POTENTIALITIES OF DNA REPLICATION AND REPAIR IN PROLIFERATING AND DIFFERENTIATING CHICK CELLS

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要　旨

動物胚の組織分化に際して，増殖と分化にともなうニワトリ細胞のDNAの複製能および修復能に注目し，以下の研究をおこなった。(1) 無殻培養したニワトリ2日胚に発癌剤である[^{3}H]-4HAQOを投与したところ，投与1時間のうちに[^{3}H]-4HAQOは無害な化合物へと代謝され，また，[^{3}H]-4HAQOとDNAとの結合比は投与2時間後に最大(18.2，μmol/mol P of nucleotide)となったのち減少した。すなわちニワトリ胚においても4HAQOの代謝経路は哺乳類と同様であり，かつ4HAQOによるDNA損傷に対する修復系も存在すること，また発生過程において分化をはじめようとする組織が4HAQOに高い感受性を示し，形態異常を生じやすいことも示唆された。(2) 松影らの作製したニワトリ胚DNAポリメラーゼαおよびβに対する抗体を用いた間接蛍光抗体法により，ニワトリ胚のレンズ，網膜，脊髄における両酵素の分布と変遷をしらべた。DNAポリメラ

ーゼαは増殖能を保持している細胞の核内に見出されたが，増殖能を失ない，分化しつつある細胞では検出されず，この酵素の消失はDNA複製能の消失ときわめてよく一致していた。一方，DNAの修複に関与していると考えられているDNAポリメラーゼβはしらべた限りのいずれの発生段階，組織においても，ほぼすべての細胞核において見出された。DNAポリメラーゼαに対する抗体を用いた今回の方法によってニワトリの胚について細胞を個々に，分化を開始しはじめているか否かを検出することが可能となった。(3) そこで次に細胞再生系の例として，小腸上皮と雄の生殖細胞をとりあげ，両酵素の分布をしらべることとした。ニワトリ胚十二指腸において，DNAポリメラーゼαは，①8日胚ではほぼすべての腸上皮細胞に見出されたが，胚発生とともにその存在が検出される細胞は，②12日胚では前絨毛隆起の基部側の細胞，③18日胚からふ化直前の胚では絨毛の基部側の約半分

の細胞，④ふ化後2週令のひなでは腺窩の細胞に限られ，この酵素の消失はDNA複製能の消失ときわめてよく一致していた。またこの研究によって長年論議されてきたニワトリの腺窩におけるパネス細胞の存否について，これが見出されないことを示すことができたと思われる。一方，DNAポリメラーゼβは，しらべたすべての段階のほぼすべての細胞核に見出された。9か月令の成鶏の精巣では，DNAポリメラーゼαは精原細胞と第1次精母細胞に見出されたが，それより分化段階の進んだ諸細胞には見出されず，一方，DNAポリメラーゼβは精子核をのぞくほぼすべての精巣の細胞核に見出された。精子核にβの見出されぬ理由についても論議した。

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PART I.

METABOLISM OF 4-HYDROXYAMINOQUINOLINE-1-OXIDE AND ITS BINDING TO DNA IN CHICK EMBRYOS

SUMMARY

The metabolic pathway of 4-hydroxyaminoquinoline-1-oxide (4HAQO) and its binding to DNA was studied in 2-day chick embryos administered [G-^{3}H]4HAQO in a shell-less culture. The 4HAQO rapidly metabolized into non-carcinogenic compounds and 1 h after administration only very small amounts of free 4HAQO could be detected in the embryo cells. The amount of DNA-bound 4HAQO in the embryo cells reached a maximum 2 h after administration, then began to decrease. The maximum extent (μ mol/mol P of nucleotide) was 18.2, equivalent to 1 molecule of 4HAQO-purine adducts per 2.8×10^4 base pairs of DNA. It was possible to detect removal of 4HAQO-purine adducts from DNA in chick embryo cells in a shell-less culture. A dose-response relationship for the killing effect of 4HAQO on 2-day embryos was observed in the range of 0.24–24 nmol 4HAQO per embryo. The practicality of the present method of administration of 4HAQO for 'flash administration' of compounds to chick embryo and the advantages of the shell-less culture method which provides access for biochemical and developmental studies of chick embryos were also discussed.

Key words: 4-Hydroxyaminoquinoline-1-oxide – Chick embryo – DNA – Shell-less culture – Hydroxyapatite column

Abbreviations: 4AQ, 4-aminoquinoline; 4AQO, 4-aminoquinoline-1-oxide; 4HAQO, 4-hydroxyaminoquinoline-1-oxide; 4HAQ, 4-hydroxyaminoquinoline; 4HQO, 4-hydroxyquinoline-1-oxide; 4NQO, 4-nitroquinoline-1-oxide; PB, sodium phosphate buffer; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

INTRODUCTION

Nakahara et al. [1], for the first time, reported that 4-nitroquinoline-1-oxide (4NQO) was capable of producing cancer in the mouse. Kondo et al. [2] has pointed out that UV-sensitive strains of *Escherichia coli* were also highly sensitive to killing and mutagenic action of 4NQO. Kato et al. [3] showed that in various rat and mouse organs 4NQO was first reduced by cytosol enzymes to 4HAQO. Tada and Tada [4–6] found that 4HAQO was converted to an aminoacylated metabolite of 4HAQO through the action of certain species of aminoacyl synthetase, and that the metabolite binds covalently with both the guanine and the adenine residues of DNA to form 4NQO-purine adducts. Ikenaga et al. [7] demonstrated that the major causes of 4NQO killing and mutagenic action of 4NQO in *E. coli* were the 4NQO-purine adducts, and that these adducts were removed from DNA by the excision repair system. Ikenaga et al. [8] also found a similar removal repair mechanism in normal human cells. Almost all of the studies on applied 4NQO have been carried out in bacteria and cultured mammalian cells; few have been done on developing embryos. Mammalian embryos developing in the uterus were difficult to deal with and were not as advantageous to our purposes as were avian embryos. As a first step in studying the effects of 4HAQO, a proximate metabolite of 4NQO, on developing embryos, we investigated the metabolism of 4HAQO, the binding of 4HAQO to DNA and the killing effect of 4HAQO on early chick embryos cultured outside the shell.

This paper gives the results of this study as well as discusses an experimental system which should be very useful in biochemical and developmental studies involving administration of carcinogens or other chemicals to whole chick embryos.

MATERIALS AND METHODS

Culture of chick embryos

Dunn's shell-less culture technique [9] with slight modifications was applied in order to prepare chick embryos cultured outside the shell. Fertile, white leghorn eggs from a local breeder were placed in a humidified incubator at 38°C for 48 h, by which time the embryos had reached Hamburger and Hamilton stage 11–12 (13–16 somite stage) [10]. After the shell surfaces had been sterilized with 70% ethanol, egg contents were removed and suspended in a sheet of plastic wrap (approx. 30 × 30 cm) supported by a rubber band in a sterile paper cup (7 cm, diam., 8 cm, height and 200 ml, vol.) as shown in Fig. 1. The paper cup was covered with a sterile culture dish which allowed clearance for gas exchange and then placed under normal atmospheric conditions at 38°C in a saturation humidity incubator to prevent evaporation. No culture media or antibiotics were added.

Treatment of [G-^{3}H]4HAQO to the embryos

[G-^{3}H]4HAQO was obtained through the courtesy of Dr. Y. Kawazoe of

Fig. 1. A shell-less chick embryo culture. The egg contents (ec) are suspended in a sheet of plastic wrap (pw) supported in a paper cup (pc) and covered with a culture dish (cd). The sketch in the lower corner shows a vertical plane of the culture.

Nagoya City University. The stock solution, 0.1 M of [G-^{3}H]4HAQO (spec. act., 28 mCi/mmol) in dimethylsulfoxide, was kept in the dark below 5°C and then diluted with phosphate buffered saline (pH 7.4) immediately before use. A 50-μl quantity of the diluted solution containing 10 nmol of [G-^{3}H]4HAQO was dripped from a micropipette (Gilson Pipetman P-200) over each chick embryo (0.28 μCi/embryo) immediately after it was transferred to the shell-less culture. After the [G-^{3}H]4HAQO has been added, the embryos were placed in a humidified incubator at 38°C.

At 1,2,4 and 8 h after 4HAQO administration, the embryos were separated from the yolk under a binocular dissecting microscope and washed by passing through several baths of phosphate buffered saline.

Analysis of 4HAQO metabolites

At 1 h and 2 h after [G-^{3}H]4HAQO administration, 5 embryos each were

suspended in 0.5–1 ml of distilled water and homogenized by sonication for 1 min in a Kontes Micro Ultrasonic Cell Disrupter under maximum power output. Three volumes of methanol were added to the homogenate, the mixture was shaken well and left for 1 h at 4°C. After centrifugation at 3000 rev./min for 20 min at 4°C, the supernatant (the methanol soluble fraction) was collected and condensed to 5 μl by evaporation. The condensate, together with authentic 4HAQO, 4-aminoquinoline-1-oxide (4AQO), 4-aminoquinoline (4AQ) and 4-hydroxyquinoline-1-oxide (4HQO) (Wako Chemical Co. Ltd. Osaka) was applied to a silica gel plate (Tokyo Kasei KK, Tokyo). The gel plates were developed in a mixture of ethyl-acetate/methanol/acetic acid (6:3:1) by ascending chromatography for 90 min. After development, the fluorescence of the 4HAQO metabolites was used to locate them under 260 nm or 320 nm UV light. The gel plate was cut into individual lanes, each lane was cut from the origin into 5 mm segments and the segments were placed in scintillation vials. A liquid scintillation counter (Beckman, LS8000) was used to count the radioactivity of the segments in the scintillation mixture (PCS: Amersham, U.S.A.).

Extraction and estimation of DNA in chick embryos

DNA was isolated by hydroxyapatite column chromatography according to the method of Meinke et al. [11], as modified by Shoyab [12]. From 15 to 20 embryos, individually administered 0.28 μCi of [G-^{3}H]4HAQO, were homogenized by sonication in 1–2 ml of lysing solution (8 M urea, 1% sodium dodecyl sulfate (SDS), 0.002 M EDTA in 0.24 M sodium phosphate buffer (PB)) for 1 min at room temperature. The entire cell lysate so prepared was applied to a column which had been previously packed with 2–3 ml bed volume of hydroxyapatite (DNA Grade Bio-Gel HTP, Bio-Rad) and equilibrated with 2–3 ml of lysing solution. Proteins and RNA were removed from the column by washing with 8 M urea in 0.24 M PB; then both the urea and the SDS were washed out with 0.12 M PB. Finally, the DNA was eluted with 0.48 M PB. An ISCO UV monitor was used to monitor the absorbance of the eluate at 254 nm.

UV absorbance at 260 nm was determined for the 0.48 M PB eluted fraction and the DNA content of the eluate was estimated using the relationship 1 mg/ml of DNA = 20 $O.D._{260}$ unit. Carrier DNA (25 μg of calf thymus DNA) and 10% cold trichloroacetic acid (TCA) were added to the 0.48 M PB eluate to make a final TCA concentration of 5%. This was allowed to stand for 30 min at 4°C. The DNA was collected as the precipitate on a glass microfiber filter (Whatman, GF/C 2.5 cm). This was followed by washing with 5% cold TCA and again with cold ethanol. It was then air-dried. In order to determine the amount of DNA-bound [G-^{3}H]4HAQO, radioactivity of the filter was measured using a liquid scintillation counter.

Examination of 4HAQO effects on embryonic development

Gross observation of the embryos was carried out under a binocular dissecting microscope at 3 days after administration of 4HAQO (0.24–

24 nmol/embryo, i.e., 0.05–5 μg/embryo) in order to examine killing and malformation effects. Embryos showing no heart pulsation and with or without tissue degeneration were regarded as dead, whereas those showing microcephalia, myeloschisis and/or any other abnormal appearance were regarded as malformed. After gross observation, all the embryos were fixed in Bouin's fluid. For some of them, this was followed by routine histological sectioning and staining in preparation for more detailed observations.

RESULTS AND DISCUSSION

Metabolism of 4HAQO in 2-day chick embryo

In order to analyze the 4HAQO metabolites, the methanol soluble fractions from the chick embryos were subjected to thin-layer chromatography at 1 h and 2 h after [G-^{3}H]4HAQO administration. Figure 2 shows the radiochromatographic patterns of the methanol soluble fractions from these embryos. It was found that radioactivity was high in the 4AQO region, low in both the 4HAQO and the 4AQ regions, and barely detectable in the 4HQO region for embryos at 1 h and 2 h after administration. In comparison with the embryos cultured at 38°C, radioactivity in the 4HQO region was more readily detectable in embryos cultured at 20°C (data not shown). The metabolic rate was low at the later temperature. These results suggest that: (1) 4HAQO in chick embryos may be metabolized as shown in Fig. 3, this is

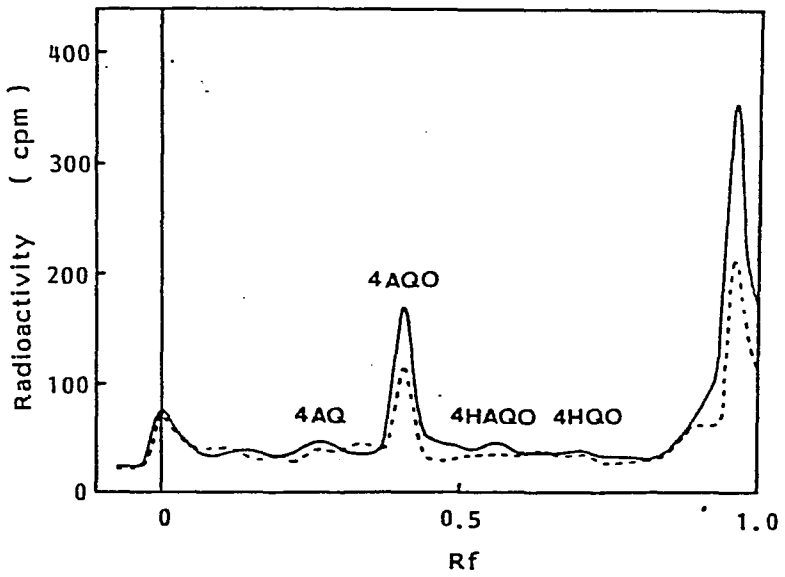

Fig. 2. Results of radiochromatography of methanol soluble fractions obtained from 2-day embryos 1 h (solid line) and 2 h (dashed line) after administration of [G-^{3}H]4HAQO. Preparation of the methanol soluble fraction and radiochromatographic procedures were conducted as described under Materials and Methods. Radioactivity is expresssed as cpm per 5-mm silica gel plate segment.

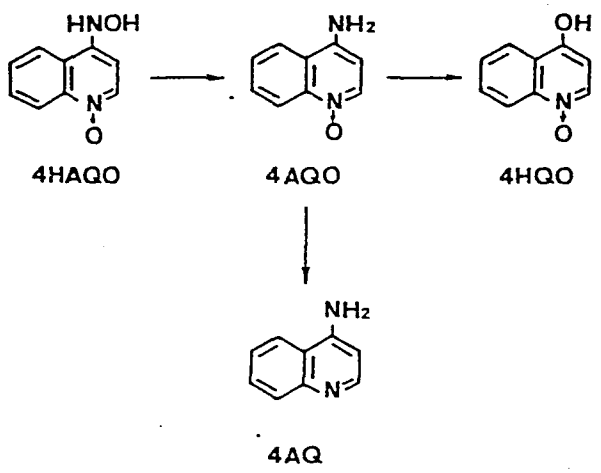

Fig. 3. Suggested metabolic pathway for 4HAQO in chick embryos.

similar to metabolism in the mouse [13] and (2) conversion of 4HAQO (a carcinogenic compound) to 4AQO (a non-carcinogenic compound) in chick embryo cells may be very rapid while conversion of 4AQO to 4AQ and 4AQO to 4HQO may be slow. Therefore, the potentiality of 4HAQO to produce DNA lesions in chick embryo cells may be almost completely lost within 1–2 h after administration of 4HAQO. Also the present method of drip administration of 4HAQO can be a practical 'flash administration method' for embryos in a shell-less culture.

Binding of 4HAQO to DNA

Figure 4 shows the chromatographic profile of cell lysates from 2-day chick embryos on a hydroxyapatite column. The DNA was eluted with 0.48 M

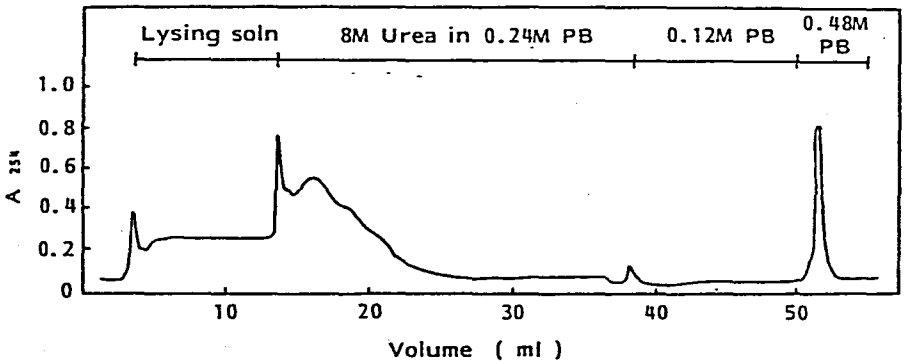

Fig. 4. Chromatographic profile of 2-day embryo lysates on a hydroxyapatite column. Lysates obtained from 15–20 embryos were applied to a 2–3-ml bed volume of hydroxyapatite column as described under Materials and Methods. The optical density of the eluate was monitored at 254 nm using an ISCO UV monitor. The last peak is the DNA fraction from the hydroxyapatite column eluted with 0.48 M PB. Elution rate was approx. 0.5 ml/min.

PB as a clear peak. From twenty 2-day embryos (approx. 370 mg total wet wt.), 48 μg of DNA was isolated by this chromatographic technique.

Solomon [14] reported that the average amount of DNA per nucleus in the chick embryo is 7.5×10^{-13} g. Adopting this value, 48 μg of DNA (from twenty 2-day embryos) corresponded to 6.4×10^{7} cells and the average total number of cells per 2-day embryo was estimated as approx. 3.2×10^{6}. This is quite consistent with the number of cells reported by Solomon [14]. This may indicate that the present method gives a good yield of DNA in nearly pure form, and therefore the radioactivity associated with the DNA in this study, even though apparently low, may be significant. We also found that the hydroxyapatite column method is very useful for DNA isolation from even a small amount of material.

The amount of 4HAQO molecule covalently bound to the DNA, i.e., the amount of 4HAQO-purine adducts, was estimated on the basis of the DNA-associated radioactivity. Figure 5 shows the time course of the extent of 4HAQO bound to DNA in 2-day embryos administered [G-^{3}H]4HAQO. Each of the values in Fig. 5 represents the mean of 2 measurements from duplicated experiments. Each measurement was carried out using 15–20 embryos.

As shown in Fig. 5, the amount of DNA-bound 4HAQO per embryo reached a maximum 2 h after administration. The DNA content per embryo was 2.7 μg; and the amount of DNA-bound 4HAQO per embryo was 0.16 pmol. Accordingly, the extent of DNA-bound 4HAQO (μmol/mol P of nucleotide) was 18.2, which was equivalent to 1 molecule of 4HAQO purine adduct per 2.8×10^{4} base pairs of DNA. These values, obtained from the chick embryos,

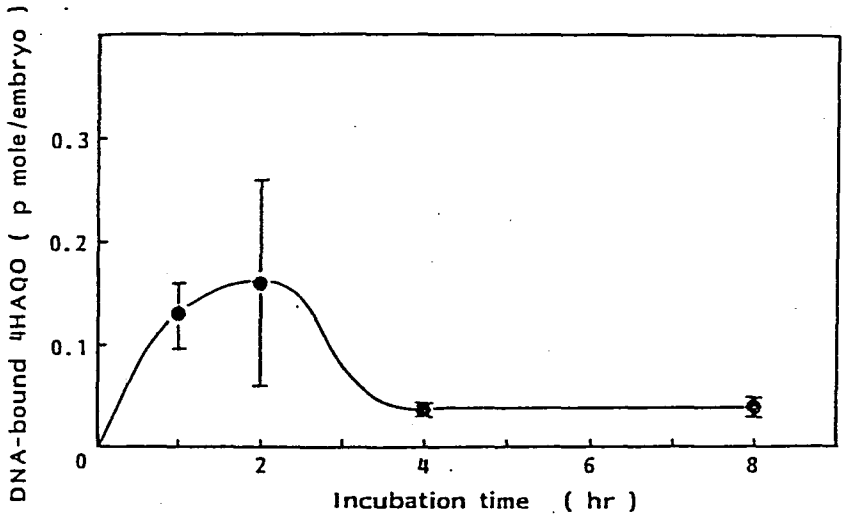

Fig. 5. Kinetics of DNA-bound 4HAQO in 2-day chick embryos in a shell-less culture at 38°C. DNA isolation and radioactivity assays were carried out as described under Materials and Methods. The amount of DNA-bound 4HAQO is expressed in pmol/embryo. Closed dots and bars indicate means and ranges, respectively, of measurements from duplicated experiments.

were for all practical purposes consistent with the values obtained from cultured mammalian cells by Morita et al. [15].

The extent of DNA-bound 4HAQO (μmol/mol P of nucleotide) 1, 2, 4 and 8 h after administration was 15.6, 18.2, 4.2 and 3.6, respectively, while DNA content per embryo (μg) was respectively 2.4, 2.7, 2.9 and 3.3. Between 2 h and 4 h following administration, the amount of DNA-bound [G-^{3}H]4HAQO per embryo decreased by 75%, whereas the DNA content per embryo increased by only 7%. This decrease may suggest that the DNA-bound 4HAQO was removed from the DNA in chick embryos.

In our preliminary experiments, when the embryos were kept at 20°C after administration of [G-^{3}H]4HAQO, no reduction or removal of DNA-bound [G-^{3}H]4HAQO was found. At 20°C, the embryos ceased development and both per embryo DNA and protein content remained nearly constant. These embryos began to develop again after the culture temperature was brought back up to 38°C.

Paterson et al. [16], who studied repair of UV-induced pyrimidine dimers (UV-dimers) in 10-day embryo chick fibroblasts, found that in the dark the UV-dimers were hardly removed from the DNA of the fibroblasts. On the contrary, Wade and Lohman [17] reported that the excision of UV-dimers in 11-day embryo chick fibroblasts progressed more slowly than it did in human fibroblasts. Recently, Bootsma et al. [18] found that in heterokaryons obtained from fusion of chick erythrocytes with UV-irradiated xeroderma pigmentosum cells, the chick erythrocytes were able to provide factors which may be complementary to the defects in the DNA repair system of the xeroderma pigmentosum cells. The function of the excision repair system in chick embryo cells was or was not affected depending upon the experimental conditions. Taking these together, one may speculate that the excision repair system is probably involved in the removal of the 4HAQO-purine adducts in chick embryos. Further investigation into the repair mechanisms in chick embryo cells is necessary.

The effect of 4HAQO on embryonic development

Table I shows the effects of 4HAQO on 2-day chick embryos. When a dose of 24 nmol of 4HAQO was dripped on the embryos, only 11% of them survived and just 10% of the survivals showed a normal appearance. The others were malformed. When the dose was 2.4 nmol/embryo, 76% of the embryos survived and when it was 0.24 nmol/embryo, no killing effect at all was observed. These results indicated a dose-response relationship for the killing effect of 4HAQO on 2-day chick embryos in a shell-less culture. Additional morphological examinations are now being undertaken on the effects of 4HAQO on embryonic development.

Shell-less chick embryo cultures as an experimental system

The shell-less culture method used in this study has several advantages over the window method. These include: (1) the procedure is so simple that it is possible to prepare a large number of embryos in a relatively short time,

TABLE I

EFFECTS OF 4HAQO ON EMBRYONIC DEVELOPMENT

4HAQO was administered to 2-day chick embryo. Examinations were conducted 3 days later.

Dose (nmol/ embryo)	No. of embryo(%)	No. of dead embryo[a](%)	No. of malformed embryo[b](%)	No. of normal embryo(%)
0.00	58 (100)	4 (7)	3 (5)	51 (88)
0.24	38 (100)	3 (8)	1 (3)	34 (89)
2.40	38 (100)	9 (24)	1 (3)	28 (73)
24.00	71 (100)	63 (89)[c]	7 (10)	1 (1)

[a] Embryos showing no heart pulsation and with or without tissue degeneration were considered dead.

[b] Embryos showing microcephalia, myeloschisis and/or other abnormal appearances were considered malformed.

[c] Application of χ^2-test with Yates' correction yielded a P-value <0.001, in comparison with the control group.

(2) it permits normal chick embryo growth in paper cups for about 10 days, from the 2nd day of incubation when the embryos have 13–16 somites (Hamburger and Hamilton Stage 11–12) to the time when the embryos are approx. 30 mm in crown-rump length and have conspicuous flight feathers and just visible claw primodia on the toes (Hamburger and Hamilton Stage 36), (3) carcinogens or other drugs can be readily administered and UV or X-ray irradiation and surgical operations can be accurately performed on the embryo and its membranes and (4) the entire embryo is visible. The chick embryo in a shell-less culture, therefore, provides easy access for studies of morphogenesis, growth and cell proliferation and differentiation as well as for studies in developmental biochemistry.

ACKNOWLEDGEMENTS

We are grateful to Dr. Y. Kawazoe of Nagoya City University for providing the [G-^{3}H]4HAQO. We are also indebted to both Mr. Y. Minoura of Aichi Cancer Center Research Institute, and Miss K. Maeda of College of General Education, Osaka University, for their kind help.

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PART II. IMMUNOCYTOHEMICAL LOCALIZATION OF DNA POLYMERASE α AND β IN THE DEVELOPMENT OF THE CHICK EMBRYO LENS, RETINA AND SPINAL CORD

SUMMARY

Intracellular localization of DNA polymerase α and β in the developing chick embryo lens, retina and spinal cord was surveyed by indirect immunofluorescent methods with antibodies against chick embryo DNA polymerase α and β, in addition to autoradiographic studies to detect DNA synthesis in these cells. DNA polymerase α, namely responsible for DNA replication, was detected in the nuclei of the cells capable of proliferating but not detected in the nuclei of the cells which began to differentiate in all tissues and organs examined. The disappearance of DNA polymerase α closely coincided with the loss of DNA replicating capacity in these cells. On the other hand, DNA polymerase β, responsible for DNA excision repair, was detected in almost all the cell nuclei at all developmental stages examined. The availability of the present immunofluorescent method using the antibody against DNA polymerase α in studies of developmental biology, especially to distinguish proliferative cells from non-proliferative cells in the chick embryo has been discussed.

INTRODUCTION

The onset of cellular differentiation is generally accompanied by the progressive loss of cellular proliferative activity. The biochemical studies of DNA polymerase α have shown that DNA polymerase α, mainly responsible for DNA replication (1-3), declines in the course of cellular differentiation (4-8), while DNA polymerase β, responsible for DNA excision repair (1-3), is detectable in various tissues and cells (4-9). However, it is difficult to clarify the intracellular localization of DNA polymerases in growing tissue cells by biochemical methods.

Recently, mouse monoclonal antibodies have been prepared against DNA polymerase α of human KB cell (10), calf thymus (11) and chick embryo (12); and also the intracellular localization of DNA polymerase α has been revealed by immunocytochemical methods using these antibodies (13-15). These studies showed that DNA polymerase α was detectable in the proliferating cells but not in the quiscent cells in culture. This means that the differential distribution of DNA polymerase α in the proliferating and the quiscent cells in culture can be immunnocytochemically observed.

Almost all the immunocytochemical studies of DNA polymerase α, however, have been carried out in cultured cells; few studies have been done on the embryo cells in situ. The developing chick embryo is considered one of the very

proper materials for the present subject to aim at distinguishing the proliferating cells from the postmitotic, differentiating cells by means of immunocytochemical detection of DNA polymerase α, if possible, because a large number of morphological works and reviews to be referred have been done on the chick embryos by many experimental zoologists and embryologists (16). Matsukage et al. (12) have opportunely prepared mouse monoclonal antibodies against chick embryo DNA polymerase α (12) and a specific rabbit antibody against chick embryo DNA polymerase β (17). By the use of these antibodies, it is possible to reveal clearly that the onset of cellular differentiation is concomitant with the disappearance of DNA replication potentiality in chick embryo cells.

The present studies were designed to find out the distribution of DNA polymerase α and β in the chick lens, retina and spinal cord during development by indirect immunofluorescent methods with these antibodies.

MATERIALS AND METHODS

Chick embryos and [^{3}H]thymidine labeling

Fertile white leghorn eggs from a local breeder were incubated in a humidified incubator at 38°C.

In order to identify which cells in the developing embryo are synthesizing DNA, a 0.4 ml of Ringer's solution containing 40 µCi of [methyl-^{3}H]thymidine (20 mCi/m mole, New England Nuclear, Boston, Mass.) was dripped from a micropipette (Gilson Pipetman P-200) through a window opened on the egg shell over the chorioallantoic membrane of each of the chick embryos at 8,12,18 days of incubation.* After [^{3}H]thymidine administration, the window was sealed with a piece of scotch tape and eggs were back to a humidified incubator at 38°C. The embryos were separated from the yolk and the extraembryonic membranes at 1 hr and 24 hr after [^{3}H]thymidine administration, washed by passing several baths of phosphate buffered saline (PBS, pH 7.2), fixed and embedded as follows.

Tissue fixation and preparation of tissue sections

Embryos were fixed by 3.5% formaldehyde in 0.1 M sodium-phosphate buffer (pH 7.2) for 2-4 hrs at 4°C, rinsed in 0.1 M sodium-phosphate buffer at 4°C for 12 hrs by changing of buffer solution several times to remove formaldehyde.

* Ages of the embryos were given either as days after the initiation of incubation or as the Hamburger and Hamilton stage.

The polyester wax embedding and sectioning technique was embloyed for the immunofluorescent staining of DNA polymerase α and β, according to Kusakabe et al. (18). Briefly, whole embryos were dehydrated through an ethanol series and embedded in polyester wax (BDH Chemicals Ltd., Broom Road, Poole BH 12, 4NN, England) without passing any toluene bath. Embryos in the wax were cut serially at 4 μm at 4-8°C by a conventional microtome and sections were mounted on ovalbumin coated glass slides. The wax was removed and rehydrated through an ethanol series, the sections were rinsed in cold 0.01 M phosphate buffered saline (PBS) at pH 7.2, before the following staining procedures were applied.

Immunofluorescent microscopy

Both the mouse monoclonal antibody against chick embryo DNA polymerase α and the rabbit antiserum against chick embryo DNA polymerase β were obtained through the courtesy of Dr. A. Matsukage of Aichi Cancer Center Research Institute. The former antibody was prepared from the culture spernatant of a mouse hybrydoma clone, 4-8H, which was established by Matsukage et al (12). This antibody is γ_1-type IgG and is specific to the 135000 dalton polypeptide of chick DNA polymerase α. The latter anti-DNA polymerase β rabbit antibody was prepared by Yamaguchi et al. (17), using a homogenous preparation of

chick embryo DNA polymerase β as an antigen. This antibody specifically immunoprecipitated the 40000 dalton polypeptide of chick DNA polymerase β.

For immunofluorescent staining of DNA polymerase α, the sections on slide glasses were incubated with PBS containig 5% normal goat serum to mask non-specific binding sites, incubated again with the mouse monoclonal antibody against chick DNA polymerase α at 37°C for 30 min, rinsed by 5 baths of cold PBS, followed by incubation with the second antibody, a rabbit antibody against mouse IgG (25 μg/ml, Zymed Lab., South San Francisco, Calif.), and finally with the third antibody, a fluorescein isothiocyanate (FITC) conjugated goat antibody against rabbit IgG (25 μg/ml, TAGO, Burlingame, Calif.).

For immunofluorescet staining of DNA polymerase β, the sections on slide glasses were incubated with diluted the rabbit antiserum against chick DNA polymerase β (usually 1:50 dilution with the antiserum), rinsed in cold PBS and again incubated with the FITC conjugated goat antibody against rabbit IgG,which was used as the third antibody for the staining of DNA polymerase α.

As for the counter staining, 0.02% Evans Blue (Merk) in PBS was applied for 2 min. The cytoplasm and non-fluorescent nuclei could be easily recognized as stained deep orange with Evans Blue when the dye was excited by a mercury lamp.

After rinsing briefly in PBS, sections were mounted in 80%

non-fluorescent glycerol (Merk) in PBS and covered with a piece of cover glass to observe under a fluorescent microscope (Olympus, model BH-2RFK, Tokyo), using a BV excitation system. Photographs were taken with Kodak VR-400 and/or Tri-X film.

Autoradiography

After fluorescent microscopy, the slide glass on which the sections were mounted, was dipped into PBS containing 60% glycerol to remove the coverglass, rinsed in PBS and followed by immersion in a Sakura NR-M2 emulsion (Konishiroku Photo, Tokyo) to coat the surface of the section with a thin film of the emulsion. After 2-4 week exposure, the sections were applied photographic procedures and lightly stained with Nuclear fast red (Merk) to identify the nuclei.

RESULTS

Localization of DNA polymerase α and β in the developing lens

In the 2-day embryo (stage 12), almost all the nuclei of the lens placode cells were shown distinct immunofluorescence for either DNA polymerase α or DNA polymerase β (fig. 1a,d).

In the 3-day embryo (stage 17), immunofluorescence for DNA polymerase α was detected in the nuclei of cells in the anterior wall of the lens vesicle;but not in the nuclei of the primary lens fiber cells, just before beggining to elongate, being located in the center of the posterior wall of the lens vesicle (fig. 1b). Immunofluorescence for DNA polymerase β was detected in the nuclei of both the anterior wall and the primary lens fiber cells (fig. 1e).

In the 5-day embryo (stage 25), when the basic architecture of the lens has been already established, immunofluorescence for DNA polymerase α was detected in the nuclei of the lens epithelial cells;but the strength of the fluorescence declined sharply in the nuclei of the cells at the equatorial margin of the lens, as compared with the nuclei of the epithelial cells. No immunofluorescence for DNA polymerase α was detected in the nuclei of the lens fiber cells (fig. 1c). Immunofluorescence for DNA polymerase β was observed in the nuclei of both lens epithelial cells and lens fiber cells

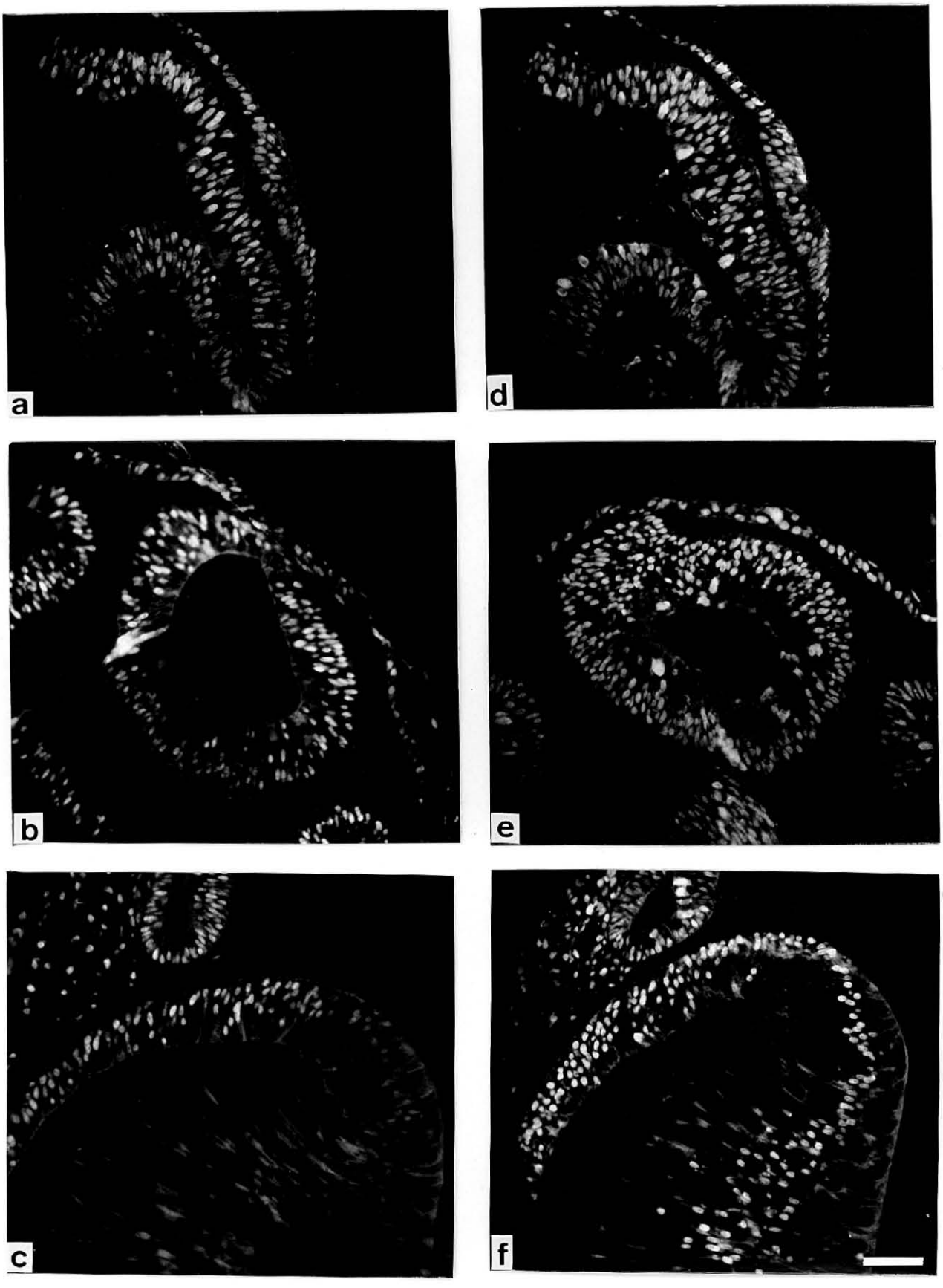
a
d
b
e
c
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Fig. 1. Immunofluorescent microphotographs showing the localization of DNA polymerase α in a transverse section of the 2-day embryo lens placode (a), the 3-day embryo lens vesicle (b) and the 5-day embryo lens (c); and the localization of DNA polymerase β in a transverse section of the 2-day embryo lens placode (d), the 3-day embryo lens vesicle (e) and the 5-day embryo lens (f). Bar, 50 μm.

(fig. 1f).

In the 8-day embryo (stage 33), faint immunofluorescence for DNA polymerase α was detected in the nuclei of lens epithelial cells, but not in the nuclei of lens fiber cells (fig. 2a). Immunofluorescence for DNA polymerase β was detected in almost all the lens cell nuclei except for the lens fiber cell nuclei near the central region of the lens body where lens fiber denucleation had begun from this stage (fig. 2c).

In order to compare the DNA polymerase α distribution with the DNA replicating capacity among the chick embryo lens cells, [^{3}H]thymidine was administered to the 8-day chick embryo. In autoradiograms of the 8-day embryo lens at 1 hr and 24 hr after [^{3}H]thymidine administration, silver grains were observed only on the nuclei of the lens epithelial cells and the grain was hardly observed on the nuclei of the lens fiber cells (fig. 2b,d). The distribution of the grain-positive nuclei shown in figs. 2b and 2d coincided closely with the distribution of DNA polymerase α-positive cell nuclei shown in fig. 2a.

These results suggest that DNA polymerase α would be retained only in the proliferating cell nuclei of developing chick embryo lens, and that DNA polymerase α would not be retained in the nuclei of lens cells which are destined to differentiate into lens fiber.

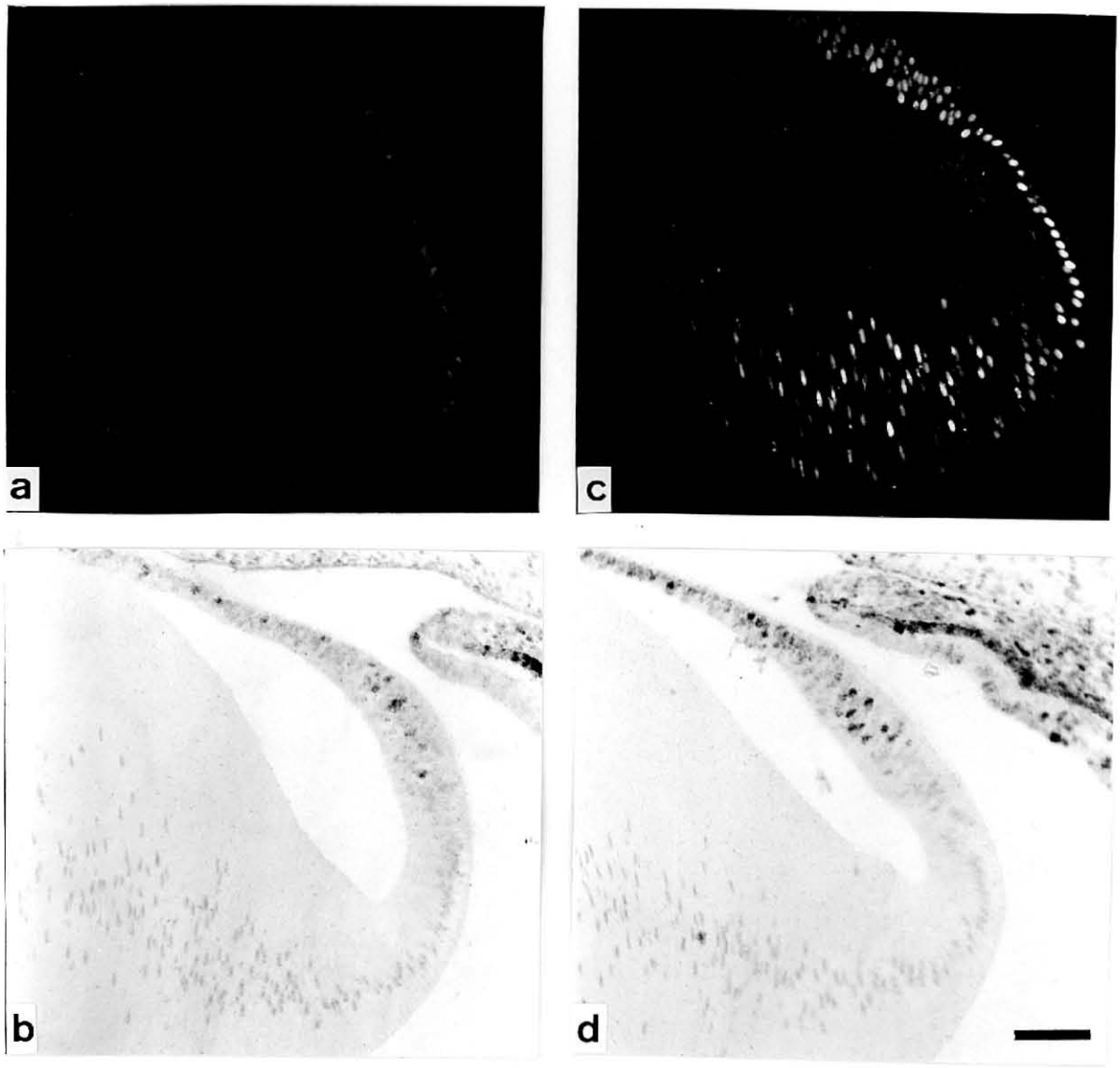

Fig. 2. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (c) in 8-day embryo lens sections and autoradiograms of 8-day embryo lens sections at 1 hr (b) and 24 hr (d) after [^{3}H]thymidine administration. Bar, 50 μm.

Localization of DNA polymerase α and β in the developing retina

The chick retina is composed of two layers, the outer retinal pigment epithelium and the inner neural retina. The latter includes several types of neuronal cells and these neuronal cells are separated into discrete strata in an orderly temporal and spatial sequence during its development (19). This allows us to determine the type of neuronal cells from their position in the neural retina at any stage of development.

Fig. 3a showed the intracellular localization of DNA polymerase α in the optic cup of 3-day embryo. DNA polymerase α was detected in almost all the nuclei of cells in both the inner and the outer layer of the optic cup.

Fig. 3b showed the intracellular localization of DNA polymerase α in the fundic region of the optic cup of 5-day embryo. DNA polymerase α was detected in almost all the nuclei of cells in the inner, thicker layer of the optic cup, differentiating into the neural retina but not detected in the nuclei of cells in the outer, thinner layer of the optic cup, differentiating into the retinal pigment epithelium. At this stage, many mitotic figures were found in the inner layer (next the outer layer), whereas mitotic figures were rarely found in the outer layer of the optic cup. Thus, in the 5-day embryo retina, DNA polymerase α was detected in the proliferative thicker layer cells;but not in the thinner layer cells which had fin-

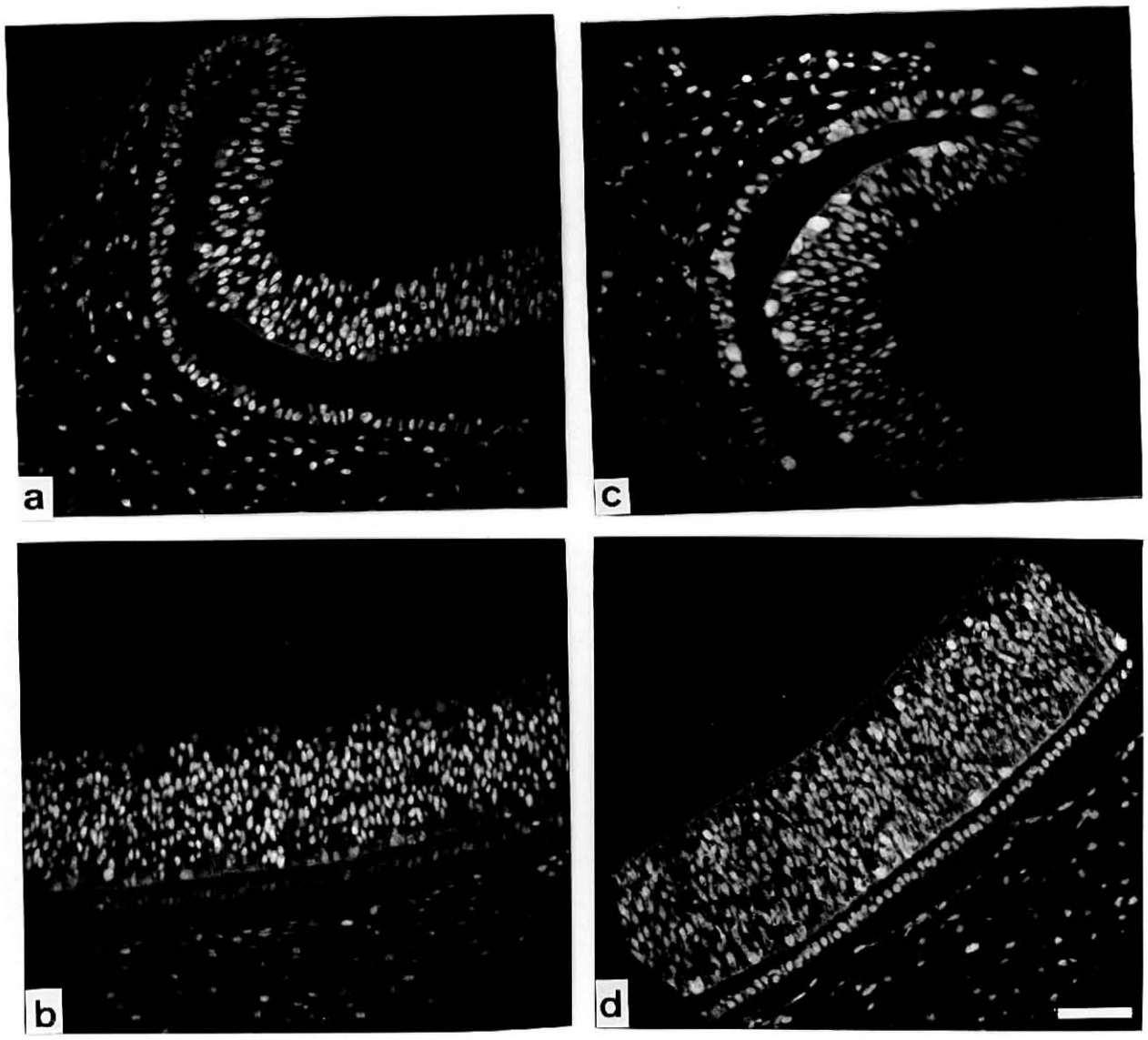

Fig. 3. Immunofluorescent microphotographs showing the localization of DNA polymerase α in a transverse section of the 3-day embryo optic cup (a) and the 5-day embryo retina (b); and the localization of DNA polymerase β in a transverse section of the 3-day embryo optic cup (c) and the 5-day embryo retina (d). Bar, 50 μm.

ished their proliferation.

Fig. 4a showed the intracellular localization of DNA polymerase α in the 8-day embryo neural retina. In the neural retina at this stage, the ganglionic cell layer and the inner nuclear layer could be distinguished at the fundic region. DNA polymerase α was detected in the spindle-shaped nuclei of cells which was located in the middle zone of the inner nuclear layer, as well as in the mitotic cells which was found just below the outer limiting membrane. However, DNA polymerase α was not detected in the spheroid-shaped nuclei of the ganglionic cells.

The autoradiogram shown in fig. 4b was obtained from the same section that was applied to the indirect immunofluorescent staining to show DNA polymerase α localization (fig. 4a). Namely, at 1 hr after [^{3}H]thymidine administration, an embryo was sacrificed to get a retinal section and the section was photographed after the immunofluorescent staining for DNA polymerase α, and then the section was processed for autoradiography. By these procedures it became possible of realization to examine whether DNA polymerase α or β could be detected in the nuclei taken up [^{3}H]thymidine. Detailed examinations of the photographs obtained showed some of nuclei in the inner nuclear layer were DNA polymerase α-positive and covered with silver grains, while, some of nuclei were DNA polymerase α-positive but not

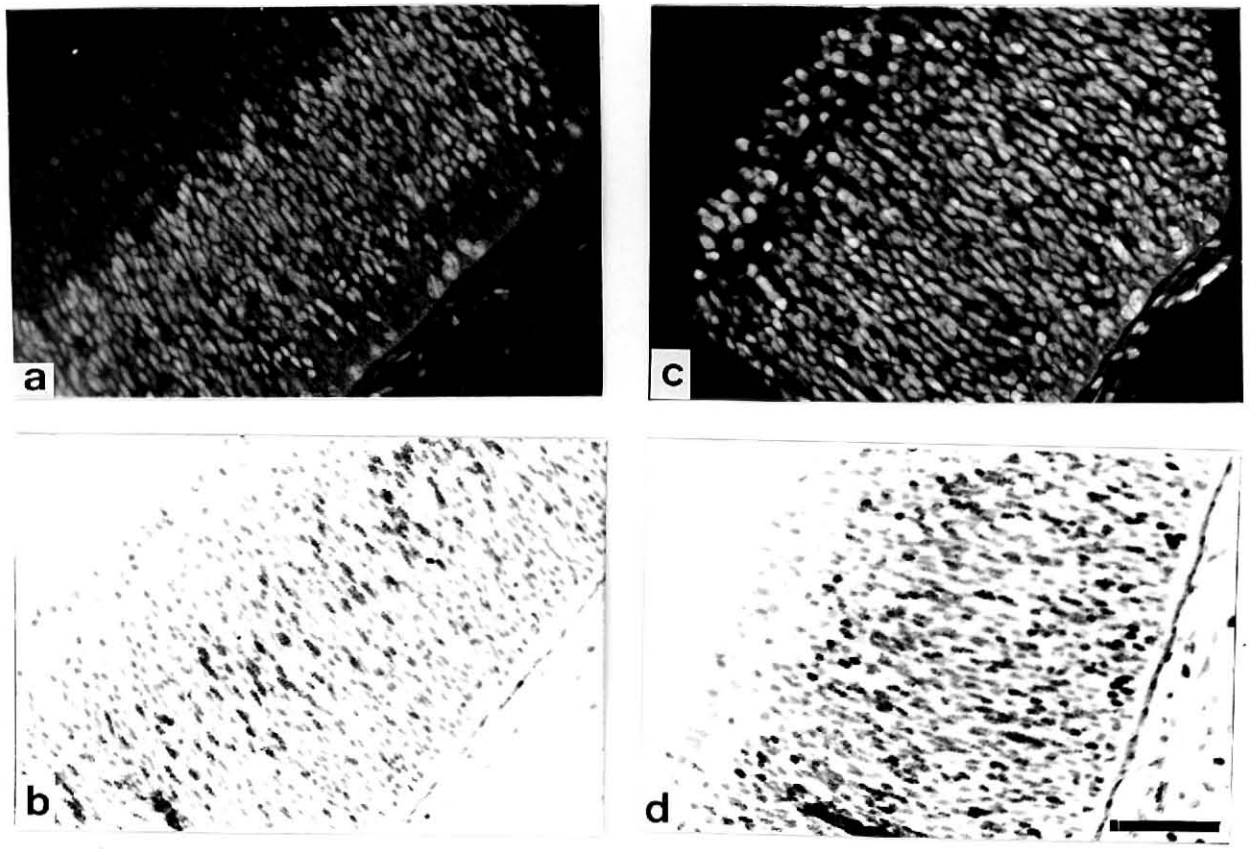

Fig. 4. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (c) in the sections of 8-day embryo neural retina; and autoradiograms of the sections of 8-day embryo neural retina at 1 hr (b) and 24 hr (d) after [^{3}H]thymidine administration. An area shown in fig.4a is the same that is taken in fig,4b (see text). Bar, 50 μm.

with silver grains. Each of these cells including grain-positive and DNA polymerase α-positive nucleus, therefore, would be regarded as being in S phase in the cell cycle, while the cells including grain-negative but DNA polymerase α-positive nucleus would be regarded as being in either G_1 or G_2 phase in the cell cycle. No ganglionic cell having become already negative for DNA polymerase α at this stage was labelled with [^{3}H]thymidine in this autoradiogram.
Fig. 4d also showed the autoradiogram of the 8-day embryo neural retina, but in this case the embryo was fixed to get autoradiograms at 24 hr after [^{3}H]thymidine administration. Silver grains were observed over almost all the DNA polymerase α-positive nuclei which were located in the inner nuclear layer, as well as over almost all the mitotic figures. However, the ganglionic cell nuclei were still free of silver grains. Fujita and Horii (20) pointed out that mitotic cells found in the receptor cell layer of the chick embryo neural retina during the second week of incubation, were nothing but the representation of a mitotic phase of the cells in the inner nuclear layer. Therefore, in the neural retina of 8-day embryo, DNA polymerase α-positive cells including the spindle shaped nuclei and the cells in the course of mitosis were regarded as the " matrix cells " (21, 22). DNA polymerase α would be retained in the nuclei of proliferating matrix cells,

but not in the nuclei of ganglionic cells which had ceased to turn round the cell cycle by this stage.

Fig. 5a showed the intracellular localization of DNA polymerase α in the 12-day embryo retina. At this stage, the ganglionic cell layer, the inner nuclear layer and the outer nuclear layer have been clearly distinguished at the fundic region. DNA polymerase α was detected only in a few spindle-shaped nuclei of the neural retinal cells which were scattered in the inner nuclear layer. Except for them, almost all nuclei of neural retinal cells, as well as those of retinal pigment epithelial cells were practically immunofluorescent-negative for DNA polymerase α, although all nuclei were still DNA polymerase β positive (fig. 5c).

Only a few spindle-shaped nuclei in the inner nuclear layer were covered with silver grains as shown in fig. 5b and fig. 5d. These findings showed that in the 12-day embryo neural retina, only a few cells were capable of replicating DNA, and the majority of the neural retinal cells had finished their DNA replication by this stage.

In the 18-day embryo, histogenesis of the retina has nearly finished and its histological architecture is almost as same as the adult one. Neither the neural retinal cells nor the retinal pigment epithelial cells of the 18-day embryo retina were immunofluorescent-positive for DNA polymerase α, as shown in fig. 6a. Fig. 6b and fig. 6d showed the autoradiograms

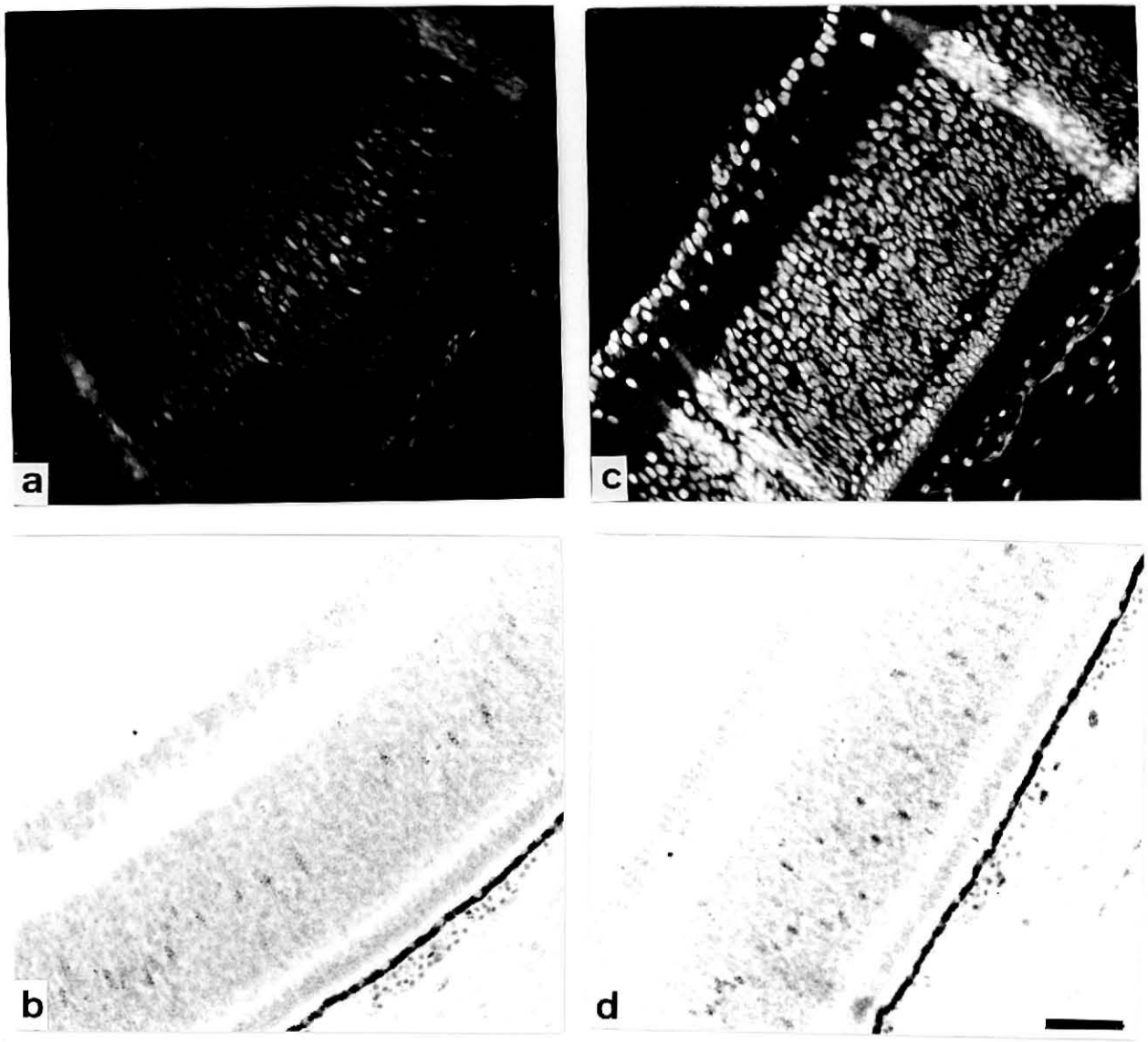

Fig. 5. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (c) in the sections of 12-day embryo retina; and autoradiograms of the sections of 12-day embryo retina at 1 hr (b) and 24 hr (d) after [^{3}H]thymidine administration. Bar 50 μm.

of the 18-day embryo retinae at 1 hr and 24 hr after [^{3}H]thymidine administration. In these autoradiograms, labelled cells were not found in both the neural retina and the retinal pigment epithelium. The neural retinal cells of the 18-day embryo could not replicate DNA for lack of DNA polymerase α.

From these results it would be concluded that distribution of nuclei being DNA polymerase α-positive closely coincides with the distribution of nuclei capable of replicating DNA in the developing chick retina, and that DNA polymerase α was retained in the nuclei of proliferative cells but not in the nuclei of postmitotic cells which had ceased proliferating.

On the other hand, immunofluorescence for DNA polymerase β was detected in almost all the neural retinal and retinal pigment epithelial cells in all stages examined as shown in figs. 3c, 3d, 4c, 5c, 6c. Thus DNA polymerase β would be retained not only in proliferative cells but also in postmitotic cells in the chick embryo retina.

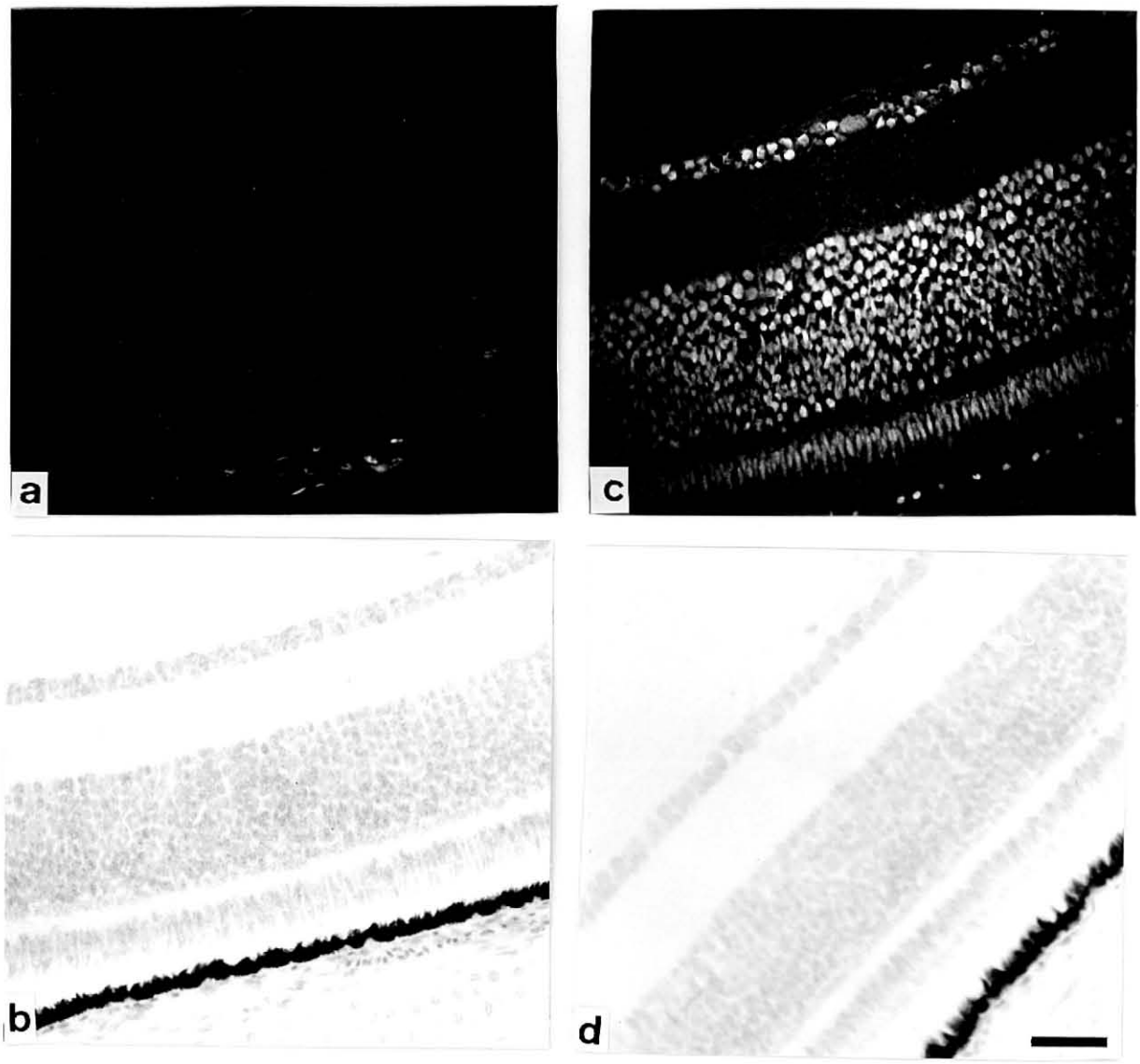

Fig. 6. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (c) in the sections of 18-day embryo retina; and autoradiograms of the sections of 18-day embryo retina at 1 hr (b) and 24 hr (d) after [^{3}H]thymidine administration. Bar 50 μm.

Localization of DNApolymerase α and β in the developing spinal cord

Fig. 7a showed the intracellular localization of DNA polymerase α in the 3-day embryo spinal cord, which was composed of the matrix cells proliferating vigorously as the progenitor cells of all neurons and glias (21-23). DNA polymerase α was retained in the nuclei of these rapidly proliferating matrix cells in the 3-day embryo spinal cord.

Fig. 7b showed the intracellular localization of DNA polymerase α in the 5-day embryo spinal cord. DNA polymerase α was detected in the nuclei of neuroepithelial cells located in the inner half of the spinal cord, but not in the nuclei of neuroepithelial cells located in the outer half of the spinal cord. At this stage, the matrix cell layer became thinner and the neuroblast migration into the mantle layer occured in the basal plate, while in the alar plate the matrix cells were still actively proliferating (22,23). Thus, DNA polymerase α would be retained in the proliferating matrix cells but not in the postmitotic neuroblasts in the 5-day embryo spinal cord.

Fig. 8a showed the intracellular localization of DNA polymerase α in the 12-day embryo spinal cord. At this stage, a few nuclei of matrix cells lining along the ventricular lumen of the spinal cord gave DNA polymerase α immunofluorescence-

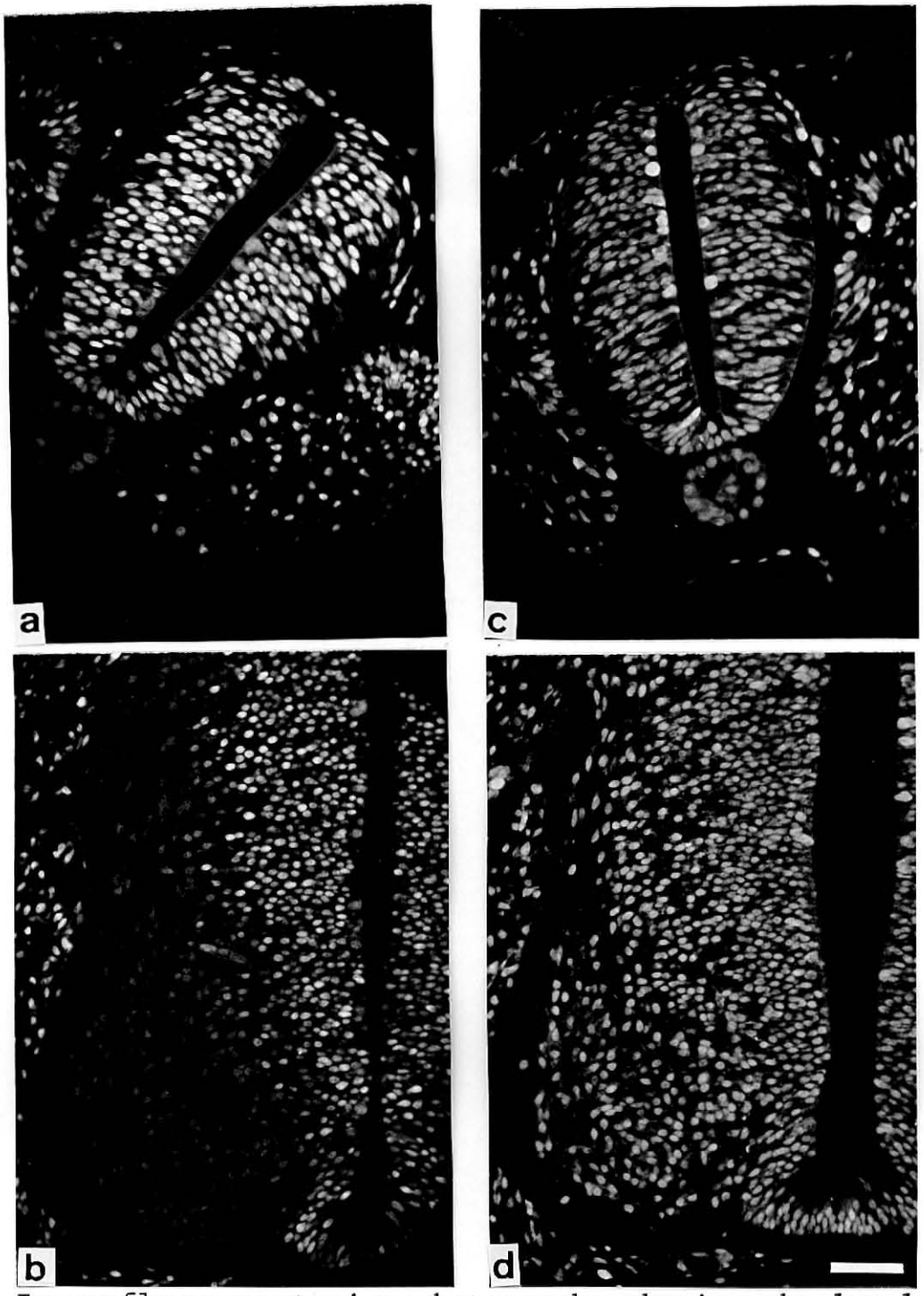

Fig. 7. Immunofluorescent microphotographs showing the localization of DNA polymerase α in a transverse section of the 3-day embryo spinal cord (a) and the 5-day embryo spinal cord (b); and the localization of DNA polymerase β in a transverse section of the 3-day embryo spinal cord (c) and the 5-day embryo spinal cord (d). Bar, 50 μm.

positive, whereas the nuclei of neuroblasts or young neurons located in the mantle layer gave DNA polymerase α immunofluorescence-negative. Immunofluorescence for DNA polymerase α was also detected in the small cell nuclei being interspersed among the neuroblasts in the mantle layer. Figs. 8c and 8d showed the autoradiograms of the 12-day embryo spinal cord taken at 1 hr and 24 hr after [^{3}H]thymidine administration, respectively. In these autoradiograms, a few nuclei of the matrix cells located at the ventricular surface were labelled, but the nuclei of the neuroblasts or young neurons in the mantle layer were practically unlabelled. Several small nuclei with silver grains, showing DNA replication in the mantle layer would be the nuclei of glioblasts which had been derived from the matrix cells and were capable of differentiating all types of glial cells in the spinal cord (24,25). Thus, in the 12-day embryo spinal cord, a few matrix cells and also the glioblasts would be proliferative and DNA polymerase α was detected only in these cells. The neuroblasts or the young neurons had ceased proliferating in 12-day embryo and these cells were negative in immunofluorescence for DNA polymerase α.

These findings indicate that intranuclear DNA polymerase α would be retained in the matrix cells and also in the glioblasts, but not in the neuroblasts or neurons in the chick embryo spinal cord.

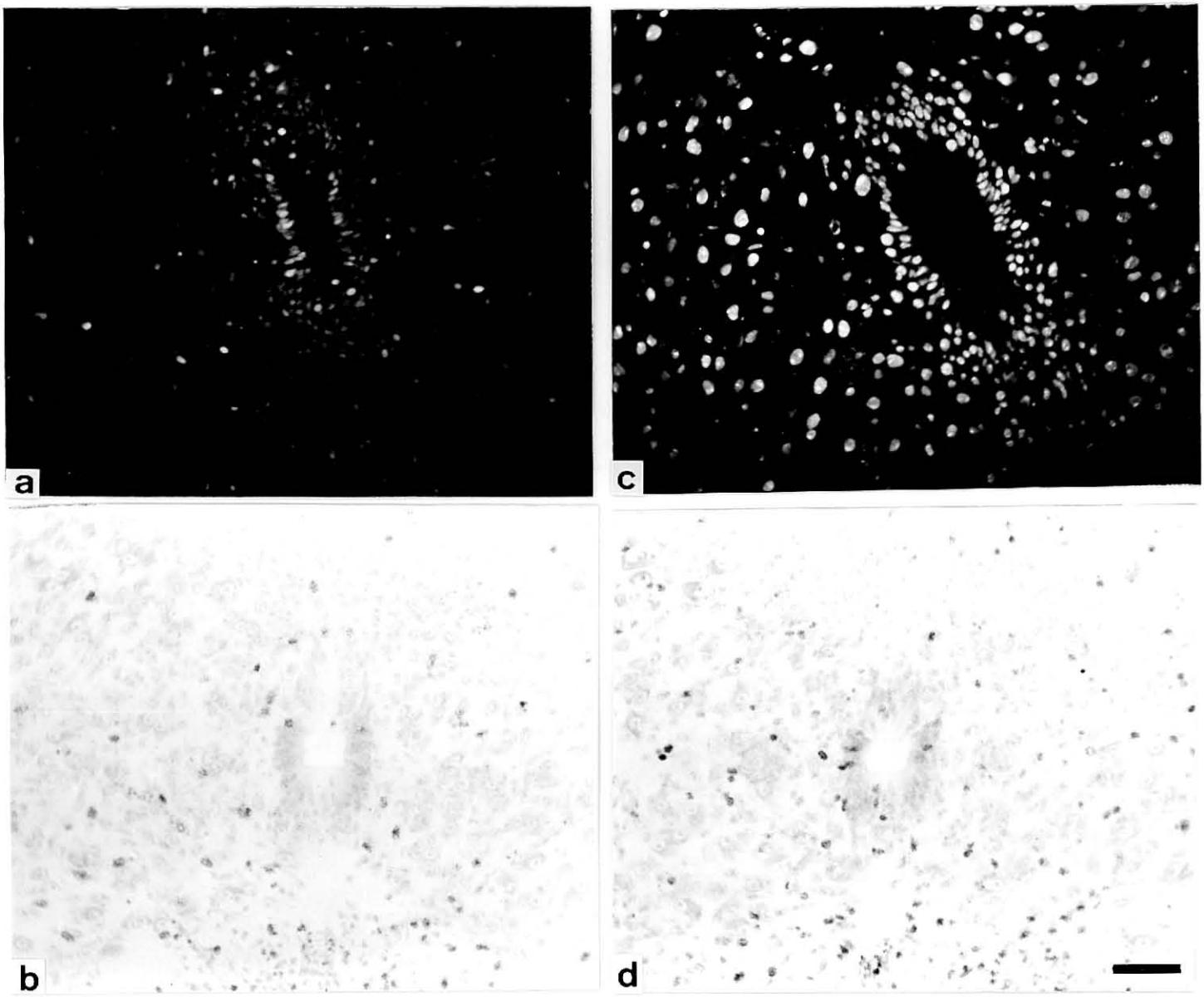

Fig. 8. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (c) in the sections of 12-day embryo spinal cord; and autoradiograms of the sections of 12-day embryo spinal cord at 1 hr (b) and 24 hr (d) after [^{3}H]thymidine administration. Bar, 50 μm.

On the other hand, immunofluorescence for DNA polymerase β was detected in almost all nuclei of neuroepithelial cells found in spinal cord at any stage examined (figs. 7c, 7d and 8c). The matrix cells, the glioblasts, the neuroblasts and the young neurons showed positive immunoflourescence for DNA polymerase β, that is, DNA polymerase β would be retained in the muclei of both proliferative and the postmitotic cells.

DISCUSSION

By [^{3}H]thymidine autoradiography in addition to histological studies in the chick embryo lens, several previous authors (26-31) reported that all of the lens placode cells were proliferative and capable of synthesizing DNA at the earliest stage of the lens development; that a high labelling index was shown in the anterior wall of the lens vesicle, whereas the index decreased progressively in the posterior wall; that the primary lens fiber differentiation occured when labelled cells in the early lens vesicle had disappeared completely; that DNA replication and proliferation were confined in the lens epithelium and that neither DNA replication nor proliferation occured in the lens fiber after the secondary lens fiber formation began. It has been regarded, therefore, that the lens fiber differentiation of the primary or of the secondary, is accompanied by the progressive loss of DNA replicating and proliferative activity of individual lens cells. However, it is impossible to identify which cells are capable of replicating DNA by autoradiography, unless they are in S phase of cell cycle. The present immunocytochemical method to detect DNA polymerase α is available to cells not only in S phase but also in G_1 or G_2 phase of cell cycle to show potentiality of DNA replication in their nuclei.

In the present studies, [^{3}H]thymidine autoradiography was

applied to ascertain whether the cell having DNA polymerase α-positive nucleus is capable of replicating DNA or not. It was found that the presence of DNA polymerase α were concomitantly with the DNA replication or the cell proliferation in the chick embryo tissues, but not DNA polymerase β. For example, as described above, during the lens development, DNA polymerase α was detected in almost all the lens placode cell nuclei in the 2-day embryo (stage 12); in the cell nuclei located in the anterior wall of the lens vesicle, but not in the cell nuclei located in the posterior wall of the lens vesicle in the 3-day embryo (stage 17); in the lens epithelial cell nuclei, but not in the lens fiber cell nuclei in both the 5-day (stage 25) and the 8-day (stage 33) embryo. In fact, a sharp contrast of the immunofluorescence for DNA polymerase α at the equatorial margin of the lens as shown in fig. 1c would reflect a rapid loss of this enzyme in the lens cells which have finished the last DNA replication for cell proliferation and are destined to become the lens fiber. Similar coincidence between the disapperance of DNA polymerase α and the loss of proliferative potentiality was revealed in the retinal cells as well as in the cells of spinal cord in the chick embryo.

Several previous authors determined, by autoradiographic studies using [^{3}H]thymidine, the timing and the order of the retinal cell differentiation in the developing chick neural

retina (20, 32-34). According to these studies, some of the presumptive ganglion cells began to cease proliferating as early as on the 3rd day of incubation and the majority of them have completed their proliferation period by the 8th day of incubation; next the presumptive receptor cells and followed by the presumptive amacrine and horizontal cells to complete their proliferation period by the 12th day of incubation, while the precursors of the bipolar and/or Müller cells were still proliferative at this developmental stage; and bipolar and/or Müller cells were the last cells to complete their proliferation period in the chick neural retina development. The present immunocytochemical detection of DNA polymerase α in the neural retina indicated also that the presumptive ganglionic cells were the cells to become DNA polymerase α-negative at the earliest stage of development and followed by the presumptive ganglionic cells, the precursors of the receptor, amacrine and horizontal cells, to become negative and finally the presumptive bipolar and/or Müller cells to become negative. The timing of the loss of proliferative potentiality in neural retinal cells was very closely coincided with the timing of the disappearance of intracellular DNA polymerase α in the neural retinal cells during development. Therefore, the present immunocytochemical technique using anti-DNA polymerase α antibody could reveal which cells are capable of proliferating or which cells

are going to differentiate at a developmental stage.

Carré and Pieau (35) studied the change of both DNA polymerase α and β activities in the chick neural retina during development, and found that the activity of DNA polymerase α rapidly decreased between 7th and 11th day of incubation. The present immunocytochemical study of DNA polymerase α coincided with their results obtained biochemically. Carré and Pieau (35) also found a detectable activity of DNA polymerase α in the neural retina of chicken just hatched, and even of 5-year-old adults; and suggested that the DNA polymerase α activity found in the adult neural retina may be due to either a few glial cells keeping their replication capacity or the retinal neurons. However, the present studies showed that no DNA synthesis and no immunofluorescence-positive cells for DNA polymerase α was found in the neural retina of 18-day embryos. The discrepancy between my findings and those of Carré and Pieau remained to be explained.

Fujita (21, 22, 24, 25) has distinguished three developmental stages in the cytogenesis of neurons and neuroglia in the chick spinal cord as follows: the first stage (until the third day of incubation) that the chick spinal cord is composed of only the matrix cells, which are vigorousry proliferating and capable of differentiating the neuronal and glial cells, the second stage (from the third to eighth day of

incubation) that the neuroblasts are differentiating from the matrix cells, the third stage (from the nineth day of incubation) that the matrix cells are differentiating into the ependymal cells and the glioblasts, which migrate into the mantle layer, actively synthesize DNA and further transform into the glial cells. In either the first or second stage it is necessary for the matrix cells to proliferate actively, and in the third stage it is necessary for the matrix cells as well as for the glioblasts to proliferate in the mantle layer. Findings of Fujita was supported by the present results.

As contrasted strikingly in DNA polymerase α, DNA polymerase β, responsible for DNA excision repair was detected at any stage of development in all proliferative and postmitotic cells of the lens, the retina and the spinal cord, except in the lens fiber cells near the central region of the lens body in the 8-day embryo. According to Romanoff (16), these lens fiber cells begin to denucleate from this stage on. The disappearance of DNA polymerase β in these cells would due to the denucleation.

Yamaguchi et al. (17) estimated that the half life of DNA polymerase β polypeptide was about 10hr in the cultured chick fibroblasts. Although few studies have been done on the metabolism of DNA polymerase α in higher eukaryotic cells, the half life of DNA polymerase α might be much shorter than

that of DNA polymerase β , because a rapid loss of DNA polymerase α from the postmitotic cells may be associated with prompt degradation of this enzyme, the imunological inactivation of this enzyme and/or the structural change of the DNA replication complex in which DNA polymerase α is considered to be integrated (36).

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PART III. IMMUNOCYTOCHEMICAL LOCALIZATION OF DNA POLYMERASE α AND β IN THE DEVELOPMENT OF THE CHICK EMBRYO INTESTINE AND IN THE ADULT CHICKEN TESTIS

SUMMARY

Intracellular localization of DNA polymerase α and β in the developing chick intestine and in the adult testis was investigated by indirect immunofluorescent methods using the antibodies against chick embryo DNA polymerase α and β. In the intestine, DNA polymerase α was detected (1) in almost all the epithelial cells of the duodenum in the 8-day embryos, (2) in the epithelial cells located in both basal and middle part of the previllous ridges,but not in the tips of the ridges in the 12-day embryos, (3) in the epithelial cells located in the lower half of the villi just formed, but not in the upper half of the villi in the 18-day embryos, and (4) only in the cells of the crypts in 2-weeks after hatching. On the other hand, DNA polymerase β was detected in the cells of the all parts of the duodenum at any stage of the development. In addition, almost all the epithelial cells detectable DNA polymerase α were also found labelled with [^{3}H]-thymidine, if administered. In the adult chick testis, DNA polymerase α was detected in the spermatogonia and the pre-leptotene spermatocytes, but not detected in the mature spermatozoa. DNA polymerase β was detected in almost all the germ cell nuclei except for the spermatozoan nuclei.

INTRODUCTION

In the previous part of this thesis it was shown that an immunofluorescent method using the antibody against chick embryo DNA polymerase α was very useful to distinguish each of proliferative cells in the chick embryo, and by this immunofluorescent method and autoradigraphy using [^{3}H]thymidine as a DNA precursor that cellular differentiation in chick embryo began just after the cells had finished their last mitotic division in their respective cell cycle. Then in this part these techniques applied to aim to detect intracellular localization of both DNA polymerase α and β and also DNA synthesis in the typical cells belonging to a cell renewal system. In such a system, cellular proliferation is balanced by cell loss; and stem cells and their pregenitor cells should be distinguished. The chick intestinal epithelium in development and the adult chick testis were chosen as the present materials to be investigated.

MATERIALS AND METHODS

The chick embryos and [^{3}H]thymidine labeling, tissue fixation, prepatation of tissue sections, immunofluorescent microscopy and autoradiography were carreid out as described in the MATERIALS AND METHODS in PART II.

For chickens and adults, they were fed by commercial chick food and water ad libitum in cages of our laboratory. As a dose of [^{3}H]thymidine for the chickens and fowls, a 0.4 ml of the Ringer's solution containig 80 μCi of [^{3}H]thymidine was injected into the abdominal cavity to each of them. At 1 hr and 24 hr after [^{3}H]thymidine injection they were sacrificed, and the duodenum or the testis was fixed and embedded as described previously. The chick duodenum is the most proximal part of the small intestine, next to the gizzad and occupies the whole first main loop of the intestine accompanied by pancreas. By this morphological characteristic, the duodenum can be identified in the chick embryos at any stage of development or in the chickens and adult fowls, and this was the reason why the duodenum was chosen as the material for the present study.

RESULTS

Localization of DNA polymerase A and B in the developing duodenum

The duodenum of the 8-day chick embryo showed a cylinder like structure and any evidence of villus formation was not yet found in the intestinal epithelium. As shown in fig. 1a, DNA polymerase α was detected in the nuclei of epithelial cells at this stage. Fig. 1b is the autoradiogram of the same section shown in fig. 1a. The section was obtained from an embryo fixed at 1hr after [^{3}H]thymidine administration. In this autoradiogram, silver grains were found over several epithelial cells but not over dividing ones. Fig. 1d is an autoradiogram of duodenum obtained from a 8-day embryo which was fixed at 24hr after [^{3}H]thymidine administration. Silver grains were found over almost all of the epithelial cells, as well as over the dividing cells in this autoradiogram. These findings indicated that in the 8-day embryo, almost all the epithelial cells of the duodenum are proliferative and capable of replicating DNA.

In the duodenum of the 12-day embryo, zigzag-form previllous ridges appeared and also villi had been formed at the top of the ridges (1, 2). DNA polymerase α was detected in the nuclei of epithelial cells located in both basal and middle regions of the ridges, but not detected in the nuclei of cells located in the upper regions as shown in fig. 2a. Fig. 2b was an autoradiogram of the same section shown in

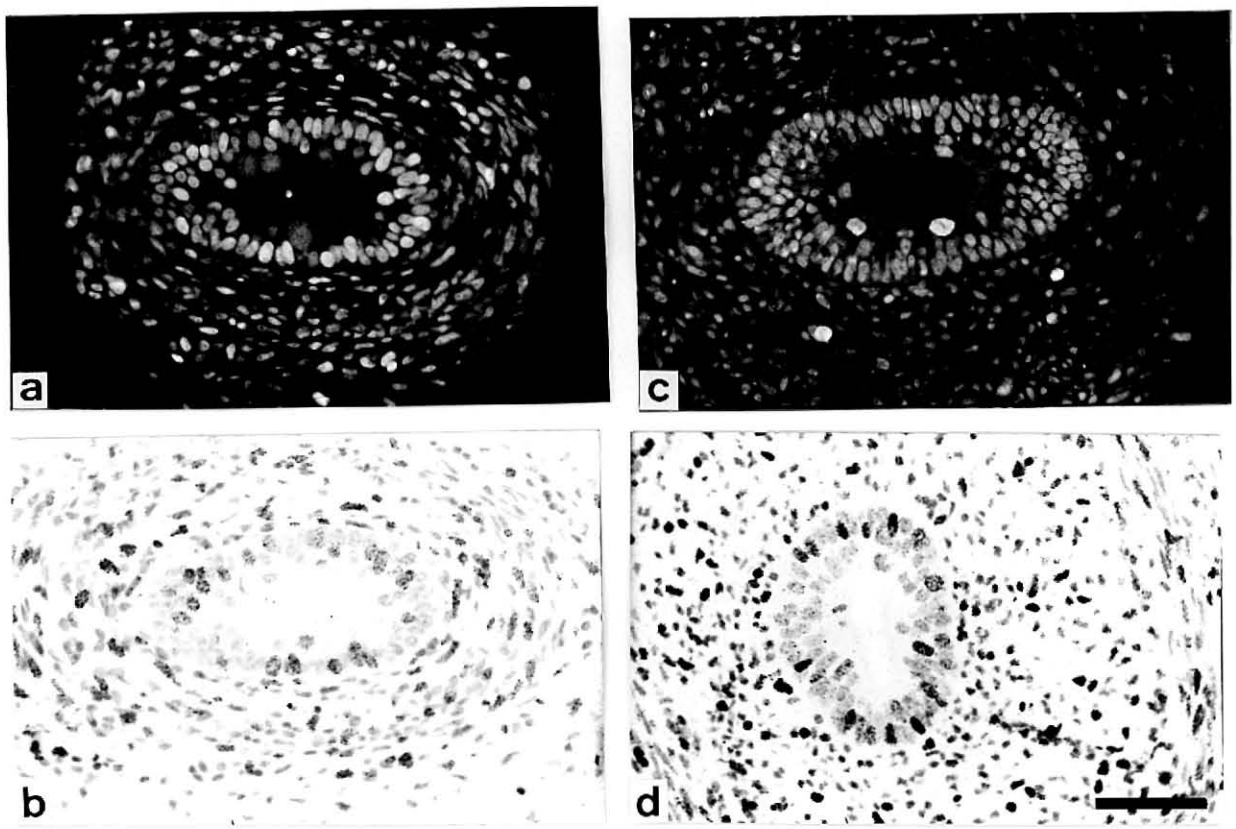

Fig. 1. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (c) in the sections of 8-day embryo duodenum; and autoradiograms of the sections of 8-day embryo duodenum at 1 hr (b) and 24 hr (d) after [^{3}H]thymidine administration. An area shown in fig.1a is the same that is taken in fig.1b (see text). Bar, 50 μm.

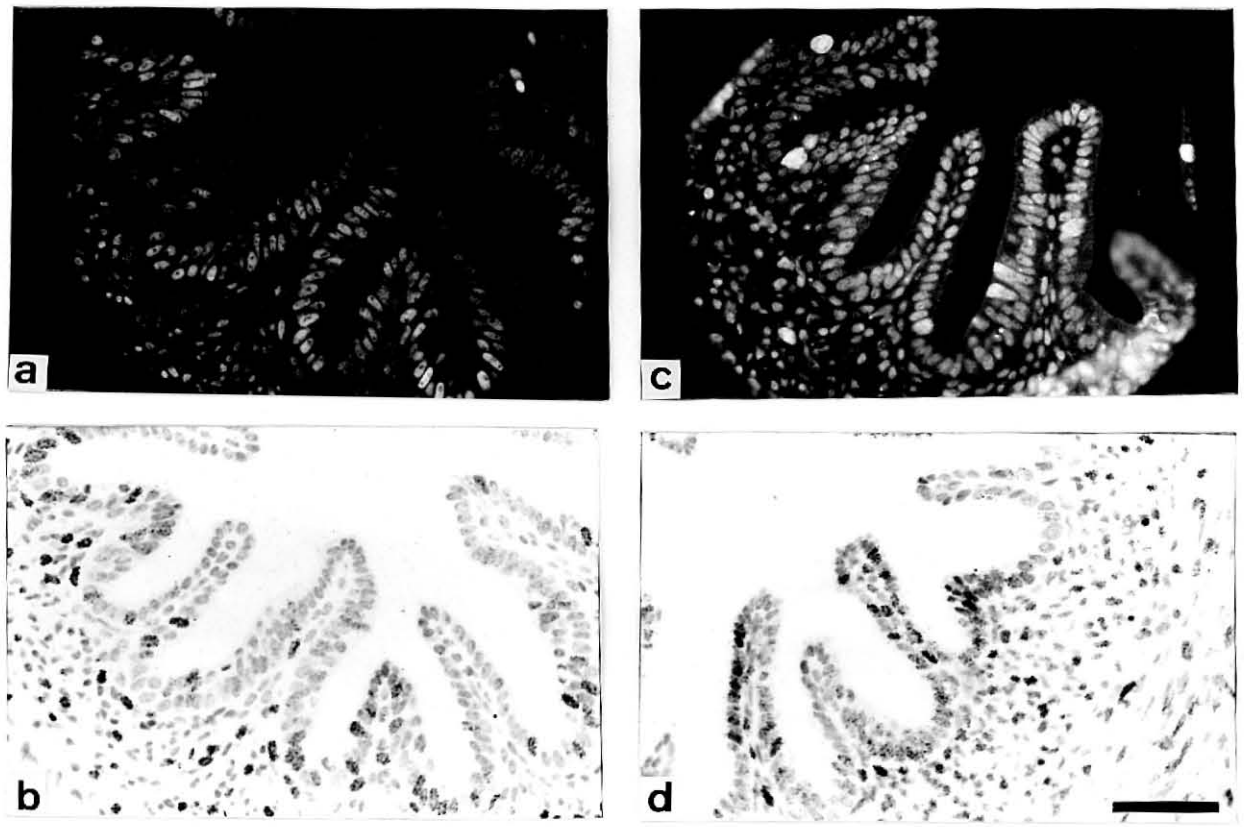

Fig. 2. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (c) in the sections of 12-day embryo duodenum; and autoradiograms of the sections of 12-day embryo duodenum at 1 hr (b) and 24 hr (d) after [^{3}H]thymidine administration. An area shown in fig.2a is the same that is taken in fig.2b (see text). Bar, 50 μm.

fig. 2a. In this autoradiogram obtained from an embryo section prepared at 1hr after [^{3}H]thymidine administration, epithelial cells with silver grains were located in the basal and middle regions of the ridges, whereas no epithelial cell with silver grains was found in the upper region of the ridges. Silver grains were not found over the epithelial cells in the upper region of the ridges in the autoradiogram obtained from the section of the 12-day embryo duodenum which were fixed at 24hr after [^{3}H]thymidine administration (fig. 2d).

In the duodenum of the 18-day embryo, many definitive villi were found and DNA polymerase α was detected in the nuclei of epithelial cells located in the proximal half of the villi, but not detected in the nuclei of cells located in the distal half of the villi (fig. 3a). Figs. 3b and 3d were autoradiograms of the duodenum obtained from the 18-day embryos at 1hr and 24hr after [^{3}H]thymidine administration, respectively. In both autoradiograms, labelled epithelial cells were found in the proximal half of the villi, whereas no cells was labelled in the distal half of the villi.

Fig. 4a showed the intracellular localization of DNA polymerase α in the duodenum of 21-day embryo, immediately before hatching. Well-developed villi were abundant in the duodenum at this stage, although distinct crypts had not yet occurred at the base of the villi. DNA polymerase α was detected in the nuclei of epithelial cells located in the

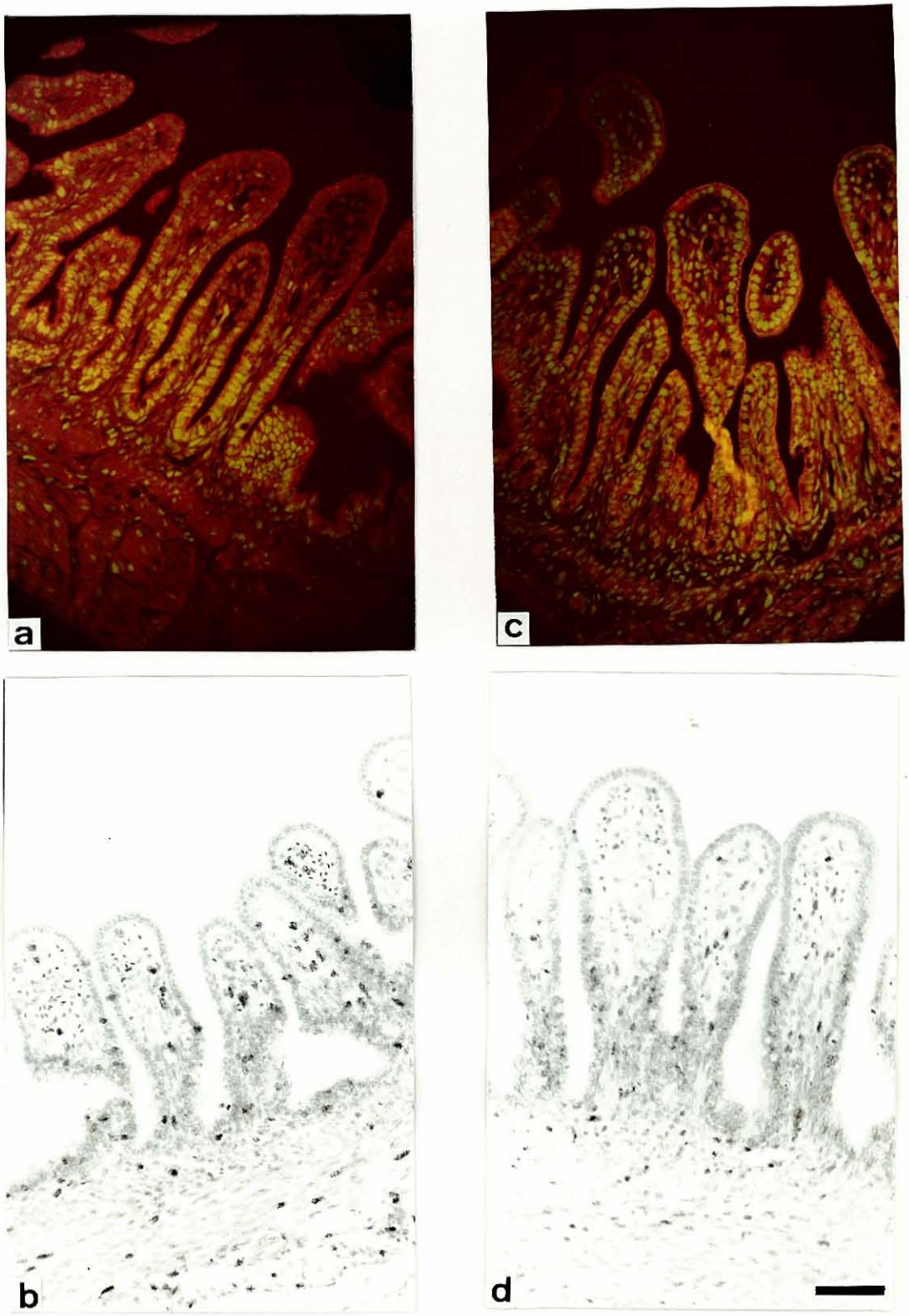

Fig. 3. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (c) in the sections of 18-day embryo duodenum; and autoradiograms of the sections of 18-day embryo duodenum at 1 hr (b) and 24 hr (d) after [^{3}H]thymidine administration. Bar, 50 μm.

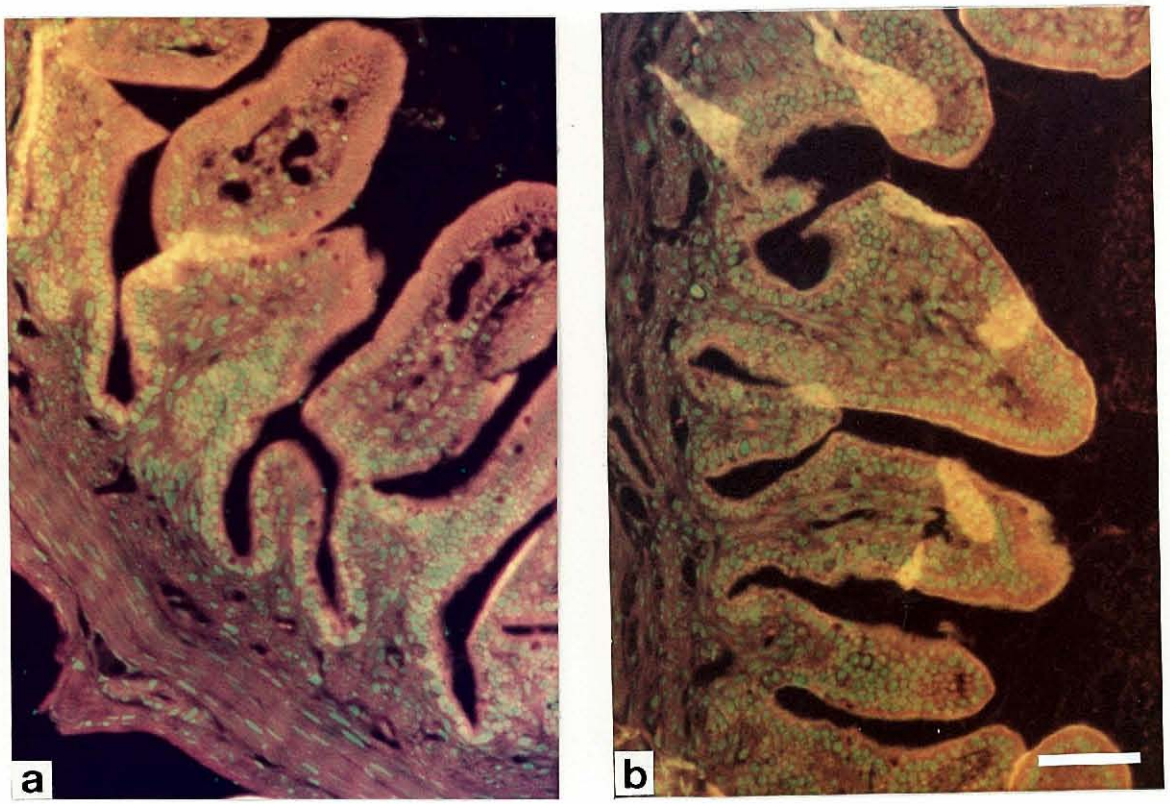

Fig. 4. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (b) in the sections of 21-day embryo duodenum. Bar, 50 μm.

proximal half of the villi but not detected in the nuclei of cells located in the distal half of the villi.

In the duodenum of a chicken 2 weeks after hatching, only the epithelial cells consisting in the crypts became immunofluorescence-positive for DNA polymerase α as shown in figs. 5a and 6a. Autoradiograms of these duodenum at 1hr and 24hr after [^{3}H]thymidine injection were shown figs. 5b and 5d, respectively. At 1hr after [^{3}H]thymidine injection, labelled cells were found only in the crypt, and the epithelial cells in villi were unlabelled. However, at 24hr after [^{3}H]thymidine injection, labelled cells were found in the crypt as well as near the base of the villi. The latter labelled cells had migrated no doubt from the crypt where [^{3}H]thymidine was taken up. Thus, in the duodenum, the region in a villus including epithelial cells showing positive immunofluorescence for DNA polymerase α has become to localize as the developmental stage proceeds until the region becomes limited in the crypts.

On the other hand, DNA polymerase β was detected in almost all cell nuclei in every section of duodenum, irrespective of its developmental stage and potentiality of cellular DNA replication and proliferation as shown in figs. 1c, 2c, 3c, 4b, 5c and 6b.

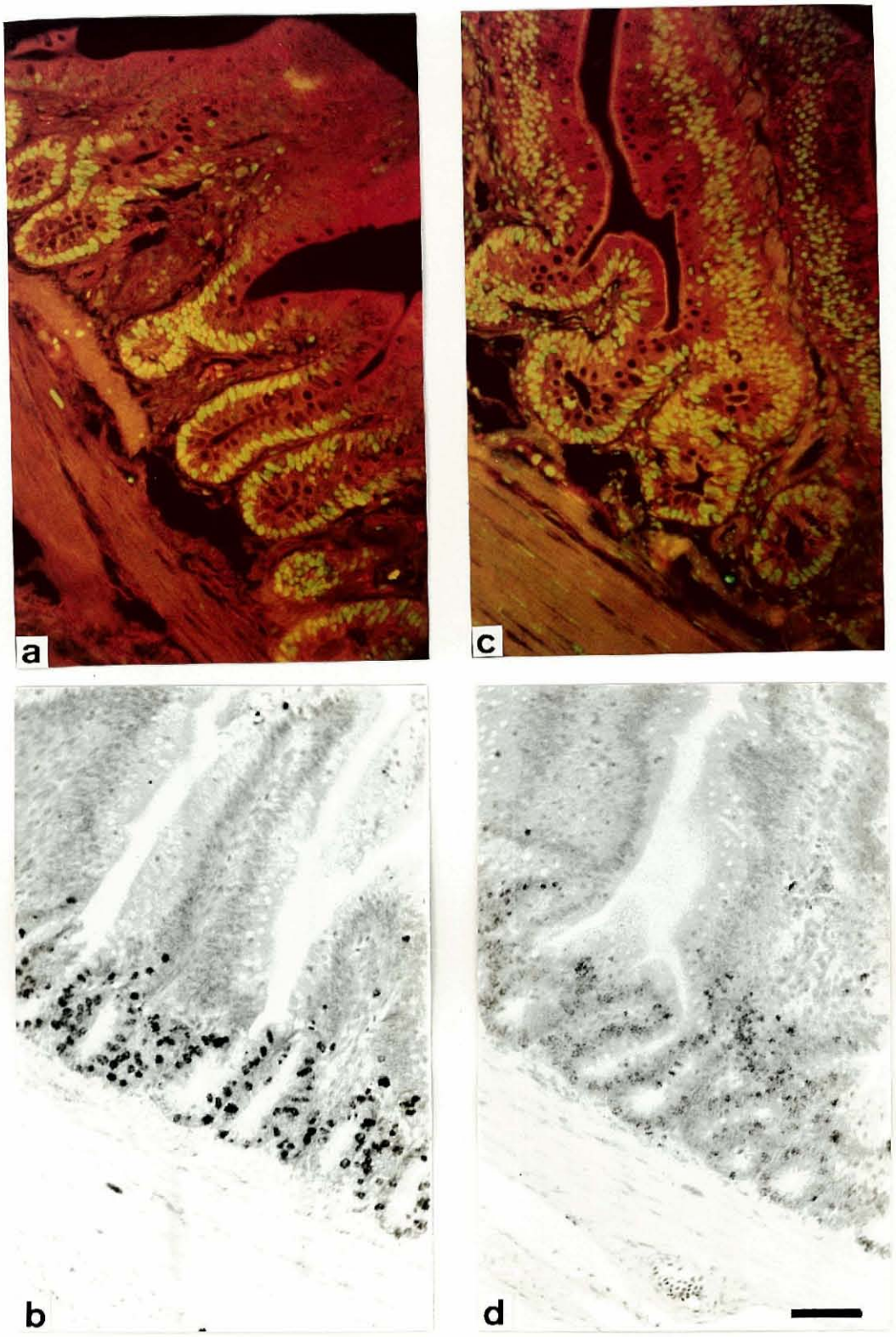

Fig. 5. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (c) in the sections of duodunum of a 2-weeks old chicken; and autoradiograms of the sections of a 2-weeks old chicken duodenum at 1 hr (b) and 24 hr (d) after [^{3}H]thymidine injection. Bar, 50 μm.

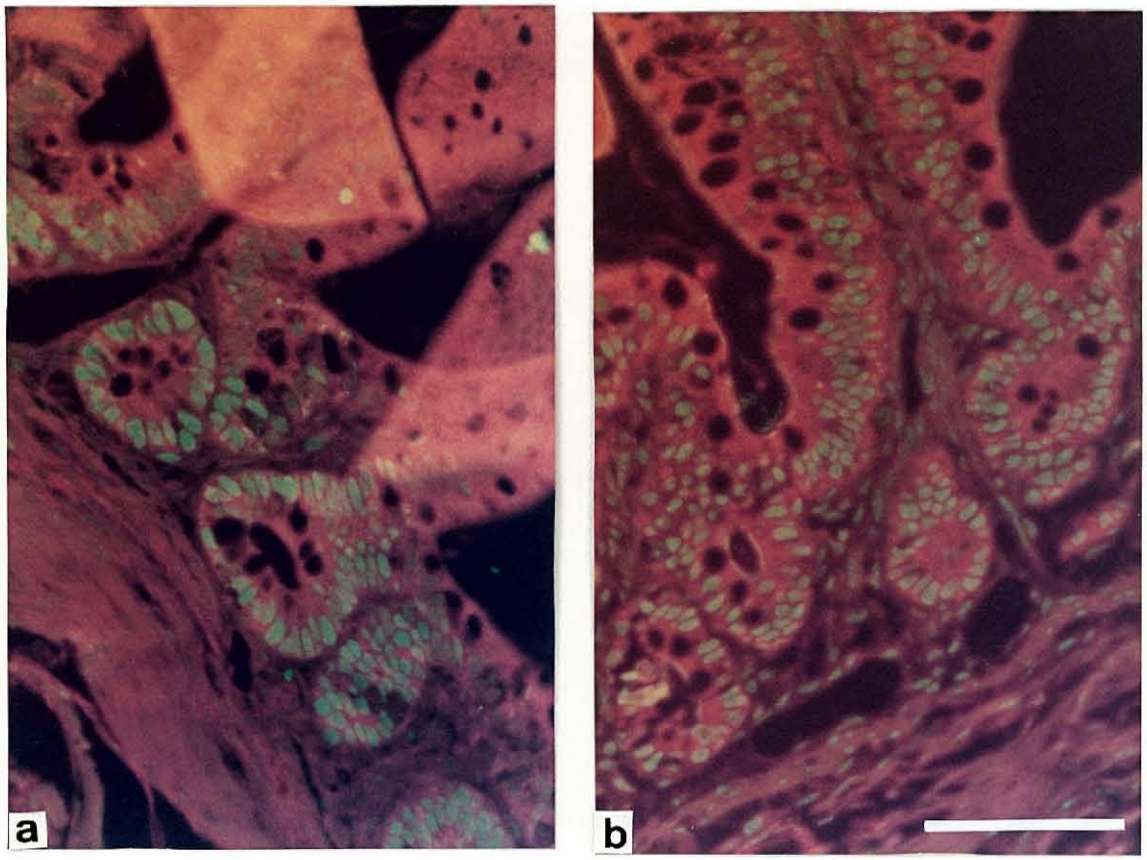

Fig. 6. Immunofluorescent microphotographs showing the localizations of DNA polymerase α (a) and β (b) in the sections of crypt regions of the duodenum of a 2-weeks old chicken at a higher magnification. Bar, 50 μm.

Localization of DNA polymerase α and β in the adult testis

The adult testis, a sperical compound tubular gland, is divided into lobules by a number of septa which are projected from the capsule of the testis. Within the lobules are distributed the twisted seminiferous tubules, which end blindly. The seminiferous tubule consists of a tunic of fibrous connective tissue, a well-defined basal membrane and a complex stratified epithelium. The epithelium comprises of Sertoli cells and germ cells which are stacked in several layers that occupy the space between the basement membrane and the lumen of the tubule. The process of spermatogenesis can be observed usually in these cell layers. The spermatogonia, relatively small cells, are situated next to the basal membrane; the primary and secondary spermatocytes, larger than the spermatogonia, are located nearer the lumen of the tubule; the spermatids can be distinguished by their small size, condensed chromatin, their location almost in the center of the tubules; and the mature spermatozoa occupy the center of the tubules.

In figs. 7a and 7c were shown the intracellular localizations of DNA polymerase α and β in the cross sections of the testis obtained from an adult male white leghorn; and figs. 7b and 7d were demonstrated to show the detailed localization of DNA polymerase α and β, respectively. DNA polymerase α was detected only in oval nuclei of cells forming a

single layer along the basement membrane of the seminiferous epithelium, but not in the nuclei of cells which located near the lumen of the seminiferous tubules.

On the other hand, DNA polymerase β was detected in almost all nuclei of cells in the seminiferous epithelium. It was very contrary to the findings as described above, however, neither DNA polymerase α nor DNA polymerase β was detected in the spermatozoan nuclei stayed in the lumen of seminiferous tubule.

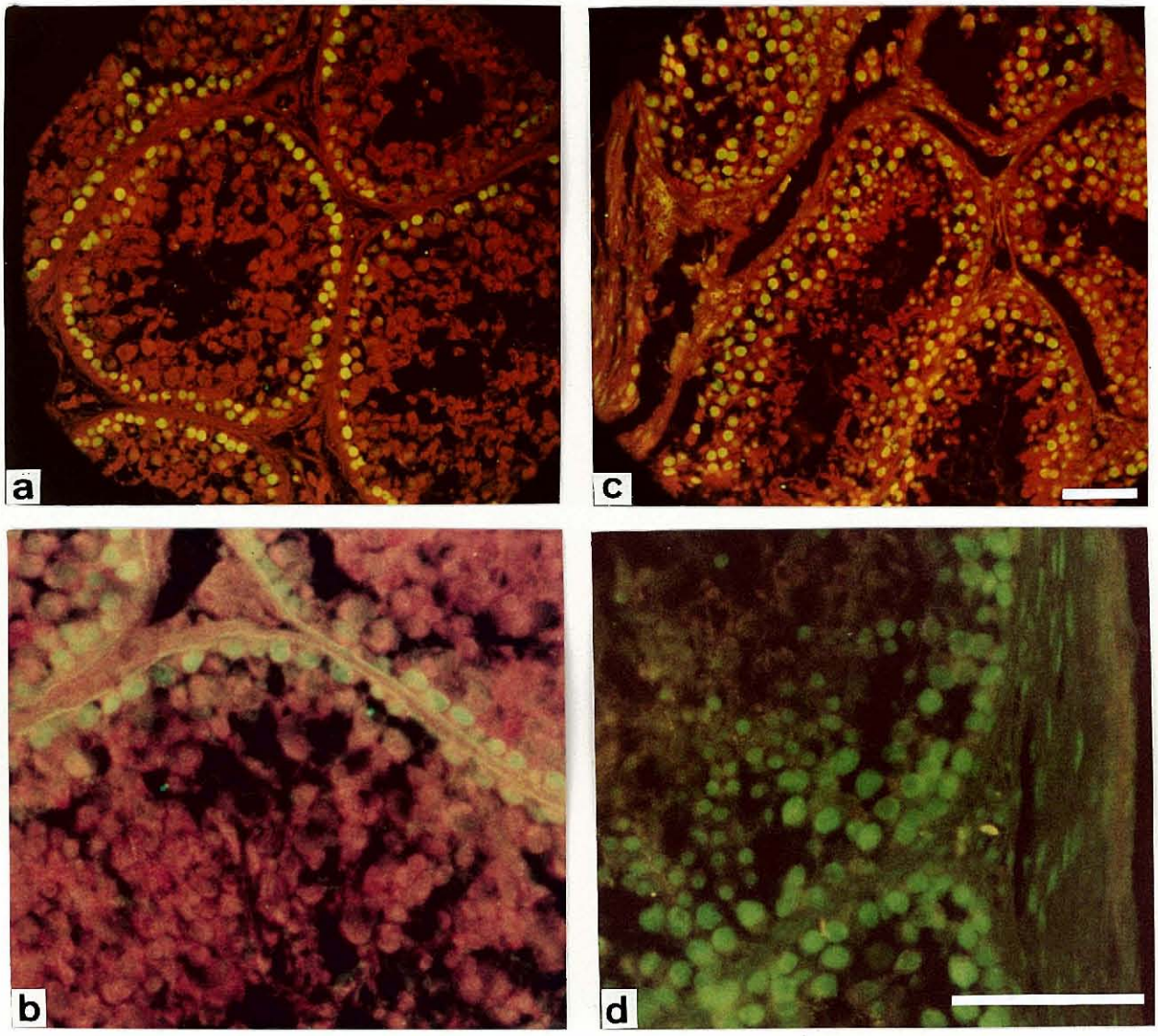

Fig. 7. Immunofluorescent microphotographs showing the localization of DNA polymerase α (a) and β (c) in the sections of adult testis. Localization of DNA polymerase α (b) and β (d) in the seminiferous epithelium were also shown at a higher magnification. Bar, 50 μm.

DISCUSSION

Hermos et al. (3) reported that DNA synthesis and cell division had occurred in epithelium along the entire length of the villi in the duodenum of the fetal rats until one day before their birth, but the site of cell division was restricted within the lower part of the villi. The present observations confirmed that in the developing chick duodenum, DNA polymerase α was detectable in the epithelial cells consisting of the entire inner surface of the duodenum at the early stage of development, that the cells being DNA polymerase α detectable had become restricted to the proximal part of the previllous ridges and later of the immature villi, as the progress of the duodenal morphogenesis in the embryos; and that in chicken 2 weeks after hatching, DNA polymerase α was detected only in the cells of crypts, simple tubular glands occurring between the bases of the villi. The sites of cell division in the chick duodenal epithelium had become also restricted during development, as was described the case with cells being DNA polymerase α detectable in the epithelium.

In the mammalian small intestine, the Paneath cells containing acidophilic granules are found in the base of the crypts (4). However, in the chicken small intestine, no Paneath cell can be identified morphologically (5, 6). By the present immunocytochemical detection of DNA polymerase α,

in the duodenum of the 2-week-old chicken, DNA polymerase α was shown in almost all cells of the crypts, even in the cells located in the part where Paneath cells would be found, if present. This finding supports strongly that no Paneath cell, which is no longer proliferative, exists in the crypts of the chick duodenum.

Matsukage et al. (7) reported that in the testis of a 4-week old chicken, DNA polymerase α was detected only in the large rounded nuclei of cells considered to be spermatogonia and/or spermatocytes in the seminiferous tubule, whereas DNA polymerase β was detected in the nuclei of almost all the testicular cells. However, the histological feature of the 4-weeks old chicken testis is far from the mature state, and the adult fowl testis was examined to clarify the difference of the distribution of DNA polymerase α and β among the cell nuclei during spermatogenesis.

In the 9-month old adult chicken testis, examined in my present studies, DNA polymerase α was detected only in the oval nuclei of the cells in the layer next to the basement membrane of the seminiferous tubule. According to the localization, size and shape of these cells, they were surely regarded as the spermatogonia proliferative for giving rise to the spermatocytes, and/or the pre-leptotene spermatocytes capable of replicating DNA for the meiotic division.

It is well established by biochemical anylysis that in the

mouse the fractions containing the spermatogonia and/or the pre-leptotene spermatocytes exhibited a high activity of DNA polymerase α. The present findings obtained from my immunocytochemical study of DNA polymerase α in the adult fowl testis agreed well with the biochemical analysis by the previous reseacheres (8,9). Moreover, by the present immunocytochemical study both the timing and the site of the loss of DNA polymerase α from the cell nuclei during spermatogenesis in the adult fowl testis could be shown.

Again, DNA polymerase β was detected in almost all the nuclei of the cells in the seminiferous tubules. However, it should be emphasized that DNA polymerase β was not found in the nuclei of mature spermatozoa in the present study. DNA polymerase β was detected in the nuclei of germ cells during the first half of the spermatogenesis up to the second meiotic division, but became undetectable after the spermatid was transformed into mature spermatozoa. This finding suggests presumably that DNA polymerase β has been eliminated from the spermatozoan nuclei during spermiogenesis. If so, the lost of potentiality of DNA excision repair would be considered rather signnificant or effective for a spermatozoon as a carrier of genes for the next generation; because it would be rather unnecessary to risk failure in repair of damaged DNA, even the probability of the failure is not so high, in such

a stage and in the nucleus being heteropicnotic.

Several autoradiographic studies have revealed" unscheduled " DNA synthesis in mammalian spermatogenic cells, except spermatozoa, after the irradiation of UV light or X-ray (10-12) or after the treatment of chemical mutagen (13). The " unscheduled " DNA synthesis would be considered a repair synthesis of DNA, damaged by irradiation or chemicals in the spermatogenic cell nuclei. It would be supported by the the present finding that DNA polymerase β was expected to be mainly responsible for such a DNA synthesis in birds.

A small amount of " natural " DNA synthesis also has been shown to occur at the zygotent/pachytene stages of prophase I in the mammalian testis (10, 11, 14, 15). This kind of DNA synthesis during the pachytene stage is considered to be associated with the recombination process resulting in crossing over (10, 11, 14). The present finding showing the pachytene spermatocytes were detectable DNA polymerase β would strongly suggest a possible involvement of DNA polymerase β in the recombination process in the meiotic division in birds.

In order to access the mechanism of histogenesis in the developing embryo or in the adult cell renewal system, it would be important to manifest the distribution of both the proliferative and the postmitotic cells in the tissue architecture. [^{3}H]thymidine autoradiography has been widely used for detecting the proliferative cells in vivo. However, a lot of

laborious works should be necessary to distinguish all the proliferative cells in the tissue by [^{3}H]thymidine autoradiography. The present immunocytochemical technique for DNA polymerase α and β detection, therefore, would be very useful : to investigate histogenesis in the embryo or the cell renewal system in the adult;and also may be applicable to the studies on the cell proliferation in the tumour tissues in future.

AKNOWLEDGEMENTS

The author is grateful to Dr. Akio Matsukage of Aichi Cancer Center Research Institute for providing the antibodies against DNA polymerase α and β, to Dr. Moriaki Kusakabe of Nagoya University, School of Medicine for kind advice on the polyesterwax sectioning method, and to Dr. Ikuko Ishikawa and Mr. Shinri Horiuchi of Osaka University for permitting to use their laboratory facilities. The author is also indebted to Dr. Toshiteru Morita of Aichi Cancer Center Research Institute for providing the convinience to use his laboratory facilities for the radioisotope and for his many valuable suggestions. The author also would like to express my great appreciation to Professor Yutaka Koshida of Osaka University for his valuable advice and continuous encouragement during the course of this work, and to Mrs. Kazumi Tsutsui of Osaka University for her kind help.

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