Table 1
Comparison of DNA Yields Between Unfixed or Ethanol-Fixed Frozen Tissues and Methacarn-Fixed Wax-Embedded Tissues[a]

Tissue condition	No. of sample	DNA yield (μg/mg wet tissue)
Unfixed frozen	5	1.05 ± 0.10
Ethanol-fixed frozen	5	1.15 ± 0.11
Methacarn-fixed wax-embedded	4	0.76 ± 0.06[b]

[a]Rat liver tissue was used as described previously in **ref. *15***. After dewaxing, extraction of DNA from methacarn-fixed wax-embedded small liver tissue blocks was performed by digestion with 500 μL of 10 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 10 m*M* ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate, and 1 U of proteinase K at 55°C for 2 h. The film was removed from the tube at this time point. Then 500 μL of Tris buffer-saturated phenol was added, mixed well, and centrifuged at 10,000*g* for 15 min. The supernatant was further extracted again with 500 μL of Tris-phenol/chloroform (1:1), and the separated aqueous portion after centrifugation was transferred to a new tube and treated with 0.5 U of RNase A at 37°C for 1 h. The solution was extracted with 500 μL of phenol/chloroform (1:1) and then treated with ether. Extracted DNA was precipitated by adding 1 μL of cold 99.5% ethanol, and after storing at –20°C overnight, centrifuged at 5000*g* for 5 min. The pellet was washed twice with 75% ethanol, dried, and resuspended in 10 μL of water. One milliliter of sample was used to measure DNA concentration by Hoechst 33258 and a fluorescence spectrophotometer. Extraction of DNA from methacarn-fixed wax-embedded small liver tissue blocks was performed after dewaxing. Frozen tissue blocks of unfixed or ethanol-fixed liver were directly subjected to DNA extraction, and dewaxed blocks of methacarn-fixed wax-embedded liver tissue were air-dried before extraction.

[b]Significantly different from unfixed frozen sample ($p < 0.01$ by ANOVA).

(Reproduced with permission from **ref. *15***.)

The yield and quality of extracted DNA are critical for the subsequent analysis of microdissected cells. DNA yield from ethanol-fixed frozen tissues is similar to that from unfixed frozen tissues (*see* **Table 1**) ***(15)***. On the other hand, DNA yield from methacarn-fixed wax-embedded tissue is slightly reduced, the mean value being about 70% of that from unfixed frozen tissues (**Table 1**). The integrity of extracted DNA from methacarn-fixed wax-embedded tissue is assessed by electrophoresis on 1.5% agarose gel (*see* **Fig. 1**). Although a slightly greater intensity at the top of the DNA smear is observed in unfixed or ethanol-fixed frozen samples, DNA in every case distributes within the high molecular weight range, suggesting good preservation of extracted DNA.

3.1.2. Preparation of Methacarn-Fixed Wax-Embedded Tissue Sections

1. Section at 5–10 μm in thickness using a microtome.
2. Stretch sections with slide warmer (Sakura Finetek Japan).
3. Mount stretched section onto a 1.35-μm thin polyethylene film (PALM GmbH) overlaid on a glass slide.

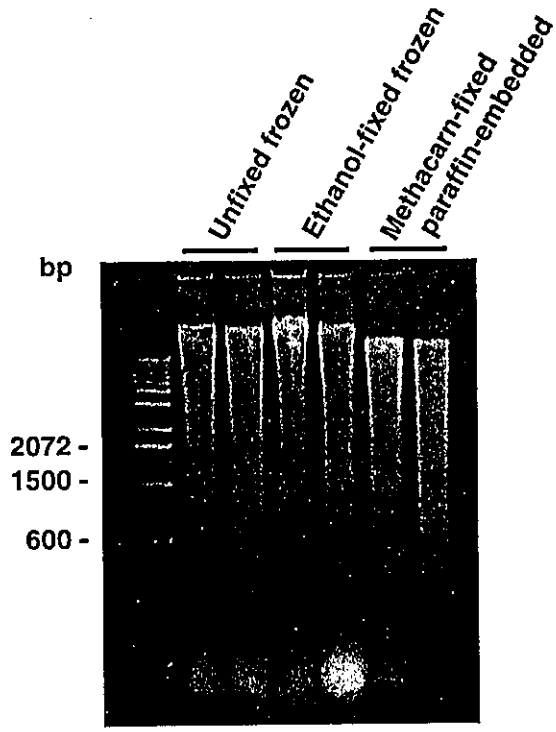

Fig. 1. Comparison of the integrity of DNA extracted from rat liver of unfixed or ethanol-fixed frozen tissue blocks and dewaxed methacarn-fixed tissue blocks. Tissue blocks were directly subjected to DNA extraction as described in the footnote of **Table 1**, and 2.0 μg of extracted DNA was subjected to electrophoresis in 1.5% agarose gel and stained with ethidium bromide as previously described *(15)*. (Reproduced with permission from **ref. 15**.)

4. Dry sections overnight at 37°C in an incubator.
5. Store sections at 4°C until use.

3.2. Tissue Staining

Dewax sections by immersing in xylene for 3 × 2 min followed by 99.5% ethanol for 2 × 2 min. Tissue sections can be stained with cresyl violet or H&E, or immunostained (*see* **Note 2**).

3.2.1. Cresyl Violet Staining

1. Dissolve 0.5 g cresyl violet in 500 mL water.
2. Add 8 drops of acetic acid.
3. If necessary, boil the solution until completely dissolved.
4. Filter the solution before using.
5. Immerse dewaxed sections briefly in water.

6. Incubate sections in cresyl violet solution for 20 min.
7. Wash sections once with 95% ethanol that contains 0.5% acetic acid, and then with 99.5% ethanol twice.
8. Air-dry.

3.2.2. Hematoxylin and Eosin Staining

1. Immerse dewaxed sections briefly in water.
2. Immerse sections in hematoxylin solution (Tissue-Tek® Hematoxylin 3G) for 10 s.
3. Wash sections briefly with water.
4. Immerse sections with eosin solution (Tissue-Tek® Eosin) for 10 s.
5. Wash sections briefly with 99.5% ethanol.
6. Air-dry.

3.2.3. Immunostaining

1. Treat dewaxed sections with 1% periodic acid solution for 10 min.
2. Wash sections briefly with water and 1X phosphate-buffered saline (PBS; pH 7.4).
3. Block nonspecific binding sites with 0.5% casein in PBS for 30 min.
4. Incubate with primary antibody of appropriate dilution for 2 h.
5. Wash sections with PBS for 5 min × 3.
6. Incubate sections with biotin-labeled secondary antibody.
7. Repeat **step 5**.
8. Incubate sections with avidin-biotin complex utilizing Vectastain Elite kit (Vector Laboratories).
9. Repeat **step 5**.
10. Visualize immunoreaction using the avidin/biotin system with 0.004% hydrogen peroxide as substrate and DAB as chromogen.
11. Rinse sections with water.
12. Perform nuclear staining with hematoxylin if desired (*see* **Subheading 3.2.2.**).
13. Air-dry.

Tissue staining can affect the yield and quality of extracted DNA with methacarn-fixed wax-embedded tissue sections (*see* **Table 2** and **Fig. 2**) *(15)*. **Table 2** shows the DNA yield from methacarn-fixed wax-embedded tissue sec-

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Fig. 2. (*opposite page*) Integrity of DNA extracted from stained sections of methacarn-fixed wax-embedded tissue. Liver of a rat treated with thioacetamide at the promotion stage in the two-stage hepatocarcinogenesis model *(23,24)* was used. Tissue blocks were trimmed to obtain sections of 100 mm^2 in area before sectioning, and sectioned at 10 μm in thickness. Serial sections were randomized and mounted onto polyethylene film overlaid on a glass slide. Dewaxed sections were either unstained, stained with H&E, or immunostained with glutathione-*S*-transferase placental form

Table 2
Effect of Staining on Yield of Extracted DNA From Methacarn-Fixed Rat Liver Wax-Embedded Tissue Sections[a]

Tissue condition	No. of samples	Yield of DNA (ng/100 mm^2 area)[b]	Ratio of unstained section (%)
Unstained	10	2705.1 ± 853.4	100
H&E-stained	5	2687.4 ± 632.4	99.3
Immunostained[c]	13	314.5 ±85.2[d]	11.6

[a]Liver of a rat treated with thioacetamide at the promotion stage in the two-stage hepatocarcinogenesis model *(23,24)* was used as described previously in **ref. *15***.

[b]Tissue blocks were trimmed to 100 mm^2 before sectioning, and sectioned at 10 µm. DNA extraction and estimation of its concentration were performed according to the methods described in **Table 1**.

[c]Sections were immunostained with GST-P.

[d]Significantly different from the unstained and H&E-stained samples ($p < 0.0001$ by ANOVA).

(Reproduced with permission from **ref. *15***.)

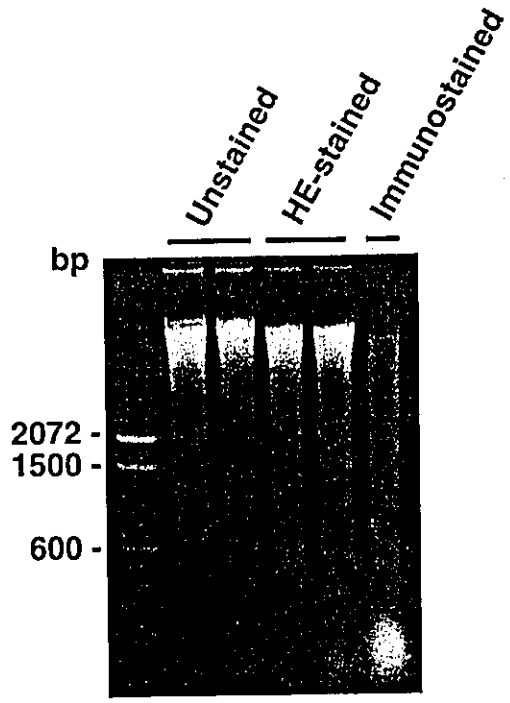

Fig. 2. (*continued*) (GST-P) (*see* **Subheadings 3.2.2.** and **3.2.3.**). One µg of extracted DNA from whole tissue section was subjected to electrophoresis in 1.5% agarose gel and stained with ethidium bromide as described previously *(15)*. (Reproduced with permission from **ref. *15***.)

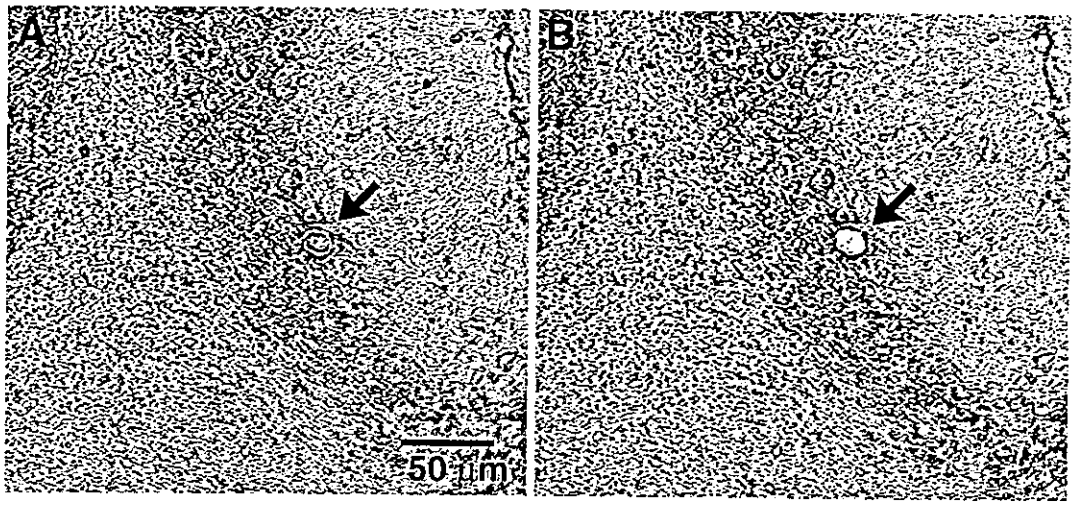

Fig. 3. Microdissection of single Purkinje cells from cresyl violet-stained rat cerebellum section as described previously in **ref. *15***. **(A)** Wax-embedded rat cerebellum sectioned at 10 µm in thickness was mounted on a film, dewaxed, and stained with cresyl violet (*see* **Subheading 3.2.1.**). A single Purkinje cell was selected and microdissected from the surrounding tissue with a laser beam (arrow). **(B)** The Purkinje cell has been cut out and catapulted by laser pressure (arrow). (Reproduced with permission from **ref. *15***.)

tions after tissue staining. The yield recovered from H&E-stained sections is similar to that from unstained sections. Immunostained tissue sections, on the other hand, result in very low DNA yield, values being 12% those of unstained sections. **Fig. 2** shows the integrity of DNA extracted from stained sections as visualized by electrophoresis on 1.5% agarose gel. DNA from unstained sections distributes mainly within the high molecular weight range. Similar to the unstained tissue section, H&E-stained sections show good preservation of the extracted DNA. As compared to unstained and H&E-stained cases, DNA extracted after immunostaining shows significant degradation of the DNA with small DNA fragments of approx 100 bp size (*see* **Note 3**).

3.3. Microdissection

Microdissection is performed with PALM Robot-MicroBeam equipment (Carl Zeiss Co., Ltd.) as described previously (*see* **Note 4**) *(25)*. Briefly, the film with the attached specimen is mounted in reverse (film side up) onto a new cover slip (26 × 76 mm) by adhering the film to the cover slip with nail polish. The specimens are then subjected to Robot-MicroBeam dissection by laser beam and the selected cells are catapulted by laser pressure into mineral oil-coated PCR tube caps (**Fig. 3**) *(15)*. In case of large specimens (circle areas of 150–200 µm in radius or square areas larger than 60 × 60 µm), the excised

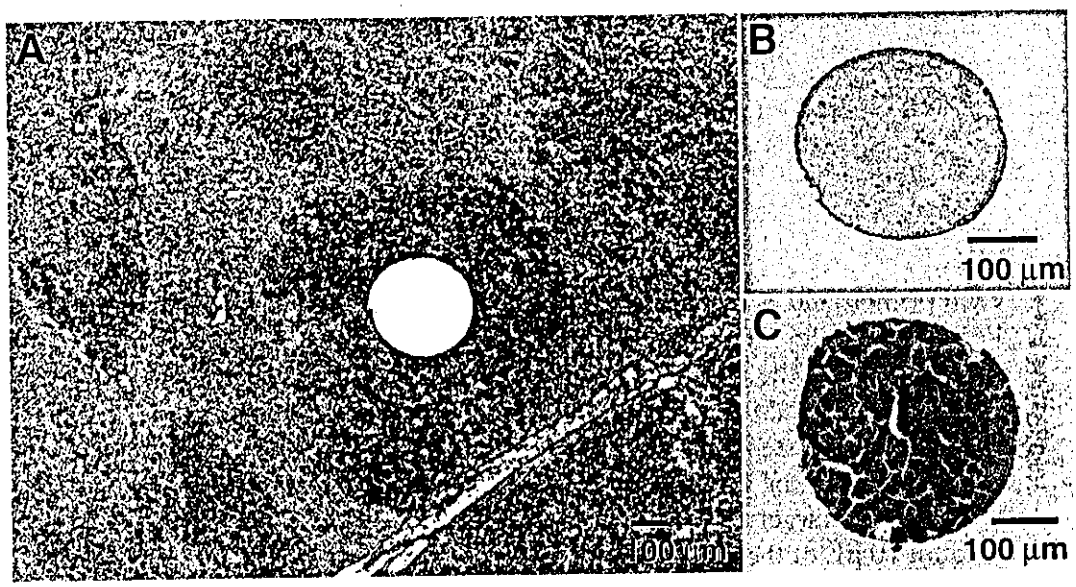

Fig. 4. Microdissection using a liver section immunostained with GST-P from a thioacetamide-treated rat in the two-stage hepatocarcinogenesis model as described previously in **ref. *15***. **(A)** A liver section (10-μm thick) mounted on polyethylene film was immunostained with GST-P (*see* **Subheading 3.2.3.**) and a circle area of 150 μm radius within a GST-P-positive focus was microdissected. **(B)** Removed GST-P-positive cellular area from the section shown in **Fig. 4A**. **(C)** Identical portions of circle area in GST-P-positive foci were microdissected from H&E-stained adjacent section. A mean of 150 cells were contained in the circle area ($n = 5$). (Reproduced with permission from **ref. *15***.)

cells can be picked up with a thin needle tip (**Fig. 4**) ***(15)***. Transfer of microdissected specimen on the cap of a PCR tube should be verified under a microscope.

3.4. DNA Extraction From Microdissected Cells

Microdissected cells or tissue areas on PCR tube caps are subjected to extraction with 4 μL of TaKaRa DEXPAT™ (*see* **Note 5**) at 95°C for 10 min, and the entire extracts are used as a template for PCR by adding to the master mix of total 50 μL directly as described in **Subheadings 3.5.1.** and **3.5.2.** ***(15)***. In the case of a large cellular area such as 1 × 1 mm area, tissue specimens are extracted with 40 μL of DEXPAT.

3.5. PCR

PCR is the major tool for analysis of genomic DNA; cycle numbers should be minimized to avoid amplification-derived DNA-polymerization errors. Hot-start PCR of the genomic sequence of the gene of interest is performed with

PLATINUM *Taq* DNA polymerase in a 50-µL total reaction volume *(15)*. If nested PCR is intended, 1 µL of the first PCR product is used as a template in a 20-µL total volume (*see* **Note 6**).

3.5.1. Amplification by Nested PCR

1. Aliquot 4 µL of extracted DNA and mix with PCR reaction mixture contained 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 0.2 m*M* dNTP, 1.5 m*M* $MgCl_2$, 0.2 µ*M* each primer, and 2.5 U of *Taq* DNA polymerase in a 50-µL total volume.
2. Perform first-step PCR of 20–35 cycles.
3. Aliquot 1 µL of the first PCR product and mix with PCR reaction mixture with 1 U of PLATINUM *Taq* DNA polymerase in a 20-µL total volume.
4. Perform second-step PCR of 20–35 cycles.
5. Aliquot 8 µL of the PCR product and run agarose gel electrophoresis to identify the amplified target fragment (*see* **Fig. 5**).
6. Aliquot 10 µL of the PCR product for direct sequencing.

By nested PCR, the 522-bp DNA fragment of the α_{2u}-globulin gene is successfully amplified in 20% of the PCR attempts of single Purkinje cells with a total of 70 PCR cycles from cresyl violet-stained rat cerebellum sections (*see* **Table 3**) *(15)*. Similar, but less effective, amplification can be obtained with microdissected areas of hippocampal CA1 region, in which a successful detection is obtained in 15% of 20 × 20 µm samples (corresponding to 2.4 cells). The frequency of PCR detection increases with the area microdissected, but does not reach 100% even in a 60 × 60 µm area.

3.5.2. Amplification by Single-Step PCR

1. Aliquot 4 µL of extracted DNA and mix with PCR reaction mixture as described in **Subheading 3.5.1.** and 2.5 U of *Taq* DNA polymerase in a 50-µL total volume.
2. Perform PCR of 35 cycles.
3. Aliquot 8 µL of the PCR product and run agarose gel electrophoresis to identify the amplified target fragment.
4. Aliquot 10 µL of the PCR product for direct sequencing.

In the PALM system, either a rectangle or a circle of any size can be microdissected in automated mode. In H&E-stained rat liver sections as described in **Fig. 4**, a 522-bp fragment can be amplified by single-step PCR of 35 cycles with both 150- and 200-µm-radius samples after DEXPAT extraction, although the amplification of 969-bp fragments is unsuccessful even with 200-µm-radius samples (*see* **Table 4**) *(15)*. Liver samples of 150- and 200-µm-radius areas in this case contain 150 and 270 cells, respectively. In immunostained tissue, a weak 522-bp band can be amplified only with 200-µm-radius samples. In the case of 150-µm-radius samples, only a 184-bp fragment can be amplified.

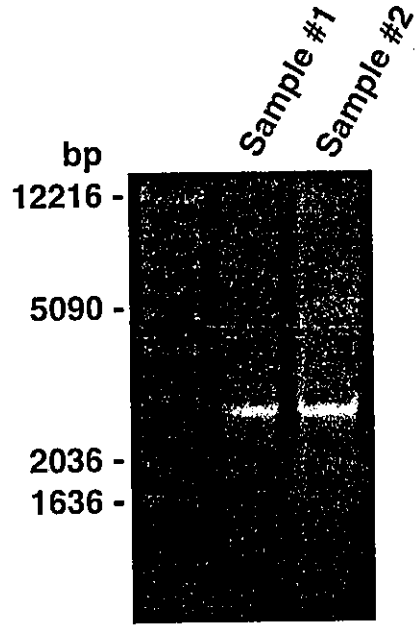

Fig. 5. Nested PCR results for the α_{2u}-globulin genomic sequence (Accession no. M24108 in GenBank/EMBL Data Bank), sized 2.8 kb, with DNA extracted from methacarn-fixed wax-embedded rat cerebral cortex as described previously *(15)*. From 10-μm thick, cresyl violet-stained brain sections, 1 × 1 mm areas of cerebral cortex were microdissected and extracted with 40 μL of DEXPAT to extract DNA. Four-μL aliquots of cell extracts were directly applied for the first PCR reaction with upstream-outside primer, 5'-ACGGATCCAG GCTTCAAGTT CCGTATTA-3' and downstream primer for the 2954-bp fragment, 5'-TGAAATCCTG AGACTAAGCT-3'. With 1 μL of the first PCR product, second-step PCR was performed to amplify a 2.8-kb fraction with a combination of upstream-inside primer, 5'-AAAGTTAAAT GGAATCAGAA-3', and the downstream primer used for the 2954-bp fragment in the first PCR. Nested PCR in a 20-μL total volume was performed using 1 μL of the first PCR product as a template. This figure shows results of two different samples. (Reproduced with permission from **ref.** *15*.)

4. Notes

1. Exposure of tissues to saline prior to fixation may cause a severe tissue shrinkage artifact *(10)*. Glassware for the preparation of methacarn should be autoclaved before use. Do not use disposable plasticware that can be damaged by chloroform for the preparation and/or storage of methacarn solution. For fixation, the ratio of the fixative volume to tissue volume should be 20:1–30:1. If necessary, tissue processing can be stopped at the step of ethanol dehydration, and tissue blocks can be kept in ethanol at 4°C for several days after fixation.
2. Immersion of the tissue section in aqueous solution for a long time may increase the risk of degradation of DNA *(15)*. If nuclear staining is intended, the staining

Table 3
Detection of 522-bp DNA Fragment From Microdissected Single Cells or Cellular Areas by Nested PCR[a]

Cell or cellular area	Microdissected	No. of samples	PCR-detection (%)
Purkinje cell	single cell	15	20
Hippocampus,	20 × 20 μm (2.4 cells)[b]	26	15
CA1 region	40 × 40 μm (9.5 cells)[b]	15	67
	60 × 60 μm (21.3 cells)[b]	15	87
Cerebral cortex[c]	1 × 1 mm	24	100

[a]Rat α_{2u}-globulin gene was used for PCR as described previously in **ref. *15***. First-step PCR was performed to amplify a 969-bp fragment with an upstream-outside primer, 5'-ACGGATCCAG GCTTCAAGTT CCGTATTA-3', and a downstream primer, 5'-CGTCATCTGT GGAGGAAATT-3'. With 1 μL of the first PCR product, second-step was performed to amplify a 522-bp fragment with an upstream-inside primer, 5'-AAAGTTAAAT GGAATCAGAA-3', and a downstream-inside primer, 5'-TAAGTCCGTC TCACATGGCT-3'.

[b]Mean cell number in a 60 × 60 μm area (n = 18) was estimated with the aid of an objective micrometer, and the mean cell number in each square area was calculated.

[c]Extract from 1 × 1 mm area of cerebral cortex was further diluted with DEXPAT to adjust the concentration of template to correspond to a 60 × 60 μm area for first-step PCR.

(Reproduced with permission from **ref. *15***.)

solution should be autoclaved or filtrated if possible. Methacarn-fixed tissue sections can be stained more quickly than formalin-fixed tissue sections, and therefore the time of the histological staining procedure can be reduced.

3. Immunostaining of methacarn-fixed wax-embedded tissue results in a substantial decrease in DNA yield, in particular the loss of high molecular weight DNA. There is progressive decrease in DNA yield in proportion to the length of the immunostaining process *(15)*.
4. There are two major techniques for microdissection utilizing the precision of lasers. One technique is laser microbeam microdissection as employed in our laboratory; this system is based on a pulsed UV laser with a small beam focus to cut out areas or cells of interest by photoablation of adjacent tissue. Another technique is laser capture microdissection, which uses a low-energy infrared laser pulse to capture the targeted cells by focal melting of the thermoplastic membrane through laser activation. Advantages and disadvantages of these systems are described elsewhere *(26)*.
5. TaKaRa DEXPAT is a reagent originally designed for one-step extraction of DNA from wax-embedded tissue fixed with 10% formalin. DEXPAT is designed to optimize DNA extraction from wax embedded tissue; it utilizes ion exchange resin and detergents, and DNA is extracted in the supernatant. We use only the detergent component.With methacarn-fixed wax-embedded tissues, the time for the preparation of PCR-ready DNA from wax-embedded tissue is dramatically reduced from 2 to 3 d required for a conventional method for formalin-fixed tissues to 25 min.

Table 4
Detection of Genomic DNA Fragment by Single-Step PCR in H&E-Stained or Immunostained Tissue Areas From Methacarn-Fixed Rat Liver Wax-Embedded Tissue Sections[a]

Stain	Tissue area (μm in radius)	Fragment size (bp)	Positive detection
H&E-stained	200	184[b]	8/8 (100%)
	200	522[c]	9/9 (100%)
	200	969[c]	0/8 (0%)
	150	184	5/5 (100%)
	150	522	5/5 (100%)
Immunostained[d]	200	184	5/5 (100%)
	200	522	5/5 (100%)
	150	184	10/10 (100%)
	150	522	0/5 (0%)

[a]Liver of a rat treated with thioacetamide at the promotion stage in the two-stage hepatocarcinogenesis model was used as described previously in **ref. *15***. Circle areas of 150 or 200 μm in radius were microdissected and solubilized with 4 μL of DEXPAT solution in PCR tubes at 95°C for 10 min and whole extracts were subjected to PCR directly. PCR with 50 μL reaction volume was performed to amplify 184-, 522-, and 969-bp fragments with the same cycle parameters of 95°C for 2 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 30 s, and final extension at 72°C for 7 min. Eight microliters of PCR product was applied to 2.0% agarose gel electrophoresis.

[b]Rat GST-P gene (Accession no. L29427 in GenBank/EMBL Data Bank) was amplified using upstream primer, 5'-GGAGCAGGAC CCAAAAATGA-3', and downstream primer, 5'-GCA GACGAAT AAAGGCCCCA-3'.

[c]Rat α_{2u}-globulin gene was used for amplification. Primer pairs for each DNA fragment were similar to those described in the footnote of **Table 3.**

[d]Sections were immunostained with GST-P.

(Reproduced with permission from **ref. *15***.)

6. To amplify target fragment sizes smaller than 1 kb, PCR was performed with cycle parameters of 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 30 s. The extension time for 2, 3, and 4 kb is 1.5, 2.5, and 3.5 min respectively. Although the source of cells and the detection system are different from those in the present study, similar performance was obtained when DNA from 25 cells of alcohol-fixed cytology specimens was used in the multiplex PCR *(27)*.

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ARTICLE

Microdissected Region-specific Gene Expression Analysis with Methacarn-fixed, Paraffin-embedded Tissues by Real-time RT-PCR

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SUMMARY We have previously shown methacarn to be a versatile fixative for analysis of proteins, DNA, and RNA in paraffin-embedded tissues (PETs). In this study we analyzed its suitability for quantitative mRNA expression analysis of microdissected PET specimens using a real-time RT-PCR technique. Fidelity of expression in the methacarn-fixed PET sections, with reference to dose-dependent induction of cytochrome P450 2B1 in the phenobarbital-treated rat liver, was high in comparison with the unfixed frozen tissue case, even after hematoxylin staining. RNA yield from methacarn-fixed PET sections was equivalent to that in unfixed cryosections and was also not significantly affected by hematoxylin staining. Correlations between the expression levels of target genes and input amounts of extracted RNA in the range of 1–1000 pg were very high (correlation coefficients >0.98), the regression curves being similar to those with unfixed cryosections. Although cell numbers should be optimized for each target gene/tissue, >200 cells were necessary for accurate measurement in 10-μm-thick rat liver sections judging from the variation of measured value in small microdissected areas. These results indicate high performance with methacarn, close to that of unfixed tissues, regarding quantitative expression analysis of mRNAs in microdissected PET-specimens. (J Histochem Cytochem 52:903–913, 2004)

KEY WORDS
methacarn
paraffin-embedded tissue
mRNA expression
real-time RT-PCR
microdissection
hematoxylin staining

THE RECENT DEVELOPMENT of microdissection techniques has enabled us to perform biochemical or molecular biological analyses of small tissue areas (Emmert-Buck et al. 1996; Schütze and Lahr 1998). For this purpose, use of cryosections from unfixed frozen tissues has become the gold standard because molecules to be analyzed remain intact. However, preparation of cryosections from unfixed frozen tissue for the purpose of microdissection may not be optimal for routine samples because of the inconvenience in terms of tissue storage and the skill required for preparation and subsequent microdissection. Therefore, tissue embedding after fixation is preferable for microdissected tissue preparations if high yield and quality of molecules can be guaranteed.

For histological assessment, tissue fixation and subsequent paraffin embedding are routinely employed because of the ease of handling tissues and subsequent staining, as well as the good preservation of morphology. Usually, formaldehyde-based fixatives, such as buffered formalin, are used for this purpose. However, with such crosslinking agents there is limited performance in terms of the yield and quality of extracted RNA (reviewed by Srinivasan et al. 2002), protein (Ikeda et al. 1998; Shibutani et al. 2000), and genomic DNA (Srinivasan et al. 2002), with consequent difficulty in the analysis of microdissected, histologically defined tissue areas. Extraction efficiency and quality of molecules are critical for analysis in microdissected cells. Recently, we found that methacarn, a non-crosslinking organic solvent fixative (Puchtler et al. 1970), meets critical criteria for analysis of RNAs, proteins,

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and DNAs in microdissected defined areas of paraffin-embedded tissue (PET) sections by simple extraction protocols (Shibutani et al. 2000; Shibutani and Uneyama 2002; Uneyama et al. 2002). With regard to RNA expression analysis using RT-PCR, long RNA fragments as well as rare RNA species can successfully be amplified from methacarn-fixed PET sections (Shibutani et al. 2000).

For RNA expression analysis in microdissected tissue samples, PCR-based techniques are suitable because of their sensitivity with samples having as few as 10 copies of a specific transcript. In this study we examined the suitability of methacarn fixation for measurement of mRNA expression levels in microdissected PET specimens using real-time PCR (Higuchi et al. 1992,1993). For this purpose, we assessed (a) fidelity of mRNA expression in comparison with unfixed frozen tissue, (b) abundance of amplifiable mRNAs in comparison with unfixed cryosections, (c) linearity between the input amount of extracted total RNA and the expression level, (d) effect of tissue staining with hematoxylin, and (e) cell numbers required for practical measurement of mRNA expression in hematoxylin-stained tissue.

Materials and Methods

Animals and Experimental Design

Sprague–Dawley rats from Charles River Japan (Kanagawa, Japan) were used. They were maintained in an air-conditioned animal room (temperature 24 ± 1C; relative humidity 55 ± 5%) with a 12-hr light/dark cycle and allowed ad libitum access to feed and tap water. All animals, including pregnant rats, were housed individually in polycarbonate cages with wood chip bedding.

To measure the dose-dependent induction of cytochrome P450 (CYP) 2B1 mRNA in the liver by treatment with sodium phenobarbital (PB; Wako Pure Chemical Industries, Osaka, Japan), female rats received daily IP injections of PB at doses of 0 (vehicle saline), 1.25, 5, 20, or 80 mg/kg body weight/day for 3 days and were sacrificed 24 hr after the last injection. The highest dose was selected according to the PB-specific enzyme induction protocol described by Kocarek et al. (1998). For practical assessment in microdissected areas, region-specific expression of mRNAs was measured in the hypothalamic medial preoptic area (MPOA) in male and female pups at postnatal day 10, the time point for the late stage of brain sexual differentiation in rats (Rhees et al. 1990a,b).

All animals used in the present study were sacrificed by exsanguination from the abdominal aorta under ether anesthesia. The animal protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Tissue Fixation

Methacarn solution consisting of 60% (v/v) absolute methanol, 30% chloroform, and 10% glacial acetic acid was freshly prepared before fixation and stored at 4C until use. At autopsy, livers were removed and 3-mm-thick slices or 5 × 5 × 3 mm-sized tissue blocks were prepared from the left lateral lobe and fixed in methacarn for 2 hr at 4C with gentle agitation. Whole brains of rat pups were also removed and subjected to methacarn fixation. For embedding, liver slices/blocks and coronal brain slices, including the hypothalamus, were dehydrated three times for 1 hr in fresh 99.5% ethanol at 4C, immersed in xylene for 1 hr and then three times for 30 min at room temperature (RT), and immersed in hot paraffin (60C) four times for 1 hr, for a total of 4 hr. Embedded tissues were stored at 4C for up to 6 months until tissue sectioning. Unfixed liver tissue samples, either 3 × 3 × 1 mm or 5 × 5 × 3 mm, were also prepared from portions adjacent to the tissue samples for methacarn fixation and immersed in RNAlater (Ambion; Austin, TX) overnight at 4C, or embedded in Tissue-Tek 4583 OCT compound (Sakura Finetek Japan; Tokyo, Japan) by quick freezing on dry ice. They were stored at −80C until direct extraction of RNA or sectioning before RNA extraction, respectively. For immunohistochemical analysis of MPOA in pups, brains were immersed in 10% neutral buffered formalin (pH 7.4) overnight at RT with gentle agitation. Coronal brain slices that included the hypothalamus were then routinely embedded in paraffin.

Preparation of Tissue Specimens and Microdissection

For assessment of dose-dependent induction of CYP2B1 in the rat liver by PB treatment, methacarn-fixed PETs were sectioned at 10 μm and mounted on 2.5-μm PEN-foil film (Leica Microsystems; Tokyo, Japan) overlaid on a glass slide that had been treated with 3% H_2O_2 for 10 min, rinsed with absolute ethanol, and then dried in an incubator overnight at 37C. The sections were deparaffinized by immersion in xylene twice for 2 min, followed by 99.5% ethanol once for 30 sec. Sections were either unstained or stained with Tissue Tek Hematoxylin 3G (Sakura Finetek Japan) for 10 sec, rinsed briefly with water, and air-dried. For assessment of linearity between the input amounts of total RNA and expression levels of target genes, as well as for estimation of the relative abundance of amplifiable mRNAs, series of 20 10-μm-thick sections were prepared from 5 × 5 × 3-mm unfixed frozen tissues and methacarn-fixed PETs and were collected into 1.5-ml tubes. Integrity of extracted total RNA was also examined in these preparations by judging the resolution of rRNAs in agarose gel. In this experiment, effect of fixation itself on the integrity was also examined with fresh-frozen sections fixed with methacarn for 10 min at 4C. For preparation of tissue sections and hematoxylin staining, RNase-free ultrapure water, prefiltrated with a Gengard filter attached to an Elix 3 ultrapure water system (Millipore; Billerica, MA) was employed. Whole tissue areas of methacarn-fixed PET sections were dissected together with PEN-foil film and collected into 1.5-ml tubes for the dose-dependent expression analysis. With microdissected small tissue areas, hematoxylin-stained 10-μm-thick sections were used and circles of 30-, 50-, and 100-μm radius were microdissected from mid-zonal areas of hepatic lobules using PALM Robot-MicroBeam equipment (Carl Zeiss; Tokyo, Japan). In addition, for assessment of the relationship between the cell number and the amount of extracted total RNA, square ar-

eas of 250 × 250, 500 × 500, and 1000 × 1000 μm were also microdissected.

For microdissection of MPOA, 6-μm-thick sections between pairs of 20-μm-thick sections were prepared from methacarn-fixed rat brain PETs. The 20-μm sections were mounted on PEN-foil film. As shown in Figure 1, localization of the sexually dimorphic nucleus of the preoptic area (SDN-POA), identified as an intensely stained cellular region, was determined under microscopic observation of 6-μm-thick sections stained with hematoxylin and eosin, and the bilateral portions of the MPOA (1000 × 600 μm) containing SDN-POA were microdissected from the adjacent unstained 20-μm-thick sections. Because of sexual dimorphism in the volume of SDN-POA, six to ten sections in males and four to six sections in females were used for microdissection.

RNA Extraction

Quantitative mRNA expression analysis of target genes was performed with a real-time RT-PCR system. In cases of unfixed frozen liver tissue blocks (3 × 3 × 1 mm), whole tissue sections of liver PETs, and microdissected MPOAs from the brain PET sections, total RNA was extracted using RNA STAT-60 (Tel-Test "B"; Friendswood, TX), precipitated with isopropanol in the presence of 2 μg/ml glycogen as a carrier, and reconstituted with 10 μl of ultrapure water treated with diethylpyrocarbonate (Ambion). Unfixed frozen tissue blocks were disintegrated in RNA STAT-60 solution with a Mixer Mill MM300 (QIAGEN; Tokyo, Japan) before extraction. For liver tissue sections of 5 × 5 mm from 5 × 5 × 3-mm unfixed frozen tissues and methacarn-fixed PETs, total RNA was extracted with RNeasy Mini (QIAGEN) according to the manufacturer's protocol, and the final elution volume was set at 30 μl. Contaminating genomic DNA was digested with DNase I (Ambion) at the end of the extraction according to the manufacturer's protocol. One μl of isolated RNA was labeled with a RiboGreen RNA Quantitation kit (Molecular Probes; Eugene, Oregon) and concentrations were estimated with a fluorescence spectrophotometer F2500 (Hitachi; Tokyo, Japan) in 1 ml of total volume with water.

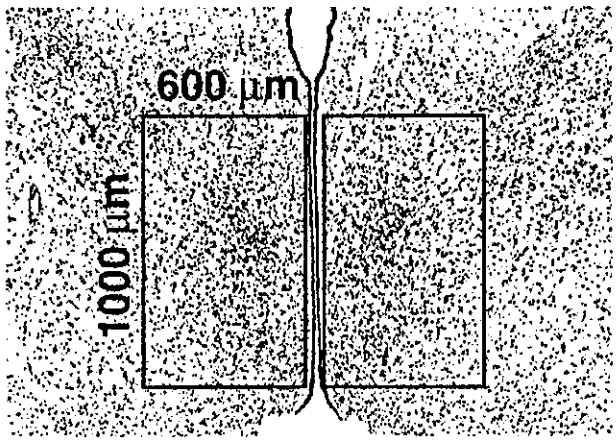

Figure 1 The microdissected area for expression analysis in the MPOA of rat pups at postnatal day 10. The intensely stained cellular regions near the center of each rectangle area are SDN-POAs.

In cases of small tissue areas microdissected from liver PET sections, RNAqueous-Micro (Ambion) was used for total RNA extraction and the final elution volume was set at 20 μl. Contaminating genomic DNA was digested with DNase I included in the kit and the final volume was set to be 25.3 μl.

Real-time RT-PCR

When two-step real-time RT-PCR was planned, RT was performed using 1 μl (200 U) of SuperScript II RNase H⁻ Reverse Transcriptase with 2 μl of 50 μg/ml random hexamers, 1 μl of 10 mM dNTP mix, 2 μl of 10 × PCR buffer, 1.2 μl of 50 mM $MgCl_2$, 2 μl of 0.1 M dithiothreitol, 1 μl of RNase inhibitor, and 9.8 μl of RNA solution in a 20-μl total reaction volume (all reagents were purchased from Invitrogen; Carlsbad, CA). After treatment with 1 μl of RNase H, 1 μl of RT product was subjected to real-time PCR in a 25 μl of total reaction volume with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems Japan; Tokyo, Japan) using either QuantiTect SYBR Green PCR Kit (QIAGEN) or TaqMan Universal PCR Master Mix (Applied Biosystems Japan). With this two-step RT-PCR, mRNA expression levels of CYP2B1, estrogen receptor (ER)α, ERβ, γ-aminobutyric acid transporter type 1 (GAT-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured. Primer Express software (Version 2.0; Applied Biosystems Japan) was used for the design of primer sequences and TaqMan probes. For expression analysis of GAPDH, either SYBR Green or TaqMan probe system was applied. In the latter case, TaqMan Rodent GAPDH Control Reagents (Applied Biosystems Japan) were used. The sequences of primers and probes are listed in Table 1.

With the SYBR Green detection system, mRNA levels of CYP2B1, ERβ, GAT-1, and GAPDH were measured (1 μl of RT product, 12.5 μl of 2 × QuantiTect SYBR Green PCR Master Mix, and 300 nM of primers in a 25-μl total reaction volume). Cycle parameters in this system were as follows: initial activation at 95C for 15 min; 50 cycles of 15 sec at 94C, 30 sec for annealing, and 30 sec at 72C. Annealing temperatures for CYP2B1, ERβ, GAT-1, and GAPDH were 53C, 54C, 54C, and 59C, respectively. With the TaqMan probe detection system, mRNA levels of ERα and GAPDH were measured (1 μl of RT product, 12.5 μl of 2 × TaqMan Universal PCR Master Mix, 900 nM of primers, and 250 nM of TaqMan probe in a 25-μl total reaction volume). Cycle parameters with this system for both genes were: single step of 50C for 2 min, initial activation at 95C for 10 min; 50 cycles of 15 sec at 95C and 60 sec at 60C.

When RT and following real-time PCR were intended to be performed sequentially in one tube, one-step kits, such as the QuantiTect SYBR Green RT-PCR Kit (QIAGEN; for CYP2B1) and the QuantiTect Probe RT-PCR Kit (QIAGEN; for GAPDH) were used with 5 μl of total RNA in a 50-μl total reaction volume according to the manufacturer's protocols. Cycle parameters for CYP2B1 were similar to the above described two-step case, and a RT step at 50C for 30 min was preceded for the initial activation step at 95C for 10 min. In the case of GAPDH, cycle parameters were as follows: single step of 50C for 30 min; single step of 95C for 15 min; and 50 cycles of 94C for 15 sec followed by 60C for 60 sec.

Table 1 Sequences of primers and probes used for real-time RT-PCR

Gene	Accession No.		Sequence	Product size
CYP2B1	M37134	Sense	5'-TTGGCTCCCAAGGACATTG-3'	72 bp
		Antisense	5'-GATCTGGTACGTTGGAGGTATTTTTC-3'	
ERα	Y00102	Sense	5'-GGGCTTCCCCAACACCAT-3'	65 bp
		Antisense	5'-CGTTTCAGGGATTCGCAGAA-3'	
		Probe	5'-TGAGAACTCCCAGGCTCCCCACAA-3'	
ERβ	U57439	Sense	5'-TGCTGGATGGAGGTGCTAATG-3'	82 bp
		Antisense	5'-CGAGGTCGGGAGCGAAA-3'	
GAT-1	NM_024371	Sense	5'-CCTCTGAGATGTTTGGCAAGAA-3'	82 bp
		Antisense	5'-AATTGTACGACCCTTAACGTTGTG-3'	
GAPDH*	M17701	Sense	5'-GGCCGAGGGCCCACTA-3'	88 bp
		Antisense	5'-TGTTGAAGTCACAGGAGACAACCT-3'	

*For TaqMan PCR, a commercially available TaqMan Rodent GAPDH Control Reagents (Applied Biosystems) was used (sequence information is not available).

As a negative control for RT, reverse transcriptase (−) mock RT samples were included in each PCR experiment.

Immunohistochemical Analysis

Because ERα mRNA is differentially expressed in the MPOA depending on the gender, the corresponding protein expression was also examined immunohistochemically. A series of five 3-μm-thick sections were prepared at 30-μm intervals through the MPOA and the first of each series was stained with hematoxylin and eosin. These sections were examined microscopically and one showing the maximum size of SDN-POA was identified and selected for IHC with ERα in each animal. Deparaffinized and hydrated sections were treated with microwaving for 9 min in 0.01 M citrate buffer (pH 6.0) and treated with 1% periodic acid solution for 10 min. After incubation with mouse anti-ERα monoclonal antibody (Novocastra Laboratories, Newcastle upon Tyne, UK; × 40 dilution), immunodetection was performed using a VECTASTAIN Elite ABC KIT (Vector Laboratories; Burlingame, CA) with a standard protocol using diaminobenzidine as chromogen. The sections were then counterstained with hematoxylin. Digital photomicrographs at a magnification of ×180 were taken with a Fujix Digital Camera system (Fujifilm; Tokyo, Japan), and the numbers of immunostained nuclei within the MPOA (600 × 1000-μm areas) were counted using MacSCOPE (version 2.65; Mitani, Fukui, Japan).

Statistical Analysis

Comparison of data of mRNA expression levels and ERα-immunoreactive cell numbers in MPOA was performed with the Student's *t*-test after confirmation of equal variance of values. Pearson's correlation coefficients were calculated between the input amounts of RNA and the target gene expression levels in the validation studies in the liver and MPOA. Variability was expressed as coefficient of variation (CV).

Results

Integrity of Total RNA

Figure 2 shows the integrity of extracted total RNA from methacarn-fixed PET sections. Judging from the resolution of 18S and 28S rRNAs, integrity of total RNA was well preserved in the methacarn-fixed frozen sections (Figure 2, Lane 2) similar to that from unfixed frozen sections (Figure 2, Lane 1). In the methacarn-fixed PET sections (Figure 2, Lane 3), integrity of both rRNA bands was largely retained, but slight reduction of the band intensity of 28S rRNA was also observed as well as a slight increase of background smearing at the position below the 28S band.

Fidelity of mRNA Expression

Figure 3 shows data for mRNA expression in unfixed frozen tissue and methacarn-fixed PET sections, unstained or stained with hematoxylin. Dose-dependent induction of CYP2B1 mRNA was evident in the livers of rats treated with PB for 3 days. In the unfixed tissue, a clear dose-dependent induction of CYP2B1 was detected, and expression levels relative to that at 80 mg/kg PB were 27.5, 4.95, 1.10, and 0.33%, at 20, 5, 1.25, and 0 mg/kg, respectively. Similar dose-dependent expression was observed in the methacarn-fixed

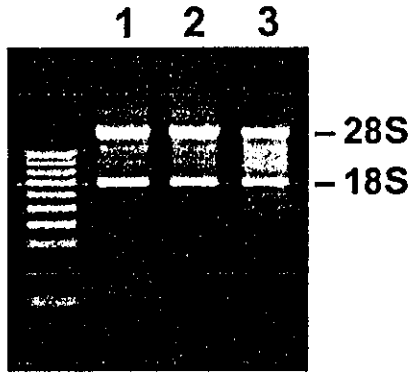

Figure 2 Integrity of total RNA extracted from methacarn-fixed rat liver PET sections. One-μg total RNA samples were resolved in a 1.0% agarose gel and visualized with ethidium bromide. Lane 1, unfixed frozen sections; Lane 2, methacarn-fixed frozen sections; Lane 3, methacarn-fixed PET sections.

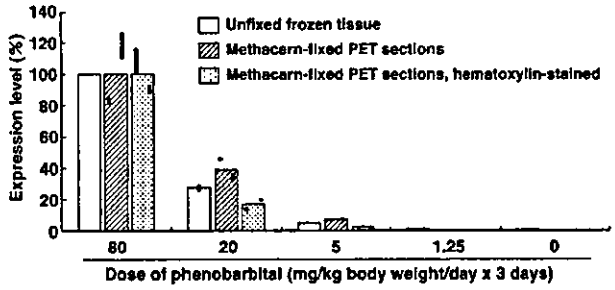

Figure 3 Comparison of gene expression pattern between the unfixed frozen tissue and stained or unstained methacarn-fixed PET sections. For analysis of dose-dependent CYP2B1 expression in the rat liver by PB, one animal was examined at each dose, and therefore the tissue source was set to be identical between preparations at each dose level. In the unfixed frozen tissue, RT was performed with 2 μg of total RNA. For methacarn-fixed PET sections, RT was performed with 335 ng of total RNA. Real-time PCR was performed in duplicate on each RT product. The upper and lower ends of the bars on each column of the graph represent the expression levels measured in duplicate from the same cDNA template.

PET sections irrespective of staining with hematoxylin, although slight suppression was noted at doses of 1.25 and 0 mg/kg. With regard to variability, there was a maximal 47% difference between the values in each RT sample at 80 mg/kg PB (unstained sections). The relative expression levels at 20, 5, 1.25, and 0 mg/kg PB were 38.6, 7.27, 0.21, and 0.06% in unstained sections and 18.1, 2.49, 0.01, and 0.02 in hematoxylin-stained sections, respectively. Reverse transcriptase (−) mock RT samples did not show any amplification on the PCR.

Relative Abundance of Amplifiable mRNA Molecules

To examine the relative abundance of amplifiable mRNA molecules in the methacarn-fixed PET sections, gene expression levels were compared with those in unfixed cryosections using liver of a rat treated with PB at 80 mg/kg body weight/day for 3 days. RNA yields from unfixed cryosections and methacarn-fixed PET sections were determined to be 35.4 ± 11.3, and 42.1 ± 6.0 ng/mm^2 area in 10-μm-thick sections, respectively (n=5). With extracted total RNAs in the range of 1–1000 pg, relative expression of CYP2B1 and GAPDH was determined (Table 2). Although expression signals for both genes could be detected with 1 pg total RNA, values varied when input amount of total RNA was decreased in both tissue section preparations. With 100 and 1000 pg of total RNA, variability of expression data, as judged by the values of CV, was reduced in both unfixed and methacarn-fixed PET sections, with a small reduction of amplifiable mRNAs for both genes in the latter compared with unfixed cryosections (88.2~98.5% for CYP2B1 with statistical difference of $p<0.05$ at 100 pg; 76.5~86.3% for GAPDH with $p<0.05$ at 1000 pg).

Linearity Assessment of mRNA Expression with Input Amount of Total RNA

Figure 4 shows comparisons of regression curves between cryosections and methacarn-fixed PET sections from the liver of a PB-treated rat, based on the data shown in Table 2. In the methacarn-fixed PET sections, the linearity between input amounts of total RNA and expression levels was very high for both CYP2B1 and GAPDH genes and the curves were almost identical with those for unfixed sections. The correlation coefficients in the analysis of CYP2B1 and GAPDH with unfixed frozen sections were 0.997 and 0.990, respectively. Similarly, correlation coefficients in the methacarn-fixed PET sections were 0.991 and 0.982, respectively.

Variability of the mRNA expression data during the processes of RT and after real-time PCR was assessed for four genes with the same RNA sample de-

Table 2 Relative abundance of amplifiable mRNAs in methacarn-fixed PET sections[a]

	No. of samples	Extracted total RNA (pg)			
		1000	100	10	1
CYP2B1					
Unfixed cryosections	5	100.0 ± 7.1[b] (7.1)[c]	8.08 ± 0.41 (5.1)	0.61 ± 0.10 (16.4)	0.12 ± 0.04 (33.3)
Methacarn-fixed PET sections	5	98.5 ± 12.4 (12.6)	7.13 ± 0.69[d] (9.7)	0.64 ± 0.09 (14.1)	0.10 ± 0.04 (40.0)
GAPDH					
Unfixed cryosections	5	100.0 ± 13.3 (13.3)	8.54 ± 0.46 (5.4)	0.79 ± 0.15 (19.0)	0.06 ± 0.04 (66.7)
Methacarn-fixed PET sections	5	76.5 ± 13.6[d] (17.8)	7.37 ± 1.10 (14.9)	0.83 ± 0.37 (44.6)	0.04 ± 0.03 (75.0)

[a]Liver of a rat treated with phenobarbital (80 mg/kg body weight/day IP, once daily for 3 days). One to 1000 pg of total RNA extracted from 10-μm-thick sections by RNeasy Mini was subjected to one-step RT-PCR of GAPDH with the TaqMan probe detection system and CYP2B1 with the SYBR Green detection system.
[b]Relative expression (% of the level at 1000 pg of total RNA from unfixed cryosections). Values are expressed as mean ± SD.
[c]Values in parentheses represent the CV.
[d]Significantly different from the corresponding unfixed frozen section ($p<0.05$ by Student's t-test).

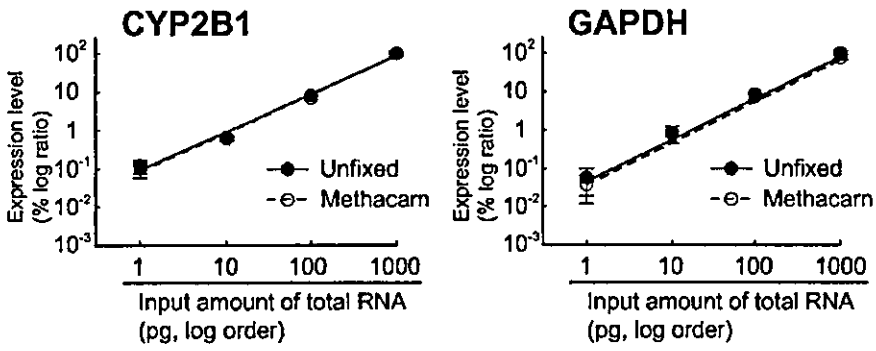

Figure 4 Linearity assessment of mRNA expression in the methacarn-fixed PET sections with the input amount of extracted total RNA in the range of 1–1000 pg. Expression levels of CYP2B1 and GAPDH were analyzed with one-step real-time RT-PCR. Relative expression levels were calculated when the values for the 1000 pg template RNA was accounted as 100% (mean ± SD; n=5). Pearson's correlation coefficients between the input amount of RNA and the expression of CYP2B1 or GAPDH were 0.997 and 0.990 in the unfixed frozen sections, and 0.991 and 0.982 in the methacarn-fixed PET sections.

rived from the MPOA of a male rat on postnatal day 10 (Figure 5). With the total RNA in the range of 5–45 ng, variability in the expression for each gene expressed as CV was mostly within 20%. In addition, high correlation of the expression levels to the input amount of total RNA was observed for all genes examined (0.972, 0.985, 0.965 and 0.985, respectively, for ERα, ERβ, GAT-1, and GAPDH).

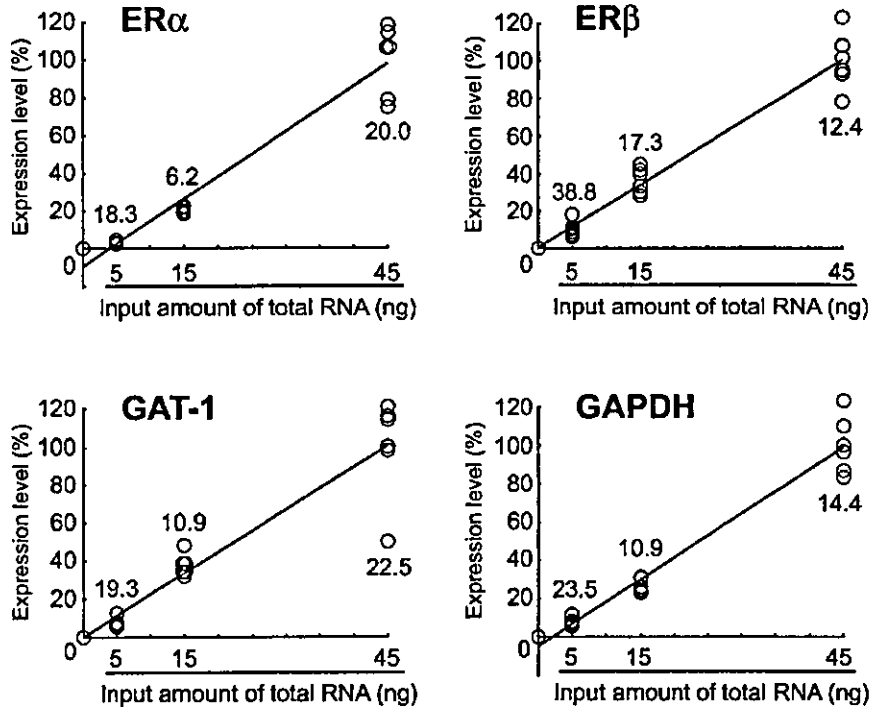

Figure 5 Variability of mRNA expression during the processes of RT and real-time PCR in the microdissected MPOA from methacarn-fixed, paraffin-embedded rat brain tissue. Total RNA extracted from microdissected specimens of MPOA from one male pup was divided to make triplicate samples for each of 5, 15, and 45 ng. RT was performed in a 20 μl of total volume. With 1 μl of RT product in duplicate, real-time PCR was performed for each gene utilizing the TaqMan probe detection system (ERα) or SYBR Green detection system (ERβ, GAT-1, and GAPDH). Relative expression levels were calculated when the values of the 45 ng template RNA was accounted as 100% (n=3). The values in the reverse transcriptase (−) mock RT product using 45 ng total RNA was used as those for zero template. Open circles represent expression levels in each sample. Numerical values in the graphs represent the CV of three samples in duplicate. Pearson's correlation coefficients between the input amount of total RNA and expression level of target gene were 0.972, 0.985, 0.965 and 0.985 for ERα, ERβ, GAT-1 and GAPDH, respectively.

Relative Abundance of mRNAs in the Hematoxylin-stained Sections

Table 3 shows the relative abundance of mRNA molecules retained in the hematoxylin-stained, methacarn-fixed PET sections in comparison with the unstained sections. RNA yields of unstained or hematoxylin-stained sections (ng/section, 10 μm in thickness) were 873 ± 276 (n=6), and 1136 ± 354 (n=5), respectively. Expression levels of CYP2B1 and GAPDH were examined with 1 or 10 ng of template total RNA. After hematoxylin staining, 0–20% reduction was observed in the relative abundance of amplifiable mRNAs for the genes, with statistical difference in CYP2B1 (p<0.05). The 1/10 reduction of the input amount of RNA (from 10 to 1 ng) reduced the expression levels of both CYP2B1 and GAPDH proportionally to ~1/10, irrespective of the tissue staining.

Gene Expression Analysis in the Microdissected MPOA of Rat Pups

Because sexual dimorphism in the expression of ERα in the developing rat MPOA has been demonstrated by IHC (Yokosuka et al. 1997), expression level of ERα mRNA was examined in the microdissected MPOA of rat pups at postnatal day 10 (Figure 6), along with the ERβ mRNA expression level, for which no substantial sexual dimorphism in the MPOA was found both by IHC and in situ hybridization (Orikasa et al. 2002). Expression of ERα mRNA in females was higher than that in males (Figure 6), even when the expression level was normalized to the GAPDH value. The ERβ mRNA expression level, on the other hand, did not differ between males and females. IHC of ERα in the brains at the same age demonstrated intense nuclear staining in the hypothalamic brain region (Figure 7A). Numbers of ERα-immunoreactive nuclei counted in the MPOA corresponding to the area used for mRNA expression analysis (600 × 1000 μm) were higher in females than in males (Figures 7A and 7B).

Table 3 Relative abundance of mRNAs in unstained and hematoxylin-stained, methacarn-fixed PET sections[a]

	No. of samples	Extracted total RNA (ng)	
		10	1
CYP2B1			
Unstained	6	100.0 ± 14.8[b]	100.0 ± 18.3 (9.0)[c]
Hematoxylin-stained	5	77.6 ± 5.1[d]	79.3 ± 6.5[d] (9.2)
GAPDH			
Unstained	6	100.0 ± 17.4	100.0 ± 18.1 (10.5)
Hematoxylin-stained	5	100.3 ± 17.1	85.0 ± 16.7 (8.9)

[a]Liver of a rat treated with phenobarbital (80 mg/kg body weight/day IP, once daily for 3 days). A 1- or 10-ng aliquot of total RNA extracted from 10-μm-thick sections by RNeasy Mini was subjected to one-step RT-PCR of GAPDH with TaqMan probe detection system and CYP2B1 with SYBR Green detection system.
[b]Expression level (%) relative to corresponding unstained sections. Values are mean ± SD.
[c]Values in parentheses represent (%) expression levels of corresponding tissue section with 10 ng input amount of RNA.
[d]Significantly different from the corresponding unstained section (p<0.05 by Student's t-test).

Cell Numbers Required for mRNA Expression

To determine the cell numbers required for quantitative measurement of mRNA expression in methacarn-fixed PET sections, hematoxylin-stained 10-μm-thick liver sections of a rat treated with 80 mg/kg PB were used and tissue areas up to 100 μm in radius were randomly microdissected from the mid-zonal areas of liver lobules. One-step real-time RT-PCR for CYP2B1 and GAPDH was performed. Table 4 shows the difference in the threshold cycle (C_T: fractional cycle number at which the fluorescent signal passes the fixed threshold) between the microdissected samples and standard samples, showing the lower limit within the dynamic range of amplification in each gene. For CYP2B1 expression, one or more circle tissues of 100 μm in radius, corresponding to >52 ± 3 liver cells (n=10), showed a C_T within the dynamic range of amplification. For GAPDH expression, most tissue-samples of more than 50 μm in radius showed a C_T within the dynamic range of amplification. Data variability between samples of identical tissue size was ex-

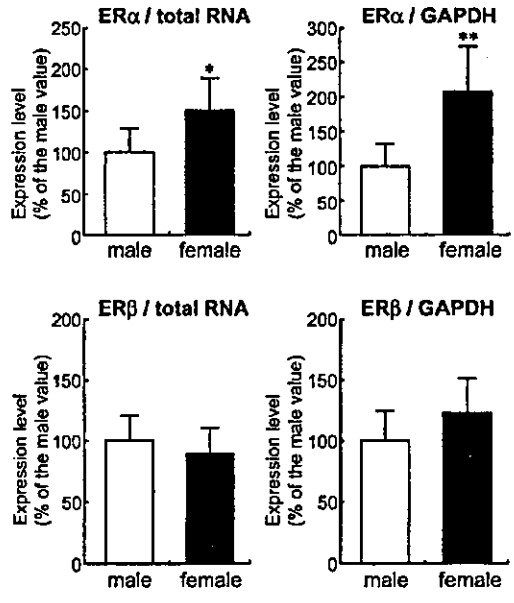

Figure 6 Measurement of mRNA expression levels of ERα and ERβ in the MPOA of male and female rat pups at postnatal day 10 using methacarn-fixed PET specimens. Numbers of animals examined were 5 for males and 6 for females. RT was performed with 24 ng of total RNA in a 20-μl total volume. With 1 μl of RT product, real-time PCR of each gene was performed. The values of ERα and ERβ were normalized for the amount of total RNA (left panels) or the expression level of GAPDH (right panels), and expressed as mean + SD. **, *: Significantly different from the male expression levels (*p<0.05, ** p<0.01 by Student's t-test).

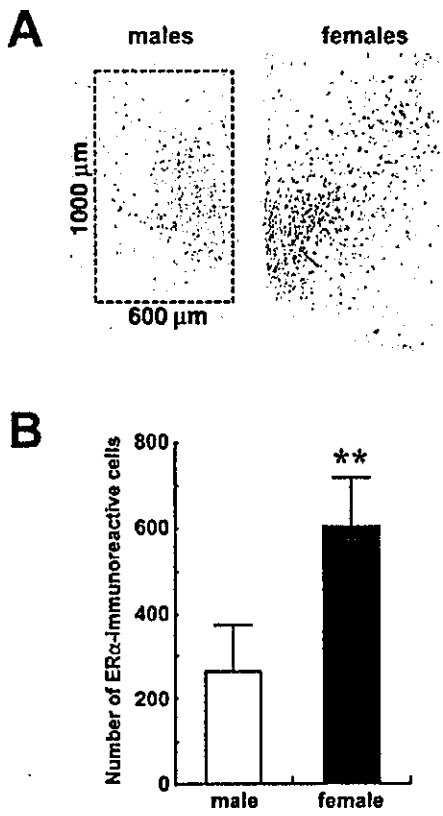

Figure 7 Sex difference of the ERα-immunoreactive cell population in the MPOA of rat pups at postnatal day 10. (A) Number of immunoreactive nuclei was counted in one MPOA (1000 × 600 µm) of one section in each animal. Left, male; right, female. (B) Mean numbers of immunoreactive cells in the MPOA (mean + SD; $n=3$ for both males and females). **: Significantly different from the male value ($p<0.01$ by Student's t-test).

pressed as CV for the "difference in C_T." With both genes, variability of data was decreased with an increase in the area of microdissected tissue, and less variable data were obtained with samples corresponding to the 208 cell/area. Variability between samples was greater with CYP2B1 compared with GAPDH.

Total RNA Yields in the Microdissected Tissue Areas

Table 5 shows RNA yields for microdissected unit areas of hematoxylin-stained rat liver PET sections. With increase in tissue size, RNA yield was proportionally increased. Because 1 ng/ml is the lower detection limit of RNA quantitation with RiboGreen fluorescent dye (manufacturer's instructions), in the rat liver a 250 × 250-µm area (corresponding to 104 cells) was the detection limit of RNA quantitation.

Discussion

Methacarn is an organic solvent fixative and therefore would not be expected to modify nucleotides or polypeptides as reported for crosslinking fixatives, such as formaldehyde (reviewed by Srinivasan et al. 2002). Previously, we have found that methacarn fixation followed by paraffin embedding does not affect the integrity of extracted total RNA, but results in halving of the RNA yield from unfixed frozen samples (Shibutani et al. 2000). However, we could here extract total RNA from methacarn-fixed rat liver sections with an efficiency equivalent to that with unfixed cryosections. Although we could not identify the reason for the observed difference between the previous and present studies, the tissue condition (tissue blocks in the previous study vs sectioned tissues in the present study), normalization of RNA yield (wet weight vs unit area), or extraction tool (RNASTAT-60 utilizing isopropanol precipitation for RNA isolation vs RNeasy Mini utilizing selective binding properties of silica gel-based membrane for RNA isolation) might have exerted an influence. We also observed only a small reduction in the relative abundance of amplifiable mRNAs retained in the methacarn-fixed PET sections compared with unfixed cryosections. Slight reduction of the integrity of extracted total RNA in the methacarn-fixed PET sections may be parallel to the results of the relative abundance of amplifiable mRNAs. On the other hand, methacarn itself does not appear to affect the quality of RNA molecules because fixation of fresh frozen sections for 10 min with this solution well preserved the integrity of total RNA in the present study. Considering the advantages in tissue handling during sectioning and after microdissection steps with PET specimens, as well as the disadvantages with unfixed frozen tissue in terms of tissue handling and instability of RNA molecules in sectioned specimens, methacarn fixation in combination with paraffin embedding has clear benefits for mRNA expression analysis of microdissected specimens.

Generally speaking, tissue staining with hematoxylin appears to affect both extraction efficiency and PCR amplification of genomic DNA (Murase et al. 2000; Serth et al. 2000), although in the present study the influence of brief staining with hematoxylin was marginal. Polynucleotides in tissue sections fixed with organic solvent fixatives, such as Carnoy's solution or methacarn, may be released into solution during IHC or ISH (Urieli-Shoval et al. 1992; Uneyama et al. 2002). However, methacarn-fixed PET sections could quickly be stained with hematoxylin in a period of 1–10 sec (Uneyama et al. 2002), and this might have contributed to the limited loss of extractable RNA after hematoxylin staining in the present study. We also observed only slight reduction (0–20%) in the relative abundance of amplifiable mRNAs after hematoxylin staining. We previously noted that staining with hematoxylin and eosin affected PCR of genomic DNA in methacarn-fixed PET sections, despite no deteriora-