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Sampling Technique to Discriminate the Different Tissue Layers of Growing Antler Tips for Gene Discovery

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ABSTRACT

The utilization of a deer antler model to study gene expression in tissues undergoing rapid growth has been hampered by an inability to sample the different tissue types. We report here a standardized procedure to identify different tissue types in growing antler tips and demonstrate that it can help in the classification of expressed sequence tags (ESTs). The procedure was developed using observable morphological markers within the unstained tissue at collection, and was validated by histological assessments and virtual Northern blotting. Four red deer antlers were collected at 60 days of growth and the tips (top 5 cm) were then removed. The following observable markers were identified distoproximally: the dermis (4.86 mm), the subdermal bulge (2.90 mm), the discrete columns (6.50 mm), the transition zone (a mixture of discrete and continuous columns) (3.22 mm), and the continuous columns (8.00 mm). The histological examination showed that these markers corresponded to the dermis, reserve mesenchyme, precartilage, transitional tissue from precartilage to cartilage, and cartilage, respectively. The gene expression studies revealed that these morphologically identified layers were functionally distinct tissue types and had distinct gene expression profiles. We believe that precisely defining these tissue types in growing antler tips will greatly facilitate new discoveries in this exciting field. Anat Rec 268:125–130, 2002.

Key words: antler; gene expression; histology; dissection; cartilage; growth

The gene expression profiling of tissues undergoing rapid growth is not yet fully understood. Systems in which normal growth occurs at an astonishing rate, such as in the placenta, embryo, and deer antler, can give significant insights into tissue growth and repair in general. Antler is a prime example of rapid growth, with rates during late spring and early summer reaching 12.5 mm/day in sika deer (Gao and Li, 1988) to 27.5 mm/day in elk (Goss, 1970). In the growth region at the tip of the antler, multiple tissue types are represented, from skin on the surface to the area where stem cell proliferation occurs, and then to cartilage growth and transformation to bone. While models for soft-tissue growth exist, relatively little is known about cartilage and bone growth. Deer antler provides the unique opportunity to study these processes in a system in which the growth rate is accentuated. However, gene expression studies aimed at revealing the mechanism underlying rapid antler growth have been hampered by an inability to sample different tissue types. We report here a standardized procedure to overcome this problem and demonstrate that it can help in the classification of expressed sequence tags (ESTs) from antler.

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It is known that the antler growth center is located in the region of the tip (Chapman, 1975). The histological makeup of the growth center has been partially understood for over a century (Banks and Newbrey, 1982). Based on histology, regions identified from the distal to the proximal have been called the zones of proliferation, maturation, hypertrophy, and calcification. Antler's rapid growth is mainly achieved through the activity of the cells residing in the proliferation zone. The proliferation zone consists of three layers. These layers are distoproximally the reserve mesenchyme, precartilage, and cartilage. Because the histological classification of growing antler tips from Banks and Newbrey (1982) permits direct correlation to structures and events that characterize typical mammalian endochondral ossification, it has been adopted for many following studies (Goss, 1983; Kierdorf et al., 1994; Li and Suttie, 1994, 1998).

In recent years, with the advent of molecular biology, some fundamental questions regarding antler growth rates are being addressed. For instance, what is the molecular basis for this fast growth? Is this rapid growth driven by unique growth factor(s), or achieved through a unique regulating system? To answer these questions, gene expression studies have been carried out and ESTs have been isolated from the antler tip. In order to obtain tissues suitable for such studies, a new approach to sampling tissue from the antler tip is required. Although the layers of the proliferation zone in a growing antler tip are well classified (see above) and are readily identified by histological methods, we recognized a need to locate and sample these layers from a piece of unstained antler tip tissue. Any process designed to facilitate identification of functionally distinct layers would need to be rapid, thus ensuring timely and effective processing of labile substances in the tissue, and must also result in the tissue being suitable for gene expression studies.

In previous relevant studies, different approaches have been taken to sample tissues from a growing antler tip. Different tissue types were located for in vitro studies by distance measurement: the tissue 0.75 cm distal to the antler tip was classified as proliferative, and the tissue 0.75–1.5 cm distal to the tip was defined as mature (Sadighi et al., 1994). Price et al. (1994) classified antler tip into three zones by vascularity macroscopically for in vitro studies: a thin strip of vascular zone was called undifferentiated tissue; the relatively avascular zone was defined as the reserve mesenchyme and the cartilage zone. In their gene expression studies, Francis and Suttie (1998) collected tissues of reserve mesenchyme, precartilage, and cartilage from antler tips by color discrimination on the cut surface. All of the above tissue sampling methods, which differed among the studies, are not only arbitrary but are also not comparable with each other.

We have developed a generally applicable and standardized procedure to meet the sampling requirements for gene expression and in vitro studies. This procedure was confirmed by histological assessment and the probing of velvet antler tip ESTs to virtual Northern blots.

MATERIALS AND METHODS

Collection and Dissection of Antler Tissue Samples

Four antlers, grown for 60 days, were collected from 3-year-old red deer stags. The antler removal procedure was in accordance with regulations set by the New Zealand National Velveting Standard Board. The distal 5 cm of tip was removed (Fig. 1A) and sectioned sagittally (Fig. 1B) along the longitudinal axis. The tip was then cut into 5-mm-thick slices along the same plane. The slices were further cut into strips 1–2 cm across (Fig. 1C). Only the strips from the center regions were used for the tissue layer dissections.

Determination of Tissue Layers

To determine whether we could accurately identify the layers, stitches (purple 4.0 coated vicryl surgical thread; Ethicon Inc., Somerville, NJ) were sewn into the strips of antler under a dissecting microscope (Fig. 1C). The positioning of the stitches was as follows. The most distal stitch was at the junction of the dermis and the subdermal bulge. This bulge can be observed macroscopically on the surface of a longitudinally cut antler (Fig. 2). The bulge becomes more pronounced if blood is forced proximo-distally toward the tip. A stitch was then placed approximately 3.0 mm proximally, at the base of the subdermal bulge. Under the dissecting microscope discrete columns were readily identified on the cut surface of each strip just proximal to the subdermal bulge. Further proximally continuous columns were found. Between the discrete and continuous columns a zone consisting of both types of columns were located. This mixed zone covers the area in Figure 1B wherein the blood becomes visible, and a stitch was placed at the upper and lower limits of this area. The last stitch was placed at the bottom of the continuous columns, where a deep red color was visible. The antler strips were histologically processed and embedded in paraffin wax. Subsequently, the embedded blocks were sectioned at 5 μm and the sections were stained with hematoxylin and eosin (H&E)/Alcian blue (AB).

The distances between the stitches on the fresh antler strips (fresh tissue), embedded blocks (paraffin blocks), and stained sections (on slides) were measured using a digital micrometer. This allowed any adjustments for differential shrinkage of the layers to be taken into account. These data and percentage changes between stages were analyzed by analysis of variance (ANOVA), with the animal as the block structure and the layer as the treatment structure, ensuring that there was no spatial correlation.

Tissue Sampling and mRNA Preparation

Velvet antler tissue was obtained from the growing tip and dissected into distinct zones as described above. Samples of body tissues were obtained from adult red deer after they were slaughtered in an abattoir. All tissue samples were stored at −80°C until use. Total RNA was prepared from tissue samples using Trizol Reagent (Life Technologies, Bethesda, MD) and mRNA was isolated from the total RNA preparations using the MicroPoly (A) Pure Kit (Ambion Inc., Austin, TX).

Suppression Subtractive Hybridization (SSH)

A cDNA library enriched for partial sequences of differentially expressed velvet antler tip genes was constructed using the PCR-Select cDNA Subtraction Kit (Clontech Laboratories Inc., Palo Alto, CA) (Diatchenko et al., 1996). Tester cDNA (transcripts of interest) were derived from an equal volume mixture of mRNA isolated from each of the six velvet antler tip tissue zones. Driver cDNA (refer-

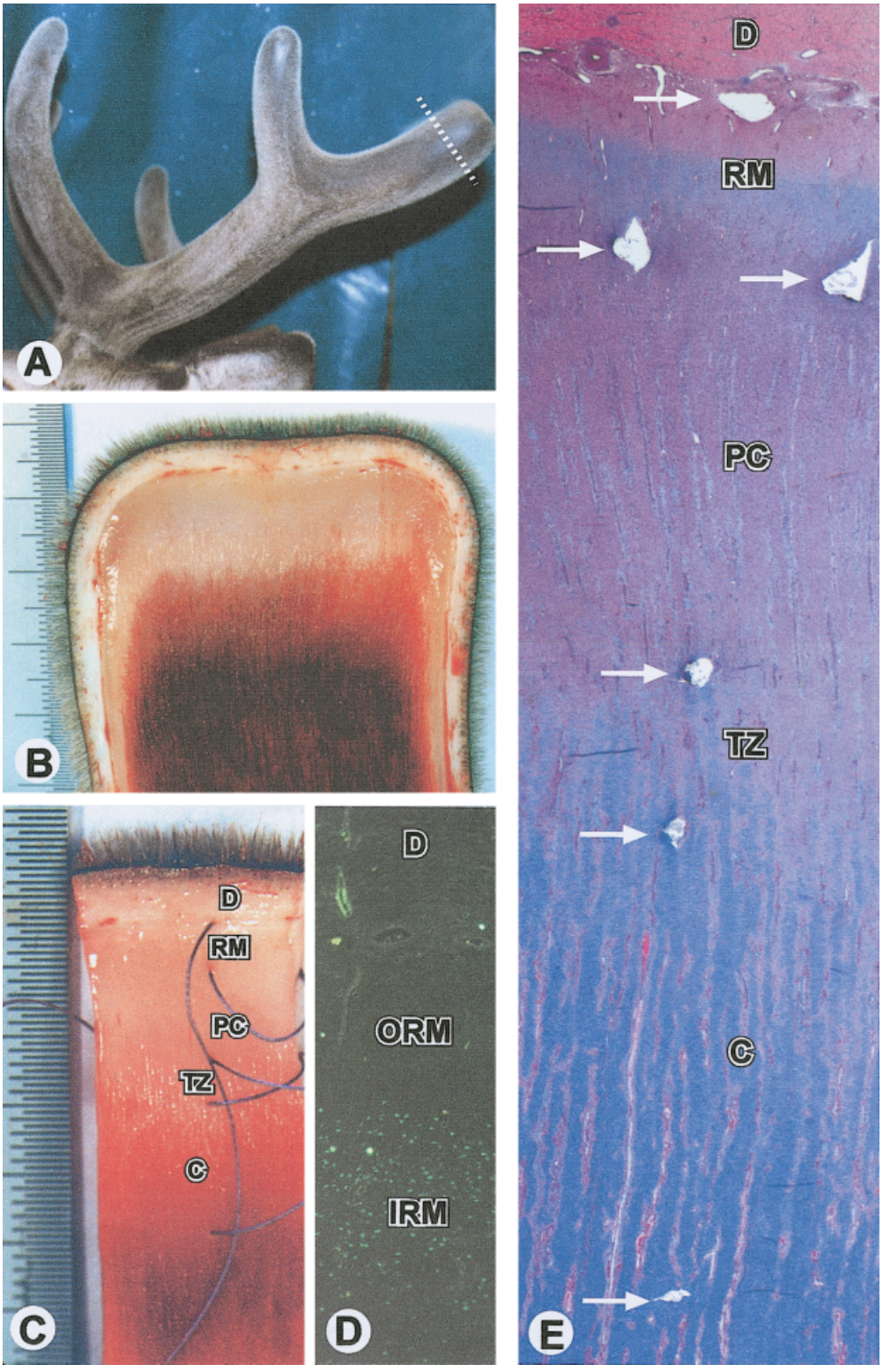

Fig. 1. **A:** Antler ready to be harvested, and line indicating amount of tip removed. **B:** Tip after being cut sagittally. **C:** Layers as identified by the distinct morphological markers and marked by the stitches. **D:** BrdU incorporation in the dermis (D), outer reserve mesenchyme (ORM), and inner reserve mesenchyme (IRM). **E:** Histological section of the antler tip, with holes from the stitches evident (arrows). D, dermis; RM, reserve mesenchyme; PC, precartilage; TZ, transition zone; C, cartilage.

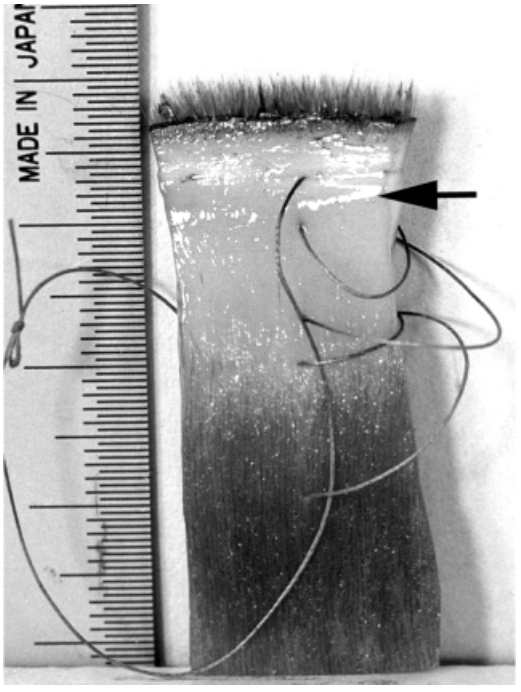

Fig. 2. A mid block of antler from a sagittally-cut tip. The bulge region is indicated by the arrow.

ence transcripts) were derived from an equal volume mixture of mRNA isolated from liver, spleen, kidney, cartilage, muscle, heart, lung, skin, and spinal cord of adult red deer. PCR products, representing differentially expressed cDNAs, were ligated into the pGEM T vector (Promega Corp., Madison, WI) and then transformed into *E. coli* DH5α cells. Colonies were grown on Luria broth (LB) agar plates containing ampicillin and X-gal in a 37°C incubator.

Cultures of individual colonies were set up in 5 ml LB/ampicillin and grown overnight at 37°C with shaking. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, CA).

Virtual Northern Blots

Double-stranded cDNA was synthesized (Sambrook et al., 1989) from the following mRNA mixtures: velvet skin; inner and outer reserve mesenchyme; precartilage, transition, and cartilage; liver, spleen, and kidney; cartilage, muscle, and heart; and lung, skin, and spinal cord. These cDNA mixtures were electrophoresed in 1% agarose gels containing ethidium bromide and transferred by alkaline Southern blotting (Sambrook et al., 1989) to Hybond N+ (Amersham Pharmacia Biotech, Auckland, New Zealand).

Probe DNA was generated from the plasmid DNA of SSH clones by PCR with the NP1 and NP2R primers (Clontech Laboratories Inc.) and gel purified using the Concert Rapid Gel Extraction System (Life Technologies). Probes were labeled with $\alpha(^{32}P)$-dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech) using the Rediprime II DNA Labeling Kit (Amersham Pharmacia Biotech). Hybridization to virtual Northern blots was performed overnight using Rapid-Hyb Buffer (Amersham Pharmacia Biotech) at 65°C. The filters were washed in 2× SSC/0.1% SDS for 20 min at room temperature and in 0.2× SSC/0.1% SDS for 15 min at 65°C. They were then autoradiographed with Kodak XAR film and intensifying screens at −80°C.

TABLE 1. Mean (n = 4) length (mm) of the five distinct tissue layers at the three processing stages with pooled SEM for each stage

	Fresh tissue	Paraffin block	On slide
Dermis	4.86	4.26	4.77
Mesenchyme	2.90	2.29	2.46
Precartilage	6.50	5.43	6.53
Transition	3.22	2.87	3.23
Cartilage	8.0	6.92	7.94
	SEM 0.461	SEM 0.381	SEM 0.450

RESULTS

Development of a Sampling Procedure

In Figure 1E, the positions of the stitches are indicated by holes, some of which still contain the silk fibers. Histological examination of the location of the stitches indicates that the regions have been very accurately marked, which confirms the validity of the dissection technique. The dermis lies in the most distal regions and is easily distinguished macroscopically (Fig. 1B and C). The reserve mesenchyme is observable by H&E/AB staining (Fig. 1E) as the area that is below the dermis and has the AB staining. The precartilage region is evidenced by the less mature vascular spaces and the chondroblasts. Stitches placed to mark the transition zone indicate a region where both precartilage and cartilage are present. Determination of the extent of the cartilage zone is more difficult. However, by shortening the region it is possible to ensure that only cartilage is collected and that the calcified and/or remodeling region is excluded.

The length of each layer in the proliferation zone was recorded throughout the procedure. These results are shown in Table 1.

The mean layer lengths at the three processing stages are given in Table 1. A shrinkage of 14.6% (SEM 1.4; $P < 0.001$) was observed when the fresh tissue was compared to the paraffin block. When the fresh tissue was compared to the tissue sections on the slides, a difference of 3.1% (SEM 1.9; $P > 0.05$) was detected, indicating a recovery to approximately the original size. In both cases no one layer was affected more significantly than any other. The mesenchyme was the narrowest layer at 2.90 mm. Figure 3 shows a diagrammatic representation of the layers.

Virtual Northern Blots

The SSH procedure was successfully enriched for cDNA sequences specific to the antler tip. The virtual Northern blots show that the ESTs are expressed in velvet antler tissue, but not in adult body tissues (Fig. 4), as would be expected with truly differentially expressed cDNA clones. For example, clone A49 is expressed in all zones of the velvet antler tip, with two transcripts of approximately 2.0 kilo-basepairs (kb) and 2.4 kb (Fig. 4A). Expression of the smaller A49 transcript appears to be variable across the velvet antler tip zones, being most abundant in the reserve mesenchyme, intermediate in the precartilage to cartilage zones, and least abundant in the skin (Fig. 4A).

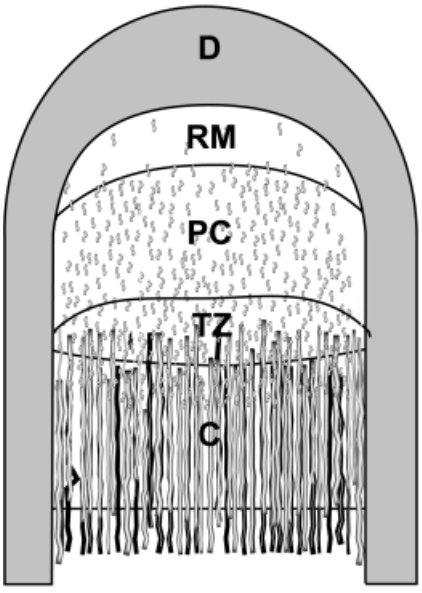

Fig. 3. Diagram of layers on a histological section of a growing antler tip. D, dermis; RM, reserve mesenchyme; PC, precartilage; TZ, transition zone; C, cartilage.

Expression of the larger A49 transcript is intermediate across all velvet antler tip zones (Fig. 4A).

Clone A39 is only expressed in the precartilage to cartilage zones as a single transcript of approximately 800 bp (Fig. 4B). Clone A44 is expressed in the skin and precartilage to cartilage zones, but not in the reserve mesenchyme (Fig. 4C). The highly abundant A44 transcript in the precartilage to cartilage zones at approximately 1.7 kb is slightly smaller than the less abundant transcript in the skin at approximately 1.9 kb (Fig. 4C).

These results confirm that RNA species can be specifically assigned to histologically and functionally distinct tissue areas within the antler.

DISCUSSION

The astonishing growth rate of deer antlers offers an opportunity for discovering new potent growth factors and unique regulating systems. Multiple systems can be studied, including cartilage growth, bone transformation, vascular, nerve, and soft-tissue growth. However, the molecular approach toward these findings has been hampered by an inability to rapidly and precisely locate different tissue types from an unstained growing antler tip. The antler is well organized, with the dermis, mesenchyme, precartilage, and cartilage areas contributing and responding to growth and differentiation in different ways. In this study we aimed to strengthen our gene discovery program by establishing a way to accurately determine which layers are dissected at the time of tissue collection.

Although the histological identification of the various layers is clear (Fig. 1E), it is difficult to identify them in the fresh antler so that various regions can be collected for molecular analysis.

To do this we developed a standardized procedure that will make comparison between different studies possible. This procedure has been confirmed by hybridization of velvet antler genes to virtual Northern blots. We have thus far successfully used this procedure for studying the expression of genes in growing antler tips using RT-PCR, Northern blot analysis, and in situ hybridization. The findings demonstrate that the techniques described for tissue dissection allow for the identification of genes regulating antler growth. Antler provides a unique model for the discovery of known genes involved in rapid growth processes. The fact that it grows at such a dramatic rate suggests that the factors operating to drive this system may be expressed in large quantities, making the discovery process more straightforward. Understanding these regulatory processes has important implications for understanding and controlling growth in antlers, as well as in other tissues in which the control of growth rate is important. Furthermore, unique growth systems operate in antler, thus presenting a unique opportunity to discover novel genes and regulatory pathways. This study demonstrates that antler is a valuable model that will facilitate new discoveries in this exciting field.

In a previous study, Banks and Newbrey (1982) showed that the proliferation zone could be divided into three layers based on H&E/AB staining: the reserve mesenchyme (light blue staining), precartilage (no blue staining), and cartilage (deep blue staining) (Fig. 1E). We have found that the reserve mesenchyme can be further divided into two sublayers, based on our results from BrdU (5-bromo-2′-deoxyuridine) localization (Fig. 1D). These sublayers are termed the outer reserve mesenchyme (ORM) and the inner reserve mesenchyme (IRM). BrdU is a synthetic analogue of thymidine and can be taken up only by dividing cells. The results showed that the ORM is nearly devoid of BrdU-labeled cells, whereas the IRM consists of intensively BrdU-labeled cells. Therefore, as each sublayer is clearly different, the ORM and IRM could be investigated separately. However, it is problematic to accurately dissect such a small amount of tissue, so for the purposes of this work they are considered together.

Between the discrete column precartilage and continuous column cartilage, a narrow zone containing both tissue types can be observed. We call this the transition zone (Fig. 1C and E). Its inclusion ensures that the true precartilage and cartilage tissues are sampled. Below the cartilage region, calcification and remodeling occur, so by slightly truncating the amount of tissue collected it is possible to exclude this process. Conversely, by collecting tissue below this region the process of calcification can be studied.

We have shown that different tissue types can be rapidly and accurately sampled using the measurement of layer length for 60-day-old antlers from 3-year-old red deer stags. Although the lengths recorded can be used on similar animals, the measurements may not be applicable to antlers at different stages of growth. However, we are confident that the morphological markers are accurate and thus can be generalized—at least in red deer. It is possible that the measurements and the morphological markers defined in this study are valid only for red deer, although the histologically classified zones and layers are widely applicable to nearly all major deer species, including white-tailed deer, mule deer, fallow deer, red deer, American elk, reindeer, caribou (Banks and Newbrey, 1982), and sika deer (Li et al., 1989). However, even if the measurements and markers presented herein were valid only for red deer, our established techniques would certainly be helpful for developing other species-specific methods. In the present study, we have also confirmed

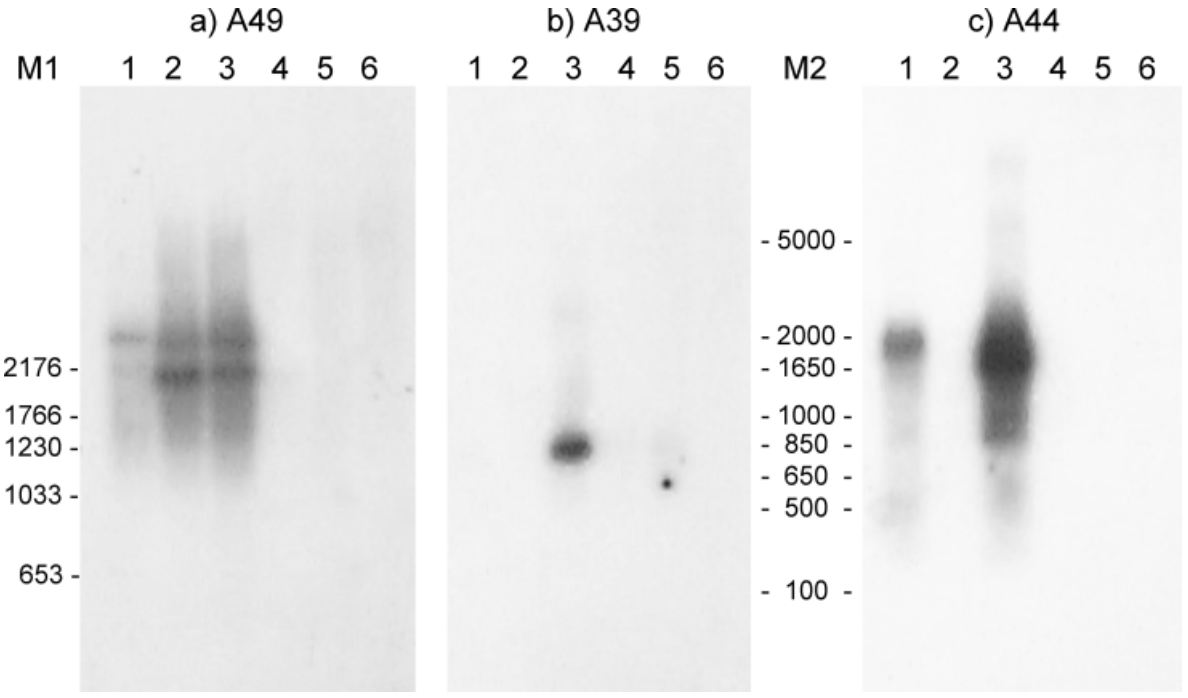

Fig. 4. Virtual Northern blots probed with three different ESTs. Lane 1: Red deer velvet antler tip dermis cDNA. Lane 2: Red deer velvet antler tip mesenchymal cDNA. Lane 3: Red deer velvet antler tip precartilage and cartilage cDNA. Lane 4: Red deer adult liver, spleen, and kidney cDNA. Lane 5: Red deer adult cartilage, muscle, and heart cDNA. Lane 6: Red deer adult lung, skin, and spinal cord cDNA. Marker M1: Roche marker VI in kb. Marker M2: LifeTechnologies 1 kb plus DNA ladder in kb.

that the layer measurements of fresh antler are similar to those of tissue sections, although not of embedded tissue. This helps provide a guide as to how much tissue is collected from each layer (Table 1).

The present findings enable further progress to be made with the molecular aspects of this work by increasing our knowledge of the histology and cell biology of the tissues under investigation. The virtual Northern analysis in Figure 4 clearly shows the localization of A49, A39, and A44 in different areas of the antler. Knowing that a gene, such as A39, is expressed only in precartilage and cartilage areas has implications as to its function, and can indicate potential bioassays for studying that function. It also aids in experiments to localize the mRNA and protein. The dissection of the antler into functional layers is a powerful technique that enables the collection of tissue for molecular and functional analysis.

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