Localization of Minoxidil Sulfotransferase in Rat Liver and the Outer Root Sheath of Anagen Pelage and Vibrissa Follicles

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The precise biochemical mechanism and site(s) of action by which minoxidil stimulates hair growth are not yet clear. Minoxidil sulfate is the active metabolite of minoxidil, with regard to smooth muscle vasodilation and hair growth. Formation of minoxidil sulfate is catalyzed by specific PAPS-dependent sulfotransferase(s) and minoxidil-sulfating activities have been previously reported to be present in liver and hair follicles. One of these minoxidil-sulfating enzymes has been purified from rat liver (rat minoxidil sulfotransferase, MST) and a rabbit anti-MST antibody has been prepared. Using this anti-MST antibody, we have immunohistochemically localized minoxidil sulfotransferase in the liver and anagen hair follicles from rat. In rat pelage and vibrissa follicles, this enzyme is localized within the cytoplasm of epithelial cells in the lower outer root sheath. Although the immunolocalization of MST might not necessarily correlate with the MST activity known to be present in anagen follicles, the results of this study strongly suggest that the lower outer root sheath of the hair follicle may serve as a site for the sulfation of topically applied minoxidil. *J Invest Dermatol 96:65–70, 1991*

Minoxidil (MNX) acts pharmacologically as both a vasodilator [1,2] and a hair growth stimulant [3,4]. It has been proposed that the in vivo ability of MNX to relax vascular smooth muscle is dependent on the formation of an active metabolite, minoxidil sulfate [1,2]. The formation of MNX sulfate is catalyzed by specific 3′-phosphoadenosine 5′-phosphosulfate (PAPS)-dependent sulfotransferases, two of which have been purified and characterized in some detail [5–8]. Human phenol-specific phenol sulfotransferase (P-PST) [7,8] and rat minoxidil sulfotransferase (MST) [5,6] are liver enzymes capable of transferring the sulfate from PAPS to many phenolic substrates and to the N-oxide of MNX. The P-PST enzyme, also referred to as thermostable PST, recognizes many low-molecular-weight phenolic molecules with the exception of the monoamines [7–9]. Catecholamines are sulfated by a different enzyme, namely thermolabile PST or monoamine-specific PST, M-PST [9]. Human P-PST (32 KDa) sulfates minoxidil with a Km of 890 μM, and p-nitrophenol with a Km of 0.5 μM [7,8]. This enzyme is closely related to rat liver MST, based on molecular weight, substrate recognition, kinetic constants, cytoplasmic localization, and common antigenic determinants [6]. Thus, we hypothesize that these two homologous enzymes perform similar metabolic functions in both rat liver and human liver. Although these enzymes apparently play significant roles in drug metabolism, the natural endogenous substrates for these enzymes have yet to be fully defined. To date, neither the complete peptide sequences nor the cDNA genes for these MNX-metabolizing sulfotransferases have been identified. Furthermore, the minoxidil-sulfating sulfotransferases are distinctly different from the enzymes that sulfate steroid substrates ([8,14–16]; C. Falany, unpublished results).

The ability of MNX to stimulate hair growth is a well-documented but poorly understood phenomenon [3]. One hypothesis attributes minoxidil's action to modulation of dermal blood flow [10], whereas an alternative hypothesis suggests that MNX can act directly on hair follicles [4]. In support of the latter hypothesis, in vitro studies on dissected mouse anagen hair follicles have demonstrated that MNX is active in stimulating growth and differentiation of hair epithelial cells [4]. This activity is also dependent on the formation of MNX sulfate, and MNX sulfate exhibits a greater potency than MNX in this system [22].* Attempts to localize minoxidil by immunohistochemistry and autoradiography in treated skin and follicles have not been successful [23]†, indicating that MNX is not preferentially bound to specific receptors within the hair follicle. Furthermore, dissected hair follicles have been shown to contain relatively high amounts of minoxidil sulfotransferase

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Abbreviations:

DHEA-ST: dehydroepiandrosterone sulfotransferase
IgG: immunoglobulin G
M-PST: monoamine-specific phenol sulfotransferase
MNX: minoxidil
MST: minoxidil sulfotransferase
ORS: outer root sheath
PAPS: 3′-phosphoadenosine 5′-phosphosulfate
P-PST: phenol-specific phenol sulfotransferase

* Buhl AE, Waldon DJ, Baker CA, Johnson GA: Minoxidil sulfate is the active metabolite that stimulates hair follicles (abstr). J Invest Dermatol 92:538, 1989

† Buhl AE, Kawabe TT, Waldon DJ, Knight KA, Johnson GA, Walker CJ, Diani AR: Pigmentation affects the distribution of MNX in hair follicles but does not influence its activity (abstr). J Invest Dermatol 92:409, 1989; and Zelei BV, Diani AR, Walker CJ, Kawabe TT, Buhl AE: Immunohistochemical studies with a polyclonal minoxidil antibody suggest that minoxidil is not localized in any cell type of the isolated mouse vibrissa follicle (abstr). Meeting of the Histochemical Society, 1990

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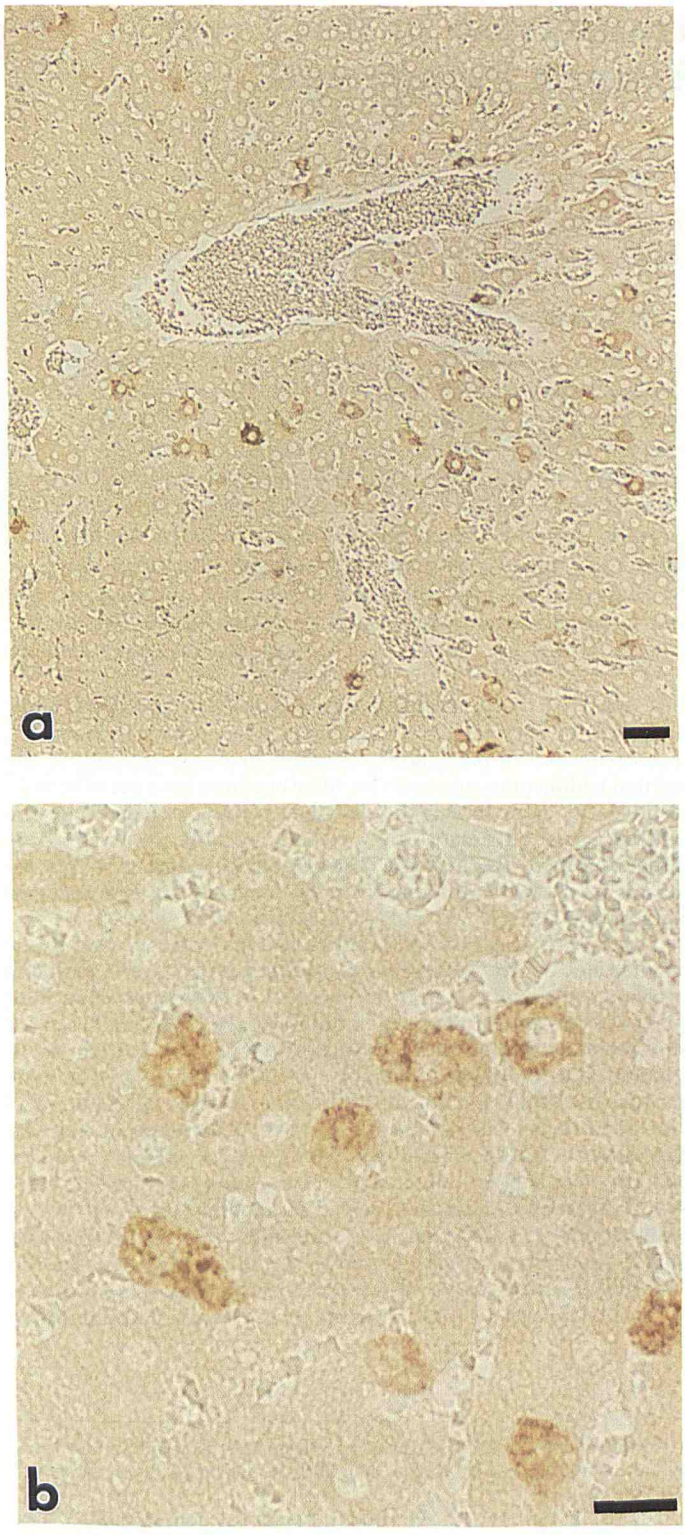


Figure 1. Rat liver section incubated with 1:2000 dilution of rabbit anti-rat MST. Immunoreactive MST is evident in the cytosol of hepatocytes, with some cells intensely stained. *a) bar,* 0.05 mm; *b) bar,* 0.02 mm.

activity [11]*, but the precise sites of MNX sulfation and pharmacologic activity within the hair follicle have not been elucidated. This paper reports the localization of MST within rat liver and anagen pelage and vibrissa follicles. Our results indicate that minoxidil sulfotransferase is present in rat hepatocytes and the lower outer root sheath (ORS) of rat anagen hair follicles.

MATERIALS AND METHODS

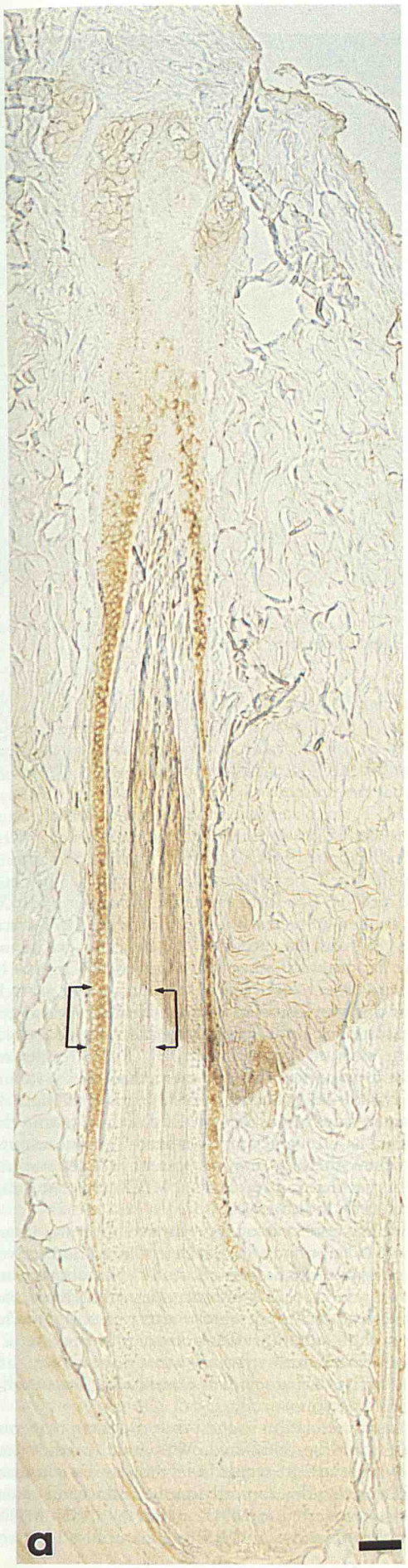
Reagents Preparation of both PAP-affinity purified rat liver MNX sulfotransferase and rabbit polyclonal anti-MST IgG have been described by Hirshey and Falany [6]. This antibody preparation has been previously utilized in Western blot experiments to demonstrate specificity for liver cytosolic MST, whereas MST was absent from the cytosols of kidney, intestine, lung, and brain from rats [6]. The stock concentrations of MST and rabbit anti-MST IgG were determined to be 20 μg/ml and 0.5 mg/ml, respectively.

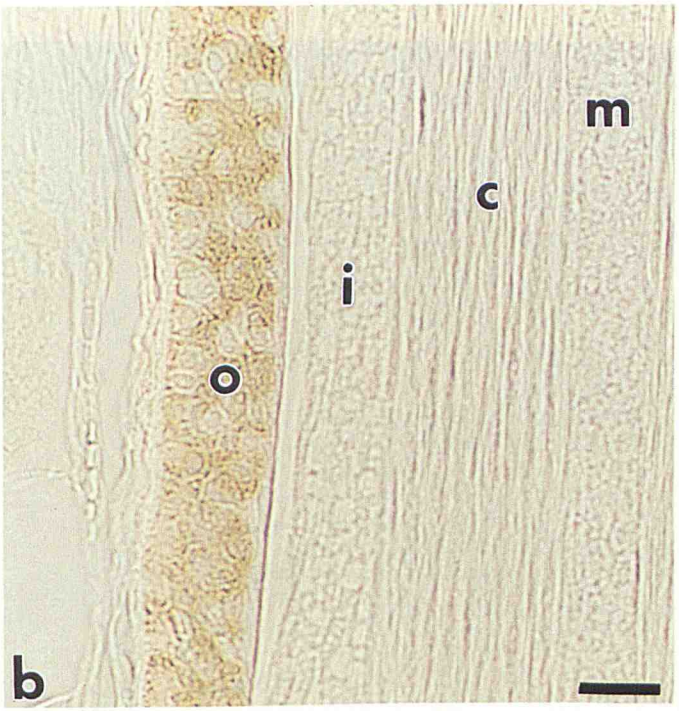
Immunohistochemical Detection of MST Liver, whisker pad, and skin from the lumbodorsal region of aged male rats (CDF F-344/Cr1BR, Charles River Laboratories) were fixed for 24 h in 10% formalin, dehydrated through ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin. Six-micron sections were mounted on albumin-coated slides and heated at 50°C for 30 min. The slides were deparaffinized in xylene, rehydrated in descending concentrations of ethanol (3 min each), and rinsed in distilled water. Endogenous peroxidase was inactivated for 30 min in 3% H_2O_2, followed by a water rinse, and nonspecific binding was blocked in 5% bovine serum albumin in phosphate-buffered saline (PBS), pH 7.2 for 20 min. After a 10-min rinse in PBS, the slides were blocked in 5% normal goat serum and were then incubated overnight at 4°C with 1:2000 dilution of rabbit anti-rat MST in PBS. Controls included normal rabbit serum, 90 μl diluted anti-MST IgG plus 10 μl of PBS, and 90 μl diluted anti-MST plus 10 μl purified MST incubated together for 1 h at 4°C, prior to reaction with the slide (preabsorption control). Sections were rinsed in PBS, incubated for 30 min with diluted biotinylated goat anti-rabbit IgG solution (Vector Laboratories), rinsed in PBS, and incubated for 45 min in peroxidase-labeled avidin (VECTASTAIN ABC reagent; refer to methods in [12,13]). Finally, the slides were rinsed in PBS, incubated for 4 min in 0.05% diaminobenzidine tetrahydrochloride (DAB; Polysciences, Inc., Warrington, PA) in PBS with 0.01% H_2O_2, rinsed in distilled water, and coverslips were mounted with Aquamount (Lerner Laboratories, Pittsburgh, PA).

RESULTS

In order to identify the site(s) of minoxidil sulfotransferase (MST) in rat liver, we performed immunohistochemical localization with a rabbit polyclonal antibody directed against purified rat liver MST. Immunoreactive MST was detected in the cytosol of rat liver hepatocytes with this anti-MST IgG preparation (Fig 1*A,B*). MST appeared to be present at modest levels in most hepatocytes throughout the liver sections, as visualized by a diffuse staining pattern. However, certain hepatocytes were heavily stained, indicating very high concentrations of cytoplasmic MST in a low percentage of the cells (estimated to be less than 5%). To indicate that the pattern of localization was specific to endogenous liver MST, primary antibody controls using both normal rabbit serum (non-immunized) and anti-MST antibody preabsorbed with purified rat liver MST were utilized. As expected, staining was not observed in liver sections that were incubated with the normal rabbit serum control and the MST-preabsorption of the anti-MST IgG dramatically reduced the cytoplasmic staining of rat liver hepatocytes (data not shown).

Recent demonstrations of the presence of MST activity in dissected rodent anagen hair follicles [11]* prompted us to determine whether an enzyme similar or identical to liver MST could be localized to any specific cell types within anagen hair follicles. A longitudinal section of a rat anagen pelage follicle stained with anti-MST IgG is shown in Fig 2*A,B*. Cross-sections of rat pelage follicles were also stained immunohistochemically (Fig 2*C*). Normal rabbit serum and MST preabsorption controls are included for comparison (Fig 3*A,C,* respectively). Immunoreactive MST is abundant and detectable only in the cytoplasm of epithelial cells in the lower outer root sheath (ORS), spanning from the apex of the dermal papilla to approximately the middle of the length of the follicle. Immunoreactive MST was not observed in the upper ORS or in any other epithelial or mesenchymal components of the hair follicle (i.e.,





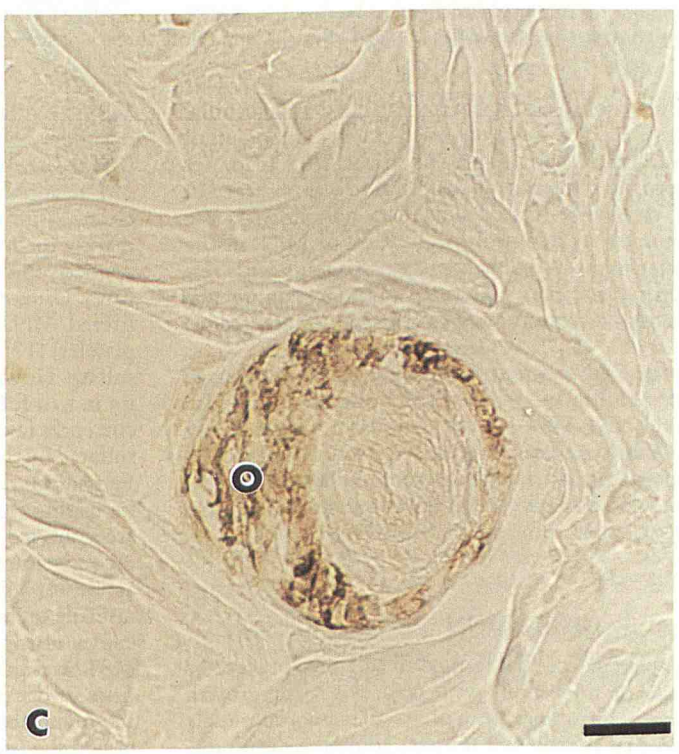


Figure 2. Localization of immunoreactive MST in rat pelage follicles. *a)* Representative longitudinal section of dorsal pelage follicle (guard hair) incubated with 1 : 2000 dilution of rabbit anti-rat MST. Note intense staining in the outer root sheath, and absence of staining in viable epidermis. *Bar,* 0.037 mm. Area within *brackets* is enlarged in *(b). b)* High magnification of same section illustrating cytoplasmic staining in the outer root sheath *(o)* and absence of immunoreactive MST in inner root sheath *(i),* cortex *(c),* and medulla *(m). Bar,* 0.02 mm. *c)* Cross-section of pelage follicle (coat hair) reacted with 1 : 500 dilution of anti-MST IgG. Outer root sheath *(o)* exhibits pronounced staining. *Bar,* 0.02 mm.

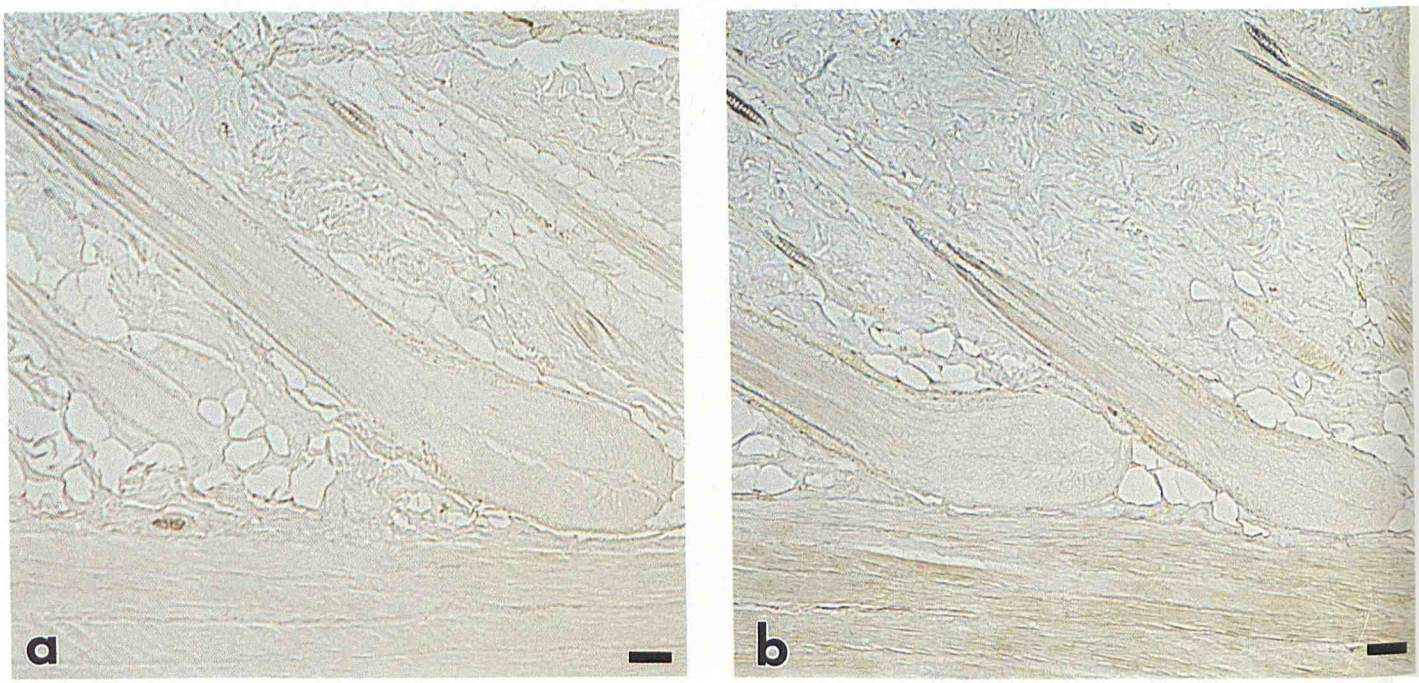


Figure 3. Control sections for immunohistochemical localization of MST in rat pelage follicles. *a)* Normal rabbit serum negative control, undiluted. *Bar,* 0.1 mm. *b)* Preabsorption control with purified rat MST. *Bar,* 0.1 mm. Note absence of staining in *a* and *b* compared to Fig 2*a*.

germinal matrix, inner root sheath, sebaceous gland, dermal papilla, etc.). Immunoreactive MST was not observed in any component of rat telogen pelage follicles, which lack the lower ORS (data not shown). Furthermore, no difference in localization was observed in anagen follicles of minoxidil-treated skin (data not shown). We have observed immunohistochemical localization of MST in the lower ORS in greater than 20 independent experiments with this anti-MST IgG preparation. This pattern of immunostaining was confirmed by an alternative technique, FITC-immunofluorescence (data not shown).

Consistent with the ORS-restricted pattern of staining in pelage follicles, the follicles within the vibrissa pad from rat (Fig 4) also contained immunoreactive-MST in the cytoplasm in the lower outer root sheath. Vibrissae from rats lacked MST in non-ORS tissues (germinal matrix, inner root sheath, cortex, dermal papilla, etc.). Thus, MST was detected only in the lower outer root sheath in whiskers, similar to the pattern in pelage follicles.

DISCUSSION

Recent findings have suggested that minoxidil sulfate appears to be the active metabolite of minoxidil, with regards to hair growth stimulation [22]* and in vitro relaxation of vascular smooth muscle [2]. The observation that rodent hair follicles contain a sulfotransferase capable of sulfating MNX [11,22]* stimulated our interest in localizing this enzyme within hair follicles. We have shown that only the lower outer root sheath cells of rodent anagen follicles contain cytoplasmic immunoreactive-minoxidil sulfotransferase (or a closely related enzyme). These data were obtained by immunohistochemical studies using an anti-MST antibody. A possible alternative method for localizing this enzyme would involve using a cloned MST cDNA probe in situ to determine where the mRNA to MST occurred. However, this alternative approach is not currently feasible, because, to date, none of the genes have been cloned to the enzymes capable of sulfating minoxidil (i.e., MST and P-PST). At this time, the only mammalian cytoplasmic sulfotransferase cDNA genes that have been cloned and reported are for those enzymes that utilize steroid substrates, such as bovine placental estrogen ST [14] and rat hydroxysteroid ST [15]. MNX is not a substrate for PAPS-dependent sulfation by these hydroxysteroid sulfotransferases or for purified DHEA-ST ([8,16]; C. Falany, unpublished observations).

The significance of MST localization to the lower ORS is unclear. Assuming that the presence of MST-antigen in the ORS is a marker for active MST, this observation indicates that MNX, when applied topically, is likely to be sulfated predominantly in the lower ORS. Unlike topically applied MNX, orally or systemically administered MNX would likely be metabolized by liver (or perhaps platelet) [17] MST or P-PST to form the active metabolite, MNX-sulfate. However, in view of recent findings indicating MST activity in hair follicles [11]* and data presented in this work, we do not currently favor the hypothesis that topically applied MNX must be sulfated by the liver in order to be active in stimulating hair growth. Further support for this conclusion arises from clinical observations indicating that the hypertrichotic effects of topically applied MNX are confined to the sites of application [3,18]. These results are inconsistent with the hypothesis of a liver-metabolized systemic action for MNX. On the other hand, the results presented here do not rule out alternative sites of possible MNX sulfation within the epidermis or dermis, as we have only localized immunoreactive MST. It is possible that other enzymes are present in skin keratinocytes or other cell types that can metabolize MNX and that other active metabolites of MNX also exist.

Because minoxidil is a potent vasodilator in vivo [1], it was interesting that the anti-MST antibody did not detect immunoreactive-MST in dermal blood vessels in the current study. This observation is consistent with a previous report indicating that MNX sulfate, but not MNX, is active on rabbit mesenteric artery smooth muscle, indicating that minoxidil sulfation does not occur at this site [2]. With regard to vasodilation, we hypothesize that minoxidil sulfate is formed primarily in liver hepatocytes or platelets and acts distally on smooth muscle via a systemic route.

The results of this investigation suggest that the transcutaneous delivery of MNX should be optimized. We would predict that formulations that bypass the ORS would be less effective at stimulating hair growth. We predict that topically-applied minoxidil enters the ORS, where it is converted to MNX sulfate by MST. MNX sulfate could act either directly on ORS cells or diffuse to affect

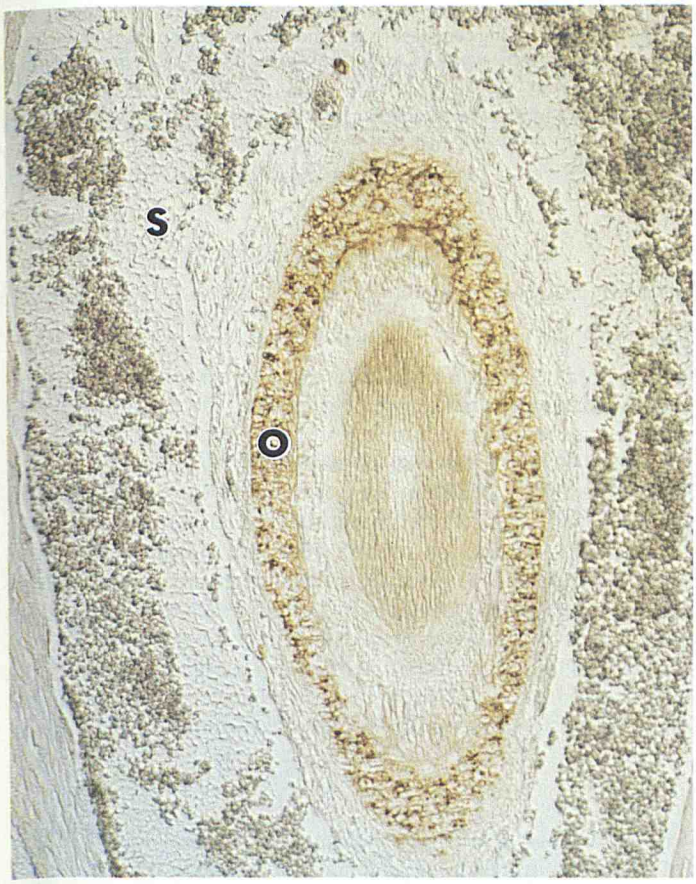


Figure 4. Localization of immunoreactive MST in rodent vibrissa follicles. Tangential section of rat vibrissa (whisker) pad, demonstrating intense staining restricted to the outer root sheath (1 : 2000 dilution of anti-MST IgG). Note absence of staining in other regions, i.e., sinus(es). *Bar,* 0.05 mm.

other target cells within the hair follicle. The sites and mechanism by which newly formed MNX sulfate acts are currently being explored.

The hair follicle is a very complex organ system, consisting of numerous cell types, most of which are epithelial in origin [19]. The outer root sheath represents only one of many concentric structures surrounding the inner root sheath layers and the hair shaft cortex. The lower ORS, where we observed MST, surrounds the epithelial cells of the inner root sheath and cortex, which are undergoing differentiation and keratinization to form the hair shaft. It is tempting to speculate that the close proximity of the MST-containing lower ORS and the keratogenous zone of the follicle indicates a causal role for MST involvement in the MNX-induced differentiation process. Interestingly, we did not observe anti-MST antibody staining in the upper portion of the ORS, which is developmentally distinct from the lower portion [19,20,24]. Fuchs and coworkers [20] have recently shown that the spatial and differential expression of keratin K14 in human follicles indicates distinct differences between the upper and lower portions of the ORS. The pattern of keratin expression in the upper ORS resembles that of the viable epidermis, whereas the region below the isthmus was unlike the epidermis. Thus, MST antigen is restricted to the portion of the outer root sheath that undergoes developmental differentiation in a pattern dissimilar to the epidermis. It is also worth noting that Oliver [21] has reported that micro-dissected follicles lacking dermal papilla but containing lower ORS cells are capable of regenerating hair follicles in vivo, suggesting these cells may play inductive roles in follicular cell proliferation and differentiation in a pleuripotent manner.

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ANNOUNCEMENT

The Second International Congress on Ultrasound and the Skin will be held September 18–21, 1991 in Modena, Italy.

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