Abstract. The tongue of the adult mouse is covered by a multilayered squamous epithelium which is continuous on the ventral surface, however interrupted on the dorsal surface by many filiform and few fungiform papillae. The filiform papillae by themselves are subdivided into an anterior and posterior unit exhibiting different forms of keratinization. Thus, the entire epithelium shows a pronounced morphological diversity of well recognizable tissue units.

We have used a highly sensitive in situ hybridization technique to investigate the differential expression of keratin mRNAs in the tongue epithelium. The hybridization probes used were cDNA restriction fragments complementary to the most specific 3'-regions of any given keratin mRNA. We could show that independent of the morphologically different tongue regions, all basal cells uniformly express the mRNA of a type I 52-kD keratin, typical also for basal cells of the epidermis. Immediately above the homogenous basal layer a vertically oriented specialization of the keratin expression occurs within the morphological tissue units. Thus the dorsal interpapillary and ventral epithelium express the mRNAs of a type II 57-kD and a type I 47-kD keratin pair. In contrast, in the anterior unit of the filiform papillae, only the 47-kD mRNA is present, indicating that this keratin may be coexpressed in tongue epithelium with different type II partners. In suprabasal cells of both, the fungiform papillae and the posterior unit of the filiform papillae, a mRNA of a type I 59-kD keratin could be detected; however, its type II 67-kD epidermal counterpart seems not to be present in these cells. Most surprisingly, in distinct cells of both types of papillae, a type I 50-kD keratin mRNA could be localized which usually is associated with epidermal hyperproliferation.

In conclusion, the in situ hybridization technique applied has been proved to be a powerful method for detailed studies of differentiation processes, especially in morphologically complex epithelia.

Keratins comprise a group of probably more than 20 related proteins that are responsible for the formation of intermediate filaments in epithelial cells. According to their charge properties the proteins can be subdivided into two distinct subclasses and one distinguishes between the basic to neutral type II keratins and the acidic type I keratins. As a rule, the complexity of a keratin pattern increases from simple to stratified epithelia, being most complex in terminally differentiating epithelia. In the epidermis, for example, the keratin expression is compartmentalized. Developmental studies (2, 19), investigations with antibodies to keratins (1, 22, 25), and analysis of sectioned epidermis (6) or epidermal cell fractions (22, 23) have shown that basal epidermal cells express a coordinate set of at least one basic and one acidic subunit which is different from the keratin pair of the living suprabasal compartment. The complexity is furthermore increased by the formation of posttranslationally derived stratum corneum equivalents mainly of the suprabasal keratin subunits (2, 3, 19, 22).

Investigations on the ordered keratin expression in stratified epithelia are difficult in cases where a continuous epithelium displays either regular, or more or less focal, morphological variations. This is the case in the adult mouse tail epidermis which exhibits an alternating pattern of para- and orthokeratotic subregions (18). By far the most heterogenous epithelium is that of the adult mouse tongue, which is basically covered by a multilayered squamous epithelium. On the dorsal surface, however, it is interrupted by a multitude of papillary projections which themselves seem to be morphologically subdivided (4, 10).

In the present study we have investigated the keratin expression in the tongue epithelium by means of in situ hybridization with various, highly specific keratin cDNAs. We have recently improved the experimental conditions of this technique (17; Knapp, B., M. Rentrop, J. Schweizer, and H. Winter, manuscript submitted for publication) and show here that especially in morphologically complex epithelia it represents an excellent tool for exact studies of differentiation processes.

Parts of this investigation have been presented at the 5th...
Materials and Methods

Dissection of Tongue

About 7-wk-old NMRI-mice were killed by cervical dislocation. The tongues were cut off at the apical region of the sulcus terminalis, so that the removed portion comprised the freely movable anterior two-thirds of the organ (4).

Keratin Analysis

The dissected tongues were placed for 20 s in 60°C hot water. Subsequently the entire epithelium could easily be peeled off as an intact cup-shaped sheet with the stratum corneum inside. Histological examination confirmed that virtually no epithelial cells remained attached to the connective tissue core of the tongue. Keratin proteins were isolated from the epithelia essentially as described (28). They were analyzed by nonequilibrium pH gradient electrophoresis according to O’Farrell et al. (16). Gels were stained with 0.1% Coomassie Blue in 40% methanol, 10% acetic acid, and destained in 40% methanol, 10% acetic acid.

Keratin cDNA Clones and Subclones

We have recently isolated and characterized the following keratin clones: (a) clones pkt 57-1 and pkt 47-1 from a library of mouse tongue epithelium poly(A)RNA. These clones are complementary to the mRNA of a type II 57-kD and a type I 47-kD keratin protein (12). (b) Clone pke 59 from a cDNA library of adult mouse footpad epidermis poly(A)RNA containing sequences coding for the type I 59-kD keratin protein of epidermal suprabasal cells (13; Knapp, B., M. Rentrop, J. Schweizer, and H. Winter, manuscript submitted for publication). (c) Clones pkSCC 52 and pkSCC 50 from a library constructed with poly(A)RNA from a transplacental squamous cell carcinoma of mouse back skin containing sequence information for a type I 52-kD keratin protein of basal epithelial cells and for a type I 50-kD keratin expressed in hyperproliferative mouse epidermis (Knapp, B., M. Rentrop, J. Schweizer, and H. Winter, manuscript submitted for publication). The five cDNA clones were digested with restriction enzymes to yield fragments comprising the 3'-noncoding regions of the corresponding mRNAs (without the poly [A] tail) and the regions (or at least parts of the regions) coding for the carboxy-terminal domain of the proteins (24). Details of these procedures are illustrated and specified in Fig. 1. The restriction fragments were subcloned into pUC 8 and used as probes for in situ hybridization experiments.

In Situ Hybridization

Removal tongues were placed on cork plates (1 cm ø), coated with embedding medium and immediately frozen in liquid nitrogen-cooled isopentane. The tongues were orientated differently on the cork plates to enable the preparation of sagittal and frontal sections, as well as samples with a more or less tangential plane of section relative to the dorsal tongue surface. Frozen sections (nominally 5 \( \mu \)m) were cut using a cryostate microtome at -20°C. They were immediately placed on aminopropyltriethoxysilane-pretreated glass slides (17), air dried, fixed in 4% paraformaldehyde in phosphate-buffered saline, 5 mM MgCl2, and stored in 70% ethanol at 4°C until further use. In situ hybridization with [\( ^{35} \)S]-labeled cDNA subclones was performed essentially as recently described (12, 17). The specificity of hybridization was checked by multiple control experiments: (a) RNase treatment of tissue sections before hybridization, (b) competition experiments with unlabeled homologous cDNA probes, (c) studies on cDNA probe-concentration dependence, (d) kinetic studies on hybridization time, and (e) wash temperature kinetics.

Results

Morphology of the Tongue Epithelium

The ventral side of the mouse tongue is covered by a keratinizing epithelium that consists of a row of cuboidal basal cells, three to four layers of spinous cells, a thin granular cell layer with sparsely scattered, small keratohyalin granules, and a prominent stratum corneum (Fig. 2, a and b). Unlike the ventral epithelium, the dorsal epithelium of the tongue exhibits a very complex morphology in that numerous papillary projections are distributed within an interpapillary epithelium. Morphologically, the latter resembles the ventral epithelium except it has a markedly less condensed stratum corneum (Fig. 2, c and d). By far the most preponderant papillae in the anterior two-thirds of the tongue are of the filiform type. They possess a complex three-dimensional structure (4) and in sagittal sections show up as hook-shaped posteriorly pointing projections whose basal cell layer is folded over a dermal papilla of connective tissue (Fig. 2, c and d). Apparently, the filiform papilla consists of two morphological units. The anterior unit is clearly orthokeratinizing, whereas the cells of the posterior unit do not pass through a granular stage and give rise to an extremely condensed stratum corneum in which nuclear remnants frequently can be seen (10; Fig. 2 d). In carefully orientated sections the precise unit boundaries can be traced from the surface down to the epithelial–dermal junction (Fig. 2 d).

A second type of papilla, the broad and dome-like fungiform papillae, are distinctly less numerous (\(~40\) papillae). Their morphology has only been poorly described in the mouse tongue (4). Sagittal sections show that they consist of an ascending wall of cells which line a prominent dermal

Figure 1. Diagram showing the specific restriction fragments of five keratin cDNA clones used as hybridization probes. Hatched bars represent the end of the regions coding for the \( \alpha \)-helical domains. Open bars indicate the regions that code for the carboxy-termini of keratin proteins (positions of stop codons marked by open arrowheads), and the 3'-noncoding regions of the mRNAs (A, poly[A] tail). For each of the clones the restriction fragment to be subcloned is indicated between arrows. The length of the individual fragments is: 553 bp (pkt 57-1), 387 bp (pkt 47-1; Mbo-I-HindII fragment; details of this fragment given in Knapp, B., M. Rentrop, J. Schweizer, and H. Winter, manuscript submitted for publication), 255 bp (pkSCC 50), 292 bp (pkSCC 52), 392 bp (pke 59).

Figure 2. In situ hybridization with the specific fragment of clone pkt 57-1 (a, c, and e) and pkt 47-1 (b, d, and f). (a and b) Ventral epithelium; (c and d) dorsal epithelium, sagittal sections; (e and f) dorsal epithelium, tangential sections. The arrowheads in a and b indicate basal cells that are labeled above background noise by both fragments. The arrowheads in d point to the demarcations of the different morphological units. The dashed line outlines the boundary between the anterior and posterior unit of the filiform papilla. \( fp \), Filiform papilla; ac, anterior compartment; pc, posterior compartment; isc, interpapillary stratum corneum; ipe, interpapillary epithelium; dp, dermal papilla; bc, basal cells; khg, keratohyalin granules. Bar, 60 \( \mu \)m.

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Two-dimensional Keratin Pattern of the Tongue Epithelium

Non-equilibrium pH gradient electrophoresis resolution of tongue epithelium keratins yields a complex pattern of ~10 subunits (Fig. 3). The most prominent proteins are a type II 57-kD and a type I 47-kD subunit, a keratin pair which is also present in the esophageal and forestomach epithelium (unpublished results). The remaining minor proteins are only faintly visible and have not been mentioned in previous investigations (5). Based on their positions in relation to the 57-kD and 47-kD proteins or on the coelectrophoresis of tongue keratins with keratins from both newborn mouse epidermis and epidermal cells in culture (i.e., probes that allow a good visualization of the suprabasal and basal epidermal keratin subunits [results not shown]), it could be deduced that some of these minor keratin proteins in the mouse tongue epithelium may correspond to epidermal keratin subunits. Apparently, the suprabasal epidermal 59-kD subunit, the basal epidermal 60-kD and 52-kD keratin pair (22) as well as a 50-kD subunit typical for hyperproliferative epidermis, epidermal tumors, and cultured epidermal cells (20, 21, 29) are present in the tongue epithelium. In contrast, the type II 65-kD and 62-kD subunits seem to have no epidermal counterpart.

In Situ Hybridization

We have recently shown that in situ hybridization with a specific cDNA fragment of clone pkt 57-1 leads to a deposition of silver grains in suprabasal cells of both the entire ventral epithelium and the dorsal interpapillary epithelium of the tongue (12, 17; Fig. 2, a and c). Within these epithelial regions there is a clear-cut density gradient of grains from the spinous to the granular layer, and in the stratum corneum no hybridization signals could be detected. The observed label is RNase-sensitive and is dependent on stringency conditions (12, 17) and the amount of probe used for hybridization. Moreover the hybridization signals decrease after the addition of unlabeled homologous, but not heterologous, DNA (results not shown).

Essentially the same distribution of silver grains was found after hybridization with the specific fragment of clone pkt 47-1 (Fig. 2, b and d). In addition, both cDNA fragments occasionally labeled basal cells which were especially well visible in the ventral epithelium (arrowheads in Fig. 2, a and b; [22]). However, unlike clone pkt 57-1, the pkt 47-1 fragment also recognized the suprabasal living cell compartment of the orthokeratinized anterior unit of the filiform papillae (compare Fig. 2, c and d). The labeling characteristics of both cDNAs are best visualized in tangential sections. De-
pending on the plane of sections the filiform papillae appear as more or less oval, well demarcated structures within the inter papillary epithelium, and their anterior and posterior units can easily be distinguished by their different type of keratinization. Fig. 2 e shows that the entire papillary areas are free of pkt 57-1 label, whereas pkt 47-1 hybridization signals are clearly detectable in the suprabasal compartment of the anterior units (Fig. 2 f).

Recently we could show that clone pkSCC 52 contains sequence information for a 52-kD keratin expressed in basal cells of the epidermis (Knapp, B., M. Rentrop, J. Schweizer, and H. Winter, manuscript submitted for publication). In situ hybridization of the specific cDNA fragment of this clone to tongue sections also results in a uniform labeling of the entire basal layer irrespective of ventral (Fig. 4 a), dorsal inter papillary or papillary regions (Fig. 4 b). The label is, however, not uniquely restricted to the very basal cells, but can also be detected, although with reduced intensity, in few parabasal cells. In contrast, in cells of the upper strata, a significant deposition of silver grains above background noise can virtually be excluded.

Clone pke 59, which contains sequences complementary to the mRNA of the type I 59-kD keratin protein (13; Knapp, B., M. Rentrop, J. Schweizer, and H. Winter, manuscript submitted for publication), has been shown to specifically label the suprabasal living cell layers (Knapp, B., M. Rentrop, J. Schweizer, and H. Winter, manuscript submitted for publication). In situ hybridization with the specific cDNA fragment of this clone to sagittal tongue sections reveals small labeled areas corresponding to suprabasal living cells of the posterior unit of the filiform papillae (Fig. 5 a). Again, tangential sections are ideally suited to unequivocally confirm the localization of the hybridization signals in these areas (Fig. 5 b).

Clone pkSCC 50 contains sequences that code for a 50-kD keratin protein expressed in cultured epidermal cells, epidermal tumors, and hyperproliferative epidermises (20, 21, 29). Hence, this protein corresponds to the human 48-kD keratin (28; keratin 17 [14]) and together with a type II 59.5-kD keratin protein forms the "hyperproliferative" keratin pair of stimulated mouse epidermises (unpublished results). A priori we therefore did not expect the presence of the 50-kD mRNA in cells of the tongue epithelium. Surprisingly, however, we observed distinct labeled areas within the dorsal tongue epithelium after in situ hybridization of the specific fragment of clone pkSCC 50 to tissue sections (Fig. 6). Apparently the transcript is present in those basal and parabasal cells of the filiform papillae that surround the apex of the dermal papillae, and the label seems to be particularly dense in basal cells that belong to the posterior unit of the filiform papillae (Fig. 6).

The distribution of hybridization signals obtained with the five cDNA probes within the fungiform papillae is visualized in Fig. 7. The specific probes of clones pkt57-1 (Fig. 7 a) and pkt 47-1 (not shown) did not lead to detectable label in any part of this type of papilla. However, as already observed for the ventral and dorsal inter papillary and papillary epithelia, the cDNA fragment of pkSCC 52 strongly labels the basal cells of the fungiform papilla (Fig. 7 b). Thus the basal pkSCC 52 label is virtually continuous along the entire tongue epithelium. The specific fragment of clone pke 59
leads to a cap-like deposition of silver grains in the differenti-
tiated cell layers of the flattened papillary surface epithelium
(Fig. 7 c). This suprabasal expression of the 59-kD mRNA
is especially well documented by the complete absence of la-
bel in a tangentially cut fungiform papilla (Fig. 7 d) in which
the plane of section has obviously traversed the basal cell
compartment. The most surprising observation was made af-
fter in situ hybridization with the specific pkSCC 50 frag-
ment. As shown in Fig. 7, e and f, the corresponding mRNA
is present in the entire epithelium of the fungiform papilla.
The specific labeling characteristics of the five cDNA clones
in the different morphological units of the tongue epithelium
are summarized in Table I.

Discussion
The aim of the present study was to elucidate the differen-
tiation pathways in a morphologically particularly complex epi-
thelium by means of RNA-cDNA in situ hybridization with
a variety of keratin cDNAs. A precautionary measure to en-
sure the selective cellular recognition of individual keratin
mRNAs consisted in the consequent use of cDNA restriction
fragments that comprised sequences corresponding to the
most specific region of any given keratin mRNA. Thus, each
fragment contained the 3'-noncoding tract (without the
poly[A]tail), generally unique for individual members of
various multigene families (30). Regarding the adjacent se-
cquences coding for the carboxy-terminal domain of the pro-
teins, the pkt 57-1 fragment lacked the sequences that code
for the highly conserved H 2-subdomain of type II keratins
(24). Similarly, the pke 59 fragment was devoid of sequences
coding for the glycine-serine-rich V 2-subdomains (24). In
two cases (clones pkt 47-1 and pkSCC 52) the cDNA frag-
ments still contained short stretches (maximally 30 bp) cod-
ing for the terminal part of the a-helical domain. However,
these fragments also represent highly specific hybridization
probes since they did not lead to detectable cross-hybridiza-
tion under the stringency conditions used. Moreover, recent
investigations on the differential location of collagen mRNAs
have demonstrated that only probes exhibiting amazingly
high sequence homologies (>70%) lead to substantial cross-
hybridizations (8).

The in situ hybridization experiments have shown that the
various morphologically discernable units of the mouse
tongue epithelium also display unique forms of keratin ex-
pression. Each unit consistently exhibits a compartmental-
azation into basal and suprabasal keratins. Surprisingly, the
entire basal cell layer of the complex tongue epithelium uni-
formly expresses a mRNA that codes for a type I 52-kD ker-
atin. The same mRNA (as well as the corresponding type II
60-kD keratin mRNA; results not shown) is also located in
the basal cell compartment of epidermis, palate (Knapp,
B., M. Rentrop, J. Schweizer, and H. Winter, manuscript
submitted for publication), forestomach, and esophagus epi-
thelium (manuscript in preparation), suggesting that it rep-
resents an ubiquitous basal component of mouse stratified
epithelia, independent of the keratin expression in the dif-
ferentiating compartments. The present investigation does
not permit us to decide whether the 52-kD mRNAs detect-
able more or less frequently in parabasal cells represent in-
tact or already partially degraded transcripts. Collectively, it
may be concluded that in terminally differentiating epithelia,
no significant suprabasal expression of basal cell keratin
mRNAs takes place. Immediately above the homogeneous
basal cell layer of the tongue epithelium an impressive diver-
gence and specialization of the keratin expression is ob-
served. In effect, as soon as the cells begin to differentiate
into the various morphologically recognizable tissue units,
they express a unique and unit-specific keratin spectrum,
thus lending biochemical support to the suggestions that, for
example, the anterior and posterior parts of the filiform
papillae represent independent and sharply defined prolifera-
tion units (10).

Comparative analysis of the morphology and the keratin
pattern in the suprabasal compartments of the tongue epithe-
lium may be of interest for general understanding of epithe-
lial keratin expression. Morphologically, both the ventral
epithelium and the dorsal interpapillary epithelium of the
tongue clearly display all basic features of a terminally
differentiating tissue. In fact, in many aspects both resemble
the epidermis of newborn mice. It has recently been stated that the size of keratin subunits increases from the inner to the outer cell layers as a function of the complexity of the tissue in which they are expressed (26). However, not only is the 57-kD/47-kD keratin pair expressed in the ventral and dorsal interpapillary tongue epithelium different from and smaller than that of the epidermis (67 kD/59 kD) but, more importantly, this keratin pair is also distinctly smaller than the basal cell keratin pair (60 kD/52 kD). Thus, pronounced epithelial complexity and terminal differentiation must not generally be associated with the expression of only high molecular weight keratin subunits.

Furthermore, the presence of the 47-kD keratin mRNA and the definitive lack of the 57-kD keratin mRNA in the suprabasal cells of the anterior part of the filiform papillae implies that the 47-kD keratin is able to associate with different type II partners in adjacent regions of the same tissue. Similar aspects hold true for the 59-kD keratin whose mRNA is expressed in both the suprabasal compartment of the fungiform papillae and the posterior part of the filiform papillae. Since even in overloaded gels no traces of a 67-kD keratin (the type II partner of the 59-kD keratin in epidermis [22]) can be detected, it seems likely that the 59-kD subunit is coexpressed with a still unidentified type II keratin of
tongue epithelium. It remains to be seen whether the basic 65-kD and 62-kD keratin subunits present in the tongue keratin pattern may act as possible complementary type II proteins of the 47-kD and 59-kD subunits. In both cases, however, any of the resulting combinations would not comply with the proposal that “within each keratin pair the basic member is always larger than the acidic member by approximately 8 kD” ([26]; see, however, reference 7).

A particularly intriguing result is the expression of the mRNA of the 50-kD keratin in the filament and fungiform papillae. This keratin subunit is commonly associated with terminal differentiation in mouse and human epidermis. [20, 21, 29]; see also reference 19. Proteolytic modification of acidic and basic keratins during terminal differentiation in mouse and human epidermis. Eur. J. Biochem. 142:29-36.

References

11. Deleted in press.
12. Knapp, B., M. Rentrop, J. Schweizer, and H. Winter, manuscript submitted for publication). Since the proliferative rate of this follicular region is basically similar to that of the adjacent normal epidermis (15), it appears that this keratin subunit may also be expressed under nonhyperproliferative conditions.

In conclusion, our experiments have shown that the in situ hybridization with specific keratin cDNAs is particularly suited to reveal even minor details of complex epithelial differentiation pathways. A priori the method allows a more precise cellular localization of the site of biosynthesis of individual keratins than protein immunohistochemistry (which also detects transported and stored keratin proteins and can be influenced by masking and secondary modification of proteins). The RNA-cDNA in situ hybridization would therefore be an essential tool for studies of developmentally, pathologically, and experimentally induced modulations of epithelial keratin gene expression.

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Table I. cDNA Clone-specific Localization of Hybridization Signals in Tongue Epithelium

<table>
<thead>
<tr>
<th>Tissue portion</th>
<th>pkS57-1</th>
<th>pkS47-1</th>
<th>pkSCC52</th>
<th>pkE59</th>
<th>pkSCC50</th>
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<tbody>
<tr>
<td>Ventral epithelium</td>
<td></td>
<td></td>
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<tr>
<td>Basal cells</td>
<td>+</td>
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<tr>
<td>Suprabasal cells</td>
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<tr>
<td>Dorsal interpapillary epithelium</td>
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<tr>
<td>Basal cells</td>
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<tr>
<td>Suprabasal cells</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Filiform papillae</td>
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<td></td>
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<tr>
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<td>+</td>
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<tr>
<td>Anterior unit, suprabasal cells</td>
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<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Posterior unit, basal cells</td>
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<td></td>
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<td>+</td>
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<tr>
<td>Basal cells</td>
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<tr>
<td>Suprabasal cells</td>
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* The label is restricted to cells surrounding the apex of the dermal papillae.