Developmental Expression of the Embryonic Chicken Brain DNA Polymerase $\alpha$ and Its Binding with Monoclonal Antibodies against Human KB Cell DNA Polymerase $\alpha$

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Abstract

Changes in DNA polymerase $\alpha$ activity accompanying tissue development have been well established in several systems. In most cases, DNA polymerase $\alpha$ activity decreases with development. Here, we report observed changes in DNA polymerase $\alpha$ activity throughout embryonic chicken brain (ECB) development. The level of DNA polymerase $\alpha$ activity was found to gradually decrease by 60% (2.3 to 0.8 nmol of $[^{3}H]$dCMP incorporated/mg protein/h) between 9- and 19-day-old ECB. An enzyme-linked immunosorbent assay of DNA polymerase $\alpha$ utilizing monoclonal antibody SJK 237-71 (human KB cell DNA pol-$\alpha$ binder) also demonstrated a gradual decrease (up to 60%) of antigen over this same range of development. Analysis of DNA polymerase $\alpha$ from 11- and 19-day-old ECB by a 10 to 30% glycerol density gradient revealed a high molecular weight peak sedimenting near catalase (11.3 S) with activity at the 11th day being approximately 3-fold greater than activity at the 19th day. A Western immunoblot analysis utilizing monoclonal antibody SJK 237-71 (against human KB cell DNA polymerase $\alpha$) showed a decrease in DNA polymerase $\alpha$ from 186 kilodaltons in 9- and 11-day ECB cell-free extracts to 120 kilodaltons in extracts from 13- to 19-day ECB. The conversion of DNA polymerase $\alpha$ from a higher to a lower molecular weight form may be a regulatory mechanism in eukaryotic DNA replication.

Introduction

DNA polymerase $\alpha$ has been unequivocally identified as the main enzyme for extramitochondrial DNA replication in the eukaryotic system (1, 2). A tightly associated complex of DNA polymerase $\alpha$ and DNA primase has been reported to initiate DNA replication at the SV-40 replication origin sequences (3). Many reports have appeared in the literature relating the level of DNA polymerase $\alpha$ activity to the rate of DNA synthesis during cell proliferation, but only a few investigations have addressed whether the changes in DNA polymerase $\alpha$ activity are caused by an altered level of the catalytic subunit or are due to the appearance or disappearance of a regulatory factor for DNA polymerase $\alpha$ (4, 5). Inhibitors of protein synthesis, such as cycloheximide, and inhibitors of RNA transcription, such as actinomycin D, were found to inhibit the increase of DNA polymerase $\alpha$ activity in regenerating rat liver (6). These observations provided indirect evidence for the requirement of de novo protein synthesis for the maintenance and regulation of DNA polymerase $\alpha$ activity. Whether this protein synthesis is required for the synthesis of a DNA polymerase $\alpha$ polypeptide or of some regulatory protein factor for DNA polymerase $\alpha$ is not clear. In rat giant trophoblasts, decrease in DNA polymerase $\alpha$ activity was found to be due to the disappearance of a stimulatory protein factor (7). We have previously reported the resolution of DNA polymerase $\alpha$-primase complex and primase-free DNA polymerase $\alpha$ from embryonic chicken brain (8). Here, we report that the decrease in the activity level of DNA polymerase $\alpha$ in embryonic chicken brain during embryonic development is indeed due to the decrease in the catalytic polypeptide of DNA polymerase $\alpha$. The progressive disappearance of high molecular weight DNA polymerase $\alpha$ polypeptides with increasing embryonic age in chicken brain was also observed.

Results

Inhibition of DNA Polymerase $\alpha$ Activity by SJK 132-20. The monoclonal antibody SJK 132-20 (9) was found to be completely inhibitory to DNA polymerase $\alpha$ activity from embryonic chicken brain. One to 2 $\mu$g of SJK 132-20 were sufficient to neutralize the activity of 12 $\mu$g of partially purified DNA polymerase $\alpha$ from embryonic chicken brain (Fig. 1). This monoclonal antibody has been reported to inhibit DNA polymerase $\alpha$ activity from various experimental systems as well (10-12).

Level of DNA Polymerase $\alpha$ Activity in Embryonic Chicken Brain with Embryonic Development. The level of DNA polymerase $\alpha$ activity was found to decline progressively between 9- and 19-day-old embryonic chicken brains. Specific activity decreased by as much as 60% (from 2.3 to 0.8 nmol/mg protein/h) from the 9th...
to the 19th day (Fig. 2). The activity of 9-day ECB was taken as 100%, and the activity at other embryonic ages was expressed relative to the activity level in 9-day-old ECB. This result clearly indicated that decrease of DNA polymerase α activity is correlated with embryonic development. However, it was not apparent whether this decrease in DNA polymerase α activity was caused by the presence of a lower amount of a catalytically active DNA polymerase α or due to an embryonic development-specific expression of a regulatory factor for DNA polymerase α.

ELISA for the Comparison of the Relative Level of DNA Polymerase α Antigen at Various Embryonic Ages of Chicken Brain. ELISA of DNA polymerase α antigen from ECB cell-free extracts of different ages with the nonneutralizing monoclonal antibody SJK 237-71 (9) was carried out as described in "Materials and Methods." The results (Fig. 3) showed a gradual decrease in the relative level of DNA polymerase α antigen with increasing embryonic age. This observation was in close agreement with the results obtained from the measurement of the catalytic activity level of DNA polymerase α in chicken brain of different embryonic ages (Figs. 2 and 3). The absorbance readings for 9-day ECB were taken as 100%, and the absorbance values for other embryonic ages were expressed relative to those values for 9-day-old ECB. Both methods showed a 60% reduction in the level of DNA polymerase α between 9- and 19-day-old ECB. It is apparent from these results that the decrease in DNA polymerase α activity with increasing embryonic age was probably due to the decrease of the DNA polymerase α protein itself and perhaps not to an age-specific expression of an inhibitor for DNA polymerase α. On the other hand, in rat giant trophoblasts, a decrease in DNA polymerase α activity was found to result from the disappearance of a stimulatory factor for DNA polymerase α (7).

Isolation of Polymerase α/Primase Complex from Embryonic Chicken Brain by DE-23 Cellulose Column Chromatography. The complex containing both DNA polymerase α and primase activities from various embryonic ages of chicken brain was isolated by ion-exchange chromatography on a DE-23 cellulose (8). Irrespective of embryonic age, both activities were found to coelute at a concentration of 200 to 250 mM potassium phosphate (Fig. 4). For 9-day-old ECB, three peaks of DNA polymerase α activity were observed, primase activity being associated with the first of the three peaks of DNA polymerase α activity (8). However, in higher embryonic ages (9-day to 19-day), primase and DNA polymerase α activities were found to be coincident (Fig. 4). The isolated complex was found to sediment near catalase marker on a continuous glycerol velocity gradient as described below. The ammonium sulfate (0 to 45%, w/v) precipitated fraction of ECB cell-free extract, when subjected to DE-23 cellulose chromatography, showed the presence of high primase activity in the wash fractions and little or no DNA polymerase α associated with it. Column-bound primase and DNA polymerase α activities were eluted at 250 mM potassium phosphate concentration, as was observed during the DE-23 cellulose column chromatography of the untreated ECB cell-free extract (results not shown). This result indicated that ammonium sulfate treatment of the cell-free extract might be causing a partial separation of primase activity from the DNA.

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8 The abbreviations used are: ECB, embryonic chicken brain; ELISA, enzyme-linked immunosorbent assay; kDa, kilodalton(s); SDS, sodium dodecyl sulfate; DTT, dithiothreitol; BSA, bovine serum albumin; dNTP, deoxyribonucleotide triphosphate; PBS, phosphate-buffered saline.
polymerase α/primase complex. Use of highly hydrophobic conditions or high ionic strength for the partial separation of primase from the DNA polymerase α/primase complex has been reported from mouse FM3A cells (13), HeLa cells (11, 14), calf thymus (15), and Drosophila melanogaster embryos (16, 17).

Glycerol Density Gradient Analysis of DNA Polymerase α Activities from 11- and 19-Day-Old Embryonic Chicken Brain. Continuous glycerol density gradient (10 to 30%) analysis of DNA polymerase α activities from 11- and 19-day-old embryonic chicken brains showed the presence of a high molecular weight peak of DNA polymerase α activity sedimenting near the catalase (11.3 S) marker (Fig. 5), but the magnitude of the activity in 11-day-old embryonic chicken brain was nearly 3-fold higher than that in 19-day-old embryonic chicken brain, an observation consistent with other experiments.

Western Blot Analysis of DNA Polymerase α Polypeptides from Embryonic Chicken Brain of Different Ages. Western blot analysis of DNA polymerase α polypeptides from different ages of embryonic chicken brain was carried out as described in “Materials and Methods.” Nondenaturing conditions were used to ensure that DNA polymerase α polypeptides were in their catalytically active configuration. Immunostaining with monoclonal antibody SJK 237-71 showed the presence of a 186 kDa high molecular weight band in 9- and 11-day-old embryonic chicken brains (Fig. 6). In contrast, a 120 kDa band appeared at 13 to 19 days (Fig. 6).

Discussion

Involvement of DNA polymerase α in tissue growth has been demonstrated in many developing systems, such as regenerating rat liver (18), rat brains (19), rat forebrain neuron (20), D. melanogaster embryos (21), sea urchin embryos (22), chick neural retina (23), and rabbit mammary glands (24). In almost all cases, DNA polymerase α activity was found to decrease with development, but the basis for the observation was not apparent. Prominent growth pattern accompanied by extensive diversification with embryonic development has made embryonic chicken brain an ideal system for the study of developmental changes of DNA polymerase α (25-29). Our laboratory has utilized embryonic chicken brains in developmental studies of glycosyltransferases for two decades (26-29). Because of the cross-reactivity of the monoclonal antibodies SJK 132-20 and SJK 237-71 (raised against human KB cell DNA polymerase α) with embryonic chicken brain DNA polymerase α, both were used as tools to measure the relative levels of DNA polymerase α protein in different embryonic ages in order to understand whether the decrease in catalytic activity is really caused by the lower level of DNA polymerase α in higher embryonic ages. A positive correlation between the two was obtained from the results. Whether the decrease in the DNA polymerase α in higher embryonic ages is due to the degradation of the enzyme to inactive forms by proteolysis could be determined, at least in part, from these observations. ELISA with a nonneutralizing monoclonal antibody (SJK 237-71) showed (Fig. 3) a decrease in the DNA polymerase α level with embryonic development. Had the epitope for the recognition of SJK 237-71 (a nonneutralizing antibody not directed to the catalytic site of the enzyme) not been destroyed by proteolysis, then in order to maintain a constant level of activity throughout development, it would have shown the same or an elevated level of DNA polymerase α antigen with development.

Immunostaining of DNA polymerase α polypeptides, separated by SDS-gel electrophoresis followed by electrophoretic blotting on a nylon membrane, also showed a similar trend of lower molecular weight DNA polymerase α polypeptides appearing in the latter part of embryonic development and a gradual disappearance of higher molecular weight polypeptides with increasing embryonic development.
age. Immunostaining of electroblotted DNA polymerase α polypeptides separated by SDS-gel electrophoresis appeared to be more sensitive for the detection of higher molecular weight polypeptides and thus made possible a better visualization of the relative intensities. Also, this experiment was performed with cell-free extract as opposed to purified DNA polymerase α to eliminate the possibility of artificial DNA polymerase α degradation. This method showed the presence of a 186 kDa high molecular weight band in both 9- and 11-day-old embryonic chicken brains (Fig. 6). A 120 kDa band was detected from 13- to 19-day ECB (Fig. 6). The ability of the monoclonal antibody SJK 237-71 to detect the lower molecular weight polypeptides of DNA polymerase α also ruled out the possibility of destroying an epitope of recognition by proteolysis. Under the present experimental conditions, no catalytically active DNA polymerase α polypeptides greater than 120 kDa could be detected by activity gel analysis. Detection of catalytic activity in 190 kDa polypeptide from monkey BSC-1 cells was reported to be possible only after the elution of the polypeptide from the polyacrylamide gel (30). These results strongly favored the possibility of a programmed proteolysis of DNA polymerase α with increasing embryonic age. The absence of lower molecular weight forms in earlier embryonic ages could be used to argue against the possibility of uncontrolled proteolysis during isolation. Moreover, freshly prepared cell-free extracts in the presence of a variety of protease inhibitors were used during the experiments, thereby eliminating the possibility of generating lower molecular weight forms during storage (25, 31). The appearance of lower molecular weight forms of DNA polymerase α, which were detected in the mouse neuroblastoma cell-free extract prepared by cell lysis in the presence of boiling SDS, also points toward eliminating the possibility of uncontrolled proteolysis during isolation (31). The conversion of a DNA polymerase α precursor to lower molecular weight by a genetically programmed proteolysis (20, 32) or dissociation of glycoprotein-binding subunits (33) as a regulatory mechanism for DNA replication have been suggested.

In addition to the primase complexed with DNA polymerase α (Fig. 4), a free primase activity was isolated by DE-23 cellulose column chromatography of ammonium sulfate (0 to 45%, w/v) -precipitated fraction of embryonic chicken brain (34, 35). This free primase activity was partially purified by blue agarose column chromatography. Primase activity was not inhibited by a high concentration of α-amrinin (200 μg/ml). Although the levels of both DNA polymerase α and primase activity with DNA polymerase α were found to decrease, an increase in the level of free primase activity was observed with increased embryonic age (35).

The occurrence of DNA polymerase α with different molecular weights in mouse neuroblastoma cells, N-18 (31, 36), and human neuroblastoma cells, IMR-32 (37, 38), under different growth conditions has been observed. Recently, the stability of the DNA polymerase α/primase complex in rat prostate tumor PA-3 cells during different phases of the cell cycle has been studied (39, 40). Using activity gel analysis, the molecular weight of PA-3 polymerase α was detected as 180 kDa and 120 kDa during S and G1 phases, respectively (39, 40). The relation between changes in molecular weight of polymerase α and the stability of the DNA polymerase α/primase complex is not well understood and is under study (33).

Materials and Methods

Preparation of a Cell-free Extract from Embryonic Chicken Brain. Embryonic chicken brains were collected from fertilized chicken eggs, incubated in an egg incubator between 9 and 19 days of development, and kept...
frozen at -20°C in 20% glycerol. The aliquots of tissue frozen were of equal wet weights in order to have similar thawing times. The frozen brains were thawed and homogenized in 2 volumes of 50 mM Tris-HCl (pH 8.0) buffer containing 5 mM DTT, 6 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 50 μg/ml soybean trypsin inhibitor, 5 μg/ml each of leupeptin and pepstatin A, 5 mM aminoacetonitrile bisulfate, and 0.1% polyethylene glycol (MW = 8000). Protease inhibitors such as soybean trypsin inhibitor, leupeptin, pepstatin A, and aminoacetonitrile bisulfate were added to the buffer just before homogenization. The homogenate was prepared by 30 strokes with a Potter-Elvehjem glass-Teflon homogenizer. The post-microsomal supernatant, obtained by centrifugation of the homogenate at 105,000 x g for 1 h, was used as the enzyme source.

**Assay of DNA Polymerase α Activity.** DNA polymerase α activity was assayed by the extent of incorporation of [³H]dCMP into acid-insoluble activated calf thymus DNA (45) in the presence and absence of 15 mM N-ethylmaleimide (38, 39).

The following components were present in the assay mixture in a total volume of 0.1 ml: 400 μM/ml activated calf thymus DNA, 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 20 mM KCl, 5 mM DTT, 50 μg BSA, 50 μM each unlabeled dNTP, 50 μM [³H]dCTP (specific activity, 150 to 250 cpm/pmol), and enzyme (10 to 25 μg protein). Incubation was carried out at 37°C for 45 min. The reaction was stopped by the addition of 50 μg BSA and 1 ml ice-cold 10% trichloroacetic acid containing 50 mM sodium pyrophosphate. After mild vortexing, the mixture was kept at 4°C for 30 min to complete the precipitation. Precipitates were collected on GF/C glass fiber filters and washed with 15 ml ice-cold 5% trichloroacetic acid containing 50 mM sodium pyrophosphate and 5 ml each of water, 95% ethanol, and acetone for the removal of unincorporated dNTPs. After washing, the glass fiber filters were dried in a microwave oven, and the radioactivity incorporated into acid-precipitable material was quantitated by liquid scintillation counting in a Beckman model LS 3801 multichannel liquid scintillation counter.

**Assay for Primase Activity.** For the assay of primase activity (41), short riboprokers (oligo C₃₀₋₁₅) synthesized on single-stranded polydeoxyribonucleotide (polydeoxycytidylic acid) by primase were used in situ by the primase-associated DNA polymerase α or by added Klenow fragment of *Escherichia coli* DNA polymerase I. The formation of radiolabeled DNA chain as a function of primase activity was found to constitute a sensitive assay for primase (42).

The following components were present in the assay mixture in a total volume of 0.05 ml: polydeoxycytidylic acid or a single-stranded DNA template (10 μg), 50 mM Tris-HCl (pH 7.4), 8 mM MgCl₂, 3 mM DTT, 0.1 mg/ml BSA, 4 mM GTP or nucleotide triphosphate complementary to the template sequence, 10% glycerol, 0.1 mM [³H]GTP or complementary dNTP (specific activity, 50 cpm/pmol), and enzyme (0.005 to 0.015 mg protein). For the assay of free primase activity, 0.2 unit of Klenow was added in the incubation mixture. Following the incubation at 37°C for 30 to 45 min, the reaction mixtures were chilled at 4°C and spotted on pencil-marked DE-81 paper strips (3 x 2 cm). After air-drying, the strips were washed five times with 3% Na₂HPO₄ (w/v) (250 ml solution for 10 to 30 strips), twice with water (250 ml) and once with 95% ethanol (250 ml). After washing, the strips were dried, and the amount of radioactivity incorporated was quantitated by counting in a toluene scintillation system by a Beckman LS3801 liquid scintillation spectrometer.

**Glycerol Density Gradient Centrifugation Analysis of DNA Polymerase α from Embryonic Chicken Brain.** Three to 4 mg of soluble protein from cell-free extracts from embryonic chicken brains (11 and 19 days old) were layered on the top of a 4.5-ml linear gradient of 10 to 30% glycerol in 10 mM Tris-HCl (pH 8.0), 5 mM DTT, and 100 mM KCl. Parallel gradients contained catalase (11.3 S), IgG (7.5 S), and BSA (4.4 S) as sedimentation markers. Centrifugation was carried out in a SW 50.1-type rotor (Beckman) at 105,000 x g for 16 h at 4°C. Fractions of approximately 400 μl were collected from the bottom of the tube with a peristaltic pump and were assayed for DNA polymerase α activity.

**Isolation and Purification of Monoclonal Antibodies (SIK 132-20 and SJK 237-71).** Monoclonal antibodies SJK 132-20 and SJK 237-71 against human KB cell DNA polymerase α were isolated from the growth medium supporting the growth of the hybridoma cell lines and were purified by Protein A-Sepharose 4B affinity chromatography as described (9). Monoclonal antibodies were precipitated from the growth medium by 50% saturation with ammonium sulfate at room temperature, followed by stirring at 4°C. The precipitate was collected by centrifugation at 10,000 x g, dissolved, and dialyzed against 50 mM Tris-HCl (pH 8.6) containing 150 mM KCl. The dialyzed sample was loaded on a 1-ml Protein A-Sepharose 4B column equilibrated with 50 mM Tris-HCl (pH 8.6) containing 150 mM KCl. After washing off the unadsorbed proteins with 5 column volumes of the equilibration buffer, antibodies were eluted from the column with 5 column volumes of 50 mM sodium acetate (pH 4.0) containing 150 mM KCl. The eluted antibodies were concentrated by precipitation with 70% saturation of ammonium sulfate. The precipitate was collected by centrifugation at 100,000 x g for 1 h at 4°C and was then dissolved and dialyzed against 50 mM potassium phosphate (pH 7.2) containing 150 mM KCl. Affinity-purified monoclonal antibodies were divided into small aliquots and stored at -20°C.

**Inhibition of DNA Polymerase α Activity by the Monoclonal Antibody SJK 132-20.** Inhibition of DNA polymerase α by the monoclonal antibody SJK 132-20 was determined by measuring the catalytic activity of DNA polymerase α in the presence of different concentrations of purified SJK 132-20 in the incubation mixture. Recently, using an SJK 132-20 immunoaffinity column, we have purified DNA polymerase α from 11-day-old ECB (33, 43).

**Comparison of the Relative Level of DNA Polymerase α Antigen at Different Embryonic Ages by ELISA.** DNA polymerase α antigen (2 to 800 μg/ml) in 0.2 M Na₂CO₃- NaHCO₃ buffer (pH 9.6) was applied in 50-μl quantities to the wells of a 96-well polystyrene microtiter plate and incubated at room temperature for 4 h. After removal of the unbound proteins, residual binding sites were blocked with 100 μl/well of radioimmunoassay grade BSA for 30 min at room temperature. After removal of unbound BSA, the wells were washed with 200-μl aliquots of PBS containing 0.1% Tween 20 and 0.01% thimerosal (wash buffer) three times for a total of 30 min. Fifty μl/well of monoclonal antibody (SIK 237-71, 20 μg/ml) were
added to the wells and incubated at room temperature for 2 h. After removal of the monoclonal antibody, the wells were washed again with the wash buffer as before. Then a goat anti-mouse IgG-horseradish peroxidase conjugate from Bio-Rad (1:5000 dilution) was added to the wells and incubated for 1 h at room temperature. After removal of the unbound antibody, the wells were washed as mentioned above. Horseradish peroxidase was then assayed with o-phenylenediamine and 3% H2O2 in 0.1 M sodium citrate buffer (pH 4.5), 50 µl/well, and incubated for 30 min at room temperature in the absence of light. Intensity of color development was determined at 450 nm with a Titertek Multiskan PLUS microtiter plate reader.

**Western Blot-Immunoblot Analysis of DNA Polymerase α Polypeptides.** After activity gel electrophoresis of cell-free extracts (45 µg protein/well), protein was transferred onto a nylon membrane and immunodetectected by monoclonal antibodies against DNA polymerase α. Subsequent to the renaturation step in activity gel, the gel was soaked in 25 mM Tris-HCl (pH 8.5) containing 192 mM glycine (running buffer for electroblot) for 30 min at 4°C with one change in buffer. The gel was transferred onto a nylon membrane presoaked with the running buffer. Electroblot was carried out at 4°C with Bio-Rad blotting apparatus for 16 h at 150 mA. Residual binding sites on the membrane were blocked by soaking with 10% Carnation instant milk (defatted) in 10 mM sodium phosphate (pH 7.2) containing 0.9% NaCl (PBS buffer) and 0.1% SDS. The membrane was incubated with monoclonal antibody solution (80 µg/ml) in PBS buffer for 3 to 5 h, followed by washing with PBS buffer for 30 min to remove any unbound monoclonal antibody. Subsequent to washing, the membrane was incubated with a second antibody solution (anti-mouse IgG conjugated with horseradish peroxidase; 1:2000 dilution) in PBS buffer for 30 min. As before, the membrane was washed for 30 min. Color development was achieved by soaking the membrane in freshly prepared developing solution (0.3 mM 4-chloro-1-naphthol in PBS buffer with 0.1% hydrogen peroxide) until the desired color was obtained (30 to 60 min). Color development was stopped by rinsing the membrane with deionized water, and it was stored in the absence of light.

**Protein Estimation.** Protein concentrations of the different samples were measured by the method of Bradford (44) using crystalline BSA as standard.

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**References**


