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Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples

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ABSTRACT

Formalin-fixed archival samples are known to be poor materials for molecular biological applications. We conducted a series of experiments to understand the alterations in RNA in fixed tissue. We found that formalin-fixed tissue was resistant to solubilization by chaotropic agents. However, proteinase K completely solubilized the fixed tissue and enabled the extraction of almost the same amount of RNA as from a fresh sample. The extracted RNA did not show apparent degradation. However, as reported, successful PCR amplification was limited to short targets. The nature of such 'fixed' RNA was analyzed using synthetic homo-oligo RNAs. The heterogeneous increase in molecular weight of the RNAs, measured by MALDI-TOF mass spectrometry, showed that all four bases showed addition of mono-methylol (-CH_2OH) groups at various rates. The modification rate varied from 40% for adenine to 4% for uracil. In addition, some adenines underwent dimerization through methylene bridging. The majority of the methylol groups, however, could be removed from bases by simply elevating the temperature in formalin-free buffer. This demodification proved effective in restoring the template activity of RNA from fixed tissue. The improvement in PCR results suggested that more than half of the modification was removed by this demodification.

INTRODUCTION

Messenger RNA in a cell is a snapshot of the real time activity of its genome, depicting what genes are expressed and to what extent. Accumulation of human gene sequences and advances in transcript detection methods such as RT–PCR (1–4) and differential display (5) have enabled researchers to see a part of this picture. Moreover, the recent innovation of microarray based-hybridization techniques has shown the possibility of reading a significant part of this picture at one time (6–8). Application of such new methods to pathological specimens is expected to greatly facilitate categorizing diseases and understanding disease processes (9,10). However, the availability of fresh disease tissue specimens is very limited, and they are used only for prospective studies. Accordingly, formalin-fixed samples, which have been archived in quantity in pathology departments, along with their clinical histories and prognoses, are attracting increasing attention as RNA sources.

Since Rupp *et al.* (11) first reported northern hybridization of formalin-fixed samples in 1988, significant efforts have been made toward recovery of RNA from formalin-fixed tissues. After von Weizsäcker *et al.* (12) successfully amplified endogenous mRNA from archival material, various modifications were made to the extraction steps, using RT–PCR to evaluate the outcome (13–18). In all reports, successful amplifications were limited to small fragments and sensitivities in transcript detection were much worse than with fresh material, although their alterations to the protocols did improve the results somewhat. The following three possibilities have been stated as the reasons for the poor results: RNA was degraded in the tissue before, during or after fixation (19); the RNA was resistant to extraction (20) probably due to cross-linking with proteins (21); the extracted RNA from fixed specimens was chemically modified by formalin in a way that is still elusive (22,23). However, direct evidence for each of these possibilities and thoughtful investigation regarding the contribution of these three possibilities to the overall results has been lacking.

In this study, we conducted experiments to evaluate these three factors independently and optimized the procedure to extract RNA suitable for molecular biology applications from formalin-fixed archival specimens.

MATERIALS AND METHODS

Fixation of tissue samples

Liver tissues excised from female weaning mice were used. After resection, liver was dissected into uniform samples weighing ~100 mg. Some pieces were immediately frozen in liquid nitrogen and stored at –80°C to serve as control fresh material; the others were soaked in neutral phosphate-buffered formalin (10%, pH 7.0; Nakarai Tec.) and incubated at 4°C for varying lengths: 16 h or 2, 3, 5 or 7 days. After fixation, the

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tissues were dehydrated with increasing concentrations of ethanol with the final step in 100% ethanol at least overnight, and stored at 4°C for 1 month before RNA extraction.

RNA extraction

The acid guanidinium/phenol/chloroform (AGPC) method. A modified protocol described by Chomczynski and Sacchi (24) was used. Briefly, 100 mg of tissue were homogenized in 2.0 ml of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol and incubated for 10–60 min with occasional vortexing at room temperature. Then, 0.2 ml of 2 M sodium acetate (pH 4.0), 2.0 ml of phenol (water saturated) and 0.4 ml of chloroform/isoamyl alcohol (49:1) were added and mixed. Prior to centrifugation at 10 000 *g* for 20 min, the solution was chilled on ice for 15 min. After centrifugation and precipitation by ethanol, the resulting RNA pellet was dissolved in 400 µl of RNase-free water.

Proteinase K digestion. A modified protocol described by Shibata *et al*. (25) and Jackson *et al*. (26) was used for proteinase K digestion. The 100 mg samples of tissue were homogenized in 1.0 ml of digestion buffer (200 mM Tris–HCl, 200 mM NaCl, 1.5 mM $MgCl_2$, 2% SDS, pH 7.5) with 500 µg proteinase K® (Sigma Chemical Co.) and incubated at 45°C for 1 h. Tissue lysate was extracted with 1.0 ml of liquified phenol/chloroform/isoamyl alcohol (25:24:1) followed by 1.0 ml of chloroform. After precipitation by ethanol, the RNA pellet was dissolved in 400 µl of RNase-free water. To ensure elimination of genomic DNA, each sample was then incubated for 1 h at 37°C with 1 U DNase I (Life Technologies) per µg of total RNA, followed by inactivation at 75°C for 5 min.

Detection of specific transcripts by PCR

With the help of the program Oligo v.4.0 (National Biosciences Inc.) primers were designed on mRNA of apolipoprotein A (accession no. L04151), apolipoprotein E (M12414), and α2-macroglobulin (M93264), which are abundant in mouse liver. For each cDNA, one antisense primer (23mer) that anneals in the 3′-untranslated region with its 5′-end at nucleotide position either 812, 957 or 4513, respectively, was made. Altogether 16 sense primers (23mers) were made to amplify 133–4035 bp cDNA fragments with one of these antisense primers.

PCR was performed in a 20 µl volume containing 1× PCR buffer (10 mM Tris, pH 9.0, 50 mM KCl, 0.01% w/v gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 0.4 µM each specific primer, 144 µM each dATP, dCTP, dGTP and dTTP, 1 U of AmpliTaq® DNA polymerase (Perkin Elmer), and 1 µl of cDNA from the RT reaction. After an initial incubation at 94°C for 5 min, the reaction mixtures were subjected to 35 cycles of amplification using the following sequence: 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. This was followed by a final extension step at 72°C for 10 min. Five microliters of the reaction mixture were run on a 2% agarose gel and visualized with ethidium bromide.

Formalin treatment of synthetic oligo RNAs and cellular RNAs

Four kinds of homopolymeric octamers of ribonucleotides, rA8, rU8, rG8 and rC8, were synthesized (Genset SA). Each oligo RNA was adjusted to 200 pmol/µl with distilled water. An equal volume of 20% buffered formalin was added to 100 µl of each of these oligo RNAs, and the mixture was incubated under various conditions (4°C for 16 h and 4°C for 7 days). After incubation, RNAs were pelleted by adding 2 vol of cold ethanol and were then centrifuged at 15 000 r.p.m. (12 000 *g*) in a microfuge for 30 min. RNA pellets were dissolved in 200 µl of distilled water.

One hundred micrograms of purified total RNA extracted from fresh mouse liver were incubated in 200 µl of 10% buffered formalin at 4°C for 16 h. This concentration is similar to the RNA concentration in tissue (RNA amount per unit volume of liver). RNA was pelleted by ethanol precipitation and dissolved in distilled water.

Mass spectrometric analysis

The changes in molecular weights of oligo RNAs were observed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Voyager™-RP MALDI-TOF/MS; Perkin Elmer Biosystems). Samples were prepared according to the manufacturer's instructions. Briefly, residual salts in oligo RNA solution were removed by passing them through a micro cation exchange column (AG®50W-X8 resin; BioRad Laboratories). For each run, 1 ml of sample, ~100 pmol/ml, was mixed with 4 ml of matrix (3-hydroxypicolinic acid), applied to the sample plate and air dried.

Complementary DNA synthesis

One microgram of total RNA and 0.5 µg of oligo$(dT)_{12–18}$ primers (Life Technologies) were heated in 10 µl of RNase-free water at 70°C for 5 min and then chilled on ice. The reaction mixture was made up to a final volume of 20 µl, containing 4 µl of 5× RT buffer (consisting of 250 mM Tris, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 2 µl of DTT (100 mM), 1 µl of dNTPs (10 mM each dATP, dCTP, dGTP and dTTP), and 200 U of SuperScript II (Life Technologies). The reaction mixture was incubated at 42°C for 60 min and 72°C for 15 min. Then, 2 U of RNase H (Life Technologies) were added to the mixture, and it was incubated for 20 min at 37°C.

To monitor the extent of cDNA synthesis, reverse transcription was performed as described above, except that the final concentration of dTTP was reduced to 0.2 mM and 1 µl of [methyl,1′,2′-^{3}H]thymidine 5′-triphosphate (1 mCi/ml) (Amersham) was added to the reaction mixture. The reaction products were electrophoresed on a 1% alkaline agarose gel containing 50 mM NaOH, and the gel was soaked in 10% trichloroacetic acid for 30 min. The size marker lane was separated and stained with SYBR®Green I (FMC), and a photograph was taken for later comparison. After complete dehydration, the gel was exposed to a BAS-1800™ imaging plate reader (Fuji Photo Film Co. Ltd), and radioactivity was measured. The preheating demodification process in which RNAs were incubated in TE (10 mM Tris–HCl, pH 7.0, 1 mM EDTA) buffer at 70°C for various times (10, 20, 30 and 60 min) was done before annealing with the oligo(dT) primers.

Calculation of modification rate

The probability of modification for each nucleoside (modified base/total base) in homo-oligo RNA was determined as follows. Assuming equal reactivity at all positions of bases, the fraction of octamer possessing mono-methylol additions at a multiplicity of n (F_n) is given by the equation $F_n = {}_8C_n \times a^n \times (1 - a)^{8-n}$, where a represents the probability of modification

for each nucleoside. For a series of *a* values from 0.001 to 0.8 with an increment of 0.001, F_0–F_8 were calculated. The modification rate *a* was determined as the value that gives the calculated F_0–F_8 closest to the observed values.

The changes in the modification ratio of cellular RNA were determined as follows. The probability (*P*) of a region longer than *L* being free from modification is given by the equation $P(L) = (1 - m)^L$, where the modification rate of unit length is *m*. By assuming that positive PCR results require a certain amount of unmodified fragment, an *n*-fold increase in positive PCR amplicon size by a reduction of the modification rate to *m′* indicates the relation $P(L) = P(nL)$. Using the equation above, this can be written as $(1 - m)^L = (1 - m')^{nL}$. When *m* is small enough, this equation can be approximated as $mL = m'nL$. This equation means that an *n*-fold increase in amplicon size (*L*) corresponds to an *n*-fold decrease in modification rate (*m*).

RESULTS

Comparison of RNA extraction methods from formalin-fixed tissue

We first tested the most popular two methods, the proteinase K-based method and the AGPC method, for extracting RNA from formalin-fixed tissues. As a material, we used a liver block fixed in 10% buffered formalin (BF) and stored for 1 month after fixation at 4°C. A slice of tissue, ~100 mg, from this block was subjected to each of these extraction methods. With the AGPC method, even after extensive homogenization or prolonged incubation in guanidinium thiocyanate solution for up to 1 h, small tissue fragments remained visible. In contrast, another slice was completely digested in proteinase K lysis buffer by incubation at 45°C for 1 h. Reflecting the difference in tissue solubilization ability, the yield of RNA was 10-fold more with the proteinase K digestion method than that with the AGPC method (Fig. 1A). The RNA yield with the proteinase K method was not affected by prolonged incubation in BF (Fig. 1B). With the proteinase K method, no apparent difference was found in the amount and integrity of extracted RNA between the fixed and fresh frozen specimens of similar sizes. From these results, we concluded that the fixation process, i.e. incubation in BF and ethanol dehydration, did not degrade RNA and that sufficient RNA was extractable if the proper method for tissue solubilization was selected.

Impairment of RT–PCR by formalin fixation

The applicability of these RNAs from formalin-fixed tissues (*in situ* fixed RNA) to molecular biological analyses was assessed by RT–PCR. As targets, mRNAs for apolipoprotein A, apolipoprotein E and α2-microglobulin were selected. Sixteen primer pairs spaced from 133 to 4035 bp designed on one of these three transcripts were used to evaluate template ability. In most cases, the pairs were set on different exons to easily discriminate between the products and contaminating genomic DNA. Under conditions that allow PCR amplification of all 16 fragments from RNA of fresh liver samples, long amplicons could not be amplified from *in situ* fixed RNAs. This impairment of amplification by fixation appeared to be positively correlated with the size of the amplicon and the length of incubation in BF (Fig. 2).

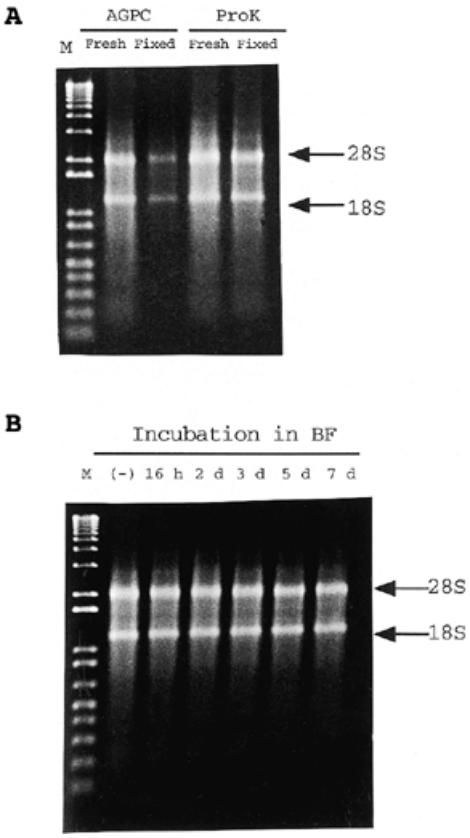

Figure 1. (**A**) Total RNA extracted by the AGPC and proteinase K methods from mouse fresh-frozen or formalin-fixed liver tissues. Incubation in BF was for 16 h at 4°C, and the tissue block was dehydrated in alcohol and stored at 4°C for 1 month before RNA extraction. Starting with 100 mg tissue slices, 1/200 of the RNA extracted was fractionated in 1% TBE agarose and stained with ethidium bromide. M, 1 kb DNA ladder (Life Technologies). (**B**) Effect of the length of incubation in BF on the extracted RNA. Tissue slices (100 mg) were soaked in BF for from 16 h to 7 days as indicated, and RNAs were extracted by a proteinase K-based method. Sections were processed as above: 1/200 of the yield was applied to 1% agarose along with RNA from a fresh liver slice. M, 1 kb DNA ladder.

These correlations have been pointed out before (19,27–29), but, in our present studies, the effect of RNA fragmentation and insufficient extraction of RNA was ruled out, as shown above (Fig. 1B). Contamination by some unknown inhibitory factor in the RNA preparation (30) was unlikely because adding a solution of *in situ* fixed RNA to fresh RNA did not interfere with amplification of all the amplicons (data not shown). Thus, the remaining possibility was that RT–PCR was impaired because RNA was modified by tissue fixation.

Modification of synthetic oligo RNA by formalin

To assess the modification that occurred on *in situ* fixed RNA, we first used synthetic homo-octamers of ribonucleotides (rA8, rC8, rG8, and rU8) as model reactants. These homo-oligo RNAs were treated with formalin under conditions that mimic tissue fixation (100 pmol/μl in BF at 4°C), and the changes in the molecular weights were observed by time-of-flight mass spectrometry.

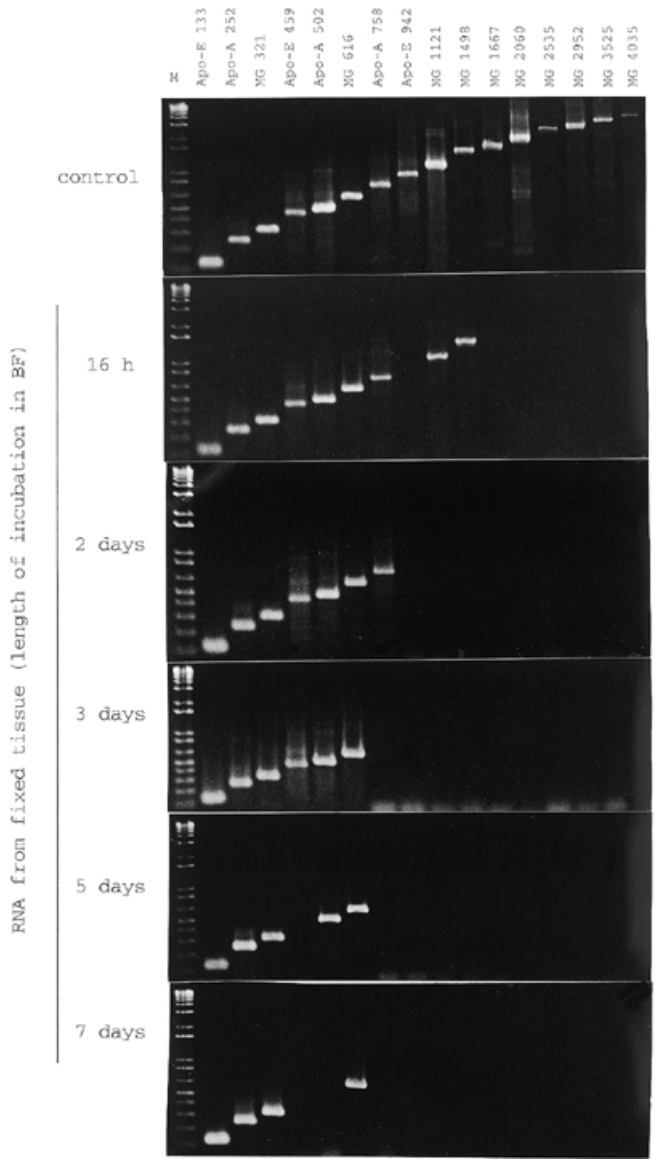

Figure 2. Examples of RT–PCR results using RNAs from liver tissue incubated in BF for various lengths of time. Sixteen primer pairs, which amplify 133–4035 bp from three mRNAs (apolipoprotein A, Apo-A; apolipoprotein E, Apo-E; α2-macroglobulin, MG), were tested. The target mRNA and expected product size are given above each lane. Each panel represents the indicated length of incubation in BF before dehydration and storing at 4°C for 1 month. The RNA from fresh-frozen liver tissue was used as a control. M, 1 kb DNA ladder.

Formalin treatment made the oligo RNA of homogeneous weight into a mixture of products with heterogeneous molecular weights larger than the original oligo. In the mass spectrogram of formalin-treated oligos, the major peaks were spaced 30 a.m.u. (atomic mass units) apart, indicating multiple additions of a moiety of 30 a.m.u. (Fig. 3b and d). The multiplicities of these additions varied from one to eight. The most likely interpretation of these patterns was mono-methylol addition to amino groups of bases (N-CH_2OH). Using the ratio of variously added products, the average addition rate for a base was calculated for each oligo (Fig. 4). In the case of rA8, the addition rate was 39.2% after formalin treatment for 16 h at 4°C, which was increased to 62.1% by formalin treatment for 7 days. For the least active uracil group, these addition rates were 3.8 and 4.0%, respectively. Only in the reaction with rA8, 12 a.m.u. heavier peaks accompanied each of the major peaks. These minor peaks probably represented methylene bridging products between neighboring bases (N-CH_2-N) formed by condensation of amino bases and *N*-methylols. The heights of minor peaks were about a quarter of the nearest major peaks, suggesting that one-fifth of the mono-methylol derivatives underwent condensation.

It was easily imagined that reverse transcription was impeded at such modified bases and dimers. Expecting a reversible nature of the methylol addition, these formalin-treated RNAs were incubated at 70°C for 1 h after replacing the solvent with TE buffer by ethanol precipitation. The temperature was determined as the highest temperature that would not hydrolyze the phosphate backbone. As expected, the majority of methylol groups were released by this procedure (Fig. 3c and e). For example, the modification rate of adenine estimated from the peak pattern decreased from 39.2 to 10.3% (Fig. 4).

Modification of fixed cellular RNA

We then investigated the modification of cellular RNA fixed either *in vitro* or *in situ*. The extent of modification was monitored by cDNA synthesis and RT–PCR. Complementary DNA synthesized on purified RNA incubated in BF for 16 h (*in vitro* fixed RNA) was considerably shorter in length and less in amount than the original RNA (Fig. 5). Such impeded cDNA synthesis was in part recovered in a time-dependent manner by heating the RNA at 70°C (Fig. 5). The improvement in cDNA synthesis by preheating was further demonstrated by PCR amplification of fragments of various sizes. Preheating the fixed RNA clearly made the size limit longer (Fig. 6A).

Next, the effect of preheating on the *in situ* fixed RNA was evaluated by RT–PCR (Fig. 6B). Despite the presence of other reactive groups in a cell, the results were basically the same as those with *in vitro* fixed RNA. The impaired synthesis first observed was partially recovered by heating the RNA. The difference from *in vitro* fixed RNA was that the changes, impediment by fixation and recovery by heating, were less with *in situ* fixed RNA.

DISCUSSION

To date, various methods have been employed to extract RNA from formalin-fixed materials. Digestion with proteinase K followed by alcohol precipitation is the classic method (11,26) and the AGPC method has become popular, mainly due to its simplicity (14,17,27). In our hands, formalin-fixed tissue was resistant to solubilization by saturated guanidinium thiocyanate solution. In contrast, a proteinase K-based buffer completely solubilized the same tissue sample resulting in nearly complete recovery of RNA. The RNA was not degraded on prolonged fixation nor during storage at 4°C for 1 month after fixation. However, the resulting RNA was a poor template for cDNA synthesis.

The reaction between formaldehyde and nucleotide monomers has been shown to progress in two steps. The first step is addition of a formaldehyde group to a base in the form of

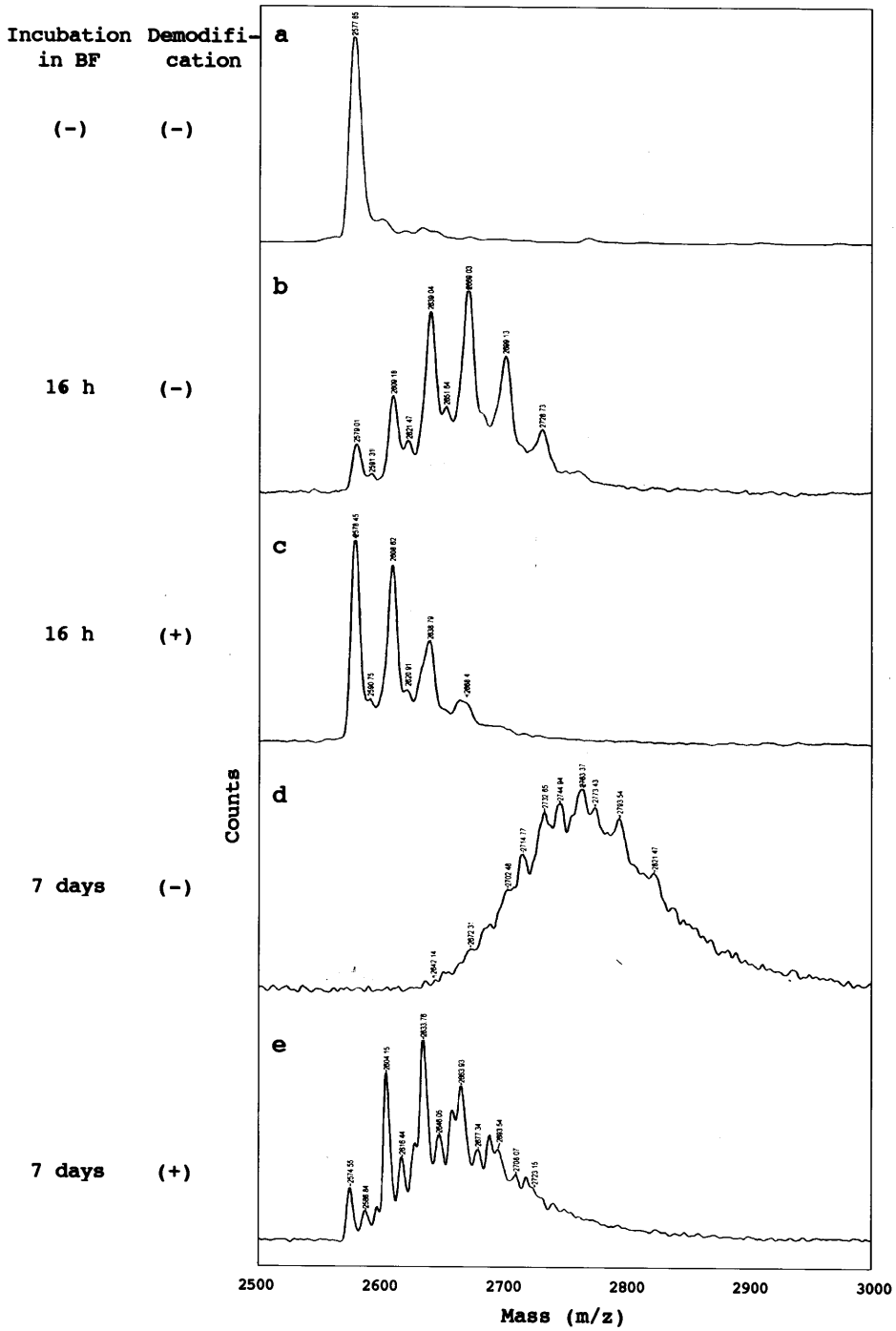

Figure 3. Examples of TOF mass spectrograms of oligo RNA (rA8) before (**a**) and after incubation in BF at 4°C for 16 h (**b**) or for 7 days (**c**). (**d** and **e**) Spectrograms of BF-treated RNAs after demodification at 70°C for 1 h in TE. Numbers on the peaks indicate the molecular weights assigned by mass spectrometry.

N-methylol (N-CH_2OH). The second slow step is electrophilic attack of *N*-methylol on an amino base to form a methylene bridge between two amino groups (22,23). The reaction of formaldehyde with RNA has been speculated to progress in the same manner. However, no direct evidence has been provided and the extent of the progression of these two reactions has never been evaluated. In this study, we have demonstrated these two reactions as molecular weight changes of oligo RNA (Fig. 3) and determined the extent of progression of these two steps. The reactivity of the bases of RNA was in the order A, C >> G > U, which suggests that the tertiary amino group was the primary target for formalin attack. Nearly 40% of adenines received mono-methylol

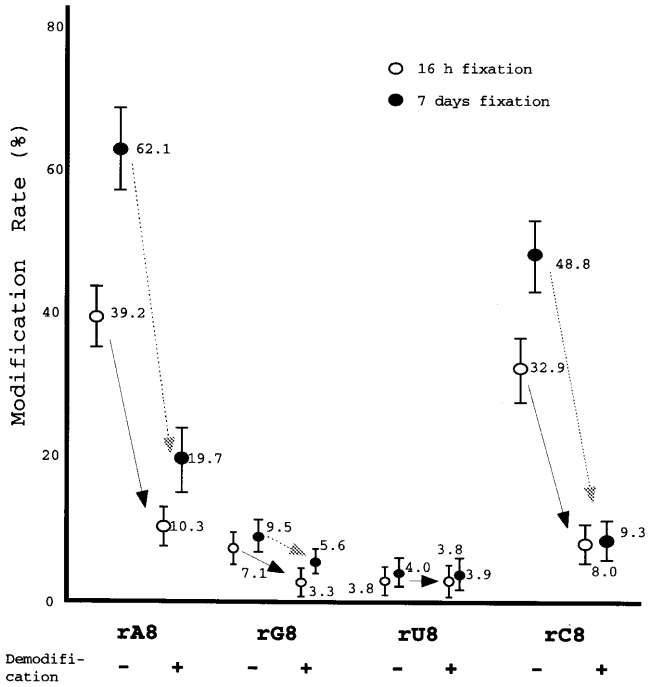

Figure 4. Modification rate of bases on oligo RNAs (rA8, rG8, rU8 and rC8). Samples were treated in BF at 4°C for 16 h (open circle) or for 7 days (closed circle). For each oligo, modification rate of a base (modified bases/total bases) was calculated from the peak heights of mass spectrograms taken before and after the demodification process. Mean values ± SDs were obtained from results of five repeated runs of TOF mass spectrometry.

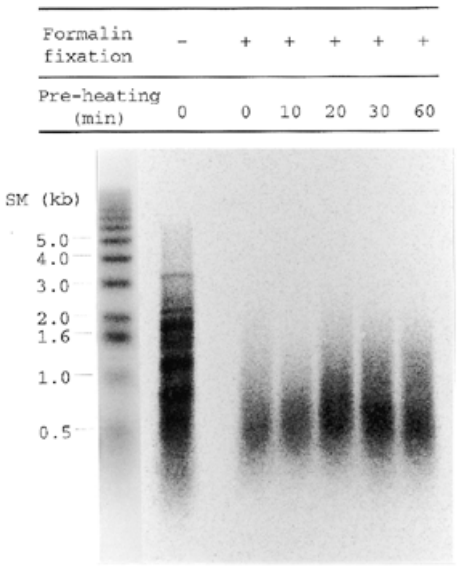

Figure 5. Complementary DNA synthesis on fresh, *in vitro* fixed and demodified RNAs visualized by [d^3H]TTP incorporation. Processes before reverse transcription, i.e. fixation at 4°C for 16 h and demodification at 70°C, are indicated above the lanes. Mobility of the size marker (1 kb DNA ladder) is given in the left margin.

addition after incubation in BF for 16 h at 4°C. We further showed that the majority of the methylol additions to oligo RNA were reversed by heating in TE buffer (Fig. 3).

In the cellular RNA, either fixed *in vitro* or *in situ*, the same methylol addition appeared to be involved in the interruption of reverse transcription because cDNA synthesis and RT–PCR results were improved by heating RNA prior to cDNA synthesis (Figs 5 and 6A and B). Although the extent of methylol addition in cellular RNA cannot be determined from our results, the proportion of methylol addition to total modification is evaluated from the size limits in successful PCR amplification. For example, after *in vitro* fixation for 16 h at 4°C, the longest positive amplicon was 459 bp, but preheating increased it to 1667 bp. Assuming that a certain fraction of the mRNA was unmodified and gave positive amplification, the recovery rates indicated that 72.5% of the modification was removed from the *in vitro* RNA (see Materials and Methods for calculation). Since other types of reversible modification were unlikely, this fraction was regarded as methylol additions. The rest presumably represent other types of modification, such as bridging dimers of amino bases. Compared with *in vitro* fixed RNA, the modification of *in situ* fixed RNA appeared less frequent but less reversible (Fig. 6A and B). With *in situ* fixed RNA, the longest positive amplicon was initially 758 bp, which increased to 1667 bp after heating. Thus, the reversible fraction, probably methylol derivatives, was 54.5% in *in situ* treated RNA. In fixation *in situ*, amino bases of proteins can be targets for attack by mono-methylol bases of RNA, disregarding DNA, which is separated and condensed in the nucleus. The presence of such extra targets, which possibly promote the second step of the reaction, may explain the less reversible nature of modification *in situ*. Conversely, proper folding of RNA or binding of proteins to RNA, which will reduce the exposed area susceptible to electrophilic attack by formaldehyde, may be the reason for less frequent modifications *in situ*.

Although we strongly suspected that modifications resistant to incubation at 70°C were impeding reverse transcription, the fragile nature of the phosphodiester bond of RNA precluded employing harsher conditions such as a higher temperature, different pH or longer incubation. Incorporation of neutral nucleotides such as inosine triphosphate or testing other available enzymes may improve cDNA synthesis.

Among the bases of RNA, adenine appeared the most susceptible to electrophilic attack in both the first and second steps of the reaction (Fig. 4). We speculate that the poly(A) tail of fixed mRNA is also heavily modified by these attacks. Accordingly, without demodification, oligo(dT) would not anneal well to the poly(A) tail nor extend cDNA synthesis by reverse transcriptase properly. Batch isolation of mRNA using oligo(dT) particles would not provide a good yield. The employment of random priming in cDNA synthesis may well improve the RT–PCR result (31–33).

In summary, our observations show that the majority of RNA can be extracted from properly processed archival samples. Although chemical modification by formalin does not allow the direct application of extracted RNA to cDNA synthesis and RT–PCR, more than half of the modification is simple methylol addition, which is reversed by simply heating in TE buffer. Our recommendations for researchers who make

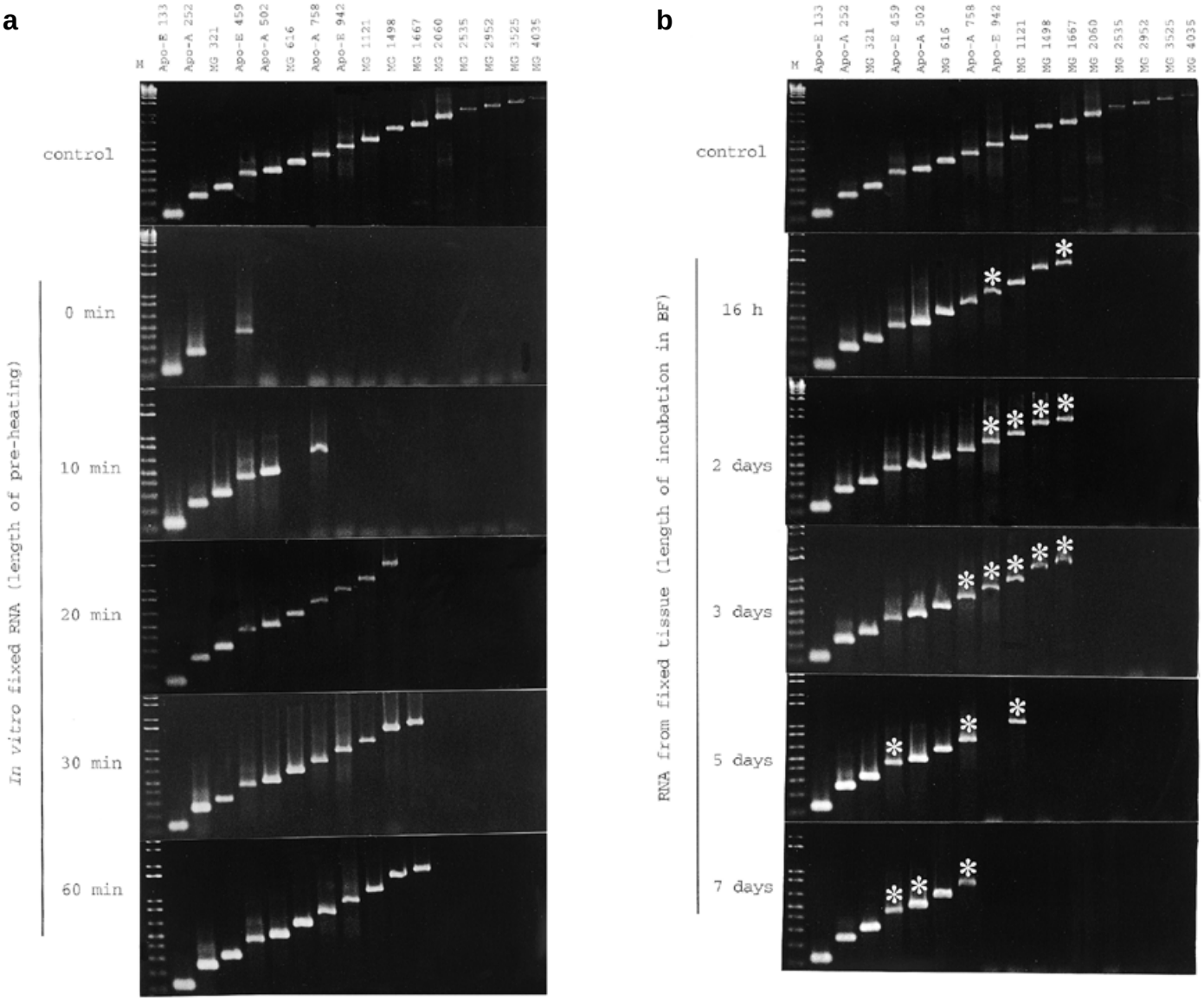

Figure 6. (**a**) Effect of demodification on RT–PCR results of *in vitro* fixed RNAs. Sixteen primer pairs were tested that amplify 133–4035 bp from three mRNAs (apolipoprotein A, Apo-A; apolipoprotein E, Apo-E; α2-macroglobulin, MG). The target mRNA and expected product size are given above each lane. Length of incubation at 70°C before cDNA synthesis in TE buffer is indicated on each panel. The RNA from fresh-frozen liver tissue was used as a control. M, 1 kb DNA ladder. (**b**) Effect of demodification on RT–PCR results of *in situ* fixed RNAs. The length of incubation in BF is indicated on each panel. All tissue blocks were dehydrated after fixation and kept at 4°C for 1 month before RNA extraction. The RNAs were all incubated at 70°C in TE for 1 h before RT–PCR. The positive amplicons that were absent in RT–PCR without demodification (Fig. 2) are marked with asterisks (*).

or use archival samples are as follows: (i) avoid prolonged fixation that results in irreversible modification; (ii) use a proteinase K-based protocol to extract RNA rather than a chaotropic agent-based one; (iii) introduce a heating step prior to hybridization or reverse transcription.

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