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IN SITU LOCALIZATION OF c-*MYC* mRNA IN HL-60 CELLS USING NON-RADIOACTIVE SYNTHETIC OLIGODEOXYNUCLEOTIDE PROBES

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To analyze specific mRNA at individual cell level, non-radioactive *in situ* hybridization has been a powerful technique. *In situ* hybridization is most commonly done using double-stranded cDNA probes. We have already reported the usefulness of thymine-thymine (T-T) dimerized double-stranded cDNA as a non-radioactive probe. In this study, we extended this approach to use synthetic oligodeoxynucleotide (oligo-DNA). Two oligo-DNAs (I; 65-mer, II; 57-mer) complementary to the different regions of the human c-*myc* mRNA were synthesized and ultraviolet (UV)-irradiated (7,000 J/m^2). When the T-T dimerized oligo-DNAs were dot-blotted to nitrocellulose filters, at least 40 pg of both oligo-DNAs was detected immunohistochemically using anti-T-T antibody. By dot-blot hybridization, about 80 pg of sense strand of c-*myc* DNA was specifically detected with T-T dimerized c-*myc* oligo-DNA (I). When the oligo-DNA (II) was used as a probe, however, the sensitivity of signal detection was significantly lower. Furthermore, the c-*myc* antisense oligo-DNAs reacted with the total RNA from HL-60 cells, but not from normal rat liver. Finally, using these oligo-DNAs, c-*myc* mRNA was detected in HL-60 cells.

For the better understanding of physiological states of cells and tissues, the study of expression of specific mRNA of individual cells rather than the study of a mass of cells or tissues is required, since the expression of mRNA varies considerably from cell to cell. To accomplish this task, the localization of mRNA by means of *in situ* hybridization was found to be the most effective method (6, 22, 39).

As probes for the *in situ* hybridization, there are several choices. Double-stranded cloned cDNA is most commonly used, and recently the probes of single-stranded cDNA (45) and cRNA (9, 18) have been introduced. When double-stranded cDNA is used as a probe, one must denature the probe DNA by boiling before it is applied to

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cells or tissue sections. Moreover during hybridization, the re-annealing between dissociated strands of probe DNA will occur, resulting in a decrease in the effective concentration of probe DNA (9). The use of single-stranded probes avoids the above problem. However, for the construction and handling of single-stranded cDNA or cRNA, expertise in molecular technology is required.

The use of automatically synthesized oligodeoxynucleotide (oligo-DNA) probe enables us to access more the *in situ* hybridization method by avoiding the above various difficulties. A more important advantage in the use of oligo-DNA probe is that one can construct probes complementary to unique or highly variable regions of RNA and DNA sequence, which allows us to use probes of great specificity.

Most of the studies with oligo-DNA probes used ^{32}P-labelling (12, 28, 34, 36, 44) or ^{125}I-labelling (7) for signal detection though the resolution was not always sufficient for the analysis of individual cells. Recently, in order to obtain a better resolution and to minimize the cumbersome procedures associated with the handling of radioactive compounds, non-radioactive labels have been developed (13, 19, 26, 35, 42), and one of them has been applied to the *in situ* hybridization with oligo-DNA probe (15, 25). Recently we have developed a new method using thymine-thymine (T-T) dimerized DNA as a non-radioactive probe (21, 22, 33), which is thought to be the simplest in terms of no requirement for a separation of labelled DNA from unreacted labelling compounds. Thus the combination of oligo-DNA with T-T dimer labelling seems to favor the practice of *in situ* hybridization.

In this study, two oligo-DNAs ((I) 65-mer and (II) 57-mer) complementary to the different regions of the human c-*myc* mRNA corresponding to the third exon of the gene were synthesized and T-T dimerized by ultraviolet (UV) irradiation. Then we examined whether c-*myc* mRNA can be detected immunohistochemically both on nitrocellulose filters and in HL-60 cells by using the T-T dimerized antisense c-*myc* oligo-DNA probe. c-*myc* is a normal cellular counterpart of retroviral oncogene (v-*myc*), highly conserved beyond species (4) and is expected to be involved in the regulation of cell proliferation (17, 40, 48) and differentiation (14, 24, 47). In HL-60 cells, c-*myc* gene was amplified and the expression of the gene was changed depending on the states of differentiation (8, 47), thus providing us the appropriate experimental system to evaluate the efficacy of *in situ* hybridization using T-T dimerized oligo-DNA probe.

MATERIALS AND METHODS

Materials: Following reagents were purchased; formamide from Nakarai Chemical Co., Japan, 3,3′-diaminobenzidine/4 HCl from Dojin Chemical Co., Japan, bovine serum albumin (BSA) (98–99% pure), proteinase K (18–20 units/mg protein), horseradish peroxidase (type VI) (HRP) and polyvinylpyrrolidone (MW 360,000) from Sigma Chemical Co., USA, Ficoll-400 from Pharmacia Co., Sweden, salmon sperm DNA from Wako Pure Chemical, Co., Japan, RPMI 1640 medium, antibiotics and fetal bovine serum from Gibco, S1 nuclease from Takara Shuzo Co., Japan. All chemicals and biochemicals used were of analytical grade.

Cells and tissue: HL-60 cells (a cell line from human promyelocytic leukemia) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml of penicillin G and 100 μg/ml of streptomycin under 5% CO_2 in air. The ratio of differentiated cells (8) was less than 4% of

total cells by nitroblue-tetrazolium reduction and Wright-Giemsa's staining. Normal livers were obtained from adult male Wistar rats weighing 250–350 g. The rats were killed by cervical dislocation.

DNA probes used: Two oligo-DNAs (Fig. 1) complementary to the different regions of the human c-*myc* mRNA corresponding to the third exon region of the gene (3) ((I) 803–867 and (II) 1,110–1,166 of nucleotide sequence (46)) were synthesized by β-cyanoethylphosphoramidite method on a DNA synthesizer (Cyclon, Biosearch Inc.) and purified by reverse-phase HPLC. For the preparation of single-stranded c-*myc* DNA, the 1.4 kb *Cla* I-*Eco* RI fragment from the plasmid, pMC41-3RC (3, 10), including the third exon of the human c-*myc* gene was cloned into the *Pst* I site of the plasmid, M13mp7 (31), using *Pst* I linker. The single-stranded DNA was separated from the vector by agarose gel electrophoresis after digestion of the recombinant clone which was partially annealed around the palindromic polylinker region with *Pst* I. Then the DNA fragment was purified by phenol extraction and ethanol precipitation (30).

The DNAs were dissolved in 10 mM Tris/HCl buffer (pH 7.4) containing 1 mM EDTA (TE) and kept at −20°C.

Labelling of probe DNA: T-T dimer was introduced into DNAs by UV-irradiation as described in detail previously (22, 33). The optimal UV dose for c-*myc* oligo-DNA probe was 7,000 J/m^2 when it was determined by dot-blot hybridization.

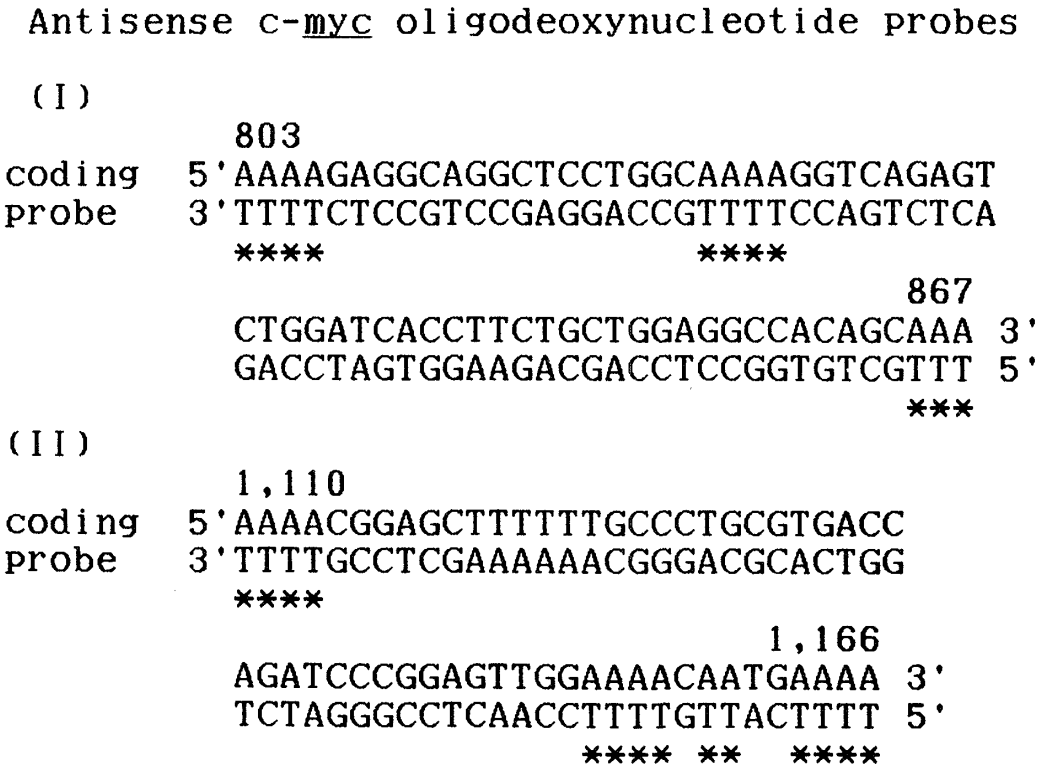

FIG. 1. Antisense c-*myc* oligo-DNA probes used in this paper. The sequences of c-*myc* oligo-DNA (I) and (II), which are complementary to the region of human c-*myc* mRNA corresponding to the third exon portion of the gene, are shown. The symbols (*) indicate T-T dimerizable region. The other details are described in MATERIALS AND METHODS.

Digestion of single-stranded DNA by S1 nuclease: The sense strand of single-stranded cDNA was digested with S1 nuclease to reduce the size, according to the textbook of molecular cloning (30). The size of the DNA was less than about 100 nucleotides by gel-electrophoresis.

Isolation of total RNA: Total RNA was isolated from HL-60 cells and rat livers according to the method of Chirgwin *et al.* (5). After the RNA fraction was precipitated with ethanol and dried up, the final pellet was suspended in TE.

Antibody used: Rabbit anti-T-T dimer was prepared by immunizing rabbits with a mixture of T-T dimerized salmon sperm DNA and methyl-bovine serum albumin (27). IgG from the rabbit serum was isolated and used as the first antibody. Fab fragment of goat IgG against rabbit IgG conjugated with HRP was used as the second antibody.

Immunohistochemical detection of T-T dimerized oligo-DNA: Unless otherwise specified, the following experiments were carried out at room temperature (RT; 25–28°C). Two μl of the c-*myc* oligo-DNA solution was applied onto nitrocellulose filters which were pretreated with 20×SSC (1×SSC; 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for at least 30 min and air-dried. Each spot contained 4 pg to 40 ng of DNA. After being air-dried, the filters were baked at 80°C for 2 hr. For the detection of T-T dimer, the filters were treated with phosphate buffered saline (PBS) (pH 7.2, unless otherwise specified) containing 5% BSA, 500 μg/ml normal goat IgG, 100 μg/ml salmon sperm DNA, 100 μg/ml yeast tRNA and 0.05% NaN_3 for 1 hr in order to block non-specific reaction of the antibody with the filters. Then the filters were reacted with rabbit anti-T-T IgG (40 μg/ml) dissolved in PBS containing 5% BSA, 100 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA and 0.05% NaN_3 for 3 hr. As a control, normal rabbit IgG was used at the same concentration. After overnight wash with PBS, the filters were reacted for 3 hr with the second antibody dissolved in PBS containing 5% BSA, 100 μg/ml yeast tRNA and 100 μg/ml salmon sperm DNA and then washed with PBS for 3 hr. The sites of HRP were visualized using 0.5 mg/ml 3,3′-diaminobenzidine/4 HCl in the presence of nickel and cobalt ions (1).

Dot-blot hybridization: Two μl of the solution of single-stranded sense or antisense c-*myc* DNA (8 pg to 8 ng per spot) or of the solution of the total RNA from HL-60 cells or normal rat liver (100 ng to 500 ng per spot) was applied onto nitrocellulose filters and baked as described above. 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, 50% (v/v) deionized formamide, 500 μg/ml yeast tRNA, 250 μg/ml salmon sperm DNA at 42°C for 2 hr. Then the filters were incubated in the hybridization mixture containing 10 mM Tris/HCl (pH 7.3), 1 mM EDTA, 0.6 M NaCl, 1×Denhardt's solution, 40% (v/v) deionized formamide, 250 μg/ml yeast tRNA, 125 μg/ml salmon sperm DNA and 0.8 μg/ml T-T dimerized c-*myc* oligo-DNA at 42°C for 15–17 hr. For the RNA-DNA hybridization, a mixture of oligo-DNA (I) (0.4 μg/ml) and (II) (0.4 μg/ml) was used as a probe. The filters were washed twice with 2×SSC (30 min each), twice with 1×SSC (30 min each) and with 0.1×SSC (30 min), successively. The detection of T-T dimer was done as described above.

In situ hybridization:

Cell preparation; HL-60 cells were washed once with PBS (pH 7.4) and suspended in 2% paraformaldehyde (PFA) in PBS (pH 7.4) at a concentration of about 5×10^5 cells/ml. An aliquot (0.2 ml) of the suspension was spun down onto a gelatin-coated

glass slide (22) by Cytospin (Shandon) and fixed further in 4% PFA in PBS (pH 7.4) at 4°C for 10 min. After fixation, the cells were air-dried for 1 hr and baked for 2–4 hr at 45°C. Then the cells were kept under −80 to −60°C until use.

Pretreatment; at the time of *in situ* hybridization, the cells were rehydrated with PBS. The specimens were treated with 0.3% H_2O_2 in methanol for 10 min and were treated with 0.2 N HCl for 20 min. After digestion with 1 μg/ml of proteinase K dissolved in PBS at 37°C for 15 min and washing 3 times with PBS (5 min each), the specimens were fixed in 4% PFA in PBS (pH 7.4) for 5 min and washed twice with PBS (5 min each), and the remaining aldehyde was quenched twice by immersion in 2 mg/ml glycine in PBS for 15 min each. Some of the specimens were not treated with proteinase K. Then the specimens were washed twice with PBS (5 min each), immersed in 40% (v/v) deionized formamide in 2 × SSC and kept in the formamide solution until hybridized. The other details were described previously (22).

Hybridization; Twenty μl of the hybridization mixture containing T-T dimerized DNA probe at various concentrations of 0.8–3.2 μg/ml was applied to each slide, mixed and incubated in a moist chamber at 42°C for 15 hr. Then the slides were washed 4 times with 50% (v/v) formamide in 2 × SSC at 37°C for 1 hr each, and twice with 2 × SSC at RT for 15 min each.

Enzyme-immunohistochemistry; for immunohistochemical detection of T-T dimerized DNA, similar procedures as done with filters were taken and the other details were reported elsewhere (21, 22, 33).

RESULTS

Immunohistochemical detection of T-T dimerized oligo-DNA fixed onto nitrocellulose filters

As shown in Fig. 2, at least 40 pg of T-T dimerized oligo-DNA was detected us-

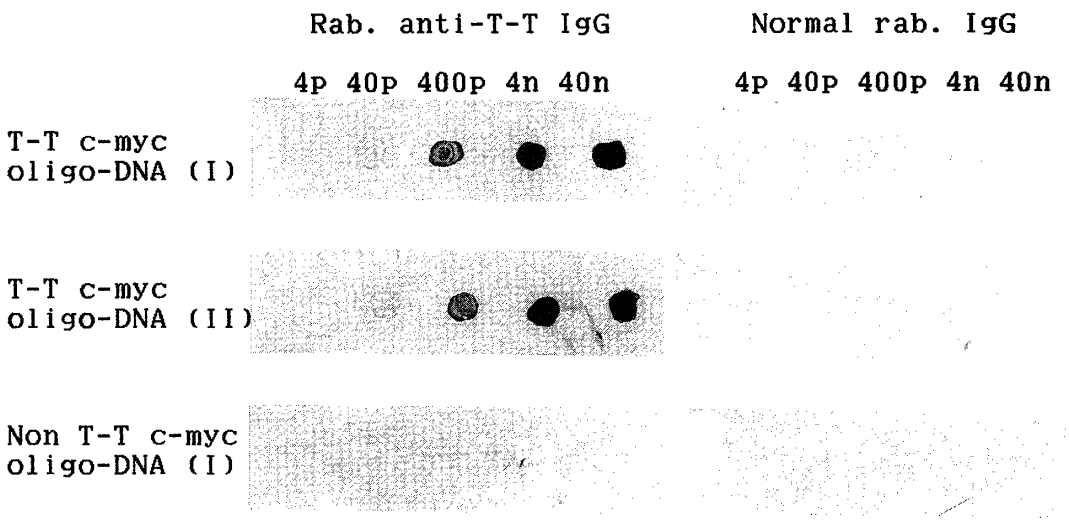

FIG. 2. Immunohistochemical detection of T-T dimerized c-*myc* oligo-DNAs on nitrocellulose filters. T-T dimerized antisense c-*myc* oligo-DNA (I) and (II), or non T-T dimerized c-*myc* oligo-DNA (I) were fixed nitrocellulose filters at the amount of 4 pg, 40 pg, 400 pg, 4 ng and 40 ng per spot. Then the filters were reacted with rabbit anti-T-T IgG or normal rabbit IgG at the same concentration (40 μg/ml).

ing anti-T-T IgG. When the filters were reacted with normal rabbit IgG in place of anti-T-T IgG, no staining was observed. Also there was no staining with non T-T dimerized DNA when reacted with anti-T-T IgG. It should be noted that we detected both T-T dimerized c-*myc* oligo-DNA (I) and (II) at the similar sensitivity.

Immunohistochemical dot-blot hybridization

In order to examine whether T-T dimerized oligo-DNA probe can detect a specific sequence of nucleic acids, T-T dimerized antisense c-*myc* oligo-DNAs were hybridized with sense or antisense single-stranded c-*myc* DNA fixed onto nitrocellulose filters and the hybrids were detected immunohistochemically. As shown in Fig. 3a, using the c-*myc* oligo-DNA probe (I) at least 80 pg of sense c-*myc* DNA was specifically detected. When the c-*myc* oligo-DNA (II) was used as a probe, a specific signal was also found, but the sensitivity of detection (about 800 pg of sense c-*myc* DNA) was significantly lower than that with the c-*myc* oligo-DNA (I). When the c-*myc* oligo-DNA (I) and (II) were mixed at a concentration of 0.8 μg/ml each and used as a probe, no significant increase in sensitivity was observed (data not shown).

Next, the total RNAs from HL-60 cells and normal rat livers were fixed onto nitrocellulose filters and hybridized with a mixture of T-T dimerized antisense c-*myc* oligo-DNA (I) and (II). As shown in Fig. 3b, the spots of RNA from HL-60 cells were specifically stained. These results were consistent with the previous findings that the RNA from HL-60 cells contain c-*myc* mRNA (47), but not the RNA from normal rat liver (29). In addition, the use of the mixture of oligo-DNA (I) and (II)

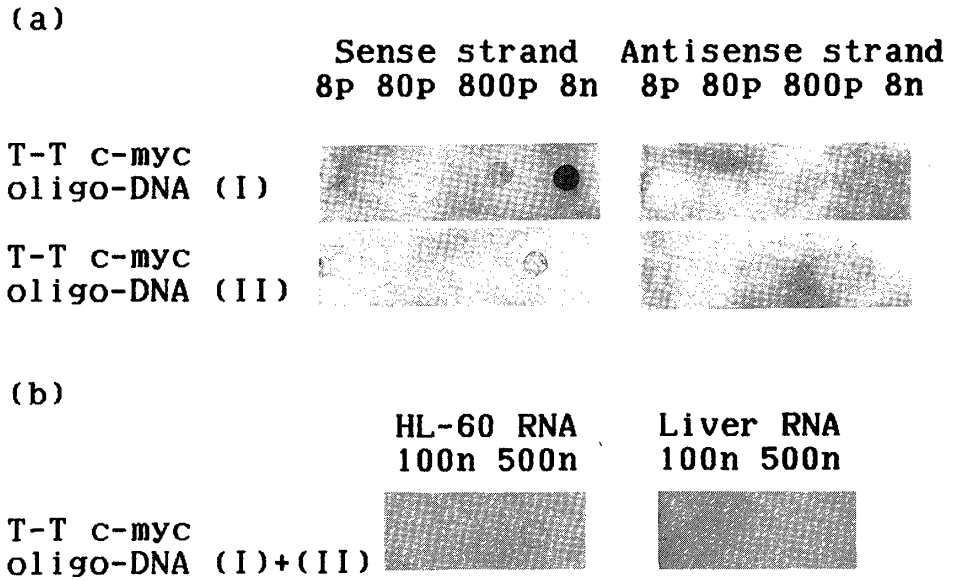

FIG. 3. Dot-blot hybridization using T-T dimerized antisense c-*myc* oligo-DNA probes. a) The sense or antisense strand of c-*myc* DNA was fixed onto nitrocellulose filters at the amount of 8 pg, 80 pg, 800 pg and 8 ng per spot. After the filters were hybridized with T-T dimerized antisense c-*myc* oligo-DNA (I) and (II), the hybrids were detected immunohistochemically. b) Total RNAs from HL-60 cells or normal rat liver were fixed onto nitrocellulose filters at the amount of 100 ng and 500 ng per spot. After the filters were hybridized with a mixture of T-T dimerized antisense c-*myc* oligo-DNA (I) and (II), the hybrids were detected immunohistochemically.

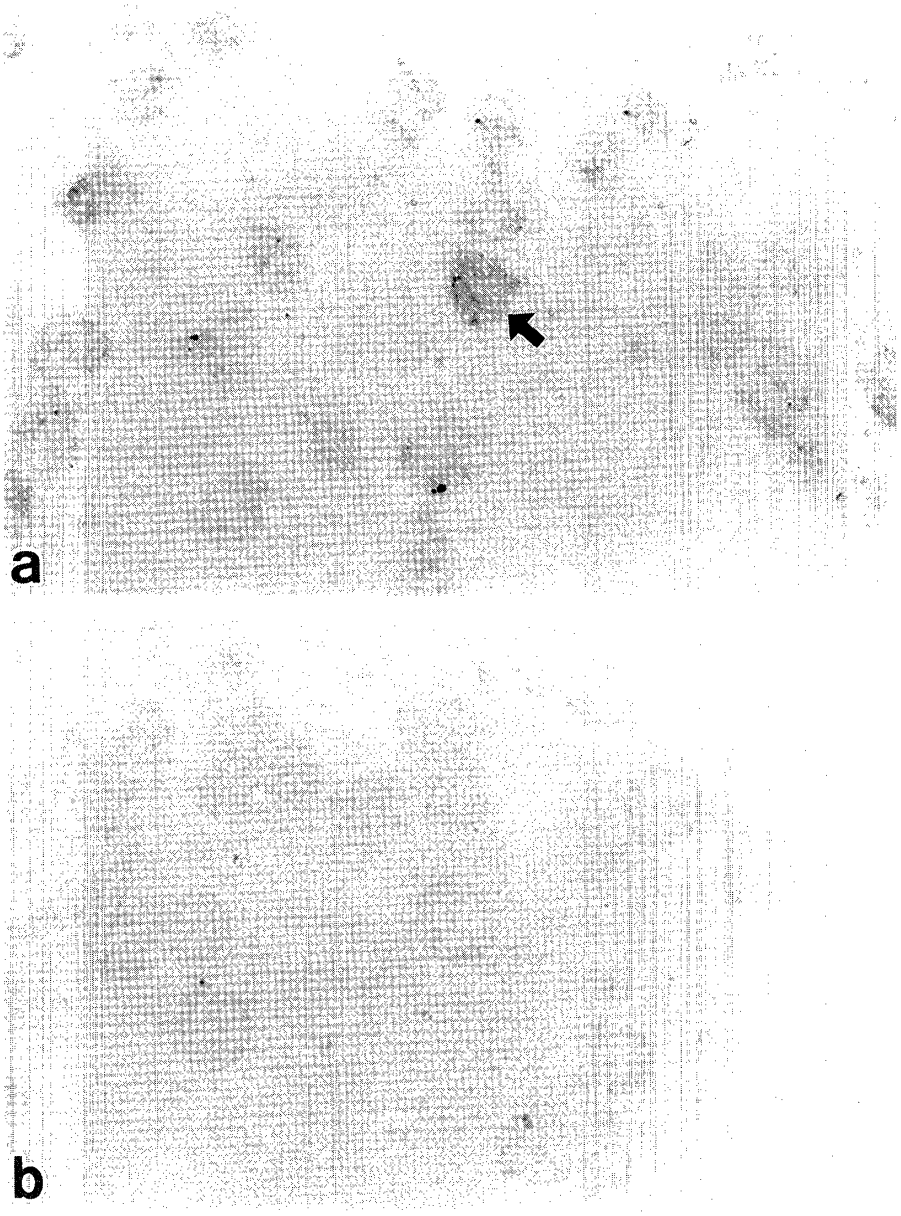

FIG. 4. *In situ* localization of c-*myc* mRNA in HL-60 cells using T-T dimerized c-*myc* oligo-DNA (I) with or without proteinase K digestion. The HL-60 cells were hybridized *in situ* with antisense c-*myc* oligo-DNA (I) probe (1.6 μg/ml) (a, c) and with the S1-nuclease digests of sense-strand of c-*myc* DNA (1.6 μg/ml) (b, d). Proteinase K digestion (37°C, 15 min) was carried out (c, d) at a concentration of 1 μg/ml. The arrows indicate strongly positive cells. ×400

resulted in no significant increase in sensitivity also in RNA-DNA hybridization, as compared to that with oligo-DNA (I) (0.4 μg/ml) alone.

In situ localization of c-myc mRNA in HL-60 cells

As our final purpose, we examined whether or not c-*myc* mRNA can be detected in HL-60 cells using T-T dimerized antisense c-*myc* oligo-DNA probe (I). As shown in Fig. 4c, the specific strong staining of c-*myc* mRNA was observed in about 10% of

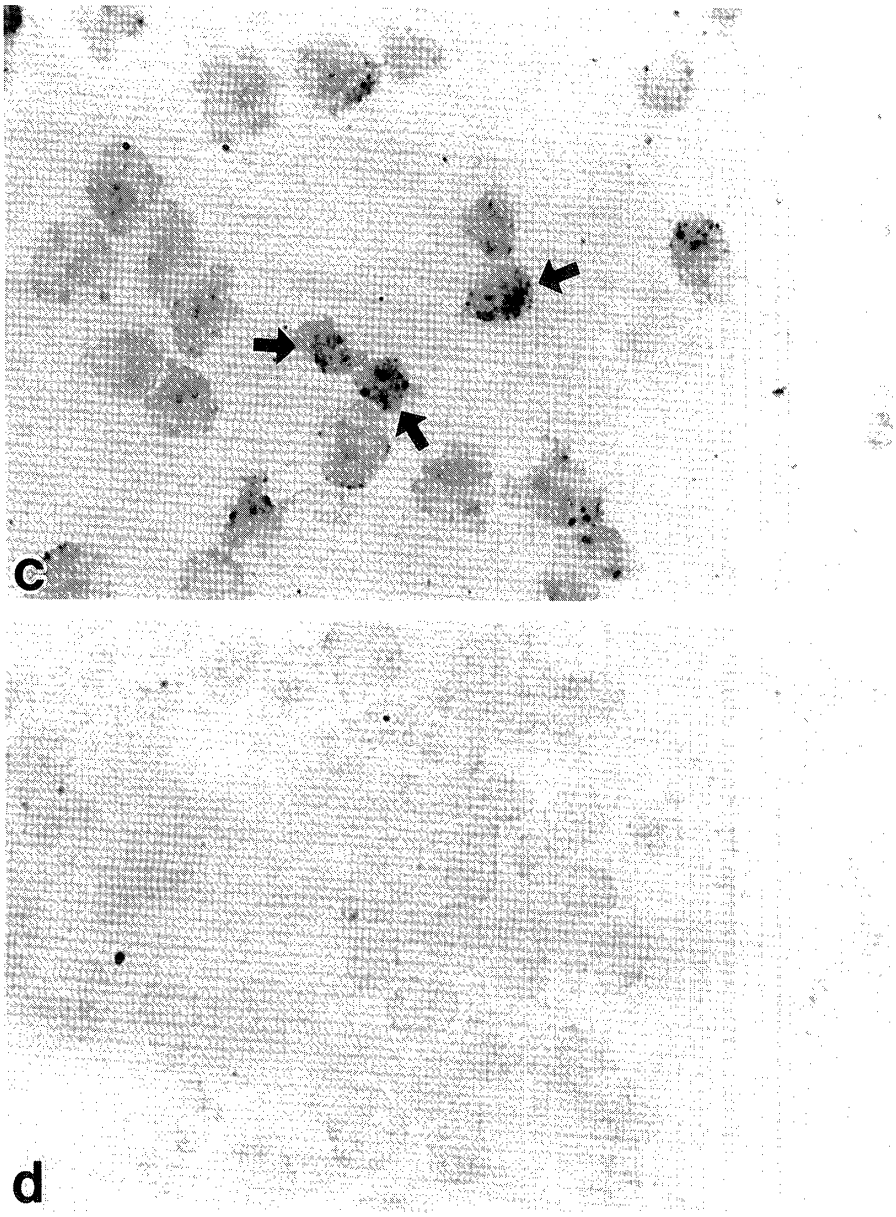

FIG. 4.

HL-60 cells, and the other cells were also stained, but weakly. With S1 nuclease digests of T-T dimerized single-stranded sense c-*myc* DNA, little or no staining was found (Figs. 4b, d). The intensity of the specific staining and the ratio of strongly positive cells were increased by protease treatment without affecting the background staining (Figs. 4c, d). However, when the concentration of probe DNA was increased from 0.8 μg/ml to 3.2 μg/ml, no significant increase in the intensity of staining was observed (data not shown).

DISCUSSION

Non-radioactive *in situ* hybridization is thought to be the most appropriate technique to analyze the expression of specific mRNA sequences at individual cell level. Conventionally using cloned double-stranded cDNA as a probe, a lot of studies to establish a tissue processing procedure best suited for *in situ* hybridization have been done (6, 22, 39). As already stated, however, the advantage of the use of double-stranded cDNA is limited by the presence of sense strand, whose sequence is identical to that of the corresponding mRNA. Moreover, we have sometimes encountered that the impurity in probe DNA preparation isolated from bacterial plasmid is a major reason for a low signal/noise ratio. Thus there is a potential importance of use of non-radioactive synthetic oligo-DNA probe.

Recently, two groups (15, 25) have reported the use of biotinylated oligo-DNA probes for *in situ* hybridization. Both groups obtained good results using the oligo-DNA labelled in the 3′-end with biotin-11-dUTP by use of terminal deoxynucleotidyl transferase and using avidin-alkaline phosphatase system for signal detection. In this method, however, after the labelling of oligo-DNA with biotinylated nucleotides, a separation of labelled probes from unincorporated nucleotides is required. To avoid these cumbersome and expensive procedures, we applied the T-T dimer method to *in situ* hybridization with synthetic oligo-DNA probe in this study.

T-T dimerized c-*myc* oligo-DNA was specifically detected (Fig. 2) and when hybridized with sense single-stranded c-*myc* DNA or total RNA from HL-60 cells (Fig. 3a and b), specific staining was clearly demonstrated. Finally, in Cytospin preparations of HL-60 cells fixed with 4% PFA in PBS, c-*myc* mRNA was detected (Fig. 4), indicating usefulness of T-T dimerized oligo-DNA probe in localization *in situ* of specific mRNA. However, in order to introduce T-T dimer by UV irradiation, more than two thymine residues have to be present as neighbours. Furthermore the formation of one T-T dimer results in an occurrence of 4 mismatched base pairs in hybrids (16). Thus selecting the region used for a probe is very important process. It seems better to select the region which has T-T dimer forming sites only at both ends to minimize the effect of base mismatches on stability of hybrids. In addition, as shown in Fig. 3a, c-*myc* oligo-DNA (II) probe was less sensitive than oligo-DNA (I) probe. As both T-T dimerized oligo-DNAs were immunohistochemically detected at a similar sensitivity (Fig. 2), we may expect a similar number of T-T dimer in both probe DNAs. The lower sensitivity of oligo-DNA (II) probe to detect complementary sequence may be due to the presence of the sequence (AAAAAA) in the probe DNA, which is able to cause intramolecular and/or intermolecular hybridization with T-enriched region.

In molecular hybridization, it is very important to set the appropriate conditions of hybridization and washing in order to avoid non-specific hybridization and to remove non-specific unstable hybrids. Among papers published previously (7, 12, 15, 25, 28, 34, 36, 44, 45), various conditions concerning hybridization and washing were employed in *in situ* hybridization with synthetic oligo-DNA probes. These situations were made more complicated by the fact that the theoretical formula does not always work in this area (2). Thus, the best conditions should be explored empirically in practice.

As reported previously (22, 32), in order to implement *in situ* hybridization especially with non-radioactive cloned double-stranded cDNA, the ability of probe

DNA to penetrate into cells was one of the essential factors. Oligo-DNA probes, the sizes of which were less than 100 nucleotides, were expected to penetrate relatively easily into cells. Actually, Priestly *et al.* (36) reported no beneficial effects of protease treatment using ^{32}P-labelled oligo-DNA probe. In our hands, however, protease digestion resulted in an increase in signal intensity (Fig. 4), indicating that the unmasking of target mRNA by proteolysis is still important even in using oligo-DNA probe, in agreement with the previous reports (15, 25). The discrepancy may be due to the difference in the fixatives used.

As to the states of expression of c-*myc* mRNA in HL-60 cells, all cells were not homogeneously stained with antisense c-*myc* oligo-DNA probe. The ratio of strongly positive cells in a random culture was at most 10%, in consistent with our previous results obtained by using T-T dimerized c-*myc* double-stranded cDNA (23) and with the immunohistochemical results of expression of c-*myc* protein (20, 41). Moreover, the expression of c-*myc* mRNA at least in strongly positive cells was dependent on cell cycle (41). These accumulated results apparently conflict with the results by Thompson *et al.* (43). They reported a constant expression of c-*myc* mRNA throughout cell cycle using HL-60 cells fractionated by counterflow centrifugation. Taken together with these results, our results seem to indicate that during cell separation the states of c-*myc* expression are changed, and/or that *in situ* hybridization is more sensitive to demonstrate a heterogeneity in the states of gene expression of cells than Northern blot analysis (21, 22). The former possibility is also raised by the fact that half-lives of c-*myc* mRNA (11) and protein (37) are very short.

Finally, to fully implement the *in situ* hybridization method with synthetic oligo-DNA probe, more detailed analysis on various conditions is required. Now we are making efforts especially on setting the hybridization and washing conditions best suited for *in situ* hybridization with synthetic oligo-DNA probes.

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