Mitochondrial DNA Polymerase from *Drosophila melanogaster* Embryos: Kinetics, Processivity, and Fidelity of DNA Polymerization†

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ABSTRACT: The mitochondrial DNA polymerase from embryos of *Drosophila melanogaster* has been examined with regard to template-primer utilization, processivity, and fidelity of nucleotide polymerization. The enzyme replicates predominantly single-stranded and double-stranded DNAs: the rate of DNA synthesis is greatest on the gapped homopolymeric template poly(dA)·oligo(dT), while the highest substrate specificity is observed on single-stranded DNA templates of natural DNA sequence. Kinetic experiments and direct physical analysis of DNA synthetic products indicate that the *Drosophila* DNA polymerase γ polymerizes nucleotides by a quasi-processive mechanism. The mitochondrial enzyme demonstrates a high degree of accuracy in nucleotide incorporation which is nearly identical with that of the replicative DNA polymerase α from *Drosophila* embryos. Thus, the catalytic properties of the near-homogeneous *Drosophila* DNA polymerase γ are consistent with the in vivo requirements for mitochondrial DNA synthesis as described in a variety of animal systems.

Drosophila mitochondrial DNAs (mtDNAs),[1] like all animal mtDNAs, are circular duplex molecules (Clayton, 1982). The genome size varies among species from 15.7 to 19.5 kilobase pairs almost exclusively as a result of sequence variation in a single region termed the A + T region (Wolstenholme et al., 1979). Electron microscopic studies have shown that replication of *Drosophila* mtDNAs initiates in the A + T region and proceeds unidirectionally (Goddard & Wolstenholme, 1980). Synthesis of the leading DNA strand is most frequently 87–98% complete before complementary DNA strand synthesis ensues, although in a small fraction of molecules lagging DNA strand synthesis may be initiated earlier (Wolstenholme et al., 1979; Goddard & Wolstenholme, 1980). Thus, *Drosophila* mtDNA is replicated by a highly asymmetric mechanism as observed in a variety of organisms, both in vivo and under tissue culture conditions (Clayton, 1982).

In mammalian systems, the mode of replication has been described in detail by studies of replication intermediates (Clayton, 1982). Although less is known about *Drosophila* or insect mtDNA replication by comparison, the available data suggest similar enzymatic requirements. In an effort to define the biochemical and genetic requirements for mtDNA replication in *Drosophila*, we have described the purification and partial characterization of the mitochondrial DNA polymerase (Pol γ) from *Drosophila melanogaster* embryos (Wernette & Kaguni, 1986). The enzyme consists of two polypeptides of 125 000 and 35 000 daltons as judged by SDS–polyacrylamide gel electrophoresis, and is most likely a heterodimer. While the 125 000-dalton subunit is the catalytic core of the enzyme, no function has yet been assigned to the 35 000-dalton subunit.

In this report, we describe the further mechanistic characterization of the *Drosophila* γ-polymerase including a comparison of the rate and specificity of DNA synthesis on several template-primers and an examination of the products of DNA synthesis. Analyses of the processivity and fidelity of nucleotide polymerization on single-stranded DNA templates are presented. The findings are examined in comparison with well-characterized DNA polymerases from both procaryotic and eucaryotic sources and discussed with regard to the requirements for mitochondrial DNA replication as defined by in vivo studies.

Experimental Procedures

Materials

Nucleotides and Nucleic Acids. Unlabeled deoxy-, dideoxy-, and ribonucleoside triphosphates were purchased from P-L Biochemicals. [^{3}H]dTTP, [α-^{32}P]dCTP, and [α-^{32}P]dATP were from New England Nuclear. Calf thymus DNA (highly polymerized type I) was purchased from Sigma and was activated by partial digestion with DNase I (Boehringer-Mannheim; Fansler & Loeb, 1974). Poly(dA)$_{700}$·p(dT)$_{10}$ was purchased from P-L Biochemicals and contains adenine and thymine in a molar ratio of 20:1, respectively, such that the calculated average single-stranded DNA region between primers is 200 nucleotides.

ϕX174*am*3 DNA was prepared essentially as described (Cunningham et al., 1980). A synthetic oligodeoxynucleotide homologous to the sequence extending from position 778 to position 764 in ϕX174 DNA (Sanger et al., 1978) was prepared in an Applied Biosystems Model 477 oligonucleotide synthesizer. Singly primed ϕX174*am*3 DNA was prepared by annealing the synthetic primer to ϕX174 DNA for 35 min at 65 °C using a 6:1 molar ratio of primer to homologous ϕX174 DNA in 10 mM Tris·HCl (pH 8.1), 0.3 M NaCl, and 0.03 M sodium citrate. The primer terminus is 177 nucleotides upstream from the *am*3 mutation which is located at position 587.

Multiprimed M13 DNA was prepared by a modification of the method of Matson et al. (1980). The replicative form of an M13 recombinant DNA (10 907 nucleotides) was digested with DNase I (1.2 μg/mL) for 60 min at 37 °C in 10 mM potassium phosphate (pH 7.6), 20 mM potassium chloride, 5 mM $MgCl_2$, and 4 mM 2-mercaptoethanol to generate DNA fragments ranging in size from 50 to 250 nucleotides.

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[1] Abbreviations: mtDNA, mitochondrial DNA; pol γ, DNA polymerase γ; BSA, bovine serum albumin; nt, nucleotide(s); Pol I, *Escherichia coli* DNA polymerase I; Pol α, DNA polymerase α; RF, replicative form.

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The average fragment size was 150 nucleotides as determined by fractionation of the products of digestion in a 2% agarose gel, staining with ethidium bromide, and subsequent densitometric scanning. The M13 DNA fragments were purified, denatured, and annealed to the homologous M13 viral DNA for 90 min at 65 °C in 10 mM Tris·HCl (pH 8.1), 0.3 M NaCl, and 0.03 M sodium citrate. The number of primer fragments per M13 single-stranded DNA molecule was determined by complete replication of the template-primer (35 pmol as nucleotide) in enzyme excess (*Escherichia coli* DNA polymerase I Klenow fragment, 5 units) and subsequent analysis of the DNA product strands by densitometric scanning of an 0.8% alkaline agarose gel which was dried and autoradiographed. The average product size was 1800 nucleotides, corresponding to an average of 6 primer fragments per M13 DNA molecule.

Enzymes. *Drosophila* DNA polymerases α (fraction VI, 5.2×10^4 units/mg) and γ (fraction VI, 2.7×10^4 units/mg) were prepared as described (Kaguni et al., 1983; Wernette & Kaguni, 1986). *E. coli* DNA polymerase I (lot 41/2101) and its Klenow fragment (lot 29) were purchased from Amersham and New England Biolabs, respectively; units were as defined by the manufacturers.

Methods

DNA Polymerase γ Assay. Reaction mixtures (0.05 mL) contained 50 mM Tris·HCl (pH 8.5), 5 mM $MgCl_2$, 20 mM dithiothreitol, 110–200 mM KCl, 400 μg/mL bovine serum albumin (BSA), 60 μM each of dATP, dCTP, and dGTP, 30 μM [^{3}H]dTTP (500–2000 cpm/pmol), saturating levels of DNA template-primer, and enzyme, unless otherwise indicated. The saturating concentration for the DNA template-primers examined and the optimal concentration of KCl used were as follows: 180 μM DNase I activated calf thymus DNA at 200 mM KCl, 180 μM poly(dA)·p(dT)$_{10}$ at 120 mM KCl, 48 μM singly primed φX174 DNA at 120 mM KCl, and 48 μM multiprimed M13 DNA at 110 mM KCl. Incubation was for 10 min at 30 °C unless otherwise indicated. Because the activity of the enzyme varies on different preparations of DNase I activated calf thymus DNA, we have redefined the unit of DNA polymerase γ activity as that amount of enzyme that catalyzes the incorporation of 1 nmol of deoxyribonucleoside triphosphate into acid-insoluble material in 60 min at 30 °C on the poly(dA)·p(dT)$_{10}$ (20:1) template-primer.

Steady-State Kinetic Analysis of Template-Primer Utilization. Reaction mixtures were as described in the legend to Table I. For each template-primer examined, two or more determinations were made at eight DNA concentrations over the indicated ranges: poly(dA)·p(dT), 5–80 μM; activated calf thymus DNA, 3–80 μM; singly primed φX174 and multiprimed M13 DNA, 0.25–20 μM. Reactions at each DNA concentration were performed in triplicate. Incubation was for 5 min at 30 °C—control experiments indicated that the reaction rate was linear for at least 10 min at all of the substrate concentrations examined. The data were analyzed by an enzyme kinetics analysis progrm (EDATA v1.1, 1985 EMF Software) in an IBM XT computer; the kinetic values listed in Table I are presented as means and standard deviations.

Quantitative Kinetic Analysis of Processivity. Processivity was determined by the quantitative kinetic method of Bambara et al. (1978). The DNA polymerase γ reaction mixtures (0.05 mL) contained 50 mM Tris·HCl (pH 8.5), 5 mM $MgCl_2$, 110 mM KCl, 20 mM dithiothreitol, 400 μg/mL BSA, 10 μM dNTPs, 100 μM multiprimed M13 DNA, and enzyme (0.20–0.26 unit). The *E. coli* DNA polymerase I reaction mixtures (0.05 mL) contained 50 mM Tris·HCl (pH 8.5), 10 mM $MgCl_2$, 4 mM dithiothreitol, 200 μg/mL BSA, 10 μM dNTPs, 100 μM multiprimed M13 DNA, and enzyme (0.01 unit). In the limited reactions where three deoxynucleoside triphosphates were used, mixtures contained 10 μM each of dATP, dTTP, and [α-^{32}P]dCTP (7×10^4 cpm/pmol). The complete reactions contained 10 μM each of dATP, dTTP, dGTP, and [α-^{32}P]dCTP (4×10^3 cpm/pmol). The inhibited reactions were altered to contain 0.4 μM multiprimed M13 DNA and 100 μM poly(dA)·p(dT)$_{10}$.

All determinations were performed in triplicate and were incubated for 10 min at 30 °C. The data obtained were analyzed by a computer program kindly provided by Dr. R. A. Bambara (University of Rochester).

Analysis of Products of DNA Synthesis by Polyacrylamide Gel Electrophoresis. Products to be analyzed by polyacrylamide gel electrophoresis were made 1% in sodium dodecyl sulfate and 10 mM in EDTA, heated for 4 min at 80 °C, adjusted to 0.15 N NaOH, incubated for 2 h at 37 °C, neutralized with HCl, and precipitated with ethanol in the presence of 1 μg of sonicated calf thymus DNA as carrier. In the gel analysis of processivity, the incubation in 0.15 N NaOH was omitted. The ethanol precipitates were resuspended in 80% formamide, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue. Aliquots were denatured for 4 min at 100 °C and electrophoresed in a 4%, 6%, or 8% polyacrylamide slab gel (13 × 30 × 0.15 cm) containing 7 M urea in 90 mM Tris–borate (pH 8.3) and 25 mM EDTA. The gel was then washed in distilled water for 20 min to remove the urea, dried under vacuum, and exposed for ~100 h at −80 °C to Kodak XAR-5 X-ray film using a Du Pont Quanta III itensifying screen.

Quantitation of the data presented in Figures 2, 5, and 6 was performed by densitometric scanning of the autoradiographs using a Hoefer Model GS 300 densitometer. The area under the peaks was determined either by computer integration analysis or by the cut and weigh method which produced identical results. The calculated area (or weight) was normalized to the nucleotide level to correct for the uniform labeling of the DNA products. The comparison of DNA products of ~600 nt to those of 110 and 90 nt in Figure 2 in the Pol γ and Pol I analyses, respectively, was accomplished by quantitation of the relative abundance of products in the region between the markers of 543 and 652 nt (lane 15, average length ~600 nt) and of those at the 110- and 90-nt positions.

Fidelity of DNA Synthesis. DNA synthesis reactions were as described for the standard DNA polymerase assay with the following modifications. The DNA template-primer was singly primed φX174*am*3 DNA (235 pmol as nucleotide); the reaction volume was 0.1 μL; and [^{3}H]dTTP was present at 3000 cpm/pmol. In the unbiased nucleotide pool determinations, dATP, dCTP, dTTP, and dGTP were present at 40 μM each. In the biased nucleotide pool determinations, the biased deoxynucleoside triphosphates were present at 1000 and 10 μM each, and the remaining two at 40 μM each. Incubation was for 40 min with 0.72 unit of DNA polymerase γ, or 4.6 units of DNA polymerase α.

Spheroplasts of *E. coli* C600 (CR34) were prepared, and assay of progeny phage was performed as described by Kunkel and Loeb (1979) using *E. coli* CR and *E. coli* C as permissive and nonpermissive hosts, respectively.

RESULTS

Template-Primer Specificity and Rate of Nucleotide Polymerization. Preliminary template utilization studies indi-

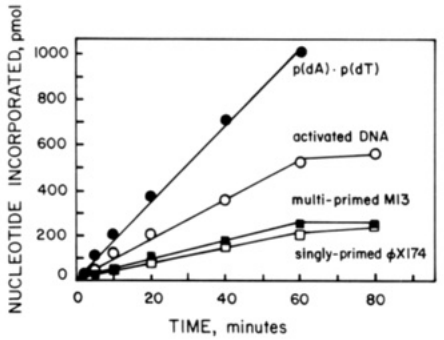

FIGURE 1: Time course of DNA synthesis in template-primer excess. DNA polymerase γ fraction VI (1.26 units) was assayed on poly-$(dA)_{700}$·$p(dT)_{10}$ (180 μM), DNase I activated calf thymus DNA (180 μM), multiprimed M13 DNA (48 μM), and singly primed ΦX174 DNA (48 μM).

Table I: Kinetic Parameters of Template-Primer Utilization by DNA Polymerase γ[a]

template-primer	KCl optimum concn (mM)	K_m (μM)	k_{cat} [nt incorporated s^{-1} (enzyme molecule)$^{-1}$]
poly(dA)·p(dT)	120	30.3 ± 6.8	6.75 ± 0.81
activated calf thymus DNA	200	12.5 ± 3.0	3.08 ± 0.32
multiprimed M13 DNA	110	0.57 ± 0.17	2.41 ± 0.21
singly primed ϕX174 DNA	120	1.06 ± 0.27	1.66 ± 0.81

[a] Pol γ (fraction VI) was assayed under standard conditions except that the template-primer concentration was varied, and the optimal KCl concentration for each template-primer was used. Incubation was for 5 min at 30 °C. Other experimental details were as described under Experimental Procedures.

cated that the *D. melanogaster* mitochondrial DNA polymerase catalyzes efficient DNA synthesis on single-stranded as compared to double-stranded DNA templates (Wernette & Kaguni, 1986). To further evaluate this finding, the reaction conditions with each of four template-primers were optimized with regard to KCl concentration, and the kinetic parameters of DNA polymerization were determined.

Two DNAs of high primer density (the ratio of primer termini to single-stranded DNA template) were used as substrates. The synthetic homopolymer poly$(dA)_{700}$·$p(dT)_{10}$ contains deoxyadenylate and thymidylate residues in a molar ratio of 20:1 to yield interprimer regions of single-stranded DNA of ~200 nucleotides (nt). DNase I activated calf thymus DNA contains nicks and short single-stranded DNA gaps, and although it is structurally uncharacterized, it is the substrate used for purification of the enzyme primarily because of its high primer density and natural DNA sequence. In addition, two DNAs of low primer density were examined. Bacteriophage ϕX174 DNA (5386 nt) was primed with a single synthetic 15-mer at a unique site. A bacteriophage M13 DNA (10907 nt) was primed with M13 DNA fragments at random sites to yield interprimer regions of ~1650 nt.

DNA synthesis proceeded linearly for 60 min on all template-primers and to the greatest extent on poly(dA)·oligo(dT) (Figure 1). Enzyme activity was ~2-fold less at all time points on DNase I activated DNA, but the affinity of the enzyme for the latter substrate is higher than for the former (Table I). As a consequence, Pol γ exhibits a relative substrate specificity (k_{cat}/K_m) of ~1.0 for these two template-primers of high primer density. DNA polymerase activity on activated DNA was approximately 2-fold greater than that observed on the two natural single-stranded DNAs for which the enzyme exhibited a significantly higher affinity (lower K_m values), resulting in an ~10-fold greater substrate specificity.

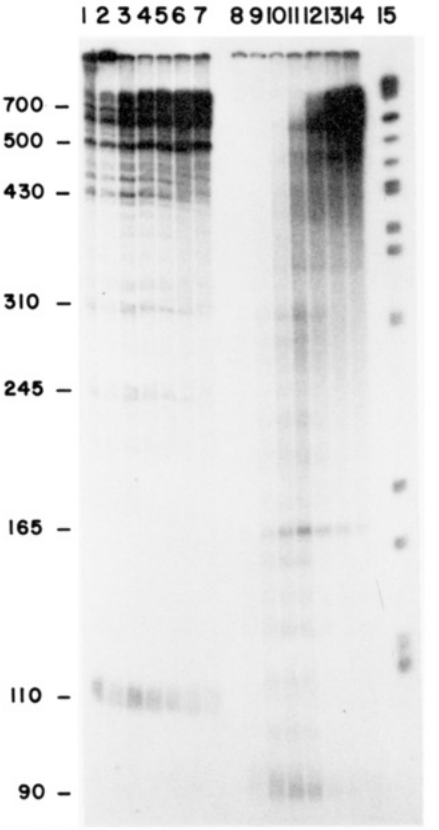

FIGURE 2: Analysis of the products of DNA synthesis on singly primed ΦX174 DNA. The DNA products obtained upon incubation of *Drosophila* DNA polymerase γ (0.63 unit) or *E. coli* DNA poymerase I (0.008 unit) with singly primed ΦX174 DNA (12 μM) were isolated, denatured, and electrophoresed in a denaturing 6% polyacrylamide gel as described under Experimental Procedures. The time intervals and extents of synthesis for the Pol γ reactions were as follows: 2 min, 0.3%; 5 min, 1%; 10 min, 2%; 20 min, 5%; 40 min, 7%; 60 min, 9%; and 80 min, 12% (lanes 1–7, respectively). Those for the Pol I reactions were as follows: 2 min, 0.3%; 5 min, 0.7%; 10 min, 1.4%; 20 min, 3%; 40 min, 6%; 60 min, 8%; and 80 min, 11% (lanes 8–14, respectively). Lane 15 contains *Hpa*II restriction fragments of M13G*ori*1 replicative form (RF) DNA (25): fragments larger than 900 nt were not fractionated under these conditions and migrated together in the first band; the sizes of the other fragments are 818, 652, 543, 472, 545, 381, 357, 272, 176, 156, 129, and 123 nt, respectively. The sizes of several major product classes are indicated on the left. Fragments smaller than ~75 nt migrated off the gel. No full-length products (5386 nucleotides) were observed.

The data suggest that the template features of nucleotide composition (or DNA sequence specificity), single-strandness and primer density influence catalysis by DNA polymerase γ. DNA polymerization on singly primed ϕX174 DNA by γ-polymerase is approximately 50% relative to that on activated calf thymus DNA. This is dramatically higher than that observed with the replicative α-polymerase which exhibits only a 3–8% relative efficiency (Kaguni et al., 1984; this study).

The products of DNA synthesis on the singly primed ϕX174 DNA were examined by polyacrylamide gel electrophoresis under denaturing conditions and compared to those obtained upon incubation with *E. coli* DNA polymerase I (Pol I) which exhibits similar template usage properties. Under conditions of substrate excess (~25 primer termini per enzyme molecule), the extents of DNA synthesis for the two enzymes were similar at each time point: after incubation for 80 min, 12.2% of the available template was copied by Pol γ and 10.8% by Pol I (Figure 2 legend). At the same time, a remarkable difference in the mechanism of DNA synthesis by the two enzymes was evident. DNA strands greater than 700 nucleotides in length

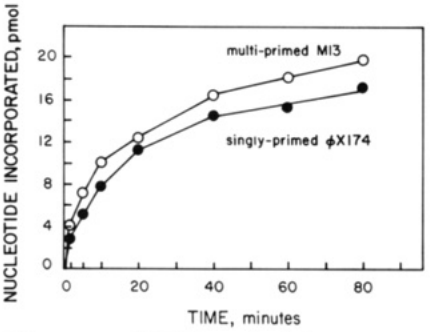

FIGURE 3: Time course of DNA synthesis in enzyme excess. DNA polymerase γ fraction VI (1.26 units) was assayed on singly primed ΦX174 DNA (0.4 μM) and multiprimed M13 DNA (0.4 μM).

were synthesized by Pol γ after only 2 min of incubation, when the extent of DNA synthesis was 0.3%, and the calculated average product size was 16 nt (Figure 2, lane 1). In contrast, nascent DNA strands larger than 600 nucleotides in length were not observed until 20 min of incubation with Pol I when the extent of DNA synthesis was nearly 10-fold greater, at 2.6% (Figure 2, lane 11). In the Pol γ reaction, the abundance of the smallest observed product (110 nt) relative to large products (~600 nt) was similar at the 2- and 20-min time points, ratios of 2.2 and 1.3, respectively. On the other hand, in the Pol I reaction, the relative abundance of large products increased dramatically in the same time interval; the ratio of the 90-nt product to large products (~600 nt) was >78 at 2 min and 3.7 at 20 min. The data suggest that the *Drosophila* mitochondrial DNA polymerase preferentially utilizes previously extended 3′-OH termini, perhaps by a mechanism involving incomplete dissociation after a polymerization cycle. Further, the presence of discrete product classes suggests that DNA polymerase γ is sensitive to template secondary structure, a feature characteristic of DNA polymerase α (Pol α) from *D. melanogaster* (Kaguni & Clayton, 1982) and from vertebrate sources (Weaver & DePamphilis, 1982).

Pol γ exhibited a lower turnover number on the multiprimed and singly primed single-stranded DNA templates as compared to the DNAs of high primer density under conditions of substrate excess (Table I). We examined the possibility that impediments such as template secondary structure in the natural single-stranded DNAs might influence template-primer utilization under conditions of substrate limitation. The rate and extent of DNA synthesis were compared on the M13 DNA containing multiple primers at random sites and on the φX174 DNA containing a single primer at a unique site. The data in Figure 3 indicate that the rate of DNA synthesis on both template-primers is nearly equal as was observed under conditions of substrate excess. Further, the extents of DNA synthesis of 85–100% after 80 min of incubation suggest that both template-primers can be copied completely by Pol γ. Previous work with the vaccinia virus (Challberg & Englund, 1979) and bacteriophage T4 (Roth et al., 1982; Huang & Hearst, 1980) DNA polymerases has indicated that several regions of the bacteriophage φX174 and fd (homologous to M13) DNA genomes which may contain stable dyads cause dramatic decreases in the overall rate of DNA synthesis. Remarkably, catalysis by Pol γ on φX174 DNA is unaffected relative to polymerization on the multiprimed M13 DNA which has a 3-fold higher primer density and, because the primers are located at random sites, is a substrate in which the potential inhibitory effects of stable dyads are minimized (Fay et al., 1981). Because both template-primers contain extensive single-stranded DNA stretches and because Pol γ has a high affinity for single-stranded DNAs of natural sequence (Table I), nonproductive binding to regions without primer termini might occupy most of the enzyme molecules in either case. Thus, perhaps neither primer binding nor pausing during elongation is the rate-limiting process, but rather dissociation of the enzyme from the single-stranded DNA regions.

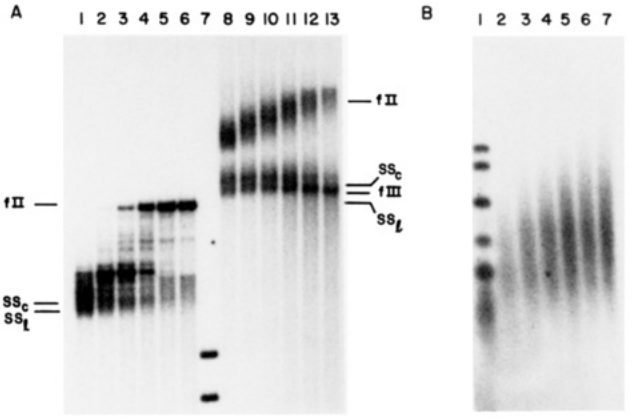

FIGURE 4: Analysis of the products of DNA synthesis under conditions of template-primer limitation. (A) The DNA products obtained upon incubation of Pol γ fraction VI (1.26 units) with singly primed ΦX174 DNA (0.4 μM) and multiprimed M13 DNA (0.4 μM) were isolated and electrophoresed in a 0.8% agarose gel. The time intervals and extents of DNA synthesis on the singly primed ΦX174 DNA were the following: 2 min, 14%; 5 min, 26%; 10 min, 39%; 20 min, 56%; 40 min, 73%; and 80 min, 86% (lanes 1–6, respectively). Those on the multiprimed M13 DNA were as follows: 2 min, 20%; 5 min, 36%; 10 min, 50%; 2 min, 62%; 40 min, 83%; and 80 min, 99% (lanes 8–13, respectively). Lane 7 contains *Hpa*II restriction fragments of M13G*ori*1 RF DNA whose sizes are 1925 and 1207 nt. SS_c is single-stranded circular DNA; SS_l is single-stranded linear DNA; fII and fIII are duplex circular and linear DNA, respectively. (B) The DNA products obtained in the reaction on the multiprimed M13 DNA were denatured and electrophoresed in an 0.8% alkaline agarose gel (lanes 2–7). Lane 1 contains restriction fragments of M13 RF DNA (*Cla*I, 3512 and 2895 nt) and M13G*ori*1 RF DNA (*Hpa*II, 1925, 1207, and 829/819 nt; the fragments smaller than 800 nucleotides were not resolved).

The products of DNA synthesis on the two template-primers were analyzed by gel electrophoresis under native and denaturing conditions. Analysis of the reaction products on singly primed φX174 DNA under nondenaturing conditions revealed a complex array of products, indicating a variety of nascent DNA strand sizes (Figure 4A). In addition, accumulation of discrete products after 2, 5, and 10 min of incubation (lanes 1–3) indicated the formation of either complex structures or templates containing product strands of discrete lengths. Form II molecules representing complete replication were observed after only 5 min of incubation (lane 2) where they represented ~1% of the total products. After 40 min of incubation, the completed form II molecules predominated (lane 5), consistent with the extent of replication of 73% as determined by acid precipitation.

Notwithstanding the formation of complex structures and/or discrete products on singly primed φX174 DNA resulting from the presence of stable dyads in the template, the rate of DNA synthesis was nearly identical with that observed on the multiprimed M13 DNA (Figure 3). However, in the analysis of the products of DNA synthesis on the M13 template under both native (Figure 4A, lanes 8–13) and denaturing (Figure 4B, lanes 2–7) conditions, there was no indication of the formation of discrete products, but rather a continuous array of partially replicated molecules (Figure 4A) and nascent DNA strand lengths (Figure 4B) as expected for a template primed at random positions. In this case, the input DNA contained circular and linear molecules in approximately equal

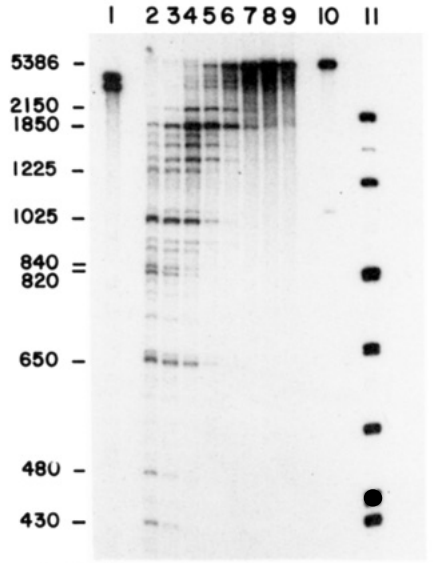

FIGURE 5: Analysis of the products of DNA synthesis in the presence of limiting amounts of singly primed ΦX174 DNA. The DNA products obtained upon incubation of Pol γ fraction (1.26 units) with singly primed ΦX174 DNA (0.4 μM) were isolated, denatured, and electrophoresed in a denaturing 4% polyacrylamide gel as described under Experimental Procedures. The time intervals and extents of DNA synthesis were as follows: 1 min, 7%; 2 min, 14%; 5 min, 26%; 10 min, 39%; 20 min, 56%; 40 min, 73%; 60 min, 77%; 80 min, 86% (lanes 2–9, respectively). The restriction fragment markers were the following: lane 1, M13 RF DNA (*Cla*I, 3512 and 2895 nt); lane 10, ΦX174 RF DNA (*Xho*I, 5386 nt); and lane 11, M13G*ori*1 RF DNA (*Hpa*II, 1925, 1207, 829/818, 652, 543, 472, and 454 nt). The lengths in nucleotides of major product classes are indicated on the left. No products smaller than ~400 nucleotides were detected. To correlate DNA product classes with helical regions in the DNA template, the positions of predicted stable dyads in ΦX174 DNA were determined by using the Beckman Microgenie program in an IBM XT computer.

amounts, such that the expected products upon native gel electrophoresis are form II and form III DNAs, respectively, as shown (Figure 4A).

Because the M13 template is multiprimed, genome-length product strands are not expected upon denaturation. The data in Figure 4B show that the average size of the nascent DNA strands increased from ~500 nt after 2 min of incubation (lane 1) to the calculated maximum average size of ~1800 nt at 80 min (lane 7).

In comparison, we examined the nascent DNA strands synthesized by Pol γ on the singly primed φX174 DNA by polyacrylamide gel electrophoresis under denaturing conditions (Figure 5). As predicted from the native gel analysis, products of discrete lengths were obtained. Notably, of the discrete products observed, several could be directly correlated with the positions of the predicted stable dyads which presented major impediments to DNA synthesis by the vaccinia virus (Challberg & Englund, 1979) and bacteriophage T4 (Roth et al., 1982) DNA polymerases: the 3′ ends of product strands of 2175, 3130, 3775, and 1800 nt in the Pol γ analysis map to barrier A (nucleotide positions 3974–3956 in the φX174 DNA sequence), barrier B (3020–2997), barrier C (2373–2308), and barrier D (4357–4329), respectively. In addition, 14 of 20 of the discrete products could be correlated with computer-predicted helical regions in φX174 DNA. The remainder could represent sites where primary sequence determinants are responsible for template-directed pausing. Such sites were found to inhibit α-polymerases from *Drosophila* (Kaguni & Clayton, 1982) and from vertebrate sources (Weaver & DePamphilis, 1982). These studies also demonstrated that *E. coli* DNA polymerase I is less sensitive to template secondary structure and relatively insensitive to primary sequence determinants. Taken together, the data in Figures 2 and 5 suggest that γ-polymerase is sensitive to both primary sequence and secondary structure in the DNA template, accounting for the dramatic differences in the pausing patterns observed for Pol γ as compared to Pol I.

Table II: Kinetic Assessment of the Processivity of Nucleotide Polymerization by DNA Polymerase γ

enzyme	no. of determinations[a]	relative cycling time	processivity
Pol γ	6	4.4 ± 1.6	29.1 ± 6.2
Pol I	2	11.9 ± 2.1	13.7 ± 0.5

[a] Reactions were performed in triplicate as described under Experimental Procedures.

In this analysis under conditions of enzyme excess, full-length species appeared after only 2 min of incubation, representing 0.2% of the total product strands (lane 2). At this time, 14% of the input DNA had been replicated, and the calculated average product size was only 750 nt. Although the most abundant product was 1025 nucleotides, representing 19% of the total products, discrete products ranging from 430 to 5386 nt were observed. In the interval between 2 and 20 min of incubation (lanes 3–6), the increase in abundance of an 1850-nt intermediate was 7-fold, whereas that of the 5386-nt full-length product increased 125-fold. After 40 min of incubation when the extent of replication was 73%, the predominant product was full length (5386 nt).

As with the analysis in substrate excess, the data suggest that Pol γ preferentially utilizes previously extended primer termini. Further, the enzyme has the capacity to replicate completely and efficiently a single-stranded DNA which has substantial secondary structure.

Processivity of Nucleotide Polymerization. The high rate of DNA synthesis on singly primed φX174 DNA relative to that on activated calf thymus DNA suggested that the mitochondrial DNA polymerase might replicate long stretches of single-stranded DNA without dissociation. However, the presence of discrete short products in the gel analyses of the reactions carried out both in substrate excess and in limitation indicates that Pol γ is not a highly processive enzyme. To examine this issue, the processivity of Pol γ in replicating single-stranded DNA templates was measured on multiprimed M13 DNA by the kinetic method of Bambara et al. (1978), and on singly primed φX174 DNA by direct analysis of the reaction products in a denaturing polyacrylamide gel.

Processivity, defined as the number of nucleotides polymerized in a single binding event, was measured kinetically on the multiprimed M13 DNA in large substrate excess to avoid any potential negative contribution of template secondary structure (Fay et al., 1981). Poly(dA)·oligo(dT) was used as the competitive inhibitor (K_i = 10 μM). The data in Table II indicate that DNA polymerase γ is a moderately processive enzyme, polymerizing on average 2-fold more nucleotides than *E. coli* DNA polymerase I. The relative cycling time is a measure of the time between enzyme binding cycles in the absence and in the presence of DNA polymerization, and indicates the static versus kinetic affinity of the enzyme for the template-primer (Bambara et al., 1978). The value for Pol γ is ~3-fold less than for Pol I, indicating that in the absence of DNA synthesis Pol γ recycles at a greater rate than Pol I. Mechanistically, this would allow the mitochondrial enzyme to complete more polymerization cycles per unit time on a per molecule basis than Pol I.

While the kinetic method yields a value representing the average number of nucleotides added per binding event, the

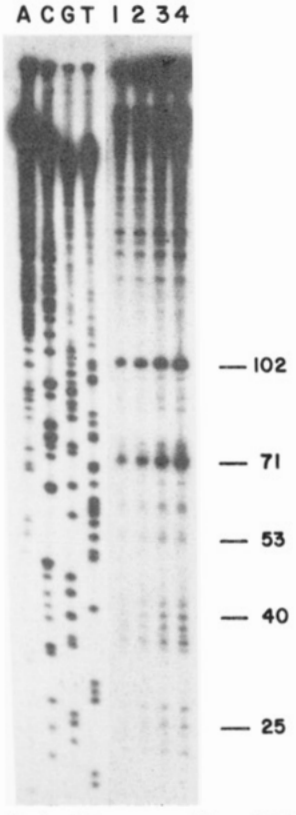

FIGURE 6: Gel analysis of the processivity of DNA polymerase γ. Pol γ fraction VI (0.03 unit) was assayed on the singly primed ΦX174 DNA (50 μM). The reaction products after 5, 10, 20, and 40 min of incubation (lanes 1–4, respectively) were isolated, denatured, and electrophoresed in an 8% denaturing polyacryamide gel as described under Experimental Procedures. The extents of DNA synthesis were the following: 5 min, 0.003%; 10 min, 0.01%; 20 min, 0.02%; and 40 min, 0.04%. The product sizes indicated were determined by comparison with a dideoxy sequencing ladder generated by incubation of the singly primed ΦX174 DNA with Pol I Klenow fragment (26, lanes A, C, G, and T).

direct measurement of processivity by denaturing polyacrylamide gel electrophoresis demonstrates the shortest DNA product actually synthesized. Gel electrophoresis of the Pol γ reaction products on singly primed φX174 DNA yielded a minimum processivity value of 25–40 nt (Figure 6), in agreement with the kinetic measurement. However, the appearance of a complex mixture of products >200 nucleotides in length at the very low extents of DNA synthesis, indicated in the legend to Figure 6, suggests that a significant number of enzyme–substrate interactions actually result in highly processive synthesis catalyzed by Pol γ. Because of the lack of resolution of the large products in this analysis, we cannot make a definitive statement regarding their abundance. In subsequent experiments in which all of the products have been accurately quantified, we find that products >200 nucleotides in length comprise ≤20% of the total DNA product strands and that the average product length determined by gel analysis is ~150 nt (data not shown). Neither the fraction of longer products nor the average product length changes over an 8-fold range of primer concentration. In contrast to the result with Pol γ, the processivity value obtained in the gel analysis for Pol I is 15–30 nt; a significant fraction of products longer than 100 nucleotides is not observed (data not shown).

Fidelity of in Vitro DNA Synthesis. The fidelity of DNA synthesis is a major factor in determining the overall mutation rate in vivo. Because the accumulation of nucleotide substitutions in animal mitochondrial DNAs is 5–10-fold greater than in nuclear DNA genomes (Dawid, 1972; Brown et al., 1979), an evaluation of the accuracy of nucleotide polymerization by DNA polymerase γ is of particular significance.

Table III: Reversion Frequency of φX174*am*3 DNA Synthesized in Vitro by DNA Polymerase γ

reaction condition[a]	nucleotides polymerized[b]	phage titer[c] *am*3 (×10⁻¹)	phage titer[c] wild type (×10⁻²)	reversion frequency[d]
unbiased pool	1126	13.3	0.24	1.8×10^{-6}
A>T	1178	16.9	23.7	1.4×10^{-4} (78)[e]
G>A	1398	21.4	0.45	2.1×10^{-6} (1.2)
G>C	1427	20.5	0.39	1.9×10^{-6} (1.1)
C>T	1183	23.5	1.9	8.1×10^{-6} (4.5)

[a] The experiments were performed as described under Experimental Procedures. In the unbiased pool determination, the concentration of all four dNTPs was 40 μM. In the biased pool determinations, the concentrations of the indicated nucleotides were 1000 and 10 μM, respectively. [b] The average number of nucleotides added per primer terminus. [c] The phage titers listed are averages derived from 10 experiments. Variation in the data between experiments was ≤2-fold. [d] The reversion frequency of the uncopied control was 1.1×10^{-6}. [e] Numbers in parentheses represent biased/unbiased values.

To examine the fidelity of DNA synthesis by Pol γ, we have utilized the method of Weymouth and Loeb (1978) to measure the reversion of the φX174*am*3 mutation to wild type and pseudo wild type as a result of nucleotide misincorporation during DNA synthesis in vitro. Reactions were performed with both unbiased and biased deoxynucleotide pools. Parallel experiments were carried out with *D. melanogaster* DNA polymerase α. As indicated in Table III, the average number of nucleotides polymerized by Pol γ on each primer terminus was ~7-fold greater than the distance from the primer terminus to the *am*3 mutation which lies 177 nucleotides downstream. The extent of DNA synthesis was unaffected by the various dNTP pool bias conditions.

Transfection with DNA replicated by Pol γ under conditions where all four dNTPs were present at equal concentrations (unbiased pool) yielded revertants at a level of $\simeq 2 \times 10^{-6}$ (Table III). These values were consistently 2-fold higher than those determined for the uncopied DNA control. Notably, the reversion frequency of φX174 DNA synthesized by DNA polymerase γ was nearly identical with that obtained in parallel with DNA polymerase α (data not shown)—the control experiments with Pol α were in complete agreement with our earlier determinations (Kaguni et al., 1984). Likewise, with a pool bias where dATP was in 100-fold excess over dTTP during in vitro DNA synthesis, the number of revertants was increased dramatically and by a similar value when polymerization was catalyzed by *Drosophila* Pol γ or Pol α. In pool bias experiments where the concentrations of other pairs of nucleotides were altered, no large increase in the number of revertants was observed with either DNA polymerase. Because it is possible that some dUTP is present during in vitro DNA synthesis as a result of spontaneous deamination of dCTP, the C>T pool bias may result in an underestimate of the reversion frequency for both enzymes (Baas et al., 1980). Nevertheless, in the φX174 *am*3 reversion assay, the accuracy of polymerization on single-stranded DNA in vitro by the near-homogeneous mitochondrial DNA polymerase is nearly identical with that of the replicative DNA polymerase from the same source—*Drosophila melanogaster* embryos. At the same time, the *Drosophila* DNA polymerase α is one of the most accurate α-polymerases which has been examined (Kaguni et al., 1984; Reyland & Loeb, 1987). Further, the fidelity of these eucaryotic DNA polymerases is remarkably similar to that of *E. coli* DNA polymerase III holoenzyme (Fersht, 1979; Kaguni et al., 1984), even though the subunit structure, reaction

requirements, kinetics, and processivities of the three enzymes differ greatly.

Discussion

Comparative studies have demonstrated that vertebrate mitochondrial DNA polymerases from a variety of tissues and cell types exhibit differential template-primer usage in vitro. Partially purified γ-polymerases from mouse myeloma (Matsukage et al., 1975), human cells (Knopf et al., 1976; Bolden et al., 1977; Robert-Guroff et al., 1977), rat brain (Hübscher et al., 1977), rat liver (Bolden et al., 1977; Tanaka & Koike, 1977), and chick embryos (Bertazzoni et al., 1977) catalyze DNA synthesis on template-primers of high primer density with the general order of substrate preference being poly(rA)·oligo(dT) > poly(dA)·oligo(dT) > activated DNA > denatured DNA (lower primer density). Further, enzyme preparations from human placenta (Kollek & Goulian, 1981) and calf liver (Robertson et al., 1983) were shown to replicate single-stranded viral DNAs in vitro. Our previous work with the near-homogeneous DNA polymerase γ from *Drosophila melanogaster* embryos demonstrated that this invertebrate mitochondrial enzyme exhibits similar properties with regard to template-primer utilization (Wernette & Kaguni, 1986). In this report, we show that the *Drosophila* enzyme exhibits K_m values similar to highly purified mouse myeloma Pol γ (Matsukage et al., 1983) for two template-primers: the K_m value on activated calf thymus DNA was 12.5 μM as compared to 40.6 μM (as nt), and that on poly(dA)·oligo(dT) was 0.15 μM as compared to 0.07 μM (as primer termini), respectively. We have further investigated DNA polymerase/template-primer interactions in vitro to determine what factors may contribute to efficient enzyme function in vivo. In an examination of DNA synthesis on several template-primers, both natural and synthetic, we have identified template features which may affect catalysis by Pol γ: nucleotide composition, primer density, and single-strandedness.

Drosophila DNA polymerase γ replicates both predominantly single-stranded and double-stranded DNAs. The rate of DNA synthesis is highest on the synthetic homopolymer poly(dA)·p(dT)$_{10}$. Notably, *Drosophila* mtDNA genomes are 74–80% A + T (Fauron & Wolstenholme, 1976). In this regard, Wolstenholme and Clary (1985) have proposed that mtDNAs may have become A + T rich during long-term evolution as a result of a functional requirement (or preference) of mtDNA and/or RNA polymerase for A + T rich DNA. Our data indicate that the *D. melanogaster* mtDNA polymerase is a versatile enzyme with regard to template-primer usage. In fact, the ability of Pol γ to copy single-stranded DNA with a G + C content of 45% (ϕX174 DNA, 7) is 5–10-fold greater than that of Pol α (Kaguni et al., 1984; this study) relative to the rate at which these enzymes replicate predominantly double-stranded calf thymus DNA.

In exhibiting a high relative efficiency in the replication of single-stranded DNA, Pol γ suits the unique requirements for DNA polymerization in mitochondria. Because of the highly asymmetric mode of mtDNA replication in vivo, leading DNA strand synthesis occurs on a duplex template while lagging DNA strand synthesis, which occurs on the displaced parental DNA strand, involves replication of a predominantly (or entirely) single-stranded DNA template. We have shown that γ-polymerase is sensitive to template secondary structure, which might imply a requirement for other protein factors to facilitate lagging DNA strand synthesis in vivo. At the same time, the enzyme's ability to replicate completely the ϕX174 DNA genome under conditions of moderate enzyme excess indicates that a high local concentration of Pol γ would in itself be sufficient to replicate completely mtDNA. Furthermore, the formation of stable secondary structures in the template strand for lagging DNA strand synthesis in vivo is perhaps minimal, as a result of the high A + T content of the mitochondrial genome.

Pol γ as purified from *Drosophila* embryos does not copy very long stretches of natural single-stranded DNA without the formation of intermediates: kinetic and direct analysis of enzyme processivity indicates that Pol γ incorporates ~30 nucleotides before pausing and/or dissociating from the template. As determined by the kinetic method, the average processivity of Pol γ is similar to that of the replicative Pol α and *E. coli* Pol I which incorporate 10–20 nucleotides per binding event (Villani et al., 1981; Bambara et al., 1978). On the other hand, although it is not a highly processive enzyme as is *E. coli* polymerase III holoenzyme, which can polymerize several thousand nucleotides on natural DNA without dissociation (Fay et al., 1981; Burgers & Kornberg, 1982), the direct product analysis demonstrates that Pol γ has the capacity for preferential extension of previously utilized primer termini. This is evidenced both by the appearance of a substantial fraction of replication products which are much longer than would be expected if all primer ends were extended equally and by the abundance of products which are multiples of the minimal unit length product under conditions of large template-primer excess. Taken together, the results suggest a quasi-processive DNA synthetic mechanism for *Drosophila* γ-polymerase.

Several possible mechanisms for quasi-processive synthesis are plausible. First, the near-homogenous enzyme may consist of two distinct classes of enzyme, processive and less processive. We have proposed that the *Drosophila* γ-polymerase is a heterodimer which comprises a 125 000-dalton catalytic subunit and a 35 000-dalton subunit of unknown function (Wernette & Kaguni, 1986). The processivity of the heterodimer may be greater than that of the catalytic subunit. Further, it is possible that the catalytic subunit may exist in several subunit stoichiometries resulting in, for example, a processive holoenzyme, a quasi-processive subassembly, and a core enzyme of low processivity. This situation has been observed in analyses of *E. coli* DNA polymerase III (Fay et al., 1981). Alternatively, two enzyme classes may be generated by a specific chemical modification or from the presence of a limiting "template association" factor in the enzyme preparation which interacts with the enzyme during polymerization. Second, Pol γ may be capable of kinetic pausing without dissociation or may release the primer terminus transiently but remain bound to the template strand. Third, two classes of template molecules may be distinguishable by Pol γ—those with and without structural constraints to replication. In this regard, a highly purified mitochondrial DNA polymerase from mouse myeloma (Matsukage et al., 1975) and the near-homogeneous enzyme from chick embryos (Yamaguchi et al., 1980a,b) were shown to be highly processive on the ribohomopolymer poly(rA)·oligo(dT). Even though this synthetic polyribonucleotide bears no resemblance to the in vivo template, it may allow the evaluation of the intrinsic processivity of the enzyme in the absence of DNA context effects. Experiments to test each of these possibilities are under way.

In any case, the current studies of Pol γ/template-primer interactions indicate that the catalytic properties of the near-homogeneous mitochondrial enzyme exhibited in vitro correlate well with in vivo requirements for mtDNA replication.

The high rate of evolution of animal mtDNAs and, in particular, the high frequency of A ↔ T transversions in *Drosophila* mtDNA have led to the suggestion that there is continuous selection for A + T nucleotides at all sites in mtDNA where it is compatible with function (Wolstenholme & Clary, 1985). This might occur either by an increased mutation rate resulting from infidelity during replication or by an increased frequency of fixation of mutations, or both. We have demonstrated that the overall fidelity of replication catalyzed by γ-polymerase is nearly identical with that of the replicative α-polymerase from *Drosophila* (Kaguni et al., 1984). Specifically, *Drosophila* Pol γ does not misincorporate to yield A → T transversions at a higher rate than Pol α. However, we have examined base substitution only at a single site—the *am*3 locus in φX174 DNA, and it is possible that the *Drosophila* Pol γ would be less accurate in another assay system. For example, *E. coli* DNA polymerase III holoenzyme exhibits an accuracy similar to that of the *Drosophila* enzymes in unbiased (Fersht, 1979; Kaguni et al., 1984) and A>T (Kaguni et al., 1984) biased pools in the *am3* reversion assay, while its accuracy is severalfold higher in an A>T-biased pool in the φX174 *am16* reversion assay (Fersht & Knill-Jones, 1983).

The mitochondrial DNA polymerase from *Drosophila* embryos exhibits a 10-fold higher fidelity than the HeLa cell γ-polymerase as measured in the same assay system (Kunkel & Loeb, 1981). Likewise, direct measurement of nucleotide misincorporation into several synthetic template-primers under a variety of conditions indicated that partially purified γ-polymerases from human placenta (Krauss & Linn, 1980) and human fibroblasts and HeLa cells (Krauss & Linn, 1982) were relatively inaccurate and exhibited error rates 3–20-fold higher than the homologous α-polymerase. In contrast, in a forward mutational assay capable of detecting a spectrum of base substitution and frameshift mutations, Kunkel has demonstrated that the near-homogenous chick embryo and a partially purified calf liver γ-polymerase are highly accurate (Kunkel, 1985; Kunkel & Alexander, 1986). Clearly, the issue of the accuracy of the mitochondrial DNA polymerase and its role in the evolution of animal mtDNA warrant further study.

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Registry No. DNA polymerase, 9012-90-2; poly(dA)·p(dT), 24939-09-1.

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Sequence of Cloned Enzyme II$^{N\text{-acetylglucosamine}}$ of the Phosphoenolpyruvate:*N*-Acetylglucosamine Phosphotransferase System of *Escherichia coli*†

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ABSTRACT: In *Escherichia coli*, *N*-acetylglucosamine (nag) metabolism is joined to glycolysis via three specific enzymes that are the products of the *nag* operon. The three genes of the operon, *nagA*, *nagB*, and *nagE*, were found to be carried by a colicin plasmid, pLC5-21, from a genomic library of *E. coli* [Clarke, L., & Carbon, J. (1976) *Cell (Cambridge, Mass.) 9*, 91-99]. The *nagE* gene that codes for enzyme II$^{N\text{-acetylglucosamine}}$ of the phosphoenolpyruvate:sugar phosphotranferase system (PTS) was sequenced. The *nagE* sequence is preceded by a catabolite gene activator protein binding site and ends in a putative rho-independent termination site. The amino acid sequence determined from this DNA sequence shows 44% homology to enzymes II^{glucose} and III^{glucose} of the PTS. Enzyme II$^{N\text{-acetylglucosamine}}$, which has 648 amino acids and a molecular weight of 68 356, contains a histidine at residue 569 which is homologous to the active site of III^{glc}. Sequence homologies with enzymes II^{glucose}, II$^{\beta\text{-glucoside}}$, and II^{sucrose} indicate that residues His-190, His-213, and His-295 of enzyme II^{nag} are also conserved and that His-190 is probably the second active site histidine. Other sequence homologies among these enzymes II suggest that they contain several sequence transpositions. Preliminary models of the enzymes II are proposed.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] carries out the concomitant translocation and phosphorylation of various sugars in a variety of bacterial species. The PTS was first described in *Escherichia coli* by Kundig et al. (1964), and considerable information about the components of the PTS and their interactions has since been gathered. The PTS in *E. coli* is important as the predominant way by which many hexoses enter the cell as carbon sources for metabolism; as a system involved in the regulation of adenylate cyclase, various permeases, and other enzymes; and as a system involved in chemotaxis [see review by Postma and Lengeler (1985)].

The PTS has both soluble and membrane-bound components. The integral membrane component, enzyme II^{sugar}, is often an inducible sugar-specific enzyme that carries out both the translocation and phosphorylation of the sugar. There are two classes of enzyme II^{sugar}: those that interact directly with the common phosphocarrier protein, HPr, and those that interact with specific phosphocarrier proteins, III^{sugar}. Citing several lines of evidence, Saier et al. (1985) proposed that these two clases are functionally and evolutionarily related. The enzymes II of the former class have molecular weights close to 65 000, while the latter class has a similar molecular weight when the enzyme II^{sugar} and III^{sugar} pair are considered together. It was proposed that the former class is a fused protein with dual function of the latter class, a single gene split into two. Protein phosphorylation (Waygood et al., 1984; Peri et al., 1984) and phosphoryl-transfer experiments (Begley et al., 1982) indicated that all the PTS components, and in particular the enzymes II, could be phosphorylated. This led to the proposal that an enzyme II that interacts directly with P-HPr has a triple-displacement mechanism for phosphoryl transfer and thus should contain two phosphorylation sites (Saier et al. 1985).

Enzyme II$^{N\text{-acetylglucosamine}}$ has been identified as an integral membrane enzyme (M_r 65 000), which interacted directly with P-HPr (Waygood et al, 1984). Enzyme II^{mannitol} interacts directly with P-HPr and forms a phosphoprotein similar to enzyme II^{nag} (Waygood et al, 1984). Enzyme II^{nag} therefore

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[1] Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase; HPr, histidine-containing phosphocarrier protein of the PTS; NAG or nag, *N*-acetylglucosamine; III^{sugar}, a sugar-specific phosphocarrier protein (also called enzyme III or factor III). Enzymes II^{sugar} have sugars abbreviated: glc, glucose; man, mannose; mtl, mannitol; gut, glucitol; scr, sucrose; bgl, β-glucoside.

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