METHOD

Dramatically improved RNA in situ hybridization signals using LNA-modified probes

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ABSTRACT

In situ detection of RNA by hybridization with complementary probes is a powerful technique. Probe design is a critical parameter in successful target detection. We have evaluated the efficiency of fluorescent DNA oligonucleotides modified to contain locked nucleic acid (LNA) residues. This increases the thermal stability of hybrids formed with RNA. The LNA-based probes detect specific RNAs in fixed yeast cells with an efficiency far better than conventional DNA oligonucleotide probes of the same sequence. Using this probe design, we were also able to detect poly(A)⁺ RNA accumulation within the nucleus/nucleolus of wild-type cells. LNA-based probes should be readily applicable to a diverse array of cells and tissue samples.

Keywords: RNA-FISH; LNA; nuclear foci; poly(A)⁺ RNA

In situ hybridization (ISH) offers the advantage of studying the expression level and localization of RNA within single cells. The technique is applicable to virtually all types of eukaryotic cells ranging from yeast to human origin, and is also successfully used for probing of tissue sections or whole-mount preparations. With the rapid development of microscopes and software for image acquisition, the usage of fluorescence is nowadays the preferred method of detection. DNA oligonucleotides have become popular probes for RNA fluorescent ISH (RNA-FISH) analysis, as these can be synthesized chemically, allowing for incorporation of amino-modified nucleotides, which can subsequently be directly coupled to a fluorophore of interest (Chartrand et al. 2000). On top of this facile and reproducible probe preparation method, oligonucleotides often produce high signal-to-noise ratios because of their relatively low sequence complexity. However, this is clearly counterproductive in terms of binding affinity when compared to longer probes.

The usage of locked nucleic acid (LNA)-modified oligonucleotide probes has been shown to significantly improve the sensitivity and specificity of microRNA detection (Valoczi et al. 2004; Wienholds et al. 2005). LNA oligonu-

cleotides are a new class of bicyclic RNA analogs that exercise an unprecedented high affinity for their complementary DNA or RNA targets (Koshkin et al. 1998). By using a design in which several positions in a conventional DNA oligonucleotide were substituted by LNAs, the sensitivity in detecting mature miRNAs by Northern blotting was increased by at least one order of magnitude (Valoczi et al. 2004). More recently, LNA-modified DNA-oligonucleotides were also used as FISH-probes on whole-mount zebrafish embryos to detect the temporal and spatial expression pattern of 115 conserved vertebrate miRNAs (Wienholds et al. 2005).

To evaluate the feasibility of LNA-modified probes for mRNA-FISH on fixed yeast cells, we compared the intensity of heat-shock *SSA4* RNA staining using progressively shorter LNA-modified or DNA probes containing a single fluorescent Cy3 label at their 5'-ends (Fig. 1A; Table 1). To be able to also examine signal specificity, the *Δrip1* mRNA export mutant strain was used. At permissive growth conditions of 25°C, *SSA4* transcripts are undetectable, whereas at nonpermissive conditions of 42°C, *SSA4* transcription is induced and the strain accumulates *SSA4* RNA in a single nuclear dot at or near its site of transcription. This accumulation has previously been detected by a combination of a 51-mer (KD199) and a 54-mer (KD200) DNA oligonucleotide probe, each harboring five amino-modified thymidine residues for attachment of Cy3 fluorophores (Jensen et al. 2001; Thomsen et al. 2003).

Both singly labeled 54-mer DNA-KD200 and LNA-KD200 probes reliably detected the *SSA4* RNA signal in *Δrip1* cells

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FIGURE 1. Short LNA-modified SSA4 RNA-FISH probes yield robust signals. (A) Schematic representation of the LNA/DNA probe design. The first, and fluorescently labeled, nucleotide of the probes is in all cases positioned at the SSA4 nucleotide immediately prior to the major poly(A)-tail addition-site [denoted p(A)]. A_n indicates the SSA4 RNA poly(A) tail. (B) SSA4 RNA-FISH analysis in the $\Delta rip1$ mutant. Cells were grown at 25°C and fixed after a 20-min temperature shift to 42°C. This shift concomitantly induces SSA4 transcription and the $\Delta rip1$ -induced mRNA export phenotype. Fixed cells were analyzed by RNA-FISH using Cy3 5'-end-labeled LNA-modified or DNA probes (Fig. 1A; Table 1) and progressively more stringent hybridization conditions as indicated.

subjected to a 20-min heat pulse at 42°C (Fig. 1B, upper row, two first columns). However, with increasing hybridization stringencies, the DNA-KD200 signal was lost (Fig. 1B, upper row). In contrast, the LNA-KD200 probe still produced a robust signal even after hybridization in 70% formamide, reflecting the superior thermal stability of the LNA–RNA hybrid compared to the DNA–RNA hybrid. This relationship was underscored when analyzing progressively shorter LNA-modified probes. Remarkably, the short 25-mer LNA-THJ200 probe still produced an excellent SSA4-FISH signal even when subjected to the most stringent hybridization conditions of 70% formamide (Fig. 1B, lower row, two last columns). In fact, under these conditions, much less background stain was detectable “outside the dot domain,” indicating that the majority of SSA4 RNA in $\Delta rip1$ after a 20-min heat pulse at 42°C is localized to this nuclear

focus. Importantly, all utilized probes detected SSA4 RNA specifically, as FISH signals under inducing conditions (42°C, 20 min) always overlapped the DAPI-stained DNA signal as expected and under noninducing conditions (25°C) were absent from cells (data not shown).

TABLE 1. Oligonucleotide probes used in this study

Probe name	Probe sequence (5'–3')	T_m^a (°C)	%LNA	T_m^b (°C)
KD200	gagaacgtacaatatagtagtcatttgctgaattactgattgtatcttatatat	67	33.3	77
THJ202	gagaacgtacaatatagtagtcatttgctgaattactgattgtgt	68	32.6	79
THJ201	gagaacgtacaatatagtagtcatttgctgaattac	64	32.4	75
THJ200	gagaacgtacaatatagtagtcattt	59	32.0	71
THJ790	tttttttttttttttttt	40	35.0	61

The melting temperature (T_m) of individual probes (except for THJ790) was estimated using the T_m prediction tool accessible at <http://www.exiqon.com>. T_m^a represents the T_m for DNA probes, whereas T_m^b represents T_m for LNA-modified probes. Note that T_m predictions are based on hybridization to perfect-match DNA nucleotides. T_m for probe THJ790 is based on actual measurements against a ribo-A₂₀ complement (Jacobsen et al. 2004). The degree of LNA modification is given in percentage of probe length.

FIGURE 2. Poly(A)⁺ RNA-FISH analysis of the wild-type (WT), *pap1-1* and Δ *trf4* strains. Cells grown at 25°C or temperature-shifted for 30 min to 37°C were fixed and analyzed by RNA-FISH using a Cy3 5'-end-labeled LNA-modified dT₂₀ probe (THJ790). For comparison, wild-type cells fixed after a temperature shift to 37°C for 30 min were also subjected to RNA-FISH using a dT₇₀ DNA-oligonucleotide probe containing seven amino-modified thymidine residues for attachment of Cy3 fluorophores. To evaluate subnuclear localization, signals obtained using the THJ790 probe were overlaid with DAPI.

We also wished to evaluate the utility of an LNA-modified probe to detect poly(A)⁺ RNA. For this purpose, a dT₂₀ probe (THJ790), harboring a single Cy3 label, was synthesized (Table 1). In pilot experiments using the Δ *rip1* strain, this probe yielded overwhelmingly brighter nuclear signals than our conventionally used DNA-based dT₇₀ probe harboring seven Cy3-labeling sites (data not shown). We therefore decided to also test the effect of THJ790 staining of wild-type cells. Without precedent, a nuclear signal, which could not be detected when using the dT₇₀ probe, was detectable from fixed cells (Fig. 2, upper row). Interestingly, THJ790 signal intensity increased when these cells were temperature-shifted to 37°C for 30 min as compared to growth at 25°C. Consistent with this observation, DNA microarray experiments show that the fraction of polyadenylated RNAs in a wild-type yeast cell increases at elevated temperatures (K. Abruzzi and M. Rosbash, pers. comm.). In addition, a substantial fraction of the THJ790 signal is positioned next to the DAPI stain, suggesting a nucleolar localization (Fig. 2, upper row). Which species of polyadenylated RNA(s) is detected by the THJ790 probe is not clear. Passage of polyadenylated mRNA through the nucleolus has previously been suggested (Schneiter et al. 1995). Consistently, introduction of the temperature-sensitive *pap1-1* mutation of the conventional yeast mRNA poly(A) polymerase considerably decreases the THJ790 signal at the nonpermissive temperature (Fig. 2, middle row). However, a fraction of the nuclear pool of stable RNAs (snRNAs, snoRNAs, rRNAs, and tRNAs) has recently been shown to be turned over by the so-called TRAMP complex in a polyadenylation-dependent process requiring one of

two newly identified poly(A)-polymerases, Trf4p or Trf5p (Kadaba et al. 2004; LaCava et al. 2005; Vanacova et al. 2005; Wyers et al. 2005). Deletion of the *TRF4* gene also lowers the 37°C THJ790 signal (Fig. 2, lower row). Future studies will aim to clarify exactly which polyadenylated RNAs contribute to the THJ790-derived signal.

In this paper, we have shown that LNA-modified FISH probes readily outperform conventional DNA probes in the detection of RNA in fixed yeast cells. In addition to their superior thermal properties in RNA binding, the increased efficiency of LNA-based FISH probes is supposedly due to their short sizes, resulting in better cell permeability and target accessibility. The latter might provide a clear advantage when targets are short; for example, oligo(A)-tails produced by TRAMP. As already shown for zebrafish embryos, this methodology should be readily adoptable to other cells and tissues.

MATERIALS AND METHODS

Yeast strains

The yeast strains used in this study are derived from W303 and have all been previously described: wild type (WT) and Δ *rip1* (Stutz et al. 1997), Δ *trf4* (Wyers et al. 2005), and *pap1-1* (Dower et al. 2004).

Preparation of yeast cells for RNA-FISH

Growth, fixation, and preparation of cells as well as processing of these for FISH analysis were done as previously described

(Jensen et al. 2001). Briefly, 10 mL cell cultures were grown at 25°C to an OD₆₀₀ of 0.1–0.3 and subjected to an instantaneous temperature shift by addition of pre-heated media. After incubation at the desired temperature, cells were fixed in 4% formaldehyde for 15 min at the experimental temperature followed by 30 min at 20°C. Fixed cells were pelleted by centrifugation and washed three times in 1 mL, and subsequently resuspended in 200–500 µL, of wash buffer (1.2 M sorbitol, 0.1 M KH₂PO₄/K₂HPO₄ at pH 6.5). Then 10 µL of the individual cell suspensions were plated on 14-well, 0.1% poly-L-lysine coated glass slides (Immuno-Cell Int.) and washed in wash buffer containing 1% β-mercaptoethanol before spheroplasting for 10–15 min at 30°C in 10 µL of oxalyticase solution (20 mM vanadyl-ribonucleoside, 0.2% β-mercaptoethanol, 0.1 U/µL RNasin, 1.2 M sorbitol, 0.1 M KH₂PO₄/K₂HPO₄ at pH 6.5, 0.1 mg/mL oxalyticase [Enzogenetics]). Subsequently, cells were washed for 5 min at 20°C; twice in wash buffer, once in 0.1 M KH₂PO₄/K₂HPO₄ (pH 6.5), 0.1% NP-40 and once in 0.1 M KH₂PO₄/K₂HPO₄ (pH 6.5), and finally incubated with cold 70% ethanol for 15–30 min at –20°C.

Probe preparation and RNA-FISH

Probe preparation was done by direct labeling of 10–20 µg of oligonucleotide with 300 µg of Cy3 (Amersham Pharmacia) in 0.1 M NaHCO₃/Na₂CO₃ (pH 9.0) overnight at 25°C in the dark followed by G-50 spin column purification. For each well, 100 ng of probe was mixed with 10 µg of salmon sperm DNA and 10 µg of yeast tRNA, lyophilized and resuspended in 5 µL of solution I (80% formamide, 10 mM NaHPO₄ at pH 7.0), denatured for 5 min at 95°C, and finally mixed with 5 µL of solution II (4× SSC, 20 mM Vanadyl-ribonucleoside, 4 µg/µL BSA, 0.1 U/µL RNasin).

Prior to probe addition, cells were drained for ethanol and washed at 20°C twice for 5 min in 2× SSC and once for 10 min in 40% formamide/2× SSC, 0.1% Triton X-100, before overnight incubation at 37°C with 10 µL of probe mix. Probe removal was followed by washing steps: (1) twice in 40% formamide/2× SSC for 10 min at 37°C; (2) once in 2× SSC/0.1% Triton X-100 for 10 min at 20°C; (3) twice in 1× SSC for 10 min at 20°C; and (4) twice in 1× PBS for 5 min at 20°C. Finally, 2.5 µL of mounting solution (1× PBS at pH 8.0, 80% glycerol, 0.1 µg/mL DAPI) was applied to air-dried wells, which were subsequently covered with a coverslip and ready for analysis by fluorescent microscopy.

Increased hybridization stringencies were achieved by increasing the formamide concentration (normally 40%) in hybridization and all appropriate wash buffers to 50%, 60%, and 70%, respectively. The probes used are listed in Table 1. The LNA-modified probes are available from Exiqon (<http://www.exiqon.com>).

Imaging

Images were acquired on an Olympus BX51 microscope equipped with a cooled Olympus DP50 CCD camera and analysis software. For signal intensity comparison, individual experiments were performed in different wells on the same glass slide. Furthermore, exposure times, gain settings and so on were kept constant during image acquisition. Image handling was done in Adobe Photoshop.

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