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Assembly of pre-mRNA splicing complex is cap dependent

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ABSTRACT

To study the influence of the ubiquitous cap structure of nuclear pre-mRNAs on the assembly of a functional splicing complex, the *in vitro* splicing of a truncated human metallothionein pre-mRNA was examined in the presence of the cap analogue m^7GTP. Significant inhibition of splicing was observed at a concentration as low as 5 µM m^7GTP. Analysis of the splicing reaction on glycerol density gradients showed two complexes sedimenting at 45S and 22S. When the reaction was carried out in presence of m^7GTP a marked decrease of the material sedimenting at 45S, representing the active splicing complex, was observed. When capped pre-mRNA was replaced by uncapped pre-mRNA, complex formation was significantly reduced. These data indicate that the cap structure plays an important yet unknown role in the assembly of spliceosomes.

INTRODUCTION

Much progress in the understanding of pre-mRNA processing has been achieved by the development of efficient *in vitro* splicing systems (1-3). Recent studies with yeast extracts and nuclear extracts from HeLa cells have shown that the formation of high molecular weight multicomponent complexes is an early event of the splicing process (4-7). The active splicing complex sediments at 40-60S and contains pre-mRNA, RNA intermediates and splicing products. Smaller complexes with 22S and 35S in HeLa extracts and 15-35S in yeast extracts have also been observed. These complexes, which are formed prior to the assembly of the 40-60 S complex contain the precursor RNA but no splicing intermediates.

Assembly of the active splicing complex requires ATP, U-snRNPs, and hnRNP core proteins, together with pre-mRNA which contains at least an intact 5' splice site and the poly-

pyrimidine stretch adjacent to the 3' splice site (6,8,9). The involvement of other structural features of the RNA is not well understood at present. One of these is the cap structure, $m^7G(5')ppp(5')N$, a modification of the 5' end of all eucaryotic cellular mRNAs which is added immediately after the initiation of transcription (10). The cap structure is known to have an important function in the initiation of protein synthesis (11). Recently, however, considerable evidence has accumulated to support the notion that the cap structure might play a role in pre-mRNA splicing. Using *in vitro* RNA processing extracts it has been shown that capped pre-mRNAs are spliced more efficiently and that they produce fewer aberrant splicing products (3,12). Furthermore, cap analogues reduce the efficiency of the splicing reaction in HeLa whole-cell extracts as well as in nuclear extracts (12,13). These findings suggest that some factor(s) of the splicing complex interact(s) with the cap structure of precursor mRNAs. Using photoaffinity labelling techniques we have previously identified three nuclear proteins in HeLa cells which recognize the cap structure (14). One or more of these nuclear cap binding proteins (NCBP 1, 2 and 3) might be somehow involved in the splicing process. Therefore we decided to investigate in more detail the role of the cap in *in vitro* RNA processing systems.

In this report we show that the *in vitro* assembly of a 45S pre-mRNA splicing complex is inhibited by a cap analogue. Furthermore, formation of this complex is strongly reduced when RNA substrates lacking the 7-methylated cap structure are used in the *in vitro* reaction. These results suggest an important function of the cap structure in the assembly of the active splicing complex.

MATERIALS AND METHODS

Construction of SP6 template

The Bam HI fragment of the human metallothionein-II_A gene containing a part of exon 1, intron 1, exon 2 and 15 additional nucleotides of intron 2 was inserted into the Bam HI site of pSP65. The resulting plasmid (pSP65 ΔhMT-II_A) was linearized by digestion with HindIII and was used as a template for *in vitro*

SP6 transcription (Fig. 1). The complete hMT-II_A gene cloned in pBR322 was kindly provided by Dr. M. Karin (15).

Preparation of RNA precursors

In vitro transcriptions of pSP65 ΔhMT-II_A were performed as described by Melton et al. (16) with the following modifications: 2 µg linearized DNA template, 625 µM each of ATP and CTP, 62.5 µM each of GTP and UTP, 1.25 mM GpppG (for synthesis of capped pre-mRNA), 32 units RNasin, 12.5 mM dithiothreitol, 10 units SP6-polymerase, 60 µCi (α-^{32}P) UTP in a 20 µl reaction volume was incubated at 40°C for 2 hrs. For the synthesis of uncapped RNA, GpppG was omitted and the concentration of GTP was increased to 625 µM. The transcripts were purified as described (16) and stored in 50% ethanol at -20°C.

In vitro splicing reaction

The HeLa cell nuclear extract used for the splicing reactions was prepared according to a modified protocol of Dignam et al. (17) and will be described elsewhere (18).
Standard splicing reactions (15 µl) contained 1 mM ATP, 20 mM creatine phosphate, 2.5 mM $MgCl_2$, 600 U/ml RNasin, 66% nuclear extract and 1 x 10^5 Cerenkov cpm of ^{32}P labelled pre-mRNA (20-30 fmol). The reaction mixture was preincubated in the presence or in the absence of m^7GTP for 30 min at 30°C. Subsequently the pre-mRNA was added and the incubation continued for 2 hrs at 30°C. The reactions were stopped by incubation with 0.4 mg/ml proteinase K for 30 min at 37°C. After phenol / chloroform extraction and precipitation with ethanol, the RNA was analyzed on 8% polyacrylamide / 8 M urea gels.
For the study of splicing complexes on glycerol gradients in vitro reactions were carried out in 30 µl in the presence of 0.86 mM ATP, 17.33 mM creatine phosphate, 2.1 mM $MgCl_2$, 400 U/ml RNasin, 66% nuclear extract and 1.5 x 10^6 Cerenkov cpm ^{32}P RNA substrate (0.4 pmol). Conditions of incubation were the same as above. The reactions were stopped by addition of an equal volume of 40 mM HEPES-potassium salt (pH 7.9), 100 mM KCl and by quick freezing in liquid nitrogen. Storage was usually overnight at -70°C.

Debranching reaction

The lariats were eluted from gel slices with 0.5 M sodium-acetate, 0.5% SDS for 4 hrs at 65°C and precipitated with ethanol in presence of 10 µg carrier tRNA. After phenol extraction and ethanol precipitation the RNA was incubated with cytoplasmic S100 extract under the standard conditions for enzymatic debranching as described (19).

Glycerol gradient centrifugation

Diluted splicing reactions (60 µl) were thawed and loaded onto 10-30% glycerol gradients containing 40 mM HEPES-potassium salt (pH 7.9), 100 mM KCl and 1 mM $MgCl_2$. Centrifugation was performed at 35,000 rpm at 4°C in a Beckman SW 40 rotor for 5 hrs. Radioactivity of the fractions (400 µl) was determined by Cerenkov counting. The sedimentation values of the peaks were estimated by comparison with procaryotic ribosomal subunits run on parallel gradients. For polyacrylamide gel electrophoresis, the fractions were incubated overnight with 200 µg/ml proteinase K at 4°C in presence of 4 µg carrier tRNA. The RNA was prepared by phenol / chloroform extraction and precipitation with ethanol and analyzed on 8% polyacrylamide / 8M urea gels.

RESULTS

The pre-mRNA substrate used for the in vitro splicing reaction was derived from the human metallothionein-II_A gene (15). The truncated gene was inserted into the plasmid pSP65, and ^{32}P-labelled RNA was produced by in vitro transcription using the SP6 system. Capped RNA was obtained by priming the transcription with the dinucleotide GpppG. The unmethylated cap structure is known to be converted to the methylated form during incubation in the splicing extract (12). The plasmid construction, the pre-mRNA and the expected intermediates and products of the splicing reaction are schematically shown in Fig. 1. The pre-mRNA substrate is 459 nucleotides in length and contains pSP65 sequences at both ends, part of exon 1, intron 1 and exon 2 with a few additional nucleotides from intron 2.

In order to test the ability of the in vitro system to carry out processing of the capped ΔhMT pre-mRNA substrate the

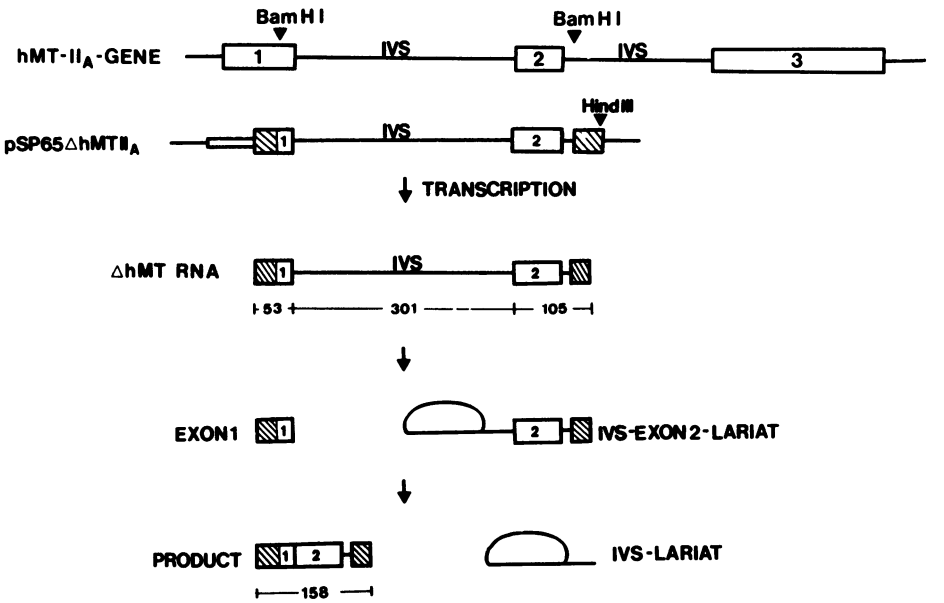

Fig.1: Construction of the plasmid pSP65ΔhMTII$_A$ from the human metallothionein-II$_A$ gene and processing scheme of the derived precursor RNA. Exons are shown as boxes, the intervening sequences (IVS) are shown as lines. Dashed boxes represent sequences derived from the SP6 plasmid. Numbers of nucleotides of the various parts of the RNA are indicated.

RNA was incubated with HeLa nuclear extract under conditions specified in the methods section. The reaction products were analyzed on a polyacrylamide gel (Fig. 2, lane 2) and compared to the pre-mRNA substrate (lane 1). The different RNA molecules were identified using DNA size markers. The structure of the products and intermediates of the reaction is schematically drawn on the right (Fig. 2, a-e). The RNA obtained after excision of the intron and religation of both exons is expected to contain 158 nucleotides corresponding to the migrational distance of band d, lane 2. Exon 1 with 53 nucleotides can be seen near the bottom of the gel (band e). It is known that RNA lariat forms have abnormal electrophoretic mobilities (20,21). In the gel system used they are expected to migrate more slowly than the pre-mRNA. Thus the intron-exon lariat can be identified as band a. The two bands with slightly different

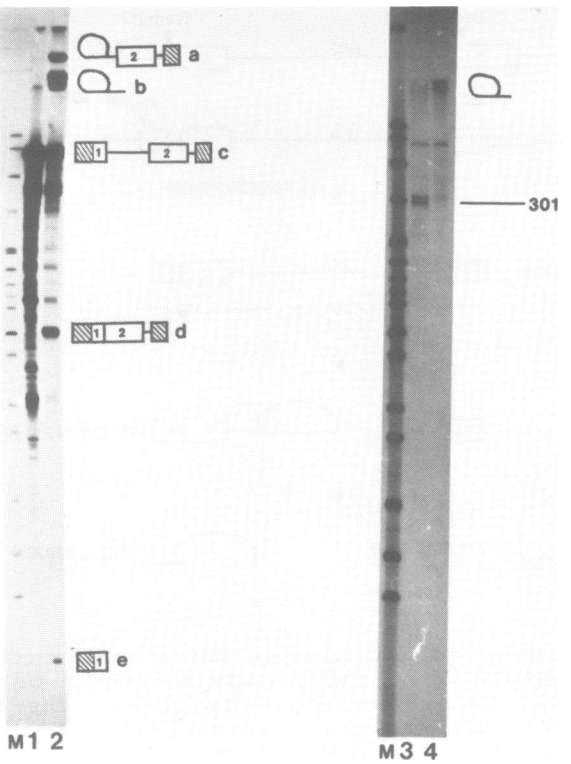

Fig.2: Analysis of the in vitro splicing products of ΔhMT pre-mRNA on 8% polyacrylamide / 8M urea gels. The various RNA intermediates and end products are shown as drawings; a: intron-exon lariat; b: intron lariat; c: pre-mRNA; d: ligated exon 1 and exon 2; e: exon 1. M: ^{32}P labelled HpaII restriction fragments of pBR322. Lane 1, pre-mRNA; lane 2, splicing reaction; lane 3, intron lariat (b) after elution from lane 2 and debranching reaction; lane 4, intron lariat (b) after elution from lane 2.

mobilities at position b, can be identified as two forms of the intron lariat as both RNAs are converted to linear molecules upon incubation with debranching extract. For this purpose the RNAs migrating at position b of lane 2 were eluted from the gel. Part of the material was incubated with debranching extract (lane 3) and compared to the original material on a polyacrylamide gel (lane 4). The result of the debranching reaction (lane 3) demonstrates that the linear RNAs migrate at a position corresponding to a length of about 300

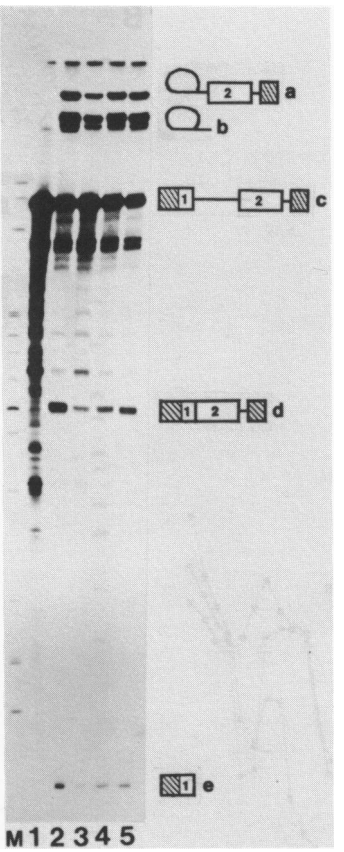

Fig.3: Inhibition of the splicing reaction by various concentrations of m^7GTP. The reaction mixture was analyzed on a 8% polyacrylamide / 8M urea gel. M: as in figure 2. Lane 1, pre-mRNA; lane 2, splicing reaction in absence of m^7GTP (control); Splicing reactions in presence of m^7GTP at concentrations of 100 µM (lane 3), 10 µM (lane 4) and 5 µM (lane 5).

nucleotides. The faster migrating band probably represents the product of nucleolytic degradation from the 3' end (19).

In order to investigate the cap dependence of this *in vitro* splicing system, the extracts were preincubated for 30 min with various concentrations of the cap analogue m^7GTP prior to the addition of the pre-mRNA. Analysis of the RNA molecules obtained under these conditions clearly shows that 100 µM m^7GTP drastically reduces the amount of the spliced product (Fig. 3,

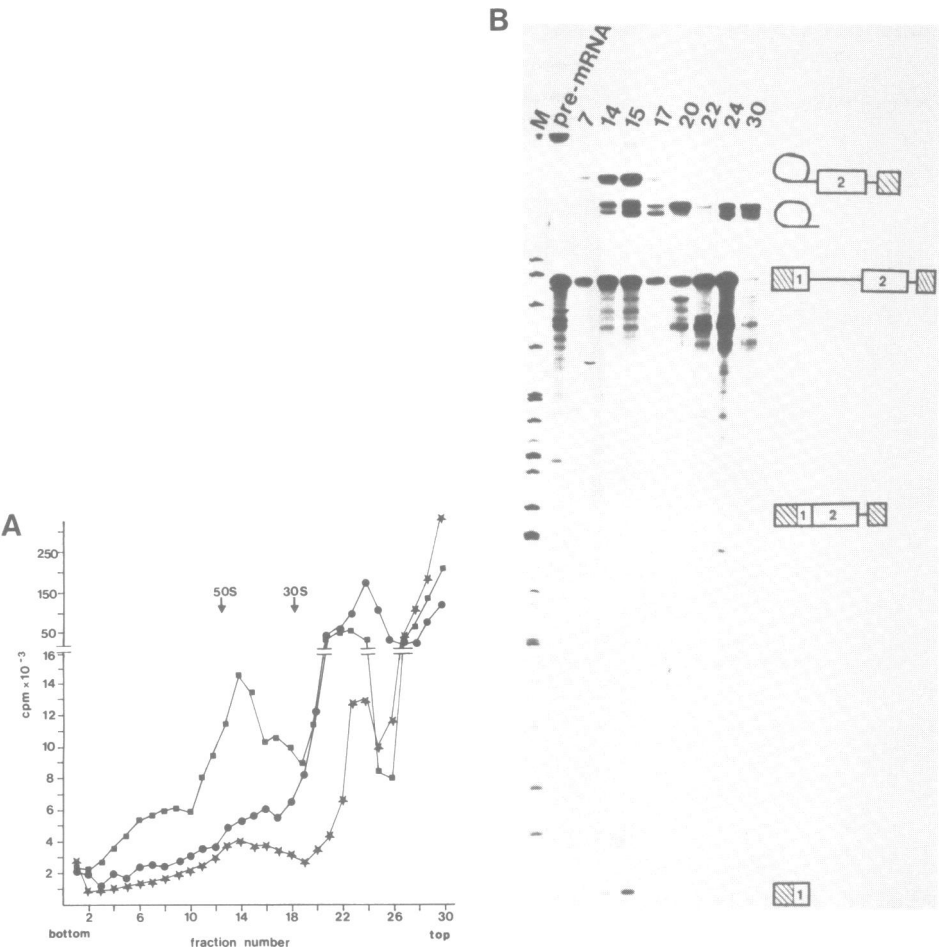

Fig.4: Sedimentation analysis of the in vitro splicing reaction.
(A) Profile of glycerol density gradient centrifugation. Capped ^{32}P-labelled ΔhMT pre-mRNA was incubated with HeLa nuclear extract in absence (gradient 1, squares) or in presence of 100 μM m^{7}GTP (gradient 2, circles). The incubation was also performed with uncapped pre-mRNA (gradient 3, stars). The reaction mixtures were centrifuged through a 10-30% glycerol gradient and the radioactivity of the fractions was determined by Cerenkov counting. The position of procaryotic ribosomal subunits as determined on a parallel gradient is indicated.
(B) Gel analysis of the RNA extracted from the peak fractions of gradient 1. The RNAs were analyzed on a 8% polyacrylamide/ 8M urea gel and visualized by autoradiography. Fractions analyzed are indicated by numbers above the gel lanes. M: as in figure 2.

lane 3) when compared to the control (lane 2). 10 μM and 5 μM m^7GTP still inhibit splicing significantly, but to a lesser extent (lane 4 and lane 5 respectively, see band d and e).

The assembly of a complex with sedimentation values between 40 and 60 S is an absolute requirement for splicing to occur (4-6). We therefore examined the effect of m^7GTP on the formation of splicing complexes on density gradients. HeLa nuclear extract was preincubated for 30 min at 30 °C either in presence of 100 μM m^7GTP or in its absence (control). After addition of pre-mRNA the incubation was continued for additional 90 min. The samples were diluted and frozen in liquid nitrogen to stop the reaction. The reaction mixtures were run on 10-30 % glycerol gradients, and the radioactivity of the fractions was determined (Fig. 4A). The control (gradient 1) shows two well-separated peaks sedimenting at approximately 22 S and 45 S. Analysis of the RNA obtained from the material sedimenting at 45S on 8% polyacrylamide gels shows the presence of the intron-exon lariat together with exon 1 indicative for an active splicing complex (Fig.4B, fractions 14 and 15), whereas these intermediates are missing in the peak at 22S (fractions 22-24). When ATP and phosphocreatine were omitted in the splicing reaction, formation of the 45 S peak was not observed (data not shown). This is in accordance with the observation that assembly of the active splicing complex is energy dependent (5,6). The region at 22 S contains almost exclusively pre-mRNA and the intron lariat (Fig. 4B, fractions 22-24).

Preincubation of the nuclear extract with 100 μM m^7GTP resulted in an inhibition of the formation of the 45 S peak concomitant with an increase of the peak at 22 S (Fig. 4A, gradient 2). When the splicing reaction was carried out using the same amount of uncapped pre-mRNA 45 S complex formation was drastically reduced (gradient 3). This is in line with the observation of a lower splicing efficiency of uncapped pre-mRNA (3,12). In addition the total radioactivity recovered in the 22S and 45S peak fractions was less than that obtained with capped pre-mRNA. This is probably due to exonucleolytic degradation of the RNA from the unprotected 5' end. As expected

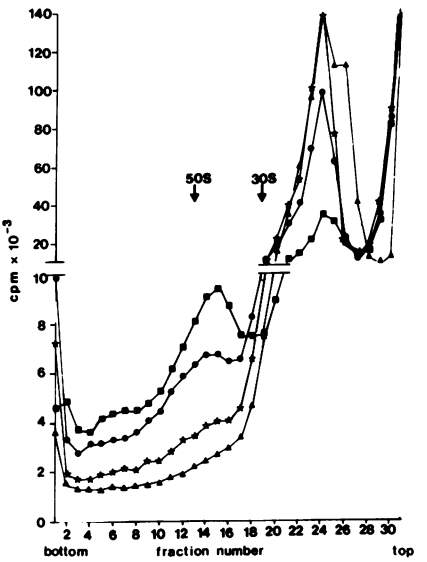

Fig. 5: Inhibition of 45S-complex formation by different concentrations of m^7GTP. Capped ^{32}P-pre-mRNA was preincubated with HeLa nuclear extract in presence of different concentrations of m^7GTP: gradient 1 (squares), 0 µM; gradient 2 (circles), 10 µM; gradient 3 (stars), 100 µM; gradient 4 (triangles), 1000 µM. The reactions were analyzed as described in figure 4A.

the same profile was obtained when the splicing reaction with uncapped pre-mRNA was carried out in the presence of 100 µM m^7GTP (data not shown).

In order to assess the inhibition of the 45S-complex formation in a more quantitative way the experiment outlined above was repeated at different concentrations of m^7GTP. As seen on Fig. 5 a gradual decrease of the 45S peak with increasing concentrations of the cap analogue was observed (see gradients 1 to 4, corresponding to 0, 10, 100, 1000 µM m^7GTP respectively). Even at 10 µM m^7GTP the assembly of the 45S complex was significantly inhibited. When m^7GTP was replaced by GTP the gradient profile was identical to the control (data not shown).

DISCUSSION

Evidence for a role of the cap structure in the processing of pre-mRNA has recently been provided by several groups. A decrease in both the efficiency and the accuracy of *in vitro* splicing has been observed when a pre-mRNA lacking the cap structure was employed (3) or when the reaction mixture was preincubated with cap analogues (12,13). This effect has been attributed to an involvement of the cap in an early step of pre-mRNA processing. However, a direct effect of m^7GTP or other cap analogues on the formation of spliceosomes has not been demonstrated so far. At least two different splicing complexes defined by their sedimentation constants on density gradients have been described in different systems (4-6). Analysis of constituents of these particles has revealed that the smaller complex contains predominantly pre-mRNA whereas the faster sedimenting complex contains the various splicing intermediates. The final products - the ligated exons and the intron lariat - are released from the spliceosome.

In the present work we have studied the formation of splicing complexes in the presence and in the absence of m^7GTP. The data presented show a strong inhibition of the assembly of the 45S complex by m^7GTP (Fig. 4A and Fig. 5). In contrast the amount of RNA in the slowly sedimenting particle (22S) was increased when the reaction was carried out in the presence of the cap analogue. Similarly, the 45S peak was considerably reduced when uncapped pre-mRNA was used as a substrate for the *in vitro* splicing reaction. This clearly shows the requirement of the cap for the progression of the pre-mRNA from a possibly unspecific ribonucleoprotein complex to the final complex which carries out the various reactions involved in splicing. The role of the cap in pre-mRNA processing must therefore be a highly specific one rather than simply a protective effect against nucleolytic degradation. The experiments suggest that protein(s) analogous to the cap binding protein required for the initiation of protein synthesis might be involved in some early step, e.g.

nucleation, during the assembly of the multicomponent splicing complex.

Several techniques have been used to investigate the role of the proteins present in the splicing complexes. Removal of hnRNP C proteins from nuclear extracts with monoclonal antibodies led to a decrease of the amount of the active splicing complex formed (8). Moreover similar proteins have been found to be tightly bound to pre-mRNA in the splicing complex by UV crosslinking (9). We have previously identified three nuclear cap binding proteins in HeLa cells using photoaffinity labelling (14). The role of these proteins is not clear at present. They might, however, represent potential candidates for factors involved in cap recognition during the assembly of the active splicing complex. Experiments designed to clarify this issue are currently underway in our laboratory.

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REFERENCES

1. Padgett, R.A., Hardy, S.F. and Sharp, P.A. (1983) Proc. Natl. Acad. Sci. USA 80, 5230-5234.
2. Hernandez, N. and Keller, W. (1983) Cell 35, 89-99.
3. Krainer, A.R., Maniatis, T., Ruskin, B. and Green, M.R. (1984) Cell 36, 993-1005.
4. Brody, E. and Abelson, J. (1985) Science 228, 963-967.
5. Grabowski, P.J., Seiler, S.R. and Sharp, P.A. (1985) Cell 42, 345-353.
6. Frendewey, D. and Keller, W. (1985) Cell 42, 355-367.
7. Konarska, M.M. and Sharp, P.A. (1986) Cell 46, 845-855.
8. Choi, Y.D., Grabowski, P.J., Sharp, P.A. and Dreyfuss, G. (1986) Science 231, 1534-1539.
9. Mayrand, S.H., Pedersen, N. and Pederson, T. (1986) Proc. Natl. Acad. Sci. USA 83, 3718-3722.
10. Salditt-Georgieff, M., Harpold, M., Chen-Kiang, S. and Darnell, Jr.J.E. (1980) Cell 19, 69-78.
11. Banerjee, A.K. (1980) Microbiological Reviews 44, 175-205.

12. Konarska, M.M., Padgett, R.A. and Sharp, P.A. (1984) Cell 38, 731-736.
13. Edery, I. and Sonenberg, N. (1985) Proc. Natl. Acad. Sci. USA 82, 7590-7594.
14. Patzelt, E., Blaas, D. and Kuechler E. (1983) Nucleic Acids Res. 11, 5821-5835.
15. Karin, M. and Richards R.I. (1982) Nature 299, 797-802.
16. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. 12, 7035-7056.
17. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475-1489.
18. Hartmuth, K. and Barta, A. (1987) submitted
19. Ruskin, B. and Green, M. (1985) Science 229, 135-140.
20. Grabowski, P.J., Padgett, R.A. and Sharp, P.A. (1984), Cell 37, 415-427.
21. Ruskin, B., Krainer, A.R., Maniatis, T. and Green, M.R. (1984) Cell 38, 317-331.