5, 8, and 12). These results, together with those of the Northern analyses and hybrid selection, demonstrate that the P/C gene is functionally bicistronic. During the course of measles infection a single mRNA was synthesized that encodes the phosphoprotein and the C protein from independently initiated overlapping reading frames.

DISCUSSION

The use of overlapping reading frames appears to be a simple and efficient means by which several of the negative-strand viruses can functionally extend their genetic information without physically expanding their genome size. There are, however, several mechanisms for accessing this genetic information. Influenza virus transcribes the various genome segments in the nucleus of the infected cell. Transcripts of segments 7 and 8 are "edited" in the nucleus by a splicing mechanism which gives rise to two different sizes of mRNAs



for both segments 7 and 8. The spliced mRNAs from segment 7 encode two distinct M proteins, but share the 5′-proximal AUG initiation codon and the nucleotide sequence for the first nine amino acids, after which the sequence diverges (21, 26). Essentially identical mechanisms lead to the synthesis of NS_1 and NS_2 proteins from the spliced transcripts of segment 8 (25). In both cases, the 5′-proximal AUG is maintained as the unique initiation codon, whereas the splicing mechanism is responsible for accessing the second open reading frames.

In the present study, we demonstrated that the phosphoprotein gene of measles virus encodes a single mRNA, which is read in two independently initiated overlapping reading frames. Northern blot analysis with a cDNA probe complementary to the 5′-proximal 630 nucleotides of the P/C mRNA failed to detect a smaller mRNA which could uniquely encode the C protein. Hybrid selection of the P/C mRNA with a cDNA clone lying well outside the coding region of C resulted in the in vitro translation of both the P and C proteins. Finally, heteroduplexes were formed between cRNA probes, containing the complementary sequence for the first 211 nucleotides of the P/C mRNA and poly(A)$^+$ mRNA from measles-infected cells. Subsequent ribonuclease trimming yielded a nested set of protected fragments of ca. 211 to 220 nucleotides in length, but no smaller fragments, which would have been expected if a splice site were present. This dispelled the notion that two messages of essentially identical size independently encoded the P and C proteins. In contrast to influenza virus segments 7 and 8, the acquisition of the two reading frames within the P-C mRNA of measles virus must occur through independent initiations of ribosomes at the respective AUG codons. A similar situation has been recently described for Sendai virus (15) and influenza B (44). Two bunyaviruses, La Crosse and snow shoe hare, appear to encode the N protein and NSs protein from overlapping reading frames present within the S genome segment (1). However, it is not certain that a single mRNA encodes for both proteins. Bos et al. (5) have shown that a mRNA transcript from the E1b region of adenovirus is translated into two distinct tumor antigens from overlapping reading frames.

Measles and Sendai viruses are strikingly similar with respect to the structural make up of the P/C cistrons. Neither of the AUG initiation codons for the P and C reading frames

FIG. 7. Mapping of the 5′ end of the P/C mRNA. Top, Schematic diagrams showing the various probes synthesized from the Riboprobe constructs for the mapping studies. Bottom, Measles mRNA (1 μg) or mRNA from uninfected cells was hybridized to each of the RNA probes. Subsequent to RNAse treatment, the protected fragments were denatured in 80% formamide, heated for 3 min at 90°C, and applied to an 8% sequencing gel. Lanes 1 and 2, Guanine and guanine plus adenine ladders of pWB9D8 fragment (300 nucleotides) 3′ end labeled at the *Pst*I site and extending past the *Xba*I site to an *Rsa*I site 17 nucleotides further into the *N* gene. Numbers appearing on the left indicate the precise nucleotide distance from the *Pst*I site to the *Rsa*I site for the single-stranded DNA. Lane 3, pSP64-7(297) untreated probe; lane 4, pSP64-7(297) probe hybridized with measles mRNA and trimmed; lane 5, same as lane 4, but hybridized with mock mRNA; lane 6, pSP65-32(310) untreated probe; lane 7, pSP65-32(310) probe hybridized with measles mRNA and trimmed; lane 8, same as lane 7, except hybridized with mock mRNA; lane 9, guanine plus adenine ladder as in lane 2; lane 10, pSP64-7(228) untreated probe; lane 11, pSP64-7(228) hybridized with measles mRNA and trimmed; and lane 12, same as lane 11, but hybridized with mock mRNA.

of these viruses are in the favored context with respect to flanking nucleotide sequences for optimal ribosome initiation (23). Presumably, this would permit ribosomes to initiate independently at both AUGs since neither would be favored over the other. Both viruses contain the AUG codons of their respective P and C cistrons near the 5′ end of the mRNA, and the two initiation codons are separated by 11 nucleotides in the case of Sendai virus and 22 nucleotides in the case of measles virus. Both C proteins are read from the +2 reading frame relative to that employed for reading the P protein. In addition, the identical trinucleotide CTT directly follows the polyadenylation signal of the adjacent N cistron, and three of the four nucleotides at the 5′-proximal end of the mRNAs for the P and C proteins of measles and Sendai viruses are also identical. Although the strategy of expression for the C polypeptide of both viruses is similar, there is a considerable difference in their amino acid sequences (15).

Rima (39), Rima and Martin (40), and Miller (31) have previously described an 18- to 20-kd protein in measles-infected cells. In addition, this protein was observed in in vitro translation studies with mRNA extracted from measles infected cells (45, 48). The synthesis of this protein was not affected by the addition of proteolytic enzyme inhibitors, and it was thought to be a primary translation product rather than a degradation product of a larger protein (47). The 20-kd protein was not observed in the purified virions and was considered to be a nonstructural protein encoded by measles virus or a host protein induced during virus infection.

The novel use of synthetic peptide antibody to probe for the predicted *C* gene product has allowed the unequivocal identification of the 21-kd C protein in cell-free translation assays with measles mRNA, as well as in cells acutely and persistently infected with measles virus. Moreover, these studies establish the C protein as a unique virally encoded product.

Using antibodies directed to the C_1 peptide of measles, we have been successful in immunoprecipitating a 20-kd protein from the in vitro translation products programmed by canine distemper virus mRNA (C. D. Richardson, W. J. Bellini, and S. Rozenblatt, unpublished data). Measles and canine distemper virus are closely related morbilliviruses for which immunological cross-reactivity of the structural proteins is well documented (17, 35). The fact that there is an antigenically related protein (C) synthesized by canine distemper virus and measles and a similar protein synthesized by Sendai virus argues in favor of the conservation of the C protein with the paramyxoviruses. In addition, C-like nonstructural proteins have been observed in SV5 (36), respiratory syncytial virus (49), Newcastle disease virus (9), and mumps virus (19, 41). In the case of respiratory syncytial virus, the nonstructural proteins are encoded by genes separate from the *P* gene, and apparently no second open reading frame exists (49). Thus, it appears that genes coding for the nonstructural proteins may be carried as separate genes or as overlapping genes within the P cistron.

The functions of the P and C proteins of measles virus are, to date, unknown. Immunofluorescent antibody studies have shown that the P protein colocalizes with cytoplasmic inclusions which also contain viral nucleocapsids (20, 34). This protein has also been found associated with nucleocapsids extracted from virions (33). If such cytoplasmic inclusions represent transcription or replication complexes, or both, within the infected cell, then these localization studies might suggest a role for the P protein in transcription or replication, or both. Indeed, a role for the P proteins of Newcastle disease virus and Sendai virus in transcription has been suggested from results of in vitro transcription and *ts* mutant studies (8, 32, 43).

Although both C_1 and C_2 antibodies efficiently immunoprecipitate the C protein of measles, their reactivities in the immunofluorescent labeling studies differed somewhat—the C_1 antibody was more efficient in detecting the C protein in cytoplasmic inclusions, whereas the C_2 antibody was more efficient at detecting nuclear inclusions of the C protein. Such results most likely reflect the availability of the epitopes defined by the C_1 and C_2 oligopeptides. Conformational alterations or protein masking may restrict the binding of C_1 and C_2 antibodies to their respective epitopes. Nevertheless, the C protein could be found both in the cytoplasmic inclusions as early as 6 h postinfection and in the nucleus late (24 to 36 h) in infection.

The localization of the C protein within intranuclear inclusions of lytically infected cells and the apparent colocalization of the N protein raises suspicions that measles replication may have an obligatory nuclear phase. Pennington and Pringle (37) provided evidence for an early nuclear dependent phase, i.e., enucleation of cells 1 h before or within the first 4 h after infection dramatically reduced the yields of infectious virus. However, enucleation 6 h postinfection had little effect on the viral yield. We observed the accumulation of the C protein in the cytoplasm early in infection, but in the nucleus only after 24 h. It is possible that small amounts of the C protein are present in the nucleus early in infection, but go undetected by immunofluorescence either because the epitope is masked or because of the limited sensitivity of this assay. We have not yet attempted to isolate the C protein from purified nuclei of infected cells, but it is interesting to note that a 20-kd protein has been observed in nuclear preparations of measles infected CV-1 cells (50).

Clearly, the production of the C protein is conserved in infections by measles, canine distemper virus, and Sendai viruses. We have noted 56 positions within the C reading frame of measles where a single point mutation could generate a termination codon. Some 25 of these would either not affect the amino acid specified in the P reading frame or give rise to a similar amino acid as originally encoded in this reading frame. Thus, there appears to be some selective pressure for maintaining the *C* gene product. Further insight into the function of the C protein, and for that matter the P protein, awaits an in vitro transcription-replication system and especially high-quality temperature-sensitive mutants with which complementation studies can be performed.

ACKNOWLEDGMENTS

We especially thank Robert A. Lazzarini, in whose laboratory this work was carried out, for his insight, advice, and encouragement during the course of these studies. We also thank David Powell for lending his computer expertise and time to this work. The excellent secretarial skills of Charlene French are also gratefully acknowledged.

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