Fluorescence Energy Transfer between the Primer and the $\beta$ Subunit of the DNA Polymerase III Holoenzyme*

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We report here our initial success in using fluorescence energy transfer to map the position of the subunits of the DNA polymerase III holoenzyme within initiation complexes formed on primed DNA. Using primers containing a fluorescent derivative 3 nucleotides from the 3'-terminus and acceptors of fluorescence energy transfer located on Cys333 of the $\beta$ subunit, a donor-acceptor distance of 65 Å was measured. Coupling this distance with other information enabled us to propose a model for the positioning of $\beta$ within initiation complexes.

Examination of the fluorescence properties of a labeled primer with the unlabeled $\beta$ subunit and other assemblies of DNA polymerase III holoenzyme subunits allowed us to distinguish all of the known intermediates of the holoenzyme-catalyzed reaction. Specific fluorescence changes could be assigned for primer annealing, Escherichia coli single-stranded DNA-binding protein binding, 3'→5' exonucleolytic hydrolysis of the primer, DNA polymerase III* binding, initiation complex formation upon the addition of $\beta$ in the presence of ATP, and DNA elongation. These fluorescence changes are sufficiently large to support future detailed kinetic studies. Particularly interesting was the difference in fluorescence changes accompanying initiation complex formation as compared to binding of DNA polymerase III holoenzyme subunit assemblies. Initiation complex formation resulted in a strong fluorescence enhancement. Binding of DNA polymerase III* led to a fluorescence quenching, and transfer of $\beta$ to primed DNA by the $\gamma\delta$ complex did not change the fluorescence. This demonstrates a rearrangement of subunits accompanying initiation complex formation. Monitoring fluorescence changes with labeled $\beta$, we have determined that $\beta$ binds with a stoichiometry of one monomer/primer terminus.

The DNA polymerase III holoenzyme is the multisubunit complex that is responsible for the majority of replicative DNA synthesis in Escherichia coli (for review, see McHenry (1988) 1). The $\beta$ subunit can be chromatographically resolved from the holoenzyme to yield pol III* 2, a subassembly of the remaining subunits (Wickner and Kornberg, 1973; McHenry and Kornberg, 1977). The $\beta$ subunit plays a significant role in the processivity of the holoenzyme (Fay et al., 1981, 1982; LaDuca et al., 1986). The holoenzyme can remain bound to a template for >40 min (Johanson and McHenry, 1982). If the dynamic affinity for the template during elongation is the same as the static affinity in isolated complexes, the holoenzyme is sufficiently processive to replicate the entire E. coli chromosome without dissociating. Experimentally, incorporation of >150,000 nucleotides in a single processive event has been demonstrated (Mok and Marians, 1987). In contrast, pol III* adds only 200 nucleotides to a primer before it dissociates from the template (Fay et al., 1982).

$\beta$ is required for initiation complex formation, the step preceding processive elongation. Initiation complexes are sufficiently stable that they can be isolated by gel filtration (Wickner and Kornberg, 1973; Wickner, 1976; Burgess and Kornberg, 1982a, 1982b; Johanson and McHenry, 1980, 1982). Upon the addition of a deoxyribonucleoside triphosphate mixture, the entire template is processively replicated. $\beta$ becomes buried within the initiation and elongation complexes, sterically precluding antibody attachment (Johanson and McHenry, 1980, 1982). It is also possible to clamp $\beta$ onto the primer in an ATP-dependent reaction that requires only the $\gamma$ and $\delta$ holoenzyme subunits (Wickner, 1976; O'Donnell, 1987). The DNA polymerase III core can then interact with this subassembly to form a processive complex.

The subunit arrangement within the holoenzyme is currently defined by genetic evidence and the ability to isolate certain active subassemblies (see McHenry (1988) 2). To refine our knowledge of the structure, we have initiated a study using fluorescence energy transfer to map out the distances between selected sites within the holoenzyme and to orient these sites with regard to the primer. This approach demands that a subunit be site-specifically labeled with a fluorescence donor or acceptor and retain its ability to reconstitute into the holoenzyme. The $\beta$ subunit presented a good choice since conditions have been found that permit selective labeling of cysteine 333 with fluorescent maleimides with retention of activity (Griep and McHenry, 1988, 1990). In this study, we used fluorescent primers and $\beta$ labeled with a fluorescence acceptor at cysteine 333 to determine primer-$\beta$ distances. In control experiments using unlabeled $\beta$ and fluorescent

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2 The abbreviations used are: pol III*, form of DNA polymerase III that contains all of the holoenzyme subunits except $\beta$; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; SSB, E. coli single-stranded DNA-binding protein; RXM-$\beta$, $\beta$ labeled at cysteine 333 with rodamine X maleimide; TMX-$\beta$, $\beta$ labeled at cysteine 333 with tetramethylrhodamine maleimide; ssDNA, single-stranded DNA; dNTP, deoxynucleoside triphosphate; dAMPoS, deoxyadenosine 5'-O-(1-thiomonophosphate).
primes, we found that we could distinguish all of the known intermediates and products of the holoenzyme reaction.

**EXPERIMENTAL PROCEDURES**

**Proteins and DNA Template**—The following proteins were isolated by standard procedures: holoenzyme (8.5 × 10⁷ units/mg) (Ohno, F., and McHenry, 1987); pol II′ (2.9 × 10⁶ units/mg) (Fay et al., 1982); anti-β IgG (8.4 mg/ml) (Johnson and McHenry, 1980); SSB (Grieb and McHenry, 1988); β (2.3 × 10⁶ units/mg) (Joehan et al., 1986); RMX-β (75% active) (Grieb and McHenry, 1988); and TMRM-β (24% active) (Grieb and McHenry, 1988). The labeled β subunits had homogeneity with the unlabeled β subunits because of increased susceptibility to cyanateinactivation when stored at −70 °C. The DNA polymerase III core (7.1 × 10⁶ units/mg) and the γ complex (6.8 × 10⁵ units/mg) were gifts of JoAnna Reems and Dr. John A. Hughes, Jr. (of this laboratory). T7 DNA polymerase with genetically inactivated 3′ → 5′ exonuclease activity (Tabor and Richard, 1989) was purchased as Sequasyme from U. S. Biochemical Corp. The 11,036-nucleotide ssDNA template used in the fluorescence studies was pDSS2-46 (Tomasiewicz and McHenry, 1987). The template was a gift of Mary Ann Franzen (of this laboratory); it was isolated using standard procedures (Johnson and McHenry, 1984). An extinction coefficient of 7370 (m nucleotide)⁻¹ cm⁻¹ at 260 nm (Berkovitz and Darnell, 1973) was determined to determine the concentration of template.

The activities of the holoenzyme and the holoenzyme reconstituted from pol III and β were determined in the usual manner (Johnson and McHenry, 1984), except that the enzyme dilution buffer was 50 mM HEPES, pH 7.5, 100 mM potassium glutamate, 10 mM dithiothreitol, and 10 mM magnesium acetate, which more closely reflected the conditions in the fluorescence experiments. The activity of the holoenzyme obtained with this buffer was ∼1.4 times greater than that obtained with the standard buffer.

**Fluorescent Primer-Template**—A 24-mer oligonucleotide (complementary to M13mp18 at nucleotides 991–1015) (Yanisch-Perron et al., 1985) was synthesized on a BioSearch 8600 DNA synthesizer by Janine Mills. Cytofine Controlled-Pore Glass (1 µmol) was the support for the synthesis and, as such, became the 3′-ribonucleotide terminus to an otherwise deoxynucleotide primer. Instead of a thymidine at the penultimate position, a 5-amino-propydeoxyuridine was incorporated. The β-cyanoethyl phosphoramidite of this molecule was prepared by JoAnna Reems by modification of the methods of Gibson and Benkovic (1987); Allen and Benkovic (1989); Allen et al. (1989), and Catalano et al. (1990). The 5′-dimethoxytrityl group was left on the oligonucleotide, but the other protecting groups were removed by published procedures (Hagerman, 1985). The oligonucleotide was purified by HPLC on a Waters μBondapak C₈ column. A 40-min (1 ml/min) linear gradient was run from 10% acetonitrile, 90 mM triethylammonium acetate, pH 8.0, to 30% acetonitrile, 70 mM triethylammonium acetate, pH 7.0. The 260 nm was monitored. The triethyl-protected oligonucleotide eluted at 26% acetonitrile, well resolved from the failure sequences and the removed protecting groups (16% acetonitrile and below). The collected oligomer was dried in a Savant Speed Vac, suspended in 95% ethanol, and evaporated a second time.

The oligomer was prepared for fluorescent labeling by first removing the triethyl protecting group in a 10-min incubation of the sample in 100 µl 6% acetic acid. The sample was dried to completion in a Savant Speed Vac. Then 20 µl of H₂O and 40 µl of 0.5 M sodium carbonate, pH 9, were added; and the sample was dissolved. Sixty microliters of 600 nmol of fluorescein isothiocyanate in dimethylformamide, pH 10, was added and allowed to react for 72 h at room temperature before it was purified on a Waters C₈ reversed-phase HPLC column (3.9 × 300 mm). The absorbance at 260 nm was monitored on a Kratos Spectroflow 757 detector, and the fluorescence was monitored with a Kratos Spectroflow 980 fluorescence detector by excitation at 426 nm and emission through a Schott OG-515 long-pass filter. A 20-ml unlabeled linear gradient was run from 10% acetonitrile, 90 mM triethylammonium acetate, pH 8.0, to 20% acetonitrile, 80% triethylammonium acetate. The fractions were collected for the peak at 19% acetonitrile, which was both absorbing and fluorescent. This fluorescent peak was resolved from the unlabeled oligomer that eluted at 17% acetonitrile. Even though only 73% of the total oligomer was labeled under these conditions, this procedure gave 16% acetic acid. Add 100% of the labeled oligomer, which is important in our experiments. The fluorescent oligomer was dried down in a Savant Speed Vac; resuspended in 100 µl of 100 mM Tris-HCl, pH 7.5, 1 mM EDTA; and stored at −20 °C until used.

**Fluorescent Primer-Template Resistant to Exonuclease**—The fluorescent 24-mer oligonucleotide was annealed to the template, and 3′-terminal [³²P]dAMP·OS incorporated onto the primer by the following procedure. Thirty picomoles of fluorescent oligomer (1.0 µM final concentration) and 30 pmol of template (0.5 µM final concentration) were added to a buffer consisting of 7.5 mM Tris, 40 mM HEPES, pH 7.5, 1 mM NaCl, 0.8 mM EDTA. The solution was incubated at 75 °C for 5 min and allowed to slowly cool to room temperature over 30 min. The primer-template solution was brought to a final volume of 200 µl and consisted of the following (final concentrations): 15 nm primer-template, 1.0 µM [³²P]dATP·OS (specific activity of 330 cpm/ fmol), 60 units of T7 DNA polymerase (4 units/pmoldomin template) in a buffer of 50 mM HEPES, pH 7.5, 65 or 120 mM NaCl, 10 mM magnesium acetate at 27 °C for 30 min; the reaction was quenched by addition of 20 mM EDTA (final concentration); and the enzyme was inactivated by heating for 10 min at 65 °C. The primer-template was separated from the free primer, unreacted nucleotide, and inactivated enzyme by gel filtration on a 2-ml Bio-Gel A-5m column (Bio-Rad). The fluorescent and radioactive primer-template was eluted in a buffer of 50 mM HEPES, pH 7.5, 100 mM potassium glutamate, 0.1 mM EDTA, 10 mM dithiothreitol. From five runs, the yield of templates on gel filtration was 87–47%. The ratio of fluorescence intensity to moles of template was the same in three runs at 31 ± 2 relative fluorescence units/pmool template, indicating that the number of annealed primer-template was constant. The ability of the labeled primer to resist the potent 3′ → 5′ exonuclease of the holoenzyme was determined as previously described (Grieb et al., 1990). The ability of the primer to be elongated was determined by adding a 45 µM dNTP mixture to a cuvette containing the initiation complex. After 1 min at 30 °C, triplicate samples were withdrawn, urea was added, and the samples were heated at 100 °C for 5 min. The products of the reaction were resolved by 8 M urea, 20% polyacrylamide (1:19 bisacrylamide: N,N-diacrylamide) gel electrophoresis. Following electrophoresis, the gel was washed for 15 min in 10% methanol, 10% acetic acid to remove the urea. The gel was dried and then exposed to Kodak XAR-5 film. The film was scanned on a Molecular Dynamics densitometer, and the radioactive intensity in each lane was quantitated by comparison to standards run on the same gel.

**Fluorescence Measurements**—The fluorescence measurements were made using a SLM-Aminco 48000 spectrofluorometer controlled by an IBM AT computer. The photomultiplier tubes had water-cooled thermoelectric housings to reduce the background signal. The sample compartment was equipped with a stirring assembly; the temperature was maintained at 30.0 ± 0.1 °C. All samples were corrected for the wavelength dependence of exciting light intensity through the use of a quartz counter, rhodamine B (Lakowicz, 1983), in the reference channel. Most measurements were performed in a buffer of 50 mM HEPES, pH 7.5, 100 mM potassium glutamate, 10 mM dithiothreitol, 1 mM magnesium acetate at 27 °C in a 2-cm quartz cuvette. The excitation and emission wavelengths were 496 and 518 nm for fluorescein, respectively. The excitation and emission bandwidths were each set at 4 nm. All measurements were corrected for the background intensity of buffer fluorescence and for dilution effects. The fluorescence energy transfer titrations were corrected for inner filter effects at the higher fluorophore concentrations and for acceptor fluorescence intensity.

**Quantum Yields**—Quantum yields were measured using the equation: ΦQ = (Fobs / Fcalc) / (Φcal / Φcalc), where Q denotes quantum yield, A absorption, F the area under the fluorescence emission curve, and the subscripts x and ref the unknown and the reference substance, respectively. The Qvalues were 0.92 for disodium fluorescein in 0.1 M NaOH (Weber and Teale, 1957).

**Fluorescence Energy Transfer**—The distance between two fluorophores, a donor and an acceptor, can be measured spectroscopically by Förster energy transfer (Streyer, 1978; Lakowicz, 1983). If the donor fluorescence (ΦD) is quenched in the presence of an acceptor fluorescence, (ΦA), then the efficiency, (E), of nonradiative energy transfer can be determined by the equation E = 1 − FD/FD. The distance (R) between the fluorophores is then obtained from the equation: R = R0 / (E1 − 1)½, where R0 is the distance at which E = 0.0. The distance (R0) can be calculated from the equation: R0 = 9786 (kQ / ΦD)½ / λ. The parameter kQ, the rate constant for the relative orientation of the transition dipoles of donor emission and acceptor absorption, and was determined by Grieb and McHenry, 1990; dos Remedios et al., 1987); n is the refractive index of the medium taken as 1.4 for protein in water, and J is the overlap integral. The spectral overlap of donor emission and acceptor
absorption was determined by the equation: \( J = \sum F_0(\lambda) a(\lambda) \lambda^4 d\lambda / \sum F_0(\lambda)d\lambda \), where \( \lambda \) is the wavelength in centimeters, \( F_0(\lambda) \) is the fluorescence at \( \lambda \) of unquenched donor, and \( a(\lambda) \) is the acceptor absorption coefficient in millimolar \(^{-1}\) centimeter \(^{-1}\). The Microsoft Multiplan program was used to calculate the \( R_0 \) values from the donor fluorescence and acceptor absorbance spectra. At 2-nm wavelength intervals, the donor fluorescence intensity was entered as were the acceptor extinction coefficients. The product of the fluorescence, the extinction coefficient, the fourth power of the wavelength in centimeters, and \( 2 \times 10^{-7} \) cm were summed together over the complete range of donor and acceptor overlap. This sum was normalized using the same procedure, yielding the contribution to \( R_0 \) from the spectral overlap.

RESULTS

Before we could determine fluorescence energy transfer between labeled primers and \( \beta \), it was necessary to determine the fluorescence changes induced by all components of our system. Thus, we embarked on a sequential study of the influence of SSB binding, polymerase binding, exonuclease digestion of the primer, and nucleotide incorporation on the fluorescence of labeled primers.

SSB Influenced Structure of Primer-Template—Direct evidence that the conformation of the primer was influenced by SSB was obtained in studies that monitored the fluorescence of the fluorescein-labeled primer when the uncomplexed ssDNA was coated with SSB (Fig. 1). The primer fluorescence decreased linearly as SSB was titrated and saturated at 31% quenching. The fluorescent quenching of the fluorescein-labeled primer-template saturated when there were 53 nucleotides bound per SSB tetramer. Consistent with an SSB-M13 ssDNA interaction, the fluorescein-labeled primer-template quenching could not be reversed by 500 mM KCl (Lohman and Overman, 1985).

In the reverse experiment, we monitored SSB intrinsic fluorescence (excitation at 300 nm, emission at 346 nm) as it was titrated with unlabeled M13-type ssDNA. The intrinsic fluorescence of SSB was linearly quenched until 45 ± 2 nucleotides of template were bound per SSB tetramer. Five different measurements were made in buffers containing various concentrations of potassium glutamate (50–250 mM). These results are consistent with SSB binding to the template in one of its high binding site size modes (Lohman and Overman, 1985; Bujalowski and Lohman, 1986; Overman et al., 1988). Thus, SSB bound to the template and altered the conformation of the fluorescein-labeled primer, resulting in fluorescence quenching.

The fluorescence quenching of the fluorescein-labeled primer following SSB binding to the template was not due to a release of the primer from the template. SSB does not induce helix melting (for a review, see Lohman et al., 1988; Glilkin et al., 1983; Langowski et al., 1985). After coating the primer-template with SSB, the complex was gel-filtered on Bio-Gel A-5m in the assay buffer. The fluorescent primer (excitation at 496 nm, emission at 518 nm) and SSB (excitation at 300 nm, emission at 346 nm) eluted in the void volume with the ssDNA (Aso), which is excluded from this gel. The primer remained bound to the template following gel filtration, indicating that SSB binding has its effect on the structure of the bound primer. The template was coated with SSB in all of the following experiments.

Holoholoenzyme Bound Primer Rapidly and Hydrolyzed It Slowly—When the fluorescence of the fluorescein-labeled primer-template was monitored after adding an excess of the reconstituted holoholoenzyme, two time-dependent enhancement phases were observed (Fig. 2). The rapid phase was half complete in 30 s and ended at a 2.0-fold fluorescence increase; this time frame corresponded to holoholoenzyme binding and formation of an active complex with the primer. The slower fluorescence increase (t<sub>1/2</sub> > 10 min (apparent from 300 to 800 s in Fig. 2)) correlated with the rate of exonuclease release of the fluoresceinlabeled nucleotide (Griep et al., 1990). The holoholoenzyme has a very potent 3'-→5' proofreading exonuclease that is capable of hydrolyzing a complete deoxynucleotide primer with a half-life of 20 s (Griep et al., 1990). For our study, a primer containing an exonuclease-resistant 3'-penultimate ribonucleotide with a phosphorothioate linkage to the terminal deoxyribonucleotide proved to be sufficiently stable. A primer with this 3'-terminus would be expected to be degraded with a half-life of ~18 min (Griep et al., 1990).

To confirm that the fluorescein-labeled primer analog actually was resistant to the 3'-→5' exonuclease of the holoholoenzyme, the 3'-terminal [55S]dAMPoS-labeled primer-template was tested under conditions identical to those described for Fig. 2. An aliquot was removed from the cuvette at specific time intervals following the addition of an excess amount of the holoholoenzyme. The moles of remaining 3'-ter-

**FIG. 1.** Titration of fluorescein-labeled primer-template with SSB. Aliquots of SSB were added to 5 nM labeled primer-template (55 μM nucleotide ssDNA) in 50 mM HEPES, pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate, 10 mM dithiothreitol, 500 μM ATP at 30°C. Fluorescence was monitored by excitation at 496 nm and emission at 518 nm.

**FIG. 2.** Time course of fluorescence changes following addition of pol III* and reconstituted holoholoenzyme to SSB-coated fluorescein-labeled primer-template. An excess of pol III* (2 units/mol of primer-template) was added to 5 nM SSB-coated labeled primer-template (concentration = primer termini) in the presence of ATP, and fluorescence quenching was measured. When excess β subunit was added, the time course was continuously monitored as described in the legend to Fig. 1. The fast phase was complete in <100 s and was attributed to initiation complex formation. The slower second phase (apparent from 300 to 800 s) was due to exonucleaseolytic primer degradation.
minal radiolabeled nucleotide were determined, and it was found that the half-life of the 3'-terminal nucleotide was 18 min (data not shown), the same as it was for the primer that lacked an attached fluorophore (Griep et al., 1990).

Since the rates of the two fluorescence enhancement phases were very different, we could determine the change resulting from the first phase of holoenzyme binding by extrapolating the linear portion of the second phase to zero time. When the holoenzyme was titrated into a solution of the fluorescently labeled primer-template, the final fluorescence enhancement saturated at 1.30 units of holoenzyme/fmol of primer-template (Fig. 3). This was close to the level of the holoenzyme required to saturate a primer as measured by our nucleotide incorporation assays (Griep and McHenry, 1989; Griep et al., 1990).

pol III* Bound to Primer-Template in Absence of β—An unexpected result was observed when pol III* was titrated into a solution of the fluorescently labeled primer-template; the primer fluorescence decreased by 16% and saturated at 1.35 units of pol III*/fmol of primer-template (Fig. 3). A stable association of pol III* with the primer has not been observed previously by physical techniques. pol III* and the holoenzyme bound to the primer-template and saturated it at similar concentrations, but caused opposite fluorescence changes.

pol III*, β, and Primer-Template Formed Fully Active Initiation Complex—When 35 nM unlabeled β was present, the binding of the reconstituted holoenzyme to the fluorescently labeled primer-template could be monitored fluorometrically (Fig. 3). The β subunit itself did not cause any fluorescence changes to the primer-template, even when present up to 180 nM. The reconstituted holoenzyme induced a final fluorescence enhancement of 1.67-fold (±0.05) and saturated at 1.25 units/fmol of primer. The fluorescence enhancement was prevented by having 320 μg/ml anti-β IgG present before the holoenzyme was reconstituted. However, if the same amount of the antibody was added after reconstitution and fluorescence enhancement had occurred, then only 2% of the fluorescence enhancement caused by holoenzyme binding was reversed (data not shown). These effects were consistent with the formation of an active initiation complex in which β becomes inaccessible to the antibody (Johanson and McHenry, 1984). We also confirmed that the reconstituted holoenzyme degraded the 3'-terminal nucleotide of the primer with a half-life of 18 min. Thus, by several criteria, the reconstituted holoenzyme acted like the intact isolated enzyme.

In contrast, no fluorescence change was observed when 5400 units of γδ complex were used to assemble 5 nM β onto 5 nM labeled primer-template. Since there was no fluorescence change, the β-γδ complex must be bound to the primer-template in a very different fashion from that of the holoenzyme or pol III*. When the holoenzyme was partially reconstituted from the β-γδ complex by adding 300 units of DNA polymerase III core (αθδ), there was a 1.12-fold fluorescence enhancement. This moderate enhancement was approximately that expected for the holoenzyme reconstituted with this limiting amount of DNA polymerase III (0.30 unit/fmol of primer-template).

Replicative Form II Duplex DNA Was More Highly Fluorescent Than Primed DNA—There was a fluorescence enhancement associated with DNA elongation. Sequential addition of TTP, dCTP, and dATP to the initiation complex should lead to primer elongation by 1, 2, and 4 nucleotides, respectively. When dGTP is added, the full template should be replicated. The time course of fluorescence intensity change was monitored while sequential additions of dNTPs were made (Fig. 4A). Only minor changes were observed when the first three nucleoside triphosphates were added. When dGTP was added to allow DNA elongation, a time-dependent fluorescence enhancement was observed. The enhancement was complete within ~15 s and reached a level 1.23-fold greater than the fluorescence intensity of a holoenzyme initiation complex.

T7 DNA polymerase is also stimulated by SSB (Tabor et al., 1987; Myers and Romano, 1988). When T7 DNA polymerase was titrated onto the SSB-coated fluorescent primer-template, only a modest fluorescence enhancement of 9.5%
was observed (data not shown). Less than 0.001 units of T7 DNA polymerase/fmol of primer-template was required to fully saturate the primer. The units of holoenzyme are different from the units of T7 DNA polymerase; and using a molar activity of 0.0017 unit/fmol, the primer becomes saturated when <6 mol of T7 DNA polymerase has been added per mole of primer-template. Like the holoenzyme, T7 DNA polymerase binds tightly to the primer-template to form a complex capable of highly processive DNA synthesis (Huber et al., 1987; Tabor et al., 1987). However, T7 DNA polymerase and the holoenzyme appear to be associated with the primer differently because they alter the fluorescence intensity of the primer to different extents.

To determine whether the fluorescence increase that accompanied DNA synthesis was polymerase-specific or due to an altered conformation in high molecular mass DNA, we performed the above experiments using T7 DNA polymerase. The processive complex of T7 DNA polymerase is composed of two proteins, the 80-kDa T7 gene 5 DNA polymerase and the 12-kDa E. coli thioredoxin (Modrich and Richardson, 1975; Mark and Richardson, 1976). During replication by T7 DNA polymerase, the accessory protein (thioredoxin) travels with the polymerase. When the four dNTPs were added to the T7 DNA polymerase-primer-template complex, the final fluorescence intensity reached the same level as it did for the DNA polymerase III holoenzyme (Fig. 4B). Even though the primer complexed with the two DNA polymerases initially exhibited very different fluorescence intensities, the fluorescence intensities were the same after replication. Thus, the fluorescence intensity following replication results from duplex DNA formation regardless of the polymerase involved. We also conclude that the changes were not due to subunits being left behind, because T7 DNA polymerase migrates along the template as an intact heterodimeric complex.

Additional evidence that the replicative form II fluorescence was not related to bound protein was obtained by salt titration. When 500 mM KCl was added to the double-stranded DNA, no change in fluorescence intensity was observed. This is in contrast to the fluorescence enhancement caused by the initiation complex, which was completely reversed by KCl, with 50% reversal at 100 mM. The elongation activity of the initiation complex is 50% inhibited by 100 mM KCl (Griep and McHenry, 1989).

Fluorescence Energy Transfer within Initiation Complex between Donor-labeled Primer-Template and Acceptor-labeled β—The initiation complex could be reconstituted using a different order of addition than that described above. When unlabeled β was added to DNA-bound pol III*, the fluorescence of the pol III*-primer-template complex increased to that characteristic of an initiation complex (Fig. 5). The fluorescence change saturated with 5.9 nM β at a 1.9-fold enhancement over the pol III* complex. This indicated that there was 1.2 mol of β bound per mol of primer-template or initiation complex. The final fluorescence intensity was the same as that for the holoenzyme bound to the primer-template, indicating that a true initiation complex had been formed. The final fluorescence intensity achieved in this way was used for Fb (the fluorescence intensity of the donor in the absence of the acceptor) in the energy transfer experiments.

For the 1:1 stoichiometry of β to primer termini to be firmly established, we needed to rule out the possibility that only one-half of the primers could be extended. This would have permitted a 2:1 stoichiometry. We incubated initiation complexes with four dNTPs at 30°C for 5 min and analyzed the DNA products by electrophoresis on a denaturing 20% polyacrylamide gel. Bands were quantitated by densitometry. Only 15% of the primers were not elongated.

When β labeled with an acceptor fluorophore, either RXM or TMRM, was titrated into the solution containing the pol III*-primer-template complex, the fluorescence increased as the initiation complex was formed (Fig. 5). Although the labeled β subunits saturated the template when ~1 mol of active labeled β was bound per initiation complex, at 5.2 nM RXM-β and 6.1 nM TMRM-β, the final fluorescence intensity of these initiation complexes was lower due to energy transfer. Within the initiation complex, the donor attached to the antepenultimate nucleotide of the primer was close enough to the acceptor on cysteine 333 of β that energy was transferred from the donor to the acceptor. Thus, the final fluorescence intensity of the initiation complex formed with acceptor-labeled β (Fb) was less than that formed with unlabeled β (Fr).

The final fluorescence intensity of the donor in the presence of acceptor (Fr) was used to calculate the distance between the two fluorophores (Table 1). The efficiency of energy transfer and R0 were calculated according to the equations given under “Experimental Procedures.” The efficiency of transfer between fluorescein located on the antepenultimate nucleotide of the primer and rhodamine X attached to cysteine 333 on β was 10 ± 4%, representing the average of four data points. The efficiency of energy transfer from the donor fluorescein to the acceptor tetramethylrhodamine was 12 ± 4% (three data points). As was expected, the R0 values that were calculated for these two donor-acceptor pairs were very similar, 46.7 Å for fluorescein-rhodamine X and 46.2 Å for fluorescein-tetramethylrhodamine. These R0 values were lower than what is usually calculated for pairs in which fluorescein is the donor because fluorescein in this system had a low quantum yield of 0.214. From the efficiency and R0, the distance was calculated to be 67 ± 3 Å from fluorescein to rhodamine X and 64 ± 3 Å to tetramethylrhodamine. Thus, within an initiation complex, the distance is on the order of 65 Å between the 3'-antepenultimate nucleotide of the primer and cysteine 333 located on the distal end of the β subunit.

**Discussion**

Current models for the mechanism of the holoenzyme-catalyzed reaction, largely based on functional data, envision...
an enzyme with a very complex and perhaps asymmetric architecture (Johanson and McHenry, 1984; McHenry, 1988; Maki et al., 1988; O'Donnell, 1987). For us to properly test these models and for us to be able to propose roles for each of the holoenzyme subunits, the structure of the holoenzyme must be determined. We have initiated a tripartite approach in our laboratory to completely determine the structure of the holoenzyme bound to primed DNA. We are using a combination of chemical subunit-subunit cross-linking, photocrosslinking of single labeled nucleotides within a primer-template to subunits, and fluorescence energy transfer between sites within initiation complexes and holoenzyme subunits. The first approach will determine most of the subunit-subunit contacts; the second will determine all of the subunit-primer-template interactions; and the third will yield distance information that will permit refinement of the two-dimensional information obtained from the first two techniques into a finite set of testable models for the three-dimensional arrangements of holoenzyme subunits within the replicative complex. In this manuscript, we report our initial success using fluorescence energy transfer to determine the distance between sites within the replicative complex.

In earlier work, we have made active derivatives of $\beta$ that contain fluorescent reporters attached specifically to cysteine 333 of $\beta$ (Griep and McHenry, 1988, 1990). In this study, we decided to instead place acceptors of fluorescence energy on $\beta$ and to place the fluorescent donor on the primer. This was done so that we could saturate the donor with the acceptor and more accurately calculate distances. We know that we can form initiation complexes on all available primers. Having the fluorescent reporter on the primer by-passes problems that would derive from having excess fluorescent $\beta$ free in solution.

Before the distance between fluorescently labeled primers and acceptor-labeled $\beta$ could be reliably determined, we needed to develop a complete understanding of all fluorescence perturbations that occurred in fluorescent primers due to interactions with unlabeled replication proteins used in our experiments. Only after understanding these changes can the differential changes caused by the introduction of an acceptor be accurately determined.

A fluorescent label on the 3'-antepenultimate nucleotide of the primer was useful for showing that properly reconstituted initiation complexes could be formed. Every femtomole of primer-template was capable of stoichiometrically binding 1.30 units of holoenzyme, 1.25 units of reconstituted holoenzyme, or 1.35 units of pol III*. That these amounts represented 1 fmol of initiation complex was indicated from the $\beta$ subunit reconstitution studies that showed that 1.2 fmol of $\beta$ bound to every femtomole of primer-template. The reconstituted holoenzyme acted like the holoenzyme in every test: DNA polymerase activity, exonuclease activity, RCI dependence, anti-$\beta$ IgG sensitivity, extent of fluorescence enhancement on the fluorescent primer-template, and the units required to fully form an initiation complex.

The binding and energy transfer experiments were made possible through the use of a primer that was resistant to the potent 3' → 5' exonuclease of the holoenzyme. The 3'-terminal nucleotides (rCp(S)dA) were chosen for this study not only because they were expected to slow the rate of removal of the 3'-terminal deoxyadenosine, but also because the terminal nucleotide could be elongated (Griep et al., 1990). The addition of a fluorescently labeled nucleotide in the 3'-antepenultimate position did not alter the degradation rate of the 3'-terminal nucleotide using either the intact or reconstituted holoenzyme. This observation indicated that this type of upstream DNA alteration (i.e. fluorescent labeling) did not serve as a target for the potent 3' → 5' exonuclease of the holoenzyme. Adding four dNTPs to the initiation complex resulted in the same final fluorescence intensity, indicating that the same amount of DNA had been replicated whether it was formed with the holoenzyme or the reconstituted holoenzyme. When the elongation products of the reconstituted holoenzyme initiation complex were examined, it was found that only 15% of the primers were not elongated.

As described under "Results" and summarized in Fig. 6, all of the known intermediates in the holoenzyme-catalyzed reaction can be distinguished by distinct fluorescence changes induced in the labeled primer. These changes will be helpful in future studies designed to describe the holoenzyme system kinetically.

As SSB bound to the ssDNA that flanks the fluorescently labeled primer, it quenched the fluorescence of the labeled primer by 31%. We believed that SSB is inducing a conformational change in the primer-template duplex region via interactions with the single-stranded regions adjacent to the primer. The alternative that SSB is binding directly to the primer-template duplex to change its conformation is unlikely. A rough estimate indicates that SSB binds to double-stranded DNA at least 1000-fold more weakly than to ssDNA (Molineux et al., 1975). Only after the ssDNA had bound all of the SSB would there be any binding to the double-stranded DNA if it occurs at all. However, we have shown that the primer conformational change parallels SSB binding to ssDNA. Furthermore, the resistance of the changes in fluorescence to salt parallels the known salt resistance of SSB binding to single-stranded DNA (Lohman and Overman, 1985).

Exonucleolytic digestion of the primer, causing release of the fluorophore as free nucleotide, results in a marked fluorescence enhancement. This change could be used in the future as a general spectrofluorometric nuclease assay or as an assay for excision of a specific nucleotide within a complex substrate.

When the holoenzyme binds to the primer-template, the final fluorescence intensity is the same whether or not the template is coated with SSB. The initiation complex has a fluorescence intensity that is higher than that of the free primer-template. Thus, the holoenzyme adjusts the primer conformation so that it is complementary to its own binding site, regardless of whether the adjacent sites are coated with SSB. However, it does not necessarily achieve this by displacing the SSB tetramers that are bound near the primer as it forms the initiation complex.

pol III* binds to the SSB-coated primer-template to form a complex with a different conformation from that of the hol-
Distance between $\beta$ and the Primer

The pol III* bound primer can be converted to the holoenzyme-induced conformation in either of two ways: by adding a stoichiometric amount of $\beta$ to bound pol III* or by adding pol III* to a solution of free $\beta$ and the primer-template.

The low processivity of pol III* has been taken as a priori evidence that it was not fully bound to the primer-template under elongation conditions (Fay et al., 1982) or was nonspecifically bound (Lasken and Kornberg, 1987). Our studies here show that pol III* binds to the primer-template at the same molar ratio as the holoenzyme and that $\beta$ converts the bound pol III* to the bound holoenzyme. Presumably, the low processivity of the bound pol III* must result from a more rapid rate of dissociation from the template that is not evident from the equilibrium measurements conducted in these experiments.

Above we describe that pol III* binds to the primer-template in a different conformation than it assumes in holoenzyme initiation complexes. Another assembly of holoenzyme subunits that can interact with primers, the preinitiation complex formed by the $\gamma6$ complex in the presence of $\beta$ and ATP (Wickner, 1976; O'Donnell, 1987), also permits the antepenultimate nucleotide of the primer to assume a different conformation than when in initiation complexes. No change results when the $\gamma6$ complex and $\beta$ interact with the primer, even though the preinitiation complexes formed were shown to be fully active upon the addition of the DNA polymerase III core (data not shown). That both pol III* and preinitiation complexes separately assume different conformations than those of complete initiation complexes, even though they collectively contain all of the holoenzyme subunits, argues for a large conformational change accompanying assembly of the intact complex.

When we used our fluorescence binding assay to monitor initiation complex formation, we found that only 1 mol of $\beta$ is required to reconstitute 1 mol of initiation complex. This observation partially resolved the question raised concerning the dimeric state of $\beta$ within the initiation complex (Griep and McHenry, 1989). Even though $\beta$ is a dimer at high concentrations (Johanson and McHenry, 1980), it is monomeric at assay concentrations in the presence of Mg$^{2+}$ (Griep and McHenry, 1989). Thus, at assay and physiological concentrations, $\beta$ could be incorporated into the initiation complex as the monomer from solution, or it could be reconstituted as a dimer. We have shown that $\beta$ is incorporated as the monomer using a direct assay for binding. Other recent publications have concluded that anywhere between two and one $\beta$ monomer is bound to holoenzyme or the initiation complex (Lasken and Kornberg, 1987; Maki and Kornberg, 1988; Maki et al., 1988). Since it is easier to overquantitate than to underquantitate this value, it appears that one $\beta$ monomer/initiation complex on a ssDNA template is consistent with all of the data. However, our results do not preclude two $\beta$ subunits interacting in a complex that contains a dimeric holoenzyme and two primer-templates.

Once the fluorescence changes of the primer-template were fully characterized, it was possible to address the additional changes that occurred when an acceptor of fluorescence energy was introduced at residue 333 of $\beta$. Two different acceptors, rhodamine and trimethylrhodamine, caused decreases in the primer quantum yield of 10 and 12%, respectively. From this information, a distance of 65 Å between $\beta$ cysteine 333 and the 3'-antepenultimate nucleotide of the primer could be calculated.

Using a distance of 65 Å between $\beta$ and the primer, a model can be generated for the orientation of $\beta$ relative to the primer-template (Fig. 7). Holding one locus fixed, a single measured distance limits the locus of a second point to a position anywhere on a sphere of a 65 Å radius. We limit these infinite possibilities by accounting for what is known about the complex. The $\beta$ monomer has a hydrated radius of 17 Å (Griep and McHenry, 1988). A hydrated spherical protein of its same molecular mass would have a radius of 16 Å. To accommodate its apparent larger size, the $\beta$ monomer must have a shape that resembles a prolate ellipsoid with an axial ratio of 2:1. Distance measurements within a free $\beta$ dimer demonstrated that cysteine 333 lies on the distal ends of the dimer and not near the dimer interface (Griep and McHenry, 1990). Estimates of the shape of the holoenzyme indicate that it is quite elongated (McHenry, 1988). The holoenzyme is likely to align its long axis parallel to the template to increase contact with the linear DNA molecule. Because it contains the polymerase active site, the $\alpha$ subunit must reside at the 3'-terminus of the primer. Since $\beta$ can stimulate the processivity of all holoenzyme subassemblies, including the DNA polymerase III core ($\alpha\theta$), it must lie adjacent to the $\alpha$ subunit (LaDuca et al., 1986). Genetic evidence for a functional $\alpha$-$\beta$ interaction was obtained while isolating repressors of a thermosensitive
α mutant (Kuwabara and Uchida, 1981). The resistance of β to anti-β IgG when it is part of an initiation or elongation complex indicates that β must be buried within those complexes (Johanson and McHenry, 1980, 1982). When the holoenzyme pauses at major barriers to elongation such as hairpins, it does not get any closer than ~4 nucleotides from the base of the hairpin (LaDuca et al., 1983). This is suggestive, but does not prove, that the major length of the holoenzyme is oriented opposite to the direction of elongation. The combined evidence suggests that β is held near the primer, behind the 3’-end of the primer, and is protected from the antibody beneath α, among other subunits. Other studies presently underway using photocross-linking of specifically labeled nucleotides within the primer and β will permit refinement of its positional assignment.

We have provided the first glimpse into the structure of the holoenzyme as it exists within the initiation complex. The multiple fluorescent signals that serve as a prelude to the actual distance measurements were crucial for showing that a proper initiation complex had been formed. By these means we have proven that β binds as a monomer within the initiation complex of a ssDNA template. This would be difficult to determine by most other means, but was possible here because we could measure binding rather than activity as is usually done. Nevertheless, it will be necessary to correlate the multiple fluorescent signals that are associated with binding to their activities for an accurate picture of the binding and rearrangements that lead to the formation of an active polymerase complex. Not only will fluorescence help elucidate the structure of the holoenzyme, but the multiple signals associated with various binding states will help elucidate the kinetics and mechanism of initiation complex formation.

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Note Added in Proof—While this work was being reviewed, O’Donnell and colleagues (Stukenberg et al., 1991) demonstrated that β could diffuse freely on double-stranded DNA, but not single-stranded, and proposed that β contacted the template behind the polymerase. This conclusion, based on functional studies, is in good agreement with our physical data.

References

Hagerman, F. J. (1985) Biochemistry 24, 7033–7037