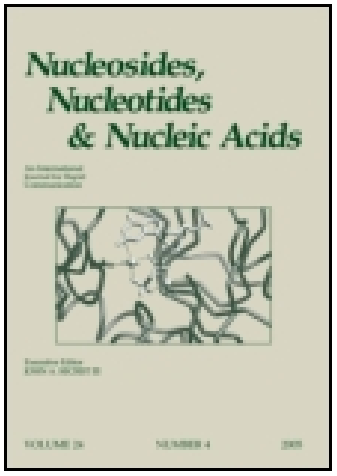

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CHEMICAL SYNTHESIS OF LNA-mCTP AND ITS APPLICATION FOR MicroRNA DETECTION

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□ *Locked nucleic acids (LNA) are being applied in hybridization studies, but current locked nucleotides cannot be transcribed into RNA probes. Here, the authors report the use of a new synthetic locked nucleotide, locMeCytidine-5′-triphosphate (LNA-mCTP), for hybridization study. This synthetic LNA-mCTP can be transcribed into a short (∼30-nt) RNA probe. Dot blot hybridization on nylon membrane suggested that the short ^{33}P-LNA RNA probes had strong binding affinity to target oligonucleotides and its detection sensitivity was approximately ∼1000 miRNAs in a 20- to 30-μm (diameter) dot area. On tissue sections, the differential expression pattern of mir-124 within different tissue regions revealed by short ^{33}P- LNA RNA probes correlated well to that analyzed by real-time RT-PCR. In addition, the specific cellular distribution of vasoactive intestinal polypeptide mRNAs in the mouse brain was the same using a 30-nt ^{33}P-LNA RNA probe and a 1.5-kb ^{33}P-RNA probe. These results suggested the high hybridization specificity of the small LNA-RNA probes to target small RNAs. Finally, the authors applied ^{33}P-LNA probes to detect miRNA let-7C expression in human cancer tissues. Let-7C was clearly present in lung, prostate, and colon cancers but undetectable in ovary and thyroid cancer samples. These results suggested that this miRNA detection method provides an alternative tool to study the cellular distribution of miRNAs in tissues.*

Keywords CTP; In situ hybridization; Locked nucleotide; miRNA; ^{33}P; Probes

Ten years ago, a new class of nucleic acid analogues, termed locked nucleic acids (LNA), was introduced.[1] Chemically synthesized locked nucleotides contain a 2′-O-CH2-4′ bicyclic structure in the ribose.[2] The LNA monomer based on the 2′-O-CH2-4′ bicyclic structure is called the oxy-LNA. There are

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also the 2′-S-CH2-4′ (thio-LNA) and the 2′-NH-CH2-4′ (amino-LNA) bicyclic analogues.[1,3,4] LNA oligonucleotides typically contain one or more 2′-O, 4′-C-methylene-β-D-ribofuranosyl nucleotide monomer(s).

The most important characteristic in locked nucleotides is the *N*-conformation of nucleic acids due to the bicyclic structure. This single nucleic acid modification shows the highest affinity obtained by Watson-Crick hydrogen bonding, so the LNA probes have very high sequence specificity for the fully matched nucleic acid target,[5,6] with greatly increased thermal stabilities. The increase in T_m per modification varies from 5 to 10°C and the highest T_m increase involves LNA oligonucleotides targeting RNA. Because of those features, LNA oligonucleotides have been widely applied in hybridization-based researches such as siRNA,[7] microarray analysis,[8] RNA isolation,[9] and comparative genome hybridization.[10] One of the main potential usages is to detect the expression of small RNAs, such as microRNAs (miRNAs), in tissue samples. To trace the LNA probes hybridized to miRNA targets, digoxigenin[11] or fluorescence dye has been used to label LNA probes.[12] These two labeling and assay methods provided useful tools to analyze the expressions of miRNAs in tissues.

MiRNAs are small regulatory RNAs,[13] which are transcribed as a ~60- to 80-nt precursor RNA containing a 4- to 15-nt loop. The precursors are then digested by Dicer to generate a single-stranded 19- to 25-nt mature miRNA.[14,15] MiRNAs have been identified in many eukaryotic organisms ranging from nematodes to humans,[16] and have been reportedly involved in multiple biological functions by regulating the translation of mRNAs to alter protein expressions.[17] Recent studies also suggest that miRNA expressions are tissue- and cell-type-specific and that miRNA expression levels were highly related to the formation and development of tumors.[18] The expression profiles of miRNAs have been deeply investigated. For instance, Wienholds et al.[19] studied the temporal and spatial expression profiles of 115 conserved vertebrate miRNAs in zebrafish embryos by in situ hybridization. Mansfield et al. applied a new method to trace the expression of miRNAs.[20] In experiment, they induced the tissue-specific expression of several miRNAs during mouse embryogenesis, including two encoded by genes embedded in homeobox (Hox) clusters, miR-10a and miR-196a. They found that these miRNAs are expressed in patterns corresponding well to those of Hox genes. Since hundreds of miRNAs have been isolated, more research is needed to clarify the in situ expression patterns of those identified miRNAs.

In situ detection of miRNA cellular distribution is important because it provides direct morphological evidence for functional studies and confirms the miRNA tissue distributions revealed by RT-PCR and microarray.[21,22] Localization of miRNAs in tissues also provides valuable clues in understanding the functions of miRNAs. However, in situ detection of miRNAs

is challenging. Due to their small sizes, only small probes can be used. Small probes usually require low hybridization temperature and weak post-hybridization wash stringency, which may increase background and reduce signal-to-noise ratio. Small probes also have fewer labeled nucleotides, leading to the weak detectable signals from each probe. To get enough visible signals, higher copies of target miRNAs in each cell are required. However, the abundance of most miRNAs in tissues is not clear. This makes it difficult to judge hybridization results.

One way to improve hybridization efficiency, specificity, and detection sensitivity is to use LNA miRNA antisense probes. Small LNA probes are able to bind target miRNAs tightly even at high temperatures and under high stringency wash conditions.[2] At present, almost all of the LNA DNA probes are made by a chemical synthetic method using LNA phosphoramidite nucleotides. Chemically synthesized LNA probes are labeled by fluorescent dye,[12] or digoxigenin,[19] at one end of the probes and have provided a useful tool for research communities to study the cellular distributions of many miRNAs. However, due to the lack of triphosphates, LNA phosphoramidite nucleotides cannot be transcribed into miRNA probes for hybridization studies.

To provide an alternative way to generate LNA miRNA probes through in vitro transcription, in this study, we chemically synthesized the triphosphate LNA-mCTP, which was then transcribed into a modified ^{33}P-labeled miRNA antisense probe. This modified antisense probe was used to detect the expression of miRNA-124 and let-7C in mouse and human tissue. Our data showed that our miRNA labeling and detection method is applicable for most research laboratories to synthesize LNA-miRNA probes.

RESULTS

In Vitro Transcription Efficiency

After we synthesized oxy-locked-mCTP, we examined whether it could be a substrate for T7 RNA polymerase and transcribed into RNA. T7 promoter-controlled templates were used to generate RNAs at expected sizes of 200, 100, and 22 bp. The ratio of LNA-CTP to normal CTP in the reaction was changed to evaluate the transcription efficiency of LNA-mCTP (Figure 1).

Our data showed that when increasing the ratio of LNA-mCTP up to 1:1, transcripts at sizes of 100 and 200 nt could not be efficiently synthesized (Figure 1a). Small aborted transcripts (>40–50 nt) were observed in 15% denaturing acrylamide (data not shown). None of the 100- or 200-nt transcripts could be made if LNA-mCTP was the only CTP source in the reaction. In contrast, the 22-nt transcripts were successfully synthesized when the ratio of LNA-mCTP to CTP was at 1:1. The total yield of the small probe was about 50% of that without the addition of LNA-mCTP in the reaction

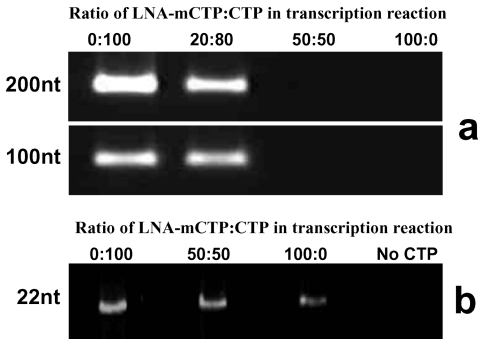


FIGURE 1 Electrophoresis pictures show the transcription efficiency of LNA-mCTP. In the reaction, when the ratio of LNA-mCTP to normal CTP reached 50%, 100- and 200-nt transcripts could not be synthesized (a). 100- or 200-nt transcripts were transcribed only when LNA-mCTP accounted for 20% of the CTP source in the reaction. However, the yield of 100- and 200-nt RNAs were much less than when transcribed with normal CTP only (a). In contrast, 22-nt transcripts were successfully generated even if LNA-mCTP accounted for 50 or 100% of the CTP sources in the reaction (b), but the yield of the transcripts was greatly reduced as compared to that without LNA-mCTP. No RNA product can be made when the reaction is lacking any form of CTP (no CTP data in lane 6).

(Figure 1b). To verify the incorporation of the LNA-mCTP in the probes, we used LNA-mCTP as the only CTP source for transcription. Analysis with gel electrophoresis showed that the 22-nt RNAs were still synthesized (Figure 1b). However, the yield of RNA probes was dramatically reduced, only ~20–30% of LNA-free probes (Figure 1b). To exclude the possible incorporation of other nucleotides into the CTP position inside synthesized probes, we removed both CTP and LNA-mCTP from the reaction and as a result we could not make any 22-nt RNA transcript.

Hybridization Conditions and Detection Sensitivity of ^{33}P-LNA Probes

To optimize the hybridization conditions, small ^{33}P-LNA let-7C antisense probes were hybridized to let-7C sense DNAs blotted on nylon membranes at different concentrations. Increasing hybridization temperatures from 40 to 60°C obviously enhanced hybridization signals on the nylon membranes (Figure 2). The maximal signals were achieved at temperatures around 55–60°C. At this hybridization temperature, the detection sensitivity of ^{33}P-labeled LNA-AMP RNA probes was at least 25-fold higher than that of ^{33}P-AMP labeled normal RNA probes (data not shown). Hybridized signals were reduced when hybridizations were done at temperatures lower than 35°C or higher than 70°C (data not shown). High stringency washes in 0.2× SSC at 45–50°C for 10–15 minutes after hybridization did not reduce specific hybridization signals, as compared to low stringency washes in 1× SSC at 45°C for 10–15 minutes.

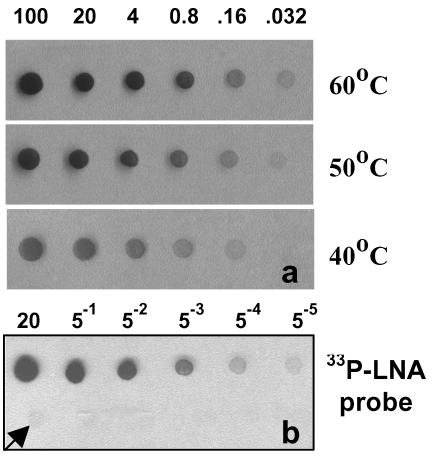


FIGURE 2 Scanned pictures show hybridization signals of ^{33}P-LNA let-7C probes at different temperatures (a) and detection sensitivity (b). The numbers above the picture denote the mass volume (ng) of let-7C sense probes blotted on the nylon membranes. Note that the signal intensity hybridized at 60°C is higher than that hybridized at 40°C (a). Numbers above (b) indicate the mass of let-7C sense DNA blotted on the membrane. The first dot is 20 ng and following dots were consecutively diluted by 5-fold. Arrow in (b) indicates the hybridization signals detected.

To determine whether ^{33}P-LNA small RNA probes are applicable to detect miRNAs in tissue samples, we evaluated its detection sensitivity on nylon membranes by dot hybridization (Figure 2b). Serial dilutions of let-7C sense DNAs were blotted onto the membranes and then hybridized with ^{33}P-LNA let-7C antisense RNA probes (30 nt). Our results showed that the dot containing approximately 1.6×10^{-3} to 3.2×10^{-4} ng let-7C sense DNA was positively detected (Figure 2b). Based on the molecular weight of let-7C oligonucleotides, 1.6×10^{-3} to 3.2×10^{-4} ng of let-7C sense probes are approximately equivalent to 3.25×10^{7} to 6.5×10^{7} copies of let-7C molecules in a 2-mm (diameter) dot area. This number is close to ~1000 copies of miRNA in a dot whose diameter is about 20–30 μm. Considering the fact that the average diameter of a tissue cell is about 20–30 μm, the expected detection limit of our method is ~1000 miRNA copies per cell.

Use of ^{33}P-LNA Probes to Detect VIP mRNAs

To test the hybridization specificity of small LNA probes, we used 30-nt ^{33}P-LNA VIP probes to localize VIP mRNA expressions in the mouse forebrain. VIP mRNAs are reportedly expressed specifically in the cerebral cortex, olfactory bulb, and hypothalamic ventral nuclei.[23] Regular long ^{33}P-VIP probes revealed a wide distribution of VIP mRNAs in the cells throughout the olfactory bulb (Figure 3a). In the cerebral cortex (Figure 3b), VIP mRNA-containing cells were usually distributed in layer I/II (surface layers) and layer V/VI (inner layer). Most hybridized cells

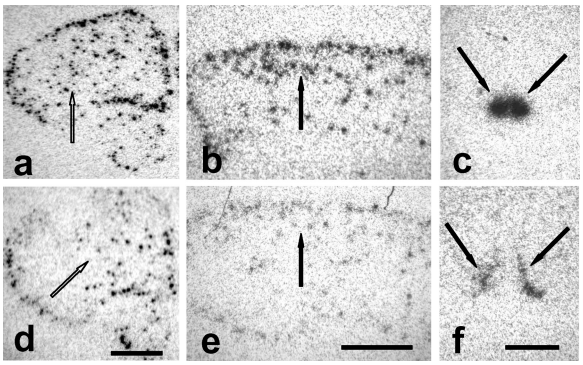


FIGURE 3 Photoradiographs show VIP mRNA expressions in the mouse brain olfactory bulb (a, d), cerebral cortex (b, e), and ventral hypothalamic nucleus (c, f). (a), (b), and (c) are in situ results revealed by large ^{33}P probes. (d), (e,) and (f) are in situ results revealed by 30-nt ^{33}P-LNA probes. Arrows indicate hybridized signals. Note that VIP mRNA distribution patterns in the olfactory bulb, brain cortex, and ventral hypothalamic nuclei detected by small VIP probes (d, e, f) are similar to that detected by large antisense probes (a, b, c). However, hybridized signals of 30-nt VIP probes are much weaker than that of large VIP probes. Bar in (d) = 200 μm (for a, d), in (e) = 2 mm (for b, e) and in (f) = 0.5 mm (for c, f).

after overnight exposure on Kodak film contain medium to high levels of VIP mRNAs. A similar distribution pattern of VIP mRNAs in the olfactory bulb and cerebral cortex was also observed in sections from the same mouse forebrain hybridized with 30-nt ^{33}P-LNA VIP probes (Figures 3d and e). VIP mRNAs are strongly detected by large VIP antisense probes in the hypothalamic ventral nuclei (Figure 3c). However, in similar brain regions from the same mouse brain, the hybridized signals were much weaker when the 30-nt probes were used for hybridization (Figure 3f). Average hybridization intensity reduced by 5- to 10-fold. Hybridization of mouse brain tissues with the 30-nt ^{33}P-LNA VIP probes did not reveal any specific hybridization spots in other brain areas. The hybridization signals revealed by small probes were much weaker than those detected by larger probes, but the background was relatively lower.

Differential Expression Profiles of mir-124 Within Different Tissues

Mir-124 in situ hybridization results showed that mir-124 was mainly detected in the cerebral cortex, hypothalamus, and cerebellum (Figure 4a). In forebrain cerebral cortex, hypothalamus, and cerebellum, mir-124 was even distributed and lacked obvious cell-type specific or region-specific distribution patterns. However, the hybridization signals of mir-124 antisense probes were very weak in the mouse medulla, while hybridization signals were stronger in the adjacent cerebellar cortex on the same section (Figure 4a). The intensity of hybridization signals in the cerebelluar cortex revealed by LNA-probes was obviously stronger than that revealed by normal ^{33}P-labeled mir-124 antisense probes (Figure 4b). Mir-124 sense probe did

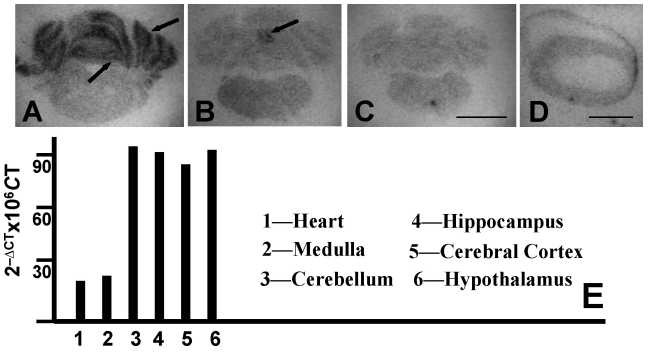


FIGURE 4 In situ hybridization (a–d) and real-time RT-PCR (e) showed the tissue distribution of mir-124 in different areas. Strong hybridization signals were present in the mouse cerebellum hybridized with LNA-pobes (arrow in (a)). However, on the same tissue section, the adjacent medulla lacked obvious hybridized mir-124 signals (open arrow in (a)). When normal ^{33}P-probes were used, mir-124 hybridization signals significantly reduced in the cortex of the cerebellum (arrow in (b)). No hybridization signals were observed when sense probes were used for hybridization (c). Mouse heart did not contain hybridized mir-124 antisene probes (d). Real-time RT-PCR revealed high $2^{-\Delta C_T}$ values of mir-124 in cerebellum, hypothalamus, hippocampus, and cerebral cortex. However, the $2^{-\Delta C_T}$ value of mir-124 were low in mouse heart and medulla (higher C_t value). Bar in (b) = 0.5 mm (for a, b) and in (c) = 1 mm.

not display any specific hybridization signals in the cerebellum region (Figure 4c). No mir-124 hybridization signals were present in mouse heart (Figure 4d). The tissue distribution patterns revealed by in situ hybridization corresponded well to that revealed by real-time RT-PCR (Figure 4e). The $2^{\Delta C_T}$ value of mir-124 was much higher in cerebral cortex, hypothalamus, and cerebellum. However, this number was lower in mouse heart and medulla (Figure 4e).

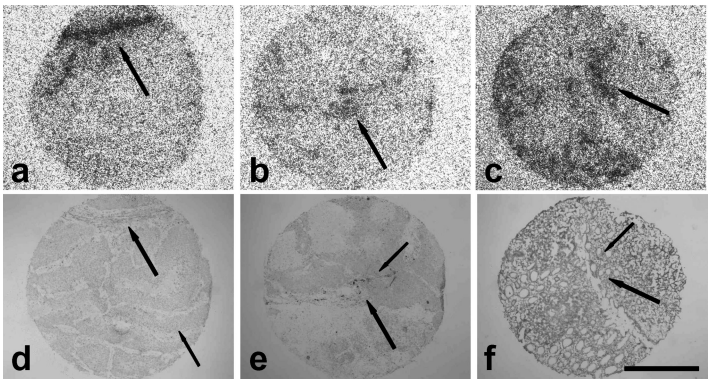


FIGURE 5 Illustrations show the expression of let-7C in cancer tissues. (a), (b), and (c) are in situ slides of let-7C in lung, pancreas, and colon cancer, respectively. (d), (e), and (f) are H&E stained same cancer tissue in (a), (b), and (c), respectively. Arrows in (a), (b), and (c) indicated the hybridized signals of let-7C in those cancer tissues. Large arrows in (d), (e), and (f) denote the tissue areas positively hybridized with let-7C antisense probes in (a), (b), and (c), respectively. Small arrows in (d), (e), and (f) indicate the regions containing cancer cells. Bar in (f) = 0.5 mm (for all pictures).

Use of ^{33}P-labeled LNA Probes to Detect let-7c Expressions in Cancer Tissues

To further test that the current method can be used to detect miRNA expression, we used ^{33}P-LNA let-7C probes to hybridize to human cancer tissue sections. In total, six different types of human cancer tissues were tested. The expression of let-7C miRNA was detected in human lung, pancreas, and colon cancer tissues (Figure 5). In human lung squamous cancer tissues, let-7C miRNA was observed mainly in the cell regions surrounding the cancer cells (Figures 5a and d). Most cancer cells have no detectable let-7C miRNA. Detectable let-7C miRNAs were also present in pancreas (Figures 5b and e) and colon cancer tissues (Figures 5c and f). In those two tumor tissues, let-7c expression was mainly observed in the cancer cell regions. Most adjacent normal tissue regions had no detectable let-7C miRNAs. Other human tumor samples (such as ovarian cancer, thyroid cancer, and uterine cancer) were also used for detecting let-7c expression. No obvious let-7C expression was identified in those cancer samples.

DISCUSSION

MiRNAs were small regulatory RNAs involved in multiple biological functions by regulating gene expression.[24] Recent studies suggest that changes in miRNA levels may be related to human cancer[25,26] and several other diseases.[27,28] Hundreds of miRNAs have been identified in different animal species, so the distribution patterns of miRNAs must be further addressed in order to understand their functional roles. Microarray[21] and RT-PCR[29] have been used to detect miRNAs in some organs, but those methods cannot be used to localize miRNAs. Hybridization is the best method to detect cellular miRNAs. However, due to the small size of the probes, high miRNA copy number per cell or a very sensitive detection method is needed.

Recently, LNA miRNA probes have been successfully used to localize miRNAs in zebrafish.[19] Since current forms of locked nucleotides cannot be used to make probes by in vitro transcription, chemical synthesis is the only way to make the probes. In this approach, one digoxigenin-UTP is linked to one end of the antisense probe. This digoxigenin-labeled synthetic LNA miRNA antisense probe is being widely used for hybridization study.[19] To provide the research community an alternative tool for detecting intracellular miRNAs, we chemically synthesized triphosphate LNA-mCTP and then use it for generating miRNAs antisense probes for hybridization.

Our transcription study showed that if LNA-mCTP accounted for more than 50% of the CTP source in the transcription reaction, 20- to 30-nt transcripts could be made. Under the same conditions, transcripts

(>100–200 nt) could not be effectively generated. LNA-mCTP is more applicable to make short miRNA probes instead of long probes. In the transcription reaction, 20- to 30-nt RNA transcripts can still be generated even if LNA-mCTP was the only CTP source in the transcription reaction. In contrast, removal of both CTP and LNA-mCTP from the reaction blocked synthesis of RNA transcripts, suggesting the incorporation of LNA-CTPs into transcripts. This conclusion was also supported by the fact that increasing hybridization temperature from 40 to 60°C did not reduce hybridization signals on dot hybridization. The hybridization stability at high temperatures may be due to the presence of LNA-mCMP inside the probe. However, the transcription efficiency of LNA-mCTP was relatively weak. Different RNA polymerase will be further tested to determine which RNA polymerase will give a better transcription efficiency. To get further insight of its transcription efficiency, other LNA NTPs without modification of bases will be synthesized and subjected for conventional and mutant RNA polymerase for its incorporation study. The total yield when only LNA-mCTP was used was about 30% compared to when only normal CTP was used. Furthermore, the number and position of LNA-mCMP in a small transcript also significantly affects probe synthesis (data not shown). A 30-nt full-length transcript was hard to make if more than 5–6 LNA-mCTPs were incorporated. The presence of consecutive LNA-CMPs in a transcript also greatly inhibited transcript yield and size. Therefore, a 20- to 30-nt cDNA sequence with evenly distributed 3–4 CMPs is the most appropriate template for making LNA probes by in vitro transcription.

Although small LNA probes have increased hybridization specificity and stability, their detection sensitivity is still not high enough to detect most miRNAs in cells. This is due to the low numbers of labeled nucleotides in the LNA probes, resulting in the weak hybridization signals from each probe. Our preliminary study on dot hybridization found that the detection sensitivity of LNA probes was almost the same as regular probes with the same RNA sequence. To increase detection sensitivity, we added 5 consecutive ^{33}P-AMPs on both ends of LNA probes, which theoretically increased detection sensitivity by 2- to 3-fold. Because the additional AMPs will not be digested by posthybridization RNase A treatment, this modification can significantly increase hybridization signals while still keeping background low. On the dot membrane hybridization, we found that the modified LNA probe could detect miRNAs approximately between 500 and 1000 copies per cell. However, due to the "stickiness" of the locked nucleotides, hybridization of small LNA probes should be done at temperatures around 55–60°C. Dot hybridization showed that signal intensity of the same LNA probes hybridized at 60°C was obviously stronger than that at 40–45°C. One explanation for this is the self-annealing of LNA probes at low temperatures, resulting in low hybridization efficiency. In this case, complete removal of self-annealing probes at higher temperature may be necessary for efficient

hybridization. The number of locked nucleotides in the probe may also affect the hybridization temperature used to denature self-annealing locked nucleotides.

To test the hybridization specificity of our ^{33}P-labeled small LNA probes, we used ^{33}P-LNA VIP probes (30 nt) to localize VIP mRNAs in mouse forebrain. VIP mRNAs are reportedly moderately expressed in the olfactory bulb, forebrain cerebral cortex, and hypothalamic nucleus as revealed by larger VIP RNA antisense probes.[17] Our 30-nt ^{33}P-LNA probes revealed a similar distribution pattern of VIP mRNA in the mouse forebrain. No hybridized cells were present outside the cerebral cortex, hypothalamic nuclei, or olfactory bulb. This result suggested that our modified LNA probes could be used to detect the expression of mRNAs or miRNAs in tissue samples. To test this, we analyzed the tissue distribution patterns of mir-124 using ^{33}P-labeled LNA mir-124 antisense probe and then we compared the in situ distribution patterns with that revealed by real-time RT-PCR. Our data clearly showed that the two results matched well. For example, on the same tissue section, in situ hybridization revealed highly expressed mir-124 in cerebellum while the adjacent region, medulla, lacked obvious hybridization signals. This phenomenon was also observed in our real-time RT-PCR study. These results suggested that small ^{33}P-LNA probes would specifically bind to target miRNA molecules on tissue sections. Since the hybridization signals of small LNA probes were 5- to 10-fold lower than those of larger probes, a relatively longer exposure time (about 3 days) is required. We should also mention that the lack of hybridization signal on tissue sections when using small LNA probe did not exclude the possibility that miRNAs were expressed at a level beyond our detection sensitivity.

We used ^{33}P-LNA let-7C probes to examine the presence of let-7C miRNA in several human cancer samples. Our data showed that let-7C was present in human lung, pancreas, and colon cancer tissues. Let-7C was undetectable in ovary, thyroid, and uterus cancer tissues. In lung squamous cancer tissues, let-7C miRNA was distributed mainly in the adjacent cells that surrounded cancer cell regions. We did not detect any obvious expression of let-7C in the tissue regions containing cancer cells. However, our results did not exclude the possibility that those cancer tissues may still express let-7C, but at a copy number lower than 500–1000 molecules. Our result agreed with the report that let-7C was downregulated in lung squamous cancer tissues.[19,29] The significance of let-7C miRNA expression was considered an inhibitory factor that affected the growth and formation of lung cancer.[29] The presence of let-7C in colon cancer and pancreas cancer cells has not yet been reported. These results suggest that our method can be widely used for the detection of our miRNA species. However, we should also mention that in tissue samples, our method may not differentiate those homologous miRNAs with one or two nucleotide differences in miRNA

sequences. Further studies are necessary to clarify whether our method can differentiate those homologous miRNA species.

In summary, we chemically synthesized oxy-locked mCTP and used it to make ^{33}P-labeled LNA probes (30 nt) by in vitro transcription. LNA-mCTP probes can be hybridized at a high temperature to increase hybridization specificity and reduce background. The detection sensitivity of our small LNA-probes might be close to 500–1000 miRNA copies per cell. Our LNA-mCTP method provides an alternative tool for general research laboratories to detect cellular miRNAs in tissue samples.

MATERIALS AND METHODS

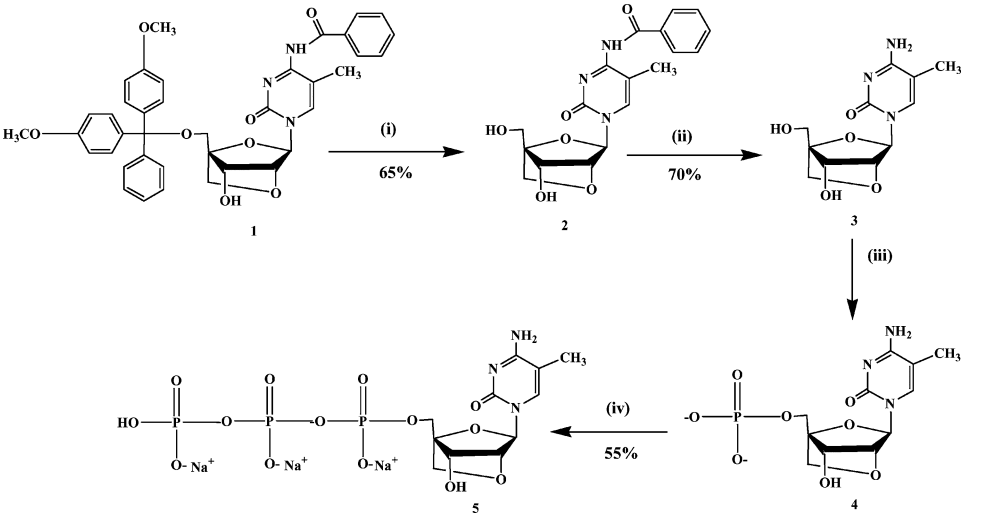
Chemical Synthesis of LNA-mCTP

Reagents

5′-DMT-locMeC(bz) was from Proligo Biochemie (Hamburg), deblocking reagent (3% trichloroacetic acid in dichloromethane) from EMT Chemicals, and other reagents (such as DMF, $POCl_3$, trimethyl phosphate, tri-*n*-butylamine, and tributylammonium pyrophosphate) from Sigma-Aldrich. The purification of LNA-mCTP was performed using an AKTA FPLC system on DEAE Sephadex A-25 column XK 50/30 (Amersham). ^{1}H-NMR (400 MHz) and ^{31}P-NMR (161 MHz) spectra were recorded using Varian 400 Inova. Reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm silica gel 60 plates impregnated with 254-nm fluorescent indicator (Merck), and by analytical HPLC (Aliance, Water's) using Hypersil SAX columns, 5 μm, 250 × 4.6 mm (Altech).

Chemical Synthesis Procedures

Scheme 1 shows the procedure for synthesis of LNA-mCTP. Detritylation of compound 1 ((*1R*,*3R*,*4R*,*7S*)-3-(4-*N*-benzoyl-5-methylcytosin-1-yl)-1-[4,4′dimethoxytrityloxymethyl]-7-hydroxy-2,5-dioxabicyclo[2.2.1] heptane) (5′-DMT-locMeC(bz) was achieved using 3% TCA in dichloromethane (DCM).[30] Compound 1 (2.6 mmol) was dissolved in 40 mL of 3% trichloroacetic acid, and the mixture was stirred at room temperature (RT). Deprotection of the dimethoxytrityl group was completed by stirring at RT for 2 hours. The solvent was then removed in vacuo. Compound 2 was purified by silica gel column chromatography (mobile phase, 0–10% MeOH in CH_2Cl_2) and then subjected to the base deprotection,[31] by incubation in 40 mL of 32% aqueous ammonia/acetonitrile (3:1) for 16 hours at 55°C in a glass vial. The aqueous ammonia was removed in vacuo and compound 3 was obtained as a white powder. Phosphorylation of compound 3 was done based on a previous method.[32] Compound 3 (2.38 mmol) was added to a mixture of trimethylphosphate (10 mL) and phosphorus oxychloride (300 μL, 3 mmol) at 0°C. The mixture was kept at 0–4°C for 3 hours



SCHEME 1 Chemical synthesis of locMeCytidine-5′-triphosphate (1) 3% TCA in DCM, r.t. 2 hours, 65%; (2) NH_4OH /ACN, 3:1, 55°C, 16 hours, 70%; (3) $[MeO]_3$ $PO/POCl_3$, 0°C, 3 hours; (4) $[Bu_3NH^+]_4$ $P_2O_7^{4-}$, DMF, 0°C, 1 minutes, 0.1 M TEAB, 75 mL, r.t., 5 hours, 55%.

with stirring. A mixture of 0.5 M bis-tri-*n*-butylammonium pyrophosphate in anhydrous DMF (34 mL) and Bu_3N (3.4 mL) was added under vigorous stirring. One minute later, the reaction was quenched with 75 mL of 0.1 M aqueous $Et_3NH_2CO_3$ (pH 7.5) at RT for 5 hours. The mixture was loaded into a DEAE-Sephadex A25 column that was washed with 5 mM triethylammonium bicarbonate and then eluted with a linear gradient of triethylammonium bicarbonate, pH 7.5 (0–0.9 M for 4 L). Fractions containing compound 5 were pooled and concentrated to dryness, and the residual bicarbonate was removed by four sequential evaporations with 50 mL of methanol. The product was dissolved in 50 mL of water and converted to its sodium salts by ion exchange on a small Dowex 50W × 8 column (Na^+ form), followed by evaporation of the elutes to a small volume, precipitation with ethanol, and centrifugation to give an amorphous compound 5 power. The spectroscopic analysis of compound 2 was in agreement with the literature data.[33] Spectroscopic data for compound 5, ^{1}H NMR (D_2O, 400 MHz) δ 7.79 (s, 1H), 5.69 (s, 1H), 4.49 (s, 1H), 4.42 (m, 2H), 4.29 (s, 1H), 4.08 (d, $J = 8.4$ Hz, 1H), 3.97 (d, $J = 8.4$ Hz, 1H), 2.05 (s, 3H); ^{31}P NMR (D_2O, 162 MHz) δ −8.65, −9.35, −21.36; MS (m/z): 505 $[M]^+$.

Enzymatic Incorporation of LNA-CTP into RNA Probes

To test if LNA-mCTP can be efficiently incorporated into small transcripts, we used it to generate RNA probes by T7 RNA promoter-driven transcription. The DNA templates used were (1) human let-7C cDNA (22 bp) with a T7 RNA promoter sequence at the 5′ end, (2) T7

promoter-driven 100-bp vasoactive intestinal polypeptide (VIP) cDNA, and (3) T7 promoter-controlled 200-bp VIP cDNA. To determine the incorporation efficiency of LNA-mCTP, we varied the ratio of normal CTP to LNA-mCTP in the transcription reaction (Figure 2). Transcription efficiency was evaluated based on the sizes and yields of the transcribed products. Transcription was performed at 37°C for 2 hours. The reaction (20 μL) contained 1 unit of T7 RNA polymerase, 1× transcription buffer, and 500 μM each of the ribonucleotides. After transcription, DNase I was used to digest DNA templates. RNA probes were precipitated by ethanol and then dissolved into DEPC-water. The sizes and yields of resulting RNA probes were analyzed and compared by spectrophotometer, and in a 1.5% denaturing agarose gel or 15% denaturing acrylamide gel.

Transcription of ^{33}P-labeled LNA Probes and Normal Probes for in situ Hybridization

Three LNA probes (30 nt) were made for hybridization study. They were (1) ^{33}P-LNA mouse VIP probes (AAAAAUUUAAUAGGACUCACU AUAGAAAAA), (2) ^{33}P-LNA mouse mir-124 probes (5′-AAAAAGGCAUUCA CCGCGUGCCUUAAAAAA-3′), and (3) let-7c probes (5′-AAAAACCAUAC AACCUACUACCUCAAAAA-3′). ^{33}P-labeled normal probe without LNA was also generated. T7 or T3 RNA polymerase controlled transcription system was used to generate antisense or sense probes, respectively. To make highly active probes, we added five more ^{33}P-labeled AMPs to both ends of the probes by in vitro transcription. In the reaction, ^{33}P-[ATP] was the only ATP source for the probes. This 30-nt probe was designed because we found that adding five more ^{33}P-AMPs in the probes significantly increased the detection sensitivity but did not affect hybridization efficiency. Furthermore, AMP is resistant to RNase A digestion, so posthybridization RNase A treatment will digest those nonhybridized probes from tissue sections to reduce background while keeping five consecutive ^{33}P-AMPs intact on the two ends of the probes specifically hybridized to the target miRNAS. A 1500-nt ^{33}P VIP antisense probe was also generated based on the method described elsewhere.[17] This probe was used to reveal the normal distribution patterns of VIP mRNAs in mouse brain.

Hybridization Conditions and Detection Sensitivity of Short LNA Probes

To determine the hybridization temperature and detection sensitivity of ^{33}P-LNA probes, we performed dot hybridization on nylon membranes. The let-7C sense DNA strand (20 nt) was blotted on the membrane in a series of 5-fold dilutions. A total of 12 dots were blotted. The first dot contained 100 ng of DNA in a round dot (diameter = 2 mm) and the last dot had

100×5^{-11} ng (~2.4 fg) of DNA. The ^{33}P-LNA let-7C antisense probes were added to the hybridization solution (1% SDS, 1 M NaCl, 10% dextran sulfate, and 50% formamide) with salmon sperm DNA, and overnight hybridization was done at 40, 50, and 60°C. After hybridization, the blot was washed in 4× SSC/0.1% SDS and 1× SCC/0.1% SDS each for 2 × 10 minutes at 45°C. The blots were then exposed for 48 hours on Kodak BioMax film. The hybridization signals were revealed on film by D-19 developer and fixer (Kodak). The detection sensitivity of ^{33}P-LNA probes was determined by tracing the last hybridized spots (containing the most diluted let-7C sense DNA) that could be detected by the ^{33}P-LNA let-7C antisense probes.

Hybridization Specificity of ^{33}P-labeled LNA Probes

To test the hybridization specificity of our LNA probes, we first used small ^{33}P-LNA probes to localize a known gene, vasoactive intestinal polypeptide (VIP) mRNA, in mouse brain. Mouse VIP mRNAs are expressed at medium to high levels specifically in the olfactory bulb, cerebral cortex, and ventral hypothalamic nuclei.[23] Detection of hybridization signals in those areas only will directly suggest the hybridization specificity of our small LNA probes.

To further demonstrate the hybridization specificity, we also compared the differential expression profiles of mir-124 in different mouse tissue regions revealed by mir-124 in situ hybridization versus real-time RT-PCR. For RT-PCR study, total RNAs were isolated from cerebral cortex, hypothalamus, cerebellum cortex, medulla, and heart using Trizol reagent (Invitrogen, California, USA). Total RNAs (1 μg) from above tissue regions were used for real-time RT-PCR to quantify mir-124 expressions, based on the method published elsewhere.[34] Reverse transcriptase reactions (20 μL) contained total RNA, 50 nM mir-124 RT primer, 1× RT buffer (Invitrogen), 0.25 mM each of dNTPs, 2U reverse transcriptase, and 1U RNase inhibitor (Promega). The reactions were incubated for 10 minutes at room temperature, 30 minutes at 42°C, 5 minutes at 95°C, and then held at 4°C. All reverse transcriptase reactions, including no-template controls and RT minus controls, were run in duplicate. Real-time PCR was performed using a standard TaqMan PCR kit protocol on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems). The 10-μL PCR included 0.67 μL RT product, 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.2 μM TaqMan probe, 1.5 μM forward primer, and 0.7 μM reverse primer. The reactions were incubated in a 384-well plate at 95°C for 1 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, a 72°C for 30 seconds. All reactions were run in triplicate. The threshold cycle (C_T) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The cycle number at which the reaction crossed an arbitrarily placed threshold (C_T) was determined for each tissue regions.

The relative amount of miRNA-124 to U6RNA in each tissue regions was described using the equation $2^{-\Delta C_T}$, where $\Delta C_T = (C_{T\text{miRNA}} - C_{18S\ RNA})$. Relative gene expression was multiplied by 10^6 to simplify the presentation of the data. Higher $2^{-\Delta C_T}$ values represent high copy number of mir-124 molecules.

MiRNA in Situ Hybridization Procedure

^{33}P-LNA VIP and mir-124 probes were used to localize VIP mRNAs and mir-123 in mouse brain and heart tissues. ^{33}P-LNA let-7C antisense probes were used to detect let-7C cellular expressions in human cancer tissues. Fixed mouse brain or heart tissues and human cancer tissues were embedded in OCT compound and then sectioned in a cryostat microtome. Sections of 15 μm were mounted on glass slides for hybridization.[35] Tissue sections were washed in 1× PBS for 3 × 5 minutes and then incubated with prehybridization buffer at 45°C for 4 hours. After prehybridization, fresh hybridization solution containing ^{33}P-LNA antisense probes at 10^7 cpm/mL was added to cover the sections. Hybridization was performed at 55–60°C overnight. Sense probes were used as negative controls in a similar fashion. After hybridization, tissue slides were washed in 4× SSC and 2× SSC at RT for 20 minutes each, and then treated with RNase A to reduce background at 37°C for 30 minutes. Slides were washed in 0.5× SSC at 45°C for 30 minutes and air-dried. The slides were then exposed on Kodak BioMax film for 72 hours and hybridization signals were revealed on film by Kodak D-19 developer and fixer.

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