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Nucleotide sequence of 5′ terminus of alfalfa mosaic virus RNA 4 leading into coat protein cistron

(RNA sequence/mRNA/ribosome binding/plant viruses sequence/two-dimensional gel electrophoresis)

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ABSTRACT **The sequence of the 5′-terminal 74 nucleotides of alfalfa mosaic virus RNA 4, the mRNA for the viral coat protein, has been deduced by using various new techniques for labeling the RNA at the 5′ end with ^{32}P and for sequencing the 5′-^{32}P-labeled RNA. The sequence is NpppGUUUUUAUUU-UUAAUUUUCUUUCAAAUACUUCCAUC*AUG*AGUUCUUC-ACAAAAGAAAGCUGGUGGGAAAGCUGG. The AUG initiator codon is located 36 nucleotides in from the 5′ end; the nucleotide sequence beyond corresponds to the amino acid sequence of the coat protein. This 5′ noncoding region is rich in U (58% U); except for the 5′-terminal G, the next G in is part of the initiator AUG codon.**

"Cap" structures of the type $m^7G(5')ppp(5')N$ are found at the 5′ terminus of many eukaryotic mRNAs and have been implicated in ribosome binding (1). The detection of other possible common signals has been hampered by the absence, until recently, of comparative sequence data on the 5′-noncoding regions of these molecules. For some time, only the sequence of the nine nucleotides between the "cap" and the AUG initiator codon in brome mosaic virus (BMV) RNA 4 was known (2). Recently, nucleotide sequences preceding the initiator codon have been published for rabbit α- and β-globin mRNA (3–5), the simian virus 40 VP1 gene (6), oncornaviral RNA (7–9), reovirus mRNA (10), vesicular stomatitis virus mRNA (11), and tobacco mosaic virus RNA (12). Comparison of these data reveals no readily identifiable recognition signals and shows that the distance between the "cap" and the first AUG codon is variable. The synthesis of these RNA species involves various different enzymes and templates, which may be either DNA or RNA, and the sequence at their 5′ end may be determined by constraints other than ribosome recognition alone.

We have analyzed the sequence of the 5′-noncoding region in RNA 4 of alfalfa mosaic virus (AlMV), which is functionally equivalent to BMV-RNA 4. AlMV, like BMV, has a genome consisting of three RNA molecules, the coat protein cistron being located in RNA 3, the smallest genome fragment (13). However, the coat protein cistron in this RNA is not open to translation. In addition to the genomic RNAs, viral preparations contain a subgenomic RNA molecule, RNA 4, that is efficiently translated into coat protein in various cell-free systems (14–16). The sequence of the 220 amino acids in AlMV coat protein is completely known (17). From molecular weight data obtained by hydrodynamic measurements, AlMV RNA 4 has been calculated to contain about 800 nucleotides (18). Consequently, extracistronic regions at the 5′ and 3′ ends of this messenger may encompass a total of about 140 nucleotides. A major difference between AlMV and BMV is that, whereas the three RNAs making up the BMV genome are infectious as such, the AlMV genome has to be supplemented with a small amount of coat protein, or the RNA 4, which presumably acts as the coat protein messenger (19). Moreover, the overall sequence homology between the genomes of the two viruses is negligible (20).

In the course of this study, we made use of a number of novel rapid sequencing techniques, including a gel sequencing method similar in principle to the DNA sequencing method of Maxam and Gilbert (21) and a "wandering spot" analysis that uses two-dimensional polyacrylamide gels (details to be published elsewhere). All the methods involve first the *in vitro* enzymatic labeling of the 5′ terminus of AlMV RNA 4, after removal of the "cap" by periodate oxidation, β-elimination, and phosphomonoesterase digestion (3). The results enable us to deduce the sequence of the 5′-terminal 74 nucleotides of AlMV RNA 4. The AUG initiator codon of the coat protein cistron was found to be preceded by 36 nucleotides. The results are discussed in relation to the available sequence data on the 5′-noncoding regions in BMV RNA 4 and other mRNAs. Implications of the sequence that we have derived with respect to some interesting features of *in vitro* translation of AlMV RNA 4 in an *Escherichia coli* system are the subject of an accompanying paper (16).

MATERIALS AND METHODS

These were mostly as described (3, 22, 23). Details of the preparation of pyrimidine specific RNase from *Bacillus cereus* will be described elsewhere. U_2 RNase was obtained from Sankyo via Calbiochem. [γ-^{32}P]ATP was prepared by using a modification of the procedure of Glynn and Chappell (24) and was used at a specific activity of 1–3 Ci/μmol.

Purification of AlMV RNA 4 (strain 425) was as described (25).

5′-End Group Labeling of AlMV RNA 4 with ^{32}P. The basic procedure was essentially the same as that used previously for rabbit globin mRNAs (3) and consisted of three steps: (*i*) removal of the 5′-terminal "cap" structure (26) in the mRNA by treatment with sodium periodate followed by β-elimination; (*ii*) conversion of the 5′-terminal triphosphate end thus generated to 5′-hydroxyl by incubation with phosphomonoesterase; and (*iii*) labeling of the 5′-hydroxyl end with ^{32}P by using [γ-^{32}P]ATP and T_4 polynucleotide kinase (27).

The AlMV [5′-^{32}P]RNA 4 was purified by electrophoresis on a 20 × 20 cm polyacrylamide (4.5%) slab gel in 7 M urea (3) for 16–20 hr at 300 V and room temperature. It was located by autoradiography and was recovered by electrophoretic elution into dialysis bags (3, 23). Recovery of the labeled mRNA from the gel bands was at least 90%.

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Abbreviations: BMV, brome mosaic virus; AlMV, alfalfa mosaic virus; pm^7G, 7-methylguanosine 5′-phosphate.

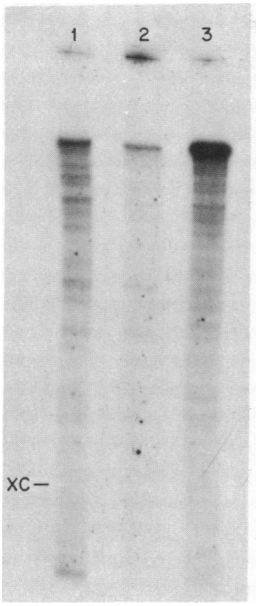

FIG. 1. Autoradiogram of AlMV [5′-^{32}P]RNA 4 electrophoresed on a 4.5% polyacrylamide slab gel in 7 M urea. AlMV RNA 4 (0.05 A_{260} unit) was incubated with T_4 polynucleotide kinase and [γ-^{32}P]ATP after various treatments. Track 1, untreated; track 2, treated with *E. coli* alkaline phosphatase; track 3, periodate oxidation followed by β-elimination and then treatment with *E. coli* alkaline phosphatase. The major radioactive bands were cut out and assayed: track 1, 13,967 Cerenkov cpm; track 2, 7931 cpm; and track 3, 79,582 cpm. XC, Location of xylene cyanole dye marker.

Partial Digestion of AlMV [5′-^{32}P]RNA 4 with Nuclease P_1. Partial digestions were carried out either at room temperature or, in the hope of obtaining a more uniform cleavage pattern within the pyrimidine clusters of the AlMV RNA, at 50°. A typical incubation mixture (8 μl) contained AlMV [5′-^{32}P]RNA 4 (300,000–750,000 cpm), 0.2 A_{260} unit of carrier tRNA in 25 mM NH_4OAc, pH 5.3, and 1.5 ng of nuclease P_1 for digestion at room temperature or 0.5 ng for digestion at 50°. Aliquots were removed at various times; after inactivation of the enzyme, the appropriate aliquots (3, 22, 23) were pooled and used for analysis by two-dimensional electrophoresis/homochromatography (28, 29). Homochromatography was either on 20-cm-long DEAE thin-layer plates with 50 mM "homomix" (22) to separate the shorter oligonucleotides or on 40-cm-long plates with 10 mM "homomix" to separate the longer oligonucleotides.

RESULTS

Labeling of AlMV-RNA 4 at the 5′ End with ^{32}P. Fig. 1 shows the results of an experiment, using a recently described procedure (3) for the removal of the m^7Gppp- "cap" structure (26) present at the 5′ end of most eukaryotic mRNAs and subsequent labeling of the mRNA with ^{32}P in which various samples of AlMV RNA 4 were incubated with T_4 polynucleotide kinase and [γ-^{32}P]ATP. The radioactive bands corresponding to the intact AlMV RNA 4 (located by staining of a separate marker track) were cut out and assayed. Although the treatment with phosphatase alone resulted in no increase in incorporation of ^{32}P into AlMV RNA 4, conditions normally used for removal of the "cap" structure resulted in approximately a 6-fold increase. The overall extent of labeling of the 5′ end of AlMV RNA (track 3) was about 60%. These results suggest that AlMV RNA 4 also has a "cap" structure at the 5′ end and support a similar conclusion reached by Roman *et al.* (30) based on the inhibition of formation of 80S initiation complex between AlMV RNA 4 and wheat germ ribosomes by the "cap" analog, 7-methylguanosine 5′-phosphate (pm^7G). Although we cannot conclude from our work alone that the nucleoside attached to the remainder of the AlMV RNA 4 through a -ppp- linkage is m^7G, this is most likely because Pinck (31) has shown that the three genomic AlMV RNAs (AlMV RNAs 1, 2, and 3) all have m^7GpppG at their 5′ termini.

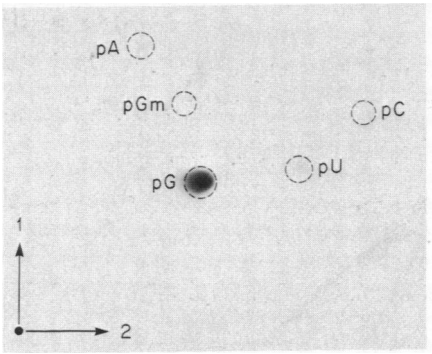

FIG. 2. Autoradiogram of a complete nuclease P_1 digestion of AlMV [5′-^{32}P]RNA 4 analyzed by two-dimensional chromatography. Dashed circles indicate the location of the ultraviolet-absorbing markers applied. Solvent systems used for the thin-layer chromatography were as in ref. 3.

Identification of G at the 5′ End of AlMV [5′-^{32}P]RNA 4. Fig. 2 shows the results of an experiment in which the AlMV [5′-^{32}P]RNA 4 obtained above was completely digested to nucleoside 5′-phosphates with nuclease P_1 (3), mixed with the appropriate nonradioactive markers (0.05 A_{260} unit each), and subjected to two-dimensional thin-layer chromatography. The predominant radioactive spot coincided with a marker of pG. Quantitative analysis (32) of radioactivity present in the various spots indicated that 87.6% was present in pG, the remainder being in pA (3.1%), pU (7.9%), and pC (1.3%); no radioactivity was found in pGm.

Sequence Analysis of AlMV [5′-^{32}P]RNA 4 by Partial Digestion with Nuclease P_1. Partial digestion of 5′-^{32}P-labeled RNA with nuclease P_1, a relatively random endonuclease that cleaves RNA or DNA phosphodiester bonds to produce 5′-phosphate and 3′-hydroxyl ends, yields a series of radioactive products ranging in size from the 5′-labeled mononucleotide to successively larger oligonucleotides. Most of these 5′-^{32}P-labeled oligonucleotides (up to 20–30 long) can be separated by two-dimensional electrophoresis/homochromatography, and the sequence at the 5′ terminus can be read directly by following the angular mobility shifts between the successively longer oligonucleotides, all of which contain the same 5′-terminal end (refs. 22 and 23; unpublished data).

Figs. 3 and 4 show the patterns obtained upon two-dimensional electrophoresis/homochromatography of such partial digests of AlMV [5′-^{32}P]RNA 4 along with the schematic derivation of the sequence of the 5′-terminal 26 nucleotides. The 5′-terminal sequence derived from Fig. 3 is GUUUUUAUUUU, and this can be extended to GUUUUUAUUUUUAAUUUUCUUUCAAA by using the results shown in Fig. 4. The radioactive spots marked X, which are on the right of the main pattern of spots, arise from a specific cleavage of pyrimidine-A bonds occurring to a small extent due to trace contamination of the 5′-^{32}P-labeled AlMV RNA 4, possibly with a "pancreatic RNase-like" enzyme during its work-up (see also Fig. 5).

Further Sequence Analysis of AlMV [5′-^{32}P]RNA 4 by

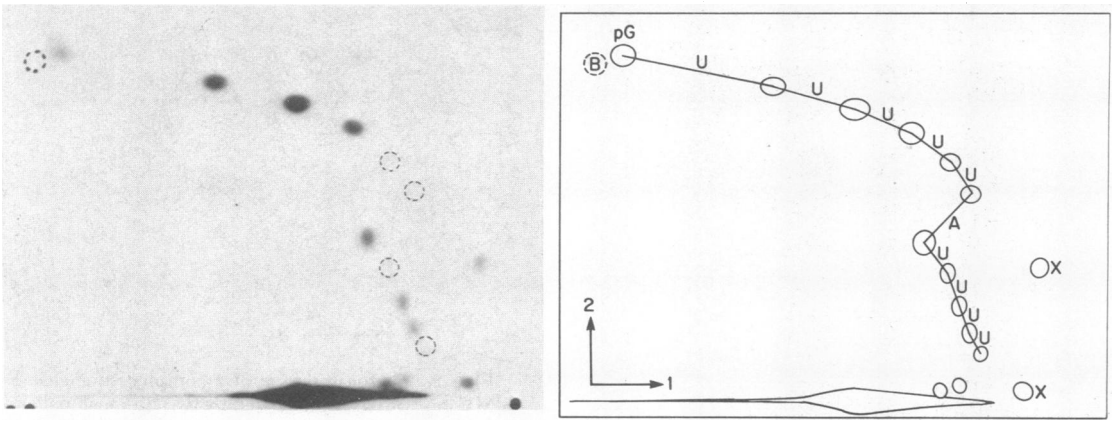

FIG. 3. Autoradiogram of a partial nuclease P_1 digestion (at room temperature) of AlMV [5′-^{32}P]RNA 4. Approximately 150,000 cpm of the partially digested AlMV RNA 4 was used. First dimension was electrophoresis on cellulose acetate at 5 kV until the blue dye (xylene cyanole FF) had migrated 8 cm. Second dimension was homochromatography on a 20 × 20 cm DEAE-cellulose plate with 50 mM "homomix." Circled B indicates the position of the blue dye.

Polyacrylamide Gel Electrophoresis of Partial Digests. Further sequence information relative to the 5′ end of AlMV RNA 4 was obtained by partial chemical and enzymatic digestion of the AlMV [5′-^{32}P]RNA 4 with enzymes of different base specificity followed by separation of the partial digestion products by polyacrylamide gel electrophoresis. This procedure, described recently by Donis-Keller *et al.* (33), included use of T_1 RNase for obtaining partial cleavage at G residues, U_2 RNase for cleavage at A residues, and alkali for cleavage at all four residues. The 5′-^{32}P-labeled partial fragments were separated according to their size by polyacrylamide gel electrophoresis under denaturing conditions and the relative locations of G, A, and, by difference, pyrimidine residues from the 5′ end were determined. For additional evidence in locating the pyrimidine residues, we used an enzyme from *B. cereus* which, unlike pancreatic RNase, will cleave within almost every pyrimidine residue under the partial digestion conditions used. In addition, we used two-dimensional polyacrylamide gel electrophoretic analysis of partial alkali digests to distinguish among the pyrimidine residues.

Fig. 5 shows the results obtained when AlMV [5′-^{32}P]RNA 4 was partially digested with T_1 RNase, alkali, U_2 RNase, and the pyrimidine specific RNase (Y) from *B. cereus* and the digests were electrophoresed on polyacrylamide gel. In this particular instance, the first 19 nucleotides from the 5′ end were run off the gel, and the sequence that can be read off this electropherogram includes nucleotides 20–74 from the 5′ end. The background radioactive bands present in the control—AlMV [5′-^{32}P]RNA 4 (−enzyme)—and hence in most other tracks, occur only in specific positions and in all cases where a Y-A sequence is found. This is possibly due to partial cleavage at Y-A bonds by trace contamination of the labeled AlMV RNA 4 with either pancreatic RNase or an enzyme similar to pancreatic RNase with a marked preference for such linkages.

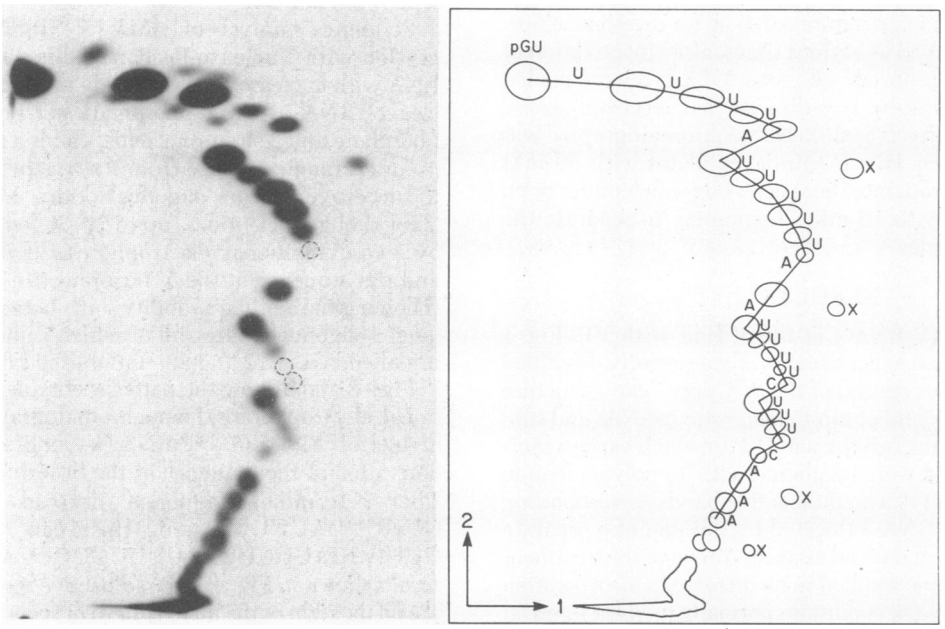

FIG. 4. Autoradiogram of a partial nuclease P_1 digestion (at 50°) of AlMV [5′-^{32}P]RNA 4. First dimension was electrophoresis on cellulose acetate until the blue dye had migrated 16 cm. Second dimension was homochromatography on a 20 × 40 cm DEAE-cellulose plate and was carried out until a freshly added marker blue dye had moved 35 cm. During the transfer of oligonucleotides from the cellulose acetate to the DEAE-cellulose plate, the region of cellulose acetate containing the blue dye originally applied was not included, and consequently the 5′-terminal ^{32}pG which migrates close to the blue dye (see Fig. 3) is not seen in this autoradiogram.

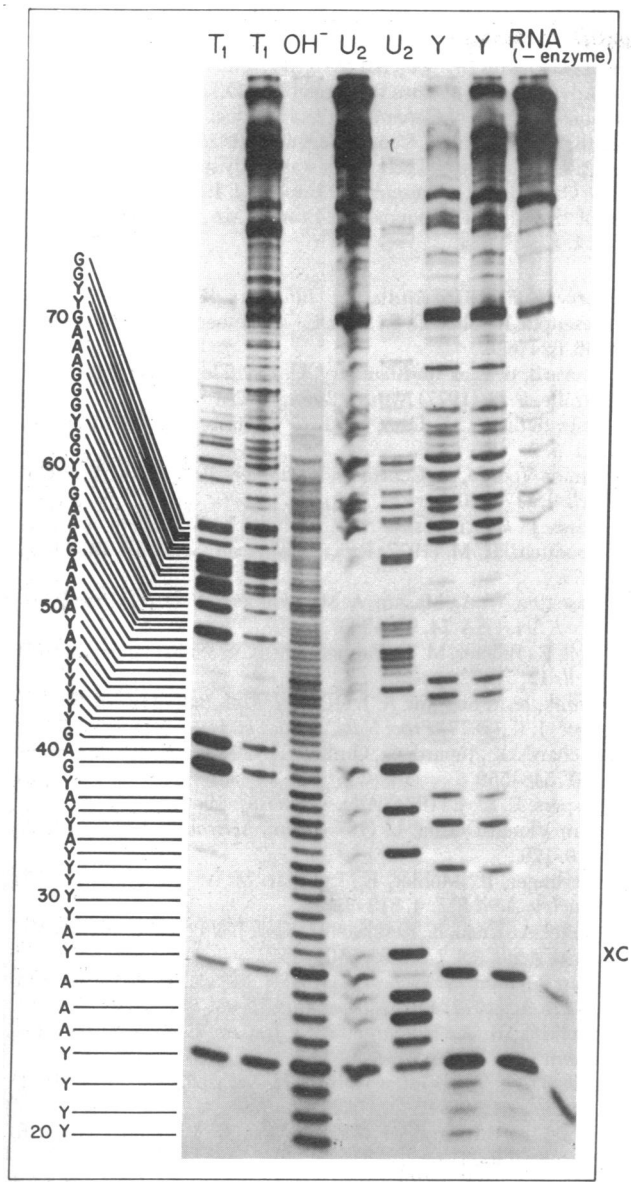

FIG. 5. Autoradiogram of partial digests of AlMV [5′-^{32}P]RNA 4. Electrophoresis was on 20% polyacrylamide gel slab (0.15 × 20 × 40 cm) in the presence of 7 M urea. Running buffer was 50 mM Tris borate, pH 8.3/1 mM EDTA (33). Electrophoresis was carried out at 1000 V at approximately 12 mA for 20 hr. Tracks: T_1, partial digestion with T_1 RNase at two different enzyme/substrate ratios; OH, partial digestion with alkali; U_2, partial digestion with U_2 RNase at two different enzyme/substrate ratios; Y, partial digestion with the pyrimidine specific enzyme from *B. cereus* at two different enzyme/substrate ratios; RNA (−enzyme), control. Each of the tracks contained approximately 120,000 cpm. XC, Location of xylene cyanole dye.

Combining the results of Fig. 5 with the 5′-terminal 26 nucleotide sequence derived above (Fig. 4), the 5′-terminal sequence of AlMV RNA 4 is deduced as

GUUUUUAUUUUUAAUUUUCUU
UCAAAYAYYYYYAYYAYGAGYYYYYYYAYAAAAGAAA
GYYGGYGGGAAAGYYGG.

After the 5′-terminal G, the next G residue is number 39, and this is part of the initiator codon for the coat protein (see below). Because the amino acid sequence of the AlMV coat protein is known (17), most of the pyrimidine residues in the above sequence could be tentatively assigned. An unambiguous identification of the individual pyrimidine residues was made possible by two-dimensional electrophoretic analysis of partial alkali digests of AlMV [5′-^{32}P]RNA 4. In such a system, mobility shifts between two successive oligonucleotide homologues which differ by a C residue can be distinguished from those which differ by a U residue. Fig. 6 shows an example of this method as applied to AlMV [5′-^{32}P]RNA 4.

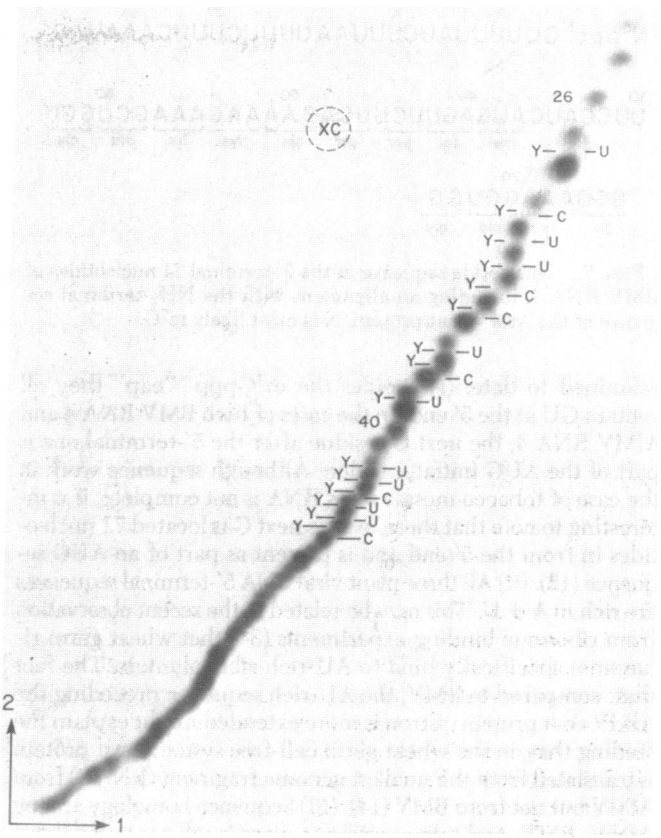

FIG. 6. Autoradiogram of a partial alkali digest of AlMV [5′-^{32}P]RNA 4. First dimension was electrophoresis on 10% polyacrylamide gel at pH 3.5. Second dimension was electrophoresis on 20% polyacrylamide gel at pH 8.3. XC, Xylene cyanole dye. Numbers 26 and 40 indicate length of the oligonucleotides. The letter Y between two radioactive spots indicates that the two oligonucleotides differ by a pyrimidine residue (using the data shown in Fig. 5); C and U indicate identification of these pyrimidines as either C or U based on the mobility shifts in this system (details will be published elsewhere).

DISCUSSION

In the sequence of the 5′-terminal 74 nucleotides of AlMV RNA 4 that we have derived, the sequence following the first AUG corresponds to the NH_2-terminal amino acid sequence of AlMV coat protein (Fig. 7). Therefore, it is unlikely that the coat protein of AlMV strain 425 is synthesized as a slightly longer precursor upon initiation with methionine, as has been suggested recently for AlMV strain S (15).

Although AlMV and BMV have a similar genomic structure, the distance of the AUG initiation codon from the 5′-terminal "cap" in the respective subgenomic mRNAs for coat protein is quite different (AlMV, 36 nucleotides; BMV, 9 nucleotides). Thus, the close proximity of AUG to the 5′-terminal "cap" found in the case of BMV RNA 4 is not a feature common to all plant viral coat protein mRNAs.

In spite of the above differences, there are certain interesting similarities in the 5′-terminal sequence of the plant viral RNAs

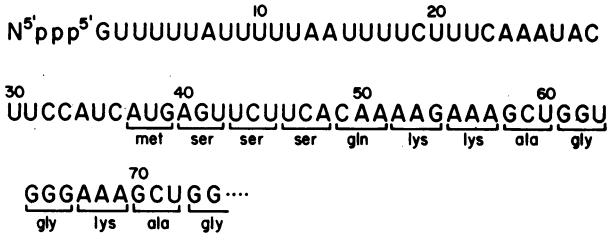

FIG. 7. Nucleotide sequence of the 5′-terminal 74 nucleotides of AlMV RNA 4, including an alignment with the NH_2-terminal sequence of the AlMV coat protein. N is most likely m^7G.

examined to date. (*i*) Besides the m^7Gppp- "cap" they all contain GU at the 5′ end. In the cases of both BMV RNA 4 and AlMV RNA 4, the next G residue after the 5′-terminal one is part of the AUG initiator codon. Although sequence work in the case of tobacco mosaic virus RNA is not complete, it is interesting to note that there too the next G is located 71 nucleotides in from the 5′ end and is present as part of an AUG sequence (12). (*ii*) All three plant viral RNA 5′-terminal sequences are rich in A + U. This may be related to the recent observation from ribosome binding experiments (34) that wheat germ ribosomes specifically bind to AU-rich ribopolymers. The fact that, compared to BMV, the AU-rich sequence preceding the AlMV coat protein cistron is more extended might explain the finding that, in the wheat germ cell-free system, coat protein is translated from the smallest genome fragment (RNA 3) from AlMV but not from BMV (14). (*iii*) Sequence homology among AlMV, BMV, and tobacco mosaic virus is substantially above that expected from random sequences. The sequences UAUU and AAUA, comprising together the BMV noncoding region, are both found in AlMV RNA (nucleotides 6–9 and 25–28, respectively). The longest stretch of homologous nucleotides between the noncoding regions of AlMV and BMV is the sequence UUAAU (AlMV, nucleotides 11–15; BMV, nucleotides 4–8). AlMV RNA 4 and tobacco mosaic virus RNA share a longer stretch of sequence homology, nucleotides 2–9 in the latter (UAUUUUUA) being identical to nucleotides 6–13 in the former. Although these homologies may be fortuitous, similarities *i* and *ii* clearly distinguish the three plant viral mRNAs from the animal viral or cellular mRNA analyzed in this respect.

As most other mRNAs whose 5′-terminal sequences have been recently established, AlMV RNA 4 also contains a sequence (in this case UAAU, nucleotides 12–15) that is complementary to the 3′ end of 18S rRNA. In the linear sequence of eukaryotic mRNAs, sequences complementary to the 18S rRNA 3′ end are located in different positions, with respect to both the 5′-terminal "cap" and the AUG initiator codon. The significance of the presence of such a sequence in the messenger function of these mRNAs remains unknown.

AlMV RNA 3, one of the three genomic RNAs, contains two cistrons, one for a protein of molecular weight 35,000 presumably located at the 5′ end, and the coat protein cistron. Under certain conditions, a read-through translation product can be obtained *in vitro* containing the total informational content of RNA 3 (14). This suggests that the intercistronic region is a translatable reading frame in phase with both cistrons. The sequence that we have derived for the noncoding region of AlMV RNA 4 represents a translatable reading frame in phase with the coat protein cistron and is in agreement with this expectation. In addition, it explains some aspects of the *in vitro* translation of AlMV RNA 4 in an *E. coli* cell-free system being reported in an accompanying paper (16).

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