Lightfoote, M. M., Coligan, J. E., Folks, T. M., Fauci, A. S., Martin, M. A., & Sundararajan, V. (1986) *J. Virol. 60*, 771–775.
Mitsuya, H., & Broder, S. (1987) *Nature 325*, 773–778.
Que, B. G., Downey, K. M., & So, A. G. (1978) *Biochemistry 17*, 1603–1606.
Roth, M. J., Tanese, N., & Goff, S. P. (1985) *J. Biol. Chem. 260*, 9326–9335.
Sandstrom, E. G., Bryington, R. E., Kaplan, J. C., & Hirsh, M. S. (1985) *Lancet 1*, 1480–1482.
Sarin, P. S., Taguchi, Y., Sun, D., Thorton, A., Gallo, R. C., & Oberg, B. (1985) *Biochem. Pharmacol. 34*, 4075–4078.
Schatz, O., Cromme, F., Gruninger-Leitch, R., & LeGrice, S. F. J. (1989) *FEBS Lett. 257*, 311–314.
Schatz, O., Mous, J., & Le Grice, S. F. J. (1990) *EMBO J. 9*, 1171–1176.
Starnes, M. C., & Cheng, Y.-C. (1989) *J. Biol. Chem. 264*, 7073–7077.
Tisdale, M., Ertl, P., Larder, B. A., Purifoy, D. J. M., Darby, G., & Powell, K. L. (1988) *J. Virol. 62*, 3662–3667.
Towbin, H., Staelhin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Vrang, L., & Oberg, B. (1986) *Antimicrob. Agents Chemother. 29*, 867–872.

# Characterization of DNA Metabolizing Enzymes in Situ following Polyacrylamide Gel Electrophoresis†

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ABSTRACT: We have detected the in situ activities of DNA glycosylase, endonuclease, exonuclease, DNA polymerase, and DNA ligase using a novel polyacrylamide activity gel electrophoresis procedure. DNA metabolizing enzymes were resolved through either native or SDS–polyacrylamide gels containing defined $^{32}$P-labeled oligonucleotides annealed to M13 DNA. After electrophoresis, these enzymes catalyzed in situ reactions and their [$^{32}$P]DNA products were resolved from the gel by a second dimension of electrophoresis through a denaturing DNA sequencing gel. Detection of modified (degraded or elongated) oligonucleotide chains was used to locate various enzyme activities. The catalytic and physical properties of Novikoff hepatoma DNA polymerase $\beta$ were found to be similar under both in vitro and in situ conditions. With 3′-terminally matched and mismatched [$^{32}$P]DNA substrates in the same activity gel, DNA polymerase and/or 3′ to 5′ exonuclease activities of *Escherichia coli* DNA polymerase I (large fragment), DNA polymerase III (holoenzyme), and exonuclease III were detected and characterized. In addition, use of matched and mismatched DNA primers permitted the uncoupling of mismatch excision and chain extension steps. Activities first detected in nondenaturing activity gels as either multifunctional or multimeric enzymes were also identified in denaturing activity gels, and assignment of activities to specific polypeptides suggested subunit composition. Furthermore, DNA substrates cast within polyacrylamide gels were successfully modified by the exogenous enzymes polynucleotide kinase and alkaline phosphatase before and after in situ detection of *E. coli* DNA ligase activity, respectively. Several restriction endonucleases and the tripeptide (Lys-Trp-Lys), which acts as an apurinic/apyrimidinic endonuclease, were able to diffuse into gels and modify DNA. This ability to create intermediate substrates within activity gels could prove extremely useful in delineating the steps of DNA replication and repair pathways.

Many DNA replication, repair, and restriction processes involve enzyme complexes composed of multifunctional and multimeric subunits. For exmaple, *Escherichia coli* DNA polymerase I is a monomeric protein ($M_r$ = 103 000) that contains three distinct enzyme activities: polymerase, 3′ to 5′ exonuclease, and 5′ to 3′ exonuclease (Jovin et al., 1969). In contrast, *E. coli* DNA polymerase III holoenzyme contains polymerase and 3′ to 5′ exonuclease activities as separate subunits, designated $\alpha$ and $\epsilon$ with molecular weights of 140 000 and 27 000, respectively (Maki & Kornberg, 1985; Scheuermann & Echols, 1984). Recently, all five classes of eukaryotic DNA polymerases, $\alpha$ (Cotterill et al., 1987), $\beta$ (Mosbaugh & Meyer, 1980; Mosbaugh & Linn, 1983), $\gamma$ (Kunkel & Soni, 1988; Kunkel & Mosbaugh, 1989; Kaguni & Olson, 1989; Insdorf & Bogenhagen, 1989), $\delta$ (Byrnes et al., 1976; Crute et al., 1986), and $\epsilon$ (Burgers et al., 1989, 1990), have been reported to contain polymerase and 3′ to 5′ exonuclease activities; however, their catalytic subunit compositions have not been determined in all cases. Many DNA metabolizing enzymes function in multistep biochemical pathways that are coordinated by interactive catalytic activities. In some cases multiple enzyme activities are exhibited by a single polypeptide whereas in others a multimeric enzyme functions as a complex (Weiss, 1981; Sancar & Sancar, 1988; Modrich, 1989).

Assignment of catalytic activities to specific polypeptides or complexes remains an important objective for elucidating the mechanisms of DNA replication and repair. Resolution and detection of enzyme activities in situ following polyacrylamide gel electrophoresis are powerful tools in this regard (Spanos et al., 1981; Mezzina et al., 1984, 1987). Methods described within this paper and elsewhere (Blank et al., 1982,

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1983) have allowed efficient renaturation of numerous enzymes from SDS[1]–polyacrylamide gels containing a DNA substrate. Thus, application of the activity gel technique to many biochemical problems would appear potentially beneficial. Several types of DNA metabolizing enzymes have been individually detected by using separate DNA substrates and assays [for a review see Bertazzoni et al. (1986)]. This prompted us to develop a technique for the simultaneous detection of DNA polymerase and associated exonucleases following SDS–polyacrylamide gel electrophoresis (Longley & Mosbaugh, 1989). This system relied upon the use of a defined $^{32}$P-labeled oligonucleotide/M13 DNA substrate cast within the SDS–polyacrylamide gel. Following enzyme activity in situ, DNA products of polymerization or degradation were resolved according to size in a second dimension of electrophoresis through a denaturing DNA sequencing gel.

In this paper, we (i) describe both nondenaturing and SDS–polyacrylamide activity gel systems for simultaneously detecting activities in an enzyme complex, (ii) utilize complex DNA substrates for detecting five different catalytic activities in SDS–polyacrylamide gels, (iii) characterize DNA polymerase $\beta$ activity both in vitro and in situ, and (iv) describe a procedure for modifying DNA substrates within the polyacrylamide gel before or after in situ enzyme reactions.

## Experimental Procedures

### *Materials*

Bovine fibrinogen, prestained molecular weight protein markers (SDS-7B), lysyltryptophyl-$\alpha$-lysine (Lys-Trp-Lys), unlabeled 2′-deoxyribonucleoside 5′-triphosphates, ATP, and $NAD^+$ were obtained from Sigma. Sodium dodecyl sulfate (SDS, Lot 44215) came from Gallard-Schlessinger, and Pharmacia was the source of 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP). [$\gamma$-$^{32}$P]ATP and [$\alpha$-$^{32}$P]dATP were purchased from Amersham, and [$^3$H]dTTP was from New England Nuclear.

M13mp2, M13mp2(-C106), and M13mp19 DNA were isolated as described by Kunkel et al. (1987). Oligodeoxyribonucleotides TAACGCCAGGGT (12-mer), GGCGATTAAGTTGGG (15-mer), GTGCTGCAAGGCGATTAAGTTGGT (24-mer), and CGGCCAGTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTG (50-mer) were prepared with an Applied Biosystems Model 380A DNA synthesizer by the Gene Research Center (Oregon State University).

*E. coli* DNA polymerase I (LF), *E. coli* DNA ligase, and restriction endonucleases *Eco*RI, *Kpn*I, *Sma*I, *Xma*I, and *Xba*I were purchased from New England Biolabs. One unit of DNA ligase catalyzes 50% ligation of 6 $\mu$g of *Hin*dIII-digested $\lambda$DNA in 30 min at 16 °C under standard conditions. One unit of *Eco*RI, *Kpn*I, *Xma*I, or *Xba*I completely digests 1 $\mu$g of $\lambda$DNA in 1 h at 37 °C. One unit of *Sma*I is defined as above but at 25 °C. T4 polynucleotide kinase and *E. coli* exonuclease III were from Bethesda Research Laboratories. One unit of exonuclease III produces 1 nmol of acid-soluble nucleotide from sonicated DNA in 30 min at 37 °C. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim. Homogeneous Novikoff hepatoma DNA polymerase $\beta$ (fraction VI) was purified as previously described (Stalker et al., 1976). *E. coli* DNA polymerase III holoenzyme (fractions IV and V) prepared as described (Oberfelder & McHenry, 1987) was a gift from C. S. McHenry (University of Colorado). *E. coli* uracil–DNA glycosylase (fraction V) was purified according to Lindahl et al. (1977) as modified by Domena et al. (1988).

### *Methods*

*Preparation of DNA Substrates.* Deblocked/deprotected oligonucleotides were purified and labeled at the 5′-end by T4 polynucleotide kinase with [$\gamma$-$^{32}$P]ATP (5000 Ci/mmol) as described by Longley and Mosbaugh (1989). Analysis by 20% polyacrylamide DNA sequencing gel electrophoresis indicated that labeled oligonucleotides were >95% pure and had specific activities of (3.0–3.5) × $10^6$ cpm/pmol of 5′-ends. Defined DNA substrates were constructed by annealing oligonucleotides to various single-stranded M13 DNA molecules. Hybridization of each oligonucleotide to M13 DNA (0.27 pmol of 5′-ends/$\mu$g of DNA) was performed by heating to 70 °C with slow cooling to 25 °C as previously described (Longley & Mosbaugh, 1989).

*SDS–Polyacrylamide Gel Electrophoresis.* Separation of enzymes by SDS–polyacrylamide gel electrophoresis, extraction of SDS from the gel, and the in situ renaturation of proteins were performed essentially as described by Longley and Mosbaugh (1989). Briefly, enzyme preparations were resolved through 7.5%, 10%, or 12.5% polyacrylamide resolving gels [acrylamide/*N*,*N*′-methylenebis(acrylamide), 37.5:1 ratio] that also contained 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 50 $\mu$g/mL bovine fibrinogen, 2 mM EDTA, and 1.9 $\mu$g/mL $^{32}$P-labeled oligonucleotide/M13 DNA. Samples were prepared by heating at 37 °C for 3 min in 54 mM Tris-HCl (pH 6.8), 1.7 mM EDTA, 120 mM 2-mercaptoethanol, 6.25% (w/v) glycerol, 0.8% SDS, and 0.03% bromophenol blue. Electrophoresis was performed at 4 °C and 100 V until the bromophenol blue dye migrated through the stacking gel. The stacking gel was composed of 4.5% acrylamide, 0.2% *N*,*N*′-methylenebis(acrylamide), 125 mM Tris-HCl (pH 6.8), 0.1% SDS, and 2 mM EDTA. After stacking occurred, the potential was increased to 200 V, and migration continued until the dye approached the bottom of the resolving gel. Polypeptide molecular weights were determined by using the prestained proteins $\alpha_2$-macroglobulin (180 000), $\beta$-galactosidase (116 000), fructose-6-phosphate kinase (84 000), pyruvate kinase (58 000), fumarase (48 500), lactic dehydrogenase (36 500), and triosephosphate isomerase (26 600), which were resolved in an adjacent lane. Immediately following electrophoresis, the SDS was extracted from the resolving gel at 25 °C by agitation for 30 min in 33 gel volumes of 10 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, and 25% (v/v) 2-propanol. After the SDS extraction step was repeated, resolving gels were incubated with agitation for 18 to 25 h at 4 °C in 27 gel volumes of an enzyme renaturation buffer, as described in the figures.

*Nondenaturing Polyacrylamide Gel Electrophoresis.* Polyacrylamide gel electrophoresis under nondenaturing conditions was performed by a modification of that described by Davis (1964). The resolving gel contained 10% acrylamide, 0.37% *N*,*N*′-methylenebis(acrylamide), 375 mM Tris-HCl (pH 8.8), 50 $\mu$g/mL bovine fibrinogen, 2 mM EDTA, 1.9 $\mu$g/mL $^{32}$P-labeled oligonucleotide/M13 DNA, 0.07% ammonium persulfate, and 0.03% TEMED. Stacking gels were composed of 2.5% acrylamide, 0.625% *N*,*N*′-methylenebis(acrylamide), 62.5 mM Tris-HCl (pH 6.8), 20% sucrose, 2 mM EDTA, 5

[1] Abbreviations: LF, large fragment of *Escherichia coli* DNA polymerase I; Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate adjusted to pH 8.0; DTT, dithiothreitol; BSA, bovine serum albumin; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; dNTP, four (A,G,T,C) deoxyribonucleoside triphosphates; SDS, sodium dodecyl sulfate; Lys-Trp-Lys, lysyltryptophyl-$\alpha$-lysine; TCA, trichloroacetic acid; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

μg/mL riboflavin, and 0.1% TEMED. The stacking gel was polymerized by exposure to fluorescent light (Sylvania Daylight F15T8/D bulbs), and during this period (15 min) the resolving gel was shielded from the light. Samples were adjusted to final concentrations of 30% (w/v) glycerol and 0.008% bromophenol blue before being loaded into wells. Electrophoresis was carried out at 4 °C in running buffer (25 mM Tris–base, 192 mM glycine, pH 8.3) as described above.

Two gel configurations (8.5 × 0.075 cm) were used for both SDS–polyacrylamide and nondenaturing polyacrylamide gel electrophoresis. The first gel format ("pilot activity gel") had multiple sample wells (0.5 × 1.3 cm), a 1 cm long stacking gel, and a relatively short resolving gel (1 cm). The pilot activity gel was designed for direct comparison of enzyme activity in individual lanes. This format was suited for determining levels of sensitivity or optimizing renaturation and/or reaction conditions for individual enzymes. The second gel format ("analytical activity gel") had a single, wide sample well (1.3 × 1.3 cm), a 1 cm long stacking gel, and a longer resolving gel (5 cm). Analytical activity gels afforded greater resolution of proteins and molecular weight determinations under denaturing conditions.

*In Situ Enzyme Reactions.* Following nondenaturing polyacrylamide gel electrophoresis or after renaturation of enzymes within SDS–polyacrylamide gels, in situ enzyme reactions were initiated by immersing resolving gels into the reaction buffers described in the figures. Pilot activity gels were placed directly into reaction mixtures; however, the lane of analytical activity gels was first sliced longitudinally into six to eight identical gel strips (~0.2 × 5 cm) before the reactions were carried out. After the strips were sliced, each gel strip was placed into a separate reaction tube containing 5 mL of reaction buffer. In situ reactions were performed at 25 °C for 60 min, unless otherwise indicated. Reactions were terminated by substituting reaction buffer (4 °C) containing 10 mM EDTA but lacking diffusible cofactors and substrates. Following incubation at 4 °C for 30 min, $^{32}$P-labeled DNA reaction products within the gels were analyzed by electrophoresis through a DNA sequencing gel.

*DNA Sequencing Gel Analysis.* Pilot or analytical activity gels containing [$^{32}$P]DNA products were cast within 20% polyacrylamide (29:1), 8.3 M urea DNA sequencing gels as described by Longley and Mosbaugh (1989). While pilot activity gels were kept in the same orientation for both protein and oligonucleotide separation, analytical activity gel slices were rotated 90° prior to the second electrophoretic analysis (DNA sequencing gel). In all cases, enzyme migration through the SDS–polyacrylamide gel was from left to right as displayed in the figures. Reorientation of analytical gels permitted separation of DNA metabolizing enzymes in the first dimension of electrophoresis and a size analysis of $^{32}$P-labeled products in the second dimension. Electrophoresis was conducted in buffer (134 mM Tris base, 44.5 mM boric acid, 2.6 mM EDTA) at 1200 V until the bromophenol blue dye had migrated ~18 cm. Autoradiography was performed at room temperature with Kodak XAR-5 film, and radioactive bands were quantified with a Biomed Instruments Model SL-504-XL soft laser scanning densitometer.

*Enzyme Assays.* Novikoff hepatoma DNA polymerase β activity was measured in reaction mixtures (100 μL) containing 25 mM Tris-HCl (pH 8.4), 5 mM 2-mercaptoethanol, 7 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, 15% (w/v) glycerol, 400 μg/mL BSA, 100 μM each dATP, dCTP, dGTP and [α-$^{32}$P]dTTP (3000–9900 cpm/pmol), 0.19 μg of unlabeled 15-mer primer/M13mp2 DNA template, and 0.03–0.3 units of DNA polymerase β. Reactions were incubated at 37 °C for 60 min and TCA-insoluble radioactivity was measured (Mosbaugh, 1988). A similar assay for polymerase β utilized the same reaction components except the incorporation of [$^3$H]dTMP (110 cpm/pmol) into activated calf thymus DNA (10 μg/reaction) was measured when indicated. *E. coli* DNA polymerase I (LF) reactions (100 μL) contained 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 7 mM $MgCl_2$, 50 mM KCl, 16% (w/v) glycerol, 400 μg/mL BSA, 100 μM each dATP, dCTP, dGTP, and [$^3$H]dTTP (100 cpm/pmol), 10 μg of activated calf thymus DNA, and 0.003–0.3 unit of polymerase. After incubation at 37 °C for 30 min, samples were processed as above to detect TCA-insoluble radioactivity. *E. coli* uracil–DNA glycosylase was assayed as previously described (Domena & Mosbaugh, 1985). One unit of uracil–DNA glycosylase is defined as the amount of enzyme required to release 1 nmol of uracil from DNA per hour at 37 °C.

## Results

*Detection of Various DNA Polymerases following Polyacrylamide Gel Electrophoresis.* A novel activity gel technique was developed to simultaneously detect several DNA metabolizing enzyme activities including DNA polymerases. An outline of this procedure using both denaturing and nondenaturing polyacrylamide gels is summarized in Figure 1. The ability to efficiently detect DNA polymerase activities in situ depends on several factors, including the optimization of reaction conditions, the efficiency of primer/template utilization within the context of the gel, and the intrinsic property of each enzyme to retain a catalytically active conformation. Since these variables cannot be predicted for a given enzyme, we initially determined the level of detection for various DNA polymerases. The level of sensitivity for detecting Novikoff hepatoma DNA polymerase β and *E. coli* DNA polymerases I (LF) and III was determined by using both denaturing and nondenaturing pilot activity gels (Figure 2). With gels containing [$^{32}$P]15-mer/M13mp2 DNA as the primer/template, DNA polymerase β was minimally detected with 0.0046 and 0.0005 unit in denaturing and nondenaturing activity gels, respectively (Figure 2A). This activity was proportional to polymerase concentration in both systems; however, the detection in SDS–polyacrylamide gels was ~9-fold less efficient. In contrast, with DNA polymerase I (LF) the threshold of detection was 0.00025 unit,[2] and nonlinear recovery of activity was observed in both gel systems (Figure 2B). Although ≥16 units of *E. coli* DNA polymerase III holoenzyme could be linearly detected in nondenaturing activity gels, up to 27 times this amount was undetectable following SDS–polyacrylamide gel electrophoresis (Figure 2C).

*Characterization of DNA Polymerase β Using Activity Gels. (A) Optimal in Situ Reaction Conditions.* DNA polymerase β samples (0.1 unit/lane) were applied to several lanes of a

[2] One unit of DNA polymerase β incorporates 1 nmol of total nucleotide into activated DNA per hour at 37 °C under standard reaction conditions. One unit of DNA polymerase I, large fragment, incorporates 10 nmol of dNMP into DNA per 30 min at 37 °C. One unit of DNA polymerase III holoenzyme incorporates 1 pmol of dNMP into DNA per minute at 30 °C. Normalization of units indicates that the minimum amounts of DNA polymerase β and polymerase I (LF) detectable by denaturing activity gels are roughly equal (4.6 and 5.0 pmol of dNMP incorporated/h, respectively). However, the minimum amount of polymerase β (0.51 pmol of dNMP incorporated/h) required to detect activity on native gels was almost 10-fold less than that of polymerase I, large fragment (5.0 pmol of dNMP incorporated/h), and the amount of polymerase III (960 pmol of dNMP incorporated/h) was almost 1900 times greater than that of polymerase β.

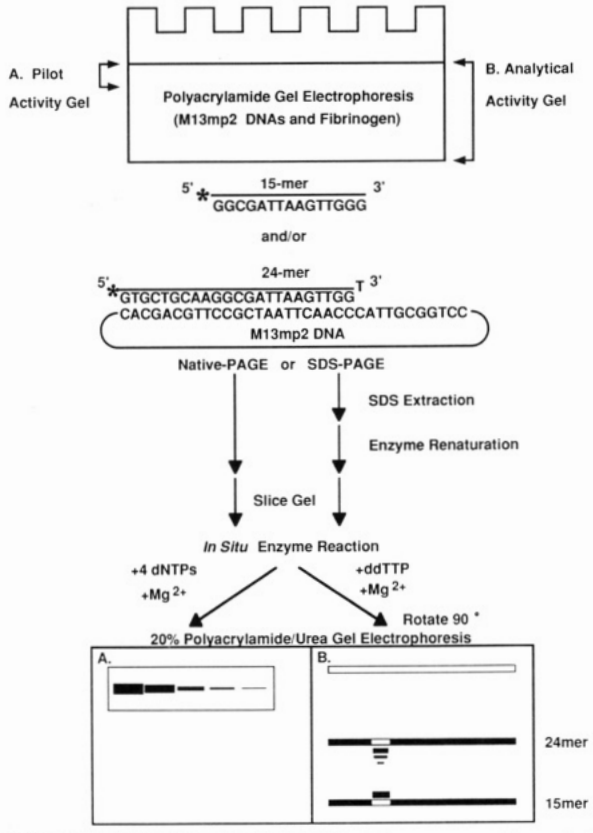


FIGURE 1: Scheme for detecting DNA polymerase and associated exonuclease activities in situ following polyacrylamide gel electrophoresis. Either denaturing or nondenaturing polyacrylamide gels containing 5'-end $^{32}$P-labeled primer/templates were cast by using one of two formats as described under Experimental Procedures. (A) Pilot activity gels consisted of a 1-cm resolving gel matrix and were used to determine the detection limit or to characterize DNA polymerase activity. (B) Analytical activity gels consisted of a 5-cm resolving gel matrix, but unlike above, these activity gels were sliced longitudinally before in situ enzyme reactions were performed. In addition, each slice was rotated 90° and cast within the DNA sequencing gel. In both formats, $^{32}$P-labeled DNA primers were resolved by electrophoresis and [$^{32}$P]DNA was detected by autoradiography. For denaturing SDS–polyacrylamide gels only, SDS was extracted with 2-propanol prior to polypeptide renaturation. Gels were incubated in a DNA polymerase reaction mixture, the in situ reactions were terminated with buffer (4 °C) containing 10 mM EDTA and then cast within a 20% polyacrylamide DNA sequencing gel, and electrophoresis was performed.

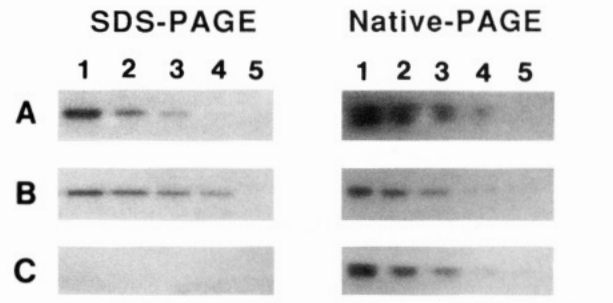


FIGURE 2: Comparison of in situ DNA polymerase activity following SDS–polyacrylamide and nondenaturating polyacrylamide gel electrophoresis. (A) Novikoff hepatoma DNA polymerase $\beta$ was applied to a 10% SDS–polyacrylamide pilot activity gel containing [$^{32}$P]15-mer/M13mp2 DNA. Samples corresponding to lanes 1–5 contained 0.12, 0.041, 0.014, 0.0046, and 0.0015 unit of DNA polymerase $\beta$, respectively. For the nondenaturing gel, lanes 1–5 corresponded to 0.014, 0.0046, 0.0015, 0.0005, and 0.00017 unit, respectively. Electrophoresis and processing of the gels were as described under Experimental Procedures. DNA polymerase $\beta$ renaturation buffer contained 25 mM Tris-HCl (pH 8.4), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 15% (w/v) glycerol, 400 $\mu$g/mL BSA, and 500 mM NaCl. The DNA polymerase $\beta$ reaction mixture was identical except that 7 mM $MgCl_2$ and 100 $\mu$M each dATP, dCTP, dGTP, and dTTP were added and the NaCl concentration was reduced to 50 mM. (B) *E. coli* DNA polymerase I (LF) was analyzed as in (A) except that the renaturation buffer contained 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 16% (w/v) glycerol, 400 $\mu$g/mL BSA, and 50 mM KCl. The reaction mixture was the same as the renaturation buffer supplemented with 7 mM $MgCl_2$ and 100 $\mu$M each deoxyribonucleoside triphosphate. Lanes 1–5 for both denaturing and nondenaturing gels contained 0.0068, 0.0023, 0.00076, 0.00025, and 0.000084 unit of DNA polymerase I (LF), respectively. (C) *E. coli* DNA polymerase III holoenzyme (fraction V) was analyzed as in (A) except that the renaturation buffer was composed of 35 mM HEPES–KOH (pH 7.6), 7 mM DTT, 15% (w/v) glycerol, 400 $\mu$g/mL BSA, and 50 mM KCl. The reaction mixture was composed of renaturation buffer supplemented with 15 mM magnesium acetate and 100 $\mu$M of each deoxyribonucleoside triphosphate. Lanes 1–5 of both gels contained 438, 146, 49, 16, and 5.4 units of DNA polymerase III holoenzyme, respectively. In situ DNA polymerase reactions (40 mL/pilot gel) were incubated at 25 °C for 60 min.

10% SDS–polyacrylamide pilot activity gel containing [$^{32}$P]-15-mer/M13mp2 DNA. Following electrophoresis, SDS extraction, and enzyme renaturation, individual gel lanes were incubated in reaction buffer containing various concentrations of NaCl, $MgCl_2$, and deoxyribonucleoside triphosphates. After in situ DNA polymerase $\beta$ reactions were terminated, pilot gels were reassembled and cast within a 20% polyacrylamide/urea DNA sequencing gel. Then unutilized $^{32}$P-labeled primers were removed by electrophoresis and extended $^{32}$P-labeled primers, too large to enter the DNA sequencing gel, were quantified by densitometric scanning of autoradiograms. As a comparison, standard in vitro DNA polymerase $\beta$ assays were performed under in situ reaction conditions. Consistent with previous in vitro results (Stalker et al., 1976), NaCl was shown to inhibit polymerase $\beta$ above 50 mM NaCl; whereas, we observed inhibition in situ above 100 mM NaCl (data not shown). Optimal cofactor requirements were determined to be 7.5 and 10 mM $MgCl_2$ for in vitro and in situ reactions, respectively. Saturation with deoxyribonucleoside triphosphates was comparable for both reactions (e.g., 40 $\mu$M each dNTP = 59% and 71% of control activity in vitro and in situ, respectively), and activity plateaued at $\sim$100 $\mu$M. To determine the optimal substrate concentration of primer/template DNA, in situ denaturing pilot activity gels that contained [$^{32}$P]15-mer/M13mp2 DNA (0.5–7.6 $\mu$g/mL) and 0.03 unit of polymerase $\beta$ were analyzed. Maximal activity was detected at $\geq$1.9 $\mu$g/mL DNA, and proportionally lower activity was observed at reduced concentrations of DNA (data not shown).

(*B*) *Heat Inactivation.* To determine whether activity could be recovered from thermally denatured enzyme, as observed for enzyme denatured by SDS, heat inactivation rates were measured. From this experiment we wished to also examine whether inactive enzymes within a preparation could be detected as cryptic activities following enzyme renaturation. After heating DNA polymerase $\beta$ at 37 °C for 0–30 min, identical samples were analyzed by in vitro assays and on denaturing pilot activity gels. Each detection method showed a linear rate of inactivation with similar half-lives of 7 min (in vitro) and 4.5 min (in situ). These results indicate that thermally inactivated DNA polymerase $\beta$ was not reactivated by the SDS-polyacrylamide gel procedure. Hence, the recovery of activity following denaturation by SDS and the inability to renature activity following heat treatment suggest fundamental differences in these two mechanisms of inactivation.

(*C*) *Molecular Weight Determination.* DNA polymerase $\beta$ was analyzed on separate 7.5%, 10%, and 12.5% polyacrylamide denaturing analytical activity gels containing

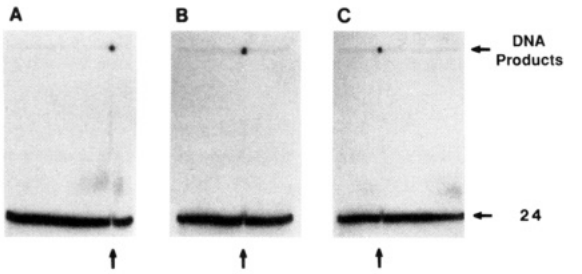


FIGURE 3: Analysis of DNA polymerase $\beta$ on analytical activity gels containing various concentrations of polyacrylamide. Samples (0.6 unit) of Novikoff hepatoma DNA polymerase $\beta$ were separately analyzed on (A) 7.5%, (B) 10%, and (C) 12.5% polyacrylamide denaturing analytical activity gels containing [$^{32}$P]24-mer/M13mp2(-C106) DNA. Electrophoresis and SDS extraction were described under Experimental Procedures. DNA polymerase $\beta$ renaturation and reaction buffers were as described in Figure 2. Single longitudinal slices ($\sim$0.2 $\times$ 5 cm) of each gel were incubated for 30 min at 25 °C in reaction buffer. Gel slices were then cast into a 20% polyacrylamide DNA sequencing gel before electrophoresis and autoradiography were performed. Unutilized [$^{32}$P]DNA primers resolved during electrophoresis in the denaturing sequencing gel are indicated as the 24-mer oligonucleotide band (arrow, 24). Migration of DNA polymerase $\beta$ through the SDS–polyacrylamide gel (arrow, DNA products) was from left to right, and its position in each panel is indicated by the vertical arrow.

[$^{32}$P]24-mer/M13mp2(-C106) DNA. Following electrophoresis and enzyme renaturation, a longitudinal slice from each gel was incubated in a DNA polymerase reaction mixture. Subsequently, [$^{32}$P]DNA reaction products within the gels were then analyzed by a second dimension of electrophoresis through denaturing DNA sequencing gels. Extended $^{32}$P-labeled primers too large to enter the DNA sequencing gel denoted the location of polymerase activity, while unextended primers migrated as a uniform band, 24 nucleotides in length (Figure 3). Compared with the mobilities of prestained molecular weight protein standards run in adjacent lanes, the molecular weights determined for DNA polymerase $\beta$ were 34 000, 38 000, and 39 000 (Figure 3A–C, respectively). Furthermore, similar amounts of [$^{32}$P]DNA were retained in each protein gel strip suggesting that the combined renaturation and reaction efficiencies were also unaffected by polyacrylamide concentration.

*Detection of 3′ to 5′ Exonucleases Using Activity Gels.* The 3′ to 5′ exonuclease activities of *E. coli* exonuclease III, DNA polymerase I (LF), and DNA polymerase III holoenzyme were analyzed by denaturing analytical activity gels containing an equal molar mixture of [$^{32}$P]15-mer/M13mp2 DNA and [$^{32}$P]24-mer/M13mp2 DNA. As schematically illustrated in Figure 4, the 15-mer was fully complementary to positions 106–120 of the *lacZ*$_\alpha$ DNA sequence of M13mp2 DNA, producing a substrate with a matched 3′-terminal nucleotide. In contrast, the 24-mer was complementary to positions 107–129 but formed a 3′-terminal (T·C) mispair at position 106. After these two substrates were mixed in an analytical activity gel, the specificity of various exonucleases for hydrolyzing matched and mismatched nucleotides was determined. Following in situ reactions, [$^{32}$P]DNA products were separated according to size. Unaltered [$^{32}$P]15-mer and [$^{32}$P]24-mer migrated as uniform bands, and degraded [$^{32}$P]oligonucleotides were observed at positions corresponding to each exonuclease. Exonuclease III was observed to degrade both the matched 15-mer and the mismatched 24-mer (Figure 4A). This result was expected since exonuclease III shows specificity for degrading double-stranded DNA whether or not the 3′-terminus contains a mispair (Brutlag & Kornberg, 1972). On the other hand, the 3′ to 5′ exonuclease activities

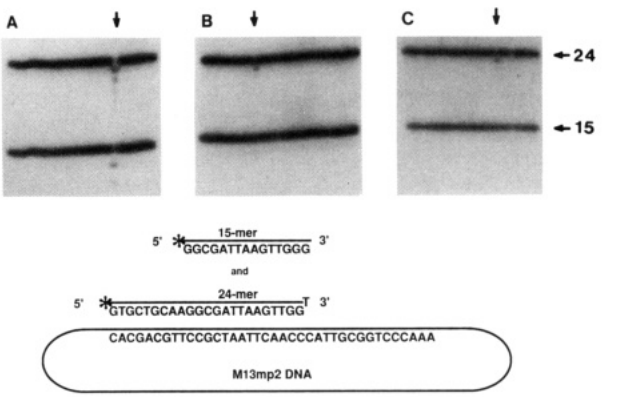


FIGURE 4: Detection of 3′ to 5′ exonucleases following SDS–polyacrylamide gel electrophoresis. (A) *E. coli* exonuclease III (0.54 unit), (B) DNA polymerase I, large fragment (0.026 unit of polymerase), and (C) DNA polymerase III holoenzyme (15 600 units of polymerase) were separately resolved by denaturing analytical activity gel electrophoresis. This 10% polyacrylamide gel was prepared, electrophoresis was performed, and SDS extraction was carried out as described under Experimental Procedures except that both [$^{32}$P]15-mer/M13mp2 DNA and [$^{32}$P]24-mer/M13mp2 DNA were included each at 0.95 $\mu$g/mL. The nucleotide sequence of these substrates is shown above. Exonuclease III was renatured in buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 400 $\mu$g/mL BSA, 50 mM KCl, and 15% (w/v) glycerol. DNA polymerases I and III were renatured as described in Figure 2. In situ reactions were conducted in renaturation buffer supplemented with 5 mM $MgCl_2$ (A), 7 mM $MgCl_2$ (B), or 15 mM magnesium acetate (C). [$^{32}$P]DNA products were resolved by denaturing DNA sequencing gel electrophoresis, and the locations of the 3′-terminally mismatched (arrow, 24) and matched (arrow, 15) oligonucleotides are indicated. The position of each enzyme is indicated with a vertical arrow.

of DNA polymerase I (LF) and the $\epsilon$-subunit of DNA polymerase III exhibited a significant preference for mismatched over matched DNA substrates (Figure 4B, C). This preference was even more apparent when the time course of the reaction was monitored (data not shown). The matched 23-mer accumulated almost exclusively prior to the generation of 22-mer or 14-mer from the fully complementary 15-mer.

*Limited DNA Synthesis in Situ by DNA Polymerase I (LF) from Terminally Matched and Mismatched Primer/Template DNA.* After determining the 3′ to 5′ exonuclease substrate specificity, we analyzed the in situ ability of DNA polymerase I (LF) to utilize these matched and mismatched DNA substrates as primer/templates. DNA polymerase I (LF) was analyzed on a denaturing analytical activity gel containing the mixed [$^{32}$P]DNA substrates, as described above. Following DNA synthesis reactions containing only dTTP, $^{32}$P-labeled reaction products were resolved by DNA sequencing gel electrophoresis and the autoradiogram was densitometrically scanned (Figure 5A). As expected, the 3′ to 5′ exonuclease of DNA polymerase I (LF) removed the 3′-terminal mismatch from the 24-mer. The newly generated 23 nucleotide long product was not anticipated to be extended because of the absence of a complementary deoxyribonucleoside triphosphate. On the other hand, the 15-mer was elongated primarily to a 16-mer by complementary addition of a single dTMP residue; 29% of the 16-mer was further converted to a 17-mer, presumably by the formation of a T·T mispair. In a reaction that contained dTTP and dGTP, DNA polymerase I (LF) elongated both [$^{32}$P]15-mer and [$^{32}$P]24-mer primers (Figure 5B). Unlike before, extension of the mismatched 24-mer was expected since dGMP incorporation could occur after excision of the 3′-terminal mismatch. This would regenerate a substrate with a 3′-terminal DNA sequence equivalent to the 15-mer. Both primers were maximally extended by two nucleotides to generate 17-mer and 26-mer products, presumably by non-

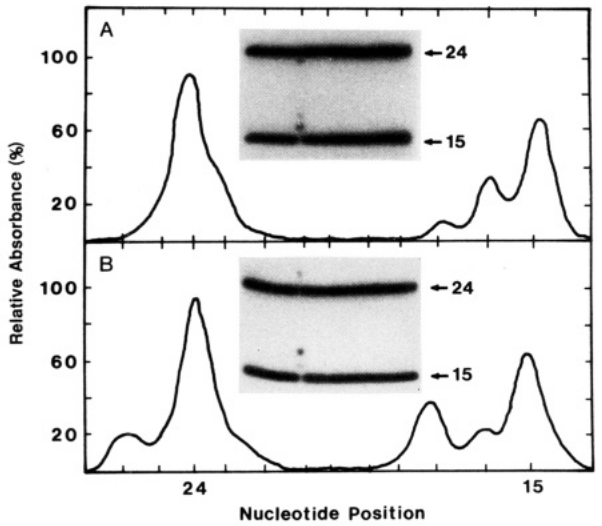


FIGURE 5: Limited DNA synthesis by DNA polymerase I (LF) with 3′-terminally mismatched and matched primer/template DNAs. SDS–polyacrylamide gel electrophoresis and renaturation of DNA polymerase I (LF) was as described in Figure 4B. In situ DNA polymerase reactions were performed as in Figure 2B excpt that only 100 $\mu$M dTTP (A) or 100 $\mu$M dTTP and 100 $\mu$M dGTP (B) were included. [$^{32}$P]DNA products were then resolved by denaturing DNA sequencing gel electrophoresis as described under Experimental Procedures. The location of the 3′-terminally mismatched (24-mer) and matched (15-mer) oligonucleotides are indicated by arrows. Scanning densitometry of autoradiograms at the location of DNA polymerase activity was performed and relative absorbance was plotted.

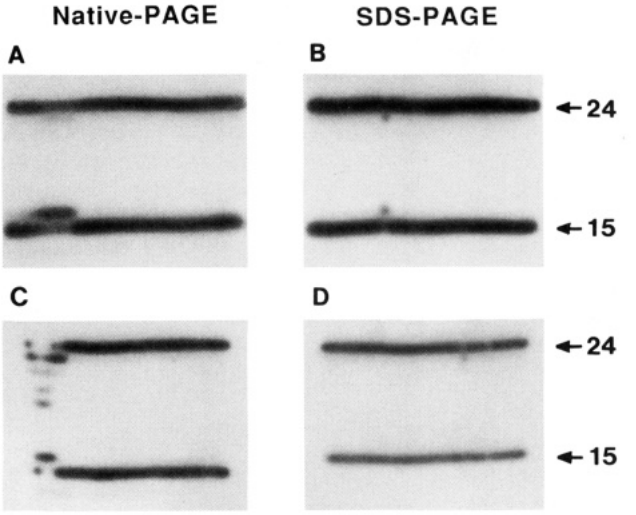


FIGURE 6: Nondenaturing and denaturing analytical activity gel analysis of DNA polymerase I (LF) and DNA polymerase III (holoenzyme) with 3′-terminally mismatched and matched primer/templates. Nondenaturing and denaturing 10% polyacrylamide gels were prepared as described under Experimental Procedures except that both [$^{32}$P]15-mer/M13mp2 DNA (matched) and [$^{32}$P]24-mer/M13mp2 DNA (mismatched) were each included at 0.95 $\mu$g/mL. DNA polymerase I, large fragment (0.026 unit/lane), was resolved by native polyacrylamide (A) and SDS–polyacrylamide (B) gels electrophoresis. Similarly, DNA polymerase III holoenzyme was resolved by native (C) and SDS–polyacrylamide (D) gels by using 1080 (fraction V) and 15 600 units (fraction IV), respectively. Electrophoresis and SDS extraction were performed as described under Experimental Procedures. Renaturation buffers and DNA polymerase reaction buffers were as described in Figure 2 except that 100 $\mu$M dideoxythymidine triphosphate was included as the only precursor for DNA synthesis. In situ reactions were carried out at 25 °C for either 15 min (A) or 60 min (B–D) before [$^{32}$P]DNA products were resolved by denaturing DNA sequencing gel electrophoresis. The location of the mismatched (24-mer) and matched (15-mer) oligonucleotides are indicated by arrows.

complementary (G/T·T) addition.

*Catalytic Subunit Composition of Multifunctional and Multimeric Enzymes.* From the results presented above, it became apparent that the catalytic subunit composition of DNA metabolizing enzymes could be determined by analytical activity gel analysis using a combination of DNA substrates. Utilizing both nondenaturing and denaturing activity gels, enzyme complexes or individual polypeptides possessing various activities could be detected. Such an analysis was performed by using DNA polymerase I (LF) and polymerase III (holoenzyme). Both the polymerase and 3′ to 5′ exonuclease activities of DNA polymerase I (LF) were observed to comigrate in nondenaturing ($R_f$ = 0.21) and denaturing ($M_r$ = 68 000) activity gels (panels A and B of Figure 6, respectively). In this experiment, in situ reactions contained only dideoxythymidine triphosphate, which alleviated the problem associated with mispairing during the primer extension reaction. Hence, DNA polymerase activity was detected by the appearance of [$^{32}$P]16-mer and 3′ to 5′ exonuclease activity by degradation to a [$^{32}$P]23-mer. Though fully complementary to M13mp2 DNA, the product of the exonuclease reaction could not be faithfully extended since dGTP was not present in the reaction. A similar analysis of DNA polymerase III (holoenzyme) indicated that both the polymerase ($\alpha$-subunit) and 3′ to 5′ exonuclease ($\epsilon$-subunit) migrated as a complex ($R_f$ = 0.08) in the nondenaturing activity gel (Figure 6C). As expected, when analyzed by SDS–polyacrylamide activity gels, the holoenzyme subunits were dissociated; however, only the 3′ to 5′ exonuclease activity was detected. The activity of the $\epsilon$-subunit had an apparent polypeptide molecular weight of 27 000 (Figure 6D). Unfortunately, the polymerase activity of the isolated $\alpha$-subunit was not active. Despite this shortcoming, in situ activity gel analysis using mixed [$^{32}$P]DNA substrates clearly affords the ability to assign catalytic activities to individual polypeptides or enzyme complexes.

*Detection of Other DNA Metabolizing Enzymes in Activity Gels. (A) E. coli Uracil–DNA Glycosylase.* To analyze uracil–DNA glycosylase in an SDS–polyacrylamide gel, a $^{32}$P-labeled oligonucleotide (24-mer) containing a site-specific uracil residue was hybridized to M13mp2(–C106) DNA and cast within the gel (Figure 7). Following electrophoresis and enzyme renaturation, in situ uracil–DNA glycosylase activity would be expected to cleave the N-glycosidic bond between uracil$_{15}$ and the deoxyribose phosphate backbone of the [$^{32}$P]24-mer. This would generate an apyrimidinic site (AP) at residue 15 but leave the oligonucleotide chain intact. To facilitate oligonucleotide cleavage, the tripeptide Lys-Trp-Lys was diffused into the polyacrylamide gel matrix. This tripeptide has been shown to bind DNA (Behmoaras et al., 1981a,b) and to promote breakage of the phosphodiester bond specifically on the 3′-side of the AP sites (Weiss & Grossman, 1987; Bailly & Verly, 1987) like a class I AP endonuclease (Mosbaugh & Linn, 1980). Therefore, uracil–DNA glycosylase activity should be detected by the generation of a [$^{32}$P]15-mer containing a 3′-terminal AP site. These DNA fragments would be expected to exhibit anomalous electrophoretic mobility (Haseltine et al., 1980; Gordon & Haseltine, 1980). As observed in Figure 7, such an oligonucleotide was indeed produced from a polypeptide of 24 000 molecular weight. This molecular weight agrees extremely well with that of the *E. coli ung* gene product (Lindahl et al., 1977; Varshney et al., 1988). In addition, another activity was observed ($M_r$ ~20 000) that produced two major $^{32}$P-labeled DNA products of approximately 14 and 15 nucleotides in length. As a control, this experiment was repeated with [$^{32}$P]24-mer/M13mp2-(–C106) DNA containing thymine in place of uracil at residue 15, and neither activity was detected.

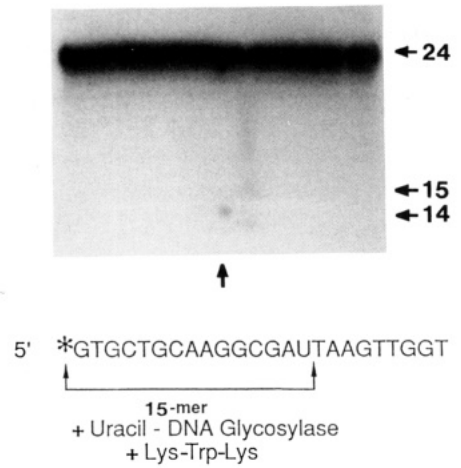


FIGURE 7: Detection of *E. coli* uracil–DNA glycosylase following SDS–polyacrylamide gel electrophoresis. A denaturing 12.5% polyacrylamide analytical activity gel containing [$^{32}$P]24-mer/M13mp2(-C106) DNA with a uracil residue at nucleotide position 15 was prepared as described under Experimental Procedures. *E. coli* uracil–DNA glycosylase (26 units) was then subjected to electrophoresis, SDS extraction, and enzyme renaturation. The renaturation and reaction buffers were both composed of 70 mM HEPES–KOH (pH 7.5), 5 mM 2-mercaptoethanol, 1 mM EDTA, 400 $\mu$g/mL BSA, and 15% (w/v) glycerol. Following the in situ glycosylase reaction the gel strip was incubated at 25 °C for 45 min in buffer containing 1 mM sodium cacodylate (pH 6.5), 0.1 mM EDTA, 1 mM NaCl, and 100 $\mu$M Lys-Trp-Lys. [$^{32}$P]DNA products were then resolved according to size by denaturing DNA sequencing gel electrophoresis. The locations of the [$^{32}$P]DNA (14-, 15-, and 24-mer) are indicated by horizontal arrows and the glycosylase is indicated by a vertical arrow.

(*B*) *Restriction Endonucleases.* To detect various restriction endonucleases, a 5′-end-labeled oligonucleotide ([$^{32}$P]50-mer) was annealed to the complementary multiple cloning region of M13mp19 DNA. This double-stranded DNA substrate contained recognition sites for *Eco*RI, *Kpn*I, *Sma*I, and *Xba*I (Figure 8). A mixture of *Eco*R1 and *Sma*I was resolved in a denaturing analytical activity gel containing [$^{32}$P]50-mer/M12mp19 DNA. After DNA restriction products were generated and resolved from the gel according to nucleotide length by using denaturing DNA sequencing gel electrophoresis, both endonucleases were detected. As shown in Figure 8A, the presence of a [$^{32}$P]9-mer designated the position of *Eco*RI ($M_r$ = 39 000), and the [$^{32}$P]27-mer indicated the location of *Sma*I ($M_r$ = 33 000). Several lines of evidence suggest that *Eco*RI exhibits biological activity as a homodimer (Modrich & Zabel, 1976; Lu et al., 1981; Frederick et al., 1984). Hence, this result suggested that in situ protein–protein oligomerization occurred following enzyme renaturation. In addition to resolving the mixture of *Eco*RI and *Sma*I, these two activities were also individually detected in situ as well as the activities of *Kpn*I, *Xba*I, and *Xma*I[3] (data not shown).

Being successful in introducing the tripeptide Lys-Trp-Lys into the gel matrix (Figure 7), we attempted to determine whether enzymes could similarly penetrate the gel and modify a [$^{32}$P]DNA substrate. Small sections of an activity gel containing the [$^{32}$P]50-mer/M13mp19 DNA were separately incubated with *Eco*RI, *Kpn*I, *Sma*I, or *Xba*I. Following DNA sequencing gel analysis of the treated gel sections, the predicted (9-mer, 25-mer, 27-mer, and 36-mer) $^{32}$P-labeled oligonucleotide fragment was observed for each restriction endonuclease (Figure 8B, lanes 1–4, respectively). Undigested [$^{32}$P]50-mer was too large to denature and/or to enter the 20% polyacrylamide gel and remained in the original section of the SDS–polyacrylamide gel.

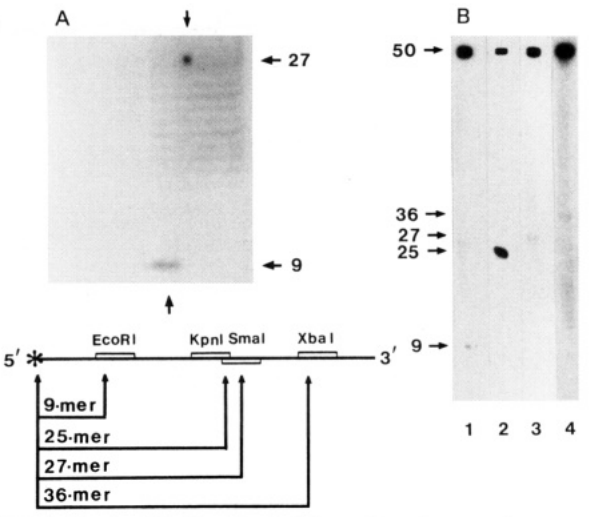


FIGURE 8: In situ detection of restriction endonucleases and exogenous modification of M13 DNA following SDS–polyacrylamide gel electrophoresis. A denaturing 10% polyacrylamide analytical activity gel containing [$^{32}$P]50-mer/M13mp19 DNA was prepared as described under Experimental Procedures. The locations of various restriction endonuclease recognition sites contained within the duplex region of the DNA molecule are indicated above. (A) A mixture of *Sma*I (94 units) and *Eco*RI (400 units) was resolved by electrophoresis, SDS was extracted from the gel, and renaturation of enzyme activites was performed as described under Experimental Procedures. The renaturation buffer consisted of 20 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 400 $\mu$g/mL BSA, 50 mM NaCl, and 15% (w/v) glycerol. In situ enzyme reactions were carried out at 25 °C for 90 min in renaturation buffer containing 25 mM NaCl and 8 mM $MgCl_2$. [$^{32}$P]DNA products were analyzed by denaturing DNA sequencing gel electrophoresis; the position of *Sma*I (27-mer) and *Eco*RI (9-mer) products are indicated with horizontal arrows, and *Eco*RI (upward arrow) and *Sma*I (downward arrow) are located. (B) Following SDS removal, a lane of the activity gel not containing restriction enzyme was sliced into sections (5 × 2 mm). Individual sections were incubated in reaction buffer (750 $\mu$L) to which (lane 1) 480 units of *Eco*RI, (lane 2) 50 units of *Kpn*I, (lane 3) 60 units of *Sma*I, or (lane 4) 100 units of *Xba*I was added. Reactions were carried out at 25 °C for either 2 h (lanes 1–3) or 16 h (lane 4) after which [$^{32}$P]DNA products were resolved by denaturing DNA sequencing gel electrophoresis. The positions of the undigested 50-mer and restriction endonuclease reaction products (9-, 25-, 27-, and 36-mer) are indicated with arrows.

*Modification of DNA Substrates before and after in Situ DNA Ligase Reactions.* We extended our experiments on modifying in situ DNA substrates to analyze a sequence of enzymatic reactions. In this experiment, we altered an oligonucleotide within the SDS–polyacrylamide gel, generating a substrate for in situ detection of *E. coli* DNA ligase. As outlined in Figure 9, a 12-mer lacking a 5′-terminal phosphate and a 5′-end-labeled [$^{32}$P]15-mer were hybridized to M13mp2 DNA. These two oligonucleotides were juxtaposed; however, the absence of a 5′-phosphate on the 12-mer was expected to prevent DNA ligase from catalyzing phosphodiester bond formation. This substrate was cast within an analytical activity gel and electrophoresis of DNA ligase carried out. After enzyme renaturation, no DNA ligase activity was detected (Figure 9A). However, upon preincubation of the gel strip with T4 polynucleotide kinase and ATP, DNA ligase activity was detected ($M_r$ = 71 000) by the formation of the [$^{32}$P]-27-mer (Figure 9B).

After illustrating that this in situ reaction was dependent on prior modification of the DNA substrate, we characterized the ligase reaction product in situ. In a similar experiment,

[3] The hexameric restriction endonuclease recognition sequence (CCCGGG) is identical for *Sma*I and *Xma*I; however, cleavage by the latter enzyme generated the expected [$^{32}$P]25-mer.

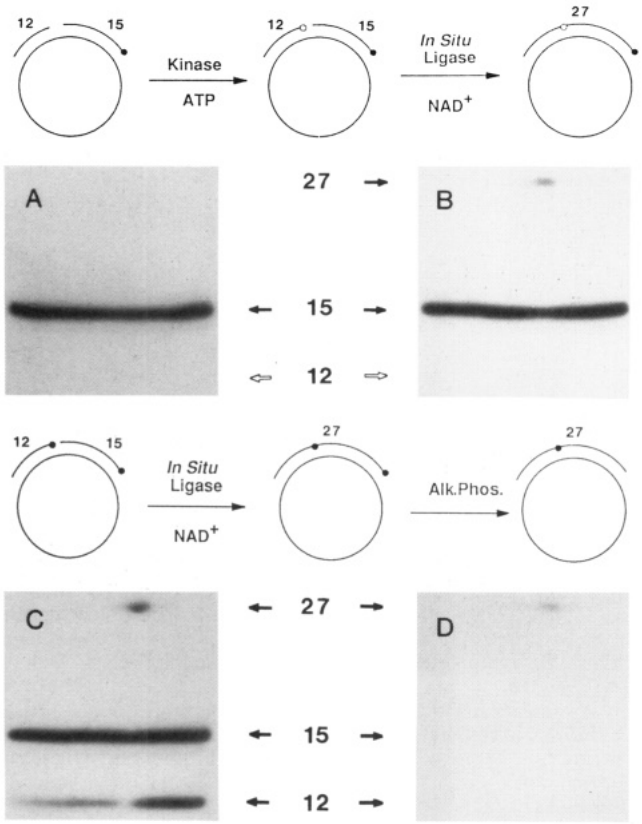


FIGURE 9: Modification of DNA substrates before and after an in situ DNA ligase reaction. Two denaturing 7.5% polyacrylamide analytical activity gels containing equal quantities of 12-mer and 15-mer (0.27 pmol/$\mu$g of DNA) both annealed to M13mp12 DNA were prepared as described under Experimental Procedures. The first gel initially contained [$^{32}$P]15-mer phosphorated at the 5′-end (●) and 12-mer containing a 5′-hydroxyl (—). *E. coli* DNA ligase (4.8 units) was applied to the gel followed by electrophoresis, SDS extraction, and enzyme renaturation. Ligase renaturation buffer contained 40 mM Tris-HCl (pH 8.0), 20 mM DTT, 50 mM KCl, 400 $\mu$g/mL BSA, and 5% (w/v) glycerol. (A) After an in situ ligase reaction at 25 °C for 30 min, [$^{32}$P]DNA products were resolved by denaturing DNA sequencing gel electrophoresis. DNA ligase reaction buffer consisted of renaturation buffer supplemented with 4.6 mM $MgCl_2$, 10 mM ammonium sulfate, and 150 $\mu$M $NAD^+$. (B) The in situ DNA ligase reaction above was preceded by incubation of the gel strip with T4 polynucleotide kinase plus ATP. Phosphorylation reactions (2 mL) were carried out at 37 °C for 60 min in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 50 mM KCl, 400 $\mu$g/mL BSA, 5% (w/v) glycerol, 1 mM ATP, and 40 units of T4 polynucleotide kinase. The addition of unlabeled phosphate to the 5′-end is represented by an open circle (○). One unit of polynucleotide kinase incorporates 1 nmol of phosphate from [$\gamma$-$^{32}$P]ATP into acid-insoluble DNA per 30 min at 37 °C. In the second gel, both 12-mer and 15-mer initially contained a $^{32}$P labels at their 5′-ends (●). Following the in situ ligase reaction, as before, the gel strip was incubated at 37 °C for 180 min in a solution (2 mL) containing 80 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$ 0.1 $MgCl_2$, $ZnCl_2$, and 40 units of calf intestinal alkaline phosphatase. One unit of phosphatase hydrolyzes 1 $\mu$mol of *p*-nitrophenol phosphate per minute at 37 °C. [$^{32}$P]DNA products were analyzed before (C) and after (D) alkaline phosphatase treatment by denaturing DNA sequencing gel electrophoresis. The locations of oligonucleotides (12-, 15-, and 27-mer) are indicated on the autoradiograms with arrows. For (A) and (B), the position of the unlabeled 12-mer was determined relative to a [$^{32}$P]12-mer standard.

5′-end-labeled [$^{32}$P]12-mer and [$^{32}$P]15-mer were hybridized to M13mp2 DNA and cast within the gel. As before, DNA ligase was subjected to electrophoresis and activity detected in situ (Figure 9C). In this experiment the two oligonucleotides formed an efficient substrate for ligase since both contained 5′-terminal phosphates. Thus, the [$^{32}$P]27-mer contained two $^{32}$P labels (one at the 5′-terminus and the other internalized between residues 15 and 16). Following this identical reaction, we treated a gel strip with exogenous calf intestinal alkaline phosphatase. As observed in Figure 9D, alkaline phosphatase treatment removed the terminal $^{32}$P label from both the unligated 12-mer and 15-mer. In contrast, the [$^{32}$P]27-mer was still detected although the $^{32}$P signal appeared to be reduced. This was anticipated since only one of the two $^{32}$P labels (5′-terminal phosphate) was hydrolyzed by phosphatase treatment.

## Discussion

We have designed a novel procedure for detecting DNA glycosylase, endonuclease, exonuclease, polymerase, and ligase activities in situ following polyacrylamide gel electrophoresis. Examples of 13 different activities were successfully detected, including Novikoff hepatoma DNA polymerase $\beta$, *E. coli* DNA polymerase I, large fragment (polymerase and 3′ to 5′ exonuclease), DNA polymerase III holoenzyme (polymerase and 3′ to 5′ exonuclease), exonuclease III, uracil–DNA glycosylase, DNA ligase, and the restriction endonucleases *Eco*RI, *Kpn*I, *Sma*I, *Xma*I, and *Xba*I. Unfortunately, we were unable to detect DNA polymerase activity from the isolated $\alpha$-subunit of DNA polymerase III holoenzyme following SDS–polyacrylamide gel electrophoresis. Recently, Studwell and O'Donnell (1990) have shown that the highly processive DNA synthesis exhibited by holoenzyme is contingent on the presence of the $\epsilon$-subunit. Since detection of DNA polymerase activity in pilot activity gels depends on significant extension of oligonucleotide primers (Longley & Mosbaugh, 1989), one explanation for this failure might involve reduced processivity. However, DNA polymerase $\beta$ is a highly distributive enzyme and it was detected by using a pilot activity gel. Thus, our inability to detect the $\alpha$-subunit more probably reflects low renaturation efficiency. Alternatively, this result could be explained by the inability of the $\alpha$-subunit to recognize the primer/template within the context of the gel. Maki and Kornberg (1985, 1987) have shown that the isolated $\alpha$-subunit exhibits reduced activity on a defined oligonucleotide primer/template compared to that of the core enzyme ($\alpha$-, $\epsilon$-, and $\theta$-subunits). Nevertheless, having detected a variety of DNA metabolizing enzymes following SDS–polyacrylamide gel electrophoresis, the inability to recover activity from the $\alpha$-subunit appears to be an exception and not the general rule.

Using nondenaturing polyacrylamide activity gels, we observed comigration of the DNA polymerase and 3′ to 5′ exonuclease activities of *E. coli* DNA polymerase I (LF) and III (holoenzyme), respectively. Whereas under denaturing conditions the activities of DNA polymerase I (LF) were contained in a single polypeptide, DNA polymerase III holoenzyme was clearly resolved into subunits. The evidence for subunit dissociation arises from the migration of the 3′ to 5′ exonuclease activity with the expected molecular weight ($M_r$ = 27 000) for the $\epsilon$ subunit (Scheuermann & Echols, 1984). Thus, the combination of these two activity gel systems should prove extremely useful in assigning catalytic activities to multifunctional or multimeric enzymes. Recently, we have utilized a similar approach on the *Thermus aquaticus* (*Taq*) DNA polymerase and observed that polymerase and 5′ to 3′ exonuclease activities appear to reside in a single polypeptide of approximately 90 000 molecular weight (Longley et al., 1991).

Several catalytic properties of DNA polymerase $\beta$ were examined under in vitro and in situ reaction conditions, and these reactions showed similar but not identical properties. Optimal in situ conditions generally required slightly higher concentrations of reaction components. Furthermore, the

inclusion of DNA in SDS–polyacrylamide activity gels did not appear to adversely affect molecular weight determinations since the molecular weight of Novikoff hepatoma DNA polymerase $\beta$ on 7.5%, 10%, and 12.5% polyacrylamide activity gels confirmed an earlier report of 32000 (Stalker et al., 1976).

Uracil–DNA glycosylase catalyzes the initial step of the base (uracil) excision repair pathway. The enzyme specifically removes uracil from DNA by cleaving the N-glycosidic bond linking the base to the deoxyribose phosphate backbone producing an AP site in DNA. Thus using a site-specific uracil-containing $^{32}$P-labeled oligonucleotide (24-mer) as substrate, the product of this reaction was expected to retain the same length. When detecting uracil–DNA glycosylase activity in situ we observed an accumulation of oligonucleotide at the location of the enzyme that migrated slightly faster than the 24-mer in the denaturing DNA sequencing gel (Figure 7). We assume that this material represented the 24-mer containing an intact AP site. To more clearly detect uracil–DNA glycosylase in situ we devised a secondary step for converting the product of the glycosylase reaction to a smaller oligonucleotide. The tripeptide Lys-Trp-Lys was allowed to penetrate the gel matrix to act like a class I AP endonuclease. Cleavage of the neighboring phosphodiester bond has been suggested to occur via $\beta$-elimination with incision expected 3′ to the AP site (Weiss & Grossman, 1987; Bailly & Verly, 1987). Thus, the combined action of uracil–DNA glycosylase and Lys-Trp-Lys produced a [$^{32}$P]15-mer containing a 3′-terminal AP site. Another minor activity ($M_r$ = 20000) was also detected in this enzyme preparation that specifically cleaved the [$^{32}$P]-24-mer on either side of the uracil residue, generating both 14-mer and 15-mer oligonucleotides. Perhaps this activity could be explained by an enzyme like *E. coli* endonuclease V ($M_r$ = 17000–27000) that degrades uracil-containing duplex DNA most efficiently (Gates & Linn, 1977) and reportedly (Demple & Linn, 1982) incised on one or the other side of DNA lesions (5′-side of 7-(bromomethyl)benz[*a*]anthracene and 3′-side of AP sites). Unfortunately, the exact cleavage site or sites of endonuclease V on uracil-containing DNA have not been determined. Nevertheless, the versatility for identifying new activities by this technique was clearly demonstrated.

In addition to modifying DNA with Lys-Trp-Lys, we showed that several enzymes (restriction endonucleases, polynucleotide kinase, and alkaline phosphatase) could permeate the gel matrix and catalytically alter the immobilized DNA. By arranging the order of substrate modifications, intermediate reaction products can be substrates for subsequent in situ reactions in a multistep pathway. In fact, the assays described within have illustrated an approach for detecting each individual step of base excision repair, including (i) removal of uracil residues from duplex DNA, (ii) cleavage at AP sites, (iii) excision of nucleotides, (iv) primer extension, and (v) phosphodiester bond formation. Thus, these activities could be sequentially identified following electrophoresis of crude preparations and modification(s) of the in situ DNA substrate. Alternatively, several different DNA substrates of varying length could be cast within a gel to detect these catalytic activities. With only simple modifications of substrate design, this technique could be applied to detect other enzymes in DNA metabolizing pathways.

**Registry No.** Lys-Trp-Lys, 38579-27-0; Mg, 7439-95-4; uracil–DNA glycosylase, 59088-21-0; DNA ligase, 9015-85-4; DNA polymerase I, 9012-90-2; exodeoxyribonuclease III, 9037-44-9; polynucleotide kinase, 37211-65-7; alkaline phosphatase, 9001-78-9; restriction endodeoxyribonuclease, 9075-08-5; apurinic/apyrimidinic endodeoxyribonuclease, 61811-29-8; DNA polymerase $\beta$, 9012-90-2; DNA polymerase III, 37217-33-7.

## References


Bailly, V., & Verly, W. G. (1987) *Biochem. J. 242*, 565–572.

Behmoaras, T., Toulme, J. J., & Helene, C. (1981a) *Nature 292*, 858–859.

Behmoaras, T., Toulme, J. J., & Helene, C. (1981b) *Proc. Natl. Acad. Sci. U.S.A. 78*, 926–930.

Bertazzoni, U., Scovassi, A. I., Mezzina, M., Sarasin, A., Franchi, E., & Izzo, R. (1986) *Trends Genet. 2*, 67–72.

Blank, A., Sugiyama, R. H., & Dekker, C. A. (1982) *Anal. Biochem. 120*, 267–275.

Blank, A., Silber, J. R., Thelen, M. P., & Dekker, C. A. (1983) *Anal. Biochem. 135*, 423–430.

Brutlag, D., & Kornberg, A. (1972) *J. Biol. Chem. 247*, 241–248.

Burgers, P. M. J. (1989) *Prog. Nucleic Acid Res. Mol. Biol. 37*, 235–280.

Burgers, P. M. J., Bambara, R. A., Campbell, J. L., Chang, L. M. S., Downey, K. M., Hubscher, U., Lee, M. Y. W. T., Linn, S. M., So, A. G., & Spadari, S. (1990) *Eur. J. Biochem. 191*, 617–618.

Byrnes, J. J., Downey, K. M., Black, V. L., & So, A. G. (1976) *Biochemistry 15*, 2817–2823.

Cotterill, S. M., Reyland, M. E., Loeb, L. A., & Lehman, I. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5635–5639.

Crute, J. J., Wahl, A. F., & Bambara, R. A. (1986) *Biochemistry 25*, 26–36.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Demple, B., & Linn, S. (1982) *J. Biol. Chem. 257*, 2848–2855.

Domena, J. D., & Mosbaugh, D. W. (1985) *Biochemistry 24*, 7320–7328.

Domena, J. D., Timmer, R. T., Dicharry, S. A., & Mosbaugh, D. W. (1988) *Biochemistry 27*, 6742–6751.

Frederick, C. A., Grable, J., Melia, M., Samudzi, C., Jen-Jacobson, L., Wang, B. C., Greene, P., Boyer, H. W., & Rosenberg, J. M. (1984) *Nature 309*, 327–331.

Gates, F. T., & Linn, S. (1977) *J. Biol. Chem. 252*, 1647–1653.

Gordon, L. K., & Haseltine, W. A. (1980) *J. Biol. Chem. 255*, 12047–12050.

Haseltine, W. A., Gordon, L. K., Lindan, C. P., Grafstrom, R. H., Shaper, N. L., & Grossman, L. (1980) *Nature 285*, 634–640.

Insdorf, N. F., & Bogenhagen, D. F. (1989) *J. Biol. Chem. 264*, 21498–21503.

Jovin, T. M., Englund, P. T., & Bertsch, L. L. (1969) *J. Biol. Chem. 244*, 2996–3008.

Kaguni, L. S., & Olson, M. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6469–6473.

Kunkel, T. A., & Soni, A. (1988) *J. Biol. Chem. 263*, 4450–4459.

Kunkel, T. A., & Mosbaugh, D. W. (1989) *Biochemistry 28*, 988–995.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol.* 154, 367–382.

Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B., & Sperens, B. (1977) *J. Biol. Chem. 252*, 3286–3294.

Longley, M. J., & Mosbaugh, D. W. (1989) *Methods Mol. Cell. Biol. 1*, 79–94.

Longley, M. L., Bennett, S. E., & Mosebaugh, D. W. (1991) *Nucleic Acids Res. 18*, 7317–7322.

Lu, A. L., Jack, W. E., & Modrich, P. (1981) *J. Biol. Chem. 256*, 13200–13206.

Maki, H., & Kornberg, A. (1985) *J. Biol. Chem. 260*, 12987–12992.

Maki, H., & Kornberg, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4389-4392.
Mezzina, M., Sarasin, A., Politi, N., & Bertazzoni, U. (1984) *Nucleic Acids Res. 12*, 5109-5122.
Mezzina, M., Rossignol, J. M., Philippe, M., Izzo, R., Bertazzoni, U., & Sarasin, A. (1987) *Eur. J. Biochem. 162*, 325-332.
Modrich, P. (1989) *J. Biol. Chem. 264*, 6597-6600.
Modrich, P., & Zabel, D. (1976) *J. Biol. Chem. 251*, 5866-5874.
Mosbaugh, D. W. (1988) *Nucleic Acids Res. 16*, 5645-5659.
Mosbaugh, D. W., & Linn, S. (1980) *J. Biol. Chem. 255*, 11743-11752.
Mosbaugh, D. W., & Meyer, R. R. (1980) *J. Biol. Chem. 255*, 10239-10247.
Mosbaugh, D. W., & Linn, S. (1983) *J. Biol. Chem. 258*, 108-118.
Oberfelder, R., & McHenry, C. S. (1987) *J. Biol. Chem. 262*, 4190-4194.
Sancar, A., & Sancar, G. B. (1988) *Annu. Rev. Biochem. 57*, 29-67.
Scheuermann, R. H., & Echols, H. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7747-7751.
Spanos, A., Sedgwick, S. G., Yarranton, G. T., Hubscher, U., & Banks, G. R. (1981) *Nucleic Acids Res. 9*, 1825-1839.
Stalker, D. M., Mosbaugh, D. W., & Meyer, R. R. (1976) *Biochemistry 15*, 3114-3121.
Studwell, P. S., & O'Donnell, M. (1990) *J. Biol. Chem. 265*, 1171-1178.
Varshney, U., Hutcheon, T., & van de Sande, J. H. (1988) *J. Biol. Chem. 263*, 7776-7784.
Weiss, B. (1981) *Enzymes (3rd Ed.) 14*, 203-231.
Weiss, B., & Grossman, L. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol. 60*, 1-34.

# Differential Sequence Dynamics of Homopolymeric and Alternating AT Tracts in a Small Plasmid DNA†

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ABSTRACT: The location of $OsO_4$ bispyridine hyper- and hyporeactivity in a small deletion derivative of plasmid ColE1 (PTC12, 1727 bp) has been determined for approximately 70% of the molecule. Thymine bases in homopolymeric $(dA)_n \cdot (dT)_n$ tracts ($n \geq 4$) were always found to be resistant toward $OsO_4$ modification. DNA supercoiling did not destabilize these tracts. The extent of $OsO_4$ bispyridine reactivity of homopolymeric $(dA)_n \cdot (dT)_n$ tracts, where $n = 3$, was found to be dependent on the rate of base unpairing of the sequence immediately 5′ and 3′ to the tract. Repressed $OsO_4$ reactivity of thymine bases in $(dA)_3 \cdot (dT)_3$ tracts was observed if immediately both 5′ and 3′ to the tract were stable DNA sequences composed of GC base pairs and/or a homopolymeric $(dA)_n \cdot (dT)_n$ tract ($n \geq 4$). Homopolymeric tracts of $n = 3$ not having adjacent sequences with repressed unpairing rates did not show reduced levels of $OsO_4$ bispyridine reactivity. Alternating $d(TA)_n$ tracts ($n \geq 2$) were found to exhibit hyperreactivity with $OsO_4$. The extent of this hyperreactivity was dependent on the length of the tract and superhelical torsional stress. The distribution and frequency of homopolymeric $(dA)_n \cdot (dT)_n$ ($n \geq 4$) tracts in *Escherichia coli* promoter sequences were examined, and the possible implications of these tracts on promoter function are discussed.

Patterns of local nucleotide sequence within promoters and regulatory sequences may be important determinants of biological activity, and we are interested in evaluating the structural dynamics of different sequence motifs that are frequently represented in *Escherichia coli* promoters. Homopolymeric and alternating AT sequences fall into this category (Hawley & McClure, 1983; Pivec et al., 1985; Galas et al., 1985; Harley & Reynolds, 1987; Travers, 1989). Hexamer sequences partly composed of alternating AT sequences are a key feature of the −10 region of prokaryotic promoters, and these sequences are involved in unpairing events leading to RNA polymerase–promoter open complex formation (Hawley & McClure, 1983; Pivec et al., 1985; Galas et al., 1985; Harley & Reynolds, 1987). Homopolymeric dA·dT sequences are present among prokaryotic promoters, particularly 5′ to the −35 hexamer recognition sequence (Galas et al., 1985; Deuschle et al., 1986; Plaskon & Wartell, 1987); however, these sequences are also found at a lower frequency in the spacer region between the −35 and the −10 recognition sequences of the promoter and within the −10 region of a small number of *E. coli* promoters (Hawley & McClure, 1983; Harley & Reynolds, 1987).

Homopolymeric and alternating AT sequences differ dramatically in structure and dynamic properties. The characteristics of homopolymeric $(dA)_n \cdot (dT)_n$ sequences are as follows: (1) short, phased runs of $(dA)_n \cdot (dT)_n$ tracts (where $n \geq 4$) result in DNA bending (Wu & Crothers, 1984; Koo et al., 1986); (2) there is a resistance toward nucleosome reconstitution (Kunkel & Martinson, 1981) and DNase I cleavage (Drew & Travers, 1984); (3) hydroxyl radical cleavage of the adenine strand (Burkhoff & Tullius, 1987) progressively decreases in a 5′ to 3′ direction; (4) there is a reduced binding affinity for the intercalative drugs ethidium bromide (Bresloff & Crothers, 1981), propidium (Jones et al., 1986), and daunomycin (Chaires, 1983) compared to most other DNA sequences including alternating AT sequences; (5)

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