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Preferential excision of the 5′ proximal intron from mRNA precursors with two introns as mediated by the cap structure

(*in vitro* splicing/cap analogues/cap recognition)

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ABSTRACT **We have studied the effect of the 5′ cap structure on the splicing of precursor mRNAs containing three exons and two introns within a single molecule in a HeLa nuclear extract. When a precursor mRNA was capped, the upstream intron was spliced out more efficiently than the downstream intron. The differential splicing reactions of the two introns are not due to differences in the intrinsic efficiency of splicing of each intron, since the preferential excision of the upstream intron was also observed when the positions of the two introns relative to the cap structure were reversed. When uncapped precursor mRNA was used as substrate, the downstream intron was spliced out appreciably, but splicing of the upstream intron was greatly reduced. Preincubation of the extract with cap analogues inhibited splicing of the upstream intron but not the downstream intron. Thus, the cap structure exerts its effect primarily on the 5′ proximal intron.**

The recent development of *in vitro* splicing systems has led to considerable progress in understanding the mechanism of nuclear precursor mRNA (pre-mRNA) splicing (refs. 1–9; for review, see ref. 10). It has been shown that a cap structure, $m^7G(5')ppp(5')N$, blocking the 5′ termini of many eukaryotic mRNAs may play an important role in pre-mRNA splicing (5, 6, 11). It appears, however, that the requirement of the cap structure differs between whole cell and nuclear extracts. In the case of the whole cell extract, the efficiency of splicing was greatly enhanced if the transcripts were capped, and the splicing reaction was inhibited up to 90% by cap analogues at relatively low levels (6). This inhibition was observed only when the analogues were added before the lag period of the splicing reaction. On the other hand, the splicing reaction was only partially inhibited by cap analogues in the nuclear extract system (5). Although uncapped transcripts of the truncated globin gene were spliced, the efficiency was considerably lower than that obtained with the capped counterparts, and some aberrant products were formed. However, Edery and Sonenberg demonstrated that a HeLa nuclear extract could be sensitized to cap analogues if the extract was preincubated with them in the presence of Mg^{2+} prior to the addition of substrate RNA (11). These results obtained with both whole cell and nuclear extracts indicate that cap recognition is an important event in the early process of the splicing reaction. The aforementioned studies were conducted with pre-mRNAs containing single introns. It is not known how the cap structure affects splicing of pre-mRNA containing multiple introns. We undertook a close examination of this problem using pre-mRNAs containing two introns within a single molecule. Here we show that the cap structure exerts its effect on excision of an intron proximal to the structure.

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MATERIALS AND METHODS

Chemicals and Enzymes. ^{32}P-labeled nucleotides were obtained from Amersham. Other nonradioactive nucleotides, including cap analogues, were purchased from Pharmacia. RNA polymerase was purified to near homogeneity from *Escherichia coli* A19 as described previously (12). Restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan). Vaccinia virus guanylyltransferase and micrococcal nuclease were from Bethesda Research Laboratories and Boehringer Mannheim, respectively.

***In Vitro* Splicing Reaction.** A HeLa nuclear extract was prepared as described by Dignam *et al.* (13). Unless otherwise indicated, splicing reactions were carried out as described by Krainer *et al.* (5). Usually 10 fmol of ^{32}P-labeled pre-mRNA ($2–3 \times 10^4$ cpm) was added per incubation. After incubation, the reaction was terminated, and the products were recovered as described previously (5).

RESULTS

Preparation of Pre-mRNAs. We have developed an efficient *in vitro* transcription system using the regulatory regions of the *supB-E* operon (12) of *E. coli* (unpublished data). In short, DNA fragments containing the promoter region (237 base pairs) and the terminator region (103 base pairs) of the operon were introduced into pBR322, and the multicloning sequences from M13mp18 were inserted between the two regulatory sequences. This plasmid, designated pSE182, was used to clone various portions of the chicken δ-crystallin gene (14). The plasmid containing a portion of the crystallin gene was linearized by digestion with an appropriate restriction enzyme (*Xmn* I or *Sma* I), and the resulting DNA fragment was transcribed with *E. coli* RNA polymerase in the presence of [α-^{32}P]GTP. The transcript containing exons and introns of the crystallin gene was purified by polyacrylamide gel electrophoresis and was subsequently capped, if necessary, with vaccinia virus guanylyltransferase. Alternatively, instead of enzymatic capping, a cap analogue, G(5′)ppp(5′)G or m^7G-(5′)ppp(5′)G, was added as a primer in the transcription reaction to cap the 5′ termini of pre-mRNA molecules.

The pre-mRNAs used in the present study are schematically illustrated in Fig. 1. In all cases, the 5′ and 3′ terminal exons contain short non-crystallin sequences derived from the plasmid.

***In Vitro* Splicing of Pre-mRNAs Containing Two Introns.** We have shown that δEX14–15 pre-mRNA is precisely spliced *in vitro* in a HeLa nuclear extract and that the reaction intermediates detected are consistent with those observed in other *in vitro* splicing systems (refs. 3–9; unpublished results). For example, when δEX13–14 and δEX14–15 pre-mRNAs were incubated with a HeLa nuclear extract, the electrophoretic gel patterns shown in Fig. 2 *A* and *B* were obtained, and the structures of the RNA products were characterized as de-

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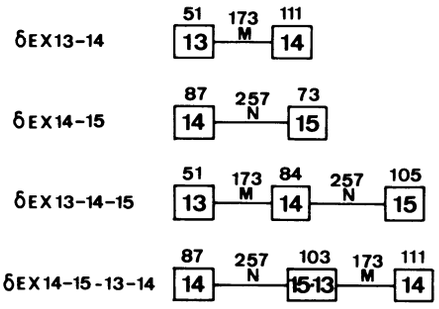

FIG. 1. Schematic representation of the pre-mRNAs. The boxes represent exon sequences, and the lines between them show intron sequences. The numbers within the boxes and the letters above the lines represent specific exons and introns, respectively, in the δ-crystallin gene (13). The 5′ terminus of each pre-mRNA is at the left side of the diagram. The lengths (in nucleotides) of the exons and introns are indicated above them. The plasmid used for the *in vitro* synthesis of δEX14–15-13–14 pre-mRNA was constructed as follows: the *Hin*dIII–*Eco*RI fragment containing exon 14, intron N, and a 5′ portion of exon 15 (71 nucleotides) was joined with the *Eco*RI–*Bam*HI fragment containing a 3′ portion of exon 13 (32 nucleotides), intron M, and exon 14, and was inserted into the *Hin*dIII–*Bam*HI sites of pSE182.

picted. In both cases, the production of the spliced products was greatly reduced if the pre-mRNAs were uncapped (data not shown).

When δEX13–14–15 pre-mRNA was incubated with the same extract, more complicated gel patterns were encountered (Fig. 2*C*). Various RNA molecules in the gel were characterized on the basis of molecular size, hybridization with DNA probes specific for exons or introns, S1 mapping, and primer extension analysis (data not shown). Their structures are schematically illustrated on the right in Fig. 2*C*; as shown, the final spliced product in which the three exons were joined in order was produced appreciably after 60 min.

Effect of the Cap Structure on Splicing of δEX13–14–15 Pre-mRNA. When δEX13–14–15 pre-mRNA was incubated with a HeLa nuclear extract, the final spliced product was efficiently produced if the pre-mRNA was capped (Fig. 3). It appears that methylation of the terminal guanosine residue did not affect the efficiency of the splicing reaction, since the pre-mRNA primed with GpppG was not spliced less efficiently than that primed with m^7GpppG (Fig. 3 *A* and *B*). When uncapped δEX13–14–15 pre-mRNA was incubated with the same extract, the splicing reaction was greatly inhibited, and the final spliced product was undetectable (Fig. 3*C*).

There were two kinds of RNA molecules in which only one of the introns was spliced out. When δEX13–14–15 pre-mRNA was capped, the molecule in which only the upstream intron was excised (designated RNA A) was produced appreciably after 30 min but gradually reduced afterward (Fig. 3 *A* and *B*). A decrease in the amount of this molecule appears to coincide with an increase in the amount of the final spliced product. The molecule in which only the downstream intron was removed (designated RNA B) appeared somewhat later and remained at essentially the same level after 60 min. When uncapped δEX13–14–15 pre-mRNA was employed, the production of RNA B was slightly increased, whereas RNA A was greatly reduced (Fig. 3*C*). Thus, it seems as if there are two pathways in the splicing reaction of δEX13–14–15 pre-mRNA—one by way of RNA A and the other by way of RNA B. It is unlikely, however, that the latter RNA represents an immediate precursor of the final product, as is described below.

These results obtained with δEX13–14–15 pre-mRNA show that the cap structure enhances the splicing reaction leading to excision of the upstream intron but not the downstream intron. Thus, the upstream intron appears to be spliced out more efficiently than the downstream intron when the pre-mRNA is capped. However, it is possible that this is due to a difference in the intrinsic efficiency of the splicing reactions of the two introns. When the two splicing reactions excising the upstream and downstream introns were com-

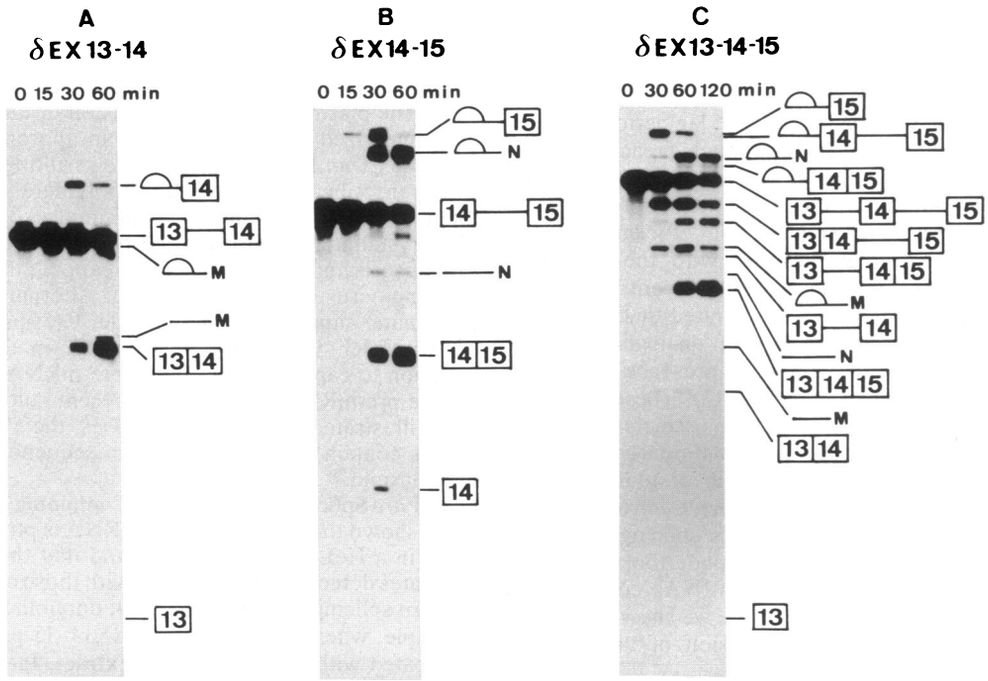

FIG. 2. *In vitro* splicing of δEX13–14 (*A*), δEX14–15 (*B*), and δEX13–14–15 (*C*) pre-mRNAs. The pre-mRNAs labeled with [α-^{32}P]GTP were incubated with a HeLa nuclear extract at 30°C, and the reactions were terminated at the times indicated at the top of each lane. The RNA products were electrophoresed on a 6% polyacrylamide/8 M urea gel and were autoradiographed. The structure of each RNA species determined as described in the text is schematically illustrated on the right in *A–C*.

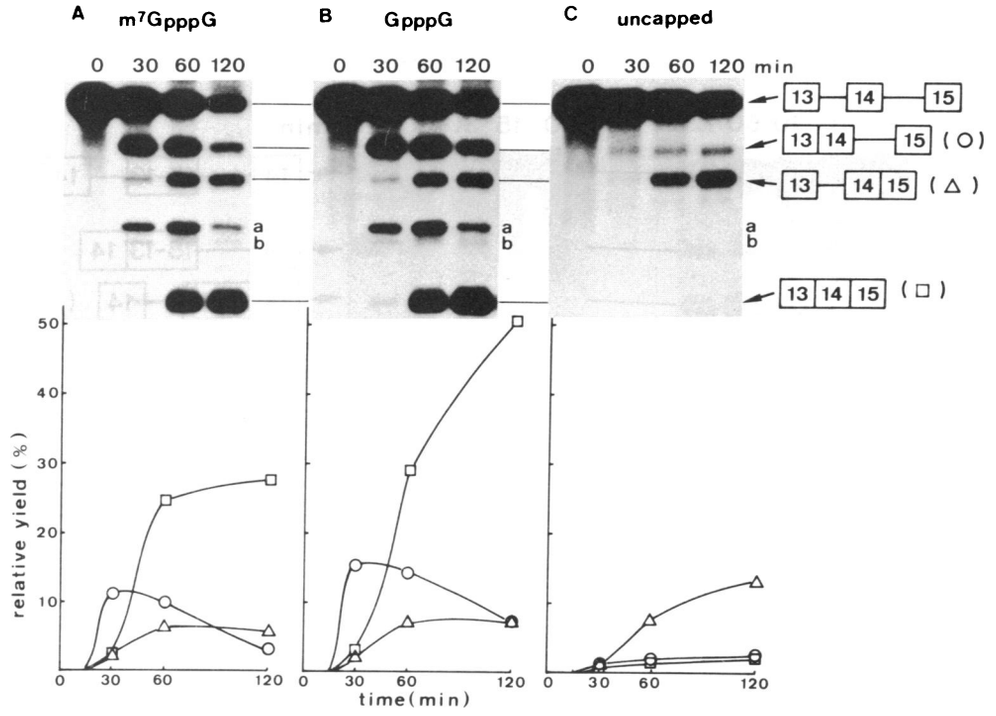

FIG. 3. Kinetics of the *in vitro* splicing reactions of δEX13–14–15 pre-mRNAs of different 5′ terminal structures. The ^{32}P-labeled pre-mRNAs were prepared by the primed synthesis method using m^{7}GpppG (*A*) or GpppG (*B*) as described in the text. (*C*) The uncapped pre-mRNA was synthesized without primer. The pre-mRNAs were individually incubated in a HeLa nuclear extract for the times indicated on the top of each lane. (*Upper*) Portions of the autoradiograms between the pre-mRNA and the final spliced product are shown. Band a, lariat form of intron M; band b, linear molecule containing exon 13, intron M, and exon 14 (see Fig. 2*C*). The gel pieces corresponding to the three RNA species indicated were cut out, and their radioactivities were measured by Cerenkov counting. (*Lower*) The molar ratios (%) of each of the three RNA species to the input pre-mRNA were calculated and plotted. ○, Molecule in which only the upstream intron was excised; △, molecule in which only the downstream intron was excised; □, final spliced product.

pared using pre-mRNAs containing single introns (δEX13–14 and δEX14–15 pre-mRNAs), the splicing efficiency of δEX14–15 pre-mRNA was 1.5–2 times as high as that of δEX13–14 pre-mRNA (data not shown). Furthermore, when splicing of the two introns was tested using δEX14–15-13–14 pre-mRNA in which the relative order of the two introns of δEX13–14–15 pre-mRNA was reversed, the upstream intron (between exons 14 and 15) was spliced out more efficiently than the downstream intron (between exons 13 and 14) if the pre-mRNA was capped (Fig. 4*A*). If the pre-mRNA was uncapped, the splicing reaction of the upstream intron was greatly diminished, and very little final spliced product was detected (Fig. 4*B*). However, the splicing reaction of the downstream intron occurred appreciably with the uncapped pre-mRNA as was the case with uncapped δEX13–14–15 pre-mRNA.

Thus, efficient splicing of the upstream intron of capped δEX13–14–15 pre-mRNA as compared to the downstream intron is not due to a difference in the intrinsic efficiency of the splicing reaction of the two introns.

Inhibition of Splicing of δEX13–14–15 Pre-mRNA by Cap Analogues. Since the cap structure affects the splicing reaction of only the upstream intron of δEX13–14–15 pre-mRNA, it is of interest to examine whether the splicing reactions of the two introns of the pre-mRNA are differentially inhibited by cap analogues. A HeLa nuclear extract was preincubated with m^{7}GpppG or GpppG and was subsequently incubated with δEX13–14–15 pre-mRNA. As shown in Fig. 5*A*, the splicing reaction of the upstream intron was inhibited by both dinucleotides but that of the downstream intron was not if the pre-mRNA was capped. When the splicing reaction was inhibited by m^{7}GpppG, the electrophoretic profile of the reaction products was indistinguishable from that of the reaction products from the uncapped pre-mRNA. This result also implies that the stability of the RNA products from the uncapped pre-mRNA was comparable with that of the products from the capped counterpart in the reaction mixture. In the case of the uncapped pre-mRNA, the cap analogues did not significantly affect the splicing reaction.

Of the two dinucleotides, m^{7}GpppG was more inhibitory than GpppG, as judged by the production of RNA A and the final spliced product at 60 min (Fig. 5*A*). Consistent with this result is the following experiment. When *S*-adenosyl homocysteine, an inhibitor of methylation, was added to the splicing mixture in which δEX13–14–15 pre-mRNA primed with GpppG was employed as the substrate, the production of the final spliced product and RNA A was inhibited, whereas the production of RNA B was relatively insensitive to the inhibitor (data not shown). In the presence of 50 μM inhibitor, the production of the final spliced product and RNA A was inhibited by 60% and 30%, respectively, whereas that of RNA B was virtually unaffected. The splicing reaction of δEX13–14–15 pre-mRNA was markedly inhibited by m^{7}GpppG at concentrations above 8 μM in the present reaction system (Fig. 5*B*).

DISCUSSION

We have shown that pre-mRNAs containing three exons and two introns are efficiently spliced *in vitro* if their 5′ termini are capped. We could not detect any RNA molecules in which the two terminal exons were directly joined and the middle exon was skipped. In addition to the final spliced product consisting of three covalently linked exons, two kinds of RNAs in which either the upstream or downstream intron was spliced out were detected. Of the two RNAs, the one in which the upstream intron alone was removed (RNA A) appears to represent a major precursor of the final spliced product. When the production of this RNA was inhibited by

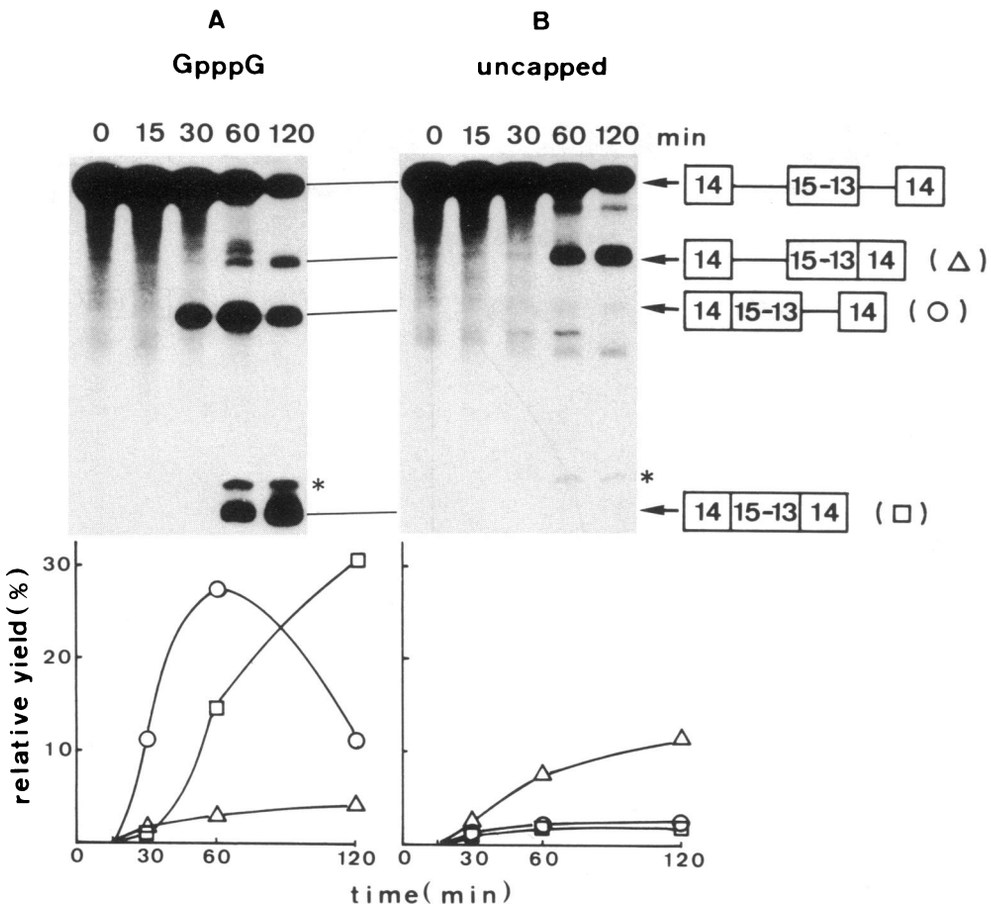

FIG. 4. Kinetics of the *in vitro* splicing reaction of capped or uncapped δEX14–15-13–14 pre-mRNA. The splicing reactions were analyzed as described in Fig. 3. (*A*) GpppG primed pre-mRNA. (*B*) Uncapped pre-mRNA. ○, Molecule in which only the upstream intron was excised; △, molecule in which only the downstream intron was excised; □, final spliced product. The band indicated by an asterisk is a lariat form of the downstream intron. Portions of the autoradiogram above the pre-mRNA and below the final spliced product are not shown.

cap analogues or by using an uncapped pre-mRNA as the substrate, the final spliced product was not detectable, while the production of the other RNA (RNA B) was unaffected. The mechanism for the production of the latter RNA is not well understood. It appears that the latter RNA is produced as a result of a splicing reaction independent of the cap structure, while the upstream intron remains unspliced. It is likely that a splicing complex is formed, albeit rather inefficiently, at the splice junctions of the downstream intron independently from the cap-mediated formation of the complex at the upstream intron.

Although splicing of the GpppG primed pre-mRNA did not occur less efficiently than that of the m^7GpppG primed pre-mRNA, the splicing reaction of the former substrate was inhibited by *S*-adenosyl homocysteine. In addition, the cap analogue inhibition experiments show that the methylated dinucleotide is more effective as an inhibitor of the splicing reaction than the unmethylated dinucleotide. It is likely that the terminal guanosine of the GpppG primed pre-mRNA was rapidly methylated during incubation, even though *S*-adenosyl methionine was not added exogenously to the reaction mixture.

We have demonstrated that the enhancing effect of the cap structure is restricted to the splicing reaction of the intron proximal to the structure. Thus, when the pre-mRNAs containing two introns were capped, the splicing reaction of the upstream intron took place more efficiently than the reaction of the downstream intron. This was also the case with pre-mRNAs containing different sets of exons and introns of the crystallin gene (for example, δEX8–9–10 pre-mRNA). It has recently been shown that exon sequences as well as the proximity of the 5′ and 3′ splice sites play an important role in pre-mRNA splicing (15). However, the differential effect of the cap structure on the splicing of the two introns of a pre-mRNA is apparently not related to the effect of exon sequences, as the cap effect was seen with any pre-mRNAs so far tested, and, in addition, the exons of the substrates employed were essentially full length. Even with δEX14–15-13–14 pre-mRNA, which was derived from a chimeric gene, we could not detect any exon jumping, thereby indicating that the splice site selection was normal. As a consequence of the effect of the cap structure, it appears as if the overall splicing reaction proceeds in a processive manner from the 5′ side of a pre-mRNA. It has generally been believed that there is no strict order for the excision of multiple introns from a pre-mRNA (16–18). The order of removal appears to be determined kinetically as if some introns are excised faster than others. It is true that the splicing reactions of a few introns of the δ-crystallin gene (for instance, the fifth and ninth introns from the 5′ end) are extremely slow compared to those of other introns (data not shown). However, in the case of the pre-mRNAs examined in this study, the 5′ proximal intron is spliced out more efficiently than the second intron if the pre-mRNAs are capped, and this differential excision of the two introns is not due to a difference in the intrinsic splicing efficiency of the introns.

It is likely that the effect of the cap structure on the splicing reaction described above is mediated by a factor(s) that specifically recognizes the structure of the pre-mRNA. It is

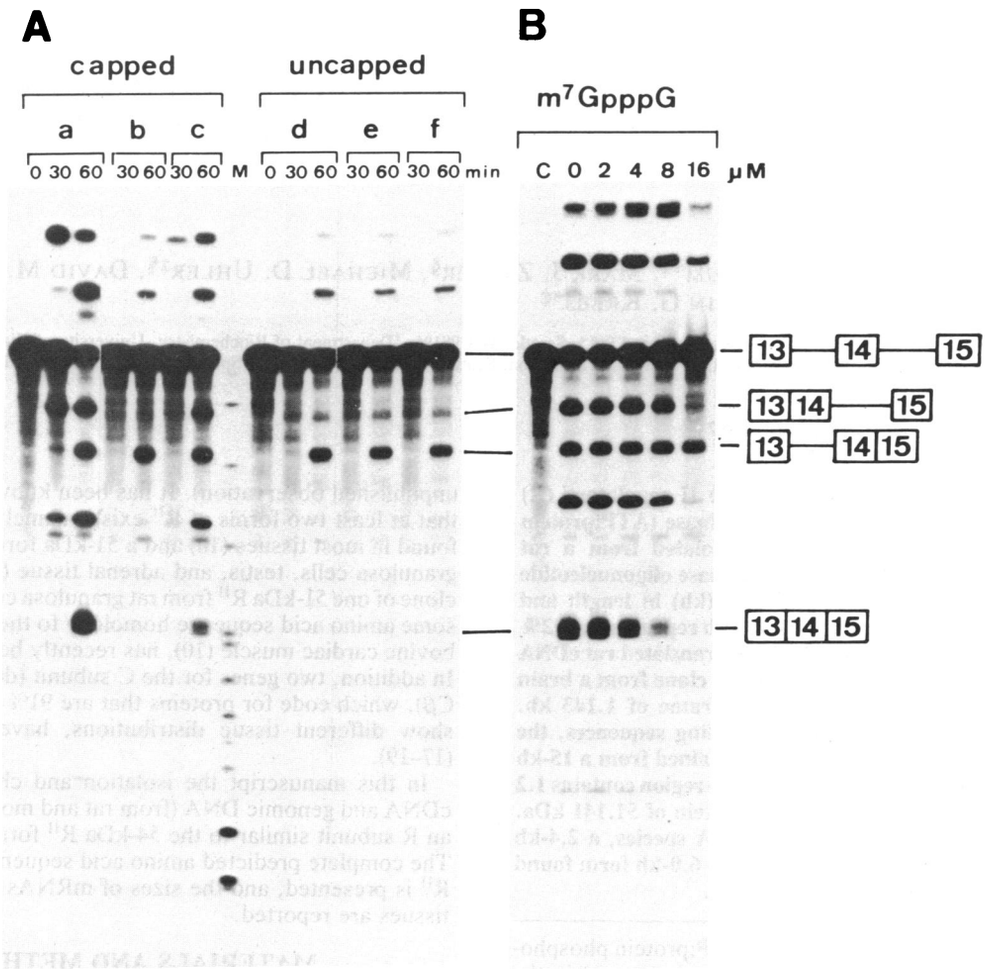

FIG. 5. Inhibition of splicing of δEX13–14–15 pre-mRNA by cap analogues. A HeLa nuclear extract was preincubated in the presence of cap analogues at 30°C for 15 min, and then the capped or uncapped pre-mRNA was added to initiate the splicing reaction. RNA products were analyzed as described in Fig. 2. (*A*) The GpppG primed pre-mRNA (lanes a–c) and the uncapped pre-mRNA (lanes d–f) were used as substrate. Incubation times of the splicing reactions are indicated above each lane. Lanes: a and d, control (preincubation in the absence of cap analogues); b and e, preincubation in the presence of 50 μM m^7GpppG; c and f, preincubation in the presence of 50 μM GpppG; M, pBR322 digested with *Hpa* II. (*B*) Preincubation was carried out in the presence of increasing concentrations of m^7GpppG as indicated on the top of each lane; subsequently, the GpppG primed pre-mRNA was added, and the samples were incubated for 60 min except for lane c (no incubation).

conceivable that through this recognition the assembly of a splicing complex at the splice site(s) of the 5′ proximal intron or the positioning of the preformed complex at the site(s) is mediated in the early process of the splicing reaction. In this connection, we have evidence indicating that the formation of a spliceosome is inhibited by m^7GpppG (unpublished data). We have identified, in a HeLa nuclear extract, a protein that binds to the 5′ cap structure. This binding is inhibited by the m^7GpppG dinucleotide (unpublished data). It is possible that this protein is the factor responsible for cap recognition in the splicing reaction.

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1. Kole, R. & Weissman, S. M. (1982) *Nucleic Acids Res.* **10,** 5429–5445.
2. Goldenberg, C. J. & Hauser, S. D. (1983) *Nucleic Acids Res.* **11,** 1337–1348.
3. Padgett, R. A., Hardy, S. F. & Sharp, P. A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5230–5234.
4. Hernandez, N. & Keller, W. (1983) *Cell* **35,** 89–99.
5. Krainer, A. R., Maniatis, T., Ruskin, B. & Green, M. R. (1984) *Cell* **36,** 993–1005.
6. Konarska, M. M., Padgett, R. A. & Sharp, P. A. (1984) *Cell* **38,** 731–736.
7. Hardy, S. F., Grabowski, P. J., Padgett, R. A. & Sharp, P. A. (1984) *Nature (London)* **308,** 375–377.
8. Konarska, M. M., Grabowski, P. J., Padgett, R. A. & Sharp, P. A. (1985) *Nature (London)* **313,** 552–557.
9. Lin, R.-J., Newman, A. J., Cheng, S.-C. & Abelson, J. (1985) *J. Biol. Chem.* **260,** 14780–14792.
10. Padgett, R. A., Grabowski, P. J., Konarska, M. M., Seiler, S. & Sharp, P. A. (1986) *Annu. Rev. Biochem.* **55,** 1119–1150.
11. Edery, I. & Sonenberg, N. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7590–7594.
12. Nakajima, N., Ozeki, H. & Shimura, Y. (1981) *Cell* **23,** 239–249.
13. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nuclear Acids Res.* **11,** 1475–1489.
14. Ohno, M., Sakamoto, H., Yasuda, K., Okada, T. S. & Shimura, Y. (1985) *Nucleic Acids Res.* **13,** 1593–1606.
15. Reed, R. & Maniatis, T. (1986) *Cell* **46,** 681–690.
16. Roop, D. R., Nordstrom, J. L., Tsai, S.-Y., Tsai, M. J. & O'Malley, B. W. (1978) *Cell* **15,** 651–685.
17. Nordstrom, J. L., Roop, D. R., Tsai, M. J. & O'Malley, B. W. (1979) *Nature (London)* **278,** 328–330.
18. Berget, S. M. & Sharp, P. A. (1979) *J. Mol. Biol.* **129,** 547–565.