J. Biosci., Vol. 11, Numbers 1–4, March 1987, pp. 361-378. © Printed in India.

Resolution of DNA polymerase-α-primase complex and primase free DNA polymerase α from embryonic chicken brain

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Abstract DNA polymerase-α from embryonic chicken brain was resolved on DEAE-cellulose into 3 component activities that remained distinct upon rechromatography. Product formation by each activity required exogenously added template-primer DNA, all 4 deoxynucleoside triphosphates, and a divalent metal cation. Each form incorporated [^{3}H]-dTTP or [^{3}H]-dCTP into a high molecular weight product that was identified as DNA by its chromatographic behavior and its sensitivity to DNase. High ionic strength, N-ethylmaleimide, and the polymerase-α-specific inhibitor aphidicolin inhibited each activity; the apparent K_i, value of aphidicolin was 3·0 μM in each case. Based on these results, the 3 activities were identified as multiple forms of DNA polymerase-α . Experiments using embryonic chicken brains of various ages indicated that polymerase-α_1, and polymerase-α3 reached maximal activity in 9-day-old embryos, while polymerase-α_2 activity was elevated at a slightly later developmental stage. Using poly (dC) as template, high primase activity was detected in polymerase-α_1, fractions.

Keywords. DNA polymerase-α; aphidicolin inhibition; primase; N-ethylmaleimide inhibition; DNA chain initiation.

Introduction

DNA polymerase-α (pol-α) has been the focus of considerable attention because of its apparent involvement in cellular replicative DNA synthesis. In many studies of pol-α techniques used for partial purification of the enzyme have yielded reproducible multiphasic activity profiles suggestive of enzyme heterogeneity. While such results often were dismissed as artifactual, improved characterizations of the resolved activities revealed distinctions among their properties inconsistent with artificial separations of single enzymatic activities. Consequently, heterogeneity has been accepted as a feature characteristic of pol-α's (Hubscher, 1983).

Two forms of pol-α were first identified in extracts of calf thymus (Momparler *et al*., 1973). Subsequent investigations using this tissue described 3 (Yoshida *et al.,* 1974; Masaki and Yoshida, 1978) or 4 (Holmes *et al*., 1974) pol-α's. Multiple forms of pol-α also have been found in rat liver (Holmes *et al*., 1974), mouse myeloma (Hachmann and Lezius, 1975; Matsukage *et al*., 1976; Chen *et al*., 1979), *Drosophila* embryos (Brakel and Blumenthal, 1977, 1978), yeast (Chang, 1977; Wintersberger, 1978), HeLa cells (Ono *et al*., 1978, 1979; Lamothe *et al*., 1981), mouse mastocytoma (Bieri-Bonniot and Schurch, 1978), wheat embryos (Castroviejo *et al*., 1979), human

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Abbreviations used: Pol-α, Polymerase-α; PEG, polyethylene glycol; 2-ME, 2-mercaptoethanol; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethyl-sulphonyl fluoride; NEM, N-ethylmaleimide; DMSO, dimethyl sulphoxide.

neuroblastoma IMR-32 cells (Bhattacharya *et al*., 1981), and KB cells (Filpula *et al*., 1982). The detection of multiple forms in clonal cell lines was particularly significant because it eliminated the possibility that multiple forms of pol-α in tissues were contributed by different cell types within the tissues.

Despite the abundance of information regarding the heterogeneity of pol-α, no relationship between multiple forms and cell or tissue development has been established. Approaches to this question have been hindered by the static natures (complete differentiation or continuous growth) of most of the enzyme sources used. The growth dependent variation in total extractable pol-α activity (Simet, 1983) prompted this investigation of multiple forms of pol-α in developing embryonic chicken brain.

The multiple forms of DNA polymerases from various eukaryotic sources appear to contain a primase activity in addition to other peptides with unknown functions. The primase subunit closely associated with DNA polymerase-α initiates RNA primers before any DNA chain initiation in mouse tumor cells (Yagura *et al*., 1982; Kozu *et al*., 1986), *Drosophila* (Kaguni *et al*., 1984), *Xenopus laevis* (Shioda *et al*., 1982), human KB cells (Wang *et al*., 1984), Yeast (Plevani *et al*., 1985), calf thymus (Grosse and Kraus, 1985; Holmes *et al*., 1985), and human IMR-32 neuroblastoma cells (Takada *et al.,* 1986a, b).

Materials and methods

The following materials were acquired from the indicated commercial source: poly (rA) $(dT)_{12–18}$ (20:1), P-L Biochemicals, Milwaukee, Wisconsin, USA; DE-23 diethylaminoethylcellulose, Whatman Ltd., Springfield, England; polyethylene glycol (PEG; average molecular weight 20,000), and *Streptomyces griseus* Pronase (type VI), Sigma Chemical Company, St. Louis, Missouri, USA; Sephadex G-100 and blue dextran 2000, Pharmacia Fine Chemicals, Piscataway, New Jersey, USA; bovine pancreatic ribonuclease (RNase), Worthington Biochemicals, Freehold, New Jersey, USA; [*methy*-^{3}H] -thymidine 5'-monophosphate (dTMP; 18 Ci/mmol), Amersham Corporation Arlington Heights, Illinois, USA; [^{3}H]-DNA (IMR-32), gift from Dr. Prabir Bhattacharya, Notre Dame, Indiana, USA; diphenylamine, J. T. Baker Chemical Company, Phillipsburg, New Jersey, USA; and aphidicolin, gift from the Drug Synthesis and Chemistry Branch, Department of Therapeutic Pharmaceuticals, Division of Cancer Treatment of the National Cancer Institute, Bethesda, Maryland, USA. All other reagents were of the highest purity available.

Assays for DN A polymerases

The complete assay incubation mixture for pol-α contained the following components in a total volume of 0·1 ml; 10 μg of activated calf thymus DNA; 50 mM Tris-HCl, p H8·5; 5 mM $MgCl_2$; 20 mM KCl; 5 mM 2-mercaptoethanol (2-ME); 50 μg of bovine serum albumin (BSA); 50 μM [^{3}H] -dTTP [specific activity, 120-200 cpm/pmol], dATP, dCTP, and dGTP; and enzyme (10–25 μg of protein as estimated by the method of Lowry *et al.* (1951)). The mixtures were incubated at 37°C for 30 min, after which the reaction was stopped by adding 50 μg of herring sperm DNA followed by ice-cold 10% trichloroacetic acid containing 0·1 M sodium

pyrophosphate. The mixtures were kept at 4°C for 15 min and then filtered through Whatman GF/C borosilicate glass discs in a Millipore apparatus. The discs were dried at 80°C, and [^{3}H]-dTMP incorporation was quantitated using a toluene-based mixture in a Beckman LS-3300-T liquid scintillation counter.

For pol -*β,* the above reaction mixture was used except that the 2-ME was omitted and 10 mM N-ethylmaleimide was added. For pol-γ, the activated calf thymus DNA was replaced with 2·5 μg of poly(rA)·(dT) $_{12–18,}$ the KCl concentration was increased to 50 mM, the nonradioactive dNTPs were omitted, and 50 mM K–PO_4, pH 8·5, was added. In assays for terminal deoxynucleotidyltransferase, 2·5 μg of poly(dA) replaced the activated DNA; 50 mM Tris-HCl, pH 7·0, replaced 50mM Tris-HCl, pH 8·5; 0·5 mM $MnCl_2$ replaced the $MgCl_2$; KCl and the unlabelled dNTPs were omitted; and [^{3}H]-dGTP (specific activity, 200 cpm/pmol) was used as substrate. The complete assay mixture for pol-γ contained the following components in a total volume of 0·1 ml: 2·5 μg of poly(rA)·(dT) $_{12–18;}$ 50 mM Tris-HCl, pH 8 ·5; 5 mM $MgCl_2$; 50 mM KCl; 5 mM 2-ME; 50 μg BSA; 50 μM [^{3}H] -dTTP (specific activity, 120 to 250cpm/pmol); 50mM potassium phosphate (K-PO_4), pH 8·5 (to suppress pol-*β* activity); and 10–25 μg enzyme protein [estimated according to Lowry *et al.* (1951)]. The reaction mixture was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 50 μg herring sperm DNA and ice-cold 10% (w:v) trichloroacetic acid containing 0·1 M sodium pyrophosphate. After chilling in ice for 20 min, the reaction mixtures were filtered through DE-81 diethylaminoethyl paper filter disks (Whatman Ltd.) in a Millipore apparatus. The disks were dried and counted as described for pol-α and pol -*β*. Counting efficiency was approximately 10%.

Reaction rates observed with each resolved activity were proportional to all protein concentrations used and remained constant with incubation times up to 45 min.

Separation of multiple forms of DNA polymerase-α

A portion of sonicated (100,000 *g)* supernatant [50mM Tris-HCl buffer, pH 8·0; 6mM KCl; 2mM $MgCl_2$; 5 mM dithiothreitol (DTT); 1 mM ethylenediaminetetracetate (EDTA); 0·5 mM phenyl methyl sulphonylfluoride (PMSF)] prepared from 9-day-old embryonic chicken brains, containing 10 to 14 mg protein, was applied to a DEAE-cellulose column (2 × 11 cm) that had been equilibrated with 50 mM K-PO_4, pH 7·6, containing 1 mM DTT. After nonadsorbed protein had been washed from the column with 100 ml of the equilibration buffer, the multiple forms of pol-α were eluted with a 40-ml linear buffer gradient of 50–350 mM K- P O_4, pH 7·6, containing 1 mM DTT. A flow rate of 1 ml per min was maintained while fractions of approximately 1 ml were collected. Each fraction was assayed for DNA-polymerizing activities as described above. To fractions containing pol-α activity were added 15 μg herring sperm DNA. These fractions then were made 50% (v: v) in glycerol or 40% (v:v) in ethylene glycol before storage at –18°C.

Rechromatography of multiple forms of DNA polymerase-α.

Pol-α-containing fractions to be rechromatographed were used immediately after

resolution and therefore contained no added DNA, glycerol, or ethylene glycol. After the fractions had been diluted with H_2O to adjust their K-PO4 concentrations to 50 mM, each was applied separately to a DEAE-cellulose column (1×8 cm) prepared as described above. The column was washed and developed as described above except that buffer volumes were halved. Fractions were assayed for pol-α activity.

Isolation of reaction products

Incubations using [^{3}H]-dTTP-containing pol-α reaction mixtures were stopped by addition of EDTA, pH 7·6, to a final concentration of 10 mM. The mixtures were pooled and applied to a Sephadex G-100 column (1×52 cm; void volume, 17 ml, determined from an elution profile of blue dextran 2000) that had been equilibrated with 15 mM NaCl. The column was developed with the same eluent at a flow rate of 0·2 ml per min. Fractions (1 ml each) were assayed for radioactivity by spotting aliquots on GF/B borosilicate glass fiber disks (Whatman Ltd.) and quantitating [^{3}H]-label as described for DNA polymerase assays. Void volume fractions were pooled and concentrated by dialysis against soild PEG.

Paper chromatography of reaction products

Samples containing concentrated reaction products were spotted on SG-81 silica gel-impregnated paper (Whatman Ltd.) and dried. [^{3}H] -DNA from IMR-32 (10 μg), [^{3}H] -dTMP (approximately 1 nmol), and [^{3}H]-dTTP (approximately 1 nmol) were run concurrently as standards. Chromatograms were developed in an ascending manner with 95% ethanol/l M ammonium acetate, pH 7·3 (7:3, v:v). The chromatogram was dried and each lane was cut into 1 " squares. Radioactivity was quantitated using the toluene based mixture described for DNA polymerase assays.

Enzymatic hydrolysis of reaction products

The complete reaction mixtures contained the following components in a final volume of 0·1 ml: reaction product, 400–900 cpm; Tris-HCl, pH 7·5, 50 mM; $MgCl_2$, 5 mM; $CaCl_2$, 5 mM (used only with Pronase); and enzyme, 1–10 μg per ml. The mixtures were incubated at 37°C for 5–30 min, and reactions were stopped by the addition of 50 μg herring sperm DNA and ice-cold 10% (w: v) trichloroacetic acid containing 0·1 M sodium pyrophosphate. The remaining acid-precipitable product was quantitated on GF/C disks as described for DNA polymerase assays.

Analytical Methods

Protein was quantitated according to Lowry *et al.* (1951) using BSA as a standard. The DNA contents of protein-free mixtures were estimated from absorbance at 260 nm after the mixtures had been heated at 90°C for 10 min to denature the DNA; an absorbance of 20·0 was assumed to represent 1 mg DNA per ml. The DNA contents of less pure samples were assessed by the method of Burton (1956), using herring sperm DNA as a Standard.

Results

Separation of multiple forms of DNA polymerase-α

Chromatography on DEAE-cellulose resolved several DNA-polymerizing activities in embryonic chicken brain extracts. The pol-*β* resistant to N-ethylmaleimide (NEM) was not adsorbed to the resin and was recovered in the wash fraction. Pol-γ, identified by its efficient copying of poly (rA)·$(dT)_{12–18,}$ was eluted at approximately 140 mM K-PO_4. Three other forms of DNA-polymerizing activity also were identified. Pol-α_1 was eluted at 160–165 mM K-PO_4, pol-α_2 at 220 mM K-PO_4, and pol-α_3 at 260–270 mM K–PO_4 (figure 1). Concentrations of K–PO_4 up to 500 mM failed to elute any additional enzymatic activities. Extracts from both 9-and 13-day-old embryonic chicken brains yielded this chromatographic profile. Separation of the 3 forms resulted in a 30- to 40-fold purification of the DNA polymerase activities, with a total yield of 60–70% (table 1).

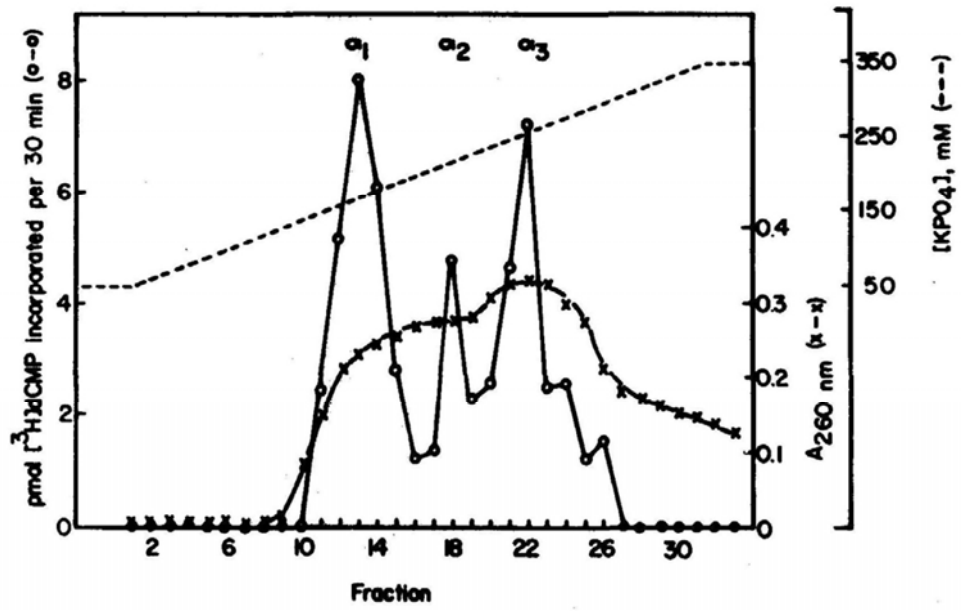


Figure 1. Resolution of multiple forms of DNA pol-α from 9-day-old embryonic chicken brain. An aliquot of 9-day-old embryonic chicken brain sonicated supernatant containing 14 mg protein was applied to a DEAE-cellulose column (2×11 cm) that had been equilibrated with 50 mM K-PO_4 pH 7·6, containing 1 mM DTT. After nonadsorbed protein had been washed from the column with the same buffer, the 3 pol-αs were eluted with a 40-ml linear buffer gradient of 50–350 mM K- PO_4, pH 7·6, containing 1 mM DTT. Fractions of 1·1 ml were collected and assayed for pol-α activity. (o), Pol-α activity (in picomoles of [^{3}H]-dCM P incorporated into activated DNA per 30 min); (x), absorbance at 260 nm; (- - -), K–PO_4 concentration.

A fourth activity, infrequently observed, was eluted at 195 mM K– PO_4. In each instance, the presence of this activity was accompanied by a decline in at least one of the other forms. The fourth activity was assumed to have been generated artificially from the other activities and therefore was not investigated further.

Table 1. Separation of DNA polymerases from 9-day-old embryonic chicken brain.

Fraction	Volume (ml)	Total units	Units per mg protein	Yield (%)
Homogenate	6·8	6195	71·6	100
Sonicate	6·8	8969	108·0	145
Sonicated supernatant	5·1	5600	195·8	90
Pellet	1·0	774	35·6	12
Pol-α_1	3·3	3058	2315·0	49
Pol-α_2	1·1	757	1146·3	12
Pol-α_3	2·2	1888	2861·0	30
Total DEAE fractions		5703		91

Activities were measured using the activated calf thymus DNA-based assay for pol-α described in 'materials and methods'. Protein was quantitated according to Lowry *et al.* (1951).

Enhancement of purification by selective enrichment of pol-α activity was attempted using a PEG precipitation procedure (Duguet *et al.*, 1978). The sonicated supernatant was made 2 M in NaCl and a high molecular weight protein fraction was precipitated by addition of PEG to 10% (w:v). The precipitate was collected, redissolved, and dialyzed to remove NaCl and PEG. This procedure reduced the yields of pol-α by 70–90% and therefore was not included in subsequent preparations.

Extract preparation in buffers of pH 8·5 or in the presence of EDTA or sucrose had no effect on yields or elution profiles. Also, no differences in elutive strengths or relative peak areas accompanied omission of the serine protease inhibitor PMSF from the homogenizing buffers. All 3 forms of enzymatic activity were present in both sonicated and unsonicated homogenates.

*Rechromatography of multiple forms of DNA polymerase-*α

Each activity yielded a single major peak of activity upon rechromatography on DEAE-cellulose (figure 2). No evidence of any significant secondary activities was apparent. The K–PO_4 concentrations required for elution of each of the forms were unchanged upon rechromatography. The recoveries of enzyme units applied were 78% for pol-α_1, 59% for pol-α_2, and 52% for pol-α_3.

*Stability of multiple forms of DNA polymerase-*α

DNA-polymerizing activity in the supernatant was fairly stable at 4°C. At – 70°C, the resolved activities were completely stable for 2-3 weeks in the presence of 0·1% 8K PEG and 20% glycerol. Addition of 2 mM $MgCl_2$ or 0·5 mM PM SF contributed little additional stabilization. However, the 3 forms individually were markedly unstable at 4°C with nearly total loss of activity within 20 h in the absence of 0·1% PEG. Addition of BSA to the fractions had no effect on the life-times of the activities. Virtually all of the initial enzymatic activities in the supernatant could be recovered after 2–4 weeks' storage at — 18°C if the fractions were made 50% (v: v) in glycerol

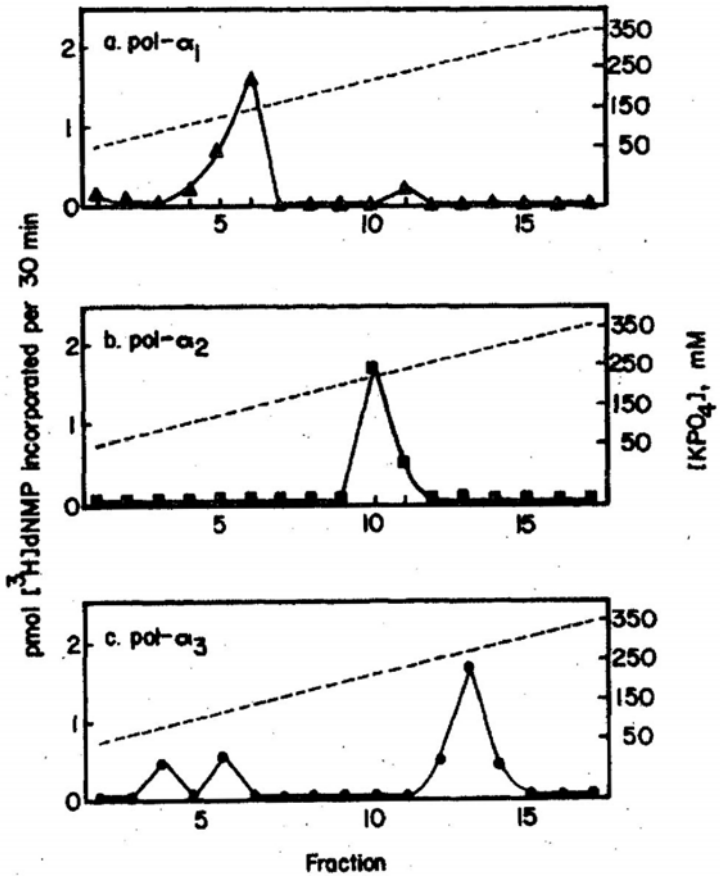


Figure 2. Rechromatography on DEAE-cellulose of multiple forms of DNA pol-α from embryonic chicken brain. Fractions containing multiple forms of embryonic chicken brain pol-α were diluted with H_2O to adjust their K-PO_4 concentrations to 50 mM and were applied separately to a DEAE-cellulose column (1 × 8 cm) that had been equilibrated with 50 mM K–PO_4, pH 7·6, containing 1 mM DTT. The column was washed and developed as described in figure 1 except that a 20-ml gradient volume was used, (a), Pol-α_1 ▲; (b), pol-α_2 ■; (c), pol-α_3 ●;(– – –),K–PO_4.concentration.

or 40% (v:v) in ethylene glycol prior to storage. Moreover, addition of herring sperm DNA to a concentration of 15 μg per ml helped maintain initial activity levels in the glycerolic fractions. The additional stabilization was not due to the increased concentration of potential substrate DNA in the assay mixtures, as the endogenous activities of the fractions remained negligible.

Requirements for enzymatic activity

The requirements for product formation were found to differ little among the 3 forms (table 2). Each activity was strictly dependent on exogenously added activated DNA. Also, omission of a single dNTP reduced DNA synthesis by 55–80%, and essentially no enzymatic activity was observed if 2–3 dNTPs were lacking. Dependence of the

Table 2. Requirements for enzymatic activity.

Reaction mixture	[^{3}H]-dNMP incorporated pol-α_1	pol-α_2	pol-α_3
	(pmol/mg protein/h)		
Complete	480·8	465·6	1258·0
— Activated DNA	0·0	0·0	0·0
— KCl	0·0	283·8	402·8
— $MgCl_2$	446·8	454·8	359·4
— $MgCl_2$, + $MnCl_2$ (1 mM)	257·4	113·0	375·4
— $MgCl_2$, + EDTA (10 mM)	38·6	0·0	0·0
— 2 – ME	271·4	204·8	568·0
— 2 – ME, + NEM (10 mM)	29·2	139·6	0·0
— BSA	43·2	0·0	127·6

The complete incubation mixtures contained the following components in a total volume of 0·1 ml: 20 μg activated calf thymus DNA; 20 mM KCl; 5 mM $MgCl_2$; 50 mM Tris-HCl, pH 8·5; 5 mM 2-ME; 50 μg BSA; and 50 μm [3H]-dTTP[2] and each unlabeled dNTP.

enzymatic activities on a divalent metal cation was suggested by their complete sensitivities to EDTA, although similar decreases in DNA synthesis could not be obtained by omission of exogenously added $MgCl_2$. Each enzymatic form was most active with $MgCl_2$, and addition of $MnCl_2$ to reaction mixtures prepared without $MgCl_2$ failed to restore optimal activity.

*Characterization of [^{3}H]-labelled products synthesized by the multiple forms of DNA polymerase-*α

Two components with strong absorbances at 260 nm were resolved by Sephadex G-100 chromatography of the reaction mixtures (figure 3a). Over 95% of the initial radioactivity of the reaction mixtures was recovered in the included component, which was identified as a mixture of deoxynucleoside mono- and tri-phosphates by paper chromatography. The excluded material contained radioactivity only if enzyme had been added to the reaction mixture; enzyme-free control reaction mixtures yielded a non-radioactive excluded fraction of material absorbing at 260 nm (figure 3b). Like [^{3}H] -DNA from IMR-32 cells, the labelled products failed to move from the origin upon paper chromatography on SG-81 paper in ethanol/ammonium acetate. The excluded fractions therefore were identified as DNA into which [^{3}H]-labelled dNMP residues had been incorporated by the enzymatic activities.

Following concentration, each of the excluded components was treated with hydrolytic enzymes. DNase rendered 70–80% of the [^{3}H]-labelled materials acid-soluble after 5 min at 37°C; only slightly more solubilization was effected by longer incubation (table 3). Neither RNase nor Pronase solubilized more than 5–7% of the [^{3}H] -labelled materials, even at 10-fold greater concentrations and with longer incubations.

*Inhibition of multiple forms of DNA pol-*α *by high ionic strength*

Each of the 3 forms was inhibited by increasing concentrations of NaCl (figure 4).

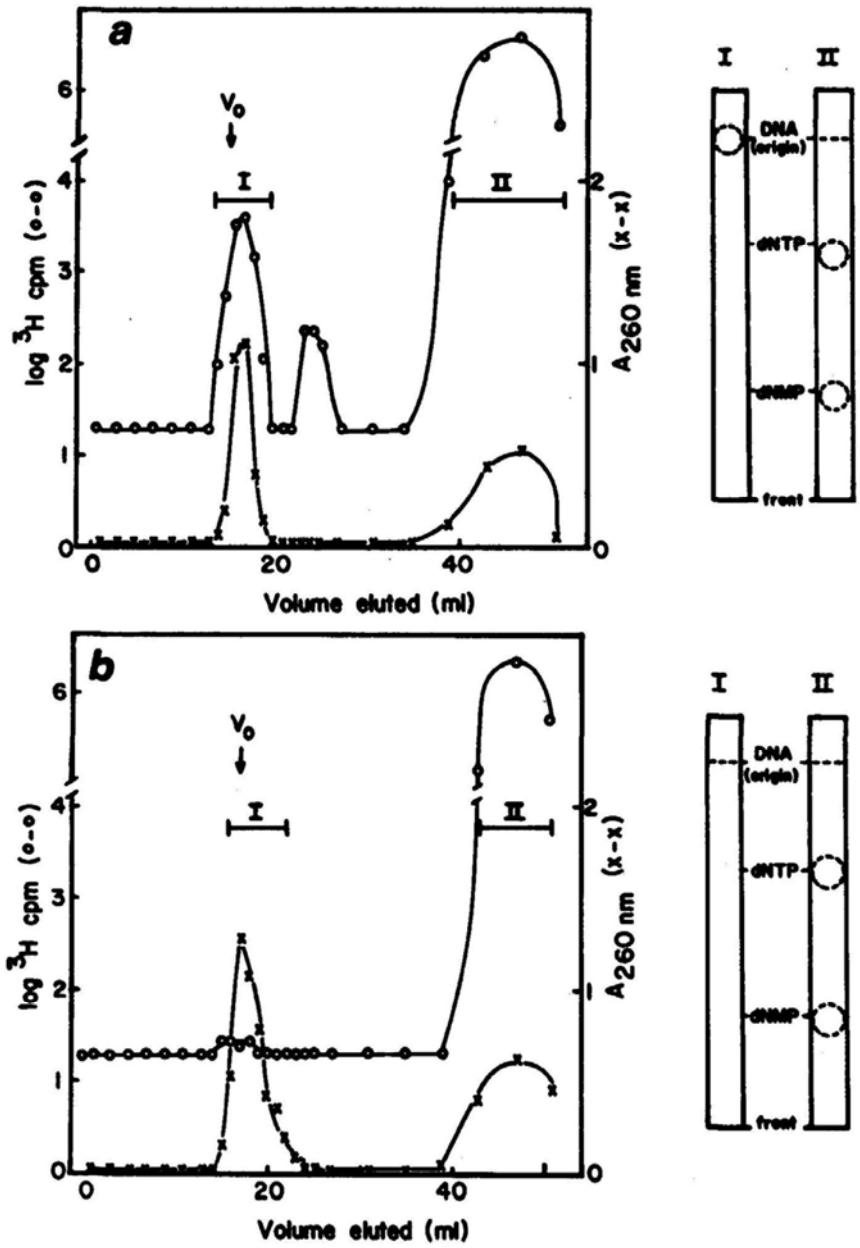
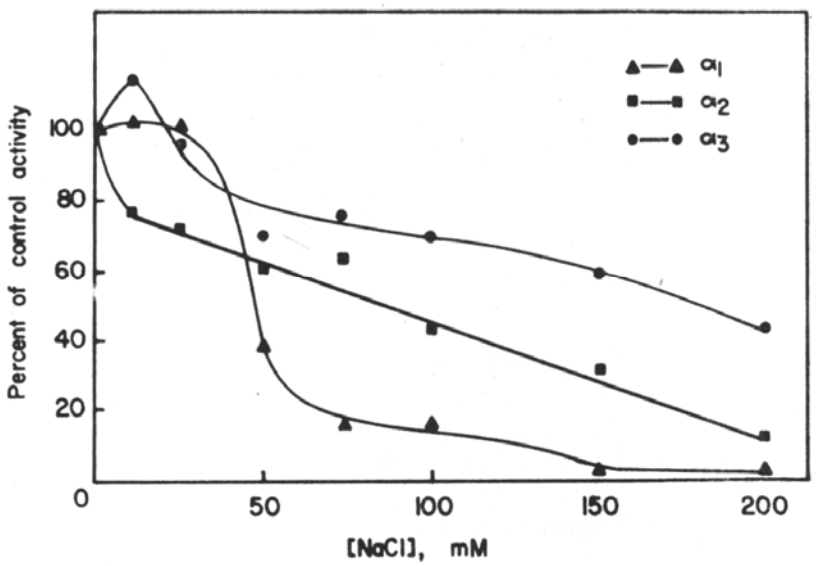


Figure 3. Isolation and partial characterization of ^{3}H-labelled products synthesized by DNA pol-α_1 from embryonic chicken brain. Pol-α assay mixtures stopped by the addition of EDTA were chromatographed on Sephadex G-100, using 15 mM NaCl as the eluent. Excluded peaks (I) and included peaks (II) absorbing at 260 nm were pooled as indicated by the bars. After concentration of the excluded material, samples of peaks I and II were chromatographed on SG-81 paper in an ascending fashion using 95% ethanol/1 M ammonium acetate, pH 7·3 (7:3, v: v), (a), Pol-α , product; (b), enzyme-free reaction mixture. (V_o), void volume [^{3}H]-counts per 0·1 ml; (x), absorbance at 260 nm. The resulting profiles of [^{3}H]-label are illustrated at the right of the panels, with the migrations of standards indicated.

Table 3. Enzymatic digestions of [3H] -labelled reaction products.

Enzyme added	Concentration	Rendered acid-soluble Pol-α_1	Pol-α_2	Pol-α_3
			%	
None		0	0	0
DNase I	1 μg/ml	72	82	77
Pancreatic RNase	10 μg/ml	7	6	2
Pronase	10 μg/ml	4	3	5

Partially purified [^{3}H]-labelled reaction products were concentrated by dialysis against solid PEG and then were treated with hydrolytic enzymes for 15 min at 37°. Total acid-precipitable materials in the untreated samples were: pol-α_1 product, 442 cpm; pol-α_2 product, 908 cpm; pol-α_3 product, 817 cpm.



Figured 4. Inhibition by NaCl of multiple forms of DNA pol-α from embryonic chicken brain. One hundred percent activity represents the following values, in picomoles of [^{3}H]-dTMP incorporated into activated DNA per mg protein per h. ▲650 cpm for pol-α_1; (■), 500 cpm for pol-α_2 ;(●)1200 cpm for pol-α_3.

Pol-α_1 activity was abolished completely by 150 mM NaCl. At 200 mM NaCl, pol α_2 and pol-α_3 were inhibited 85 and 50%, respectively. For each form, 50% of maximal inhibition was observed at 50 mM NaCl.

A mixture of the 3 forms was inhibited 50% by 25 mM K-PO_4 and 100% by 100 mM K-PO_4; in contrast, pol -β, obtained from the wash fraction during DE-23 chromatography, was completely inhibited by 25 mM K-PO_4.

Inhibition of multiples forms of DNA polymerase α by N-ethylmaleimide

The efects of NEM on the enzymatic activities were assessed using assay mixtures lacking 2-ME to avoid neutralization of the NEM. Ally 3 forms were inhibited by NEM (figure 5). Pol-α_3 activity was the most sensitive, exhibiting 50% inhibition at 0·25 mM NEM and inhibition at 2·5 mM NEM. Pol-α_2 and pol-α_3 activities were inhibited 100% by 15 mM NEM, while pol-α_2 could not be totally inhibited by any NEM concentration up to 15 mM. All 3 enzymatic activities were completely abolished by pre-incubation with 2·5 mM NEM at 4°C for 10 min (data not shown).

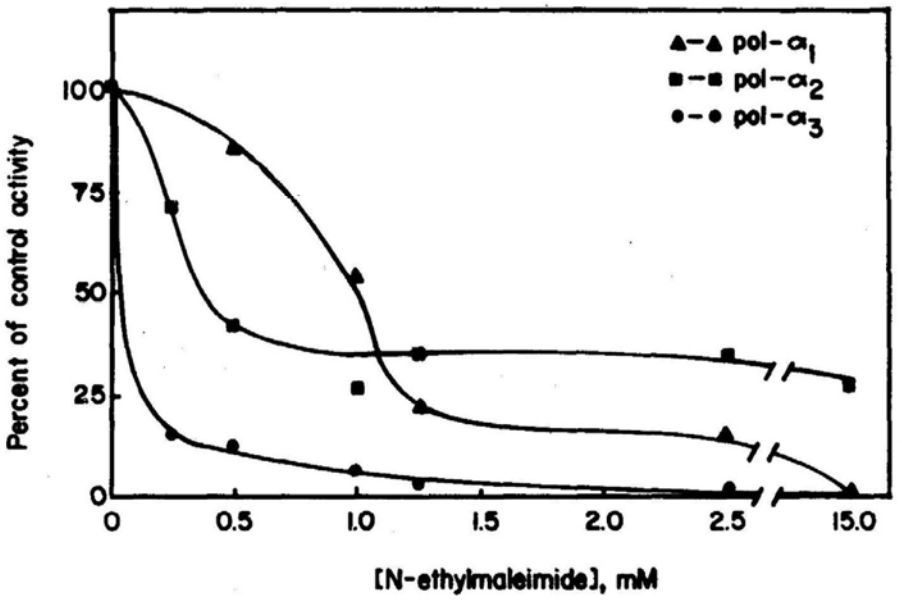


Figure 5. Inhibition by NEM of multiple forms of DNA pol -á from embryonic chicken brain. Assay mixtures were prepared without 2-ME to avoid neutralization of the NEM. One hundred percent activity presents the following values picomoles of [^{3}H]-dTMP incorporated into activated DNA per mg protein per h. (▲), 660 cpm for pol-á$_1$; (■), 500 cpm for pol-α_2; (●), 2240 cpm for pol-α_3.

Inhibition of multiple forms of DNA polymerase α by aphidicolin

In initial experiments with aphidicolin, aliquots from a stock solution of 400 μg aphidicolin per ml of 25% (v: v) aqueous dimethyl sulphoxide were added to assay mixtures to yield the desired final concentrations. Under these conditions, dimethyl sulphoxide had little effect on low levels of DNA syntheses. However, 2·5% DMSO inhibited DNA-polymerizing activities by 20–40% at saturating dCTP concentrations. Similar but less pronounced inhibition of *Xenopus* ovarian pol-α has been reported (Zimmerman *et al*., 1980). To circumvent this problem, a stock solution of 400 μg aphidicolin per ml of 10% (v:v) aqueous DMSO was used, and all assay

mixtures were adjusted to 0·25% DMSO. Inhibition of pol-α_1 by 0·25% DMSO averaged 7% and did not exceed 13%, and neither pol-α_2 nor pol-α_3 was affected.

Under the latter conditions, aphidicolin inhibited each of the 3 activities to similar extents (figure 6a). For each form of pol-α, enzymatic activity was measured in the presence of 6 concentrations of aphidicolin and 5 dCTP concentrations; representative data are illustrated. Each activity was reduced 50% by 6–10 μm aphidicolin at 1 μm dCTP and by 20–30 μM aphidicolin at 5 μM dCTP. At 50 μM dCTP, none of the activities was inhibited more than 30% by aphidicolin concentrations up to 30 μM.

In double-reciprocal plots of data from the aphidicolin inhibition experiments, aphidicolin appeared to be a competitive inhibitor of each of the enzymatic activiites (Figure 6b). The slopes of the double reciprocal lines, obtained from a line-fitting program based on least squares analysis run on an Apple II computer, were directly proportional to inhibitor concentrations, indicating that the mode of inhibition in each instance was linearly competitive. The K_i values of aphidicolin, calculated from the double-reciprocal plots, were 2·5μm for pol-α_1, 3·1 μM for pol-α_2, and 3·0 μM for pol-α_3.

Characterization of DNA pol-α_1 primase complex

The presence of primase activity in the 3 active DNA poly-α forms were tested using poly (dC) template and rGTP as a donor for the primer synthesis followed by DNA chain extension with [^{3}H]-dGTP (Takada *et al*., 1986b). It appeared that the column fraction that contained peak activity of DNA pol -α_1 (tested with ACT-DNA) also had the highest primase activity (figure 7).

Discussion

The association of elevated pol-α activity with rapid growth has stimulated the study of this enzyme in developing and differentiating systems. In only one of these systems, *Drosophila* embryos with multiple forms have been described (Brakel and Blumenthal, 1977, 1978). *Drosophila* enzyme III, which sedimented at 9·0.S, was enriched in early embryonic development (Brakel and Blumenthal, 1977), when total extractable pol-α activity was at a maximum (Margulies and Chargaff, 1973; Karkas *et al*., 1975). Conversion of the 9·0.S enzyme to smaller and possibly less active species therefore appeared to accompany a decline in pol-α activity throughout development.

To address further the involvement of multiple forms of pol-α in development, investigations of embryonic chicken brain pol-α were undertaken. Only a microsomal DNA-polymerizing activity that could not be identified unambiguously as pol-α had been isolated from this tissue (Smith and Soriana, 1977). The elevation and subsequent decline in pol-α levels during embryonic chicken brain development (Simet, 1983) suggested that this source would be well-suited for developmental studies. Previous reports of pol-α in whole chicken embryos (Brun *et al*., 1974; Yamaguchi *et al*., 1982) had contained some indications, not pursued in detail, that the enzyme might be heterogeneous. In addition, both pol-β (Stavrianopoulos *et al*., 1971,1972a,b) and pol-α (Bertazzoni *et al*., 1977; Yamaguchi *et al*., 1980) from whole

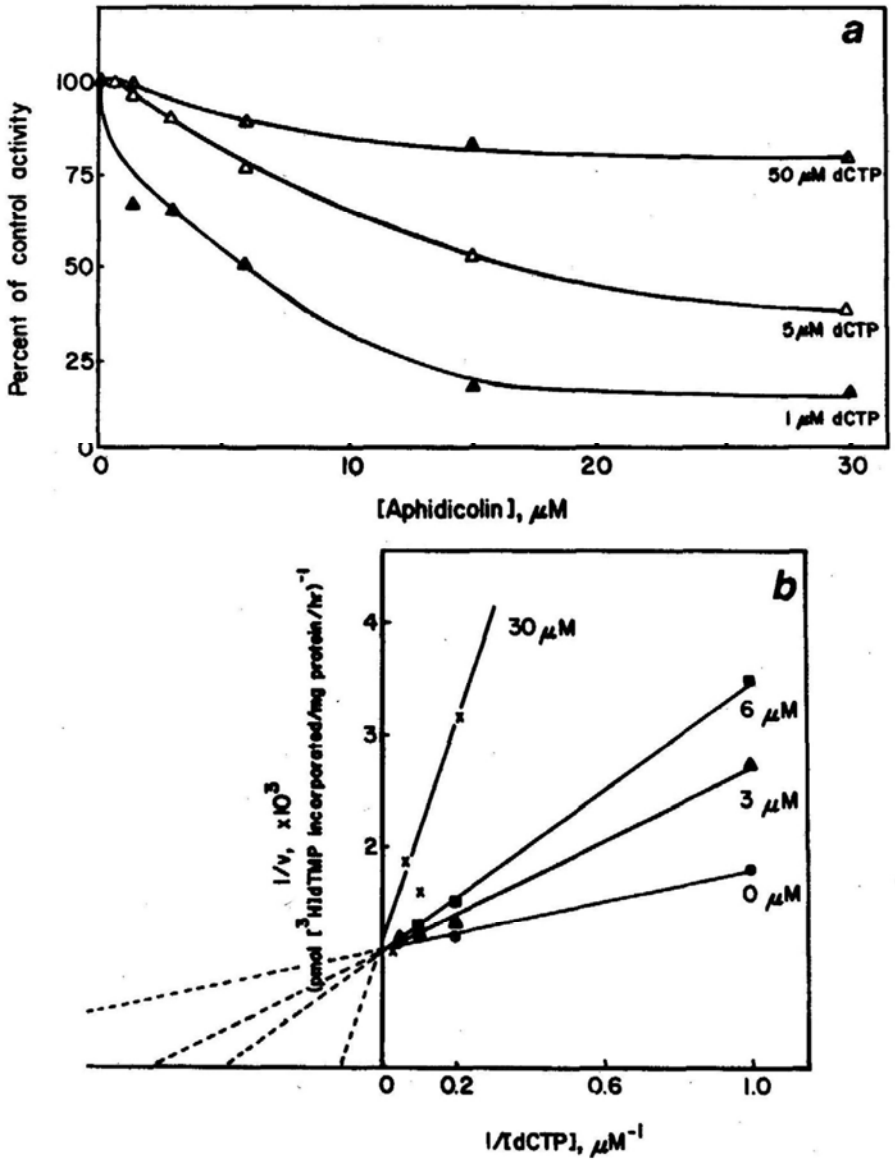


Figure 6. Inhibition by aphidicolin of multiple forms of DNA pol-α from embryonic chicken brain. All assay mixtures were adjusted to 0·25% (v:v) DMSO. Enzymatic activity in the presence of 6 concentrations of aphidicolin and 5 concentrations of dCTP was measured for each form, with representative data illustrated.

(a), Pol-α_1. One hundred percent activity represents the following values, in picomoles of [^{3}H]-dTMP incorporated into activated DNA per mg protein per h. (Δ), 550 for 1 μm dCTP; (▲), 850 for 5 μM dCTP; (▲), 990 for 50 μM dCTP. (b), Double-reciprocal plots of data obtained for figure 6a. A line-fitting program based on least-squares analysis was used to obtain slope and intercept values. (●), no aphidicolin; (▲), 3 μ M aphidicolin; ;((■), 6 μM aphidicolin; (x), 30 μM aphidicolin.

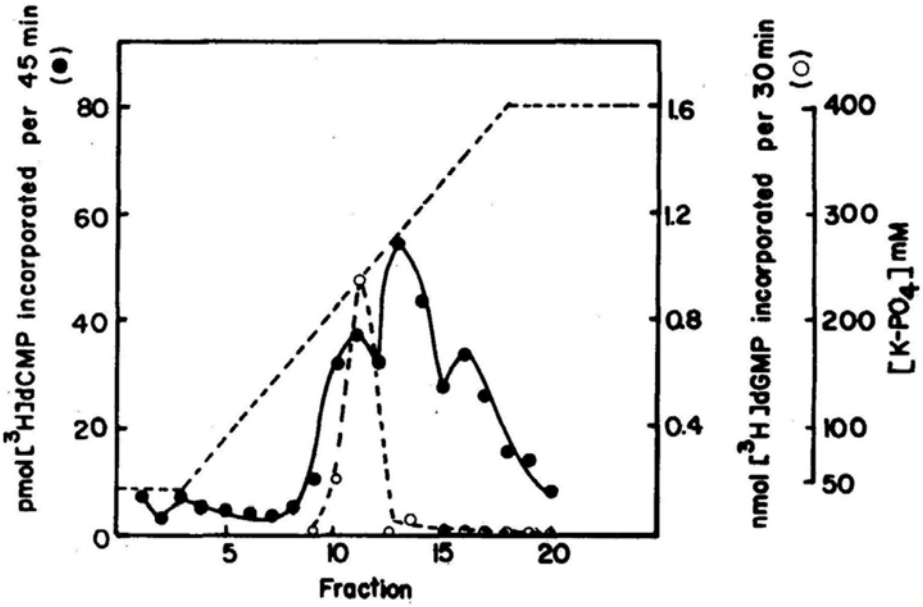


Figure 7. Resolution of DNA polymerase-primase complex on DEAE-23. Frozen embryonic brains (9-day-old) were homogenized in 3 volumes of 50mM Tris-HCl (pH 8·0) buffer containing 6 mM KCl, 2mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 0·5 mM PMSF, 2·5 mM benzamidine, 50 μg/ml soybean trypsion inhibitor, 5 μg/ml each of leupeptin and pepstatin, 1 mM aminoacetonitrile bisulphate, and 0·1 % PEG (M = 8,000). The homogenate was centrifuged at 105,000 *g* for 2 h. The supernatant containing 40 mg protein was applied to a DEAE-23 a cellulose column (1·5 ×9 cm) preequilibrated with 50 mM potassium phosphate-buffer (pH 7·6) containing 5 mM DTT and 0·1% PEG (8K). After washing of non-adsorbed proteins with the column buffer, primase (0) and DNA polymerase α (●) activities were eluted with a 40 ml linear buffer gradient of 50–400 mM K-PO_4, pH 7·6, containing 5 mM DTT and 0·1 % PE G (8K) at a rate of 1 ml/min. Fractions were collected in 20% glycerol (final concentrations) and were stored at −70° before assay. Primase activity was assayed using poly (dC) template and [^{3}H]-dGTP; pol-α was assayed using activated calf thymus and [^{3}H]-dCTP. Reaction conditions were the same as described in the text.

embryos had been characterized, facilitating comparisons of any resolved enzymatic species with known activities.

Because pol-α was the predominant DNA-polymerizing activity in 9 to 13-day-old embryonic chicken brain, initial studies were conducted using the tissue at this developmental stage. Attempts to enrich selectively the pol-α contents of embryonic chicken brain extracts by PEG precipitation were unsuccessful, probably because of the extensive loss of the unstable activity during the lengthy dialysis step required for the removal of NaCl and PEG. However, ion-exchange chromatography on DEAE-cellulose successfully resolved pol -*β*, pol-ã, and 3 additional forms of DNA-polymerizing activity (figure 1). Nonadsorption of pol -*β* was expected because of its known basicity (Stavrianopoulos *et al.,* 1972a; Simet, 1983) and the elution of pol-γ by 140 mM K–PO_4 agreed well with its reported elution from microgranular DEAE-cellulose at 120–150 mM K–PO_4 (Spadari and Weissbach, 1974; Hubscher *et al*., 1977; Bolden *et al*., 1977; Bertazzoni *et al*., 1977).

The 3 other activities were eluted at 165, 220, and 265 mM K–PO_4. To establish that each of these activities was a DNA polymerase, the [^{3}H] -labelled products of the enzymatic reactions were isolated by chromatography on Sephadex G-100. The exclusion of the products by the resin (figure 3a) indicated that they were of high molecular weight. Paper chromatography demonstrated that the [^{3}H]-label in the excluded fractions did not reside on single deoxynucleotides. The sensitivities of the products to DNase and their resistances to RNase and Pronase (table 3) confirmed that the multiple forms were incorporating [^{3}H]-dNMP residues into DNA.

The requirements for enzymatic activity provided additional evidence that the multiple forms were DNA polymerases. All 3 species required a DNA tempate-primer, all 4 dNTPs, and a divalent metal cation for optimal product synthesis (table 2).

Because heterogeneity of chick embryo pol-*α* had not been emphasized in previous studies, the possibility that the 3 activities were merely artifactual species was raised. Artificial fractionation of a single enzymatic species by ion-exchange chromatography appeared unlikely because each of the forms was eluted at its initial elutive ionic strength upon rechromatography, and no secondary activities were generated by the second passage over DEAE-cellulose (figure 2). Alternatively, the 3 forms might have represented complexes of a single enzymatic activity with varying amounts of DNA, with complexes of higher DNA content adsorbing more tightly to the resin. DNA had been detected in a DEAE-cellulose-purified *Drosophila* pol-*α* fraction (Karkas *et al*., 1975), but retention of the DNA of DNA-protein complexes by DEAE-cellulose at concentrations in excess of 300 mM K–PO_4 had also been reported (Brakel and Blumenthal, 1977). The unchanged elution patterns of the 3 forms upon rechromatography suggested that the initial resolution had not been based on differing DNA contents.

Extensive evidence of sensitivity of pol-αs to proteolysis (Hubscher, 1983) suggested that proteolytic degradation during extraction of a single pol-α might have been responsible for the observed enzymatic heterogeneity. However, omission of PMSF from extraction buffers had no effect on the elution profiled or the relative ratios of the pol-*α* multiple forms, precluding an involvement of serine proteases in non-specific generation of the activities during purification. Similar results have been reported for mouse mastocytoma (Bieri-Bonniot and Schurch, 1978), mouse myeloma (Chen *et al*., 1979), and rat liver (Mechali *et al*., 1980) pol-αs. Proteolysis by PMSF-resistant proteases cannot be ruled out at present, but the reproducibility of the ratios of the 3 forms in sonicated supernatants argues against their derivation from a single species through uncontrolled proteolysis during isolation. The 3 enzymatic activities therefore appear to be distinct components of the total embryonic chicken brain pol-*α* fraction rather than artifacts of preparation or resolution.

Attempts to classify the multiple forms as pol-*α*s by sedimentation velocity or isoelectric focusing experiments were thwarted by the remarkable instability of the resolved activities. The apparent preferences of the activities for Mg^{2+} over Mn^{2+} in activated DNA-supported reactions (table 2) implied that the 3 forms were pol-*α*s, but further substantiation was needed. Consequently, the sensitivities of the forms to known inhibitors of the eukaryotic DNA polymerases were assessed. Like the majority of previously characterized pol-*α*s, each of the 3 embryonic chicken brain activities was inhibited both by high ionic strength (figure 4) and by NEM (figure 5). Each form also was distinguished from pol -*β* by its activity in the presence of K-PO_4

concentrations greater than 30 mM, conditions under which pol-*β* activity was suppressed completely.

The effects of aphidicolin on each enzymatic species demonstrated convincingly that the multiple forms all were pol-*α*s. Each of the activities was reduced dramatically by this compound (figure 6a), which specifically inhibits pol-*α* (Ohashi *et al*., 1978; Ikegami *et al*., 1978; Pedrali-Noy and Spadari, 1979). Moreover, aphidicolin-induced inhibition of DNA synthesis was linearly competitive with dCTP (figure 6b), in accord with earlier characterizations of this inhibitor's mode of action (Oguro *et al*., 1979; Sala *et al*., 1980).

During the last decade a more refined understanding of bacteriophage DNA replication has evolved using a protein complex consisting of atleast 7 coded proteins (Alberts, 1984). All the accessary proteins of *Escherichia coli* holoenzyme pol III have recently been purified and cloned (Maki *et al*., 1986). Out of 9 subunits it is the *α*-subunit (140 *k*D) which is the catalytic subunit. However other subunits help in the highly processive replication of a prokaryotic DNA (*e.g.* On C. Plasmid). Our present attempts led us to resolve a complex that consists of DNA pol-*α* and primase activities. It is possible that this complex also contains a similar factor (NPF-1) which we have recently isolated from rat liver (Takada *et al*., 1986) and it showed to stimulate the DNA chain initiation step (figure 8). Our results (Takada *et al*., 1986a,b) suggest that this type of low molecular weight nonhistone proteins perhaps play important roles in the stabilization of the initiation complex (figure 8). Beside these accessory proteins very little is known about the chemical nature of the primases isolated from various animal tissues. Further characterization of this primase activity and its role in the embryonic development is under investigation.

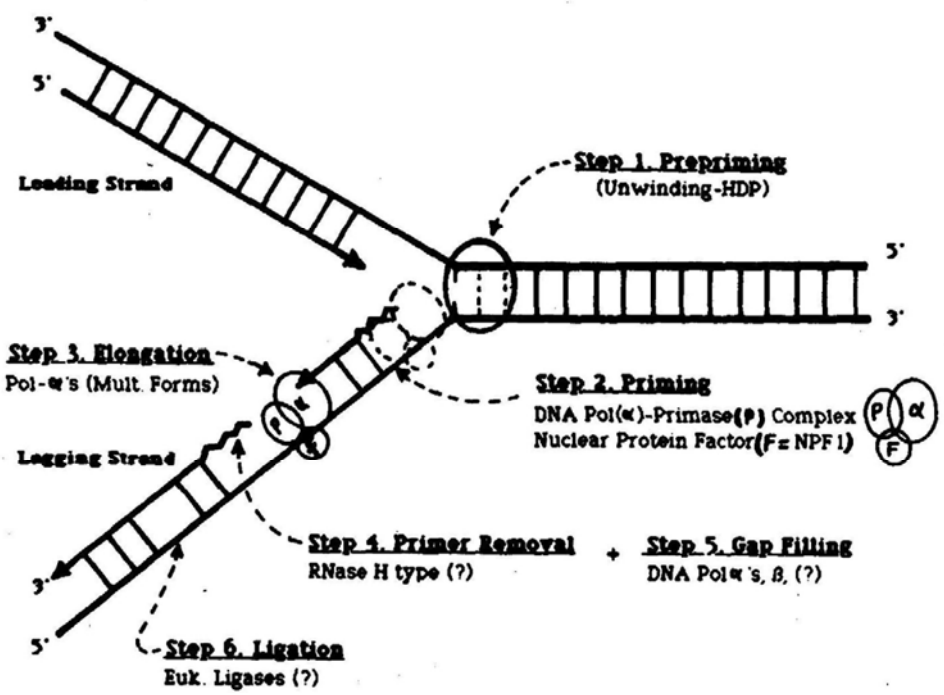


Figure 8. Proposed steps for discontinuous replication of eukaryotic DNA.

Acknowledgements

We wish to thank Dr. John Douros of the NIH for his help in securing for us a sample of aphidicolin and Dr. Shigeo Takada of the University of Notre Dame for the primase assay system used during this work. We thank Dr. Stephen Campion for his assistance in drawing some of the crucial figures. This work was supported by US Public Health Service Grants NS–18005 and CA-14764 to S.B.

References

Alberts, B. M. (1984) *Cold Spring Harb. Symp. Quant. Bio*., **49,** 1.
Bertazzoni, U., Scovassi, A. I. and Brun, G. M. (1977) *Eur. J. Biochem*., **81,** 237.
Bhattacharya, P., Simet, I. and Basu, S. (1981) *Proc. Natl. Acad. Sci. USA*, **78,** 2683.
Bieri-Bonniot, F. and Schurch, A. R. (1978) *FEBS Lett.,* **96,** 192.
Bolden, A., Pedrali-Noy, G. and Weissbach, A. (1977) *J. Biol. Chem*., **252,** 3351.
Brakel, C. L. and Blumenthal, A. B. (1977) *Biochemistry*, **16,** 3137.
Brakel, C. L. and Blumenthal, A. B. (1978) *Eur. J. Biochem.,* **88,** 351.
Brun, G., Rougeon, F., Lauber, M. and Chapeville, F. (1974) *Eur. J. Biochem.,* **41,** 241.
Burton, K. (1956) *Biochem. J*., **62,** 315.
Castroviejo, M., Tharaud, D., Tarrago-Litvak, L. and Litvak, S. (1979) *Biochem. J.,* **181,** 183.
Chang, L. M. S. (1977) *J. Biol. Chem.* **252,** 1873.
Chen, Y.-C., Bohn, E. W., Planck, S. R. and Wilson, S. H. (1979) *J. Biol. Chem.,* **254,** 11678.
Duguet, M., Mechali, M. and Rossignol, J. M. (1978) *Anal. Biochem*., **88,** 399.
Filpula, D., Fisher, P. A. and Korn, D. (1982) *J. Biol. Chem*., **257,** 2029.
Grosse, F. and Krauss, G. (1985) *J. Biol. Chem*., **260,** 1881.
Hachmann, H. I. and Lezius, A. G. (1975) *Eur. J. Biochem*., **50,** 357.
Holmes, A. M., Cherialthundam, E., Bolllum, F. J. and Chang, L. M. S. (1985) *J. Biol. Chem.*, **260,**10840.
Holmes, A. M., Hesslewood, I. P. and Johnston, I. R. (1974) *Eur. J. Biochem*., **81,** 249.
Hubscher, U. (1983) *Experientia,* **39,** 1.
Hubscher, U., Kuenzle, C. C. and Spadari, S. (1977) *Eur. J. Biochem.,* **81,** 249.
Hubscher, U., Spanos, A., Albert, W., Grummt, F. and Banks, G. R. (1981) *Proc. Natl. Acad. Sci. USA,* **78,** 6771.
Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H. and Mano, Y. (1978) *Nature* (*London*)*,* **275,** 458.
Kozu, T., Seno, T. and Yaguru, T. (1986) *Eur. J. Biochem*., **157,** 251.
Kaguni, L. S., DiFranascesco, R. A. and Lehman, I. R. (1984) *J. Biochem*., **259,** 9314.
Karkas, J. D., Marulies, L. and Chargaff, E. (1975) *J. Biol. Chem.,* **250,** 8657.
Lamothe, P., Baril, B., Chi, A., Lee, L. and Baril, E. (1981) *Proc. Natl. Acad. Sci. USA,* **78,** 4723.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.,* **193,** 265.
Maki, H., Maki, S., Lasken, R. and Kornberg, A. (1986) *J. Cell Biochem., Suppl.* **10B,** 125.
Masaki, S. and Yoshida, S. (1978) *Biochem. Biophys. Acta,* **521,** 74.
Matsukage, A., Sivarajan, M. and Wilson, S. H. (1976) *Biochemistry*, **15,** 5305.
Mechali, A., Abadiedebat, J. and deRecondo, A. M. (1980) *J. Biol. Chem*., **255,** 2114.
Momparler, R. L., Rossi, M. and Labitan, A. (1973) *J. Biol. Chem.*, **248,** 285.
Oguro, M., Suzuki-Hori, C, Nagano, H., Mano, Y. and Ikegami, S. (1979) *Eur. J. Biochem.* **97,** 603.
Ohashi, M., Taguchi, T. and Ikegami, S. (1978) *Biochem. Biophys. Res. Commun.,* **82,** 1084.
Ono, Y., Enomoto, T., Hanaoka, F. and Yamada, M. (1978) *Gann,* **69,** 207.
Ono, Y., Enomoto, T. and Yamada, M. (1979) *Gann,* **70,** 527.
Pedrali-Noy, G. and Spadari, S. (1979) *Biochem. Biophys. Res. Commun.,* **88,** 1194.
Plevani, P., Foriani, M., Valsasnini, P., Badaracco, G., Cherialthundam, E. and Chang, L. M. S. (1985) *J. Biol. Chem.,* **260,** 7102.
Sala, F., Parisi, B., Burroni, D., Amileni, A. R., Pedrali-Noy, G. and Spadari, S. (1980) *FEBS Lett.,* **117,**93.
Shioda, M., Nelson, E. M., Bayne, M. L. and Benbow, R. M. (1982) *Proc. Natl. Acad. Sci. USA,* **79,** 7209.
Simet, I. (1983) *Multiple forms of DNA polymerase-α from embryonic chicken brain,* Ph.D. Thesis, Univ. of Notre Dame, Indiana, USA.

Smith, J. and Soriano, L. (1977) *Nucleic Acids Res.* **4** 641.
Spadari, S. and Weissbach, A. (1974) *J. Biol. Chem*., **249,** 5809.
Stavrianopoulos, J. G., Karkas, J. D. and Chargaff, E. (1971) *Proc. Natl. Acad. Sci. USA,* **68,** 2207.
Stavrianopoulos, J. G., Karkas, J. D. and Chargaff, E. (1972a) *Proc. Natl. Acad. Sci. USA,* **69,** 1781.
Stavrianopoulos, J. G., Karkas, J. D. and Chargaff, E. (1972b) *Proc. Natl. Acad. Sci. USA,* **69,** 2609.
Takada, S., Torres-Rosado, A., Ray, S. and Basu, S. (1986a) *Proc. Fed. Am. Soc. Exp. Biol.,* **45,** 1779.
Takada, S., Torres-Rosado, A., Ray, S. and Basu, S. (1986b) *Proc. Natl. Acad. Sci. USA*, **83,** 9348.
Wang, T. S.-F., Hu, S.-Z. and Koran, D. (1986) *J. Biol. Chem.,* **259,** 1854.
Wintersberger, E. (1978) *Eur. J. Biochem.,* **84,** 167.
Yagura, T, Kozu, T. and Seno, T. (1982) *J. Biol. Chem*., **257,** 1121.
Yamaguchi, M., Matsukage, A. and Takahashi, T. (1980) *J. Biol. Chem.,* **255,** 7002.
Yamaguchi, M., Tanabe, K., Takahashi, T. and Matsukage, A. (1982) *J. Biol. Chem*., **257,** 4484.
Yoshida, S., Kondo, T. and Ando, T, (1974) *Biochem. Biophys. Acta,* **353,** 463.
Zimmerman, W., Chem, S. M., Holden, A. and Weissbach, A. (1980) *J. Biol. Chem*., **255,** 11847.