DETECTION AND SEQUENCE OF AN INTERNAL A-RICH T_1 OLIGONUCLEOTIDE SERIES IN BROME MOSAIC VIRAL RNA3

Edison R. FOWLKS and Yuan Fon LEE
Department of Life Sciences, Bishop College, Dallas, TX 75241, USA

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1. Introduction

Subsequent to the discovery of 4 RNA molecules [1] in brome mosaic virus (BMV) it was shown that the genome of this virus was actually distributed among several nucleoprotein particles [2]. In vitro studies indicate that BMV RNAs 1 and 2 each direct the synthesis of a large protein [3]. BMV RNA3, although it contains the coat protein cistron, is translated into a single protein unrelated to the coat protein and BMV RNA4 directs the synthesis of the coat protein [4]. These findings were the first demonstrations that the apparently homogeneous BMV contained a divided multipartite genome. However, during the last decade, numerous other viruses have been shown to have divided genomes [5–7].

The divided multipartite viral genomes lend themselves quite easily to studies on structural and functional relationships of viruses of higher organisms. Thus, BMV is an ideal model with which to probe fundamental aspects of RNA replication, processing and translation in viruses. Although RNA3 is dicistronic, it nevertheless codes for only one protein; the coat protein cistron is untranslatable [4]. Moreover, RNA4 is contained within RNA3 [8,9]. For these reasons, it seemed to us that there may possibly exist a unique sequence in RNA3 that regulates not only the expression of the coat protein cistron but also the processing of the subgenomic coat protein messenger RNA4. Reinterpretation of the findings in [10–12] on the localization of polyadenylated messenger RNA in the cell and its relationship to the onset of certain important biological events led us to predict that this 'unique' sequence may be internal oligo(A).

To test this hypothesis, we fingerprinted each of the BMV RNAs. A slanted series of spots was observed on the bottom of the RNA3 fingerprint. This series was absent in fingerprints from RNAs 1, 2 and 4. Preliminary analysis of certain spots in the series showed that the slanted series was internal poly(A) with Gp at the 3′-end [13]. However, direct sequence analysis clearly reveals that also Up, but not Cp is present. Here we describe the detection and sequence of an internal A-rich T_1 oligonucleotide series in RNA3, and propose a model to explain its possible regulatory role in translation and the production of the subgenomic coat protein cistron, RNA4.

2. Materials and methods

2.1. *^{32}P-Labeling of virus*

Type strain brome mosaic virus (BMV) was used throughout this work. Ten-day-old Moore barley plants were inoculated with a purified virus suspension in 50 mM sodium acetate, 10 mM magnesium acetate (pH 5.0) (virus buffer). Three days after inoculation, 30 plants were cut at the base of the stem and immersed in 25 ml distilled water containing 50 mCi ^{32}P (New England Nuclear-NEX 054) adjusted to pH 7 with NaOH. After all the solution had been taken up, additional distilled water was added and plants were allowed to remain for 72 h at 24°C in the growth chamber; then, the plants were frozen at −70°C.

The frozen plants were homogenized in a Waring blender with 300 ml virus buffer containing 0.3 ml thioglycolic acid. The homogenate was centrifuged for 10 min in a Sorvall GSA rotor at 10 000 rev./min. Polyethylene glycol (10%, w/v) was added to the supernatant, stirred for 1 h at 4°C, then centrifuged 10 min at 10 000 rev./min. The pellet was suspended in 20 ml virus buffer and centrifuged through a 25%

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sucrose solution for 3 h at 35 000 rev./min in a Beckman Type 42.1 rotor. Further purification was on a 5–25% step sucrose gradient for 2 h at 26 000 rev./min in a Beckman SW27 rotor. The purified virus band was removed, pelleted, and suspended in virus buffer.

2.2. *Extraction of ^{32}P-labeled RNA*

The virus was disrupted by adding 0.5 ml 10% SDS and 5 ml water-saturated phenol to 4.5 ml virus suspension (5–10 mg/ml). After shaking for 10 min, 5 ml chloroform were added and shaken again for 5 min. The phases were separated by centrifugation (Beckman J21B, JA20 type rotor) for 5 min at 10 000 rev./min. The phenol and chloroform treatment steps were repeated. The aqeous phase was removed and treated with 10 ml chloroform and then centrifuged. The chloroform treatment step was repeated until the interphase was clear. Finally, the aqeous phase was treated with ether and precipitated with ethanol. After washing with cold absolute ethanol, the RNA pellet was dissolved in either water or TNE buffer (10 mM Tris, 100 mM NaCl and 1 mM EDTA, pH 7.5). The yield from 30 plants was usually ~5 mg with spec. act. 0.04×10^6 cpm/mg.

2.3. *Oligonucleotide fingerprinting of brome mosaic viral RNA*

The BMV RNA components were separated on a slab gel (20 cm × 40 cm × 1.6 mm) containing 3% acrylamide, 0.2% bisacrylamide, 0.1% SDS, 1 mM ETDA and 7 M urea in 0.04 M Tris–borate (pH 8.0). The sample was boiled for 1 min in the above acrylamide gel buffer, chilled in ice bath, and electrophoresed at 200 V until the dye marker (xylene cyanide) migrated 30 cm from origin. Each of the 4 BMV RNA components was fingerprinted. The materials and procedures for two-dimensional gel electrophoresis and autoradiography have been described [14–16]. BMV [^{32}P]RNA was digested with RNase T_1 (Calbiochem-Behring) in the ratio of 10 μg RNA to 1 unit of enzyme and incubated in 30 μl 2 mM EDTA, 0.05 M Tris–HCl (pH 7.5) for 40 min at 37°C.

2.4. *Elution of oligonucleotides from acrylamide gel*

The elution procedure for short oligonucleotides has been described [14]. For larger oligonucleotides (>25 *N*) 4 ml 0.5 M LiCl, 0.05 Tris–borate (pH 7.5), 1 mM EDTA, and 0.1% SDS were added to the pulverized gel power [14], shaken at room temperature for 3 h or overnight, and centrifuged (Sorvall, rotor SS34) at 2000 rev./min for 5 min. The pellet was re-extracted twice, supernatant adjusted to 2 M with LiCl, 3 vol. cold absolute ethanol added and placed in −70°C freezer for at least 30 min. The precipitate was centrifuged (Sorvall) at 11 000 rev./min for 30 min; the pellet was dissolved in 2 M LiCl, 0.05 M Tris–HCl (pH 7.5) and 1 mM EDTA, and reprecipitated at −70°C for 30 min in 3 vol. cold absolute ethanol. The precipitate was centrifuged (Brinkman microcentrifuge 5412) for 30 min at 4°C.

2.5. *Analysis of oligonucleotides*

T_1 oligonucleotides eluted from fingerprints were incubated at 37°C for 30 min with 10 μl RNase A (0.1 mg/ml), 2 mM EDTA, and 0.05 M Tris–HCl (pH 7.5). The digests were separated by electrophoresis (10 V/cm for 5 h) on DEAE-Paper (Whatman DE 81) at pH 3.5 in 5% acetic acid and 0.5% pyridine [17]. The separated oligonucleotides were located by autoradiography and identified with appropriate markers by their migration distance on DEAE paper. They were then eluted from DEAE paper with 1 M triethylamide carbonate (pH 8.9) and lyophilized. Complete hydrolysis was carried out at 37°C for 1 h in a solution containing RNase T_2 (0.2 unit/μl) in 10 μl 0.05 M ammonium acetate (pH 4.5) and 1 mM EDTA. The products were separated by electrophoresis (10 V/cm for 5 h) on Whatman no. 1 paper (3MM) at pH 3.5 in pyridine acetate.

2.6. *5′-In vitro labeling and column chromatography of [5′-^{32}P]pUp*

RNase T_1 oligonucleotides eluted from two-dimensional fingerprints were 5′-^{32}P-labeled [16,18] by incubating at 50°C for 30 min in 69 μl 15 mM Tris–HCl (pH 9.0), 1 mM spermidine and 0.1 mM EDTA. The sample was chilled at 4°C; then 30 μl 0.2 M Tris–HCl (pH 7.5), 0.04 M $MgCl_2$, 0.02 M DTT, and 1 μl kinase (5 units/μl) were added, and transferred to an Eppendorf tube containing 0.1 mCi [γ-^{32}P]ATP (NEN no. NEG-002H) pre-lyophilized. After incubating at 37°C for 10 min 100 μl H_2O were added, twice treated with phenol–chloroform (1:1) followed by ether. The sample was precipitated with 2 M LiCl and 3 vol. absolute ethanol −70°C for at least 30 min. The precipitate was centrifuged (Brinkman microcentrifuge 5412) at 4°C for 30 min, dissolved in 50 μl 0.05 ammonium acetate (pH 4.5), 1 mM EDTA, and RNase T_2 (0.05 unit/μl) and incubated at 37°C for

1 h. The digest was diluted with 5 ml triethylamide acetate (TEA) (pH 5.0), 100 μg UDP and CDP markers added, and applied to a column (1.5 × 20 cm) containing 10 ml DEAE cellulose (Whatman DE52). The column was developed with a linear gradient of 0.05–0.4 M TEA (pH 5.0) (total vol 200 ml). Fractions were monitored for ^{32}P-counts and absorbance.

2.7. *Purifications of 5′-labeled sample for partial U2 and alkaline digestion*

Samples eluted from fingerprints were labeled with polynucleotide kinase and [γ-^{32}P]ATP (see section 2.6). After 10 min incubation at 37°C, the samples were precipitated, centrifuged and washed with 0°C absolute ethanol. The pellet was further purified on a 10% acrylamide slab gel (12 cm × 45 cm × 1.6 mm) with 0.04 M Tris–borate (pH 8.1), 7 M urea and 1 mM EDTA. Samples were electrophoresed (300 V constant voltage) until the bromophenol blue dye migrated 15 cm from the origin. The 5′-end labeled oligonucleotide was eluted from the gel and concentrated as above.

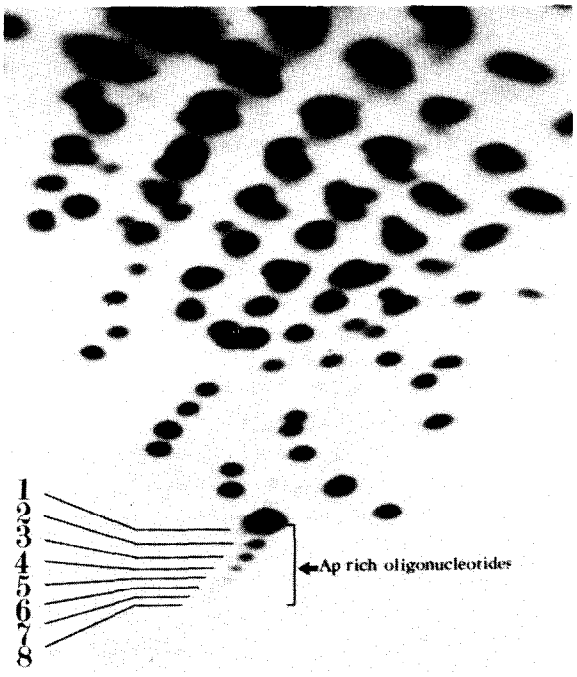

Fig.1. Two-dimensional gel electrophoresis fingerprint of BMV[^{32}P]RNA. Lower portion shows series of 8 Ap-rich oligonucleotide spots.

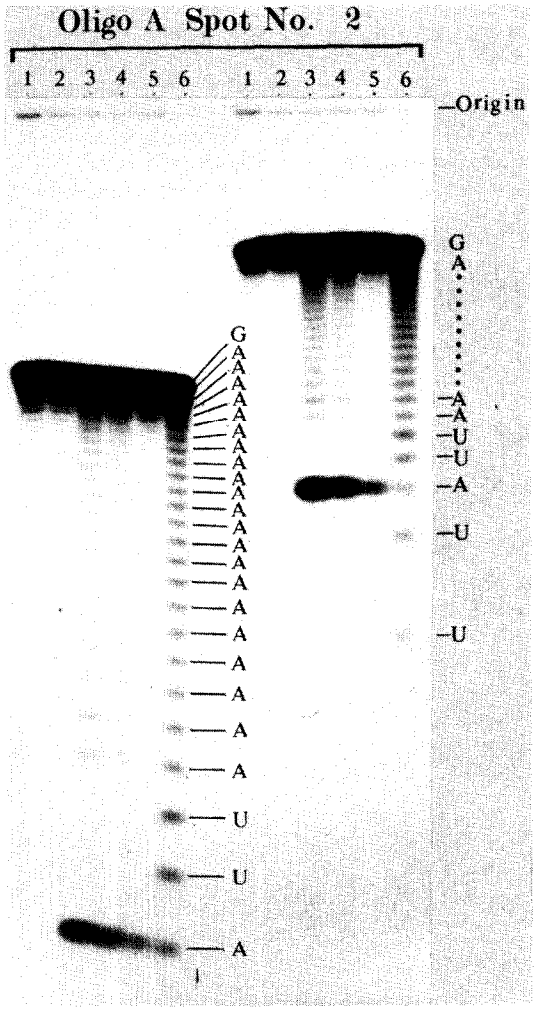

Fig.2. Rapid sequencing gel electrophoresis of Ap-rich nucleotide no. 2. Samples: (1) control, without incubation; (2) control, incubated without enzymes; (3–5) partial digestion with RNase U_2; Enzyme/substrate ratios were 3 units/μg, 2 units/μg, 1 unit/μg in series; (6) partial alkaline digestion.

Spots

```
                ⊢----------18 ------⊣
1   UUAUUAAAAAAAAAAAAAAAAAAGp
2   UUAUUAAAAAAAAAAAAAAAAAAGp
3   UUAUUAAAAAAAAAAAAAAAAAAGp
4   UUAUUAAAAAAAAAAAAAAAAAAGp
5   UUAUUAAAAAAAAAAAAAAAAAAGp
6   UUAUUAAAAAAAAAAAAAAAAAAGp
7   UUAUUAAAAAAAAAAAAAAAAAAGp
8   UUAUUAAAAAAAAAAAAAAAAAAGp
                ⊢----------25----------⊣
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Fig.3. Complete sequences of Ap-rich RNase T_1 oligonucleotide series of BMV RNA3.

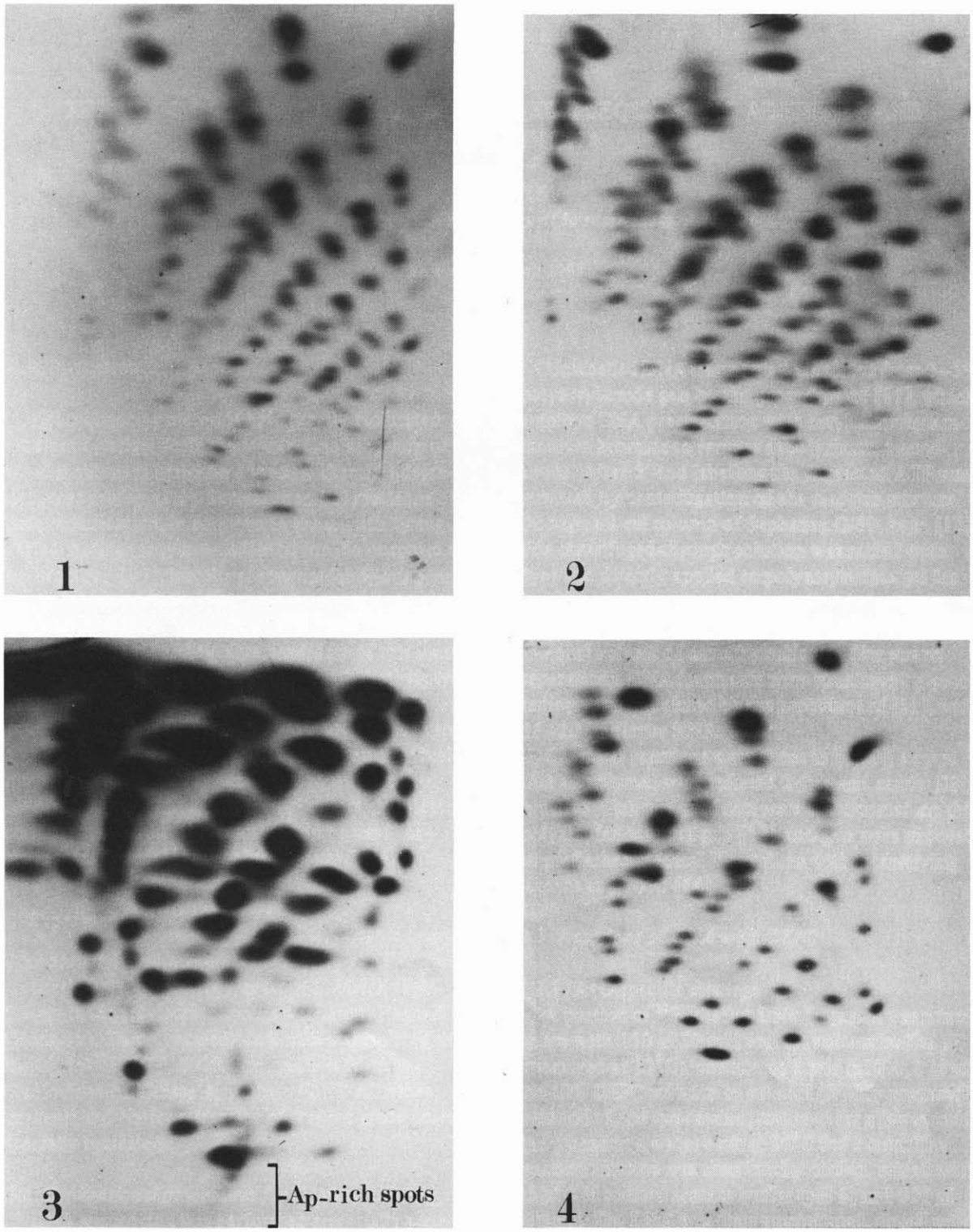

Fig.4. Two-dimensional gel electrophoresis of 4 BMV[^{32}P]RNA components. Third fingerprint shows that only BMV3 contains Ap-rich oligonucleotides.

3. Results

In vivo ^{32}P-labeled BMV RNA (30×10^6 cpm, 4×10^4 cpm/μg) was digested with RNase T_1 and applied to two-dimensional polyacrylamide gel electrophoresis. A series of slanted spots was found at the lower portion of the autoradiography fingerprint (fig.1). These spots were eluted and further digested with RNase A and applied to DEAE paper and electrophoresed at pH 3.5 [17]. The autoradiography revealed that most of the radioactive counts remained at the origin. A trace amount of an A_2G band found in the autoradiograph could be an artifact since a similar band was observed in the control column. However, there was a minor band which was shown to be ApUp and Up. The RNase T_1–A-resistant molecule which remained at the origin of the DEAE paper was eluted, and further confirmed with RNase T_2 digestion and electrophoresis on Whatman no. 1 paper at pH 3.5. The band from the origin of DEAE paper from spots 1,2,3,4 and 5 contained only Ap and Gp in the ratio of 18:1, 19:1, 20:1, 21:1 and 22:1, respectively. Thus, the series of slanted spots is Ap-rich. These T_1 oligonucleotides contain Ap, Up, Gp but lack Cp.

The approximate nucleotide chain length of the slanted series of spots was estimated by the location of spots on the fingerprints [15] to be between 25–30 nucleotides. With the composition of 19–23 Ap nucleotides, it was predicted that these Ap-rich oligonucleotides would selectively bind to oligo(dT)-cellulose [19]. Complete RNase T_1 digests of total BMV RNA were equilibrated with high salt buffer (0.5 M) and loaded onto oligo(dT)-cellulose columns. Only the Ap-rich serial oligonucleotides should be retained. Over 99% of the counts were eluted in high salt washing. Upon RNase T_2 digestion and electrophoresis of the RNA retained by the column the base composition was found to be 4Up, 20Ap, 1Gp.

Spots 1–5 were further examined by 5′-labeling with [γ-^{32}P]ATP and polynucleotide kinase followed by RNase T_2 digestion and chromatography on a DEAE-cellulose column with a linear gradient of triethylammonium acetate buffer. The data indicate that only Up was labeled with the resultant [5′-^{32}P]-UDP. Thus, the oligonucleotides (spots 1–5) are Up-(A_{19-23},U_3G)p.

The rapid sequencing method [20] was used to further confirm the sequence of the Ap-rich series. Aliquots eluted from the 2nd-dimension gel of the fingerprint were 5′-labeled with [γ-^{32}P]ATP and polynucleotide kinase. The samples (spots 1–5) were purified on a slab gel and eluted for partial RNase U_2 and alkaline digestion as in [20]. The autoradiograph (fig.2) reveals that the sequence of the T_1 resistant oligonucleotide series (spots 1–8) has a common pattern of $UpUpApUpUpAp_{18-25}Gp$. This reconfirmed our [13]. The sequence of each serial spot is shown in fig.3.

BMV contains 4 RNA molecules [1] which can be resolved by polyacrylamide gel electrophoresis. Uniformly ^{32}P-labeled total BMV RNA (50×10^6 cpm) was electrophoresed on 3% SDS–urea gel and eluted for RNase T_1 two-dimensional gel fingerprinting. The autoradiograph of each component shows the relative degree of complexity by the number of spots on each fingerprint. This is in agreement with reports on the size of these molecules [1,2]. The slanted series of A-rich RNase T_1 resistant oligonucleotides was found only in RNA3 (fig.4).

4. Discussion

The 3′- and 5′-terminal nucleotide sequence of BMV RNAs was reported [9,21] without any detection of poly(A) or oligo(A) nucleotides. However, these results confirm the existence of a series of oligo(A) sequences [13] in RNA3. The length of these oligo(A)s varies from 18–25 nucleotides which indicates heterogeneity in the RNA3 population. Different oligo(A) lengths may exist in different RNA molecules since the radioactive counts of the oligo(A) spots reveal lower molarity in comparison to the other unique long oligonucleotide spots. The 3′Gp in each oligo(A) of the series indicates that they are internal nucleotides.

In vitro studies have demonstrated that RNAs 1 and 2 each directs the synthesis of a large protein [3]. RNA3 is dicistronic and is translated into a single protein while the coat protein cistron in untranslatable [4]. The entire sequence of RNA4 which is encoded by the 3′-end of RNA3 [8,9] is an active coat protein cistron [4]. The only inactive cistron is located at the 3′ portion of RNA3 which is also the only BMV RNA component containing the oligo(A) series. Moreover, these A-rich oligonucleotides contain a common sequence (UUAUUAA ——— AG). The UAA contained in this sequence may play a role in translation as a 5′ termination codon. Thus, this A-rich oligonucleotide series may be intercistronic and located after the

3′-end of the 5′ cistron and possibly controls, both the expression of the 'silent' coat protein cistron (the 3′ cistron in RNA3) and the processing of the subgenomic coat protein messenger RNA (RNA4) which may be cleaved and released from RNA3.

Thirty nine nucleotides from the 5′-end of the coat protein cistron of RNAs 3 and 4 of alfalfa mosaic viral (AMV) RNA have been sequenced [22]. Although there is no oligo(A) sequence in this portion of the intercistronic region, such a sequence may exist at the 5′-end of the intercistronic region in AMV RNA3.

The correlation of oligo(A) with the expression of an identical coat protein cistron in RNAs 3 and 4 supports our proposed model to explain the role of poly(A) or oligo(A) sequences in messenger RNA. This model states that small oligo(A) sequences inhibit translation and that this inhibition can be removed by polyadenylation or cleavage of oligo(A). Implicit in this model is that messenger RNA with or without long poly(A) sequences are active messengers; those with short oligo(A) are inactive. The length of the Ap chain may possibly control the level of activity, and thus regulate translation.

This model is compatible with published data on messenger RNA translation. Histone messenger RNA which lacks poly(A) is translated in vitro [23,24] as well as in vivo [25]. Globin messenger RNA freed of poly(A) can also be translated in vitro [26–28]. Moreover, polyadenylated and nonpolyadenylated messenger RNA from sea urchin embryos codes for similar proteins [29]. Intergenic sequences in each messenger RNA of the vesicular stomatitis viral genome show that the short internal oligo(A) occurs at the messenger RNA–poly(A) junction which presumably signals polyadenylation of each messenger RNA [30]. A class of heterogenous nuclear RNA of Hela cells contains an internal oligo(A) sequence (~25 Ap nucleotides) which is absent in cytoplasmic messenger RNA except those containing the larger 3′-terminal poly(A) sequence [31]. Fertilization dramatically increases protein synthesis in sea urchin eggs [32], which is accompanied by large increases in the poly(A) content of the total RNA [10,11]. Studies on sporulation in *Bactillus subtilis* [12] have shown a drastic increase of polyadenylated RNA which appears to be specifically associated with sporulation. It was also shown that 20–25% of the 3′-terminal poly(A) is detected as oligo(A) (10–15 nucleotides). The above studies raise the possibility of the existence in inactive messenger RNA of a short oligo(A) sequence that inhibits translation, and is difficult to detect. Conceivably this inhibition may be removed partially or completely by poly(A) elongation or by cleavage of oligo(A).

Note Added

After the completion of this manuscript, Ahlquist, et al. [33], using a different method, found an internal oligo(A) which corresponds to the internal oligo(A) in [13], and the Ap-rich series here.

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References

[1] Fowlks, E. and Young, R. J. (1970) Virology 42, 548–550.
[2] Lane, L. C. and Kaesberg, P. (1971) Nature New Biol. 232, 40–43.
[3] Shih, D. S. and Kaesberg, P. (1976) J. Mol. Biol. 103, 77–88.
[4] Shih, D. S. and Kaesberg, P. (1973) Proc. Natl. Acad. Sci. USA 70, 1799–1803.
[5] Hull, R. (1972) J. Gen. Virol. 17, 111–117.
[6] Bancroft, J. B. and Flack, I. H. (1972) J. Gen Virol. 15, 247–251.
[7] Peden, K. W. C. and Symons, R. H. (1973) Virology 53, 487–492.
[8] Shih, D. S., Lane, L. C. and Kaesberg, P. (1972) J. Mol. Biol. 64, 353–362.
[9] Bastin, M., Dasgupta, R., Hall, T. C. and Kaesberg, P. (1976) J. Mol. Biol. 103, 737–745.
[10] Slater, I., Gillespie, D. and Slater, D. W. (1973) Proc. Natl. Acad. Sci. 70, 406–411.
[11] Wilt, F. H. (1973) Proc. Natl. Acad. Sci. USA 70, 2345–2349.
[12] Kerjan, P. and Szulmajster, J. (1980) Biochem. Biophys. Res. Commun. 93, 201–208.
[13] Manning, P. and Fowlks, E. R. (1980) in: 8th Ann. MBS Symp., p. 126, Division of Research Resources, National Institutes of Health, Bethesda MD.
[14] Lee, Y. F. and Wimmer, E. (1976) Nucleic Acids Res. 3, 1647–1658.
[15] Lee, Y. F., Kitamura, N., Nomoto, A. and Wimmer, E. (1979) J. Gen. Virol. 44, 311–322.
[16] Lee, Y. F. and Fowlks, E. R. (1981) submitted.

[17] Brownlee, G. G. (1972) in: Determination of Sequences in RNA, Lab. Tech. Biochem. Mol. Biol. (Work, T. S. and Work, E. eds) vol. 3/I, Elsevier/North-Holland, Amsterdam, New York.
[18] Szekely, M. and Sanger, F. (1969) J. Mol. Biol. 43, 607–617.
[19] Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA, 69, 1408–1412.
[20] Donis-Keller, H., Maxam, A. M. and Gilbert, W. (1977) Nucleic Acid Res. 4, 2527–2538.
[21] Glitz, D. G. and Eichler, D. (1971) Biochim. Biophys. Acta 238, 224–232.
[22] Brederode, F., Koper-Zwarthoff, E. and Bol, J. (1980) Nucleic Acids Res. 8, 2213–2223.
[23] Jacobs-Lorena, M. and Baglioni, G. (1972) Proc. Natl. Acad. Sci. USA 69, 1425–1428.
[24] Borun, T. W., Gabrielle, F., Ajiro, K., Zweidler, A. and Bajlioni, C. (1975) Cell. Vol. 4, 59–67.
[25] Huez, G. and Marbaix, G., Gallwitz, D., Weinberg, E., Devos, R., Hubert, E. and Cleuter, Y. (1978) Nature 271, 572–573.
[26] Huez, G., Marbaix, G., Hubert, E., Leclercq, M., Nudel, U., Soreq, H., Salomon, R., Lebleu, B., Revel, M. and Littauer, U. Z. (1974) Proc. Natl. Acad. Sci. USA 71, 3143–3146.
[27] Sipple, A. E., Stavrianopoulos, J. G., Schutz, G. and Feigelson, P. (1974) Proc. Natl. Acad. Sci. USA 71, 4635–4639.
[28] Williamson, R., Crossley, J. and Humphries, S. (1974) Biochemistry 13, 703–707.
[29] Brandsorst, B. P., Desh, P. S. V. and Fromson, D. (1979) Dev. Biol. 71, 128–141.
[30] Rose, J. K. (1980) Cell 19, 415–421.
[31] Venkatesan, S., Nakazato, H., Kopp, D. W. and Edmonds, M. (1979) Nucleic Acids Res. 6, 1097–1116.
[32] Epel, D., Pressman, B. C., Elsaesser, S. and Weaver, A. M. (1960) in: The Cell Cycle: Gene–Enzyme Interactions (Padilla, G. M. et al. eds) pp. 279–298, Academic Press, London, New York.
[33] Ahlquist, P., Dasqupta, R. and Kaesberg, P. (1981) Cell 23, 183–189.