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Differential Gene Expression Analysis Using Paraffin-Embedded Tissues After Laser Microdissection

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Abstract Recent advances in laser microdissection allow for precise removal of pure cell populations from morphologically preserved tissue sections. However, RNA from paraffin-embedded samples is usually degraded during microdissection. The purpose of this study is to determine the optimal fixative for RNA extractions from laser microdissected paraffin-embedded samples. The integrity of RNA was evaluated with the intactness of 18S and 28S ribosomal RNA by electrophoresis and by the length of individual gene transcripts using RT-PCR. The various fixatives were methacarn (a combination of methanol, chloroform, and acetic acid) and several concentrations of ethanol and isopropanol. Methacarn was the optimal fixative for RNA preservation in paraffin-embedded tissues, which included liver, lung, kidney, muscle, and limb. Based on RT-PCR analysis, methacarn fixed samples exhibited the expected RNA sizes for individual genes such as glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and bone-related genes (e.g., alkaline phosphatase and osteonectin). The laser microdissection technique with methacarn fixation was then applied to analyze the differential gene expression between hypertrophic and proliferative chondrocytes in the growth plate of long bone. The expression of type X collagen, a specific gene for hypertrophic chondrocytes, was only observed in hypertrophic chondrocytes, while type II collagen was observed more broadly in the growth plate as anticipated. Thus, combining laser microdissection with methacarn fixation facilitates the examination of differentially expressed genes from various tissues. J. Cell. Biochem. 90: 998–1006, 2003.

Key words: laser microdissection; paraffin; RNA; methacarn; chondrocyte

Laser microdissection (LM) is a particularly useful tool for converting morphological information into molecular biological information by recovering specific cell samples from tissue sections [Bonner et al., 1997]. The procedure involves the membrane-assisted transfer of selected cells from tissue sections to microcentrifuge tubes for extraction of nucleic acids and subsequent analysis.

Paraffin embedding has been routinely used for tissue sections because of the convenience of handling tissues and subsequent staining, as well as preservation of morphology [Shibutani et al., 2000]. Until now, paraffin-embedded tissue fixation has been extensively performed using formalin. Although tissue architecture and proteins are preserved in formalin-fixed paraffin-embedded tissue, extraction of RNA

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from the embedded tissue is difficult and usually degrades the RNA. Of the available tissue-processing methods, frozen tissues are preferred over paraffin-embedded tissues because frozen tissues yield higher quality RNA and more RT-PCR product than paraffin-embedded tissues [Goldsworthy et al., 1999]. However, frozen tissues are more difficult to transport and store. Therefore, it is important to develop and optimize new protocols for RNA extraction from paraffin-embedded tissues.

To identify specific cells desired for LM, a histochemical fixative must be used to preserve tissue morphology. However, while preserving tissue morphology, histochemical fixatives function by altering the structure of macromolecules [Sato et al., 1991; Stanta and Schneider, 1991] which may affect the integrity of RNA. Precipitating fixatives such as ethanol and acetone, consistently produce more RT-PCR amplification product than cross-linking fixatives such as formalin [Goldsworthy et al., 1999]. Ethanol and methanol-based solutions including Carnoy's fixative and methacarn are commonly used during fixation of nucleic acids. The precipitating fixatives have the advantage of minimizing chemical modifications [Hopwood, 2002]. Optimal protocols must provide acceptable morphology, allow proper laser capture of selected cells, and preserve the integrity of the RNA.

In this study, the integrity of RNA was monitored by examining the intactness of ribosomal RNA and the length of individual gene transcripts after RT-PCR in paraffin-embedded tissues treated with various fixatives after LM. In addition, expression of type II and type X collagens in mouse epiphyseal growth plates was assessed. We present data showing that laser microdissection combined with methacarn fixation represents the most optimal method for the characterization of differentially expressed genes in various tissues.

MATERIALS AND METHODS

Materials

Methacarn solution consisting of 60% (vol/vol) absolute methanol, 30% chloroform, and 10% glacial acetic acid was freshly prepared before fixation as previously described [Puchtler et al., 1970; Meloan et al., 1975]. Other fixatives used for assessing the efficiency or integrity of RNA extraction were as follows: 70% ethanol, 100% ethanol, 50% isopropanol, and 100% isopropanol. All solvents were HPLC grade and purchased from Sigma (St. Louis, MO). Diethyl pyrocarbonate (DEPC) (Sigma) treated MilliQ water and phosphate buffered saline (PBS) were used as diluents for the fixatives.

Tissue Preparation and Paraffin Embedding

The tissues were collected from 2-months old ICR mice. Kyungpook National University's stringent guidelines for animal care procedures and use were strictly adhered to. The tissues were fixed for 1 h at 4°C in one of the following fixatives: 70% ethanol, 100% ethanol, 50% isopropanol, 100% isopropanol, 10% neutral-buffered formalin, 4% paraformaldehyde, acetone or methacarn. The embedding procedure included, dehydrating the tissue sample in three changes of fresh 100% ethanol at 4°C for 1 h, immersion in xylene for 1 h, followed by three changes for 10 min each at room temperature, and finally immersed in hot paraffin (60°C) for three changes in 1 h.

RNA Extraction

There were two types of sample preparations prior to RNA extraction. First, paraffin-embedded tissue from the diverse fixatives was cut 5–10 μm in thickness. We placed sections of each paraffin-embedded tissue in a microcentrifuge tube, added 1,200 μl xylene, vortexed vigorously and centrifuged at 14,000 rpm for 2 min at room temperature. After removal of supernatant by pipetting, 1,200 μl 100% ethanol was added to the pellet, mixed gently by vortexing and centrifuged at 14,000 rpm for 2 min at room temperature. It was repeated one more time for 5 min. The supernatant was removed and the pellet was air-dried for 10 min at 37°C. Total RNA was extracted by Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's recommendation.

Second, tissue samples were prepared after LM. The RNA was extracted using micro RNA isolation kit (Stratagene, La Jolla, CA). The RNA was resuspended in DEPC-treated water. The resolution of 18S and 28S ribosomal RNAs was confirmed by RNA gel electrophoresis and reverse transcription (RT)-PCR.

Laser Microdissection

The paraffin-embedded tissues were cut at 5–10 μm sections using rotary microtomes

(Leica, Germany). A section was then transferred to a Superfrost/Plus slide (Fisher scientific, Pittsburgh, PA) with a 1.35 μm polyethylene transfer membrane. The transfer membrane was mounted onto the glass slide with DEPC treated water and further fixed in Fixogum (Marabuwerke, Germany) [Imamichi et al., 2001]. The paraffin sections were deparaffinized with xylene and dehydrated with two changes of 100% ethanol for 1 min each. The sections were then fixed with 85% ethanol for 3 min, stained with hematoxylin for 3 min, washed with 85% ethanol, and rapidly counterstained with alcoholic Eosin Y solution. Sections were then serially washed with 85% ethanol, 95% ethanol, and 100% ethanol and air-dried. The Vanadyl Ribonucleoside Complex (VRC) RNase inhibitor (NEB, Beverly, MA) was added to the solution for hematoxylin and eosin (H&E) staining at a concentration of 1–2 mM. The commercially available Robot-MicroBeam (PALM, Bernried, Germany) was used in the microdissection of methacarn fixed tissues from H&E stained paraffin-embedded tissue sections as previously described [Schutze and Lahr, 1998]. After microdissection of each specimen, captured tissue was placed in a microtube. The RNA was extracted using the micro-RNA isolation kit (Stratagene).

RT-PCR

Gene expression analysis of the extracted RNA after LM was performed by RT-PCR. The cDNA synthesis was performed in 20 μl RT-mixture at 42°C for 90 min. The RT-mixture consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.25 U AMV Reverse Transcriptase XL (Takara, Kyoto, Japan), 1 U RNase inhibitor (Takara), 0.125 pM oligo-dT primer, and 1 mM dNTP mixture. PCR reactions were performed in 25 or 100 μl PCR-mixture consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.75 mM (only type II collagen (Col2α1)) or 2.5 mM $MgCl_2$, 20 pmol 5′ primer, 20 pmol 3′ primer, and 2.5 U Taq DNA polymerase. Amplification was carried out under the following condition: 2 min at 92°C: 30 s at 94°C: 45 s (only type X collagen (Col10α1)) or 30 s at annealing temperature: followed by 10 min at 72°C. Annealing temperature is 60°C for (Col2α1, alkaline phosphatase (ALP), osteonectin) and 55°C for (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Col10α1). Primers used for RT-PCR were as follows: for GAPDH, 5′-TGAGAACGGGAAGCTTGTCA-3′, 5′-GGAAGGCCATGCCAGTGA-3′: for Col2α1, 5′-CACACTGGTAAGTGGGGCAAGACCG-3′, 5′-GGATTGTGTTGTTTCAGGGTTCGGG-3′: for Col10α1, 5′-CCACCTGGGTTAGATGGAAAA-3′, 5′-AATCTCATAAATGGGATGGG-3′: for ALP, 5′-GCCCTCTCCAAGACATATA-3′, 5′-CCATGATCACGTCGATATCC-3′: for osteonectin, 5′-GATGAGGACAACAACCTTCTGAC-3′, 5′-TTAGATCACAAGATC CTTGTCGAT-3′. PCR product was visualized by gel electrophoresis and ethidium bromide staining.

Hematoxylin and Eosin (H&E) Staining

Paraffin was removed from tissue sections by rinsing in xylene three times for 2 min each. The tissue sections were hydrated with an ethanol gradient (100, 90, 80, and 70% ethanol) each for 30 s and then washed with distilled water for 20 s. Then, sections were stained with Harris' Hematoxylin (Sigma) for 2 min, washed with water for 3 min, counter-stained with alcoholic Eosin Y (Sigma) solution for 30 s and dehydrated with 70–100% serial ethanol gradients. Finally, the sections were cleaned with xylene twice for 4 min, air dried and mounted on a slide with Permount® (Fisher Scientific, Fair Lawn).

In Situ Hybridization

The details of the in situ hybridization technique used here have been described previously [Okada et al., 2003]. Digoxigenin-11-UTP-labeled RNA probes were prepared using DIG RNA Labeling Kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. The type II collagen cDNA containing plasmid was digested with *Hind*III. Antisense riboprobes were produced by T7 RNA polymerase. The collagen type X cDNA containing vector was digested with *Xba*I and antisense riboprobes were produced by T7 RNA polymerase. The sense riboprobes were used as controls.

RESULTS

Effect of Various Fixatives on RNA Integrity After Paraffin Embedding

Most tissue processing protocols encompass four basic steps prior to paraffin embedding: fixation, dehydration, dealcoholization and impregnation. The complete procedure for this study is schematically summarized in Figure 1. We optimized the fixation step as it is the most

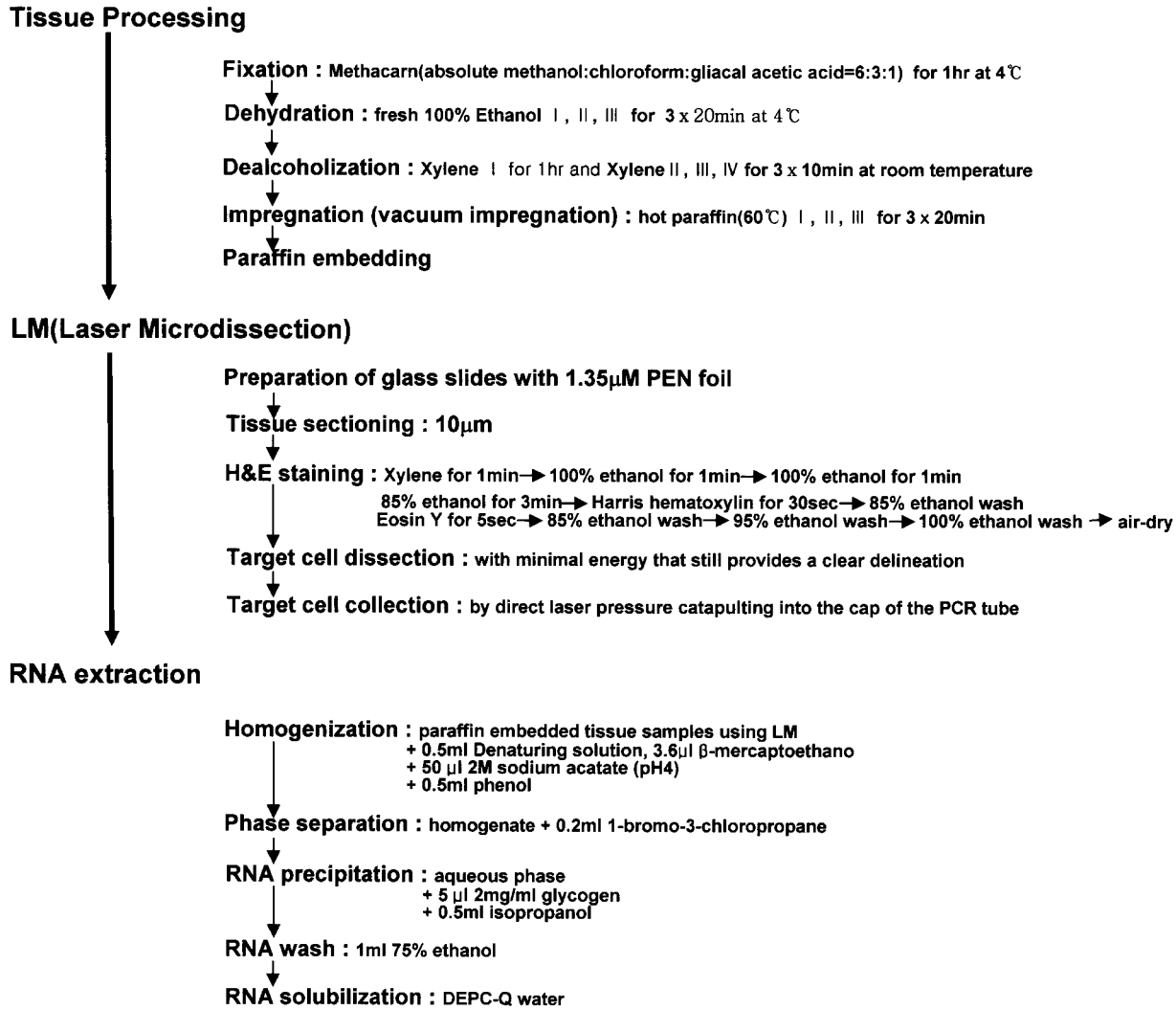

Fig. 1. Scheme of experimental procedure.

important step for RNA preservation. We tested several fixatives on a panel of tissues: such as liver, lung of adult mouse, and embryonic day 17 limb. Tissues were fixed for 1 h with various fixatives including ethanol (100%, 70%), and isopropanol (100%, 50%) and methacarn, then embedded in paraffin. The integrity of RNA was evaluated by ethidium bromide staining of ribosomal RNA (rRNA) after gel electrophoresis. Intact ribosomal RNAs exhibit two clear electrophoretic species, which represent 28S and 18S rRNAs, respectively. Typically, the signal of the 28S RNA band should be approximately twice as intense as that of 18S rRNAs. In limb, the integrity of total RNA from fixed paraffin-embedded tissue treated with methacarn was excellent (Fig. 2A) as evidenced by a twofold ratio of the 28S and 18S rRNA. However, the RNA integrity in different concentrations of ethanol and isopropanol appeared to be compromised when compared to methacarn. RNA from frozen liver sections was more intact than RNA samples obtained with the various fixatives. RNA integrity was similar in methacarn- and ethanol-treated samples, but isopropanol treatment resulted in loss of intact RNA. RNA integrity of ethanol and methacarn fixed paraffin-embedded limb was superior to that of isopropanol (Fig. 2B). In lung, the integrity of RNA from methacarn fixative, but not from ethanol (70%), was comparable to that of the frozen tissue (Fig. 2C). Interestingly, the quality of RNA was different when ethanol (70%) or isopropanol (50%) was diluted with DEPC-treated water or PBS. This observation may be analogous to the effect of salts, which

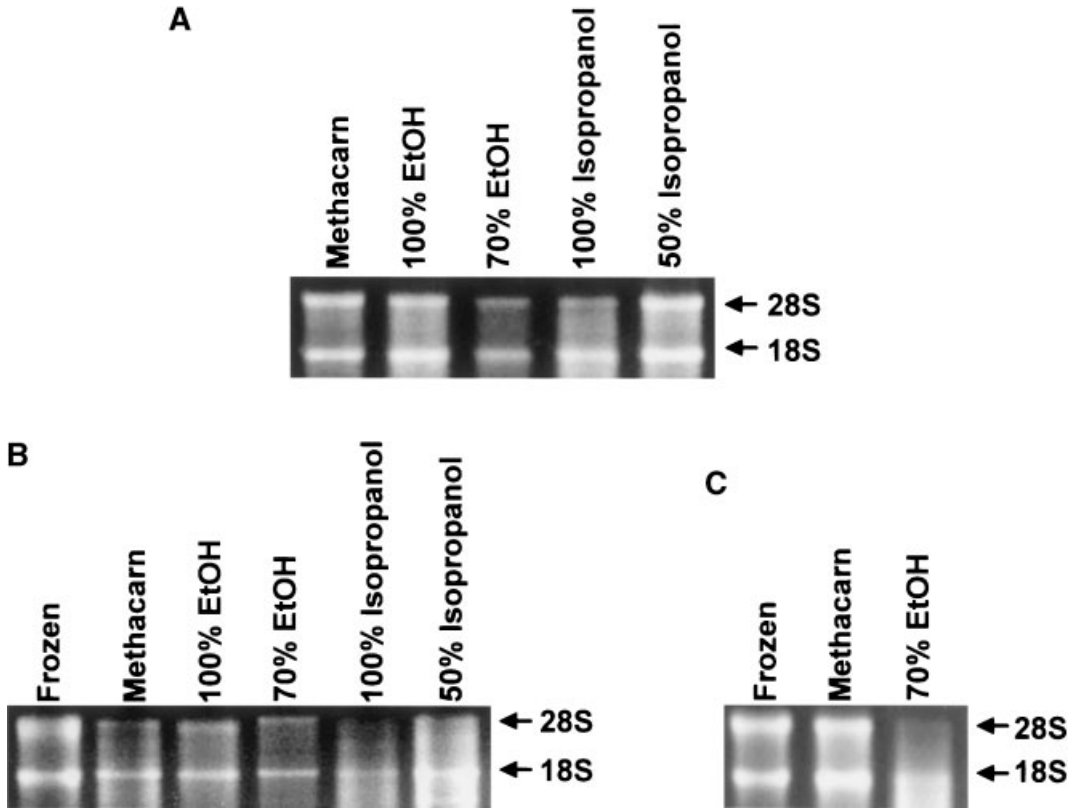

Fig. 2. Quality of RNA extracted from paraffin-embedded tissue using various fixatives. Total RNA from three different mouse tissues was extracted from paraffin-embedded tissues after various fixation with ethanol, isopropanol, and methacarn. The RNA (2 μg) was resolved in a 1% formaldehyde-agarose gel and visualized with ethidium bromide. **A**: Limb (day 17 embryo). **B**: Liver. **C**: Lung. EtOH: ethanol. The 28S and 18S ribosomal RNAs were marked (arrow).

are known to be essential for the maximum precipitation of nucleic acids from alcohol [Hopwood, 2002]. Other fixatives (acetone, methanol, 10% acetic acid, and modified precipitating fixatives such as Carnoy's solution, Clarke's solution) were used and did not yield high quality RNA (data not shown). These results indicate that RNA integrity with the same fixative is different according to the tissues and that methacarn is the most optimal choice as a fixative.

RNA Integrity in Methacarn Fixed Paraffin-Embedded Tissues

We then focused on the integrity of RNA from various paraffin-embedded tissues after methacarn fixation. The integrity of RNA from various tissues was evaluated by electrophoresis and ethidium bromide staining (Fig. 3A). Generally, RNA integrity from methacarn fixative was comparable to that of frozen tissues. The expression of GAPDH gene was detected by PCR after reverse transcription with oligo-dT primer using the same RNAs (Fig. 3B). To further confirm the integrity of RNA, we tested other bone-related genes expression in methacarn fixed paraffin-embedded mouse embryo day 17 limb by RT-PCR. We detected the expression of bone-related genes such as type II collagen, type X collagen, alkaline phosphatase, and osteonectin (Fig. 3C). The size from the poly A tail to the upper end of the amplified transcripts was more than 2 kb (Table I). These results indicate that methacarn fixation of paraffin-embedded tissues yields RNA of good quality and hence the procedure of choice to obtain intact RNA.

Gene Expression Profiles From Paraffin-Embedded Tissues After Microdissection

Hypertrophic chondrocytes are round and larger than proliferating chondrocytes in the growth plate. Type X collagen is expressed only in hypertrophic chondrocytes, while type II collagen is more broadly expressed at the growth

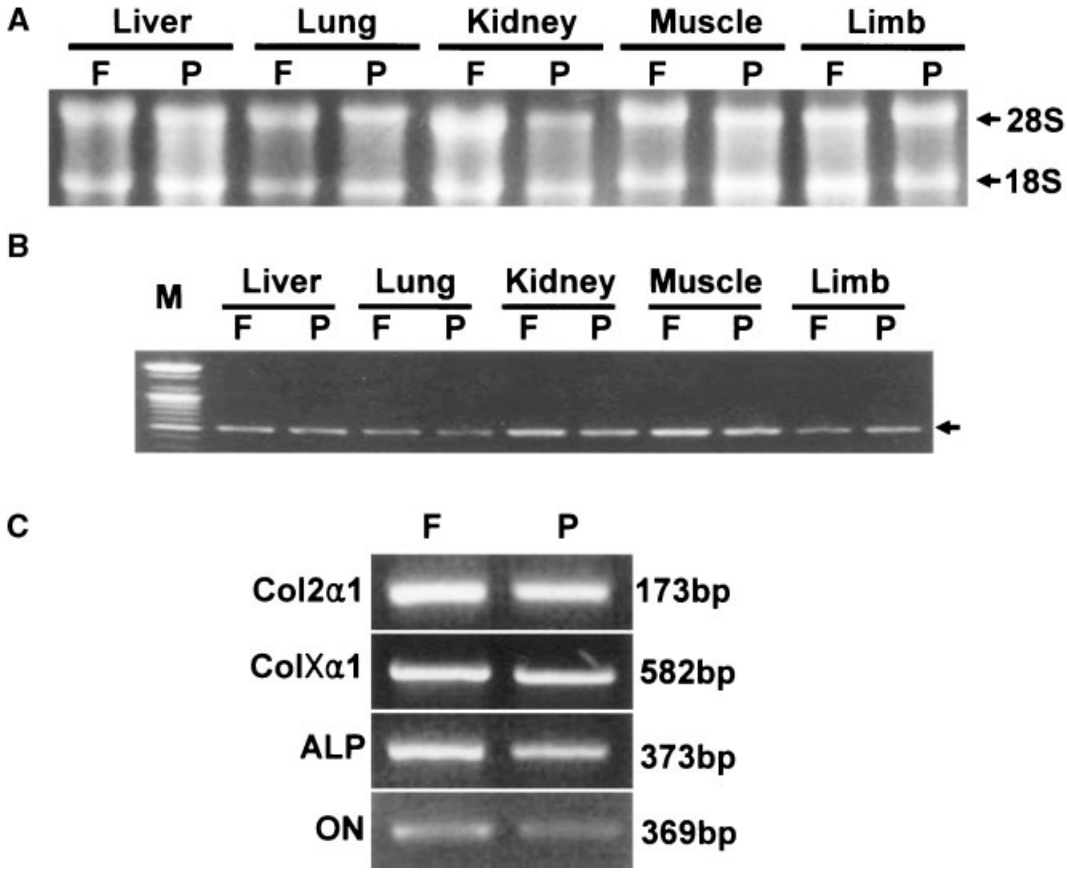

Fig. 3. RT-PCR analysis of RNA from frozen or methacarn fixed-paraffin embedded tissues. **A**: 28S and 18S rRNAs after RNA electrophoresis. **B**: The RT-PCR product of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) from mouse tissues—liver, lung, kidney, muscle, and limb. Arrow indicated GAPDH RT-PCR product (515 bp) with DNA marker (M). **C**: The gene expression of cartilage-related genes by RT-PCR. The sample was prepared from mouse embryo day 17 limb tissue with two different sample preparations. RT-PCR was performed as described in ''Materials and Methods'' for type II collagen (Col2α1), type X collagen (ColXα1), alkaline phosphatase (ALP), and osteonectin (ON). F, unfixed frozen tissue; P, Methacarn-fixed paraffin-embedded tissue.

plate as well as in the hypertrophic chondrocytes [Schmid and Linsenmayer, 1985; Gibson et al., 1986; Marriott et al., 1991; Mwale et al., 2002; Ornitz, 2002]. Tissue integrity with respect to cytoplasmic and nuclear detail was well preserved in methacarn fixed-paraffin embedded tissue (Fig. 4A,B). The expression of Type X and Type II collagen in the growth plate zone containing both proliferating and hypertrophic chondrocytes was determined by in situ hybridization (Fig. 4A). To validate the detection of expression of the two genes after

TABLE I. List of Genes for RT-PCR and Products Size

Gene	Accession No.	Combination of primers		Target size (bp)
		Upstream[a]	Downstream[a]	
GAPDH, human (1282 bp)	XM_006959	261–280	758–775	515
Col2α1, mouse (32492 bp)	M65161.1	31276–31300	31424–31448	173
ColXα1, mouse (2215 bp)	X65121.1	1032–1052	1594–1613	582
ALP, mouse (2459 bp)	J02980	468–486	821–840	373
ON, human (2133 bp)	J03040.1	601–623	946–969	369

GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; Col2α1, type II collagen; ColXα1, type X collagen; ALP, alkaline phosphatase; ON, osteonectin.
[a]Nucelotide numbers are shown for each gene according to the mRNA sequence resisted to the NCBI data bank with the accession number.

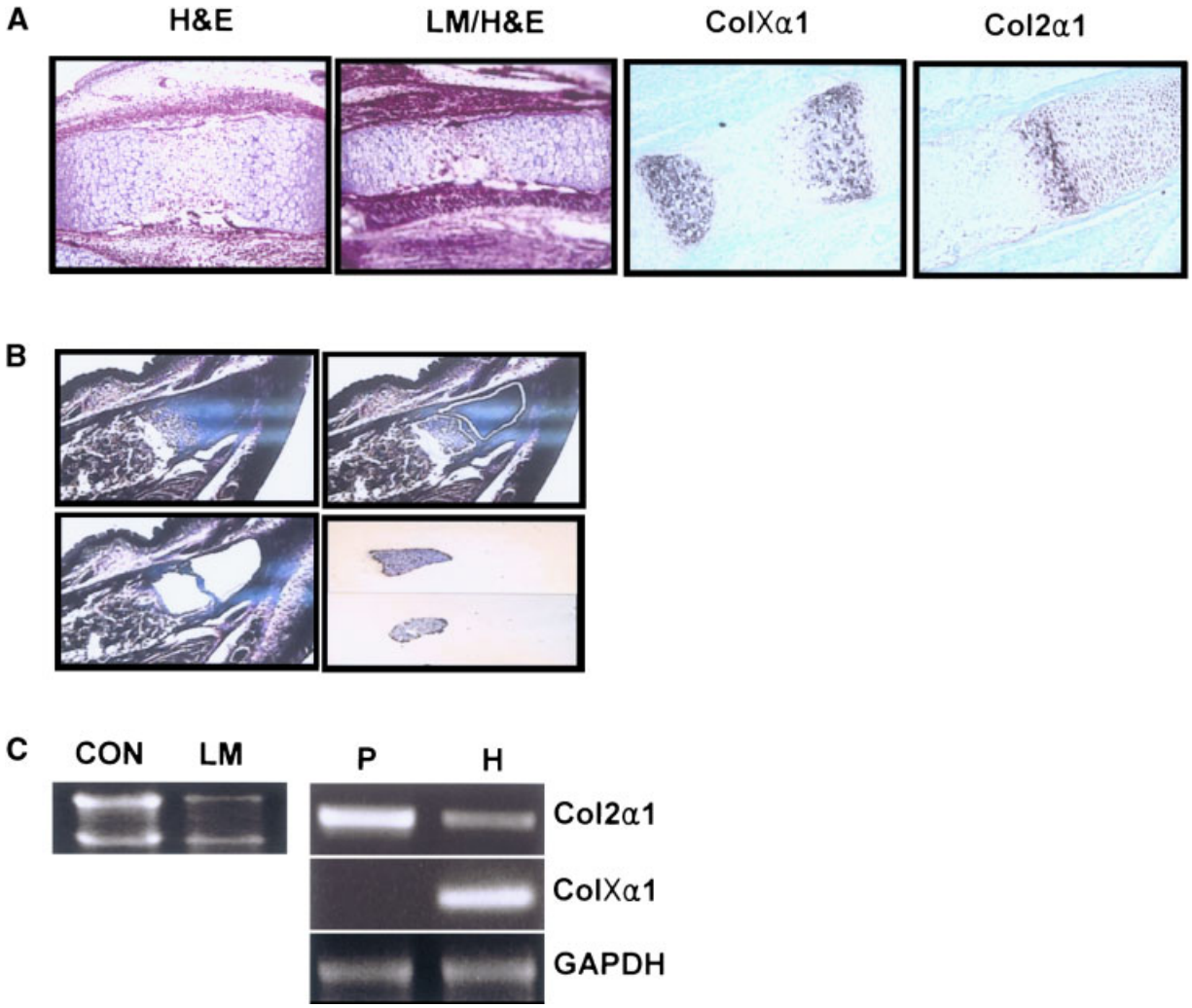

Fig. 4. Tissue-specific gene expression from paraffin-embedded tissue after microdissection. Tissue sections were prepared from mouse embryonic day 15 limb. **A**: Tissue was stained with H&E after formalin fixation (H&E), and H&E staining after methacarn fixation and LM (LM/H&E). In situ hybridization was performed using specific probes: type X collagen (ColXα1) and type II collagen (Col2α1) as described in ''Materials and Methods'' (Magnification × 20). **B**: Microdissection of tissue from methacarn fixed-paraffin embedded mouse was performed by laser microdissection system: Tissue mounted onto 1.35 μm polyethylene transfer membrane (**upper left**). Clear-cut separation between selected and nonselected tissue (**upper right**). Empty patch, corresponding to the catapulted template, leaving the surrounding tissue entirely intact (**lower left**). Catapulted specimen with preserved morphology (**lower right**) (Magnification ×10). **C**: The intactness of 28S and 18S rRNAs after extraction of RNA from frozen tissues (CON) or methacarn fixation-paraffin embedded limb using LM (LM) (**left panel**). RT-PCR analysis of tissue specific genes from proliferative or hypertrophic zones (**right panel**). RT-reaction was performed using oligo-dT primer and 500 ng of total RNA from microdissected tissue as template. The PCR products of type X collagen (ColXα1), type II collagen (Col2α1), and GADPH were shown. P, proliferative zone; H, hypertrophic zone. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

microdissection, the limbs of mouse embryos at day 15 were methacarn-fixed, paraffin-embedded, and stained with H&E. Samples were isolated by LM and gene expression was analyzed by RT-PCR. Figure 4B shows a LM captured specimen on the microtube cap. The 28S/18S rRNA ratio from methacarn fixed-paraffin embedded limbs using LM was comparable to frozen tissue. Furthermore, RT-PCR analysis revealed that the expression of type X collagen was restricted to the hypertrophic chondrocyte zone whereas type II collagen expression was detected in both proliferating and hypotrophic chondrocytes (Fig. 4C). These results establish that RNA from methacarn-fixed, paraffin-embedded, microdissected tissue

is suitable for RT-PCR based approaches to study the molecular parameters that determine bone biology.

DISCUSSION

In this study, we demonstrate that among various fixatives, the fixative of choice for the extraction of intact RNA from paraffin-embedded tissues is methacarn. The RNA integrity was confirmed using two methods. First, we showed that 18S and 28S ribosomal RNAs from methacarn fixed-paraffin embedded tissues remain intact during microdissection. Second, we demonstrated that the expression of specific mRNAs can be detected by RT-PCR in paraffin-embedded tissues.

In general, the precipitating fixatives ethanol and methanol are frequently used as fixative solutions for nucleic acids, including Carnoy's fixative and methacarn [Hopwood, 2002], and represent the recommended method for the extraction of DNA from paraffin-embedded or frozen tissues [Smith et al., 1987; Uneyama et al., 2002]. However, it has not been systemically determined whether the precipitant fixatives are useful or not for RNA extraction from paraffin-embedded specimens.

Recent studies have been able to analyze mRNA from formaldehyde-fixed, paraffin-embedded material [Schutze and Lahr, 1998; Fink et al., 2000; Heiske et al., 2000; Kasi et al., 2000; Imamichi et al., 2001; Cohen et al., 2002]. Specht and co-workers reported a protocol for the isolation of mRNA from formaldehyde-fixed, paraffin-embedded tumor tissues via the digestion of protein–RNA crosslinks by extended proteinase K incubation [Schutze and Lahr, 1998; Specht et al., 2001]. The expression of genes of interest in these studies was determined by RT-PCR. However, RNA from formaldehyde-fixed, paraffin-embedded tissue is generally degraded and it is very difficult to construct cDNA libraries and perform further analysis. In addition, to our knowledge, there are no reports showing the 28S and 18S rRNA from paraffin-embedded tissue after laser-microdissection. These results indicate that the quality of RNA may be enough to make cDNA library from paraffin-embedded tissues after laser-microdissection.

In our study, the PCR products we tested originated from oligo dT primers used for reverse transcription of mRNAs. We were able to amplify at least 2 kb of RNA upstream from the poly A tail of the mRNAs for type X collagen and type II collagen. We propose that methacarn fixation of paraffin blocks may be useful for analytical molecular analyses and the constructions of cDNA libraries, because this method yields 28S/18S rRNA ratio that is comparable to that of frozen tissue.

Methacarn is a modified Carnoy's solution in which methanol is used instead of ethanol [Puchtler et al., 1970]. Methacarn was previously used for RNA extractions from paraffin blocks [Shibutani et al., 2000]. However, data showing the quality of RNA from paraffin-embedded tissue sections were not presented. In frozen tissue sections, the fixative of choice for optimal morphology and microdissection of cells is 70% ethanol [Goldsworthy et al., 1999]. In formalin fixed-paraffin embedded tissue section, tissue integrity with respect to cytoplasmic and nuclear detail was well preserved. However, no RNA has been extracted from samples older than 20 years [Coombs et al., 1999], presumably due to fixative-related chemical modifications of the RNA. In contrast to formalin, the compounds used in methacarn do not have aldehyde groups that may influence RNA integrity in a time-dependent manner. Therefore, it may be possible to extract RNA from old samples after fixation of methacarn.

LM-based isolation of identified cell populations from tissue sections for mRNA analysis significantly expands our ability to investigate gene expression patterns at the microscopic level. Collectively, our results reveal that methacarn is an excellent fixative for samples obtained by microdissection and this fixative will not interfere with subsequent RNA analysis. Based on this method, it will be possible to assess differential expression of genes at the single cell level in both basic and clinical research settings.

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