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In situ DNA-RNA hybridization using in vivo bromodeoxyuridine-labeled DNA probe

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Summary. An in vivo 5′-bromodeoxyuridine (BrdUrd) labeled DNA probe was used for in situ DNA-RNA hybridization. BrdUrd was incorporated into plasmid DNA by inoculating *E. coli* with Luria-Bertani (LB) culture medium containing 500 mg/L of BrdUrd. After purification of the plasmid DNA, specific probes of the defined DNA fragments, which contained the cloned insert and short stretches of the vector DNA, were generated by restriction endonuclease. The enzymatic digestion pattern of the BrdUrd-labeled plasmid DNA was the same as that of the non-labeled one. BrdUrd was incorporated in 15%–20% of the total DNA, that is, about 80% of the thymidine was replaced by BrdUrd. Picogram amounts of the BrdUrd-labeled DNA probe itself and the target DNA were detectable on nitrocellulose filters in dot-blot spot and hybridization experiments using a peroxidase/diaminobenzidine combination. The BrdUrd-labeled DNA probe was efficiently hybridized with both single stranded DNA on nitrocellulose filters and cellular mRNA in in situ hybridization experiments. Through the reaction with BrdUrd in single stranded tails, hybridized probes were clearly detectable with fluorescent microscopy using a FITC-conjugated monoclonal anti-BrdUrd antibody. The in vivo labeling method did not require nick translation steps or in vitro DNA polymerase reactions. Sensitive, stable and efficient DNA probes were easily obtainable with this method.

Introduction

In situ hybridization is a powerful technique to identify specific nucleic acid sequences. Though radioisotope-labeled DNA or RNA of defined sequences is widely used as sensitive probes (Gall and Pardue 1969), they have several disadvantages: the autoradiographic analysis takes too much time, the low resolution results in poor histological visualization, and there is a risk of exposure to the radioisotope. Non-radioactive probes have been developed to overcome these disadvantages.

Bromodeoxyuridine (BrdUrd) is a thymidine analogue that is often used as a substitute for ^{3}H-thymidine in in vitro or in vivo proliferation assays. BrdUrd-labeled DNA probes have been used successfully to detect viral DNA in tissue sections (Niedobitek et al. 1988) and satellite DNA sequences on chromosomes (Frommer et al. 1988). These probes are labeled with BrdUTP by nick translation and an in vitro DNA polymerase I reaction. These methods require nick translation steps, and steps to separate the unincorporated BrdUTP from the probes. Structural alterations may be introduced by nick translation itself and a defined length of DNA is not easily prepared. Sakamoto et al. in 1987 reported the in vivo BrdUrd labeling of single stranded circular DNA, and its applications in dot hybridization experiments. However, such circular forms of DNA probes are limited to dot hybridization experiments.

In order to overcome these drawbacks, we developed BrdUrd-incorporated DNA (15%–20% of total DNA) probes of defined length with a combination of in vivo BrdUrd labeling and restriction endonuclease digestion. We demonstrated c-*myc* mRNA expression in leukemic cell lines (HL-60 and K562) as a model for in situ DNA-RNA hybridization using this BrdUrd-labeled DNA probe.

Materials and methods

Plasmid construction. The plasmid pMyc6514-2, containing the SacI 1.5 kb human genomic c-*myc* exon 2 as an inset and PBR322 as a cloned vector, was used for following in vivo labeling and hybridization probes. The plasmid pMyc6514, containing the HindIII-EcoRI 8.5 kb human genomic c-*myc* exon 1, exon 2 and exon 3, was used as a target DNA in dot-blot hybridization experiments. Both of the plasmid were originally developed by Taya et al. in 1984, and were gifts from JCRB (Japanese Cancer Research Resources Bank). The G+C content of the c-*myc* exon 2 and PBR322 was 64.4% and 53.7%, respectively. Figure 1 shows the restriction map of the pMyc6514-2 used in this experiment. As anti-BrdUrd antibodies have a low affinity for the double stranded DNA, these BrdUrd-labeled probes should have single stranded tails which do not participate in hybridization.

In vivo BrdUrd labeling and probe preparation. A single colony of an appropriate host, *E. coli*, was incubated in 500 ml of Luria-Bertani (LB) medium containing 250 mg of BrdUrd (Sigma Chemical, St. Louis, USA) and 25 mg of ampicillin. It was shaken vigor-

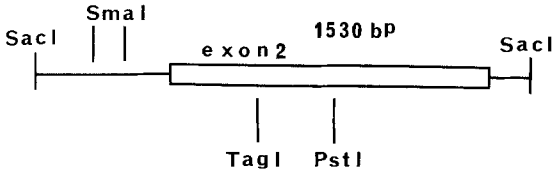

Fig. 1. Restriction map of the pMyc6514-2 used in this experiment

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ously at 37° C for 12 h to an OD_{600} of 0.5. After harvesting and lysing the bacterial cells, closed circular plasmid DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients. Specific fragments of defined lengths of the DNA sequences, which were cut at a restriction site located beyond the cloned insert by SacI endonuclease digestion, were isolated from low-melting-temperature (LMT) agarose. The BrdUrd labeling efficiency was measured by electrophoretic mobility and ultraviolet spectroscopy (Dunn and Smith 1954).

Dot-blot spotting and immunochemical staining. Serial dilutions of both double and single stranded BrdUrd-labeled DNA probes were spotted onto nitrocellulose filters (Bio-Rad, California, USA). We used non-labeled double and single stranded DNA probes as negative controls. The filters were baked at 80° C for 1.5 h and then blocked with 0.5% non-fat dry milk in 50 m*M* phosphate buffered saline (PBS, ph 7.3) for 2 h. BrdUrd was detected by the avidine biotin peroxidase complex (ABC) method using mouse anti-BrdUrd monoclonal antibody (Becton Dickinson Immunocytometry Systems) as a primary serum and biotinated anti-mouse IgG antibody as a secondary serum. Subsequently, the filters were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) without any post DAB intensification procedures.

Dot-blot hybridization and immunochemical staining. In order to estimate the hybridization efficiency and sensitivity of the in vivo BrdUrd-labeled DNA probes, a dot-blot hybridization test between the spotted single stranded DNA and single stranded BrdUrd-labeled c-*myc* exon 2 probe was performed to compare with the biotinylated c-*myc* exon 2 probe. The biotinylated DNA probe was a gift from Dr. A. Mizoguchi, Department of Anatomy of our school. Serial dilutions of the pMyc6514, containing c-*myc* exon 1, exon 2 and exon 3, were spotted on nitrocellulose filters. The filters were dried at room temperature, then baked at 80° C for 1.5 h. After denaturing the spooted DNA by alkaline treatment (1.5 *M* NaCl, 0.5 *M* NaOH), the filters were incubated for 3 h at 65° C with a prehybridization solution (5 × SSC, 0.5% w/v SDS, 5% w/v Dextran Sulfate, 5 × Denhardt's solution, 0.2% w/v non-fat dry milk, 0.1 mg/ml of sonicated and denatured salmon sperm DNA) in a sealed bag, then for 12 h at 65° C with 2 μg/ml of probe DNA in a prehybridization solution. After hybridization, the filters were washed 3 times at 65° C with washing buffer (2 × SSC, 0.1% w/v SDS) for 15 min each and 3 times with TPBS (0.05% v/v Tween 20, 50 m*M*, PBS, ph 7.3) for 15 min each. BrdUrd was detected by immunological procedures described in the dot-blot spotting experiment.

In situ DNA-RNA hybridization. We used HL-60, an acute promyelocytic leukemic cell line, as a positive control for the high level of c-*myc* transcription, and K562, a chronic myelogenous leukemic cell line, as a control for the relatively low level of c-*myc* transcription. (HL-60 expresses an approximately 10-fold greater level compared with K562 according to Eric et al. in 1982 by Northern blot experiments.) In situ DNA-RNA hybridization was performed according to the method described by Nakane et al. (1987) with slight modifications. Briefly, exponentially growing cells (K562 and HL-60) were collected onto ovalbumin-coated slides with cytospin. They were fixed with 4% paraformaldehyde (PFA) at 4° C for 10 min and then air dried. After proteinase K treatment (0.1 mg/ml, 37° C for 15 min) and post-fixation with 4% PFA at room temperature for 5 min, these specimens were incubated in a hybridization medium (10 m*M* *Tris*/HCl pH 7.3, 0.6 *M* NaCl, 1 m*M* EDTA, 1 × Denhardt's medium/250 μg/ml yeast tRNA, 125 μg/ml sonicated salmon sperm DNA, 55% (v/v) deionized formamide/2 μg/ml of probe DNA) at 37° C for 15 h in a moist chamber. At the same time, RNase pretreated (100 μg/ml, 1 h) specimens were used as negative controls. After hybridization, the slides were washed for 2 h each in 55% (v/v) deionized formamide/2 × SSC, and 50% (v/v) deionized formamide/2 × SSC, 0.01% Triton X-100. The immobilized BrdUrd was detected by an immunofluorescent technique using FITC-labeled anti-BrdUrd monoclonal antibody and fluorescent microscopy.

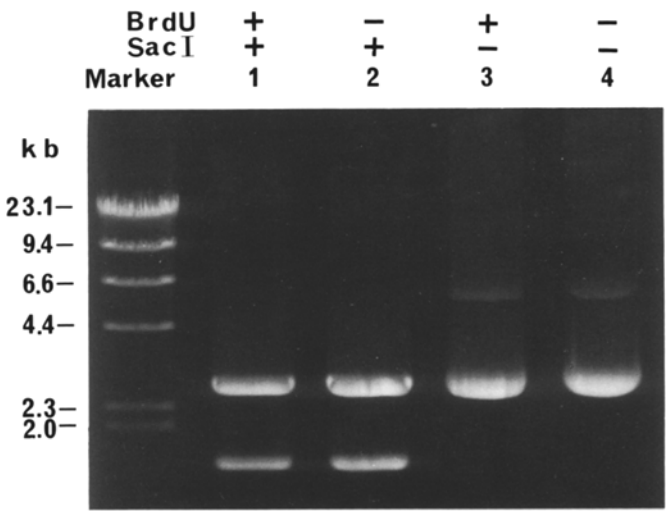

Fig. 2. SacI digestion pattern of plasmid DNA. *Lane 1,* BrdUrd-labeled plasmid DNA without SacI treatment; *lane 2,* BrdUrd-labeled plasmid DNA with SacI treatment; *lane 3,* non-labeled plasmid DNA without SacI treatment; *lane 4,* non-labeled plasmid DNA with SacI treatment. In *lane 3 and 4,* a closed circular form of the plasmid was seen around 2.5 kb and an open circular form of the plasmid was seen around 5.4 kb. In *lane 1 and 2,* SacI digested the plasmid completely and the inserted c-*myc* exon 2 (1530 bp) was released

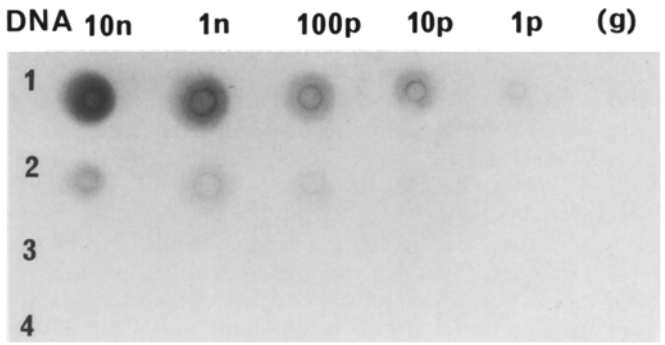

Fig. 3. Immunological detection of BrdUrd-labeled DNA on nitrocellulose filter. *Lane 1,* single stranded BrdUrd-labeled DNA; *lane 2,* double stranded BrdUrd-labeled DNA; *lane 3,* single stranded non-labeled DNA; *lane 4,* double stranded non-labeled DNA. The disks with 1 pg or more of the single stranded BrdUrd-labeled DNA were stained, whereas double stranded BrdUrd-labeled DNA showed a detection limit of 100 pg. No staining was observed with non-labeled DNA

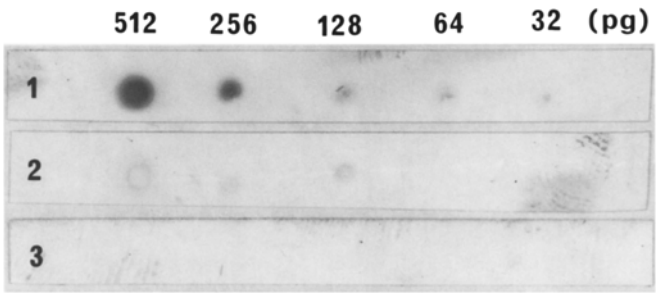

Fig. 4. Dot-hybridization using BrdUrd-labeled DNA probe (*lane 1*), biotinylated DNA probes (*lane 2*) and non-labeled DNA (*lane 3*). BrdUrd-labeled probe showed superior specificity to biotinylated probes

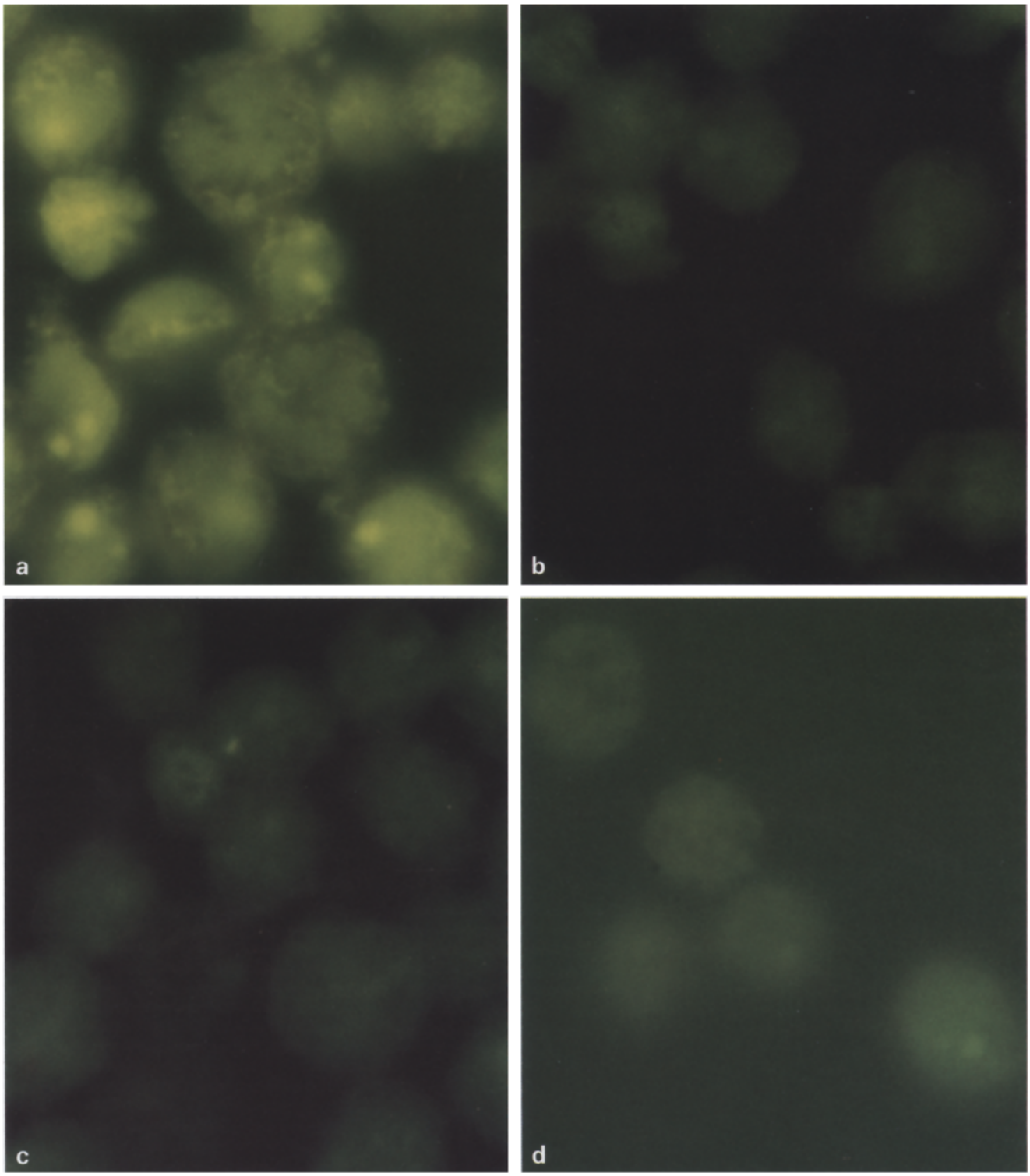

Fig. 5. a HL-60 promyelocytic leukemic cell line was hybridized in situ with in vivo BrdUrd-labeled SacI fragments of cloned c-*myc* exon 2. ×1000. **b** HL-60 promyelocytic leukemic cell line was hybridized in situ with BrdUrd labeled vector DNA. ×1000. **c** HL-60 promyelocytic leukemic cell line was pretreated with RNase before in situ hybridized with in vivo BrdUrd-labeled SacI fragments of cloned c-*myc* exon 2. ×1000. **d** K562 myelogenous leukemic cell line was hybridized in situ with BrdUrd-labeled SacI fragments of cloned c-*myc* exon 2. ×1000

Results

In vivo BrdUrd labeling

About 1 mg of plasmid DNA was purified from 500 ml of *E. coli* culture. BrdUrd had no negative effects on *E. coli* growth and plasmid production in our present study. The digestion pattern of BrdUrd-labeled plasmid with endonuclease SacI (restriction site; GAGCTC) was the same as

that of non-labeled plasmid (Fig. 2), and a specific fragment of defined length of the c-*myc* exon 2 was easily purified using the LMT agarose method. The labeling efficiency of the BrdUrd was as high as previous experiments by Dunn and Smith (1954). BrdUrd was incorporated in 15%–20% of the total DNA, that is, about 80% of the thymidine was replaced by BrdUrd.

Dot-blot spotting

Single stranded DNA probes, as small as 1 pg, were detectable on nitrocellulose filters without any intensification procedures. Double stranded DNA probes, however, showed a detection limit of 100 pg (Fig. 3). There was no cross reaction to non-labeled DNA.

Dot-blot hybridization

Dot-blot hybridization showed about 64-32 pg of spotted single stranded target DNA could be detected by BrdUrd-labeled DNA. The biotinylated probe showed a detection limit of about 256-128 pg (Fig. 4).

In situ DNA-RNA hybridization

Cellular c-*myc* transcripts were detected by fluorescent microscopy. When the HL-60 cell line was hybridized with the BrdUrd-labeled c-*myc* DNA probe, positive staining appeared mainly on the cytoplasm and the nucleoli of some clusters of the cells. Similarly prepared HL-60 cell lines remained unstained when BrdUrd-labeled vector DNA was used in the place of a BrdUrd-labeled c-*myc* DNA probe or specimens were pretreated with RNase. Some background staining on nucleoli was due to the cross reaction of the c-*myc* exon 2 to the 28S ribosomal RNA (Koji and Nakane 1988). There was 46.0% homology between the c-*myc* exon 2 and 28S ribosomal DNA by computer nucleotide sequence homology analysis (Genetyx Nucleotide Sequence Homology Data, Software Development, Tokyo, Japan). The K562 cell line was weakly positive on cytoplasma with the BrdUrd-labeled c-*myc* DNA probe and unreactive with the BrdUrd-labeled vector DNA (Fig. 5a–d).

Discussion

In this study, we demonstrated that in vivo BrdUrd-labeled DNA of defined sequence was useful as a non-radioactive probe for use in situ hybridization. In situ DNA-DNA or DNA-RNA hybridization is now a widely applied method of demonstrating specific nucleic acid sequences. A number of immunohistochemical detection systems using non-radioactive probers have been developed recently. Unlike radioactive labeled probes, they are stable, have high resolution, are safe, inexpensive and fast. At first, horseradish peroxidase was introduced directly into nucleic acids (Nakane and Wilson 1975) but enzyme-labeled probes are heat labile, and the structural alteration is great. A variety of heat stable ligands of relatively low molecular size have been used instead to modify nucleic acids chemically. 2-acetyl-aminofluorence is often used as an artificially introduced hapten for nucleic acid labeling (Landegent et al. 1984; Trask et al. 1985; Heyting et al. 1985; Mitchell et al. 1986; Cremers and Luderus 1984), but this substance is a carcinogen, and is not suitable for routine laboratory use. Sulfonated probes are also used (Morimoto et al. 1987), but often anti-sulfonated DNA antibody crossreacts with the other sulfonated substances. Other ligands such as dinitrophenyl residue (Shroyer and Nakane 1983) and Hg-sulfhydryl hapten (Hopman et al. 1986) are also used. All of these hapten-labeled probes have a common problem: the structural alteration (loss of specificity) should be minimal, while the retention of the label (sensitivity) should be maximum. Therefore, they require strict preliminary experiments. With the use of biotinylated probes (Langer et al. 1982; Forster et al. 1985), structural alterations and the presence of endogenous avidine-binding activity of some tissue are often considerable problems (Banarjee and Pettit 1984). Another labeling method, T–T dimer probe, was introduced by Nakane et al. (1987). This method does not require separating the probes from the unreacted label nor transferring the probe from one container to another. The dimerizable sites are usually a maximum of 2% of the total DNA, and the availability of stable anti-T–T dimerized nucleic acid antibody is limited. All of these modification procedures mentioned above are in vitro nucleic acid labeling methods.

We employed an in vivo nucleic acid labeling method. In this method, labeling was performed only by adding BrdUrd to a LB *E. coli* culture medium. Purification procedures of the plasmid DNA and endonuclease digestion patterns of defined DNA sequences are the same as those of non-labeled DNA. That is to say, in vivo labeling can be performed with the same procedures as the routine DNA fragment preparation. In the BrdUrd-labeled probe, the high labeling index (150–200 bases/kb) does not reduce the specificity. The detection system using FITC-conjugated anti-BrdUrd monoclonal antibody is simple and specific for the target DNA. However, the anti-BrdUrd monoclonal antibody has a low reactivity to the double stranded DNA (in our present study, about 1/100 of the single stranded DNA) and the sensitivity of the BrdUrd-labeled probe is limited. In our present experiment, detectable BrdUrd on the single stranded tails of the probe was 6% of the total DNA.

In conclusion, stable DNA probes of reliable sensitivity are readily usable in the ordinary laboratory. Further investigations of in situ histo-hybridization at both light and electron microscopic levels are possible using this method.

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