SV40 T1-mRNA *trans*-splicing and translation requires that the in vitro synthesized cRNA is capped before microinjection

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Abstract The purpose of this investigation was to study the effect on cap structure for *trans*-splicing in mammalian cells. The early SV40 Bst/Bam pre-mRNA (cRNA) was synthesized in vitro in both capped (cap-Bst/Bam-cRNA) and non-capped (Bst/Bam-cRNA) versions and microinjected into the nuclei of TC7 cells. *Trans*-splicing was monitored by immunofluorescence staining (T1-antigen) and by RT-PCR analysis. Cap-Bst/Bam-cRNA was *trans*-spliced with high efficiency, but not the Bst/Bam-cRNA molecules. Northern blot analysis revealed that both the capped and uncapped cRNA molecules had similar stability in the microinjected cells. The coinjected $m^7G(5')ppp(5')G$ cap analog did not inhibit the *trans*-splicing reaction in vivo and did not prevent nuclear export of the mRNA.

Key words: pre-mRNA synthesis in vitro; Microinjection; *Trans*-splicing in vivo; Cap structure; Nuclear export; SV40 T1-antigen

1. Introduction

Although RNA splicing has been extensively analysed during the last two decades (for review, see [1]), it is still not certain how the cell-type specific splice site selection is regulated during alternative *cis*-splicing or *trans*-splicing; nevertheless, it is evident that this requires not only the small ribonucleoprotein particles but also multiple alternative splicing factors [2–8]. Splice site selection and splicing efficiency further depend on various *cis*-acting elements. In vitro studies have revealed that the cap structure, added to all pre-mRNA molecules during transcription, positively affects *cis*-splicing. Using HeLa cell extracts to study in vitro mRNA processing, it has been demonstrated that on addition of the $m^7G(5')ppp(5')G$ cap analog the reaction mixture inhibited *cis*-splicing [9–12]. Microinjection experiments, utilizing *Xenopus* oocytes as recipients, further indicated that the cap structure preferentially facilitates excision of the 5′ terminal intron in vivo [13,14].

Furthermore, splicing and maturation of the 3′ terminal end are interrelated. Mutations within the polyadenylation signal can prevent excision of the 3′ terminal intron and RNA polyadenylation efficiency can be affected by adjacent splice sites [15,16]. Moreover, upstream introns are capable of enhancing excision of downstream introns [17] and deletion or mutation of regular splice sites frequently induce activation of cryptic splice sites [18]. As a consequence, splicing defects are frequently the cause of human genetic diseases [19].

We observed that the deletion of the first SV40 T-antigen exon caused activation of a cryptic 5′ splice site, located within the second T-antigen exon and generation of a new SV40 mRNA species by *trans*-splicing [20,21]. This was the first demonstration that mammalian cells also have the capability of generating functional mRNA molecules by *trans*-splicing (e.g. T1-mRNA).

In this study, we investigated the role of the cap structure for *trans*-splicing in mammalian cells. The T1-pre mRNA was synthesized in vitro with and without the cap structure and microinjected into the nuclei of tissue culture cells. These experiments revealed that only capped pre-mRNA molecules were efficiently *trans*-spliced in vivo but not the cap-minus RNA. However, *trans*-splicing was not inhibited when capped pre-mRNA molecules were coinjected together with the $m^7G(5')ppp(5')G$ cap analog. Furthermore, *trans*-splicing preceded polyadenylation, indicating that 3′ RNA processing is not essential for *trans*-splicing.

2. Materials and methods

2.1. DNA constructs

The p14-T7 DNA was obtained by inserting the early SV40 *Bst*XI/*Bam*HI (Bst/Bam) 2.2 kb DNA segment into the multiple cloning site (*Acc*I/*Bam*HI) of the pSPT19 DNA (Pharmacia), oriented in the same sense as the T7 promoter within the plasmid. The p14Δ5′t-T7 DNA was constructed by eliminating the consensus sequence (G/GTA changed to G/CGC) of the small t-antigen 5′ splice site of the p14-T7 DNA by site-directed mutagenesis as described elsewhere [20]. The pSVT/t-T7 DNA was generated by inserting the early SV40 *Bgl*I/Bam 2.7 kb DNA segment into the pSPT19 vector as described above and the pSVT-T7 contains the early SV40 large T-antigen cDNA.

2.2. cRNA synthesis

For runoff cRNA synthesis [22], the DNA constructs p14-T7, p14Δ5′t-T7, pT7-Bgl/Bam and pSVT-T7 were linearized by *Sma*I treatment which cuts 6 bp downstream from the *Bam*HI site; completeness of cleavage was confirmed by Southern blot analysis. In vitro transcription including 1 μg of linearised template DNA, the cap nucleotide m7G(5′)ppp(5′)G (Ambion) and T7 polymerase (T7 Cap-Scribe; Boehringer Mannheim) was performed according to the supplier's protocol. The quality and correct size of the cRNA preparations were confirmed by Northern blot analysis. The absence of any template DNA in the cRNA preparation after RQ-I-DNase (Promega) digestion was confirmed by PCR analysis with 1 ng cRNA, the SV40 specific primers **d** (5′GAAATGCCATCTAGTGAT3′) and **b** (5′GTTATGATTATAACTGTTATG3′) and 3 U Taq polymerase (BRL) after 35 PCR cycles (30 s, 94°C; 30 s, 53°C; 1 min, 74°C each cycle). Control PCRs included 1 ng reverse-transcribed (RT) cRNA. The Southern blots were hybridized with ^{32}P-labelled SV40 DNA. The primer **g** (5′GCAAAGATGGATAAAGTTT3′) was used in combination with the primer **w** (5′ACTAAACACAGCATGACT3′) to analyze T/t-antigen *cis*-splicing. The binding site (antisense) of the primer **o** (5′CCAGACATGATAAGATAC3′) is downstream from the early SV40 polyA site (5′ end of primer on SV40 is nt 2537; cleavage/polyadenylation site is nt 2586).

2.3. cRNA injection and immunofluorescence

TC7 or rat 2 cells were microinjected with capped or non-capped cRNA molecules (concentration 1 μg/μl, injection volume 10^{-8} μl/cell)

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and stained for T-antigen as described previously [23]. The cRNA was re-extracted from the injected cells and subjected to agarose gel electrophoresis under denaturing conditions (formamide/formaldehyde buffer) and further processed for Northern blot analysis as described elsewhere [22–24].

3. Results and discussion

3.1. The cap structure is essential for trans-splicing in vivo

As shown in our earlier investigations [20,21], rat cells transformed by the early SV40 Bst/Bam DNA fragment (p14) that encodes exclusively for the second SV40 T-antigen exon (aa 83–708) synthesize two truncated T-antigen molecules, the T1- and T2-antigens. The T1 antigen is generated by *trans*-splicing and contains two SV40 T-antigen segments. The first segment contains the wt T-antigen amino acid sequence 109–131 and the second one the amino acids 83–708. The T2 antigen contains the amino acid sequence 109–708. Cells which synthesize the T1-antigen are maximally transformed and cells synthesizing only the T2-antigen do not grow in soft agar and do not form tumors in nude mice (data not shown).

Since it has been demonstrated that the cap structure is essential for *cis*-splicing in vitro as well as for trypanosomal SL-RNA *trans*-splicing [9–14,25,26], we examined whether the cap structure is also required for in vivo *trans*-splicing in mammalian cells. To test this, the SV40 Bst/Bam cRNA was synthesized in vitro without having the m^7GpppG cap at its 5'end (Bst/BamΔ5't-cRNA) and microinjected into the nuclei of TC7 and rat 2 cells. About 24 h after injection cells were fixed and stained with either the Ab-2 or HPA anti T-antibodies. The Ab-2 monoclonal antibody recognizes only the T1-antigen and the polyclonal anti-hamster (HPA) recognizes both the T1- and the T2-antigens. As summarized in Table 1, none of the injected cells stained positive after injection of the Bst/BamΔ5't-cRNA. In contrast, intranuclear T1-antigen accumulation was demonstrable when the capped-cRNA (cap-Bst/BamΔ5't-cRNA) was microinjected (Fig. 1). To exclude the possibility that increased degradation of the non-capped cRNA was the reason for this negative result, the cRNA was re-extracted from the injected cells 1.5 h later and subjected to Northern blot analysis. These experiments revealed that the stability of the non-capped cRNA was not significantly lower than that of the capped cRNA (Fig. 2). This is in contrast to results obtained from studies with *Xenopus* oocytes, where non-capped RNA molecules were significantly less stable than their capped counterparts after intranuclear injection [27].

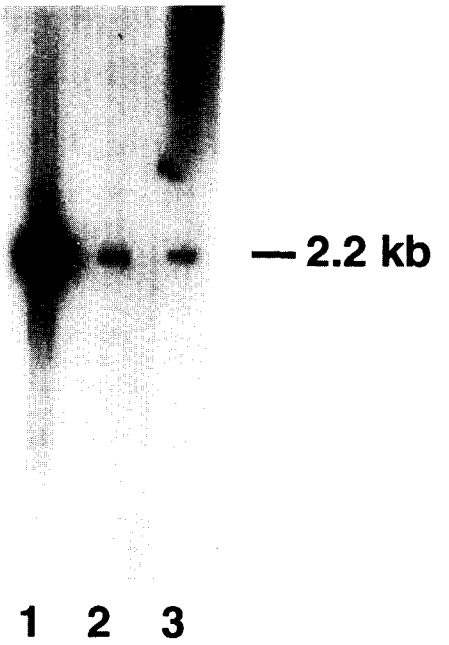

Fig. 1. Northern blot analysis. Lanes: 1, in vitro synthesized 2.2 kb Bst/BamΔ5't-cRNA; 2, cap-Bst/BamΔ5't-cRNA re-extracted from microinjected TC7 cells; 3, Bst/BamΔ5't-cRNA reextracted from microinjected TC7 cells. The blot was hybridized with the early SV40 Bgl/Bam DNA fragment.

To prove directly whether the cap structure is essential for *trans*-splicing, recipient cells were lysed 2–3 h after intranuclear cRNA transfer and the re-extracted RNA was subjected to RT-PCR analysis using the primer pair **b** and **d** for the DNA amplification reaction. These primers bind upstream and downstream form the 5' cryptic splice site at a distance of 258 bp from each other on the wt SV40 DNA. With the T1-cDNA as template the PCR generates a 405 bp DNA segment [20]. As shown in Fig. 3A (lane 2) only synthesis of the 258 bp DNA segment was demonstrable. This DNA segment was generated from the unspliced cRNA.

It has further been demonstrated that *cis*-splicing can be inhibited by an excess of the m^7GpppG cap analog [9]. The prevailing view derived from in vitro and in vivo experiments

Table 1
Percentage of T-antigen positive cells

Cells injected with	Injected into	Percentage of T-antigen positive cells	
		HPA-stained	Ab-2-stained
cap-Bst/BamΔ5't-cRNA	nuclei	50–60	20–30
Bst/BamΔ5't-cRNA	nuclei	0	0
Bst/BamΔ5't-cRNA	cytoplasm	0	0
cap-Bst/BamΔ5't-cRNA+cap analog	nuclei	50–60	20–30
cap-T-cRNA	cytoplasm	70–80	70–80
cap-T-cRNA	nuclei	60–70	60–70
T-cRNA	nuclei	2–4	2–4
T-cRNA	cytoplasm	40–50	40–50
SV40-cap-cRNA	nucleus	70–80	70–80
SV40-cap-cRNA+cap analog	nucleus	70–80	70–80

TC7 cells were fixed and stained for T-antigen 6 h after cRNA injection. The hamster polyclonal antiserum (HPA) allows detection of both the T2- and T1-antigen, whereas the mouse monoclonal antibody Ab-2 exclusively monitors T1-antigen synthesis. The wt SV40 T-antigen is recognized by both types of antisera.

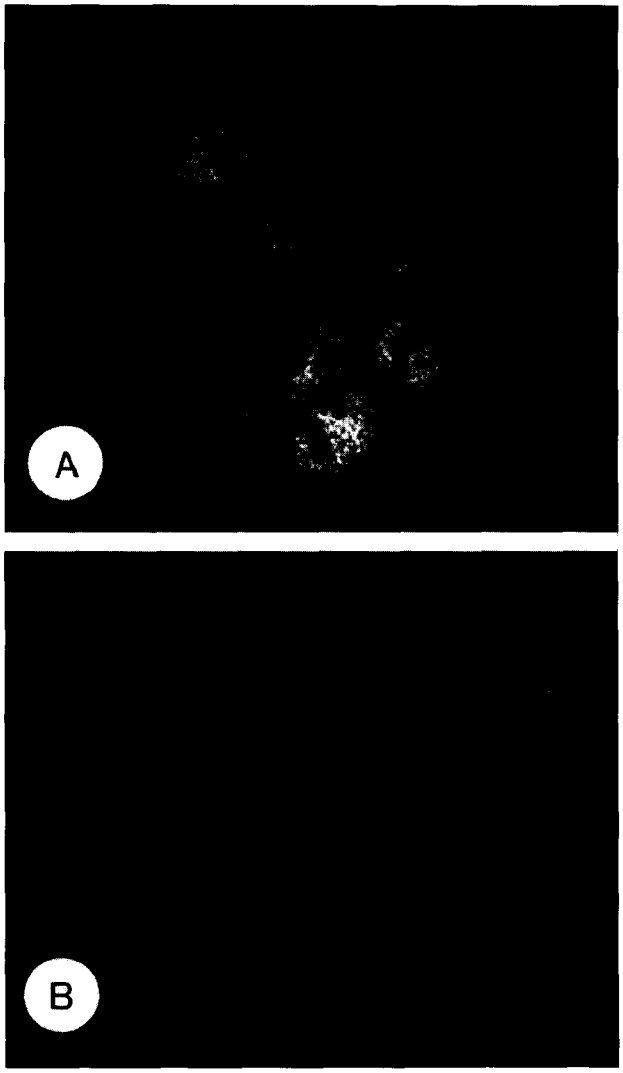

Fig. 2. (A) TC7 cells microinjected with the cap-Bst/BamΔ5′t-cRNA. (B) TC7 cells microinjected with the Bst/BamΔ5′t-cRNA. Cells were fixed and stained with the Ab-2 anti T-antigen antibodies 24 h after intranuclear cRNA injection.

is that cap-binding factors (CBP) are involved at an early stage of spliceosome assembly [13]. To test whether *trans*-splicing is also inhibited by the cap analog in mammalian cells, the m^7GpppG dinucleotide was coinjected into the nuclei of TC7 cells at a 1000–2000-fold molar excess together with the cap-Bst/BamΔ5′t-cRNA. 2 h after injection the RNA was re-extracted and subjected to RT-PCR analysis. With the primer pair **d** and **b**, PCR generated the 405 bp DNA segment with an efficiency similar to that occurring after injection of cap-Bst/BamΔ5′t-cRNA without the cap analog (Fig. 3A lanes 3,4), showing that *trans*-splicing occurred despite the high cap analog concentration (100 000–200 000 molecules).

We also investigated whether the cap analog prevents large T-antigen and small t-antigen *cis*-splicing in vivo. To this end the entire early SV40 coding region was cloned (pSVT/t-T7) and transcribed in vitro in both the capped (SV40 cap-cRNA) and non-capped (SV40-cRNA) version and microinjected into the nuclei of TC7 cells. The SV40-cRNA was then re-extracted 3 h later and converted into cDNA. For DNA amplification the primer pair **g** and **w** was used. These primers are 808 bp distant from each other at the SV40 DNA. After excision of the large T-antigen intron the distance is 462 nucleotides and with the small t-antigen-specific cDNA as template the PCR generates a 742 bp segment. As shown in Fig. 3 both *cis*-splice products were obtained after injection of the SV40 cap-cRNA, regardless of whether or not the cap analog was coinjected (Fig. 3B, lanes 3,4). However, when the non-capped SV40-cRNA was injected the large T-antigen and small t-antigen *cis*-splicing were not demonstrable (Fig. 3B, lane 2).

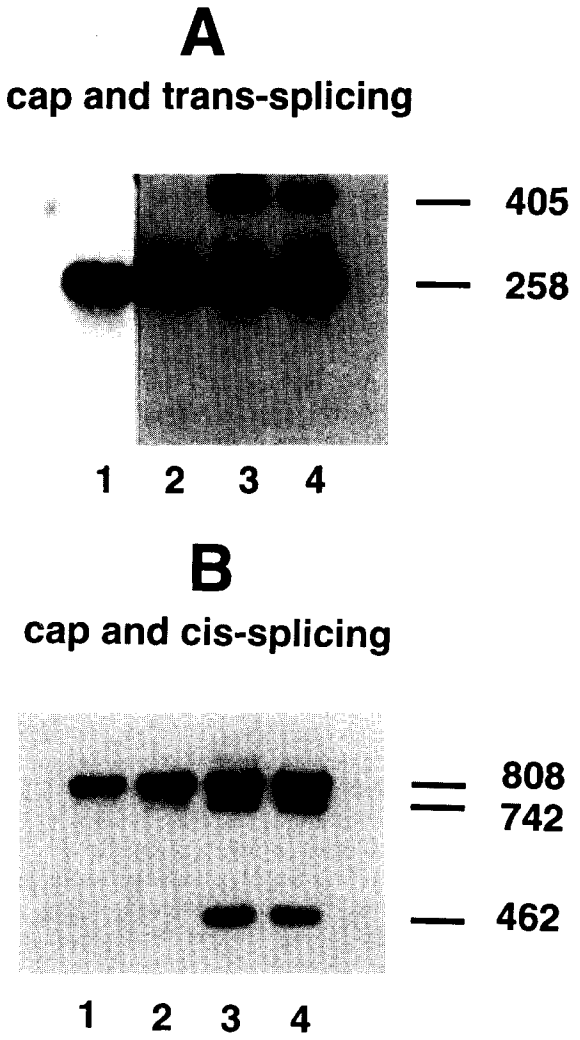

Fig. 3. (A) Correlation between the cap structure and Tl-mRNA *trans*-splicing efficiency. RT-PCR analysis. Lanes: 1, RT-PCR product obtained with Bst/BamΔ5′t-cRNA before microinjection; 2, RT-PCR product obtained with the Bst/BamΔ5′t-cRNA re-extracted from microinjected TC7 cells; 3, RT-PCR products obtained with the cap-Bst/BamΔ5′t-cRNA re-extracted from microinjected TC7 cells; 4, RT-PCR products obtained from TC7 cells after co-microinjection of the cap-Bst/BamΔ5′t-cRNA and the cap analog. (B) Correlation between the cap structure and large T-antigen and small t-antigen mRNA *cis*-splicing efficiency. RT-PCR analysis. Lanes: 1, RT-PCR product obtained with SV40 cRNA before microinjection; 2, RT-PCR product obtained with the SV40 cRNA re-extracted from microinjected TC7 cells; 3, RT-PCR products obtained with the SV40 cap-cRNA re-extracted from microinjected TC7 cells; 4, RT-PCR products obtained from TC7 cells co-microinjected with SV40 cap-cRNA and the cap analog. The RT-PCR products were separated by agarose gel electrophoresis and subjected to Southern blot analysis. The blot was hybridized with the early SV40 Bgl/Bam DNA fragment.

These results are in contrast with data obtained from in vitro splicing experiments where it was shown that the cap analog at 10 μM concentration mediated 90% inhibition of the *cis*-splicing reaction [9]. Likewise, in microinjected oocytes the cap analog acted as a strong competitor for *cis*-splicing at a 70-fold molar excess over the coinjected capped pre-mRNA [13]. It was, therefore, an unexpected result that both large T-antigen *cis*-splicing and T1-mRNA *trans*-splicing were not inhibited, although the cap analog was coinjected at a 1000–2000 molar excess. This may indicate that in TC7 cells saturation and competition were not reached at this high concentration of the cap analog. Alternatively, TC7 cells may bypass the inhibitory effect by means of an as yet undetermined mechanism.

3.2. *Nuclear export of the capped T1-mRNA was not inhibited by the cap analog*

There is experimental evidence that the cap structure serves as a positive signal for nuclear mRNA export and that the m^7GpppG cap analog can prevent the translocation of pol II transcripts from the nucleus into the cytoplasm [28]. To test whether the cap analog also inhibits export of the T1-mRNA the coinjection experiments described above were repeated. We then stained the microinjected TC7 and rat 2 cells and observed that intranuclear T-antigen accumulation occurred with the same efficiency in terms of number of T-antigen-positive cells and intensity of the immunofluorescence staining, regardless of whether or not the m^7GpppG cap analog was coinjected. This indicates that the cap analog at a high concentration did not prevent the nuclear export of the SV40 mRNA, as was the case in microinjected *Xenopus* oocytes [28].

3.3. *The cap structure is required for efficient translation of the Bst/BamΔ5′t-cRNA*

We also microinjected the Bst/BamΔ5′t-cRNA into the cytoplasm of TC7 cells and analysed T-antigen synthesis by immunofluorescence staining at different points in time after the cRNA transfer. However, neither T1-antigen nor T2-antigen synthesis was demonstrable under these conditions (Table 1). In contrast, T2-antigen synthesis occurred when the m^7GpppG cap structure was added to these cRNA molecules by guanyltransferase treatment prior to microinjection.

To determine whether the cap structure is an absolute prerequisite for translation we synthesized the cap-minus T-antigen cRNA (T-cRNA) in vitro, utilizing the early SV40 cDNA (pSV-T7) as template. The pSV-T7 DNA encodes for the intact SV40 wt large T-antigen and does not contain the large and small T-antigen intron. After cytoplasmic microinjection of the non-capped T-cRNA, 40–50% of the recipient cells exhibited a clear intranuclear T-antigen-specific fluorescence 6 h later (Table 1). Following in vitro addition of the cap structure to the T-cRNA (cap-T-cRNA), about 70–80% of the injected cells stained positive for T-antigen. It is not clear why the cap-minus T-cRNA was translated but not the Bst/BamΔ5′t-cRNA. One difference between these two kinds of cRNA molecules is the size of the 5′ untranslated leader sequence. It may be that the non-capped Bst/BamΔ5′t-cRNA, with the 269-nucleotide-long leader sequence is not translatable without having the 5′ m^7GpppG sequence, while the T-cRNA with a shorter leader sequence (70 nt) can be translated, a topic not further addressed in this investigation.

3.4. *Polyadenylation is not essential for trans-splicing*

Finally, we assessed whether polyadenylation is essential for *trans*-splicing. To this end, cRNA molecules re-extracted 1 h after injection were converted into cDNA using the primer **o** for reverse transcription and PCR amplification. The primer **o** binds at the 3′ end of the cRNA downstream from the T-antigen cleavage/polyadenylation site, allowing the differentiation between 3′ processed and unprocessed cRNA molecules. Our RT-PCR and DNA sequencing experiments revealed that non-polyadenylated cRNA molecules were *trans*-spliced as well, confirming that *trans*-splicing and polyadenylation can occur independently of each other, as in the case of cRNA *cis*-splicing (for review, see [29]).

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References

[1] Green, M.R. (1991) Annu. Rev. Cell. Biol. 7, 559–599.
[2] Chabot, B. and Steitz, J.A. (1987) Mol. Cell. Biol. 7, 698–707.
[3] Ge, H. and Manley, J.L. (1990) Cell 62, 25–34.
[4] Ge, H., Zuo, P. and Manley, J.L. (1991) Cell 66, 373–382.
[5] Krainer, A.R., Conway, G.C. and Kozak, D. (1990) Cell 62, 35–42.
[6] Zamore, P.D. and Green, M.R. (1991) EMBO J. 10, 207–214.
[7] Kohtz, J.D., Jamison, S.F., Will, C.L., Zuo, P., Luehrmann, R., Garcia-Blanco, M.A. and Manley, J.L. (1994) Nature 368, 119–124.
[8] Blumenthal, T. (1995) Trends Genet. 11, 132–136.
[9] Konarska, M.M., Padgett, R.A. and Sharp, P.A. (1984) Cell 37, 731–736.
[10] Edery, I. and Sonenberg, N. (1985) Proc. Natl. Acad. Sci. USA 82, 7590–7594.
[11] Fu, X.D. and Maniatis, T. (1992) Proc. Natl. Acad. Sci. USA 89, 1725–1729.
[12] Fu, X.D. and Maniatis, T. (1992) Science 256, 535–538.
[13] Izaurralde, E., Lewis, J., McGuigan, C., Jankowska, M., Darzynkiewicz, E. and Mattaj, I.W. (1994) Cell 78, 657–668.
[14] Inoue, K., Ohno, M., Sakamoto, H. and Shimura, Y. (1989) Genes Dev. 3, 1472–1479.
[15] Niwa, M., Rose, S.D. and Berget, S.M. (1991) Genes Dev. 4, 1552–1559.
[16] Niwa, M., MacDonald, C.C. and Berget, S.M. (1992) Nature 360, 277–280.
[17] Nesic, D. and Maquat, L. (1994) Genes Dev. 8, 363–375.
[18] Nelson, K.K. and Green, M.R. (1990) Proc. Natl. Acad. Sci. USA 87, 6253–6257.
[19] Krawczak, M., Reiss, J. and Cooper, D.N. (1992) Hum. Genet. 90, 41–54.
[20] Eul, J., Graessmann, M. and Graessmann, A. (1995) EMBO J. 14, 3226–3235.
[21] Eul, J., Graessmann, M. and Graessmann, A. (1996) Nucleic Acids Res. 24, 1653–1661.
[22] Graessmann, M., Michaels, G., Berg, B. and Graessmann, A. (1991) Nucleic Acids Res. 19, 53–59.
[23] Graessmann, M. and Graessmann, A. (1976) Proc. Natl. Acad. Sci. USA 73, 366–370.
[24] Graessmann, A., Graessmann, M. and Mueller, C. (1980) Methods Enzymol. 65, 616–626.
[25] Patzelt, E., Thalmann, E., Hartmuth, K., Blaas, D. and Kuechler, E. (1987) Nucleic Acids Res. 15, 1387–1399.
[26] Ullu, E. and Tschudi, C. (1991) Proc. Natl. Acad. Sci. USA 88, 10074–10078.
[27] Green, M.R., Maniatis, T. and Melton, D.A. (1983) Cell 32, 681–694.
[28] Hamm, J. and Mattaj, I.W. (1990) Cell 63, 109–118.
[29] Manley, J.L. and Proudfoot, N.J. (1994) Genes and Dev. 8, 259–264.