Table 1. MDA and total antioxidant status in serum samples of hypercholesterolemic patients

	Controls ($n = 30$)	Patient ($n = 30$)
MDA (nmol/ml)	3.6 ± 0.6	5.1 ± 0.8*
Total antioxidant status (mmol/L trolox equivalent)	1.68 ± 0.3	1.06 ± 0.15**

Values are given as mean ± SD; *Significantly greater than the control; **Significantly less than the control.

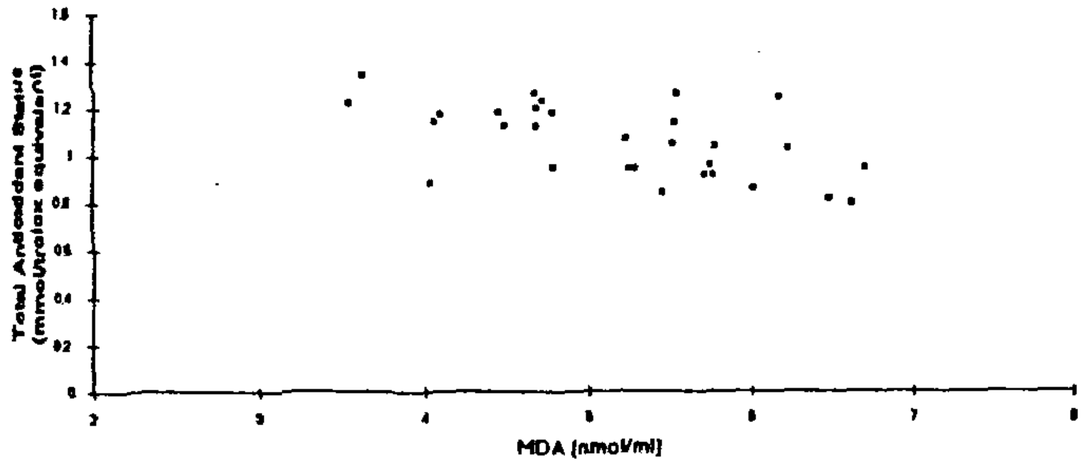

Figure 1. Negative correlation between MDA and total antioxidant status in serum samples of hypercholesterolemic patients ($r = -0.57$, $n = 30$).

depleted in comparison to the controls, thereby showing a negative correlation between the total antioxidant status and lipid peroxidation in the serum of hypercholesterolemic patients. In healthy conditions a balance exists between free-radical generation and antioxidant defence system which prevents occurrence of disease. However this study implies that hypercholesterolemia shifts the balance in favour of free-radical generation which leads to oxidative tissue damage. This in turn increases permeability and loss of membrane integrity[21,22] and thus sets the stage for atherosclerosis.

Since hypercholesterolemia and lipid peroxidation both directly relate to the severity of atherosclerosis, elimination of free-radicals in the plasma before they can be taken up by the peripheral tissues might improve the prognosis for patients. In view of our present findings, a management strategy aimed at simultaneous control of lipid peroxidation and total antioxidant status in the serum of hypercholesterolemic patients may be effective and an antioxidant therapy may be beneficial to avoid the damage that can be caused by free-radicals.

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Fluorescence resonance energy transfer: A diagnostic tool in oligonucleotide therapy

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Synthetic oligonucleotides of variable lengths and sequences are being used for treatment of viral diseases at the genetic level. Specific methods are available for monitoring these oligonucleotides *in vitro* and *in vivo*, viz. radioisotopic labelling or non-covalent/covalent attachment of fluorescent reporter groups, spectroscopic assays and of late fluorescence resonance energy transfer (FRET). This paper integrates general features of FRET and its application as a diagnostic tool in oligonucleotide therapy.

RECENTLY the use of synthetic oligonucleotides in molecular biology has gained unprecedented progress,

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specially for regulating gene expression of the cell. This may be achieved by using different therapeutic modalities[1–3] such as (i) antisense approach – using synthetic sequences complementary to target sequence of the viral genome, (ii) ribozymes – short RNA sequences possessing enzymatic and self-cleaving properties, which bring about cleavage of viral RNA at specific sites, and (iii) triplex construct – formed by sequence-specific recognition of double-stranded DNA by the oligonucleotide. Recognition is primarily achieved through Hoogsteen-type hydrogen bonds involving thymine recognition of adenine–thymine base pairs and N3-protonated cytosine (C^+) recognition of guanine–cytosine base pairs within the homopurine/homopyrimidine stretches of the duplex DNA.

Fluorescence resonance energy transfer (FRET) is an interesting example of fluorescence-related phenomenon[4]. When the fluorescence spectrum of one fluorochrome, the donor, overlaps with the excitation spectrum of another fluorochrome, the acceptor, and when the donor and acceptor are in close proximity, 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 process involves transfer of excited state energy of the donor molecule to the acceptor molecule by a resonance dipole–induced dipole interactions. The results are a decrease in donor life time, i.e. a quenching of the donor fluorescence, an enhancement of the acceptor fluorescence and depolarization of fluorescence intensity. The efficiency of energy transfer, E_t, falls off rapidly with the distance between the donor and the acceptor, R, which for most practical purposes should not exceed 70 Å. The efficiency of energy transfer is given by the following equation[5]:

$$E_t = 1/[1 + (R/R_0)^6],$$

where R_0 depends on the overlap integral of the donor emission spectrum and acceptor excitation spectrum, the index of refraction, the quantum yield of the donor and the orientation of the donor emission and the acceptor absorbance moments. The donors which are commonly used are fluorescein derivatives whereas the acceptors are rhodamine derivatives. Since FRET is extremely dependent on molecular distance between the donor and the acceptor (hence called 'the spectroscopic ruler'[6]), it can be explored for studies of intermolecular and intramolecular relationships in biophysical investigations and in cell biology. Moreover, this distance parameter is important in oligonucleotide interactions as well, since these are short sequences (≅ 20 mers) involving molecular interactions. Here, we give a critical review of application of FRET in different therapeutic modalities of oligonucleotide therapy.

The underlying principle of antisense therapy is that if any foreign DNA/RNA fragment complementary to some target sequence of the viral genome is introduced into the cell, it interacts with the target which is a growth parameter, either by hybridizing with it or degrading it in some manner, thereby inhibiting the gene expression of the cell. Such oligonucleotides are called antisense oligonucleotides[7,8] as they have the power of reverse genetics, i.e. suppressing translation. Therapeutic use of antisense oligonucleotides has been suggested earlier[9] and during the past decade a number of antisense drugs have been developed and evaluated in clinical trials for the treatment of diseases including AIDS, cancer, rheumatoid arthritis, Crohn's disease and CMV retinitis. In 1998, Vitravene was the first Food and Drug Administration approved antisense drug for the treatment of CMV retinitis. In 1999, another antisense drug Fomivirsen has been approved for the treatment of cytomegalovirus infection[10–12].

In cases of therapeutic modality it is important to monitor hybridization, *in vitro* and *in vivo*, and also assay the resistance of these oligonucleotides against the nuclease enzymes present in the cell. Hybridization can be monitored by using FRET[13]. This can be achieved by adopting different strategies: (i) By attaching the donor and the acceptor molecules covalently to the 5′ end of complementary oligonucleotides (Figure 1 *a*). (ii) In case of two oligonucleotides complementary to different regions of the target sequence separated by a distance of few monomers, FRET signals can be generated by labelling one oligonucleotide at the 5′ end and the other at 3′ end (Figure 1 *b*). (iii) Labelling one of the strands with either the acceptor or the donor and using fluorescent dyes which intercalate (such as a acridine orange) with the double helical DNA and may act as donor or acceptor molecule depending on the labelling of the strands (Figure 1 *c*). Synthesis, characterization and intercalating properties of a number of molecules have been reported from our laboratory[14–16]. This strategy is useful in cases where the oligonucleotide under investigation is long enough to render FRET ineffective (R should not exceed 70 Å). It also provides a number of fluorescent molecules at different orientations, thereby increasing the overall energy transfer efficiency. (iv) Another strategy can also be adopted by labelling the complementary strand with the donor and the acceptor at different ends so that on hybridization both the dyes come closer and strong FRET signals are observed (Figure 1 *d*). This has been used to evaluate the kinetic and thermodynamic parameters of nucleic acid hybridization[17]. These parameters (hybridization enthalpy, entropy and rate constant) can help to calculate optimal assay conditions such as temperature, hybridization equilibrium time and DNA reagent size instead of determining the same through extensive experimentation.

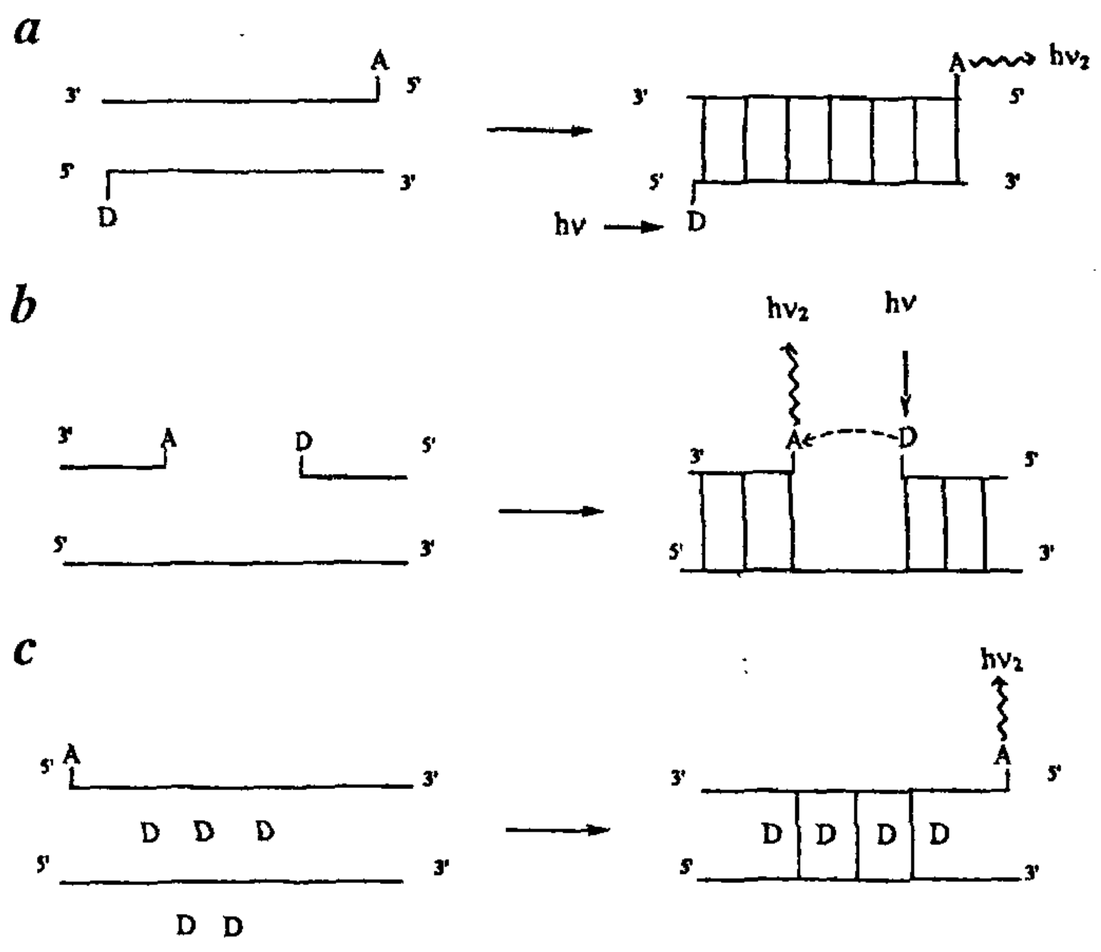

Figure 1. Different strategies for detecting oligonucleotide hybridization using FRET. *a*, Donor (fluorescein) and acceptor (rhodamine) covalently attached to 5′ end of complementary strands; *b*, Donor and acceptor covalently attached to 5′ end and 3′ end of two strands which are complementary to different regions of a single unlabelled strand; *c*, Acceptor (rhodamine) covalently attached to 5′ end of one strand and an intercalating dye (acridine orange) being used as donor; *d*, Donor (fluorescein isothiocynate) and acceptor (texas red) covalently attached to 5′ and 3′ ends of complementary strands. *D*, donor; *A*, acceptor; $h\nu$, excitation frequency of the donor; $h\nu_1$, emission frequency of the donor; $h\nu_2$, emission frequency of the acceptor after energy transfer; K_1 and K_2 are rate constants.

In all the four cases, as the hybridization ensues, a strong FRET signal is observed which manifests itself as quenching in the donor fluorescence or enhancement of fluorescence emission from the acceptor. FRET does not offer an increased sensitivity over existing methods but it has one unique feature. In all existing methods for detecting the nucleic acid hybridization, nonhybridized fragments must be removed; however FRET gives distinct signals even in the presence of nonhybridized strands. Moreover, separation of the nonhybridized fragments remaining after hybridization tends to perturb the hybridization equilibrium and hence rules out the possibility of continuously monitoring hybridization with time. Methods like NMR, colorimetry, etc. require milligram quantities of oligonucleotides which may not be desirable when working with OD amounts.

FRET technique has been applied for the study of duplex formation[18,19], branched nucleic acids[20,21], and for assessing the extent of intracellular hybridization[22].

Certain RNA molecules called ribozymes possess enzymatic, self-cleaving activity[23]. The cleavage reaction is catalytic, site-specific and dependent on magnesium ion concentration. These ribozymes possess unique secondary (possibly tertiary) structures for cleavage ability. Two structural motifs, hammerhead and hairpin have so far been described as intermediates in the cleavage reactions. This observation suggests that antisense molecules could be designed and developed which form not only RNA–RNA hybrids but also catalytically cleave the phosphodiester bonds in the target RNA strand. Because the catalytic RNA is not consumed during the cleavage reaction, a large number of substrate molecules could be processed. This makes ribozymes or their modified analogues good candidates for drugs of the future since specially-designed ribozymes can specifically cleave the viral mRNA[24]. In fact precise cleavage of human immunodeficiency virus type 1 (HIV 1) sequence in a cell-free system has been reported[25], and also modified ribozymes as potential therapeutics are established[26]. FRET can be applied to establish the three-dimensional structure of hammerhead ribozymes[27].

A major breakthrough was achieved when the FRET technique was applied to follow the ribozyme reaction, viz. ligation and site-specific cleavage in real time[28]. The ligation could be followed using FRET. This was achieved by labelling a 6-mer with the acceptor dye and labelling one end of the ribozyme with the donor dye. Ligation of the strand is followed by folding of the RNA strands producing a hairpin loop which in turn produces the FRET signals. Since ligation is followed by excision of a 3-mer labelled with the donor dye, there is manifestation of a strong FRET signal initially which falls off continuously with time[28] (Figure 2 *a*). The cleavage reaction[28] was followed by labelling the ribozyme strand with the donor (fluorescein) and the acceptor (tetramethyl rhodamine) at both the ends. With the formation of a hair loop the two ends are brought in close proximity due to folding of the RNA strand giving rise to FRET signals. The cleavage at the specific site under appropriate conditions (10% ethanol, 20 mM, pH 7.5) separates the donor and acceptor dyes apart, thereby diminishing the FRET signals with time (Figure 2 *b*).

With antisense oligonucleotides or ribozymes there is always a possibility of depolymerization/cleavage reaction by intracellular nuclease which may render it ineffective. Monitoring of oligonucleotides with fluorescent labels, the technique also being used in our laboratory[29,30], may not be that effective as any nuclease activity will yield labelled and unlabelled oligonucleotide fragments. FRET may, however, be used to monitor

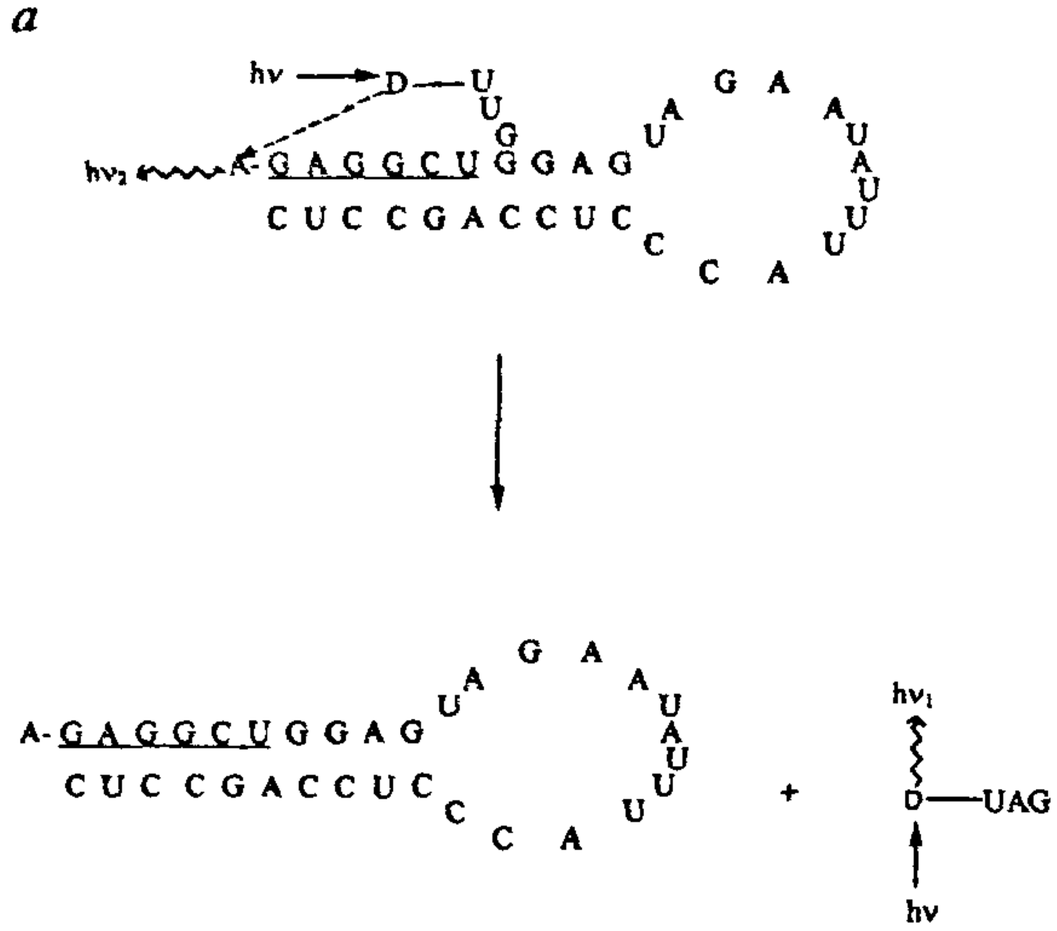

Figure 2. *a*, Strategy for monitoring the ligation reaction using FRET. *D*, donor; *A*, acceptor; the underlined sequence represents the ligating 6-mer; *b*, Monitoring of the cleavage reaction using FRET. *D*, Donor (Fluorescein); *A*, Acceptor (Tetramethyl rhodamine); $h\nu$, $h\nu_1$ and $h\nu_2$ are same as defined in Figure 1.

structural integrity of oligonucleotides *in vitro* and *in vivo*[31]. This was achieved by labelling a 10-mer with the donor fluorophore (fluorescein) at one end and the acceptor fluorophore (rhodamine-X) at the other and FRET signals were observed by quenching in the donor fluorescence emission and enhancement in the acceptor intensity. On digestion with Bal 31, (known for its endonuclease activity) the fluorescence peak at acceptor wavelength almost disappeared, showing that the

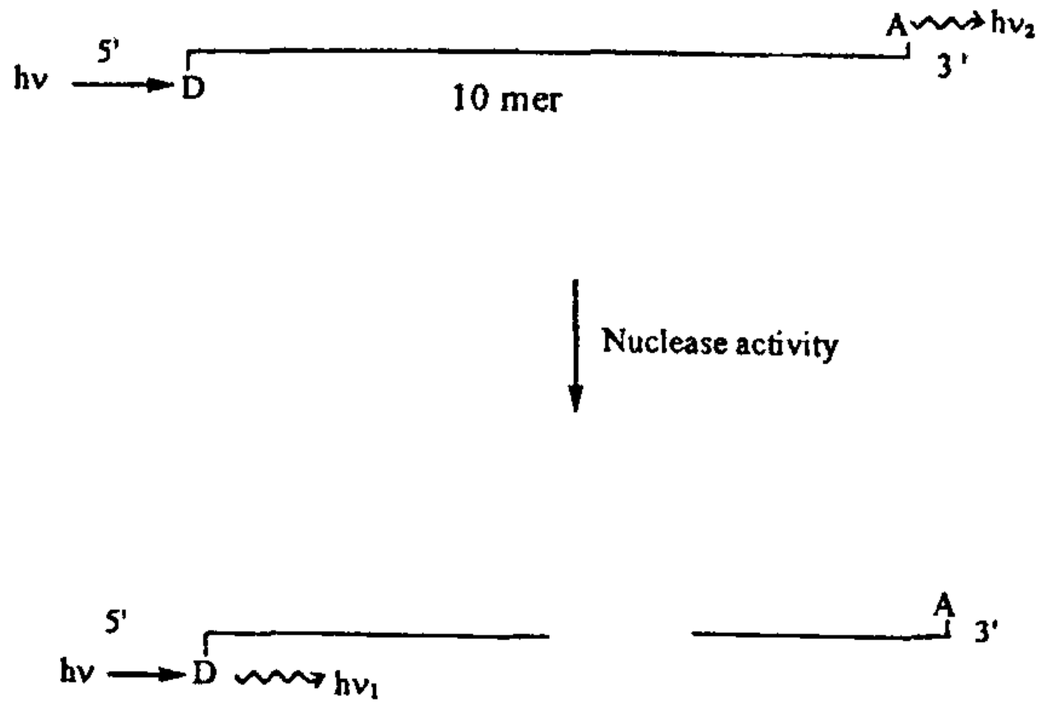

Figure 3. Detection of the structural integrity of oligonucleotide using FRET. *D*, Donor (Fluorescein); *A*, Acceptor (Rhodamine-X); $h\nu$, $h\nu_1$ and $h\nu_2$ are same as defined in Figure 1.

oligonucleotide has been cleaved (Figure 3). This effect has been examined *in vivo* by using unfertilized sea urchin eggs. The same oligomer labelled at the two ends was micro-injected into the egg and FRET signals were observed initially which diminished in due course of time (fall in the intensity of acceptor peak). These changes were not observed when eggs were microinjected with the same oligomer but with a phosphorothioate backbone (known for its resistance to nuclease activity). The *in vitro* analysis of supernatant egg in fact demonstrated the presence of nuclease.

The FRET technique is more beneficial over other techniques employed as it does not require the withdrawing of aliquots at different time intervals and continuous monitoring is possible both *in vitro* and *in vivo*.

Sequence-specific recognition of double-stranded DNA by oligonucleotide-directed triplex formation has become an active area of research in molecular biology[32]. Recognition is primarily achieved through formation of specific Hoogsteen-type hydrogen bonds involving thymine recognition of adenine–thymine base pairs and N3-protonated cytosine recognition (C^+) of guanine–cytosine base pairs within the homopurine/homopyrimidine stretches of the duplex DNA. Single triplex mismatches between an oligonucleotide and a duplex DNA have been shown to destabilize triple helix formation and FRET efficiency falls off strongly with even a single mismatch[33]. FRET was applied to evaluate thermodynamic and kinetic parameters of triple helix formation[34]. This has been carried out by labelling one of the duplex strands by the donor fluorophore (fluorescein) at the 5′ end and the oligonucleotide by the acceptor fluorophore (tetramethyl rhodamine) again at the 5′ end. Any triplex formation is accompanied by manifestation of strong FRET signals (Figure 4 *a*).

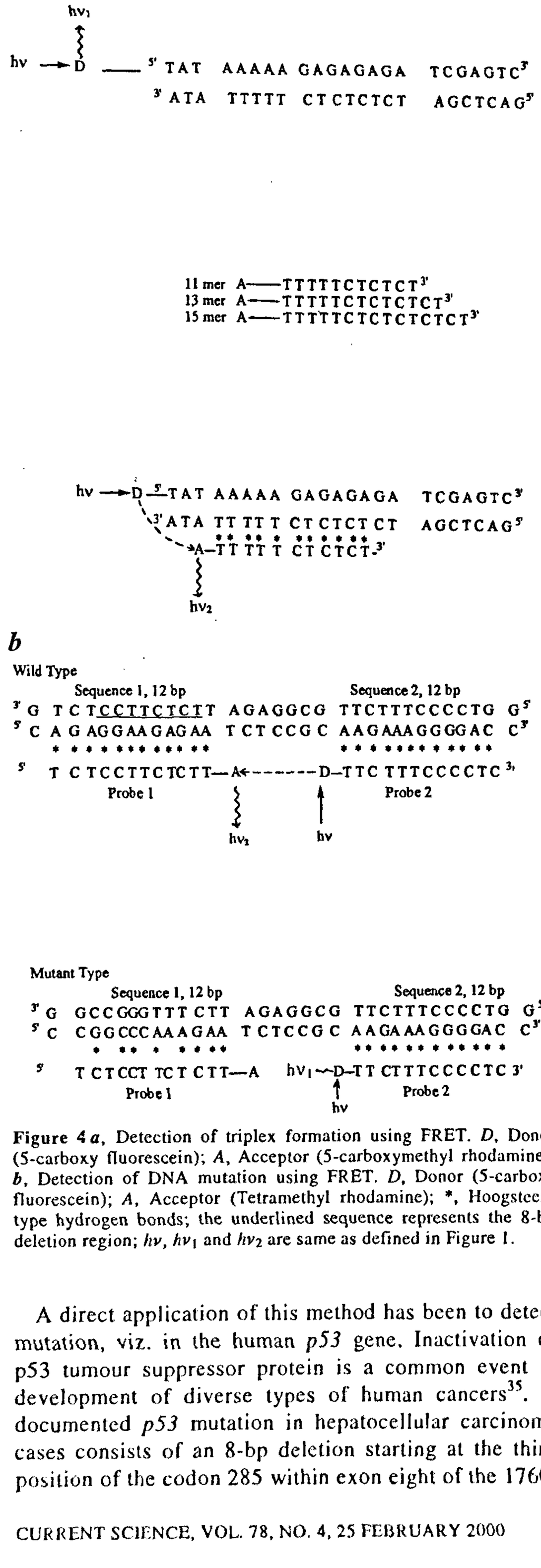

Figure 4 *a*, Detection of triplex formation using FRET. *D*, Donor (5-carboxy fluorescein); *A*, Acceptor (5-carboxymethyl rhodamine); *b*, Detection of DNA mutation using FRET. *D*, Donor (5-carboxy fluorescein); *A*, Acceptor (Tetramethyl rhodamine); *, Hoogsteen-type hydrogen bonds; the underlined sequence represents the 8-bp deletion region; $h\nu$, $h\nu_1$ and $h\nu_2$ are same as defined in Figure 1.

A direct application of this method has been to detect mutation, viz. in the human *p53* gene. Inactivation of p53 tumour suppressor protein is a common event in development of diverse types of human cancers[35]. A documented *p53* mutation in hepatocellular carcinoma cases consists of an 8-bp deletion starting at the third position of the codon 285 within exon eight of the 1760-bp *p53* gene[36]. The deletion disrupts a 12-bp homopurine/homopyrimidine sequence (sequence 1) whereas nearby 12-bp homopurine/homopyrimidine region (sequence 2) remains intact (Figure 4 *b*). It has been shown that oligonucleotides form triplex within this stretch of DNA[37,38]. The proximity of the two sites which are separated by a region of 7-bp offers possibility for FRET using acceptor labelled (probe 1) and donor labelled (probe 2) DNA probes (both at the 5′ end)[39] which were specific to sequence 1 and sequence 2, respectively. Since sequence 1 is disrupted by 8-bp deletion probe 1 forms a triplex with the wild type duplex and not with the mutant, and consequently FRET signals are observed in case of the wild duplex and not with the mutant (Figure 4 *b*). The results have been found to be consistent when tried with the clinical DNA samples obtained from patients diagnosed with hepatocellular carcinoma.

Triplex formation in fact can be monitored by other techniques such as UV melting[40], differential scanning calorimetry[41], gel electrophoresis[42] and filter binding assay[43]. These techniques invariably require either large quantity of oligonucleotide or a proper separation technique. For instance, filter binding assay suffers from low retention efficiency of single-stranded DNA, and UV melting is suitable only at temperatures near the triplex melting temperature.

In conclusion, FRET techniques can be applied in all aspects of oligonucleotide therapy be it antisense, ribozymes, structural integrity or triplex formation. From the data available in the literature it is not quite evident that FRET offers increased sensitivity or precision over other existing methods for monitoring oligonucleotides *in vitro* and *in vivo*. However, it can be safely concluded that it is one of the most important and handy tools for a molecular biologist to study genetic modulation at the cellular level. A unique feature not common to any other technique and which makes FRET more suitable is that monitoring is possible here without resorting to any separation or withdrawal of aliquots at specific intervals of time. Thus FRET is a future technique of choice for oligonucleotide therapy.

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Detection and identification of VLF seismo-electromagnetic signals

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The vertical electric field components of the electromagnetic emissions associated with earthquakes have been monitored at the Agra station (geographical lat. 27.2°N, geographical long. 78°E) since February 1998 using a borehole antenna. Analysis of one year of data has shown that large variations in the electric field occur, mostly in the form of noise bursts, both prior to and after the occurrence of major earthquakes. In the present paper some selected cases of noise bursts have been chosen to identify the waveforms of seismogenic emissions. We find that among various types of noise bursts recorded, the seismogenic noise bursts are either slowly varying, square wave patterns, or periodic variations. The long distance propagation of such signals is interpreted in terms of waveguide mode propagation through conductive channels across the main boundary seismic fault existing from the north-west to the north-east around 200 km from the observing station.

ELECTROMAGNETIC emissions of various frequencies ranging from ULF to HF have been observed both on the ground and in the ionosphere during earthquakes[1–6]. The association of electromagnetic emissions with seismic activities has been confirmed from laboratory experiments employing rock fracturing also[2,7–9]. Parrot[10] and Hayakawa[11] have reviewed thoroughly the work done in this field and Hayakawa and Fujinawa[12] and Hayakawa[13] have presented recent works in two excellent monographs.

Recently[14,15], we have shown by measuring the vertical component of electromagnetic emissions using a borehole antenna that electric field changes appear in the form of noise bursts which correspond to some major impending earthquakes. From a detailed analysis of the data obtained during 1998, we conclude that out of the total number of noise bursts associated with major earthquakes that occurred in different months, about 60% occurred prior to the earthquakes as precursors. This has indicated that monitoring of electromagnetic field components associated with earthquakes may prove to be an important tool in the field of earthquake prediction. In the present paper we make an attempt to identify the type of electromagnetic signals that are associated with seismic activities by identifying the type of signals that are responsible for peak noise bursts activities and by monitoring such signals using a vertical

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