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Cap-dependent RNA splicing in a HeLa nuclear extract

(cap analogues)

ISAAC EDERY* AND NAHUM SONENBERG*†

*Department of Biochemistry and †McGill Cancer Center, McGill University, 3655 Drummond, Montreal, Quebec, Canada H3G 1Y6

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ABSTRACT **We have studied the involvement of the 5′ cap structure in the splicing of precursor mRNAs in a HeLa nuclear extract. We show that precursor mRNAs are spliced efficiently only when they possess a cap structure and that preincubation of a HeLa nuclear extract rendered the splicing reaction highly sensitive to inhibition by cap analogues. This sensitization was dependent on exogenous Mg^{2+} but not exogenous ATP or GTP. These results demonstrate that splicing in a nuclear extract is highly dependent on the cap structure, as was demonstrated for the splicing process in a HeLa whole-cell extract [Konarska, M. M., Padget, R. A. & Sharp, P. A. (1984) *Cell* 38, 731–736], and thus support the contention that cap recognition is an important feature of eukaryotic mRNA biogenesis.**

All eukaryotic cellular mRNAs analyzed to date are blocked at their 5′ terminus by the cap structure, $m^7G(5')ppp(5')N$ (1). There is considerable evidence that the cap structure enhances translational efficiency by facilitating ribosome binding to mRNA (1). This interaction is mediated by a distinct group of proteins defined as cap-binding proteins (2, 3).

With the advent of efficient *in vitro* splicing systems (4–10), two recent studies have suggested that the cap structure may play a significant role in the splicing process as well (6, 7). Using a HeLa nuclear extract, Krainer *et al.* (7) were able to show efficient splicing of *in vitro* synthesized, truncated human β-globin transcripts. They showed that although uncapped transcripts could still be spliced, the efficiency was one-half to one-third that for their enzymatically capped counterparts. In addition, splicing of uncapped precursor mRNAs (pre-mRNAs) resulted in the production of aberrant splicing products not observed with capped transcripts. A more striking result was obtained by Konarska *et al.* (6), who used a HeLa whole-cell extract and showed that the efficiency of splicing was greatly enhanced when a capped as opposed to an uncapped precursor RNA was used. In addition, cap analogues inhibited splicing by up to 90%. The specific inhibition of splicing by cap analogues suggests that cap-recognition factors are required for precursor mRNA processing. Thus, the cap structure may play a key role in directing and regulating the assembly of mRNA processing complexes.

Here we show that when a HeLa nuclear extract is preincubated before addition of pre-mRNA, the splicing is rendered highly sensitive to cap-analogue inhibition. In addition, Mg^{2+} is required to elicit this response. We also show that precursor mRNA terminated with a 5′ nonhydrolyzable GTP analogue is a poor substrate for splicing. Thus, our data demonstrate that splicing in a HeLa nuclear extract can be cap-dependent, as in the HeLa whole-cell extract, and point to an important function of the cap structure in mRNA biogenesis.

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MATERIALS AND METHODS

Materials. Restriction enzymes and nucleoside triphosphates were from Boehringer Mannheim. SP6 RNA polymerase and [α-^{32}P]ATP were purchased from New England Nuclear. RNasin was from Promega Biotec, Madison, WI. Cap analogues were purchased from P-L Biochemicals, and polyvinyl alcohol (type II) and creatine phosphate were from Sigma.

Plasmids and SP6 Transcription. Plasmid DNA preparation and restriction enzyme digestion were carried out by standard methods (11). The plasmid pSP64-HβΔ6 (see Fig. 1; a generous gift from M. Green, Harvard) was linearized at the *Bam*HI site. *In vitro* transcriptions with SP6 RNA polymerase primed with cap analogues, in the presence of [α-^{32}P]ATP, to yield SP6/β-globin ^{32}P-labeled RNA were done under conditions previously shown to generate >95% capped transcripts (12). The transcription was primed either with 350 μM cap dinucleotide (m^7GpppG or GpppG) or with the nonhydrolyzable GTP analogue guanosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppG) in the presence of 40 μM GTP to yield substrate pre-mRNA with the appropriate 5′ termini. The transcripts were stored at −70°C in water and used directly in the splicing reaction.

***In Vitro* Splicing Reaction.** HeLa nuclear extracts were prepared as described (13). All experiments described throughout were performed using the same extract preparation. Splicing reactions were carried out in a volume of 25 μl containing 15 μl of nuclear extract at 30°C for 3.5 hr under optimized splicing conditions, exactly as described by Krainer *et al.* (7), unless otherwise indicated in the text. Typically, 1–5 ng (≈30,000 cpm) of substrate RNA was added per incubation: varying the amounts of substrate RNA in this concentration range does not affect splicing efficiency (7). Pre-mRNA processing products were analyzed directly by electrophoresis in 5% polyacrylamide/7 M urea gels followed by exposure against Fuji x-ray film as described (8). Nuclear extract was preincubated under the same conditions as for splicing reactions, for 15 min at 30°C, in the absence of pre-mRNA, except where otherwise indicated, followed by the addition of m^7GpppG-terminated pre-mRNA for the splicing reaction. Components omitted during the preincubation were added with pre-mRNA to achieve a final optimal concentration for splicing in this system (7).

Quantitation of Splicing Efficiency. Splicing efficiency is defined as a percentage based on the ratio of final spliced product relative to the sum of spliced product and input RNA remaining at the end of the incubation. Quantitation was performed by scanning x-ray films, after short exposures, with an LKB soft-laser densitometer. Numbers obtained were normalized to molar amounts.

RESULTS

To characterize the splicing activity of our HeLa nuclear extracts, we used the plasmid pSP64-HβΔ6. This plasmid and its use in the nuclear splicing system were described by Krainer *et al.* (7) and Ruskin *et al.* (8). For preparation of

substrate RNA for splicing, we linearized the plasmid with the restriction enzyme *Bam*HI (see Fig. 1) and transcribed the globin DNA with SP6 RNA polymerase in the presence of the methylated cap dinucleotide analogue m^7GpppG as a primer for transcription. Fig. 1 (lanes 1–4) shows the kinetics of splicing of these transcripts under optimized conditions over 3.5 hr. The different RNA species obtained during the reaction were identified according to their migration relative to DNA size markers and to their order of appearance during the splicing reaction as shown by Ruskin *et al.* (8). Furthermore, we have electrophoresed the RNA products of our reaction mixture, side-by-side with splicing reaction products (obtained from B. Ruskin and M. Green) that have previously been characterized (8), and found them to comigrate. The different species were assigned numbers (shown at right in Fig. 1) as follows: 1, input precursor RNA; 2, "lariat" form containing the intron and second exon; 3, spliced product; 4, first exon; 5, branched intron. The kinetics of splicing is consistent with the report of Ruskin *et al.* (8) with regard to the order of appearance of the intermediates and final product (no. 3) of the splicing reaction. Lanes 5–8 represent a time-course of the splicing reaction using a GpppG-terminated SP6 globin transcript as substrate. The kinetics and efficiency of splicing in this case were the same as with the m^7GpppG-terminated transcript. This is consistent with the results of Konarska *et al.* (6) in the whole-cell-extract splicing system; these authors also demonstrated that N^7 methylation of the GpppG structure at the 5′ end of the SP6 transcript takes place very quickly and with high efficiency in their extract. Consequently, it is very likely that methylation also takes place in our system. To determine the importance of the cap structure for splicing in our system, we have used a G(5′)pp[CH_2]p-terminated SP6 globin transcript. When this pre-mRNA was incubated in the nuclear extract and the kinetics of splicing was determined, we found that although the order of appearance of products was similar, the efficiency of splicing was markedly reduced (lane 12; 13% splicing efficiency as compared to 83% with m^7GpppG-terminated pre-mRNA, lane 4). To exclude the possibility that an inhibitor of splicing was present in the G(5′)pp[CH_2]p-terminated substrate pre-mRNA, we mixed this RNA with m^7GpppG-terminated RNA and found no inhibition of splicing (lane 13). These results show that the cap structure is required for efficient processing of pre-mRNA.

To further establish the requirement of cap recognition for pre-mRNA processing, we examined the effects of cap analogues on pre-mRNA processing in the nuclear splicing system. In contrast to the remarkable inhibitory effect of cap analogues on *in vitro* splicing in a HeLa whole-cell extract (6), addition of the cap analogue m^7GDP to the nuclear splicing system resulted only in partial inhibition [2- to 3-fold inhibition in the presence of 20 μM m^7GDP, but inhibition was not increased even when the m^7GDP concentration was increased to 1 mM (data not shown)]. Similar results were obtained by other groups (M. Konarska and P. Sharp, A. Krainer and T. Maniatis; personal communications). Thus, it was worrisome that a significant proportion of pre-mRNA processing is not affected by addition of the cap analogue m^7GDP and that these findings were different from those obtained in the HeLa whole-cell extract. The results suggested, however, that a significant fraction of the splicing machinery is inaccessible to the inhibitory action of cap analogues. Consequently, we reasoned that preincubation of the nuclear extract in the presence of cap analogues might facilitate cap-analogue recognition by the splicing machinery. As will be reported below, this was indeed the case. However, preincubation of the nuclear extract in the absence of cap analogues was also sufficient to sensitize the nuclear splicing machinery to cap analogues (see below).

To characterize the requirements for cap-analogue inhibition of splicing as a function of preincubation, we titrated the reaction against m^7GDP concentration. Preincubation (15 min at 30°C) of the nuclear extract in the absence of cap analogue had no significant effect (≈10% as compared to an extract not preincubated) on splicing efficiency (data not shown). Lane 1 in Fig. 2*A* represents a control splicing

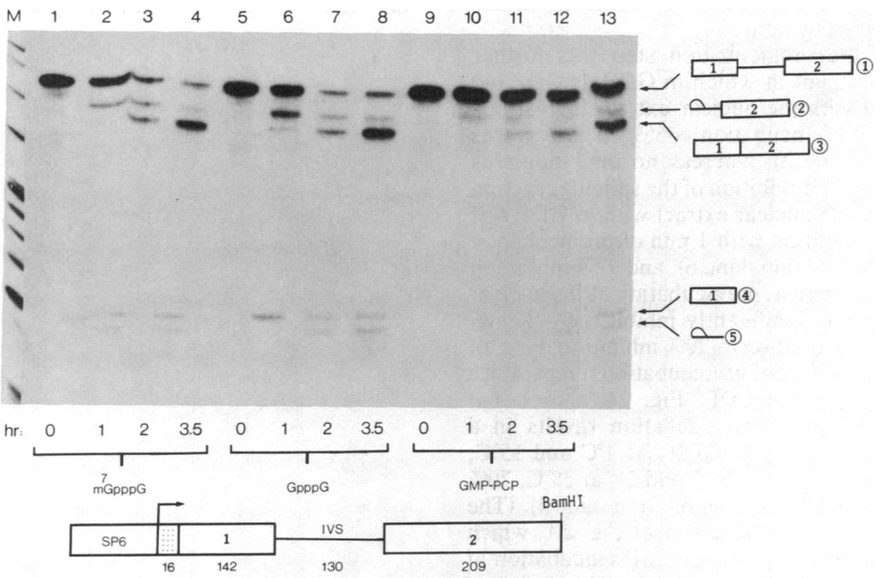

FIG. 1. Time course of *in vitro* splicing of SP6/HβΔ6-globin pre-mRNAs differing in 5′ termini. Lanes 1–12: pre-mRNA labeled with [α-^{32}P]ATP was incubated in a HeLa nuclear extract for 0, 1, 2, or 3.5 hr and the RNA products were electrophoresed in a 5% polyacrylamide/7 M urea gel and autoradiographed. Substrate RNAs used in the splicing reactions were primed with m^7GpppG (lanes 1–4), GpppG (lanes 5–8), or G(5′)pp[CH_2]p (GMP-PCP, lanes 9–12). Lane 13: products of standard splicing reaction (3.5 hr) containing equal amounts of m^7GpppG- and G(5′)pp[CH_2]p-terminated pre-mRNAs. Lane M: ^{32}P-labeled markers (*Hpa* II-digested pBR322 DNA). Structures at right represent the RNAs produced in the reaction (8). These products (numbered 1–5) are described in the text. The structure of the DNA transcription template is shown at the bottom (see ref. 8). The length of the different regions is shown in base pairs. Boxes 1 and 2 represent the first and second globin exons; IVS represents the intervening sequence; the box SP6 represents the SP6 promoter; the stippled box represents the transcribed SP6 sequence; and the arrow denotes the initiation site and direction of transcription.

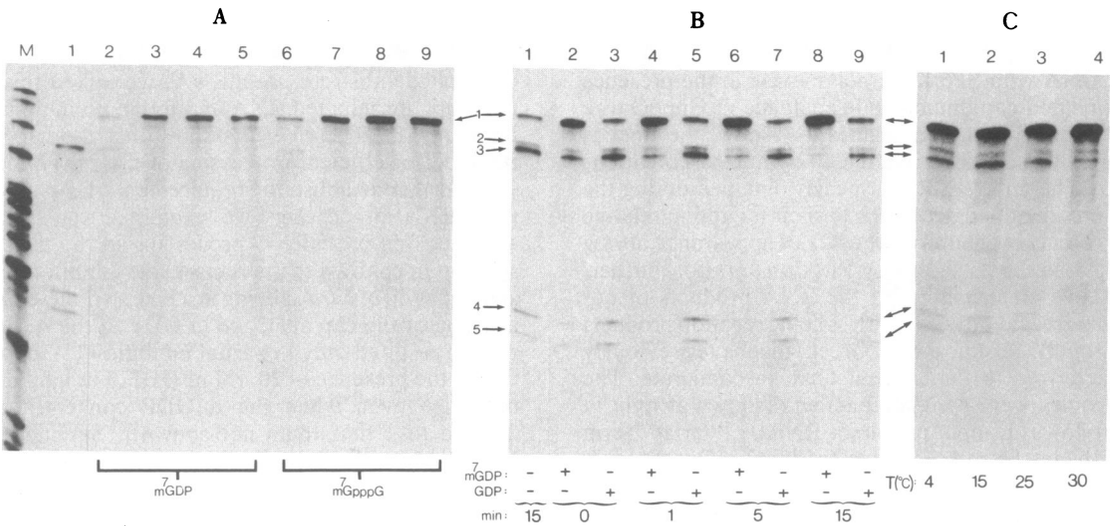

FIG. 2. Conditions influencing cap-analogue inhibition of *in vitro* splicing reaction. Nuclear extract was preincubated under various conditions; pre-mRNA then was added to initiate splicing. RNA products (indicated by arrows 1–5; see Fig. 1) were analyzed as described in *Materials and Methods*. (*A*) Preincubation in the presence of cap analogue. Lane 1: control reaction (preincubation in the absence of cap analogue). Lanes 2–9: for preincubation with m^7GDP (lanes 2–5) or m^7GpppG (lanes 6–9), the analogue concentrations and the percent splicing efficiency were as follows: 1 μM, 50% (lanes 2 and 6); 5 μM, <5% (lanes 3 and 7); 20 μM, <5% (lanes 4 and 8); 40 μM, <5% (lanes 5 and 9). Lane M: markers as in Fig. 1. Note that recovery of radioactivity in lanes 2 and 6 is only ≈60%, as determined by densitometry of the x-ray film, as compared to the other lanes. (*B*) Preincubation for various times in the presence of m^7GDP. Nuclear extract was preincubated in the presence or absence of 40 μM m^7GDP or GDP for the times indicated. Percent splicing efficiency was as follows: lane 1, 71%; 2, 22%; 3, 80%; 4, 13%; 5, 64%; 6, 8%; 7, 64%; 8, <2%; 9, 55%. (*C*) Preincubation at various temperatures with m^7GDP. Nuclear extract was preincubated with 5 μM m^7GDP at the temperatures indicated. Splicing efficiency was as follows: lane 1, 29%; 2, 29%; 3, 21%; 4, 11%.

reaction, in the absence of cap analogue (some degradation of pre-mRNA, which occurred prior to its use in the splicing reaction, was observed; see also Fig. 3*C*). When 1 μM m^7GDP was present during preincubation of the nuclear extract (Fig. 2*A*, lane 2), splicing was inhibited 50%; when 5 μM m^7GDP was used (lane 3), inhibition was >95%. A similar pattern was obtained in the presence of m^7GpppG (lanes 6–9). The 95% inhibition obtained in this experiment (compared to 50–70% inhibition without preincubation) is similar in magnitude to the inhibition by cap analogues observed in the whole-cell-extract splicing system (6).

The importance of the preincubation step was further ascertained in an experiment in which m^7GDP or GDP (40 μM) was preincubated with the nuclear extract for various times (Fig. 2*B*). Without preincubation, ≈65% inhibition was obtained with m^7GDP (lane 2), whereas no inhibition was obtained with GDP (lane 3). Inhibition of the splicing reaction due to preincubation of the nuclear extract with m^7GDP was time-dependent: 82% inhibition with 1 min of preincubation (Fig. 2*B*, lane 4), 89% with 5 min (lane 6), and 97% inhibition with 15 min (lane 8). In contrast, preincubation of the nuclear extract with GDP did not significantly inhibit the splicing, even after 15 min of preincubation (≈20% inhibition; lane 9).

We also examined the effect of preincubation temperature on the inhibitory action of m^7GDP. Fig. 2*C* shows that increasing the temperature of preincubation results in a greater inhibition of splicing by m^7GDP. At 4°C and 15°C, ≈60% inhibition was obtained (lanes 1 and 2); at 25°C, 70% inhibition (lane 3); and at 30°C, 85% inhibition (lane 4). (The control lane for this experiment is lane 1 of Fig. 2*A*, which was autoradiographed for a shorter time.) Preincubation at 37°C in the absence of cap analogue completely abolished splicing activity (data not shown).

We examined the importance of the cap-analogue phosphate groups for inhibitory activity. In a control experiment in the absence of any cap analogues, 63% splicing efficiency was obtained (Fig. 3, lane 1). Five micromolar GDP (lane 2), $p[CH_2]ppG$ (lane 4), or m^7G (lane 5) did not inhibit pre-mRNA processing. As is the case for *in vitro* translation (14), inhibition by m^7G-containing cap analogues increased with increasing number of phosphate groups [18% inhibition for m^7GMP (lane 6), in contrast to 86% inhibition for m^7GDP (lane 7)]. Preincubations were also carried out in the presence of cap dinucleotides. GpppG inhibited the splicing reaction by 51% (lane 3). The nature of the nucleotide N present in the cap analogue $m^7Gppp(p)N$ did not significantly affect the degree of inhibition (≈90% inhibition, lanes 8–11).

We investigated the potential role of several components required for *in vitro* splicing in influencing cap analogue inhibition. In this experiment, preincubation of the nuclear

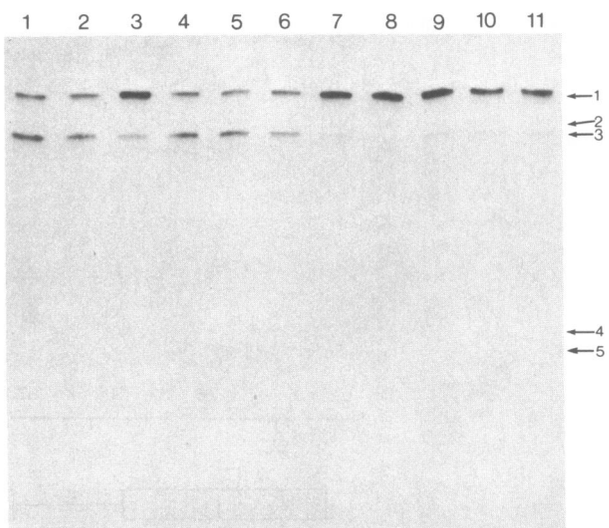

FIG. 3. Preincubation with different cap analogues. Nuclear extract was preincubated in the presence of 5 μM nucleotide or cap analogues as indicated below, followed by splicing reaction and analysis of splicing products. The analogues used and the percent splicing efficiency for each were as follows: control, 63% (lane 1); GDP, 63% (lane 2); GpppG, 31% (lane 3); $p[CH_2]ppG$, 63% (lane 4); m^7G, 62% (lane 5); m^7GMP, 52% (lane 6); m^7GDP, 9% (lane 7); m^7GpppG, 4% (lane 8); $m^7GppppG^7m$, 10% (lane 9); m^7GpppA, 10% (lane 10); m^7GpppU, 12% (lane 11).

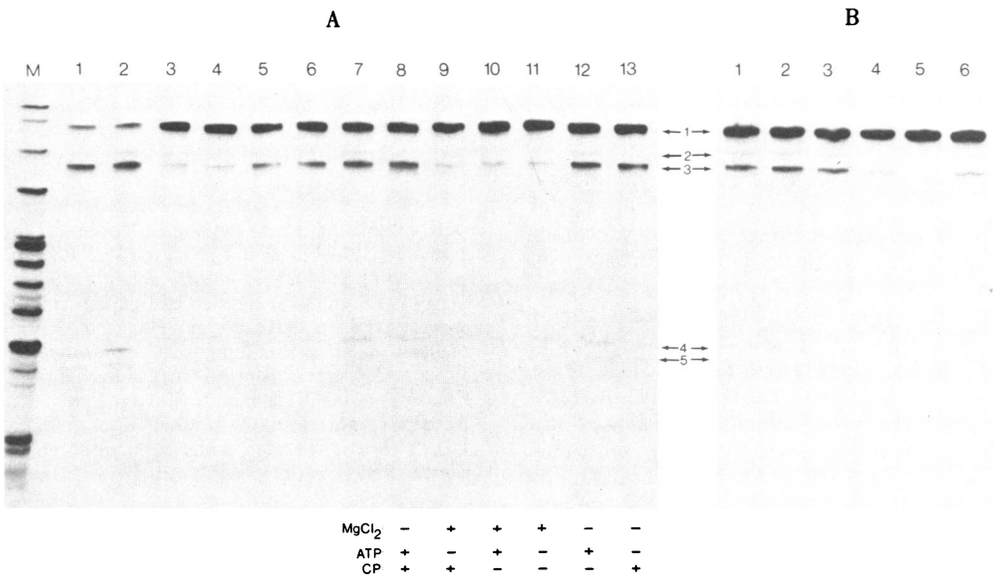

FIG. 4. Effect of the presence of splicing reaction components during preincubation on cap-analogue inhibition of splicing. (*A*) Nuclear extract was preincubated in the presence or the absence of splicing reaction mixture components [ATP, $MgCl_2$, and creatine phosphate (CP)] and in the presence of 5 μM m^7GDP. For lanes 1–3, preincubation was in the absence of m^7GDP; for lane 3, m^7GDP was added after preincubation. Pre-mRNA and the missing reaction mixture components were added to initiate the splicing reaction. Preincubation was as follows. Lanes 1, 3, 4, 6, and 7: all reaction mixture components were present; for lane 6, preincubation was at 4°C; for lane 7, preincubation was in the absence of polyvinyl alcohol. Lanes 2 and 5: the reaction mixture components ATP, $MgCl_2$, and CP were omitted. Lanes 8–13: the reaction mixture ingredients present or absent are indicated below the lanes. Percent splicing efficiency was as follows: lane 1, 70%; 2, 75%; 3, 8%; 4, 10%; 5, 34%; 6, 34%; 7, 43%; 8, 43%; 9, 10%; 10, 9%; 11, 8%; 12, 37%; 13, 37%. Lane M: markers. (*B*) Effect of $MgCl_2$ concentration during preincubation on cap-analogue inhibition of splicing. Preincubation was done in the presence of 5 μM m^7GDP and various $MgCl_2$ concentrations. For the splicing reaction, $MgCl_2$ was added to give a final concentration of 3.2 mM. $MgCl_2$ concentration and percent splicing efficiency were as follows: 1 μM, 23% (lane 1); 10 μM, 30% (lane 2); 100 μM, 26% (lane 3); 500 μM, 7% (lane 4); 1 mM, <2% (lane 5); 3.2 mM, 9% (lane 6).

extract in the absence of m^7GDP resulted in 70% splicing efficiency (Fig. 4*A*, lane 1). When the nuclear extract was preincubated in the presence of polyvinyl alcohol only, followed by addition of ATP, $MgCl_2$, and creatine phosphate, no loss in splicing activity was observed (Fig. 4*A*, lane 2, 75% splicing efficiency). This result indicates that omission of the latter components from the preincubation mixture does not affect splicing activity. Surprisingly, the extent of inhibition of splicing activity was the same (≈90%) whether m^7GDP was added prior to or after completion of the preincubation in the presence of all components (compare lanes 4 and 3, respectively). This finding is of great significance to the interpretation of the results as will be addressed in the *Discussion*. In the remaining reaction mixtures represented in Fig. 4*A*, m^7GDP was present during preincubation. When preincubation occurred in the absence of ATP, $MgCl_2$, and creatine phosphate a significant reduction in inhibition by the cap analogue was observed (51% inhibition, lane 5; compare to lane 4). The extent of inhibition in this case was comparable to the inhibition obtained when preincubation was at 4°C in the presence of all components (51% inhibition, lane 6). When polyvinyl alcohol was omitted during preincubation (lane 7), only 40% inhibition by m^7GDP was obtained. Thus, the increased inhibition by m^7GDP occurs only in the presence of polyvinyl alcohol, which is used in the splicing reaction to stimulate splicing efficiency, because of its ability to concentrate macromolecules by an excluded-volume effect (7). This compound might exert its effect during preincubation in a similar manner. To determine the other component(s) necessary for the increased inhibition, ingredients were omitted either individually (Fig. 4*A*, lanes 8–10) or in pairs (lanes 11–13). The presence of exogenous $MgCl_2$ during preincubation resulted in a cap-analogue inhibition of ≈90% (lanes 9–11). When $MgCl_2$ was omitted, the inhibition decreased to 45% (lanes 8, 12, and 13). Thus, exogenous $MgCl_2$, but not exogenous ATP, is required during preincubation to produce the increased inhibitory effect of m^7GDP.

The effect of $MgCl_2$ concentration during preincubation on cap-analogue inhibition is shown in Fig. 4*B* (lanes 1–6). After preincubation at different Mg^{2+} concentrations (in the absence of ATP and creatine phosphate) the reaction mixture was supplemented with $MgCl_2$ to 3.2 mM, the optimal concentration for *in vitro* splicing in this system (7). In a control experiment, 75% splicing efficiency was obtained (data not shown). Increasing the concentration of exogenous $MgCl_2$ in the preincubation mixture resulted in increased inhibition by m^7GDP. The addition of 1–100 μM $MgCl_2$ resulted in ≈60% inhibition (lanes 1–3), as compared to only 45% when Mg^{2+} was omitted completely (see Fig. 4*A*, lane 8). At 500 μM $MgCl_2$, a 91% reduction in pre-mRNA processing was observed (Fig. 4*B*, lane 4). Inhibition was maximal (>98%) at 1 mM $MgCl_2$ (lane 5) and then dropped at a concentration of 3.2 mM (lane 6, 88% inhibition).

The result shown in Fig. 4*A*, lane 3, prompted us to reexamine whether the presence of m^7GDP during preincubation is required to elicit the inhibitory effect on the splicing reaction. We have repeated the experiments described in Fig. 4 with the exception that m^7GDP was added only after the preincubation step. The results showed that the requirements for the increased cap-analogue inhibition, as described for Figs. 2 and 4, were identical in all respects (data not shown). Thus, the presence of m^7GDP during preincubation of the nuclear extract is not a requirement for the strong cap-analogue inhibition of splicing *in vitro*.

DISCUSSION

Our results provide strong evidence for the generality of the requirement for the cap structure for efficient splicing of eukaryotic mRNAs. Splicing in a nuclear splicing system is shown here to be cap-dependent under certain conditions, as is *in vitro* splicing in a HeLa whole-cell extract (6). Cap recognition is probably an early event in mRNA processing, since no splicing intermediates were generated in the pres-

ence of cap analogues. Thus, recognition of the pre-mRNA cap structure by a specific nuclear cap-binding protein(s) may serve as a signal for the assembly of the mRNA processing complexes that have been described recently (15, 16). The absence of a cap structure from polymerase I and III transcription products might be responsible in part for the exclusion of these RNAs from mRNA processing complexes.

We believe the strongest evidence for the important role of the cap structure in processing of eukaryotic precursor mRNA is the complete and specific inhibition of this process, which we have obtained in this study, by micromolar concentrations of cap analogues. The degree of inhibition is proportional to the number of phosphate groups in the methylated cap analogues, which is characteristic also of the inhibitory effect of cap analogues on translation (14). However, as pointed out before (6), the concentration of cap analogue required to inhibit splicing is two orders of magnitude lower than that required to inhibit mRNA translation to the same extent. This raises the possibility that the concentration of the putative nuclear cap-binding proteins required for splicing is low.

We found that cap-analogue inhibition of splicing was enhanced greatly when the HeLa nuclear extract was preincubated (even in the absence of cap analogue) before the splicing reaction. When cap analogue was added to a splicing reaction without preincubation, inhibition was ≈60% and was not increased when higher concentrations of cap analogue were used. We suggest that in an extract not preincubated, a high proportion of added pre-mRNA associates with preformed splicing complexes and that this association is not dependent on cap recognition. However, upon preincubation, splicing complexes already present are dissociated and the subsequent *de novo* assembly of complexes is dependent on cap recognition. The presence of exogenous Mg^{2+} during the preincubation was required to elicit the increased inhibition by cap analogues, lending support to the idea that a conformational change of the splicing machinery took place. The increase in inhibition by cap analogues is dependent on the duration and temperature of preincubation (Figs. 2 *B* and *C*) but not on the presence of exogenous ATP or GTP during preincubation (Fig. 4*A*). These observations imply that the putative conformational change that sensitizes the splicing machinery to cap-analogue inhibition is not dependent on energy derived from hydrolysis of a high-energy phosphate bond.

In summary, we have demonstrated that splicing in a HeLa nuclear extract can be dependent on the 5′ cap structure and that a remarkable inhibition by cap analogues can be achieved if the extract is preincubated before the splicing reaction. The development of a cap-dependent nuclear splicing system should facilitate the isolation and characterization of nuclear proteins involved in cap recognition during the early steps of eukaryotic mRNA processing.

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