Cell Tissue Res (1999) 296:511–516

REGULAR ARTICLE

Hui Wang · Jonathan Pevsner

Detection of endogenous biotin in various tissues: novel functions in the hippocampus and implications for its use in avidin-biotin technology

Received: 30 June 1998 / Accepted: 1 December 1998

Abstract Significant amounts of endogenous biotin were detected by avidin-peroxidase in fixed rat kidney, liver, and brain. The staining was indistinguishable from the true signals of immunoreactivity and could not be consistently blocked by pretreatment with avidin. The finding that certain neurons in the hippocampus contain more biotin than neurons in other areas of the brain suggests that biotin might have novel functions in the brain other than its well-known role as cofactor of carboxylases. Critical examination of published immunohistochemical localization studies on rat kidney strongly suggests that many false-positive results have been considered as true signals. Interference of endogenous biotin in any study using avidin-biotin technology must be considered if biological tissues are involved. The published data obtained by this method should therefore be reevaluated. Furthermore, appropriate controls, blockers and caution in interpreting results must be exercised, not only in immunohistochemistry but also in any applications of avidin-biotin technology.

Key words Molecular biology · Cell biology · Bioassay · Analytical chemistry · Biochemistry · Diagnosis · Therapy

This study was supported by National Institutes of Health grant IRO1NS36670-01A1 and March of Dimes Grant 5-FY98-568 to J.P.

H. Wang
Department of Neuroscience, 725 North Wolfe Street,
The Johns Hopkins University School of Medicine, Baltimore,
MD 21205, USA

J. Pevsner (✉)
Department of Neurology, Kennedy Krieger Institute,
707 North Broadway, Baltimore, MD 21205, USA
e-mail: pevsner@welchlink.welch.jhu.edu;
Tel.: +1 410 502–8615; Fax: +1 410 502–8003

Introduction

Biotin is a vitamin essential for metabolism. It is widely distributed in the body (Dakshinamurti and Chauhan 1989). Because of the extraordinarily high affinity with which biotin binds to avidin, avidin-biotin technology has been used in a variety of techniques across biomedical fields (Wilchek and Bayer 1990) ranging from molecular biology, cell biology, biochemistry, analytical chemistry, and bioassay to clinical diagnosis and therapy (Diamandis and Christopoulos 1991). However, the majority of studies employing avidin-biotin-based techniques have not been controlled for the potential interference of endogenous biotin. This problem has so far mainly been addressed in histochemical studies (including in situ hybridization) (Chevalier et al. 1997). However, it has been postulated that biotin-containing proteins are likely to be destroyed by formalin-fixation or paraffin-embedding procedures, suggesting that the use of avidin-biotin technology does not produce artifactual binding to fixed tissues or cells (Hsu 1990). Therefore, although scattered reports have pointed out the occurrence of interference of endogenous biotin in numerous tissues such as human salivary gland (Cauli et al. 1994), fish retina (Bhattacharjee et al. 1997), human ovary (Seidman et al. 1995), and rat kidney (Yi et al. 1995), the problem is still considered to be limited to particular cellular and subcellular sites (Chevalier et al. 1997). The broad relevance of endogenous biotin in virtually all biological materials has not been adequately recognized. The problem caused by interference of endogenous biotin is greater as the sensitivity of methods is increased, for example, in electron-microscopic studies (Chevalier et al. 1997). Furthermore, the potential interference of endogenous biotin in many applications of avidin-biotin technology other than immunohistochemistry has not been appreciated. This is made clear by the discovery of endogenous biotin interference in radioimmunotargeting for cancer therapy (Yao et al. 1995). In addition, although several studies address the problem of endogenous biotin in histochemistry by pretreating tissues with

avidin or biotin antibody to block any artifact as a remedy, its effectiveness has not been tested under various experimental conditions such as the different fixatives that are routinely used in the preparation of biological materials.

In view of the fact that avidin-biotin technology has a uniquely wide spectrum of biomedical applications but the broad relevance of endogenous biotin has not been appropriately noted, we have investigated (1) whether significant amounts of endogenous biotin can be detected, after various fixation procedures, in major tissues such as kidney, liver, lung, and brain of the rat by avidin-peroxidase, and (2) whether blocking by pretreatment of avidin is effective in differently fixed tissues. Additionally, analysis of statistically sampled immunohistochemical reports in the literature was carried out to determine whether artifactual results have been reported even after the problem of interference of endogenous biotin was reported (Yao et al. 1993).

Materials and methods

Experimental procedure

Sixteen Long-Evans male and female rats 1–8 months old were obtained from Charles River (Wilmington, MA) and housed under conditions of 14 h light and 10 h darkness in the Animal Facility of the Johns Hopkins School of Medicine. They received food and water ad libitum and were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were anesthetized by phenobarbital (65 mg/kg body weight, intraperitoneally) and perfused with either 4% paraformaldehyde (eight animals) or 5% glutaraldehyde (eight animals) in 0.1 M sodium phosphate buffer (pH 6.8). The brains, lungs, livers, and kidneys were dissected, immersed in the same fixative overnight at 4°C, and then cut into 100-µm-thick randomly angled liver (from each lobe), lung (from each lobe), and longitudinal kidney sections on a vibratome (see below for brain preparation). Four sets of two adjacent slices from randomly chosen areas of the tissues were collected. All sections were washed with 5% milk powder in phosphate-buffered saline (PBS), and treated sequentially for 30 min each with 1% sodium borohydride in PBS, 3% H_2O_2 and 0.1% HCl in 100% methanol, and with 1% milk powder+0.5% glycine+0.5% lysine+0.5% Triton X-100 in PBS. One of the two adjacent slices was then incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC system, Vector Laboratories, Burlingame, CA) for 1 h to overnight and the other was incubated with PBS. Staining was revealed by use of diaminobenzidine as chromogenic substrate. Alternatively, slices were incubated with avidin (0.02% w/v, Sigma, St. Louis, MO) for 1 h and adjacent slices served as control without avidin treatment. Both slices were then incubated with ABC reagent overnight and then stained with diaminobenzidine.

For all the brains, the cutting angle was adjusted so that sections would correspond to those in the stereotactic atlas of Paxinos and Watson (1982); all sections (100 µm thick) from around the interaural level 8.7–5.2 mm were collected, then adjacent sections were processed together with those of other organs as described above. All areas of the brain sections were examined by light microscopy.

Medline search and statistical analysis

The "and" combination of "rat or rats," "immunohistochemistry or immunohistochemical or immunocytochemistry or immunocytochemical," and "kidney or renal" was used to search Medline from 1966 to June 1997. Titles and abstracts of the resulting papers were examined in the order of that presented by the database. The first 100 studies (February 1994 to June 1997) involving immunohistochemical localization in rat kidney were chosen and the original articles were then investigated to determine (1) whether the avidin-biotin technology was used in immunohistochemistry and (2) whether positive signals were reported in the proximal tubule by checking both the statements in the results and the figures provided. The chi-square test was employed to evaluate whether the incidence of positive signals in the proximal tubule was associated with the use of avidin-biotin conjugate. $P<0.05$ was the minimum accepted statistically significant difference.

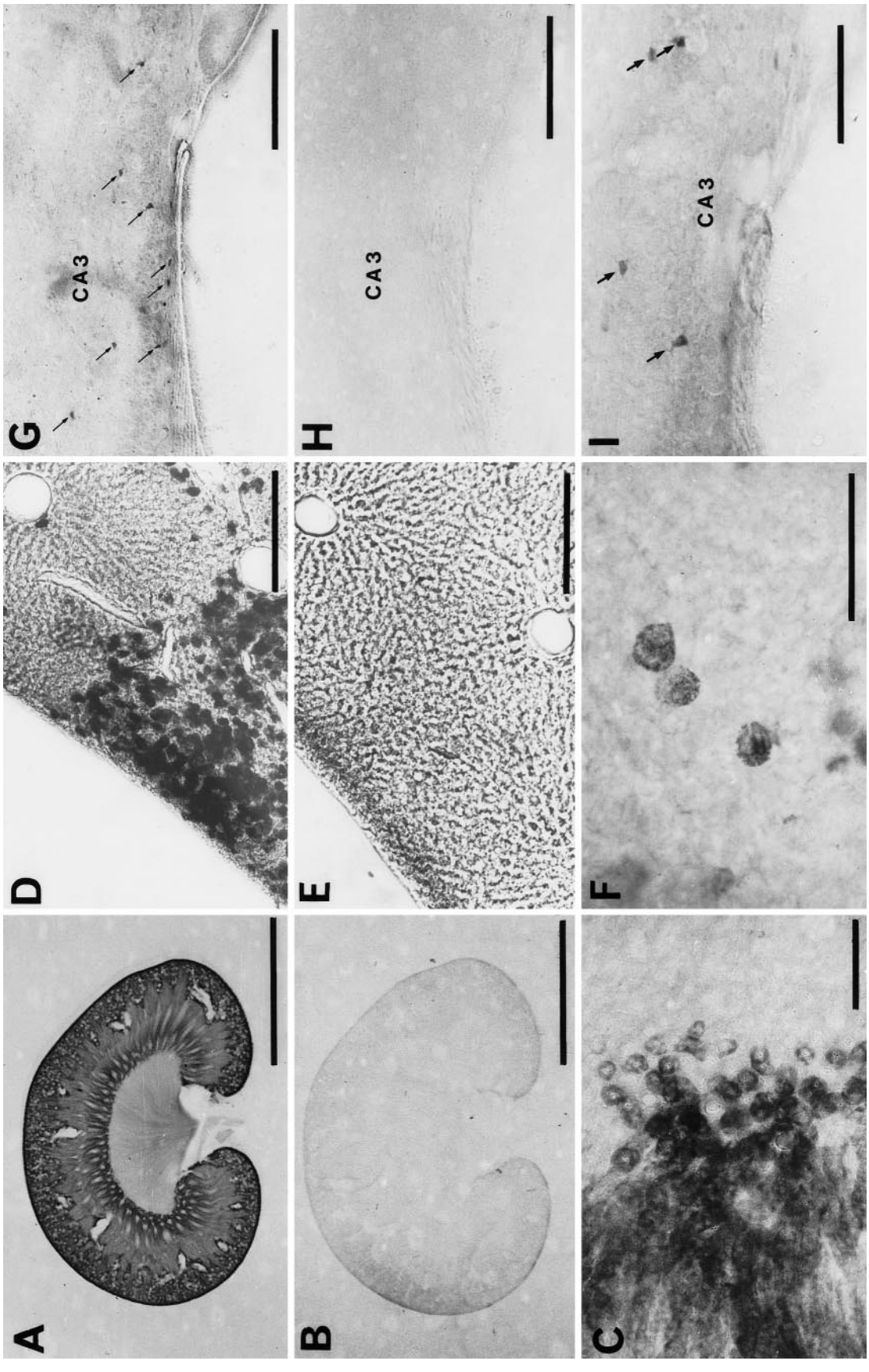
Results

Among the four organs studied, striking staining that is indistinguishable from immunostaining was observed in the kidney, liver, and brain, but not in the lung. All adjacent sections without ABC reagent incubation were completely devoid of any staining (Fig. 1B, E, H), demonstrating that the staining resulted from binding of avidin to endogenous biotin. In the kidney, the proximal tubule in the medulla exhibited the most prominent staining (Fig. 1A, C). The outer cortex showed staining inconsistently and in most cases the intensity was weaker. In the liver, the staining was scattered spots throughout the whole organ (Fig. 1D). Light microscopy indicated that the positive cells appeared to be hepatocytes (Fig. 1F). Approximately one-fourth of randomly selected liver sections showed the staining. The stained areas were not a defined structure and appeared not to be associated with the size of the sections.

All of the brain regions studied (approximately from interaural 8.7 mm to 5.2 mm) were devoid of staining except for the hippocampus, where scattered neurons particularly in the CA3 and CA4 regions were found to be positive for endogenous biotin (Fig. 1G). These neurons were pyramidal in shape (Fig. 1I) and approximately 1/20 of the whole neural population in the region. No staining was observed in other areas studied such as the cerebral cortex and magnocellular neurosecretory system.

In all organs studied, the staining revealed by peroxidase (ABC reagent) did not differ in the sections fixed by either paraformaldehyde or glutaraldehyde. Of all the tissue fixed by 4% paraformaldehyde, 1 h preincubation in avidin reduced the staining considerably as demonstrated in the sections adjacent to the endogenous biotin-positive sections (Fig. 2A, B). In the sections fixed by

Fig. 1. Adjacent sections from the kidney (**A, B**), the liver (**D, E**), and the hippocampus of brain (**G, H**). Incubation with avidin-peroxidase only resulted in intense staining indistinguishable from authentic immunohistochemical signal in particular structures of the tissues (**A**, **D**, **G**). No staining could be observed in their adjacent sections without avidin-peroxidase (**B**, **E**, **H**). Higher magnification revealed that the stained structures are proximal tubule in the kidney (**C**), hepatocytes in the liver (**F**), and pyramidal neurons in the hippocampus (**I**). (*CA3* field CA3 of hippocampus). *Arrows* (*G, I*) indicate stained pyramidal neurons in hippocampus. *Scale bars* 5 mm (**A, B**); 0.2 mm (**C, F**); 1 mm (**D, E, G, H**); 0.5 mm (**I**)


A
B
C
D
E
F
G
CA3
H
CA3
I
CA3

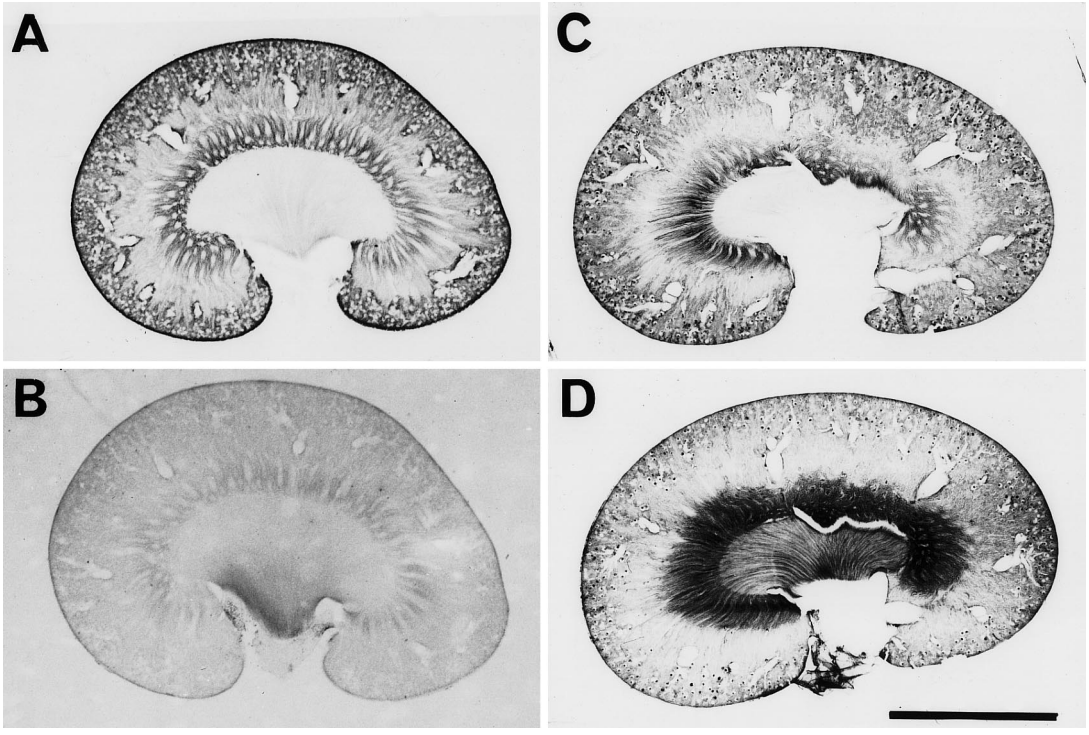


Fig. 2 Two sets of adjacent kidney sections fixed with 4% paraformaldehyde (**A, B**) and 5% glutaraldehyde (**C, D**). Pretreatment with avidin (**B, D**) considerably reduced the endogenous biotin staining in paraformaldehyde-fixed section (compare **A** and **B**) but had no effect in glutaraldehyde-fixed tissue (compare **C** and **D**). *Scale bar* 5 mm

5% glutaraldehyde, however, the avidin-blocking treatment did not effectively reduce the staining and, indeed, sometimes enhanced it (Fig. 2C, D).

Analysis of the Medline database for studies on immunolocalization of various antigens in the rat kidney revealed the fact that, for the same antigen (transforming growth factor-β), positive signals were reported in the kidney proximal tubule when the same avidin-biotin conjugate technology was used (Basile et al. 1996), whereas no staining was found in the same area when avidin-biotin conjugate technology was not employed in the immunohistochemical procedure (Paul et al. 1996), or when a biotin blocker was applied (Wright et al. 1996). One hundred statistically sampled studies (from February 1994 to June 1997) on immunolocalization in the rat kidney were further investigated. As shown in Table 1, avidin-biotin conjugate technology was the most frequently employed detection system; only 5 out of 47 (10%) studies addressed the problem of endogenous biotin by applying a biotin blocker and even that blocker was not consistently effective in our experiments. The use of an avidin-biotin conjugate system was very significantly associated with the high incidence of the positive staining in the proximal tubule, compared with that of the use of other detection methods ($P<0.001$).

Discussion

The present study demonstrates the localization of endogenous biotin by avidin-peroxidase in various tissues. Firstly, this is of biological interest. Biotin is a cofactor required by carboxylases and has obligatory roles in the metabolism of carbohydrates and lipids and in the utilization of certain amino acids (Dakshinamurti and Chauhan 1989). Therefore, it is unsurprising that organs with high rates of metabolism such as liver and kidney should have higher levels of biotin. Consistent with this, our data show that it is the proximal tubule of the kidney,

Table 1 The numbers (incidences) of immunolocalization studies on rat kidney reporting positive staining in the proximal tubule using avidin-biotin and other detection methods

	Immunostaining in proximal tubule	No immunostaining in proximal tubule	Total studies
Use of avidin-biotin without biotin blocker	36 (86%)*,**	6 (14%)	42
Use of avidin-biotin with biotin blocker	1 (20%)	4 (80%)	5
Use of other methods	17 (32%)	36 (68%)	53

*$P<0.001$, compared with "Use of other methods," chi-square test; **$P<0.001$, compared with "Use of avidin-biotin with biotin blocker," chi-square test

which has the greatest metabolism of any part of the renal tubule, in which endogenous biotin is most readily detected.

It is unexpected, however, that certain neurons in the hippocampus can be demonstrated to contain more biotin than neurons in other areas of the brain. Although neurological abnormalities are one of the better-described symptoms of biotin deficiency, the etiology is attributed to the accumulation of toxic metabolites in the brain resulting from the malfunction of the biotin-dependent carboxylases (Dakshinamurti and Chauhan 1989), which would be expected to distribute evenly in the central nervous system. It is known that pyramidal neurons are among the largest neurons and have high firing rates (Martin 1991). However, it still appears unsatisfactory if our finding is linked to metabolism in any way because (1) the hypothalamic magnocellular neurosecretory neurons which are also among the largest and consistently synthesize and release hormones do not show detectable biotin; and (2) the pyramidal cells in the cerebral cortex which are similar to those in the hippocampus in most aspects (Martin 1991) do not exhibit detectable biotin. Virtually no information links hippocampal neurons to biotin except an early study that showed impaired ability of escape and avoidance learning in the rats with biotin deficiency (Stewart et al. 1966). Thus, biotin might have specialized functions in the hippocampus other than the classical role as a cofactor of carboxylases, although the possibility that those scattered neurons shown in the present study have unusually high metabolism cannot be excluded.

In addition to the biological aspect, the present study is obviously of methodological interest not only in the field of histochemistry but also to a much wider readership. Because of the superb advantages of the avidin-biotin technology, it is indispensable for many essential techniques in biomedical research, and increasing new applications constantly lead to important discoveries (e.g. Walker et al. 1997) and advances in biotechnology (e.g., Neri et al. 1997). In addition, many clinical diagnoses and treatments rely on the technology, especially in clinical imaging and drug delivery (Rosebrough 1996). However, the interference by endogenous biotin as reported here reveals a serious problem in its application. Our critical review of the literature above indicates that many false-positive results have been interpreted as true signals in immunohistochemistry, which is only one of the many applications of the avidin-biotin technology. Although not every application of the technology is necessarily affected by endogenous biotin, the problem certainly extends beyond immunohistochemistry. Therefore, precaution should be taken by determining whether endogenous biotin interferes with avidin-biotin applications. Biotin is present in virtually every cell type and in a wide range of species from bacteria to humans (Dakshinamurti and Chauhan 1989; van den Berg 1997).

In clinical practice, the interference of endogenous biotin could potentially result in misdiagnosis and serious side-effects. For example, in cancer therapy, there is increasing interest in the administration of biotinylated antibodies that are specific for unique cancerous antigens to target the particular cells, followed by delivery of therapeutic (e.g., radio-, chemo-, or cytokine) agents via avidin to specifically eliminate tumor cells (Rihova 1997). Endogenous biotin could cause highly toxic materials to be inappropriately targeted to normal tissues and damage them. Only a few people have addressed this problem by pretreatment with a blocker and these have used avidin (Kobayashi et al. 1995; Rusckowski et al. 1997). However, in the present report we have demonstrated that such blocker is not always effective. Unfortunately, the problem of interference of endogenous biotin with the avidin-biotin technology has not been publicized adequately in either research or clinical practice. Caution must be exercised in the interpretation and further use of thousands of previous studies employing the technology.

To prevent the problems caused by the interference of endogenous biotin when applying the avidin-biotin technology in the future, we propose the following as general rules:

1. Most importantly, in all applications of avidin-biotin technology, a negative control must be included in which the biotinylated reagent is omitted. This control must be performed using the entire tissue section or other materials exactly as used in the experimental groups. Currently, in cases where the specificity of a primary agent (e.g., an antibody) is well characterized, most studies fail to also include the essential control of omitting the biotinylated reagent. No such control appears to be included in many non-detection uses of avidin-biotin technology such as purification of DNA, RNA, protein, and special types of cells.
2. If the negative control on the entire material of interest does not produce the expected negative results, alternative methods avoiding avidin-biotin technology should be considered if they are available; or
3. Blockers for biotin may be carefully used but it must be kept in mind that the blocking effects of such reagents are not robust enough to be consistently repeated under various conditions. Biotin blockers should not replace the appropriate negative controls.
4. For immunocytochemical studies, the primary antibody should be preabsorbed with the appropriate antigen as a control. The primary antibody can also be omitted. These controls would reveal artifactual labeling due to endogenous biotin.

Acknowledgements We thank Dr. Solomon H. Snyder for his constructive suggestions, and Piper Hunt for reading the manuscript.

References

Basile DP, Rovak JM, Martin DR, Hammerman MR (1996) Increased transforming growth factor-β1 expression in regenerating rat renal tubules following ischemic injury. Am J Physiol 270:F500–F509

Bhattacharjee J, Cardozo BN, Kamphuis W, Kamermans M, Vrensen GFJM (1997) Pseudo-immunolabelling with the avidin-biotin-peroxidase complex (ABC) due to the presence of endogenous biotin in retinal Müller cells of goldfish and salamander. J Neurosci Meth 77:75–82

Cauli A, Yanni G, Panayi GS (1994) Endogenous avidin-binding activity in epithelial cells of the ducts of the human salivary glands. Clin Exp Rheumatol 12:45–47

Chevalier J, Yi J, Michel O, Tang X-M (1997) Biotin and digoxigenin as labels for light and electron microscopy in situ hybridization probes: where do we stand? J Histochem Cytochem 45:481–491

Dakshinamurti K, Chauhan J (1989) Biotin. Vitam Horm 45: 337–384

Diamandis EP, Christopoulos TK (1991) The biotin-(strept) avidin system: principles and applications in biotechnology. Clin Chem 37:625–636

Hsu S-M (1990) Immunohistochemistry. Methods Enzymol 184:357–363

Kobayashi H, Sakahara H, Endo K, Yao ZS, Toyama S, Konishi J (1995) Repeating the avidin "chase" markedly improved the biodistribution of radiolabelled biotinylated antibodies and promoted the excretion of additional background radioactivity. Eur J Cancer 31A:1689–1996

Martin JH (1991) The collective electrical behavior of cortical neurons: the electroencephalogram and the mechanisms of epilepsy. In: Kandel ER, Schwartz JH, Jessell TM (eds) Principles of neural science, 3rd edn. Elsevier, New York, pp 777–791

Neri D, Carnemolla B, Nissim A, Leprini A, Querzè G, Balza E, Pini A, Tarli L, Halin C, Neri P, Zardi L, Winter G (1997) Targeting by affinity-matured recombinant antibody fragments of an angiogenesis associated fibronectin isoform. Nature Biotechnol 15:1271–1275

Paul LC, Saito K, Davidoff A, Benediktsson H (1996) Growth factor transcripts in rat renal transplants. Am J Kidney Dis 28: 441–450

Paxinos G, Watson C (1982) The rat brain in stereotaxic coordinates. Academic Press, San Diego

Rihova B (1997) Targeting of drugs to cell surface receptors. Crit Rev Biotechnol 17:149–169

Rosebrough SF (1996) Two-step immunological approaches for imaging and therapy. Q J Nucl Med 40:234–251

Rusckowski M, Fogarasi M, Fritz B, Hnatowich DJ (1997) Effect of endogenous biotin on the applications of streptavidin and biotin in mice. Nucl Med Biol 24:263–268

Seidman JD, Abbondanzo LS, Bratthauer GL (1995) Lipid cell (steroid cell) tumor of the ovary: immunophenotype with analysis of potential pitfall due to endogenous biotin-like activity. Int J Gynecol Pathol 14:331–338

Stewart CN, Bhagavan HN, Coursin DB, Dakshinamurti K (1966) Effect of biotin deficiency on escape and avoidance learning in rats. J Nutr 88:427–433

van den Berg H (1997) Bioavailability of biotin. Eur J Clin Nutr 51 Suppl 1:S60–61

Walker SA, Kennedy MT, Zasadzinski JA (1997) Encapsulation of bilayer vesicles by self-assembly. Nature 387:61–64

Wilchek M, Bayer EA (eds) (1990) Methods Enzymol: Avidin-Biotin-containing reagents. Technol 184:123–138

Wright EJ, McCaffrey TA, Robertson AP, Vaughan ED, Felsen D (1996) Chronic unilateral ureteral obstruction is associated with interstitial fibrosis and tubular expression of transforming growth factor-β. Lab Invest 74:528–537

Yao C-H, Kitazawa S, Fujimori T, Maeda S (1993) In situ hybridization at the electron microscopic level using a bromodeoxyuridine labeled DNA probe. Biotech Histochem 68: 169–174

Yao Z, Zhang M, Kobayashi H, Sakahara H, Nakada H, Yamashina I, Konishi J (1995) Improved targeting of radiolabeled streptavidin in tumors pretargeted with biotinylated monoclonal antibodies through an avidin chase. J Nucl Med 36: 837–841

Yi J, Michel O, Sassy-Prigent C, Chevalier J (1995) Electron microscopic localization of mRNA in the rat kidney: improved post-embedding in situ hybridization. J Histochem Cytochem 43:801–809