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Structure and Function of Bicistronic RNA Encoding the Phosphoprotein and Matrix Protein of Measles Virus

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Two independent full-length replicas of a bicistronic RNA species containing the complete *P* and *M* genes of measles virus arranged in tandem were isolated from an expressible cDNA library. Sequences at the 5′ and 3′ termini suggested that the bicistronic RNA was initiated and terminated at precisely the same locations as the monocistronic mRNAs of the *P* and *M* genes, respectively. The *P* and *M* cistrons were fused together via an intergenic region which was exactly colinear with and complementary to the intergenic region of the genomic RNA. This RNA species was polyadenylated at the normal polyadenylation site at the 3′ terminus of the *M* cistron, but not in the intergenic region. By DNA-mediated gene transfer, these cDNA clones were expressed into bicistronic RNA containing both *P* and *M* sequences in primate cells. RNA thus generated did not undergo nucleolytic processing but was translated into high levels of a 70,000-M_r protein immunoprecipitated by monoclonal antiserum against the measles virus *P* protein. *M* protein was not produced in the same cells even though the *M* cistron could direct *M* protein synthesis in vitro once excised from the upstream *P* cistron. These results suggested that bicistronic RNA could direct protein synthesis from the first but not the second cistron and might contribute at least in part to expression of viral genes in vivo.

The genome of nonsegmented negative-strand RNA virus typically consists of a number of linearly arranged cistrons separated by conserved intergenic regions. Upon infection of host cells, the genetic information stored in the genome is transcribed by a virion-associated polymerase complex into a series of plus-strand mRNA species (10). Transcription starts from the 3′ terminus of the genome and proceeds towards the 5′ terminus through a series of termination followed by reinitiation at specific sites in the intergenic regions (1). The products of this stop-start transcriptional process are mainly monocistronic mRNA capped at the 5′ termini and polyadenylated at the 3′ termini. However, polyadenylated RNA species which contain sequences derived from two or more adjacent cistrons have been found in cells infected by a variety of nonsegmented negative-strand RNA viruses, including vesicular stomatitis virus (14, 15), respiratory syncytial virus (11), Newcastle disease virus (34), simian virus 5 (16, 25) and measles virus (27).

These high-molecular-weight RNA species are believed to be derived from readthrough transcription which fails to terminate at the normal intergenic termination sites. The origin and possible function of the bicistronic RNA species remain unclear. Herman et al. (14, 15) originally proposed that bicistronic RNA might serve as precursors to monocistronic mRNA. Others viewed bicistronic RNA as aberrant transcription products which serve no function in virus replication. In Newcastle disease virus-infected cells, bicistronic RNA species could make up up to 25% of the total polyadenylated RNA and were associated with polysomes. The researchers thus postulated that these RNA species might be functional and could contribute to viral gene expression (34). However, the protein-coding potential of the bicistronic RNA species and the putative gene products, if any, have not been demonstrated or characterized.

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The genome of paramyxovirus measles virus contains at least seven genes. The *N* gene located near the 3′ terminus of the genome encodes an RNA-binding nucleoprotein (7, 26, 27, 35). The neighboring *P* gene encodes both the phosphoprotein and a nonstructural protein C in two different reading frames (6, 35). The *M* gene encodes the matrix protein postulated to be involved in maturation (5; T. C. Wong, G. Wipf, and A. Hirano, submitted for publication). The envelope-associated hemagglutinin and fusion protein are encoded by the *H* and *F* genes, respectively (2). The 5′-proximal *L* gene encodes a large polypeptide believed to be part of the polymerase complex (10, 17, 22). In measles virus-infected cells, six electrophoretically distinct species of virus-specific RNA could be detected (13). In addition, hybridization with gene-specific DNA probes revealed polyadenylated RNA species twice the size of the normal mRNA (27). These probably represented bicistronic RNA species analogous to those in other negative-strand RNA viruses.

To clearly understand the nature of the bicistronic RNA of measles virus, we isolated two independent full-length cDNA clones representing the complete sequence of a bicistronic RNA species from an expressible cDNA library. By high-efficiency cDNA cloning techniques, the gene expression products in Edmonston strain measles virus-infected cells were converted into over 3×10^4 full-length and expressible cDNA clones (35). After screening about 0.3% of the library with a DNA probe representing the 3′ region of the mRNA of the *M* gene (cl-*M*) (28), two classes of *M*-related cDNA clones were isolated. The majority of these were monocistronic clones which contained cDNA inserts about 1.65 kilobase pairs (kbp) long. A representative clone of this class designated as pcD-M3g is shown in Fig. 1 (left panel, lane M-3g). The 1.65-kbp inserts in these clones contained the complete *M* gene, which could be expressed into the corresponding *M* protein in vivo (Wong et al., submitted). In addition to these typical *M* clones, a second class of *M*-related cDNA clones which contained inserts

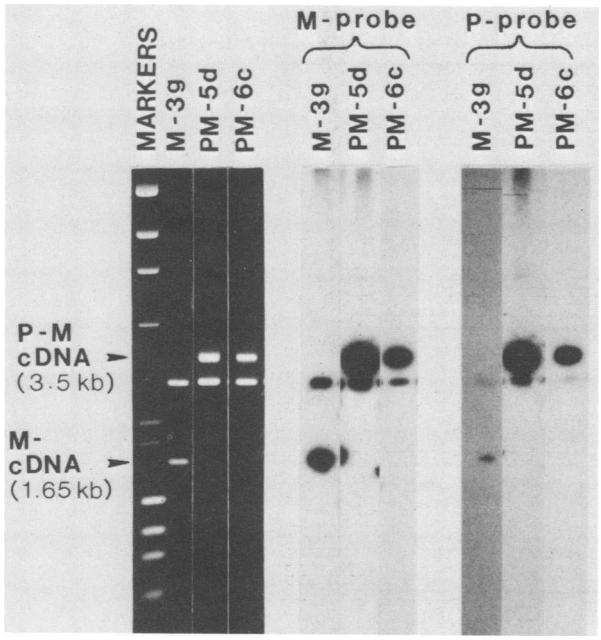

FIG. 1. cDNA clones pcD-PM5d and pcD-PM6c contained both *P* and *M* sequences. The cDNA inserts of clones pcD-M3g, pcD-PM5d, and pcD-PM6c were released from the vector by digestion with *Xho*I enzyme and analyzed by electrophoresis in a 0.7% agarose gel. Clone pcD-M3g was an expressible monocistronic clone which contained the complete sequence of the *M* gene (Wong et al., submitted). The resolved DNA fragments were stained with ethidium bromide (left panel), transferred onto nitrocellulose filter paper (30), and hybridized with either a ^{32}P-labeled *M*-specific DNA probe (*Bam*HI fragment from clone pcD-M2i [Fig. 2]) (middle panel) or a *P*-specific DNA probe (*Xho*I fragment from pcD-P8) (right panel). Markers consisted of double-strand DNA from bacteriophages λ and φX174 digested with *Hin*dIII and *Hae*III, respectively. cDNA inserts are indicated by arrows. The common fragment about 2.9 kbp long was the linearized vector.

roughly 3.5 kbp long were isolated (Fig. 1, left panel). Analysis of two clones from this latter class (pcD-PM5d and pcD-PM6c) revealed that they were bicistronic. Southern hybridization analysis showed that, whereas the 1.65-kbp insert from a typical full-length *M* clone (pcD-M3g) hybridized only to an *M*-specific DNA probe, the 3.5-kbp inserts from clones pcD-PM5d and pcD-PM6c hybridized to both *M*- and *P*-specific probes. (Fig. 1, middle and right panels, lanes M-3g, PM-5d, and PM-6c). Restriction enzyme analysis confirmed that pcD-PM5d and pcD-PM6c clones contained landmark restriction enzyme sites of both the *P* and the *M* genes.

To define the structure and possible origin of the bicistronic RNA, clones pcD-PM5d and pcD-PM6c were analyzed by DNA sequencing (29). Both clones contained the complete *P* and *M* sequences arranged in tandem (Fig. 2; only clone pcD-PM5d is shown). The 5′ termini of these clones began with the tetranucleotide 5′-AGGA-3′, a sequence identical to that at the 5′ terminus of a full-length monocistronic cDNA clone of the *P* gene (pcD-P8 [Fig. 2]) (35). The protein-coding regions of the bicistronic clones were identical to those of the monocistronic pcD-P8 clone and the genomic clones previously reported (6). However, when the intergenic regions of the bicistronic clones were compared with the 3′ terminus of the monocistronic clone pcD-P8, distinct differences were revealed. The highly conserved intergenic regions of the measles viral genome contained six uridine residues followed by the trinucleotide 3′-GAA-5′ (Fig. 2) (26). The $(U)_6$ sequence denoted the site for polyadenylation during mRNA synthesis; whereas the trinucleotide 3′-GAA-5′ was not transcribed during mRNA synthesis. Thus, the monocistronic clone pcD-P8 terminated with a polyadenylated sequence at a position corresponding to the $(U)_6$ sequence; and the sequence complementary to the intergenic trinucleotide 3′-GAA-5′ was not represented in pcD-P8 or any other monocistronic clone (Fig. 2). In contrast, the intergenic regions of the bicistronic clones pcD-PM5d and pcD-PM6c were exactly colinear with and complementary to the intergenic sequence in the genomic RNA. The *P* and *M* cistrons in these clones were separated by six A residues followed by the trinucleotide 5′-CTT-3′ (Fig. 2). Moreover, the 5′-AGGA-3′ sequence signifying the beginning of the following *M* cistron was immediately adjacent to this intergenic sequence with no omission or interruption by polyadenylated regions. The protein-coding region in the *M* cistron was identical to that of a full-length monocistronic *M* clone, pcD-M2i, which has been completely sequenced (Wong et al., submitted). Finally, both the bicistronic *P-M* clones and the monocistronic *M* clone terminated at the normal transcription termination site with a 3′ sequence, 5′-TAAAC$(A)_n$-3′. The structural arrangement and pertinent sequence of the bicistronic clone pcD-PM5d in comparison with the monocistronic *P* clone pcD-P8 and the *M* clone pcD-M2i are summarized in Fig. 2.

These results provided insight into the possible origin of the bicistronic RNA. The sequence arrangement suggested that the bicistronic *P-M* RNA species might be produced in a similar manner as a normal monocistronic mRNA. Initiation and termination of transcription occurred precisely at the respective 3′ and 5′ termini of the *P* and *M* cistrons on the RNA template. The bicistronic transcripts appeared to be derived from a failure to observe the polyadenylation and termination signals at the intergenic region. Similar to the bicistronic RNA observed in vesicular stomatitis virus (15), the intergenic trinucleotide normally untranscribed during production of monocistronic mRNA was faithfully represented in the bicistronic *P-M* RNA of measles virus. However, unlike the bicistronic RNA in vesicular stomatitis virus, the *P-M* RNA of measles virus was polyadenylated only at the 3′ terminus, not at the intergenic region. In this regard, the bicistronic RNA of measles virus was similar to that of Newcastle disease virus, which also possessed no intergenic polyadenylated sequences hybridizable to oligo(dT) (34). Interestingly, another paramyxovirus, simian virus 5, produced bicistronic RNA both with and without polyadenylated sequences in the intergenic regions (16, 25).

Bicistronic RNA has been postulated to serve as either a precursor to monocistronic mRNA or a dead-end byproduct of transcription. The sequence of the bicistronic RNA of measles virus provided no clue to its putative function. The precursor hypothesis invoked a virus- or host-derived RNase activity which could cleave the bicistronic RNA specifically at the intergenic regions. The newly formed 3′ termini could in turn serve as substrates for a putative poly(A) polymeraselike activity. Such an RNA-processing mechanism has not been demonstrated. To test whether host cells could carry out this putative nucleolytic process in the absence of other viral functions, we analyzed RNA expressed from the bicistronic cDNA clones via DNA-mediated gene transfer.

The monocistronic pcD-M2i and bicistronic pcD-PM5d clones were transfected into simian virus 40-transformed

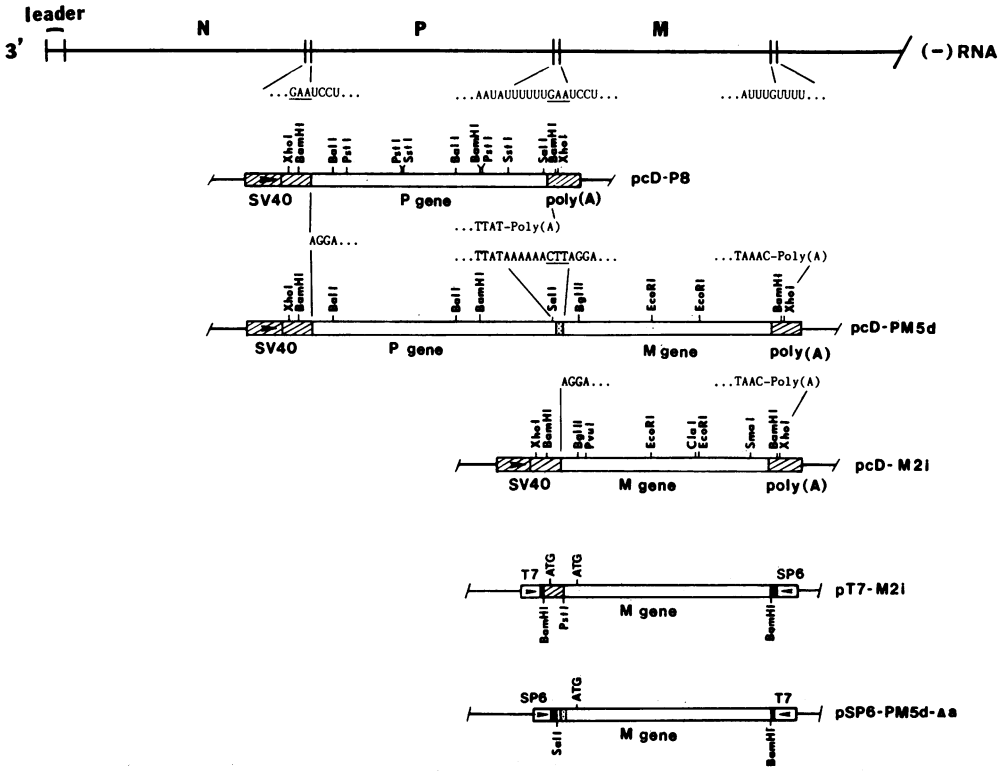

FIG. 2. Sequence comparison among bicistronic and monocistronic clones and their derivatives for in vitro expression. DNA sequences of monocistronic cDNA clones representing the complete *P* (pcD-P8) and *M* (pcD-M2i) genes of measles virus (35; Wong et al., submitted) were compared with bicistronic clone pcD-PM5d. The pertinent sequences and major restriction enzyme sites tested are depicted, showing the exact locations of initiation, polyadenylation, and termination of the plus-strand RNA with respect to the negative-strand RNA genome. Cross-hatched boxes represent sequences derived from the Okayama-Berg cloning vector, which contains the simian virus 40 (SV40) early promoter and splice signals on the 5′ side and polyadenylation sequences on the 3′ side of the cDNA inserts (24, 35). Stippled boxes represent sequences derived from the intergenic 3′-$(U)_6$-GAA-5′ sequence of the genomic RNA. The intergenic trinucleotide present only in the bicistronic clones, not in the monocistronic clones, is underlined. Clone pT7-M2i was derived from pcD-M2i by inserting the complete *M* gene defined by the two *Bam*HI sites into the *Bam*HI site in the cloning region (filled boxes) of an in vitro expression vector, pGEM-3 (Promega Biotec), such that the *M* gene was colinear with a promoter derived from bacteriophage T7. This construct retained part of the SV40 sequences (hatched box) including an in-frame ATG codon located in a short intron (24; Wong et al., submitted). Clone pSP6-PM5d-Δa was derived from pcD-PM5d by excising the *M* cistron defined by the *Sal*I and *Bam*HI sites and cloning it into the *Sal*I and *Bam*HI sites in the cloning region of pGEM-3, such that the *M* cistron was colinear with a promoter derived from bacteriophage SP6. This construct retained the extreme 3′ terminus of the *P* cistron and the intergenic region (stippled box) but contained no upstream ATG codon preceding the ATG codon of the *M* gene.

African green monkey kidney cells (COS cells) (12). At 48 h posttransfection, cellular RNA was purified and analyzed by electrophoresis in agarose gel in the presence of methylmercuric hydroxide (4). As a control, RNA purified from measles virus-infected cells was analyzed in parallel.

Hybridization with *P* and *M* gene-specific DNA probes revealed at least three size classes of virus-specific RNA in measles virus-infected cells (Fig. 3). The high-molecular-weight genome-length RNA was accompanied by heterogeneous subgenomic RNA (Fig. 3, lanes A and B), which was also observed in purified preparations of genomic RNA of measles virus (20, 31) unless removed by extensive gradient centrifugation, nuclease treatment, or both (3). A second class of intracellular RNA consisted of monocistronic RNA which migrated faster than the 18S ribosomal RNA marker (Fig. 3, lanes A and B). In addition, distinct RNA species which migrated in between the 18S and 28S marker RNAs were detected by both *P*- and *M*-specific probes. These represented bicistronic RNA. In cells transfected with *M* clone pcD-M2i, a single species of RNA with electrophoretic mobility similar to that of the 18S RNA marker was expressed (Fig. 3, lane D). This RNA species hybridized only to an *M*-specific probe, not to a *P*-specific probe (Fig. 3, lanes C and D). In contrast, bicistronic clone pcD-PM5d was expressed into RNA which comigrated with the bicistronic RNA from measles virus-infected cells and hybridized to both *P*- and *M*-specific probes (Fig. 3, lanes E and F). Moreover, virus-specific RNA of monocistronic size was not detected in cultures transfected with clone pcD-PM5d by either gene-specific probe (Fig. 3, lanes E and F). These results did not support nucleolytic processing of bicistronic RNA in transfected cells. However, these experiments did not rule out the possibility that the hypothetical processing pathway might be carried out by a viral function not present in the transfected cells.

All bicistronic RNA species in Newcastle disease virus-infected cells were associated with polysomes (34). This raised the interesting question of whether bicistronic RNA was translationally functional and, if so, what kinds of products could be produced. To answer these questions, we examined the in vivo gene products of the bicistronic clones at the protein level.

COS cells transfected with monocistronic *M* clone pcD-M2i produced a 38,000-M_r protein recognized by monoclonal

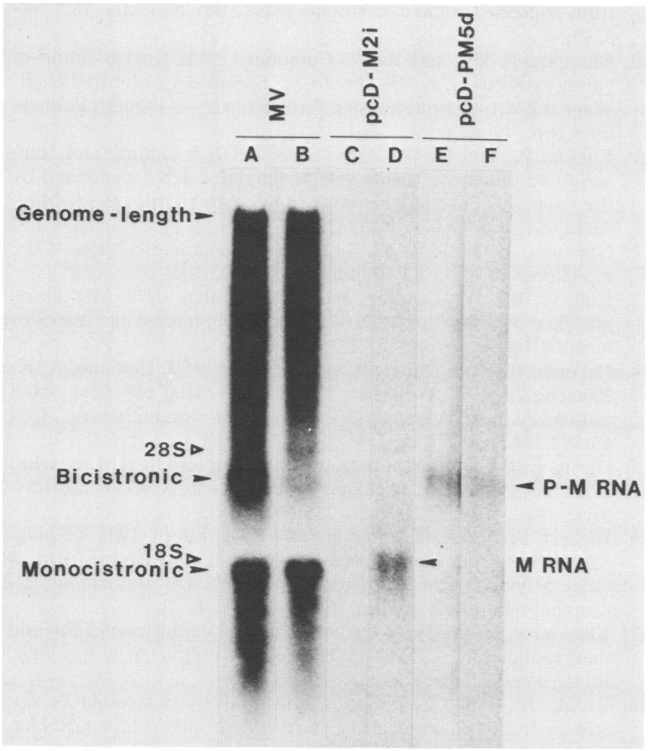

FIG. 3. Expression of monocistronic and bicistronic RNAs by DNA-mediated gene transfer. Cultures of 1.5×10^6 COS cells were transfected with 30 μg of cloned DNA pcD-M2i (lanes C and D) or pcD-PM5d (lanes E and F) by a modified calcium phosphate coprecipitation method (33, 35). At 48 h posttransfection, total intracellular RNA was extracted with 4 M guanidine thiocyanate and purified by centrifugation through a CsCl cushion (9). Amounts of 20 μg of the purified RNA were resolved by electrophoresis in a 1% agarose gel in the presence of 7.5 mM methylmercuric hydroxide (4), transferred onto nitrocellulose filter paper (32), and hybridized with a ^{32}P-labeled *P*-specific probe (*Xho*I fragment of pcD-P8) (lanes A, C, and E) or an *M*-specific probe (*Bam*HI fragment of pcD-M2i) (lanes B, D, and F). For comparison, an RNA preparation containing genome-length, bicistronic and monocistronic RNAs purified from measles virus-infected COS cells was similarly analyzed in parallel (lanes A and B). This preparation also contained heterogeneous sub-genome-length RNA characteristic of measles virus (20). Open arrows indicate the positions of 28S and 18S ribosomal RNA markers.

antiserum against *M* protein of measles virus (Fig. 4, lane B). This protein has been shown to be the genuine *M* gene product (Wong et al., submitted). An additional minor protein of 42,000 M_r was derived from aberrant translation with an upstream in-frame ATG codon located in the vector sequence (Wong et al., submitted). The bicistronic clone pcD-PM5d produced high levels of a 70,000-M_r protein immunoprecipitated by monoclonal antiserum against measles virus *P* protein (Fig. 4, lane D). No *M*-specific protein was detected in the same cells transfected with this bicistronic clone (Fig. 4, lane C). To rule out a possible defect in the protein-coding function of the *M* cistron in this clone, the *M* cistron was excised from pcD-PM5d by digestion with *Sal*I and *Bam*HI and inserted into an in vitro expression vector which contained promoters derived from bacteriophages SP6 and T7 (pGEM-3) (Promega Biotec). The resulting clone, pSP6-PM5d-Δa, contained the complete *M* gene preceded by 31 nucleotides derived from the 3′ terminus of the *P* gene joined via the intergenic region, such that the *M* cistron in this clone was not preceded by any upstream ATG codon (See Fig. 2 for details of construction). For comparison, the complete *M* gene derived from monocistronic clone pcD-M2i was inserted into the same vector downstream of the T7 promoter to generate pT7-M2i (Fig. 2). These constructed clones were transcribed by SP6 or T7 polymerase into RNA for in vitro translation (19).

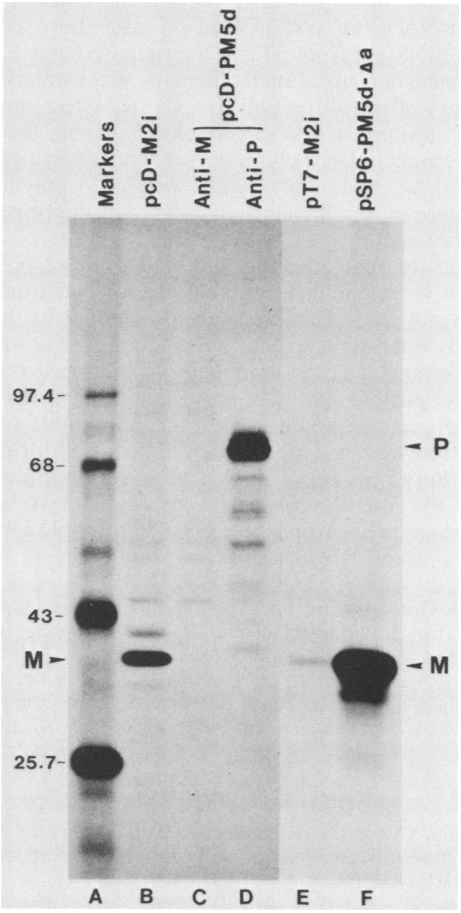

FIG. 4. Expression of protein products from monocistronic and bicistronic clones in vivo and in vitro. Cultures of 1.5×10^6 COS cells were transfected with 30 μg of cloned DNA as previously described (35). At 30 h posttransfection, cells were starved for 30 min in methionine-free Eagle minimal essential medium and labeled with 30 μCi of [^{35}S]methionine per culture. At 48 h posttransfection, cell lysates were prepared and immunoprecipitated with 5 μl of monoclonal antiserum against the *M* or *P* protein of measles virus (8, 23). Lane B represents proteins immunoprecipitated from pcD-M2i-transfected cells by antiserum against *M* protein. Lanes C and D represent proteins immunoprecipitated from pcD-PM5d-transfected cells by antisera against the *M* and *P* proteins, respectively. For in vitro expression, the complete *M* gene from a monocistronic clone or the *M* cistron from a bicistronic clone was inserted into expression vector pGEM-3 to generate clones pT7-M2i and pSP6-PM5d-Δa, respectively (see Fig. 2 for details). These constructs were linearized at a restriction enzyme site in the cloning region downstream from the inserted *M* gene and transcribed in vitro by T7 or SP6 polymerase (21). Equal amounts (1 μg) of in-vitro-generated RNA were translated in rabbit reticulocyte lysates (19). Lane E represents [^{35}S]methionine-labeled proteins translated from RNA derived from pT7-M2i. Lane F represents translation products from pSP6-PM5d-Δa. The molecular mass markers in lane A are in kilodaltons.

RNA transcribed from the monocistronic clone pcD-M2i was translated in vitro into a 38,000-M_r protein which comigrated with the *M* protein produced in vivo in transfected cells (lanes E). This in-vitro-generated *M* protein was also recognized by monoclonal antiserum against the *M* protein of measles virus (Wong et al., submitted). A 42,000-M_r protein derived from the upstream in-frame ATG codon as described above was also produced by in vitro translation. RNA generated from clone pSP6-PM5d-Δa was translated into the 38,000-M_r protein (Fig. 4, lane F). Interestingly, RNA from pSP6-PM5d-Δa was more efficiently translated than that from pT7-M2i (compare lanes E and F). The difference in translational efficiency was probably due to an inhibitory effect of the poly(G · C) sequences introduced into the 5′ termini of the cDNA clones during the cloning procedure, since these polymeric sequences were deleted in pSP6-PM5d-Δa, and clones with shorter 5′ polymeric sequences were more efficiently expressed at the protein level (unpublished data).

These results indicated that the bicistronic cDNA clones of measles virus could be expressed into bicistronic RNA which did not undergo detectable processing in the transfected cells but was translationally functional in vivo. The tandemly arranged *P* and *M* cistrons in the bicistronic RNA could direct protein synthesis only from the first (*P*), not the second (*M*), cistron, even though the AUG start codon for the *M* cistron was in a more favorable context for initiation of translation (5, 6, 18). This last observation underlies the need for the highly conserved mechanism to generate monocistronic mRNA for expression of multiple viral genes in negative-strand RNA viruses.

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