CHAPTER V
PROXIMITY RELATIONSHIP BETWEEN THE INITIATING SUBSTRATE
AND RIFAMPICIN BOUND TO THE RNA POLYMERASE
5.1. INTRODUCTION

The earlier reports on rifampicin action and the studies discussed in the previous chapter still leave some uncertainties about the mode of action of the antibiotic on RNA polymerase. The results discussed in Chapter IV favour the idea that rifampicin reduces the binding affinity of the RNA polymerase for the substrates and the nascent oligomer products. But the precise way in which it affects the binding site is not clear. The Lineweaver-Burk plot analysis of the synthesis of pppApU at the T7A1 promoter (Chapter IV) and the λPR' promoter (McClure and Cech, 1978) apparently indicated a competitive mode of inhibition by the antibiotic. But the fact that RNA polymerase binds rifampicin with an affinity constant several orders of magnitude higher than that for the nucleotides does not favour a competitive mode of inhibition, because in that case the antibiotic should completely inhibit the enzyme at much less concentration than was used in these experiments. The steric hindrance model of rifampicin action proposed by McClure and Cech (1978) requires that the antibiotic should be bound to a site on the RNA polymerase very close to the nucleotide binding sites.

A final solution to these complexities was the mapping of the physical location of the rifampicin binding site on the RNA polymerase in relation to the substrate binding site. Genetic and biochemical evidences indicate that the binding sites for the nucleotide substrates and rifampicin are located on the β subunit (Grachev et al., 1987, Jin and Gross, 1988; Chatterji et al., 1984). But their spatial relationship on the active holoenzyme is not established. Any attempt to map the physical relationship among different sites on the enzyme would require a technique that would fulfil several criteria. Firstly, X-ray
analysis of RNA polymerase is still very far away due to the reasons discussed in Chapter I. Nuclear magnetic resonance spectroscopy or nuclear Overhausser (nOe) enhancement analysis are also not possible because of the large size of the enzyme.

However, fluorescence resonance energy transfer study (FRET) between a suitably placed donor and acceptor is a technique of choice in such cases. The limits of this technique is usually within 15 to 75 Å and therefore can act as a specific "spectroscopic ruler" (Stryer, 1978). This has been used in a few cases to determine the distances among various positions on *E. coli* RNA polymerase (Wu *et al*., 1976; Chatterji *et al*., 1986; Wu and Tyagi, 1987). However, the main requirement for the energy transfer measurements to be carried out between two distinct sites on a macromolecule is the precise location of such sites, and secondly two probes in which one would act as a donor of fluorescence energy, which would be accepted by an acceptor through dipolar energy transfer. As the main intention here was to study the proximity relationship between the substrate and inhibitor binding sites of the enzyme, the precise location of these sites on the enzyme was not the primary concern. In this case, rifampicin is non-fluorescent with two absorption peaks, thereby almost fixing its role as an acceptor on the enzyme. If the substrate was to act as a donor, it has to be labelled with a fluorescent probe.

The distance calculation by FRET technique requires the correct estimation of a factor called orientation factor "K²", which relates the distance dependence with the orientation of the donor and acceptor with respect to each other (Hillel and Wu, 1976). However, if they are fixed on a particular position or if their mobility is restricted, then certain uncertainty arises resulting from the erroneous estimation of "K²". However, due to the degeneracy of the excited
state, such orientation problems have no major effect in FRET when metal ions are used as donor or acceptors (Stryer, 1978). The lanthanide metal ion Tb(III) is a very good donor in that respect. Although it forms complexes with all the nucleotides, it is GTP alone or other xanthine-containing nucleotides which shows remarkable fluorescence signals with Tb(III) (Fig. 1). Free terbium exhibits a very low level of intrinsic fluorescence and the binding of guanosine nucleotide enhances this fluorescence tremendously with two major emission peaks at 488 and 545nm (Formoso, 1973; Chatterji, 1986; 1988). The two emission peaks are generated from the $5D_4 \rightarrow 6F_6$ and $5D_4 \rightarrow 7F_0$ transition of the metal ion (Horrocks and Albin, 1984; Fig.2). The enhancement of fluorescence occurs because of the resonance energy transfer from the excited triplet state of the nucleotide to the lanthanide ion (Formoso, 1973, Gross and Simpkins, 1981). Other nucleotides such as ATP, CTP and UTP do not enhance the terbium fluorescence significantly (Formoso, 1973; Topal and Fresco, 1980). This may be because of the different electronic properties of bases or structural differences in the bases or both (Formoso, 1973). The excitation spectrum for terbium emission is always characteristic of the ligand (Morley et al., 1981).

Terbium complexes of free nucleotides are much more fluorescent than their polymers, and the base paired nucleotides as in double stranded DNA gives no fluorescence (Topal and Fresco, 1980). The fluorescence intensities of different guanosine nucleotides with terbium are in the order GTP> GDP> GMP (Topal and Fresco, 1980). The fact that a mixture of guanosine and terbium is not fluorescent suggests that the actual binding of terbium and the nucleotide is necessary for fluorescence enhancement, and the binding is mainly to the phosphate group. The terbium chelate of GTP was made use of in the present study as a fluorescent analogue of GTP.
**Figure 1.** The emission and corrected excitation spectra of EDTA complex of Tb(III). $E_x$, excitation spectrum at $\lambda_{em} = 545\text{nm}$; $E_m$, emission spectrum at $\lambda_{ex} = 250\text{nm}$. For Tb-GTP complex, the excitation wavelength used was $295\text{nm}$.


**Figure 2.** Energy level diagram of the lanthanide metal ions. —, lowest luminescent level; ---, highest level of the ground state. Fluorescent emission in Terbium occurs mainly during the transition from 5D$_4$ to 7F$_0$ and 5D$_4$ to 7F$_6$. 
The measurement of spatial separation between two sites on a macromolecule is based on the resonance transfer of energy from the excited singlet state of a fluorophore (donor) at one site to an acceptor group at the other site (Forster, 1948). Since the rate of energy transfer is dependent on the donor-acceptor separation, energy transfer can be used to measure distances in the range of 15 - 75 Å (Stryer and Haugland, 1967).

An important requirement for the resonance interaction resulting in energy transfer is that the absorption spectrum of the acceptor must overlap the emission spectrum of the donor (Cantor and Shimmel, 1980). In the present system, the separation has to be measured between rifampicin and a suitable nucleotide substrate bound in their respective sites on the RNA polymerase. Rifampicin can act as a very good acceptor in a broad range of the visible spectrum (Bahr et al., 1976). Interestingly the emission bands of the Tb-GTP complex overlaps with one of the absorption bands of rifampicin forming a Forster's donor-acceptor pair. This has facilitated the measurement of the separation between rifampicin and the Tb-GTP complex bound to the RNA polymerase.

5.2. MATERIALS

Terbium chloride was purchased from Aldrich, and its concentration in the prepared solution was determined by fluorimetric titration with standard dipicolinic acid solution as described by Barela and Sherry (1976). All the other materials were procured as mentioned in the previous chapters.
5.3. METHODS

5.3.1. Abortive initiation reaction using Tb-GTP as substrate: The abortive synthesis of pppGpC at the T7A2 promoter was described in the previous chapter. To confirm that the terbium chelate of GTP can bind to the initiation site and can serve as a substrate for the dinucleotide synthesis, the same abortive initiation reaction was carried out in the presence of 200μM GTP, 200μM terbium chloride and 6μM α-32P-CTP(1μCi/nmol). The Tb-GTP was found to be incorporated in the place of GTP and the product, a terbium complex of pppGpC could be separated from the mononucleotides in a Bio-Gel P2 column (0.7 cm x 45 cm) developed with Tris.Cl (pH 7.5), 0.1mM EDTA buffer.

5.3.2. Fluorescence measurement: All fluorescence measurements were performed in the buffer containing 10 mM Tris.Cl(pH7.5) and 100mM KCl. Just before each experiment, the enzyme was dialysed against the above buffer to minimise the concentration of glycerol, which otherwise interferes with the reproducibility of the Tb(III) emission signal. It was also found that the optimum pH of the medium was 7.5 with respect to the enzyme activity and the stability of Tb(III) in the solution. At higher pH, Tb(III) tends to precipitate out as Tb(OH)₃ on long standing. Complexes between Tb(III) and GTP were prepared by mixing them at appropriate pH. Fluorescence measurements were carried out in a Hitachi-F4000 spectrofluorimeter with spectral correction. A few uncorrected spectra were also recorded by using a Hitachi 650-10S spectrofluorimeter. The concentration of the samples were adjusted to keep the absorption at the excitation wave length low in order to avoid inner filter quenching. All spectral measurements were carried out at 24°C.
5.3.3. **Quantum yields of the samples:** The quantum yield, $Q_s$ of a sample was calculated from the absorbance ($A$) and the area enclosed by the corrected emission spectrum by using the formula

$$Q_s = \frac{Q_R (1-10^{-A_R \text{area}_s}) n^2}{\text{area}_R (1-10^{-A_s} n_s^2)}$$

where $n$ is the refractive index of the solvent and the subscripts $S$ and $R$ refer to the sample and reference respectively. Both quinine sulfate and fluorescein in 1N H$_2$SO$_4$ and 0.1N NaOH respectively were taken as references (Cantor and Shimmel, 1980) and the internal consistency of the result was checked by measuring the quantum yield of one with respect to the other. The areas of the corrected emission spectra were calculated by using the computer paired with the spectrofluorimeter.

5.3.4. **Measurement of the distance between the donor and the acceptor by the application of Forster's theory:** The donor in this case was the Tb-GTP complex bound to RNA polymerase, and rifampicin was the acceptor. In Forster's theory of dipole--dipole energy transfer (Forster, 1948), the transfer efficiency ($T$) is related to the distance '$r$' (angstroms) between the donor and the acceptor according to the equation

$$r = R_o [(1/T) - 1]^{1/6} \quad \text{or} \quad T = r^{-6}/(r^{-6} + R_o^{-6})$$

$T$ can be experimentally determined from the quantum yields according to the relation

$$T = 1 - (Q/Q_o)$$
where $Q$ and $Q_0$ are the quantum yields of the donor in the presence and absence of the acceptor.

$R_0$, called the characteristic transfer distance is the distance (in angstroms) at which the transfer efficiency equals 50%, and is given by

$$R_0 = 9.79 \times 10^3 \left[ \langle J \rangle Q(n^4)(K^2) \right]^{1/6}$$

(4)

where $n$ is the refractive index of the medium between the donor and the acceptor, and was taken as 1.4. $J'$, the spectral overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor, was approximated by the summation

$$J(M^{-1} \, cm^3) = \frac{\int F_d(\lambda) \epsilon_a(\lambda) \lambda^4 \, d\lambda}{\int F_d(\lambda) \, d\lambda}$$

(5)

where $F_d(\lambda)$ and $\epsilon(\lambda)$ are the relative fluorescence intensity (%) of the donor and the molar extinction coefficient $(M^{-1} \, cm^{-1})$ of the acceptor respectively and $\lambda$ is the wavelength at nanometer interval $d\lambda$. A small FORTRAN program written for the calculation of $J$ is given at the end of the chapter as an appendix.

The major uncertainty with the calculation of $R_0$ is the orientation factor $K^2$ which is not usually known, and is taken as an average value of $2/3$ applied for a spherical geometry. However, if either the donor or the acceptor is a metal ion as in our case, then because of the degeneracy of the excited state, the $K^2$ value of $2/3$ is a very good approximation, and the maximum error limit in the distance measurement lies within 10% (Stryer, 1978; Holmquist, 1980). It
should also be emphasized that the discrepancy in the values of $K^2$ or $n$ is not reflected in $R_0$ as much because of the sixth root dependence.

5.3.5. NMR measurements: Both the $^1$H and $^{31}$P NMR measurements were carried out by using a Varian CFT-20 or a Bruker AM-300 NMR machine operating at 80 and 300 MHz respectively for proton.

5.4. RESULTS

5.4.1. Binding of Tb-GTP to RNA polymerase: Although there are various reports on the nature of terbium binding with guanosine nucleotides either as free nucleotides or as part of DNA and RNA (Topal and Fresco, 1980; Gross and Simpkin, 1981; Chatterji, 1986, 1988), no quantitation has so far been attempted on the stability of Tb-GTP by fluorimetric assay. Knowledge about the stability of the complex was necessary for use as a substrate analogue for RNA polymerase. Therefore a detailed analysis of the binding of Tb(III) to GTP using sensitized lanthanide fluorescence was undertaken. TbCl$_3$ in 10mM Tris.Cl(pH 7.5) when excited at 295nm does not show any appreciable fluorescence in the visible region. But, as mentioned earlier, it gives two major peaks at 488nm and 545nm upon complexation with GTP. The binding of Tb(III) to GTP was quantitated by titrating a fixed concentration of GTP with varying concentrations of Tb(III) and monitoring the fluorescence emission at 545nm (Fig. 3a). Analysis of the titration data was carried out in the following way (Topal and Fresco, 1980): if there are $n$ number of binding sites available on GTP for Tb(III), then the mean association constant $K$ between Tb(III) and GTP would be
\[ K = \frac{[\text{bound GTP}]}{\text{free GTP} \cdot (n[Tb(III)] - [\text{bound GTP}])} \]  

(6)

which on rearranging becomes

\[ \frac{[\text{Tb(III)}]}{[\text{bound GTP}]} = \frac{1}{nK[\text{free GTP}]} + \frac{1}{n} \]  

(7)

In Fig. 3a, the point at which the fluorescence intensity curve reaches saturation is taken as the concentration of Tb(III) at which all the GTP molecules are terbium-bound. The concentration of bound and free GTP at the lower concentrations of Tb(III) were then calculated from the fluorescence emission. According to equation (7), when \( \text{Tb(III)}/[\text{bound GTP}] \) is plotted against \( 1/[\text{free GTP}] \) (Fig. 3b), the intercept of the curve on the Y-axis gives the value of \( n \), and the \( K_d \) value for Tb-GTP complex can be calculated by dividing the slope of the curve by the intercept. Accordingly, a \( K_d \) value of 0.2\( \mu \)M was obtained with \( n \) equal to 3. Similar values were obtained when the titration was performed with another fixed concentration of GTP. It should be mentioned here that the complexometric titration carried out earlier between various lanthanides and nucleotides estimated a \( K_d \) value of the same order (Morrison and Cleland, 1983). Here, the dissociation constant of 0.2\( \mu \)M with three binding sites represents an average value. However, the affinities between each of these sites and Tb(III) may not be very different from each other if the nature of the coordinating ligand is the same. When the \(^1\)H and \(^{31}\)P NMR spectra of GTP and Tb-GTP complex were recorded, the broadening of all the phosphorus peaks was observed in the complex without much change in the resonance of
Figure 3. Fluorimetric titration of GTP with terbium: (a) fluorescence emission at 545 nm of Tb-GTP complex formed at different concentration of Tb (III) and a fixed concentration (10 μM) of GTP. The excitation wavelength is 295. (b) Analysis of the titration curve in (a) according to equations 6-7 in the text.
Fig. 3
ring protons (Fig. 4). These experiments indicated that Tb(III) binds to GTP mainly through phosphate co-ordination. It should be mentioned here that although a high concentration of GTP was necessary for clarity of the signals, the 3:1 stoichiometry of the Tb-GTP complex was maintained as in the fluorescence experiments. However, a 1:1 complex of Tb-GTP also showed broadening of the phosphate resonances.

When RNA polymerase was added to the Tb-GTP (3:1) complex, it was observed that the intensity of fluorescence emission diminished. As RNA polymerase alone in this concentration range did not induce any enhancement of the emission signals of Tb(III), it is proposed that the decrease in Tb-GTP emission signals in the presence of the enzyme is due to the complex formation between the GTP and RNA polymerase. Fig. 5 shows the emission peaks of Tb-GTP with varying concentrations of the enzyme. Both the curves represent the formation of the same complex monitored at 488 and 545 nm emission bands respectively, and the $K_{ct}$ value of the complex obtained from the half-maximal saturation was 4 µM. Thus the stability of the Tb-GTP complex ($K_d = 0.2$ µM) is much more than that of the second complex between Tb-GTP and RNA polymerase ($K_d = 4$ µM) and the dissociation of the Tb-GTP in the presence of the enzyme is expected to be insignificant. However, the value of $K_d$ between purine nucleotides and RNA polymerase was reported to be 150 µM in the absence of a template (Wu and Goldthwait, 1969a; Chatterji, et al., 1984). This apparent discrepancy in the $K_d$ values raised the question whether Tb-GTP binds to RNA polymerase at the same site as GTP.
Figure 4. $^{31}$P - Nuclear magnetic resonance spectroscopic analysis of GTP (top) and 3:1 Tb-GTP complex (bottom). The Tb-GTP complex shows broadening of all the three phosphate peaks ($\alpha$, $\beta$ and $\gamma$) of the GTP.
Figure 5: Fluorescence quenching titration of Tb-GTP (3μM Tb (III) and 1μM GTP) with varying concentrations of RNA polymerase in 10 mM Tris.Cl (pH 7.5) and 100 mM KCl. (O---O) 545 nm emission of Tb-GTP; (●---●) 488 nm emission bond of Tb-GTP. Excitation wavelength is 295nm.
Fig. 5
5.4.2. Abortive initiation of transcription with Tb-GTP: In order to solve the above discrepancy, the Tb-GTP complex was used in the abortive synthesis of pppGpC at the T7A2 promoter. Fig. 6 shows the Bio-Gel P2 profile of the abortive initiation reaction in the presence of only Tb-GTP and radiolabelled CTP. It can be seen from the figure that the faster moving peak, presumed to be a Tb(III) complex of pppGpC is fluorescent, indicating that Tb-GTP binds to the initiation site of the RNA polymerase. As the abortive initiation of transcription needs to be carried out at pH 7.9, we have used 1:1 Tb-GTP complex in this case to avoid precipitation of Tb(III).

5.4.3. Binding of rifampicin to Tb-GTP-RNA polymerase: Rifampicin has two absorption peaks, one around 330nm and the other at the longer wave length of 470nm (Bahr et al., 1976). Therefore, in principle it can form a Forster's donor-acceptor pair with Tb-GTP provided the two are located at suitable distances. It was indeed observed that upon addition of rifampicin to Tb-GTP-RNA polymerase complex, both the emission bands of Tb-GTP were quenched as shown in Figure 7. Rifampicin is known to form a 1:1 complex with E. coli RNA polymerase (Gurgo, 1980). It was observed that the titration of Tb-GTP-RNA polymerase emission band with rifampicin was complete when rifampicin:Tb-GTP-RNA polymerase ratio was around 0.7:1. Deviation from the stoichiometric quenching may arise due to the presence of only 60-70% of active RNA polymerase molecules in any given preparation (vide Chapter II).

5.4.4. Calculation of the distance between the donor (Tb-GTP) and the acceptor (rifampicin) on RNA polymerase: In the present system, the donor of fluorescence emission was the Tb-GTP complexed with RNA polymerase and
Figure 6: Synthesis of the Tb(III) complex of pppGpC by RNA polymerase at the T7A2 promoter. The reaction was carried out in a 100μl solution containing 40mM Tris.Cl (pH 7.9), 5mM MgCl₂, 200μM GTP, 200μM TbCl₃, 3μM α-³²P-CTP (1μCi/nmol), 0.2mM DNA-phosphorus, and 40μg/ml RNA polymerase, for 30min at 37°C. (a) The whole reaction mixture was loaded into a Bio-Gel P2 column and fractionated with TE buffer. The radioactivity peak I corresponds to the product, and II, the unincorporated substrates. (b) Fluorescence spectra of the pooled fractions when excited at 295 nm.
Fig. 6
Figure 7: Titration of the corrected fluorescence emission of Tb-GTP-RNA polymerase complex (3μM Tb(III), 1μM GTP, 10μM RNA polymerase) with increasing concentrations of rifampicin at 545 nm. The excitation wavelength was 295 nm. FI indicates the relative fluorescence intensity.
Fig. 7

(Rifampicin: Tb GTP RNA Polymerase)
not the Tb-GTP alone, as the enzyme has quenched the fluorescence signal of Tb-GTP partly. But the actual emission occurs from the bound terbium. From the $K_d$ values described earlier, the percent of GTP bound to Tb(III) at 3$\mu$M Tb(III) and 1$\mu$M GTP (see Fig. 3b) is calculated to be 80%. Similarly the percentage of substrate bound on the enzyme at the saturation concentration of RNA polymerase (10$\mu$M see Fig. 5) is calculated to be 70%. Therefore all the relative fluorescences (percentage) have been corrected for 100% occupancy of substrate on RNA polymerase. Fig. 8 shows the spectral overlap between the fluorescence bands of the donor and the absorption band of the acceptor. It can be noted that both the emission bands of Tb(III) shows finite spectral overlap with rifampicin absorption. Although the emission band at 545nm had much less overlap in comparison to the 488nm band the fluorescence intensity at 545nm was much higher and thus made the overlap integral value significant. The values of quantum yield, overlap integral, $R_0$, energy transfer efficiency and the distance between the donor and the acceptor calculated from equations, 1-5 are given in Table I.

5.5. DISCUSSION

Resonance energy levels of Tb(III) fortuitously overlap with the triplet energy state of the ligand GTP, irradiated with ultraviolet light. As a result, an intermolecular energy transfer from the organic ligand to the central metal atom takes place along with a slight radiationless deactivation of the ions themselves. Thus, when excited with appropriate light (\( \lambda = 295\text{nm} \)), the Tb-GTP complex shows two emission signals at 488 nm and 545nm. Tb (III) also shows enhanced emission signals with various proteins, provided its binding site is located near one of the aromatic amino acids. This has been efficiently
Figure 8: Spectral overlap between the absorption spectrum of rifampicin (●—●) and the corrected fluorescence emission spectrum of Tb-GTP-RNA polymerase complex (----), when excited at 295 nm. The rifampicin absorption spectrum was recorded in a buffer containing 10 mM Tris.Cl (pH 7.5) and 100 mM KCl.
Fig. 8

(——) Extinction coefficient $\times 10^{-3}$ (M$^{-1}$ cm$^{-1}$)

(-----) Relative Fluorescence Intensity

$\lambda$ (nm)

400 420 440 460 480 500 520 540 560 580 600
Table I  Spectral overlap integral between the donor (Tb-GTP-RNA polymerase complex) and the acceptor rifampicin, quantum yield of the donor, energy-transfer efficiencies, and distance between the donor and the acceptor in the RNA polymerase-rifampicin complex

<table>
<thead>
<tr>
<th>Sample</th>
<th>Emission wavelength (nm)</th>
<th>J (M⁻¹cm⁻³) spectral overlap integral</th>
<th>Q (Quantum yield)</th>
<th>R₀(Å) (distance at 50% energy-transfer efficiency)</th>
<th>T (energy-transfer efficiency)</th>
<th>r(Å) (distance between donor and acceptor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tb-GTP-RPase</td>
<td>488</td>
<td>-</td>
<td>0.046</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tb-GTP-RPase + rifampicin</td>
<td>488</td>
<td>6.56 x 10¹⁴</td>
<td>0.034</td>
<td>27.8</td>
<td>0.26</td>
<td>33.1</td>
</tr>
<tr>
<td>Tb-GTP-RPase</td>
<td>545</td>
<td>-</td>
<td>0.072</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tb-GTP-RPase + rifampicin</td>
<td>545</td>
<td>1.52 x 10¹⁴</td>
<td>0.058</td>
<td>23.5</td>
<td>0.19</td>
<td>29.9</td>
</tr>
</tbody>
</table>

*Energy-transfer measurements were carried out with the Tb-GTP complex (3μM Tb, 1μMGTP) bound to 10μM RNA polymerase; rifampicin concentration was 10μM. RPase=RNA polymerase.*
utilized in the past to map the terbium binding sites on proteins with respect to other chromophores (Luk, 1971, Berner et al., 1975; Horrocks et al., 1975). However, the present study shows the overlap of absorption of the acceptor with both the emission peaks of the donor. Therefore it was possible to calculate the $R_0$ values from the quenching of both the emission signals of the donor independently in the presence of the acceptor. Ideally in such cases, both $R_0$ and $r$ calculated from the two emission peaks should be the same, as they represent the distance between the same two points. Table I reveals that the discrepancy in the values of $R_0$ and $r$ from the 488nm and 545nm bands are within 10-20%, which is the experimental and theoretical limit of fluorescence energy transfer.

Meares and Rice (1981) applied their rapid-diffusion limit energy transfer technique to determine how accessible the RNA polymerase bound rifampicin and the dye cibacron blue were to solvent by using freely diffusing neutral complexes of Tb(III) as luminescent energy donors. These studies have shown that the rate of energy transfer from the Tb(III) chelate to the enzyme-bound rifampicin was about half as large as for free rifampicin in solution. For the transfer of energy from the freely diffusing energy donors to acceptors, intermolecular contact between the donor and the acceptor is necessary. This mechanism has a very sharp distance dependence; it drops off much more rapidly than the dipole-dipole mechanism. However, in our case the donor is not an inert complex of Tb(III) and it binds strongly to the protein. Moreover the direct collision between the donor and the acceptor is not possible as the rifampicin binding domain of RNA polymerase is thought to be within a crevice formed by subunit-interaction (Rice and Meares, 1981; Jin and Gross,
Therefore the diffusion-enhanced energy transfer in this case is extremely unlikely, and hence the data is interpreted in terms of a static model. As was mentioned earlier, Tb(III) has three binding sites on GTP and therefore a 3:1 complex between Tb(III) and GTP (3μM Tb(III) and 1μM GTP) is the best suited for the energy transfer measurements, with 80% of the GTP bound to Tb(III). The free metal ion is non-fluorescent and does not show any appreciable emission in the presence of the enzyme alone. The 70% complexation of the labelled substrate with the enzyme, and its incorporation into the template-directed synthesis of RNA again indicate that the interpretation of the data on the basis of dipole-dipole energy transfer is probably correct. Moreover, the observation that the titration curve of the emission signals of Tb-GTP-RNA polymerase reached saturation in the presence of a stoichiometric amount of rifampicin support this contention.

The most striking result emerging from the present study is the large distance of around 30Å between the initiation nucleotide binding site and the rifampicin binding site on the RNA polymerase. By assuming a spherical shape to the β subunit, its radius has been estimated to be 37Å (Wu et al., 1976). Therefore it appears that the initiation nucleotide site and rifampicin binding site are located on opposite sides of the β subunit. Such a large distance indicates that rifampicin-induced inhibition of transcription initiation by RNA polymerase is not competitive. Rather, the antibiotic may probably induce a conformational change in the enzyme thereby altering the geometry of the substrate binding site. This distance also does not favour a steric hindrance by the antibiotic on the translocation step that occurs after the formation of the first phosphodiester bond during transcription initiation.
Figure 9: Schematic representation of the different functional domains on the RNA polymerase (see Table II). I, initiation site; E, elongation site; Zn, intrinsic Zn in the β subunit; S, surface of the σ subunit; R, rifampicin binding site.
I = Initiation site
E = Elongation site
Zn = Intrinsic "Zn" in β subunit
S = Surface of σ subunit
R = Rifampicin binding site

Fig. 9
Table II  The various distances obtained between specific points on the RNA polymerase by different techniques

<table>
<thead>
<tr>
<th>system</th>
<th>distance(Å)</th>
<th>technique</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Rifampicin binding site - surface of σ subunit</td>
<td>10</td>
<td>fluorescence energy transfer</td>
<td>Wu et al. (1976)</td>
</tr>
<tr>
<td>(ii) Intrinsic metal in β subunit - 132-SH of σ subunit</td>
<td>22</td>
<td>fluorescence energy transfer</td>
<td>Chatterji et al. (1986)</td>
</tr>
<tr>
<td>(iii) Intrinic metal in β subunit - initiation site</td>
<td>17</td>
<td>fluorescence energy transfer</td>
<td>Wu and Tyagi (1987)</td>
</tr>
<tr>
<td>(iv) Rifampicin binding site - substrate binding site</td>
<td>30</td>
<td>fluorescence energy transfer</td>
<td>the present study</td>
</tr>
<tr>
<td>(v) Intrinsic metal in β subunit - substrate binding site</td>
<td>4</td>
<td>NMR</td>
<td>Chatterji et al. (1984)</td>
</tr>
<tr>
<td>(vi) Initiation site - elongation site</td>
<td>6</td>
<td>EPR</td>
<td>Chuknyiski et al. (1986)</td>
</tr>
</tbody>
</table>
Earlier, fluorescence energy transfer measurements by Wu et al. (1976) had shown the rifampicin binding site on the *E. coli* RNA polymerase to be within 10Å from the surface of the σ subunit. Similarly, the distance from the σ subunit to the intrinsic metal-containing initiation site has been measured to be 22Å (Chatterji et al., 1986). Considering that the metal is located about 4Å away from the substrate-binding site (Chatterji et al., 1984), the distance calculated in this study may be represented as a schematic diagram as shown in Fig. 9. But this is only a representation of the various distances in two dimension and their orientations can be different. Such a depiction of *E. coli* RNA polymerase was reported earlier by Hillel and Wu (1977). Over the years various spectroscopic studies have measured the distances among various functional domains on RNA polymerase (Table II) which are also shown in Fig.9.

APPENDIX

The following FORTRAN program was used to calculate the spectral overlap integral J.

```fortran
REAL WL (999), EA (999), FI (999)

Z = 470
DO 101 I = 1, 41
   WL (I) = Z + 1
101  Z = WL (I)
DO 102 I = 1, 41
102  Read (1, *) EA (I), EI (1)
   OV = 0
   AO = 0
   DO 103 I = 1, 41
```

93
\[ OV = OV + (FI(I) \times EA(I) \times (WL(I) \times 4 \times 1E-35) \]

103 \[ AV = AV + FI(I) \times 0.0000001 \]

\[ AJ = OV/AV \]

WRITE (G, *) 'J factor (cm + 3m^-1) = ', AJ

STOP