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Messenger RNA sorting in enterocytes

Co-localization with encoded proteins

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This study describes the intracellular compartmentalization of three different mRNAs in the polarized rat fetal enterocyte. They encode proteins that are known to be localized within different regions of the epithelial cell namely (i) the apical, membrane-bound glycoprotein, lactase-phlorizin hydrolase (lactase), (ii) the mitochondrially localized enzyme, carbamoylphosphate synthetase (CPS), and (iii) the cytoplasmically localized enzyme, phospho*enol*pyruvate carboxykinase (PEPCK). These mRNAs are found in close proximity to their respective protein products, i.e. the apical membrane, mitochondria and cytoplasm, respectively. The significance of these observations is twofold; (i) they indicate that mRNAs are sorted into specific domains of the cytosol of intestinal epithelial cells; and (ii) they imply the presence of two distinct pathways of mRNA targeting one that allows transport of mRNAs that are translated on ribosomes associated with the rough endoplasmic reticulum (lactase mRNA), and the other that allows sorting of mRNAs that are translated on free polysomes (CPS and PEPCK mRNA).

Carbamoylphosphate synthetase; Lactase-phlorizin hydrolase; Phospho*enol*pyruvate carboxykinase

1. INTRODUCTION

Intracellular targeting of mRNAs could provide an efficient mechanism for the facilitated distribution of proteins. Evidence for intracellular heterogeneity of mRNA localization has so far only been recognized for mRNAs encoding cytoskeletal proteins [1–4]. However, no information is available about the intracellular localization of mRNAs encoding proteins with an enzymic function. Since the intestinal epithelium consists of a polarized monolayer of tall columnar cells, it provides a useful system for studying the topographical aspects of in vivo gene expression in single cells [2].

The plasma membranes of polarized epithelial cells are divided by junctional complexes into apical and basolateral domains which differ both biochemically and morphologically [5]. This polarization is illustrated in the enterocyte by the distribution of specific proteins. Lactase (EC 3.2.1.23 – EC 3.2.1.62) is exclusively incorporated into the apical plasma membrane after synthesis and co-translational translocation into the rough endoplasmic reticulum [6]. Nuclear-encoded mitochondrial proteins such as the urea-cycle enzyme CPS (E.C. 6.3.4.16), are generally accepted to be made in the cytoplasm on free polyribosomes and subsequently transported to the mitochondria [7]. CPS is found in the mitochondria of both hepatocytes and enterocytes [8]. Proteins of carbohydrate metabolism, like phospho*enol*pyruvate carboxykinase (PEPCK) (EC 4.1.1.32), which is also synthesized on free polyribosomes, are delivered into the cytosol without translocation [9]. We have studied the intracellular distribution of the mRNAs of these proteins to gain insight into the mechanisms underlying these distinct forms of protein segregation.

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2. MATERIALS AND METHODS

2.1. *Immunohistochemistry*

Immunohistochemistry was performed using the indirect unconjugated peroxidase-anti-peroxidase (PAP) technique [10]. Fixation, tissue processing, and immunohistochemistry was performed according to a previously published protocol [10]. Incubations were done on closely adjacent 7 μm-thick sections to allow easy comparison of the patterns. After deparaffination, reduction of endogenous peroxidase activity and non-specific binding sites, sections were incubated with ascites containing the anti-lactase monoclonal antibody (diluted 1:6400 in PBS), or with serum containing monospecific antibodies directed against CPS [11] (diluted 1:2000 in PBS), or against PEPCK (diluted 1:500 in PBS). Monoclonal antibody binding was detected using rabbit anti-mouse immunoglobulin, goat anti-rabbit immunoglobulin and

rabbit PAP complex, respectively. Polyclonal antibodies were detected using goat anti-rabbit immunoglobulin and rabbit PAP complex, respectively. The immune complex formed was visualized by incubation in 0.5 mg/ml 3,3'-diaminobenzidine, 0.02% hydrogen peroxide in 30 mM imidazole, 1 mM EDTA (pH 7.0). Cross-sections of total fetal rats revealed high expression of lactase, PEPCK and CPS protein in intestinal epithelium, and of CPS in liver parenchyma (data not shown). Negative controls included incubations without primary antibodies.

2.2. *In situ hybridization*

Messenger RNA localization in rat small intestine was performed by in situ hybridization using previously published protocols [8]. The 7 μm-thick closely adjacent sections were hybridized at 44°C to one of the labeled probes. Probe concentration was approximately 0.5 ng/μl and contained 5×10^5 cpm/μl. The emulsion-dipped slides were developed after 5 days.

Lactase mRNA was detected using a 1.8 kb *Eco*RI–*Pst*I fragment, derived from the 2.3 kb rat lactase cDNA [12]. This probe contains three *Hin*fI restriction sites, yielding four fragments of about 1,000, 450, 250 and 150 bp, respectively. The three largest fragments were independently used for the detection of lactase mRNA. As probes for detection of CPS and PEPCK mRNA, several *Pst*I and *Eco*RI fragments of the 5.6 kb rat CPS cDNA [8], and a 1,081 bp *Pst*I fragment of the 2.8 kb rat PEPCK cDNA [13] were used, respectively. The pBR322 vector DNA served as a negative control and underwent equal labeling and hybridization conditions. The agarose-purified DNA fragments of the different probes were all labeled overnight at 15°C with [α-^{35}S]dCTP, according to the multiprime labeling method, resulting in fragments of approximately 100 nucleotides long, which were analyzed by gel electrophoresis [14]. In situ hybridization was also performed under more stringent conditions (hybridization and washing temperature at 50°C). Data is presented from the lower stringency hybridizations.

3. RESULTS

3.1. *Localization of the respective proteins*

Lactase protein (Fig. 1a) was exclusively found on the apical (microvillus) membrane of the enterocyte, using a mouse anti-rat lactase monoclonal antibody [6]. CPS protein (Fig. 1b) was detected using a rabbit anti-rat CPS polyclonal antibody [15]. The immunostaining showed a granular distribution both apically and basally in relation to the nucleus, i.e. with the same distribution as mitochondria [5]. The apical membrane, basolateral membrane and nuclei remained free of coloring. PEPCK protein (Fig. 1c) was detected all over the cytosol of the enterocyte, using a rabbit anti-rat PEPCK polyclonal antibody [16]. Nuclei were not stained.

3.2. *Localization of the respective mRNAs*

The lactase cDNA probe only hybridized with the epithelium lining the villi. The grains were predominantly visible in the apical domains of these polarized cells (Fig. 2a). In situ hybridization with the CPS cDNA probe revealed both an apical as well as a basal localization of mRNA (Fig. 2b), a pattern comparable to the localization of CPS protein (Fig. 1b). Autoradiographic grains were equally present in both domains, and this pattern was consistently found in every enterocyte lining the intestinal villi. In situ hybridization with the cDNA probe for PEPCK revealed grains throughout the cytosol of the villus enterocyte, and an equal distribution of intensity of grains was observed (Fig. 2c). Different control experiments for the in situ hybridization were carried out: a probe for albumin was hybridized to the samples. Sections examined showed a strong signal for albumin in the liver, but no signal in intestinal tissue (data not shown). In cross-sections of total fetal rat, hybridization with lactase cDNA revealed only reaction in the gut (Fig. 2d), while hybridization with the CPS cDNA revealed also reaction in liver parenchyma (Fig. 2e), indicating the specificity of the cDNAs.

Furthermore, mRNAs for lactase, CPS and PEPCK were detected in the epithelium of all villi examined, as shown for CPS mRNA (Fig. 2f). In order to exclude possible non-specific hybridization, the data were verified by hybridization to smaller probes prepared from fragments of each above-described cDNA. Patterns obtained were identical to those obtained with the original probes (data not shown). Hybridization with the labeled pBR322 vector DNA, which served as a negative control, did not give any signal under the described conditions (data not shown). In addition, it was found that increasing the hybridization temperature and washing temperature up to 50°C did not change the hybridization pattern or diminish the intensity of the positive signals (data not shown). Haematoxylin and azophloxine staining (Fig. 2g) has been performed in order to compare the classical staining pattern of fetal intestinal epithelium with that after immunocytochemistry (Fig. 1) or in situ hybridization (Fig. 2). At this stage of development the enterocytes are polarized, and their nucleus is situated at the base of the cell. Microvilli have already been developed at the apical membrane.

4. DISCUSSION

The lactase gene, encoding a microvillous membrane enzyme, is expressed just before birth [17], and its protein product appears on the apical surface of the polarized enterocyte [6]. As expected, at fetal day 20, lactase was found to be localized at the apical periphery of the enterocyte (Fig. 1a). In contrast, CPS protein is known to be expressed beginning at day 14 in the stratified intestinal epithelium before formation of villi and a columnar monolayer [15]. Fig. 1b shows CPS protein expression at fetal day 20 localized to the basal and apical regions around the nucleus [8], those domains of the intestinal epithelial cell where the mitochondria are positioned [5]. The PEPCK gene, encoding the cytosolic isoenzyme, is reported to be expressed in the small intestine just before birth [18]. In agreement, as shown in Fig. 1c, PEPCK was found to be uniformly distributed throughout the cell [18].

A strikingly similar pattern of distribution was found

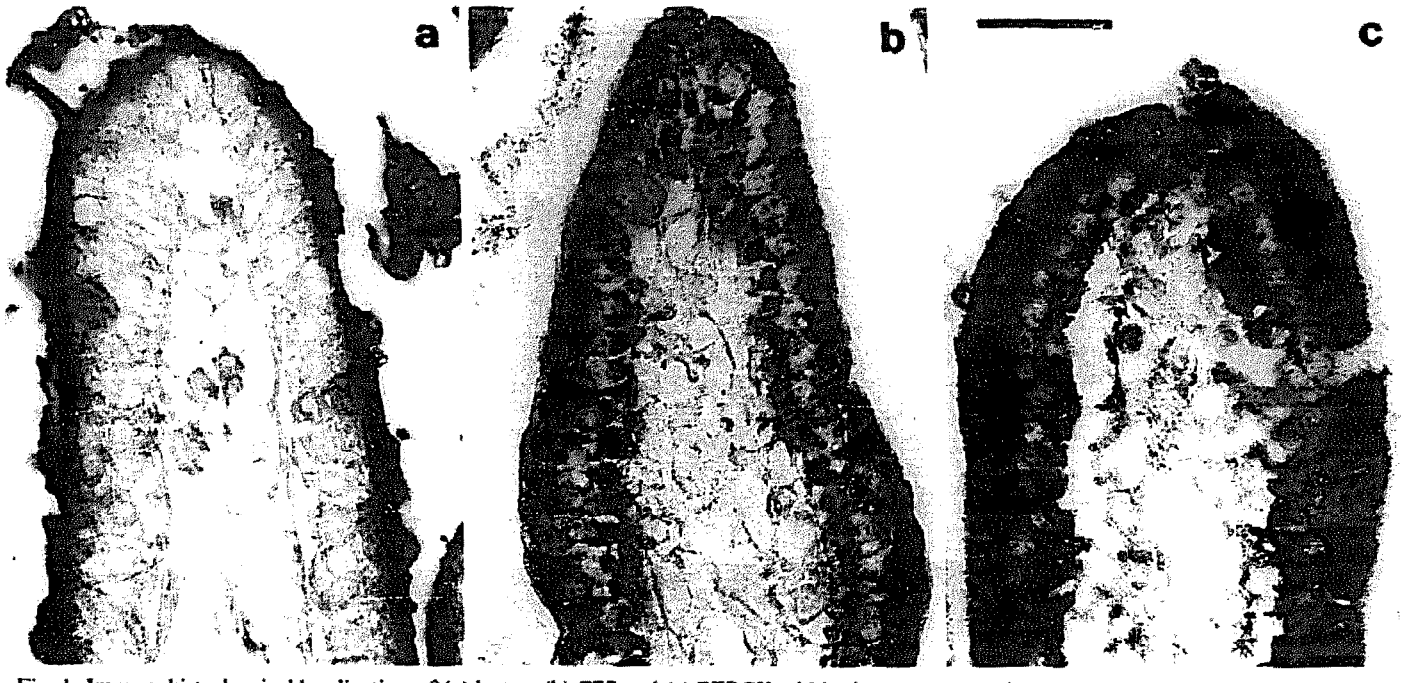

Fig. 1. Immunohistochemical localization of (a) lactase (b) CPS and (c) PEPCK within the enterocytes of the intestine of fetal rats of 20 days of development (2 days before birth). Bar = 25 μm.

for the respective mRNAs encoding these proteins: the mRNAs accumulated predominantly in close proximity to the location of their final protein products (Fig. 2). Thus, the cDNA for lactase localized apically, the probe for CPS was restricted to the mitochondrial region, and the cDNA for PEPCK was localized cytoplasmically. The existence of similar mechanisms for mRNAs encoding basolateral proteins is the subject of further study.

The use of the respective cDNA probes on closely adjacent paraffin sections allowed the recognition of the different locations of mRNAs within one relatively small cell (approximately 25 μm in height). The distinct spatial hybridization patterns within one cell provide, in itself, a localization-specific control and demonstrates that specific hybridization is being achieved (for additional controls see Fig. 2).

Fetal enterocytes were studied for the following reason. Enterocyte function is determined by the location of the epithelial cells on the crypt–villus axis. In the rat, crypts develop between the villi immediately after birth, and cell proliferation subsequently becomes confined to the crypts [19]. Anchored stem cells located in those crypts form the source of this epithelial monolayer [20]. Enterocytes migrate within approximately three days to the top of the villi [21], where they are extruded into the intestinal lumen. This rapid turnover of enterocytes constitutes a potential pitfall in establishing the intracellular distribution of mRNAs in mature animals. However, fetal enterocytes, which have a much slower turnover, provide a model which avoids this difficulty in the study of mRNA accumulation. By day 19 of fetal rat development the lumen of the small intestine expands, villi are formed, and the undifferentiated stratified epithelium is converted into columnar cells lining the villi [22]. At day 20 of development the intestinal epithelium is polarized and forms a monolayer of well-developed enterocytes with slow migration and minimal turnover. Hence, these fetal enterocytes are more appropriate for studies of intracellular localization of proteins and mRNAs than adult enterocytes.

Expression of several mRNAs has recently been described in the small intestine, including cytochrome $P\text{-}450_{IIb1}$ [23], aminopeptidase N [24], sucrase-isomaltase [25], α-fetoprotein [26] and fatty acid-binding protein [27]. These studies focused mainly on gene expression along the crypt–villus axis, no detailed information was provided regarding a possible heterogeneous intracellular localization of these mRNAs. One of the reasons for the previous lack of recognition of distinct distribution patterns might be the use of dark-field microscopy, that makes use of scattered light. Bright-field illumination, as used in the present study, although less sensitive, provides superior resolving power, which is an essential prerequisite to visualizing intracellular heterogeneity.

Recently, immunocytochemistry and in situ hybridization have shown that, in response to mechanical injury, β actin and its mRNA became positioned at the membrane involved in repair [3]. This process of asymmetric placement of mRNA suggests a sorting mechanism similar to the previously observed non-homogeneous distribution of actin mRNA in embryonic

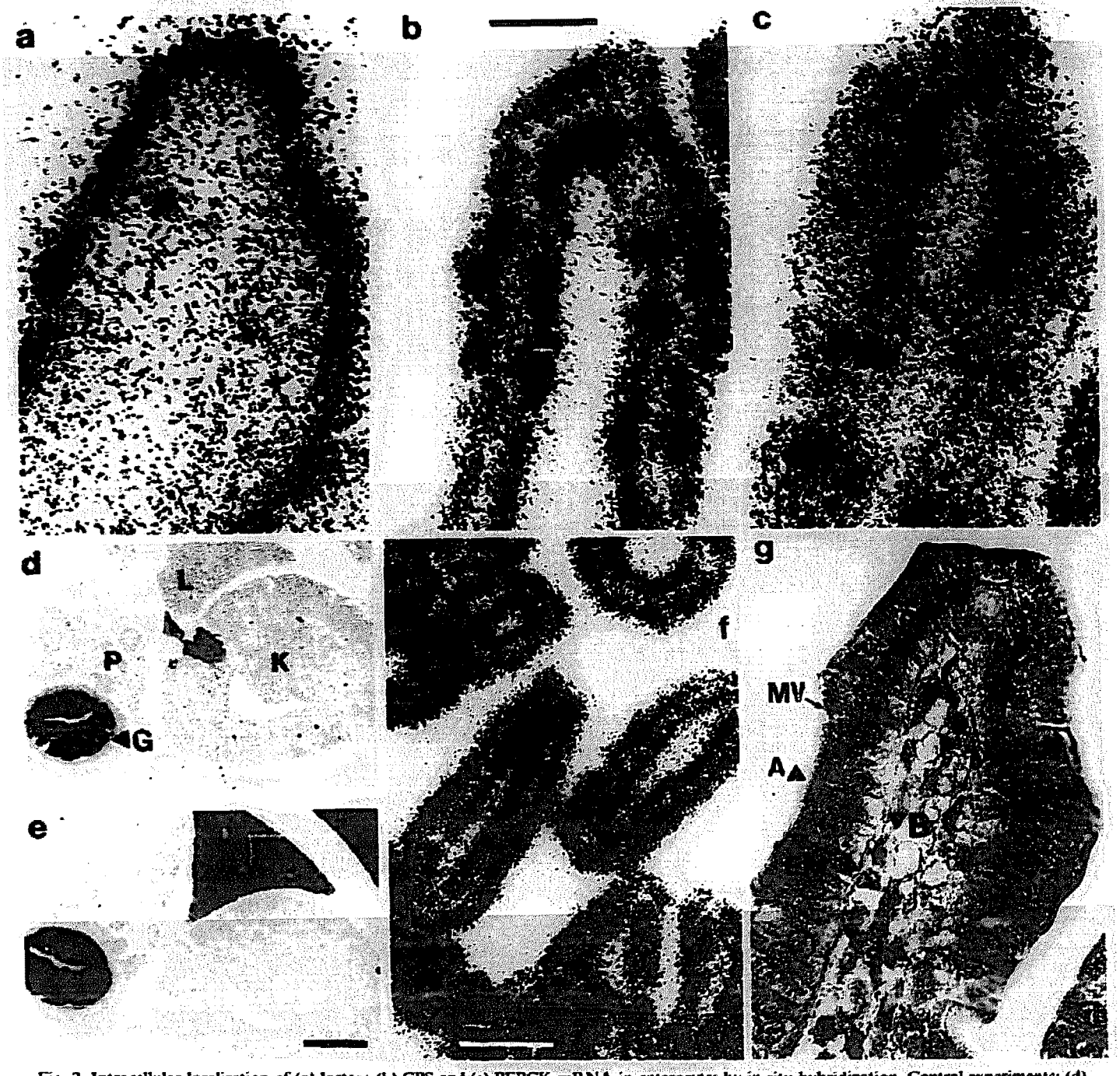

Fig. 2. Intracellular localization of (a) lactase (b) CPS and (c) PEPCK mRNA in enterocytes by in situ hybridization. Control experiments: (d) lactase mRNA, (e,f) CPS mRNA in cross-sections of a total fetal rat at 20 days of development. (g) Routine histological staining. Bars: a–c,g = 25 μm; d,e = 0.5 mm; f = 50 μm. Abbreviations: A, apical membrane; B, basal membrane; EM, epithelial monolayer; G, gut; K, kidney; L, liver; MV, microvilli; N, Nuclei; P, pancreas; S, stroma.

myoblasts and fibroblasts in vitro [1], or similar to the localized maternal mRNA at the vegetal pole of *Xenopus* oocytes [28]. Heterogeneous mRNA accumulation along the myofiber in so-called nuclear domains has been reported and is unique for this multinucleated cell [29]. Furthermore, mRNAs in myofibers are localized at the periphery of the cells, the only area where ribosomes are localized. In contrast, the ribosomes in enterocytes are spread throughout the cytoplasm [5], while the mRNAs analyzed in this study are distinctly compartmentalized.

The co-localization of mRNAs and their encoded proteins strongly suggests that protein synthesis occurs in close proximity to the site where the protein eventually accumulates and functions. Furthermore, the presence of ER throughout the enterocyte justifies this

assumption [5]. Co-localization would be an efficient process because one mRNA molecule can be translated repeatedly at a specific site, and would also be an efficient method of delivering proteins to where they are required in order to minimize protein targeting problems. It underscores the notion of the presence of a special class of polyribosomes associated with mitochondria to ensure a proper delivery of cytoplasmically made mitochondrial proteins [30].

Two explanations to achieve a non-homogeneous distribution of mRNA can be envisioned. Firstly, the sorting of the mRNA can directly depend on its nucleotide sequence and be mediated by binding of cytosolic or nuclear 'addresser' proteins to the mRNA. Secondly, the amino terminal end of the growing polypeptide may include amino acid sequences that target the mRNA and the adhering translational machinery to its final position within the cell. This could occur through autonomous signals in the growing peptide itself, resembling the mechanism involved in directing of the polymeric immunoglobulin receptor [31], or through the binding of 'addresser' protein factors to the growing peptide.

One might speculate that asymmetric placement of mRNAs within one cell might apply to other cell types as well, as it seems efficient to synthesize proteins near the site where they eventually accumulate. In order to localize mRNAs intracellularly in (non-)polarized cells, detailed examination by in situ hybridization at the ultrastructural level will be required [4].

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REFERENCES

[1] Lawerence, J.B. and Singer, R.H. (1986) Cell 45, 407–415.
[2] Cheng, H. and Bjerknes, M. (1989) J. Mol. Biol. 210, 541–549.
[3] Hoock, T.C., Newcomb, P.M. and Herman, I.M. (1991) J. Cell Biol. 112, 653–664.
[4] Singer, R.H., Langevin, G.L. and Lawrence, J.B. (1989) J. Cell Biol. 108, 2343–2353.
[5] Trier, J.S. (1968) Handbook of Physiology vol III, Williams & Wilkins, Baltimore, pp. 1125–1175.
[6] Büller, H.A., Montgomery, R.K., Sasak, W.V. and Grand, R.J. (1987) J. Biol. Chem. 262, 17206–17211.
[7] Bendayan, M. and Shore, G.C. (1982) J. Histochem. Cytochem. 30, 139–147.
[8] Moorman, A.F.M., de Boer, P.A.J., Geerts, W.J.C., van de Zande, L.P.G.W., Charles, R. and Lamers, W.H. (1988) J. Histochem. Cytochem. 36, 751–755.
[9] Iynedjian, P.B. and Hanson, R.W. (1977) J. Biol. Chem. 252, 655–662.
[10] Wessels, A., Vermeulen, J.L.M., Virágh, Sz., Kálmán, F., Morris, G.E., Nguen thi, M. Lamers, W.H. and Moorman, A.F.M. (1990) Anat. Rec. 228, 163–176.
[11] Charles, R., de Graaf, A. and Moorman, A.F.M. (1980) Biochim. Biophys. Acta 629, 36–40.
[12] Büller, H.A., Kothe, M.J.C., Goldman, D.A., Grubman, S.A., Sasak, W.V., Matsudaira, P.T., Montgomery, R.K. and Grand, R.J. (1990) J. Biol. Chem. 265, 6978–6983.
[13] Yoo-Warren, H., Monahan, J.E., Short, J., Short, H., Bruzel, A., Wynshaw-Borr's, A., Meisner, H.M., Samols, D. and Hanson, R.W. (1983) Proc. Natl. Acad. Sci. USA 80, 3656–3660.
[14] de Boer, P.A.J. and Labruyère, W.Th. (1990) Boehringer-Mannheim Biochem. 1, 5.
[15] Gaasbeek Janzen, J.W., Westenend, P.J., Charles, R., Lamers, W.H. and Moorman, A.F.M. (1988) J. Histochem. Cytochem. 36, 1223–1230.
[16] Lamers, W.H., van Roon, M., Mooren, P.G., de Graaf, A. and Charles, R. (1985) In Vitro Cell. Dev. Biol. 21, 606–611.
[17] Montgomery, R.K., Büller, H.A., Rings, E.H.H.M. and Grand, R.J. (1991) FASEB J. 5, 2824–2832.
[18] Zimmer, D.B. and Magnuson, M.A. (1990) J. Histochem. Cytochem. 38, 171–178.
[19] Potten, C.S. and Loeffler, M. (1990) Development 110, 1001–1020.
[20] Gordon, J.I. (1989) J. Cell Biol. 108, 1187–1194.
[21] Potten, C.S. and Hendry, J.H. (1983) Stem Cells, Churchill Livingstone, Edinburgh, Scotland, pp. 155–199.
[22] Hermos, J.A., Mathan, M. and Trier, J.S. (1971) J. Cell Biol. 50, 255–258.
[23] Traber, P.G., Chianale, J., Florence, R., Kim, K., Wojcik, E. and Gumucio, J.J. (1988) J. Biol. Chem. 263, 9449–9455.
[24] Norén, O., Dabelsteen, E., Høyer, P.E., Olson, J., Sjöström, H. and Hansen, G.H. (1989) FEBS Lett. 259, 107–112.
[25] Traber, P.G. (1990) Biochem. Biophys. Res. Commun. 173, 765–773.
[26] Tyner, A.L., Godbout, R., Compton, R.S. and Tilghman, S.M. (1990) J. Cell Biol. 110, 915–927.
[27] Iseki, S. and Kondo, H. (1990) J. Histochem. Cytochem. 38, 111–115.
[28] Melton, D.A. (1987) Nature 328, 80–82.
[29] Dix, D.J. and Eisenberg, B.R. (1991) Cell Tissue Res. 263, 61–69.
[30] Kellems, R.E. and Butow, R.A. (1972) J. Biol. Chem. 247, 8043–8050.
[31] Casanova, J.E., Apodaca, G. and Mostov, K.E. (1991) Cell 66, 65–75.