In vitro splicing of pre-mRNA containing bromouridine

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Abstract

The artificial UTP-analogue 5-bromouridine 5′-triphosphate (BrUTP) has been used to label pre-mRNA *in vitro* and *in vivo* [1, 2]. We have investigated the effect of bromouridine (BrU) in pre-mRNA on the efficiency of splicing. An adenovirus major late II construct was used to prepare four different transcripts, each containing a different amount of BrU. These four transcripts were tested in an *in vitro* splicing assay. We found that splicing is strongly inhibited if all uridines (U) in the transcript were substituted for BrU. Splicing was restored to some extent if 50% of the Us were replaced by BrU. The splicing efficiency returned to an almost normal level if only 1 out of every 10 Us was substituted for BrU. This demonstrates that only a pre-mRNA containing a small amount of BrU can be spliced normally *in vitro*. Furthermore, these results strongly suggest that some Us in the adenoviral transcript, probably those at the splice sites, cannot be replaced by BrU and are therefore critical in the splicing reaction.

Abbreviations: BrU = bromouridine; BrUTP = 5-bromouridine 5′-triphosphate; snRNP = small nuclear ribonucleoprotein particle; U = uridine.

Introduction

Splicing includes the removal of introns and the subsequent ligation of exons of pre-mRNAs. Splicing takes place in so-called spliceosomes, which are complexes composed of small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP proteins [reviewed in 3, 4, 5]. The splicing process consists of two transesterification steps and involves the successive binding of several snRNPs and non-snRNP proteins to specific sites of the pre-mRNA. Several conserved sequences, which are essential for splicing, have been characterized in pre-mRNAs [3, 5]. These conserved sequences include the 5′-splice site, the branch point, the polypyrimidine tract, and the 3′ splice site (see consensus sequences in Fig. 1).

Recently, we and others have reported an immunofluorescence method to visualize active sites of transcription in the interphase cell nucleus *in vitro* and *in vivo* [1, 2]. This method is based on the incorporation of the UTP-analogue 5-bromouridine 5′-triphosphate (BrUTP) into nascent RNA and the subsequent detection of the nucleotide with a specific monoclonal antibody. We have shown that BrUTP is efficiently incorporated by RNA polymerase II and does hardly affect the kinetics of nuclear run-on transcription when it replaces UTP [2]. Likewise, labeling pre-mRNA with BrU may also be useful in studying RNA processing in the nucleus. A prerequisite would be that BrU-labeled pre-mRNA is properly spliced. Therefore, in this study we have investigated the effect of BrU on the efficiency of splicing *in vitro*. Transcripts containing BrU were synthesized and splicing was tested in an *in vitro* splicing assay. We find that splicing is strongly inhibited if all Us in the pre-mRNA are replaced by BrU. However, substitution of only a limited fraction of the Us in a transcript hardly affects the efficiency of *in vitro* splicing.

Materials and methods

BrUTP

BrUTP was obtained from Sigma Chemical Co. (St. Louis, MO, USA). A sample was tested for contaminating nucleotides by HPLC on an ion exchange column as described earlier [6]. Two contaminants were found, probably corresponding to BrUDP (12%) and BrUMP (1%). No UTP could be detected.

In vitro transcription

BrUTP is an artificial nucleotide that substitutes for UTP during RNA synthesis [2, 7]. An adenovirus major late II DNA construct, linearized with Sca I [8] (see Fig. 2), was used to synthesize BrU-labeled RNA in vitro using the SP6 RNA polymerase system. Four transcription reactions were done with SP6 RNA polymerase in a final volume of 50 μl to obtain pre-mRNAs containing different amounts of BrU. The transcription reaction was performed in the presence of 0.6 mM GpppG, 1 mM CTP, 1 mM GTP, 24 μM [α-^{32}P]ATP, and either 1 mM BrUTP, 0.5 mM BrUTP + 0.5 mM UTP, 0.1 mM BrUTP + 0.9 mM UTP, or 1 mM UTP. It is expected that SP6 RNA polymerase has, like eukaryotic RNA polymerases, about the same K_m for BrUTP as for UTP [2, 7]. As a result four different types of transcripts were obtained, containing 100%, ~50%, ~10% and 0% BrUTP (percentage of the total UTP content), respectively.

The RNA transcription products were separated on a 3.5% preparative polyacrylamide gel (0.5×TBE, 7 M urea). A piece of the gel containing the full length pre-mRNA was cut out and the pre-mRNA was eluted (16 h, 4 °C) in 1 ml elution buffer (0.5 M ammonium acetate, pH 7.5; 10 mM MgCl$_2$; 0.1% SDS). After phenol/chloroform extraction and ethanol precipitation the pre-mRNA pellet was dissolved in 25 μl H$_2$O.

In vitro splicing

HeLa S100 splicing extracts were prepared from HeLa cells as described previously [9]. Our standard in vitro splicing reaction (12.5 μl) contained 6 μl HeLa splicing extract, 2 μl adenovirus major late II pre-mRNA, 10% glycerol, 10 mM Hepes, pH 7.9, 2.5 mM MgCl$_2$, 50 mM KCl, 2 mM ATP, 10 mM creatine phosphate, 0.25 mM DTE, and 0.1 mM EDTA. Reaction mixtures were incubated at 30 °C for 45 or 120 min. The reactions were terminated by adding 90

μl ice-cold stop mix (0.3 M sodium acetate, pH 5.2; 100 μg/ml E. coli tRNA; 0.1% SDS), immediately followed by phenol/chloroform extraction and ethanol precipitation. ^{32}P-labeled RNAs were separated on a 10% polyacrylamide gel (0.5×TBE, 7 M urea) and visualized by autoradiography (Kodak Xomat).

Results and discussion

To investigate the effect of BrU in pre-mRNA on the efficiency of splicing, we have tested the splicing of BrU-labeled transcripts in an in vitro splicing assay. As substrate a ^{32}P-labeled adenovirus major late II transcript (253 nt), containing two exons and one intron, was used (Fig. 2). Four different types of this transcript were synthesized, characterized by the substitution of 100%, ~50%, ~10%, or 0% of all Us for BrU, respectively (see Materials and methods). These transcripts were incubated in a splicing assay with HeLa cell splicing extract for 45 or 120 min. Subsequently, RNA splicing products and intermediates were separated by gel electrophoresis and visualized by autoradiography (Fig. 3).

Adenoviral transcripts containing no BrU served as a control in the splicing reaction (i.e. 0% BrU; Fig. 3, lanes 4). After 45 min of incubation, splicing had taken place as is illustrated by the presence of the spliced product and all reaction intermediates. After 2 h more spliced product was formed, but still a lot of pre-mRNA was present. Obviously, pre-mRNA was present in an excess amount and splicing had not completely finished after 2 h of incubation.

Almost no spliced product was detected when pre-mRNA, in which all Us were replaced by BrU, was incubated in the splicing assay (i.e. 100% BrU; lanes 1). The free 5'-exon was the only splicing intermediate that was observed suggesting that the intron-3'-exon lariat and the intron lariat were degraded. To exclude the possibility that the small amount of spliced product was the result of a small UTP contamination in the BrUTP starting material, we have tested the purity of this reagent by HPLC (see Materials and methods). No UTP could be detected, which implies that pre-mRNA used in lanes 1 does not contain any U. In conclusion, BrUTP incorporation strongly inhibited splicing of the adenoviral major late II transcript. Still, adenoviral major late II transcripts in which all Us were replaced by BrU were spliced in vitro, albeit at an extremely low rate.
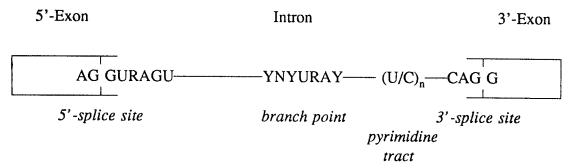
5'-Exon   Intron   3'-Exon

AG GURAGU————————YNYURAY——— (U/C)$_n$—CAG G

5'-splice site   *branch point*   *3'-splice site*
                             pyrimidine
                             tract

Fig. 1. Consensus splice site sequences in a mammalian intron [3, 5]. N is any nucleotide, R is any purine, Y is any pyrimidine.

Adenovirus major late II construct (253 nt)

5'-Exon	Intron	3'-Exon
102 nt	113 nt	38 nt

5'-████████████████GU———————————————AG███-3'

5'-GAAUAGAAGU AUAGUGCCGU UCUUUUGAGC GGCCUAUUAC CCAAGCUUGG

CGUUCGUCCU CACUCUCUUC CGCAUCGCUG UCUGCGAGGG CCAGCUGUUG

GGGUGAGUCU GAUCAGCUUG UUGACCAGCU CGGCGGUGAC CUGCACGUCU

AGGGCGCAGU AGUCCAGGGU UUCCUUGAUG AUGUCAUACU UAUCCUGUCC

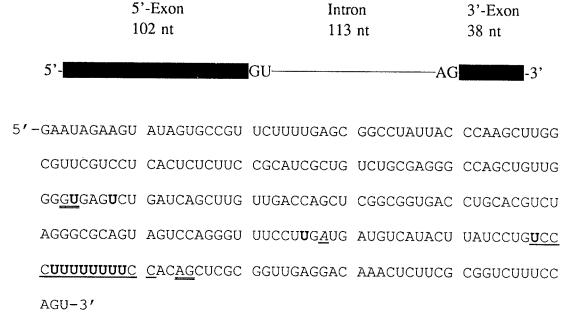CUUUUUUUUC CACAGCUCGC GGUUGAGGAC AAACUCUUCG CGGUCUUUCC

AGU-3'

Fig. 2. Sequence of the adenovirus major late II transcript that was used in the *in vitro* splicing assay [8]. The transcript contains one intron and two exons as depicted. The pyrimidine tract is underlined. The adenosine residue at the branch site is in italic and underlined. The 5'- and 3'-splice sites of the intron are doubly underlined. Uridine residues at consensus sites are depicted in bold. Note that uridines may be replaced by bromouridines as is described in Materials and methods.

If at random 50% or 10% of the Us were replaced by BrU the splicing efficiency increased significantly (Fig. 3, lanes 2 and lanes 3). The rate of splicing was still lower than in the control reaction (lanes 4), but all splicing intermediates and the spliced product were formed. Comparing the relative amounts of the splicing intermediates and spliced product in lanes 4 with those in lanes 2 and 3 may indicate the mode of inhibition of BrU. Since [free 5'-exon] + [spliced product] is proportional to the rate of the first step of splicing, and [spliced product]/[free 5'-exon] is proportional to the rate of the second step of the splicing reaction, we conclude that both transesterification steps are inhibited by the incorporation of BrU. In the case of 50% BrU RNA (lanes 2) the intron-3'-exon lariat was present in somewhat smaller amounts than the free 5'-exon. This again indicates that the intron-3'-exon lariat is relatively unstable or actively degraded.
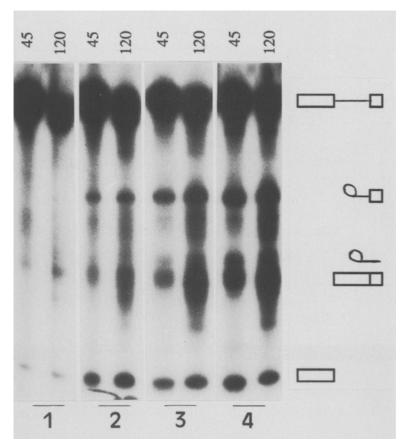
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Fig. 3. In vitro splicing of RNA. Adenovirus major late II pre-mRNAs containing a certain amount of BrU were incubated in a HeLa S100 splicing extract as described in Materials and methods. Lanes 1, RNA containing 100% BrU (percentage of total U amount); lanes 2, 50% BrU/50% U; lanes 3, 10% BrU/90% U; lanes 4, 100% U (control). The different pre-mRNAs were incubated in the splicing assay for 45 or 120 min as indicated at the top of the figure. A schematic representation of the adenovirus major late II pre-mRNA (see Fig. 2), splicing intermediates, and spliced product is indicated on the right of the autoradiograph.

Although BrU-labeled pre-mRNA does not seem to be unstable (Fig. 3), we cannot exclude the possibility that instability of BrU-containing splicing intermediates plays a role in the overall splicing efficiency of BrU-containing pre-mRNA.

The consensus splice site sequences of a mammalian intron contain a few Us, which are probably important in the splicing reaction (Fig. 1). The corresponding Us in the sequence of the adenovirus major late II transcript are depicted bold in Figure 2. We cannot conclude that any of these Us, when replaced by BrU, is responsible for the inhibition of splicing. The incorporation of BrU may modify or disturb specific RNA-protein interactions or base pairs, but may also result in a general change in RNA conformation. Especially prominent is the polypyrimidine tract, which contains eight successive Us. This sequence may be responsible for the strong inhibition of the second step of the splicing reaction [see also 10].

Summarizing, we have shown that the presence of BrU in pre-mRNA inhibits splicing *in vitro*. The degree of inhibition depends strongly on the number of BrUs that are present in the pre-mRNA. Probably, some Us in the pre-mRNA are important for splicing and can therefore not be replaced by BrU. Pre-mRNA labeled with a limited amount of BrU is apparently spliced properly. This pre-mRNA may be used to study the dynamics of pre-mRNA metabolism in the interphase cell nucleus in pulse chase experiments. BrU-labeled RNA can also be useful in immunoprecipitation experiments, and in crosslinking experiments to study RNA-protein interactions, as has been described earlier [11, 12].

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