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DNA polymerase gamma has been purified over 67,500-fold from human trophoblast tissue. Purification was achieved through successive chromatographies on phosphocellulose, DEAE-cellulose, DNA-cellulose, and high pressure gel exclusion through Fractogel TSK HW55. This preparation yielded a specific activity of 81 units/mg of protein using the preferred template-primer, poly (A)-oligo $(dT)_{12-18}$. Analysis by SDS-polyacrylamide gel electrophoresis revealed a single polypeptide band with molecular weight 94,000 daltons, confirming the molecular weight estimate determined by gel exclusion. Sedimentation analysis in high ionic strength revealed an enzyme with molecular weight of 160-180,000 daltons, suggesting the enzyme exist as a dimer of the 94,000 dalton polypeptide. The enzyme exhibits optimal activity with poly (A)-oligo $(dT)_{12-18}$ in the presence of 10 mM KPO_4, 90 mM KCl and 0.5 mM Mn^{2+}. This demonstrates the first structural analysis of a DNA polymerase gamma from a human origin.

Purification and Characterization of a DNA Polymerase Gamma from Human Trophoblast Tissue

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INTRODUCTION

Eukaryotic cells contain at least three distinct classes of DNA polymerases which have been named α, β and γ (1.2). The three classes can be differentiated from one another by their chromatographic properties, response to various inhibitors, primer-template preferences, and molecular weights. The DNA polymerases α and β are found in the nuclei, and are thought to participate in the replication and repair of nuclear DNA. DNA polymerase γ is seen predominantly in the mitochondria, where it is assumed to function in the replication of mitochondrial DNA through a displacement-type synthesis (3-7). DNA polymerase γ is also found in the nuclei where its role has not yet been determined (8-13). It has been postulated that DNA polymerase γ may be responsible for the strand displacement synthesis seen in the nuclei of developing oocytes from Xenopus and Dytiscid beetles (6). However, direct evidence linking DNA polymerase γ with this nuclear DNA synthesis has not been established.

DNA polymerase γ has been prepared to various degrees of purity by several investigators. The DNA polymerase γ from chick embryo tissue has been purified over 1,500,000 fold to a specific activity of 570,000 units/mg of protein (14). The active form of this enzyme has a molecular weight of 180,000 daltons and is composed of 4

polypeptides of 47,000 daltons. Matsukage et al. (15) demonstrated that mouse myeloma cells have a DNA polymerase γ in which the active polypeptide has an apparent molecular weight of 47,000 daltons. Moreover, the mouse enzyme was structurally related to the chick embryo enzyme by tryptic peptide mapping. Human DNA polymerase γ has been isolated from both HeLa cells and lymphoblast cells. Knopf et al. (16) was able to purify the DNA polymerase γ of HeLa cells 60,000 fold to a specific activity of 25,000 units/mg of protein. However, the polypeptide composition of this enzyme preparation was not established although the enzyme had an apparent molecular weight of 160,000 by zone sedimentation. DNA polymerase γ from the human lymphoblast cell line, NC-37, has an apparent molecular weight of approximately 120,000 daltons as determined by sedimentation in glycerol (17). Thus, the polypeptide composition for the human DNA polymerase γ was not established in these previous reports.

Our interest in the human enzymes stems from our observations that the human placenta contains a reverse transcriptase activity as well as DNA polymerases α, β and γ (18,19). In order to distinguish these DNA polymerases, the purification of these enzymes was necessary. Moreover, any comparison of the enzymological and physical properties of these enzymes requires an analysis of the polypeptide composition. For this reason, we have purified our DNA polymerase γ from human placenta.

The present paper describes the purification and structural analysis of DNA polymerase γ from human placental trophoblast

tissue. The enzyme has been purified over 67,500-fold. The molecular weight of one active form of the enzyme is 180,000 as determined by zone sedimentation under high ionic strength conditions. SDS-polyacrylamide gel electrophoresis of the highly purified preparation indicates that human placental DNA polymerase γ is composed of at least two identical polypeptides of M_r = 94,000.

REVIEW OF LITERATURE

In 1953, Watson and Crick formulated a model for the structure of DNA which was based on two complementary strands of DNA in a double-helix formation. Later that year they proposed a mechanism for the replication of that DNA structure. They postulated that the DNA precursors could orient themselves along the template in a complementary fashion and then be zipped together to form two new molecules. Although this principle of complementarity was attractive, their model did not gain wide acceptance by biochemists of that day because no enzyme capable of catalyzing the process had been described. It was in 1958 that the first report of a DNA polymerase was made for E. coli (20). This initial report led the way for further investigation into the mechanisms of DNA replication in prokaryotes and eukaryotes.

The early work on eukaryotic DNA polymerases resulted in a multitude of DNA polymerases randomly named according to tissue and cellular location. Then, in 1975 a group of forty-eight scientists assembled the currently available information on these DNA polymerases and developed a system to identify the classes of eukaryotic DNA polymerases. The Greek letters α, β, and γ were employed to name the three DNA polymerases of the cells (1). The enzyme involved in DNA replication, DNA polymerase α, was first identified in 1960. The enzymes, DNA polymerase β and γ, were described later. A mitochondrial DNA polymerase was also identified; however, it was later established that DNA polymerase γ and mitochondrial DNA

polymerase were the same (3,13,21). These three classes of eukaryotic DNA polymerases will be discussed in further detail.

DNA Polymerase α

DNA polymerase α was first characterized and partially purified from calf thymus in 1960 (22). Since then it has been found ubiquitously among eukaryotes, comprising 80-90% of all polymerase activity in actively dividing cells (23-26). Because of its prominent activity, investigators have since referred to DNA polymerase α as the enzyme responsible for DNA replication in the nucleus.

The enzyme's association with DNA replication was first noted by Chang and Bollum (27) and Baril et al. (28) who shared a predominant rise in the level of this enzyme when cells were stimulated to divide. Direct evidence for the involvement of DNA polymerase α became available when the antibiotic aphidocolin was found to be a specific inhibitor of DNA polymerase α (29). Using aphidocolin, Krokan et al. demonstrated a potent inhibition of DNA synthesis in isolated HeLa cell nuclei (30).

The involvement of the DNA polymerase α in DNA replication prompted many to assume its intracellular location to be the nucleus. However, early attempts to verify this intracellular location revealed 80% of the activity to be confined to the cytoplasm. With the development of extraction procedures capable of maintaining the integrity of the nucleus, investigators have now reported the localization of the polymerase in the nucleus (31-34). A more recent

technique involving monoclonal antibodies directed against DNA polymerase α (35) also demonstrated that DNA polymerase α is localized in the nucleus (36).

The molecular weight of DNA polymerase α has been reported to range from 100,000 daltons to 1,000,000 daltons depending on the ionic strength of the conditions used to estimate its mass (37-39). Purer preparations from human KB cells show that the enzyme is composed of a catalytic subunit of 140,000 daltons (40). Under normal ionic conditions, the enzyme exists as a dimer of this subunit. The 140,000 dalton subunit was found to be composed of two dissimilar polypeptides of 76,000 and 66,000 daltons; these two polypeptides are common to the three different forms of DNA polymerase seen in human KB cells (41). These three forms have been designated nuclear α, cytoplasmic α and cytoplasmic α' and differ in their specific activities. Similar high molecular weight polypeptides from regenerating rat liver, 156,000 daltons (42), Drosophila melanogaster embryos, 148,000 daltons (43) and spinach, 160,000 daltons (44) have been reported to possess DNA polymerase α activity. A heterogeneous range of molecular weights have been reported for DNA polymerase α isolated from calf thymus (45-48). Masaki et al. suggests the heterogeneity may be due to the proteolytic degradation of the enzyme during purification, recommending the use of protease inhibitors to obtain purified preparations.

The sensitivity of DNA polymerases to various inhibitor have been used to distinguish the three classes of these enzymes. The

DNA polymerase α group is characteristically inhibited by salt concentration as low as 25 mN NaCl, and by aphidocolin at 1 ug/ml (1,29,30). Sulfhydrl blocking agents such as N-ethylmaleimide are also found to inhibit this class of enzymes (49). However, DNA polymerase α is relatively insensitive to dideoxynucleotides at molar ratios of 20:1 with deoxynucleotides. This insensitivity differentiates DNA polymerase α from DNA polymerase β and γ, which are inhibited at molar ratios of 1:1 (50).

The preferred template-primer of DNA polymerase α is activated DNA that contains gaps of twenty to seventy nucleotides (51,52). Deoxyhomopolymers are also utilized but not as efficiently. Although native RNA or ribohomopolymers are not utilized, an RNA primed DNA template can be utilized efficiently by DNA polymerase α (53). The enzyme like the other two classes has an absolute requirement for a divalent cation such as Mg^{2+} or Mn^{2+}. Optimal activity proceeds at pH 7.5, and the enzyme displays an acidic isoelectric point (pI 4.5 - 5.0).

Associated with DNA polymerase α is an RNA polymerase or primase activity. First described for mouse DNA polymerase α, this enzymatic activity appears to be involved in the synthesis of an initiator RNA of 12-18 bases (54-57). This RNA synthesis requires a single stranded DNA template and is thought to provide the primer for DNA synthesis during replication. Reports indicate that nascent strand DNA is covalently attached to the 3' end of the initiator RNA by DNA polymerase α. DNA polymerase α has been implicated in the

synthesis of Okazaki fragments (50,58-61).

There is some concern that the primase activity associated with DNA polymerase α is a contaminating RNA polymerase II. However, the primase activity is not inhibited by high concentrations of α-amanitin, a potent RNA polymerase II inhibitor. In addition the primase is further distinguished from RNA polymerase II because it can utilize both deoxyribo and ribonucleotides. Conclusive evidence that primase activity is associated structurally with DNA polymerase α has come from a number of studies (62-64). Kozu et al. report that the DNA polymerase α associated with the primase is capable of synthesizing DNA of approximately 600 nucleotides after synthesis of initiator RNA. Recently immunoabsorption with two different monoclonal antibodies that bind to DNA polymerase α have shown that the DNA primase activity is in the DNA polymerase α complex (65). The monoclonal antibodies that inhibits polymerase activity binds to an active site different from the site required for primase activity.

Among its enzymatic activities, DNA polymerase α has been implicated in the replication of some DNA viruses in the nuclei. Both Edenberg et al. and Balabanova et al. have demonstrated the involvement of DNA polymerase α in SV40 viral DNA replication (50, 66). DNA polymerase α does not contain any nuclease activity.

DNA Polymerase β

In 1971, three separate investigators reported the isolation of a low molecular weight DNA polymerase from nuclei in HeLa cells, rat

liver, and rabbit bone marrow (67-69). This DNA polymerase, designated now as DNA polymerase β, has since been characterized in cells from adult Drosophila, slime mold Dictyostelium discoideum, human KB cells, calf thymus, chick embryos, and sea urchins (70-75). All of these cells have DNA polymerase β activity in the nucleus with one exception, developing sea urchins contain the DNA polymerase β activity in the cytoplasm (76).

Levels of DNA polymerase β activity amount to approximately 5% of the total DNA polymerase activity in growing cells. In quiescent cells it accounts for the major DNA synthetic activity (77). It appears to have a role in DNA repair synthesis since DNA polymerase β activity is increased after ultraviolet irradiation of rat neuronal nuclei (5). Additional evidence for this function comes from studies which show that DNA polymerase β is the only eukaryotic DNA polymerase capable of utilizing UV-irradiated DNA incised by correndonuclease as a template (78). More recently, DNA polymerase β has been shown to be capable of limited strand displacement synthesis from a 3' hydroxyl terminus preceding a single nucleotide gap (79). Larger regions of DNA synthesis were produced with DNA polymerase and HeLa DNase V in a "nick translation" reaction.

The structural features of DNA polymerase β have been well characterized. The enzyme has been isolated and purified to homogeneity from chick embryos, Novikoff hepatoma cells, calf thymus tissue, and cultured human cells. These studies indicate that the enzyme is a single polypeptide with a molecular weight of

approximately 30-40,000 daltons. The polymerase purified from Novikoff hepatoma nuclei has a molecular weight of 31,000 and is associated with a 12,500 dalton polypeptide which enhances enzymatic activity (80). A stimulatory protein of 85,000 daltons has also been found for HeLa cell DNA polymerase β (81).

A phylogenetic study of DNA polymerase β indicated that the enzyme is ubiquitous among eukaryotes. DNA polymerase β activity was defined by its insensitivity to N-ethylmaleimide in these studies (34). In 1981, rabbit antisera prepared against DNA polymerase β was used to demonstrate that DNA polymerase β from several different species possess common antigenic determinants. Thus, the DNA polymerase β structure appears to be highly conserved among eukaryotic cells. Supporting evidence for this structural homology comes from studies comparing the tryptic peptide maps of DNA polymerase β isolated from several different mammalian cells (82). An even more rigorous demonstration of the evolutionary conservation of this enzyme structure was made by Chang et al. (83). In their studies, DNA polymerase β from several different sources was first identified in an SDS-polyacrylamide gel by an *in situ* polymerase assay. Then the protein from these gels were transferred to nitrocellulose filter paper and subsequently reacted with antibody prepared against DNA polymerase β from calf thymus. Their results indicated that DNA polymerase β from calf liver, human liver, rat liver, rabbit liver and chick embryo were similar in size and antigen structure.

The β -group of DNA polymerases can be classified by their sensitivity or resistance to various compounds. For example, DNA polymerase β is resistant to sulfhydryl blocking agents such as N-ethylmaleimide when activated DNA is the template-primer (1,84). However, with the ribohomopolymer template poly (A)-oligo $(dT)_{12-18}$, the enzyme exhibits marked sensitivity to N-ethylmaleimide (85). The enzyme is also sensitive to dideoxynucleotides and resistant to aphidocolin (see Table 1). Unlike DNA polymerase γ , when assayed with poly (A)-oligo $(dT)_{12-18}$ DNA polymerase β is extremely sensitive to inorganic phosphate, P_i. This difference in sensitivity has been used as the primary means of distinguishing between DNA polymerase β and γ . The sensitivity of DNA polymerase β to P_i is probably the result of its inability to utilize the tripartite complex, Mn-dNTP-P_i, which is formed in the presence of P_i (86). DNA polymerase β is distinguished from DNA polymerase α by its resistance to 0.2 M salt. In fact, the enzymatic activity is stimulated by salt. The enzyme exhibits an alkaline isoelectric point (pI - 8.5) and a pH optimum of 8.4 - 9.2 (77).

The preferred template-primer for DNA polymerase β is nicked or gapped DNA. The ribohomopolymer poly (A)-oligo $(dT)_{12-18}$ and deoxyribohomopolymers are also efficiently utilized (85,87,88). Like other DNA polymerases, DNA polymerase β requires the presence of a divalent cation for enzymatic activity. When activated DNA is used as the substance, both Mn^{2+} and Mg^{2+} can be utilized by DNA polymerase (87). In the presence of Mn^{2+}, the apparent K_m for activated DNA is

less than 10uM, while in the present of Mg^{2+} the apparent K_m is 300 uM. This unusual effect of Mn^{2+} suggests that divalent cations may act as more than chelating agents for deoxyribonucleotides. Other cations such as copper, lead and cadmium can not participate in the same manner and are actually inhibitory (89).

Among its other activities, DNA polymerase β is thought to be involved in DNA endoreduplication, a process in which the entire genome is replicated without subsequent mitosis and cell division. Siegel and Kalf (90) using isolated nuclei from non-dividing rat trophoblast cells, found DNA synthesis to be resistant to N-ethylmaleimide and inhibited 50mM phosphate and 2-3 dideoxythymdine triphosphate. This activity in the presence of these DNA polymerase inhibitors identifies DNA polymerase β as the enzyme responsible for endoreduplication in these non-dividing cells. Its involvement here suggests DNA polymerase β role in the nucleus is not limited to DNA repair.

There are no reported exonuclease activities associated with DNA polymerase β . However, there has been a report that both 3' - 5' and 5' - 3' exonuclease activity is associated with the 12,500 dalton stimulatory protein found in rat liver (91).

DNA Polymerase γ

DNA polymerase γ is primarily found in the cytoplasm and mitochondria of cells and is thought to function in the replication of mitochondrial DNA (3,6). It has also been found in the nucleus where its function remains unclear. However, it has been implicated

in the nuclear replication of the viral DNAs from adenovirus (92,94).

Reports of the molecular weight for DNA polymerase γ yield widely varying estimates. This is due to the tendency of the enzyme to aggregate in low ionic strength conditions. That same tendency is exhibited by DNA polymerase α. For HeLa cells, molecular weight estimates for DNA polymerase γ have ranged from 150,000 to 330,000 daltons (16). In human lymphoblasts, DNA polymerase γ has a molecular weight of 120,000 daltons (17,95). Sedimentation analysis of the DNA polymerase γ from rat brain synaptosome ends which are devoid of nuclei and abundant in mitochondria, demonstrated that this DNA polymerase γ has a molecular weight of 180,000 daltons (4). Values of 150, and 180,000 daltons have also been reported for the DNA polymerase γ isolated from chick embryos (10,14). In the later report, Yamaguchi et al. furnish evidence that the 180,000 dalton enzyme is a tetramer composed of a single polypeptide of 47,000 daltons. The same authors later report that an identical polypeptide is the major component of mouse myeloma DNA polymerase γ (15). These polypeptides were shown to be identical by tryptic peptide mapping.

Although DNA polymerase γ is the polymerase responsible for mitochondrial DNA synthesis, it does not efficiently utilize DNA templates in vitro. In fact, the DNA polymerase γ prefers ribohomopolymers as templates in vitro (96,97). This ability of DNA polymerase γ to efficiently utilize ribohomopolymers led many to believe that normal cells also contained a "reverse transcriptase"

thought only to occur in RNA tumor virus infected cells. However, reverse transcriptase activity can be readily differentiated from DNA polymerase γ since DNA polymerase γ is incapable of utilizing natural RNA templates and the ribopolymer poly (2'-O-methylcytidylate) (98). The two enzymes are also antigenically unrelated (99).

DNA polymerase γ prefers ribohomopolymers, specifically poly riboadenylate-oligodeoxythymidylate in the presence of 0.5 mM Mn^{2+} and 100-200 mM KCl. Although DNA polymerase β is capable of utilizing this template-primer, its activity can be completely inhibited by phosphate (86), whereas DNA polymerase γ is stimulated in the presence of phosphate. With this template, DNA polymerase γ has a Km for dTTP of approximately 1 uM, and a pH optimum of 8 - 9.0.

The enzyme's involvement in mitochondrial DNA replication suggest DNA polymerase replicates via a strand displacement mechanism (100,101). This same mechanism of replication is seen in adenovirus DNA replication (102). van der Vliet and Kwant have linked DNA polymerase γ to adenovirus replication since the nucleotide analogue 2'3' dideoxythymidine triphosphate was capable of arresting adenovirus DNA replication in both intact cells and isolated nuclei. More recently, a claim was made that a different DNA polymerase distinct from α , β , and γ is responsible for adenovirus replication (103). The suspected adeno-coded polymerase apparently binds to an 80 kdalton protein to form a replicating complex. This 80 kdalton protein is the precursor protein to the terminal protein found associated with the 5' end of adenovirus DNA (104). Tananoi and

Stillman have shown that the terminal protein initiates DNA replication via a terminal protein dCMP complex (105). Stillman et al. have now claimed to have purified a 140 kdalton protein encoded by adenovirus that possesses DNA polymerase activity (106). The polymerase is tightly associated with the 80 kdalton precursor terminal protein as described by Enomoto et al. (104).

With the present finding of the adeno-coded DNA polymerase, the function of DNA polymerase γ in the nucleus still remains obscure. However, Weissbach hypothesizes that DNA polymerase γ's function in the nucleus may involve gene amplification (16). One method in which this can occur is via the rolling circle mechanism which involves strand displacement DNA synthesis. This method of replication has been implicated in the amplification of ribosomal DNA in developing Xenopus oocytes and Dytiscid beetle oocytes (107,108) where four or more copies of the rDNA coding sequences have been synthesized.

DNA polymerase β has also been shown to carry out strand displacement synthesis. However, it is not capable of synthesizing long DNA strands like DNA polymerase γ (109). Since DNA polymerase α is incapable of strand displacement DNA synthesis, it would strongly suggest the participation of DNA polymerase γ in this hypothetical model and the function of DNA polymerase γ inside the nucleus.

Table 1

Distinctive Features of Eukaryotic DNA Polymerases α, β, and γ

	α	β	γ
Location	nucleus	nucleus	mitochondria, nucleus
Proposed function	replication, nDNA	repair	replication, mtDNA
Mass, kdal	120-220	30-50	150-300
pH optimum	7.2	8.5	8.0
Preferred cation	Mg	Mg or Mn	Mg or Mn
Template-Primer preferred	gapped DNA	gapped DNA	ribohomo-polymers
ribo template-deoxy primer	no	yes	yes
deoxy template-ribo primer	yes	no	no
Relative activity,%			
growing cells	80-90	10	2
resting cells	0-5	80-90	10
Inhibitory effects			
sulfhydrl (NEM)	yes	no	yes
salt (0.2 M NaCl)	yes	stimulates	stimulates
phosphate (0.1 M)	no	yes	stimulates
dideoxy NTPs	no	yes	yes
aphidocolin	yes	no	no

Kornberg, A. (1980) DNA Replication p. 204. W.H. Freeman and Company, San Francisco.

MATERIALS AND METHODS

Reagents

Deoxyribonucleoside triphosphates were obtained from P-L Biochemicals (Milwaukie, WI). (^{3}H)dTTP (77 Ci/mmol), (^{3}H)dGTP (16 Ci/mmol) and (α-^{32}P)dNTPS (3200 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Poly rA and oligo dT, along with other synthetic template-primers were also purchased from P-L Biochemicals. Calf thymus DNA was obtained from CalBiochem (LaJolla, CA). DEAE-cellulose and cellulose CF-11 were purchased from Whatman Ltd. (Clifton, NJ), cellulose phosphate was from Sigma Chemical Co. (St. Louis, MO), poly rA-Sepharose was from Parmacia Fine Chemicals (Piscataway, NJ) and Fractogel TSK-HW55 was purchased from MCB Manufacturing Chemists Inc. (Norwood, OH). Aphidocolin, prepared in dimethylsulfoxide, was obtained through the Developmental Therapeutic Program, Chemotherapy, National Cancer Institute with the assistance of Dr. John Douros. Acrylamide, methylene-bis-acrylamide, TEMED (N,N,N',N'-tetramethylethylenediamine) and ammonium persulfate were purchased from Bio-Rad Laboratories (Richmond, CA). Pharmalytes were from Pharmacia Fine Chemicals. Trasylol, 10,000 KIU (KalliKrein Inactivation Units)/ml, was purchased from FBA Pharmaceuticals (New York, NY). Polyethylene glycol 8000 (lot 144522) was from J. T. Baker (Phillipsburg, NJ). Activated DNA was prepared according to the method of Fansler and Loeb (110). Activated DNA cellulose was prepared as described by Alberts and Herrick (111). Neutralizing antibody from SJK-132-20 (3.655 mgs/mL) to DNA polymerase α from

human KB cells was a generous gift from Drs. Teresa Wang and David Korn (Stanford University).

DNA Polymerase Assays

All incubations were carried out for 30 min. at 37°C unless stated otherwise. DNA polymerase γ assay was carried out in a reaction volume of 50 ul containing the following: 20 mM Tris-HCl, pH 7.5; 10 mM KPO_4; 90 mM KCl; 1 mM dithiothreitol; 0.5 mM $MnCl_2$; 250 ug ovalbumin; 43.5 ug $(rA)_n$ $(dT)_{12-18}$ (3.5:1); 1.29 uM (^{3}H)dTTP (77 Ci/mmol); 2-10% glycerol. The DNA polymerase assay designed for detecting all DNA polymerase activity was carried out in 100 ul reaction volumes containing the following: 50 mM Tris-HCl, pH 7.6; 8 mM $MgCl_2$; 0.5 mM $MnCl_2$; 0.1 mM EGTA; 0.8 mM 2-mercaptoethanol; 12 uM dATP, dCTP, and dGTP; 1.29 uM (^{3}H)dTTP (77 Ci/mmol); 75 ug activated calf thymus DNA. The counting efficiency was 55,000 cpm/pmol of TMP.

All reactions were stopped by the addition of 2 mls of cold 5% tricholoracetic acid containing 10 mM sodium pyrophosphate and collected on Whatman GF/C filters. The precipitate was washed first with 15 mls of 0.01 N HCl, 0.1 M sodium pyrophosphate and then 15 mls of ethanol, 4°C. Filters were dried under a heat lamp and the radioactivity measured in 10 mls of toluene-Omnifluor (New England Nuclear) in a Beckman LS-233 scintillation counter.

One unit of DNA polymerase activity catalyzes the incorporation of 1 nmol of deoxyribonucleoside monophosphate per hour at 37°C.

Purification of DNA Polymerase γ

All steps were carried out at 0-4°C. DNA polymerase activity was monitored using poly (A)-oligo $(dT)_{12-18}$ as template-primer.

Preparation of Tissue Homogenate

Approximately 200 g of term-delivered human placenta was homogenized in 2.5 volumes of Buffer A (50 mM Tris-HCl, pH 7.6; 1 mM EDTA, 0.5 M KCl; 10% glycerol (v/v), 4 mM 2-mercaptoethanol and 10 KIU/ml Trasylol, in a Waring blender at high speed for six episodes of 20 sec. The homogenate was centrifuged at 2,000 x g for 20 min. to remove tissue debris and nuclei. The detergent, Nonidet P40 (NP40), was added to the resulting supernatant (Fraction I) to a final concentration of 1%, and stirred at 4°C for 1 hour on a magnetic stirrer. The solution was centrifuted at 10,000 x g for 30 min. in a Sorvall GSA rotor at 4°C. The resulting supernatant was again centrifuged at 100,000 x g for 30 min. in a Beckman Type 35 rotor to remove the particulate debris. The clarified supernatant was decanted and saved (Fraction II).

DEAE-Cellulose Chromatography

Fraction II was adsorbed to DEAE-cellulose equilibrated in Buffer A and stirred gently for 12 hours at 4°C in a glass beaker. This adsorbed material was then poured into 10 cm x 12 cm column and washed with one column of Buffer A. Under these conditions, the DEAE-cellulose preferentially binds nucleic acids and allows proteins to be eluted in Buffer A.

Ammonium Sulfate Fractionation

Ammonium sulfate crystals were added to the DEAE-cellulose elutant to a concentration of 25% and the mixture was stirred for 1 hour at 4°C by a magnetic stirrer. The resulting protein precipitate was removed by centrifugation at 10,000 x g for 30 min. (Sorvall GSA rotor) at 4°C. The supernatant was retained and solid ammonium sulfate was again added to the supernatant to a final concentration of 55%. The mixture was stirred for 1 hour at 4°C and the precipitate recovered by centrifugation as described above. The 25-55% ammonium sulfate precipitate (Fraction III) was resuspended in 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 4 mM 2-mercaptoethanol, 20% glycerol (v/v) and 10 KIU/ml Trasylol and dialyzed extensively against Buffer B (50 mM Tris-HCl, pH 7.6; 1 mM EDTA, 4 mM 2-mercaptoethanol; 0.1 M KCl; 10% glycerol (v/v) and 10 KIU/ml Trasylol) to remove the ammonium sulfate.

Phosphocellulose Chromatography

The dialyzed Fraction III was adsorbed onto a column of phosphocellulose (4 cm x 14 cm) which had been previously equilibrated in Buffer B. The column was washed with 3 column volumes of Buffer B. The polymerase activity was eluted with a 3 column volume linear gradient (0.1 - 0.6M) in Buffer B. The major peak of activity appeared between 0.2 - 0.35 M KCl. Active fractions were pooled into dialysis bags and concentrated with solid polyethylene glycol for 4 hours at 4°C. The concentrated sample was dialyzed against Buffer B in 20% glycerol (Fraction IV).

DEAE-Cellulose Chromatography

Fraction IV was adsorbed onto a DEAE-cellulose column (4 cm x 6 cm) equilibrated in Buffer B with 20% glycerol. The column was washed with 2 column volumes, followed with a linear gradient of KCl (0.1 - 0.6 M) in Buffer B. Enzyme activity was found in the wash fractions with no additional activity seen in the gradient fractions. Active fractions were pooled, concentrated with polyethylene glycol as described above and dialyzed against Buffer B in 20% glycerol (Fraction V).

Nicked DNA-Cellulose Chromatography

Fraction V was loaded onto a DNA-Cellulose column (1.5 cm x 5 cm) equilibrated in Buffer B in 20% glycerol at a rate of 2 ml per hour. The column was washed with a 4 column volume linear gradient of KCl (0.1 - 0.6 M) in Buffer B. The active fractions were again pooled, concentrated and dialyzed as above in Buffer B with 20% glycerol (Fraction VI). This fraction was either used for subsequent purifications or dialyzed against Buffer B in 50% glycerol and stored at -20°C. This enzyme preparation is stable for 12 months.

High Pressure Gel Exclusion Chromatography

Fraction VI was dialyzed against 50 mM Tris-HCl buffer pH 7.6 containing 1 mM EDTA, 0.5 M KCl, 10 KIU/ml Trasylol, and 4 mM 2-mercaptoethanol, and later applied to a 2.2 x 3.0 cm column of Fractogel TSK-HW55 equilibrated in the same buffer. The column was pumped at the rate of 35 mls/hr. and the elutant was monitored

with a U.V. light source. Fractions of 1 ml each were collected into tubes containing 1 ml of the same buffer in 50% (v/v) glycerol. These fractions were assayed for DNA polymerase gamma activity as previously described.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Slab gels of 0.8 mm thickness were prepared as described by Laemmli (112). A 5% stacking and a 10% separating acrylamide gel was used for determination of enzyme size. Prior to electrophoresis, samples were denatured at 100°C for 1 min. in sample buffer containing the following: 2.3% Sodium dodecyl sulfate (SDS); 0.0625 M Tris base; 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol. Following electrophoresis, gels were fixed for 1 hour in a solution containing 10% trichloroacetic acid, 30% methanol and 10% acetic acid. The gels were then stained with 0.4% Coomassie brilliant blue G-250 (Bio-Rad) or silver nitrate to visualize the polypeptide bands (113).

Detection of Polymerase Activity in SDS-Gels

The procedure used to detect DNA polymerase activity in SDS-gels was modified from Spanos et al. (114). The SDS-acrylamide slab gels were bonded to GelBond (Marine Colloids, FMC) before use. Enzyme extracts in 65 mM Tris base, pH 6.8; 2.3% SDS; 10% (v/v) glycerol; 5% (v/v) 2-mercaptoethanol and 2 mM EDTA were heated at 37°C for 3 min. and subjected to electrophoresis in a 5% stacking and 7.5% (v/v) separating acrylamide gel containing 0.1% SDS and 100 ug/ml activated calf thymus DNA. After electrophoresis, the gels were

incubated in 25% (v/v) isopropanol, 50 mM Tris buffer, pH 7.6, for 1.5 hour at room temperature. The isopropanol washing solution was changed twice during this period to facilitate the removal of SDS. The separated proteins were allowed to renature for 20 hours at $4^{o}C$ mM Tris buffer, pH 7.6; 1 mM EDTA and 4 mM 2-mercapoethanol. This buffer was changed 4-5 times during this 20 hour period. Gels were then incubated at $37^{o}C$ for 24 hours in a solution containing 50 mM Tris buffer, pH 7.6; 8 mM $MgCl_2$; 0.1 mM EGTA; 0.8 mM 2-mercaptoethanol; 12 mM each of dATP, dCTP, and dGTP and 250 uCi (α-^{32}P)dTTP (3200 Ci/mmol). Unincorporated (α-^{32}P)dTTP was removed by extensive washing with 5% trichloroacetic acid with 1% sodium pyrophosphate. These gels were then exposed to X-ray film (Kodak X-OMAT) for 10-24 hours at $4^{o}C$ and later stained with silver.

Isoelectric Focusing

Isoelectric focusing using 1% (w/v) ampholytes (pH 3-10) in a gradient of 0-60% glycerol was run in an LKB 8100-1 column. Approximately 2 mls of sample was dialyzed against 60% (w/v) glycerol for 3 hours before application to the column. The pH gradient was formed at a constant power setting of 5 W for 15 hours at $4^{o}C$. Upon completion of the isoelectric focusing, 55 equal fractions were collected from the bottom of the column and placed on ice. Fractions were immediately assayed for DNA polymerase activity.

Glycerol Gradient Sedimentation

Sedimentation velocity in glycerol was used to estimate enzyme

size. The 10-30% glycerol (v/v) gradients were run in an SW 50.1 at 42,000 rpm for 24 hours at $4^{o}C$. The gradients were prepared in 0.15 M or 0.60 M KCl; 50 mM Tris-HCl, pH 7.6; 1 mM EDTA; 4 mM 2-mercaptoethanol.

Immunological Studies

Approximately 0.19 ug of DNA polymerase gamma (Fraction VII) was incubated with various amounts of monoclonal immune (SJK-132-20) or non immune (P3) immunoglobulin in 50 mM Tris, pH 7.6, 100 mM KCl, 10 mM KPO_4, 1 mM dithiothreitol, 0.5 mM $MnCl_2$, and 0.5 mg/ml ovalbumin for 1 hour at $0-4^{o}C$. After 1 hour, 1.3 uM (^{3}H)-dTTP (77 Ci/mmol) and 43.5 ug poly (A)-oligo $(dT)_{12-18}$ were added to a final volume of 50 ul and incubated at $37^{o}C$ for 30 min. Neutralizing activity was computed as percentage of polymerase activity remaining compared to the controls where no immunoglobulin was present.

Protein Determinations

The concentration of protein in samples was measured by the Coomassie blue R technique (Bio-Rad) with bovine serum albumin as the standard. Samples and standards were measured at 595 nm.

RESULTS

Purification of DNA Polymerase γ From Human Placental Tissue

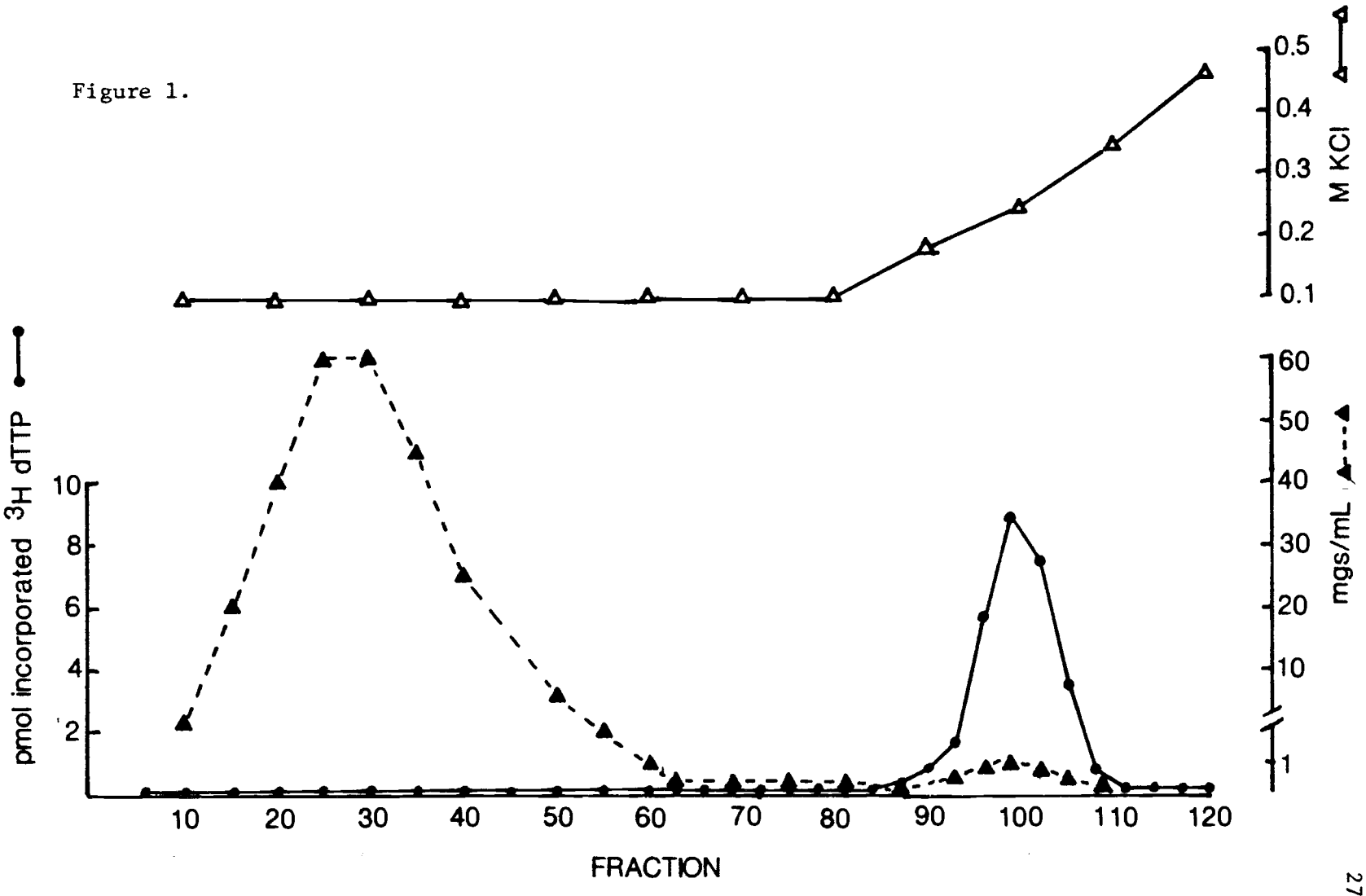
The initial tissue extract was prepared from a nuclei-free fraction from the trophoblast layer of the human placenta. This extract was treated with NP40 to lyse the mitochondria and further clarified by centrifugation. DNA polymerase γ was purified about 67,500-fold by sequential chromatography through phosphocellulose, microgranular DEAE-cellulose, DNA cellulose and Fractogel. After phosphocellulose chromatography there was a hundred-fold increase in specific activity as a substantial amount of protein was washed through the column before the peak of enzyme activity (Fig. 1). The enzyme containing fractions correspond to 3.5% of the total protein (Table I).

After concentration and dialysis, the phosphocellulose bound fraction (Fraction IV) was applied to a DEAE-cellulose column. The column was washed with Buffer B containing 0.1 M KCl and enzyme activity appeared in the wash fractions (Figure 2). Apparently the human placental enzyme does not bind tightly to DEAE-cellulose as found by Lewis et al. (115), where 0.3 M KCl was used to elute DNA polymerase γ isolated from human lymphoblastoid cells.

Enzyme-containing fractions from the DEAE-cellulose column (Fraction V) were prepared for DNA cellulose chromatography as described in the Materials and Methods. DNA polymerase γ activity eluted at 0.22 to 0.38 M KCl (Figure 3) and a four-fold increase in specific activity was obtained. The enzyme is extremely stable at

Figure 1. Phosphocellulose chromatography. Elution of DNA polymerase on phosphocellulose. Five microliter aliquots from the fractions were assayed for DNA polymerase activity as described in the Materials and Methods, using the template-primer poly (A)-oligo $(dT)_{12-18}$.

Figure 1.


0.5
0.4
0.3
0.2
0.1
M KCl
pmol incorporated ^{3}H dTTP
10
8
6
4
2
60
50
40
30
20
10
1
mgs/mL
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20
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40
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110
120
FRACTION

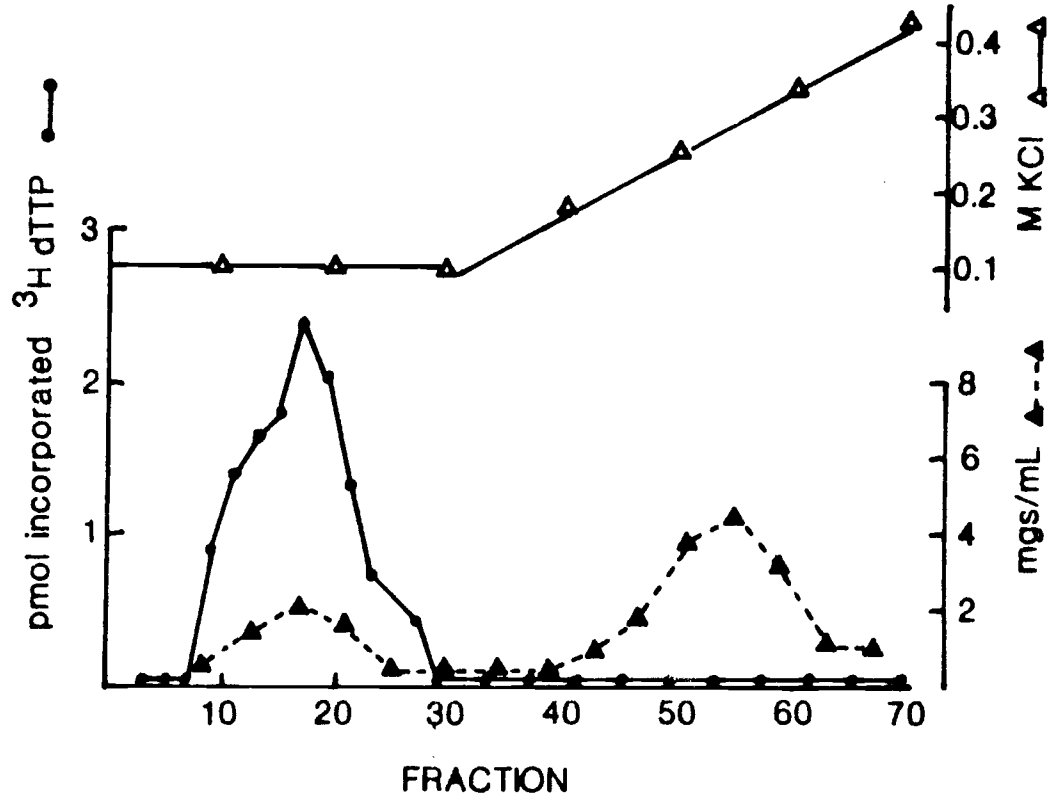


Figure 2. DEAE-cellulose chromatography. Elution of DNA polymerase γ on DEAE-cellulose. Fractions were assayed as stated earlier.

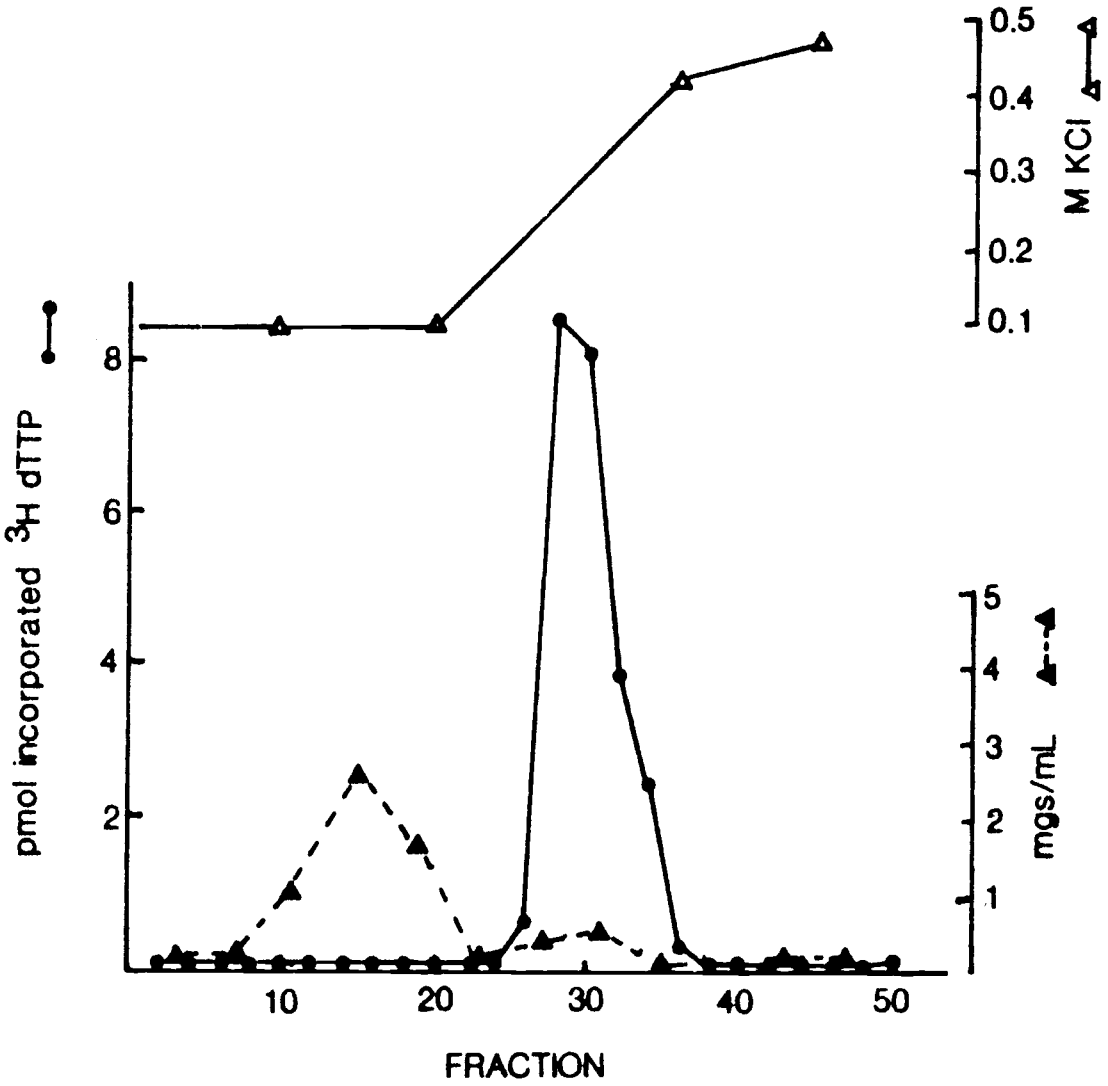


Figure 3. DNA-cellulose chromatography. Elution of DNA polymerase γ on DNA-cellulose. Fractions were assayed as stated earlier.

this point and it can be stored at -20°C in 50% glycerol for at least one year.

Final purification of the enzyme was achieved by high pressure gel exclusion chromatography through Fractogel TSK HW55. As depicted in Figure 4, the enzyme coincided with a protein having a molecular weight of 94,000. The active fractions were pooled and concentrated in 50 mM Tris-HCl, pH 7.6; 0.1 M KCl; 1 mM EDTA; 4 mM 2-mercapto-ethanol; 10 KIM/ml Trasylol; and 50% glycerol (v/v) for storage at -20°C. The enzyme was found to be extremely unstable following Fractogel purification. Enzyme activity was markedly reduced after 10-20 days at -20°C. At this step, the purified enzyme was found to have a specific activity of 81 units/mg protein (Table I).

Polypeptide Analysis of DNA Polymerase Gamma By SDS Gel Electrophoresis

Fraction VI from the DNA cellulose column and Fraction VII from the Fractogel column were analyzed for polypeptide composition by SDS-PAGE. Identical gels were developed by Coomassie blue staining and silver staining. A comparison of the staining patterns obtained by either method is shown in Figs. 5 and 6. It is clear that the Coomassie blue stain is not sensitive enough to detect all contaminating polypeptide bands in Fraction VI (Fig. 5). Although the preparation appears to be composed of a single polypeptide band at 94,000 with the Coomassie blue stain, silver staining of the same preparation revealed 7 additional bands at 63,000, 60,000, 54,000, 49,000, 40,000, 34,000, and 28,000 daltons. It was only after

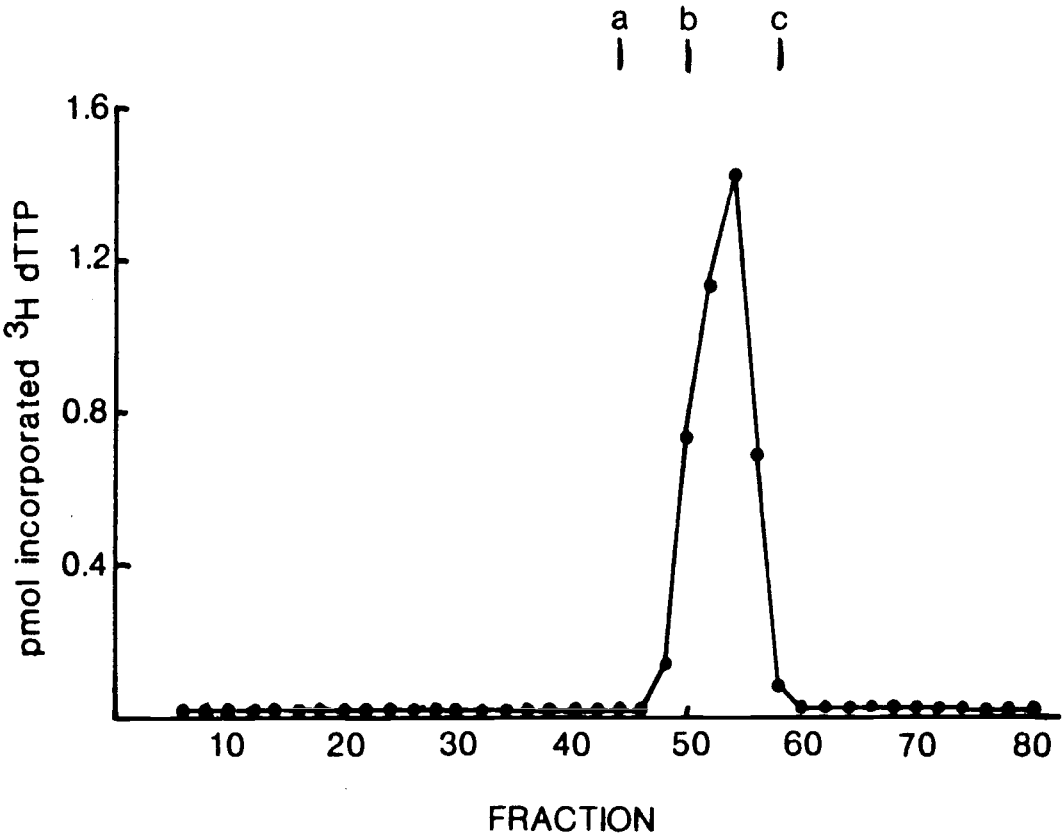


Figure 4. Fractogel chromatography. High pressure gel exclusion chromatography of DNA polymerase through Fractogel TSK HW55. Five microliter aliquots from the fractions were assayed for DNA polymerase activity using poly (A)-oligo $(dT)_{12-18}$. The column was calibrated with the following standard proteins: a) bovine liver catalase, MW = 250,000; b) rabbit muscle aldolase, MW = 153,000; and c) chicken ovalbumin, MW = 43,000.

Table 2

Purification of DNA Polymerase From Human Placenta[1]

Procedure	Fraction No.	Vol. (ml)	Total Units	Protein mg/ml	Units / mg prot.	Purification
Crude	I	420		20.8	1.2×10^{-3}	0
100,000 x g super	II	400		20.0	2.1×10^{-3}	1.8
25-55% $(NH_4)_2SO_4$	III	100		20.6	8.0×10^{-3}	6.7
Phosphocellulose	IV	35	63.1	2.12	0.85	708
DEAE-cellulose	V	16	30.8	1.48	1.3	1,083
DNA-cellulose	VI	8	6.7	0.17	4.9	4,083
Fractogel TSK	VII	2.0	4.9	3.0×10^{-2}	81.0	67,500

[1]One unit corresponds to the incorporation of 1 mmol of dTTP in 1 hour at 37°C with poly (A)-oligo $(dT)_{12-18}$ as substrate.

Figure 5. SDS-polyacrylamide gel electrophoresis of placental DNA polymerase γ purification. Purification steps of placental polymerase on a 10% denaturing gel stained with 0.4% Coomassie blue. Lane 1 contains molecular weight markers: myosin (200,000), phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (45,000), α-chymotrypsinogen (24,000), β -lactoglobulin (18,000) and cytochrome C (12,300). Lane 2, 5.0 ug of Fraction V; lane 4, 5.0 ug of Fraction VI. Samples were prepared as described in Materials and Methods.

Figure 6. Silver stained denaturing gel of DNA polymerase γ purification. Lane 1, molecular weight markers, bovine serum albumin (68,000), ovalbumin (45,000) α-chymotrypsinogen (24,000) and β -lactoglobulin (18,000). Lane 2, 1.0 ug of Fraction VI; Lane 3, 1.0 ug of Fraction VII. Gel was stained as previously described (113).

Figure 5.

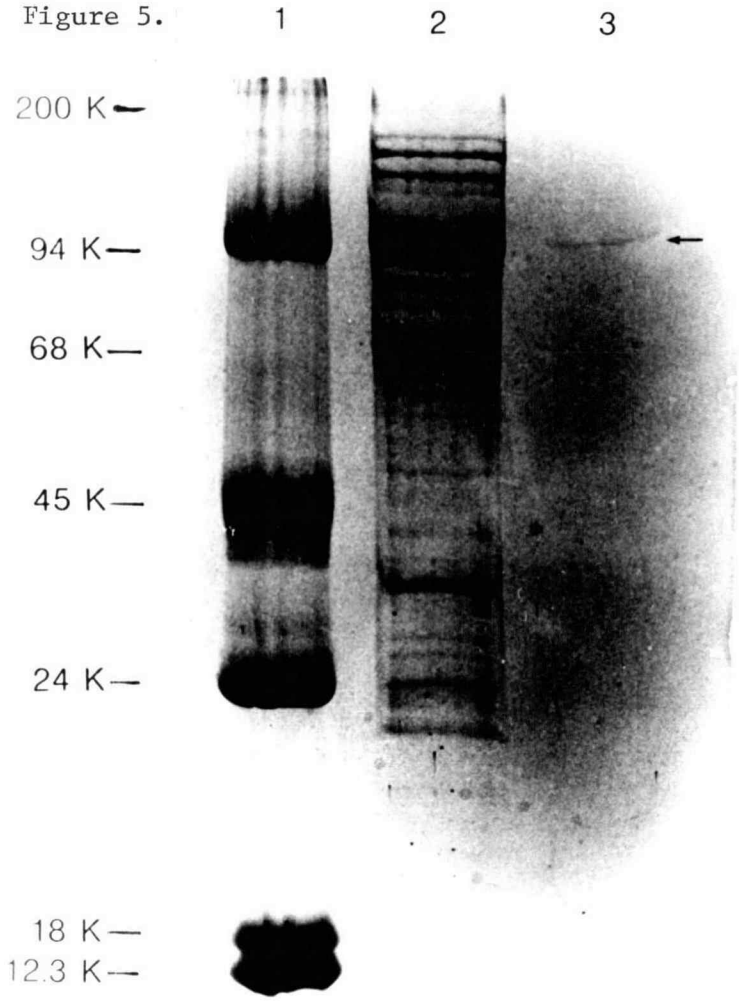
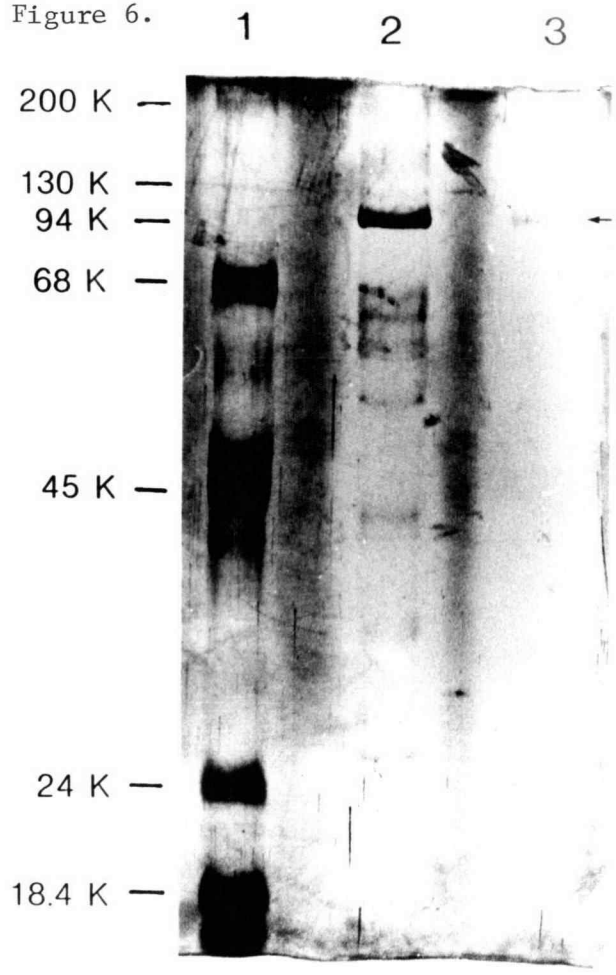


Figure 6.



Fractogel purification that a single polypeptide at 94,000 was found in the enzyme active preparation with silver staining (Fig. 6). This molecular weight estimate corresponds with the estimated molecular weight of the enzyme obtained by Fractogel chromatography and indicates that the human DNA polymerase γ is composed of a single polypeptide of 94,000.

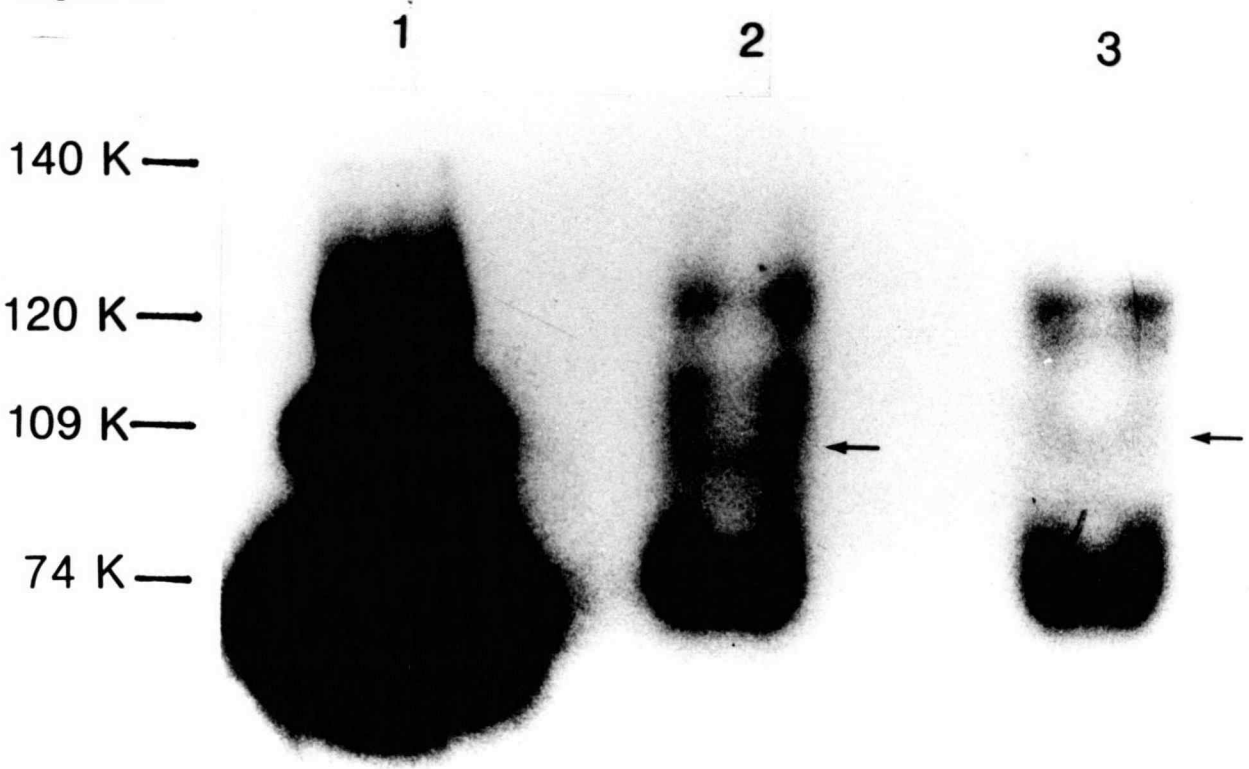
Detection of DNA Polymerase Activity By In Situ Assay

In order to identify the DNA polymerase γ activity with the 94,000 dalton polypeptide, we assayed for DNA polymerase activity *in situ* after SDS-gel electrophoresis (114,116,117). There were several bands of activity in Fraction VI (Figure 7, Lane 3). The preparation exhibited DNA polymerase activities of 123,000, 116,000, 94,000, 82,000, and 73,000 daltons. No activity was found at the lower molecular weight region although polypeptide bands were present by silver staining (see above). The only polypeptide band associated with enzyme activity was the 94,000 dalton band. The four other enzyme activities were found at positions in the gel where no polypeptide band was detectable by silver staining. When this enzyme preparation was further purified by high pressure gel exclusion, we were unable to detect any *in situ* DNA polymerase activity. This result may reflect the decreased stability of the enzyme after Fractogel purification or the fact that the enzyme does not prefer activated DNA as a template.

Control DNA polymerase activities shown in Fig. 7, Lane A consisted of *E. coli* DNA polymerase III (140,000), DNA polymerase II

Figure 7. In situ polymerase assay for DNA polymerase γ
Fractions from the DNA polymerase γ purification process were electrophoresed for 4 hours at 150 volts and treated as described in the Materials and Methods. Lanes 1, homogeneous E. coli DNA polymerase I (10 units) and large fragment of E. coli pol I (10 units); 2, mouse myeloma cell extract (10 ug); 3, fraction VI γ polymerase (8 ug); 4, Fraction VII γ polymerase (5 ug). Molecular weight markers were myosin (200,000, β-galatcosidase (130,000), phosphorylase (94,000), bovine serum albumin (68,000), ovalbumin (45,000), E. coli pol I (109,000), Klenow fragment (74,000), E. coli pol II (120,000) and E. coli pol III (140,000). The gel was then exposed to Kodak X-OMAT x-ray film for 1 hour to locate pol I and the Klenow fragment. The gel was again reexposed to detect the other polymerase activities.

Figure 7.



(120,000), polymerase I (109,000), and the large Klenow fragment of polymerase I (75,000). Enzyme activity was found for the higher molecular weight DNA polymerases even though no polypeptide bands were detected at that molecular weight range in the gels by silver staining. In addition, we prepared a cellular extract as a control for eukaryotic DNA polymerases. Mouse myeloma cells, SP-2, were lysed and the nuclei and cellular debris were separated from the extract by centrifugation. Six bands of DNA polymerase activity were identified at 136,000, 123,000, 108,000, 96,000, 82,000 and 73,000. This activity profile is very similar to that obtained by Hubscher et al. (116).

Properties Of The Purified DNA Polymerase γ

The following studies were performed on Fraction VII of the human placental enzyme (Table 2), using a Tris-HCl (6.8 - 8.8) with the pH optimum between pH 6.8 and 7.4 (Fig. 8).

The optimal Mn^{2+} requirement was determined for the purified gamma polymerase with the template-primer $(rA)_n$ $(dT)_{12-18}$. As shown in Fig. 9, activity proceeds optimally at a concentration of 0.5 mM. Attempts to substitute Mg^{++} up to 20 mM did not stimulate enzyme activity. This result differs from other reports where Mg^{2+} was used by DNA polymerase γ even though activity in the presence of Mg^{2+} proceeded at a reduced rate.

The optimal ionic strength required for the human enzyme was determined for KCl. Maximum enzyme activity was found at 90 mM KCl (Fig. 10). There was a sharp rise in enzyme activity from 30 to 90

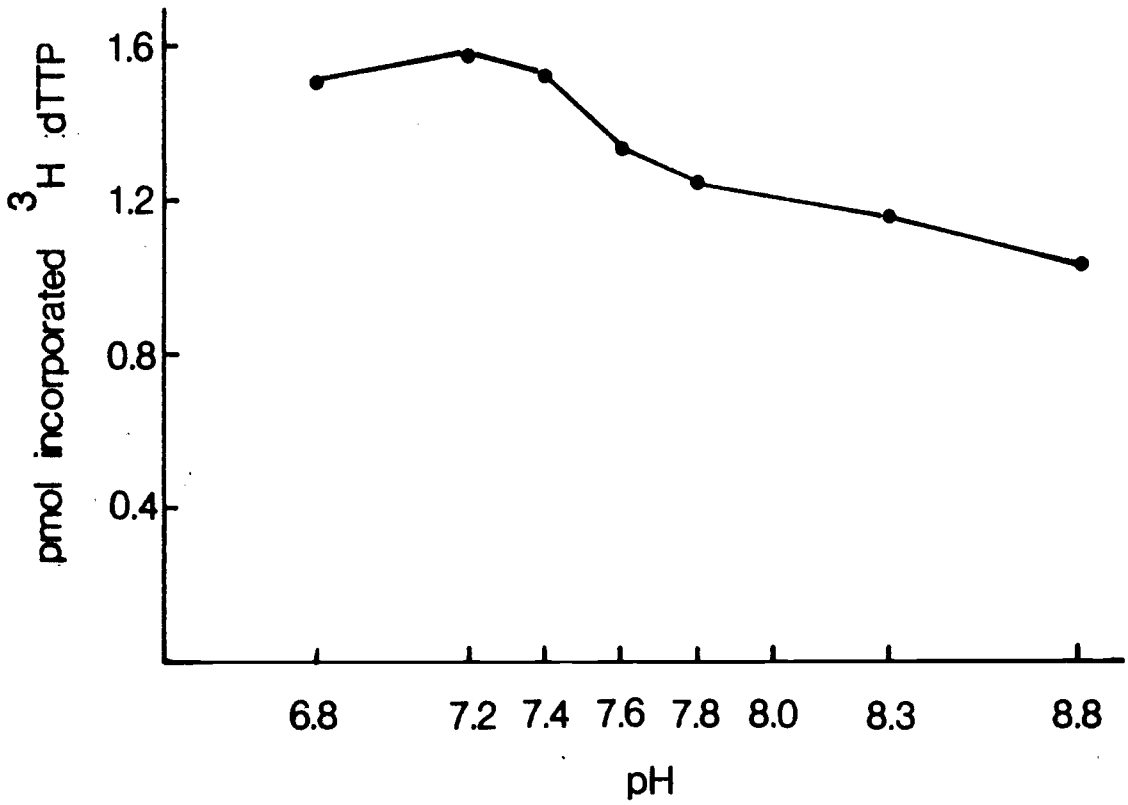


Figure 8. Optimal pH for DNA polymerase activity. Approximately 0.195 ug of Fraction VII were assayed for DNA polymerase activity at each point using poly (A)-oligo $(dT)_{12-18}$.

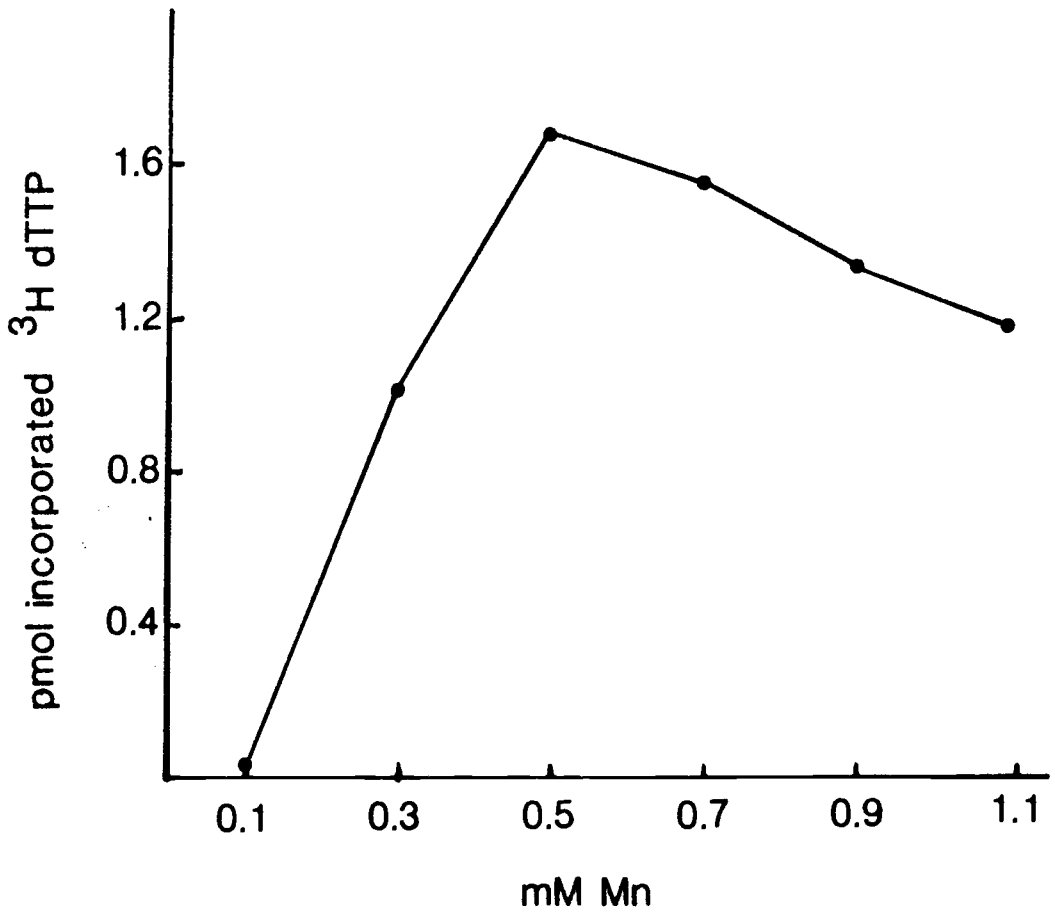


Figure 9. Optical divalent cation concentration for DNA polymerase activity. DNA polymerase activity was assayed as described earlier using the divalent cation, Mn^{2+}.

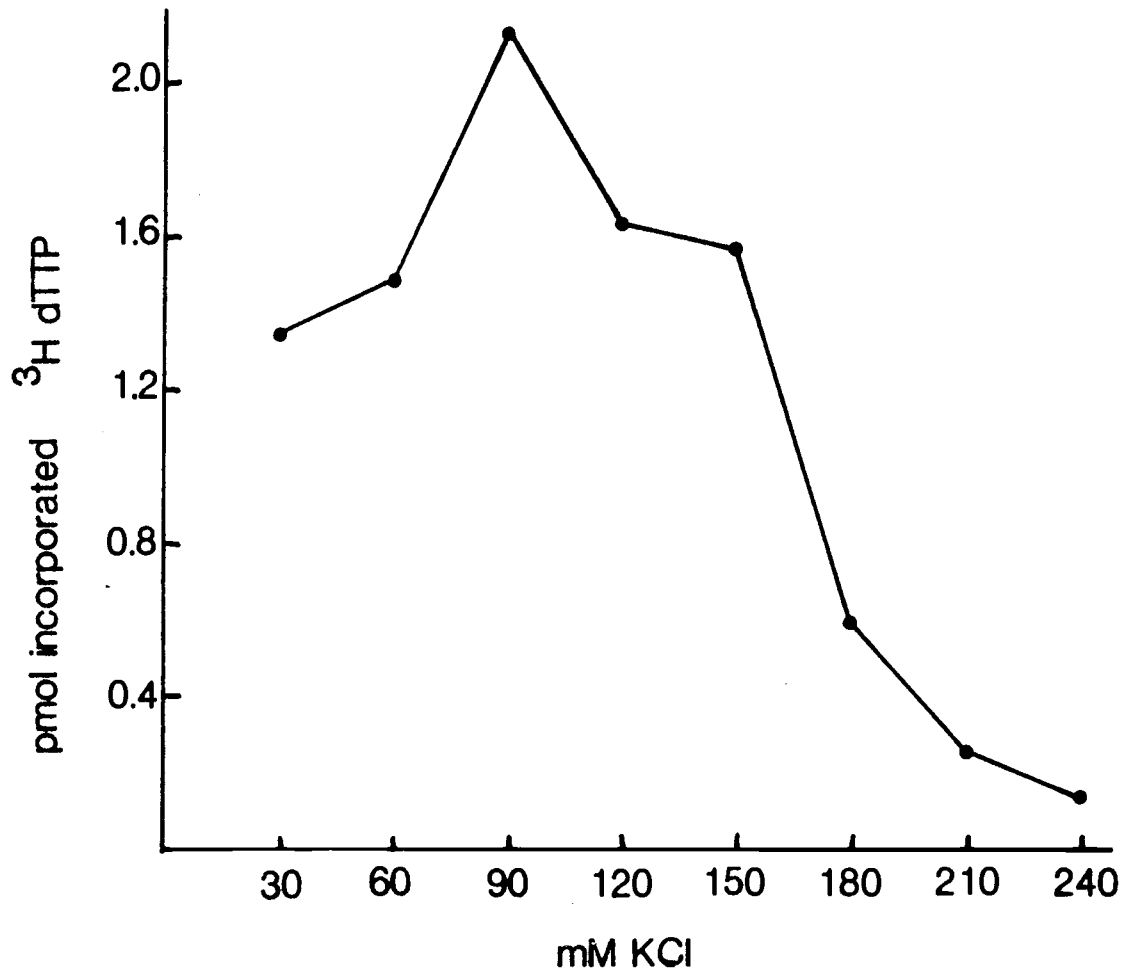


Figure 10. Optimal ionic strength requirement for DNA polymerase activity. The salt KCl was used to determine the optimal ionic strength for DNA polymerase activity. DNA polymerase activity was assayed as described in the Materials and Methods.

mM KCl and with KCl concentrations higher than 150 mM, there was a sharp drop in enzyme activity. The sensitivity of DNA polymerase γ to high salt concentrations has been reported by others (3,118).

The efficiency of the purified placental DNA polymerase γ to read synthetic and natural templates decreased in order: poly (A)-oligo$(dT)_{12-18}$, poly (dA)-oligo$(dT)_{12-18}$, oligo (dT)-primed-globin mRNA, activated salmon sperm DNA, poly (dC)-oligo$(dG)_{12-18}$, and poly G (see Table 3). The preference for poly (A)-oligo$(dT)_{12-18}$, exhibited by the placental enzyme is characteristic of DNA polymerase γ. When the reaction using oligo (dT)-primed-globin mRNA was repeated with ^{3}H-dGTP as the nucleotide substrate, no incorporation of label into acid precipitable material was evident. This result suggests that the DNA polymerase γ is capable of copying only the poly (A) region of the rabbit globin mRNA. When the oligo (dT) primer was omitted, no incorporation of ^{3}H-dTTP was observed.

The inhibitors of DNA polymerase activity such as aphidocolin, N-ethylmaleimide, dideoxythymidine triphosphate (ddTTP), and phosphate are used to define and identify the various eukaryotic DNA polymerases (1,118). The placental enzyme is extremely sensitive to ddTTP at 2 uM in the presence of dTTP (molar ratio of 1.5:1) (Fig. 11). Animal DNA polymerase β and γ are very sensitive to ddTTP:dTTP ratios as low as 1:1 (50). However, unlike DNA polymerase β the placental DNA polymerase γ is relatively insensitive to increasing phosphate concentrations (Fig. 12). In

Table 3

Template Primer Utilization of Human Placenta DNA Polymerase γ

Template	^{3}H-labelled substrate	Polymerase activity %
poly (A)-$(dT)_{12-18}$ (43.5 ug/ml)	^{3}H-dTTP	100.0
poly (dA)-$(dT)_{12-18}$ (40 ug/ml)	^{3}H-dTTP	22.8
poly (C)-$(dG)_{12-18}$ (40 ug/ml)	^{3}H-dGTP	0.5
poly (dC)-$(dG)_{12-18}$ (40 ug/ml)	^{3}H-dGTP	2.9
poly (dG) (40 ug/ml)	^{3}H-dGTP	0.5
Activated DNA (150 ug/ml)	^{3}H-dTTP	8.1
mRNA-oligo $(dT)_{12-18}$ (40 ug/ml)	^{3}H-dTTP ^{3}H-dGTP	23.1 0.5
mRNA (20 ug/ml)	^{3}H-dTTP	0.5

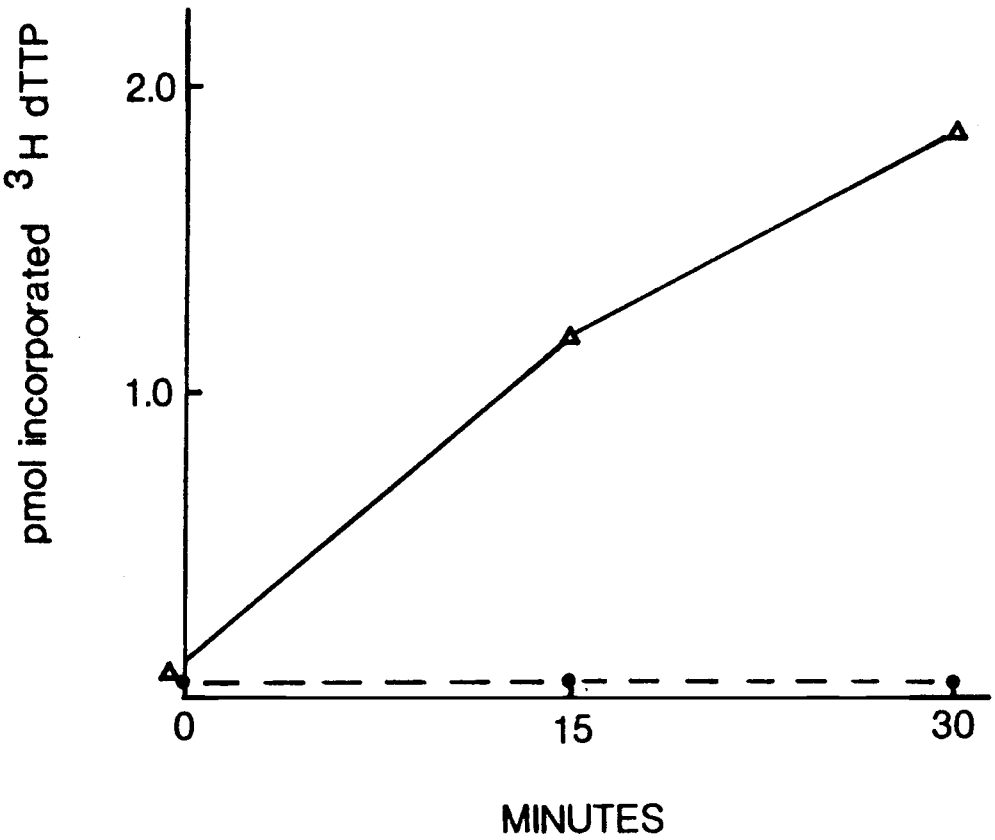


Figure 11. Effect of dideoxythymidine triphosphate (ddTTP) on DNA polymerase activity. Sensitivity of DNA polymerase to 2 uM ddTTP at various time points. Figures are depicted as follows: ▲—▲, control; and ●- - -●, 2 uM ddTTP.

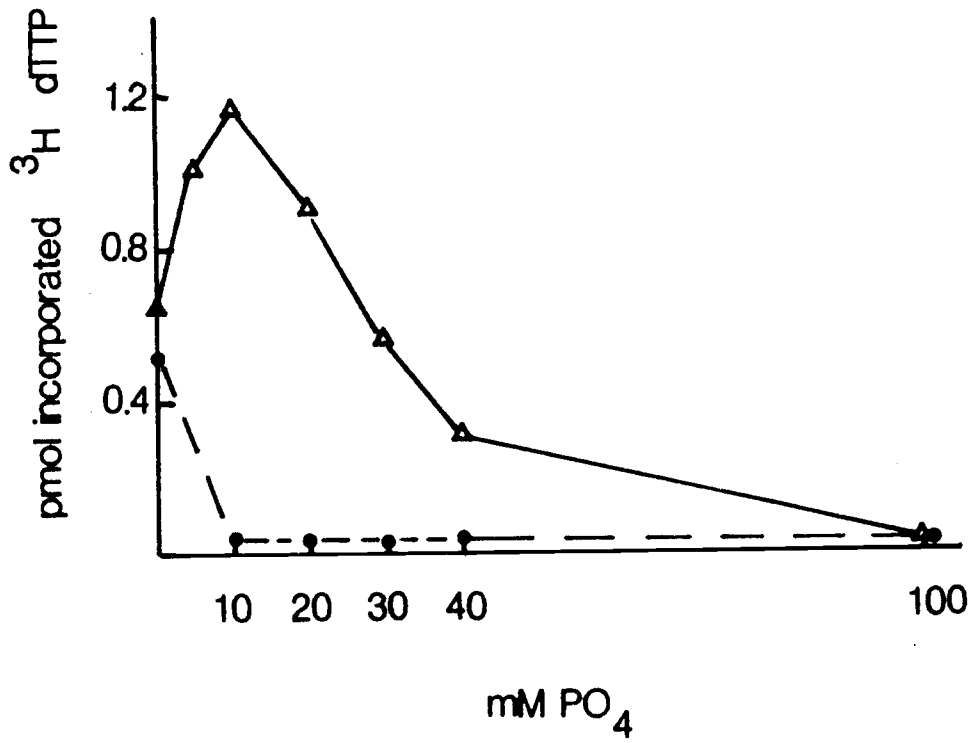


Figure 12. Effect of inorganic phosphate (PO_4) on DNA polymerase β and γ activity. Effect of increasing concentrations of PO_4 to DNA polymerase β (●--●), and DNA polymerase γ (Δ—Δ) activity.

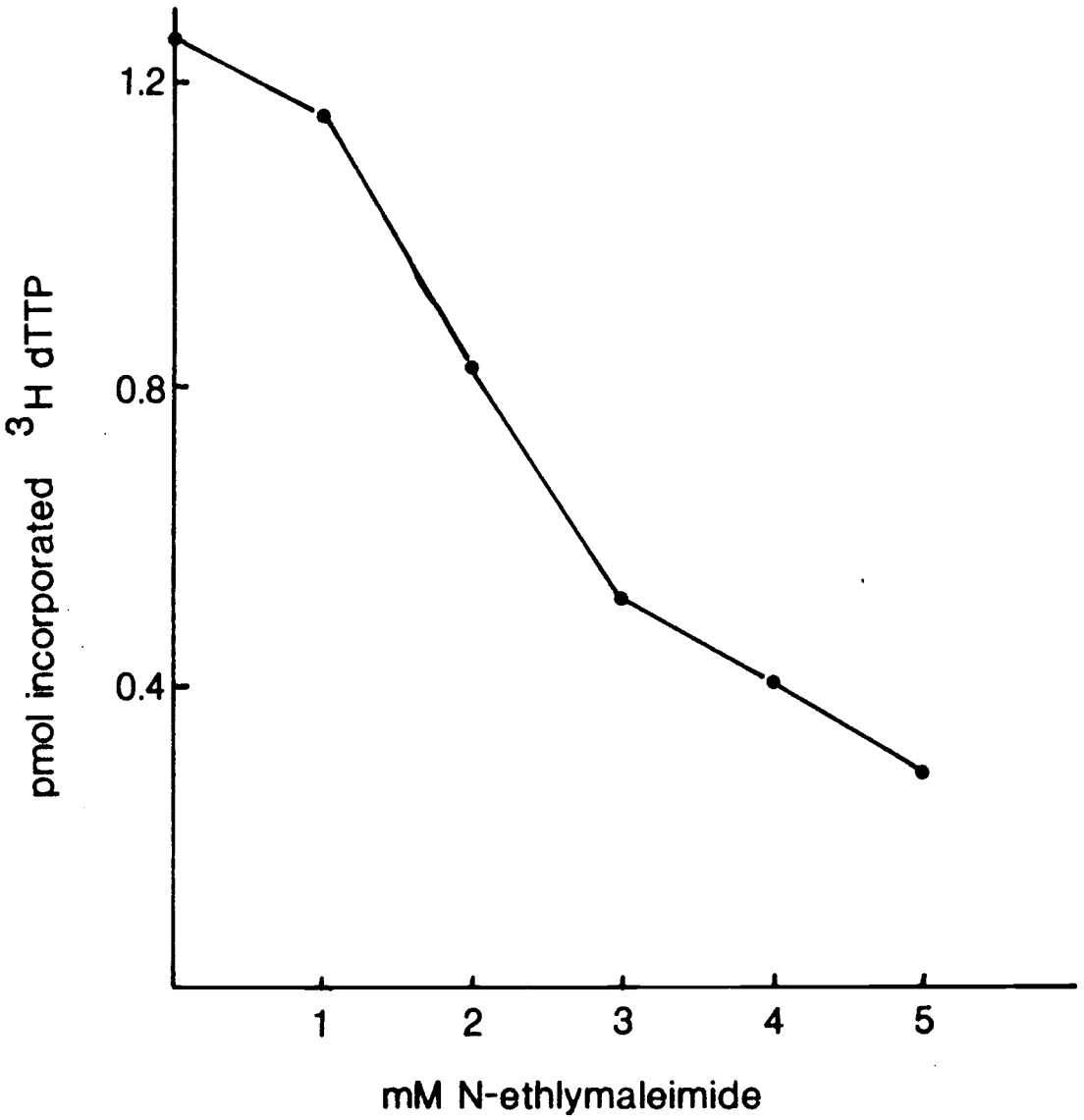


Figure 13. Effect of N-ethylmaleimide on DNA polymerase activity. Sensitivity of DNA polymerase to increasing concentrations of N-ethylmaleimide. All concentrations contained 0.195 ug of Fraction VII in 50 ul reactions using poly (A)-oligo $(dT)_{12-18}$.

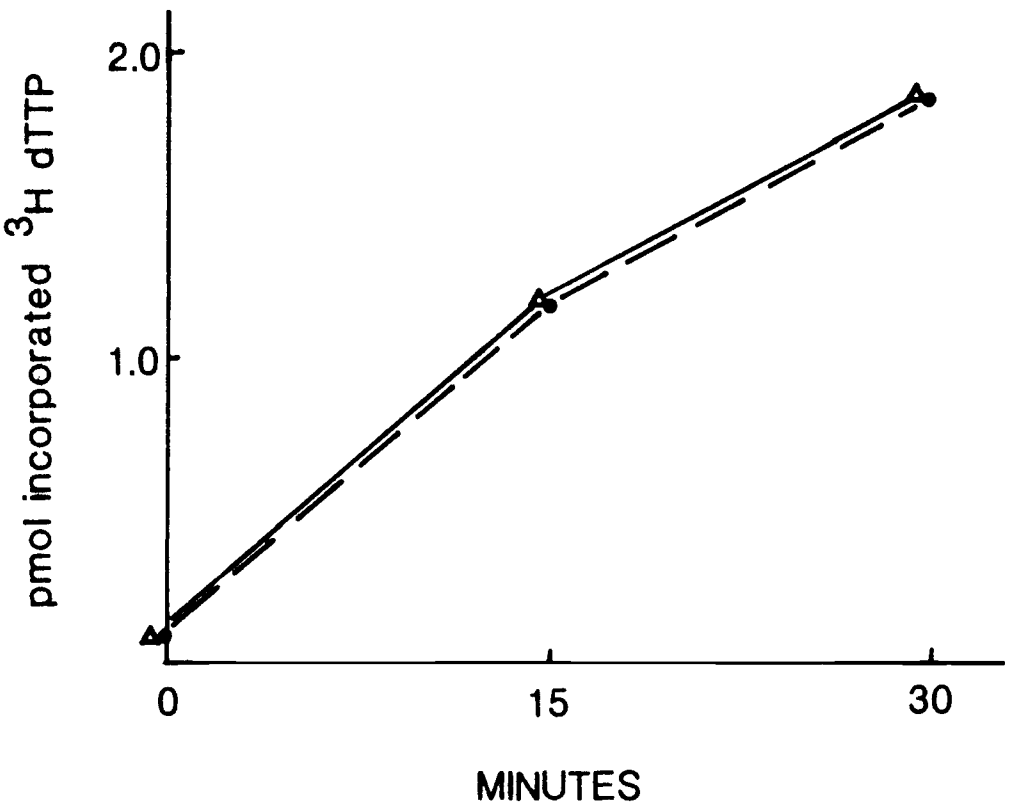


Figure 14. Effect of aphidocolin on DNA polymerase activity. In sensitivity of DNA polymerase to 5 ug/ml aphidocolin at various time points. Figures are depicted as follows: Δ—Δ, control; and •- - •, 5 ug/ml aphidocolin.

fact, 10 mM phosphate is actually stimulatory. The human placental DNA polymerase β was purified and tested for enzyme activity with poly (A)-oligo $(dT)_{12-18}$ (unpublished results). Although it does utilize this template efficiently, its activity is completely inhibited in the presence of 10 mM phosphate (Fig. 12).

The placental DNA polymerase γ is relatively insensitive to N-ethylmaleimide. At 1 mM N-ethylmaleimide the enzyme is inhibited by approximately 20% and at 5 mM the enzyme still exhibits 20% of the control activity (Fig. 13). Aphidocolin, a known specific inhibitor of the α class of eukaryotic DNA polymerases, had no effect on this polymerase (Fig. 14). These inhibitor studies strongly indicate that the purified human placental enzyme is a DNA polymerase γ .

The apparent K_m of purified human DNA polymerase γ for substrates was analyzed by the Hanes-Wolf method (Figs. 15 and 16). The K_m for dTTP was estimated to be 0.61 uM. This value is in agreement with the K_m values reported for γ polymerase isolated from chick embryos (14). The apparent K_m value for poly (A)-oligo $(dT)_{12-18}$ in the presence of two different concentrations of dTTP was 27 ug/ml.

Size Analysis Of DNA Polymerase γ By Glycerol Gradient Centrifugation

Glycerol gradients were run in order to estimate the size of the active placental enzyme. As seen in Fig. 17, the enzyme has a

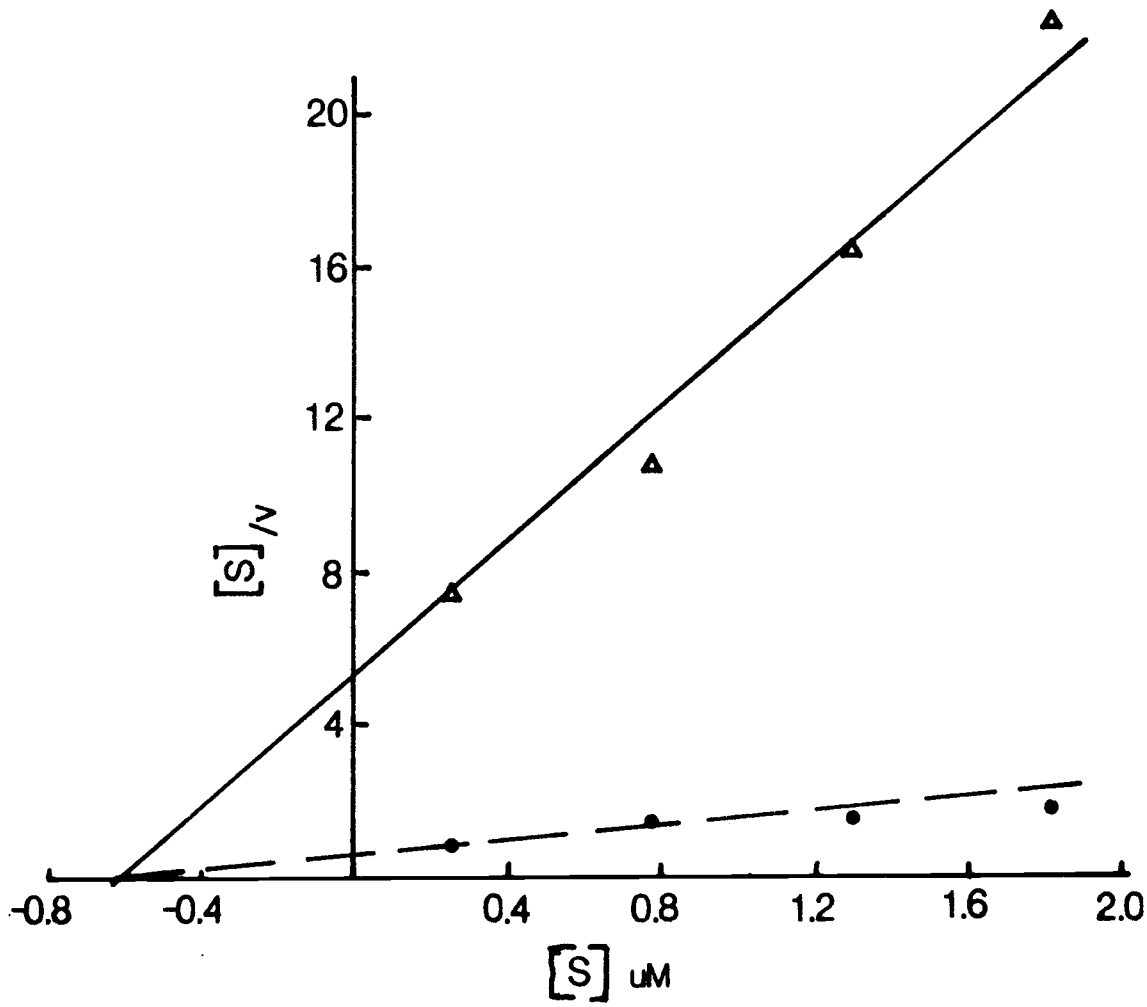


Figure 15. Hanes-Wolf plot for dTTP Km determination. The Km for dTTP was determined for DNA polymerase at two concentrations of poly (A)-oligo $(dT)_{12-18}$: 8.7 ug/ml (Δ—Δ), and 130 ug/ml (•- - -•).

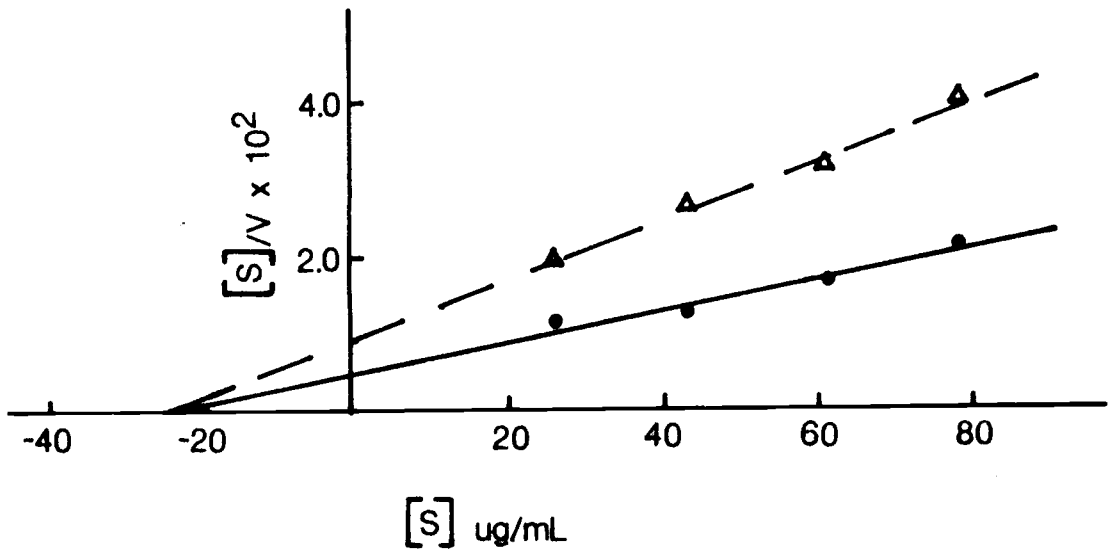


Figure 16. Hanes-Wolf plot for poly (A)-oligo $(dT)_{12-18}$ Km determination. The Km for poly (A)-oligo $(dT)_{12-18}$ was determined for DNA polymerase γ at two concentrations of dTTP: 0.26 uM (Δ--Δ) and 1.3 um (●—●).

sedimentation value of 9.7S in glycerol gradients prepared in 0.15 M KCl. This corresponds to a relative molecular weight of 400,000 for a globular protein using bovine serum albumin (4.2S) and rabbit muscle adolase (7.3S) as molecular weight standards. In an identical gradient containing 0.5 M KCl the enzyme sediments at 7.3S as shown in Fig. 18. It appears that in high salt conditions the enzyme has a relative molecular weight of 160,000 daltons. When both samples were analyzed in SDS-polyacrylamide gels, the 94,000 dalton band was the most prominent band in the active fractions (data not shown). Attempts were made to determine the sedimentation value of the enzyme in Fraction VII. However, no activity was recovered in the gradient fractions after centrifugation. This result may reflect the instability of the enzyme in Fraction VII.

Immunological Assessment

Purified DNA polymerase γ from Fraction VII was analyzed with neutralizing antibody prepared against human DNA polymerase α. The antibody was obtained from the hybridoma cell line, SJK 132-20, as described by Tanaka et al, (35). Antibody concentrations as high as 0.5 ug per reaction did not inhibit enzyme activity. At this concentration, DNA polymerase α from human KB cells is neutralized 80% of its original activity (30).

Isoelectric Focusing

Fraction VI was dialyzed in 60% (w/v) glycerol and applied to an isoelectric focusing column as described in Materials and

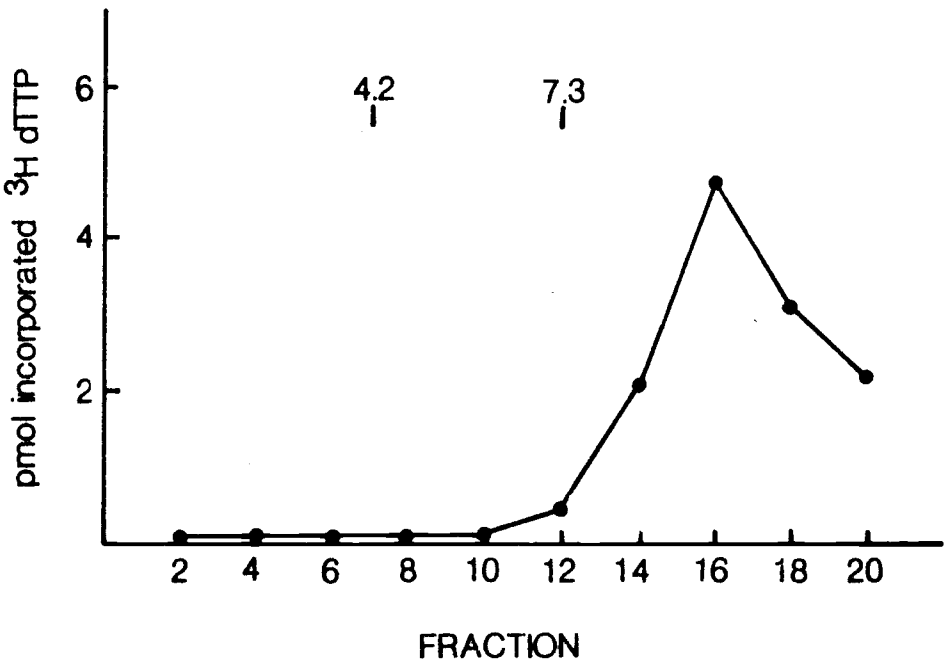


Figure 17. Low salt glycerol gradient centrifugation. Samples of Fraction VI were centrifuged in 10-30% glycerol gradients containing 0.15 M KCl as described in the Materials and Methods. Ten microliter aliquots from the fractions were assayed for DNA polymerase activity with poly (A)-oligo $(dT)_{12-18}$. The markers, bovine serum albumin (4.2S) and rabbit muscle aldolase (7.3S), were run in parallel gradients.

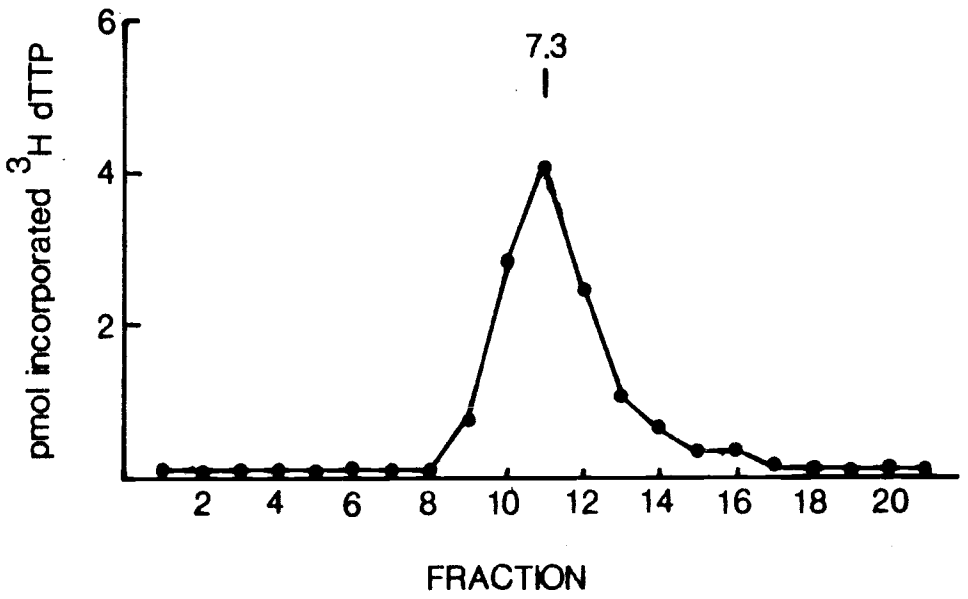


Figure 18. High salt glycerol gradient centrifugation. Samples of Fraction VI were centrifuged in 10-30% glycerol gradients containing 0.5 M KCl as described in the Materials and Methods. Ten microliter aliquots from the fractions were assayed as described earlier. Rabbit muscle aldolase (7.3S) was used as a size marker in a parallel gradient.

Methods. After 15 hours, fractions of 2 mls each were collected and assayed with poly (A)-oligo $(dT)_{12-18}$ (Fig. 19). Two peaks of activity were found at pH 8.0 and 6.8. To determine which peak corresponds to DNA polymerase γ, the sensitivity of each enzyme to increasing concentrations of inorganic phosphate was determined. The phosphate curve for the enzyme with pI = 8.0 was identical to that of DNA polymerase γ, while the enzyme activity at pI = 6.8 was extremely sensitive to phosphate.

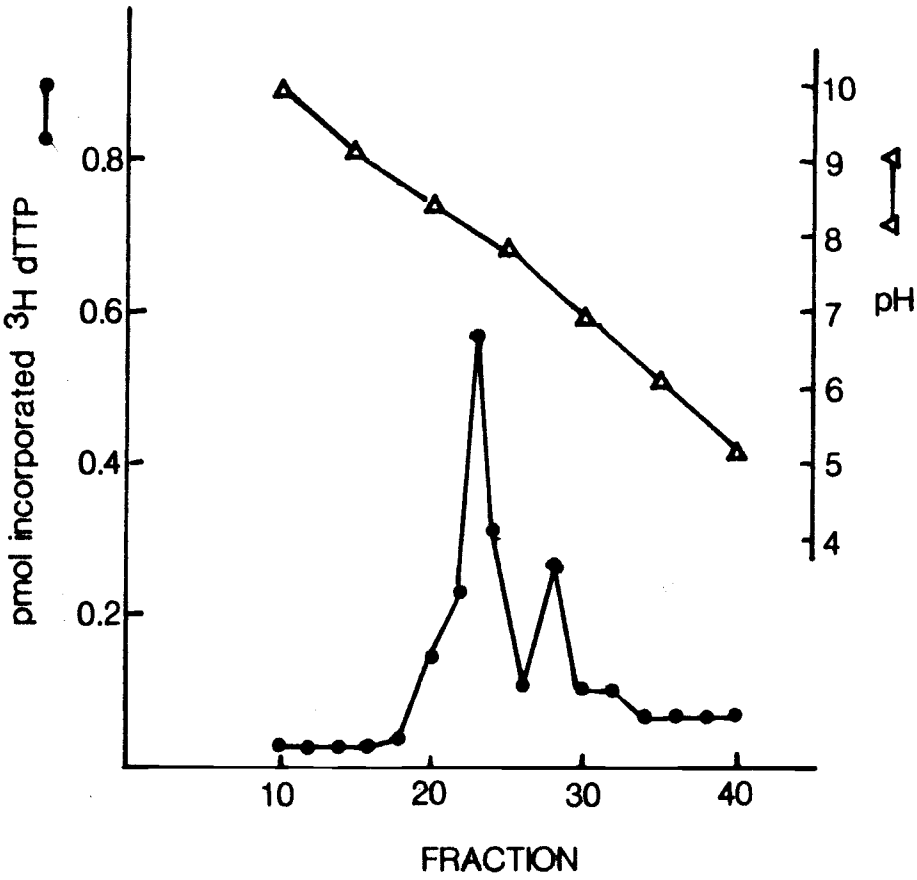


Figure 19. Isoelectric focusing. Isoelectric focusing of Fraction VI in a 0.60% (w/v) glycerol gradient, pH range 4-10. Ten microliter aliquots from each fraction were assayed for DNA polymerase activity with poly (A)-oligo $(dT)_{12-18}$ as described in the Materials and Methods.

DISCUSSION

The characterization of eukaryotic DNA polymerases requires pure enzyme preparations. Studies involving fidelity of transcription, template preferences, polymerase interaction with stimulatory or inhibitory factors, are difficult to interpret when the DNA polymerase used in these assays is not homogeneous. For this reason, we have purified the human placental DNA polymerase γ so that a single polypeptide band appears after SDS-polyacrylamide gel electrophoresis of the final enzyme extract. Our results indicate that the enzyme is active as a 94,000 dalton polypeptide.

Many purification schemes for eukaryotic DNA polymerases involve ammonium sulfate precipitation, DEAE- and phosphocellulose chromatography, and finally, DNA-cellulose chromatography (115). Presumably, these procedures lead to the effective separation of DNA polymerases α, β, and γ, and most reported purifications are complete after DNA-cellulose chromatography (10,16,115). However, we find that the Fraction VI enzyme preparation after DNA cellulose is contaminated with other enzyme activities and other polypeptides. In situ polymerase assays of the Fraction VI preparation after SDS-PAGE revealed 5 separate bands of activity (Fig. 7). When the same gel was stained with silver to detect the polypeptide bands, 8 bands developed. Only the 94,000 dalton polypeptide band coincided with a band of polymerase activity. The other 7 polypeptide bands did not have a corresponding DNA polymerase activity band. These results indicate that the in situ polymerase assay is extremely sensitive

because DNA polymerase activity can be detected even in the absence of a visible polypeptide band. The control studies show that commercial preparations of E. coli DNA polymerase I and the Klenow fragment is contaminated with DNA pol II and III. All four DNA polymerase activities are shown in the in situ polymerase gel. However, only DNA pol I and the Klenow fragment appeared as silver-stained polypeptides in the same gel. It is clear that in our hands, the DNA polymerase γ preparations are not homogenous after DNA-cellulose chromatography.

Further attempts at purification of Fraction VI was made by affinity chromatography of the extract through poly rA-sepharose. A homogeneous enzyme preparation was not achieved by this method since SDS-PAGE of the extract revealed 8 polypeptide bands (data not shown). The procedure did increase the specific activity of the enzyme by five-fold. However, the enzyme activity was extremely labile after this procedure and thus it was not used routinely in the purification of DNA polymerase γ .

Final purification was achieved with high pressure gel exclusion in Fractogel TSK. A 67,500-fold purification of the enzyme was achieved with this procedure. Analysis of this enzyme preparation by SDS-PAGE revealed a single polypeptide of 94,000 daltons after silver staining of the gel. The enzyme is moderately labile at this point and can be stored at -20°C in 50% glycerol for 10 - 20 days. We were unable to obtain DNA activity in situ after gel electrophoresis with this preparation. This result may be due to the enzyme

instability or due to the fact that the in situ polymerase reaction is done in the presence of activated DNA as a template. The purified enzyme may be in such low concentrations in the gel that its activity cannot be detected with the activated DNA template. The enzyme is more than ten times as active with poly (A)-oligo $(dT)_{12-18}$ than with activated DNA. We are in the process of developing conditions for running the in situ polymerase reaction in a mini-gel so that the costs for such studies are not prohibitive.

The human placental DNA polymerase γ resembles previously described γ polymerases in its: sensitivity to dideoxynucleotides and N-ethylmaleimide, resistance to aphidocolin, and stimulation by inorganic phosphate. Other characteristics such as pH optimum, ionic strength, divalent cation requirement and template-primer preferences are also similar to that reported for other γ polymerases (3,10,11,16). The K_m values for dTTP and poly (A)-oligo $(dT)_{12-18}$ are lower than that reported for the DNA polymerase γ isolated from chick embryo (14). This result may be due to the increased homogeneity of the placenta DNA polymerase preparation. This DNA polymerase γ does differ from other mammalian γ -polymerases by its alkaline pI of 8.0. Previously rat liver DNA polymerase was reported to be an acidic protein with a pI of 5.6 (4).

The molecular weight estimates for human DNA polymerase γ have ranged from 120,000 to 330,000 daltons (16,17). These discrepancies in size may be due to the enzyme's tendency to form aggregates in low salt. In high salt (0.5 M NaCl), DNA polymerase

from human placenta has a reported molecular weight of 150,000 daltons (119) by glycerol gradient analysis. Under similar conditions, human lymphoblast and HeLa cell DNA polymerase γ have molecular weights of 120,000 and 160,000 daltons respectively. In this study we report the placental polymerase to have a relative molecular weight of 160,000 daltons. Further analysis of the placental polymerase demonstrated that the enzyme activity was associated with a 94,000 dalton polypeptide and suggests that the enzyme exists as a dimer of this polypeptide during sedimentation in glycerol at 0.5 M KCl.

A 47,000 dalton polypeptide has been identified as the DNA polymerase in chick embryos (14) and mouse myeloma cells (15). A similar polypeptide has not been detected in the human placental enzyme preparations. This difference may be due to phylogenetic differences or it may be the result of using protease inhibitors in the human enzyme extraction. Further studies comparing these enzymes by tryptic peptide analysis and immunological reactivity should be conducted.

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