Effects of Benzo[*a*]pyrene-DNA Adducts on a Reconstituted Replication System†

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ABSTRACT: We have used a partially reconstituted replication system consisting of T7 DNA polymerase and T7 gene 4 protein to examine the effect of benzo[*a*]pyrene (B[*a*]P) adducts on DNA synthesis and gene 4 protein activities. The gene 4 protein is required for T7 DNA replication because of its ability to act as both a primase and helicase. We show here that total synthesis decreases as the level of adducts per molecule of DNA increases, suggesting that the B[*a*]P adducts are blocking an aspect of the replication process. Polyacrylamide gels indicate that a shorter DNA product is produced on modified templates and this is confirmed by determining the average chain lengths from the ratio of chain initiations to chain elongation. Gene 4 protein primed synthesis reactions display a greater sensitivity to the presence of B[*a*]P adducts than do oligonucleotide-primed reactions. By challenging synthesis on oligonucleotide-primed B[*a*]P-modified DNA with unmodified DNA, we present evidence that the T7 DNA polymerase freely dissociates after encountering an adduct. Prior studies [Brown, W. C., & Romano, L. J. (1989) *J. Biol. Chem. 264*, 6748-6754] have shown that the gene 4 protein alone does not dissociate from the template during translocation upon encountering an adduct. However, when gene 4 protein primed DNA synthesis is challenged, we observe an increase in synthesis but to a lesser extent than observed on oligonucleotide-primed synthesis. Finally, we have examined DNA synthesis on duplex templates and show that B[*a*]P adducts inhibit synthesis by the T7 DNA polymerase and gene 4 protein to the same extent regardless of whether the adducts are positioned in the leading or lagging strand, while synthesis by the polymerase alone is inhibited only when the adducts are in the template strand.

Benzo[*a*]pyrene (B[*a*]P)[1] is an important environmental carcinogen that is derived from the incomplete combustion of organic materials. It is one of the best studied carcinogenic agents and has served an important role in the determination of several of the steps involved in the induction of cancer by chemicals: specifically, the requirement for metabolic activation to an electrophilic species, the stereochemical considerations involved in DNA adduct formation, and the relationship between DNA adducts formation and cytotoxicity and mutagenicity. Metabolic activation leads to the formation of a diol epoxide derivative and one isomer of this species, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydroxybenzo[*a*]pyrene (BPDE I), is believed to be the ultimate carcinogenic form of B[*a*]P (Singer & Grunberger, 1984; Selkirk et al., 1982). BPDE I can covalently bind to several sites on deoxyguanosine or deoxyadenosine residues in DNA, but the major adduct formed results from the binding of the N2-exocyclic amino group of guanine to the C-10 position of BPDE I (Ivanovic et al., 1976; Meehan et al., 1976, 1977; Weinstein et al., 1976). Several groups have attempted to determine the effect of this adduct on DNA or RNA synthesis and have concluded that these adducts pose strong blocks to the progression of a polymerase (Moore & Strauss, 1979; Moore et al., 1981; Chan et al. 1985; Nath et al., 1987).

Our goal in this study is to more fully understand how DNA damage affects the process of DNA replication. The relative simplicity and detailed knowledge of T7 DNA replication makes this an ideal model to study the effect of DNA damage on replication. It is possible to reconstitute all the enzymatic activities occurring at the replication fork during bacteriophage T7 DNA replication (Richardson, 1984) by using just three proteins: the T7 gene 5 protein, which provides the core polymerase functions (Grippo & Richardson, 1971); *Escherichia coli* thioredoxin, which enhances polymerase processivity (Huber et al., 1987; Tabor et al., 1987); and the gene 4 protein. The gene 4 protein plays a key role in T7 DNA replication in vivo and in vitro. It has been shown to function as both a helicase, capable of unwinding duplex DNA at the replication fork (Kolodner & Richardson, 1977; Matson et al., 1983), and a primase, synthesizing discrete tetraribonucleotide primers necessary for lagging-strand synthesis (Scherzinger et al., 1977; Romano & Richardson, 1979a,b). We have previously shown (Brown & Romano, 1989) that B[*a*]P-DNA adducts act as blocks to the bacteriophage T7 gene 4 protein translocation and that this protein is irreversibly bound at the adduct site. In the present study, we would like to extend our initial results on the effect of B[*a*]P adducts on the gene 4 protein to this reconstituted in vitro replication system. By examining the effects of these adducts on synthesis by the T7 DNA polymerase and T7 gene 4 protein using both single- and double-stranded B[*a*]P-modified M13 DNA, we provide data that not only indicate that replication is strongly inhibited by these adducts but that also provide additional support for the model that suggests that the polymerase and

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[1] Abbreviations: B[*a*]P, benzo[*a*]pyrene; BPDE I, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydroxybenzo[*a*]pyrene; SSB, single-stranded DNA binding protein; TLC, thin-layer chromatography.

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gene 4 protein carry out DNA replication as a complex.

Experimental Procedures

Bacterial Strains and Bacteriophages. *E. coli* D110 *Su thy end pol A1* has been previously described (Romano & Richardson, 1979b). *E. coli* 011′ *Su*$^+$ *thy*, T7 wild-type phage, and T7 amber mutants were obtained from Dr. F. W. Studier (Brookhaven National Laboratory). T7 amber mutants are designated by subscript notation indicating the mutant gene only. The mutations used as the following: gene 3, *am29*; gene 4, *am20*; gene 5, *am28*; gene 6, *am147*. T7 phage was grown on *E. coli* 011′ *Su*$^+$ *thy* as described (Studier 1969, 1973). *E. coli* JM103 and M13 mp9 bacteriophages have been described (Messing & Vieira, 1982) and were obtained from Dr. J. E. LeClerc (Rochester University).

DNA. Bacteriophage T7 DNA (Richardson, 1966) and single- and double-stranded M13 mp9 DNA (Hayes & LeClerc, 1983) were prepared as described.

Nucleotides. Unlabeled nucleotides were purchased from P. L. Biochemicals. Radioactive nucleotides were obtained from ICN Radiochemicals. HPLC-purified radionucleotides were from New England Nuclear.

Enzymes and Protein. Wild-type T7 DNA polymerase (form II) (95% pure) was purified from the mixture of two extracts prepared from two derivatives of *E. coli* K38 that contained either pGP5-3, an expression vector containing T7 gene 5, or pTrx-2, an expression vector containing the gene for thioredoxin (Tabor et al., 1987). These strains were obtained from Dr. Charles Richardson (Harvard University). The specific activities of the final fraction were, polymerase activity, 7800 units/mg and, exonuclease activity, 7600 units/mg. Conversion of the T7 DNA polymerase to the exonuclease-deficient form (form I) was performed as described (Engler et al., 1983). The specific activities of the preparation used in these experiments were, polymerase activity, 9400 units/mg and, exonuclease activity, 900 units/mg.

T7 gene 4 protein (95% pure) was prepared from $T7_{356}$-infected *E. coli* D110 (Fischer & Hinkle, 1980). The specific activity of the most purified fraction of gene 4 protein was 8900 units/mg. *E. coli* single-stranded DNA binding protein was previously purified by the method of Weiner et al. (1975). The purity of the above proteins was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Other Materials. Centricon 30 microconcentrators were from Amicon. [ring-^{3}H]-(±)-7β,8α-Dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydroxybenzo[a]pyrene (BPDE I) was obtained from the NCI Chemical Carcinogen Reference Standard Repository, a function of the Division of Cancer Etiology, NCI, NIH, Bethesda, MD 20205.

Benzo[a]pyrene Modification of M13 DNA. Modification of M13 mp9 DNA with [^{3}H]BPDE I was performed as described (Ridder et al., 1984). Modification reaction mixtures were extracted 10 times with water-saturated diethyl ether to remove unbound BPDE and its hydrolysis products followed by three consecutive ethanol precipitations of the DNA from the aqueous phase. The DNA concentration was determined from the absorbance at 260 nm and the number of adducts per DNA molecule was calculated from the ratio of radioactive decays to DNA concentration.

Gene 4 Protein Primed DNA Synthesis. DNA synthesis reactions (50 μL) primed by gene 4 protein synthesized RNA primers contained 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 μg/mL bovine serum albumin, 300 μM [^{3}H]dTTP (99 cpm/pmol), dATP, dCTP, dGTP, rCTP, rATP, rUTP, rGTP, 69 μM single-stranded unmodified or B[a]P-modified M13 mp9 and 4.25 or 10.6 ng of exonuclease-deficient T7 DNA polymerase. Gene 4 protein was added as indicated. Incubation was carried out in the dark at 37 °C for the time specified in each figure and was terminated by the addition of 3 mL of ice-cold 100 mM $Na_2P_2O_7$ in 1 M HCl. The precipitate was collected on a Whatman GF/C filter and rinsed six times with 3 mL of the acid solution and once with 5 mL of 95% ethanol. Acid-precipitable counts were measured on a Beckman LS 7500 liquid scintillation counter. Blanks were treated the same except no enzyme was added. Each reported value represents a minimum of four determinations that were averaged.

Oligonucleotide-Primed DNA Synthesis. M13 mp9 DNA and a 3-fold molar excess of pentadecamer primer were annealed in the dark, first at 65 °C for 15 min and then at room temperature for 18–20 h in a solution containing 60 mM KCl. The pentadecamer-primed DNA (0.12 pmol) was added to a reaction mixture as above with the exception of the rNTPs. Exonuclease-deficient T7 DNA polymerase was added at 0.11 pmol (10.6 ng) so that the ratio of polymerase molecules to primed template molecules was 0.92. Incubation and product determination were carried out as described above. Each reported values represents a minimum of four determinations that were averaged.

Simultaneous Labeling of Primers and Nascent DNA Chains. DNA synthesis reaction conditions were identical with those using RNA-primed synthesis with the following exceptions. [γ-^{32}P]rATP was 100 μM and had a specific activity of 31 000 cpm/pmol; [^{3}H]dTTP was 300 μM and had a specific activity of 114 cpm/pmol. Reactions were run with 224 ng of gene 4 protein and 4.25 ng of T7 DNA polymerase. These were incubated at 37 °C for 25 min and then taken to 20 mM with EDTA and placed on ice. The reaction mixtures were then passed through a 1.5-mL Sephadex G-50 column equilibrated with 20 mM Tris-HCl (pH 7.5), with 0.2 mM EDTA to remove unincorporated nucleotides. Those fractions (0.15 mL) containing synthesis products as determined by Cerenkov counting were pooled, precipitated with acid, and counted as described above.

Polyacrylamide Gels. DNA synthesis was carried out as described above except that [α-^{32}P]dATP (1400 cpm/pmol) was used to label the nascent DNA chains and dTTP was unlabeled. Reactions contained 224 ng of gene 4 protein and 4.25 ng of T7 DNA polymerase and were incubated for 25 min at 37 °C. They were stopped by addition of EDTA to a final concentration of 20 mM and then phenol-extracted. An aliquot of each reaction was loaded onto a 3.5% denaturing (7 M urea) polyacrylamide gel and electrophoresed until the bromopheol blue migrated to the end of the gel.

Preparation of a Double-Stranded Nicked Template. Nicked double-stranded M13 mp9 DNA was prepared by the addition of 0.04 ng of DNase I per microgram of DNA followed by incubation at 16 °C for 30 min. Reactions were stopped by the addition of EDTA to 20 mM and extraction with phenol. The DNA was ethanol-precipitated, the pellet resuspended in TE, and the concentration determined from the absorbance at 260 nm. Complete conversion to the nicked form was determined by 0.8% agarose gel electrophoresis against double-stranded standards. Quantitation of nicks per molecule was performed as described (Weiss et al., 1968). Synthesis conditions on these templates were identical with those for single-stranded ones listed above.

Preparation of Strand Specifically Modified NHDs. M13 RF DNA was linearized by digestion with *Bam*HI. This was mixed with a 5-fold excess of single-stranded circular M13

and diluted to a final concentration of 0.006 mg/mL DNA with 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.1 mM EDTA. To prepare positive strand-modified nicked heteroduplexes (NHD), the viral DNA was pretreated with [^{3}H]-BPDE I and purified as described above. For negative strand-modified NHDs, the linearized RF DNA was pretreated with [^{3}H]BPDE I. The dilute DNA mixtures were then incubated at 100 °C for 6 min and then placed in an 80 °C water bath that was allowed to cool to 65 °C. The temperature was held at 65 °C for 30 min and then allowed to cool to room temperature. The volume of the mixture was reduced to 2 mL by butanol extraction and then further reduced to 800 μL by filtration in a Centricon 30. This was layered onto a 5–25% sucrose gradient containing 1 M NaCl and centrifuged in an SW 41 rotor at 4 °C for 14 h at 30000 rpm. The gradient was then fractionated and fractions (200 μL) containing the NHD DNA were identified by agarose gel electrophoresis. These fractions were pooled, the volume was reduced, and the buffer was changed to 10 mM Tris-HCl (pH 7.5) with 0.1 mM EDTA by filtration in a Centricon 30. The adducts per molecule were determined as above. Synthesis reaction conditions were identical with those for single-stranded DNA. Synthesis by T7 DNA polymerase alone on negative or positive strand-modified NHDs contained 10.6 ng of polymerase.

Thin-Layer Chromatography of Hydrolysis Products. The hydrolysis of nucleoside 5′-triphosphates by gene 4 protein was assayed by measuring the formation of nucleoside diphosphates from ^{3}H-labeled nucleoside 5′-triphosphates (Kolodner & Richardson, 1977). Aliquots (5 μL) of synthesis reaction mixtures were removed and mixed with 5 μL of a 40 mM EDTA solution that was 6 mM in both dTTP and dTDP. These were then spotted onto PEI–cellulose TLC plates (10 × 20 cm) and developed in 1.0 M acetic acid and 0.8 M LiCl. The position of the dTTP and dTDP standards was determined by visualization under UV light. One-centimeter strips were scraped from the plates in these regions and the radioactivity was determined by scintillation counting. Each reported value represents a minimum of four determinations that were averaged.

Results

As previously stated, T7 gene 4 protein functions in vivo as a primase synthesizing tetraribonucleotide primers necessary for lagging-strand synthesis. Gene 4 protein is thought to directly interact with T7 DNA polymerase in the initiation of synthesis from these primers and several models detailing possible steps in this interaction have been proposed (Nakai & Richardson, 1986b). Since the gene 4 position is so intimately involved in the replication of T7 DNA and a gene 4 protein primed synthesis reaction more closely approximates an in vivo system than an oligonucleotide-primed reaction, we have examined the effect of B[*a*]P modification on synthesis by these two proteins.

Characterization of DNA Synthesis on BP-Modified Single-Stranded DNA. Previous studies have shown that DNA synthesis decreases on printed DNA templates as the number of bulky adducts per molecule increases (Moore et al., 1980; Michaelis et al., 1986). This is presumed to occur because the adducted nucleotide distorts the DNA structure in such a way that renders the base noninstructional or because the adduct itself presents a physical block to the progression of the polymerase molecule. For example, extensive polyacrylamide gel analysis using B[*a*]P-modified single- or double-stranded templates has been shown to cause a variety of DNA polymerases to terminate synthesis one nucleotide 3′ to the adduct on the template strand (Moore & Strauss, 1979; Larsen & Strauss, 1987).

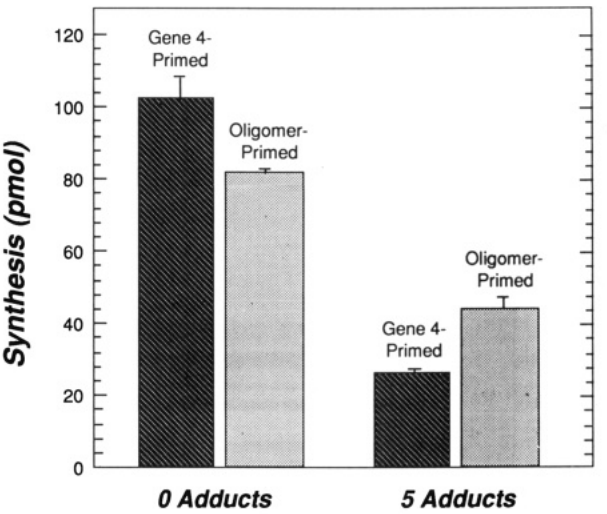

FIGURE 1: Comparison of gene 4 protein primed synthesis with oligonucleotide-primed synthesis on native and B[*a*]P-modified templates. DNA synthesis by the T7 DNA polymerase (10.6 ng) was performed on single-stranded M13 mp9 templates (20 μM) containing zero or five B[*a*]P adducts per molecule, primed by either a synthetic oligonucleotide or 28 ng of gene 4 protein, as described under Experimental Procedures. Incubation was at 37 °C for 25 min and DNA synthesis levels were measured by the incorporation of [^{3}H]dTTP into acid-precipitable DNA.

To compare the effect of B[*a*]P adducts on synthesis by the T7 DNA polymerase and gene 4 protein with their effect on the polymerase alone, we measured DNA synthesis on native or B[*a*]P-modified single-stranded templates by using either oligonucleotide primers or gene 4 protein synthesized primers. DNA synthesis in both cases was inhibited by the presence of the B[*a*]P adducts; however, the inhibition induced by these bulky adducts was greater when synthesis was primed by the gene 4 protein (Figure 1). Prior studies (Brown & Romano, 1989) have shown that translocation by the gene 4 protein is inhibited by the presence of B[*a*]P adducts in the template. The most straightforward explanation of this result is that the B[*a*]P adducts are preventing the gene 4 protein from reaching recognition sequences in the template where primer synthesis occurs—consequently there are fewer primers for extension by T7 DNA polymerase, less initiation of synthesis, and less total synthesis compared with oligonucleotide-primed synthesis. However, it is also possible that the polymerase and gene 4 protein form a complex during synthesis (Nakai & Richardson, 1986b) and that the adducts inhibit this complex to a greater extent than the polymerase alone.

To further characterize the effect of B[*a*]P adducts on DNA synthesis catalyzed by the T7 DNA polymerase and gene 4 protein, synthesis was carried out on single-stranded templates containing various average levels of B[*a*]P adducts (Figure 2). Synthesis on these templates was dependent on priming by the gene 4 protein as evidenced by the lack of synthesis when rNTPs are excluded from the reaction mix (data not shown). As has been found for synthesis by various other DNA polymerases, DNA synthesis by this replication system was found to be inhibited by the presence of the B[*a*]P adducts in the template and the level of inhibition increased as the number of adducts per template increased. Furthermore, DNA synthesis reached maximum levels at progressively earlier times as the degree of template modification increases.

The effect of these adducts on the average chain length synthesized was determined by two complementary procedures. First, the average chain length was calculated from the ratio

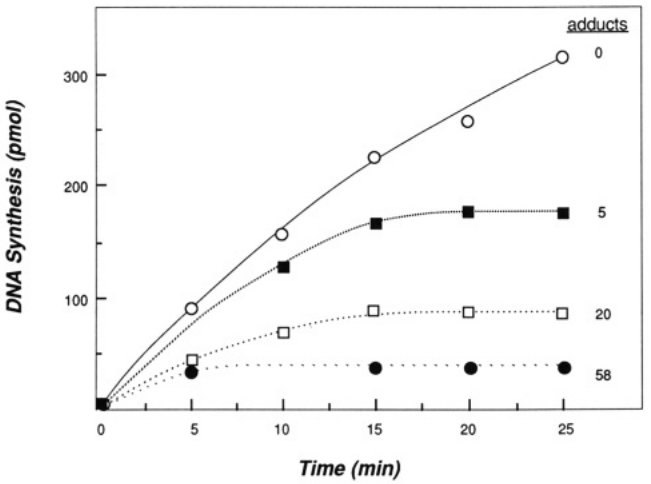

FIGURE 2: Time course of gene 4 protein primed synthesis on native and B[a]P-modified DNA. DNA synthesis was performed on single-stranded M13 mp9 templates (69 μM) containing an average of 0, 5, 20, and 58 B[a]P adducts per molecule by 4.25 ng of T7 DNA polymerase and 224 ng of gene 4 protein as described under Experimental Procedures. DNA synthesis levels were measured by the incorporation of [^{3}H]dTTP into acid precipitable DNA.

of gene 4 protein synthesized primer incorporated into acid precipitable DNA to the total extent of DNA synthesis. The former level was determined by measuring the incorporation of [γ-^{32}P]ATP, which is the first nucleotide of the RNA-primed DNA synthesis, while total synthesis was determined by [^{3}H]dTTP incorporation (Figure 3). Although it is evident that the presence of B[a]P adducts decreases the average chain length of the nascent DNA strands, the extent of inhibition is not directly proportional to the number of adducts present and the inhibition is less than expected if each adduct was an absolute block to synthesis, suggesting that the predominant effect in the reduction of synthesis may not be in preventing the gene 4 protein from reaching primer sites. We have confirmed the chain lengths determined in this manner by running the ^{32}P-labeled product on a polyacrylamide gel (Figure 3). The average chain lengths determined from the gel are in rough agreement with those determined above. It should be noted that because there are multiple sites for gene 4 protein primed DNA synthesis to initiate, the product DNA does not display discrete bands as would be seen if an oligonucleotide was used to prime synthesis.

Challenge of Synthesis on BP-Modified Templates with Native DNA. Prior studies have indicated that the gene 4 protein can become sequestered at the site of a B[a]P adduct during translocation on modified DNA (Brown & Romano, 1989). However, it is possible that the presence of the T7 DNA polymerase might affect the interaction between the gene 4 protein and the DNA and thus alter this tight binding at the adduct site. We first attempted to determine whether the T7 DNA polymerase also becomes bound to the DNA at the adduct site by priming synthesis on a modified template with an oligonucleotide and adding an excess of primed, unmodified DNA at a time in the reaction where no further DNA synthesis was occurring (Figure 4A). Following this addition, DNA synthesis again occurs such that after the 15-min challenge incubation, the amount of additional synthesis (50 pmol) equals that which occurred in the unmodified control in 15 min (Figure 4A). It is important to note that this additional synthesis is not simply due to excess polymerase present in solution, since these experiments were carried out under conditions where there was approximately stoichiometric level of template and polymerase. Similar results were generated when the modified template was present in excess (not shown). The implications of these data are that, unlike what we found with the gene 4 protein (Brown & Romano, 1989), the T7 DNA polymerase is not being sequestered at the adduct site.

	Adducts/Molecule		
	0	5	20
DNA Synthesis (pmols)	274	118	56
Primer Synthesis (fmols)	52	50	45
Avg. Chain Length (bases)	5200	2500	1200

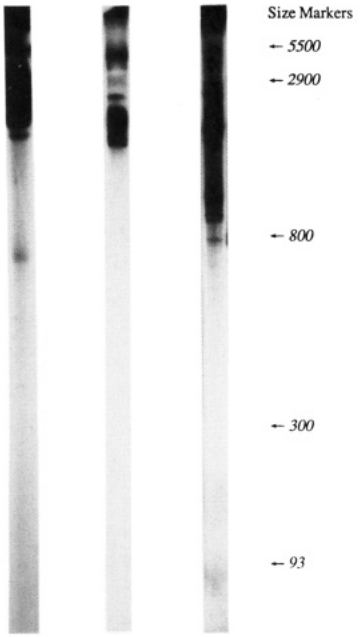

FIGURE 3: Average chain length of DNA synthesized on B[a]P-modified single-stranded templates. (Top) DNA synthesis on single-stranded M13 templates containing 5 or 20 adducts per molecule was carried out by the T7 DNA polymerase (4.25 ng) and the gene 4 protein (224 ng) as described under Experimental Procedures. Primer utilization was quantified by measuring the incorporation of [γ-^{32}P]ATP, while DNA synthesis was determined by the incorporation of [^{3}H]dTTP. The average chain length was determined from the ratio of these two values. (Bottom) Polyacrylamide gels showing the product of synthesis by the T7 DNA polymerase and gene 4 protein. Synthesis was carried out as described under Experimental Procedures using a [α-^{32}P]dATP label. Size markers were prepared from ^{32}P-labeled restriction fragments and were run in parallel lanes.

When gene 4 protein primed synthesis on this template was challenged with unmodified DNA there also was an increase in the extent of DNA synthesis. Over the 15-min incubation following the challenge, the amount of additional synthesis is 25 pmol. This increase corresponds to 50% of that which was measured at 15 min on the unmodified control (50 pmol) (Figure 4B). We do not believe that this 50% reduction is the result of diluting the enzymatic activites or separating the polymerase and gene 4 protein on the excess template since identical experiments carried out with only unmodified DNA (final DNA concentration 200 μM) gave 36% *more* synthesis than was obtained with the lower template concentration (20 μM). Furthermore, the increased synthesis observed upon the challenge is dependent on a higher DNA to protein ratio, since no reinitiation is observed without the challenge, as evidence by the leveling off of synthesis in the absence of added challenge DNA.

Experiments were also carried out in which gene 4 protein primed DNA synthesis on B[a]P-modified templates was challenged with oligonucleotide-primed DNA (Figure 4C).

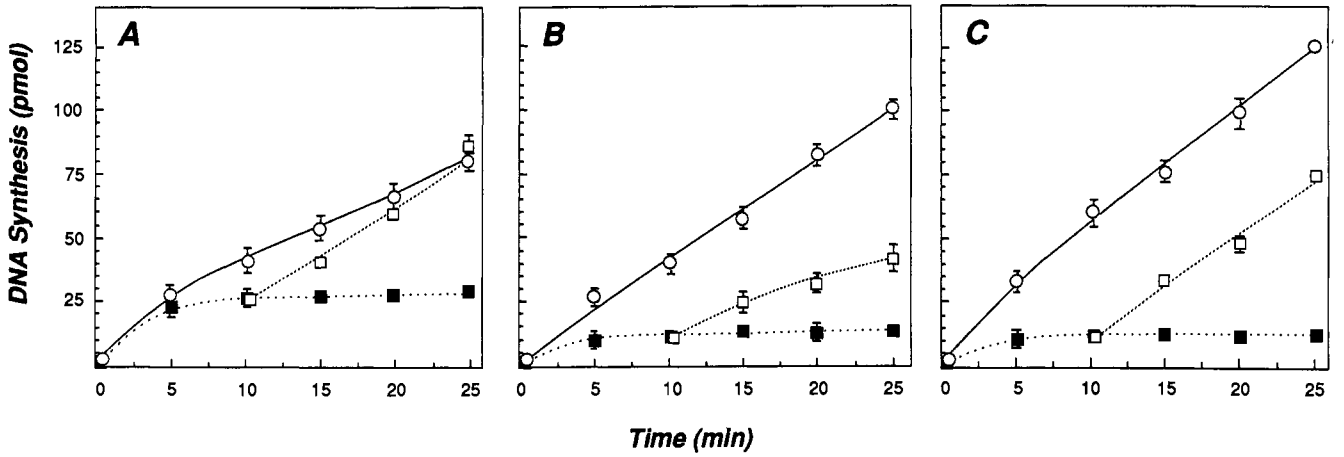

FIGURE 4: Synthesis on B[*a*]P-modified templates with excess native DNA added at 10 min. DNA synthesis by T7 DNA polymerase (10.6 ng) was performed on single-stranded M13 mp9 templates (20 μM) containing 0 (○) and 20 B[*a*]P (■) adducts per molecule. After 10 min native M13 mp9 was added to each in excess (200 μM) (□). DNA synthesis levels were measured by the incorporation of [^{3}H]dTTP into acid-precipitable DNA. Panel A is synthesis by T7 DNA polymerase alone using an oligonucleotide-primed template and challenge. Panel B is synthesis by T7 DNA polymerase with gene 4 protein (28 ng) priming for both template and challenge. Panel C is DNA synthesis by T7 DNA polymerase with gene 4 protein (28 ng) priming on the template with oligonucleotide-primed challenge.

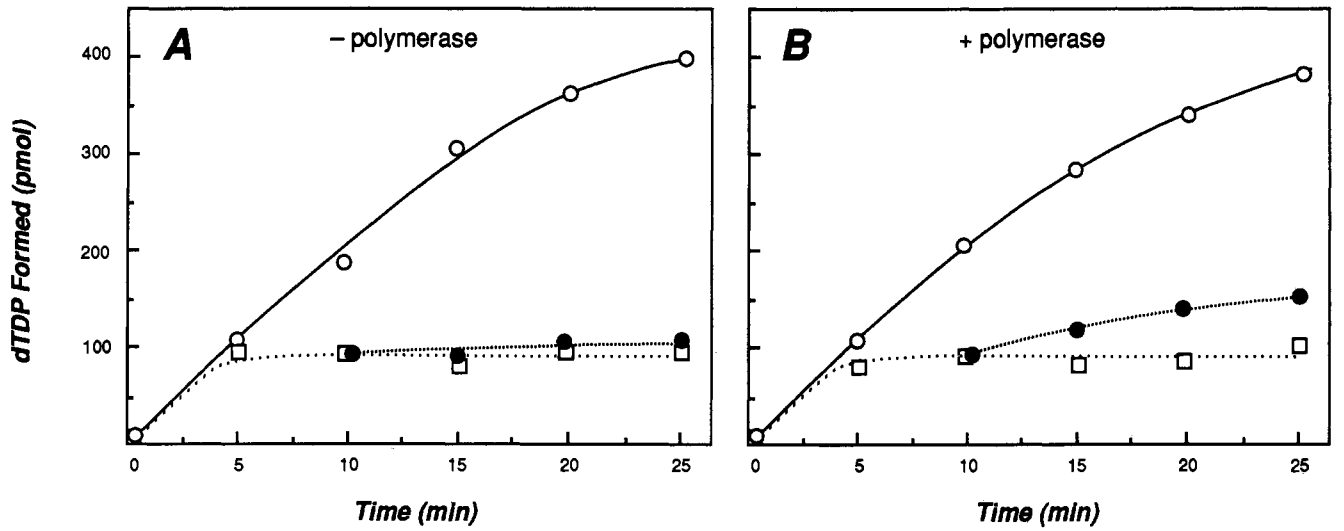

FIGURE 5: Hydrolysis of dTTP by the T7 gene 4 protein in the presence or absence of T7 DNA polymerase. Hydrolysis of dTTP by 224 ng of T7 gene 4 protein in the absence (A) or presence (B) of 89 ng of T7 DNA polymerase was measured on 0.27 μg of single-stranded M13 DNA containing no (○) or 20 (□) B[*a*]P adducts per molecule. In separate experiments (●), hydrolysis on B[*a*]P-modified DNA was carried out for 10 min, after which excess native M13 DNA was added (final concentration, 400 μM). Hydrolysis was measured as described under Experimental Procedures by the amount of ^{3}H product migrating with dTDP during TLC.

The increase in total synthesis when this DNA is added is equivalent to approximately 80% of that in the first 15 min of synthesis on *oligonucleotide-primed* unmodified control template by gene 4 protein and T7 DNA polymerase (Figure 4C). Taken together, these results indicate that reinitiation by the T7 DNA polymerase occurs to a lesser extent when gene 4 protein is present than when T7 DNA polymerase is alone.

Effect of the T7 Polymerase on Hydrolysis by the Gene 4 Protein. The implication of the experiments described in Figure 4 is that the presence of the gene 4 protein decreases the ability of the T7 DNA polymerase to dissociate from the template when it encounters a B[*a*]P adduct. However, the fact that the challenge did result in additional synthesis suggests that the binding of the polymerase and gene 4 protein to the modified template is not as strong as was found when similar challenges were carried out with the gene 4 protein alone (Brown & Romano, 1989). To determine if the presence of the T7 DNA polymerase altered the binding of the gene 4 protein to B[*a*]P-modified DNA, a challenge experiment was carried out measuring hydrolysis of dTTP by the gene 4 protein in the presence or absence of the T7 DNA polymerase (Figure 5). It is clear from these data that in the absence of the polymerase, the gene 4 protein is unable to dissociate from the modified template to begin translocating on the unmodified challenge DNA. However, in the presence of the T7 DNA polymerase, a portion of the gene 4 protein does move to the newly added unmodified DNA and resume hydrolyzing dTTP.

Synthesis on Nicked Duplex DNA by T7 DNA Polymerase and T7 Gene 4 Protein. Selective oxidation of the exonuclease domain of T7 DNA polymerase (which can occur if purification is carried out with buffers that do not include EDTA) results in an exonuclease-deficient form (form I) that lacks 95% of the 3→5′ exonuclease activity that the wild-type enzyme contains. This exonuclease-deficient form is also distinguished from the wild-type enzyme by its ability to catalyze limited strand displacement synthesis, up to 550 nucleotides, from a nick in duplex DNA without the presence of the gene 4 protein (Lechner et al., 1983), while the wild-type enzyme polymerizes fewer than five nucleotides under similar conditions. In addition, DNA synthesis by this exonuclease-deficient form of the polymerase initiating from a nick is strongly stimulated by the presence of the gene 4 protein.

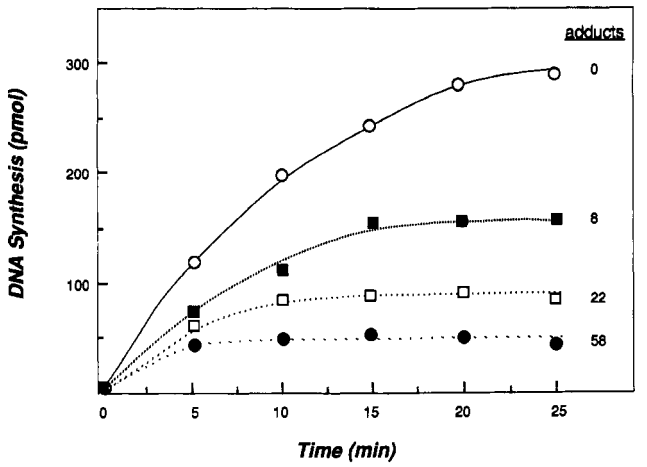

FIGURE 6: Gene 4 protein primed synthesis on native and B[a]P-modified nicked duplex DNA with increasing time. DNA synthesis was performed on nicked M13 mp9 RF template (69 μM) containing 0, 8, 22, and 58 B[a]P adducts per molecule, using 224 ng of gene 4 protein and 4.25 ng of T7 DNA polymerase with increasing time as described under Experimental Procedures. DNA synthesis levels were measured by the incorporation of [^{3}H]dTTP into acid-precipitable DNA.

A nicked, duplex M13 DNA template was prepared by limited DNase I treatment. Using the method of Weiss et al. (1968), the number of nicks per molecule was determined to be 3.75 ± 0.25. Synthesis assays using the T7 DNA polymerase and gene 4 protein were performed under conditions identical with those for the single-stranded template and the levels of synthesis obtained were roughly comparable. As was found with the single-stranded templates, synthesis levels on the modified duplex templates declined as the number of B[a]P adducts per molecule increased (Figure 6). Furthermore, the inhibition of synthesis observed per adduct is roughly equivalent to that observed with single-stranded templates (cf. Figures 2 and 6). We also find that leading- and lagging-strand syntheses are inhibited to comparable extents since the degree of inhibition obtained in the absence of rNTPs is identical with that found in their presence (data not shown).

Synthesis on Strand Specifically Modified Nicked Heteroduplex DNA. Nicked heteroduplex (NHD) M13 DNAs were constructed in which only one of the two strands contained B[a]P adducts. This was accomplished by either modifying the (+) viral single-stranded circular M13 DNA or linear duplex M13 DNA, followed by denaturing the unmodified or modified linear duplex and reannealing to the modified or unmodified (+) single-stranded circles, respectively. The polymerase initiates synthesis from the single nick in the negative (displaced) strand and thus adducts positioned specifically in the (+) strand of these DNA molecules are in the template strand while adducts in the nicked (−) strand are in the strand that is displaced during synthesis. Thus these templates can be used to determine whether adducts in either the leading or lagging strand have a differential effect on synthesis catalyzed by T7 DNA polymerase in the absence or presence of the gene 4 protein.

Larson and Strauss (1987) showed that UV photoproducts in the displaced strand have no effect on synthesis by DNA polymerase I or AMV reverse transcriptase. We have carried out similar experiments to determine if B[a]P adducts had a similar effect on the exonuclease-deficient T7 DNA polymerase in the absence of the gene 4 protein. Using 10.6 ng of T7 DNA polymerase, we obtained 72 pmol of synthesis on a nicked, unmodified template and almost identical levels of synthesis when the template contained 15 adducts in the displaced strand (Table I). However, 14 B[a]P adducts in the template strand reduced synthesis by the T7 DNA polymerase to 32 pmol. Comparable experiments in the presence of the gene 4 protein resulted in a substantial reduction of synthesis regardless of the location of the adducts. These data suggest that the inhibition of DNA synthesis that is observed when the adducts are positioned in the displaced strand is due to the presence of the gene 4 protein.

We have also used these strand specifically modified templates to simultaneously determine the effect of adduct position on DNA synthesis and dTTP hydrolysis by the gene 4 protein and T7 DNA polymerase. These experiments were carried out on templates that contained either no adducts (Figure 7A) or comparable levels of adducts positioned in either the template strand (Figure 7B,C) or displaced strand (Figure 7C,E). We find that similar numbers of adducts in *either* strand induce comparable levels of inhibition. Furthermore, both synthesis and hydrolysis are inhibited to similar extents on each template as indicated by the calculated ratios of hydrolysis to synthesis (Figure 7). These results suggest that when the replication complex reaches the site of an adduct in the template strand, which should block the polymerase, the gene 4 protein does not proceed past this site since hydrolysis on this template (Figure 7B,D) is identical with that obtained when the adducts are positioned in the displaced strand (Figure 7C,E).

Discussion

Replication of DNA is generally accomplished by a DNA polymerase acting in concert with a variety of accessory proteins that provide the necessary functions required for the orderly progression of the replication fork. The number of accessory proteins and the complexity of the interactions among proteins vary with the system. The replication machinery of bacteriophage T7 is one of the best understood systems and involves a small number of well-characterized proteins. Of the accessory proteins involved in T7 DNA replication, the gene 4 protein is without question the most important—it provides both the primase and helicase activities essential for simultaneous synthesis on the leading and lagging strands. Gene 4 protein activity must be closely coordinated with T7 DNA polymerase activity in the initiation and elongation of DNA synthesis on the lagging strand and elongation on the leading strand, and thus the effect of bulky lesions on these interactions is of great interest.

Our results, as well as those of others (Larson & Strauss, 1987), indicate that T7 DNA polymerase, in the absence of any other ancillary proteins, is blocked by B[a]P adducts (Figures 1 and 4A; Table I), leading to the accumulation of shorter nascent DNA chains then those observed for unmodified DNA templates. However, the inhibition observed for synthesis under comparable conditions but primed by the gene 4 protein is greater (Figure 1) and this reduced synthesis is less able to be challenged by added unmodified DNA than synthesis carried out in the absence of the gene 4 protein (cf. Figure 4A,B). Furthermore, the gene 4 protein is unable to switch templates once it encounters a B[a]P adduct (Brown & Romano, 1989); while in the presence of the T7 DNA polymerase, the gene 4 protein is no longer completely sequestered on the modified template (Figure 5). Taken together, these results suggest that the T7 DNA polymerase and gene 4 protein may interact to modulate their DNA binding properties.

Several models for how gene 4 protein and T7 DNA polymerase activities are coupled in the transition from RNA primer synthesis to DNA synthesis have been proposed (Nakai

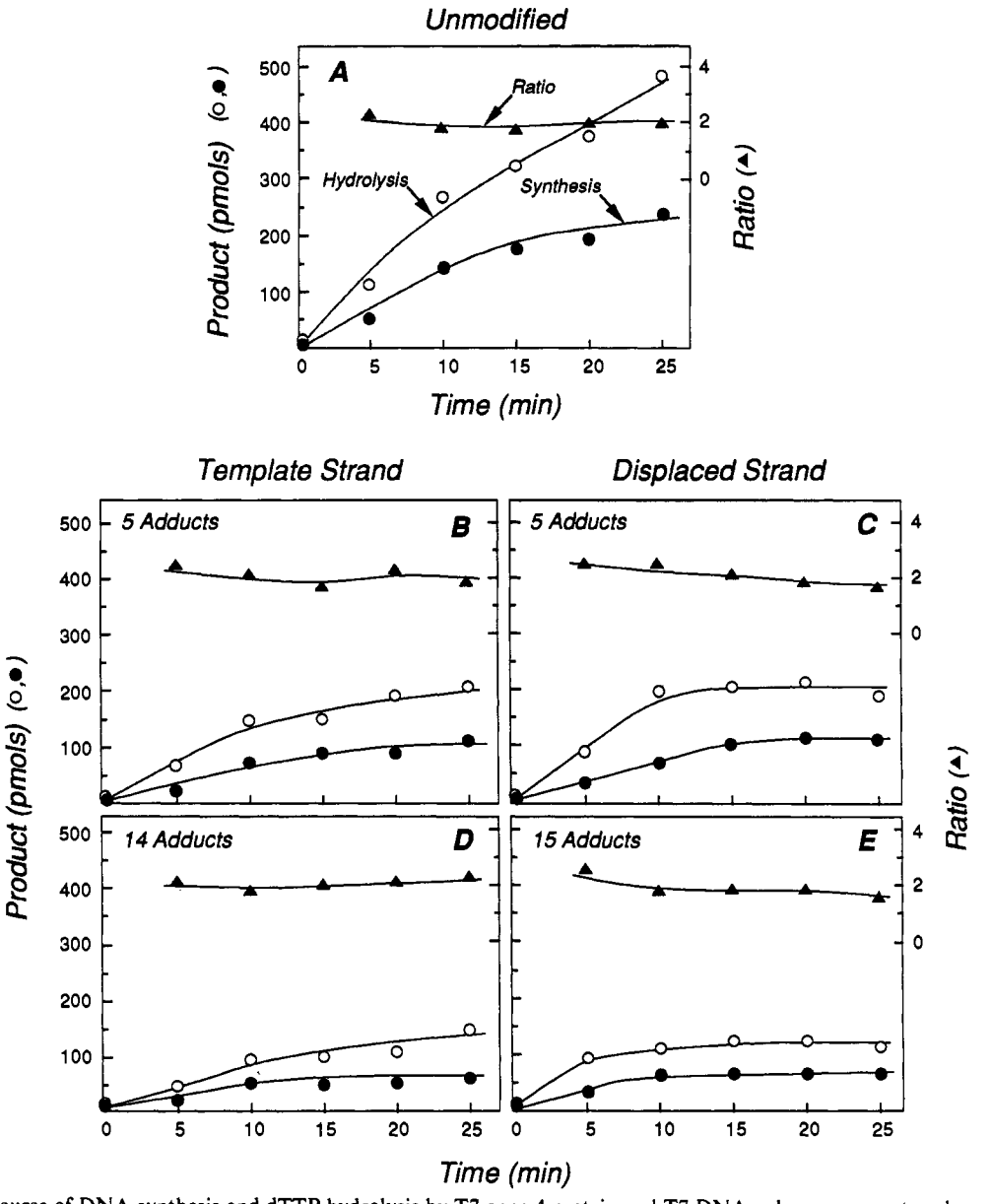

FIGURE 7: Time course of DNA synthesis and dTTP hydrolysis by T7 gene 4 protein and T7 DNA polymerase on strand specifically modified nicked heteroduplex templates. DNA synthesis was carried out as described under Experimental Procedures. The extent of DNA synthesis (●) was measured by the incorporation of [^{3}H]dTTP into acid-precipitable DNA. Hydrolysis (○) was measured by the amount of ^{3}H product migrating with dTDP during TLC. The ratio is a comparison of picomoles of dTTP hydrolysis to picomoles of nucleotide incorporated. The templates used for each experiment are as follows: (A) unmodified NHD; (B) five B[*a*]P adducts in the template strand only; (C) five B[*a*]P adducts in the displaced strand only; (D) 14 B[*a*]P adducts in the template strand only; (E) 15 B[*a*]P adducts in the displaced strand only.

& Richardson, 1986b). The models most strongly supported by the available data differ in protein association and translocation. In one model, the proteins bind separately, do not associate with one another, and translocate separately. When gene 4 protein reaches a recognition sequence it synthesizes, a primer, briefly remaining to stabilize it until a polymerase molecule arrives to extend it. In the other model, gene 4 protein and polymerase bind to the DNA independently but then associate and gene 4 protein directs polymerase translocation. When this complex reaches a recognition sequence, gene 4 protein synthesizes a primer, which may immediately be extended by DNA polymerase (Nakai & Richardson, 1986b). One interpretation of our data is that these proteins are translocating together as this latter model proposes and, when an adduct is encountered, T7 DNA polymerase dissociates from the template and influences gene 4 protein to dissociate. This is not found to occur when the gene 4 protein is alone. Of course we cannot fully exclude the possibility that the proteins are operating independently and only encounter each other at a B[*a*]P modification site. However, this alternative seems unlikely in light of the evidence presented, which demonstrates a DNA-dependent interaction between the T7 DNA polymerase and the gene 4 protein (Nakai & Richardson, 1986b).

We have also examined synthesis by T7 DNA polymerase and gene 4 protein on randomly B[*a*]P-modified duplex templates as a means of correlating our results from synthesis on B[*a*]P-modified single-stranded templates with actual occurrences at a replication fork. When identical conditions and equal concentration of template DNA are used, synthesis on either double- or single-stranded unmodified or modified template proceeds to a similar extent (cf. Figures 2 and 6). The adduct-induced decreases in synthesis levels on double-stranded templates, when compared to synthesis levels on single-stranded templates containing similar numbers of B[*a*]P adducts, are roughly the same. Since modifications occur

Table I: Adducts in the Displaced Strand Only Inhibit DNA Synthesis When the Gene 4 Protein Is Present[a]

addition	adducts in DNA template (pmol)		
	none	displaced strand[b]	template strand[c]
T7 DNA polymerase[d]	72	70	32
T7 DNA polymerase and gene 4 protein[e]	210	45	40

[a] DNA synthesis was carried out as described under Experimental Procedures for 20 min. [b] The displaced strand contained 14 adducts per molecule. [c] The template strand contained 15 adducts per molecule. [d] DNA synthesis was initiated by the addition of 10.6 ng of T7 DNA polymerase. [e] This experiment used 4.25 ng of the T7 DNA polymerase and 224 ng of gene 4 protein.

randomly (so the adducts on double-stranded templates should, on the average, be equally distributed in the template and displaced strands) this result suggests that adducts in the displaced strand are acting as blocks to either the progression of the replication complex or the helicase activity of gene 4 protein.

In order to conclusively test if adducts in the displaced strand act as blocks to the replication complex or gene 4 protein helicase activity, we have constructed templates that are strand specifically modified. Larsen & Strauss (1987) have employed similarly constructed templates in determining that the presence of thymine dimers in the displaced strand have no effect on several different DNA polymerases. Our results show that B[a]P adducts in the displaced strand do not inhibit synthesis by T7 DNA polymerase in the absence of the gene 4 protein (Table I). When these same displaced-strand-modified templates are replicated in the presence of the gene 4 protein, both DNA synthesis and dTTP hydrolysis are inhibited (Table I) and the degree of inhibition is proportional to the level of modification (Figure 7). On the other hand, adducts positioned specifically in the template strand inhibit not only DNA synthesis but also hydrolysis by the gene 4 protein with the ratio of hydrolysis to synthesis remaining constant both over time and with increasing numbers of adducts. Taken together, these data suggest that a replication complex tends to remain associated with the DNA upon encountering an adduct that blocks it. Moreover, we find no detectable uncoupling of hydrolysis and synthesis, which would have indicated that one of the proteins was continuing past the site of the adduct in the absence of the other protein.

Registry No. DNA polymerase, 9012-90-2.

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Complete Nucleotide Sequence of the Gene for Human Heparin Cofactor II and Mapping to Chromosomal Band 22q11[†,‡]

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ABSTRACT: Heparin cofactor II (HCII) is a 66-kDa plasma glycoprotein that inhibits thrombin rapidly in the presence of dermatan sulfate or heparin. Clones comprising the entire HCII gene were isolated from a human leukocyte genomic library in EMBL-3 λ phage. The sequence of the gene was determined on both strands of DNA (15 849 bp) and included 1749 bp of 5′-flanking sequence, five exons, four introns, and 476 bp of DNA 3′ to the polyadenylation site. Ten complete and one partial Alu repeats were identified in the introns and 5′-flanking region. The HCII gene was regionally mapped on chromosome 22 using rodent–human somatic cell hybrids, carrying only parts of human chromosome 22, and the chronic myelogenous leukemia cell line K562. With the cDNA probe HCII7.2, containing the entire coding region of the gene, the HCII gene was shown to be amplified 10–20-fold in K562 cells by Southern analysis and in situ hybridization. From these data, we concluded that the HCII gene is localized on the chromosomal band 22q11 proximal to the breakpoint cluster region (BCR). Analysis by pulsed-field gel electrophoresis indicated that the amplified HCII gene in K562 cells maps at least 2 Mbp proximal to BCR-1. Furthermore, the HCII7.2 cDNA probe detected two frequent restriction fragment length polymorphisms with the restriction enzymes *Bam*HI and *Hin*dIII.

Heparin cofactor II (HCII)[1] is a plasma glycoprotein that inhibits thrombin rapidly in the presence of dermatan sulfate or heparin (Tollefsen, 1989). HCII cDNA clones have been isolated, sequenced, and expressed in *Escherichia coli* (Inhorn & Tollefsen, 1986; Blinder et al., 1988; Blinder & Tollefsen, 1990; Derechin et al., 1990) and COS cells (Ragg, 1986; Ragg et al., 1990). A dysfunctional variant of HCII ($HCII_{Oslo}$) with decreased affinity for dermatan sulfate was characterized by amplification and sequence analysis of a portion of the HCII gene (Blinder et al., 1989). Restriction fragment length polymorphisms (RFLPs) with *Bam*HI and *Msp*I have been identified in the HCII gene by Southern blot analysis (Blinder et al., 1988; Turner et al., 1990). In the present investigation, we determined the complete nucleotide sequence of the HCII gene, including portions of the 5′- and 3′-flanking sequences, observed a new *Hin*dIII polymorphism, and identified sites in the gene at which the *Bam*HI and *Hin*dIII polymorphisms may occur.

The HCII gene has been assigned to human chromosome 22 by hybridization of a cDNA subclone to DNA from flow-sorted chromosomes (Blinder et al., 1988). Chromosome 22, although minute in size, is involved in inherited as well as in acquired diseases. About a dozen diseases including seven different malignancies have been characterized at the cytogenetic or phenotypic level or both (Kaplan et al., 1987; Kaplan & Emanuel, 1989). Analysis of the molecular pathology of chromosome 22 is greatly facilitated by the use of regionally assigned DNA probes that recognize RFLPs. Therefore, we regionally mapped the polymorphic HCII gene using somatic cell hybrids containing only parts of chromosome 22 and the chronic myelogenous leukemia (CML)-derived cell line K562. In addition, a more detailed analysis was achieved by using pulsed-field gel electrophoresis for a long-range map surrounding the HCII locus.

Materials and Methods

Probes. HCII7.2 and HCII7.3 are 1.6- and 0.6-kb *Eco*RI fragments subcloned into pGEMblue, corresponding to 5′ and 3′ portions of the HCII cDNA, respectively (Blinder et al.,

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[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05309.
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[1] Abbreviations: HCII, heparin cofactor II; RFLP, restriction fragment length polymorphism; serpin, serine proteinase inhibitor; PCR, polymerase chain reaction; CML, chronic myelogenous leukemia; BCR, breakpoint cluster region; Mbp, megabase pair(s).

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