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-

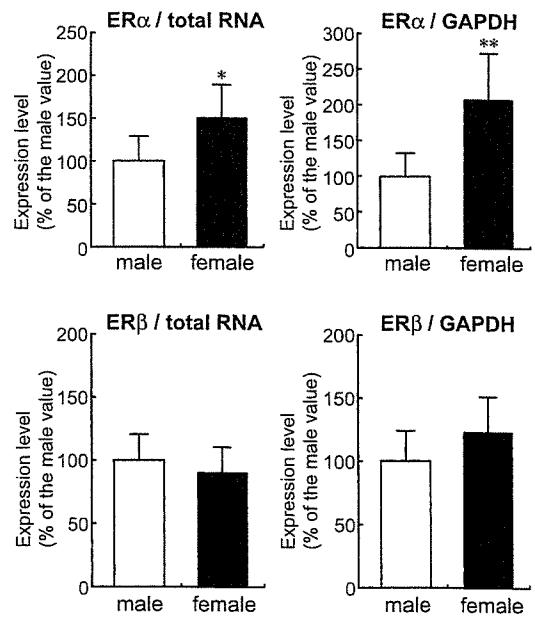

Figure 6 Measurement of mRNA expression levels of ERα and ERβ in the MPOA of male and female rat pups at postnatal day 10 using methacarn-fixed PET specimens. Numbers of animals examined were 5 for males and 6 for females. RT was performed with 24 ng of total RNA in a 20-μl total volume. With 1 μl of 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).

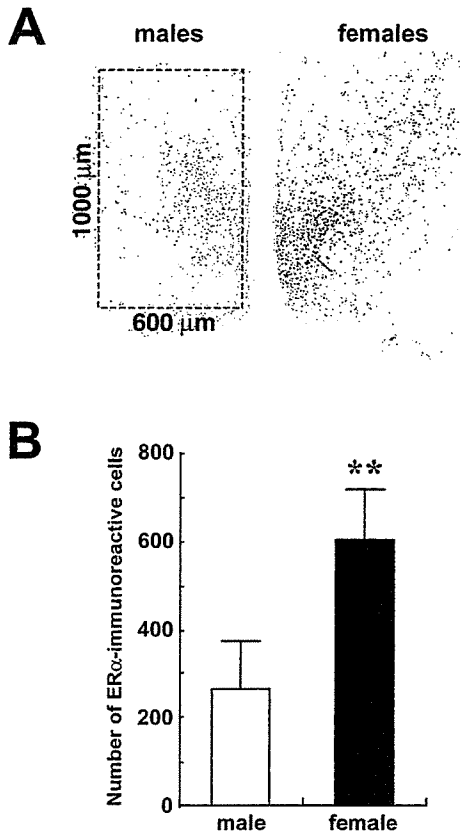

Figure 7 Sex difference of the ERα-immunoreactive cell population in the MPOA of rat pups at postnatal day 10. **(A)** Number of immunoreactive nuclei was counted in one MPOA (1000 × 600 μm) of one section in each animal. Left, male; right, female. **(B)** Mean numbers of immunoreactive cells in the 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-

Table 4 Difference in the C_T of real-time amplification in the microdissected samples from those of the lower limit of amplification in the standard samples[a]

	Tissue area (μm in radius)	Corresponding cell numbers[b]	No. of samples	Difference in C_T[c]	CV
Gene					
CYP2B1	100 × 4 pieces	208	6	3.9 ± 1.3[d]	33.3
	100 × 2 pieces	104	9	2.8 ± 2.0	71.4
	100 × 1 piece	52	8	1.6 ± 1.5	93.8
	50 × 1 piece	13	16	−1.7 ± 1.7	Not available
	30 × 1 piece	5	6	−2.1 ± 2.5	Not available
GAPDH	100 × 4 pieces	208	5	7.9 ± 0.4	5.1
	100 × 2 pieces	104	10	5.3 ± 1.5	28.3
	100 × 1 piece	52	9	1.9 ± 2.1	110.5
	50 × 1 piece	13	14	1.5 ± 1.9	126.7
	30 × 1 piece	5	5	−2.4 ± 1.3	Not available

[a]Liver tissue of a rat treated with PB at 80 mg/kg body weight/day for 3 days was used for both standard and microdissected samples. Unfixed frozen tissue was used for standard and hematoxylin-stained 10-μm-thick sections were subjected to analysis. With 5 μl of total RNA extracted by RNAqueous Micro, one-step RT-PCR was performed on GAPDH with the TaqMan probe detection system and CYP2B1 with the SYBR Green detection system.
[b]Cell numbers in each sample were calculated from the mean liver cell numbers in the circle area of 100 μm radius (52 ± 3; n=10).
[c]Values were calculated by subtracting the C_T of each sample from the C_T of the standard sample showing the lower limit of amplification. Expressed as mean ± SD.
[d]Amount of total RNA and its C_T (mean ± SD) of the standard sample at the lower limit of amplification within the dynamic range was respectively 0.4 pg and 32.4 ± 1.3 cycles for CYP2B1 (n=3), and 0.4 pg and 38.1 ± 0.9 cycles for GAPDH (n=4).

tion of the integrity and yield of extracted DNA (Uneyama et al. 2002). Hematoxylin has been reported to influence divalent cations (Mg^{2+}) that are important for maintaining Taq DNA polymerase activity (Chen et al. 1996), and this was reported to be apparent when manually dissected large tissue samples are subjected to PCR analysis (Burton et al. 1998; Murase et al. 2000). However, such an inhibitory effect might be negligible when microdissected small tissue specimens are analyzed (Ehrig et al. 2001). In line with the present study results, hematoxylin staining did not affect RT-PCR when microdissected small tissue specimens were analyzed in a previous study (Imamichi et al. 2001).

The cell number required for mRNA expression analysis in microdissected tissue specimens is primarily dependent on the expression level of the target genes of interest. In the case of cyclin D1 in primary tumor tissues, Specht et al. (2002) reported that transcripts could be measured in a minimum of 20 microdissected tumor cells from formalin-fixed PET specimens with an improved extraction protocol and the TaqMan PCR method in combination, but they also found 2000 cells to be suitable to obtain reproducible real-time PCR results. Although the expression values did not greatly vary (CV <20) from 100 pg of total RNA in the present experiment examining relative abundance of amplifiable mRNAs (Table 2), ~200 cells (corresponding to 2 ng based on the RNA yield data in Table 5) can be considered as a minimum for the practical expression analysis of mRNA species, judging from the very small variation of difference in C_T values of GAPDH gene with the corresponding tissue size (100 μm in radius × 4 pieces) reflecting homogeneity in the expression between samples as well as very small technical variation (Table 4). Expression levels of CYP2B1 varied between samples, even with a tissue area of 208 cells. With PB treatment, graded expression of CYP2B1 in the liver lobule occurs in the rat, with pronounced induction in the periportal region (Bühler et al. 1992). In the present study, midzonal areas of hepatic lobules were subjected to analysis, and therefore the variability of CYP2B1 expression in each sample might rather reflect a local event due to the graded expression profile of the transcript within this area.

In the present study, the concentration of total RNA was measured by RiboGreen fluorescent dye, with 1 ng as the lower detection limit in a 1-ml assay volume, and we could obtain ~1 ng of total RNA from 100 microdissected liver cells. If normalization of mRNA expression level to the input amount of total RNA is intended, a total of 3 ng or more (corresponding to >300 cells in a 10-μm-thick section of the rat liver)

Table 5 Total RNA yields in microdissected unit areas of methacarn-fixed rat liver PET sections[a]

Microdissected area[b]	No. of samples	RNA yield (ng/tissue)
1000 × 1000 μm (1669 cells)[c]	5	34.1 ± 5.81
500 × 500 μm (417 cells)	4	5.8 ± 2.89
250 × 250 μm (104 cells)	5	1.3 ± 0.16

[a]Liver of a rat treated with PB at 80 mg/kg body weight/day for 3 days. Total RNA (20 μl) extracted with RNAqueous-Micro was subjected to determination of RNA yield by RiboGreen RNA Quantitation kit.
[b]Tissue sections 10 μm thick were stained with hematoxylin before microdissection.
[c]Numbers of cells contained in each area were calculated from the mean cell numbers in the circle area of 100 μm radius as estimated in Table 4.

would be necessary for measurement of RNA concentration (1 ng) and after real-time RT-PCR of several genes (2 ng) under the present experimental conditions. If a fluorescence microplate reader is available, the total volume for assay of total RNA concentration could be minimized. As another normalization method, the expression level of a reference gene can be used. Housekeeping genes, such as GAPDH in the present study, are usually selected for this purpose. However, disadvantages with a single housekeeping gene were recently reported (Lee et al. 2002; Tricarico et al. 2002), and sexual dimorphism exists in GAPDH expression in several brain regions of developing rats (Perrot-Sinal et al. 2001). Sexual dimorphism of ERα expression in MPOA was apparent here, irrespective of the normalization method.

In conclusion, we have now demonstrated that methacarn-fixed PET allows practical mRNA expression analysis in microdissected areas with real-time RT-PCR after hematoxylin staining. Although recent studies have also demonstrated good performance of mRNA expression measurement with microdissected formalin-fixed PETs (Specht et al. 2001,2002), formaldehyde causes modification of nucleotides and therefore a high frequency of non-reproducible sequence alteration and amplification of only short fragments (reviewed by Srinivasan et al. 2002), suggesting limited utility for molecular analysis. Considering the availability for both DNAs and proteins in PET sections (Shibutani et al. 2000; Shibutani and Uneyama 2002; Uneyama et al. 2002), methacarn should prove to be a versatile tool for multipurpose analysis of target genes in specific cell populations. We are now applying methacarn for global gene expression analysis using a microarray technique with microdissected PET specimens. The question of how long molecules are retained intact in methacarn-fixed PET should now be addressed. Although we do not have data for archival tissues stored for several years/decades, mRNA levels could be measured with methacarn-fixed PET that had been prepared 6 months previously in the present study.

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Methacarn fixation—effects of tissue processing and storage conditions on detection of mRNAs and proteins in paraffin-embedded tissues

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Abstract

In this study, we examined suitable conditions for tissue fixation with methacarn and ethanol dehydration and storage of paraffin-embedded tissues (PETs) on gene expression analysis. With fixation and dehydration of rat liver tissues for up to 16 h (overnight) and 1 week, respectively, at 4 °C, integrity of extracted total RNAs and polypeptides did not vary, the former integrity being constantly lower than that with unfixed frozen tissue, while protein yield was slightly reduced with increasing dehydration. Retained expression levels of mRNAs and proteins were mostly unaffected by the period of fixation but slightly fluctuated with the length of dehydration. When PETs were stored for up to 12 months, integrity of both total RNAs and polypeptides was retained at 4 °C but reduced at room temperature. Reduced expression levels of mRNAs and proteins were also noted by storage at room temperature after 12 and 3 months, respectively. However, neither tissue processing nor storage affected variability in either mRNA or protein levels among samples. Thus, the results suggest that, for gene expression analysis, tissues can be fixed with methacarn and dehydrated for at least 1 day and 1 week, respectively, and PETs can be stored for at least 12 months, but a temperature of 4 °C is preferable.

Keywords: Methacarn; Molecular integrity; Paraffin-embedded tissue; Expression analysis

Molecular analysis of pathological specimens has provided insights into mechanisms underlying disease, facilitating development of diagnostic/therapeutic agents. To elucidate pathological processes in target cells involving alterations in cellular functions, application of microdissection techniques allows high performance even with a complex tissue architecture [1–3]. For molecular analysis, tissue samples should preferably be kept biochemically unmodified, and therefore unfixed frozen tissue (UFT)[2] has become the gold standard for analysis by microdissection and microbiochemical techniques. However, as compared with paraffin-embedded tissues (PETs), UFTs are inconvenient with regard to storage and skills required for preparation and subsequent microdissection itself. Therefore tissue embedding after fixation is preferable if extraction of molecules with high quality and yield can be guaranteed.

We have recently found that methacarn, an organic-solvent-based noncross-linking fixative [4], retains advantages for analysis of expression levels of mRNAs and proteins and for analysis of mutations of target genes in microdissected tissue samples from PETs, with performance close to that possible with UFTs [5–10]. Another group confirmed the suitability of methacarn among a series of fixatives for differential mRNA expression analysis using microdissected PET specimens [11]. For these earlier studies, tissue fixation with methacarn and ethanol dehydration was performed at 4 °C, default time settings for each step were

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[2] *Abbreviations used*: UFT, unfixed frozen tissue; PET, paraffin-embedded tissue; PB, sodium phenobarbital; PCNA, proliferating cell nuclear antigen; EGFR, epidermal growth factor receptor.

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empirically determined by selecting the minimal time period for each step (1 or 2 h for fixation and three times 1 h for dehydration under gentle agitation), and analyses of molecules were performed usually within 1 month after paraffin embedding. However, we did not examine the effects of fixation and following dehydration periods or the storage time and temperature after paraffin embedding.

Determination of a suitable range for lengths of fixation/dehydration steps is very important for practical molecular analysis using methacarn if large numbers of tissue samples are to be analyzed, such as in the case of animal experiments requiring time-consuming autopsy. Furthermore, conditions for long-term storage of PETs for effective molecular analysis need to be optimized. The present study was therefore performed using methacarn-fixed and paraffin-embedded rat liver tissues from one animal with differing periods of processing and storage, focusing on the integrity of extracted molecules and the relative expression levels in sections.

Materials and methods

Chemicals and experimental animals

Sodium phenobarbital (PB) was purchased from Wako Pure Chemical Industries (Osaka, Japan). A 5-week-old CD (SD)IGS male rat from Charles River Japan Inc. (Atsugi, Japan) was maintained in an air-conditioned animal room (temperature 24 ± 1 °C, relative humidity 55 ± 5%) with a 12-h light/dark cycle and allowed ad libitum access to tap water and feed, CRF-1 (Oriental Yeast Co. Ltd., Tokyo, Japan). After 1-week acclimation, the rat was intraperitoneally injected with PB at 80 mg/kg body weight, once daily for 3 days. One day (24 h) after the last injection, the animal was killed by exsanguination from the abdominal aorta under deep anesthesia, and then the liver was removed for tissue processing. The dose was selected according to the PB-specific enzyme induction protocol described by Kocarek et al. [12], and the animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Tissue preparation and storage

From the center portion of the left lobe, tissue blocks sized 5 × 5 × 3 mm were excised and subjected either to embedding in Tissue-Tek 4583 OCT compound (Sakura Finetek Japan, Tokyo, Japan) or to immersing in methacarn for tissue fixation. Methacarn solution consisting of 60% (vol/vol) absolute methanol, 30% chloroform, and 10% glacial acetic acid was freshly prepared and stored at 4 °C before fixation [5,7,9]. UFTs were quickly frozen in ethanol/dry ice and stored at −80 °C until sectioning on the next day after embedding. To examine the effects of length of time of tissue processing (Experiment 1), fixation with methacarn and subsequent dehydration with 99.5% ethanol were performed for 2 and 16 h (overnight), 5 and 16 h, 16 and 16 h, or 2 h and 1 week at 4 °C in a refrigerator. Tissue blocks were dehydrated twice in fresh ethanol solution for 1 h and then for 16 h (overnight) or 1 week. They were then paraffin embedded as described previously [9], and tissue sectioning was performed within 1 week after paraffin embedding. To examine PET storage conditions (Experiment 2), 2 h-fixed overnight-dehydrated tissues were stored for 1 month at 4 °C or 3 or 12 months either at 4 °C in a refrigerator or at room temperature in a laboratory. Both UFTs and PETs were sectioned at 10 μm and a total of 20 sections per block were collected in 1.5-ml microtubes for storage at −80 °C until extraction of molecules.

RNA analysis

Methacarn-fixed PET sections stored at −80 °C were subjected to deparaffinization with xylene three times for 5 min, immersed in 100% ethanol three times for 5 min, and immediately processed for RNA extraction using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. For UFT sections stored at −80 °C, total RNAs were immediately extracted after removal from the deep freezer. The final elution volume was set at 30 μl and contaminating genomic DNA was digested with DNase I (Ambion, Austin, TX) according to the manufacturer's protocol. For quantitation of RNA yield, 1 μl of isolated RNA was labeled with a RiboGreen RNA Quantitation kit (Molecular Probes, Eugene, OR) and concentrations were estimated with a fluorescence spectrophotometer F2500 (Hitachi Co. Ltd., Tokyo, Japan) in 1 ml of total volume with water [5]. To examine the integrity of 18S and 28S ribosomal RNAs in the extracted total RNAs, 1 μg from each sample was loaded onto a 1.0% agarose gel and visualized with ethidium bromide. The integrity was also examined by measuring the 28S/18S ribosomal RNA ratio with an RNA 6000 Nano LabChip kit (Agilent Technologies, Mountain View, CA) and RNA ladders (Ambion) in an Agilent 2100 bioanalyzer according to the manufacturer's directions.

For measurement of relative mRNA expression levels, the following four mRNA species were selected: cytochrome P450 (CYP) 2B1 (GenBank/EMBL Data Bank, Accession No. M37134), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Accession No. M17701), solute carrier family 34A2 (slc34a2; Accession No. NM_053380.1), and syntaxin 6 (stx6; Accession No. NM_031665.1). CYP2B1 is a gene that is known to show strong induction in the liver by PB treatment of rats [9], and GAPDH is a representative housekeeping gene. Slc34a2 and stx6 were found to show specific expression changes by microarray analysis at the tumor promotion stage in the experimental hepatocarcinogenesis study previously performed in our laboratory using rats, and their expression levels relative to that of GAPDH were found to be rather minor by real-time RT-PCR analysis (data not shown). For measurement of GAPDH and CYP2B1 mRNA levels, one-step real-time RT-PCR with the SYBR Green detection system was performed using the

ABI PRISM 7000 Sequence Detection System (Applied Biosystems Japan, Tokyo, Japan) in a 50-μl total reaction volume including 50 ng of total RNA, 300 nM each forward and reverse primers, 12.5 U Multiscribe Reverse Transcriptase, 10 U RNase Inhibitor, and 25 μl SYBR Green PCR Master Mix, according to the manufacturer's protocol (all reagents were purchased from Applied Biosystems Japan). Primer sets for both genes were identical with those used in our previous study [9]. Cycle parameters in this system were as follows: single step of 48 °C for 30 min, single step of 95 °C for 10 min, and 50 cycles of 95 °C for 15 s followed by 60 °C for 1 min. As for measurement of mRNA levels of slc34a2 and stx6, two-step real-time RT-PCR with the TaqMan probe detection system was performed. Gene-specific primers and the corresponding TaqMan MGB probes (6-FAM-dye-labeled) were derived from TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd.). Reverse transcription was performed using 1 μg of total RNA with a High-capacity cDNA Archive Kit (Applied Biosystems Japan Ltd.) in a 100-μl total reaction volume. Real-time PCR was performed in a 50-μl reaction volume using the TaqMan probe detection system with 25 μl of TaqMan Universal PCR Master Mix (Applied Biosystems Japan Ltd.) and 2.5 μl each of target primer mix and reverse transcription product corresponding to 50 ng total RNA. Cycle parameters with this system were single step of 50 °C for 2 min, and initial activation at 95 °C for 10 min, and 50 cycles of 15 s at 95 °C and 60 s at 60 °C. Among real-time PCR methods, SYBR Green and TaqMan assays are known to produce comparable dynamic range and sensitivity [13]. For quantitation of expression data, a standard curve method was applied using the total RNA from UFTs as a standard sample.

Protein analysis

Deparaffinized sections were treated with 10% trichloroacetic acid in saline at 4 °C for 15 min. After brief centrifugation, pellets were washed once with ice-cold saline and then sonicated and solubilized in 200 μl of 2× sodium dodecyl sulfate (SDS) gel-loading buffer excluding bromophenol blue. Protein concentrations were estimated using a Nano-Orange Protein Quantitation Kit (Molecular Probes) and a fluorescence spectrophotometer [5]. After adjusting the protein concentration with 1× SDS gel-loading buffer including bromophenol blue, samples were heat-denatured at 80 °C for 30 min in the presence of 10% (v/v) β-mercaptoethanol and applied to 10% SDS polyacrylamide gel electrophoresis. For analysis of protein integrity, 20 μg of each protein extract was loaded, and resolved polypeptides were visualized after staining with 2.5% Coomassie brilliant blue. For Western blot analysis, resolved polypeptides were transferred to a polyvinylidine difluoride membrane (Millipore, Billerica, MA). Expression of three molecules with different functions and subcellular localizations were examined. After blocking with 0.2% casein, blots were incubated with either mouse monoclonal anti-β-actin (clone AC-15; Sigma; 20,000× in dilution), proliferating cell nuclear antigen (PCNA; clone PC10; Upstate, Charlottesville, VA; 1000× in dilution), or epidermal growth factor receptor (EGFR; clone 6F1; Medical and Biological Laboratories Co., Ltd., Nagoya, Japan; 300× in dilution) antibodies. Amounts of protein extract applied were 1 μg for β-actin, 5 μg for PCNA, and 15 μg for EGFR. As the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (DakoCytomation Co., Ltd., Kyoto, Japan; 2000× in dilution) was used, and protein signals were detected with the ECL Western Blotting Detection System (Amersham Biosciences Corp., Piscataway, NJ). Relative protein levels of β-actin, PCNA, and EGFR were estimated by analyzing the band intensities using ONE-D/ZERO-Dscan Quantitative Gel and Blot Analysis software (Scanalytics, Inc., Fairfax, VA).

Statistical analysis

Comparison of 28S/18S ribosomal RNA ratios, yields of total RNA and protein, and expression levels of mRNA and protein retained in the tissues was performed with Student's *t* test when the variance was indicated to be homogeneous among groups using the test for equal variance. If a significant difference in the variance was observed, Welch's *t* test was performed. Comparison was first made between UFT and each PET preparation. In Experiment 1, comparisons were further performed between samples subjected to 2-h fixation/overnight dehydration or other fixation/dehydration conditions. Similar comparisons were also performed for Experiment 2. Variability was expressed as coefficient of variation (CV).

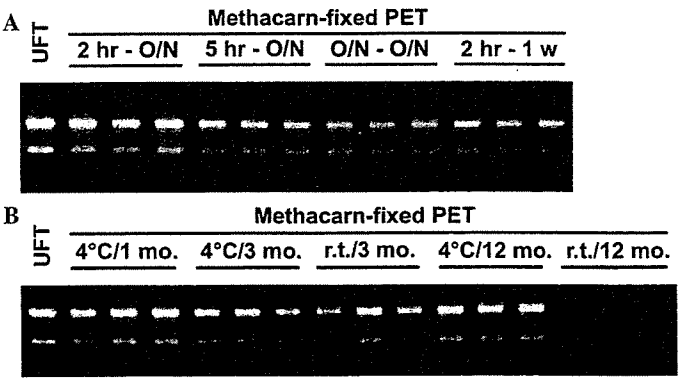

Fig. 1. Integrity of total RNAs extracted from methacarn-fixed rat liver PETs under different conditions of tissue processing or storage. One microgram of total RNA was applied to a 1% agarose gel and visualized with ethidium bromide. (A) Effects of period of tissue processing (Experiment 1): UFT, unfixed frozen tissue; 2 hr-O/N, samples fixed for 2 h followed by overnight dehydration; 5 hr-O/N, samples fixed for 5 h and dehydrated overnight; O/N-O/N, samples fixed overnight and then dehydrated overnight; 2 hr-1 w, samples fixed for 2 h and dehydrated for one week. (B) Storage effects of PETs with regard to period and temperature (Experiment 2): UFT, unfixed frozen tissue; 4 °C/1 mo., PETs stored for 1 month at 4 °C; 4 °C/3 mo., PETs stored for 3 months at 4 °C; r.t./3 mo., PETs stored for 3 months at room temperature; 4 °C/12 mo., PETs stored for 12 months at 4 °C; r.t./12 mo., PETs stored for 12 months at room temperature.

Results

Integrity and yield of total RNAs

In Experiment 1, integrity of total RNAs as judged by visual intensities for 18S and 28S ribosomal RNAs in agarose gel was not appreciably changed by the period of fixation up to overnight and dehydration up to 1 week (Fig. 1A). On measurement of the 28S/18S ribosomal RNA ratio, the integrity of total RNA was significantly reduced in methacarn-fixed PET sections compared with UFT sections (Table 1). The ribosomal RNA ratio remained unchanged from that of the 2-h fixed/overnight dehydrated case, although a tendency for reduction was observed in cases fixed for 5 h or overnight. Relative yields of total RNA per unit area were similar for all cases (Fig. 2A).

Table 1
28S/18S rRNA ratio of extracted total RNA in methacarn-fixed PETs under the different conditions for tissue processing or storage

Tissue condition	No. of samples	rRNA ratio [28S/18S][c]
Unfixed frozen	9	1.70 ± 0.45
Methacarn-fixed paraffin-embedded		
Fixation/dehydration[a]		
2 h/overnight	3	0.83 ± 0.07*
5 h/overnight	3	0.58 ± 0.04**
Overnight/overnight	3	0.62 ± 0.14**
2 h/1 week	3	0.70 ± 0.07**
Temperature/duration of storage[b]		
4 °C/1 month	6	0.75 ± 0.06**
4 °C/3 months	6	0.79 ± 0.09**
r.t./3 months	6	0.59 ± 0.14**
4 °C/12 months	6	0.66 ± 0.05**
r.t./12 months	6	Unmeasurable

*,**Significantly different from the unfixed frozen samples ($*p < 0.05$, $**p < 0.01$).

[a] Examination of the effect of time length for tissue processing (Experiment 1). Fixation and ethanol dehydration were performed at 4 °C. After dehydration, tissue blocks were paraffin embedded as described under Materials and methods. Already prepared PETs were stored at 4 °C until all of the tissues were processed for embedding.

[b] Examination of the effect of tissue storage with regard to the period and its temperature (Experiment 2). Fixation and ethanol dehydration were performed at 4 °C for 2 h and overnight, respectively. At the end of storage, tissue sections were prepared and stored at −80 °C until analysis.

[c] Data are expressed as mean ± SD.

In Experiment 2, when the visual integrity was compared among PET samples with different storage conditions, apparent reduction of both 18S and 28S ribosomal RNA bands was noted in samples stored for 12 months at room temperature (Fig. 1B), and this reduction resulted in the ribosomal RNA ratio being unmeasurable (Table 1). Although a nonsignificant reduction in the ribosomal RNA ratio was noted in PET samples stored for 3 months at room temperature as compared with those stored for 1 month at 4 °C, other PET storage conditions did not change the integrity of total RNA extracted (Fig. 1B, Table 1). Relative yields of total RNA per unit area were similar between the 1-month-stored case at 4 °C and each of the other cases (Fig. 2B).

Levels of mRNAs retained in PETs

On measurement of mRNA levels in PETs, mean threshold cycle (C_T: fractional cycle number at which the fluorescent signal passes the fixed threshold) of each gene was measured under the same input amount of total RNA using representative PET samples, i.e., 2-h-fixed/overnight-dehydrated samples in Experiment 1 and 1-month-stored samples at 4 °C in Experiment 2 (Table 2). As expected, C_T values of both GAPDH and PB-induced CYP2B1 were much smaller than those of slc34a2 and stx6, with ≥ 10-cycle differences simply reflecting ≥ 1000-fold expression differences. Judging from C_T values, relative quantity of transcripts was estimated to be in the order of CYP2B1 > GAPDH ≫ slc34a2 > stx6.

In Experiment 1, the GAPDH mRNA level retained in PETs was reduced in tissues dehydrated for 1 week to 62.2% of the 2-h-fixed/overnight-dehydrated case, but period of fixation did not affect the mRNA level (Fig. 3A). CYP2B1 mRNA levels were not apparently changed by the tissue-processing conditions. With regard to slc34a2, slight reduction of mRNA level was observed by 5-h fixation/overnight dehydration and 2-h fixation/1-week dehydration. Stx6 mRNA levels were not apparently changed irrespective of the fixation/dehydration conditions. With mRNA species examined, CV values ranged mostly within 20%, except for that of slc34a2 by overnight fixation being 40.0%.

In Experiment 2, retention of mRNAs in PETs was affected by storage for 12 months at room temperature, with reduction

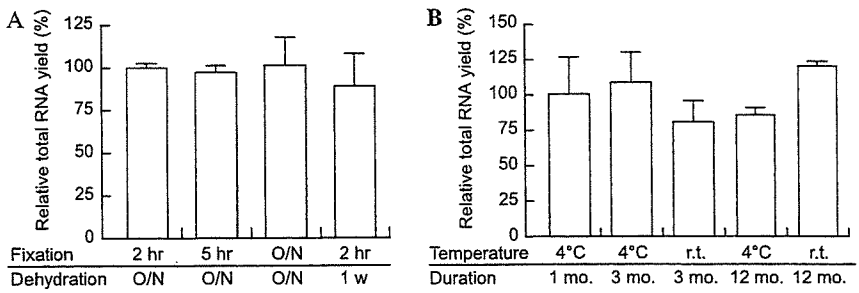

Fig. 2. Relative yields of total RNA in methacarn-fixed PETs under the different conditions of tissue processing or storage. (A) Experiment 1 ($n = 3$ for each condition). (B) Experiment 2 ($n = 6$ for each condition). Data are expressed as mean ± SD. Sample conditions were as for Fig. 1.

Table 2
Comparison of mean threshold cycle (C_T) of genes used in the present study in the methacarn-fixed paraffin-embedded rat liver tissue by real-time RT-PCR[a]

Tissue condition	No. of samples	Genes			
		GAPDH[b]	CYP2B1[b]	Slc34a2[c]	Stx6[c]
Fixation/dehydration[d]					
2 h/overnight	3	16.7	13.2	26.4	27.3
Temperature/duration of storage[e]					
4 °C/1 month	6	16.5	13.1	24.7	27.5

[a] Liver of a rat treated with PB for 3 days was used. C_T values in the 50 ng total RNA were measured at the fixed fluorescence threshold level of 0.14.
[b] Signal detection was performed by one-step real-time RT-PCR with the SYBR Green detection system.
[c] Signal detection was performed by two-step real-time RT-PCR with the TaqMan probe detection system.
[d] Examination of the effect of time length for tissue processing (Experiment 1). Already prepared PETs were stored at 4 °C until all of the tissues were processed for embedding.
[e] Examination of the effect of tissue storage with regard to the period and its temperature (Experiment 2). At the end of storage, tissue sections were prepared and stored at −80 °C until analysis.

of GAPDH, slc34a2, and stx6, to 76.1, 54.6, and 44.5%, respectively, of that for 1 month storage at 4 °C (Fig. 3B). Other storage conditions did not apparently affect the retention of mRNAs, while nonsignificant reduction was noted with slc34a2 stored for 3 months at 4 °C. CV values were mostly within 20%, except for those of slc34a2, ranging 21.3–33.8.

Integrity and yield of polypeptides

In Experiment 1, the visual pattern of resolved polypeptide bands and their intensities in polyacrylamide gels did not differ among samples with the tissue-processing conditions (Fig. 4A). However, protein yield was reduced in the 1-week dehydrated case as compared with that in the overnight dehydrated case after 2-h fixation (Fig. 5A).

In Experiment 2, the visual patterns of polypeptide bands were similar under the different storage conditions, but their intensities were rather reduced in the case of storage for 12 months at room temperature (Fig. 4B). With regard to protein yield, this was slightly reduced with storage of PETs at room temperature, reaching statistical significance in the 3-month storage case (Fig. 5B).

Levels of protein signals retained in PETs

In Experiment 1, retained levels of β-actin did not differ between the 2-h-fixed/overnight-dehydrated and other fixation/dehydration conditions, and variability in each was rather small (CV values <20%) (Fig. 6A). With PCNA, increase of relative expression was observed in samples fixed for 2 h and kept for 1 week at the dehydration step, the level being 132% of that with overnight dehydration, but the CV value was rather small (<20%) irrespective of the tissue-processing condition. In the EGFR case, a tendency for reduction of relative expression level was observed in 1-week-dehydrated PETs as compared with similar fixation for 2 h but dehydration overnight. However, this was not significant at least partly due to the variability in the expression values among samples in each condition, with CV values ranging 24.2–34.5%.

In Experiment 2, the retained expression level of β-actin was reduced in PETs stored at room temperature for more than 3 months, to 74% at 3 months and 52% at 12 months of the 1-month value at 4 °C (Fig. 6B). Slight, insignificant

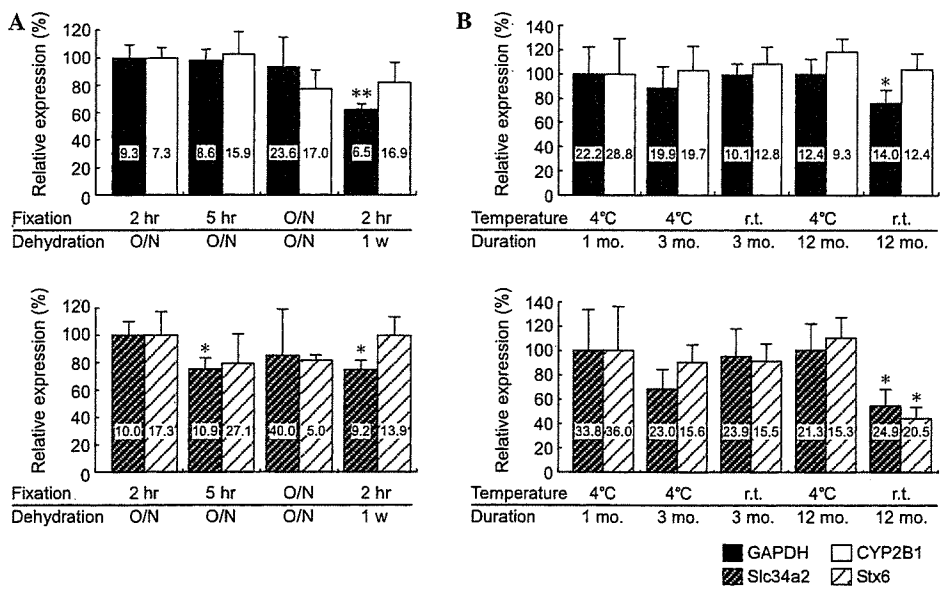

Fig. 3. Relative expression levels of GAPDH, CYP2B1, slc34a2, and stx6 mRNAs in methacarn-fixed PETs under the different conditions of tissue processing or storage. GAPDH and CYP2B1 mRNA levels were measured by one-step real-time RT-PCR with the SYBR Green detection system, and slc34a2 and stx6 mRNA levels were measured by two-step real-time RT-PCR with the TaqMan probe detection system. (A) Experiment 1 ($n = 3$ for each condition). (B) Experiment 2 ($n = 6$ for each condition). Data are mean ± SD values and coefficients of variation (CVs). *, **Significantly different from the 2-h-fixed/overnight-dehydrated case in Experiment 1, or from the 1-month-storage case at 4 °C in Experiment 2 ($^*p < 0.05$, $^{**}p < 0.01$).

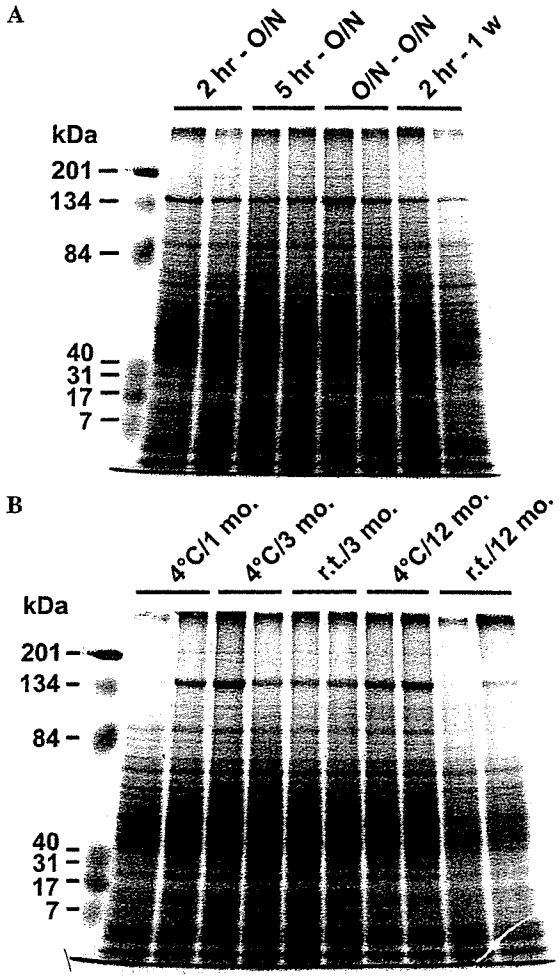

Fig. 4. Integrity of polypeptide bands in protein extracts from methacarn-fixed PETs under different conditions of tissue processing or storage. (A) Experiment 1. (B) Experiment 2.

reduction in the β-actin level was also evident in samples stored for 12 months at 4 °C, but here variability among storage conditions was relatively small, except for the case at room temperature for 12 months, with the CV value of 30.8%. With PCNA, no apparent reduction in protein signals was observed irrespective of the storage condition. For EGFR, a tendency for decrease was observed in samples stored for 3 months at room temperature and 12 months at 4 °C. Storage at room temperature for 12 months was associated with a level only 28% of the value for 1 month at 4 °C.

Discussion

Usually, formaldehyde-based cross-linking agents, such as buffered formalin, limit molecular analysis of DNAs, RNAs, and proteins due to direct interactions with these biomolecules [14]. However, in addition to methacarn, several alternative noncross-linking fixatives, such as HOPE solution [15–17], zinc-based agents [18], acetone-based AMeX [19], and alcohol or alcohol-based fixatives [20,21], have been developed for better preservation of nucleic acids and proteins in PETs. However, information on the effects of tissue processing and storage has hitherto been limited. We here revealed that fixation for a period up to overnight did not affect either the integrity or the expression levels of RNAs and proteins. On the other hand, dehydrating tissues for 1 week did result in slight fluctuations in the relative expression level of both molecular species. Integrity of both total RNAs and polypeptides was retained with storage of PETs at 4 °C but was reduced at room temperature over 12 months. Reflecting the reduction in the integrity, levels of molecules retained after storage at room temperature were also reduced. Reduction was apparent in protein levels by storage for more than 3 months, while this was evident in mRNA levels by 12 months storage.

With regard to the fixation condition in Experiment 1 of the present study, fixation for 5 h resulted in slight reduction in the slc34a2 mRNA level. Although statistical significance was not attained, a similar weak reduction was also noted on another minor mRNA species stx6. However, these reductions lacked apparent time dependency, showing no further reduction by overnight fixation. On the other hand, although most data have not been quantitatively measured and statistically analyzed, tissue fixation with a universal molecular fixative (UMFIX), composed of methanol and polyethylene glycol at a predetermined ratio (Sakura Finetek USA, Inc., Torrance, CA), for 24 h resulted in no obvious changes in either the integrity of extracted total

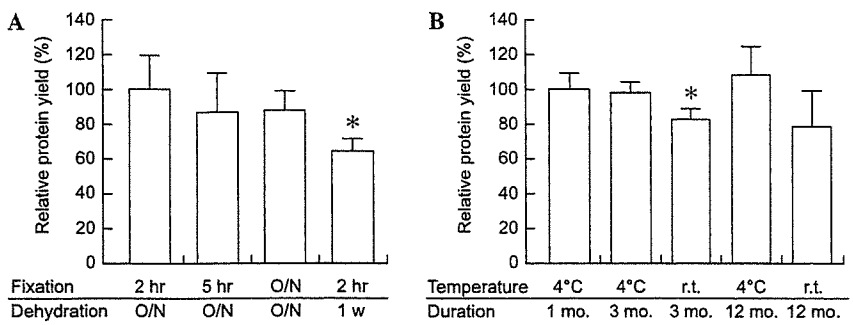

Fig. 5. Relative protein yields from methacarn-fixed PETs under different conditions for tissue processing or storage. (A) Experiment 1 ($n = 3$ for each condition). (B) Experiment 2 ($n = 3$ for each condition). Data are mean ± SD values. *Significantly different from the 2-h-fixed/overnight-dehydrated case in Experiment 1 or from the 1-month-storage case at 4 °C in Experiment 2 ($*p < 0.05$).

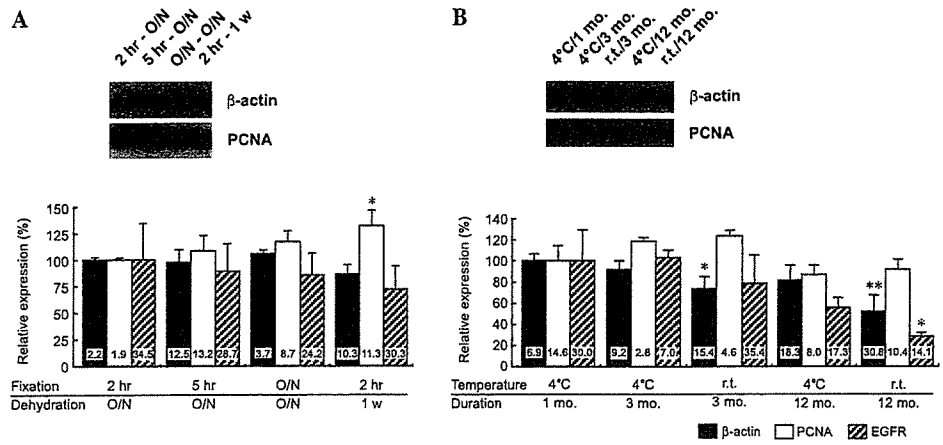

Fig. 6. Relative expression levels of β-actin, PCNA, and EGFR in methacarn-fixed PETs under different conditions of tissue processing or storage. (A) Experiment 1 ($n = 3$ for each condition). (B) Experiment 2 ($n = 3$ for each condition). Data are mean ± SD values and coefficients of variations (CVs). Each inset shows representative blot data for β-actin and PCNA. *, **Significantly different from the 2-h-fixed/overnight-dehydrated case in Experiment 1 or from the 1-month-storage case at 4 °C in Experiment 2 ($^*p < 0.05$, $^{**}p < 0.01$).

RNAs or the C_T for detection of GAPDH mRNA levels on real-time RT-PCR analysis, despite slight deterioration of protein signals being evident as compared with the 1-h-fixed case [21]. Taking all the results in combination, fixation with alcohol-based fixatives for more than 24 h may affect protein expression levels, but the effect on mRNA expression may be marginal.

Our findings for dehydration suggest the possibility of release of protein molecules into ethanol solution over time, although levels of nuclear protein PCNA and membrane-bound EGFR were increased (significant) and decreased (nonsignificant), respectively. Lipid extraction by alcohol-based protein precipitating fixatives may result in diffusion artifacts of membrane-bound proteins, causing difficulties with their immunohistochemical detection [22–24]. In our previous study, slight loss of protein yield in methacarn-fixed PETs as compared with UFTs was suggestive of diffusion artifacts [5]. While we observed high variability of EGFR levels among samples as compared with those for PCNA and cytoskeletal β-actin in Experiment 1, this was irrespective of the period of fixation and dehydration. One-week dehydration here also resulted in relative decrease of the mRNA levels of GAPDH and slc34a2, although diffusion artifacts may not have played a role because (i) the yield of total RNA was equivalent to that with other tissue-processing conditions and (ii) the mRNA expression of other genes, CYP2B1 and stx6, was not altered.

With regard to tissue storage effects, RNA degradation was evident in the study using UMFIX-fixed PETs from the visual pattern of major isoforms of ribosomal RNAs, and the cDNA array profile was changed by storage for 4 or 8 weeks at room temperature [21], although the authors argued that the extracted total RNAs were nondegraded and the cDNA array profile was comparable with results for freshly prepared PETs. In the present study, both 18S and 28S ribosomal RNAs mostly disappeared in methacarn-fixed PETs stored for 12 months at room temperature, suggesting degradation over time. On the other hand, mRNA levels in these samples were not entirely affected on measurement with real-time RT-PCR. Usually, real-time PCR utilizes target fragments sized only around 100 bp, and therefore not all RNA molecules in methacarn-fixed PETs are lost on long-term storage at room temperature. Interestingly, this reduction was apparent in mRNA species with low copy numbers, suggesting an uneven effect of long-term storage on degradation of mRNA species, although the corresponding mechanism was unclear.

Integrity of both total RNAs and proteins was well preserved at 4 °C in our study, in accordance with a previous report for the AMeX method, in which only minimal degradation of polypeptides resolved on polyacrylamide gels was documented for a period of 2 years at 4 °C [19]. On the other hand, as with total RNA, decreased intensity of polypeptides in gels was observed with our methacarn-fixed PETs stored for 12 months at room temperature, paralleling the reduced expression levels noted for two of three proteins examined. Oxidation of tissue samples during storage may cause modification of nucleic acids and proteins to affect their yield and integrity [14,25]. In the case of sectioned tissue samples, under ambient conditions they rapidly lose antigenicity; however, the magnitude of this loss differs from antigen to antigen [25]. Variation in the retained expression levels of our three proteins might reflect a similar phenomenon. For tissue sections, combined nitrogen storage and paraffin coating is a useful technique for preserving antigenicity [25]. With regard to storage of PETs, use of vacuum packing is recommended for prevention of oxidation [14]. In the context of tissue storage for future research purposes, vacuum packing may similarly be warranted.

In summary, for gene expression analysis using methacarn-fixed PETs, tissues can be fixed overnight. Moreover, considering similar expression variability among processing conditions, fixed tissues can be kept at the dehydration step for at least 1 week, and PETs can be stored for at least 12 months, but preferably at a temperature of 4 °C in a refrigerator.

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Hypothalamus Region-Specific Global Gene Expression Profiling in Early Stages of Central Endocrine Disruption in Rat Neonates Injected with Estradiol Benzoate or Flutamide

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ABSTRACT: To identify genes linked to early stages of disruption of brain sexual differentiation, hypothalamic region-specific microarray analyses were performed using a microdissection technique with neonatal rats exposed to endocrine-acting drugs. To validate the methodology, the expression fidelity of microarrays was first examined with two-round amplified antisense RNAs (aRNAs) from methacarn-fixed paraffin-embedded tissue (PET) in comparison with expression in unfixed frozen tissue (UFT). Decline of expression fidelity when compared with the 1×-amplified aRNAs from UFTs was found as a result of the preferential amplification of the 3′ side of mRNAs in the second round *in vitro* transcription. However, expression patterns for the 2×-amplified aRNAs were mostly identical between methacarn-fixed PET and UFT, suggesting no obvious influence of methacarn fixation and subsequent paraffin embedding on expression levels. Next, in the main experiment, neonatal rats at birth were treated subcutaneously either with estradiol benzoate (EB; 10 μg/pup) or flutamide (FA; 250 μg/pup), and medial preoptic area (MPOA)-specific microarray analysis was performed 24 h later using 2×-amplified aRNAs from methacarn-fixed PET. Numbers of genes showing constitutively high expression in the MPOA predominated in males, implying a link with male-type growth supported by perinatal testosterone. Around 60% of genes showing sex differences in expression demonstrated altered levels after EB treatment in females, suggesting an involvement of genes necessary for brain sexual differentiation. When compared with EB, FA affected a rather small number of genes, but fluctuation was mostly observed in females, as with EB. Moreover, many selected genes common to EB and FA showed down-regulation in females with both drugs, suggesting a common mechanism for endocrine center disruption in females, at least at early stages of post-natal development. Develop Neurobiol 67: 253–269, 2007

Keywords: brain sexual differentiation; microarray; estradiol benzoate; flutamide; microdissection

INTRODUCTION

Sex steroids play important roles in sexual differentiation of the mammalian brain (McEwen and Alves, 1999). In the rat, there is a critical period beginning

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at the last week of gestation and terminating in few days after birth, during which circulating testosterone secreted from the fetal and neonatal testes masculinizes the brain in males (Rhees et al., 1990a,b), the hormone being metabolized to estradiol by the enzyme aromatase to trigger brain sexual differentiation. Steroid-mediated processes during this period, including alterations in neuronal plasticity, myelination, and cell death, are the basis of sexual dimorphism in the structure and function of the adult brain (Matsumoto et al., 2000). For example, the medial preoptic area (MPOA) in the hypothalamus that is believed to mediate sexually dimorphic behavior in adult life (Meisel and Sachs, 1994; Numan, 1994) contains a highly cellular region, the sexually dimorphic nucleus of preoptic area (SDN-POA), that has an approximately 10 times larger volume in males than in females (Meredith et al., 2001). Inhibitory effects of steroids against the normal apoptosis that proceeds in the female SDN-POA during the critical period have been suggested to be responsible for the large size in males (Arai et al., 1996; Davis et al., 1996).

Sex steroids with their receptors are powerful regulators of gene transcription, and changes in the hormonal milieu during development can trigger reproductive dysfunction in later life by affecting molecular cascades responsible for sexual differentiation (McEwen and Alves, 1999). For instance, both α and β estrogen receptors (ERs) are strongly expressed in the hypothalamus during neonatal development, showing region-specific distributions (Orikasa et al., 2002), and neonatal hormonal manipulations can affect their expression levels and/or locations (Tena-Sempere et al., 2001; Orikasa et al., 2002), resulting in organizational changes in the brain structure and reproductive function in later life (Nagao et al., 1999; Tsukahara et al., 2003).

To elucidate mechanisms underlying disruption of brain sexual differentiation, gene screening applying global gene expression profiling in target brain region(s) is an effective approach. We recently established multipurpose genetic analysis methods with paraffin-embedded tissues (PETs) utilizing methacarn as a novel fixation tool, in combination with laser microbeam microdissection (Shibutani et al., 2000; Shibutani and Uneyama, 2002; Uneyama et al., 2002). With this system, we could achieve high performance regarding quantitative expression analysis of mRNAs using real-time RT-PCR, close to that with unfixed frozen tissue (UFT) (Takagi et al., 2004).

In the present study, we focused on region-specific gene expression analysis utilizing microarrays to identify genes linked with disruption of brain sexual differentiation in rats. With limited tissue samples, such as those collected by microdissection, multi-round amplification of mRNAs is necessary to obtain sufficient quantities of antisense RNAs (aRNAs) applicable for microarray analysis, and therefore, we first performed validation experiments using methacarn-fixed liver PETs to determine fidelity of expression profiles with 2× *in vitro* transcribed aRNAs in comparison with 1×- or 2×-amplified examples from UFTs. After confirmation of the efficacy of the methods, we then analyzed gene expression profiles in the neonatal MPOA in terms of sex differences and acute responses to neonatally injected estradiol benzoate (EB), a potent analog of endogenous estrogen, or flutamide (FA), a non-steroidal anti-androgen.

METHODS

Chemicals and Animals

Estradiol benzoate (EB; CAS# 50-50-0) and flutamide (FA; CAS# 13311-84-7) were purchased from Sigma (St. Louis, MO), sodium phenobarbital (PB; CAS# 57-30-7) from Wako Pure Chemical Industries (Osaka, Japan) and CD®(SD)IGS rats from Charles River Japan (Kanagawa, Japan). For the preliminary validation study regarding expression fidelity with microarrays using methacarn-fixed PET, one 7-week-old male rat was used, and for gene expression profiling in the early stage of disruption of brain sexual differentiation, six pregnant rats at gestational Day 3 (the day when vaginal plugs were observed was designated as GD 0). The animals were housed individually in polycarbonate cages (SK-Clean, 41.5 × 26 × 17.5 cm in size; CLEA Japan, Tokyo) with wood bedding (Soft Chip; Sankyo Lab Service, Tokyo, Japan), maintained in an air-conditioned animal room (temperature: 24°C ± 1°C, relative humidity: 55% ± 5%) with a 12-h light–dark cycle, and allowed *ad libitum* access to feed and tap water. For the rat in the preliminary validation study, CRF-1, a standard rodent diet, purchased from Oriental Yeast Co. (Tokyo, Japan), was used as the basal diet. For pregnant animals, soy-free diet (Oriental Yeast Co.) was used as a basal diet to remove possible interaction of phytoestrogens included in the regular soy-containing diet with the action of EB or FA. The formulation of the soy-free diet as well as the dietary concentrations of estrogens and phytoestrogens were as described earlier (Masutomi et al., 2003). Essentially, concentrations of phytoestrogens except for coumestrol, detected at 0.3 mg/100 g diet, were lower than the detection limit (0.05 mg/100 g diet).

Experimental Design

In the preliminary validation study, the rat received PB intraperitoneally at 80 mg/kg, once daily for three days. The dose was selected according to the PB-specific enzyme

induction protocol described by Kocarek et al. (1998). One day after the last injection, the animal was killed by exsanguination from the abdominal aorta under ether anesthesia, and the liver was removed and trimmed to make tissue blocks sized 5 × 5 × 3 mm.

For gene expression profiling in the early stage of disruption of brain sexual differentiation, offspring of two dams each were injected subcutaneously either with EB, FA, or vehicle at postnatal day (PND) 1 (the day of delivery) within 3–6 h after completion of delivery. The dose level of EB was set as 10 μg/pup, shown in our laboratory, to induce typical estrogenic effects on sexual development and the endocrine/reproductive system at the adult stage in both sexes, including reduction of the SDN volume in males (Shibutani et al., 2005). For FA, 250 μg/pup was selected on the basis of earlier study finding of retardation of male reproductive development with repeated injections of this dose (Rivas et al., 2002). Each chemical was dissolved in sesame oil to achieve a total injection volume of 20 μL. Vehicle control animals were injected with 20 μL of sesame oil. Twenty-four hours after the injection (PND 2), offspring including vehicle control pups were killed by decapitation for removal of brains.

The animal protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Preparation of Tissue Specimens and Microdissection

Liver tissues of the rat treated with PB were either quick frozen in ethanol–dry ice after embedding in Tissue-Tek 4583 OCT compound (Sakura Finetek Japan, Tokyo, Japan), or immersed in methacarn for tissue fixation. For this purpose, methacarn solution consisting of 60% (vol/vol) absolute methanol, 30% chloroform, and 10% glacial acetic acid was freshly prepared and stored at 4°C (Shibutani et al., 2000; Shibutani and Uneyama, 2002; Takagi et al., 2004), before fixation for 2 h at 4°C. Fixed tissue samples were then dehydrated three times for 1 h in fresh 99.5% ethanol at 4°C, immersed in xylene once for 1 h and then three times for 30 min at room temperature, and immersed in hot paraffin (60°C) four times for 1 h, for a total of 4 h. Both UFTs (n = 3) and methacarn-fixed PETs (n = 3) were sectioned at 10 μm and 20 sections per block were stored in 1.5 mL tubes at −80°C until RNA extraction.

For MPOA-specific microarray analysis, whole brains of rat pups were subjected to methacarn fixation (n = 3/sex/group). Before embedding, coronal brain slices including the hypothalamus were trimmed. Microdissection of the MPOA was performed based on the method described earlier (Takagi et al., 2004). After paraffin embedding, 6-μm-thick sections between three 18-μm-thick sections were prepared. The 18-μm sections were mounted onto PEN-foil film (Leica Microsystems, Tokyo, Japan) overlaid on glass slides, dried in an incubator overnight at 37°C, deparaffinized with xylene three times each for 3 min, placed in 99.5% ethanol for 1 min, and then air-dried. The localization of the SDN-POA, identified as an intensely stained cellular region, was determined by microscopic observation of the 6-μm-thick sections stained with hematoxylin and eosin (HE) (as shown in Fig. 1), and bilateral portions of the MPOA (500 × 300 μm) containing SDN-POA were microdissected from the adjacent unstained 18-μm-thick sections using PALM Robot-MicroBeam equipment (Carl Zeiss Co., Tokyo, Japan). In both sexes, 10–12 sections from one animal were used for microdissection, and the microdissected samples were stored in one 1.5 mL sample tube at −80°C until extraction of total RNA.

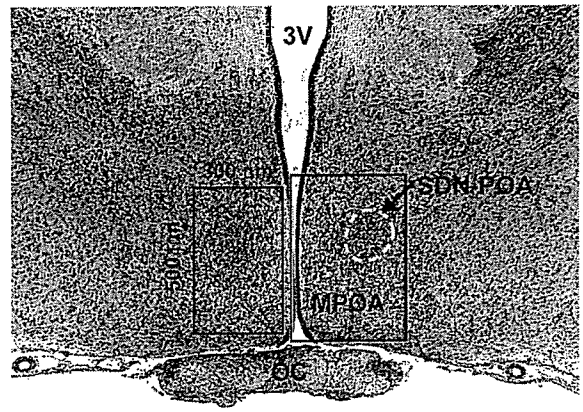

Figure 1 Overview of the hypothalamic MPOA at PND 2. Bilateral portions of MPOA as shown in the left boxed area were microdissected from sections of methacarn-fixed paraffin-embedded brain slices for gene expression analysis. The right boxed area is the anatomical location for immunohistochemical observation of protein signals shown in Table 6 and Fig. 6 (3V, third ventricle; SDN-POA, sexually dimorphic nucleus of the preoptic area; MPOA, medial preoptic area; OC, optic chiasm).

RNA Isolation, Amplification, and Microarray Analysis

Total RNAs from liver sections of UFTs and methacarn-fixed PETs were extracted with RNeasy® Mini (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, with the final elution volume set at 30 μL. Contaminating genomic DNA was digested with DNase I (Ambion, Austin, TX) at the end of the extraction. Total RNAs from microdissected MPOAs were extracted using an RNAquous®-Micro RNA isolation kit (Ambion), eluted twice with a total volume of 14 μL, and then treated with DNase according to the manufacturer's protocol.

For quantitation of RNA yield, 1 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 Co., Tokyo, Japan) in 1 mL of total volume with water.

For microarray analysis, extracted total RNA samples were subjected to amplification, consisting of reverse tran-

scription and subsequent *in vitro* transcription, using a MessageAmp™ aRNA Kit (Ambion) with an oligo dT/T7 primer, according to the manufacturer's protocol. Total RNA samples from liver UFT sections were either subjected to one- or two-step amplification, and those from methacarn-fixed liver PET sections were subjected to two-step amplification. For expression analysis with the microdissected MPOA, two-step amplification was performed. For one-step amplification, 5 μg of total RNA was subjected to one-round of aRNA amplification. For the two-step amplification, 50 ng of total RNA was subjected to first-round amplification, and resultant aRNAs of 150–200 ng were subjected to second-round amplification. During the second *in vitro* transcription, generating aRNAs were labeled with biotin-UTP and biotin-CTP (Enzo Biochem, Farmingdale, NY).

For normalization of transcript levels with reference to amplification efficiency, an *in vitro* transcribed spike RNA from pGIBS-PHE (American Type Culture Collection, Manassas, VA), a short fragment of *Bacillus subtilis* (accession no. M24537 in GenBank/EMBL data bank), was added to the extracted total RNA at 3.76 pg/μg.

After the second-round amplification, 20 μg of biotinylated aRNA was denatured at 94°C for 35 min in fragmentation buffer ($4. \times 10^{-2}$ *M* Tris-acetate, pH 8.1, 1×10^{-7} *M* KOAc, 3×10^{-2} *M* MgOAc) and subjected to hybridization in a mixture containing control cRNAs (Affymetrix, Santa Clara, CA). Aliquots of 200 μL containing approximately 15 μg of aRNA were hybridized with GeneChip® Rat Genome U34A Arrays (Affymetrix) at 45°C for 18 h, stained with streptavidin/R-phycoerythrin conjugates (Molecular Probes), and then scanned with a GeneChip® Scanner 3000 (Affymetrix). Individual samples were subjected to analysis with individual microarrays in both the validation study and the MPOA-specific gene expression profiling study ($n = 3$/group for comparison in each study).

Real-Time RT-PCR

Quantitative real-time RT-PCR was performed for confirmation of expression values obtained with microarrays using ABI Prism 7700 (Applied Biosystems Japan, Tokyo, Japan). In a separate microarray study, to investigate gene expression changes in microdissected MPOA of rat neonates that have been administered 0.01–0.5 ppm ethinylestradiol through the maternal diet, we selected two genes, i.e., thymosin β4 and GTP-binding protein (Gαi2), showing profound sex differences in basal expression. Gene specific primers for thymosin β4 (accession no. NM_031136 in the GenBank/EMBL data banks) and Gαi2 (M12672) as well as corresponding TaqMan® MGB probes (6-FAM™-dye-labeled) were obtained from Assays-on-Demand™ Gene Expression Products (Applied Biosystems Japan). Reverse transcription was performed using 100 ng of first-round aRNAs prepared for microarray analysis containing spike RNA with a high-capacity cDNA Archive Kit (Applied Biosystems Japan) in a 100 μL total reaction volume. Real-time PCR was performed in a 50 μL reaction volume using the TaqMan probe detection system with 25 μL of TaqMan® Universal PCR Master Mix (Applied Biosystems Japan) and 2.5 μL each of target primer mix and RT product. Cycle parameters with this system were: single step of 50°C for 2 min, initial activation at 95°C for 10 min, 45 cycles of 15 sec at 95°C, and 60 sec at 60°C. For quantitation of expression data, a standard curve method was applied using first-round amplified aRNAs from a male MPOA as a standard sample.

For the spike gene (*Bacillus subtilis*), *in vitro* amplified transcript levels were measured by one-step real-time RT-PCR using the SYBR® Green detection system in a 50 μL total reaction mixture containing 25 μL of 2× QuantiTect™ SYBR® Green PCR Master Mix (QIAGEN), 8 ng of first-round amplified aRNA, multiscribe RTase (17.5 units), RNase inhibitor (20 units), and 2.5×10^{-7} *M* of primers. Cycle parameters in this system were as follows: 48°C for 30 min, 95°C for 10 min, 45 cycles of 15 sec at 95°C, and 60 sec at 60°C. The primer set for the spike gene, 5′-AGCGCCCCGGACTGA-3′ (forward; nucleotides 3152–3166), and 5′-CTCTAGGCCCAAAACGACCTT-3′ (reverse; nucleotides 3107–3127), was designed using Primer Express® software (Version 2.0; Applied Biosystems Japan).

Immunohistochemical Analysis

Whole brains of male and female neonates injected with EB or vehicle at PND 1 and obtained on PND 2 were subjected to fixation in Bouin's solution overnight ($n = 4$/sex/group). Coronal brain slices including the hypothalamus were trimmed and paraffin-embedded, and 3-μm serial sections were prepared for localization of the MPOA including the SDN-POA with HE-stained sections each one prepared in every 10 sections.

Immunohistochemistry was performed with antibodies against poly(ADP-ribose) polymerase (PARP) (rabbit IgG, 50× dilution; Santa Cruz Biotechnology, Santa Cruz, CA), glutamate receptor (GluR) 1 (rabbit immunoaffinity purified IgG, 1 μg/mL; Upstate, Charlottesville, VA), GluR5 (rabbit polyclonal IgG, 100× dilution; Upstate), GluR6/7 (rabbit immunoaffinity purified IgG, 5 μg/mL; Upstate), microtubule-associated protein (MAP) 2 (mouse monoclonal IgG_1, 400× dilution; Chemicon International, Inc, Temecula, CA), and metallothionein-1/2 (MT-1/2; clone E9, mouse $IgG_{1\kappa}$, 400× dilution; DakoCytomation, Carpinteria, CA). The PARP antibody can detect PARP-1, and to a lesser extent PARP-2, according to the manufacturer's product information. For detection of GluR1, GluR5, and GluR6/7, deparaffinized sections were subjected to microwave treatment, four times for 3 min in 1×10^{-2} *M* citrate buffer (pH 6.0), according to the recommendation in the manufacturer's protocol. For MAP2, microwave treatment was performed twice for 3 min in the same citrate buffer. Nonspecific endogenous peroxidase activity was blocked by treatment with 0.9% hydrogen peroxide in absolute methanol for 10 min. After masking with normal goat (for rabbit polyclonal antibodies) or horse (for mouse monoclonal antibod-

Table 1 Comparison of the Expression Status of Genes in Microarrays Between aRNA Samples Prepared from UFT and Methacarn-Fixed PET[a]

Tissue Status	UFT		Methacarn-Fixed PET
aRNA Sample	1× amplified	2× amplified	2× amplified
Rates with gene probes for each expression status (%)			
Present	40.3	36.9	36.4
Absent	57.5	60.9	61.5
Marginal	2.2	2.2	2.1
Signal ratio with the GAPDH gene (3′/5′, × fold)	1.1	12.3	11.3

aRNA, antisense RNA; UFT, unfixed frozen tissue; PET, paraffin-embedded tissue; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
[a]Liver tissue of a rat treated daily with sodium phenobarbital (80 mg/kg body weight, s.c.) for three days was used.

ies) serum, sections were incubated with primary antibodies, overnight at 4°C, and subsequently with biotinylated secondary antibody for 60 min at room temperature. All antibodies used were diluted with 0.5% casein in PBS before application. Immunodetection was carried out with the horseradish peroxidase–avidin–biotin complex utilizing a VECTASTAIN® Elite ABC kit (Vector Laboratories, Burlingame, CA), with 3,3′-diaminobenzidine/H_2O_2 as the chromogen. Sections were counterstained with hematoxylin for microscopic examination. For quantitative measurement of the numbers of nuclei immunoreactive for PARP, bilateral portions of a 250 × 250 μm area covering the SDN region were subjected to analysis under 200× magnification. Also, nuclei immunoreactive for MT-1/2 were counted in bilateral MPOAs by randomly selecting three fields (125 × 125 μm area) on each side under 400× magnification. For each antigen (PARP and MT-1/2), mean ratios of nuclear immunoreactive cells to the total cells counted in each side were estimated.

Data Analysis

Scanned output files of microarrays were visually inspected for hybridization artifacts, and then expression signals for each gene were measured by calculating pixel intensities using a Microarray Suite software package (Version 5.0, Affymetrix). With this software, the expression status of each gene, whether present, absent, or marginally expressed, was judged. Exclusion of genes showing absence in at least three of six samples of the two groups for comparison of expression, normalization of expression data, and statistical comparisons were performed using GeneSpring® software (Version 5; Silicon Genetics; Redwood City, CA). For microarray data in the validation study of expression fidelity with methacarn-fixed PET specimens, per chip normalization was performed by dividing the signal strength for each gene with the level of the 50th percentile of the measurement in the chip, and per gene normalization with average signal strength of the identical gene of three 1×-amplified aRNAs samples from UFTs. With regard to the microarray data for microdissected MPOA, per chip normalization was performed by dividing the signal strength of each gene by the level of the spike RNA signal in each sample, and per gene normalization with average signal strength of the identical gene in three untreated control samples. Average relative expression values were determined for each gene in the treatment group, and genes showing expression changes with ≥2-fold differences were first estimated. Then, comparison of expression data between the untreated controls and each treatment group was performed using Student's *t*-test with multiple testing corrections applying Benjamini and Hochberg false positive discovery rate calculations, and genes showing statistical significance with a p value <0.05 were selected.

To assess fidelity of expression patterns in microarrays between the 2×-amplified aRNA samples from methacarn-fixed liver PETs and 1×- and 2×-amplified samples from liver UFTs, Pearson's correlation coefficients (r) for each combination were estimated using all genes included in the array.

For the real-time RT-PCR data, expression values were normalized to the amplification efficiency of the first-round *in vitro* transcription by dividing the expression values with the signal level of spike RNA included in each sample. Differences in expression levels between sexes were analyzed by the Student's *t*-test, when the variance proved to be homogeneous among groups using the test for equal variance. If a significant difference in the variance was observed, a Welch's *t*-test was performed.

Morphometrically analyzed data for nuclear immunoreactive cell ratios of PARP and MT-1/2 were compared by Student's and Welch's *t*-tests. Regarding immunoreactivities on which morphometric analysis could not be applied, total incidence of immunoreactive cases and grades of intensity were visually analyzed and statistically compared using the Fisher's exact probability test and Mann–Whitney's *U*-test, respectively.

RESULTS

Expression Fidelity in Methacarn-fixed PETs

Expression fidelity of the microdissected small tissue samples in microarray analysis might be influenced by tissue processing for microdissection and/or multiround amplification. To clarify the effect of tissue processing for microdissection (methacarn-fixation and following paraffin-embedding) on the expression

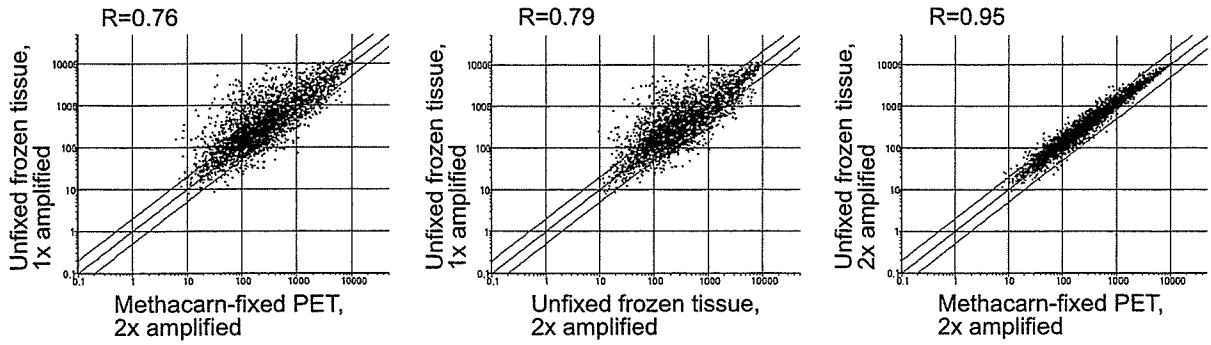

Figure 2 Scatter plot analysis of gene expression profiles of aRNA samples derived from methacarn-fixed PET and UFT of rat liver.

fidelity after 2×-amplification, expression in the second-round amplified aRNA samples was compared with that obtained from the 1×- or 2×-amplified aRNAs from UFT. With the 8799 probes included in the array used, percentages of expression status (present, absent, and marginal) were similar among aRNA-preparations irrespective of the tissue status and the amplification cycle (Table 1). However, preferential amplification of the 3′ portions was evident with 2× amplification in both UFT and methacarn-fixed PET cases (Table 1). By scatter plot analysis, high correlations were observed in the expression profiles between the 2×-amplified aRNAs from methacarn-fixed PET and UFT ($r = 0.95$, $n = 3$; Fig. 2). When the correlation of gene expression levels was examined between the 1×-amplified aRNAs from UFT and 2×-amplified ones from either methacarn-fixed PET or UFT, r values were similar, but relatively low when compared with the value for the correlation between the two 2× amplified examples, indicating a lowered expression fidelity in the methacarn-fixed PET because of the second-round amplification. When the number of genes showing presence was examined for 2×-amplified methacarn-fixed PET and 1×-amplified UFT aRNAs, 3173 probes were positive in common (as shown in Fig. 3). The numbers of probes showing presence solely in the methacarn-fixed PET (2× amplified) and UFT (1× amplified) were 373 and 822, respectively, suggesting that 10.5% of the total genes exhibiting presence in the 2×-amplified samples should be regarded to be false positive, and that 20.6% of the present genes in the 1×-amplified samples from UFT lost their signals after two-round amplification. It is possible that the distance from the poly(A) tail to the positions of the probes may affect the expression status of each gene after the second-round *in vitro* transcription due to preferential amplification of the 3′-terminal portion. Among genes showing presence solely in the methacarn-fixed PET (2× amplified) and UFT (1× amplified), sequence information including the full 3′-untranslated region from the poly(A) tail was available for six and five genes, respectively. The mean distances from the 5′-end of the probes (both 5′- and 3′-most probes) to the 5′-end of poly(A) tail expressed as the number of nucleotides were examined for these (Table 2), and as expected, they were shorter with 2×-amplified aRNA samples from methacarn-fixed PET than with their 1×-amplified counterparts from UFT. These results indicate that the decline in expression fidelity with 2×-amplified samples is mainly due to preferential amplification at the 3′-portions by the second-round amplification and methacarn fixation and paraffin-embedding did not apparently affect the fidelity.

Gene Expression Profiles of MPOA of Neonates Acutely Treated with EB or FA

In the MPOAs at PND 2, about 3600 genes showed presence in both sexes in untreated controls. Sex dif-

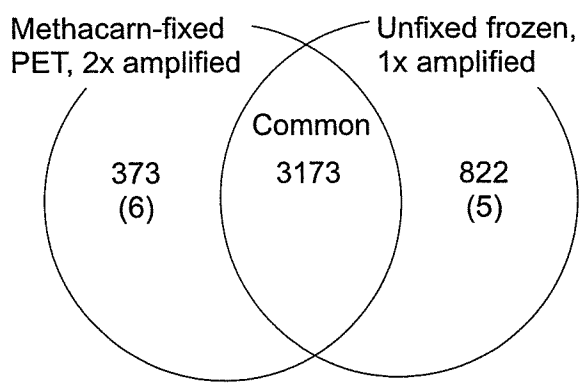

Figure 3 Gene populations showing presence with aRNA samples 2×-amplified from methacarn-fixed PET and 1×-amplified from UFT.