Localization of Trinucleotide Repeat Sequences in Myotonic Dystrophy Cells Using a Single Fluorochrome-Labeled PNA Probe

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ABSTRACT

A labeled peptide nucleic acid (PNA) antisense probe was used to study the spatial distribution of triplet repeats (CTG) in human myotonic dystrophy (DM) cells by high-resolution fluorescence in situ hybridization (FISH). It was found that transcripts containing triplet repeats were present as a number of discrete foci in the DM nuclei. Greater numbers of foci were visible with the PNA probe than a comparable DNA probe. The PNA probe was also used to visualize the triplet-repeat expansion within the DM gene located on chromosome 19. Using the intensity of the expanded triplet-repeat on the chromosomes as a reference, it was estimated there were between 15–230 RNA molecules in each focus observed in DM nuclei.

INTRODUCTION

Trinucleotide repeat sequences have been identified in many sites throughout the human genome. In a normal individual, the number of trinucleotides in a repeat sequence is typically within the range of 5–50. Triplet repeats may expand into hundreds or even thousands of copies, causing as many as ten different neurological disorders (6,10, 13,14,20).

The molecular basis of myotonic dystrophy (DM) is an extreme expansion of the trinucleotide (CTG) within the 3′ untranslated region (UTR) of the transcript for DM protein kinase (DM-PK). The normal DM-PK gene encodes a serine-threonine PK (2,4,11), is 11.5 kb, contains 14 exons (21) and is localized on chromosome 19q13.3 (8,15). In normal individuals, this gene contains a block of 5–30 CTG repeats, whereas DM patients have from 80 to more than 3000 triplet repeats, which are highly unstable (21). The severity of disease increases, and the age of the onset decreases with increasing number of repeat units (7,19). The molecular basis of DM, however, remains puzzling since the repeat occurs in the 3′ UTR of DM-PK mRNA, a region that does not encode protein.

This report describes the development of peptide nucleic acid (PNA) probes for fluorescence in situ hybridization (FISH) and detection of the expanded triplet repeats in the mutant allele in DM cells. PNA is a new type of DNA analog in which the natural phosphate deoxyribose backbone of deoxyribonucleic acid has been replaced by N-(2-aminoethyl)-glycine, a peptide-like unit (12). The achiral and electrostatically neutral PNA molecule is capable of recognizing complementary sequences in DNA, RNA and PNA according to Watson-Crick base pairing rules (3,22). Binding of PNA to its complement can occur in either a parallel or anti-parallel orientation of PNA; however, the anti-parallel duplex is much more stable (3). The melting temperature of PNA/DNA and PNA/RNA hybrids are much higher than corresponding DNA/DNA or DNA/RNA duplexes due to a lack of electrostatic repulsion in the PNA/DNA and PNA/RNA helices. Also, PNA has been shown to bind to the complementary repeat DNA sequences in double-stranded DNA by a novel mechanism, allowing the formation of stable DNA/PNA hybrids (1).

Recently, Thisted et al. (18) examined the expression of immunoglobulin κ-light chain mRNA in tissue sections using PNA probes, and Lansdorp et al. (9) have demonstrated direct detection of human telomere sequences in metaphase spreads and interphase nuclei with fluorochrome-labeled PNA. This report describes the use of fluorochrome-labeled PNA probes and FISH to study the spatial distribution of trinucleotide repeat sequences within the nucleus and on the chromosomes of the DM cells.
MATERIALS AND METHODS

Cell Culture and Fixation

Normal human fibroblasts (Detroit 551; ATCC, Rockville, MD, USA) and fibroblasts from a DM patient (No. 3132, provided by Dr. David Housman [MIT, Cambridge, MA], which contain ca. 6 kb of repeat expansion) were grown on gelatin-coated coverslips in Dulbecco’s minimal essential medium (DMEM; Life Technologies, Gaithersburg, MD, USA) low glucose supplemented with 10% fetal bovine serum (FBS).

Cells on coverslips were washed in Hank’s balanced salt solution (HBSS; Life Technologies) and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (2.7 mM KCl, 1.5 mM KH2PO4, 0.137 M NaCl and 8 mM Na2HPO4, pH 7.5) containing 5 mM MgCl2 for 15 min at room temperature (RT). After fixation, cells were washed with 70% ethanol and stored in 70% ethanol at 4°C.

Cytogenetic Preparation of Interphase Nuclei and Chromosome Spreads

Fibroblast cultures grown in DMEM supplemented with 10% FBS were exposed to 0.015 µg/mL Colcemid for 45–60 min (16). After incubation, media were removed in a 50-mL centrifuge tube, and cells were trypsinized with 0.5% trypsin-0.05 mM EDTA in HBSS. Cells were suspended in 0.075 M KCl, incubated 45–60 min (16). After incubation, the cell pellet was resuspended in 1 mL of methanol:acetic acid. Cells were dropped onto ethanol-washed slides from a distance of 1–2 ft and air-dried overnight. The slides were heated at 65°C for 1–2 h and stored at -80°C.

Probe Preparation

Fluorescein-labeled (Sigma Chemical, St. Louis, MO, USA) or cyanine-3-labeled (Cy™-3; Amersham, Arlington Heights, IL, USA) oligonucleotide probes (DNA-30) were prepared as described previously by Taneja et al. (16). The DNA-30 probe was 5′-T(F)-TCTTATCTT(F)CAGCAGCAGCAGCAGCAGCAG-3′, where T(F) represents a thymidine residue labeled at the 5-position with Cy-3. The 15-mer PNA probe (CAG)3 typically is not used in the repeat sequences was prepared by PerSeptive Biosystems (Framingham, MA, USA) and Boston Probes (Bedford, MA, USA) and was labeled at the N terminus with one molecule of Cy-3 (Figure 1).

In Situ Hybridization

Detection of Nuclear Transcripts.

Normal and DM cells fixed to slides were hydrated in PBS containing 5 mM MgCl2 for 10 min and then treated with 40% formamide, 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) for 10 min at RT. Cells were hybridized for 2 h at 37°C with fluorescein-labeled DNA-30 (10 ng) or PNA-15 (5 ng) probe in 20 µL of 40% formamide; 2× SSC; 0.2% bovine serum albumin (BSA). After hybridization, cells were washed once with 40% formamide; 1× SSC for 30 min at 37°C and twice with 1× SSC at RT for 30 min. Cells on coverslips were counterstained with 4’,6-diamino-2-phenylindole (DAPI; Sigma Chemical) and were mounted on slides using 1,4-phenylenediamine in 90% glycerol in PBS.

DNA vs. PNA probe competition and displacement. In a competition experiment, a mixture (20 µL) containing 1:1 or 2:1 molar ratios of DNA-30 and PNA-15 probes was prepared and hybridized to DM cells as described above. For the displacement experiment, cells were hybridized with the fluorescein-labeled DNA-30 probe and washed as described above. The cells were then exposed to the Cy-3-labeled PNA-15 probe for 30 min under the same hybridization conditions. After washing, images for each wavelength were obtained for comparison.

Detection of the mutant DM-PK gene. Cytogenetic preparations were denatured similar to that described by Lansdorp et al. (9). Interphase nuclei and spreads, in 40 µL of hybridization buffer (70% formamide; 0.2× SSC) containing 10 ng of Cy-3-labeled PNA-15, were heated at 70°C for 30 min in a humidified incubator. The slides were then cooled to 37°C for 60 min and washed with hybridization buffer at 37°C for 30 min. After two washes with PBS at RT (10 min each), the slides were counter-stained with DAPI and mounted as described above.

Digital Imaging Microscopy

Digital imaging microscopy was performed as described by Taneda et al. (17). Typically, twenty images were obtained at 0.25-µm intervals for each wavelength with a charge-coupled device (CCD) camera (Model 22; Photometrics, Tucson, AZ, USA). Using a mathematical algorithm, images were processed to remove out-of-focus light from each focal plane. For double-labeling, images from the same Z-plane for each wavelength were superimposed using fiduciary markers. Those voxels containing both red and green signals were colored white. Images were displayed on a Silicon Graphics workstation (Mountain View, CA, USA), were inspected using graphic software developed by CSP (Billerica, MA, USA).

Figure 1. Structure of the PNA probe. C, A and G are nucleobases, and R is Cy-3.
and were photographed using a digital-to-analog film recorder (Matrix Instrument, Orangeburg, NY, USA).

RESULTS AND DISCUSSION

Distribution of Repeat Sequences in the DM Nucleus

A 15-mer PNA probe (PNA-15), complementary to the trinucleotide repeat sequence (CAG)$_n$ was singly labeled at the amino terminus with Cy-3 (Figure 1). As a result, the fluorochrome intensity per probe molecule was defined. Using this probe, it was found that DM-PK transcripts containing expanded trinucleotide repeat sequences were post-transcriptionally accumulated as a number of discrete foci (Figure 2A) in DM patient fibroblasts, as was shown previously (16) using a Cy-3-labeled DNA-30 probe (Figure 2C). No foci were detected in the normal fibroblast cells with the PNA-15 probe (Figure 2B), indicating that signal was due to the presence of the expanded triplet repeats. After 30 min of hybridization with the PNA-15 probe, no increase in signal intensity was observed, whereas at least 2 h were required for complete hybridization using an equivalent molar concentration of DNA-30.

Measurement of the fluorescence intensity of the 50 brightest foci from different cells observed with the PNA-15 or DNA-30 probe revealed that the average spot intensity with the PNA-15 probe was approximately 3.8 times brighter than with the DNA-30 probe. I also counted the number of foci per cell in 100 cells from each hybridization with nuclear preparations of DM cells. With the DNA-30 probe, the number of foci per nucleus varied from 2–15 with an average of 5.5 foci per nucleus; whereas, with the PNA-15 probe, the number was from 3–30, and the average was 9.6 foci per nucleus. The greater signal-to-noise ratio of the PNA probe was due to a number of factors including: (i) low nonspecific binding of the PNA probe to protein or other components in the cells and (ii) the high thermal stability of the PNA/RNA hybrid, which allowed the use of high-stringency hybridization and wash conditions.

Because PNA binds in either (parallel and antiparallel) orientation (3), it was important to determine whether the PNA and DNA probes were binding to the same targets. This was demonstrated by competition and displacement experiments (Materials and Methods). When cells were hybridized simultaneously with the DNA-30 probe and PNA-15 probe, only the signal from the PNA-15 probe was detected. It was not possible to co-localize the DNA and PNA signals by performing simultaneous hybridization even using a twofold molar excess of DNA-30 probe. The results indicated that binding of the PNA-15 probe was favored, because of a faster hybridization of the PNA and a greater stability of the resulting PNA/RNA hybrid.

Figure 2. Detection of CTG repeats in DM fibroblasts. DM patient and normal (Detroit 551) fibroblasts were plated on gelatin-coated cover-slips, washed and fixed in 4% paraformaldehyde. A single PNA-15 or DNA-30 probe complementary to CTG repeats was labeled with Cy-3 and was hybridized to the cells. After washing, cells were counterstained with DAPI and mounted on slides in 1,4-phenylene diamine. (A) DM fibroblasts were hybridized to PNA-15 probe. Repeat transcripts appeared as discrete foci. (B) Normal cells hybridized to PNA-15 probe. DM fibroblasts were hybridized to DNA-30 probe.

Figure 3. Co-localization of binding of the PNA-15 and DNA-30 probes. Nuclear preparations of DM cells were hybridized to the fluorescein-labeled DNA-30 probe followed by washing and hybridization with the Cy-3-labeled PNA-15 probe under identical conditions. (A) DNA-30 probe; (B) PNA-15 probe. (C) Superimposition of Panels A and B.
In a displacement experiment, DM fibroblast cells were first hybridized with the fluorescein-labeled DNA-30 probe for 2 h, and then cells were subsequently exposed to the Cy-3-labeled PNA-15 probe for 30 min. The results presented in Figure 3 clearly showed that all the green foci (DNA-30 probe) were converted to red foci (PNA-15 probe), proving that the PNA-15 probe was detecting the same expanded trinucleotide repeat sequences as the DNA-30 probe in the DM nuclei. The presence of additional red foci that did not co-localize with green foci was consistent with the earlier observation that PNA-15 probe was able to detect lower copy number foci than the DNA-30 probe. Because I was careful to ensure that hybridization with DNA-30 probe was complete (checked by duplicate slides) before addition of the PNA-15 probe, the signal generated by the PNA-15 probe was due to displacement of the DNA-30 probe by the PNA-15 probe. This resulted in a more stable RNA/PNA complex. The ratio of red-to-green fluorochrome in each focus increased with the time of exposure to the PNA-15 probe, and only a very low level of green signal was detectable after 2 h. Further exposure to PNA-15 did not continue to reduce the green signal, indicating that equilibrium existed between the PNA-15 and DNA-30 probes.

Detection of Single Copy of Triplet Repeats on Chromosomes

Using the non-denaturing conditions derived for the detection of repeat transcripts, no signal was detected in metaphase chromosome spreads, indicating an inability of the PNA-15 probe to strand-invade dsDNA. However, denaturation of the DNA in the presence of the PNA-15 probe resulted in the appearance of two bright red spots on the sister chromatids of a single chromosome 19 within each metaphase spread (Figure 4). Examination of 20 randomly selected metaphase spreads, revealed that 17 (85%) were labeled, demonstrating that the hybridization efficiency was very high. The PNA results were consistent with the findings of others who used either a biotin-labeled oligonucleotide probe (5) or a genomic...
probe for the DM region (8,15).

The expanded trinucleotide repeat within the DM-PK gene of the fibroblasts used in this study was approximately 6 kb in length (16). Each chromosomal signal therefore maximally represented approximately 400 fluorochrome molecules if the PNA probe saturated the DNA target. Comparison of the spot intensities of the chromosome molecules if the PNA probe represented approximately 400 fluorosomal signal therefore maximally approximately 6 kb in length (16). Each chromosome used in this study was approximately 6 kb in length (16).

High signal intensity and low background are important for the detection of single-copy genes by FISH. The results obtained using a PNA probe for study of DM indicate that PNA will become an important tool in clinical medicine and in basic research. Further improvements may include an enhanced sensitivity by incorporation of additional fluorophores and differential labeling (i.e., multicolor FISH) to provide greater information per experiment.

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