Southern Blot Analysis of DNA Extracted from Formalin-fixed Pathology Specimens

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ABSTRACT

We have developed a method for the extraction of DNA from formalin-fixed, paraffin-embedded pathology specimens. High-molecular-weight DNA was recovered from well-fixed nonautolyzed samples of viable tissue. DNA recovered from samples exposed to picric acid or mercuric chloride containing fixatives was not intact. Increasing the formalin fixation time decreased the amount of intact DNA available. When these limitations were taken into consideration, the procedure allowed for the removal of degraded and chemically modified DNA from the preparation, and the final product was suitable for quantitative and qualitative analysis by Southern or dot blotting techniques. Digestion with methylation-sensitive restriction endonucleases showed that DNA methylation patterns were not altered after formalin fixation.

INTRODUCTION

The field of molecular biology has expanded rapidly over the last few years, and a large amount of new information has accumulated on the organization of the human genome. The genome is not as static as previously thought, and gene rearrangement (1), amplification (2), deletion (3), mutation (4), and altered DNA methylation patterns (5, 6) are examples of determinants now thought to be important for the regulation of gene expression. Similar modifications are also thought to take place in neoplastic processes and may have prognostic significance. For example, gene deletions (3), amplifications (7), and the presence of some restriction fragment length polymorphisms (8) have been associated with a poor prognosis for specific tumors.

The use of this new knowledge for the study of tumor biology and also as a clinical tool has been delayed because of the need to use fresh or frozen tissue as a source of DNA, making it difficult to conduct retrospective studies on a large number of patients. These restrictions would be overcome if DNA was obtainable from the vast archives of material available in pathology departments throughout the world. Since clonable and restrictable DNA has been obtained from sources as ancient as Egyptian mummies (9), we were encouraged to develop an extraction method for DNA from formalin-fixed, paraffin-embedded tissue. We are particularly interested in analyzing gene rearrangements, methylation patterns, and amplification using Southern blotting and dot blotting techniques.

Formylation of nucleic acids produces Schiff bases on free amino groups of nucleotides (10–12), and exposure of nucleo-proteins to formaldehyde results in the formation of cross-links between proteins and DNA (13). The fact that both of these processes are reversible in aqueous solutions (12–14) suggested to us that DNA could be recovered from formalin-fixed tissue. Indeed, Goelz et al. (15) have recently shown that DNA which can be cut by restriction endonucleases and hybridized to DNA probes can be obtained from embedded specimens. In the present paper, we have characterized the effect of formalin fixation and paraffin embedding on the availability of DNA for Southern blot analysis and have developed an extraction method which allows for the removal of degraded and irreversibly modified DNA from the preparation. The purified DNA is suitable for quantitative as well as qualitative Southern blot analyses.

MATERIALS AND METHODS

Source and Handling of Tissue. Fresh tissue was obtained from the Department of Pathology of the Kenneth Norris Hospital in Los Angeles. Unfixed tissue samples were frozen in isopentane and kept at −70°C until DNA was extracted using the method previously described by Jones and Taylor (16). Fixed samples were sliced, washed several times with 10% buffered formalin, fixed in an excess volume of formalin, and processed for paraffin embedding in parallel with routine surgical pathology specimens. Old blocks of paraffin-embedded, formalin-fixed tissue were obtained from the Kenneth Norris Hospital or from the archives of the Los Angeles County Hospital.

Digestion with Restriction Endonucleases. DNA was incubated overnight at 37°C using 4 units of enzyme per µg of DNA as recommended by the manufacturer (New England Biolabs, Beverly, MA). The following morning one additional unit of enzyme was added per µg of DNA, and incubation was continued for 8 h or until completeness of digestion was demonstrated by running aliquots of the digest on a minigel.

Gel Electrophoresis and Southern Blotting. Aliquots of restriction endonuclease digests were electrophoresed on 1% agarose gels in 40 mM Tris-acetate-1 mM EDTA, pH 7.4. After electrophoresis the DNA was stained with ethidium bromide and visualized by UV illumination. The DNA was then transferred to nitrocellulose filters (Millipore, Bedford, MA) using the procedure of Southern (17). The filters were air dried and baked between 2 sheets of Whatman No. 3MM paper under vacuum for 3 h at 80°C.

Preparation of [32P]-labeled DNA Probes. The c-Ha-ras probe (clone pT24-C3) was obtained from Dr. Mariano Barbacid from the National Cancer Institute. The probe was labeled with [32P]dCTP to specific activities >10⁶ cpm/µg using the procedure of Feinberg and Vogelstein (18).

DNA Hybridization Conditions. Nitrocellulose filters were prehybridized overnight at 68°C in a solution containing 6x SSC,³ 0.5% SDS, 5x Denhardt’s solution (19), and denatured salmon sperm DNA (200 µg/ml) (Sigma). The prehybridization buffer was then discarded, and the [32P]-labeled probes were hybridized to the filters for 60–65 h at 68°C in 6x SSC-0.01 M EDTA-5x Denhardt’s solution-0.5% SDS-denatured salmon sperm DNA (100 µg/ml). Hybridization mixtures were filtered through Millex-HA 0.45-µm filter units (Millipore) as recommended by Meinkoth and Wahl (20) prior to addition to the nitrocellulose filter to aid in the reduction of background. Following hybridization, the filters were washed at room temperature once in 2x SSC-0.5% SDS for 5 min, once in 2x SSC-0.1% SDS for 15 min, and then for 1–3 h at 55°C in 0.1x SSC-0.5% SDS with multiple buffer changes. The filters were exposed to Kodak XAR-5 X-ray film with 2 DuPont Lightning-Plus intensifying screens for 10–14 days at ~ 80°C.

Dot Blotting. Ten-µg samples of formalin-fixed DNA were precipitated in ethanol and resuspended in 9 µl of 0.1x SSC. One µl of 2 N NaOH was added, and the samples were boiled for 5 min. Twenty µl

³The abbreviations used are: SSC, 0.15 M NaCl-0.015 M sodium citrate; SDS, sodium dodecyl sulfate.

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of 3 M NaCl were added, and the tubes were put on ice. Each sample
was spotted onto Millipore (Bedford, MA) type HA, 0.45-μm nitrocel-
lulose paper. The paper was dried on a piece of Whatman No. 3MM
filter paper for 30 min, washed twice for 15 min in 6× SSC, and dried
for an additional 30 min. The nitrocellulose paper was baked at 80°C
under vacuum for 3 h between 2 sheets of Whatman No. 3MM paper.
Prehybridization and hybridization with a 32P-labeled c-Ha-ras probe
were done as for Southern blots.

Extraction of DNA from Formalin-fixed, Paraffin-embedded Speci-
mens. A flow diagram of the various steps used for the DNA extraction
procedure is shown in Fig. 1. Comments about each of these steps are
given in the rest of this section as well as in “Results.”

Selection of Paraffin Blocks. The single most important cause of
failure to recover intact high-molecular-weight DNA from formalin-
fixed pathology specimens is the use of inadequately fixed tissue.
Histological sections of paraffin blocks should therefore be examined
prior to DNA extraction, and the blocks should not be used if autolysis
is evident. Examination of histological sections may also reveal the
presence of tissue necrosis. Such areas should be removed with a scalpel
blade before starting the extraction. Intact DNA could not be recovered
from tissue that had been exposed to picric acid (Bouin) or mercuric
chloride (Zenker, B5) containing fixatives. The amount of intact DNA
available from the tissue decreased as the length of fixation in formalin
increased.

Tissue Homogenization. One effect of formalin fixation is to produce
a hard rubbery tissue difficult to homogenize. We have taken advantage
of the fact that the fixed tissue was embedded in paraffin blocks to cut
it into thin slices using the microtome. Five-μm-thick slices were cut
until the whole paraffin block was exhausted or enough tissue was
obtained, and the slices were collected into a centrifuge tube. Practically
every cell present in the tissue was cut at this setting, thus providing a
first homogenization step.

Solubilization of DNA. The thin tissue slices were deparaffinized and
rehydrated by resuspension in 50 ml of xylene (J. T. Baker, Phillipsburg,
NJ) and washing 3 times by centrifugation in xylene, absolute ethanol,
and SSC. The final pellet was resuspended in 5 ml of SSC containing
1% SDS and 100 μg of proteinase K per ml (E. Merck, Darmstadt,
Germany) (Solution A). If the block contained only small amounts of
tissue (less than 25% of the volume of the block), it was resuspended
in 2.5 ml of Solution A instead of 5 ml. The preparation was then
incubated at 37°C in a plastic centrifuge tube that was taped inside an
850-cm² tissue culture roller bottle (Falcon, Oxnard, CA) and rotated
at a speed of one revolution every 2.5 min. All of the DNA present in
the tissue was slowly released in a soluble form during this process
(Fig. 2). The rate of release was not the same for different types of
tissue and depended on the density and abundance of extracellular
stroma. For highly cellular tissue containing little stroma, such as
spleen (Fig. 3a), over 80% of the DNA was released within 24 h (Fig.
2). However, it took 6 days before a similar percentage of the total
DNA could be recovered from a uterine leiomyoma containing large
quantities of dense stroma (Figs. 2 and 3). DNA from a metastatic
teratoma of moderate cellularity containing areas of loose stroma was
solubilized at an intermediate rate (Fig. 2). The rate of solubilization
and the amount of total DNA recovered were not altered when the
length of time the tissue had been exposed to formalin was varied from
12 h to 5 days (results not shown).

Incubation in Solution A was done with 2 consecutive extractions in
order to remove low-molecular weight DNA from the preparation.
After 6 to 12 h of incubation (24 h for very fibrous tissue), the
preparation was centrifuged, and the supernatant was discarded. The
pellet was then resuspended in fresh Solution A and put back at 37°C.
This step also allowed removal of pigments such as hemoglobin.
The second incubation was stopped after 2 to 6 days, depending on the
density of the tissue stroma.

Extraction with Chloroform-Isomyl Alcohol. The preparation was
centrifuged to remove insoluble debris after the second incubation in
Solution A, and the supernatant was discarded. The pellet was then
resuspended in fresh Solution A and put back at 37°C. This step also
allowed removal of pigments such as hemoglobin. The second
incubation was stopped after 2 to 6 days, depending on the
density of the tissue stroma.

Fig. 2. Effect of stromal density on the rate of extraction of DNA from
formalin-fixed tissues. Paraffin blocks from the spleen (□), teratoma (△),
and uterine leiomyoma (■) shown in Fig. 3 were cut, and the tissue slices
were incubated at 37°C in Solution A after rehydration. The samples were
centrifuged at the indicated time intervals, the supernatants were saved, and the pellets were
resuspended and reincubated in fresh Solution A until the next time point. The
amount of total DNA present in each supernatant as well as in the insoluble pellet
remaining after 10 days was determined using the diphenylamine method (29).
Total DNA originally present in each sample was calculated by adding the amount
of DNA in each supernatant to the DNA left in the residual pellet after 10 days.
DNA FROM FORMALIN-FIXED TISSUE

Effect of Extraction Time. Because variable amounts of degraded DNA were always present in our preparations, we wanted to see if this degraded material was solubilized at the same rate as high-molecular-weight DNA during the incubation step in Solution A and to determine if this step could therefore be used to remove the low-molecular-weight material. Paraffin blocks from 3 different inadequately fixed breast tumors were selected in order to obtain DNA preparations with increased amounts of degraded low-molecular-weight material. The blocks were sectioned with the microtome, rehydrated, and incubated in Solution A for 24 h. The preparations were then centrifuged, and the pellets were resuspended in fresh Solution A. The supernatants and the resuspended pellets were put back at 37°C for an additional 60 h. DNAs from each tube were purified using the method described in "Materials and Methods" except that the purified samples were collected by centrifugation instead of spooling after the phenol extraction step. DNA from each purified sample was electrophoresed on a 1% agarose gel (Fig. 4). The results clearly show that, for all 3 cases, low-molecular-weight DNA with high electrophoretic mobility was recovered during the first 24 h, but not during the second incubation period. Thus, low-molecular-weight DNA unsuitable for Southern blot analysis can be removed by performing 2 consecutive incubations in Solution A and discarding the supernatant obtained after the first incubation. It should be emphasized that poorly fixed autolyzed material was used for this experiment and that most of the DNA recovered from adequately fixed tissues would not have penetrated 1% agarose gels unless it had first been digested by restriction endonucleases (results not shown).

Effect of Length of Fixation in Formalin. When tissue specimens are processed in surgical pathology laboratories, formalin fixation times often vary from a few hours up to 5 days. Certain types of tissue such as lymph nodes are routinely fixed for at least 48 h in some centers. We determined the effect of fixation time on DNA extractability, because it seemed likely that the length of fixation would be an important determinant of the quality of DNA present in a specimen. Tissue samples from a metastatic ovarian carcinoma were fixed for 12, 36, 60, and 110 h. The corresponding DNAs were spooled on a glass rod after phenol extraction. After spooling, variable quantities of precipitate were seen remaining in each sample. These precipi-
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Fig. 4. Electrophoresis of DNA extracted for different lengths of time in Solution A. Paraffin blocks of poorly fixed specimens from 3 different breast tumors (a to c) were cut with the microtome, rehydrated, and incubated for 24 h in Solution A. The 3 samples were then centrifuged, and the pellets were resuspended in fresh Solution A. The supernatants and resuspended pellets were put back at 37°C for an additional 60 h. DNA was extracted from each of the resulting 6 samples but was not spooled after the phenol extraction step. Each sample was electrophoresed through 1% agarose, and the gel was stained with ethidium bromide. For each tumor, Lane 1 is the supernatant recovered after the first incubation, and Lane 2 is the resuspended pellet. kb, kilobase(s).

Table 1 Effect of time of fixation on the ability to spool DNA

<table>
<thead>
<tr>
<th>Length of fixation (h)</th>
<th>Total spoolable DNA (μg)</th>
<th>Total unspoolable DNA (μg)</th>
<th>% of total DNA spoolable</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>829</td>
<td>619</td>
<td>57</td>
</tr>
<tr>
<td>36</td>
<td>474</td>
<td>889</td>
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<td>60</td>
<td>211</td>
<td>696</td>
<td>23</td>
</tr>
<tr>
<td>110</td>
<td>49</td>
<td>568</td>
<td>8</td>
</tr>
</tbody>
</table>

Fig. 5. Dot blots of spoolable and unspoolable DNA samples. DNA from each sample of spoolable and unspoolable material in Table 1 was dot blotted onto nitrocellulose, hybridized to 32P-labeled c-Ha-ras probe, and autoradiographed. Dots a, b, c, and d represent spooled samples fixed for 12, 36, 60, and 110 h, respectively. Dots e, f, g, and h are the corresponding unspoolable samples.

Ha-ras probe (Fig. 5). All spoolable DNAs showed good hybridization, although dot intensity of the sample fixed for 110 h was slightly decreased. Hybridization intensities from samples of unspoolable DNA were reduced but showed a gradual increase with increasing fixation time. DNA Samples a, b, c, d, and h were digested with MspI, electrophoresed on an agarose gel, stained with ethidium bromide, and visualized under UV light (Fig. 6). Although the staining intensity of the DNA entering the gel was similar for each sample of spool DNA, most of the DNA in Sample h, which is the unspoolable sample that showed the highest hybridization intensity in Fig. 5, could not penetrate the gel. When similar experiments were done using the other unspoolable samples, the data were either similar or showed mainly low-molecular-weight DNA (results not shown). Thus, it was important that samples be kept concentrated enough during the extraction procedure to allow for spooling of the DNA and that any unspoolable material be discarded.

Digestion with Restriction Endonucleases. Fig. 7 is a stained gel of DNA extracted from unfixed and fixed samples of a metastatic ovarian adenocarcinoma. A certain amount of incompletely digested material was present in the fixed sample even after taking the precautions mentioned above. Although the nature of this material is not clear, it was observed in samples from paraffin-embedded tissue that had been fixed in ethanol without exposure to formalin (results not shown). Some of this undigestible material may therefore represent single-stranded DNA generated by exposure to elevated temperatures during the embedding process. Increasing either the amount of enzyme or the length of digestion did not improve the completeness of the reaction. The amount of DNA loaded on a gel should be 2 to 3 times the amount normally used for DNA from unfixed samples to account for the presence of this undigestible material. Most of this material did not transfer to nitrocellulose filters during Southern blotting procedures (results not shown).

Southern blots of DNA obtained from fresh and fixed tissues, digested with either MspI or HpaII endonucleases and hybridized to 32P-labeled c-Ha-ras gene probe, were also compared (Fig. 8). The mobility of the fixed material was slightly slower than that of DNA from fresh tissue, as also reported by Goelz et al. (15). The reasons for this decrease in mobility are not clear, but this was found with all of the DNA samples extracted from paraffin blocks. The banding patterns of fresh and fixed tissues were otherwise very similar, and comparison of the results with the 2 enzymes showed that DNA methylation patterns were not altered by formalin fixation and could be analyzed by methylation-sensitive endonucleases.
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DISCUSSION

The results of our studies clearly show that DNA suitable for dot blotting and Southern blotting analysis can be obtained from formalin-fixed, paraffin-embedded material. The results also show that DNA methylation patterns are preserved intact during histological processing. The procedure we have developed contains the following modifications over a simpler one recently reported by Goelz et al. (15). (a) Degraded low-molecular-weight DNA was removed by sequential extractions with Solution A; this was also an effective way to remove tissue pigments (e.g., hemoglobin). (b) Chemically modified DNA unsuitable for restriction endonuclease digestion or hybridization studies was removed by spooling out the intact DNA. (c) We have brought attention to the fact that the amount of DNA usable for hybridization studies decreased markedly when the time of fixation in formalin was increased to as little as 5 days. (d) We used a simple and effective method for tissue homoge-

Fig. 6. Effect of DNA spooling on electrophoretic mobility of restriction fragments. DNA samples used for Dots a, b, c, d, and h in Fig. 5 were digested with MspI, electrophoresed, and stained with ethidium bromide. kb, kilobase(s).

Fig. 7. Electrophoresis of restriction DNA fragments from fixed and unfixed tissues. DNAs from a fresh (Lane a) or fixed (Lane b) bladder tumor specimen were digested with MspI, electrophoresed, and stained with ethidium bromide. kb, kilobase(s).
certain genes are tissue specific (see Ref. 6), methylation studies of metastatic tumors may allow us to determine their tissue of origin. Viral infections, which often produce nonspecific morphological changes only, can be readily detected by using the appropriate DNA probes. In certain cases, the method can be used to determine if 2 tumors arose from the same or different clones (28) and thus assist the pathologist in determining if the appearance of a new tumor in a cancer patient represents a new primary or a metastasis. Perhaps even much more significant is the fact that the importance of genetic alterations such as gene amplification, deletion, DNA methylation, and restriction fragment length polymorphism on tumor behavior can now be studied retrospectively and that this is likely to provide accurate and objective methods to obtain prognostic information for patients.

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