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Immunohistochemical Localization of Thymosin β_4 in Bovine Tissues

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Abstract: The histochemical localization of thymosin β_4 was investigated for the first time in several bovine tissues, using the indirect immunofluorescent technique and the newly applied alkaline phosphatase anti-alkaline phosphatase (APAAP) antibody method. A polyclonal antiserum raised against the N-terminal fragment (1-14) of thymosin β_4, showing less than 0.1% crossreactivity with the second β-thymosin in calf tissues, thymosin β_9, was used. Positive reaction for thymosin β_4 was observed in the cytoplasm of all tissues studied, i.e., bovine spleen, thymus, lung, liver, kidney, lymph nodes and cardiac muscle. The highest staining intensity was found in the cytoplasmic area of spleen and thymus, whereas in sections of muscle the reaction was very weak. In the latter case a relatively strong reaction was strictly located in the sarcolemn region of the myofibrils.

Key Words: Thymosin β_4, bovine tissues, immunofluorescent technique.

Introduction

Thymosin β_4 ($T\beta_4$), an acidic polypeptide of 43 amino acid residues long first isolated from calf thymus (1), is ubiquitously distributed in many tissues of various vertebrates, ranging from human to toads (2), but is also present in cell lines (3) and in types of human malignant tissues (4, 5, 6). Until today, eight homologues to $T\beta_4$ were isolated ($T\beta_4^{ala}$, $T\beta_4^{Xen}$, $T\beta_9$, $T\beta_9^{met}$, $T\beta_{10}$, $T\beta_{11}$, $T\beta_{12}^{Tr}$ and $T\beta_{12}^{Per}$), their primary sequences showing a high degree of conservation ranging from 72-98% (7). $T\beta_4$ is the predominant form in mammalian cells, although many species are known to produce at least two β-thymosin isoforms, in most cases both present in the same cell. Immunocrossreactive material for $T\beta_4$ was also detected in human serum (8), plasma, platelets and leukocytes (9).

The high intracellular concentration of $T\beta_4$ helped in the elucidation of its role. (10) first reported $T\beta_4$ to be an actin-sequestering polypeptide in human platelets, able to form 1:1 complexes with monomeric G-actin, thereby stabilizing the intracellular pool of unpolymerized actin, both *in vivo* and *in vitro* (11). Factors inducing the polymerization of actin from the actin: $T\beta_4$ complex are phalloidin, skeletal myosin subfragment 1, chiken intestinal myosin I and F-actin in the presence of increased salt concentration (12). The increase of $T\beta_4$ mRNA in the S, G_2 and M phase of the cell cycle also suggests a role in the morphological changes and actin redistribution occuring during cell cytokinesis (13), although a possible implication of the polypeptide in programmed cell death or "apoptosis" mediated via DNase I can not be ruled out (14).

In agreement with its role, several histochemical studies for the intracellular localization of $T\beta_4$ showed that the polypeptide was solely localized in the cytoplasm of the tissues examined (2, 15, 16, 17). Also the bulk of the $T\beta_4$ crossreactive material in bovine thymus, as measured by a specifically developed radioimmunoassay, was found in the extracellular fraction (18).

For further evaluating the subcellular localization and the physiological role of $T\beta_4$, it was of interest to expand the histochemical studies in various tissues of a phylogenetically evoluted organism like a mammal. The selection of bovine tissues enabled us: (i) to study various tissue sections, divided in three main groups according to their $T\beta_4$- content, the $T\beta_4$-rich (spleen, thymus and lung), the $T\beta_4$-intermediate (liver, kidneys and lymph nodes) and the $T\beta_4$-poor (muscle) and (ii) to make a

direct comparison of the present study with the previously reported results for the second β-thymosin present in calf, $T\beta_9$ (19).

All sections were stained by the alkaline phosphatase anti-alkaline phosphatase (APAAP) and the immunofluorescent techniques, using antibodies raised against the fragment $T\beta_4$ (1-14) (20). The APAAP technique was suggested as more suitable for detecting low numbers of antigen-bearing cells in a specimen compared to other immunostaining methods (21).

Material and Methods

Fresh bovine spleen, thymus, lung, liver, kidneys, lymph nodes and cardiac muscle were collected upon slaughter of a 12 months old calf, immediately frozen in liquid nitrogen and stored at -20°C.

All tissues were sectioned in a cryostat Leitz (1720 Digital) at -15°C in 6-12 μm thick pieces. Without fixing, the sections were freeze-dried and subsequently stained by the indirect fluorescent antibody technique (19), using rabbit anti-$T\beta_4$ (1-14) polyclonal antiserum (20) in a dilution 1: 100 and by a modification of the APAAP method (21). For the latter, the sections were carried on poly-1-lysine coated cover glasses in a moist chamber at room temperature, where several incubation steps were performed in the following order: first antibody, i.e., rabbit anti-$T\beta_4$ (1-14) polyclonal antiserum, dilution 1:600 for 1 h; second antibody, i.e., mouse anti-rabbit IgG-monoclonal serum (Sigma, Deisenhofen, Germany), dilution 1:80 for 30 min; third antibody, i.e., rabbit anti-mouse IgG-monoclonal serum (Sigma, Deisenhofen, Germany), dilution 1:80 for 1h; APAAP complex, i.e., alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase (Sigma, Deisenhofen, Germany) for 30 min; substrate (0.04% naphthol phosphate, 0.02% levamisol, 0.1% Fast Red) for 1h. After washing with PBS, the contrast staining, containing 1% hemalum was performed for ca. 3 min, followed by covering of the sections with mowiol (Kallestadt, Germany) or Kaiser's glycerin gelatin. Cell nuclei were stained with a basic hematoxylin solution.

Normal rabbit serum, heat activated anti-$T\beta_4$ (1-14) antiserum and PBS instead of the first antibody served as negative controls.

Results

In order to detect the localization of $T\beta_4$ in various bovine tissues, two immunohistochemical techniques were used, the indirect fluorescent antibody technique and the APAAP method. The antibody used was raised against the N-terminal fragment 1-14 of $T\beta_4$, obtained after tryptic cleavage of isolated bovine lung $T\beta_4$. The polyclonal antiserum, produced via rabbit immunizations, recognized, both, the $T\beta_4$ (1-14) fragment and the whole polypeptide in a ca. 1:1 ratio and showed less than 0.1% crossreactivity with the highly homologous $T\beta_9$, also present in bovine tissues (20).

As expected, positive immunostaining, observed as vivid red reaction, for $T\beta_4$ was found in sections of all tissues investigated, i.e., spleen, thymus, lung, liver, kidneys, lymph nodes and cardiac muscle, but with a different degree of intensity, most prominent in spleen and thymus tissues. In all cases, the positive reaction was in principle found inside the cells. Negative reactions were observed after incubations with non-immune rabbit serum, heat inactivated $T\beta_4$ (1-14) antiserum or PBS.

In detail, in spleen sections the staining intensity was high, indicating the high content of the tissue, in $T\beta_4$ (Fig. 1). The staining was homogeneously spread throughout the tissue, both in areas of the red and the white pulp. All cells were stained in the cytoplasm and a relatively stronger stain was noticed at the cell borders.

In sections of the thymus gland, a result similar to that of spleen was observed. Thymocytes, as well as epithelial cells and Hassall's corpuscles were strongly stained. In a panoramic view of a thymic lobule (Fig. 2A), the dense lymphoid tissue stain of the cortex area (border) contrasts the diffuse lymphoid tissue stain of the medulla (center). In a higher magnification of the cortex (Fig. 2B), an area rich in numerous small thymocytes, indicative of the high proliferation rate of the young thymus gland, a very intense stain of the thin cytoplasmic area surrounding the enlarged thymocyte nuclei of all cells is observed. The stain was strictly located in the cytoplasm, neither spreading out of the cell border nor at the interlobular trabeculae. A similar observation was made when the cortex was stained by the immunofluorescent technique (Fig. 2C). In a higher magnification of the medulla area (Fig. 2D), where, beside thymocytes, many epithelial cells are present, the latter structurally distinguished by the presence of pseudopodia (better seen by the fluorescence staining technique; Fig. 2E), a strong positive reaction for $T\beta_4$ is also observed. The Hassall's corpuscle in the middle of figure 2D is also intensively stained.

Although lung is considered to be a $T\beta_4$-rich tissue (22), in lung sections a histochemically relatively low staining activity was observed and only ca. 10% of the

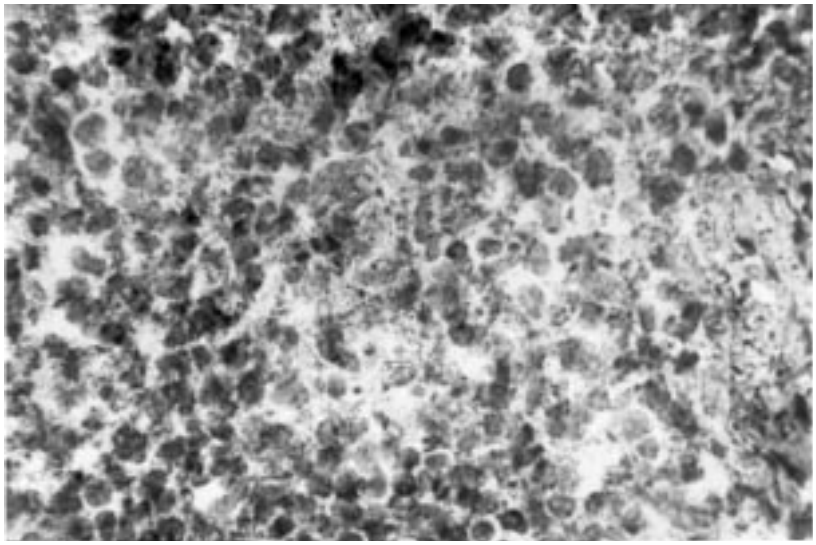
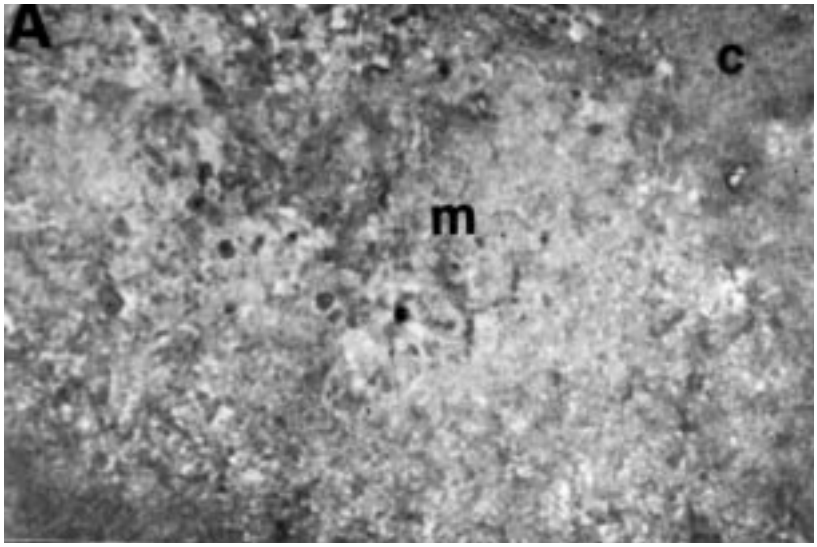


Figure 1. Immunohistochemical localization of $T\beta_4$ in bovine spleen. The APAAP staining technique is applied. Positive reactions is observed with red stain. Nuclei are stained with hematoxylin, coloured dark blue in all figures presented. Magnification 180: 1.



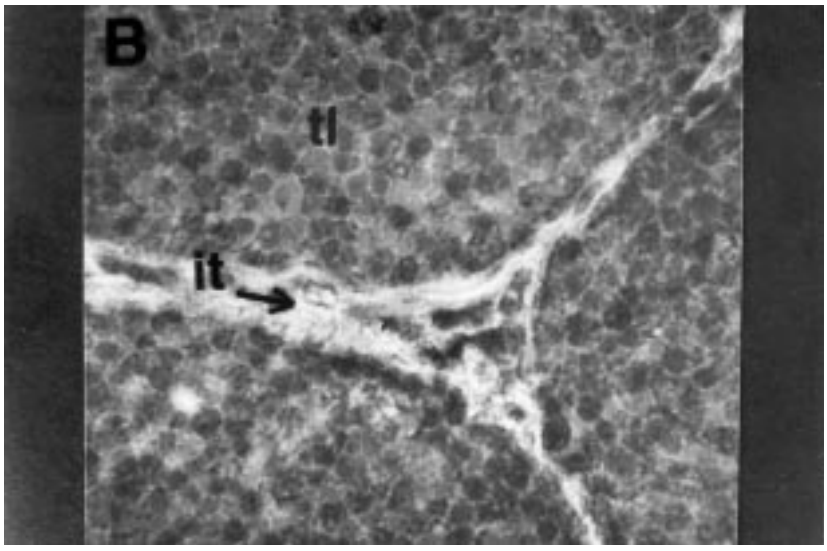


Figure 2. Immunohistochemical localization of $T\beta_4$ in bovine thymus sections. A, B and D are stained with the APAAP method, C and E are stained with the fluorescence technique. (A) panoramic view of a thymic lobule; m: medulla; c: cortex. Magnification 112: 1. (B) cortex area with numerous small thymocytes; tl: thymic lobule; it: interlobular trabeculae. Magnification 180: 1.

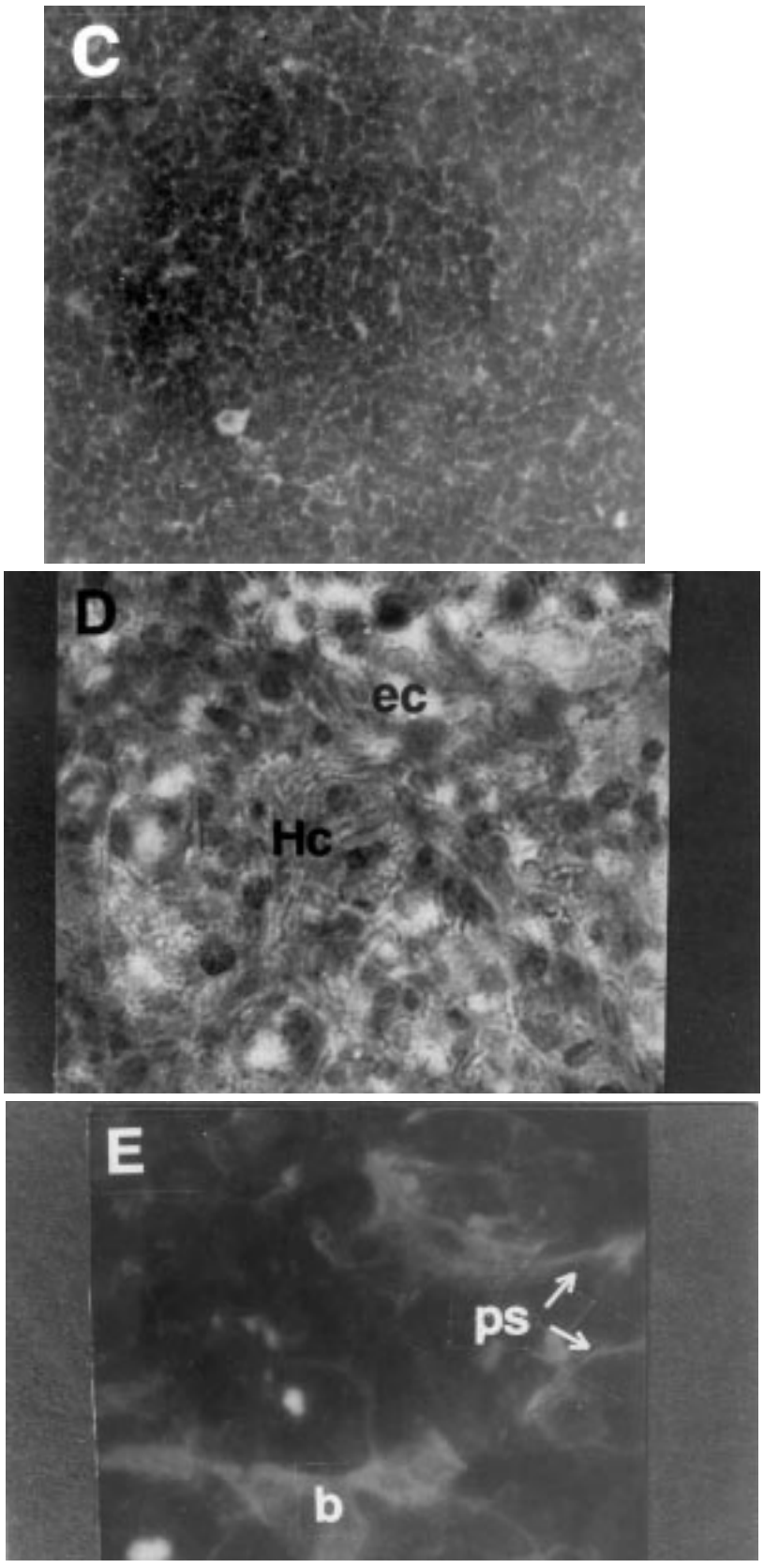


Figure 2. (C) cortex area where strong fluorescence in the cytoplasm of thymocytes is observed. Magnification 180: 1. (D) medulla area; ec: epithelial cell; Hc: Hassall's corpuscle. Magnification 450: 1. (E) medulla area where the structure of the epithelial cells is better observed; b: body of the epithelial cell; ps: pseudopodia. Magnification 1125: 1.

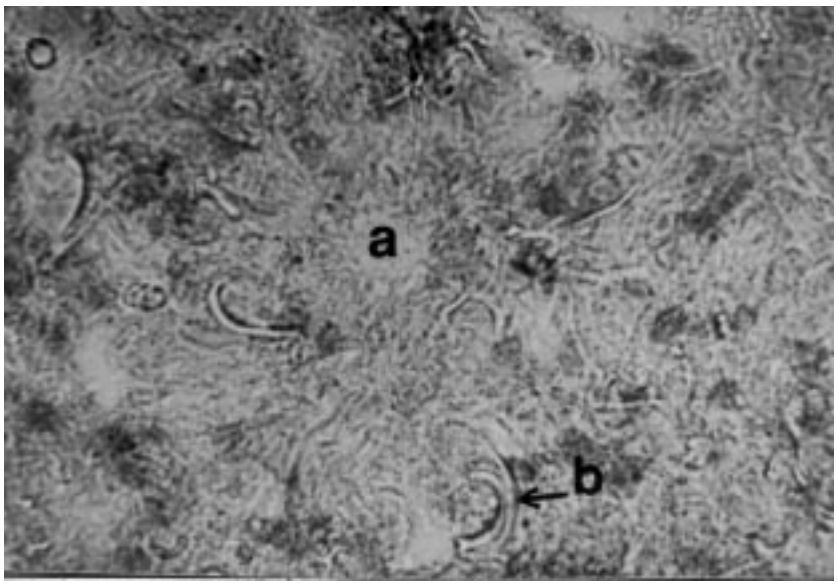


Figure 3. Immunohistochemical localization of $T\beta_4$ in a bovine lung section (APAAP stain); a: alveoli; b: bronchioli. Magnification 450: 1.

total number of cells show an important amount of positive satin (Fig. 3). The dark blue nuclei present in the section are fewer compared to spleen or thymus and the lung cells are bigger in size with a higher cytoplasmic content compared to splenocytes and thymocytes. Due to the high air content of the tissue, areas filled with air (alveoli), remaining unsatained, can also be observed.

In liver sections, the stain was relatively weak throughout the entire tissue. In a section of a hepatic lobule (Fig. 4), the polyhedral liver cells, each containing a significant amount of cytoplasm and a round central nucleus, are homogeneously, although weakly, stained. Areas with Kupffer cells present a similar staining intensity. These results are in agreement with the reported $T\beta_4$-content of the tissue.

In kidney sections, the cortex area was also weakly stained (Fig. 5A). The positive reaction in more intense in the areas of the glomeruli. The empty areas in the pictures represent either sections through the proximal and distal convoluted tubules and the upper portions of

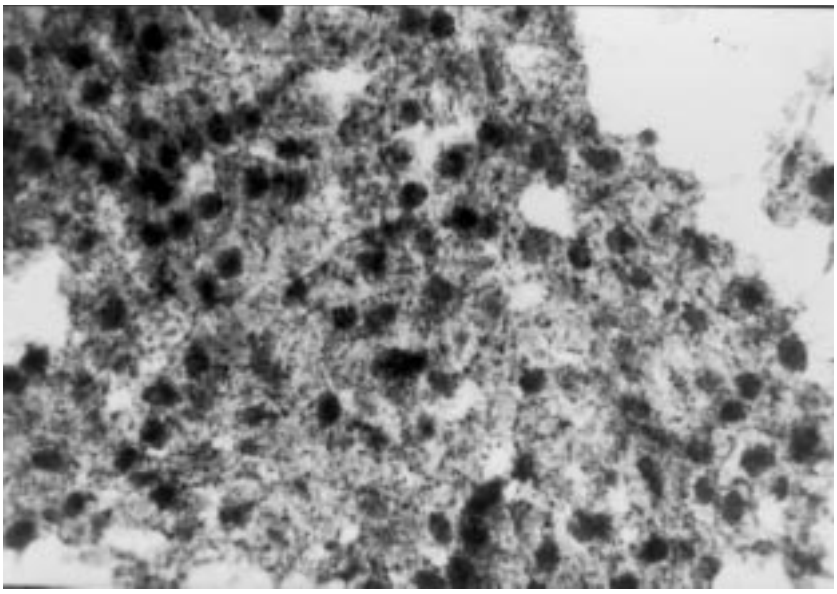


Figure 4. Immunohistochemical localization of $T\beta_4$ in a bovine liver section (APAAP stain). Magnification 450: 1.

Henle's loops, or artifacts occuring during sectioning of a non-solid tissue like kidney. In the magnification of a glomerulum (Fig. 5B)., only 5-10% of the cells show a very high staining reaction. A strong positive reaction can also be observed in the capsule of the glomerulum (Bowman's capsule) and in the interlobular artery wall (Fig. 5C), indicating the $T\beta_4$ activity present in the tunica media area of the vessel, composed predominantly of circular smooth muscle fibres.

In sections of lymph nodes, which like spleen and thymus belong to the lymphoid tissues, an intense stain was expected. In a section of the cortex area including a germinal center (Fig. 6), few cells containing a significant amount of cytoplasm are seen, the larger ones being lymphoblasts, the smaller ones medium-sized lymphocytes. In all cells, the staining intensity is much weaker compared to spleen and thymus and similar to that of the glomeruli of the kidney tissue.

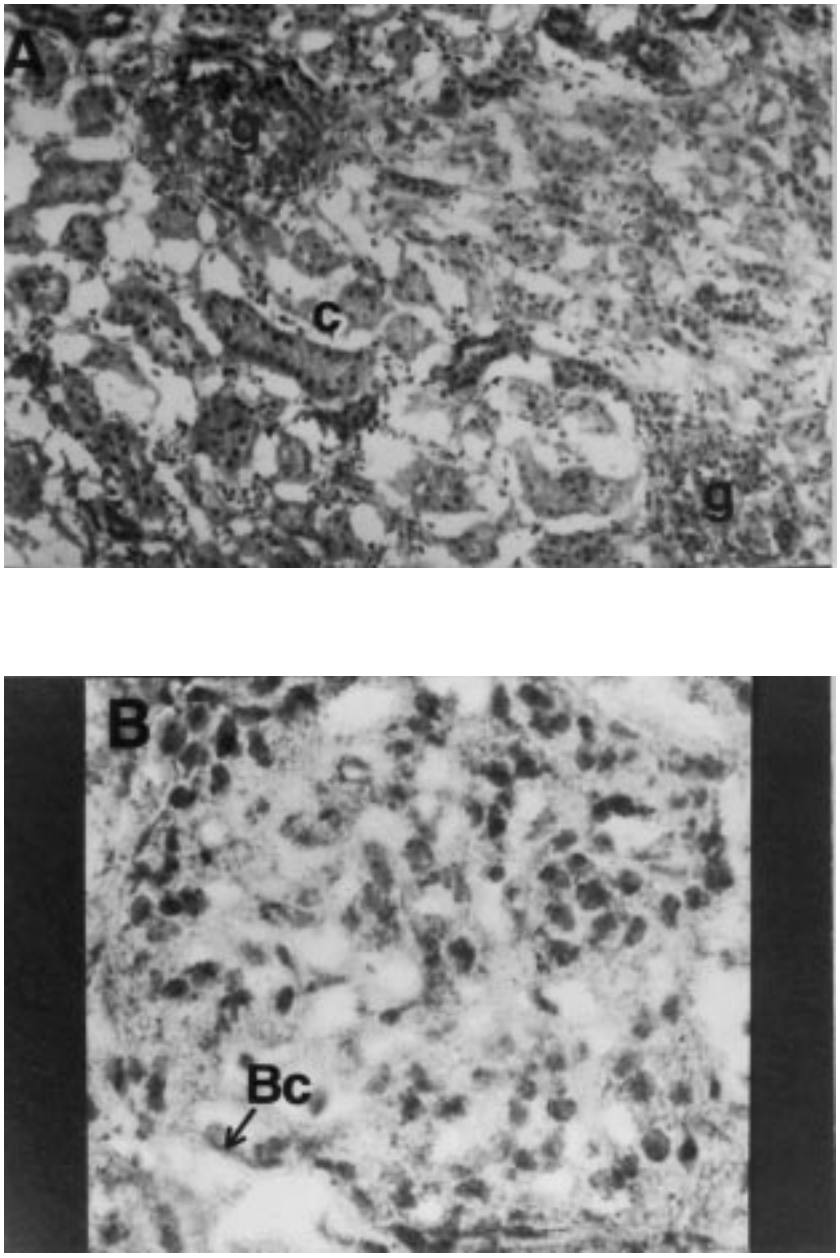


Figure 5. Immunohistochemical localization of $T\beta_4$ in bovine kidney (APAAP method). (A) panoramic view of the tissue; c: cortex; g: glomeruli. Magnification 112: 1. (B) enlargement of a glomerulum area; Bc: Bowman's capsule. Magnification 450: 1.

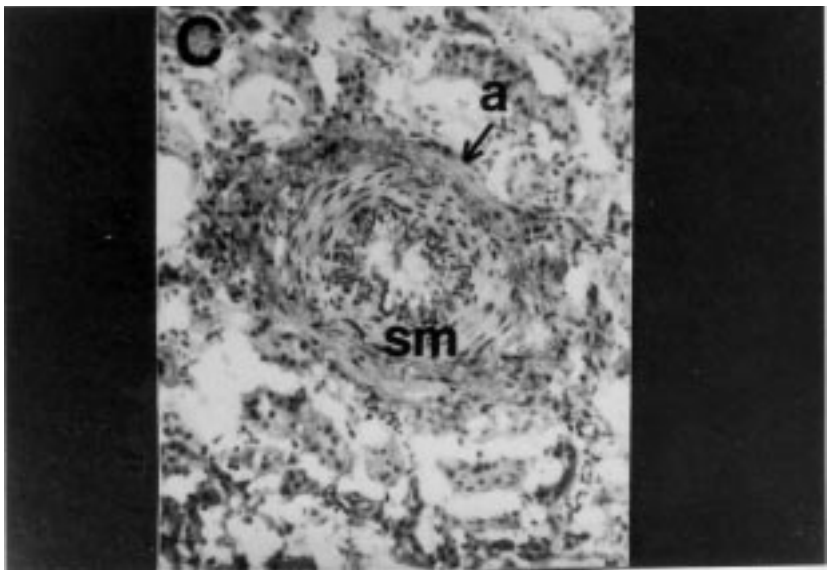


Figure 5. C) enlargement of the cortex area; a: artery; sm: smooth muscle. Magnification 112: 1.

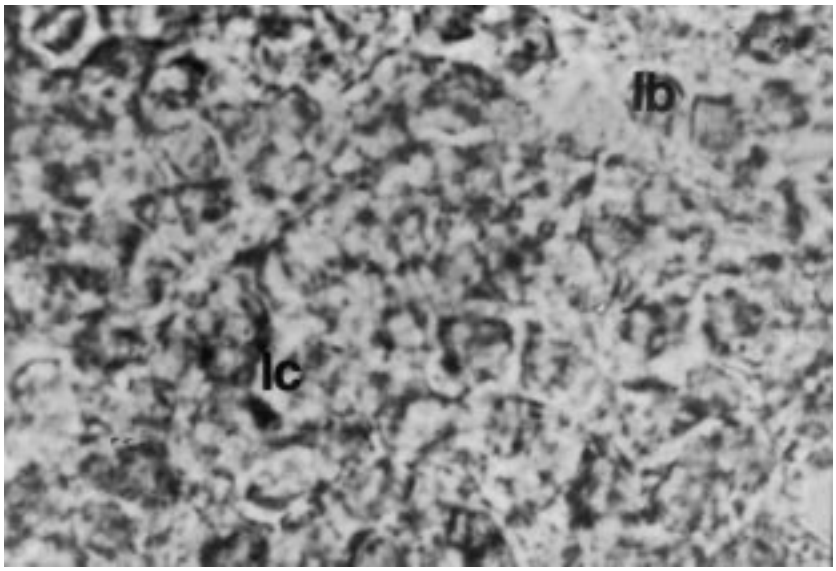


Figure 6. Immunohistochemical localization of $T\beta_4$ in bovine lymph node's germinal center (APAAP method); 1b: lymphoblast; 1c: lymphocyte. Magnification 1125: 1.

Finally, in cross-sections of striated (cardiac) muscle tissue, the weak positive stain was observed only at the sarcolemn borders, whereas inside the muscle fibre no satining was noticed (Fig. 7A). An a higher magnification of the triangular spaces where muscle fibre sarcolemns tightly approach (Fig. 7B), the positive activity looks restricted to the cell borders. It should be pointed out that the stain does not spread in the extracellular space and areas where the stain seems to slightly penetrate in the fibres should be considered as artifacts. Longitudinal sections (results not shown) present the same picture, i.e., weak positive stain is only seen at the sarcolemn borders but not in the fibre itself.

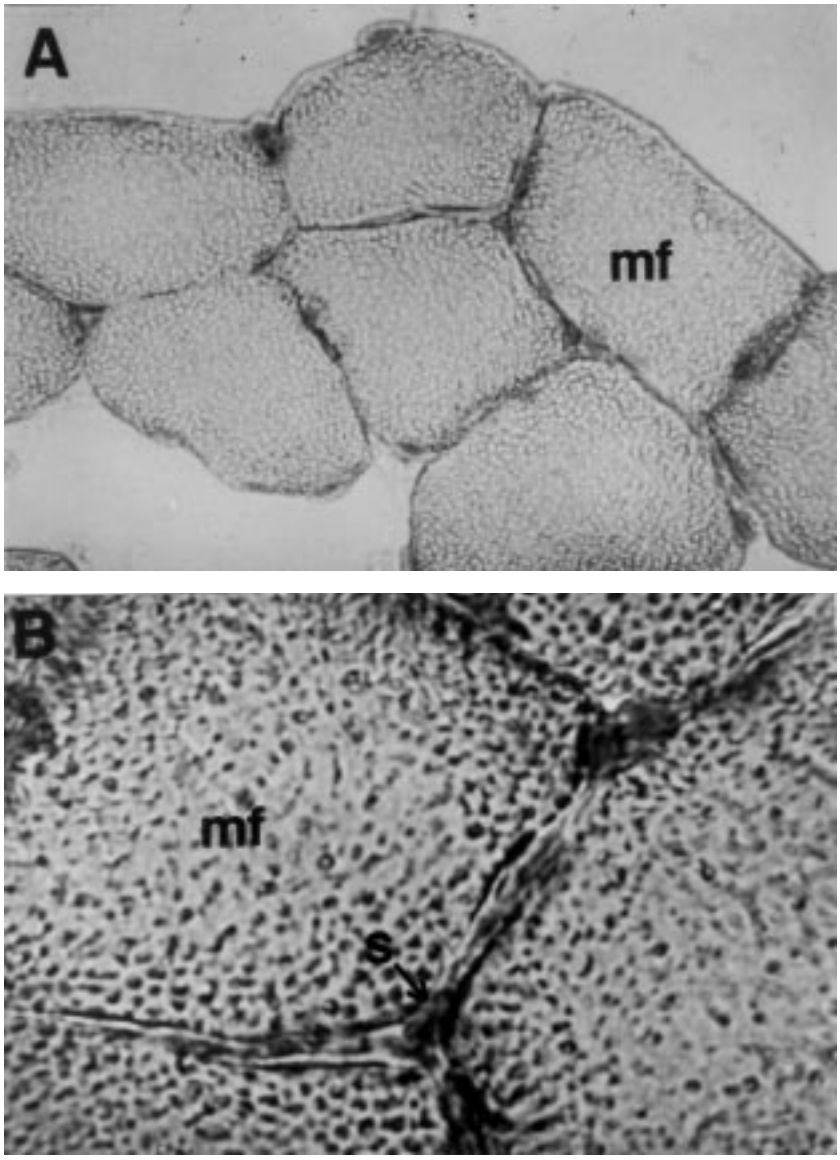


Figure 7. Immunohistochemical localization of Tβ_4 inbovine cardiac muscle secitons (APAAP method). (A) cross-section of the muscle; mf: muscle fibre. Magnification 450: 1. (B) enlargement of the area between fibres; mf: muscle fibre; s: sarcolemn. Magnification 1125: 1.

Discussion

In this study, the subcellular localization of the quantitatively most important β-thymosin in bovine tissues, thymosin β_4, was examined using, besides the classic immunofluorescence method (19), an indirect APAAP antibody technique (21), i.e., the indirect APAAP-labeling reaction with complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase as second and third antibodies respectively. The APAAP method gives excellent immunohistochemical labeling with a high degree of clarity and intensity and is suggested as particularly suitable for staining tissue sections with small numbers of positive cells. The vivid red colour of the reaction product, produced when naphtol salt as coupling agent and Fast Red as capture agent are used, makes positively labeled cells stand out with great clarity. The dark cell nuclei, stained with a basic hematoxylin solution, are also easily picked out, contrasting well with the red reaction products. The indirect fluorescent antibody technique used in parellel, enabled us to clearly distinguish several morphological details (e.g. pseudopodia in thymic epithelial cells; Fig. 2E) and to compare these results with the previously reported Tβ_9 localization in similar tissue sections (19).

The anti-thymosin β_4 antibodies used, were raised in rabbits against the fragment 1-14 of thymosin β_4. The antiserum exhibited a high titre, recognized in a 1:1 ratio the native $T\beta_4$ and showed minimal crossreactivity (less than 0.1%) with the second β-thymosin present in calf, $T\beta_9$ (20). Compared to antisera raised against intact $T\beta_4$ (20, 23), the use of N-terminal fragments for immunizations seems to produce more specific antibodies possessing the advantage of recognizing intact $T\beta_4$ and lacking the main disadvantage of crossreacting with the highly homologous $T\beta_9$ (18 ,20). This observation appears to be valid in the opposite direction as well, i.e., an antiserum raised against the N-terminal fragment (1-14) of $T\beta_9$ was reported to show less than 1% cross-reactivity with $T\beta_4$ (24).

The localization of $T\beta_4$ was investigated in seven bovine tissues, lymphoid and non-lymhoid. A positive reaction was observed in all of them, suggesting the wide distribution of the peptide, which, although first isolated from calf thymus (1), according to all previously reported studies was never considered as an organ-specific one (22, 25, 26, 27). Significant differences were noticed in the staining intensity, which was most prominent in spleen and thymus, weak in lung, liver, kidney and lymph nodes and very weak in muscle.

Compared to the previously reported $T\beta_4$-content in rat (22, 25, 26), mouse (25) and rabbit (28) tissues, as measured by specifically developed immunoassays, amino acid analyses and HPLC, the high $T\beta_4$-content of spleen and thymus was excepted. Spleen was homogenously and strongly stained, whereas in thymus sections, the difference in the positive reaction between the cortex and the medulla area can be explained by the presence of more cytoplasm and smaller nuclei in the epithelial cells, compared to the low but concentrated cytoplasmic content surrounding the enlarged nuclei of the thymocytes, the latter presenting a more intense staining.

In lung sections, a positive reaction similar to that of thymus was excepted, since lung is a $T\beta_4$-rich tissue at least in rat (214 and 203 μg of $T\beta_4$ per g of tissue for thymus and lung respectively; 22). When a cDNA for rat $T\beta_4$ was used to investigate the expression of its gene in various tissues, in lung the third (after spleen and thymus) largest degree of expression was found (27). Two possible reasons could explain the relatively low staining intensity observed in bovine lung sections, one being the fact that lung tissue normally contains a significant amount of air, whereas the measurements of its $T\beta_4$-content were expressed in μg $T\beta_4$/g of solid tissue, the second being the fact that lung cells are much bigger in size containing more cytoplasm than splenocytes and thymocytes and therefore the stain is spread in a broad area.

In liver and kidney, the low staining intensity is in agreement with the reported values in rat (22, 25, 27) and rabbit (28) tissues. On the contrary, in lymph nodes, a tissue belonging to the lymphoid organs and containing the same type of cells as spleen and thymus (i.e., lymphochytes, macrophages, monocytes), the staining intensity found was much lower than expected. Two basic histological aspects could explain this; first is that in lymph nodes the percentage of the various cell subtypes differs from that of thymus and spleen and second, lymph nodes contain many sinuses in contrast to spleen and thymus which are more homogenous and solid organs.

Finally, in cardiac muscle, the low $T\beta_4$-content reported for rat heart (22, 27) is confirmed by the very low stain. In muscle the strong stain in the sarcolemn was unexpected, although it is in agreement with the localization of $T\beta_9$ in the same tissue (19).

The subcellular localization of $T\beta_4$ in all tissues examined was similar. $T\beta_4$ was widely distributed in the extranuclear area, mainly near the cell borders and membranes, and was not found in the nucleus. The number of positively stained cells varied from over 90% (in spleen and thymus) to 5-10% (in kidney), whereas in some cells of the same tissue no staining was visible. Positive stain for $T\beta_4$ could also be detected organ-unspecifically in the connective tissue sections of thymus, kidney and muscle, as well as in blood vessels.

In contrast with the confict existing about the intracellular localization of prothymosin α and/or parathymosin α (18, 29), all indications until now even prior to the elucidation of $T\beta_4$'s role, were that it is strictly of cytoplasmic localization in various tissues studied, like human thymus (30), human and monkey brain, skin, lymph nodes, spleen, liver and lung (15), rat spleen (28), guinea pig cerebral cortex (17), peritoneal macrophages (31) and neural tissues of *Xenopus laevis* (32). Even when injected into *Xenopus laevis* oocytes, $T\beta_4$ remained in the cytosol (16). The occurance of $T\beta_4$ only in the cytoplasm is in agreement with its role as an actin-sequestering polypeptide. It is not unlikely that $T\beta_4$ plays the same role in every organ it exists in, although its concentration differs. This difference, as well as the fact that a certain number of cells in the same tissue present a stronger red stain than their neighbouring cells (Fig. 3, Fig. 6), could be explained by the speculation that cells in active movement (eg., thymic epithelial cells with pseudopodia), contain an increased amount of this

polypeptide and subsequently present an intense positive reaction.

Comparing these results with those reported for $T\beta_9$ (19), we conclude that both β-thymosins present the same subcellular localization. It would be of interest to study if $T\beta_4$ and $T\beta_{10}$, the latter present in human tissues, also present a similar localization.

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