Feinberg, M. B., Jarret, R. F., Aldovini, A., Gallo, R. C., & Wong-Staal, F. (1986) *Cell 46*, 807-817.
Felber, B. K., Hadzopoulou-Cladaras, M., Cladaras, C., Copeland, T., & Paulakis, G. N. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1495-1499.
Green, M. R., & Zapp, M. L. (1989) *Nature 238*, 200-201.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108-4116.
Hammarskjold, M. L., Heijmer, J., Hammarskjold, B., Sangwan, I., Albert, L., & Rekosh, D. (1989) *J. Virol. 63*, 1959-1966.
Harrison, S. C. (1990) in *Virology* (Fields, B. N., et al., Eds.) pp 37-61, Raven Press, Ltd., New York.
Holland, S. M., Ahmad, N., Maitra, R. K., Wingfield, P. T., & Venkatesan, S. (1990) *J. Virol. 64*, 5966-5975.
Jones, N. (1990) *Cell 61*, 9-11.
Knight, D. M., Flomerfelt, F. A., & Ghrayeb, J. (1987) *Science 236*, 837-840.
Mallim, M. H., Hauber, J., Le, S.-Y., Maizel, J. V., & Cullen, B. R. (1989) *Nature 338*, 254-257.
Marston, F. A. O. (1986) *Biochem. J. 240*, 1-12.
Mitchinson, C., & Pain, R. H. (1985) *J. Mol. Biol. 184*, 331-342.
Nalin, C. M., Purcell, R. D., Antelman, D., Mueller, D., Tomchak, L., Wegrzynski, B., McCarney, E., Toome, V., Kramer, R., & Hsu, M.-C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7593-7597.
Olsen, H. S., Nelbock, P., Cochrane, A. W., & Rosen, C. (1990) *Science 247*, 845-848.
Pomerantz, R. J., Trono, D., Feinberg, M. B., & Baltimore, D. (1990) *Cell 61*, 1271-1276.
Provencher, S. W., & Gloeckner, J. (1981) *Biochemistry 20*, 33-37.
Ratner, L., Haseltine, W., Patarca, R., Liuak, K. S., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C., & Wong-Staal, F. (1985) *Nature 313*, 277-283.
Rosen, C. A., Terwilliger, E., Dayton, A., Sodroski, J. G., & Haseltine, W. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2071-2075.
Shire, S. J., McKay, P., Leung, D. W., Cachianes, G. J., Jackson, E., & Wood, W. I. (1990) *Biochemistry 29*, 5119-5126.
Silver, P., & Goodson, H. (1989) *CRC Crit. Rev. Biochem. Mol. Biol. 24*, 419-435.
Sodroski, J., Goh, W. C., Rosen, C., Dayton, A., Terwilliger, E., & Haseltine, W. (1986) *Nature 321*, 412-417.
Starr, C. M., & Hanover, J. M. (1990) *BioEssays 12*, 323-330.
Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113-175.
Trono, D., & Baltimore, D. (1990) *EMBO J. 9*, 4155-4160.
Wingfield, P., Payton, M., Traverneir, J., Barnes, M., Shaw, A., Rose, K., Simona, M. G., Demczak, S., Williamson, K., & Dayer, J.-M. (1986) *Eur. J. Biochem. 160*, 491-497.
Winzor, D. J., & Scheraga, H. A. (1963) *Biochemistry 2*, 1263-1267.
Zapp, M. L., & Green, M. R. (1989) *Nature 342*, 714-716.

Induction of DNA Polymerase Activities in the Regenerating Rat Liver†

Chun-Li Yang,‡ Shan-Jian Zhang,‡ N. Lan Toomey,‡ T. Norman Palmer,§ and Marietta Y. W. T. Lee*,‡
Department of Medicine, University of Miami School of Medicine, Miami, Florida 33101, and Department of Biochemistry, University of Western Australia, Perth, Australia

Received June 8, 1990; Revised Manuscript Received March 13, 1991

ABSTRACT: The levels of DNA polymerase α, DNA polymerase δ, and its accessory protein, proliferating cell nuclear antigen (PCNA) were examined in the regenerating rat liver. The levels of DNA polymerase α and δ activities in regenerating liver extracts were determined by the use of the DNA polymerase α specific inhibitor, BuAdATP [2-(*p*-*n*-butylanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate], and monoclonal antibodies. These reagents showed that the total DNA polymerase activities increased ca. 4-fold during regeneration and that the fraction of DNA polymerase δ activity at the peak was 40% of the total DNA polymerase activity. Immunoblots and inhibition studies using specific antibodies showed that DNA polymerase δ and ϵ and PCNA were concomitantly induced after partial hepatectomy. The levels of both DNA polymerase δ and ϵ and PCNA reached their maxima at 24-36 h post hepatectomy, i.e., at the same time that in vivo DNA synthesis reached its peak. Partial purification and characterization of DNA polymerases δ and ϵ from the regenerating rat liver were also performed. These observations suggest that the variation of DNA polymerase δ and ϵ and PCNA during liver regeneration is closely related to DNA synthesis and is consistent with their involvement in DNA replication.

DNA replication is a complex process that requires the cooperation of multiple enzymes and protein factors. DNA polymerases are central elements in this process, as they are directly responsible for the synthesis of the DNA chains. In eukaryotic cells, two major DNA polymerase activities are now considered to be involved in the replication process. The first is DNA polymerase α, which has long been recognized as having a role in DNA synthesis (Lehman & Kaguni, 1989). The second is DNA polymerase δ, first reported in 1976 as a new type of mammalian DNA polymerase that was apparently unique among mammalian polymerases in that it possessed an associated 3′ to 5′ exonuclease activity (Byrnes

† This work was supported by NIH Grant GM31973 and was performed during the tenure of an Established Investigatorship of the American Heart Association to M.Y.W.T.L.
* Address correspondence to this author at the Department of Medicine (R-57), University of Miami School of Medicine, P.O. Box 016960, Miami, FL 33101.
‡ University of Miami School of Medicine.
§ University of Western Australia.

0006-2960/91/0430-7534$02.50/0

et al., 1976; Goscin & Byrnes, 1982; Byrnes, 1985). The existence of this enzyme became more firmly established when it was shown that it could also be isolated from calf thymus (Lee et al., 1980, 1981, 1984), the tissue in which DNA polymerase α had been intensively studied (Fry & Loeb, 1986). The purified calf thymus DNA polymerase δ contained polypeptides of 125 and 48 kDa (Lee et al., 1984). The earlier studies of DNA polymerase δ were directed toward its rigorous isolation and the demonstration that its 3′ to 5′ exonuclease activity was not a separable artifact. Thus, examination by several rigorous criteria established that the 3′ to 5′ exonuclease was associated with DNA polymerase activity for the reticulocyte and calf thymus enzymes (Byrnes et al., 1976; Lee et al., 1980, 1981, 1984). These earlier studies also revealed similarities with DNA polymerase α, e.g., DNA polymerase δ was a high molecular weight enzyme and was sensitive to aphidicolin and sulfhydryl reagents.

Evidence for the functional roles of DNA polymerase δ has also emerged. Significant evidence has emerged that DNA polymerase δ activity plays an important role in DNA replication/repair processes. The discovery that BuAdATP[1] and BuPdGTP were specific inhibitors of DNA polymerase α while DNA polymerase δ was insensitive (Byrnes, 1985; Lee et al., 1985) provided tools for the differential assay of their activities. The first ontogenic study of DNA polymerase δ activity showed that it was a significant fraction of the cellular DNA polymerase activity and that the developmental progress of DNA polymerases α and δ was similar in the neonatal rat heart (Zhang & Lee, 1987). Studies of permeabilized cells have provided evidence for a role of DNA polymerase δ in both replication and repair (Dressler & Frattini, 1986, 1988; Dressler et al., 1988; Hammond et al., 1987, 1990).

A critical role for DNA polymerase δ activity in eukaryotic DNA replication has recently been established. This evidence originates from studies of a 36-kDa factor first identified in the calf thymus (Lee et al., 1984) and that converts the enzyme from low to high processivity when acting on sparsely primed templates (Tan et al., 1986). The factor was shown to be identical with PCNA (proliferating cell nuclear antigen) (Prelich et al., 1987b), a nuclear protein whose expression is strongly tied to the S phase of the cell cycle (Bravo & Celis, 1980; Mathew et al., 1984). This finding has provided a major impetus to the study of the δ polymerases (Blow, 1989; Fairman, 1990). Both DNA polymerase δ and PCNA have been shown to be essential for the in vitro replication of the SV40 chromosome (Prelich et al., 1987a; Lee, S. H., et al., 1989). As a result, the conclusion was drawn that DNA polymerase δ must be involved in DNA replication, since it is the only known target protein of PCNA. It has been further proposed that the PCNA-dependent form of DNA polymerase δ is involved in the synthesis of the leading strand at the replication fork, while DNA polymerase α may be involved in the synthesis of the lagging strand because of its lower processivity and association with DNA primase (Prelich & Stillman, 1988; Downey et al., 1988; So & Downey, 1988; Weinberg & Kelly, 1989). The latter hypothesis is consistent also with the induced processivity of DNA polymerase δ in the presence of PCNA and the association of α with primase activity.

A second form of DNA polymerase δ was isolated from human placenta (Lee & Toomey, 1987), this having a catalytic polypeptide of 170 kDa. The relationships between this enzyme and the formerly described enzyme of ca. 125 kDa have been clarified by its isolation from human placenta and the use of polyclonal and monoclonal antibodies to both the 125- (Lee et al., 1991) and 170-kDa polypeptides (Lee, M. Y. W. T., et al., 1989). These studies show that these two forms are immunochemically distinguishable but may also be structurally related since some antibodies cross-reacted. A 215-kDa form of DNA polymerase δ has also been isolated from HeLa cells, and has been shown to function as a repair enzyme (Nishida et al., 1988; Syvaoja & Linn, 1989). Other studies, using relatively crude enzyme preparations, have also indicated the presence of multiple forms of DNA polymerase δ in calf thymus (Crute et al., 1986; Focher et al., 1988, 1989), with polypeptides from 110 to 245 kDa. An important finding first noted with the 215-kDa (Syvaoja & Linn, 1989) and later with the 170-kDa form (Lee et al., 1991) is that they are not affected by PCNA. Thus, there is a functional difference between these forms and the classical DNA polymerase δ despite the fact that they all possess 3′ to 5′ exonuclease activities. It has been recommended that the "δ" designation be reserved for the PCNA-dependent 125-kDa enzyme and that the name ϵ be given to the non-PCNA-dependent form(s) (Burgers et al., 1990). (In this work, we will accordingly refer to the PCNA-independent human placental DNA polymerase activity as DNA polymerase ϵ.) The roles of DNA polymerase ϵ activity in DNA replication are not as clear as of DNA polymerase δ, although it should be pointed out that the HeLa enzyme has been shown to function as a repair factor (Nishida et al., 1988; Syvaoja & Linn, 1989).

The finely programmed regeneration of rat liver tissue after partial hepatectomy is a well-established system for the study of the correlation of enzyme activity and DNA synthesis, behaving as a synchronized culture of hepatocytes (Nowak et al., 1989). Earlier studies have demonstrated that DNA polymerase α activity increases in the regenerating rat liver (Chang & Bollum, 1972). Thus, it is an attractive system in which to follow the induction of DNA polymerase δ during DNA synthesis. Here we present experiments that demonstrate that DNA polymerase δ, DNA polymerase ϵ, and PCNA closely follow in vivo DNA synthesis during liver regeneration.

Materials and Methods

Partial Hepatectomy. Partial (two-thirds) hepatectomies were performed on 42 Wistar rats (200–250-g body weight). At 18, 24, 36, 48, 72, and 96 h after hepatectomy, the rats were operated on and the regenerating livers were removed and collected in 2 volumes of ice-cold homogenization buffer containing a cocktail of protease inhibitors as previously described (Lee & Toomey, 1987).

Preparation of Liver Extracts. Different methods of extraction were also tested for optimal recovery of enzyme activity in the liver extracts. This included the use of Triton X-100 in the extraction buffer, high ionic strength, and the use of different homogenization procedures. The most efficient method with respect to both time and yield was found to be the method used for the purification of the DNA polymerase δ from human placenta (Lee & Toomey, 1987), which involves homogenization with 3 volumes of buffer in a Waring blender, centrifugation at 25000*g* for 30 min, resuspension of the pellet, rehomogenization, and combination of the two supernatants (Lee & Toomey, 1987). The homogenization buffer used was 50 mM Tris-HCl/1 mM dithiothreitol/1 mM $MgCl_2$/0.5 mM EDTA/0.1 mM EGTA, pH 7.5/0.25 M sucrose/10% glycerol,

[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PCNA, proliferating cell nuclear antigen; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BuAdATP, 2-(*p*-*n*-butyanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

containing the following protease inhibitors: 0.25 mg/mL soybean trypsin inhibitor, 10 mM benzamidine, 0.1 mg/mL bacitracin, 1 μg/mL pepstatin, 1 μg/mL leupeptin, 0.8 mM phenylmethanesulfonyl fluoride, and 10 mM sodium bisulfite.

Pretreatment of Liver Extracts. Liver extracts were partially purified by a miniscale DEAE-cellulose (DE-52, Whatman) batch method in a 1.5-mL microcentrifuge tube before assay for DNA polymerase activity. The DE-52 was preequilibrated in 40 mM Tris-HCl/10% glycerol/0.5 mM EDTA/0.1 mM EGTA/1 mM dithiothreitol, pH 7.8. Extracts (200 μL) were suspended by mixing with 0.5 mL of DE-52 suspension, washed twice with buffer by centrifugation, and eluted with 0.25 mL of 500 mM KCl in the same buffer by centrifugation. The supernatants were then used for assay. With use of such a procedure and 10 μL of supernatant per reaction mixture, the DNA polymerase assays were found to be linear up to 10 min by using both activated calf thymus DNA and activated poly(dA-dT) as template primers.

Assays for DNA Polymerase Activity. Assays using activated calf thymus DNA and poly(dA-dT) as templates were performed as previously described (Lee & Toomey, 1987) in a total reaction volume of 100 μL. When poly(dA)$_{2000}$/oligo(dT)$_{18}$ (20:1 ratio, 0.2 OD_{260} units/mL) was used as a template, the assay conditions were 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, pH 5.90, 5% glycerol, 10 μM [^{3}H]TTP (647 cpm/pmol), 10 mM $MgCl_2$, and 100 μg/mL heat-denatured bovine serum albumin. PCNA when added at a concentration of 100 ng/assay. Assay times were 10 min. Units of activity were expressed as nanomoles of dTTP incorporated per 60 min.

Inhibition of DNA Polymerase Activities by Antibodies. The polyclonal antibody to DNA polymerase ε was that against the human placental enzyme (Lee & Toomey, 1987); the DNA polymerase α specific monoclonal antibody, SJK 287-38 (Tanaka et al., 1982), was purchased from American Type Culture Collection and was prepared from ascites cells; monoclonal antibody IB5 was prepared as described by Lee, M. Y. W. T., et al. (1989); monoclonal antibody A was prepared as described by Lee et al. (1991). The liver extract (10 μL) and the antibody (0–1.2 μg) also in 10 μL were incubated at 0 °C for 2 h. A sample (15 μL) was taken for the assay with either activated calf thymus DNA or poly(dA-dT) as a template.

Partial Purification of Rat Liver DNA Polymerases. An extract from the livers of six rats taken 36 h after partial hepatectomy was prepared as described above. The extract was then centrifuged at 100000*g* for 120 min. The supernatant was diluted with 40 mM Tris-HCl/10% glycerol/0.5 mM EDTA/0.1 mM EGTA/1 mM dithiothreitol, pH 7.8, to a final conductivity equivalent to that of 20 mM KCl. The enzyme was adsorbed onto DE-52 columns (3 × 1.5 cm) and eluted with a gradient of 0–0.5 M KCl in 40 mL of the same buffer. The peak fractions of DNA polymerase activity from the DE-52 column (fractions 31–60) were combined and dialyzed against 50 mM Tris-HCl/5% glycerol/0.5 mM EGTA/0.1 mM EDTA/1 mM dithiothreitol, pH 7.8. The dialyzate was loaded onto an anion-exchange column (Mono Q HR5/5, Pharmacia Corp.) and eluted with a gradient of 0–1 M KCl in 30 mL of the same buffer at a flow rate of 1 mL/min on a Waters 650 HPLC. Buffers used for the purification contained the same cocktail of protease inhibitors as described above for the homogenization step.

[^{3}H]Thymidine Incorporation. In order to determine the amount of [^{3}H]thymidine incorporated at various time intervals after partial hepatectomy, three rats were injected with

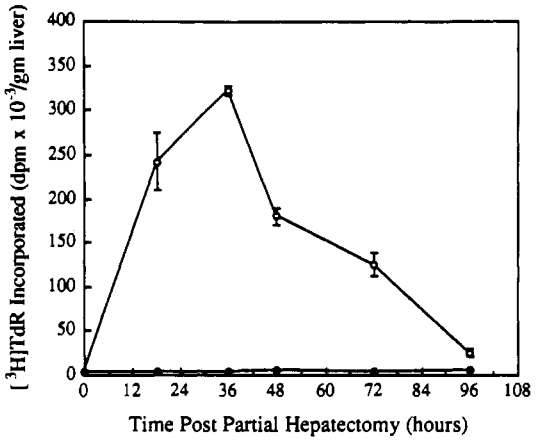

FIGURE 1: DNA synthesis during liver regeneration. [^{3}H]Thymidine incorporation was determined as described in the Materials and Methods section. Three rats were used for each time point. Data for the regenerating liver are shown as O and for livers from the sham controls as ●.

20 μCi of [^{3}H]thymidine/100 g of body weight (specific activity = 71 Ci/mmol, diluted in 0.9% NaCl to 200 μCi/mL). Sham-operated rats were also injected and used as controls. The rats were sacrificed by cervical dislocation after 60 min. The excised livers were immediately placed in 50 mM Tris-HCl, pH 7.4/0.25 M sucrose/6 mM KCl/1 mM $MgCl_2$/1 mM phenylmethane sulfonyl chloride (3 mL/g of tissue) and cut into small 1–2-cm^3 pieces. The liver pieces were then homogenized. For the determination of [^{3}H]thymidine incorporated, 250 μL of 10% perchloric acid was added to 250 μL of liver homogenate in a microcentrifuge tube. The mixture was allowed to remain at 4 °C for 10 min. The tubes were centrifuged for 5 min, and the pellet was washed three times in 5% perchloric acid. The final pellet was washed twice in 10 mL of ethyl ether (3:1). The precipitate was resuspended in 250 μL of 5% perchloric acid, heated at 70 °C for 30 min and centrifuged, and 50 μL of the supernatant was counted in 4 mL of Biofluor.

Immunoblotting. Crude extracts were used for immunoblotting experiments. The blots were performed with use of a biotinylated second antibody–streptavidin system (Amersham) as described previously (Lee, M. Y. W. T., et al., 1989). Anti-PCNA monoclonal antibody was obtained from American Biotech. Inc., Plantation, FL, as a solution of 11.25 mg/mL and used at a dilution of 1:300.

RESULTS

Changes in Total DNA Polymerase Activities. The incorporation of thymidine into DNA was determined as a function of time in groups of rats after partial hepatectomy. The results showed that in vivo DNA synthesis increased within 18 h to a peak at 36 h in the regenerating liver and was unchanged in the sham-operated rats (Figure 1). Exploratory experiments were performed to obtain methods for the optimal assay for DNA polymerase activity in the liver extracts in order to assay for the changes in DNA polymerase activity in the regenerating liver (Materials and Methods). The liver extracts (200 μL) were first partially purified by a batchwise extraction on DEAE-cellulose. This was done in order to remove DNA polymerase β activity (Falaschi & Spadari, 1987) as well as endogenous inhibitors that otherwise interfered with the DNA polymerase assay. With this procedure, the assays were found to be linear up to 10 min with either activated calf thymus DNA or activated poly(dA-dT) as template primer. The levels of DNA polymerase activity expressed as units per milligram of protein in the extracts increased during the process of liver

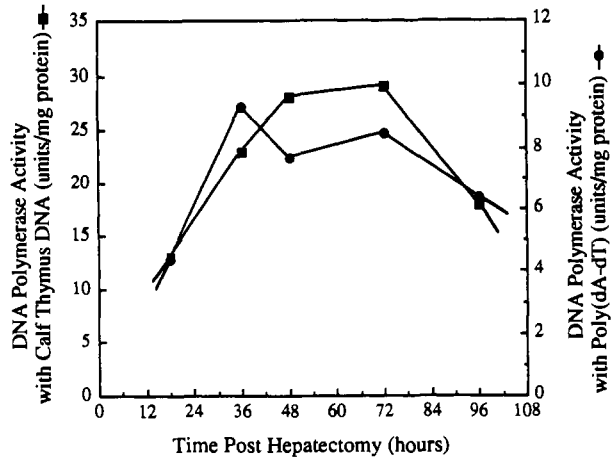

FIGURE 2: Induction of DNA polymerase activity after partial hepatectomy. Liver extracts were prepared and partially purified by a miniscale DE-52 batch method before assay (Materials and Methods). Assays were formed with use of activated calf thymus DNA (■) and activated poly(dA-dT) (●) as the template primer. [Assays of extracts for sham-operated animals did not change significantly (not shown). The data represent the average values determined for six rats.]

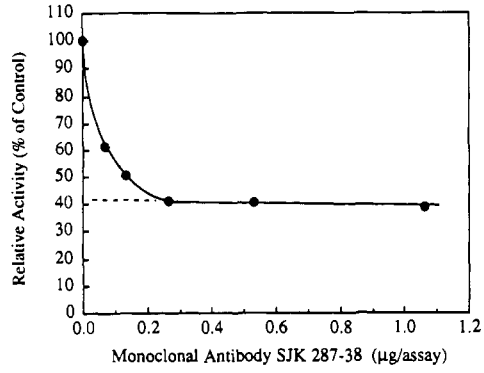

FIGURE 3: Inhibition of DNA polymerase activity in the 36-h regenerating rat liver by an anti-polymerase α monoclonal antibody. The extracts were purified by the miniscale DE-52 method and assayed with use of activated calf thymus DNA as the template after preincubation with anti-α monoclonal antibody SJK 287-38 (Materials and Methods). Values given are as the percentage of the control (incubation with control IgG).

regeneration and reached a maximum between 36 and 48 h to approximately 3–4 times the sham-operated control activity with use of either activated calf thymus DNA or poly(dA-dT) as substrates (Figure 2).

Determination of the Relative Levels of DNA Polymerase δ and α Activities. The assays commonly employed for DNA polymerase α activity do not distinguish it from DNA polymerase δ activity, and additional assay methods were used to provide a direct index of the relative levels of DNA polymerase δ and α activities. The fraction of DNA polymerase α was estimated by two methods: the use of monoclonal antibody SJK 287-38 (Tanaka et al., 1986) that is relatively specific for human DNA polymerase α and does not inhibit human DNA polymerase δ at low antibody concentrations (Lee & Toomey, 1987) and the use of BuAdATP, an inhibitor of DNA polymerase α (Khan et al., 1984) that does not inhibit DNA polymerase δ (Lee et al., 1985; Byrnes, 1985; Lee & Toomey, 1987). In both cases, DNA polymerase activity in the 36-h regenerating rat liver was inhibited to a level that plateaued at 42% of the control activity (Figures 3 and 4) with activated calf thymus DNA as the template. Similar results were obtained when poly(dA-dT) was used as the template (not shown). Thus, by two independent methods we can es-

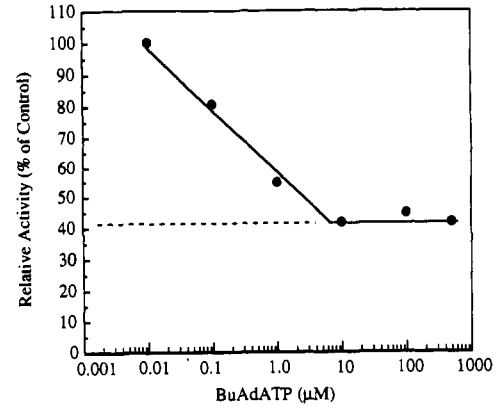

FIGURE 4: Inhibition of DNA polymerase activity in the 36-h regenerating rat liver by BuAdATP. The same extract used in Figure 3 was assayed in the presence of increasing concentrations of BuAdATP using activated calf thymus DNA as the template primer.

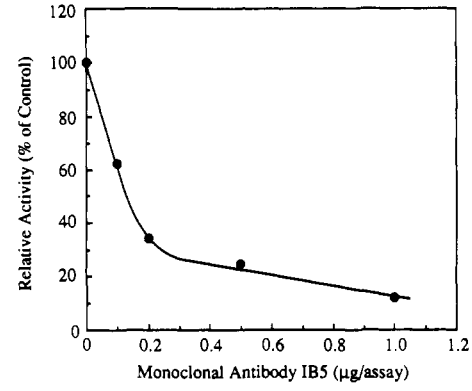

FIGURE 5: Inhibition of DNA polymerase activity in the 36-h regenerating rat liver by monoclonal antibody 1B5. Extracts were prepared and incubated with antibody 1B5 as described in Figure 3, followed by assay of the DNA polymerase activity with use of poly(dA-dT) as the template.

timate that only 58% of the assayable activity is inhibited and therefore attributable to DNA polymerase α at the peak induction of DNA polymerase activity. The difference can be attributed to DNA polymerase δ activity if the total activity is attributable only to DNA polymerase δ plus α activity. This is a reasonable assumption since most of activity in the extracts could be inhibited by aphidicolin (not shown), an inhibitor of both DNA polymerase α and δ (Lee et al., 1984, 1985). Another demonstration that the assayable activity in the liver extracts can be considered to be essentially due to DNA polymerase δ plus α activity came from the use of monoclonal antibodies to DNA polymerase δ (Lee, M. Y. W. T., et al., 1989). With antibody 1B5, which inhibits both DNA polymerase α and δ activities (Lee, M. Y. W. T., et al., 1989; Lee et al., 1991), it was found that most of the DNA polymerase activity in the 36-h regenerating liver extract could be inhibited at a concentration of 1 μg per assay (Figure 5). The finding that the anti-human polymerase δ antibody also cross-reacted with rat liver DNA polymerases is consistent with previous observations that antibodies to human DNA polymerase δ and α cross-react with the rat heart DNA polymerases (Zhang & Lee, 1987).

Demonstration That Immunoreactive Polypeptides for DNA Polymerase δ and PCNA Are Induced during Liver Regeneration. Western blot analysis was performed to determine if the activity changes of DNA polymerase δ were due to changes in enzyme protein and to determine whether the changes in this enzyme paralleled the changes in PCNA that are known to be proliferation-dependent. The changes in

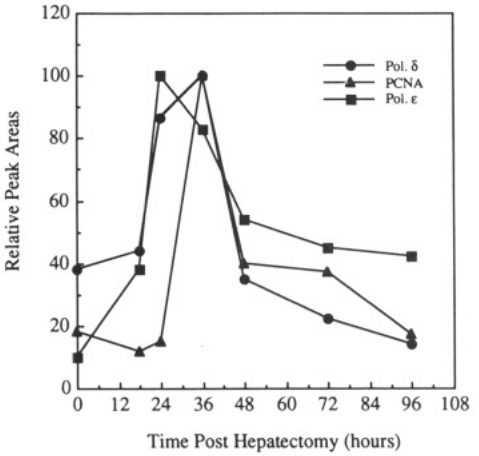

FIGURE 6: Relative changes in the immunoreactive polypeptides of DNA polymerase δ and ϵ and PCNA. The relative changes in the immunoreactive 125- and 170-kDa polypeptides for DNA polymerase δ (●) and ϵ (■), respectively, and for PCNA (▲) were determined by scanning of the photographic negatives of the Western blots with a Zeineh soft-laser densitometer. The peak areas were determined and converted to a percentage of the maximum values observed. About 2.5 μg of crude liver extract taken at various times after hepatectomy was loaded onto 5–15% polyacrylamide–SDS gradient gels. Immunoblotting was performed as described in the Materials and Methods section. Anti-PCNA monoclonal antibody was obtained from American Biotech. Inc. as a solution of 11.25 mg/mL and used at a dilution of 1:300. Undiluted cell supernatant from monoclonal antibody A (Lee et al., 1991) was used for the immunoblotting of DNA polymerase δ. Cell supernatant from monoclonal antibody no. 236 (Lee, M. Y. W. T., et al., 1989) was used for the immunoblotting of DNA polymerase ϵ.

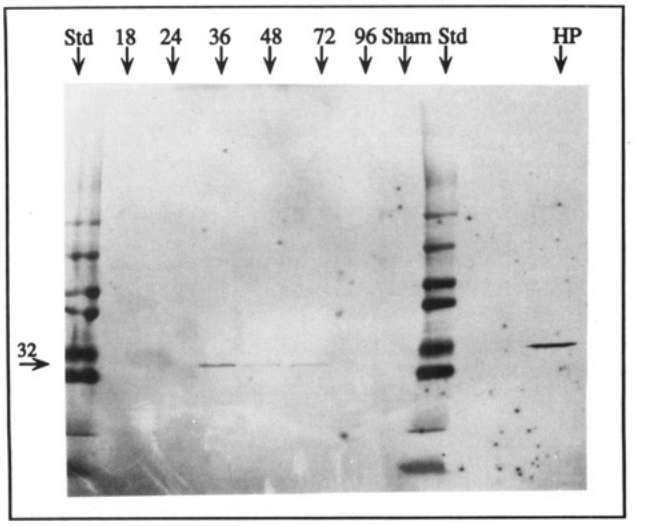

FIGURE 7: Immunoblotting of rat liver extracts with a monoclonal antibody against PCNA. About 2.5 μg of crude liver extract taken at various times after hepatectomy was loaded onto a 5–15% polyacrylamide–SDS gradient gel. Immunoblotting was performed as previously described (Lee & Toomey, 1987). Lanes marked with numerals refer to times after partial hepatectomy in hours. Sham refers to a liver extract from a 36-h sham-operated rat. HP refers to purified human PCNA (2 μg). The horizontal arrow indicates the position of the 32-kDa band. Lanes marked Std are prestained molecular mass standards (Sigma Chem. Co.) (in descending order: α_2-macroglobulin, 180 kDa; β-galactosidase, 116 kDa; fructose-6-phosphate kinase, 84 kDa; pyruvate kinase, 58 kDa; fumarase, 48.5 kDa; lactate dehydrogenase, 36.5 kDa; triosephosphate isomerase, 26.6 kDa).

immunoreactive protein for DNA polymerase ϵ were also examined, with this question being of significance since it is only the 125-kDa form of δ that is PCNA-sensitive and for which strong evidence for a critical role in DNA replication has been obtained. In order to gain some insight into this issue, liver extracts were examined by Western blotting with specific antibodies. The first was a monoclonal antibody (antibody A) that is specific for the 125-kDa polypeptide of DNA polymerase δ (Lee et al., 1991). The second was murine anti-human PCNA monoclonal antibody, and the third was a murine polyclonal antibody to human placental DNA polymerase ϵ that has been shown to inhibit the activity of DNA polymerase ϵ but not DNA polymerase α (Lee & Toomey, 1987) and also immunoblots and immunoprecipitates a 170-kDa polypeptide (Lee, 1988). With use of equal amounts of liver homogenate, the apparent changes in the immunoreactive polypeptides were examined by Western blotting (not shown); the polyclonal antibody to DNA polymerase ϵ immunoblotted a major 170-kDa band and also some smaller polypeptides, and monoclonal antibody A immunoblotted a major 125-kDa band and also some smaller polypeptides but did not immunoblot a 170-kDa band, showing that the two antibodies had the required specificity against the rat enzymes (not shown). An approximate quantitation was performed by densitometric analysis of the blots, and the relative amounts of the immunoreactive bands in the rat liver extracts at different times after regeneration are shown in Figure 6. These blots showed that the DNA polymerase polypeptides for both DNA polymerase δ and ϵ showed an increase with the maximum levels at the 24–36-h time periods, followed by a decrease. While these data can only be considered very roughly quantitative, they do support the view that the increases in activity observed by assay methods can be accounted for by increases in the immunoreactive polypeptides for both DNA polymerase δ and

The level of PCNA is maintained at a low level in nonproliferating liver but also increases sharply to a maximum at 36 h after partial hepatectomy. This time-dependent increase of PCNA during liver regeneration coincides with the pattern of in vivo DNA synthesis pattern presented above (both peak at 36 h post hepatectomy) consistent with the current views that PCNA, like DNA polymerase δ, plays a role in DNA replication. In the rat, a 32-kDa rat immunoreactive band is observed, as opposed to the 36-kDa band for human PCNA (Figure 7). The slight size difference between human and rat PCNA may be due to species variation. PCNA from various sources shows some variability on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, e.g., yeast (26 kDa; Bauer & Burgers, 1988) and soybean (34 kDa; Suzuka et al., 1989).

Characterization of the Partially Purified Rat Liver DNA Polymerases. The DNA polymerase α, δ, and ϵ activities were separated by chromatography and characterized (Materials and Methods), in order to confirm the presence of the latter two activities in the regenerating liver and to validate the reagents used for their selective determination. Extracts from the 36-h regenerating liver were centrifuged at 100000g, dialyzed, and adsorbed onto DE-52 columns and eluted with a gradient of 0–0.5 M KCl (Materials and Methods). A single major peak of enzyme activity is seen, and the activities are apparently not resolved (Figure 8). However, by assaying the column fractions in the presence of dimethyl sulfoxide, which we have shown to stimulate polymerase δ (Lee & Toomey, 1986), a minor peak of DNA polymerase activity that eluted before the major peak was detected. This minor fraction was characterized as DNA polymerase ϵ (see below); the relative amount of this fraction is ca. 5%; however, this may not reflect its relative amount in the tissue, since this is dependent on recovery. The major peak of enzyme was dialyzed and further fractionated by HPLC on a Mono Q column

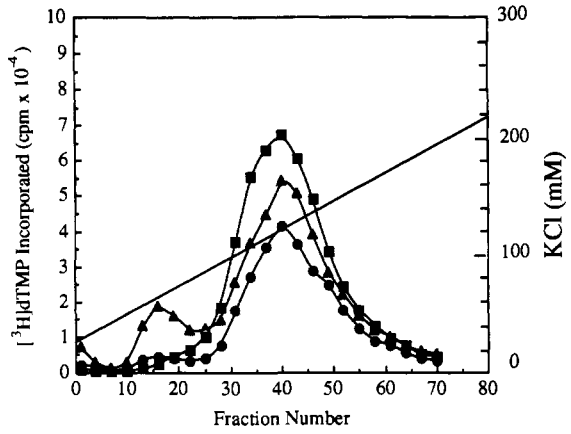

FIGURE 8: DE-52 chromatography of rat liver DNA polymerase activity. A 100000g supernatant from six rats taken 36 h after partial hepatectomy was chromatographed on a DE-52 column (Materials and Methods). The fractions were assayed for DNA polymerase activity with activated calf thymus DNA (■), activated poly(dA-dT) (●) and activated poly(dA-dT) with 15% DMSO in the assay mixture (▲).

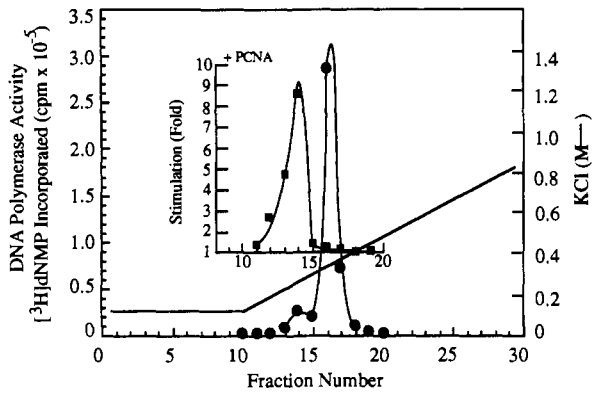

FIGURE 9: HPLC of DNA polymerase activity on a Mono Q column. The major peak of DNA polymerase activity (fractions 31–60 from the DE-52 column, Figure 8) were combined, dialyzed, and chromatographed on a Mono Q column with use of a Waters 650 HPLC system (Materials and Methods). The enzyme was eluted with a gradient of 0–1 M KCl at a flow rate of 1 mL/min. The fractions were assayed for DNA polymerase activity with activated poly(dA-dT) as a template (●). The fractions were also assayed with poly-$(dA)_{2000}$/oligo$(dT)_{18}$ (20:1) as the template in the absence and presence of PCNA (0.1 μg/assay), and the degree of stimulation (■) plotted against fraction number is shown in the inset.

(Figure 9). Two peaks of activities were resolved with use of activated poly(dA-dT) as a template. The minor peak (fraction 14) eluted at 258 mM KCl was stimulated by human placental PCNA when poly(dA)/oligo(dT) (20:1 ratio) was used as the template (Figure 9, inset). This enzyme therefore behaves as a PCNA-dependent form of DNA polymerase δ whereas the major peak (fraction 17) is insensitive to PCNA.

The three fractions of rat liver DNA polymerases described above were further confirmed as DNA polymerases δ, ε, and α by using specific reagents. Table I summarizes these experiments. Fraction 14 of the Mono Q column, in addition to its PCNA sensitivity, is identified as the 125-kDa form of DNA polymerase δ by its inhibition by monoclonal antibody A, while fraction 17 is identified as DNA polymerase α because it is inhibited by monoclonal antibody SJK 287-38 and BuAdATP. Peak I of the DE-52 column was identified as DNA polymerase ε because it is only slightly affected by PCNA (1.5-fold stimulation) and is relatively insensitive to inhibition by BuAdATP or monoclonal antibodies against DNA polymerase α and δ. The effects of the other reagents tested are also consistent with these assignments. *N*-Ethyl-

Table I: Differential Responses of Rat Liver DNA Polymerases to Specific Inhibitors and Activators[a]

reagent	relative activities		
	DE-52 peak I	Mono Q fr 14	Mono Q fr 17
control	100	100	100
Mab A (1.5 μg/assay)	80	10	85
Mab SJK 287-38 (1 μg/assay)	81	80	8
PCNA (80 ng/assay)	150	890	106
BuAdATP (4 μM)	69	75	5
dimethyl sulfoxide (15%)	160	480	124
Mab 1B5 (1.5 μg/assay)	10	15	12
N-ethylmaleimide (1.8 mM)	23	15	12
aphidcolin (2 μg/mL)	22	11	10
carbonyldiphosphonate (20 μM)[b]	75	20	100

[a] The different polymerase fractions were prepared as described in the text. Approximately 0.5 unit of DNA polymerase activities was assayed in duplicate in the presence or absence of the indicated reagents. [b] Obtained as a generous gift from Dr. C. E. McKenna, University of Southern California, Los Angeles.

maleimide, aphidicolin, and monoclonal antibody 1B5 inhibited all three enzymes as expected. This eliminates the possibility that one of the fractions might be DNA polymerase β, which is not affected by *N*-ethylmaleimide or aphidicolin (Weissbach et al., 1971; Falaschi & Silvio, 1978). The enzyme fractions cannot be attributed to DNA polymerase γ activity because the latter is not inhibited by aphidicolin (Fry & Loeb, 1986). The effects of carbonyldiphosphonate on the three enzyme fractions were also evaluated (Table I), as there are contradictory reports regarding its selectivity for inhibition of ε and δ (Talanian et al., 1989; Syvaoja et al., 1990). Our results show that only the rat liver DNA polymerase δ activity (Mono Q, fraction 14) is significantly inhibited, while DNA polymerase α is not affected and the PCNA-independent ε activity is only slightly affected. This findings differ from those of Talanian et al. (1989) but agree with those of Syvaoja et al. (1990) and suggest that carbonyldiphosphonate may be a useful reagent for the selective inhibition of the PCNA-dependent DNA polymerase δ activity.

Discussion

The regenerating rat liver is one of the first systems to be used to demonstrate the association of DNA polymerase α activity with the mitotic state (Chang & Bollum, 1972). In addition to the induction of DNA polymerase α, other activities potentially involved in DNA replication, such as DNA topoisomerase II and primase, have also been shown to be induced in the regenerating rat liver (Philippe et al., 1986). The findings here extend and complement these previous studies and provide the first examination of the induction of DNA polymerase δ, as well as of PCNA, in the regenerating rat liver. This dissection of the contribution of DNA polymerase δ activities to the rise of DNA polymerase activity during liver regeneration is important since the previously used methods for the assay of DNA polymerase α do not allow its discrimination from DNA polymerase δ. In this study we have shown that a significant fraction of the apparent DNA polymerase α activity is due to DNA polymerase δ activity. The relative levels of DNA polymerase δ and α activity are in general agreement with observations that have been made in studies of the developing rat heart (Zhang & Lee, 1987) and in permeabilized human fibroblasts (Hammond et al., 1987, 1990; Dresler & Frattini, 1986, 1988), with use of similar assay strategies. Marraccino et al. (1987) studied the changes of DNA polymerase α and δ activities during the cell cycle in CHO cells and observed an increase in DNA polymerase δ activity during the G2/M phase. It has been suggested that

DNA polymerase δ activity may in fact be responsible for the major fraction of DNA synthesis in permeabilized CHO cells synchronized in the S phase (Basnakian et al., 1989). Our findings are consistent with the current evidence that DNA polymerase δ activity is crucial to DNA replication.

The situation has recently become more complex in that PCNA-independent DNA polymerase δ activities have also been described and are now referred to as DNA polymerase ε. These have been isolated from human placenta as 170-kDa polypeptides (Lee & Toomey, 1987; Lee et al., 1991) and from HeLa cells as 215-kDa polypeptides (Syvoaja & Linn, 1989) and as partially purified preparations from calf thymus (Focher et al., 1989; Crute et al., 1986). The Western blotting studies reported here show that the levels of immunoreactive protein for DNA polymerase δ and ε are induced concomitantly after partial hepatectomy and rise to a maximum between 24 and 36 h. This is the first direct evidence for the increase in enzyme protein for both these two enzymes in an actively proliferating cell system. The levels of PCNA were also followed and, consistent with previous studies on its cell-cycle association, also peaked in parallel with that of its target polymerase.

In addition, the presence of DNA polymerase δ, ε, and α activities in the rat liver was demonstrated by their separation; the latter study also indicated that the DNA polymerase ε fraction appears to be a minor component of the total activity. Questions that still remain are the roles of the PCNA-independent DNA polymerase ε. The present study shows that the DNA polymerase ε behaves in a manner similar to that of α and δ during the regeneration process, leaving open the possibility that it is involved in DNA replication. However, DNA polymerase ε has been shown to be required for repair synthesis (Nishida et al., 1988). In addition, several studies using permeablized cells and inhibitors to DNA polymerase α have also implicated DNA polymerase δ activity in repair synthesis as well as replication (Hammond et al., 1990; Dresler & Frattini, 1986, 1988). However, no distinction was made between PCNA-dependent and PCNA-independent DNA polymerase activities. Further studies using specific antibodies to DNA polymerase δ and ε in terms of the examination of their cell-cycle behavior and potential involvement in repair processes are clearly needed.

The molecular cloning of the cDNAs for DNA polymerase α and PCNA (Wong et al., 1988; Matsumoto et al., 1987; Almendral et al., 1987; Liu et al., 1989) has provided tools for the study of their transcriptional regulation (Wahl et al., 1988; Jaskulski et al., 1988; Liu & Bambara, 1989). PCNA mRNA levels have been shown to peak in the S phase and increase many fold on serum stimulation of quiesent cells and then decline in the G2/M phase (Alemendral et al., 1987; Matsumoto et al., 1987). In the most thorough and rigorous study of the gene expression of human polymerase α to date, Wahl et al. (1988) have shown that it is a complex process, which must be differently considered for (a) cells activated from the quiescent state (G_0) after serum deprivation, (b) transformed cells, and (c) normal cells progressing through the cell cycle. This study showed that polymerase α expression (i) is increased when cells are activated, (ii) is higher in transformed cells than normal cells, and (iii) is constitutively expressed during the normal cell cycle with modest increases prior to the S phase. In all three instances the temporal and quantitative concordance of enzyme activity, protein synthesis, and steady-state mRNA levels indicates a transcriptional regulation. It would be expected that the crucial enzymatic activities involved in DNA replication are regulated by similar transcriptional mechanisms, and the future cloning of the cDNAs for the polymerases δ and ε will be required for more in-depth studies of its transcriptional regulation.

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

References

Almendral, J. M., Huebsch, D., Blundell, P. A., MacDonald-Bravo, H., & Bravo, R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1575–1579.

Basnakian, A., Banfalvi, G., & Sarkar, N. (1989) *Nucleic Acids Res. 1*, 4757–4767.

Bauer, G. A., & Burgers, P. M. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7506–7510.

Blow, J. (1989) *Trends Genet. 5*, 13–136.

Bravo, R., & Celis, J. E. (1980) *J. Cell Biol. 84*, 795–802.

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

Byrnes, J. J. (1985) *Biochem. Biophys. Res. Commun. 132*, 628–634.

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

Chang, L. M. S., & Bollum, F. J. (1972) *J. Biol. Chem. 243*, 7948–7950.

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

Downey, K. M., Tan, C.-K., Andrews, D. M., Li, X., & So, A. G. (1988) *Cancer Cells 6*, 403–410.

Dresler, S. L., & Frattini, M. G. (1986) *Nucleic Acids Res. 17*, 7093–7102.

Dresler, S. L., & Frattini, M. G. (1988) *Biochem. Pharmacol. 37*, 1033–1037.

Dresler, S. L., Frattini, M. G., & Robinson-Hill, R. M. (1988) *Biochemistry 27*, 7247–7254.

Fairman, M. P. (1990) *J. Cell Sci. 95*, 1–4.

Falaschi, A., & Silvio, S. (1978) in *DNA Synthesis: Present & Future* (Molineux, J. & Kohiyama, M., Eds.) pp 487–515, Plenum Press, New York.

Focher, F., Gassmann, M., Hafkemeyer, P., Ferrari, E., Spadari, S., & Hübscher, U. (1989) *Nucleic Acids Res. 17*, 1805–1821.

Fry, H., & Loeb, L. A. (1986) in *Animal Cell DNA Polymerases*, p 50, CRC Press, Boca Raton, FL.

Goscin, L. P., & Byrnes, J. J. (1982) *Biochemistry 21*, 2513–2518.

Hammond, R. A., Byrnes, J. J., & Miller, M. R. (1987) *Biochemistry 26*, 6817–6824.

Hammond, R. A., McClung, J. K., & Miller, M. R. (1990) *Biochemistry 29*, 286–291.

Jaskulski, D., Gatti, G., Travali, S., Calabretta, B., & Baserga, R. (1988) *J. Biol. Chem. 263*, 10175–10179.

Khan, N. N., Wright, G. E., Dudycz, L. W., & Brown, N. C. (1984) *Nucleic Acids Res. 12*, 3695–3706.

Lee, M. Y. W. T. (1988) in *DNA Replication & Mutagenesis* (Moses, R. E., & Summers, W., Eds.) pp 68–80, American Society of Microbiology, Washington, DC.

Lee, M. Y. W. T., & Toomey, N. L. (1986) *Nucleic Acids Res. 14*, 1719–1726.

Lee, M. Y. W. T., & Toomey, N. L. (1987) *Biochemistry 26*, 1076–1085.

Lee, M. Y. W. T., Tan, C.-K., Downey, K. M., & So, A. G. (1980) *Biochemistry 19*, 2096–2101.

Lee, M. Y. W. T., Tan, C.-K., Downey, K. M., & So, A. G. (1981) *Prog. Nucleic Acid Res. Mol. Biol. 26*, 83–96.

Lee, M. Y. W. T., Tan, C.-K., Downey, K. M., & So, A. G. (1984) *Biochemistry 23*, 1906–1913.

Lee, M. Y. W. T., Toomey, N. L., & Wright, G. E. (1985) *Nucleic Acids Res. 13*, 8623-8630.
Lee, M. Y. W. T., Alejandro, R., & Toomey, N. L. (1989) *Arch. Biochem. Biophys. 272*, 1-9.
Lee, M. Y. W. T., Jiang, Y., Zhang, S. J., & Toomey, N. L. (1991) *J. Biol. Chem. 266*, 2423-2429.
Lee, S. H., Eki, T., & Hurwitz, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7361-7365.
Lehman, I. R., & Kaguni, L. S. (1989) *J. Biol. Chem. 264*, 4265-4268.
Liu, Y. C., & Bambara, R. A. (1989) *Biochem. Biophys. Res. Commun. 161*, 873-882.
Liu, Y. C., Marraccino, R. L., Kang, P. C., Bambara, R. A., Lord, E. M., Chou, W. G., & Zain, S. B. (1989) *Biochemistry 28*, 2967-2974.
Marraccino, R. L., Wahl, A. F., Kang, P. C., Lord, E. M., & Bambara, R. A. (1987) *Biochemistry 26*, 7864-7870.
Mathews, M. B., Bernstein, R. M., Franza, B. R., Jr., & Garrels, J. I. (1984) *Nature (London) 309*, 374-376.
Matsumoto, K., Moriuchi, T., Koji, T., & Nakane, P. K. (1987) *EMBO J. 6*, 637-642.
Nishida, C., Reinhardt, P., & Linn, S. (1988) *J. Biol. Chem. 263*, 501-510.
Nowak, R., Siedlecki, J. A., Kocamarek, L., Zmudzha, B. Z., & Wilson, S. H. (1989) *Biochem. Biophys. Acta 1008*, 203-207.
Philippe, M., Rossignol, J. M., & DeRecondo, A. M. (1986) *Biochemistry 25*, 1611-1615.
Prelich, G., & Stillman, B. (1988) *Cell 53*, 117-126.
Prelich, G., Kostura, M., Marshak, D. R., Mathews, M. B., & Stillman, B. (1987a) *Nature (London) 326*, 471-475.
Prelich, G., Tan, C. K., Kostura, M., Mathews, M. B., So, A. G., Downey, K. M., & Stillman, B. (1987b) *Nature (London) 326*, 517-520.
So, A. G., & Downey, K. M. (1988) *Biochemistry 27*, 4591-4595.
Suzuka, I., Daidoji, H., Matsuoka, M., Kadowaki, K. I., Takasaki, Y., Nakane, P. K., & Moriuchi, T. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3189-3193.
Syvaoja, J., & Linn, S. (1989) *J. Biol. Chem. 264*, 2489-2497.
Syvaoja, J., Suomensaari, S., Nishida, C., Goldsmith, J. S., Chui, G. S. J., Jain, S., & Linn, S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6664-6668.
Talanian, R. V., Brown, N. C., McKenna, C. E., Ye, T.-G., Levy, J. N., & Wright, G. E. (1989) *Biochemistry 28*, 8270-8274.
Tan, C.-K., Castillo, C., So, A. G., & Downey, K. M. (1986) *J. Biol. Chem. 261*, 12310-12316.
Tanaka, S., Hu, S. Z., Wang, T. S.-F., & Korn, D. (1982) *J. Biol. Chem. 257*, 8386-8390.
Wahl, A. F., Geis, A. M., Spain, B. H., Wong, S. W., Korn, D., & Wang, T. S.-F. (1988) *Mol. Cell. Biol. 8*, 5016-5025.
Weinberg, D. H., & Kelly, T. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9742-9746.
Weissbach, A., Schlabach, A., Fridlander, B., & Boldin, A. (1971) *Nature (London) 231*, 167-170.
Wong, S. W., Wahl, A. F., Yuan, P.-M.; Arai, N., Pearson, B. E., Arai, K., Korn, D., Hunkapillar, M. W., & Wang, T. S.-F. (1988) *EMBO J. 7*, 37-47.
Zhang, S. J., & Lee, M. Y. W. T. (1987) *Arch. Biochem. Biophys. 252*, 24-31.

Reaction of Formate with the Fast Form of Cytochrome Oxidase: A Model for the Fast to Slow Conversion†

Jon R. Schoonover‡ and Graham Palmer*
Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77251-1892
Received November 29, 1990; Revised Manuscript Received May 3, 1991

ABSTRACT: The ability to isolate preparations of cytochrome oxidase which are highly homogeneous has facilitated a study of the effects of various reagents on the purified enzyme. The addition of either sodium formate, formamide, formaldehyde, or sodium nitrite to enzyme which reacts in a single rapid kinetic phase with cyanide causes a blue-shift of 4-6 nm of the net (cytochrome a + cytochrome a_3) Soret maximum. Only the derivative prepared by adding sodium formate demonstrates measurable intensity in the $g' = 12$ region of the low-temperature electron paramagnetic resonance (EPR) spectrum. This $g' = 12$ resonance is characteristic of cytochrome oxidase which has undergone a modification at the binuclear center and thereby reacts sluggishly with cyanide. As the site of cyanide binding in resting enzyme as been demonstrated to be Cu_B [Yoshikawa, S., & Caughey, W. S. (1990) *J. Biol. Chem. 265*, 7945-7958], it is proposed that formate can bind to Cu_B and the fast to slow transition is rationalized by using this proposal. The $g' = 12$ signal is also produced upon the addition of sodium formate to mitochondrial preparations, suggesting that the species responsible for this behavior may have possible physiological relevance. Physical properties of the formate derivative and data for other reagents reacted with the fast-reacting enzyme preparation are presented.

Preparations of cytochrome oxidase have been shown to exhibit variable spectral and kinetic properties (Baker et al., 1987; Palmer et al., 1988), and the fact that purified preparations of the enzyme are inhomogeneous is now generally accepted.

Early studies by van Buuren (1972) and Kumar et al. (1984) illustrated the problem of heterogeneity by showing multiple kinetic phases during the binding of cyanide to the purified enzyme. Kumar et al. further demonstrated that the phases of the reaction varied from preparation to preparation. Baker et al. (1987) then discovered that most purified samples consist

†Supported by grants from the National Institutes of Health (GM 21337) and the Welch Foundation (C-636).
‡Present address: Department of Chemistry, University of North Carolina, Chapel Hill, NC.

0006-2960/91/0430-7541$02.50/0