

## Direct Observation of Specific Messenger RNA in a Single Living Cell under a Fluorescence Microscope

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**ABSTRACT** We observed the expression of human *c-fos* mRNA in a living transfected Cos7 cell under a fluorescence microscope by detecting hybrid formed with two fluorescently labeled oligodeoxynucleotides (oligoDNAs) and *c-fos* mRNA in the cytoplasm. Two fluorescent oligoDNAs were prepared, each labeled with a fluorescence molecule different from the other. When two oligoDNAs hybridized to an adjacent sequence on the target mRNA, the distance between the two fluorophores became very close and fluorescence resonance energy transfer (FRET) occurred, resulting in changes in fluorescence spectra. To find sequences of high accessibility of *c-fos* RNA to oligoDNAs, several sites that included loop structures on the simulated secondary structure were selected. Each site was divided into two halves, and the pair of fluorescent oligoDNAs complementary to the sequence was synthesized. Each site was examined for the efficiency of hybridization to *c-fos* RNA by measuring changes in fluorescence spectra when *c-fos* RNA was added to the pair of oligoDNAs in solution. A 40 mer specific site was found, and the pair of oligoDNAs for the site were microinjected into Cos7 cells that expressed *c-fos* mRNA. To block oligoDNAs from accumulating in the nucleus, oligoDNA was bound to a macromolecule (streptavidin) to prevent passage of nuclear pores. Hybridization of the pair of oligoDNAs to *c-fos* mRNA in the cytoplasm was detected in fluorescence images indicating FRET.

### INTRODUCTION

Localization, movements, and degradation of mRNA within cells are important factors in posttranscriptional genetic control (St. Johnson, 1995; Beelman and Parker, 1995). Direct observation of mRNA in living cells can provide much valuable information on the motility of mRNA in the cytoplasm (Arn and Macdonald, 1998). One approach to the visualizing and tracking of mRNA is to inject fluorescently labeled RNA synthesized *in vitro* from the corresponding full-length cDNA into cells (Ainger et al., 1993; Glotzer et al., 1997). Another approach is to tag mRNA binding proteins with fluorescent markers such as green fluorescent protein and observe the expressed fusion proteins in cells (Bertrand et al., 1998; Theurkauf and Hazelrigg, 1998).

One possible method of detecting specific mRNA is to introduce fluorescently labeled oligonucleotides, which have a sequence complementary to a specific site of the target mRNA, into cells to make them hybridize to the target mRNA in the cytoplasm and then observe the formed hybrid. To detect the formed hybrid in living cells, where nonhybridizing fluorescent oligonucleotides cannot be washed out, fluorescence has to be changed by hybridization. We therefore used fluorescence resonance energy transfer (FRET). Two kinds of fluorescently labeled oligonucleotides, each labeled with a different kind of fluorescence molecule, and which were complementary to an adjacent nucleic acid sequence, were prepared. When the two

fluorescent oligonucleotides hybridize to adjacent sequences on the target nucleic acids, the distance between two fluorophores on the formed hybrid becomes close and FRET occurs (Fig. 1; Cardullo et al., 1988; Mergny et al., 1994). FRET is an interaction between two kinds of fluorescence molecules (donor and acceptor) located within a distance less than  $\sim 8$  nm from each other. The excited-state energy of a donor molecule is transferred nonradiatively to an acceptor molecule, and occurrence of FRET results in a quenching of donor fluorescence and an enhancement of acceptor fluorescence intensity (Lakowicz, 1983). FRET has been widely used to detect associations of proteins, protein conformational changes, and hybridization between donor-labeled oligodeoxynucleotides and acceptor-labeled complementary oligodeoxynucleotides (Tsien et al., 1993; Miyawaki et al., 1997; Cardullo et al., 1988; Sixou et al., 1994), using the strong dependency (inverse of sixth power) of FRET efficiency on the distance between the donor and acceptor.

In this study, we attempted to detect *c-fos* mRNA in a living Cos7 cell under a fluorescence microscope, by introducing two fluorescently labeled oligonucleotides into the cytoplasm by microinjection, making them hybridize to *c-fos* mRNA, and taking images of FRET caused by hybrid formation. As a target mRNA to detect in living cells, we chose human *c-fos* mRNA. The *c-fos* gene encodes a DNA-binding protein that functions as a component of a mammalian transcription factor AP-1, that binds to its corresponding response element site and induces the expression of various genes concerning cell cycle regulation, differentiation, and other cellular events in a variety of cell types (Sheng and Greenberg, 1990; Curran and Morgan, 1995; Hughes and Drasgunow, 1995). The expression of *c-fos*, one

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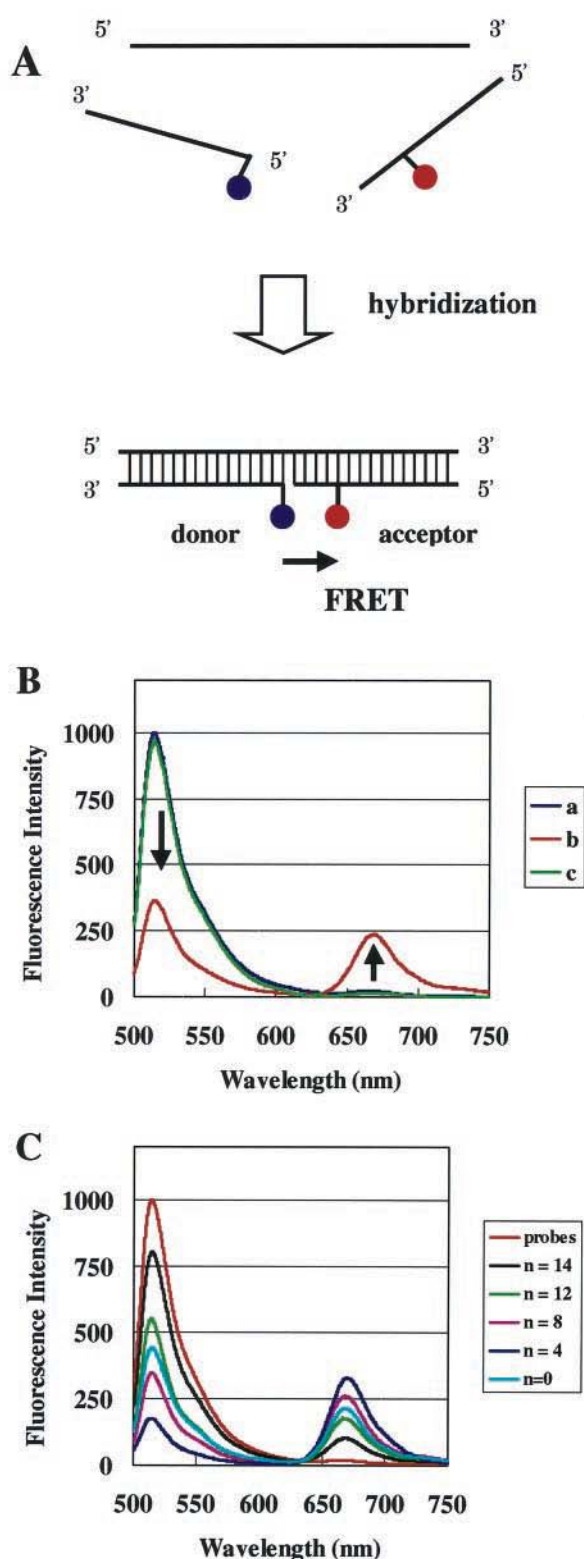


FIGURE 1 Detection by FRET of nucleic acids with a specific sequence. (A) Two fluorescently labeled oligonucleotides, one labeled with a donor fluorophore and another labeled with an acceptor fluorophore, hybridize to a target nucleic acid adjacent to each other. The distance between the donor and acceptor on the formed hybrid becomes very close and FRET occurs. (B) Changes in fluorescence spectra caused by hybrid formation. A com-

of the cellular immediate-early genes, is low in the absence of stimuli but is rapidly induced transiently to a higher level within several minutes to 1 h when various extracellular stimuli are received. Therefore, *c-fos* mRNA is an attractive target for testing whether our approach to observing specific mRNA and monitoring the expression levels and their temporal changes is effective. The method using two specific oligoDNA probes has an additional advantage of high specificity for detection of the target mRNA from among several thousand kinds of mRNAs.

To make oligonucleotides hybridize to the target mRNA efficiently, sites (sequences) on the mRNA should be selected, because mRNA has its own secondary structure and is associated with several kinds of proteins on the specific sites. We examined sequences for hybridization of oligonucleotides to human *c-fos* RNA by measuring changes in fluorescence spectra when the pair of fluorescently labeled oligoDNAs for each site was mixed with *c-fos* RNA in solution and found a 40 mer-specific site for hybridization with high efficiency. The oligoDNA probes for the sequence were microinjected into Cos7 cells that expressed *c-fos* mRNA. To avoid oligoDNAs accumulating in the nucleus, oligoDNAs were bound to a macromolecule, streptavidin, to prevent them from passing through nuclear pores, which made the oligoDNA probes localize and caused them to be stable in the cytoplasm for more than 30 min. FRET caused by hybrid formation was measured under a fluorescence microscope as ratio images of donor fluorescence intensity and acceptor fluorescence intensity with donor excitation. Hybridization of the oligoDNA probes to *c-fos* mRNA in the cytoplasm was detected.

## MATERIALS AND METHODS

### Oligonucleotides

Oligodeoxynucleotides (oligoDNAs), Bodipy493/503-labeled oligoDNAs, Bodipy493/503 and biotin-labeled oligoDNAs, and 40 mer RNA were obtained from Takara Shuzo (Ohtsu, Japan). Bodipy493/503 (Molecular Probes) was conjugated at the 5' end with TFAc-hexanolamined linker

bination of Bodipy493/503 and Cy5 was used as a donor and an acceptor. As a model experiment, 33 mer of a part of a multicloning site was used as a target sequence. A donor probe was a 16 mer DNA complementary to the 5' half of the target sequence and was labeled with Bodipy493/503 at the 5' end. An acceptor probe was a 17 mer DNA complementary to the 3' end of the target sequence and labeled with Cy5 at a position four nucleotides from the 3' end. Donor probes and acceptor probes were mixed at a molar ratio of 1:1 in  $1 \times$  SSC solution and used as a pair probe. (a) Spectrum of a pair probe. (b) Target 33 mer DNA was added to the pair probe solution at a molar ratio of 1:1. (c) Nontarget 33 mer DNA that had the same sequence as the pair probe was added to the pair probe solution at a molar ratio of 1:1. Excitation: 490 nm. (C) Fluorescence spectra of hybrids. In each spectrum, a different kind of acceptor probe, which was labeled with Cy5 at a  $[n]$  nucleotide position from the 3' end, was used (The  $[n]$  nucleotides separated two fluorophores on the formed hybrid.) Hybrids were prepared using HPLC.

(Perkin-Elmer Applied Biosystems). Biotin was conjugated at the 3' end as Biotin ON CPG (Clontech). These oligoDNAs were dissolved in diethyl pyrocarbonate (DEPC)-treated water.

For labeling oligoDNAs with Cy5, oligoDNAs containing Uni-Link AminoModifier (Clontech) at various intermediate positions of the nucleotide sequence were obtained from Takara Shuzo. In oligoDNAs conjugating biotin at the 5' end, Biotin amidite (Perkin-Elmer Applied Biosystems) was used. To label oligoDNA with Cy5, FluoroLink Cy5 Mono Reactive Dye (Amersham Pharmacia Biotech) was dissolved in 100  $\mu$ l of sterilized water and mixed with oligoDNA in 200  $\mu$ l of 0.5 M sodium bicarbonate buffer (pH 9.0) and made to react overnight. The reaction mixture was applied to a reverse-phase high-performance liquid chromatograph (HPLC), using a Capcell PAKC18 (6  $\times$  250 mm) column (Shiseido, Tokyo, Japan). The samples were eluted with a linear gradient of 15.5–33.5% CH<sub>3</sub>CN in 0.05 M triethylammonium acetate at a flow rate of 1 ml/min at 40°C. The peak fraction at 25–30 min was recovered and lyophilized (Cy5-conjugated oligoDNA). Before use, the lyophilized Cy5-conjugated oligoDNA was dissolved in DEPC-water, and the absorbance at 260 nm and at 649 nm was measured. The labeling yield (molar ratio of Cy5 to oligoDNA) was 0.9–1.0.

In the model experiments of Fig. 1, we used a part of a multicloning site as a target 33 mer sequence (Table 1). The sequences of the target DNA, donor probes (Bodipy493/503-labeled 16 mer DNA), and acceptor probes

(Cy5-labeled 17 mer DNA) are listed in Table 1. In the experiments for the detection of human *c-fos* mRNA, several target sites on *c-fos* mRNA were selected (see Results). These were 657–696, 206–245, 898–937, and 1659–1698 sites. Sequences of these sites, donor probes, and acceptor probes are shown in Table 1.

### Preparation of hybrid formed with a donor-labeled oligoDNA, an acceptor-labeled oligoDNA, and the target DNA

A pair consisting of 40 pmol of 16 mer Bodipy493/503-labeled DNA and 40 pmol of 17 mer Cy5-labeled DNA were mixed with 40 pmol of the target 33 mer DNA in 10  $\mu$ l of 10 mM Tris-HCl, 140 mM NaCl (pH 7.4) and incubated for 5 min at room temperature. The mixture solution was applied to an ion-exchange HPLC to separate hybrid and free fluorescent oligoDNAs. The HPLC was performed using a TSK-GEL DEAE (diethylaminoethyl)-NPR column (Tosoh, Tokyo, Japan) with a NaCl gradient in 20 mM Tris-HCl (pH 9.5) at 25°C. The NaCl concentration gradient was as follows: 0–2 min, 25–45%; 2–12 min, 45–55%; 12–13 min, 55–100%. The flow rate was 1 ml/min. Elution was monitored with an absorption of 260 nm and with a fluorescence intensity of 650 nm excited at 475 nm. In

**TABLE 1** Sequences of donor probes, acceptor probes, and targets

#### Model experiments of Fig. 1

target DNA	5'-GGGTAAATTGCGCGCT GTAATCATGGTCATAGC-3'
donor probe	3'-CCCAITTAACGCGCGA-(Bodipy493/503)-5'
acceptor probes	3'-CATTAGTACCAGTA(Cy5)CG-5', n=14 3'-CATTAGTACCAG(Cy5)ATCG-5', n=12 3'-CATTAGTACC(Cy5)GTATCG-5', n=10 3'-CATTAGTA(Cy5)CAGTATCG-5', n=8 3'-CATT(Cy5)GTACCAGTATCG-5', n=4 3'-(Cy5)-CATTAGTACCAGTATCG-5', n=0

#### Experiments for detection of *c-fos* mRNA

target sequence (657-696)	5'-----GCGGAGACAGACCAACUAGA AGAUGAGAAGUCUGCUUUGC-----3'
donor probe (15mer)	3'-CTGTCTGGTTGATCT-(Bodipy493/503)-5'
donor probe (20mer)	3'-CGCCTCTGTCTGGTTGATCT-(Bodipy493/503)-5'
donor probe for binding to streptavidin	3'-(Biotin)-CGCCTCTGTCTGGTTGATCT-(Bodipy493/503)-5'
donor probe containing oligonucleotide linker	3'-(Biotin)-CCCCCCCCCGCCTCTGTCTGGTTGATCT-(Bodipy493/503)-5'
acceptor probe (15mer)	3'-TCTA(Cy5)CTCTTCAGACG-5'
acceptor probe (20mer)	3'-TCTA(Cy5)CTCTTCAGACGAAACG-5'
acceptor probe for binding to streptavidin	3'-TCTA(Cy5)CTCTTCAGACGAAACG-(Biotin)-5'
acceptor probe containing oligonucleotide linker	3'-TCTA(Cy5)CTCTTCAGACGAAACGCCCCCCCCC-(Biotin)-5'
sense probe (donor)	5'-(Bodipy493/503)-AGATGAGAAGTCTGCTTTGCCCCCCCCC-(Biotin)-3'
sense probe (acceptor)	5'-Biotin-CCCCCCCCCGCGGAGACAGACCAAC(Cy5)TAGA-3'
target sequence (206-245)	5'-----UAACCGCCACGAUGAUGUUC UCGGGCAACGCAGACUACUU-----3'
donor probe	3'-ATTGGCGGTGCTACTACAAG-(Bodipy493/503)-5'
acceptor probe	3'-AGCC(Cy5)CGTTGCGTCTGATGAA-5'
target sequence (898-937)	5'-----CCAGAGGUUGCCACCCGGA GUCUGAGGAGGCCUUCACCC-----3'
donor probe	3'-GGTCTCCAACGGTGGGCCT-(Bodipy493/503)-5'
acceptor probe	3'-CAGA(Cy5)CTCCTCCGGAAGTGGG-5'
target sequence (1659-1698)	5'-----UCUCUUUCUUUAGUCCUU CUCAUAGCAUUAACUAAUCU-----3'
donor probe	3'-AGAGAAAGAGGAATCAGAA-(Bodipy493/503)
acceptor probe	3'-GAGT(Cy5)ATCGTAATTGATTAGA-5'

the chromatograph, two major peaks appeared. The peak that appeared at 5 min showed only 260-nm absorption and little fluorescence, and the peak at 7 min showed 260-nm absorption and strong fluorescence. The free probes were eluted at 5 min, and the hybrid was eluted at 7 min. The hybrid fractions were recovered, and fluorescence spectra were measured.

### Simulation of the secondary structure of *c-fos* RNA

A sequence of human *c-fos* cDNA was obtained from the DNasis database (Hitachi Softair Engineering), and polyA tail was added to the sequence. A simulation of the secondary structure was performed using DNasis software.

### Synthesis of *c-fos* RNA by in vitro transcription reactions

A pSPT plasmid containing human *c-fos* cDNA (pSPT-*c-fos*) was obtained from the Riken Gene Bank. The pSPT-*c-fos* was treated with *EcoRI* to cut off *c-fos* DNA (2.1 kb), and the *c-fos* DNA fragment was then inserted into the *EcoRI* site on pBluescriptII KS(+) (Stratagene, La Jolla, CA). The plasmid containing *c-fos* DNA (pBluescript-*c-fos*) was treated with *SmaI* to produce a linearized plasmid and used as a template for in vitro transcription reactions driven by T3 promoter to synthesize *c-fos* RNA. In vitro transcription reactions were performed with a T3 MEGAscript kit (Ambion, Austin, TX).

A linearized plasmid containing *Xenopus* elongation factor 1- $\alpha$  (Xelf1- $\alpha$ ) gene (TRIPLEscriptXef) was purchased from Ambion. The cutoff of  $\sim 1.8$  kb xelf1- $\alpha$  DNA, insertion into pBluescriptII KS(+), linearization of the plasmid, and in vitro transcription reactions for synthesis of xelf1- $\alpha$  RNA were performed in the same manner as for the *c-fos* RNA.

### Fractionation of oligoDNAs bound to *c-fos* RNA and free oligoDNAs by HPLC

When free oligoDNAs and oligoDNAs bound to *c-fos* RNA were fractionated, 20 pmol of 20 mer Bodipy493/503-labeled DNA probes or 20 pmol of 20 mer Cy5-labeled DNA probes were mixed with 20 pmol of *c-fos* RNA in  $1\times$  SSC solution (17 mM sodium citrate, 150 mM NaCl, pH 7.0) and were incubated for 20 min at room temperature. The mixture was applied to a TSK-GEL DEAE (diethylaminoethyl)-NPR column (Tosoh) with a NaCl gradient in 10 mM Tris-HCl (pH 9.5), 1 mM EDTA at 25°C. The NaCl gradient was 0.3–1.0 M for 10 min. Elution was monitored with an absorption of 260 nm and with a fluorescence intensity of 515 nm excited at 475 nm for Bodipy493/503-labeled oligoDNA probes or 667 nm excited at 650 nm for Cy5-labeled oligoDNA probes. In this HPLC condition, free oligoDNAs were eluted at 3.5–4 min and *c-fos* RNA at 6.9–7.1 min.

### Fluorescence spectra

Fluorescence spectra were measured using a Hitachi F-4500 fluorescence spectrophotometer. In the hybridization experiments of fluorescent oligoDNA probes to *c-fos* RNA, *c-fos* RNA was added to  $1\times 10^{-6}$  to  $1\times 10^{-7}$  M of fluorescent oligoDNAs in  $1\times$  SSC solution at room temperature unless otherwise indicated. Changes in fluorescence spectra caused by hybrid formation were completed within 5–10 min in the case of  $1\times 10^{-6}$  M and within 10–15 min in  $1\times 10^{-7}$  M. In the measurements at 37°C, the temperature was maintained with circulating warm water.

### Preparations and characterization of Cos7 cells that expressed *c-fos* mRNA

The pSPT-*c-fos* was treated with *EcoRI* to cut off *c-fos* DNA (2.1 kb), and the *c-fos* DNA fragment was inserted into the *EcoRI* site on pME18S expression vector driven by SR $\alpha$  promoter (pME18S-*c-fos*).

Cos7 cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in 5% CO<sub>2</sub> at 37°C. To express *c-fos* mRNA transiently in Cos7 cells, the cells were transfected with pME18S-*c-fos* by electroporation. 5  $\mu$ g of pME18S-*c-fos* was added to a cell suspension ( $1\times 10^7$  cells/ml), and a pulse voltage of 930 V was applied in a BioRad GenePulser II. The cell suspension was diluted with 20-fold DMEM medium and washed twice by centrifugation. The cells were suspended and plated to glass-bottomed dishes (P35G-0-14-C; Mat-Tek, Ashland, MA).

Expression of *c-fos* mRNA in transfected Cos7 cells was confirmed by dot blotting for total RNA extracted from the cells. At appropriate times after plating, the total RNA was extracted from transfected Cos7 cells with a QuickPrep Total RNA Extraction Kit (Pharmacia Biotech). The extracted RNA, together with *c-fos* RNA synthesized as mentioned above, were transferred onto a nylon membrane and fixed by illumination with UV light (Gene Linker; Biorad). The membrane was treated with digoxigenin (DIG)-labeled 2.1-kb RNA complementary to *c-fos* mRNA, followed by treatment with alkalinephosphatase-conjugated anti-DIG antibodies. The DIG-labeled 2.1-kb *c-fos* RNA probe was synthesized by in vitro transcription reactions driven by T7 promoter, using DIG RNA Labeling Mix (Boehringer Mannheim). After washing the nylon membrane with 0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl<sub>2</sub> (pH 9.7), the hybridized DIG-labeled RNA probes on the membrane were detected by coloration of 4-nitroblue tetrazolium catalyzed by alkalinephosphatase, using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim). The membrane was scanned with a densitometer, and quantities of cellular *c-fos* mRNA relative to the standard *c-fos* RNA were measured by weighing of the densitometer profiles.

Fluorescence in situ hybridization (FISH) was also performed to monitor the expression of *c-fos* mRNA. Digoxigenin (DIG)-labeled RNA probe complementary to *c-fos* mRNA was synthesized as described above. The RNA probes were treated in an alkaline condition to produce 100–150-bp fragments. These fragment mixtures were used as probes for hybridization to *c-fos* mRNA. Cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) (pH 7.4) for 15 min at room temperature and treated with the DIG-labeled RNA overnight at 45°C, followed by the treatment with fluorescein isothiocyanate-conjugated anti-DIG antibodies. FISH images of the transfected cells 1 day after plating showed that *c-fos* mRNA was expressed in more than 90% of the transfected cells.

### Binding of oligoDNA probes to streptavidin

To bind Bodipy493/503-labeled oligoDNA to streptavidin, 100  $\mu$ g of streptavidin (Molecular Probes) was dissolved in 10  $\mu$ l of PBS and mixed with 4.1  $\mu$ l of  $1\times 10^{-4}$  M Bodipy493/503-oligoDNA in DEPC-water (the molar ratio of streptavidin to oligoDNA was 4:1). Binding of Cy5-labeled oligoDNA to streptavidin was also performed. Two kinds of fluorescently oligoDNAs premixed with streptavidin were mixed at a molar ratio of 1:1 and used for injection experiments.

### Fluorescence microscopy and microinjection

Fluorescence images of cells were observed with an inverted fluorescence microscope (Axiovert 135TV; Carl Zeiss). Image acquisition and analysis were performed with a cooled CCD camera (Hamamatsu C4880 and Argus-50; Hamamatsu). Cos7 cells on glass-bottomed dishes were incubated on the microscope stage by circulation of Hanks' balanced salt solution at 37°C during experiments. Microinjection was performed using



a micromanipulator (model 5171; Eppendorf) and a transjector (model 5246 Plus/Basic; Eppendorf).

For each measurement, a set of three kinds of images was obtained. A fluorescence image of Bodipy493/503 with an excitation of Bodipy493/503 (DD image) was taken, using a filter set of BP450–490, FT510, and BP515–565 (Carl Zeiss), for the excitation filter, dichroic mirror, and barrier filter, respectively. A fluorescence image of Cy5 with an excitation of Bodipy493/503 (DA image) was taken using a filter set of BP450–490, FT510, and BP660–710. A fluorescence image of Cy5 with an excitation of Cy5 (AA image) was taken using a filter set of BP575–625, FT645, and BP660–710. To minimize photobleaching during experiments, the excitation light (100-W Hg lamp) was attenuated using a 3% ND filter for illuminating cells. The typical acquisition times were 4 s for DD and DA images and 1 s for AA images. Under these conditions, photobleaching of both Bodipy493/503 and Cy5 fluorescence within cells was negligible during several image acquisitions. The obtained images were provided to perform quantitative analysis after background subtractions.

## RESULTS

### Detection by FRET of nucleic acids with a specific sequence

Two oligoDNAs were used as a pair of probes to detect nucleic acids that had a specific sequence (Fig. 1 *A*). One probe was labeled with a fluorescence molecule and had a sequence hybridizing to a specific site of the target nucleic acids. The other was labeled with another kind of fluorescence molecule and had a sequence hybridizing to one adjacent site of the target nucleic acid where the first probe hybridized. When the two probes hybridized to a target nucleic acid adjacent to each other, the distance between two fluorescence molecules became very close on the formed hybrid, which made FRET occur, resulting in changes in the fluorescence spectrum (Cardullo et al., 1988). Fig. 1 *B* shows a change in fluorescence spectra caused by the hybrid formation in which Bodipy493/503 was used as a donor fluorophore and Cy5 as an acceptor fluorophore. The fluorescence spectrum of the mixture of donor and acceptor probes at a molar ratio of 1:1 showed a strong fluorescence of Bodipy493/503, which peaked at 514 nm, and a weak fluorescence of Cy5, which peaked at 670 nm (*a*). When a target 33 mer DNA was added to the probe mixture, the fluorescence intensity of Bodipy493/503 decreased and that of the Cy5 increased (*b*). When a nontarget 33 mer DNA was added to the probe mixture as a control experiment, no change in the fluorescence spectrum was observed (*c*). Moreover, no change in spectra occurred when the 33 mer target DNA was added to the donor probe solution or the acceptor probe solution. These results show that the hybrid formation of a Bodipy493/503-labeled oligoDNA and a Cy5-labeled oligoDNA with a target nucleic acid can be detected by measuring FRET as changes in fluorescence spectra. We chose a combination of Bodipy493/503 and Cy5 as a donor and an acceptor. Although this combination has a relatively small overlap between the fluorescence spectra of Bodipy493/503 and the absorption spectra of Cy5 (a Förster distance of 42 Å), the

distance between two fluorophores on the formed hybrid becomes very small, which makes FRET occur efficiently (Fig. 1, *B* and *C*). This combination has several advantages for FRET measurements, such as a good separation between Bodipy493/503 fluorescence and Cy5 fluorescence and less excitation of Cy5 at the excitation wavelength for Bodipy493/503.

To obtain the fluorescence spectrum of the hybrid, the mixture of two fluorescent oligoDNA probes and the target 33 mer DNA was applied to HPLC and fractionated into hybrid and free oligoDNAs. Fig. 1 *C* shows fluorescence spectra of the hybrids. In these experiments, different kinds of acceptor probes, each of which was labeled with Cy5 at a different position (*n*) from the 3' end on the 17 mer probe, were prepared. One of these acceptor probes was used in combination with the donor probe labeled with Bodipy493/503 at the 5' end. In these combinations, the numbers of nucleotides (*n*) that separated two fluorophores on the double strand of the formed hybrid were different. These were *n* = 0, 4, 8, 12, and 14. (On the formed hybrid, the 3' end of an acceptor probe faced the 5' end of the donor probe where Bodipy493/503 was conjugated (see Fig. 1 *A*). When the acceptor probe labeled with Cy5 at the [*n*] position from the 3' end was used, [*n*] nucleotides separated two fluorophores on the formed hybrid.)

As the number of nucleotides separating two fluorophores (*n*) decreased from 14 to 4, changes in fluorescence spectra became larger. In each spectrum, FRET efficiency (*E*) was determined from quenching of Bodipy493/503 fluorescence (Table 2). FRET efficiency was largely depen-

TABLE 2 FRET efficiency on hybrids

<i>n</i>	<i>E</i>	<i>r</i> (Å)	Expected distance between two nucleotides (Å)
4	0.83	32.2	26.2
8	0.65	37.9	31.2
10	0.50	42.0	37.9
12	0.45	43.4	47.0
14	0.20	52.9	54.8
0	0.56	ND	7.1

An analysis of fluorescence spectra of hybrids (Fig. 1 *C*) was summarized. *n*, Number of nucleotides separating two fluorophores on the hybrid. *E*, Efficiency of FRET determined from quenching of Bodipy493/503 fluorescence in the spectrum. *r*, Average distance between Bodipy493/503 and Cy5 on the hybrid, estimated according to  $E = 1/(1 + (r/R_0)^6)$ . A Förster distance (*R*<sub>0</sub>) of 42 Å was used for a combination of Bodipy493/503 and Cy5 (see text). Expected distances between two fluorophore-conjugated nucleotides on the hybrid were worked out, assuming a B-helix where the distance between nucleotides was 3.4 Å and the diameter of the helix was 20 Å. Bodipy493/503 was conjugated to a nucleotide via TFAC-hexanolaminelinker, and Cy5 was conjugated to a Uni-Link AminoModifier that was incorporated into the oligonucleotide. The extended lengths of these spacers linking fluorophore and nucleotide are ~8 Å and ~6 Å, respectively. Accordingly, the distances between two fluorophores determined from the spectra represent those that varied with the configuration of these spacers.

dent on the numbers of nucleotides separating two fluorophores on the formed hybrid and was enhanced from 20% in the case of  $n = 14$  to 83% in  $n = 4$ . The average distance ( $r$ ) between Bodipy493/503 and Cy5 on the hybrid was estimated from FRET efficiency according to  $E = 1/(1 + (r/R_0)^6)$  (Table 2). Here, a Förster distance ( $R_0$ ) of 42 Å was used, which was determined from an overlap integral of  $3.87 \times 10^{-14} \text{ M}^{-1} \text{ cm}^3$ , a fluorescence quantum yield of Bodipy493/503 of 0.90, and an assumption of free rotational mobility of fluorophores ( $\kappa^2 = 2/3$ ). Table 2 also lists the expected distances between two fluorophore-conjugated nucleotides when it was assumed that the formed hybrids were B-helix, where the distance between base pairs was 3.4 Å and the diameter of helix was 20 Å. Within a range from  $n = 14$  to  $n = 4$ , the distances between two fluorophores estimated from FRET efficiency were well correlated with the expected distances between two fluorophore-conjugated nucleotides. These results show that FRET occurred quantitatively in response to the numbers of nucleotides separating the donor and acceptor on the double-strand hybrids formed.

In the case of  $n = 0$ , FRET efficiency was 56%, which was lower than  $n = 4$  and  $n = 8$ . The distance to an adjacent nucleotide ( $n = 0$ ) on the hybrid was expected to be 7.1 Å (Table 2), which is too short a separation between two fluorophores for them to be stable. Therefore, the two fluorophores must be located further from each other via their linkers and would have some specific interactions with other components of the helical structure to be stable. In these cases, the assumption of free rotation of the fluorophores ( $\kappa^2 = 2/3$ ) is not applicable. The orientation factor ( $\kappa^2$ ) when  $n = 0$  may have a smaller value than other cases of separation distances of several bases. In addition, the interaction of Bodipy493/503 with other components of the helical structure may decrease its fluorescence quantum yield. These factors should result in a lower FRET efficiency. We used oligonucleotides labeled with Cy5 four nucleotides from the 3' end as acceptor probes in the experiments below.

### Determination of sequences for hybridization of oligoDNAs to *c-fos* RNA

Human *c-fos* mRNA was used as an example for detecting a specific mRNA in living cells under a fluorescence microscope. The secondary structure of *c-fos* mRNA was simulated. In the predicted structure, some specific sites of 40 mer that contained a loop structure were selected. These sites were 206–245, 657–696, 898–937, and 1659–1698. Each specific 40 mer site was divided into two 20 mer parts, and a 20 mer DNA with a sequence complementary to each part was prepared. One 20 mer DNA was labeled with Bodipy493/503 and another with Cy5, and two 20 mer DNAs were mixed at a molar ratio of 1:1 and used as a pair of probes. The pair probe was mixed with *c-fos* RNA

synthesized by in vitro transcription reactions in solution, and the fluorescence spectra were measured. When the pair probe with a sequence complementary to the 657–696 site of *c-fos* mRNA was used, the addition of *c-fos* RNA caused quenching of Bodipy493/503 fluorescence and enhancement of Cy5 fluorescence intensity (Fig. 2 *A*). The addition of more *c-fos* RNA caused larger changes in the spectra. No change occurred when xelf1RNA was added as a control experiment. The ratio of fluorescence intensity at 670 nm and at 514 nm of the spectrum when *c-fos* RNA was added to the probe solution at a molar ratio of 1:1 was 0.54. Fig. 2 *C* shows a calibration curve, which indicates a relation between ratios of fluorescence intensity in the spectra and molar ratios of pair probes hybridizing to the target nucleic acid. The value 0.54 indicates that ~70% of the pair probe hybridized to *c-fos* RNA. When pair probes for other sites of *c-fos* mRNA were examined, smaller changes in fluorescence spectra were observed with the addition of *c-fos* RNA to the probes (Fig. 2 *B* shows one example using a pair probe for the 898–937 site). In these sites, less than 30% of the pair probes hybridized to *c-fos* RNA (Fig. 2 *C*). To confirm that the probes for the 657–676 site and for the 677–696 site hybridize to *c-fos* RNA, one of the pair probes was mixed with *c-fos* RNA at a molar ratio of 1:1 in solution at room temperature, and the mixture was fractionated to probes bound to *c-fos* RNA and free probes in HPLC. Elution patterns were monitored with fluorescence intensity and with absorption at 260 nm. In the case of the Bodipy493/503-labeled probe for the 657–676 site, a strong fluorescence peak appeared at the position of *c-fos* RNA as well as the positions of free probes, and the ratio of probes coeluted with *c-fos* RNA was 76%. Also in the case of Cy5-labeled probe for the 677–696 site, almost the same result was obtained and the ratio of probes coeluted with *c-fos* RNA was 43%. These results show that both the donor probe for the 657–676 site and the acceptor probe for the 677–696 site hybridize to *c-fos* RNA, and the hybrid formation of both probes with *c-fos* RNA causes FRET to occur. In pair probes for other sites, hybridization assay by HPLC showed that at least one probe of each pair had a lower hybridization efficiency. Therefore, we selected the 657–696 site for hybridization of probes to *c-fos* mRNA. In most reported antisense studies for rat *c-fos* gene, target sites were around the initiation site of translation (Machwate et al., 1995; Szabo et al., 1996; Baille et al., 1997). The 657–696 site we found is far from the translation start site and within a coding region.

### Length of oligoDNA probes for use at 37°C

We examined effects of the length of oligoDNA probes on hybridization efficiency at 37°C. A pair of 20 mer probes (donor probe for the 657–676 site and acceptor probe for the 677–696 site) and a pair of 15 mer probes (donor probe for 662–676 and acceptor probe for 677–691) were used.

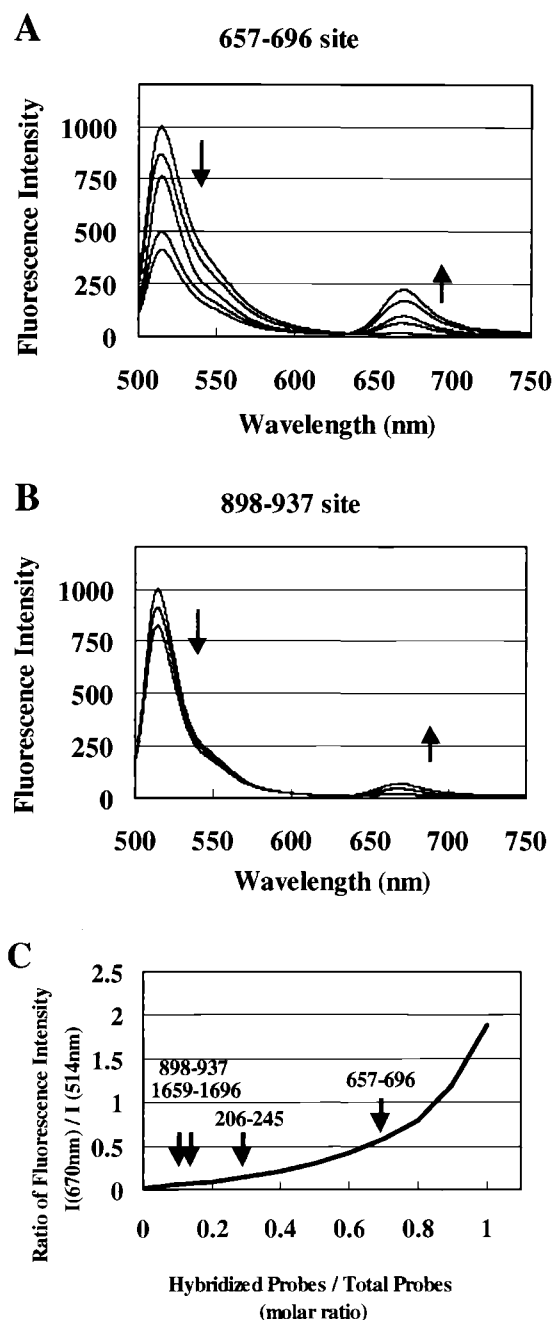


FIGURE 2 Fluorescence spectral changes by hybrid formation of the pair oligoDNA probes with *c-fos* RNA. (A) Donor probes for the 657–676 site and acceptor probes for the 677–696 site were mixed at a molar ratio of 1:1 in  $1\times$  SSC solution, and the fluorescence spectrum was measured. *c-fos* RNA synthesized by in vitro transcription reactions was added. Molar ratios of *c-fos* RNA to probes were 0.1, 0.2, 0.5, and 1.0. (B) Another pair probe, donor probes for the 898–917 site and acceptor probes for the 918–937 site, was used. Fluorescence spectra of the pair probe and mixtures of probes and *c-fos* RNA at a molar ratio of 0.5 and 1.0, respectively, are shown. (C) A calibration curve indicating a relation between ratios of fluorescence intensity in spectra and molar ratios of hybridizing probes. Fluorescence spectra at various molar ratios of hybridizing probes and free probes were made by linear combinations of fluorescence spectrum of the hybrid ( $[n = 4]$  of Fig. 1 C) and that of the pair probe ( $[probes]$  of Fig. 1 C).

Fig. 3 shows time courses of fluorescence spectral changes with the addition of *c-fos* RNA to  $1 \times 10^{-6}$  M of 15 mer or 20 mer pair probe solution at a molar ratio of 1:1 at room temperature or at  $37^\circ\text{C}$ . Hybridization was completed within 5–15 min in all cases. Changes in fluorescence spectra were larger when 20 mer probes were used rather than 15 mer probes. As mentioned above, fluorescence intensity ratios indicate fractions of oligoDNA probes hybridizing to the target RNA (Fig. 2 C). These results show that the fraction of hybridizing probes was larger when 20 mer probes were used rather than 15 mer probes. When the temperature increased from room temperature to  $37^\circ\text{C}$ , the fraction of hybridizing probes decreased for both 20 mer and 15 mer probes. The decrease was more extensive for 15 mer probes. About 58% of hybridizing fraction at room temperature decreased to a 34% fraction at  $37^\circ\text{C}$  for 15 mer probes, while 65% of the hybridizing fraction at room temperature decreased by only 60% at  $37^\circ\text{C}$  for 20 mer probes (Figs. 3 and 2 C). These results show that a 20 mer length is preferable when oligoDNAs are used as hybridization probes in living mammalian cells.

### Distribution of oligoDNA probes in living Cos7 cells

When Bodipy493/503-labeled 20 mer DNA probes were microinjected into the cytoplasm of Cos7 cells, the probes accumulated in the nucleus within 5 min (Fig. 4 B a). The same result was observed when Cy5-labeled 20 mer DNA probes were microinjected (Fig. 4 B b). Accumulation of injected deoxyoligonucleotides and deoxysulfonateoligonucleotides in the nucleus was previously reported for various types of cells (Leonetti et al., 1991; Fisher et al., 1993). 20 mer DNA would freely pass through nuclear pores from the cytoplasm into the nucleus, but it would not pass through nuclear pores from the nucleus to the cytoplasm or bind to nuclear structures (Clarenc et al., 1993; Zamecnik et al., 1994). When fluorescein-isothiocyanate-labeled dextran was injected into the cytoplasm, dextran with a molecular mass of less than 70 kDa was distributed throughout the nucleus and the cytoplasm, but dextran with a molecular mass of more than 70 kDa was localized in the cytoplasm (data not shown). Then, to avoid oligoDNAs accumulating in the nucleus, oligoDNA was bound to a macromolecule, streptavidin (60 kDa), via biotin, which was at one end of the oligoDNA (Fig. 4 A). Biotin-labeled oligoDNA probes premixed with streptavidin were injected into Cos7 cells.

Ratio of fluorescence intensity at 670 nm and at 514 nm for each spectrum was plotted against the molar ratio of hybridized probes. To evaluate the hybridization efficiency of the pair probe for each site on *c-fos* RNA, the ratio of fluorescence intensity ( $I_{670\text{nm}}/I_{514\text{nm}}$ ) in the spectrum of the mixture of the pair probe and *c-fos* RNA at a molar ratio of 1:1 was estimated. These values are indicated by arrows.

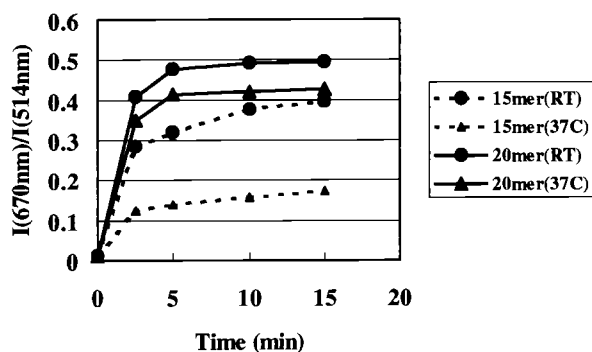


FIGURE 3 Effect of oligoDNA length on hybridization efficiency. *c-fos* RNA was added to  $1 \times 10^{-6}$  M of a pair of oligoDNA probes at a molar ratio of 1:1 at room temperature or at 37°C, and the fluorescence spectra were measured. The ratio of the fluorescence intensity at 670 nm to that at 514 nm in the spectra were plotted against the times after the addition of *c-fos* RNA. The 15 mer probes were a pair: the donor probe for the 662–676 site and the acceptor probe for 677–691 site; the 20 mer probes were a pair: the donor probe for the 657–676 site and acceptor probe for the 677–696 site.

The probes were localized and distributed diffusely in the cytoplasm, and the distribution did not change over the more than 30 min of incubation on the microscope stage (Fig. 4 B, c and d).

A pair of streptavidin-bound oligoDNA probes, which were donor probes bound to streptavidin at the 3' end and acceptor probes bound to streptavidin at the 5' end, were mixed with *c-fos* RNA in solution. Changes in fluorescence spectra were observed to be the same as when using a pair of donor and acceptor probes that were not bound to streptavidin were used, although the changes were somewhat smaller (Fig. 4 C). These results show that hybrid formation of the streptavidin-bound pair probe with *c-fos* RNA occurred in solution. Another form of a pair of streptavidin-bound oligoDNA probes, in which the donor and acceptor probes were bound to streptavidin at the 3' end, was also examined. In this case, changes in fluorescence spectra were smaller than those when the above pair of streptavidin-bound oligoDNA probes was used (data not shown), which shows that the probes hybridized to *c-fos* RNA with lower efficiency. The binding of macromolecules such as streptavidin to oligonucleotides would partly interfere with the hybridization of oligonucleotides to the target RNA in some cases. In the experiments below, a pair of streptavidin-bound probes were used, which were donor probes bound to streptavidin at the 3' end and acceptor probes bound to streptavidin at the 5' end.

### Streptavidin-bound oligoDNA probes hybridize to the target RNA in the cytoplasm of living Cos7 cells

A pair of streptavidin-bound donor and acceptor probes were microinjected into the cytoplasm of Cos7 cells, and

fluorescence images were taken. About  $10^5$  molecules of probes were injected into a cell. Donor images (DD) and acceptor images (AA) showed that both donor and acceptor probes were distributed diffusely in the cytoplasm. In DA images, little fluorescence was observed in the cytoplasm. FRET can be measured as the ratio of fluorescence intensity in the DD image to that in the DA image (DA/DD) under a fluorescence microscope. Fig. 5D shows DA/DD values of mixed solutions in various molar ratios of the pair probe and the target 40 mer RNA under the microscope set-up we used in this study. As the molar ratios of target RNA to probes increased, DA/DD values increased from 0.08 to 2.0. Values on the x axis (molar ratios of target RNA to probes) in Fig. 5 D indicate fractions of the probes participating in the hybrid formation with the target RNA. For the probe solution, DA/DD was 0.08. The DA/DD value of hybrid was about 2.0.

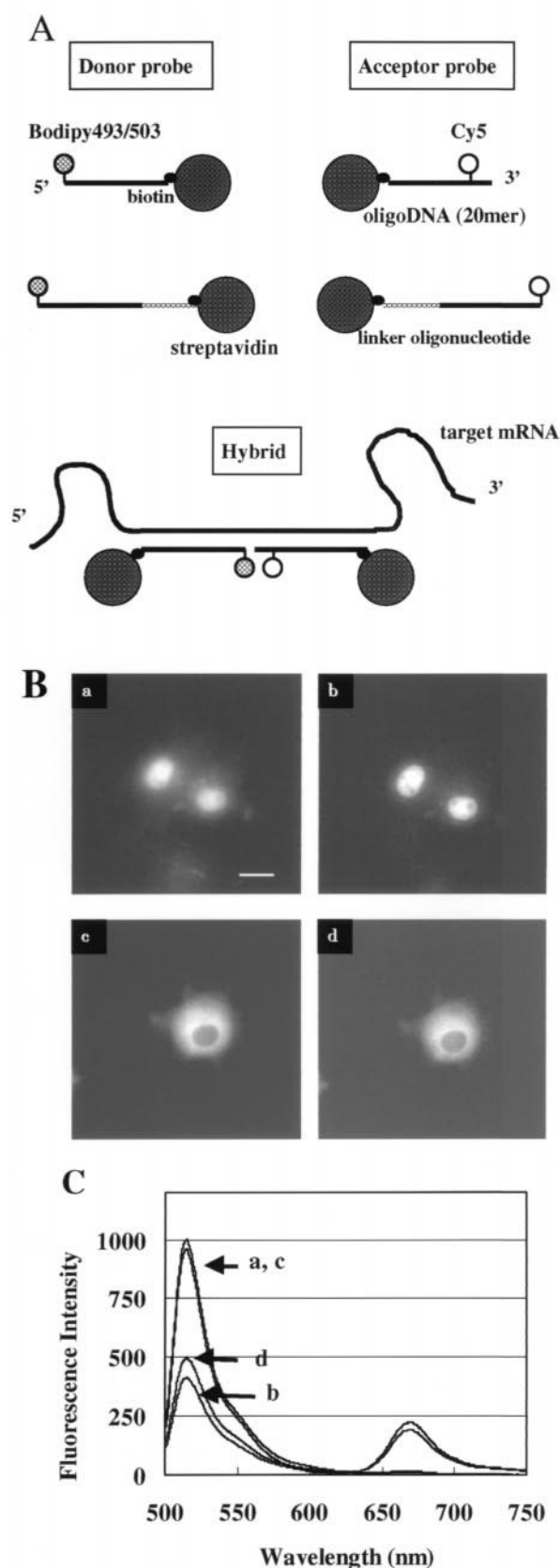
In the probe-injected Cos7 cells, the DA/DD value of the cytoplasm was  $\sim 0.08$ , the same value as that for the probe solution for injection, which showed that no FRET occurred in the cytoplasm. After 10 min of incubation, target 40 mer RNA that had the same sequence as the 657–696 site of *c-fos* mRNA was injected into the cytoplasm of the probe-injected cells. Fig. 5 A shows changes in fluorescence images after injection of the target RNA. The fluorescence intensity of the cytoplasm in the DD image decreased and that in the DA image increased. Ratio values DA/DD increased up to 1.5 (Fig. 5 C). The value of 1.5 means most probes in the cytoplasm hybridized to the target RNA. As a control experiment, 40 mer RNA that had a sequence complementary to the 657–696 site of *c-fos* mRNA (the same sequence as the pair probe) was injected into the cytoplasm. No change in fluorescence images was observed (Fig. 5, C and D). These results show that streptavidin-bound probes introduced into the cytoplasm can hybridize rapidly to the target RNA in the cytoplasm and the hybrid formation can be detected as changes in fluorescence images.

### Imaging of *c-fos* mRNA in a living Cos7 cell

Cos7 cells that expressed *c-fos* mRNA were prepared. An expression vector pME18S containing *c-fos* cDNA was introduced into Cos7 cells by electroporation. Dot blotting for total RNA extracted from the transfected cells showed that expression of *c-fos* mRNA continued for up to 3 days at high levels of more than  $10^4$  molecules per cell (see below). FISH images at 1 day after transfection showed that more than 90% of the cells expressed *c-fos* mRNA.

A pair of streptavidin-bound donor and acceptor probes was injected into the cytoplasm of transfected Cos7 cells. For 30 min after injection, DA/DD values of cytoplasm were low ( $\sim 0.1$ ), and fluorescence images did not change, which shows that no hybrid formation occurred in the cytoplasm. One interpretation of this result is that streptavidin obstructs hybridization of oligoDNA probes to *c-fos*





mRNA in the cytoplasm when intracellular proteins are bound to the mRNA. We introduced linker oligodeoxynucleotides as a spacer between 20 mer nucleotides for hybridization and biotin for binding to streptavidin (Fig. 4 A). The spacer made a spatial separation of the nucleotide for hybridization from streptavidin. For example, 10 cytosines were added to the 3' end of 20 mer oligoDNA as a linker (spacer) in a donor probe, and 10 cytosines were added to the 5' end of oligoDNA as a linker (spacer) in an acceptor probe. When these two linker-contained streptavidin-bound oligoDNA probes were mixed with *c-fos* RNA in solution, the same fluorescence spectral changes were observed as when the streptavidin-bound oligoDNA probes without linker oligodeoxynucleotides were used (data not shown).

Fig. 6 A shows an example of fluorescence images of the cells that were transfected with the expression vector containing the *c-fos* gene when the linker-added probes were injected. About  $10^5$  probe molecules were injected. Fluorescence images (DD, DA, and AA images) and a phase-contrast image (Ph) at 5 min after injection are shown. In the AA image, fluorescence was observed throughout the cytoplasm and strongly in some regions near the nucleus. Because AA images directly show the distribution of Cy5, the image shows that the acceptor probes were diffusely distributed throughout the cytoplasm and were also present in higher concentrations in some regions near the nucleus. DD images also show the distribution of Bodipy493/503, although higher concentrations of donor probes are not ob-

**FIGURE 4** Characterization of fluorescently labeled oligoDNA probes used in living cells. (A) Fluorescently labeled oligoDNA probes used in this study. Streptavidin-bound oligoDNA probes, streptavidin-bound oligoDNA probes containing linker oligodeoxynucleotides, and a hybrid formed with a pair probe and mRNA are described. Although streptavidin binds four biotins per molecule, oligoDNA was mixed with streptavidin at a molar ratio of 1:4 in the present experiments, so that most of the streptavidin molecules bind to one or no oligoDNA. (B) Distribution of oligoDNA probes in living Cos7 cells. OligoDNA probes were microinjected into the cytoplasm of Cos7 cells. (a) Bodipy493/503-labeled 20 mer DNA for the 657–676 site. (b) Cy5-labeled 20 mer DNA for the 677–696 site. (c) The 20 mer DNA labeled with Bodipy493/503 at the 5' end and with biotin at the 3' end, premixed with streptavidin. (d) The 20 mer DNA labeled with Cy5 four nucleotides from the 3' end and with biotin at the 5' end, premixed with streptavidin. Fluorescence images of Bodipy493/503 were taken using a filter set for DD images (see Materials and Methods) in a and c, and fluorescence images of Cy5 were taken using a filter set for AA images in b and d. Fluorescence images were shown at 5 min after injection in a and b and at 30 min after injection in c and d. Bar: 20  $\mu$ m. (C) Changes in fluorescence spectra caused by hybrid formation of streptavidin-bound oligoDNA probes with *c-fos* RNA in solution. (a) Mixture of Bodipy493/503-labeled oligoDNA for the 657–676 site (donor probe) and Cy5-labeled oligoDNA for the 677–696 site (acceptor probe) in  $1\times$  SSC. (b) *c-fos* RNA was added to the probe mixture at a molar ratio of 1:1. (c) Mixture of the streptavidin-bound donor probes and the streptavidin-bound acceptor probes. (d) *c-fos* RNA was added to the streptavidin-bound probe mixture at a molar ratio of 1:1. In c and d, the donor probe was labeled with biotin at the 3' end and the acceptor probe at the 5' end.

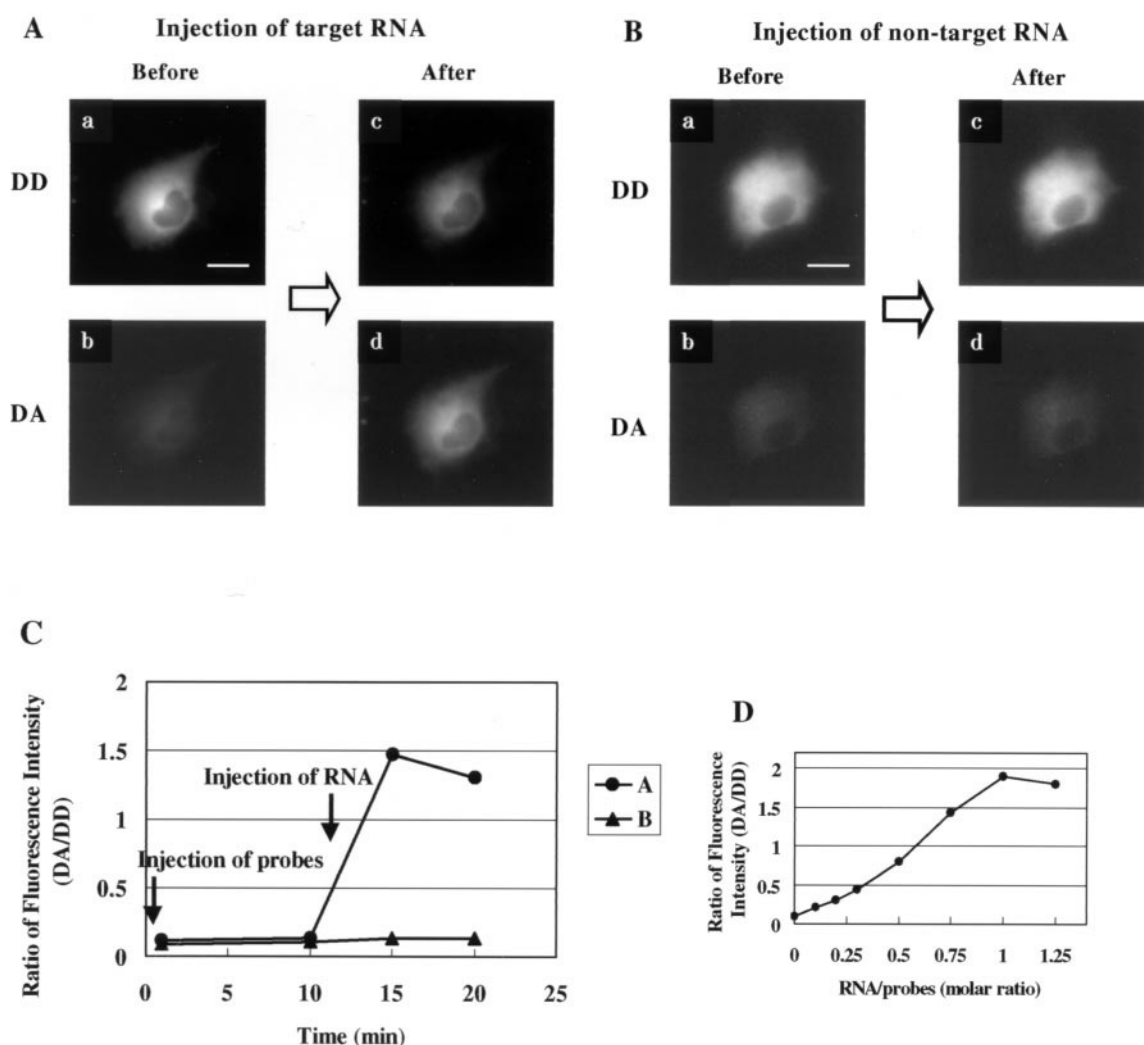
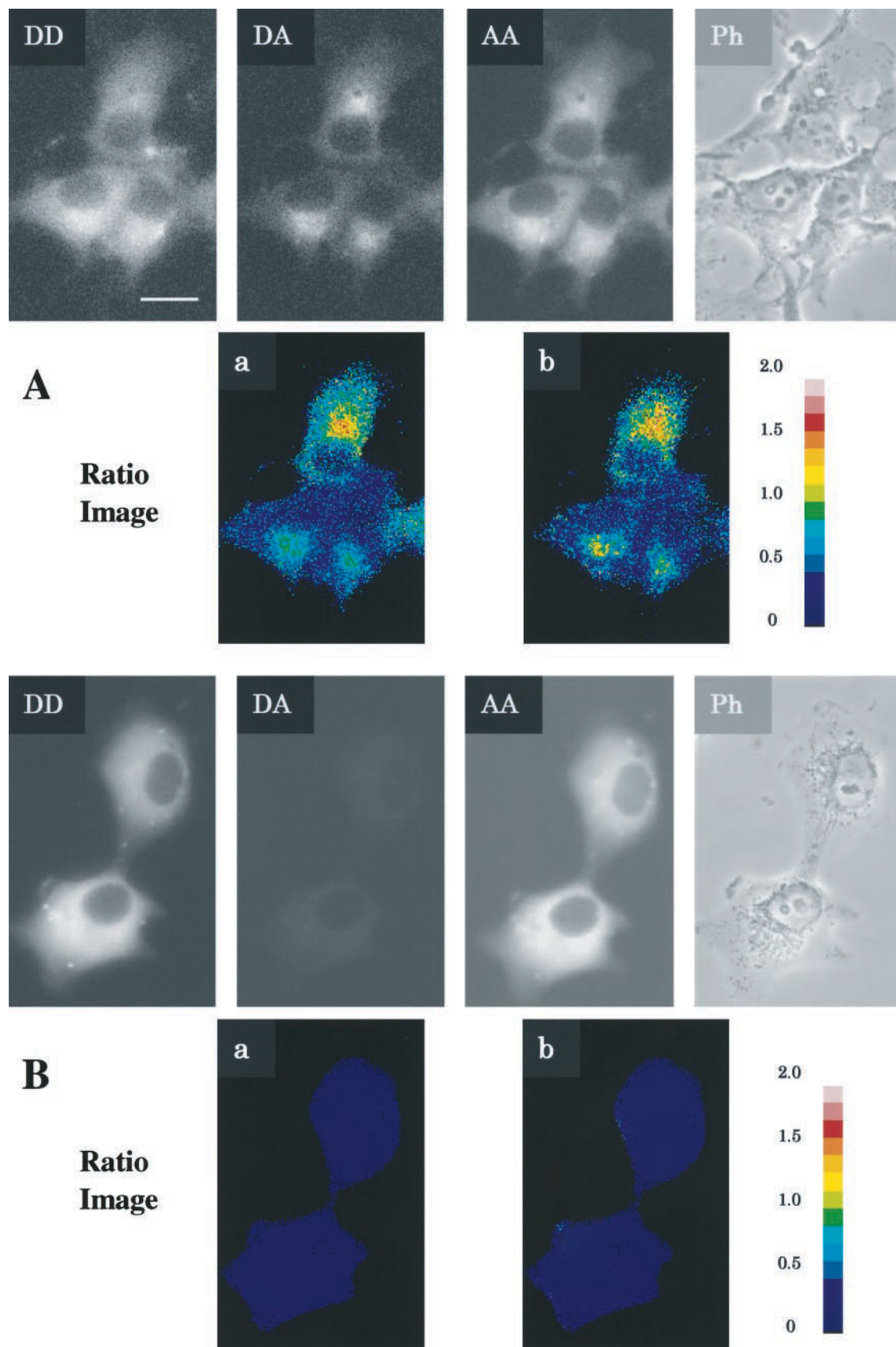


FIGURE 5 Changes in fluorescence images of Cos7 cells caused by hybrid formation in the cytoplasm. (A)  $1 \times 10^{-5}$  M of the pair probe, which was a mixture of donor probes premixed with streptavidin and acceptor probes premixed with streptavidin, was microinjected into the cytoplasm of a Cos7 cell, and fluorescence images were obtained. (a) DD image and (b) DA image 10 min after probe injection. Then  $1 \times 10^{-5}$  M of target 40 mer RNA was injected into the cytoplasm of the probe-injected cell. (c) DD image. (d) DA image. Bar: 20  $\mu$ m. (B)  $1 \times 10^{-5}$  M of the pair probe was microinjected into the cytoplasm of a Cos7 cell, as in the experiment in A. (a) DD image and (b) DA image 10 min after probe injection. Then  $1 \times 10^{-5}$  M of 40 mer RNA with a sequence complementary to the 657–696 site of *c-fos* mRNA (the same sequence of the pair probe) was injected into the cytoplasm. (c) DD image. (d) DA image. Bar: 20  $\mu$ m. (C) The ratio of fluorescence intensity in the cytoplasm in the DA image to that in the DD image (DA/DD) was plotted against incubation times. (a) Cos7 cell in A. (b) Cos7 cell in B. (D) A calibration curve under the fluorescence microscope, showing ratios of fluorescence intensity (DA/DD) versus fractions of the pair probe participating in hybrid formation with the target RNA. Streptavidin-bound donor probes and streptavidin-bound acceptor probes were mixed at a molar ratio of 1:1 in PBS and used as the pair probe. The pair probe and the target 40 mer RNA having the same sequence as the 657–696 site of *c-fos* mRNA were mixed at various molar ratios. The mixed solutions were dropped on coverslips and fluorescence images were taken. The ratio of fluorescence intensity in the DA image divided by that in the DD image (DA/DD) was plotted against molar ratios of target RNA to probes.

served directly as stronger fluorescence, as was the case in AA images when some populations of donors were in FRET, because fluorescence of the donors is quenched by FRET. The DD image of Fig. 6 A shows that fluorescence was observed diffusely throughout the cytoplasm and was also observed to be relatively strong in the regions where the strong fluorescence was observed in the AA image, although that was not as clear as in the AA image. The DA image in which fluorescence caused by FRET is present

shows that quite a strong fluorescence was present in the region where a relatively strong fluorescence was observed in the DD and the AA image. These fluorescence images show that donor and acceptor probes were present in higher concentrations in the regions near the nucleus where FRET occurred with higher efficiency than in other areas of the cytoplasm.

To evaluate FRET efficiency within cells, ratio images (a DA image divided by a DD image) were obtained and



presented in pseudocolor. Fig. 6 *A a* shows the ratio image at 5 min after injection. The cytoplasm of the cells showed high DA/DD values. The DA/DD values had a heterogeneous distribution within the cytoplasm. In the highest region near the nucleus, these were 0.7–1.2. The other cytoplasmic area had values of 0.3–0.4. These values were higher than 0.08, which was the value of the injection probe solution and the values observed in the transfected cells into which the sense probes were injected (see below and Fig. 6 *B*). These results show that FRET occurred in most regions of the cytoplasm. DA/DD values represent molar ratios of pair probes forming the hybrid with *c-fos* mRNA to free probes. The higher values show a higher ratio of probes forming hybrids. The ratio image shows that the hybrid was present throughout the cytoplasm and accumulated somewhat in the regions near the nucleus. Fig. 6 *A d* is the ratio image 20 min after injection. The images had undergone almost no change over 20 min. The DA/DD values within the cytoplasm increased gradually over 15–20 min, which shows that the formation of hybrid was progressing during that time. In most of the cells examined, nonuniform distribution of probes and the FRET efficiency within cytoplasm were observed, although the extent of these varied between individual cells. An adequate molar ratio of probes to target RNA provides a better image resolution in the experiments.

As a control experiment, a pair probe that had the same sequence as the 657–676 site and 677–696 site (sense probes) was injected into transfected cells (Fig. 6 *B*). In these cells, fluorescence was observed diffusely throughout the cytoplasm both in the DD image and in the AA image. Little fluorescence was observed in the DA image. The ratio image shows that DA/DD values of the cytoplasm were  $\sim 0.1$ , which was the same value as the probe solution for injection (Figs. 6 *B a* and 7). Distribution of fluorescence within the cell and DA/DD values in cytoplasm did not change for 20 min after injection (Fig. 6 *B b*). These results show that FRET did not occur in the cytoplasm of the cells, which indicates that no hybrid formation occurred. As another control experiment, the pair probe for the 657–676 site and the 677–696 site was injected into nontransfected Cos7 cells. In these cells, DA/DD values were also  $\sim 0.1$  (images are not shown; see Fig. 7).

### Estimation of the expressed *c-fos* mRNA in living cells

The results of dot blotting show that transfected Cos7 cells expressed more than  $10^4$  molecules of *c-fos* mRNA for 3

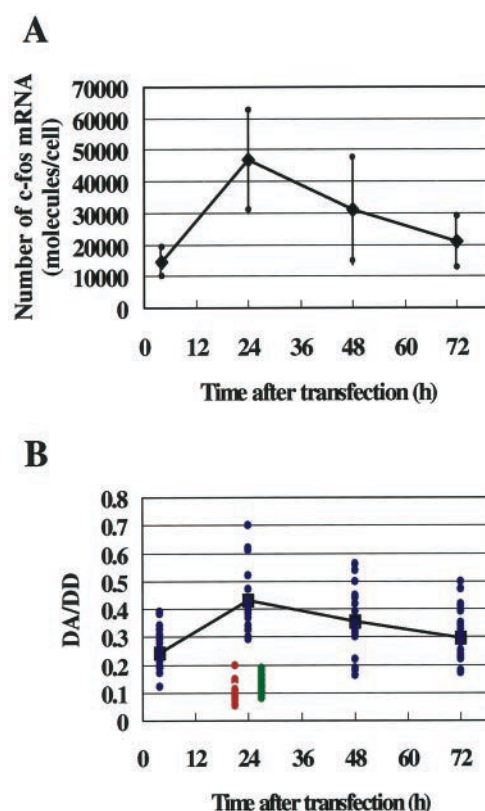


FIGURE 7 Amounts of the expressed *c-fos* mRNA after transfection. (A) Molecular numbers of the expressed *c-fos* mRNA per cell at 4, 24, 48, and 72 h after transfection were estimated by dot blotting. Each point represents the mean  $\pm$  SEM from triplicate experiments. (B) DA/DD values for the whole cytoplasm at 15 min after injection in injection experiments. About  $1 \times 10^5$  molecules of the pair probe were injected into the cytoplasm of Cos7 cells. Each marker (blue circle) represents each cell. Injection was performed at 3–5 h ( $N$  (number of cells) = 27), 24 h ( $N$  = 26), 48 h ( $N$  = 26), and 72 h ( $N$  = 24) after transfection. The average DA/DD value between cells each time is also shown (black square). As control experiments, the sense pair probe was injected into transfected cells 24 h after transfection (red circle), and the antisense pair probe was injected into nontransfected cells 24 h after plating (green circle).

days after transfection and that the average numbers of *c-fos* mRNA in cells varied at the times after transfection (Fig. 7 *A*). In the present method, the DA/DD value for the whole cytoplasm in each cell indicates the molar ratio of the probes participating in hybrids in the cell. We performed injection experiments in which  $\sim 10^5$  molecules of oligoDNA probes were injected into each cell at 3–5, 24, 48, and 72 h after transfection. The DA/DD value for the whole cytoplasmic region was obtained in each cell. The total

FIGURE 6 Imaging of hybrid of the pair oligoDNA probe with *c-fos* mRNA in the cytoplasm of living Cos7 cells.  $1 \times 10^{-5}$  M of the pair probe was injected into the cytoplasm of transfected Cos7 cells at 2 days after transfection. (A) Injection of the antisense probe (donor probe complementary to the 657–676 site and acceptor probe complementary to the 677–696 site). (B) Injection of the sense probe (the donor probe had the same sequence as the 677–696 site and the acceptor probe had the same sequence as the 657–676 site). Fluorescence images (DD image, DA image, and AA image) and a phase-contrast image (Ph) at 5 min after injection are shown. Ratio images, which were obtained by dividing the DA image by the DD image, are also presented in pseudo-color. (a) Five minutes after injection. (b) Twenty minutes after injection. Bar: 20  $\mu$ m.



fluorescence intensity in the cytoplasmic region in the DD and the DA images was estimated, and the fluorescence intensity in the DA image was divided by the intensity in the DD image. The DA/DD value for the whole cytoplasmic region in each cell was plotted in Fig. 7 B. Two kinds of control experiments were also performed. One was injection of the sense probe into transfected cells, and the other was injection of the antisense probe into nontransfected cells. These experiments were performed 24 h after transfection (Fig. 7 B).

DA/DD values of the cells in the control experiments were  $\sim 0.1$ , which was the same as the value for the probe solution for injection. In most of the transfected cells examined, the DA/DD values were higher than 0.1, and there were large divergences between cells. The average DA/DD values at the times after transfection changed in response to the average numbers of the expressed *c-fos* mRNA. The average numbers of the hybrid formed within cytoplasm in a cell at 3–5, 24, 48, and 72 h were estimated from the average DA/DD values, using the curve of Fig. 5 D and assuming  $10^5$  molecules for the total number of probes in cytoplasm. These were  $\sim 13,000$ ,  $\sim 30,000$ ,  $\sim 24,000$ , and  $\sim 19,000$  molecules at 3–5, 24, 48, and 72 h, respectively. In the cytoplasm, not all of the *c-fos* mRNA molecules could form hybrids with the probes. The above numbers of hybrids estimated from the DA/DD values are consistent with the average numbers of *c-fos* mRNA in a cell estimated by dot blotting.

There were large discrepancies in DA/DD values between cells at all examined times after transfection when the antisense probe was injected into transfected cells, while the divergences in the control experiments were relatively small. As mentioned above, DA/DD values indicate molar ratios of hybridizing probes and free probes, determined by molar ratios of probes to the target mRNA in the cytoplasm. Therefore, the large discrepancy in DA/DD values between individual cells represents a large divergence in molecular numbers of *c-fos* mRNA present in each transfected cell at the time of injection.

## DISCUSSION

### Examination of sequences for hybridization of oligonucleotides to the target mRNA

The efficiency of hybridization of oligonucleotides to the target mRNA varies widely, depending on sites on the mRNA, because accessibility of the target mRNA to the oligonucleotides is dependent on the secondary and tertiary structures of the mRNA. The efficiency of hybridization is also dependent on backbone structures and some modifications of bases of the oligonucleotides. In the present study, we demonstrated a simple method for examining sequences for hybridization of oligonucleotides to the target mRNA and measuring hybridization efficiency in solution (Fig. 2).

For sequences examined to determine whether the site has good accessibility to oligonucleotides, the site is divided into two parts, and two oligonucleotides with a sequence complementary to each half of the site are prepared. Each oligonucleotide is labeled with a fluorescence molecule different from that of the other. When both of two fluorescently labeled oligonucleotides hybridize to the site of the mRNA adjacent to each other, the distance between two fluorophores becomes very small, which causes FRET, resulting in changes in fluorescence spectra.

Many combinations of fluorescence molecules (donor and acceptor) can be used in this method. These are combinations in which sufficient fluorescence spectral changes occur by forming the hybrid. Spectral changes are mainly determined by the FRET efficiency on the formed hybrid and the fluorescence quantum yield of acceptor. Because the distance between two fluorophores becomes very small on the double strand of formed hybrid ( $32 \text{ \AA}$  when  $n = 4$ ), the FRET efficiency becomes high even when a donor-acceptor combination such as Bodipy493/503 and Cy5, which has a relatively small overlap integral between the fluorescence spectrum of donor and the absorption spectrum of acceptor, is used. Therefore, we can choose donor-acceptor combinations by other factors such as little excitation of the acceptor at the wavelength for excitation of the donor and a small contamination of donor fluorescence in the region of acceptor fluorescence wavelength.

In the present study, we used probes that were a pair of 20 mer DNA labeled with Bodipy493/503 at the 5' end and 20 mer DNA labeled with Cy5 at a position four nucleotides from the 3' end. Because fluorescence spectra change in response to ratios of probes forming hybrid with target RNA and free probes, spectral changes that occur by adding the target RNA represent the efficiency of hybridization of the pair probes to the target RNA (Fig. 2). Therefore, the parameters for determining the best mode for hybridization of oligonucleotides to the target RNA, which are sequences, backbones, length of oligonucleotides, and other factors, can easily be examined by measuring fluorescence spectral changes. Using this method, we found a 40 mer-specific site within a coding region of human *c-fos* mRNA.

### Streptavidin-bound oligonucleotides for hybridization probes used in living cells

In this study, we used oligoDNAs bound to streptavidin to form hybrid with the target mRNA in the cytoplasm of living cells. Streptavidin-bound oligoDNAs were localized in the cytoplasm for at least 30 min (Fig. 4 C). Digestion of oligoDNAs by some kinds of nucleases, which is a serious problem in achieving hybridization of oligoDNAs to the target RNA in living cells, did not occur. If any sites of the phosphodiester bond of the backbone of Bodipy493/503-labeled oligoDNA were digested with nucleases, the oligoDNAs that were less than 20 mer and labeled at the 5' end

with Bodipy493/503 were released from streptavidin, which would then move into the nucleus and accumulate there within 5 min. The results in which the nucleus had little fluorescence after 30 min of incubation show that the digestion of the oligoDNA that was bound to streptavidin did not occur in the cytoplasm for at least 30 min.

A pair of streptavidin-bound oligoDNA probes, which were one probe (donor probe) bound to streptavidin at the 3' end and another probe (acceptor probe) bound to streptavidin at the 5' end, hybridized efficiently to the target *c-fos* RNA in solution. Moreover, a pair of streptavidin-bound oligoDNA probes immediately formed hybrid with the target 40 mer RNA in the cytoplasm of living Cos7 cells after the target RNA was injected into the cytoplasm (Fig. 5). The result suggests that most of the streptavidin-bound oligoDNAs did not have any specific interaction with other components in cells and were free within the cytoplasm. Binding of oligonucleotide to macromolecules such as proteins and dextran, which do not pass through nuclear pores and do not have some specific interaction with other components in cells, is one possible way for oligonucleotide probes to hybridize efficiently to the target RNA in the cytoplasm of living cells.

The addition of a linker oligonucleotide between streptavidin and the oligonucleotide for hybridization was required for efficient hybridization of probes with *c-fos* mRNA within cytoplasm, although there was no need for hybridization in vitro. Because mRNA within cells is bound with several intracellular proteins, there may be some cases where streptavidin obstructs hybridization of oligonucleotide probes to the target mRNA, which does not occur in the absence of intracellular proteins in solution in vitro. The addition of a linker separates the oligonucleotide for hybridization from streptavidin, which removes obstruction from the streptavidin and induces efficient hybridization.

### Imaging of specific mRNA in a living cell

We demonstrated that *c-fos* mRNA was detected in a living Cos7 cell that expressed a large number of *c-fos* mRNA molecules (more than  $10^4$  molecules per cell) under a fluorescence microscope (Figs. 6 and 7). Two fluorescent oligoDNA probes hybridized to *c-fos* mRNA in the cytoplasm. Fluorescence images as well as ratio images show that heterogeneous distributions of hybrid within cells were clearly observed, suggesting that the present method reveals regional distributions of target mRNA in living cells. Because FRET efficiency indicates molar ratios of hybrid to probes, the DA/DD values in ratio images should change in response to the numbers of target mRNA in the regions. Streptavidin-bound oligoDNA probes used in the present study localized in the cytoplasm and were stable for more than 30 min. Because induction of expression of immediate early genes such as *c-fos* upon extracellular stimuli occurs within several minutes to 1 h in most cases, the present

approach may make it possible to observe the induced expression of these genes on a single-cell level. These measurements would, for instance, reveal divergences in expression levels of the genes and time courses of the expression among individual cells.

Recently, another type of fluorescent oligonucleotide probe, a "molecular beacon," the fluorescence of which changes upon hybridization, has been developed (Tyagi and Kramer, 1996; Tyagi et al., 1998). The probe is labeled with donor and acceptor (quencher for donor fluorescence) at the 5' and 3' ends, respectively, and has its own stem-and-loop structure in the presence of a micromolar range of  $Mg^{2+}$  and at appropriate temperatures. The fluorescence of the donor is strongly quenched in the stem-and-loop conformation, but the quenching is canceled when the conformation is broken by hybridization of complementary nucleic acids or under unstable conditions for the conformation. The molecular beacon was applied to visualize *vav* protooncogene mRNA in K562 human leukemia cells and to obtain images in which the *vav*-expressed cells showed fluorescence intensity that was several times higher throughout the cells (Sokol et al., 1998).

To detect a small number of specific mRNA molecules in living cells, the specificity of detection and a detection limit as well as the stability of probes in cells are key factors. In mammalian culture cells, several thousand kinds of genes are expressed. To detect a specific small number of target mRNA molecules in a mixture of several thousand kinds of mRNAs, the method offering higher specificity would be better. In the present method using two kinds of specific oligonucleotide probes, changes in fluorescence occur only in the case where both probes hybridize to the specific site on the target RNA. This method would therefore have higher specificity than other methods using only one probe. The number of hybrids that can be detected with the present method is limited mainly by the intensity of background light, such as cell autofluorescence and fluorescence from medium, coverslips, and other optical elements. The background light decreases signal-to-noise ratios for detecting hybrids. Measurement methods on a longer wavelength, such as using Cy7 as an acceptor in combination with Bodipy581/591 as a donor, would reduce background light. Another method for improving signal-to-noise ratios for detection is a time-resolved measurement in which decays of acceptor fluorescence intensity are measured after pulse excitations (Morrison, 1988). When FRET occurs, the decay of acceptor fluorescence becomes slower. Measurements of only the acceptor fluorescence to detect FRET, where the wavelength of the measurement is far from the excitation wavelength needed to illuminate cells, have the benefit of a reduced background. Another advantage of this method is separation of FRET-excited acceptor fluorescence from acceptor fluorescence excited by absorbed excitation light, because fluorescence decay curves of the FRET-excited acceptors are different from those of the

directly excited acceptors. This separation, which is not possible in fluorescence spectra, would be useful for the detection of hybrid under the condition that molar ratios of hybrid to free probes are low. We have been developing a time-resolved measurement approach to improve the present method to detect a small number of mRNA molecules (Sato et al., submitted).

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