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Localization of E2A mRNA expression in developing and adult rat tissues

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ABSTRACT **E2A helix–loop–helix proteins are involved in the control of various developmental pathways. We show here by *in situ* hybridization that E2A transcripts are present in most embryonic and adult tissues. However, no E2A expression is detectable in heart and nonproliferative regions of the brain and spinal cord. Highest levels of E2A expression are found in the ependyma cell layer surrounding the cerebral ventricles in the embryonic rat brain. In addition, in the embryo, E2A transcripts were found in secretory cells of the pancreas, the bronchial tubes of the lung, glomeruli of the kidney, and the lining of the stomach. Interestingly, high levels of E2A transcripts are selectively found in the germinal center of the lymphatic nodules in the adult rat spleen. Thus, E2A, like its *Drosophila* homolog daughterless, is expressed in most tissues. The most notable feature of the E2A expression pattern is its high levels of expression in some areas of rapid cell proliferation and differentiation and in certain epithelial cell types.**

Recently, a new class of DNA-binding proteins has been identified that have in common a region of structural similarity, designated the helix–loop–helix (HLH) domain (1). The HLH motif is a dimerization domain (1). Adjacent to the HLH domain is a basic region shown to mediate DNA binding (2). HLH proteins bind to a common DNA sequence element, the *E*-box elements (3, 4). One class of HLH proteins binds to a common DNA sequence, the *E2*-box (GCAGNTG in which N is any nucleoside) (5). HLH members of proteins binding to the *E2* box are E12 (1, 6), E47 (1, 6, 7), E2-2 (7), HEB (8), daughterless (9, 10), MyoD (11, 12), myogenin (13), myf-5 (14), and members of the achaete-scute family (15). The family of HLH proteins binding to the *E2-2* box can be further divided into different subclasses (2, 5): class I HLH molecules, including E12, E47, E2-2, HEB, and daughterless, all of which bind to DNA either as homodimers or heterodimers or both (5); and class II HLH molecules, including MyoD, myogenin, myf-5, achaete scute T3 (AS-C T3), AS-C T5, AS-CT4, and AS-C T8, all of which only bind to DNA as heterodimers (2, 5).

E12 and E47 are encoded by one gene, the *E2A* gene, and arise through differential splicing of exons encoding the DNA binding domain (1, 16). They are most closely related to *Drosophila* protein daughterless, sharing 80% identity in the region surrounding the HLH domain (1); daughterless is involved in at least two developmental pathways: sex determination and neurogenesis (9). *E2A* gene products are similarly involved in the control of a number of mammalian developmental pathways. They are present in B cells and in muscle- and pancreatic-specific complexes (17–20). In B cells, they form homodimers that bind to E2 box elements present in both the immunoglobulin heavy and light chain gene enhancers (17). In muscle they form heterodimers with MyoD and myogenin and activate muscle-specific genes (11, 18). In addition, they are involved in pancreatic-specific gene expression (19–21). Thus, the *E2A* gene products are present in a number of tissue-specific complexes that regulate tissue-specific enhancers.

Like daughterless, E2A mRNA has been found in all tissues examined, and its presence in E2 box-binding complexes suggests a ubiquitous expression pattern (1, 7, 20). However, a comprehensive analysis of the distribution of E2A transcripts has not been undertaken. We now report that the *E2A* gene is expressed in most tissues in the developing embryo except in the heart. High levels of expression are particularly evident in the ependyma cell layer surrounding the cerebral ventricles. In addition, relatively high levels of E2A expression can be found in secretory cells of the salivary gland and the pancreas, the lining of the stomach, bronchial tubes of the lung, and glomeruli of the kidney. The high levels of E2A expression in certain regions of the embryo undergoing rapid cell division suggests an important role for E2A in proliferation and differentiation.

MATERIALS AND METHODS

Animals and Tissue Preparation. Pregnant female and normal male (250 g) Sprague–Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA) and kept under standard housing, feeding, and lighting conditions (23°C, 12 hr light/12 hr dark cycle with lights on at 0600). On the appropriate day of pregnancy, rats were anesthetized with an i.m. injection of an equal-volume mixture of ketamine hydrochloride at 90 mg per kg of body weight and xylazine at 10 mg/kg and perfused transcardially with 100 ml of saline followed by 700 ml of ice-cold 4% paraformaldehyde in borate buffer (38.14 g of sodium tetraborate per liter, pH 9.5). Embryos were removed and postfixed for 1–6 months at 4°C in 10% neutral buffered formalin (Richard-Allan Medical, Richard, MI). Various tissues, removed from the adult male rats, were postfixed for 2 weeks. Sucrose (10%) was added 24 hr prior to freezing the tissue in a 1:1 (wt/vol) mixture of Aquamount (Lerner Laboratories, Pittsburgh) and OCT (Miles) embedding medium. Frozen sections were cut on a cryostat at 25-μm thickness, mounted on polylysine-coated slides, and stored in a desiccator overnight at room temperature. Tissue was then stored in slide boxes with desiccant at −20°C until used for *in situ* hybridization analysis. Three embryos for each day from 12 through 20 days postcoitum (p.c.) and tissues from two adult male rats were prepared and analyzed. Sex of the embryos was determined after tissue preparation and staining. Males and females were present and analyzed at all time points.

Generation of RNA Probes for *in Situ* Hybridization. An antisense ^{35}S-labeled RNA probe specific for the rat E2A immunoglobulin enhancer-binding proteins was generated by transcription from the T7 promoter of linearized pBluescript

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Abbreviations: HLH, helix–loop–helix; p.c., postcoitum.

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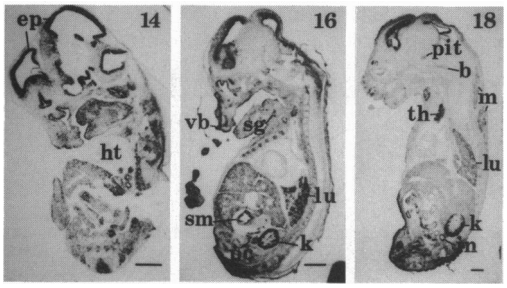

FIG. 1. Autoradiographic images of E2A mRNA expression in 14-, 16-, and 18-day p.c. midsagittal rat embryo sections. In the 14-day p.c. embryo, a weak ubiquitous mRNA signal is found throughout the embryo but is lacking in the heart (ht). The strongest signal is found in the proliferative ependyma cell layer lining the cerebral ventricles (ep). In the 16-day p.c. embryo, the intense E2A mRNA signal is still present in the ependyma cell layer and is also prominent in the vibrissae (vb), stomach (sm), salivary gland (sg), lung (lu), kidney (k), and pancreas (pc). A less intense ubiquitous signal is observed in other areas and is absent in the heart and in the nonproliferative areas of the brain and spinal cord. In the 18-day p.c. embryo, additionally strong signals are found in the pituitary (pit), thymus (th), intestine (in), muscle (m), and bone (b). (Bars = 1 μm.)

SK subclones, each to a specific activity of 10^8–10^9 cpm/mg. Maximal labeling of the probe was achieved by using an excess of uridine 5′-[α-(^{35}S)thio]triphosphate (≈1200 Ci/μmol; 1 Ci = 37 GBq). The RNA probe complementary to E2A mRNA comprised 600 nucleotides including the HLH domain (1). A sense ^{35}S-labeled RNA probe generated by transcription from the T3 promoter was used for control hybridization.

***In Situ* Hybridization.** E2A mRNA was detected by using a modification of the protocol of Simmons *et al.* (22). Mounted sections were incubated at room temperature for 30 min in the 4% paraformaldehyde/borate buffer fixative, rinsed in a 0.05 M potassium phosphate buffered saline, and then incubated for 15–30 min in proteinase K (5 or 10 μg/ml) at 37°C. After digestion, sections were rinsed in 0.1 M triethanolamine, then incubated for 10 min at room temperature with acetic anhydride in 0.1 M triethanolamine, and dehydrated by using ethanol in ascending concentrations. Vacuum-dried sections were hybridized for 16 hr at 60°C to labeled RNA probes. After RNase A (20 μg/ml) treatment to reduce nonspecific binding, desalted sections were washed in 15 mM NaCl/1.5 mM sodium citrate with 1 mM dithiothreitol at 60°C for 30 min. Dehydrated sections were exposed to Hyperfilm-βmax (Amersham) for 4 days. After film expo-

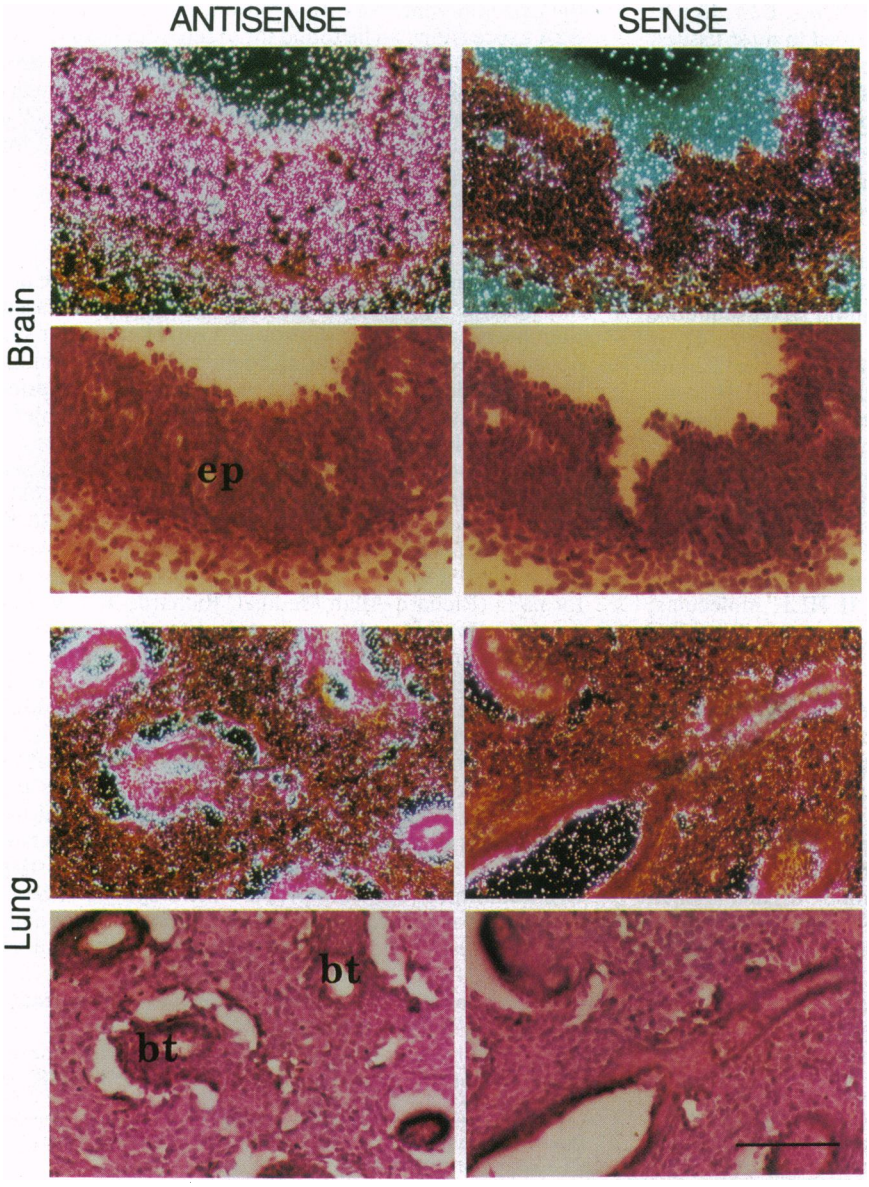

FIG. 2. E2A mRNA expression in 16-day p.c. rat embryo sections. Photomicrographs of photoemulsion-dipped sections show the morphology (bright-field; lower panels of brain and lung) and mRNA signal (white grains in dark-field; upper panels of brain and lung). Message can be seen over the ependyma cell layer lining the cerebral ventricles of the brain (ep) (*Upper*) and over the bronchial tubes of the lung (bt) (*Lower*). Adjacent sections show the lack of signal in these tissues when using a labeled sense-strand RNA control probe. Dark areas surrounding the bronchial tubes in the bright-field photomicrographs are H&E staining, not hybridization signal. (Bar = 100 μm.)

sure, mounted sections on slides were defatted, and the slides were dipped in nuclear track emulsion (NTB-2, Kodak) and exposed for 21–28 days. After development (Kodak D19) and fixation (Kodak Ektaflo), slides were stained with hematoxylin and eosin-Y (H&E), and coverslips were mounted with DPX mountant (Gallard Schlesinger). Adult brain sections were stained with bisbenzimide (Sigma), which provides a fluorescent blue ribosomal counterstain. Black and white photographs of the autoradiographic image on βmax x-ray film were taken with Ilford XP2 35-mm film. The emulsion-coated sections were photographed using Kodak Gold Kodacolor 35-mm film and printed on Fuji paper.

RESULTS

***In Situ* Hybridization of E2A mRNA in the Rat Embryo.** We examined *in situ* hybridization of E2A mRNA at three different embryonic ages: 14, 16, and 18 days p.c. At 12 days p.c., a positive mRNA signal for rat E2A was found throughout the embryo but was distinctly lacking in the heart. This pattern of expression was also observed in the 14-day p.c. embryo, with the strongest signal found in the proliferative zone lining the cerebral ventricles (Fig. 1). At 16 days p.c. the intense E2A mRNA signal was still present in the cells lining the ventricles and was also prominent in bronchial tubes of the lung (Figs. 1 and 2), the vibrissae, lining of the stomach, secretory buds of the salivary gland, glomeruli of the kidney, and the secretory acini of the pancreas (Fig. 1). A less intense signal was observed in other areas and was absent or below the level of detection in the heart and in the nonproliferative areas of the brain and spinal cord. The pattern of expression of E2A mRNA in the 18-day p.c. embryo was again remarkably similar to the 16-day p.c. embryo with additionally strong signals found in the pituitary, thymus, lining of the intestine, muscle, and bone (Fig. 1). No notable differences in the hybridization pattern were found between males and females at any time point examined.

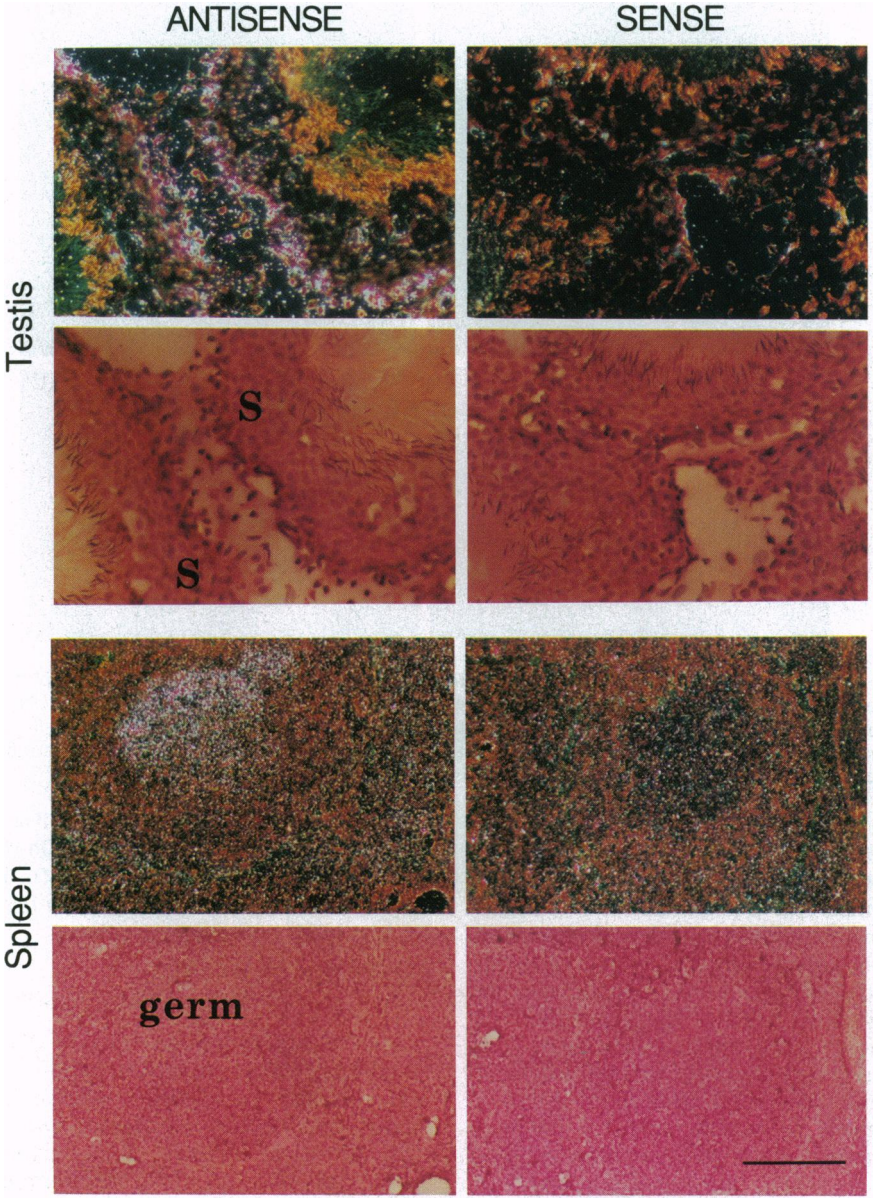

FIG. 3. E2A mRNA expression in adult rat tissue sections. Photomicrographs of photoemulsion-dipped sections show the morphology (bright-field; lower panels of testis and spleen) and mRNA signal (white grains in dark-field; upper panels of testis and spleen). Message can be seen in the testis over cells near the basement membrane of the seminiferous tubules (S) (*Upper*) and in the spleen over the germinal center of the lymphatic nodules (germ) (*Lower*). Adjacent sections show the lack of signal in these tissues when using a labeled sense-strand RNA control probe. (Bar = 100 μm.)

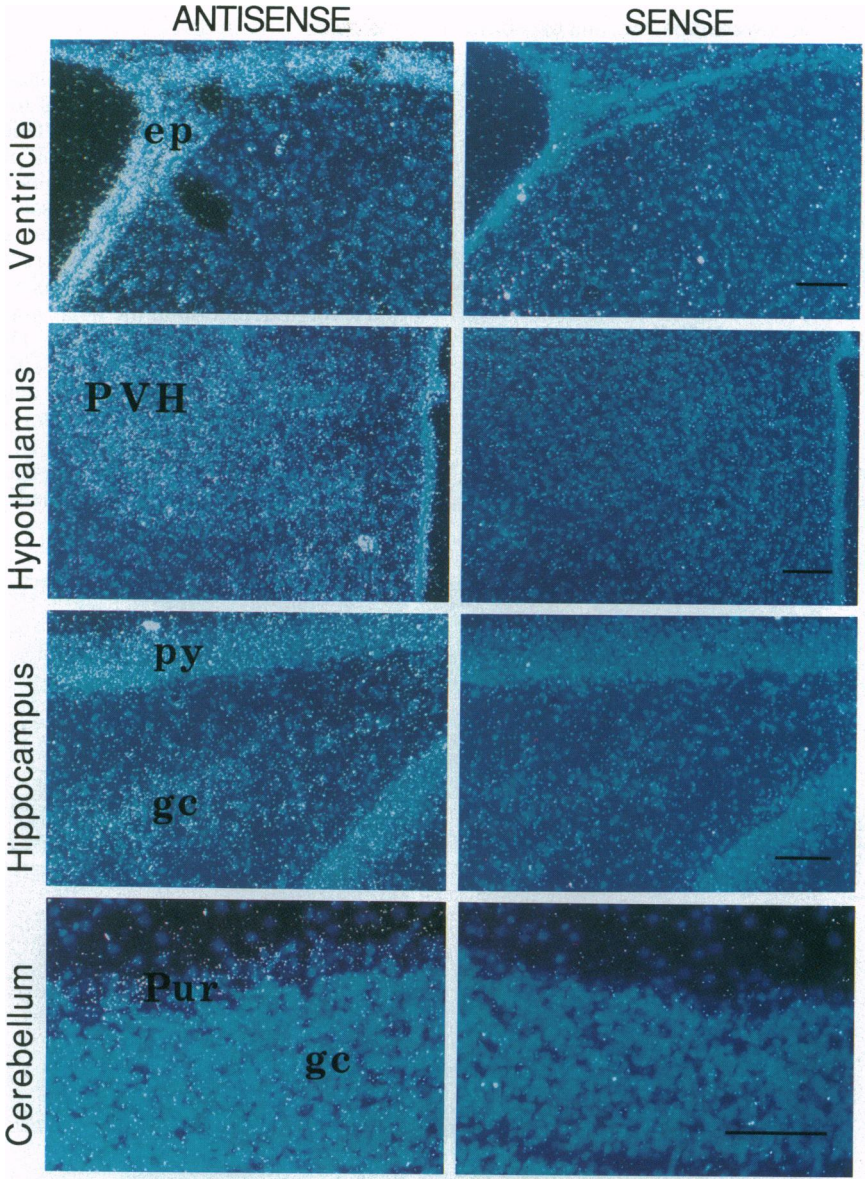

FIG. 4. E2A mRNA expression in the adult rat brain. Combination dark-field/fluorescence photomicrographs of photoemulsion-dipped sections show the morphology (blue fluorescent staining) and mRNA signal (white grains) of the ependyma cell layer lining the walls of the ventricles (ep), in the magnocellular neurons of the hypothalamic paraventricular nucleus (PVH), in the granule cell (gc) and pyramidal cell (py) layers of the hippocampus, and in the Purkinje (Pur) and granule cell (gc) layers of the cerebellum. Adjacent sections show the lack of signal in these areas using a labeled sense-strand RNA control probe. (Bars = 100 μm.)

***In Situ* Hybridization of E2A mRNA in Adult Rat Tissues.** The expression of E2A mRNA was examined in the brain, kidney, lung, liver, intestine, adrenal gland, pituitary, spleen, and testis of the adult male rat. Using *in situ* hybridization analysis, we detected mRNA signals in each of these tissues except for the liver. In the kidney, adrenal, thymus, and lung, the expression appeared ubiquitous throughout the tissue. In the pituitary, the ubiquitous E2A mRNA signal was confined to the anterior and intermediate lobes. In the testis, the mRNA signal was apparent only over cells near the basement membrane of the seminiferous tubules, while in the spleen, a weak signal was observed throughout the tissue but was strongest in the germinal center of the lymphatic nodules (Fig. 3). In the brain, E2A antisense signal intensity was strongest in the ependyma cell layer lining the walls of the ventricles (Fig. 4). In the forebrain, a positive signal was observed in the piriform cortex, the olfactory tubercle, and the islands of Calleja. Signal was apparent over the magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei, in the granule cell and pyramidal cell layers of the hippocampus, and in the Purkinje and granule cell layers of the cerebellum (Fig. 4). Background levels of hybridization were determined by using a sense E2A RNA probe in all experiments.

DISCUSSION

While Northern blot analysis of E2A transcripts indicated that E2A mRNA is present in all cell lines examined, the assay could not determine whether E2A is indeed ubiquitously expressed during development or whether there would be differences in levels of E2A expression in different rat tissues (1, 20). This study, therefore, uses high-stringency *in situ* hybridization of radiolabeled riboprobes to examine the distribution of E2A mRNA in the developing rat embryo from 12 to 18 days p.c. and in tissues of the adult male rat.

An intense signal was found surrounding the cerebral ventricles in the embryonic rat brain, which is an area of rapid

cell proliferation from which all cells that are necessary to form the brain originate (23). Eventually, these cells stop dividing and differentiate into ependymal cells with epithelial characteristics bordering the ventricles. In the adult rat brain, the E2A mRNA signal was restricted to this cell layer as well as to the Purkinje and granule-cell layers of the cerebellum, the piriform cortex, olfactoray tubercle, islands of Calleja, the granule-cell and pyramidal-cell layers of the hippocampus, and areas of magnocellular hypothalamic paraventricular and supraoptic nuclei. While the presence of mRNA signal may be due to the higher concentration of cells in some of these areas, the complete lack of signal in the control sense-strand treated sections argues against this. It is not yet clear whether E2A expression in the adult brain is in the neurons, which are postmitotic, or in the neuroglia, which are not completely static cells.

It is interesting to note that in most tissues of the 16-day p.c. embryo, abundant E2A message was confined to cells with epithelial characteristics. This includes the vibrissae, secretory cells of the salivary gland and pancreas, bronchial tubes of the lung, glomeruli of the kidney, and the lining of the stomach. By 18 days p.c. strong signals were also observed in the epithelial lining of the intestine and in the pituitary and thymus, which secrete hormones directly into the blood. In these endocrine tissues, the mRNA signals did not appear to be localized to any particular cell type. Strong signals were also found in muscle and the hematopoietic marrow of the bone at 18 days of development, when secondary fibers of muscle are increasing rapidly and hematopoietic marrow is formed.

By using *in situ* hybridization histochemistry, mRNA signals were localized to particular adult rat cell types and were found to be ubiquitous throughout the kidney, adrenal, thymus, lung, and anterior and intermediate pituitary. Signal was lacking from the adult liver, where the level of expression may be below the level of detection of this assay. In the testis, spleen, and brain as described, however, the mRNA signal was confined to particular cell types. The localization of signal in the testis near the basement membrane of the seminiferous tubules suggests that the spermatogonia may be selectively expressing E2A mRNA. These germ cells lie in the basal compartment of the seminiferous epithelium and undergo a series of mitotic divisions, with the final division producing the more apically located spermatocytes. In the spleen, the strongest E2A mRNA signal was found in the germinal center of the lymphatic nodules. The germinal center contains large lymphocytes that, like the spermatogonia, are frequently undergoing mitosis. E2A products have been shown to bind to immunoglobulin enhancer elements and activate both the immunoglobulin heavy and light chain gene enhancers (1, 6, 24).

E2A gene products activate transcription synergistically with muscle regulators like MyoD and myogenin (11, 17, 18). In addition, E2A-like proteins have been shown to form a complex with MyoD and myogenin (11, 17, 18). It is surprising, however, that E2A levels in muscle are relatively low. Even more striking is the complete absence of E2A transcripts in the heart. This raises the possibility that E2A-like proteins—for example, E2-2 and HEB—play a role in myogenesis and heart development, and their expression patterns during embryonic development should be determined.

The *Drosophila* homolog of E2A, daughterless, is ubiquitously expressed and is involved in the formation of the peripheral and central nervous system and in sex determination (16, 17). A striking characteristic of E2A proteins is that they are similarly involved in the control of a number of mammalian developmental pathways. They bind and activate the immunoglobulin enhancers in B cells (1, 17, 25), activate a number of muscle-specific enhancers in myocytes (26) and they are involved in pancreatic-specific gene expression (6, 20). We have recently detected neuronal-specific *E2* box-binding proteins that include *E2A* gene products (C.M., unpublished results). Likely, E2A is involved in other pathways as well. We now demonstrate that E2A transcripts are present in most tissues, usually with high levels of expression in cells that are rapidly proliferating and differentiating. The high levels of E2A expression in the proliferative cells lining the cerebral ventricles suggest an important function in mammalian neurogenesis. The role of E2A in mammalian development should be assessed by using mutant mice in which the *E2A* gene is disrupted by homologous recombination.

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1. Murre, C., McCaw, P. S. & Baltimore, D. (1989) *Cell* **56**, 777–783.
2. Davis, R. L., Cheng, P. F., Lassar, A. B. & Weintraub, H. (1990) *Cell* **60**, 733–746.
3. Church, G. M., Ephrussi, A., Gilbert, W. & Tonegawa, S. (1985) *Nature (London)* **313**, 798–801.
4. Ephrussi, A., Church, G., Tonegawa, S. & Gilbert, W. (1985) *Science* **227**, 134–140.
5. Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B. & Weintraub, H. (1989) *Cell* **58**, 537–544.
6. Nelson, C., Shen, L. P., Meister, A., Fodor, E. & Rutter, W. J. (1990) *Genes Dev.* **4**, 1035–1043.
7. Henthorn, P., Kiledjian, M. & Kadesh, T. (1990) *Science* **247**, 467–470.
8. Hu, J.-S., Olson, E. & Kingston, R. E. (1992) *Mol. Cell. Biol.* **12**, 1031–1042.
9. Caudy, M., Grell, E. H., Dambly-Chaudiere, C., Ghysen, A., Jan, L. Y. & Jan, Y. N. (1988) *Genes Dev.* **2**, 843–852.
10. Cronmiller, C. & Cline, T. W. (1988) *Genes Dev.* **2**, 1666–1676.
11. Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y. & Lassar, A. (1991) *Science* **251**, 761–766.
12. Davis, R. L., Weintraub, H. & Lassar, A. B. (1987) *Cell* **51**, 987–1000.
13. Brennan, T. J. & Olson, E. N. (1990) *Genes Dev.* **4**, 582–595.
14. Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. & Arnold, H. H. (1989) *EMBO J.* **8**, 701–709.
15. Sun, X.-H. & Baltimore, D. (1991) *Cell* **64**, 459–470.
16. Caudy, M., Vaessin, H., Brand, M., Tuma, R., Jan, L. Y. & Jan, Y. N. (1988) *Cell* **55**, 1061–1067.
17. Murre, C., Voronova, A. & Baltimore, D. (1991) *Mol. Cell. Biol.* **11**, 1156–1160.
18. Lassar, A. B., Davis, R. L., Wright, W. E., Kadesh, T., Murre, C., Voronova, A., Baltimore, D. & Weintraub, H. (1991) *Cell* **66**, 305–315.
19. Cordle, S. R., Henderson, E., Masuaka, H., Weil, P. A. & Stein, R. (1991) *Mol. Cell. Biol.* **11**, 1734–1738.
20. Aronheim, A., Ohlsson, H., Won Park, C., Edlund, T. & Walker, M. D. (1991) *Nucleic Acids Res.* **19**, 3893–3899.
21. Moss, L. G., Moss, J. B. & Rutter, W. J. (1988) *Mol. Cell. Biol.* **8**, 2620–2627.
22. Simmons, D. M., Arriza, J. C. & Swanson, L. W. (1984) *J. Histotechnol.* **12**, 169–181.
23. Jacobson, M. (1990) *Developmental Neurobiology*, ed. Jacobson, M. (Plenum, New York), 3rd Ed.
24. Bain, G., Grunwald, S. & Murre, C. (1993) *Mol. Cell. Biol.*, in press.
25. Ruezinsky, D., Beckmann, H. & Kadesch, T. (1991) *Genes Dev.* **5**, 29–37.
26. Lassar, A. B., Buskin, J. N., Lochshon, D., Davis, R. L., Apone, S., Hauschka, S. D. & Weintraub, H. (1989) *Cell* **58**, 823–831.