NON-METHYLATED GUANOSINE AS THE 5' TERMINUS OF CAPPED mRNA FROM INSECT OOCYTES

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Summary: Guanosine, rather than its methylated derivative, was found to be the inverted nucleoside present in the 5' terminal capping structure of insect oocyte messenger RNA. Since methylation of the terminal guanosine at the 7 position is necessary for the initiation of protein synthesis in eukaryotes, this evidence suggests that the translational inactivity of the mRNA prior to fertilization may be associated with the absence of methylation.

Introduction: Numerous recent reports have demonstrated the existence of a unique sequence at the 5' end of a wide variety of viral and eukaryotic mRNAs (cf. reviews 1, 2). This sequence was often found to consist of a 7-methyl-guanosine attached to the penultimate nucleotide via a 5'-5' triphosphate linkage. Because of the inverted position of the m^7G, this 5' structure is resistant to base hydrolysis and digestion by a number of nucleases, and thus is referred to as a "cap". Although the precise role for the cap is not yet understood, several reports have indicated that the methyl group at the 7 position of the terminal guanosine is required for the initiation of protein synthesis (3,4,12). Consequently, initiation can be prevented by inhibiting methylation of the guanosine or by chemically removing the m^7G.

Abbreviations: m^7G - 7 methyl guanosine; m^7 pG - 7 methyl guanosine 5' monophosphate; BAP - bacterial alkaline phosphatase; pG - Guanosine 5' monophosphate; G - guanosine.

The existence of the capping group provides a possible explanation for the translational inactivity of the stored maternal mRNA present in the oocytes of a number of organisms such as sea urchins (5) and Xenopus (6). In these systems, low levels of protein synthesis are observed until shortly after fertilization when the levels rise rapidly. Messenger RNA in these systems could contain a cap at the 5' terminus which would not be methylated until after fertilization. Following methylation at the 7 position of the terminal guanosine, the stored maternal mRNA would then be available for protein synthesis.

Absence of detectable protein synthesis prior to fertilization and the ease with which large amounts of labeled mRNA can be obtained make lepidopteran oocytes exceptional material for the study of stored maternal mRNA (7). In this report, we describe studies of the 5' terminus of maternal mRNA obtained from developing oocytes of the tobacco hornworm, Manduca sexta.

Materials and Methods:

Labeling and isolation of mRNA: Larvae of the tobacco hornworm (Manduca sexta) were reared on an artificial diet as described previously (8,9). Ovaries were dissected from newly emerged adults and incubated 12 hours in 1 ml Graces Insect Tissue Culture medium containing either 2 mCi [^{32}P]-orthophosphate or 200 uCi [^{3}H]-guanosine. Poly (A^+) RNA was isolated and purified by phenol extraction followed by poly (U)-Sepharose chromatography according to methods described earlier (7,10).

RNA Digestion: For T_2 RNase digestion, mRNA was dissolved in 0.5 ml of 10 mM acetate buffer pH 4.5 and incubated 5 hours at 37°C with 2.5 units enzyme/ml. To insure complete digestion, following the initial incubation, the reaction mixture was heated at 100°C for 30 seconds, cooled, and re-incubated for 1 hour with 1.25 units of fresh enzyme. For alkaline phosphatase digestion, nucleotides were incubated with 20 units enzyme/ml in 50 mM tris pH 8.5 for 30 minutes at 37°C. For nucleotide pyrophosphatase digestion, cap was incubated with .05 units enzyme/ml in 20 mM tris pH 7.5 and 1 mM $MgCl_2$ for 15 minutes at 37°C.

Column Chromatography: Columns of DEAE-cellulose (0.8 x 20 cm) were equilibrated with 50 mM tris pH 7.5 and 7 M urea. RNA digest in the same buffer was loaded onto the column and then eluted with a 150 ml gradient of NaCl in the same buffer. Markers consisted of a pancreatic RNase digest of

yeast tRNA and were located in the eluate by determining A_{260}. Pooled material was desalted on a Bio-Gel P2 column (1 x 20 cm) equilibrated with distilled H_2O.

Periodate Oxidation and Borohydride Reduction: Isolated cap was incubated with 20 mM $NaIO_4$ in 0.1 M sodium acetate pH 5.3 in the dark for 1 hour at 4°C. After 1 hour, the mixture was made 1% in glucose to rid the mixture of excess periodate. The mixture was then desalted and made 0.1 M in acetate pH 7.7. A 1000-fold molar excess of [^{3}H]-potassium borohydride was added, and the mixture was incubated for 12 hours at 4°C. The cap was then desalted and prepared for further treatment.

Chromatography: Thin layer chromatography was performed on cellulose plates using Solvent A. Ion exchange TLC was on PEI-cellulose using solvent B for 4 cm and then changing to solvent C for 10 more cm from the origin. Solvent A: isopropanol:1% $(NH_4)_2SO_4$ (2:1). Solvent B; 1 M acetic acid. Solvent C: 0.3 M LiCl.

Chemicals and enzymes: [^{32}P]-orthophosphoric acid, [^{3}H]-potassium borohydride (sp. ac. 302 mCi/mmole), and [^{3}H]-guanosine (sp. ac. 8.4 Ci/mmole) were obtained from New England Nuclear. Grace's Insect TC medium was from Gibco. T_2RNase, diphenyl carbazone, and nucleotide pyrophosphatase were obtained from Sigma; and, bacterial alkaline phosphatase was from Worthington Biochemicals. Bio-Gel P2 was from Bio-Rad Inc., and DEAE-cellulose (DE-52) was from Whatman. PEI-cellulose plates were a product of Brinkman Instruments, Inc.

Results: Purified, [^{32}P]-labeled, poly (A^+) RNA was digested with T_2 RNase and loaded onto a DEAE-cellulose column. The profile of radioactivity obtained when the column was eluted with a salt gradient (0.05 to 0.2 M NaCl) is shown in Figure 1A. The major peak of radioactivity eluted at the -2 position and represented a distribution of the four common mononucleotides (data not shown). The smaller peak of radioactivity eluted at the -3.5 position may have arisen from internal methylation and has not been examined further. The much smaller peak of radioactivity eluted at the -5.0 to -5.5 position is tentatively identified as the cap and its charge is consistent with the structure: X(5')ppp(5')Yp where X and Y are nucleosides. For further verification that this is the cap structure, and to determine how many of the negative charges (present as $(PO_4)^-$ groups) are free and susceptible to cleavage by alkaline

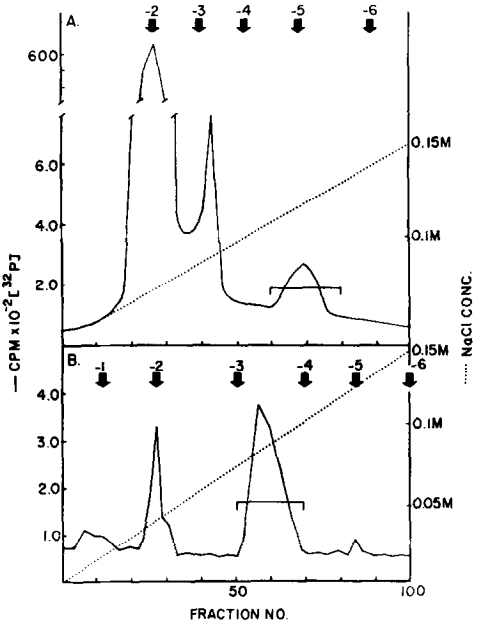

Figure 1. DEAE-cellulose column chromatography of digested [^{32}P] mRNA. Samples were adjusted to 7.0 M urea in 50 mM Tris (pH 7.5) and applied to DEAE-cellulose columns (0.8 x 20 cm). The columns were eluted with a 150 ml gradient of NaCl, and 1 ml fractions were collected. Each fraction was scanned for A_{260} to locate marker positions. The radioactivity of every second fraction was determined by liquid scintillation counting. NaCl concentration was determined by titrating every tenth fraction with $Hg(NO_3)_2$ in the presence of diphenyl carbazone indicator. Positions of marker oligonucleotides are designated as net negative charge. Bracketed peaks indicate 5' terminal structures.

A. T_2 RNase digest of [^{32}P] poly (A^+) RNA eluted with a gradient of 0.05 to 0.2 M NaCl.

B. BAP digest of the -5.5 peak from Figure 1A. Following desalting of the indicated fractions in Figure 1A, the oligonucleotides were rechromatographed on DEAE-cellulose and eluted with a gradient of 0.0 to 0.2 M NaCl.

phosphatase, these fractions were pooled and desalted. The resulting oligonucleotides were digested with bacterial alkaline phosphatase (BAP), loaded onto a DEAE-cellulose column, and eluted with a gradient of 0.0 to 0.2 M NaCl. The profile of radioactivity eluted in this manner is shown in Figure 1B. The

inorganic phosphate cleaved by the BAP is seen as a peak eluting at the -2 position, while the cap material eluted at the -3.5 position. This indicated a loss of only 2 negative charges after treatment with BAP. This loss is consistent with the cap structure in other systems where three internal phosphates are protected from cleavage by the inverted m^7G.

We next attempted to identify the terminal nucleoside present in the cap and, in particular, to determine whether it is methylated. If the terminal nucleoside were truly inverted, it would contain a free 3' hydroxyl group which would make the base susceptible to oxidation by periodate and subsequent reduction by borohydride. The -5.0 to -5.5 peak of T_2 RNase digest of poly (A^+) RNA (Figure 1A) was desalted, oxidized with $NaIO_4$, and then reduced with [^{3}H]-potassium borohydride. The product of this treatment was a tri-alcohol with tritium at the 3'end. The cap labeled in this manner was then digested with nucleotide pyrophosphatase to break the triphosphate linkage, followed by BAP to yield free nucleosides of which only the former terminal base would be tritium labeled. These bases were then separated by chromatography in a variety of solvent systems, both at acidic and basic pH, on thin layer plates. Since m^7G is particularly susceptible to ring-opening at alkaline pH, it was necessary to treat a portion of the marker nucleosides with base also. From the results of a typical thin layer chromatogram (Figure 2A), it is evident that all of the migrating radioactivity eluted in the position of guanosine while none migrated with either the ring-opened or natural forms of m^7G. The radioactivity remaining at the origin probably represented incomplete digestion and breakdown products.

The terminal nucleoside of the cap was also identified by obtaining poly (A^+) RNA which had been labeled with [^{3}H]-guanosine. Following T_2 di-

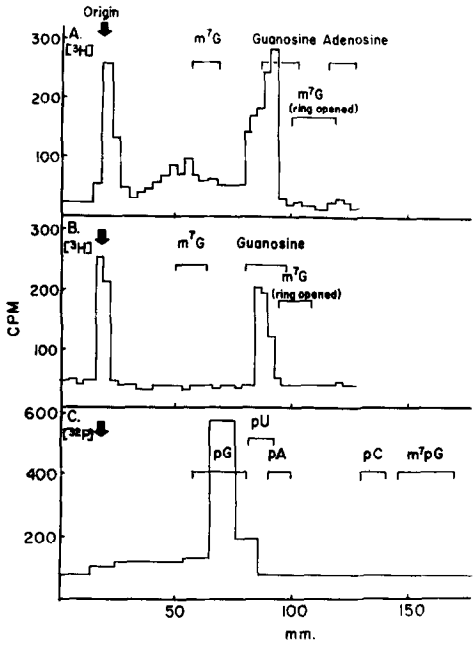

Figure 2. Thin layer chromatography of enzymatic digests of the cap structure. Following chromatography, marker positions were determined by observation under UV light, and determination of radioactivity was by liquid scintillation counting in a toluene based scintillation cocktail.

A. Chromatography of [^{3}H]-borohydride labeled nucleosides. Cap was isolated from the -5.5 position of Figure 1A, desalted, oxidized with $NaIO_3$, and reduced with [^{3}H]KBH_4. The oligonucleotide was then desalted, digested with nucleotide pyrophosphatase followed by BAP. The nucleoside mixture was spotted on cellulose thin layer plates and chromatographed with the appropriate marker nucleosides in solvent A.

B. Chromatography of [^{3}H]-guanosine labeled nucleosides. Cap was isolated from a T_2 digest of [^{3}H]-guanosine labeled mRNA chromatographed as in Figure 1A, desalted and digested with nucleotide pyrophosphatase followed by BAP. Nucleosides were then spotted on cellulose thin layer plates and chromatographed with markers in solvent A.

C. Ion exchange chromatography of [^{32}P]-nucleotides of the cap. Cap was isolated from the -3.5 position of a DEAE-cellulose column as in Figure 1B, desalted and digested with nucleotide pyrophosphatase. After desalting, the nucleotides were mixed with unlabeled marker nucleotides, spotted onto PEI-cellulose thin layer plates, and chromatographed with solvent B (4 cm.) followed by solvent C (10 cm.). Prior to solubilization in scintillation cocktail, the radioactivity of each fraction was eluted from the cellulose with 0.2 N HCl.

gestion, the cap was eluted from DEAE-cellulose as in Figure 1A, then desalted and treated with pyrophosphatase followed by BAP. The resulting nucleosides were separated on thin layer plates as before, and a typical profile of radioactivity is shown in Figure 2B. Once again, all of the radioactivity beyond the origin migrated with the guanosine marker.

Further evidence that the terminal base of the cap is guanosine came from experiments where the isolated cap was digested into nucleotide 5' monophosphates and separated on ion-exchange thin layer plates. [^{32}P]-labeled poly (A^+) RNA was digested with T_2RNase and separated on a DEAE-cellulose column. The fractions containing the cap (Figure 1A were pooled, desalted and digested with BAP. The digestion products were re-separated on DEAE-cellulose, and the peak at -3.5 (as in Figure 1B) was pooled, desalted, and treated with nucleotide pyrophosphatase to yield 5' monophosphates. The desalted product was then spotted onto PEI-cellulose thin layer plates. Figure 2C shows that all of the radioactivity migrated with the pG marker.

Discussion: The data presented above demonstrate that the stored maternal mRNA from unfertilized oocytes of Manduca contains a cap structure at its 5' terminus. It is evident that the terminal base in this cap is inverted (i.e. contains a 5'-5' linkage) because of the cap's resistance to both T_2RNase and alkaline phosphatase digestion, and because of its susceptibility to periodate oxidation. That the two nucleotides are linked by at least two, and probably three, phosphate groups is indicated by its -3.5 charge which is resistant to alkaline phosphatase but is susceptible to nucleotide pyrophosphatase.

Evidence that the terminal nucleoside is guanosine rather than its methylated derivative is derived from three different experimental procedures: di-

rect labeling of the mRNA with [^{3}H]-guanosine and [^{32}P]-orthophosphate, as well as [^{3}H]-borohydride reduction of the oxidized terminal nucleoside. Various regimes of enzymatic digestion and chromatographic separation all yielded labeled guanosine as a product. Digestion of the cap from mRNA labeled by incorporation of [^{32}P]-orthophosphate yielded only labeled guanosine, indicating that the penultimate nucleoside is also guanosine.

Whether the absence of $m^{7}G$ is responsible for the absence of mRNA translation seen in insect oocytes (7) remains to be demonstrated. In both developed and undeveloped embryos of Artemia, the terminal nucleoside of the cap was shown to be $m^{7}G$ (11). However, that study compared two embryonic forms of mRNA. We are currently analyzing polysomal mRNA from fertilized eggs of Manduca to determine whether the terminal guanosine of the cap becomes methylated following fertilization.

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