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Variation of DNA polymerases -∝, -β. and -γ during perinatal tissue growth and differentiation

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ABSTRACT

The activities of the three known DNA polymerases-α, β-, and -γ were determined in rat brain neurons, cardiac muscle and spleen, and were correlated with the rate of cell proliferation during perinatal development.

In neurons and cardiac muscle, which stop dividing before birth, DNA polymerase-α activity drops sharply from a high level with the approach of term and disappears at approximately two weeks postnatal age. In contrast, α-polymerase activity is almost absent in spleen during late gestation, when the rate of cell division is low, and increases abruptly after birth with the sudden onset of cell proliferation. These data give further evidence for an involvement of DNA polymerase-α in DNA replication.

DNA polymerase-β and -γ activities show essentially no correlation with the rate of cell division. Thus, these enzymes are probably responsible for repair type processes rather than for DNA replication.

INTRODUCTION

Three major DNA polymerases, called α, β, and γ (1-5), are present in animals cells. They are immunologically distinct (6-8) and are not inhibited by antibodies against reverse transcriptase (9-11). A fourth, minor, cellular polymerase has been assumed to be associated with mitochondria; but recent investigations (12, and U. Hübscher, C.C.Kuenzle, and S. Spadari, submitted to Eur.J.Biochem.) have shown that this enzyme closely resembles the γ-polymerase found in the cytoplasm and nucleus. Several attempts have been made to identify the specific role played by these enzymes in DNA synthesis and replication. Tissue culture cells (13), rege-

nerating rat liver (14, 15), and lymphocytes (16-18) in resting or growing states have been examined for changes in their DNA polymerase activities. Similarly, the DNA polymerase content in various phases of the cell cycle in synchronized cells has been measured (19, 20). In general, it has been found that DNA polymerase-α responds to changes in the rate of DNA replication in the cell, whereas DNA polymerase-β seems present at a more constant level throughout the cell cycle. Only two of the previous reports dealt with DNA polymerase-γ, which was found to increase in HeLa cells during S phase (19) and in phytohemagglutinin stimulated lymphocytes (17).

However, in all the systems described so far, the correlation between enzyme level and physiological state is not unambiguous (with the exception perhaps of regenerating liver). We thought it worthwhile to explore the conditions in which well defined organs (or cell types) undergo pronounced variations in physiological growth rate. We have thus studied the in vivo temporal relationship between DNA polymerase levels and the rate of cell multiplication in several rat tissues showing characteristic patterns of tissue growth and differentiation: they are isolated neurons of the forebrain, ventricular cardiac muscle and spleen during perinatal development.

MATERIALS AND METHODS

Materials

Unlabeled deoxynucleoside triphosphates, calf thymus DNA and NEM[21] were Calbiochem (Lucerne, Switzerland) products. Terminal deoxynucleotidyltransferase (EC 2.7.7.31) was a gift of Dr. S. Pestka from the Roche Institute of Molecular Biology, Nutley, N.J. Density grade sucrose was purchased from Schwarz/Mann; deoxyribonuclease I (bovine pancreas, EC 3.1.4.5) was from Worthington Biochemical Corp., Freehold, N.J.; poly(rA) and $(dT)_4$ were obtained from Miles Laboratories, Inc., Kankakee, Ill.; pancreatic ribonuclease (EC

2.7.7.16) was from Boehringer, Mannheim, Germany, and [^{3}H] deoxynucleoside triphosphates from Radiochemical Centre, Amersham, U.K. BSA was from Fluka, Buchs, Switzerland, and mithramycin was purchased from Serva, Heidelberg, Germany.

SIV-50 rats of either sex were used throughout this study. Developmental stages included fetuses of 18 and 20 days gestation (the mean duration of gestation in rats is 22 days), as well as animals of postnatal ages 0 (newborns), 2, 4, 7, 14, 23, 30, and 60 days.

METHODS

Preparation of tissues and tissue extracts. At each developmental stage, the animals were killed by decapitation, and forebrain, heart ventricles and spleen were immediately removed. Neuronal perikarya from forebrain hemispheres (fetuses and postnatal stages 0-4 days) or forebrain cortex (postnatal ages 7-60 days) were obtained by the method of Sellinger et al. (22). All tissues were used immediately or within a few days after storage at -70°. Approximately 0.2g of tissue was homogenized in 0.6 ml of 10 mM Tris·HCl (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT using a Duall ground glass homogenizer (clearance 0.1 mm). The homogenate was then made 0.5 M in KCl, 0.5% in Triton X-100, 20 mM in Tris·HCl (pH 7.5), and sonicated 2 x 5 seconds with the microtip of a Branson Sonifier at a setting of 50 W. This crude extract was used as an enzyme source. Before sucrose gradient centrifugation, the suspension was centrifuged in a Spinco 65 fixed angle rotor at 40,000 rpm for 1 h at 0°, and 0.175 ml of the supernatant was layered over 4.8 ml of a 5-20% (w/v) sucrose gradient in 0.1 M KH_2PO_4 (pH 7.2), 0.1 M KCl and 1 mM DTT. Following centrifugation in a Spinco SW 50.1 rotor at 40,000 rpm for 16 h at 0°, approximately 20 fractions were collected from the bottom of the tubes.

Protein determination. The method of Lowry et al. (23) was used with BSA as standard.

Cell-cycle analysis of neuronal tissue. Neuronal perikarya were stained with mithramycin and the relative DNA contents determined essentially as described by Crissman and Tobey (24).

DNA determination. Triplicate assays were performed on cardiac muscle and spleen by the method of Burton (25).

Assays of DNA polymerases-α, -β, and -γ. Each reaction was carried out at 37° and, when assaying crude extracts, analyses were done in triplicate at 6, 12, and 18 min for α- and β-polymerase, and at 3, 6, and 9 min for γ-polymerase. The acid precipitable radioactive material increases linearly during this time period. The α-polymerase reaction was carried out in 20 mM KH_2PO_4 (pH 7.2), 0.1 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 250 μg/ml BSA, 200 μg/ml of activated calf thymus DNA and all 4 deoxyribonucleoside triphosphates at 50 μM each, plus [^{3}H]TTP (400 cpm/pmol). Under these conditions, as shown by assays on sucrose gradient fractions (cf. Results), β-polymerase responds with a 30% efficiency to the α-polymerase assay. Therefore all α-polymerase data presented here have been corrected for the contribution of the β-enzyme. On the other hand, γ-polymerase is inactive under these conditions (26).

DNA polymerase-β was assayed in 50 mM Tris·HCl (pH 8.5), 0.1 M KCl, 10 mM $MgCl_2$, 1 mM DTT, 250 μg/ml BSA, 200 μg/ml of activated calf thymus DNA and all four deoxynucleoside triphosphates at 50 μM each, plus [^{3}H]TTP (400 cpm/pmol). When the enzyme samples were preincubated with 10 mM NEM at 0° for 30 min (to inactivate α-polymerase, ref. 3), the β-assay was strictly specific for the β-polymerase.

DNA polymerase-γ was assayed according to Knopf et al. (26). The reaction was carried out in 50 mM Tris·HCl (pH 8.5), 50 mM KH_2PO_4 (pH 8.5), 0.13 M KCl, 0.5 mM $MnCl_2$, 1 mM DTT, 250 μg/ml BSA, 50 μg/ml poly(rA)·$(dT)_{25-30}$, and 30 μM [^{3}H]TTP (1000 cpm/pmol). Under these conditions, the assay was strictly specific for γ-polymerase as β-polymerase activity on poly(rA)·$(dT)_{25-30}$ is inhibited by phosphate (26)

and α-polymerase does not utilize this polynucleotide as template-primer (1, 3, 9).

A unit is defined as 1 nmol of total deoxynucleotide incorporation into acid insoluble form in 60 min at 37°.

Preparation of templates. The oligonucleotide, $(dT)_{25-30}$, was prepared from $(dT)_4$ with terminal deoxynucleotidyltransferase according to Chang and Bollum (27). The oligomer-homopolymer complex, poly(rA)·$(dT)_{25-30}$, was hybridized as described by Spadari and Weissbach (9).

Calf thymus DNA (about 2 mg/ml) was first treated with pancreatic RNase (15 µg/ml), extracted 3 times with phenol-chloroform (1:1), and the phenol removed by ether extractions. After ethanol precipitation, the DNA was resuspended at 2 mg/ml in 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, and incubated at 37° with 1 µg of pancreatic DNase per 25 mg DNA. The incubation was carried out until 10-15% of the DNA was made acid soluble. After cooling, the solution was extracted with phenol-chloroform (1:1) and the phenol removed by ether extractions. The activated DNA was then dialyzed against 10 mM Tris·HCl (pH 8.5), 1 mM EDTA.

RESULTS

Developmental profiles of DNA polymerases-α, -β, and -γ in forebrain neurons. The results are presented in Fig.1. DNA polymerase-α shows its highest specific activity before birth and then declines progressively reaching a level of almost zero by the 23rd day after birth. The enzyme activity decreases to 25% of its original value between day -4 and 0. During the next 7 days of development assayable activity is of the order of 15% of the -4 day level. In contrast, the activities of DNA polymerases-β and -γ do not change significantly during perinatal development and are still present at measurable levels in adults.

If the relative amounts of the three DNA polymerases present in late gestation are compared, approximately 65-70% of the total activity is due to α-, 25-30% to β-, and 2% to γ-

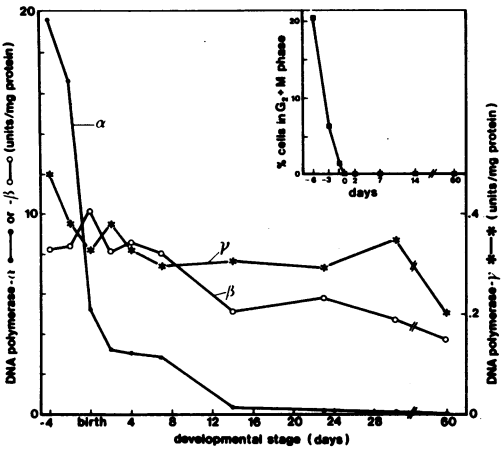

Fig.1: Developmental profiles of DNA polymerases-α, -β, and -γ in forebrain neurons. The specific assay conditions described in Methods were used in order to determine the activities of each DNA polymerase. Note different scales used for plotting DNA polymerase-α and -β activities on the one hand and DNA polymerase-γ on the other. The inset shows the percentage of cells in G_2 and M phase as determined by cell-cycle analysis.

polymerase. After birth, the relative amounts of β- and γ-polymerase increase to the adult proportions of 92-95% and 5-7%, respectively.

The loss in α-polymerase activity correlates temporally very well with the decline in mitotic activity (Fig.1, inset), which drops to zero at birth.

Developmental profiles of DNA polymerases-α, -β, and -γ in ventricular cardiac muscle. The results are reported in Fig.2. Compared to neurons, all three DNA polymerase activities in cardiac muscle are already low at day -2 ; they progressively decline during further development. Specifically, α-polymerase decreases to 14% of its original level between day -2 and 2 and reaches a value close to zero at day 7. During the same time interval, DNA polymerases-β and -γ drop only by 30-40% ; at later times, they do not change significantly. Contrary to α-polymerase, these two enzymes are still assayable in adults, even though γ-polymerase activity is reduced to levels at least one order of magnitude lower than those observed in all other tissues studied in this work.

A comparison of the three enzyme activities shows that, already before birth, α-polymerase is present in low proportions. At day -2 approximately 71% of the total activity

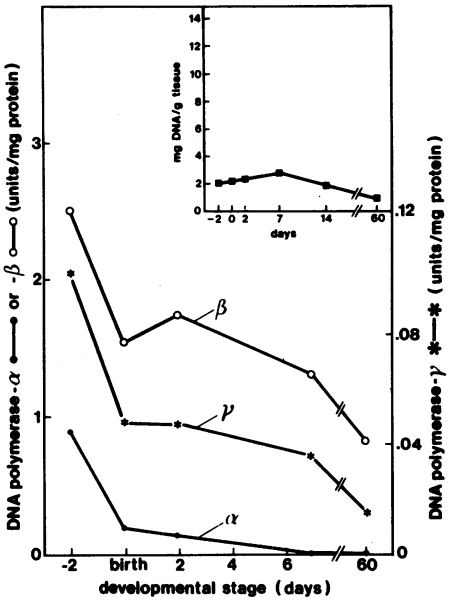

Fig.2: Developmental profiles of DNA polymerases-α, -β, and -γ in ventricular cardiac muscle. Enzyme activities were determined as described in Methods. Note different scales used for plotting DNA polymerase-α and -β activities on the one hand and DNA polymerase-γ on the other. The inset shows the amount of DNA per g tissue chosen as a parameter of the rate of cell multiplication.

is due to β-polymerase, 26% to α-polymerase, and 3% to γ-polymerase.

The low activity of α-polymerase before birth and its further perinatal decrease correlate well with the almost total arrest of cell multiplication that has occurred prior to the period under study (Fig.2, inset; in this, as well as in the following experiment, in which organs rather than isolated cells were used, we chose the amount of DNA per unit mass as a parameter of cell proliferation).

Developmental profiles of DNA polymerases-α, -β, and -γ in spleen. The results are shown in Fig.3. The levels of all three DNA polymerases are very low in late gestation and at birth. At day 2 we observe a dramatic 50 fold increase of α-polymerase activity and a 3- to 4-fold enhancement of the β- and γ- enzymes. All three activities remain high during the next two weeks. This is followed by a 70% decrease of α-polymerase activity until the age of 60 days, whereas the β- and γ-polymerases show negligible variations in activity during this period.

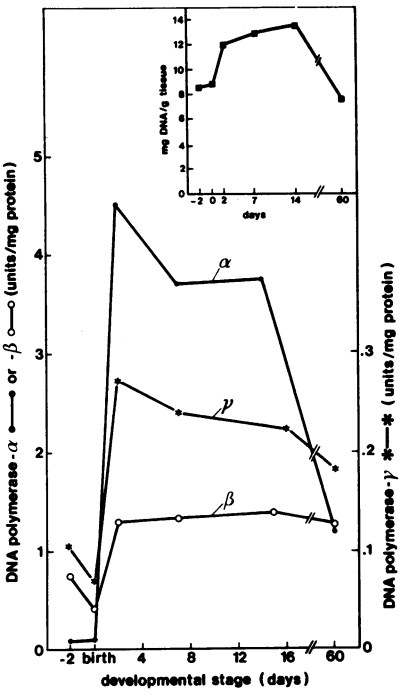

Fig.3: Developmental profiles of DNA polymerases-α, -β, and -γ in spleen. Enzyme activities were determined as described in Methods. Note different scales used for plotting DNA polymerase-α and -β activities on the one hand and DNA polymerase-γ on the other. The inset shows the amount of DNA per g tissue chosen as a parameter of the rate of cell multiplication.

The relative distribution of DNA polymerase-α, -β, and -γ activities changes respectively from 1%, 85%, and 14% prenatally (day -2) to 74%, 21%, and 5% postnatally (day 2). In adults, α-polymerase is still present in proportions (45%) much higher than in the other tissues studied (approximately 1% in neurons and cardiac muscle).

The developmental variation of α-polymerase activity in spleen closely mirrors the rate of cell multiplication in this organ (Fig.3, inset). The high activity of α-polymerase in adults seems to be ascribable mainly to dividing and differentiating lymphocytes in the germinal centers (S. Spadari, G. Villani, and N. Hardt, in preparation).

Mixing experiments. It could be argued that soluble dissociable inhibitors or activators of DNA polymerases might be present at different times during development. However, mixing experiments (data not shown) gave nearly additive results and therefore permitted to rule out such a possibility.

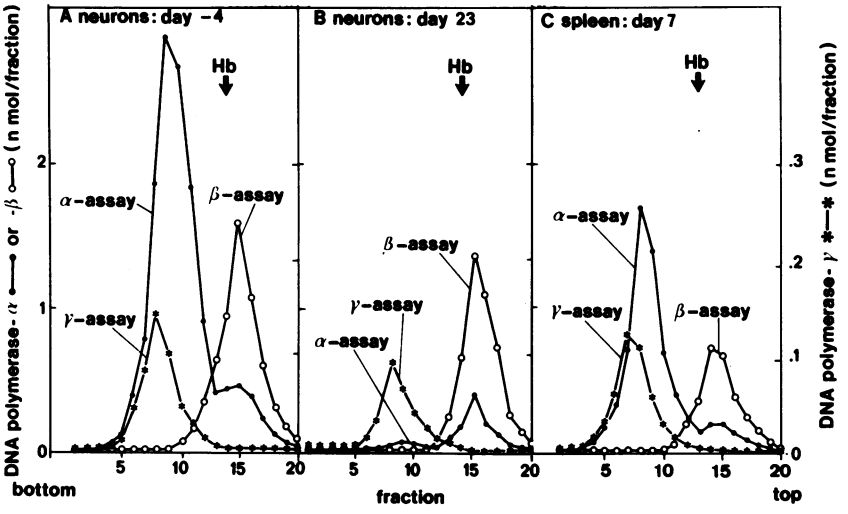

Fig.4: Visualization of DNA polymerases-α, -β, and -γ in sucrose gradients. Representative gradients are shown for neurons on day -4 (A) or 23 (B) and for spleen on day 7 (C). Gradients were run as described in Methods, and 10-20 µl per fraction were assayed for 20 min at 37°. Hemoglobin (Hb) was simultaneously run as a molecular weight marker (68,000). Note different scales used for plotting DNA polymerase-α and -β activities on the one hand and DNA polymerase-γ on the other.

Visualization of DNA polymerases-α, -β, and -γ in sucrose gradients. The results shown in Fig.4 demonstrate that, under the experimental conditions described in Methods, the β- and γ-assays are strictly specific for both enzymes: (a) the fractions in the α- and γ-polymerase region do not respond to the β-assay, and (b) the fractions containing β-polymerase do not respond to the γ-assay. In contrast, β-polymerase responds with 30% efficiency to the α-assay; but, as already said, all our measurements of α-polymerase activity were corrected for this contribution. This correction is valid even under conditions of low α-polymerase activity as exemplified by the excellent agreement between the relative polymerase levels observed when either a crude extract (Fig.1) or a sucrose gradient fraction (Fig.4B) of day 23 neurons was analyzed.

DISCUSSION

Several data on DNA synthesis and mitosis in neurons, cardiac muscle and spleen at different stages of development are available in the literature (28-35). Much less is known about the mechanism by which DNA synthesis and cell division are regulated. In view of the absence, in animal systems, of mutants defective in DNA replication, we have studied *in vivo* the temporal relationship between the activities of the cellular DNA polymerases-α, -β, and -γ and the rate of cell multiplication in a number of tissues. For this purpose, we have chosen tissues with characteristic patterns of mitotic activity during pre- or postnatal development. Forebrain neurons (28-30) and cardiac muscle cells (31-33, 36, 37) were selected for their inability to replicate after birth, while spleen is a tissue characterized by a burst of postnatal cell multiplication (34, 35). These studies, on well defined cells or organs undergoing well characterized variations in growth rate, seem more amenable to interpretations than the other less defined systems studies in the past (13-20).

The activity of DNA polymerases-α and -β has previously been studied in crude brain extracts which were not fractionated according to cell type (neurons or glial cells) (38, 39) and in cardiac muscle (36, 37, 40). The activities of α- and β-polymerase have not so far been studied in spleen during the perinatal period. None of these earlier studies included the recently discovered γ-polymerase (1). Furthermore, in all these previous studies, DNA polymerase levels were determined on native or denatured DNAs, which are not suitable templates for animal DNA polymerases (3). Utilization of such templates by DNA polymerases reflects either the presence of nucleases in the enzyme preparation or the presence of "hairpin" structures formed in denatured DNA.

Our present results show pronounced perinatal changes in the levels of DNA polymerase-α activity; they mirror

the in vivo cell multiplication rate of the tissues under study. Considering the fact that the physiological variations of the cells (or organs) under study are well understood, and that α-polymerase is localized in the nucleus (41-44), these observations bring strong support for the idea that α-polymerase is an important enzyme in DNA replication; they suggest a probable involvment of this enzyme in the regulation of cell division. DNA polymerase-α seems to be synthesized and degraded at a rapid rate in order to keep up with the requirements of the cell. In contrast, DNA polymerase-β and -γ are present at all stages of development in the tissues studied in the present work and their levels remain relatively independent of the in vivo rate of cell multiplication. Thus, it is unlikely that these two enzymes play a major role in DNA replication; rather, as already suggested by Bertazzoni et al. (18) for the β-enzyme, they might be of importance in DNA repair type processes occurring in proliferating and non-proliferating tissues.

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