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Minireview

Antisense oligonucleotides made of 2′-*O*-alkylRNA: their properties and applications in RNA biochemistry

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Oligo(2′-*O*-alkylribonucleotides) have been developed recently as novel oligonucleotide analogues with properties that enhance their use as antisense probes. They possess high chemical stability and are resistant to hydrolysis by DNA- or RNA-specific nucleases. Many forms of oligo(2′-*O*-alkylribonucleotides) hybridise specifically and efficiently to complementary RNA sequences, forming stable duplexes that are not substrates for cleavage by RNase H. In combination with prosthetic reporter groups, such as biotin, DNP or fluorophores, oligo(2′-*O*-alkylribonucleotides) have important applications in a wide range of biochemical studies on RNA function and structure.

Oligonucleotide; Antisense; 2′-*O*-Alkyl; RNA

1. INTRODUCTION

Oligo(2′-*O*-alkylribonucleotides) are analogues of oligoribonucleotides in which all the 2′-hydroxyl groups are alkylated (see Fig. 1). A major consequence of the presence of an alkyl group at the ribose 2′-hydroxyl is that the resulting polymer becomes resistant to cleavage by alkali and by a wide range of RNA and DNA specific nucleases. This is mainly due to the effect of the alkyl group reducing the nucleophilic character of the 2′ oxygen. However, steric hindrance by the bulky alkyl group also helps to reduce cleavage of the adjacent 3′–5′ phosphodiester bond. This becomes more pronounced for larger alkyl groups, as seen in the rate of digestion of oligo(2′-*O*-alkylribonucleotides) by phosphodiesterases with dual RNA/DNA cleavage specificities, such as nuclease P1. Thus, oligo(2′-*O*-methylribonucleotides) are cleaved more efficiently by nuclease P1 than the bulkier oligo(2′-*O*-{3,3-dimethylallyl}ribonucleotides) [1].

In addition to their increased resistance to degradation, certain forms of oligo(2′-*O*-alkylribonucleotides) also hybridise more stably to complementary, unmodified RNA sequences than do either DNA or RNA oligonucleotides [2,3]. This unique combination of chemical robustness and efficient hybridisation makes polymers of 2′-*O*-alkyl RNA the reagents of choice for many antisense applications in biochemistry and molecular biology where hybridisation to RNA target sequences is involved. In this minireview we will survey the properties of oligo(2′-*O*-alkylribonucleotides) and illustrate how they can be used as antisense probes.

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2. SYNTHESIS OF OLIGO(2′-*O*-ALKYLRIBONUCLEOTIDES)

The first in vitro syntheses of oligo(2′-*O*-alkylribonucleotides) were made over twenty years ago and involved the enzymatic synthesis of 2′-*O*-methyl RNA homopolymers [4–6]. This was achieved following the unexpected discovery that the enzyme polynucleotide phosphorylase could polymerise the 5′-diphosphates of 2′-*O*-methylribonucleosides [4]. However, chemical synthesis of mixed sequence oligo(2′-*O*-methylribonucleotides) was first reported only in 1985, by Inoue et al., using phosphotriester chemistry [7], while in 1989, Sproat et al. reported synthesis of mixed sequence oligo(2′-*O*-methylribonucleotides) using the more efficient phosphoramidite procedure [8].

The 2′-*O*-methyl ether of RNA is a naturally occurring modification found in vivo at selected positions in tRNAs, mRNAs, rRNAs and snRNAs. Recently, following the encouraging results obtained using oligo(2′-*O*-methylribonucleotides) as antisense probes, the synthesis of non-naturally occurring 2′-*O*-alkyl ethers of ribonucleosides has also been explored. This has included the synthesis of oligo(2′-*O*-allylribonucleotides) [1,9,10], oligo(2′-*O*-{3,3-dimethylallyl}ribonucleotides) [1], oligo(2′-*O*-butylribonucleotides) [11] and oligo(2′-*O*-ethylribonucleotides) [12,13]. The structures of these

analogues are illustrated in Fig. 1. Both the 2′-*O*-methyl and 2′-*O*-allyl derivatives are now commercially available and the corresponding oligo(2′-*O*-methyl and 2′-*O*-allylribonucleotides) can be conveniently synthesised using the standard 2-cyanoethyl phosphoramidite chemistry that is routinely employed for DNA synthesis [14]. The only significant difference from the synthesis of DNA is that the coupling time for 2′-*O*-alkyl modified ribonucleosides, using tetrazole as activator, must be extended to between 5 and 15 min, depending on the size of the 2′-*O*-alkyl group.

Many important applications of oligo(2′-*O*-alkylribonucleotides) require the synthesis of probes containing prosthetic reporter groups, such as biotin, DNP or fluorophores. As will be discussed later, the presence of such reporter groups facilitates detection and allows the oligonucleotides, together with any other molecules interacting with them, to bind to specific affinity matrices. This forms the basis of several affinity chromatography methods that are useful for the purification, depletion and characterisation of RNA or RNA–protein complexes. Attachment of reporter groups can either be done post-synthetically, using an appropriate amino-linker [8], or, more conveniently, during the solid phase synthesis using commercially available reporter-linked phosphoramidites.

3. HYBRIDISATION PROPERTIES OF OLIGO(2′-*O*-ALKYLRIBONUCLEOTIDES)

The thermal stability of duplexes formed between oligo(2′-*O*-alkylribonucleotides) and complementary RNA target sequences is of prime importance for their use as antisense probes. The development of chemical methods for synthesising 2′-*O*-alkylated polymers of defined sequence has allowed their hybridisation properties to be analysed in detail. Inoue et al. showed that short oligo(2′-*O*-methylribonucleotides) formed hybrids with complementary, unmethylated oligoribonucleotides that were similar in stability to RNA·RNA homoduplexes and more stable than the corresponding DNA·RNA ones [2]. A separate study by Kibler-Herzog et al., reported that a 14 mer 2′-*O*-methyl modified RNA oligonucleotide formed a more stable duplex with a complementary DNA strand than either unmodified RNA, unmodified DNA or phosphate-modified DNA, in the following order: 2′-*O*-methyl RNA > DNA > unmodified RNA > methylphosphonate DNA > phosphorothioate DNA [3].

Our own thermal analyses of duplex stability (previously unpublished data), comparing a variety of oligo(2′-*O*-alkylribonucleotides) as antisense probes (i.e. (−)strand), and a complementary oligoribonucleotide (sequence 5′-GAGGCUUAUCCA-3′) as the target, (i.e. (+)strand), have led to the following order of stability, measured in 0.1 M salt solution at pH 7.0: RNA·2′-*O*-methyl RNA (T_m 61.8°C) > RNA·2′-*O*-allyl RNA (T_m 57.3°C) ≥ RNA·RNA (T_m 56.6°C) > RNA·2′-*O*-butyl RNA (T_m 51.5°C) > RNA·2′-*O*-dimethylallyl RNA (T_m 46°C).

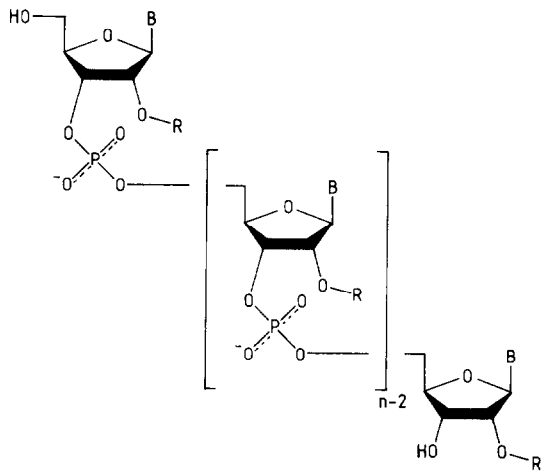

Fig. 1. The structure of an oligo(2′-*O*-alkylribonucleotide) *n* residues long is illustrated, in which R represents an alkyl group and B represents any nucleobase, generally uracil-1-yl (U), cytosin-1-yl (C), adenin-9-yl (A) or guanin-9-yl (G). The following alkyl groups have been incorporated: $-CH_3$ (methyl) [7,8], $-CH_2CH_3$ (ethyl) [12,13], $-CH_2CH{=}CH_2$ (allyl) [1,9,10], $-CH_2CH_2CH_2CH_3$ (butyl) [11], or $-CH_2CH{=}C(CH_3)_2$ (3,3-dimethylallyl) [1].

The above data show clearly that the stability of RNA·2′-*O*-alkyl RNA heteroduplexes is dependent upon the structure of the 2′-*O*-alkyl group. However, the presence of modified bases, as well as sugars, can also influence the hybridisation properties of an antisense probe [15–19]. An example of this is the incorporation within oligo(2′-*O*-alkylribonucleotides) of the modified base 2,6-diaminopurine (i.e. 2-aminoadenine), which forms three hydrogen bonds to uracil to give a base pair of similar stability to a GC pair. This increases the T_m of the hybrid formed between an oligo(2′-*O*-alkylribonucleotide) antisense probe and an unmodified RNA target by approximately 3°C per 2-aminoadenine incorporated [17]. The extra stabilisation conferred by 2-aminoadenine can be especially valuable in allowing short probes to be used effectively against U-rich target sequences [15]. Another example of duplex stabilisation through incorporation of modified bases is provided by the recent demonstration by Froehler et al. that the presence of a 5-propynyl substituent on either 2′-*O*-allyl U, or 2′-*O*-allyl C, in oligo(2′-*O*-allylribonucleotide) probes significantly increases the stability of hybrids with RNA targets; the increases in T_m per substitution being 1.7°C (2′-*O*-allyl U) and 2°C (2′-*O*-allyl C) respectively [18]. The 5-propynyl U will therefore be particularly useful for probes designed to hybridise with short, A-rich RNA targets.

In summary, several studies have established that oligo(2′-*O*-alkylribonucleotides) with unbranched, short chain alkyl groups hybridise more stably to unmodified RNA than either DNA or RNA itself. The

physical basis for this enhanced duplex stability is at present unclear. However, it is interesting to compare the stabilizing effect of alkylating the ribose 2′ hydroxyl in RNA with the corresponding decrease in the stability of hybridisation observed when phosphorothioate groups are introduced into the backbone of DNA to make it resistant to nuclease degradation [3]. The alkyl groups in oligo(2′-*O*-alkylribonucleotides) also decrease their interaction with nucleic acid binding proteins [1]. In many biological experiments this serves to increase the fraction of the probe available for binding to target sequences by minimising the formation of non-specific protein–oligonucleotide complexes that cannot hybridise. It should also be noted that RNA target sequences are not cleaved by RNase H when hybridised to oligo(2′-*O*-alkylribonucleotides) [20]: RNase H cleaves RNA specifically when it is hybridised to DNA [21]. This fortunate combination of properties, including stable hybrid formation, resistance to degradation and low non-specific interactions therefore makes 2′-*O*-alkyl RNA polymers well suited for use as antisense probes.

4. APPLICATION OF OLIGO(2′-*O*-ALKYLRIBONUCLEOTIDES) AS ANTISENSE PROBES

Antisense probes can be used either to block the function of specific RNAs, or as affinity chromatography reagents for the depletion or purification of RNA or RNA–protein complexes. The stable in situ hybridisation of an antisense probe labeled with a suitable reporter group can also be used to detect RNAs in the fluorescence or electron microscope. Antisense probes thus provide important tools for studies on RNA biochemistry and gene expression and complement techniques based on the use of immunological reagents. We discuss below recent examples illustrating these applications using antisense probes made of oligo(2′-*O*-alkylribonucleotides).

Most of the initial applications of antisense oligo(2′-*O*-alkylribonucleotides) have been directed towards the study of RNA processing. One of the major RNA processing reactions in eukaryotic cells is splicing, i.e. the removal of introns from mRNA precursors (pre-mRNA). Splicing takes place in the nucleus within a large RNA–protein complex termed the spliceosome [22]. The major subunits of the spliceosome are the U1, U2, U4/U6 and U5 small nuclear ribonucleoprotein particles (snRNPs), each of which is itself an RNA–protein complex containing one (or two in the case of U4/U6) small nuclear RNAs (snRNAs) and snRNP proteins. Each of the snRNPs are essential for pre-mRNA splicing and their functions and structures show a high level of evolutionary conservation [23].

Antisense oligo(2′-*O*-methyl and 2′-*O*-allylribonucleotides) that hybridise specifically to each of the splicing snRNAs have been developed [15,17,24–26]. In many

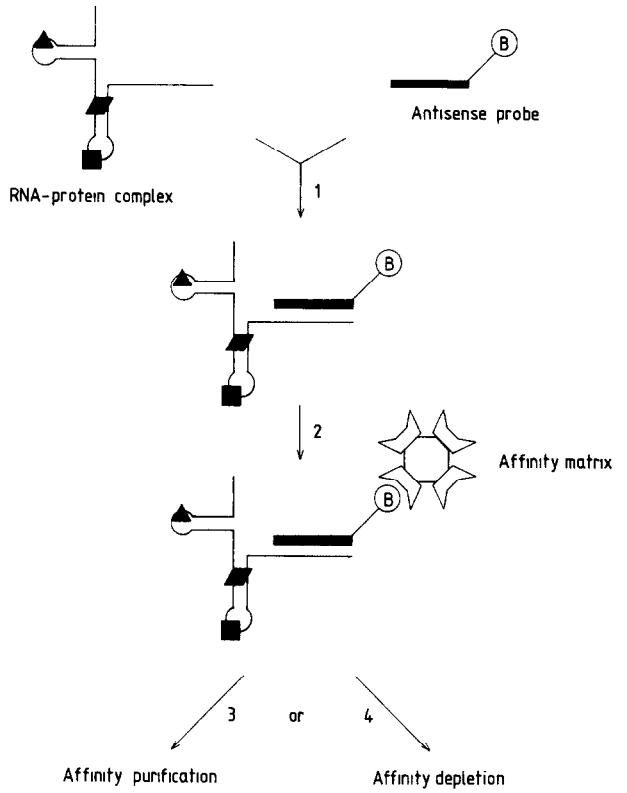

Fig. 2. The cartoon illustrates the general strategy for affinity chromatography using antisense probes. Here the antisense probe is an oligo(2′-*O*-alkylribonucleotide) linked to biotin (B), via a long, flexible spacer arm [25,26,40]. The antisense probe is complementary to an exposed RNA target sequence in the RNA–protein complex. In step 1, a stable hybrid is formed specifically between the targeted RNA and antisense probe in an in vitro extract. In step 2, the biotin on the antisense probe is bound to an appropriate affinity matrix (e.g. streptavidin, avidin or anti-biotin antibodies coupled to agarose beads). This selectively retains both the biotinylated antisense probe and any other molecules stably bound to it. This strategy can be used either to purify and characterise the targeted RNA or RNA–protein complex (step 3) [25,26,28–32], or to efficiently remove the targeted complex, allowing subsequent analysis of the depleted extract (step 4) [15,35,36].

cases, binding of an antisense probe to an snRNA inhibits its splicing. The inhibitory effect can arise either because the antisense hybrid with the snRNA blocks the snRNP base pairing to pre-mRNA (i.e. competitive hybrid formation), or because it dissociates the snRNP (i.e. disruptive hybrid formation). The binding of antisense probes to different regions of the snRNA in a snRNP particle can also inhibit different stages of the splicing reaction. For example, antisense probes binding to different regions of U2 snRNA can specifically block either stable binding of U2 snRNP to pre-mRNA, the completion of spliceosome assembly after binding of U2 snRNP or the conversion of splicing intermediates to products within the spliceosome [17]. Mayeda et al. have also shown that splicing can be inhibited in vitro by oligo(2′-*O*-methylribonucleotides) complementary to the splice sites in β-globin pre-mRNA [27].

The U7 snRNP is not involved in splicing but instead is an essential factor in the 3′ processing reaction that forms the mature 3′ termini of histone mRNAs. Cotten et al. have shown that the 3′ end processing of histone pre-mRNA can be inhibited by antisense oligonucleotides made of oligo(2′-*O*-methyl or 2′-*O*-ethylribonucleotides) that are complementary to U7 snRNA [12]. The same authors showed in addition that both forms of oligo(2′-*O*-alkylribonucleotides) were more potent inhibitors of U7 snRNP function than either RNA or phosphorothioate modified DNA probes of identical sequence [12].

The stable binding of antisense oligo(2′-*O*-alkylribonucleotide) probes containing reporter groups such as biotin has also been used to affinity purify or deplete snRNPs from in vitro extracts (illustrated in Fig. 2). Both nucleoplasmic snRNPs involved in splicing and nucleolar snRNPs involved in pre-rRNA processing have been purified through hybridisation of biotinylated antisense probes made of either oligo(2′-*O*-methyl or 2′-*O*-allylribonucleotides) [15,17,26,28]. Similarly, biotinylated oligo(2′-*O*-alkylribonucleotides) complementary to pre-mRNA sequences have also been used to affinity select assembled spliceosomes [29]. Antisense affinity selection can purify snRNPs (or other RNPs) sufficiently for the associated proteins to be analysed directly [30–32]. This has been particularly useful for studies of snRNP proteins in trypanosome extracts, where no antibodies were available that would recognise the trypanosome snRNPs [31].

Other important applications of antisense affinity selection have emerged in recent studies where it was used to enrich for rare species prior to further analysis. For example, Bruzik and Maniatis have used biotinylated oligo(2′-*O*-methylribonucleotides) to enrich for RNAs that have undergone a trans-splicing reaction, which occurs at low frequency in mammalian cells [33]. The affinity enriched, trans-spliced RNAs could then be amplified by the PCR method and characterised in detail. In a separate study, Wassarman and Steitz used biotinylated oligo(2′-*O*-methylribonucleotides) to enrich for specific snRNA complexes that had been cross-linked to pre-mRNA by treating splicing extracts with psoralen [34]. The cross-linked complexes could then be analysed by two-dimensional polyacrylamide gel electrophoresis.

The specificity and stability of hybrid formation between oligo(2′-*O*-alkylribonucleotides) and RNA target sequences also facilitates high efficiency depletion of RNP complexes from cell-free extracts using the antisense approach. For example, the concentration of either the U1, U2, U4/U6 or U5 snRNPs can be made rate limiting for splicing in vitro through antisense affinity depletion [15,35,36]. In the case of U5 snRNP, incorporation of 2-aminoadenine in place of adenine in the antisense probe was important for high efficiency depletion, since the only complementary sequence in U5 snRNA available for hybridisation is a short, U-rich loop [15]. These depletion studies demonstrated that while each snRNP is essential for splicing, spliceosome assembly was arrested at different stages according to which snRNP was depleted. This confirms that there is a specific order of snRNP binding to pre-mRNA during spliceosome assembly.

The nuclease resistance and favourable hybridisation properties of oligo(2′-*O*-alkylribonucleotides) has also been exploited to localise RNAs in mammalian cells by in situ hybridisation [28,37–39]. Antisense probes bound to RNA in situ can be visualised by fluorescence microscopy either through secondary labeling with avidin-conjugated fluorochromes, or directly through attachment of fluorochromes to the oligonucleotides. For example, nucleoplasmic and nucleolar snRNAs, and 5S and 28S rRNAs have been localised in this way [28,38,39]. Antisense oligo(2′-*O*-allylribonucleotides) coupled to fluorochromes have also been microinjected into living cells and thus used to localise RNAs in cells that have not been exposed to fixation procedures [37]. The distribution of different RNAs can also be compared in parallel by microinjecting specific oligonucleotides coupled to different fluorochromes [37].

In conclusion, the properties of oligo(2′-*O*-alkylribonucleotides) make them valuable reagents for use as antisense probes. Recent studies have established that they can be effectively used to inhibit RNA function, to purify or deplete RNAs or RNA–protein complexes and to determine RNA localisation patterns in situ. Now that phosphoramidites allowing the chemical synthesis of 2′-*O*-methyl and 2′-*O*-allyl modified polymers are commercially available, we anticipate a significant increase in the use of oligo(2′-*O*-alkylribonucleotides) for a wide range of experiments in biochemistry and molecular biology that can benefit from the application of antisense techniques.

REFERENCES

[1] Iribarren, A.M., Sproat, B.S., Neuner, P., Sulston, I., Ryder, U. and Lamond, A.I. (1990) Proc. Natl. Acad. Sci. USA 87, 7747–7751.
[2] Inoue, H., Hayase, Y., Imura, A., Iwai, S., Miura, K. and Ohtsuka, E. (1987) Nucleic Acids Res. 15, 6131–6148.
[3] Kibler-Herzog, L., Zon, G., Uznanski, B., Whittier, G. and Wilson, W.D. (1991) Nucleic Acids Res. 19, 2979–2986.
[4] Rottman, F. and Heinlein, K. (1968) Biochemistry 7, 2634–2641.
[5] Bobst, A.M., Rottman, F. and Cerutti, P.A. (1969) J. Mol. Biol. 46, 221–234.
[6] Zmudzka, B. and Shugar, D. (1970) FEBS Lett. 8, 52–54.
[7] Inoue, H., Hayase, Y., Asaka, M., Imura, A., Iwai, S., Miura, K. and Ohtsuka, E. (1985) Nucleic Acids Res. Symposium Series No. 16, 165–168.
[8] Sproat, B.S., Lamond, A.I., Beijer, B., Neuner, P. and Ryder, U. (1989) Nucleic Acids Res. 17, 3373–3386.
[9] Sproat, B.S., Iribarren, A.M., Guimil Garcia, R. and Beijer, B. (1991) Nucleic Acids Res. 19, 733–738.
[10] Sproat, B.S. (1993) in: Oligonucleotide Synthesis Protocols (Agrawal, S. ed.) chapter 6, Humana Press, Totowa, NJ.

[11] Sproat, B.S., Lamond, A.I., Guimil Garcia, R., Beijer, B., Pieles, U., Douglas, M., Bohmann, K., Carmo-Fonseca, M., Weston, S. and O'Loughlin, S. (1991) Nucleic Acids Res. Symposium Series No. 24, 59–62.
[12] Cotten, M., Oberhauser, B., Brunar, B., Holzner, A., Issakides, G., Noe, C.R., Schaffner, G., Wagner, E. and Birnstiel, M.L. (1991) Nucleic Acids Res. 19, 2629–2635.
[13] Wagner, E., Oberhauser, B., Holzner, A., Brunar, H., Issakides, G., Schaffner, G., Cotten, M., Knollmüller, M. and Noe, C.R. (1991) Nucleic Acids Res. 19, 5965–5971.
[14] Sinha, N.D., Biernat, J., McManus, J. and Köster, H. (1984) Nucleic Acids Res. 12, 4539–4557.
[15] Lamm, G.M., Blencowe, B.J., Sproat, B.S., Iribarren, A.M., Ryder, U. and Lamond, A.I. (1991) Nucleic Acids Res. 19, 3193–3198.
[16] Sproat, B.S., Iribarren, A., Beijer, B., Pieles, U. and Lamond, A.I. (1991) Nucleosides and Nucleotides 10, 25–36.
[17] Barabino, S.M.L., Sproat, B.S. and Lamond, A.I. (1992) Nucleic Acids Res. 20, 4457–4464.
[18] Froehler, B.C., Jones, R.J., Cao, X. and Terhorst, T.J. (1993) Tetrahedron Lett. 34, 1003–1006.
[19] Howard, F.B. and Miles, H.T. (1984) Biochemistry 23, 6723–6732.
[20] Inoue, H., Hayase, Y., Iwai, S. and Ohtsuka, E. (1987) FEBS Lett. 215, 327–330.
[21] Berkower, I., Leis, J. and Hurwitz, J. (1973) J. Biol. Chem. 248, 5914–5921.
[22] Lamond, A.I., Barabino, S. and Blencowe, B.J. (1990) in: Nucleic Acids and Molecular Biology (Eckstein, F. and Lilley, D.M.J. eds.) vol. 4, pp. 243–257, Springer-Verlag, Berlin/Heidelberg.
[23] Lührmann, R., Kastner, B. and Bach, M. (1990) Biochim. Biophys. Acta 1087, 265–292.
[24] Lamond, A.I., Sproat, B., Ryder, U. and Hamm, J. (1989) Cell 58, 383–390.
[25] Barabino, S., Sproat, B.S., Ryder, U., Blencowe, B.J. and Lamond, A.I. (1989) EMBO J. 8, 4171–4178.
[26] Blencowe, B.J., Sproat, B.S., Ryder, U., Barabino, S. and Lamond, A.I. (1989) Cell 59, 531–539.
[27] Mayeda, A., Hayase, Y., Inoue, H., Ohtsuka, E. and Ohshima, Y. (1990) J. Biochem. 108, 399–405.
[28] Carmo-Fonseca, M., Tollervey, D., Pepperkok, R., Barabino, S.M.L., Merdes, A., Brunner, C., Zamore, P.D., Green, M.R., Hurt, E. and Lamond, A.I. (1991) EMBO J. 10, 195–206.
[29] Ryder, U., Sproat, B.S. and Lamond, A.I. (1990) Nucleic Acids Res. 18, 7373–7379.
[30] Wassarman, D.A. and Steitz, J.A. (1991) Mol. Cell. Biol. 11, 3432–3445.
[31] Palfi, Z., Günzl, A., Cross, M. and Bindereif, A. (1991) Proc. Natl. Acad. Sci. USA 88, 9097–9101.
[32] Smith, H.O., Tabiti, K., Schaffner, G., Soldati, D., Albrecht, U. and Birnstiel, M.L. (1991) Proc. Natl. Acad. Sci. USA 88, 9784–9788.
[33] Bruzik, J. and Maniatis, T. (1992) Nature 360, 692–695.
[34] Wassarman, D.A. and Steitz, J.A. (1992) Science 257, 1918–1925.
[35] Barabino, S.M.L., Blencowe, B.J., Ryder, U., Sproat, B.S. and Lamond, A.I. (1990) Cell 63, 293–302.
[36] Wolff, T. and Bindereif, A. (1992) EMBO J. 11, 345–359.
[37] Carmo-Fonseca, M., Pepperkok, R., Sproat, B.S., Ansorge, W., Swanson, M.S. and Lamond, A.I. (1991) EMBO J. 10, 1863–1873.
[38] Carmo-Fonseca, M., Pepperkok, R., Carvalho, M.T. and Lamond, A.I. (1992) J. Cell Biol. 117, 1–14.
[39] Carmo-Fonseca, M., Ferreira, J. and Lamond, A.I. (1993) J. Cell Biol. 120, 841–852.
[40] Pieles, U., Sproat, B.S. and Lamm, G.M. (1990) Nucleic Acids Res. 18, 4355–4360.