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USE OF NUCLEOTIDES AS AN ALTERNATIVE TO FORMAMIDE IN NON-RADIOACTIVE IN SITU HYBRIDIZATION

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To analyze the expression of specific mRNA at the level of individual cells, non-radioactive *in situ* hybridization has been a most powerful technique. In the process of *in situ* hybridization, the use of formamide is usually required in order to reduce the melting temperature (Tm) of nucleic acids. However, formamide is an expensive and unstable reagent, and more importantly, formamide in itself has some deteriorative effects such as nonspecific staining and morphological damage on the results of *in situ* hybridization. In this study, we examined the use of a mixture (Nm) of nucleotides (AMP, GMP, UMP, CMP) as an alternative to formamide in our non-radioactive hybridization system with thymine-thymine (T-T) dimerized DNA probe. When the effects of Nm on re-annealing of denatured pBR 322 DNA were investigated by electrophoretic patterns on agarose gel, it was confirmed that Nm (about 20–200 mg/ml) reduced the Tm of pBR 322 DNA. On dot-blot hybridization using Nm, we obtained sensitive and specific results similar to that of formamide. Finally, on frozen sections of rat pituitary glands fixed by perfusion of 4% paraformaldehyde in phosphate buffered saline (pH 7.4), prolactin mRNA was successfully localized *in situ* using Nm instead of formamide.

Non-radioactive *in situ* hybridization has been a most effective method for localization of specific mRNA in individual cells (4, 6, 19, 20). Recently we have developed a new method (10, 11, 17) using thymine-thymine (T-T) dimerized DNA as a non-radioactive probe, which is thought to be the simplest in terms of no requirements for the separation of labeled DNA from unreacted labeling compounds, and we have further shown a wide applicability of the method (7, 8, 9, 18, 21). In parallel with the approach, using T-T dimerized DNA probes we have tried to establish a tissue processing procedure best suited to non-radioactive *in situ* hybridization (10, 16).

In molecular hybridization, in order to obtain specific hybrids between probe nucleic acids and target nucleic acids, hybridization reaction should be carrid out near the melting temperature (Tm) of the corresponding double-stranded nucleic acids, theoretically. In practice, however, it is recommended that hybridization be carried out at a temperature of (Tm-25)°C (for filter hybridization) or (Tm-30)°C (for *in situ* hybridization) (3). In most of the cases to hybridize between probe DNA and target mRNA, Tm-30 was calculated to be higher than 65–75°C (22), while to preserve the morphology of specimens hybridization should be carried out at a temperature lower than 45°C. Since it is thought to be prerequisite to preserve the morphology of cells and tissues for a full implementation of *in situ* hybridization, the use of some reagents

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to decrease Tm was required. Among the reagents, formamide is now most popularly used. Though formamide was useful in *in situ* hybridization, we have had much experience with the use of formamide, especially in the use of long stored reagent, which brought about serious deteriorative effects such as non-specific staining and morphological damage, and much care should be taken in handling formamide because of its own instability and expensiveness. In order to avoid these cumbersome and expensive procedures, the selection of an alternative reagent is a problem of great urgency.

In this study, using a non-radioactive *in situ* hybridization system with T-T dimerized DNA probe, we examined the use of a mixture (Nm) of nucleotides (AMP, GMP, UMP, CMP) as an alternative to formamide. The nucleotides are not destructive to cell morphology and are expected to act as a competitive inhibitor of the re-annealing process resulting in reduction of Tm, since a monomeric base selectively forms a stable complex with the corresponding complementary base (13). However, it is unclear how monomeric nucleotides react with polynucleotides in convenient hybridization conditions, partly because the stability of hydrogen-bonding formed between complementary bases in double-stranded polynucleotides varies depending upon base sequence as well as base composition (2). In order to confirm the reducing effect of Nm on Tm, the effects of Nm on re-annealing of denatured pBR 322 were investigated by agarose gel electrophoresis. Thereafter using Nm, we investigated whether similar sensitivity and specificity to that with formamide was obtained in both dot-blot hybridization and *in situ* hybridization systems. In the latter case, prolactin mRNA was localized using Nm and formamide on frozen sections of rat pituitary glands fixed by perfusion of 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS).

A part of this study has already been appeared in abstract form (12).

MATERIALS AND METHODS

Materials: The following materials were purchased; formamide from Nakarai Chemical Co., Japan, mixed bed resin (AG-501-X8(D)) from Bio-Rad, USA, agarose 1600 from Wako Pure Chemical, Co., Japan, 5′-AMP, 5′-GMP, 5′-UMP and 5′-CMP from Sigma Chemical Co., USA, lambda DNA, pBR 322 and *Hind* III from Toyobo Co., Japan. Nucleotides (AMP, GMP, UMP, CMP) of equal weight were dissolved in distilled water and the solution was used as Nm. All other chemicals and biochemicals used in this study were the same as in the previous papers (10, 11).

Tissue: Pituitary glands from normal adult male Wistar rats were used for *in situ* hybridization. According to the previous paper (10), the tissues were fixed by perfusion with 4% PFA in PBS (pH 7.4). The cryostat sections were taken up to gelatin-coated glass slides fixed with 1% PFA and baked at 45°C for 4 hr.

DNA used: For dot-blot hybridization, pBR 322 DNA and lambda DNA were used. To investigate the effects of Nm on re-annealing of heat-denatured, partially single-stranded DNA, linear pBR 322 DNA prepared by digestion with *Hind* III was used. Rat prolactin cDNA (823 bp) (5) and lambda DNA (as a control probe) were used for *in situ* hybridization.

Labeling of probe DNA: DNAs were haptenized by UV-irradiation (5,000 J/m^2) as described in detail previously (10, 17) and used for hybridization experiments.

Dot-blot hybridization: Two μl of the solution of heat-denatured pBR 322 DNA or

lambda DNA (1 pg to 10 ng per spot) was applied to nitrocellulose filters and baked at 80°C for 2 hr. The filters were prehybridized with 10 mM Tris/HCl buffer (pH 7.3) containing 1 mM EDTA, 0.6 M NaCl, 1 × Denhardt's solution, 500 μg/ml yeast tRNA, 250 μg/ml salmon sperm DNA at 42°C for 2 hr. When formamide was used in the following hybridization, deionized formamide was added to the prehybridization mixture at a final concentration of 50% (v/v). Then the filters were further incubated at 42°C for 15 hr in the hybridization mixture containing 10 mM Tris/HCl (pH 7.3), 1 mM EDTA, 0.6 M NaCl, 1 × Denhardt's solution, 250 μg/ml yeast tRNA, 125 μg/ml salmon sperm DNA, 10% dextran sulfate, 40% formamide or Nm at various concentrations of 0.16–160 mg/ml, and 1 μg/ml T-T dimerized pBR 322 DNA or lambda DNA. After appropriate washing, the hybrids were detected immunohistochemically with rabbit anti-T-T dimer antibody as the first antibody and Fab fragment of goat IgG against rabbit IgG conjugated with horseradish peroxidase as the second antibody, as detailed previously (10, 17). The sites of the peroxidase were visualized using 0.5 mg/ml 3, 3′-diaminobenzidine/4 HCl in the presence of nickel and cobalt ions (1).

Solution hybridization: A linear form of pBR 322 DNA was denatured by keeping it in a boiling water bath for 10 min and cooled quickly in an ice bath. The denatured DNA(40 μg/ml) was incubated in the presence of various concentrations of formamide or Nm at various temperature for 3 hr. As the final reaction mixture contained 12 mM NaCl, Tm was calculated to about 70°C without formamide or Nm. The aliquots of re-annealed DNA were used in gel-electrophoresis.

Electrophoresis of DNA: The aliquots of pBR 322 DNA, which were non-denatured, heat-denatured and successively re-annealed, or just after heat-denatured, were applied to 1% agarose gel, which was immersed in 40 mM Tris/HCl (pH 8.0) containing 5 mM sodium acetate and 1 mM EDTA and precooled at 4°C. Electrophoresis was done under a 40 V electric field at 4°C (otherwise we were not able to separate single-stranded DNA from re-annealed DNA on the gel). The running period was determined by the position of both bromophenol blue and xylene cyanol. The resulting gel was stained with ethidium bromide (10 ng/ml) for 15 min. After washing with distilled water, the gel was observed under a UV-illuminator (14).

In situ hybridization: Rat prolactin mRNA was localized *in situ* on cryostat sections of rat pituitary glands, as detailed in the previous paper (10). Briefly, the sections were treated with 0.2 HCl (20 min) and proteinase K (1 μg/ml, 37°C, 15 min), successively. After a brief post-fixation with 4% PFA in PBS, excess aldehydes were neutralized with 2 mg/ml glycine in PBS (15 min, twice) and immersed in 40% formamide/2 × SSC (for the sections which were hybridized with formamide) or in only 2 × SSC (for the sections which were hybridized with Nm in place of formamide). Hybridization was done at 42°C for 15 hr in a mixture of 10 mM Tris/HCl (pH 7.3), 1 mM EDTA, 0.6 M NaCl, 1 × Denhardt's solution, 250 μg/ml yeast tRNA, 125 μg/ml salmon sperm DNA, 10% dextran sulfate, 40% freshly deionized formamide or 160 mg/ml of Nm, and 2 μg/ml T-T dimerized prolactin cDNA. After 5 washings of 1 hr each with 50% formamide/2 × SSC (when formamide was used in hybridization) or with 2 × SSC (when Nm was used in hybridization) at 37°C, the slides were washed further with 2 × SSC and PBS (pH 7.2), and used for immunohistochemical detection of T-T dimer, as described previously (9, 10, 11). Unless otherwise specified, the experiments were done at room temperature.

RESULTS AND DISCUSSION

1. Effects of nucleotides on re-annealing of denatured pBR 322 DNA assessed by electrophoretic patterns

In order to examine the validity of electrophoretic assays and to what extent denatured DNA is re-annealed under given conditions, the electrophoretic pattern of re-annealed pBR 322 DNA at 4°C, 37°C, 50°C (near the Tm-25), and 70°C (near the Tm), was analyzed on 1% agarose gel. pBR 322 DNA denatured by heating (not re-annealed) was also applied to the same gel. As shown in Fig. 1-(I), only denatured pBR 322 DNA is split into two main bands; the lower band represents single-stranded DNA and the other (upper) band represents double-stranded DNA, estimated by relative mobility. A similar electrophoretic pattern to that of denatured DNA was obtained in the DNA re-annealed at 4–37°C. When the denatured DNA was re-annealed at a temperature near (Tm-25)°C, the temperature regarded as optimal for hybridization (14), the bands of single-stranded pBR 322 DNA were lost and the bands of DNA hybrids with higher molecular weight appeared in ladder form. When

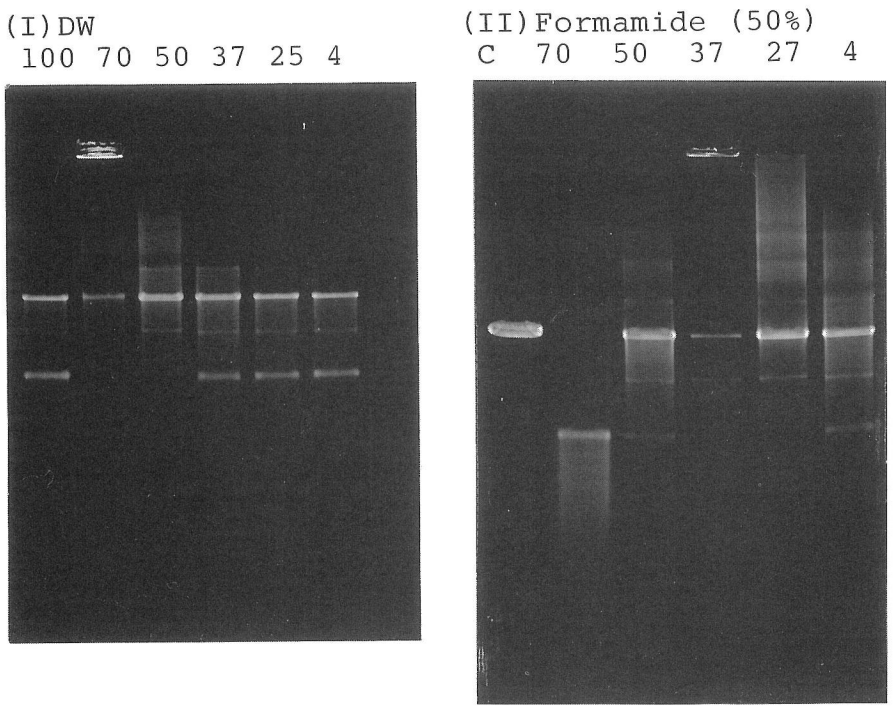


FIG. 1. Electrophoretic patterns of pBR 322 DNA re-annealed at various temperatures in the presence or absence of formamide. pBR 322 DNA digested with *Hind* III was heat-denatured and re-annealed at various temperatures (4°C, 25°C, 37°C, 50°C and 70°C) in the presence of 50% formamide (II) or deionized water (DW) (I) in place of formamide. Then the aliquots of DNA were analyzed by agarose gel electrophoresis. The denatured and non-re-annealed DNA was applied to the lane indicated as "100". Non-denatured DNA was applied to the lane indicated as "C".

re-annealed at a temperature near Tm, the DNA was detected near the origin as large DNA aggregates.

When denatured DNA was re-annealed in the presence of 50% formamide at various temperatures, the electrophoretic pattern of DNA (Fig. 1-(II)) seemed to shift to that obtained when the DNA was re-annealed at the higher temperature without formamide by about 33°C, compared to the results in Fig. 1-(I); the pattern obtained with the DNA re-annealed at 70°C in Fig. 1-(I) was similar to that of the DNA re-annealed at 37°C in Fig. 1-(II). This shift of electrophoretic pattern in the presence of formamide was reasonable, since it had been known that 10% formamide decreases the Tm by about 6°C (15). Therefore, the effects of reagents on the Tm can be easily

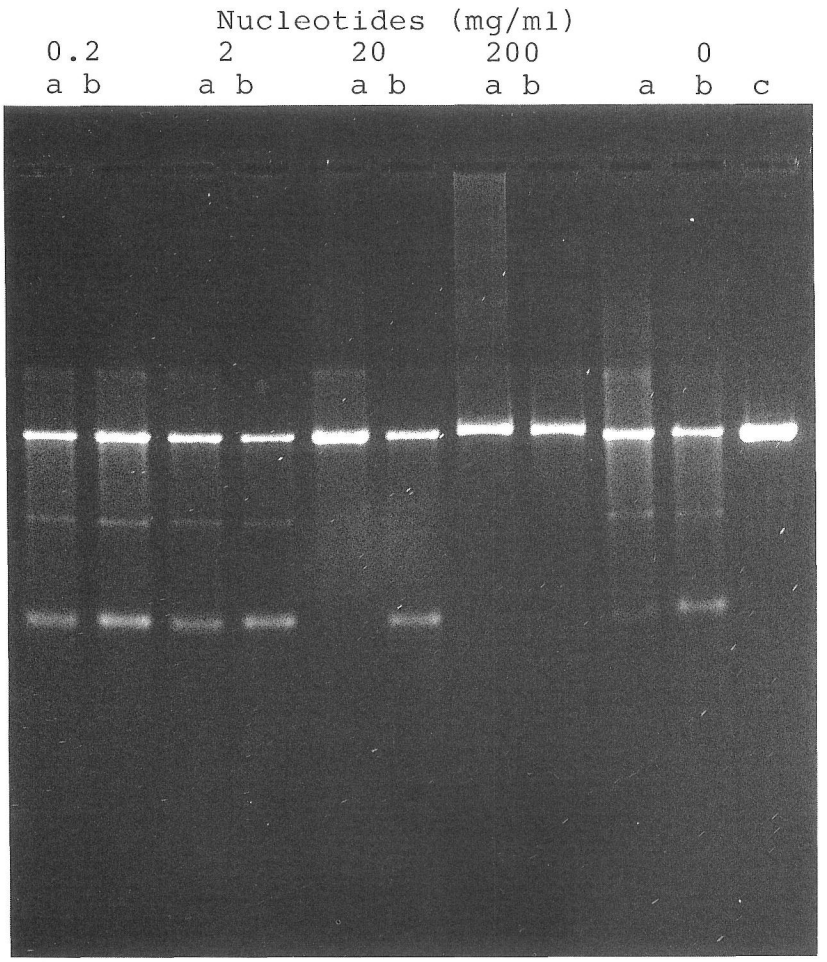


FIG. 2. Electrophoretic patterns of pBR 322 DNA re-annealed in the presence of various concentrations of nucleotides. After denatured pBR 322 DNA was re-annealed (a) or not re-annealed (b) in the presence of various concentrations (0 to 200 mg/ml) of nucleotides, electrophoretic analysis was performed. Non-denatured DNA was applied to the lane indicated as "c".

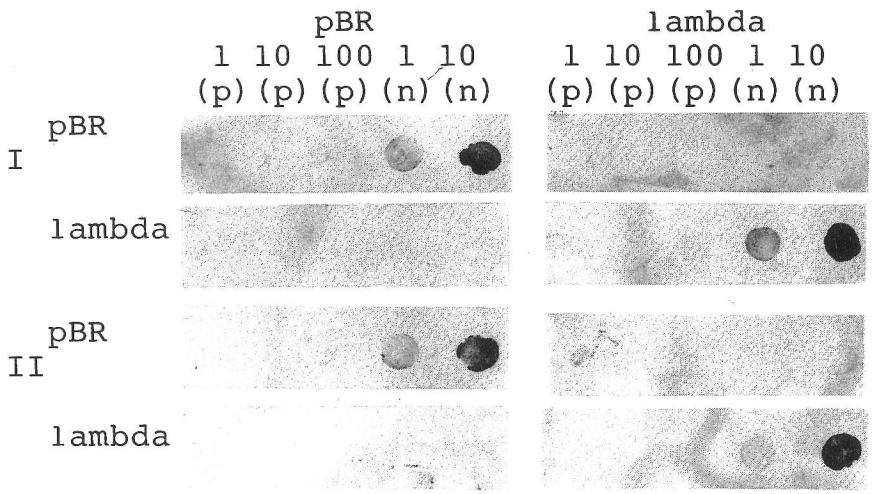


FIG. 3. Dot-blot hybridization with formamide (I) or nucleotide mixture (II). T-T dimerized pBR 322 DNA or lambda DNA was hybridized with nitrocellulose filters, which were spotted with various amounts (1 pg to 10 ng) of pBR 322 DNA or lambda DNA, in the presence of 40% formamide or of 160 mg/ml Nm at 42°C.

assessed with this type of electrophoretic assay.

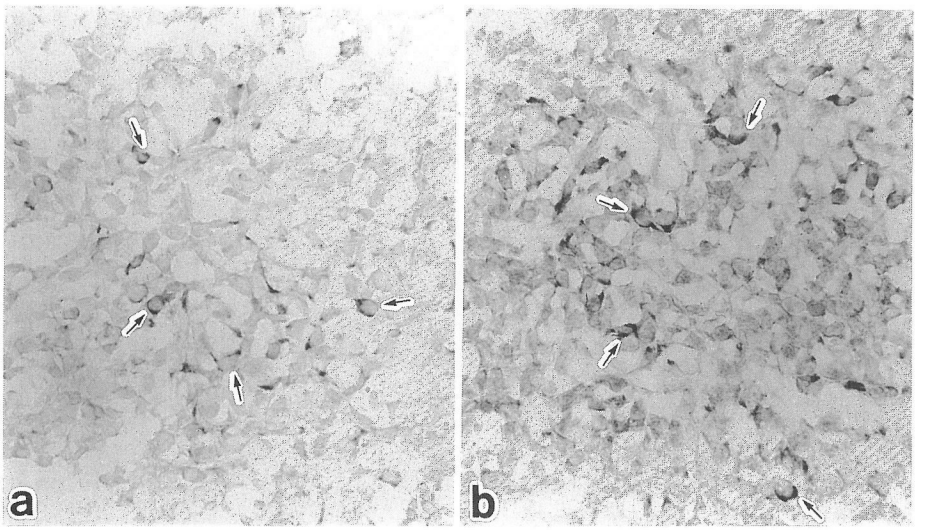
Using the above system, the effects of various concentrations of Nm on re-annealing of denatured pBR 322 DNA were examined, as shown in Fig. 2. In this experiment, the heat-denatured DNA was incubated at 4°C (lane b) or 37°C (lane a) for 3 hr in the presence of Nm (0–200 mg/ml). The disappearance of the band of single-stranded pBR 322 DNA and the formation of high molecular weight hybrids were observed at concentrations ranging from 20 to 200 mg/ml of Nm; namely, the electrophoretic pattern in that range of Nm concentration was thought to correspond to the situations observed when the denatured DNA was re-annealed around (Tm-25)°C. These results indicate that Nm at 20–200 mg/ml decreases the Tm of pBR 322 DNA. In addition, though the relative mobility of DNA on agarose gel was affected by contaminated ions in a loading solution, the effects of the sodium ion included in nucleotide crystals were negligible in this experiment.

2. Use of nucleotides in dot-blot hybridization

The usefulness of Nm in practice was examined by dot-blot hybridization. Various amounts (1 pg to 10 ng per spot) of pBR 322 DNA or lambda DNA were spotted onto nitrocellulose filters after denaturation of both DNAs, then the filters were hybridized with T-T dimerized pBR 322 or lambda DNA at 42°C in the presence of either 40% formamide (Fig. 3-(I)) or 160 mg/ml Nm (Fig. 3-(II)), and the hybridization signals were detected immunohistochemically. As is clearly shown in Fig. 3, the sensitivity and specificity in signal detection with the use of Nm were very similar to that obtained with formamide.

3. *In situ* hybridization with nucleotides

Finally, prolactin mRNA in cryostat sections of rat pituitary gland was hybridized *in situ* with T-T dimerized rat prolactin cDNA in the presence of either formamide



FIGS. 4a–b. *In situ* detection of prolactin mRNA in frozen sections of rat pituitary gland. The frozen sections were hybridized *in situ* with T-T dimerized rat prolactin cDNA in the presence of 40% formamide (a) or 160 mg/ml Nm (b). The positive cells are indicated by arrows. ×300

(Fig. 4-a) or Nm (Fig. 4-b). With similar sensitivity and specificity, prolactin mRNA was localized using Nm compared to results using extensively deionized formamide.

In summary, Nm has turned out to be a stable and economical alternative to formamide in molecular hybridization both on filters and tissue sections. Nm is especially useful in *in situ* hybridization, avoiding the deteriorative effects of formamide.

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