METHYLATION OF LATE ADENOVIRUS 2 NUCLEAR AND MESSENGER RNA

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SUMMARY: Methylation of adenovirus 2 (Ad 2) late RNA was studied. RNA was double-labeled with [^{3}H-methyl]-methionine and [^{14}C]-uridine 15-20 h postinfection. Nuclear RNA (nRNA) and cytoplasmic RNA (mRNA) was extracted, and fractionated into polyA(+) and (-) molecules using poly(U)-Sepharose. Ad 2 specific RNA was purified by 2 cycles of hybridization to and elution from Ad 2 DNA immobilized on filters. The Ad 2 polyA(+) and (-) nRNA and mRNA fractions had the same ^{3}H/^{14}C ratios, and were estimated to contain a minimum of 1.4 methylated nucleotides per 1000 bases. Viral RNA was digested with RNase T_2 and chromatographed on DEAE-Sephadex in 7 M urea at pH 7.6. All four Ad 2 RNA fractions contained methylated constituents consistent with: (1) two classes of methylated "capped" 5'-termini with general structures m^7GpppN^mpNp and $m^7GpppN^mpN^mpNp$; (2) internal base methylations; (3) minor amounts of internal ribose 2'-O-methylations. Two classes of 5'-termini have previously been reported for animal cell mRNA, but not for mRNA from a variety of viruses. Internal methylations may be unique to RNA molecules transcribed in the nucleus, since they have not been found in RNA from cytoplasmic viruses. No gross differences were observed in the DEAE-Sephadex elution profiles of the methylated constituents of the four types of Ad 2 RNA. These results suggest that the majority of methylation events occur in the nucleus, and raise the possibility that Ad 2 methylated late nRNA may differ significantly from SV40 late nRNA (Lavi, S., and Shatkin, A.J. (1975) Proc. Natl. Acad. Sci. USA 72, 2012-2016).

INTRODUCTION: It has recently been demonstrated that mRNA from animal cells (1) and viruses that replicate in eukaryotic cells (2) is methylated. With viruses that replicate without a nuclear phase the methylation is exclusively at the 5'-terminus in an oligonucleotide of the structure $m^7G(5')ppp(5')N^mpNp$..(N^m represents a ribose 2'-O-methylation) (3). This structure has been termed a "cap" and it appears necessary for binding of mRNA to polyribosomes (4). Animal cell mRNA contains the above cap (cap 1), plus an additional cap (cap 2) of the structure $m^7G(5')ppp(5')N^mpN^mpNp$.. (5-7). Unlike cytoplasmic viruses, animal cell mRNA is also methylated at internal positions in the molecule, mainly (or exclusively) as N^6-methyladenosine (N^6mA) (5-9), and possibly as 2'-O-methylated constituents (5-9).

SV40 is an oncogenic DNA virus that transcribes its genome in the cell nucleus. SV40 mRNA, isolated at late stages of infection, contains

caps plus internal N^6mA moieties (10,11), so it is methylated similarly to cell mRNA. Unlike cytoplasmic viruses, both SV40 (12) and cell mRNA (13) are thought to be derived from larger precursor molecules in the nucleus, and must be transported from the nucleus to the cytoplasm. Therefore, methylation may play a role in the processing of SV40 (10,11) and cell (3,5) mRNA. Internal methylations might be most important in this regard.

Cell nuclear RNA (nRNA) is methylated, although to a lesser extent than mRNA (1). The only type of nRNA studied in detail is from SV40, and this had a different pattern of methylation than SV40 mRNA, consisting of large amounts of 2'-O-methylguanosine (G^m) and 2'-O-methyluridine (U^m), and lesser amounts of N^6mA, and type 1 caps (10). The G^m and U^m varied between 30-80% of the total methylated constituents. It is not known whether G^m and U^m are located at internal positions in nRNA, or at the 5'-terminus in the form of type 2 caps. It was suggested that the nRNA molecules may be cleaved, resulting in loss of G^m and U^m during maturation into mRNA (10).

Adenovirus 2 (Ad 2) is also an oncogenic DNA virus that transcribes its genome in the cell nucleus. Several lines of evidence suggest that late Ad 2 mRNA may arise from larger polycistronic precursor molecules (14,15, Wold and Green, unpublished results). We are presently investigating what role, if any, methylation may play in the processing of Ad 2 mRNA. This report shows that late Ad 2 nRNA and mRNA contains internal methylations, and at least two classes of caps.

MATERIAL AND METHODS: Our methods will be described in detail elsewhere. Briefly, Ad 2 infected human KB cells were labeled 14-15 h postinfection with [^{3}H-methyl]-met (ca. 11 Ci/mmole) at 0.5-1.0 mCi/ml, plus either [^{14}C]-uridine (ca. 60 mCi/mmole) at 0.4 µCi/ml or carrier-free $H_3{}^{32}PO_4$ at 3 mCi/ml. After 4-5 h labeling, cells were harvested and lysed (16). Nuclei were collected by centrifugation and washed twice in lysis buffer. Cytoplasmic contaminants were removed from the nuclei by a double-detergent treatment (17). The nRNA was extracted by the hot-SDS-phenol procedure (18). The mRNA was obtained either by extracting (20°C) the cytoplasm directly, or by extracting polyribosomes pelleted through a 2.0 M sucrose cushion. All RNA extractions were performed in 0.5-1.0% SDS using equal volumes each of phenol and chloroform-isoamyl alcohol (24:1). RNA was collected by ethanol precipitation.

The nRNA and mRNA were fractionated into polyA(+) and (-) material using poly(U)-Sepharose. Ad 2 specific polyA(+) and (-) nRNA and mRNA was selected by hybridization to and elution from Ad 2 DNA immobilized

on nitrocellulose filters. No rRNA or tRNA was apparent as judged by polyacrylamide gel electrophoresis of hybridization-purified Ad 2 RNA. Ad 2 RNA was eluted (16), ethanol precipitated, rehybridized to fresh Ad 2 DNA filters, and eluted again. Hybridizations were overnight in 0.72 M NaCl, 1 mM EDTA, 0.05% SDS, 10 mm PIPES, pH 6.7, in 50% formamide at 37°C, or in aqueous buffer at 68°C. Purified Ad 2 RNA samples were digested with RNase T_2, and chromatographed on DEAE-Sephadex in 7 M urea and 10 mM Tris-Cl, pH 7.6. RNase A digested yeast RNA was used as oligonucleotide markers. Columns were developed by linear gradients of 0 - 0.35 M NaCl, followed by a 1.0 M NaCl wash.

RESULTS: It is possible to estimate the extent of methylation of different RNA preparations by double-labeling with [^{3}H-methyl]-met and [^{14}C]-uridine, and comparing the $^3H/^{14}C$ ratios. $^3H/^{14}C$ ratios of various RNA fractions obtained from one experiment are given in Table 1. The Ad 2 specific total nRNA (polyA(+) plus (-)), polyA(+) mRNA, and polyA(-) mRNA samples were obtained by two cycles of hybridization-purification. The $^3H/^{14}C$ ratios of these RNAs were identical. No significant difference in the $^3H/^{14}C$ ratios were observed when the polyA(+) and polyA(-) nRNA were analyzed independently (not shown in Table I). The unhybridized material presumably represented a composite of rRNA, tRNA, cell mRNA, and unhybridized viral RNA. In our experiments, rRNA and tRNA were extensively labeled by [^{3}H-methyl]-met, [^{14}C]-uridine, and [^{32}P]-phosphate.

TABLE 1: $^3H/^{14}C$ ratios of RNA samples.

RNA fraction	RNA that did not hybridize	Ad 2 specific RNA after two cycles of hybridization-purification
Total nuclear	6.7[a]	2.1[b]
polyA(+) cytoplasm	4.5	2.0
polyA(-) cytoplasm	18.9	2.1

[a] Based on trichloroacetic acid insoluble cpm.

[b] ^{3}H-counts hybridized: total nuclear, 8,400; polyA(+) cytoplasm, 12,100; polyA(-) cytoplasm, 28,800.

Assuming that all unhybridized material from the polyA(-) mRNA fraction was rRNA, and based upon a comparison of the $^{3}H/^{14}C$ ratios of this rRNA with the purified Ad 2 RNA, we conclude that all types of Ad 2 RNA contain 1.4 methylated residues per 1000 nucleotides (see ref. 1 for calculation). This is a minimum value, because the unhybridized RNA from this fraction also contains tRNA, which is more extensively methylated than rRNA. Thus 1.4 is in reasonable agreement with the values 2.2 and 1.8 calculated for L cell (1) and SV40 mRNA (11) respectively.

All four types of Ad 2 RNA preparations (polyA(+) and (-) nRNA and mRNA) had similar elution profiles of methylated constituents upon DEAE-Sephadex chromatography of RNase T_2 digests. Fig. 1 illustrates a typical elution profile of methylated constituents. Under these chromatographic conditions, oligonucleotides are eluted by NaCl essentially according to the net charge of the phosphate groups. T_2 hydrolyzes phosphodiester bonds through a 2', 3'-cyclic intermediate, so phosphodiester bonds in caps and internal 2'-O-methylations are enzyme-stable. A major peak of methylated material (base methylated mononucleotides) eluted at charge -2, and two major peaks between charges -5 to -7. The methyl label was not incorporated into purine ring structures, since radioactivity was not

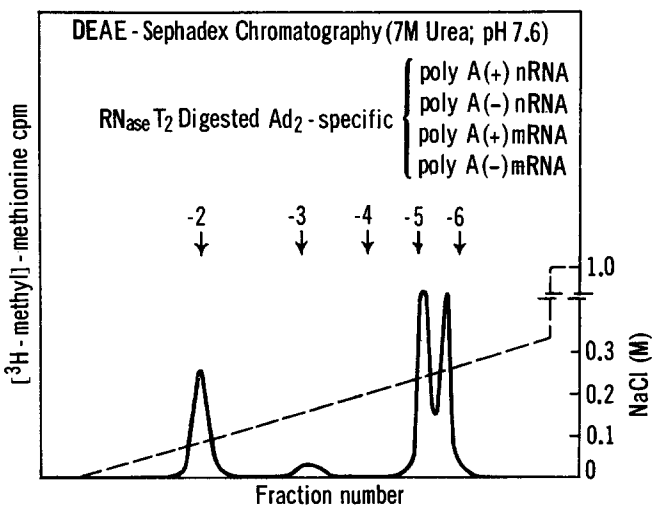

Figure 1. Typical elution of profile based on four separate experiments of Ad 2 specific polyA(+) and (-) nRNA and mRNA. Radioactivity in the column fractions varied between zero and 5000 cpm, depending upon the experiment. RNA was digested with RNase T_2, mixed with oligonucleotide markers, and chromatographed on DEAE-Sephadex A-25 in 7 M urea, 10 mM Tris-Cl, pH 7.6. Columns (3 x 30 mm) were developed by 200 ml linear NaCl gradients. No radioactivity eluted at charges >-7, indicating that RNase digestion was complete.

found in adenosine or guanosine by thin layer chromatography of mononucleotides. The -5 to -7 charged material most likely consisted of 5'-termini, since they contained m^7G (not shown), and since caps elute at these positions (eg. ref. 5). A small amount of radioactivity also eluted at charge -3. Analyses indicate that these are 2'-O-methylated constituents. The -3 material may be due to small amounts of rRNA contaminants, since rRNA contains a large number of internal 2'-O-methylations. However, the -3 material was present in all four types of Ad 2 RNA, including polyA(+) material processed through two cycles of hybridization-purification. Therefore, they may indeed be Ad 2 specific.

The ratios of radioactivity in the mononucleotide peak to the termini varied somewhat from experiment to experiment with each RNA type. Since the caps are at the 5'-end, the mononucleotides are internal, and poly(A) is at the 3'-end, this variation could be due to the poly(U)-Sepharose fractionation step if the RNA was degraded to any extent. The ratios of the first major cap peak to the second major cap peak were also variable. The first and second major cap peaks may represent oligonucleotides of the cap 1 and cap 2 types alluded to above. Furuichi et al. (5) commented that the ratios of cap 1 to cap 2 in HeLa mRNA seemed to vary with the "cell growth conditions", and a similar explanation may apply to the variability we observe.

DISCUSSION: Our results provide presumptive evidence that in common with other eukaryotic mRNA molecules, late Ad 2 mRNA contains methylated caps. The presence of two major cap peaks indicates that Ad 2 mRNA contains at least two types of 5'-termini, possibly of the cap 1 and cap 2 structures described for HeLa cell mRNA (5). As with other mRNA molecules transcribed in the nucleus, late Ad 2 mRNA is methylated at internal positions, mainly as base methylations, and possibly as minor amounts of 2'-O-methylations.

The distinction between polyA(+) and (-) material is not clear, since most late Ad 2 RNA has been reported to be polyadenylated (19). Thus, polyA(-) fractions may represent degraded molecules. However, polyacrylamide gel electrophoresis of polyA(+) and (-) nRNA and mRNA revealed similar size distributions, and of that expected from published work (14, 15, 20). When polyA(-) RNA was passed through a second poly(U)-Sepharose column (or oligo dT-cellulose), nothing bound (although 95% of [^{3}H]-polyA bound), suggesting that the material indeed did not contain polyA. When polyA(+) RNA was rechromatographed on poly(U)-Sepharose more than 90% bound.

An interesting finding is that large differences were not observed

in the methylation of nRNA and mRNA, either in the $^{3}H/^{14}C$ ratios, or the DEAE-Sephadex elution profiles. This may or may not be significant with regard to mRNA maturation. If mRNA matures from a larger nRNA precursor, most nRNA should be in terminal stages of maturation, and "non-mRNA" portions of precursor should be relatively scarce (because of processing). Since RNA was labeled for 4-5 h, the majority of label in nRNA would be in abundant matured molecules. Hybridization-purification would preferentially select these "matured" RNA sequences, which might be identical to, or closely resemble mRNA. Alternatively, perhaps Ad 2 mRNA does not arise from a precursor but in in a manner similar to prokaryotic mRNA as unique transcripts corresponding to essentially one structural gene. Regardless, posttranscriptional methylation and polyadenylation apparently does occur, and some or all of this takes place in the cell nucleus. Only complete characterization of all the methylated constituents in nRNA and mRNA, and the analysis of unprocessed putative mRNA precursor molecules will establish the role of methylation in Ad 2 RNA metabolism. However, the existence of internal methylations in Ad 2, SV40, and animal cell RNA molecules transcribed in the nucleus, and the absence of these in mRNA transcribed in the cytoplasm, (eg. certain RNA viruses) suggests a role for internal methylations in mRNA processing.

Most intriguing, it is possible that the methylation of Ad 2 nRNA may differ from SV40. With SV40, U^{m} and G^{m} represent the majority of methylated constituents (10). These may be either associated with type 2 cap structures, or may be located at internal positions, since experiments were not done to distinguish these possibilities. With late Ad 2 nRNA, cap 2 structures represent less than one-third of the methylated constituents, and there are very few, if any, internal 2'-O-methylated residues. Whether these results reflect genuine differences in the metabolism of late SV40 and Ad 2 nRNA, or differences due to the selection of different abundance classes of viral nRNA (processed versus unprocessed transcripts) remains to be established.

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