Detection of Undegraded Oligonucleotides in Vivo by Fluorescence Resonance Energy Transfer

NUCLEASE ACTIVITIES IN LIVING SEA URCHIN EGGS*

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A method was investigated for monitoring the integrity of oligonucleotides in solution and in cells using fluorescence resonance energy transfer between two different fluorochromes attached to a single oligonucleotide. Ten-mer oligodeoxyribonucleotides labeled with fluorescein at one end and with rhodamine X at the other end were used. The oligomer had a specific absorption spectrum with peaks at 497 and 586 nm, which corresponded to fluorescein and rhodamine X, respectively. When excited at 494 nm, the oligomer had a specific fluorescence spectrum with peaks at 523 and 610 nm. The fluorescence intensity at 610 nm was 6–8 times higher than that at 523 nm. After digestion of the oligomer with an endonuclease, the fluorescence at 523 nm increased more than 12–15-fold but its fluorescence peak at 610 nm almost completely disappeared. To examine effects in vivo, sea urchin eggs were injected with a solution of the oligomer and excited with blue light at 470–490 nm. Two fluorescent images, a green image at 520–560 nm and a red image at above 580 nm, were obtained when a single egg was viewed under a fluorescence microscope. The ratio of the intensities of red to green fluorescence decreased in dependence on time after injection of the oligomer. These changes were not observed in eggs that had been injected with a solution of similarly double-labeled, phosphorothioate oligomer. These results indicated that unfertilized sea urchin eggs had nucleolytic activity. Analysis in vitro on supernatant of the egg homogenate indeed demonstrated the existence of nucleases. All together, our results indicate that the integrity of oligonucleotides can be estimated in living cells by monitoring the fluorescence resonance energy transfer of the double-labeled oligonucleotide.

The injection of oligonucleotides into living cells is an effective method for control of the expression of target genes (1, 2). Oligodeoxyribonucleotides have been used to analyze the functions of various genes, since such short DNA molecules can inhibit the synthesis of particular proteins in injected cells by binding to the corresponding mRNA that includes the complementary sequence (3, 4). Introduction of a functional gene with transposable elements into target cells would allow us to produce functionally important cell lines (5). The fate of single-stranded oligonucleotides in vivo has been monitored by use of materials labeled with a fluorescent dye at one end (6). In the search for a treatment for AIDS, ribozymes or their artificially modified analogues appear to be good candidates for drugs of the future since specially designed ribozymes can specifically cleave the mRNA of the AIDS virus (7–13). In studies of such possible therapeutic modalities, the structural integrity of oligonucleotides merits careful consideration because of the dependence of specific binding activity on the length of the oligonucleotide sequence. There is always the undesirable possibility of depolymerization or cleavage by digestion by intracellular nucleases. Ribozymes are especially sensitive to intracellular ribonucleases because their active sites are composed of RNA (14). If we are to monitor the fate of ribozyme in vivo by use of conventional materials labeled with a fluorescent dye at one end, we are likely to detect degraded RNAs labeled with a fluorescent dye at their end. To address these issues, we investigated methods for estimating the integrity of injected oligonucleotides and detecting the nucleolytic activity in living cells using oligonucleotides that had been double-labeled with two fluorescent dyes. We examined the fluorescence characteristics of and the fluorescence resonance energy transfer (FRET)1 within these double-labeled oligonucleotides.

FRET is an interesting example of a fluorescence-related phenomenon (15, 16). When the fluorescence spectrum of one fluorochrome, the donor, overlaps with the excitation spectrum of another fluorochrome, the acceptor, and when the donor and the acceptor are in close physical proximity, the excitation of the donor induces the emission of fluorescence from the acceptor as if the acceptor has been excited directly and the intensity of fluorescence from the donor decreases. The extent of FRET is extremely sensitive to the distance between the donor and the acceptor, being inversely proportional to the sixth power of the distance. This phenomenon can be explored for studies of intermolecular and intramolecular relationships in biophysical investigations and in cell biology and has been used, for example, to examine the structure in solution of a hammerhead ribozyme (17), the concentration of cAMP in vivo (18), membrane fusion (19), retroviral proteases (20), nucleic acid structures and sequences (21), and the extent of intracellular oligonucleotide hybridization (22), kinetic studies of hybridization to the oligonucleotide (23), donor-acceptor distance distributions in a double-labeled fluorescent oligonucleotide (24).

To investigate the integrity of oligonucleotides and the detection of nucleolytic activity in living cells, we used fluores-
cently double-labeled, single-stranded oligodeoxyribonucleotides. FRET was demonstrated spectroscopically in solutions of the modified oligonucleotides and was visualized under the fluorescence microscope in sea urchin eggs after microinjection of the modified oligonucleotides. Enzymatic digestion eliminated the efficiency of FRET both in solutions and in eggs. These results indicate that it is possible to detect only the intact oligonucleotides in living cells by monitoring FRET. We also found nucleolytic activity in living, unfertilized sea urchin eggs by measuring the time-dependent decrease of efficiency of the FRET.

MATERIALS AND METHODS

Double-labeled Oligodeoxyribonucleotides—Double-labeled oligodeoxyribonucleotides were synthesized chemically or with the aid of an enzyme. For enzymatic synthesis, fluorescein 12-dideoxyuridine-5'-triphosphate was purchased from Boehringer Mannheim (Germany). A 9-mer oligodeoxyribonucleotide conjugated with rhodamine X at its 5'-end (R-ODN) was purchased from Takara Shuzo (Kyoto, J. apan). The sequence of the oligonucleotide was 5'-TGAATTGTT-3'. To synthesize the 10-mer oligonucleotide (R-ODN-F) with fluorescein and rhodamine X at its 3' and 5' ends, respectively, 0.1 mM fluorescein 12-dideoxyuridine-5'-triphosphate and 0.05 mM R-ODN were incubated with 20 units of terminal deoxynucleotidyl transferase (Life Technologies, Inc.) for 2 h at 37°C in a solution that contained 100 mM potassium cacodylate buffer (pH 7.2), 2 mM CaCl2, and 1 mM dithiotreitol (25). The product, R-ODN-F, was purified by high performance liquid chromatography (HPLC; Tosoh model 8100; Tokyo, J apan) with an ion-exchange column (TSK gel DEAE-5PW, Tosoh). The column was eluted with a linear gradient of NaCl (0.1 to 1 M) in 20 mM Tris-HCl buffer (pH 9.0) at a flow rate of 1.0 ml/min at room temperature (26).

Alternatively, we also used a chemically synthesized 10-mer oligodeoxyribonucleotide (5'-TGAATTGTT-3') that was double-labeled with fluorescein at its 5'-end and rhodamine X at its 3'-end, respectively (F-ODN-R) and we also used another chemically synthesized 10-mer phosphorothioate oligodeoxyribonucleotide (5'-TGAATTGTT-3') that was double-labeled with rhodamine X at its 5'-end and fluorescein at its 3'-end, respectively (R-S-ODN-F). F-ODN-R was supplied by Takara Shuzo (Kyoto, J apan) and R-S-ODN-F was supplied by Bex (Tokyo, J apan). The purity of F-ODN-R was confirmed by HPLC under the conditions described above. These fluorescent oligomers were confirmed to have the same spectrometric characteristics as the enzymatically synthesized oligomers.

Absorption and Fluorescence Spectroscopy—The oligonucleotides were diluted to a solution (Medium A) containing 50 mM Tris-HCl buffer (pH 8.0), 0.1 mM NaCl, 1 mM CaCl2, and 1 mM MgCl2 to concentrations appropriate for absorption and fluorescence spectroscopy (27). Absorption spectra were recorded with a spectrophotometer (Hitachi model 557, Hitachi, Ltd., Tokyo, Japan), and the fluorescence spectra were recorded with a fluorescence spectrophotometer (model 850, Hitachi).

Enzymatic Digestion of Oligonucleotides—Oligonucleotides with regular phosphodiester linkages were digested with Bal-31 nuclease (Boehringer Mannheim) which functions as an endonuclease when presented with single-stranded DNA as substrate (28). Phosphorothioate oligonucleotide was digested with nuclease P1 (Boehringer Mannheim) which functions as a nuclease against phosphorothioate oligonucleotides with a 3'-phosphate group (29). For measurements in vitro, F-ODN-R (34 pmol) was incubated with Bal-31 nuclease (1000 units) in reaction buffer solution (25) containing 20 mM Tris-HCl (pH 8.0), 600 mM KCl, 12 mM MgCl2, 1 mM EDTA for 60 min at 30°C, and then resuspended in 2 ml of medium A; and R-S-ODN-F (240 pmol) was incubated with nuclease P1 (1.6 units) in reaction buffer solution (20) containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 0.1 mM EDTA for 60 min at 30°C and then resuspended in 2 ml of medium A. For measurement in vivo, F-ODN-R (200 pmol) was incubated with Bal-31 (0.3 units) and R-S-ODN-F (4 pmol) for 30 min at 30°C. Digestion was stopped by addition of 2 μl of 0.1 M EGTA to 4 μl of the reaction mixture.

Sea Urchin Eggs and Microinjection—Eggs of the sea urchin Hemi- centrotus pulcherrimus were collected by intracoelomic injection of 0.55 mM KCl. They were washed twice with seawater. Eggs were set on a poly-L-lysine-coated glass coverslip to which a Lucite frame had been attached to produce a chamber with a volume of about 3 ml. Oligonucleotides were suspended at a concentration of 67–240 μM in 100 mM Hepes-KOH (pH 7.2) in distilled water. The solution of oligonucleotides (about 1–2% of the eggs volume) was microinjected into eggs by the method described elsewhere (30).

Microscopic Visualization of FRET—To investigate the FRET from eggs that had been injected with oligonucleotides, the components of the fluorescence were separated with two types of filter attached to the fluorescence microscope (Diaphot-TMD: Nikon, Tokyo, J apan). Green images were observed through a band-pass filter at 520–560 nm, and red images were observed through a sharp cutoff filter at wavelengths above 580 nm. For both types of imaging, infrared light was eliminated with a heat-absorbing filter. The excitation light, obtained at 470–490 nm by passage through a band-pass filter, was removed from the fluorescence signal by a dichroic mirror with a cut-off wavelengths of 510 nm. All filters and the mirror were purchased from Nikon. To decrease the extent of photo-bleaching of dyes by strong excitation light, we used a 100-W halogen lamp at 6 volts that was regulated by a stabilizing DC power supply (NP-150Z; Nissyo Industry, J apan).

The green and red components of the fluorescent images were accumulated alternatively for 5 s with a high-sensitivity ICCD camera (c2400–80; Hamamatsu Photonics, Hamamatsu city, J apan), and the fluorescence intensity was analyzed with an image processor (ARGUS-50; Hamamatsu Photonics).

RESULTS AND DISCUSSION

Double-labeled Oligonucleotide with Two Fluorescent Dyes—The integrity of oligonucleotides is important for their function when they are injected into cells to regulate transcription or translation. Since the conventional fluorescent single-end-labeling method cannot be used to monitor the fate of nuclease-sensitive oligomers, we investigated methods for estimating the integrity of injected oligonucleotides in living cells using, at first, an oligonucleotide that had been double-labeled with two fluorescent dyes. The fluorescence characteristics of and the FRET within these double-labeled oligonucleotides were examined in vitro and in vivo. Since the purity of the double-labeled oligonucleotide was important for measurements of FRET in solution and in cells, we analyzed the components of the synthesized oligomer by HPLC. As shown in Fig. 1, only one peak was observed in the chromatogram of F-ODN-R after chromatography on an ion-exchange column when absorption of the eluent was monitored at 260 nm. A single peak also appeared in the elution profile after HPLC when fluorescence was monitored at 606 nm with excitation at 494 nm. The presence of the single peak in each case demonstrated that the synthesized product consisted exclusively of a 10-mer oligonucleotide, the two ends of which were separately labeled with fluorescein and rhodamine X.

FRET in Vitro—The spectroscopic characteristics of F-ODN-R were analyzed to determine the appropriate conditions for measurements of FRET. The absorption spectrum recorded with a spectrophotometer had peaks at 497 and 586 nm (Fig. 2), indicating that the solution included fluorescein and rhodamine X. The fluorescence spectrum of the F-ODN-R was also recorded and peaks at 523 and 610 nm were found upon excitation at 494 nm (Fig. 3A). These wavelengths correspond to the peaks of fluorescence emitted by fluorescein and rhodamine X, respectively. The fluorescence intensity at 610
nm was 8 times higher than that at 523 nm. The excitation spectrum, detected at 606 nm, is shown in Fig. 3B. Two peaks can be seen which correspond to fluorescein and rhodamine X, respectively. These spectra indicate that considerable energy from the excited fluorescein fluorochrome was transferred to rhodamine X.

To confirm that the observed FRET occurred between the dyes attached to one and the same molecule of the oligonucleotide, we digested the oligonucleotide with Bal-31, which functions as an endonuclease against single-stranded DNA. After incubation with the enzyme, the digested material was analyzed spectroscopically. The fluorescence intensity at 523 nm was 15 times higher than that before endonucleolytic digestion (Fig. 4). The peak of red fluorescence at 610 nm disappeared. These results indicate that the FRET of F-ODN-R was due to the relationship between intramolecular fluorochromes.

FRET in Vivo—To examine the possibility of visualization of FRET in cells, F-ODN-R was microinjected into unfertilized sea urchin eggs. These eggs were illuminated with blue light at 470–490 nm to photoexcite the fluorescein fluorochrome of the F-ODN-R. The green and red components of the emitted fluorescence were separately monitored with a fluorescence microscope equipped with appropriate sets of filters. The results are shown in Fig. 5. The eggs injected with the intact F-ODN-R emitted bright red light in addition to the green light from fluorescein (Fig. 5, A and B). By contrast, no red fluorescence was emitted from eggs that had been injected with the previously Bal-31-digested oligonucleotide (Fig. 5E); only green fluorescence was emitted (Fig. 5D). Similarly, only green fluorescence was emitted from eggs that had been injected with a solution of mixture of a fluoresceinated mononucleotide and a rhodamine X-labeled 9-mer oligonucleotide (data not shown). These results are consistent with the results of the experiments in vitro with the fluorescence spectrophotometer.
confirm this possibility, we observed the solution of the double-labeled oligonucleotide F-ODN-R. Unfertilized eggs were injected with the intact oligomer (A, B, and F) or with the enzymatically digested oligomer (D and E). The paired images of fluorescent components in the green-light region (A and D) and the red region (B and E) were recorded separately from the same eggs. The ratio image (C) of red to green was computed from the fluorescent components in photographs A and B. These photographs are shown in pseudocolors. Photograph F shows the transmitted-light image of the egg. Bar equals 50 μm.

The intensity of fluorescence varied among different regions of the egg. The middle region of the egg was intensely fluorescent and the intensity of fluorescence decreased from the center to the periphery in both red- and green-light images. The absence of uniformity can be ascribed to the length of the light path within the spherical sea urchin egg. The ratio intensities of the two qualitatively different images could be used to eliminate the effect of cell volume, as in the case of calculations of intracellular concentrations of calcium ions using the fluorescent dyes Fura-2 (31) and Indo-1 (32). We "divided" the red image by the green image with an image processor and the result is shown in Fig. 5C. The new image shows the uniformity of FRET within the cytoplasm of an egg injected with F-ODN-R. The cytoplasmic margin of the egg surface, namely, the egg cortex, gave a higher value for the ratio than the inner cytoplasm. This higher ratio might be the result of the higher viscosity in the narrow cortex region as compared with the inner region (33). Such a difference causes the disparity in the refraction between green and red light at the surface, giving rise to an error in the estimation of FRET. While we were unable to clarify the discontinuity in the extent of FRET at the cortex, the ratio of the images demonstrates that the double-labeled fluorescent oligonucleotide exhibited uniform FRET throughout the cytoplasm of the living egg.

**Time-dependent Decrease of FRET in Living Eggs**—The FRET in the egg was different from that measured in solution by fluorescence spectroscopy. As shown in Fig. 5 (A and B), the fluorescence intensity of the green component was nearly identical to that of the red component, whereas the ratio of the intensity at 610 nm to that at 523 nm in solution was 6.72 (Table I). The marked discrepancy between these two ratios might be caused by the imaging instrumentation of the microscope and the video camera, in particular since the camera was sensitive to wavelength. The camera used here was about 1.5 times more sensitive to light at 520 nm than at 600 nm. To confirm this possibility, we observed the solution of the double-labeled oligo's enclosed by liquid paraffin in a glass capillary, using the same microscope-video camera system under the same conditions as for the accumulation of image of fluorescence from the egg. The image analysis of this sample yielded a ratio of red to green fluorescence of 6.1. This value for the solution in the capillary was of the same order as that for the same sample measured with the fluorescence spectrophotometer and was entirely different from the FRET in the egg. The results suggest that the discrepancy in the extent of FRET between the egg and the solution might be due mainly to the condition of the F-ODN-R molecule in the intracellular environment and not to the detection system.

The elimination of FRET after cleavage of the oligonucleotide was demonstrated by injection of F-ODN-R that had been pre-treated with endonuclease (Fig. 5, D and E). If the sea urchin egg contains nuclease, the injected double-labeled oligomer should be degraded and the extent of FRET should decrease with the time after the injection. Therefore, we measured time-dependent changes in the fluorescence ratio of the red to the green component from a single living egg under the microscope (Fig. 6A). When the oligonucleotide was injected, FRET rapidly decreased and reached plateau in about 1 h. The ratio decreased from 2.3 at 1 min to 0.42 at 60 min. This time-dependent decrease of FRET was not observed in the eggs injected

| TABLE I  |
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| Ratio of the red to the green components of the fluorescence |  
| Average values from independent three experiments. |
| Fluorescence spectrophotometer | Microscope system |
| No treatment | Nuclease digestion | In eggs |
| R-ODN-F | 6.72 | 0.16 | 0.42 |
| R-S-ODN-F | 7.27 | 0.18 | 5.09 |

*The ratio was calculated from the intensities of fluorescence at 523 nm and 610 nm.  
*R-ODN-F was digested by BAL31 nuclease, R-S-ODN-F was digested by Nuclease P1.  
*60 min after injection.
with double-labeled phosphorothioate, R-S-ODN-F, which is known to be resistant to digestion by general nucleases. This modified R-S-ODN-F had the same FRET property as that of the oligomer with regular phosphodiester linkages (Table I). note that the fluorescence ratio of R-S-ODN-F decreased by the treatment with nuclease P1, a nucleolytic enzyme that can digest phosphodiester linkages with $S_3$ configuration (29). The incubation of the eggs injected with phosphorothioate R-S-ODN-F for 60 min showed no change in the ratio (Fig. 6B). These results indicate that the lower extent of FRET in the eggs for the double-labeled oligomer with natural phosphodiester linkages was mainly due to the enzymatic digestion of the oligonucleotide.

Nuclease Activity in the Egg Homogenate—Microscopic measurements of FRET suggested that sea urchin eggs might have nuclease activity against single-stranded oligonucleotides. An endonuclease in sea urchin eggs had been reported by Koide et al. (34). They have demonstrated the Ca$^{2+}$- and Mg$^{2+}$-dependent nuclease activity in the ammonium sulfate precipitate of egg homogenate against sperm DNA, whereas the possibility of existence of nucleases against single-stranded oligonucleotides in the eggs remained obscure. Since our data (Fig. 6A and Table I) indicated the existence of such nucleases, we analyzed the ability of the supernatant from the sea urchin egg homogenate to digest single-stranded oligonucleotides. A 9-mer oligonucleotide labeled with rhodamine X at the 5′-end (R-ODN) was used as substrate. The HPLC analysis with fluorescence detection revealed that the reaction mixture, after incubation at 30 °C for 60 min, was composed of 8 peaks (Fig. 7A). Considering nine constituent nucleotides of R-ODN, the number of the peaks was compatible with the assumed number of fluorescent fragments produced after digestion with nucleases. An incubation of the same reaction mixture, except for the presence of EDTA, showed only one peak (Fig. 7B), which coincided with a single peak of an R-ODN solution without the supernatant. This indicated that divalent metal ions are necessary for the nuclease activity in the supernatant. These results indicate that unfertilized sea urchin eggs have metal ion-dependent nucleases specific for single-stranded oligonucleotides, and suggest that the FRET decrease of the double-labeled oligonucleotide after the injection into the eggs was due to the digestion with an endogenous, nuclease activity of the eggs.

Conclusion—The newly synthesized oligodeoxyribonucleotide that was labeled with fluorescein and rhodamine X at different ends exhibited FRET in vitro and in vivo. Enzymatic digestion eliminated the efficiency of FRET both in solutions and in eggs. Fluorescence ratio calculation of a red component to a green with the microscopic system revealed the time-dependent decrease of FRET of the oligomer after microinjection into sea urchin eggs; the presence of a nuclease activity in the egg cytoplasm was confirmed. Thus, the FRET technique should be useful for studies in vitro and in vivo of nuclease activity and the integrity of nuclease-sensitive oligonucleotides.

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