Capping of U6 Small Nuclear RNA in Vitro Can Be Uncoupled from Transcription*

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U6 small nuclear RNA (snRNA) is a required component in the splicing of eukaryotic pre-mRNAs. Mammalian U6 snRNA was synthesized in vitro by T7 RNA polymerase and purified on polyacrylamide gels. This U6 RNA, with pppG on its 5' end, was accurately capped to CH3-O-pppG, when incubated with HeLa cell extract and this capping was dependent on the capping signal present within the U6 snRNA. When 32P-labeled U6 RNA was used as a substrate, the U6 cap formed in vitro retained this labeled 32P-phosphate, indicating that the cap formation involves the methylation of the 3' phosphate incorporated during transcription. U6 snRNAs with ppG or pG as their 5' ends, were not capped in this in vitro capping system. Capping of U6 snRNA in vitro requires at least two components, a heat-labile component and S-adenosylmethionine as a methyl group donor. The data presented here show that capping of U6 snRNA can be uncoupled from transcription and that the mechanism of U6 snRNA cap formation differs markedly from the capping mechanism of mRNAs and other U snRNAs where capping is cotranscriptional. While many methyltransferases have been characterized earlier, this is the first report of a methyltransferase that is specific to phosphate residues. This in vitro capping system will be useful for purification and studies on the U6 snRNA sequence-dependent methyltransferase activity.

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MATERIALS AND METHODS

Fine Chemicals—The nucleases P1, alkaline phosphatase, AdoMet, and S-adenosylhomocysteine were from Sigma; T2 RNase was from Sankyo; T7 polymerase was from Stratagene; polynucleotide kinase was from New England Biolabs; [α-32P]GTP, [γ-32P]GTP, and [γ-32P]ATP were obtained from Amersham Corp.; methyl GTP standard was synthesized from [α-32P]GTP and methanol (Darzykowski et al., 1985), and pGp was synthesized by labeling Gp with [γ-32P]ATP and polynucleotide kinase.

Construction of U6 Mutants—The mouse U6 RNA gene (Das et al., 1988) and Syrian hamster 5 S RNA gene (Hart and Folk, 1982) were used as controls. Construction of altered U6 genes was carried out using standard recombinant DNA techniques. The U6 RNA used for in vitro capping studies was constructed in the following manner. The DNA sequence corresponding to the mammalian U6 RNA was ligated to the T7 promoter obtained by annealing two synthetic...
oligonucleotides and was then cloned into BamHI/HindIII sites of the pUC19 vector. When this DNA was truncated with EcoRV and transcribed with T7 polymerase, the resulting RNA was 208 nt long; Nts 1-106 corresponded to the mammalian U6 RNA, nts 107-190 corresponded to 3' non-transcribed spacer of the mouse U6 gene (Das et al., 1988) and nts 191-208 were from the vector DNA (Fig. 1). The 208 nt-long RNA was fractionated on polyacrylamide gels, extracted, precipitated with ethanol and used for studies on capping in vitro.

In Vitro Transcription of 5S, U6, and Mutated U6 Genes—The appropriate plasmid DNA's were transcribed in HeLa cell extract prepared according to Well et al. (1979). In a typical transcription reaction, a 50-μl reaction mixture containing 2 μg of supercoiled plasmid DNA was incubated at 30 °C for 3 h. The reaction mixture contained 50% (v/v) HeLa cell extract, 0.6 mM each of unlabeled CTP, UTP, ATP, 0.025 mM GTP, 5 μCi of [α-32P]GTP, 6 mM creatine phosphate, 20 mM KCl, 1 mM DTT, and 10 mM Tris, pH 8.1. The transcribed RNAs were phenol extracted, precipitated with ethanol, and purified by electrophoresing on 5 or 10% polyacrylamide gels. U6 RNA or other RNA transcripts were extracted from the gels and utilized for further studies.

Fractionation of HeLa Extract—The HeLa cell extract was loaded on a DEAE-Sephadex-A25 column in 10 mM Tris buffer, pH 8, containing 100 mM KCl, and was washed with the above buffer. The bound material was eluted step-wise with buffer containing 0.5, 1, 1.5, 2, and 3 mM KCl. The eluted material was concentrated and resuspended in buffer containing 100 mM KCl.

In Vitro Capping—Labeled RNA transcripts (usually 10,000-100,000 cpm by Cerenkov counting) were incubated in a 50-μl reaction mixture at 30 °C for 30-45 min. The reaction mixture contained the substrate RNA, 150 mM KCl, 50 mM Tris, pH 8, and 35 μl of HeLa cell extract. The reaction was terminated by the addition of sodium dodecyl sulfate/phenol, and the RNA was precipitated from the aqueous phase with ethanol. The RNAs were digested with nuclease P1 or T2 RNase. In some instances the nuclease P1 digests were also treated with bacterial alkaline phosphatase at pH 8. These digests were fractionated by electrophoresing on DEAE-cellulose paper at pH 3.5 (Epstein et al., 1980). Autoradiography was done for 1-7 days at −70 °C using XAR-5 film with Lightning Plus screens.

Capping Efficiency—The autoradiograms were scanned with a LKB laser beam densitometer. The capping efficiency was determined from the relative amounts of pppG and CH3-0-pppG at the end of the capping reaction.

RESULTS

Transcription and Capping of U6 RNA Can Be Uncoupled—When the mammalian U6 gene was transcribed in vitro using HeLa cell extract, a fraction of the synthesized U6 RNA was accurately capped (Fig. 2, lane 2 and Singh et al., 1990). To see whether U6 RNA can be capped post-transcriptionally, we prepared U6 RNA using T7 polymerase and purified it on polyacrylamide gels; this RNA is referred to as T7-U6 RNA. The T7-U6 RNA contained pppG as its 5' end (Fig. 2, lane 3) and when incubated with HeLa cell extract for 30 min, a fraction of the T7-U6 RNA was capped accurately (Fig. 2, lane 4). These data show that transcription and capping of U6 snRNA can be uncoupled. The T7-U6 transcript used in this study contained 102 extra nucleotides on its 3' end. These additional sequences are unlikely to have any significant effect on the efficiency of capping, since our earlier studies showed that the 5' 25 nucleotides of U6 snRNA are required and sufficient for capping; additional sequences of up to 350 nucleotides had no detectable effect on capping (Singh et al., 1990).

The optimum conditions for capping U6 snRNA in vitro were determined and are given under "Materials and Methods." The efficiency of conversion ranged from 30 to 80% in a typical 30 min reaction; there was detectable cap structure formed after 2 min incubation, the shortest time period tested (results not shown). The capping efficiency varied depending on the conditions used for this reaction. In some experiments, the reaction mixture also contained nucleotides, creatine phosphate, and low salt concentration and under these conditions the capping efficiency was less. Both KCl and Mg2+ were required as there was no detectable capping in their absence. The capping reaction was not dependent on ATP and was not affected by the addition of EGTA, indicating that Ca2+ ions are not required for the capping reaction. Addition of EDTA resulted in extensive degradation of added labeled RNA, and there was no detectable pppG at the end of incubation (results not shown).

Post-transcriptional Capping in Vitro Was Dependent on a Capping Signal in U6 RNA—We tested whether the cap structure formed post-transcriptionally requires the capping determinant characterized previously (Singh et al., 1990). While the T7-U6 RNA was capped after incubation with HeLa cell extract (Fig. 2, lane 4), the 5 S RNA (Fig. 2, lane 6) or U6 1(k)26 RNA in which the capping signal (nucleotides between 1-26 of U6 snRNA) was deleted (Fig. 2, lanes 8 and 9) were not capped. These data show that the capping observed in this in vitro system is not random but is specific to U6 snRNA containing the capping signal.

The γ-Phosphate of T7-U6 RNA Was Retained in the Cap Structure—During the m7G cap formation in mRNAs and other U RNAs, the γ-phosphate from the pppN is removed from the pre-mRNA transcript and the α-phosphate of the capping G is retained (reviewed in Banerjee, 1980; Shatkin, 1976). To understand the origin of the three phosphate residues in the U6 snRNA cap, the RNA was labeled with [γ-32P]GTP and the fate of this labeled γ-phosphate was followed during the in vitro capping reaction. As shown earlier (Singh et al., 1988), the T7-U6 transcript used in this capping reaction was accurately capped (Fig. 2, lane 2 and Singh et al., 1990). The γ-phosphate of T7-U6 RNA labeled in vitro was retained in the cap structure (Fig. 2, lane 2) and was not affected by the addition of EDTA, indicating that Ca2+ ions are not required for the capping reaction. Addition of EGTA resulted in extensive degradation of added labeled RNA, and there was no detectable pppG at the end of incubation (results not shown).
et al., 1990), the cap was labeled when U6 RNA with label in the α-phosphate was used as substrate (Fig. 3, lane 3). The cap structure formed with the γ-phosphate-labeled U6 RNA, was also labeled (Fig. 3, lane 6). This cap structure had the same electrophoretic mobility as the cap formed with other U6 RNA substrates (Fig. 3, lanes 1 and 3), and it was resistant to digestion with alkaline phosphatase (Fig. 3, lane 7). These data show that during U6 RNA cap formation, the γ-phosphate is not removed from the substrate; instead, the γ-phosphate gets methylated.

U6 snRNAs Containing pG or ppG on Their 5' Ends Are Not Recognized as Substrates for Methylation—When the U6 RNA gene or 5 S RNA gene was transcribed in vitro using the HeLa cell extract, the RNAs initially made with ppG were found to be converted partly to ppG and pG (Fig. 4, lanes 1, 2, 7, and 9). This is presumably due to phosphatase activity present in the extract. If the capping machinery is able to cap U6 RNA at α or β positions, these U6 RNA substrates with ppG or pG termini present during transcription would have been converted to CH₂-O-ppG and CH₂-O-pG, respectively. However, there were no detectable spots corresponding to CH₂-O-ppG or CH₂-O-pG (Fig. 4, lane 4), indicating that ppG and pG-containing U6 RNAs are not substrates for capping and the capping machinery appears to be specific to the γ-phosphate of U6 snRNA.

To directly test whether mono- or diphosphate-containing U6 RNA can be capped in vitro, α-³²P-labeled T7-U6 RNA and U6 RNA transcribed from U6 gene were incubated with HeLa cell extract. Again only CH₂-O-pppG was detectable and there was no detectable CH₂-O-ppG or CH₂-O-pG (Fig. 4, lanes 11 and 12), suggesting that the capping machinery is rather stringent in requiring γ-phosphate for capping.

Effect of Distance between ppG and the Capping Signal—We carried out studies with three different T7 polymerase-transcribed RNAs, where the ppG was shifted 32, 51, or 361 nts away with reference to the capping determinant. In all these cases, there was no readily detectable cap structure formed (results not shown). These data confirm our earlier data and show that the γ-phosphate has to be in close proximity to the capping determinant for optimal capping.

Factors Involved in the Capping of U6 RNA—Since the γ-phosphate is retained and gets methylated during the capping of U6 RNA, we carried out studies to identify the methyl group donor and the factor(s) involved in this methylation reaction. The HeLa extract was heated for 5 min at 65 °C and tested for its ability to cap U6 snRNA. While the normal HeLa cell extract capped T7-U6 snRNA in vitro (Fig. 5, lane 1), the heat-treated extract was not capable of capping the T7-U6 snRNA (Fig. 5, lane 3), indicating that one or more heat-labile factor(s) are required for the capping reaction.

When a U6 capping-permissive extract was fractionated on a DEAE-Sephadex column, several fractions were obtained upon elution with different concentrations of KCl. These fractions were tested for their ability to cap T7-U6 snRNA in vitro. The fraction eluted with 0.5 M KCl was not capable of capping the T7-U6 RNA (Fig. 5, lane 5); however, this fraction when supplemented with AdoMet supported the capping reaction (Fig. 5, lane 6). The cap structure formed in the presence of AdoMet was resistant to digestion by alkaline phosphatase (Fig. 5, lane 7). In addition, the U6 snRNA capped with the addition of S-adenosylhomocysteine, a known inhibitor of methylation reactions mediated by AdoMet (Fig. 5, lane 8). These data show that AdoMet serves as a methyl group donor during in vitro capping of U6 snRNA.

**FIG. 3.** Analysis of cap structures formed from α- and γ-labeled T7-U6 RNAs. Lane 1, RNA obtained by transcribing mouse U6 DNA in vitro; lane 2, T7-U6 RNA labeled with [α-³²P]GTP; lane 3, α-³²P-labeled T7-U6 RNA incubated with HeLa cell extract; lane 4, same as in lane 3 but also digested with alkaline phosphatase; lane 5, T7-U6 RNA labeled with [γ-³²P]GTP; lane 6, γ-³²P-labeled T7-U6 RNA incubated with HeLa cell extract; lane 7, same as in lane 6 but also digested with alkaline phosphatase. The RNAs were digested with nuclease P1 (and also with alkaline phosphatase in lanes 4 and 7), electrophoresed on DEAE-cellulose paper, and subjected to autoradiography. 0', RNAs before incubation with HeLa extract; 30', period of incubation with HeLa extract.

**FIG. 4.** Analysis of cap structure from U6 snRNA containing mono-, di-, or triphosphates on their 5' termini. Lane 1, U6 DNA transcribed in vitro and digested with nuclease P1; lane 2, 5 S RNA transcribed from Syrian hamster 5 S DNA transcribed in vitro and digested with nuclease P1; lanes 3 and 10, synthetic methyl GTP as standard; lane 4, same as lane 1 but also digested with alkaline phosphatase; lane 5, same as in lane 2 but digested also with alkaline phosphatase; lane 6, standard pGp; lane 7, same as lane 1 but digested with T2 RNase; lane 8, T7-U6 RNA digested with T2 RNase; lane 9, same as lane 2 but digested with T2 RNase; lane 11, U6 RNA transcribed from mouse U6 DNA in vitro, U6 RNA purified and incubated with HeLa cell extract and digested with nuclease P1 and alkaline phosphatase. Lane 12, T7-U6 RNA, labeled with [α-³²P] GTP, was incubated with HeLa cell extract and digested with nuclease P1 and alkaline phosphatase. The digested RNAs were electrophoresed on DEAE-cellulose paper and subjected to autoradiography. The positions of CH₂-O-ppG and CH₂-O-pG are indicated from the observed mobility of radioactive standards run in earlier experiments. In lanes 7, 8, and 9, only mononucleotides Gp, Ap, and Cp are included; Up, which migrates faster, is not included in this figure.

**DISCUSSION**

Data presented in this study show that presynthesized U6 snRNA can be accurately capped in vitro by HeLa cell extract. During this capping reaction the γ-phosphate is retained; AdoMet serves as the methyl group donor for the U6 snRNA sequence-dependent methylation of the γ-phosphate.

There are several lines of evidence to suggest that transcription and capping of mRNAs and U snRNAs are concurrent.
In RNA polymerase II transcripts, capping is cotranscriptional as studied in viral and cellular mRNAs (Furuichi, 1978 and references therein; also reviewed in Banerjee, 1980), as well as in U snRNAs (Skuzeski et al., 1984). Furthermore, only 5–10% of the T7 mRNA transcripts in mammalian cells contained terminal cap structure presumably because of the lack of coupling between capping enzyme and heterologous T7 polymerase (Fuerst and Moss, 1989). These data further contained terminal cap structure presumably because of the cell extracts post-transcriptionally. In this respect, the U6 snRNA-capping mechanism differs from the capping mechanism of pre-mRNAs and other characterized U snRNAs.

Recently, one U4/U6 snRNPs protein has been characterized (Banroques and Abelson, 1989), and SP6-transcribed U6 RNA was shown to reconstitute into snRNPs particles and incorporate into spliceosomes (Pikielny et al., 1989). It is not known whether the capping enzyme is part of the U6 snRNPs particle or whether cap structure is required for U6 snRNPs function in the spliceosome. Since the T7-U6 snRNA is inhibited by S-adenosylhomocysteine (Fig. 5), it is possible to study the requirement of cap structure for U6 snRNPs function. If the caps in U6 RNA are required for spliceosome assembly and splicing of pre-mRNAs, addition of S-adenosylhomocysteine should result in inhibiting these reactions.

Where in the cell does U6 snRNA get capped? The capping of other U snRNAs takes place in two stages: the addition of m'G is coupled to transcription in the nucleus (Skuzeski et al., 1984) and the trimethylation of the m'G cap is cytoplasmic (Mattaj, 1986). The capping of U6 snRNA appears to be cytoplasmic. When T7-U6 snRNA was injected into nucleated frog oocytes, accurate capping of U6 RNA was observed. These data suggest that capping of U6 snRNA occurs post-transcriptionally in the cytoplasm.

The DNA and RNA methylation reactions involving AdoMet characterized earlier do not require magnesium ions (Martin and Moss, 1975; Yuan and Hamilton, 1984); however, some methylation reactions are stimulated in the presence of Mg2+ ions (reviewed in Yuan and Hamilton, 1984). The methylation reaction characterized here requires magnesium ions, and there was no detectable capping with HeLa extract depleted of Mg2+ ions. Since the stem-loop structure in U6 snRNA is required for the U6 snRNA cap formation (Singh et al., 1990), it is likely that the Mg2+ ions are necessary to facilitate U6 RNA in acquiring the appropriate secondary structure required to direct the cap formation. It is also possible that the U6 RNA sequence-dependent methyltransferase is different from other methyltransferases in requiring magnesium ions.

During the mRNA cap formation from mRNA and ppG, the γ-phosphate of the mRNA is removed and does not appear in the mature mRNA. In contrast, data obtained in this study show that the γ-phosphate of U6 snRNA is not removed during cap formation. Therefore, the mechanism of U6 cap formation is very different from the mechanism that has been well characterized for mRNAs (reviewed in Banerjee, 1980).

In addition to the requirement for the capping signal present within the U6 snRNA (Singh et al., 1990, and Fig. 2), the capping machinery methylated only the γ-phosphate although U6 snRNAs with pG and ppG on their 5' ends were also present. This suggests that the γ-phosphate has to be in the right context with reference to the capping signal. Whenever the ppG was moved away from the capping signal, the efficiency of capping was <1%. These data suggest that the only requirement in the U6 snRNA substrate for the capping is the capping signal and the γ-phosphate in the right position.

Many DNA sequence-dependent methyltransferases have been purified and characterized (Holliday, 1989; Syzfy et al., 1989; Yuan and Hamilton, 1984, and references therein). Since most cellular RNAs, including ribosomal, transfer, and messenger RNAs, are methylated post-transcriptionally (Perry, 1981), many RNA sequence-dependent methyltransferases must exist in the cells. Recently, RNA sequence-dependent accurate methylation of internal adenosine residues in messenger RNA was reported (Narayan and Rottman, 1988); however, all the known methyltransferases modify either the base or the sugar moieties in nucleic acids. This is the first instance of RNA sequence-dependent methyltransferase that modifies the phosphate residue. The ability to reconstitute the capping with a DEAE-Sephadex fraction and AdoMet provides evidence that there are at least two components necessary for capping the T7-U6 snRNA. This assay system will be useful in purifying and studying the mechanism of action of the capping enzyme.

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References—The capping signal and the γ-phosphate in the right position.

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