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Nuclear localization of alkaline phosphatase in cultured human cancer cells

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Abstract The nucleolar localization of alkaline phosphatase (ALPase) was observed by electron microscopic cytochemistry. The culture cells used in this study were normal human cells (fibroblast, WI-38) and human cancer cells (hepatocellular carcinoma, Hep-G2; malignant melanoma, A-375; pancreatic carcinoma, BxPC-3). Cultured cells in almost all strains contained high ALPase activity in the nucleolus, and the localization of ALPase changed during the cell cycle stages. The pattern of ALPase localization during the interphase was divided into three groups: cytoplasmic type, nucleus type, and both types. Moreover, at the mitotic phase, the reaction products were observed on the chromosome. In the cultured malignant melanoma cells, the appearance ratio of ALPase reaction products on the nucleolus (33.9%) showed a higher ratio compared with normal cultured fibroblasts (6.3%). This phenomenon suggests that the high level of the ALPase reaction product may be related to the high level of proliferation of cancer cells.

Key words ALPase · Nucleolus · Chromosome · Cell cycle · Electron microscopy

Introduction

Alkaline phosphatase (ALPase) has been demonstrated in almost all mammalian tissues by subcellular analysis and ultrastructural histochemistry. The localization of ALPase has been observed on the plasma membrane, nuclear membrane, and in the endoplasmic reticulum,[1,2] the Golgi apparatus,[3] and mitochondria[4] in normal and cancer cells.[5] High ALPase activity in the nucleus has been also revealed in several cells, for example, HeLa cells,[6] chondrocytes,[7–9] and human neutrophils.[10] However, the nuclear membrane was the only site at which nuclear ALPase activity was observed. Although Slor and Bustan[11] indicated an inverse relationship between DNA synthesis and ALPase activation in synchronized HeLa S3 cells, the question remains in what part of the nucleus ALPase exists at its times of high activity. Furthermore, it was necessary to clarify whether ALPase change was dependent on the cell cycle stage.

We report here the nucleolar localization of ALPase in cultured human normal and cancer cells by transmission electron microscopic cytochemistry. It is the purpose of this study to confirm the more detailed localization of ALPase within the nucleus and to clarify whether the localization of ALPase change during the cell cycle.

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Materials and methods

Cell culture

Human normal cells (fibroblast, WI-38 cells) and human cancer cells (hepatocellular carcinoma, Hep-G2 cells; malignant melanoma, A-375 cells; pancreatic carcinoma, BxPC-3 cells) were used in this study. These cells were obtained from the American Type Culture Collection (ATCC). After these cells were cultured on Petri dishes with Eagle's minimum essential medium containing 10% fetal calf serum for 10 days under 5% CO_2 in air at 37°C, the intracellular localization of ALPase was examined by electron microscopic cytochemistry.

Electron microscopic cytochemistry for nonspecific ALPase

A method described by Mayahara et al.[12] was employed for the ultrastructural nonspecific ALPase localization in the

nucleus. This method is the most reliable and provided the best results because the incubating medium is stable and clear without the addition of a chelating agent at high alkaline pH. The cultured cells were fixed for 15 min in 1.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 8% sucrose, and the cells were rinsed in the same buffer solution containing 8% sucrose for 60 min. The samples were incubated for 60 min at room temperature in a substrate medium that contained 1.4 ml 0.2 M Tris-HCl buffer (pH 8.5), 2 ml 3% sodium β-gly cerophosphate, 2.6 ml 15 mM magnesium sulfate, and 4 ml 0.5% lead citrate (pH 10.0). As cytochemical controls, the samples were incubated for 60 min in sodium β-glycerophosphate-free substrate medium. For electron microscopy, the cells were briefly rinsed in distilled water following incubation, postfixed in 1% OsO_4 solution for 60 min, dehydrated in an ascending ethanol series, and embedded in epoxy resin. Ultrathin sections were stained with 4% uranyl acetate solution for 10 min and observed with a JEM-1200EX (JEOL, Tokyo, Japan) transmission electron microscope.

Results

In the WI-38, Hep-G2, A375, and BxPC-3 cells, the localization of nonspecific ALPase was examined by electron microscopic cytochemistry. ALPase reactions were often observed on the cell membrane, and in the endoplasmic reticulum, mitochondria, the Golgi apparatus, and nucleoli in all cultured cells (Figs. 1,2). Although strong ALPase reactions were seen on the microvilli of the bile canaliculi between cultured cells in the Hep-G2 cells (Fig. 3), ALPase reactions were not observed in the samples without sodium β-glycerophosphate (Fig. 4). In the interphase, the pattern of localization was divided into three groups. One group

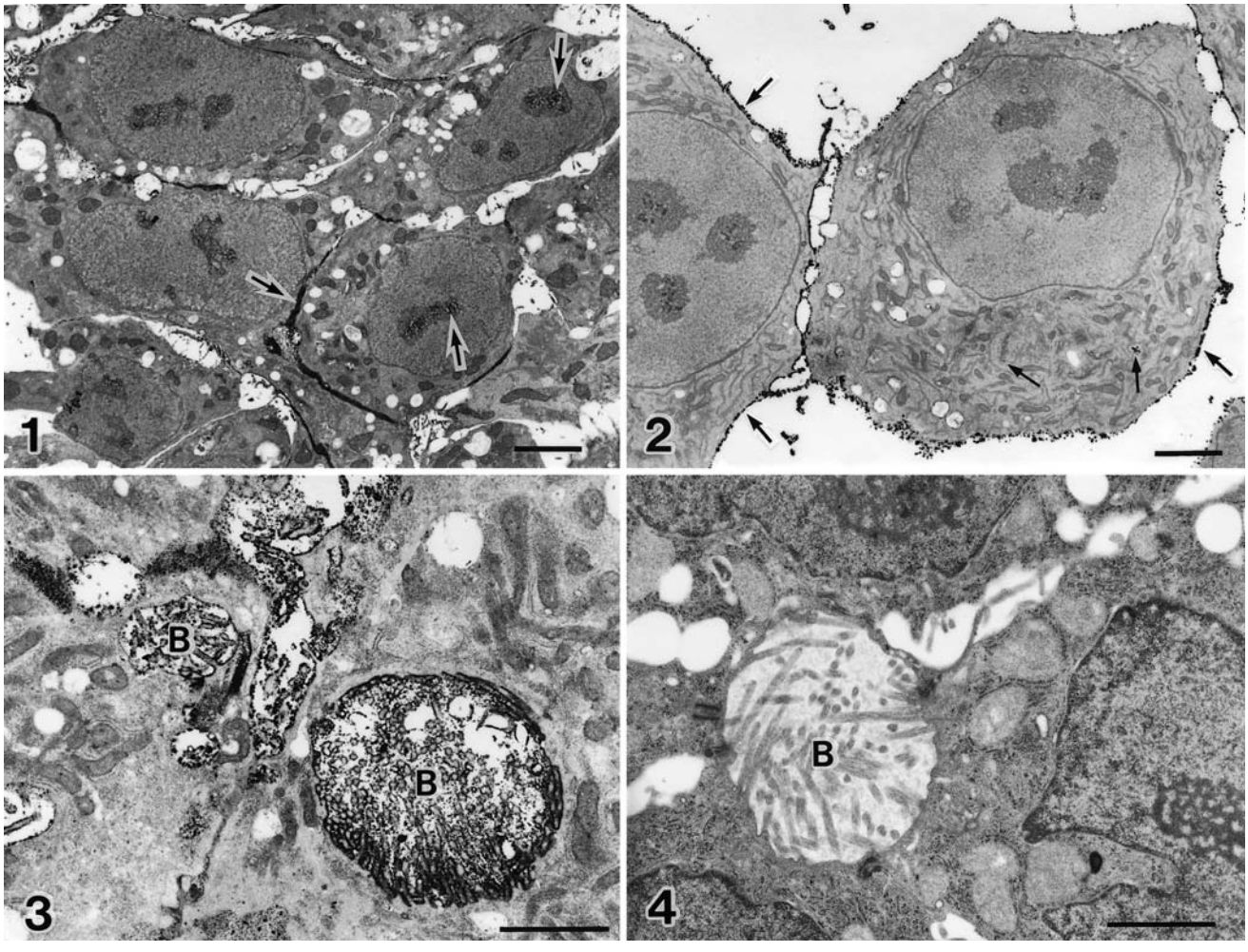


Fig. 1. Reaction products of alkaline phosphatase (ALPase) (*arrows*) were seen on the cell membrane and in the nucleolus of human cultured hepatocarcinoma (Hep-G2 cells). *Bar* 2 μm

Fig. 2. ALPase staining of Hep-G2 cells. Although strong reaction products (*arrows*) were seen on the cell organelle and cell membrane, reaction deposits were not completely observed in the cell organellae except for nucleolus. *Bar* 3 μm

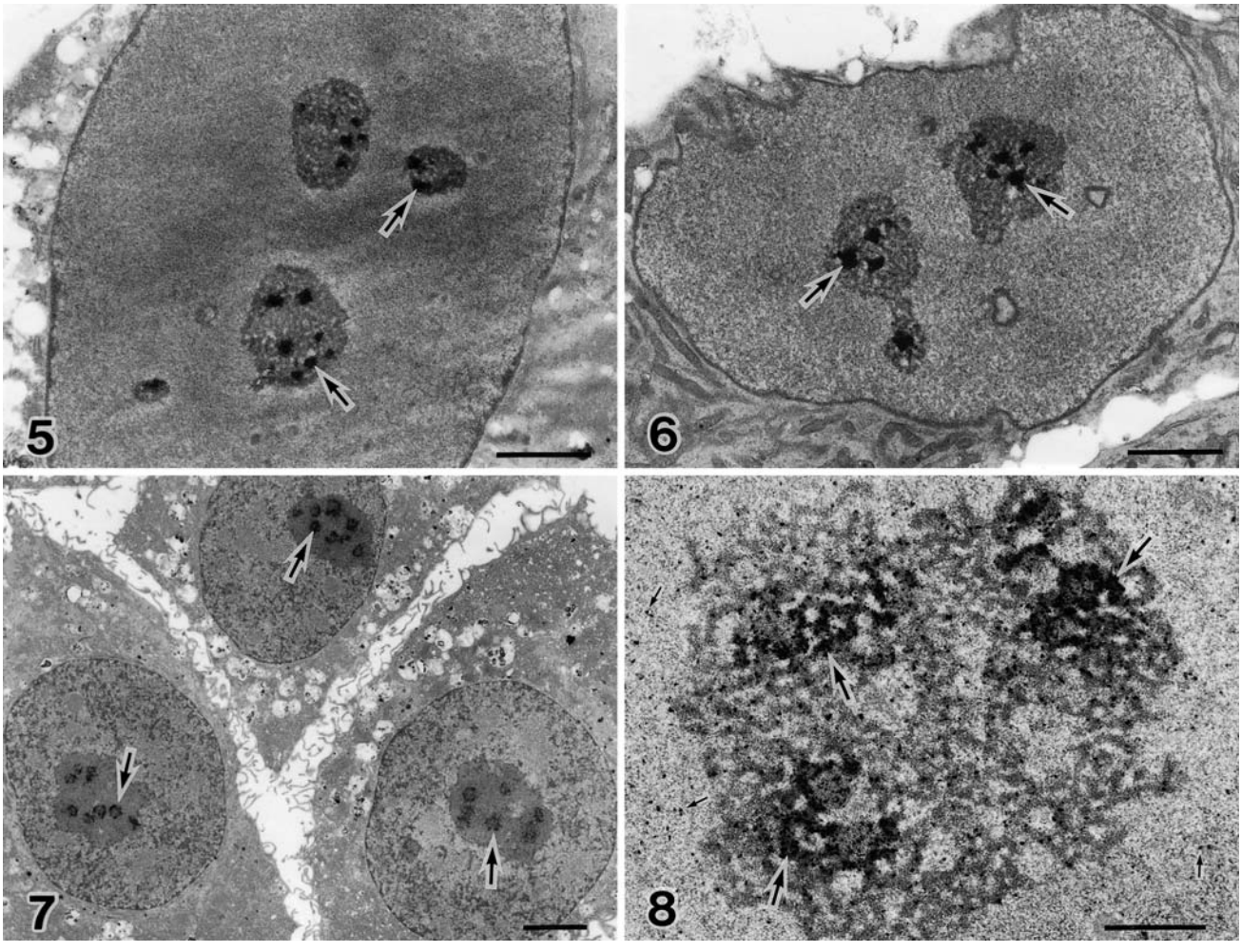
Fig. 3. ALPase staining of Hep-G2 cells. The reaction products of ALPase were localized on the microvilli of the newly formed bile canaliculi (*B*). *Bar* 1 μm

Fig. 4. ALPase reaction products were not observed on the microvilli and cell organelles of Hep-G2 cells without sodium β-glycerophosphate. *B*, Bile canaliculi. *Bar* 1 μm

showed reaction sites in the cytoplasm only, the second group showed aggregation of almost all ALPase in the nucleus, and in the third group ALPase appeared in both cytoplasm and nucleus. Moreover, in the mitotic phase, the reaction sites were always observed in the chromosome. Although the lead products of ALPase were weakly diffused on the euchromatin of the nucleus, we found the chief site of ALPase localization to be at a nucleolus (Figs. 5–7). The nucleolus is generally composed of five ultrastructural components: nucleolar chromatin, fibrillar centers, dense fibrillar components, granular components, and nucleolar interstices.[13] Strong reactions were especially observed on the dense fibrillar components (Fig. 8). By counting under electron microscopy, the appearance ratio of ALPase reaction in the cultured malignant melanoma cell nucleoli (A-375) was 33.9% (407 cells per 1199 cells), whereas the ratio in normal cultured fibroblasts (WI-38) was 6.3% (35 cells per 552 cells). This average appearance of ALPase in the nucleoli was significantly fivefold higher than the level of the normal cells. On the other hand, the reaction sites in the mitotic phase appeared on the chromosome (Figs. 9–11). However, we also found that a few cells were not staining for ALPase reaction in every cell strain.

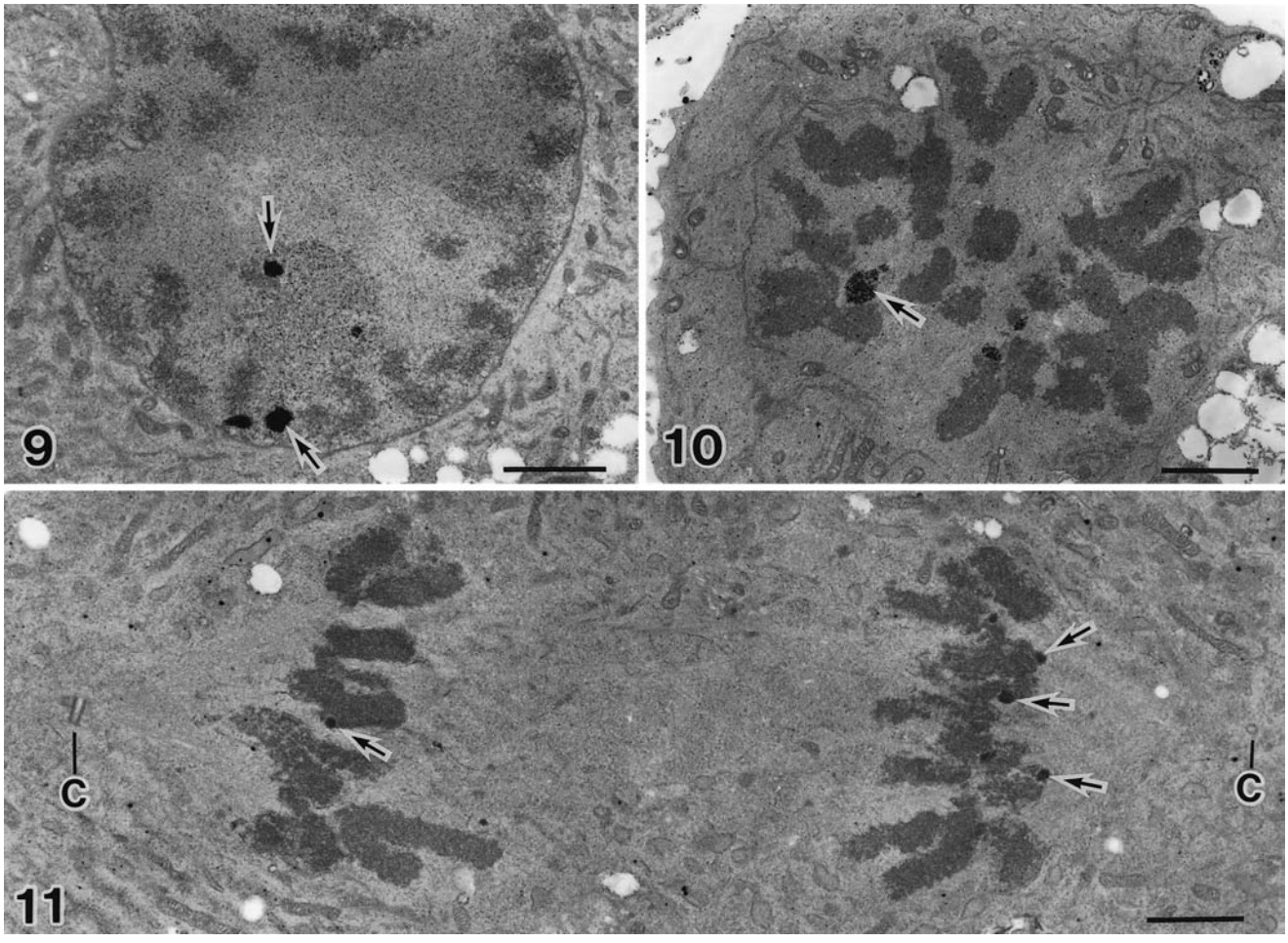
Discussion

Cancerous ALPase isoenzymes have been classified as nonspecific type, intestinal type, placental type, and liver/bone/kidney type from tissues of patients with malignant tumors.[5,14–16] Intracellular localization of ALPase in human cancer cells has been recognized on the plasma membrane and in the other cell organellae such as endoplasmic reticulum, Golgi apparatus, and mitochondria.[5] It is generally known that the principal function of ALPase is transportation of substances, because the intestinal type increases in activity when amino acids, lipids, and glucose are absorbed



Figs. 5–7. ALPase staining in nucleoli of human cultured normal cells (fibroblast, WI-38) and human cancer cells (hepatocarcinoma, Hep-G2; malignant melanoma, A-375; pancreatic carcinoma, BxPC-3). **5** Fibroblast (WI-38 cell). **6** Malignant melanoma (A-375 cell). **7** Pancreatic carcinoma (BxPC-3 cell). *Arrows*, reaction products of ALPase. *Bar* **5**, **6** 2 μm; **7** 3 μm

Fig. 8. ALPase staining on the nucleolus of Hep-G2 cells. The reaction lead products of ALPase were revealed on the dense fibrillar components (*large arrows*) and euchromatin (*small arrows*). *Bar* 0.5 μm



Figs. 9–11. ALPase staining of the chromosomes at the mitotic phase of A-375 cells. **9** Prophase. **10** Metaphase. **11** Anaphase. *Arrows*, reaction products of ALPase, *C*, centrosome. *Bar* 2 μm

into the epithelial cell. The bone type has functions such as raising the concentration of inorganic phosphate, promoting the deposition of calcium phosphate, and disassembling pyrophosphate acid, which checks ossification.[17] In this study, we found ALPase to be localized in the dense fibrillar components of the nucleolus and a part of the chromosome at the mitotic phase in both normal cells (WI-38) and cancer cells (Hep-G2, A375, and BxPC-3). Although the nucleolus generally contains large amounts of RNA, NAD pyrophosphatase, and RNA polymerase,[18,19] ALPase activity in the nucleolus has not yet been reported, as far as we know.

Since Henderson et al.[20] and Hsu et al.[21] revealed that the genes for ribosomal RNA are located at the chromosomal nucleolar organizer regions (NORs) by using an in situ hybridization method, many investigators[22] have demonstrated a relationship between nucleoli and chromosomal NORs by the silver staining method. The chromosomal NORs are also closely associated with secondary constriction of the chromosome. These secondary constrictions were specifically stained with an Ag-As staining method by Goodpasture and Bloom.[23] Furthermore, the fibrillar centers and dense fibrillar components of nucleoli were the substrata for the silver staining reaction.[24] In addition, placenta-type ALPase has protein phosphatase activity and acts for dephosphorylation of the phosphate protein that is chromatin-composing protein. We assumed that the reaction site of ALPase exists at the same place as the NOR part or around the NOR. Wachtler and coworkers[25–27] showed that the dense fibrillar component was the site of rDNA location and transcription in nucleoli by light and electron microscopic autoradiography on human Sertoli cells and lymphocyte nucleoli. We presumed that ALPase acts on the chromosomal NOR in which rDNA existed and regulated gene expression.

The reaction ratio of nucleolar ALPase in Hep-G2 cells indicated approximately fivefold higher frequency than that of the normal cells. This phenomenon meant that the high level of appearance of ALPase reaction was caused by a high proliferation of cancer cells, and we suggested that the cells required ALPase at that specific time in the interphase. Although we were unable to confirm the specific time in this study, it was assumed that the specific time was the G_1 phase of the cell cycle stage.

References

1. Tokumitsu S, Tokumitsu K, Fishman WH (1981) Intracellular alkaline phosphatase activity in cultured human cancer cells. Histochemistry 73:1–13
2. Tokumitsu S, Fishman WH (1983) Alkaline phosphatase biosynthesis in the endoplasmic reticulum and its transport through the Golgi apparatus to the plasma membrane: cytochemical evidence. J Histochem Cytochem 31:647–655
3. Wetzel BK, Spicer SS, Horn RG (1967) Fine structural localization of acid and alkaline phosphatases in cells of rabbit blood and bone marrow. J Histochem Cytochem 15:311–334
4. Mori S, Nagano M (1985) Electron-microscopic cytochemistry of alkaline-phosphatase activity in endothelium, pericytes and oligodendrocytes in the rat brain. Histochemistry 82:225–231
5. Sasaki M, Fishman WH (1973) Ultrastructural studies on Regan and non-Regan isoenzymes of alkaline phosphatase in human ovarian cancer cells. Cancer Res 33:3008–3018
6. Griffin MJ, Cox RP, Grujic N (1967) A chemical method for the isolation of HeLa cell nuclei and the nuclear localization of HeLa cell alkaline phosphatase. J Cell Biol 33:200–203
7. Lewinson D, Toister Z, Silbermann M (1982) Quantitative and distributional changes in the activity of alkaline phosphatase during the maturation of cartilage. J Histochem Cytochem 30:261–269
8. Ishizeki K, Takigawa M, Nawa T, Suzuki F (1996) Mouse Meckel's cartilage chondrocytes evoke bone-like matrix and further transform into osteocyte-like cells in culture. Anat Rec 245:25–35
9. Sasaki T, Amizuka N, Irie K, Ejiri S, Ozawa H (2000) Localization of alkaline phosphatase and osteopontin during matrix mineralization in the developing cartilage of coccygeal vertebrate. Arch Histol Cytol 63:271–284
10. Vergnes HA, Brisson-Lougarre A, Grozdea JG, Blum GC, Kihn Y, Sevely J (1992) Nuclear localization and characterization of alkaline phosphatase in neutrophils from normal controls and pregnant women. Am J Hematol 39:249–256
11. Slor H, Bustan H (1973) Alkaline phosphatase activity in relation to DNA synthesis in synchronized HeLa S3 cells. Experientia (Basel) 29:1214–1215
12. Mayahara H, Hirano H, Saitoh T, Ogawa K (1967) The new lead citrate method for the ultrachemical demonstration of activity of non-specific alkaline phosphatase. Histochemie (Paris) 11:88–96
13. Raška I, Ochs RL, Salamin-Michel L (1990) Immunocytochemistry of the nucleus. Electron Microsc Rev 3:301–353
14. Seargeant LE, Stinson RA (1979) Evidence that three structural genes code for human alkaline phosphatase. Nature (Lond) 281:152–154
15. McKenna MJ, Hamilton TA, Sussaman HH (1979) Comparison of human alkaline phosphatase isoenzymes. Structural evidence for three protein classes. Biochem J 181:67–73
16. Henthorn PS, Raducha M, Edwards YH, Weiss MJ, Slaughter C, Lafferty MA, Harris H (1987) Nucleotide and amino acid sequences of human intestinal alkaline phosphatase: close homology to placental alkaline phosphatase. Proc Natl Acad Sci USA 84:1234–1238
17. Meyer JL, Reddi AH (1985) Changes in pyrophosphatase activity during the de novo mineralization associated with cartilage and bone formation. Arch Biochem Biophys 242:532–539
18. Marinozzi V (1964) Cytochemie ultrastructurale du nucleole-RNA et proteines intranucleolaires. J Ultrastruct Res 10:433–456
19. Granboulan N, Granboulan P (1965) Cytochemie ultrastructurale du nucleole. II. Etude des sites de synthese du RNA dans le nucleole et le noyau. Exp Cell Res 38:604–619
20. Henderson AA, Warburton D, Atwood KC (1972) Location of ribosomal DNA in the human chromosome complement. Proc Natl Acad Sci USA 69:3394–3398
21. Hsu TC, Spirito SE, Pardue ML (1975) Distribution of 18 + 28S ribosomal genes in mammalian genomes. Chromosoma (Berl) 53:25–36
22. Loftus BM, Gilmartin LG, O'Brien MJ, Carney DN, Dervan PA (1990) Intratubular germ cell neoplasia of the testis: identification by placental alkaline phosphatase immunostaining and argyrophilic nucleolar organizer region quantification. Hum Pathol 21:941–948
23. Goodpasture C, Bloom SE (1975) Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma (Berl) 53:37–50
24. Goessens G (1976) High resolution autoradiographic studies of Ehrlich tumor cell nucleoli. Nucleolar labeling after [^{3}H] actinomycin D binding to DNA or after [^{3}H] TdR or [^{3}H] uridine incorporation in nucleic acids. Exp Cell Res 100:88–94
25. Wachtler F, Hartung H, Devictor M, Wiegant J, Stahl A, Schwarzacher HG (1989) Ribosomal DNA is located and transcribed in the dense fibrillar component of human Sertoli cell nucleoli. Exp Cell Res 184:61–71
26. Wachtler F, Mosgöller M, Schwarzacher HG (1990) Electron microscopic in situ hybridization and autoradiography: localization and transcription of rDNA in human lymphocyte nucleoli. Exp Cell Res 187:346–348
27. Wachtler F, Stahl A (1993) The nucleolus: a structural and functional interpretation. Micron 24:473–505