Promoter-Specific Synthetic Photoendonuclease: Rose Bengal-Labeled T7 RNA Polymerase†

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ABSTRACT: We have constructed a synthetic photoendonuclease composed of T7 RNA polymerase linked to rose bengal. The promoter-specific polymerase confers site-specific binding, and the photosensitizer rose bengal allows light-induced DNA cleavage. Using a gentle labeling procedure, we find that the polymerase can be labeled with 1–30 rose bengals. Polymerase labeled to about 8 rose bengals per molecule retains the same efficiency and specificity of binding to promoter-containing DNA as unlabeled polymerase. At this level of rose bengal substitution, the synthetic endonuclease, in the presence of visible light, specifically cleaves linear or supercoiled DNA containing a T7 promoter. It induces frank single-strand breaks, rather than labile sites convertible to breaks upon additional treatments. Neither the free rose bengal moiety not bonded to polymerase nor the free (not bound to DNA) rose bengal-substituted polymerase cleaves DNA. Although rose bengal is an efficient generator of singlet oxygen, depletion of oxygen from reaction mixtures *increases* the cleavage rate. This indicates that singlet oxygen cleavage is not a major mechanism of DNA nicking by the synthetic endonuclease. At higher levels of rose bengal substitution, the labeled polymerase shows decreased binding efficiency and increased nonspecific binding to DNA without a T7 promoter; the specificity of DNA cleavage also decreases. These results indicate that the site specificity of rose bengal photocleavage by the synthetic endonuclease results from specific binding of the polymerase, and thus rose bengal photonicking reflects polymerase binding.

Construction of synthetic endonucleases for cleavage at specific, rare sites in DNA could aid chromosome mapping, permit alteration of regulation of gene expression, and be useful in DNA lesion quantitation. Approaches to designing synthetic endonucleases include using small molecules which bind preferentially to different DNA conformations (Barton & Raphael, 1984), linking oligonucleotides to cleaving moieties (Chu & Orgel, 1985; Dreyer & Dervan, 1985; Chen & Sigman, 1986; Francois et al., 1989; Corey & Schultz, 1987), or labeling DNA binding peptides or proteins with activatable cleaving groups (Sutherland, B. M., et al., 1987; Sluka et al., 1987; Chen & Sigman, 1987; Ebright et al., 1990). Major limitations of many such synthetic endonucleases are the lack of stability of the complex (Sun et al., 1989; Mergny et al., 1992) and nonspecific cleavages induced by the hybrid molecules (Chu & Orgel, 1985; Beal & Dervan, 1992). Further, the specific recognition of only DNA sequences by oligonucleotides precludes recognition of DNA functional classes, structures, or lesions.

One solution would be the construction of synthetic endonucleases using DNA binding proteins for site recognition. However, existing methods for protein labeling are harsh, and lead to significant losses in activity of the protein (Sutherland, B. M., et al., 1987; Ebright et al., 1990; Epling et al., unpublished results). We have used a gentle labeling method developed by Epling et al. (unpublished experiments) to add the photosensitizer rose bengal to T7 RNA polymerase, a labile, site-specific DNA binding protein. Polymerase labeled with 1–8 rose bengals retains the efficiency and specificity of binding of unlabeled polymerase. We show that such a rose bengal–T7 RNA polymerase (RB–RNAP)[1] synthetic endonuclease introduces single-strand breaks specifically into linear or supercoiled DNA containing a T7 promoter sequence, but does not cleave DNA lacking a T7 promoter.

MATERIALS AND METHODS

T7 RNA Polymerase. DNA-dependent T7 RNA polymerase, purified to >95% homogeneity, was the gift of J. J. Dunn.

Linkers and Cleaving Groups. Three activated linked photosensitizers were utilized in the preparation of photosensitizer-linker protein conjugates. All used rose bengal as the photosensitizer, with the linker being either a hexanoic acid, an undecanoic acid, or a trioxyethylene hemisuccinate. Rose bengal hexanoic acid was prepared by reacting rose bengal and 6-bromohexanoic acid in 70% acetone/water at reflux overnight. The product was isolated by flash chromatography on silica gel. The activated ester was prepared by carbodiimide coupling of the acid with *N*-hydroxysuccinimide in dimethylformamide (DMF) at room temperaure, and purified by flash chromatography on silica gel. Activated linked rose bengal undecanoate was similarly prepared, except substituting 11-bromoundecanoic acid for 6-bromohexanoic acid. The ester of rose bengal and triethylene glycol was prepared from rose bengal and 2-[2-(2-chloroethoxy)ethoxy]-ethanol in DMF. This ester was treated with succinyl chloride in methylene chloride to give the trioxyethylene hemisuccinate, which was activated with dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide for use in protein coupling. Full synthetic details are provided elsewhere (Epling et al., unpublished results).

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[1] Abbreviations: DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; RB, rose bengal; RNAP, RNA polymerase.

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Labeling T7 RNA Polymerase. Protein labeling was carried out as described elsewhere (Epling, unpublished results). In brief, the activated linked rose bengal was suspended in reagent-grade anhydrous tetrahydrofuran (Pierce), and diluted just before use into 1× labeling buffer (20 mM sodium phosphate, buffer, pH 7.7, and 50 mM NaCl) containing 5 mM dithiothreitol (DTT). T7 RNA polymerase was diluted to 1.6 mg/mL into the same buffer and incubated on ice for 5 min. The activated linked photosensitizer was mixed with polymerase at a final protein concentration of 1 mg/mL, and the solution was incubated at 30 °C for 15 min. The labeled polymerase was then applied to a spin column of lipophilic Sephadex (LH60-120 Sigma) in labeling buffer containing 1 mM DTT to remove photosensitizer not conjugated to protein; in some experiments, the labeling was terminated by the addition of DTT to 10 mM. Protein concentrations were determined by a microLowry reaction (Lowry et al., 1951), and the rose bengal concentration by the absorbance at 560 nm.

DNAs. Supercoiled pET-2 (Rosenberg et al., 1987) and pUC18 DNAs were prepared by detergent lysis, heat treatment, centrifugation, and poly(ethylene glycol) precipitation, followed by extraction with chloroform–isoamyl alcohol, phenol, and chloroform. Plasmid pUC18 was treated with *Bgl*I (Boehringer-Mannheim) using the manufacturer's conditions. The restriction digestion was terminated by the addition of EDTA to 10 mM and heating to 65 °C for 20 min. The DNA was dialyzed into 10 mM sodium phosphate buffer, pH 7.2.

Quantitation of Polymerase–Promoter Binding. Polymerase binding to nonradioactive plasmid DNA was quantitated using the normalizing DNA method of Sutherland et al. (1992). In brief, pET-2 DNA (5–15 ng) which contains the strong T7 promoter $\phi 10$ was mixed with the same quantity of *Bgl*I-digested pUC18 in binding buffer (20 mM sodium phosphate, pH 7.7, 50 mM NaCl, 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, and 0.1 mg/mL gelatin), or (for detection of nonspecific binding) supercoiled pUC18 and *Bgl*I-restricted pUC18 in the same buffer. In some assays, 5 mM GTP was added to the binding buffer (see text). Polymerase was diluted into binding buffer and incubated 30 min on ice, and then added to the DNA mixture, and the samples were incubated at 37 °C for 15 min. Agarose (Sigma type II) (1%) gels were prepared in 50 mM NaCl, 1 mM EDTA, and 1 mM DTT, and after the gels solidified, they were soaked in electrophoresis buffer (60 mM sodium phosphate buffer, pH 6.5, 2 mM EDTA, 18 mM NaCl, and 1 mM DTT). The wells were rinsed with binding buffer immediately before sample loading. Horizontal agarose gels were electrophoresed in electrophoresis buffer in a Bio-Rad Mini-Sub cell for 1 h at 60 V; gels were maintained in an ice bath, and the buffer was recirculated after samples had entered the gel. After electrophoresis, the gel was stained for 10 min in ethidium bromide (1 μg/mL in water) and destained in water, and a quantitative electronic image of the ethidium fluorescence on the gel was obtained using either a charge-coupled device (CCD)-based imaging system (Sutherland, J. C., et al., 1987) or a "second-generation" CCD-based system that will be described elsewhere. The quantity of ethidium fluorescence corresponding to DNA bands of interest was determined in each gel lane.

Quantitation of Synthetic Endonuclease Cleavage. Cleavage of promoter-containing supercoiled DNA was measured using the normalizing DNA method (Sutherland et al., 1992). Polymerase was added to a DNA mix containing (for detection of promoter-specific cleavage) 5–15 ng of supercoiled pET-2 DNA and the same quantity of *Bgl*I-restricted pUC18 in binding buffer or (for detection of nonspecific cleavage) supercoiled pUC18 and *Bgl*I-restricted pUC18 in the same buffer. Each sample was divided into two aliquots; all samples were incubated for 15 min at 37 °C with or without argon or nitrogen treatment; then one aliquot was exposed at 37 °C to cool white fluorescent light for 30 min while a companion aliquot was kept in the dark at 37 °C for 30 min. A neutral stop mixture containing 0.125% bromophenol blue and 0.05% sodium dodecyl sulfate (SDS) in 50% glycerol was added to each sample before electrophoresis. DNAs were electrophoresed in a cooled gel with buffer recirculation, stained, and destained, and an image was obtained as described above. The quantity of ethidium fluorescence corresponding to DNA bands of interest was determined in each gel lane, and ϕ, the fraction of supercoiled DNA, was calculated.

Cleavage of T7 DNA by the RB-labeled polymerase was determined by alkaline gel electrophoresis and analysis using the method of moments (Freeman et al., 1986). RB-polymerase was incubated with T7 DNA; then the complex (in translucent microcentrifuge tubes) was exposed to a 60-W cool white fluorescent light for increasing times. The samples were then mixed with an alkaline stop solution [50% (v/v) glycerol, 0.25% (w/v) bromocresol green, and 0.5 N NaOH] and electrophoresed along with molecular length standards [λ and T7 DNAs, and *Bgl*I restriction fragments of T7 DNA (21, 14, and 4 kb)] on 0.4% alkaline agarose gels as previously described. A quantitative electronic image was obtained, and the number-average molecular length was calculated for each sample. The frequency of strand breaks was calculated as previously described (Freeman et al., 1986).

RESULTS

Derivatizing labile proteins such as T7 RNA polymerase requires a rapid, effective labeling procedure which can be carried out in mild conditions. Since polymerase is available in limited quantities, we used commercially available immunoglobulin G (IgG) as a model protein in initial labeling experiments. We labeled IgG with the activated esters to determine labeling conditions which would give adequate derivatization. We found that IgG could be labeled with the rose bengal hexanoate, trioxyethylene hemisuccinate, or undecanoate-activated esters in 20 mM sodium phosphate buffer, pH 7.7, and 50 mM NaCl. The extent of labeling depended strongly on temperature, time, and concentration of activated ester. In 15 min at 30 °C, IgG at 1 mg/mL could be labeled with 1–30 rose bengals per protein molecule, with the extent of labeling determined by varying the concentration of the activated linked rose bengal up to about 0.5 mg/mL.

Figure 1 shows that T7 RNA polymerase is also readily labeled under these reaction conditions. In addition to time, temperature, and concentration of activated linked rose bengal, the extent of labeling also depended on the nature of the hydrocarbon linker. The trioxyethylene hemisuccinate-rose bengal gave intermediate labeling, about 10 RB/polymerase at a concentration of 0.15 mg/mL rose bengal-linker. The RB-hexanoate appeared to label with equal or higher efficiency, but the rose bengal-undecanoate gave less efficient labeling over the entire concentration range.

We then determined whether the rose bengal-labeled polymerase could nick T7 DNA, which contains 17 promoters for T7 RNA polymerase. Polymerase labeled with rose bengal hexanoate was incubated with T7 DNA and then exposed to visible light for increasing times. The samples were then electrophoresed on alkaline agarose gels (McDonell et al.,

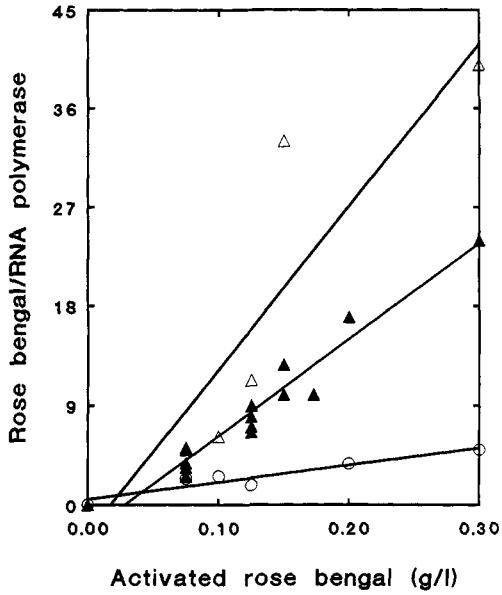

FIGURE 1: Effect of the concentration of the activated linked rose bengal moieties on the labeling of T7 RNA polymerase. The activated linked rose bengals (△, hexanoate; ▲, trioxyethylene hemisuccinate; ○, undecanoate) suspended in THF were diluted and added to the polymerase, and the solution was incubated at 30 °C for 15 min. The labeled protein was separated from the unreacted linker by chromatography on an LH60 spin column. The lines were fit to the data by linear least-squares analysis.

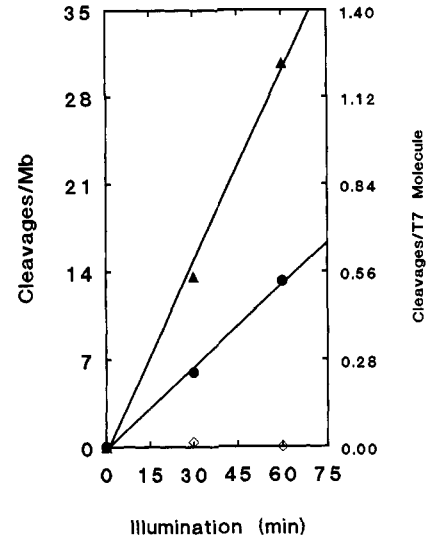

FIGURE 2: Cleavage of T7 DNA by T7 RNA polymerase [18.7 (▲) or 9.4 ng (●)] labeled with rose bengal hexanoate (0.08 mg/mL), and absence of cleavage by rose bengal hexanoate not bonded to polymerase (◊). For the latter, rose bengal hexanoate was diluted to 0.08 mg/mL into the labeling buffer without polymerase, and incubated for 10 min; DTT was added to terminate the reaction, the solution was diluted as for the polymerase-bound samples and added to T7 DNA. After 10-min incubation, all mixtures were exposed to visible light for increasing times or kept in the dark. Alkaline stop mixture was added, and the samples were electrophoresed on alkaline agarose gels along with molecular length standards. The frequency of single-strand breaks was determined as previously described (Freeman et al., 1986). The DNA is cleaved by the rose bengal-labeled polymerase in the presence of light, but the unconjugated rose bengal hexanoate is inactive in DNA cleavage. The lines were fit to the data by linear least-squares analysis.

1977), a quantitative electronic image obtained (Sutherland, J. C., et al., 1987), and the number average molecular length calculated for each sample using the method of moments. The frequency of strand breaks was calculated as previously described (Freeman et al., 1986). Figure 2 shows that the RB-labeled polymerase indeed induced nicks in the T7 DNA; the reaction appeared linear over the 60-min illumination period, and was proportional to the quantity of polymerase added to the reaction. The inactivated rose bengal hexanoate thio ester (not linked to protein) was inactive in DNA cleavage.

The T7 cleavage results showed that, upon exposure to light, the RB-based synthetic endonuclease induced either breaks or DNA lesions convertible to breaks upon treatment with alkali. Since the detection of breaks in the T7 system requires strand separation by alkaline treatment and electrophoresis on alkaline gels, the breaks we measured might reflect either true single-strand breaks or sites converted to breaks upon treatment with alkali. We thus tested RB-RNAP-mediated cleavage of supercoiled DNA, in which breaks can be detected without treatment with alkali. Induction of single-strand breaks in supercoiled DNA yields nicked circles (relaxed DNA), while double-strand break induction results in linear molecules. Supercoiled DNA, nicked circles, and linear molecules are easily resolved by electrophoresis under neutral conditions.

Quantitation of nonradioactive supercoiled DNA conversion has been difficult due to the differential binding of ethidium bromide to supercoiled and relaxed DNA [which varies from a ratio of 1 to 1.66 under different experimental conditions (Masnyk & Minton, 1991; Roots et al., 1985; Lloyd et al., 1978; Bauer & Vinograd, 1968; Ciulla et al., 1989)] and to the nonlinear response of photographic film (Pulleyblank et al., 1977; Prunell et al., 1977; Laskey & Mills, 1975). However, we have recently developed a method for quantitation of conversion of nonradioactive supercoiled DNA to other molecular forms which circumvents these difficulties (Sutherland et al., 1992). We used this method to examine cleavage by the RB-based endonuclease.

RB-RNAP was incubated with supercoiled pET-2 (Rosenberg et al., 1987) DNA containing the *ϕ10* T7 promoter and a linear normalizing DNA which serves as a mass standard for supercoil cleavage quantitation (Sutherland et al., 1992). Then one sample of the polymerase–DNA complex was exposed to visible light while a companion sample was incubated in the dark. After cleavage, samples were electrophoresed on neutral agarose gels, stained with ethidium, and destained, and an electronic image was obtained using a charge-coupled device camera (Sutherland, J. C., et al., 1987). The supercoiled or relaxed DNA and the mass standard DNA were quantitated, and the cleavage of supercoiled DNA was calculated (Sutherland et al., 1992). Figure 3A shows that in the presence of light, the RB-polymerase effectively cleaves the supercoiled DNA containing the T7 promoter (●), with no cleavage occurring in the absence of light (○). Since the sample remained in neutral conditions during incubation, cleavage, and electrophoresis, the nicks induced by the RB-RNAP were true breaks, not alkali-labile sites which could be converted to breaks upon additional treatment. Further, since only relaxed circles and no detectable linear DNA molecules were produced upon cleavage, the synthetic endonuclease induced single- rather than double-strand breaks during light-induced DNA cleavage.

Since rose bengal is a well-known generator of singlet oxygen (Gandin et al., 1983), we tested whether singlet oxygen was involved in the DNA cleavage reaction. Singlet oxygen-mediated reactions are less efficient at lowered oxygen concentrations due to unimolecular radiationless decay of the sensitizer competing with biomolecular energy transfer to oxygen to form excited singlet oxygen. Water-saturated argon was blown gently over the surface of reaction mixtures containing rose bengal-labeled polymerase and supercoiled

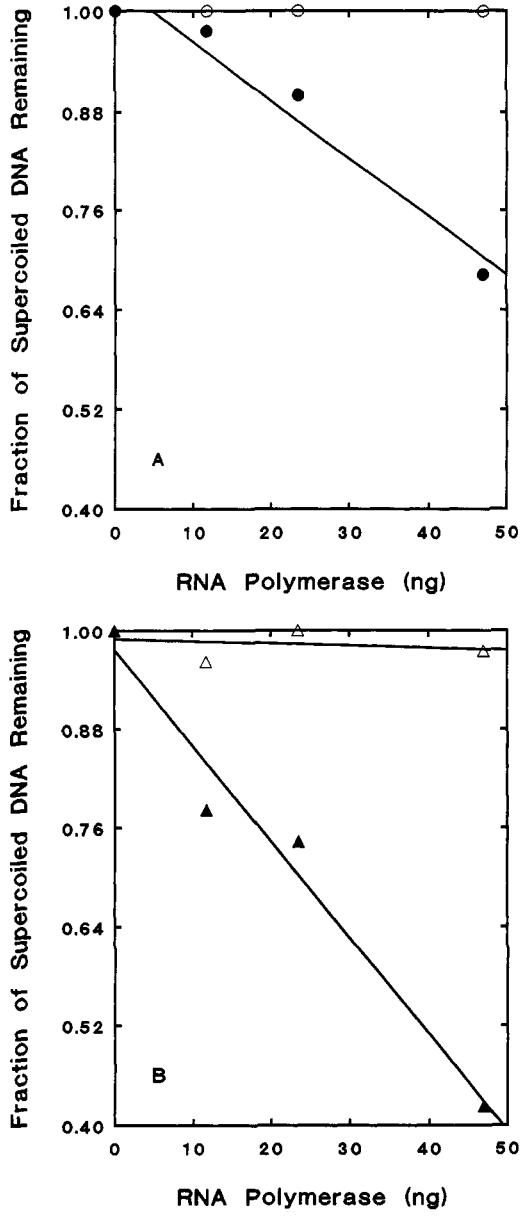

FIGURE 3: Cleavage of promoter-containing DNA by polymerase labeled with rose bengal hexanoate (0.28 mg/mL) in air (panel A) or after argon treatment (panel B). Polymerase was diluted into binding buffer for 30 min on ice and then added to a mixture containing 21 ng each of supercoiled pET-2 and linear pUC-18 DNAs. The solution was incubated for 15 min at 37 °C without (panel A) or with (panel B) argon treatment. The tubes were then capped and exposed to visible light for 90 min (filled symbols), while companion samples were kept in the dark (open symbols). After electrophoresis, ethidium staining, and electronic imaging, the fraction of supercoiled DNA cleaved by the synthetic endonuclease in the light was calculated as by the normalizing method previously described (Sutherland et al., 1992). Depletion of oxygen by argon blanketing (panel B) increases promoter-specific cleavage in the presence of light (▲), with little cleavage in the dark (△). Less cleavage (relative to that shown in panel A) occurs in companion samples illuminated without prior argon treatment (●, light; ○, dark). The lines were fit to the data by linear least-squares analysis.

DNA containing a T7 promoter (and a normalizing DNA). After light exposure, gel electrophoresis, staining with ethidium bromide, and imaging, the cleavage of pET-2 DNA was calculated. Comparison of panels A and B in Figure 3 shows that argon treatment actually increases DNA cleavage by the rose bengal-RNA polymerase. This result indicates that singlet oxygen is unlikely to play a major role in cleavage by this synthetic endonuclease.

Although the RB-RNAP endonuclease gave detectable cleavage, the reaction was inefficient, with more than 10 polymerase molecules per promoter required to cleave 10% of the supercoiled DNA molecules in 90 min. Effective cleavage by an endonuclease requires both good cutting by the cleaving moiety and high, specific binding by the protein portion. We therefore investigated the binding efficiency and specificity of unlabeled RNA polymerase and of RB-RNAP. Unlabeled polymerase was incubated under the standard conditions described above with a DNA containing a promoter and a normalizing DNA without a promoter. After complex formation, the samples were electrophoresed under neutral conditions, the gel was stained with ethidium, a quantitative electronic image was obtained, and the fraction of free DNA was determined (Sutherland et al., 1992). Panel A of Figure 4 shows that, in standard binding conditions, the unlabeled polymerase bound to promoter-containing DNA, but required large numbers of polymerase molecules for formation of substantial levels of detectable complexes.

Although RNA polymerase binds weakly to DNA in buffer solution, it forms a tight complex with the promoter in the presence of GTP, the first nucleotide incorporated by the polymerase (Chamberlin & Ring, 1973; Oakley et al., 1975). We tested the effect of GTP on the efficiency and specificity of binding. Panel B of Figure 4 shows the binding of unlabeled polymerase in the presence of GTP to DNA containing (▲) or lacking (△) a T7 promoter. Comparison with panel A (Figure 4) clearly indicates the higher efficiency of specific binding in the presence of GTP.

Since cleavage by the endonuclease depends on both the DNA scission capacity of the cleaving group and the specific binding of the protein, we determined the effect of rose bengal substitution on the binding properties of the polymerase. Panel C of Figure 4 shows that polymerase labeled with moderate levels of rose bengal (5–8 RB/RNAP) retains good binding activity and specificity. However, at higher levels of rose bengal derivatization, the binding to DNA containing the T7 promoter decreases, while binding to DNA without a T7 promoter increases significantly (Figure 4D).

The binding experiments shown in Figure 4 suggested that, if binding by the polymerase were an important determinant of the cleavage efficiency and specificity, the RB-polymerase might cleave DNA more efficiently and specifically in the presence of GTP. Panel A of Figure 5 shows that the RB-hexanoic acid–RNAP cleaves plasmid DNA containing the T7 promoter at low ratios of polymerase to DNA molecules. This RB-RNAP, containing about eight rose bengal molecules per polymerase, showed virtually no cleavage of DNA lacking a promoter at the polymerase concentrations used in this experiment. Polymerase labeled with 10 or 13 RB/protein molecule shows increasing efficiency of DNA cleavage (solid symbols in Figure 5A,B), but little if any preference for promoter-containing DNA, reflecting the loss of binding specificity (cf. Figure 4D). These data indicate that rose bengal cleavage reflects polymerase binding. High specificity and efficiency of binding are thus essential for effective promoter-specific DNA cleavage.

We also investigated factors which might limit cleavage by the RB-RNAP. Figure 6 shows that in the presence of argon, the reaction is not linear with time, and the rate of cleavage decreased significantly at longer irradiation times (cf. Figure 2, which shows cleavage in the presence of oxygen). Binding assays showed that the polymerase was still bound to the promoter-containing DNA at these reaction times. We tested the possibility that photobleaching of the rose bengal led to

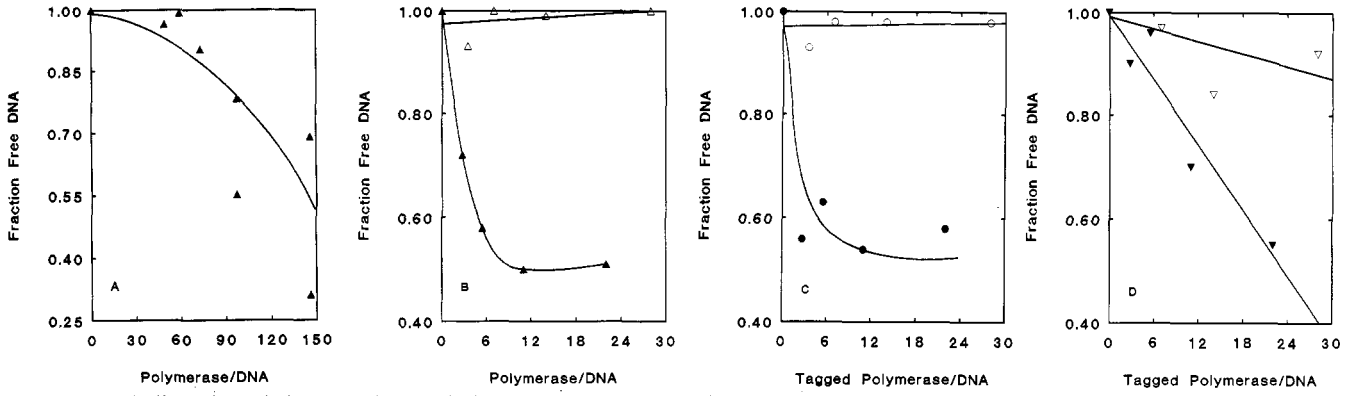

FIGURE 4: Binding of unlabeled (panels A and B) and rose bengal-labeled T7 RNA polymerase (panels C and D) to DNA containing (filled symbols) or lacking (open symbols) a T7 promoter. (A) In standard binding buffer (20 mM sodium phosphate, pH 7.7, 50 mM NaCl, 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, and 0.1 mg/mL gelatin), polymerase was mixed with pET-2 containing a T7 promoter and pUC18 (which does not contain a promoter) DNAs, incubated 15 min at 37 °C, then electrophoresed, stained, and imaged, and the fraction of free DNA was computed. (B) In standard binding buffer plus 5 mM GTP, polymerase was assayed for binding to DNA with a promoter (▲) or without a promoter (△), and the fraction of free DNA in each case was computed as in panel A. (C) Polymerase labeled with five rose bengal-trioxyethylene hemisuccinates per molecule was assayed for promoter-specific (●) and nonspecific (○) binding in the presence of GTP. (D) As in panel C, but the polymerase was labeled with 10 RB-trioxyethylene hemisuccinates molecules per protein. Binding to promoter-containing DNA (▼) is decreased, and to DNA without a promoter (▽) increased. The curve in (B) was fit by the spline method; all other curves were fit by eye to the data. All lines were fit to the data by linear least-squares analysis.

the decreasing rate of DNA photocleavage at long irradiation times. A photosensitizer-linked protein conjugate was prepared from rose bengal hexanoate activated ester and rabbit IgG. A purified solution of this conjugate in labeling buffer was irradiated in the presence or absence of oxygen using two 15-W fluorescent bulbs at a distance of 2 cm. The linked rose bengal bleached rapidly in the absence of oxygen, while in the presence of oxygen the rose bengal bleached more slowly (Figure 6B).

DISCUSSION

To be an effective site-specific cleaving agent, the RNA polymerase-based synthetic endonuclease must retain efficient, specific binding at sufficient levels of labeling for DNA cleavage. We therefore evaluated the efficiency and specificity of the polymerase at different levels of labeling. We first asked whether a labile protein like T7 RNA polymerase could be labeled with rose bengal and still retain promoter-specific binding. Although methods for labeling proteins with cleaving groups had been developed (Sutherland, B. M., et al., 1987; Ebright et al., 1990), these methods resulted in severe alterations of the protein: the catabolite gene activator protein tagged with one 1,10-phenanthroline per molecule showed a K_{obs} of 1×10^8 M^{-1}, compared to 4×10^{10} M^{-1} for the unmodified protein. Although we had developed a carbodiimide coupling method for rose bengal derivatization of proteins, we found that the polymerase was inactivated during labeling by this procedure. We therefore developed a labeling procedure in which an activated ester moiety serves to couple the rose bengal-linker to the protein (Epling et al., unpublished results). This procedure allowed effective labeling using mild conditions and brief treatment, and afforded easily controlled levels of rose bengal substitution (Figure 1). We used lipophilic Sephadex to separate the unreacted activated linked rose bengal (which remained as a band at the top of the column) from the labeled polymerase (which, at low levels of labeling, was not retained by the column). At very high levels of substitution (> ~30 RB/protein), the labeled polymerase was retained by the column.

Evaluation of the binding properties of the labeled polymerase provided insight into the effects of labeling. We measured the binding to promoter-containing DNA as a measure of binding efficiency, and binding to DNA without a promoter as an indication of nonspecific binding. Up to about 4 RB/RNAP, the polymerase showed equal efficiency and specificity of binding as the unlabeled enzyme. At intermediate levels of labeling (5–8 RB/RNAP), the RB-polymerase showed little or no loss of efficiency of binding, and similar specificity to unlabeled polymerase. At higher levels of rose bengal substitution, we found two changes in the properties of the polymerase: first, the specific binding efficiency decreased, perhaps from addition of a rose bengal to the DNA binding site of the polymerase; second, nonspecific binding to DNA lacking a promoter increased, which might be a consequence either of rose bengal addition to a site contributing to the specificity of binding, such as a nucleotide binding site, or a general conformational change. Oakley et al. (1975) found that T7 RNA polymerase could be inactivated by alkylation of a single free sulfhydryl group; this alkylation did not affect binding (as measured by retention on nitrocellulose filters), but did affect binding of GTP to the enzyme.

Are the binding properties useful predictors of cleavage by this synthetic endonuclease? At intermediate and higher levels of rose bengal substitution, they correlate well with the cleavage results: Figure 5 shows that RB-RNAP which binds effectively and with high specificity for promoter-containing DNA also cleaves efficiently and specifically; conversely, more highly substituted polymerase, which shows decreased binding to promoter-containing DNA and increased binding to DNA without a promoter, also cleaves DNA less efficiently and with lowered specificity.

At very low levels of rose bengal addition, binding properties of the RB-RNAP are not good predictors of cleaving activity. Although polymerase molecules containing about 1–4 RB/molecule bind DNA efficiently and specifically, they cleave DNA ineffectively. This probably reflects the addition of rose bengal to sites on the protein which do not allow proximity of the rose bengal moiety to DNA. On occasion, rose bengal-polymerase preparations failed to cleave DNA effectively although they contained quantities of rose bengal expected to yield effective cleavage. Electrophoresis of these preparations on SDS–polyacrylamide gels indicated that much of the rose bengal was not associated with the protein, suggesting hydrolysis of the rose bengal–protein linkage. These data indicate that a successful synthetic endonuclease must not only bind specifically but also contain cleaving groups which

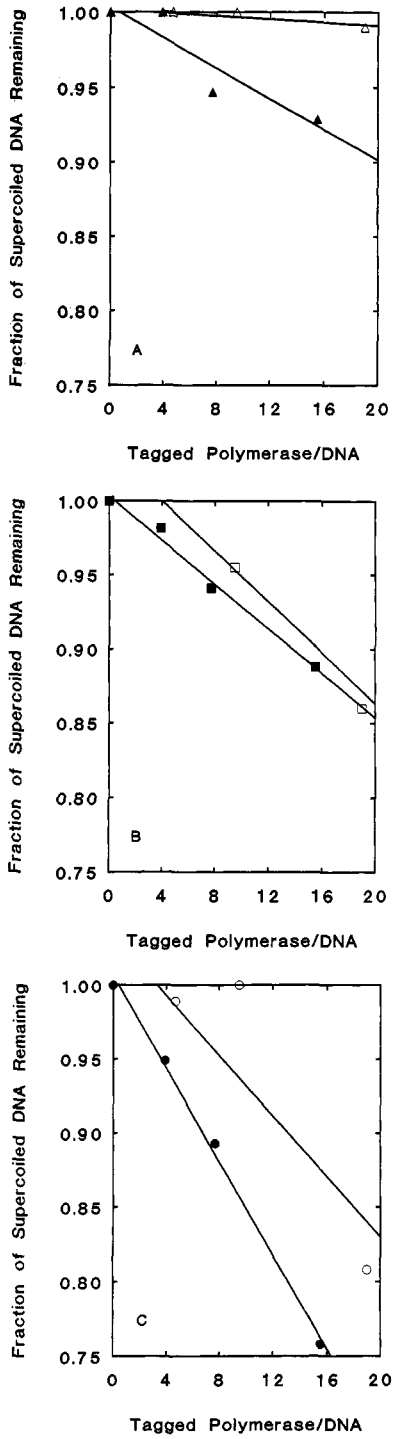

FIGURE 5: Cleavage of DNA by the RB-RNAP containing 8 (panel A), 10 (panel B), or 17 (panel C) rose bengal hexanoate moieties per polymerase. Polymerase was incubated in standard binding buffer plus 5 mM GTP with supercoiled, promoter-containing substrate and linear normalizing DNAs and was blanketed with water-saturated argon; samples were exposed to visible light while companion samples were incubated in the dark. DNAs were separated on neutral agarose gels, and the fraction of intact supercoiled DNA was computed as described. For polymerase labeled with 8 rose bengals (panel A), cleavage of DNA containing (▲) a T7 promoter is much greater than of DNA lacking (△) the promoter. However, polymerase labeled with 10 (panel B) or 17 (panel C) rose bengals cleaves DNA without a T7 promoter (open symbols) almost as well as promoter-containing DNA (closed symbols).

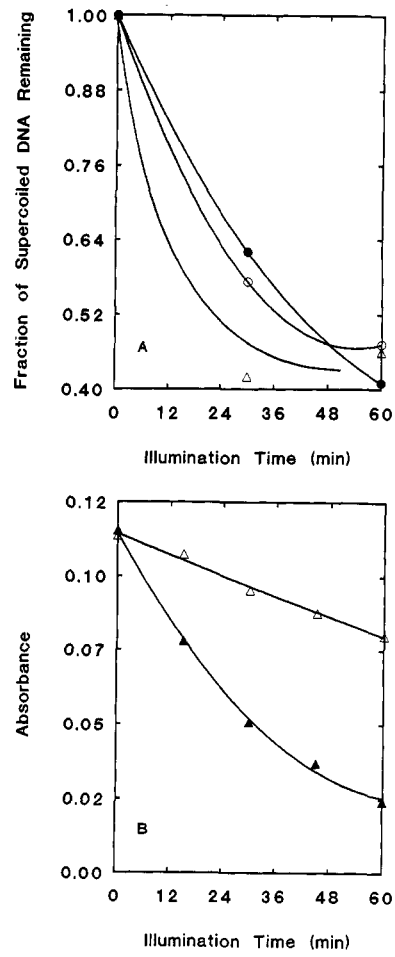

FIGURE 6: (A) Cleavage of supercoiled DNA containing a T7 promoter by polymerase tagged with eight rose bengal trioxyethylene hemisuccinate moieties per molecule. Polymerase [2.5 RB-polymerase molecules/DNA (●); 5 RB-polymerase/DNA (○); 10 RB-polymerase/DNA (△)] was incubated with pET-2 supercoiled DNA and linear normalizing pUC18 DNA, and exposed to visible light for increasing times. The fraction of supercoiled DNA molecules remaining was determined by electrophoresis, imaging, and quantitation as previously described (Sutherland et al., 1992). (B) Bleaching of rose bengal chromophore, followed by the absorbance at 571 nm in a rose bengal–hexanoate–protein conjugate by irradiation with fluorescent lamps; irradiation under oxygen (△); irradiation under nitrogen (▲).

have access to susceptible DNA components during the lifetime of the cleaving species.

RB-RNAP can cleave linear double-stranded DNA, producing breaks detectable on alkaline agarose gels. It can also cleave supercoiled DNA, converting it to nicked circular DNA without alkali or heat treatment. Its low nonspecific cleavage frequency is demonstrated by its lack of nicking of supercoiled DNA which does not contain a T7 promoter. The unactivated linked rose bengals alone, or unactivated linked rose bengals mixed with (but not covalently bonded to) polymerase, do not cleave the DNA. These data indicate that rose bengal induces frank breaks, not alkali- or heat-labile sites which are later converted to breaks. The breaks are single- not double-strand breaks, since no linear molecules appear in the supercoiled DNA cleavage system.

Attempts to evaluate the position of cleavage by the RB-RNAP have been impeded by the poor binding of the labeled polymerase, even in the presence of GTP, to promoter-containing defined-sequence oligonucleotides containing a T7 promoter site. Even unlabeled T7 RNA polymerase binds only weakly to its promoters, with binding constants of $<10^7$–10^8 M^{-1} measured under different conditions (Chamberlin & Ring, 1973; Oakley et al., 1975; Gunderson et al., 1987). Thus, high enzyme concentrations are required for DNA footprinting on a promoter-containing oligonucleotide (Ikeda & Richardson, 1986; Gunderson et al., 1987). We found that

about 50–100 RB-RNAP/promoter were required for significant complex formation with a ^{32}P-labeled 210-mer as detected by a mobility shift assay. Cleavage by the polymerase was not observed routinely, probably due to enzyme aggregation or photo-cross-linking of DNA at higher protein concentrations.

Although rose bengal is an efficient generator of singlet oxygen, $^1O_2$ does not seem to play a major role in DNA cleavage by the rose bengal-based synthetic endonuclease: depletion of oxygen in the reaction mixture by blanketing the solution with argon or nitrogen actually increased the cleavage rate rather than decreasing it as would be expected in a reaction in which oxygen is a reactant. This observation points to the existence of a non-oxygen-dependent photochemical mechanism for rose bengal-induced DNA cleavage. We believe that a photoinduced electron transfer to excited rose bengal initiates the formation of reactive free radicals which lead to subsequent DNA cleavage. Such a mechanism of cleavage by unstable radicals would be unlikely to nick DNA that was not in close proximity to the photosensitizer generating the radicals. This would likely lead to much better site-specific cleavage of the DNA than would a mechanism involving singlet oxygen, a comparatively more stable intermediate which can diffuse a great distance before deactivating.

Several investigators have used different approaches to selection or construction of synthetic endonucleases: activatable small molecules with preference for a particular DNA conformation gave increased cleavage of that conformation (Barton & Raphael, 1984); complementary oligonucleotides with chemically activatable cleaving groups gave site-specific cutting at the binding site (Francois et al., 1989). Cleaving agents based on oligonucleotides offer the advantages of stable and easily synthesized reactants, and sequence-specific location. However, the binding constants are frequently low; in some cases, intercalating agents have been used to provide additional stabilization of the DNA–oligonucleotide complex (Sun et al., 1989; Mergny et al., 1992).

Synthetic endonucleases based on site-specific DNA binding proteins offer opportunities for cleaving at functional classes of DNA sites, for example, promoters. These systems not only may provide positional information for mapping such sites but could also give information on the strength of the interaction of the protein with that site. In addition, protein-based synthetic endonucleases based on proteins recognizing specific DNA structures or lesions could report the frequency and location of such sites in DNA.

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