METHYLATION OF CYTOPLASMIC ADENOVIRUS RNA SYNTHESIZED EARLY AND LATE IN PRODUCTIVE INFECTION

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SUMMARY

[^{3}H]-methyl methionine was incorporated into poly A containing cytoplasmic RNA synthesized during productive infection with adenovirus 2. Polyacrylamide gel electrophoresis of cytoplasmic RNA lacking poly A demonstrated that the methionine methyl was not incorporated into purine rings: Preparations labeled with [^{3}H]-uridine contained peaks of cellular 5S RNA and viral 5.5S RNA in addition to tRNA. In contrast RNA labeled with [^{3}H]-methyl methionine yielded only tRNA, with no detectable incorporation into the unmethylated 5S and 5.5S RNAs. The poly A containing RNA labeled with [^{3}H]-methyl methionine was annealed to adenovirus DNA; 39% of RNA synthesized early in infection formed hybrid and more than 35% of late RNA was virus specific.

INTRODUCTION

Recent studies have demonstrated the existence of methylated ribonucleotides in animal virus (1-5) and eukaryotic (6-10) mRNAs. Structural analyses of RNAs transcribed *in vitro* from reovirus RNA (1) and vaccinia virus DNA (5) have shown that the 5' termini of these viral RNAs contain a blocked, methylated structure. Similar structures have also been proposed for the 5' termini of mRNAs isolated from HeLa and mouse L cells (6,7). *In vitro* studies of protein synthesis with reovirus and VSV viral RNAs (11) and reticulocyte mRNA (12) indicate that methylation of viral mRNAs is required for translation *in vivo*.

Because of the possible roles of methylated nucleotides in mRNA metabolism and translation, experiments were performed to determine if mRNA transcribed from a nuclear DNA virus is methylated. Cultured human cells (KB) infected with adenovirus 2 were labeled with [^{3}H]-methyl methionine, and the label in RNA was characterized by hybridization to viral DNA. The results provide evidence that viral RNA synthesized at both early and late times in infection is methylated.

METHODS

Cell culture and virus infection

Exponentially growing KB suspension cultures were concentrated to 1.2 x 10^7 cells/ml and infected with adenovirus 2 as previously described (13,14). To label RNA synthesized early in infection, the culture was diluted to 9 x 10^5 cells/ml after the 1 hr adsorption period. One hour later the culture was centrifuged again at room temperature and resuspended at 9 x 10^5 cells/ml in Joklik minimum essential medium containing no serum, 20 µM L-methionine (1/5 the normal methionine concentration), 20 mM sodium formate, 20 µM adenosine and guanosine each, and 30 µCi/ml of [^{3}H]-methyl methionine (11 Ci/mmole, New England Nuclear Corp.) (8). For all early RNA preparations, cycloheximide was present in the medium at concentrations of 25 µg/ml (15). For the preparation of late RNA, cultures were labeled with 20 µCi/ml of [^{3}H]-methyl methionine in the same medium, beginning 18 hours after infection. Control RNAs were prepared by labeling for 3 hrs in the presence of 12.5 µCi/ml [^{3}H]-uridine (40 Ci/mmol, New England Nuclear).

Cell fractionation, RNA purification and hybridization

Purification of cytoplasmic RNA, selection of poly A containing molecules by oligo-dT-cellulose chromatography, and hybridization to adenovirus 2 DNA were performed as previously described (15).

Electrophoresis of RNA

Electrophoresis of RNA was performed with polyacrylamide gels cross-linked with ethylene diacrylate (14); after electrophoresis, gels were frozen, sliced, solubilized with 0.1 ml concentrated NH_4OH and counted in a scintillation counter.

RESULTS AND DISCUSSION

Different portions of the adenovirus 2 genome code for

cytoplasmic viral RNA synthesized early and late in productive infection. At early times, before viral DNA synthesis begins, approximately 25% of the viral genome is transcribed into cytoplasmic RNA; late in infection transcripts of a much larger portion of the genome are present as functional mRNA (16-18). Most if not all the viral mRNAs synthesized at early and late times contain poly A (17,19,20).

To isolate early RNA, cultures were labeled in the presence of cycloheximide, an inhibitor of protein synthesis, which prevents the onset of viral DNA synthesis (21). Early RNA was prepared from cultures labeled 2-5 hrs after infection, and late RNA was isolated from cultures labeled 18-21 hrs. To determine if the [^{3}H]-methyl methionine present in purified RNA preparations was indeed incorporated into RNA molecules, cytoplasmic RNA preparations were treated with either DNAse or pronase (Table 1). Neither treatment reduced the acid-precipitable radioactivity whereas RNAse or KOH degraded the incorporated [^{3}H]-methyl label to acid-soluble material (Table 1).

A lower percent of RNA preparations labeled with [^{3}H]-methyl methionine bound to oligo-dT-cellulose as compared to control RNAs labeled with [^{3}H]-uridine (Table 2). This observation is consistent with evidence that the level of methylation in mRNA is lower than that in ribosomal and transfer RNA (8), RNA molecules that do not bind to oligo-dT-cellulose (22). Some of the [^{3}H]-label in RNA not binding to oligo-dT-cellulose might be [^{3}H]-methionyl-tRNA. To test this possibility, the RNA excluded from oligo-dT-cellulose was deacylated by incubating in 1.8M Tris HCl, pH 8.1, for 100' at 37° (9); this procedure reduced the acid-precipitable radioactivity 25-35%.

Electrophoretic analysis of the [^{3}H]-methyl labeled RNA

Table 1

PROPERTIES OF [^{3}H]-METHYL LABEL INCORPORATED INTO RNA PREPARED FROM CULTURES HARVESTED EARLY AND LATE IN INFECTION

Treatment	Percent Resistant Early RNA	Late RNA
None	100	100
KOH	0.91	2.1
None	100	100
DNAse I	99.5	80.5
RNAse A	0.54	1.3
Pronase	94.5	89.5

Cultures were labeled with [^{3}H]-methyl methionine as described in the Methods section. Cytoplasmic RNA was isolated by phenol-chloroform-isoamyl alcohol extraction and dissolved in 10 mM Tris buffer pH 8.0. Early cytoplasmic RNA (input 16,219 cpm) and late cytoplasmic RNA (input 2588 cpm) were incubated with RNAse A (10 µg/ml); pronase (10 µg/ml), or DNAse I (5 µg/ml in 5 mM Mg) at 37° for 1 hr. Aliquots were also digested with 1 N KOH for 1 hr at 80°. Following each treatment the acid-precipitable radioactivity was determined.

which did not bind to oligo-dT-cellulose provided evidence that there was not significant incorporation of the methionine methyl into purine groups. Late in adenovirus infection there is extensive synthesis of a 5.5S RNA which is virus specified (23,24,25) as well as continued synthesis of cellular 5S RNA (25). Neither of these RNAs is methylated (26,27). RNA labeled late in infection with [^{3}H]-methyl methionine contained a radioactive tRNA peak (Fig. 1A); radioactive 5S and 5.5S RNA were not present, the expected result when labeling with a precursor utilized only for

Table 2

YIELD OF [^{3}H]-METHYL LABELED AND [^{3}H]-URIDINE LABELED CYTOPLASMIC RNAs SYNTHESIZED EARLY AND LATE IN PRODUCTIVE INFECTION

Labeling Time	Precursor	Yield (cpm/3x10^8 cells)	Percent binding to oligo-dT-cellulose
late	uridine	32.5 x 10^6	8.3
late	methionine	4.4 x 10^6	2.1
late	methionine	2.3 x 10^6	3.1
early	uridine	25.4 x 10^6	17.2
early	methionine	5.3 x 10^6	0.3
early	methionine	7.5 x 10^6	1.2

Early RNAs were isolated from cultures labeled with either [^{3}H]-uridine (12.5 μCi/ml) or [^{3}H]-methyl methionine (30 μCi/ml) from 2-6 hours after infection. Later RNAs were prepared from cultures labeled 18-21 hours after infection with either [^{3}H]-uridine (12.5 μCi/ml) or [^{3}H]-methyl methionine (20 μCi/ml).

the methyl groups of nucleotides. A control RNA preparation labeled with [^{3}H]-uridine contained a single peak of 5.5S and 5S RNA in addition to tRNA (Fig. 1B). Since ribosomal RNA synthesis is reduced more than 85% by the infection (28), only small amounts of 28S and 18S RNA were detected in RNA labeled with both [^{3}H]-uridine and [^{3}H]-methyl methionine.

RNA-DNA hybridization demonstrated that a significant portion of the [^{3}H]-methyl methionine incorporated into mRNA was virus specified (Fig. 2). Poly A containing molecules were selected by oligo-dT-cellulose chromatography and then annealed to filters containing increasing amounts of viral DNA. Early RNA labeled with [^{3}H]-uridine annealed 45% (Fig. 2A); a parallel preparation labeled with [^{3}H]-methyl methionine annealed 39%

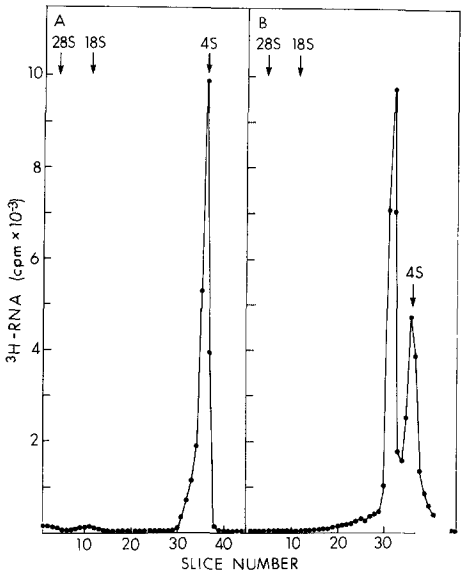

Figure 1: Polyacrylamide gel electrophoresis of nonpolyadenylated RNA from infected cells labeled with [^{3}H]-methyl methionine or [^{3}H]-uridine. Cytoplasmic RNA prepared from cultures labeled late in infection was fractionated by chromatography on oligo-dT-cellulose. The excluded RNA was concentrated by ethanol precipitation and analyzed on 3.2% polyacrylamide gels cross-linked with ethylene diacrylate. Electrophoresis was at room temperature for 4 hrs, 5 mA/gel. The positions of [^{14}C] ribosomal RNA and tRNA markers are indicated. (A) [^{3}H]-methyl RNA; (B) [^{3}H]-uridine RNA.

(Fig. 2B). Of the poly A containing RNA synthesized at late times in infection, at least 80% is virus specified (19,20). When [^{3}H]-methyl RNA was prepared from cultures labeled at 18 hrs after infection, 35% of the poly A containing RNA annealed to 16 μg of viral DNA (Fig. 2C). Since this amount of DNA was not sufficient for exhaustive hybridization, it is likely that a high percent of the [^{3}H]-methyl label incorporated into mRNA synthesized late in infection is virus specified.

These observations demonstrate the incorporation of [^{3}H]-methyl label into cytoplasmic adenovirus RNA. However, the structure and location of the methylated nucleotides, as well

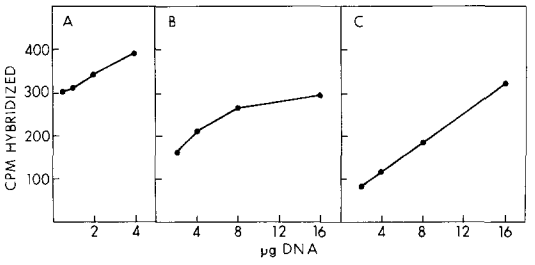

Figure 2: Hybridization of cytoplasmic poly A containing RNAs labeled with [^{3}H]-uridine or [^{3}H]-methyl methionine. Cytoplasmic RNA was prepared from cultures labeled either early or late in productive infection. The poly A containing RNA was purified by oligo-dT-cellulose chromatography and annealed to increasing amounts of adenovirus 2 DNA. Hybridizations were performed with 6.5 mm cellulose nitrate filters at 66°C for 20 hrs. Panel A shows the results of hybridization of 860 cpm early RNA labeled with [^{3}H]-uridine. For the hybridization of [^{3}H]-methyl labeled early RNA 758 cpm were used (B) and for [^{3}H]-methyl labeled late RNA the input was 915 cpm (C).

as the level of methylation, remain to be determined. In the present study the amount of methylation in viral RNA relative to ribosomal RNA could not be determined, for the early RNA was synthesized in the presence of cycloheximide, a drug which inhibits ribosomal RNA synthesis (29,30), and late in adenovirus infection 28S and 18S ribosomal RNA synthesis is inhibited at least 85% (28). In this initial study, we have not used specific adenovirus 2 DNA fragments to determine if individual viral mRNA species derived from different regions of the genome (31) all contain methylated nucleotides. However, the fact that early RNAs labeled with [^{3}H]-uridine and [^{3}H]-methyl methionine annealed to the same extent to adenovirus DNA suggests that most early viral mRNAs must be methylated. Since adenovirus RNAs undergo processing prior to exit from the nucleus (32-35), the methylation of specific nucleotides may play an important role in the maturation of viral mRNA as well as in its translation.

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