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IMMUNOHISTOCHEMICAL DETECTION OF HUMAN CELLULAR YES GENE (c-*yes*-1) MESSENGER RNA BY A NON-RADIOACTIVE SYNTHESIZED OLIGODEOXYNUCLEOTIDE PROBE

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A synthetic 39-mer anti-sense strand oligodeoxynucleotide complementary to the region of human cellular *yes* gene (c-*yes*-1) mRNA that codes for a polypeptide of 543 amino acids (Mr: 60, 801) and the corresponding sense strand 39-mer oligodeoxynucleotide were chemically synthesized by β-cyanoethyl phosphoramidite method. The probes thymine-thymine (T-T) dimerized by UV irradiation were used for *in situ* hybridization studies. Various cells were fixed with ethanol-acetic acid (3 : 1) at room temperature for 20 min and hybridization was carried out in 50% formamide for 36 hr at 37°C. The good signal was obtained by utilizing anti-T-T dimer serum (IgG frafction), peroxidase-labeled goat anti-rabbit IgG antibody, and diaminobenzidine-4-HCl and H_2O_2 as substrates. The anti-sense strand synthetic probe hybridized with cellular RNA of cultured cells with c-*yes*-1 mRNA, while the sense strand synthetic probe did not. The c-*yes*-1 mRNA was clearly detected in the cytoplasm of c-*yes*-1 gene product-expressing cultured cells by *in situ* hybridization using a T-T dimerized synthetic probe. Synthetic T-T dimerized oligodeoxynucleotides appear to constitute attractive reagents for *in situ* hybridization studies when supported by appropriate control procedures.

The *in situ* hybridization (ISH) has recently become a widely used procedure for detection and localization of messenger RNAs (mRNA) in cells or tissue sections (1, 3, 6, 8–10, 12, 14, 16, 18, 22–24, 26). The most commonly used probes to detect mRNAs by ISH are radiolabeled cloned complementary DNAs (cDNA), complementary RNAs (cRNAs), and radiolabeled synthetic oligonucleotides. The signal provided by these probes is detected by autoradiography. Other methods of mRNA detection by ISH use non-radioactive probes. The most efficient probes are cDNAs or cRNAs into which biotinylated nucleotides are incorporated and are secondarily revealed by using a histochemical or an immunohistochemical reaction. Development of non-radioactive probes for ISH is profitable because they have a long shelf-life, they avoid the biohazards and the autoradiographic procedure linked to the use of radio-isotopes, they give optimal histological resolution, and they provide results

within hours after the completion of ISH (7, 18).

In situ localization of mRNA using a thymine-thymine (T-T) dimerized cDNA was initially developed by Nakane *et al.* (17). The reason we adopted this technique is that the preparation of a probe with T-T dimer can be easily done in a conventional environment, while photobiotin requires the separation steps, and the labeling procedures must be carried out in the dark (4).

In view of the high potential of oligonucleotide probes for investigating oncogene expression in cells and tissues, we attempted to design a method for histological detection of mRNA with T-T dimerized synthetic deoxyoligonucleotides. We have chemically synthesized sense and anti-sense strand 39-mer probes of cellular yes gene (c-*yes*-1) mRNA for ISH. We were able to successfully localize c-*yes*-1 mRNA in the cytoplasm of various cultured cells.

MATERIALS AND METHODS

Cell lines

TIG-1-20 (human fibroblast) and Marcus (human glioblastoma) were supplied by Japanese Cell Resources Bank (JCRB), Tokyo, Japan. NIH/3T3 cells transfected with c-*yes*-1 gene (containing several copies) were prepared by one of the present authors.

Synthetic probes

A synthetic 39-mer anti-sense strand deoxyoligonucleotides complementary to the region of human cellular yes gene (c-*yes*-1) mRNA (TTTTA^{286}-T^{300}TT) and a synthetic 39-mer sense strand deoxyoligonucleotides were synthesized by β-cyanoethyl phosphoramidite method (5, 20) on a DNA synthesizer (Cyclone, Biosearch, Inc., USA), purified by polyacrylamide electrophoresis, and lyophilized.

Several thymines were added at its 5′ and 3′ end for thymine-thymine (T-T) dimerization by UV irradiation (Fig. 1).

Antibodies

Murine monoclonal antibody (1B7) against c-*yes*-1 gene product was prepared and characterized by one of the present authors (Manuscript in preparation). Briefly, bacterial phosphate binding protein (PBP)-c-*yes*-1 fusion protein of ca. 40 kDa emulsified in complete Freund's adjuvant was injected subcutaneously into BALB/c mice several times. After the somatic cell hybridization, hybrid cells were selected in HAT medium and the culture supernatants were assayed for their anti-PBP-c-yes-1 fusion protein activity by ELISA method. Finally we obtained a hybridoma reacting with c-

FIG. 1. Sequences of synthesized oligodeoxynucleotides. Number over A or T represents nucleotides of the human c-*yes*-1 cDNA (20).

yes-1 gene product.

Rabbit anti-T-T dimer antibody was prepared by immunizing rabbits with a mixture of T-T dimerized salmon sperm DNA and methyl-bovine serum albumin (a gift from Dr. H. Yoshida, Kyoto University School of Medicine) (13). This rabbit anti-T-T dimer antibody reacts only with T-T dimerized DNAs, and not with salmon sperm DNAs, yeast transfer RNA and cellular RNAs of K562 (human myelogenous leukemia) containing mainly ribosomal RNAs. IgG from the rabbit serum was isolated by salting out and used as the first antibody.

Fab fragment of goat IgG against rabbit IgG conjugated with horseradish peroxidase was prepared according to the method of Wilson and Nakane (25).

T-T dimerization of synthetic probes

T-T dimer was introduced into synthetic probes by UV irradiation as described in detail previously (9, 17). The optimal UV dose for the synthetic probes was 7,000 J/m^2 when they were determined by the dot blot hybridization method.

Detection of T-T dimerized synthetic probes

Anti-sense and sense strand synthetic probes were irradiated at the rate of 5 J/m^2/sec for 10 min (3000 J/m^2), ca. 17 min (5000 J/m^2), or ca. 23 min (7000 J/m^2). Ten pg to 10 ng of either anti-sense strand or sense strand synthetic probe was baked on nitrocellulose membranes. The membranes were first reacted with 200 μl of 32 μg/ml anti-T-T IgG for 3 hr and followed by the reaction with 200 μl of 1 μg/ml of peroxidase-labeled goat anti-rabbit IgG for 3 hr. The membranes were then histochemically stained for peroxidase using diaminobenzidine-4 HCl and H_2O_2 as substrates.

Isolation of cellular RNA from Marcus cells

Cellular RNA was isolated by the method of Maniatis *et al.* (15). Marcus cells were washed twice with phosphate-buffered saline (PBS), lysed with NP-40 lysis buffer (5 M NaCl, 1 M $MgCl_2$, 1 M Tris, ph 8.6, 0.5% NP-40, 10 mM vanadyl-ribonucleoside complex, and gently mixed. The solution was centrifuged at 5,000 rpm at 4°C for 5 min. The supernatant in 0.1% SDS and 5 mM EDTA/PBS and chloroform/phenol (1 : 1) with equal volumes was rotated at room temperature (RT) for 20 min, and centrifuged at 3,000 rpm at RT for 10 min. The upper layer was mixed with equal volumes of chloroform/isoamylalcohol (99 : 1), rotated at RT for 20 min, and centrifuged at 3,000 rpm at RT for 10 min. The upper layer was mixed with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol, and was allowed to stand at −20°C overnight. Thereafter, the mixture was centrifuged at 5,000 rpm at 0°C for 10 min. The precipitate was dissolved in TE buffer (0.01 M Tris-HCl, pH 7.4, 1 mM EDTA).

Dot blot hybridization

Various quantities (10 ng to 1 μg of RNA in 2 μl of TE buffer) of cellular RNA were dotted on strips of nitrocellulose filters. The filters were dried, heated at 80°C for 2 hr, and prehybridized at 37°C for 2 hr. Hybridization was performed with 1 μg/ml synthetic probes at 37°C for 36 hr as described elsewhere (9, 17).

In situ hybridization

About one hour prior to the start of the procedure, the cells were spotted on Denhardt's solution-coated slide glasses (2) with a Cytospin centrifuge (Shandon Instruments Inc., USA) or the cells were cultured in small chambers. The cells were fixed with ethanol-acetic acid (3 : 1) mixture for 20 min at RT. *In situ* hybridization was

carried out with 1 μg/ml synthetic probes at 37°C for 36 hr as described elsewhere (17).

Immunohistochemistry

For comparison with the data by *in situ* hybridization at mRNA level, immunohistochemistry of the cells (ABC-PO method) was performed. As the first antibody, 1B7 monoclonal antibody recognizing c-*yes*-1 gene product was utilized. The staining procedure is the same as one described elsewhere (19).

RESULTS

Determination of optimal conditions for dot blot and in situ hybridization

As shown in Fig. 2, the optimal UV dose for the synthetic probes was 7,000 J/m^2 as determined by the dot blot hybridization method. The disks with 10 pg or more of T-T dimerized synthetic probe were stained.

Dot blot hybridization

Strips of nitrocellulose dotted with various amounts of cellular RNA from Marcus (human glioblastoma) cells were used to determine the sensitivity of hybridization. When these strips were hybridized with 250 μl of 1 μg/ml of T-T dimerized synthetic oligodeoxynucleotides (sense and anti-sense strand), those dotted with 1 μg or

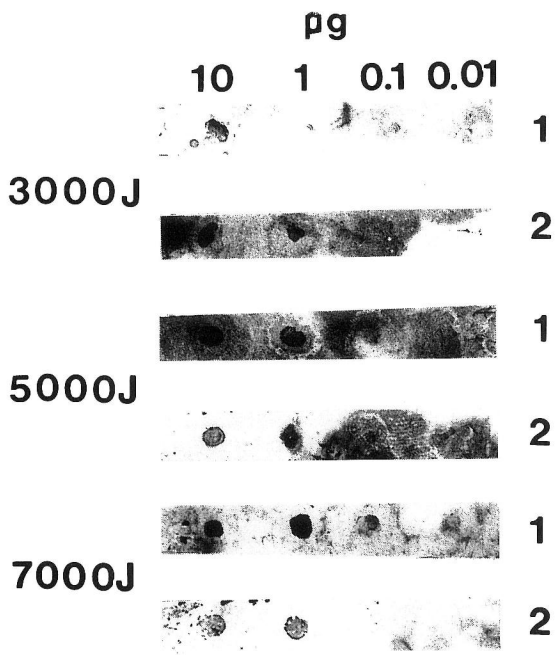


FIG. 2. Immunocytochemical detection of T-T dimerized synthetic probes on nitrocellulose membrane.

1. Anti-sense strand synthetic probe. 2. Sense strand synthetic probe.
3,000 J=3,000 J/m^2. 5,000 J=5,000 J/m^2. 7,000 J=7,000 J/m^2.

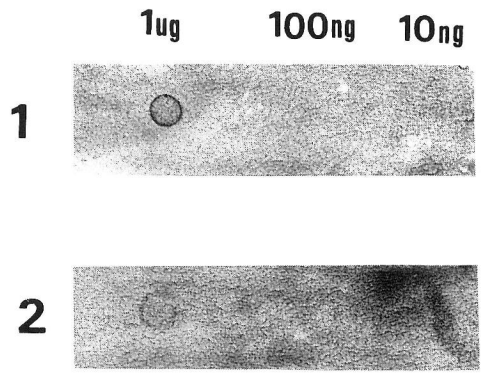


FIG. 3. Dot blot hybridization by T-T dimerized synthetic oligodeoxynucleotides
1. Anti-sense strand synthetic probe. 2. Sense strand synthetic probe.

more of cellular RNA of Marcus cells stained consistently with increasing intensity as the amount of dotted cellular RNA of Marcus cells increased. T-T dimerized anti-sense strand oligodeoxynucleotides hybridized with cellular RNA of Marcus cells. T-T dimerized sense strand oligodeoxynucleotides seemed to hybridize very weakly with the cellular RNA for an unknown reason (Fig. 3).

In situ hybridization

Marcus cells have c-*yes*-1 gene products as revealed by a murine monoclonal antibody (1B7) (Fig. 4). When Marcus cells were hybridized *in situ* with T-T dimerized anti-sense strand synthetic c-*yes*-1 oligodeoxynucleotides, the cytoplasm of the Marcus cells were stained, but the Marcus cells were not hybridized with a T-T dimerized sense strand synthetic probe (Fig. 4). Furthermore, TIG-1-20 cells also expressing c-*yes*-1 gene products were hybridized *in situ* with anti-sense or sense strand T-T dimerized synthetic probe. The cytoplasm of the TIG-1-20 cells was stained with anti-sense strand T-T dimerized oligodeoxynucleotides, although the positive signal was located near the nuclear membrane (Fig. 5). NIH/3T3 cells transfected with c-*yes*-1 gene were also stained with an anti-sense strand T-T dimerized synthetic probe (Fig. 6), but not with a sense strand T-T dimerized synthetic probe. Interestingly, nucleoli in TIG-1-20 and NIH/3T3 cells transfected with c-*yes*-1 gene were hybridized with an anti-sense strand synthetic probe.

FIGS. 4A–D. *In situ* hybridization of Marcus cells with T-T dimerized synthetic oligodeoxynucleotides and immunohistochemistry.
A. Anti-sense strand synthetic probe (1 μg/ml). ×600
B. Sense strand synthetic probe (1 μg/ml).
C. Monoclonal antibody specific to c-*yes*-1 gene product (1B7) (10 μg/ml). ABC-PO method. ×600
D. Non-immune mouse serum (10 μg/ml).

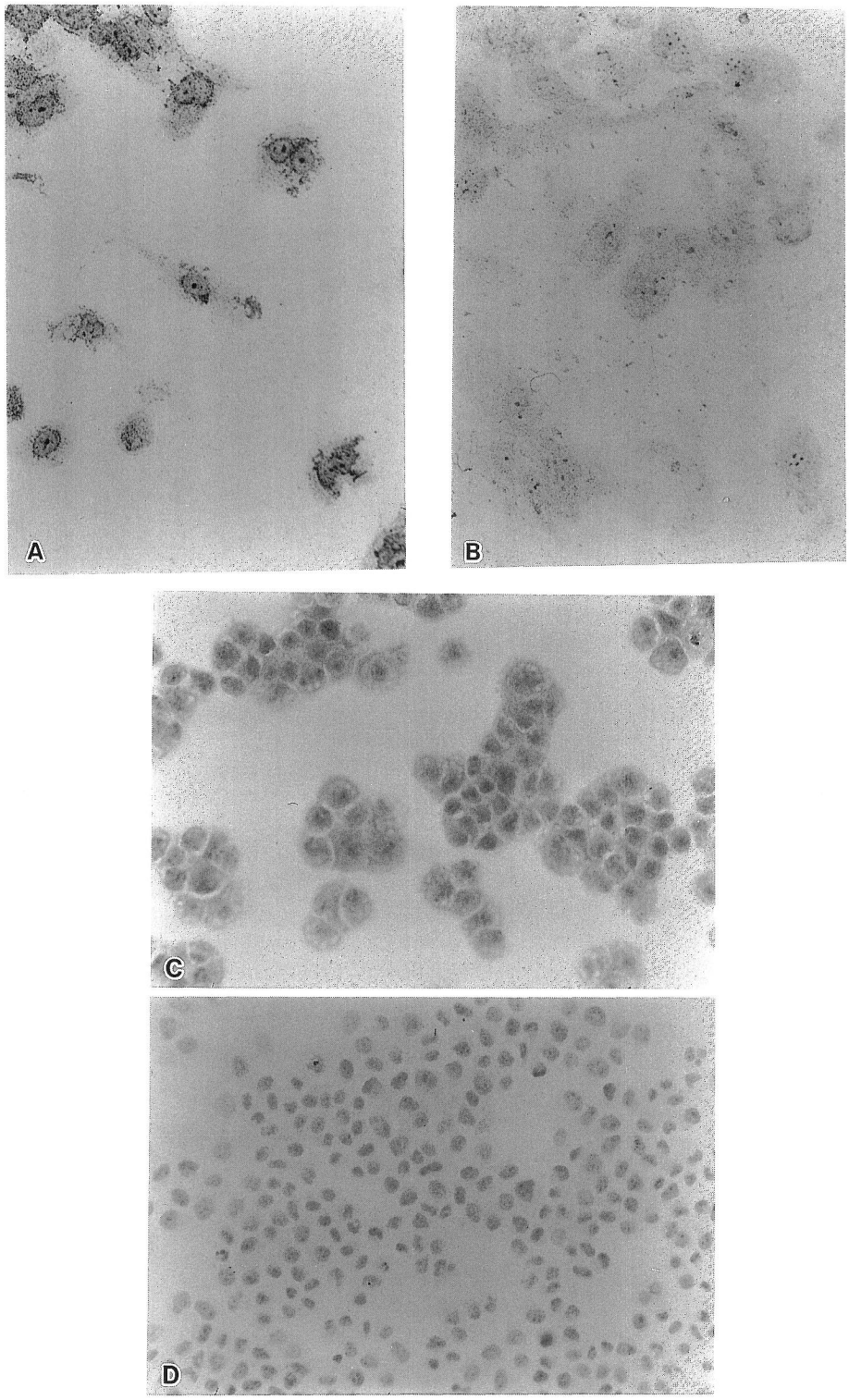
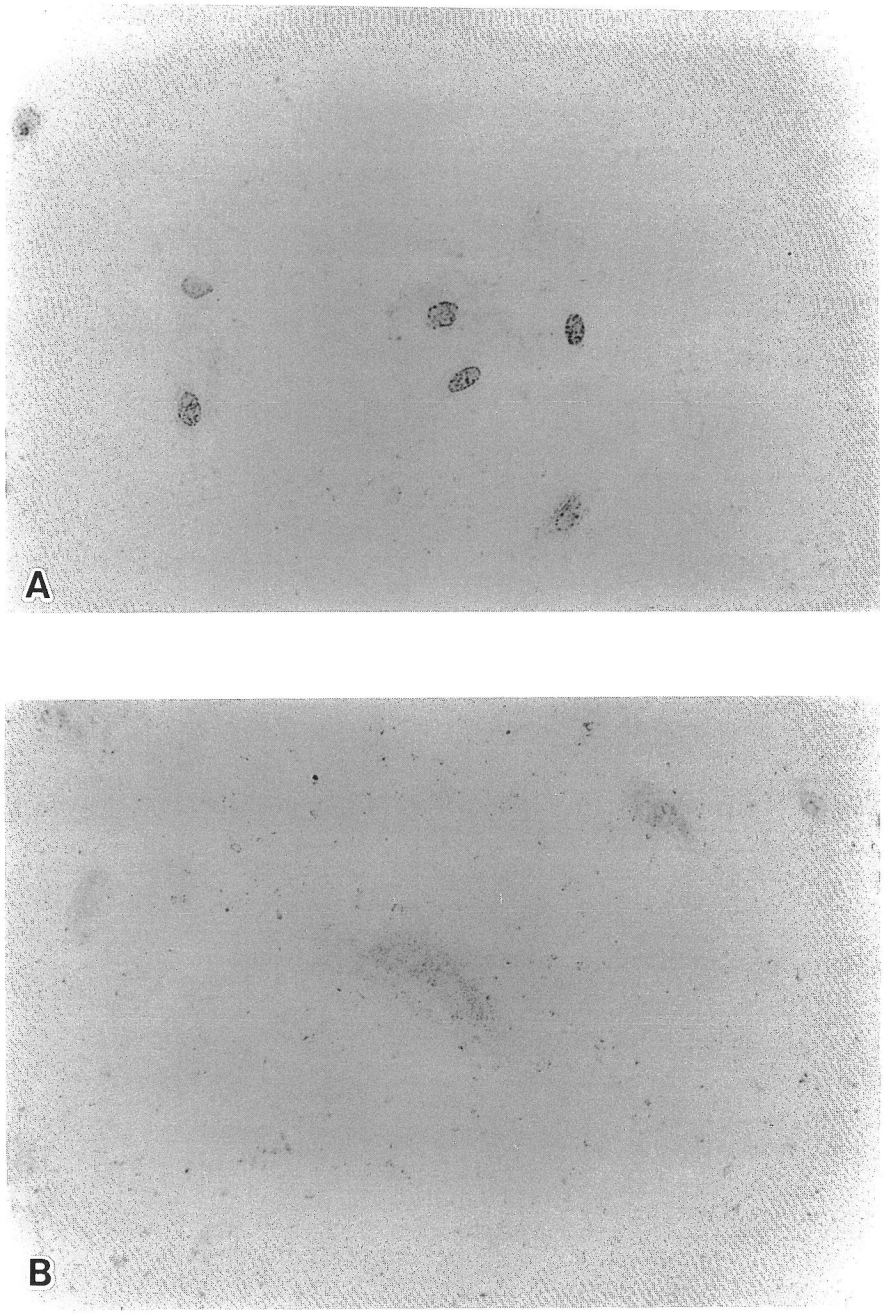
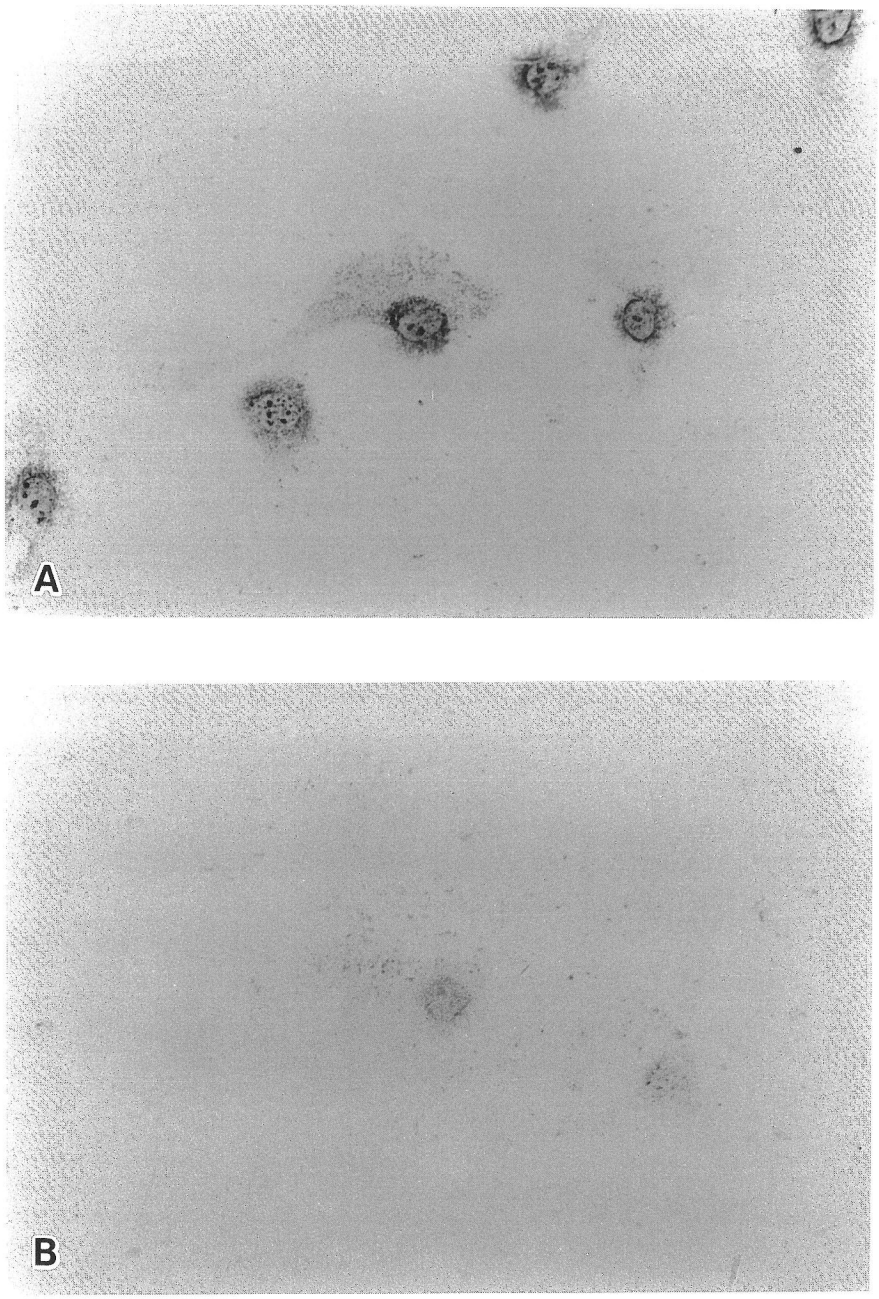


FIG. 4.



FIGS. 5A–B. *In situ* hybridization of TIG-1-20 cells with T-T dimerized synthetic oligodeoxynucleotides. ×600

A. Anti-sense strand synthetic probe. B. Sense strand synthetic probe.



FIGS. 6A–B. *In situ* hybridization of NIH/3T3 cells transfected with c-*yes*-1 gene. ×600
A. Anti-sense strand synthetic probe. B. Sense strand synthetic probe.

DISCUSSION

The usefulness of T-T dimer as a hapten marker for the non-radioactive synthetic oligodeoxynucleotide probe was demonstrated in this study. Our method has several advantages over other ligands used for non-radioactive probes (5-allylaminobiotin-labeled deoxyuridine triphosphate, acetylaminofluorene, and photobiotin) (4, 11, 21). In our method, there is only one required step for the preparation of the probe with T-T dimer, that is to UV irradiate the probe DNA, and this can be easily done in a conventional environment (17). We have also shown that synthetic oligodeoxynucleotides are useful in *in situ* hybridization by T-T dimerized cDNA probes.

One of the major advantages of the synthetic probes is that they can be easily synthesized using a DNA synthesizer and can be prepared in large quantity. Several thymines were added at 5′ and 3′ and because we thought that several thymines were required for efficient T-T dimerization by UV irradiation.

As rabbit anti-T-T antibody was not commercially available, we used anti-T-T antibody provided by Dr. Yoshida. However, we recently succeeded in preparing rabbit anti-T-T antibody by injecting T-T dimerized oligothymidylic acid, $d(pt)_{30}$ and methylated bovine serum albumin (BSA) subcutaneously into rabbits several times (13) (Manuscript in preparation). This antibody did not react with salmon sperm DNAs, yeast transfer RNA and cellular RNAs from K562 (human myelogenous leukemia). Thus, it is relatively easy to prepare rabbit polyclonal antibody against T-T dimers.

Our method is simple and reliable and is especially applicable for the localization of a particular oncogene mRNA. Nucleoli of TIG-1-20 and NIH/3T3 transfeced with c-*yes*-1 gene were *in situ* hybridized with an anti-sense strand syntetic probe. Further study will be required to clarify whether it is an actual signal or not. A lot of oncogene cDNA have been cloned and it is easy to determine the particular oncogene-specific region for chemical DNA synthesis by computer-assisted homology search of cloned cDNAs. We believe that our method will provide a useful means for analyzing oncogene expression.

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