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Original Article

Tissue distribution of the DNA binding oncoprotein Maf during chicken development

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ABSTRACT To assess the normal function of the c-*maf* protooncogene product, we examined its tissue distribution during fetal growth and organogenesis of the chicken using an immunohistochemical technique. Nuclei of neuronal cells in the optic lobe and in the outer granular layer of the cerebral cortex were efficiently stained by anti-Maf serum throughout all developmental stages examined. In the lung, mesenchymal cells were stained intensely by the antiserum, with the most intense staining around embryonic day 15. During the embryonic period, nuclei of mesenchymal cells in the perichondrial and periosteal tissues as well as intestinal submucosa were also stained specifically by the antiserum. In addition, we detected substantial Maf immunoreactivity in nuclei of renal glomerular and proximal-tubular epithelial cells. These results suggest that c-Maf protein plays some important roles in the development of the central nervous system and tissues of mesodermal origin such as connective and renal tissues.

KEY WORDS: *chicken, development, immunohistochemistry, Maf*

Introduction

maf is an oncogene of an acutely oncogenic avian retrovirus, AS42, that was isolated from a chicken musculoaponeurotic fibrosarcoma (Nishizawa *et al.*, 1989; Kawai *et al.*, 1992). Its product, v-Maf contains a structural motif common to transcription factors, the basic-leucine zipper (bZip) in its carboxy-terminal region. By structural analysis of chicken c-*maf* cDNA clones, the deduced c-Maf structure was shown to be almost identical to its viral counterpart with only two exceptions; fusion of viral Gag sequence in v-Maf just at amino-terminal of c-Maf and an amino acid substitution (Kawai *et al.*, 1992). We also showed that, under control of a strong viral promoter, the c-*maf* gene itself is equally effective in cell transformation (Kataoka *et al.*, 1993). As deduced from its structure, the Maf protein forms a homodimer, specifically recognizes palindromic DNA sequences, and can act as a transcriptional activator (Nishizawa *et al.*, 1989; Kataoka *et al.*, 1993, 1994a). The consensus recognition sequences of Maf (TGCTGACTCAGCA and TGCTGACGTCAGCA) are, in their central parts, identical to binding sequences of AP-1 (TGACTCA) and ATF/CREB family transcription factors (TGACGTCA), respectively (Kataoka *et al.*, 1994a). It was also recently reported that Maf can associate with two major components of the AP-1 transcription factors, Fos and Jun (Kataoka *et al.*, 1994a; Kerppola and Curran, 1994). Interestingly, DNA-binding specificities of Maf/Jun and Maf/Fos heterodimers are distinct from Maf homodimer or AP-1, suggesting that the interaction of these bZip factors plays a critical role in transcriptional control of their target genes.

Utilizing the relatively long recognition sequences of Maf, we previously screened for candidates as target genes of Maf by surveying DNA sequence databases (Kataoka *et al.*, 1994a). Among the candidates identified, some cellular genes expressed specifically in erythroid cells were found to be actually regulated by Maf-related proteins. Small bZip proteins encoded by three *maf*-related genes (*maf*K, *maf*F and *maf*G) efficiently heterodimerize with another bZip protein, p45, to form an erythroid-specific transcription factor, NF-E2 (Andrews *et al.*, 1993; Igarashi *et al.*, 1994). In addition, another *maf*-related gene, *nrl*, is expressed specifically in neural retina cells, suggesting that it regulates expression of neural retina-specific genes (Swaroop *et al.*, 1992). On the other hand, the normal function of c-Maf is still unclear and remains to be studied.

Previously we examined the distribution of its transcript in adult chicken tissues by RNase protection assay (Fujiwara *et al.*, 1993). The c-*maf* mRNA was detected in almost all tissues examined but its highest level of expression was observed in the kidney followed by the brain, lung and intestine.

Many researchers have analyzed expression profiles of AP-1 factors in normal adult and embryonic tissues as well as in many established cell lines by blot hybridization, *in situ* hybridization

Abbreviations used in this paper: AMeX, aceton-methylbenzoate-xylene; DAB, 3,3'-diaminobenzidine tetrahydrochloride.

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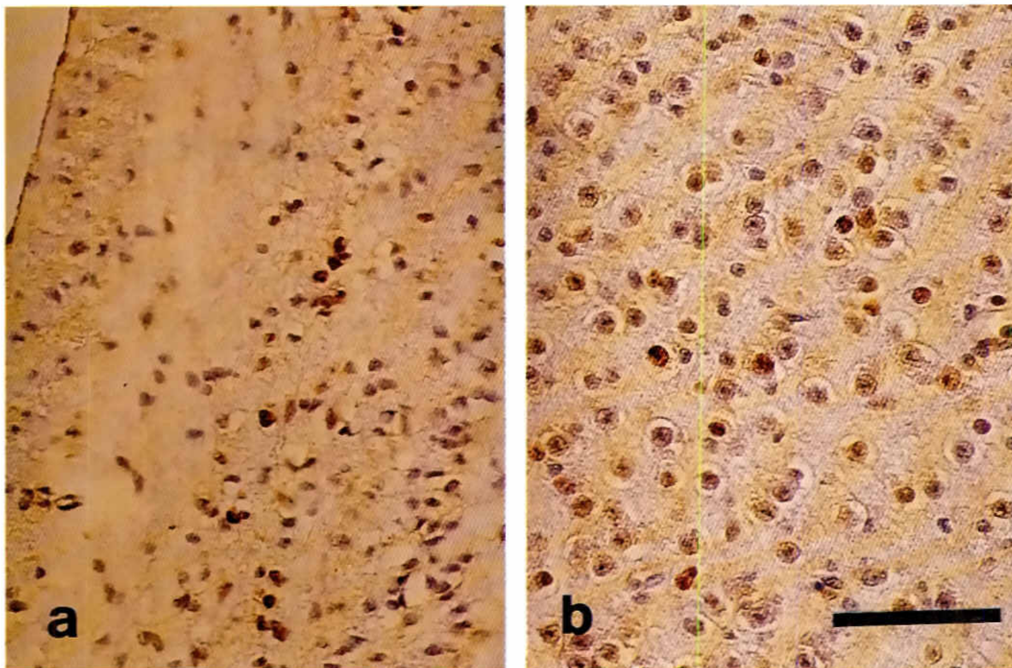


Fig. 1. Immunostained brain tissues; the cerebral cortex (a) and optic lobe (b) from an embryo at ED 15. *Many nerve cell nuclei showed positive immunoreactivity for Maf. Bar, 50 µm.*

and immunohistochemical staining. For instance, in 4- to 6-week-old mice, constitutive expression of the c-*fos* gene was reported in hematopoietic cells (Müller *et al., 1984*). However, at the embryonic stage, relatively high levels of constitutive c-*fos* expression were detected in neuronal cells (Gubits *et al.*, 1988; Caubet, 1989). In addition, the liver, bone, tooth, cartilage, skin and blood cells were observed to express c-*fos* mRNA during their development (Müller *et al.*, 1982; Caubet, 1989). In the case of the c-*jun* protooncogene, substantial expression was reported in the developing cartilage, intestine and central nervous system (Wilkinson *et al.*, 1989; Mellström *et al.*, 1991).

In this study, as a first step towards determining the normal function of the c-*maf* protooncogene product, we examined immunohistochemically the distribution of Maf in avian embryos and juvenile chickens. Maf immunoreactivity was most intense in the mid to late stage of organogenesis in brain neuronal cells, and mesenchymal cells of the lung, intestine, and perichondrial and periosteal tissues. In renal epithelial cells, Maf immunoreactivity was also detected constantly throughout all developmental stages examined. These results suggest the functional importance of c-Maf protein in cell proliferation and differentiation during fetal development.

Results

In this study we used an anti-Maf rabbit serum as primary antibody for immunohistochemical staining of tissue sections. As we have previously reported (Kataoka *et al.*, 1993), the antiserum was prepared by immunizing a rabbit with bacterially synthesized v-Maf protein. The antiserum reacted specifically with nuclei of chicken embryonic fibroblast cells infected with the AS42 virus in which v-Maf protein is overexpressed, but did not react with nuclei of uninfected cells, showing its specificity against Maf. In an immunoprecipitation experiment, the antibody specifically precipitated the v-Maf protein from the virus-infected cell lysate, also indicating its specificity (Kataoka *et al.*, 1993). The nucleotide sequence of the v-*maf* is identical to c-*maf* except for addition of the viral *gag* component (Kawai *et al.*, 1992). The antiserum against Maf, therefore, could recognize c-Maf as well as v-Maf. Tissue sections were prepared from tissues of chicken embryos at embryonic days (ED) 10, 15 and 21 and at post-hatching days (PHD) 0, 2, 5 and 40, and subjected to immunostaining.

Most prominently, the anti-Maf serum reacted with nuclei of two particular parts of the brain, outer granular layer of the cerebral cortex (Fig. 1a) and the optic lobe (Fig. 1b). By the image analysis technique, in the cerebral cortex, the number of positive cells and staining intensity was shown to increase from marginal levels at ED 10 (Fig. 2a) to the highest level at ED 15 (Fig. 2b). In chicken, the layer formation of the cerebral cortex is known to finish at this developmental stage (ED 15). However, both the number of positive cells and staining intensity decreased to the lowest levels around the day of hatching (ED 21 and PHD 0; Fig. 2c and d), they increased again after that (Fig. 2e and f) but did not exceed the level at ED 15. In the optic lobe, which is the equivalent of the mammalian mesencephalic tectum and is important for processing visual information, Maf-positive neuronal cells were detected as early as ED 7 (Fig. 3a). The highest levels of expression were observed at ED 10 (Fig. 3b) and 15 (Fig. 3c). Similarly to the cerebral cortex, after a transient decrease in the number of Maf-positive nuclei around the day of hatching (Fig. 3d), the expression of Maf in the optic lobe again increased to a higher level at PHD 5 (Fig. 3e) and 40 (Fig. 3f). These results were confirmed by the quantitative analysis using the numerical data for the image-analyzed pictures (Figs. 4 and 5). Neuronal cell nuclei in the spinal cord of embryos at ED 15 also reacted with the anti-Maf serum. We detected no c-Maf expression in cerebellar neuronal cells including Purkinje cells.

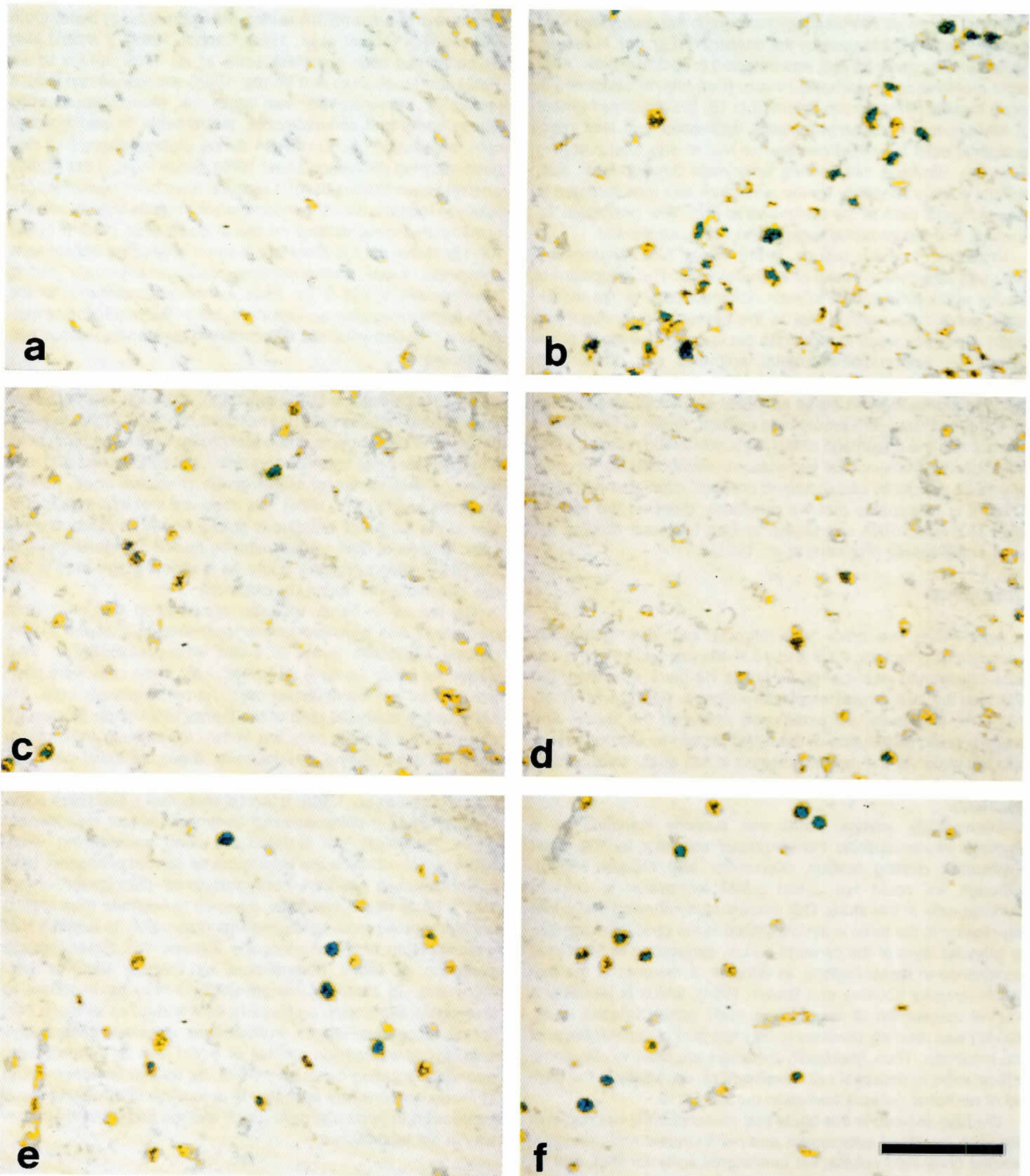


Fig. 2. Computer analyzed images of nuclear expression of Maf. *Immunostained sections were analyzed and shown by a pseudocolor system according to the relative intensity of the reaction. The most intense reaction was shown in green, followed by yellow and grey. Cerebral cortex from chick embryos at ED 10* **(a)**, *15* **(b)** *and 21* **(c)** *and PHD 0* **(d)**, *5* **(e)** *and 40* **(f)**. *Bar, 40 μm.*

Nuclei of almost all mesenchymal cells in the embryonal lung were also stained intensely by the antiserum (Fig. 6a). However, no immunoreactivity for Maf was detected in epithelial cells. After rapid proliferation of epithelial cells to form tubular structures in loose mesenchymal tissue around ED 15, the absolute number of Maf-positive mesenchymal cells decreased and less mesenchymal cells remained positive for Maf at and after hatching (Fig. 6b). Similarly, only during embryonic development, substantial nuclear reactivity for the antiserum was also detected in mesenchymal cells of the perichondrial (Fig. 7) or periosteal tissues, and of the intestinal submucosa (data not shown).

In addition to these tissues, in the kidney, Maf immunoreactivity was detected in nuclei of epithelial cells. The cytoplasm of tubular epithelia is known to stain nonspecifically by the avidin-biotin peroxidase method due to the presence of endogenous biotin. However, weak to moderate but specific staining of some glomerular and proximal-tubular epithelial cell nuclei was observed with the anti-Maf antibody (Fig. 8). We confirmed that the immunoreactivity in these epithelial cell nuclei was specific by the peroxidase-anti-peroxidase method, which is less sensitive but has the advantage of lacking avidin-biotin reaction (data not shown). Notably, c-Maf expression in renal cells did not show any stage specificity and remained constant even after hatching. Thus, it is reasonable that we previously detected the highest level of c-*maf* mRNA expression among the adult chicken tissues in the kidney (Fujiwara *et al.*, 1993).

Discussion

Apart from two other well-characterized viral oncogenes encoding bZip factors, v-*fos* and v-*jun*, the expression of the cellular counterpart of v-*maf* is, as far as we have examined, not changed by cell growth stimulatory signals (Nishizawa *et al.*, 1989). In this study, we extensively analyzed the tissue- and stage-specific distribution of the c-Maf protein by utilizing a specific antibody. All the results obtained in this study were consistent with our previous RNase protection assay data (Fujiwara *et al.*, 1993).

Interestingly, mouse c-Maf was recently identified as a Purkinje neuron-specific transcriptional regulator by the yeast expression cloning system (Kurschner and Morgan, 1995), although we could not detect c-Maf expression in chicken Purkinje cells in this study. Our present study showed that c-Maf expression in the brain is almost limited to the optic lobe and outer granular layer of the cerebral cortex, suggesting its functional importance in these regions. In addition, a member of the *maf* family, *kreisler* (Cordes and Barsh, 1994), which is probably a murine counterpart of the chicken *maf*B gene (Kataoka *et al.*, 1994b) was recently revealed to be important in segmentation of the hindbrain. Thus, Maf family members are likely to play some critical roles in neuronal cell development, especially in the period of neuronal network formation around ED15.

We also showed in this study that mesenchymal cell nuclei of the lung, intestinal submucosa and perichondrial and periosteal tissues contained substantial immunoreactivity for Maf, and its level of expression in these tissues was highest during embryonic development. Among these tissues, perichondrial and periosteal tissues may be especially notable because connective tissue cells had been previously reported to express considerable levels of c-*fos* mRNA during development into bone, tooth and cartilage (Müller *et al.*, 1982; Caubet, 1989). It should also be noted that both *maf* (Nishizawa *et al.*, 1989; Kawai *et al.*, 1992) and *fos* (Curran and Verma, 1984) oncogenes can induce tumors of mesenchymal cell origin, i.e. musculoaponeurotic fibrosarcoma and osteosarcoma, respectively. In perichondrial cells, expression of c-*jun* mRNA during organogenesis has also been reported (Wilkinson *et al.*, 1989). Since most of the studies on expression profiles of AP-1 proteins have been performed primarily in rodents, we cannot compare our results directly with the accumulated observations on tissue distribution pattern of the AP-1 proteins, but it is likely that in some connective tissues cells coexpress these protooncogene products especially during development. If this is the case, concerted expression of the bZip factors may play an important role in the regulation of multipotent mesenchymal cell differentiation by forming various heterodimers.

In contrast, in mesenchymal cells of the embryonic lung, we detected significant c-Maf expression, although distribution of Fos and Jun in these cells has not been reported. In the absence of the AP-1 components, the c-Maf protein should be present as a homodimer or as heterodimers with other bZip factors and may induce a distinct set of target genes (Kataoka *et al.*, 1994a; Kerppola and Curran, 1994). We observed that expression of c-Maf in the lung and intestine is strictly limited to mesenchymal cells. Control of DNA transcription by homo- or heterodimers of the DNA-binding proteins would be a critical stage- and/or tissue-specific event during development.

On the other hand, we also observed in this study that glomerular and proximal tubular epithelial cells of the kidney contain significant levels of c-Maf immunoreactivity in their nuclei. As far as we have examined, only these cells were positive for Maf among epithelial cells. As mesenchymal cells, the Maf-positive epithelial cells of the permanent kidney, glomerular and proximal tubular cells are known to originate from mesoderm. At least during development, these epithelial cells are known to be profoundly related to surrounding mesenchymal cells (Koseki *et al.*, 1992). It is thus likely that c-Maf plays some critical role(s) in developmental control of cells of mesodermal origin. Previously, we isolated an avian transforming virus, NK24, which contains the v-*fos* gene as its viral oncogene, from a spontaneous chicken nephroblastoma (Nishizawa *et al.*, 1987). Since nephroblastoma is known to originate from resting undifferentiated renal cells, it seems reasonable to assume that overexpresion of v-*fos* oncogene induces this tumor through depletion of c-Maf homodimers by forming Maf/Fos heterodimers. To examine this possibility, it may be important to investigate expression profiles of c-Maf and c-Fos at much earlier developmental stages. Furthermore, extensive comparative analyses of expression profiles of c-Maf and its heterodimeric partners, including c-Jun and c-Fos, by double immunostaining of tissue sections, are essential to determine their simultaneous expression in particular cells. Such studies are currently underway in our laboratories.

Materials and Methods

Chicken embryos and juvenile chickens used were White Leghorns raised under specific pathogen-free (Line M, Nisseiken Laboratory,

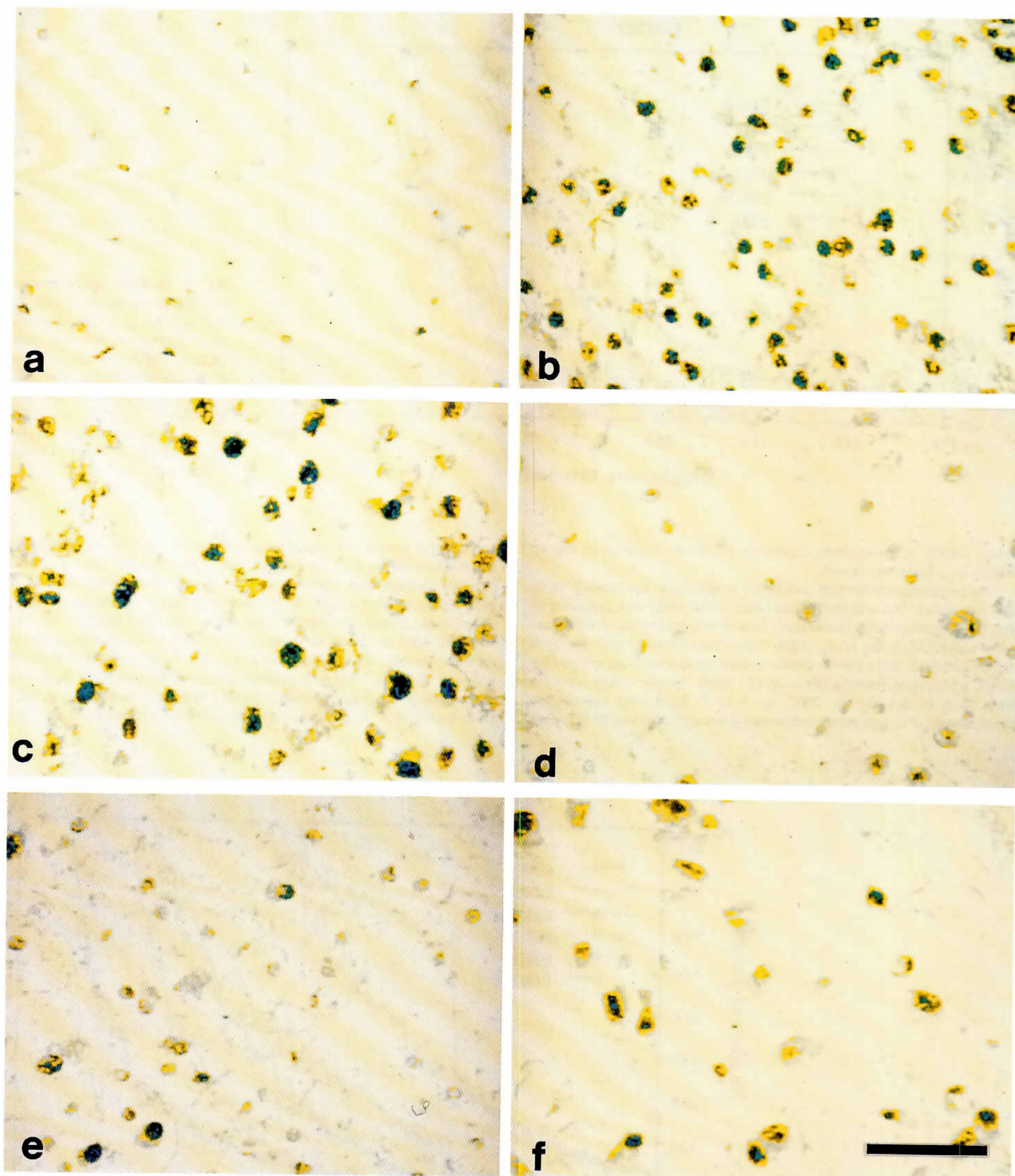


Fig. 3. Images analyzed similarly to those in Figure 2. *Optic lobe from chick embryos at ED 7* **(a)**, *10* **(b)** *and 15* **(c)** *and PHD 0* **(d)**, *5* **(e)** *and 40* **(f)**. *Bar, 40 μm.*

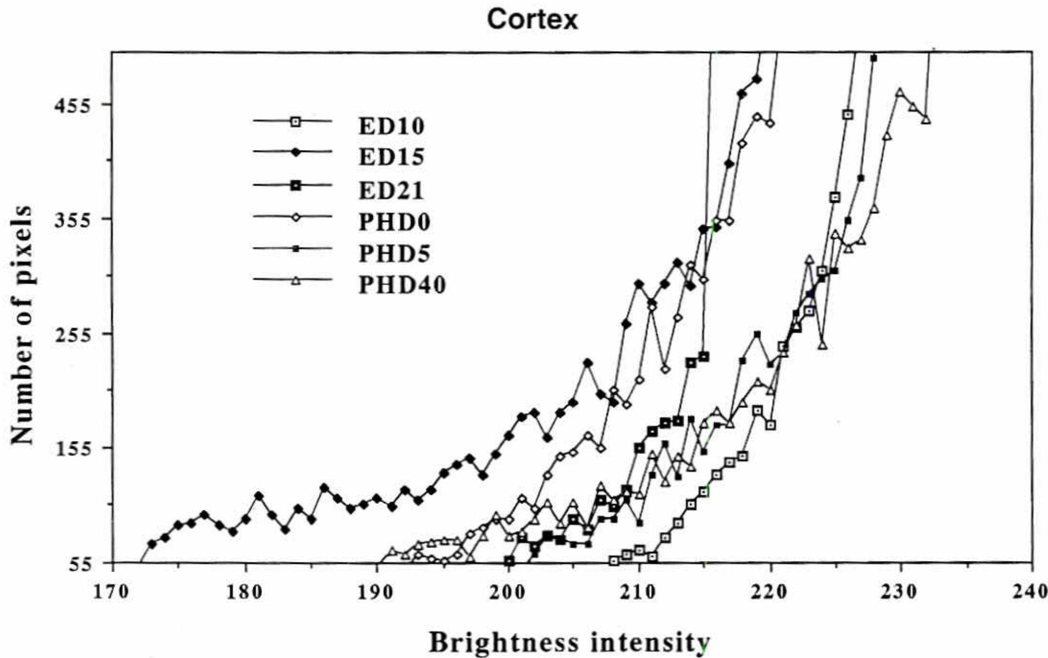


Fig. 4. Quantitative analysis for the pictures of Figure 2. *The number of pixels showing each brightness intensity was plotted. The brightness intensity was graded 0 (black) to 255 (white). Lower value of the intensity means stronger immunoreaction and higher pixel number means more positive cells in a picture.*

Tokyo, Japan) or conventional conditions (Saitama Experimental Animal Supply Co., Saitama, Japan).

The immunohistochemical procedure employed in the present study was as follows. The anti-Maf serum used in this study was prepared as described previously by immunizing rabbits with bacterially synthesized v-Maf protein (Kataoka *et al.*, 1993). Tissue slices from chicken embryos or from newborn and juvenile chickens were embedded in paraffin by the AMeX embedding method (Sato *et al.*, 1986). Briefly, tissue samples were fixed in acetone at -20°C for 1 or 2 days, and sequentially processed at 4°C and room temperature through acetone, methylbenzonate, and xylene. We then embedded them in paraffin, keeping their temperature below 58°C to prevent loss of antigenicity. Four µm thick paraffin sections were deparaffinized in xylene and passed through a descending ethanol series. To prevent nonspecific reactions, sections were preincubated with normal goat serum and then reacted with the anti-Maf rabbit serum at an appropriate dilution at 4°C overnight. As a negative control, we used preimmune rabbit serum instead of the first antibody. Sections were then allowed to react with biotinylated goat antibody against rabbit IgG (Kirkegaad and Perry Lab., Gaithersburg, MD, USA) for 1 h at 37°C, and then subsequently with avidin-peroxidase con-

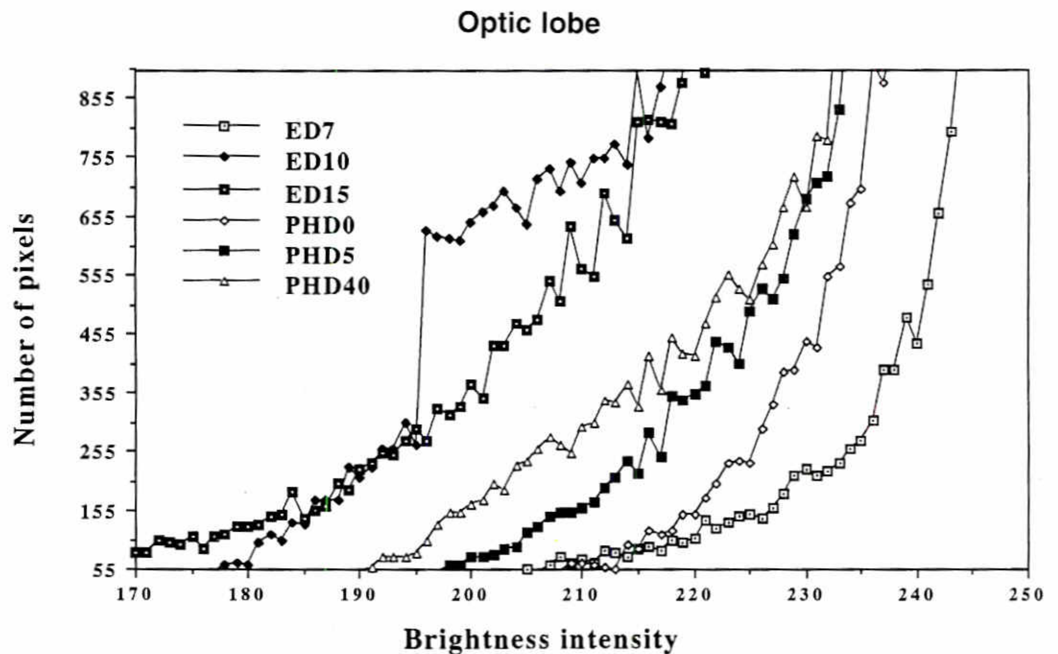
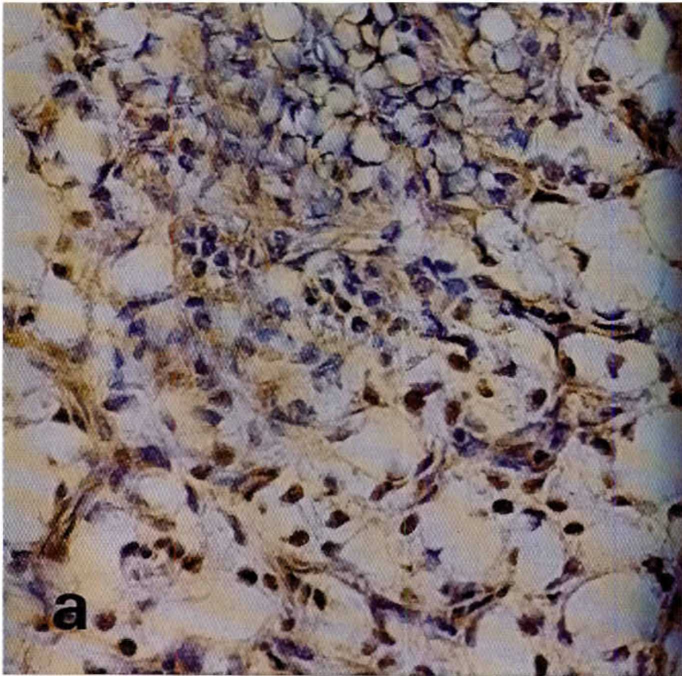


Fig. 5. Quantitative analysis for the pictures of Figure 3. *This figure was drawn by the same fashion as Figure 4.*



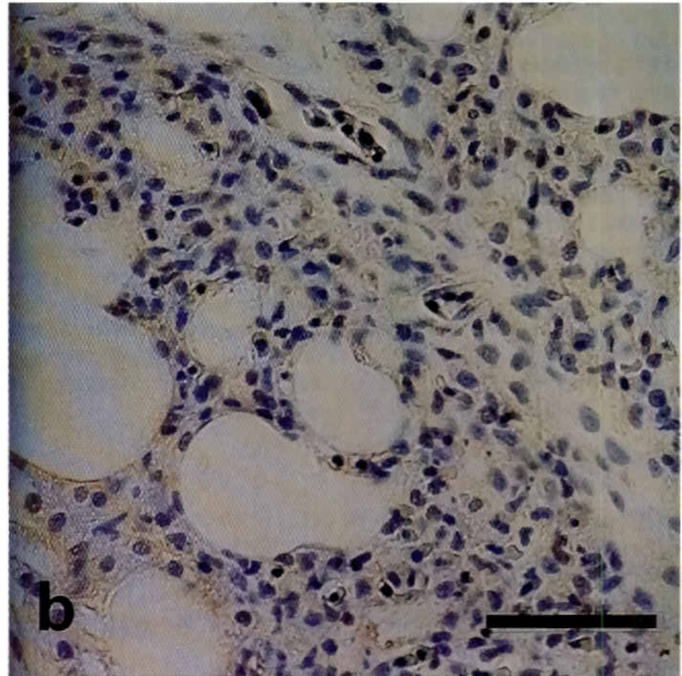


Fig. 6. Immunostained sections of lungs at ED 15 (a) and PHD 2 (b). *There were many mesenchymal cells with Maf-positive nuclei at the peak of intensity* **(a)**. *The numbers of immunoreactive mesenchymal cells, however, decreased at and after the day of hatching, and Maf-negative epithelial cells proliferated to form a tubular or alveolar pattern* **(b)**. *Counterstained with hematoxylin. Bar, 35 μm.*

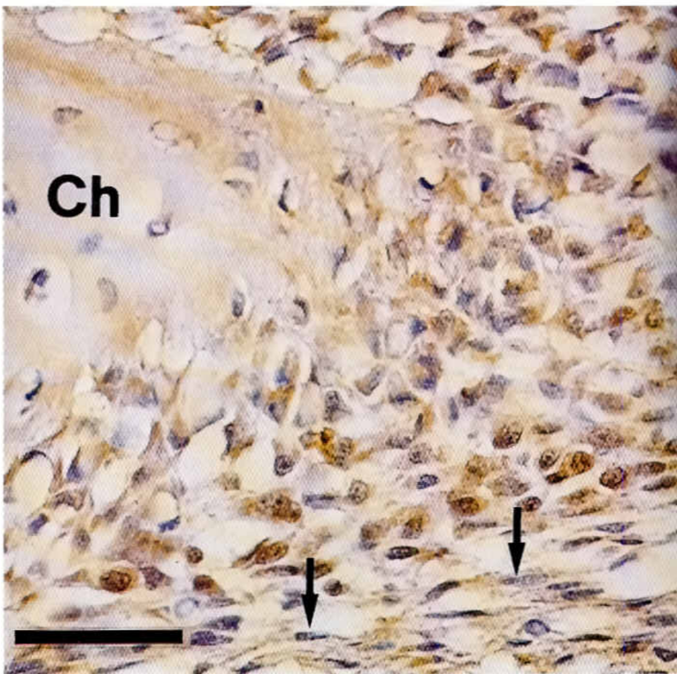


Fig. 7. Immunostained perichondrial tissue from an embryo at ED 15. *Nuclei of the perichondrial cells showed positive immunoreactivity for Maf. Nuclei of mature chondrial (Ch) and fibroblastic (arrows) cells were negative. Bar, 30 μm.*

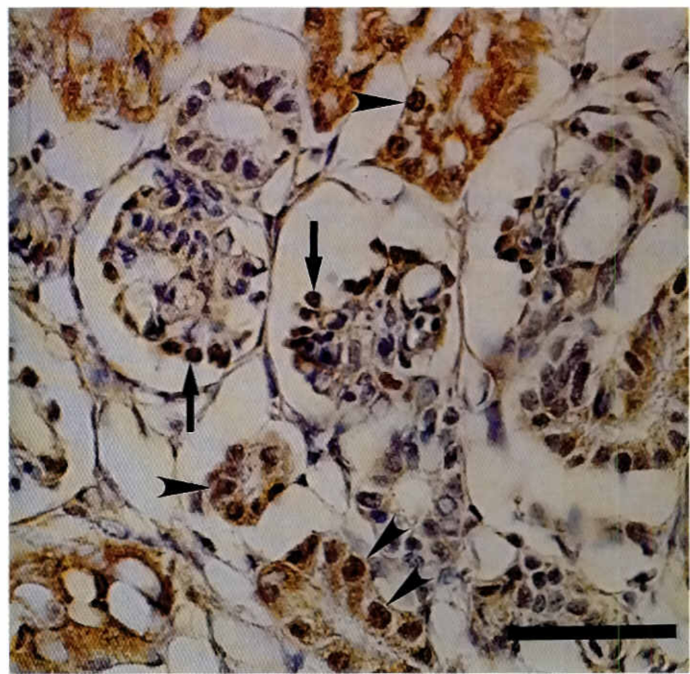


Fig. 8. Immunostained kidney section from an embryo at ED 15. *Nuclei of glomerular (arrows) and proximal tubular (arrowheads) epithelial cells were stained positively for Maf. Note unstained nuclei of glomerular mesangial and distal tubular cells. Cytoplasm of the proximal tubular cells showed positive immunoreactivity probably due to endogenous biotin. Bar, 30 μm.*

jugate (Vectastain ABC kit, Vector labs., Burlingame, CA, USA) for 1 h at room temperature. Finally, we placed the sections in 0.05% DAB solution (0.01% hydrogen peroxide-0.2 M Tris-HC, pH 7.6) to allow visualization of the immunocomplex. Sections were counterstained with hematoxylin.

For semi-quantitative analysis of stage- or tissue-specific distributions of c-Maf immunoreactivity, immunostained sections were analyzed using the PIAS III Image Analyzing System (PIAS, Osaka, Japan) on a PC 9801 computer (NEC, Tokyo, Japan). Full color image of the sections was taken into the system and the brightness information of the blue color element was output, in which the brown color of the DAB product was the most contrasted. The brightness value of the background in each section was, then, standardized to compare the authentic intensity of the immunoreaction. The resulting black and white pictures were painted with the PIAS pseudocolor system. For quantitative analysis, the standardized bright value (0 to 255) of each pixel was measured and the number of pixels showing a bright value was plotted.

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