Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 118-126

PURIFICATION OF DNA FROM FORMALDEHYDE FIXED AND PARAFFIN EMBEDDED HUMAN TISSUE

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Received May 28, 1985

SUMMARY: The ability to isolate DNA from preserved human tissues would provide numerous experimental opportunities. In this report it is shown that DNA can be extracted from tissues prepared for routine histopathological examination (i.e., fixed with formaldehyde and embedded in paraffin). Although the extracted DNA is not intact, it is double stranded, cleavable with restriction endonucleases, and suitable for a variety of standard techniques used in molecular biology.

INTRODUCTION: One of the most exciting developments in cancer research has been the discovery of some of the genetic changes that occur in human cancer. Studies from numerous laboratories have demonstrated that certain cellular genes, generally called oncogenes, are sometimes mutated (1,2), amplified (3,4), rearranged (5,6) or aberrantly methylated (7,8) in cancer cells. Other studies have shown that viral genomes are present in certain human tumors and that these viral genomes apparently are involved in the process of carcinogenesis (e.g., refs. 9,10).

Among the many questions raised by these studies are what role these genomic changes play in the development, progression and clinical course of the tumors in which they are found. It is possible, for example, that the clinical behavior of a tumor is dependent on the nature of the genetic alterations occuring in the malignancy. To answer such questions, it is necessary to examine the DNA from a large number of tumors of a given type in an attempt to correlate specific genetic alterations with histologic patterns

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0006-291X/85 $1.50

and clinical parameters. However, obtaining large numbers of fresh samples of human tumor tissue, even from relatively common tumors, is time consuming and difficult. Furthermore, many years must often elapse between the time of excision of the tumor and knowledge of the patient's clinical outcome.

To circumvent these difficulties, we have tried to determine whether DNA can be prepared from tissue which has been fixed and embedded for routine histopathological examination. Pathology archives contain literally hundreds of thousands of such samples, representing surgical pathology specimens collected over many years. We report here that DNA can be extracted from such specimens. Although the DNA is not completely intact, it is double stranded, cleavable with restriction endonucleases, and hybridizes efficiently with labeled probes. Therefore, DNA from routinely fixed and embedded tissue is suitable for studying many of the genomic changes that have been detected in human cancer cells.

MATERIALS AND METHODS

Tissue Samples

To compare DNA extraction from fresh-frozen, fixed, and fixed and embedded tissue, samples of human placenta and leiomyoma were frozen in liquid nitrogen immediately after surgical removal and then stored at -80°C. A portion of the frozen tissue was fixed in phosphate buffered formaldehyde (formaldehyde concentration 3.7-4.0%, methanol concentration 1-1.5%, buffered osmolality 200 +/- 9 msm/kg, pH 7.0 +/- 0.2, [Columbia Diagnostics Inc., Springfield, VA]) at room temperature for at least 16 hours. Use of unbuffered 4% formaldehyde in water as a fixative (rather than buffered formaldehyde) resulted in marked degradation of DNA. Part of the fixed tissue was embedded using an automated tissue processor, as follows: The formaldehyde fixed tissue was rinsed with water for 2-3 hours and placed in 70% ethanol for 12-24 hrs. It was then transferred sequentially to 80% ethanol for 3 hrs, 80% ethanol for 2 hrs, 95% ethanol for 2 hrs, 95% ethanol for 2 hrs, 100% ethanol for 1 hr, and 100% ethanol for 1 hr. It was then sequentially placed in xylene for 2 hrs, 1 hr and 1 hr and then in paraffin heated to 60°C for 1 1/2 hrs, 1 hr and a final period which ranged from 1 hr to 2-3 days. Histopathologic sections 6 microns thick were made from the blocks, and the remainder of the blocks was stored at room temperature.

Blocks of embedded tissue retrieved from the Surgical Pathology files of The Johns Hopkins Hospital were also examined. Blocks had been prepared for routine histopathologic examination of surgical resection specimens for colorectal carcinoma between 1979 and 1984. This preparation was exactly as described above for the placenta and leiomyoma samples, except that tissues were not frozen and that a variable amount of time elapsed between surgical removal and fixation in formaldehyde. Histopathologic sections were cut from the blocks to provide a guide for removal of pieces of tumor (see Fig. 1).

DNA Extraction

DNA was extracted from unfixed, fixed, or fixed and embedded tissue as follows: tissue was weighed, finely minced with a razor blade, and suspended in TE9 (500 mM Tris, 20 mM EDTA, 10 mM NaCl, pH 9.0) containing 1% SDS and 500

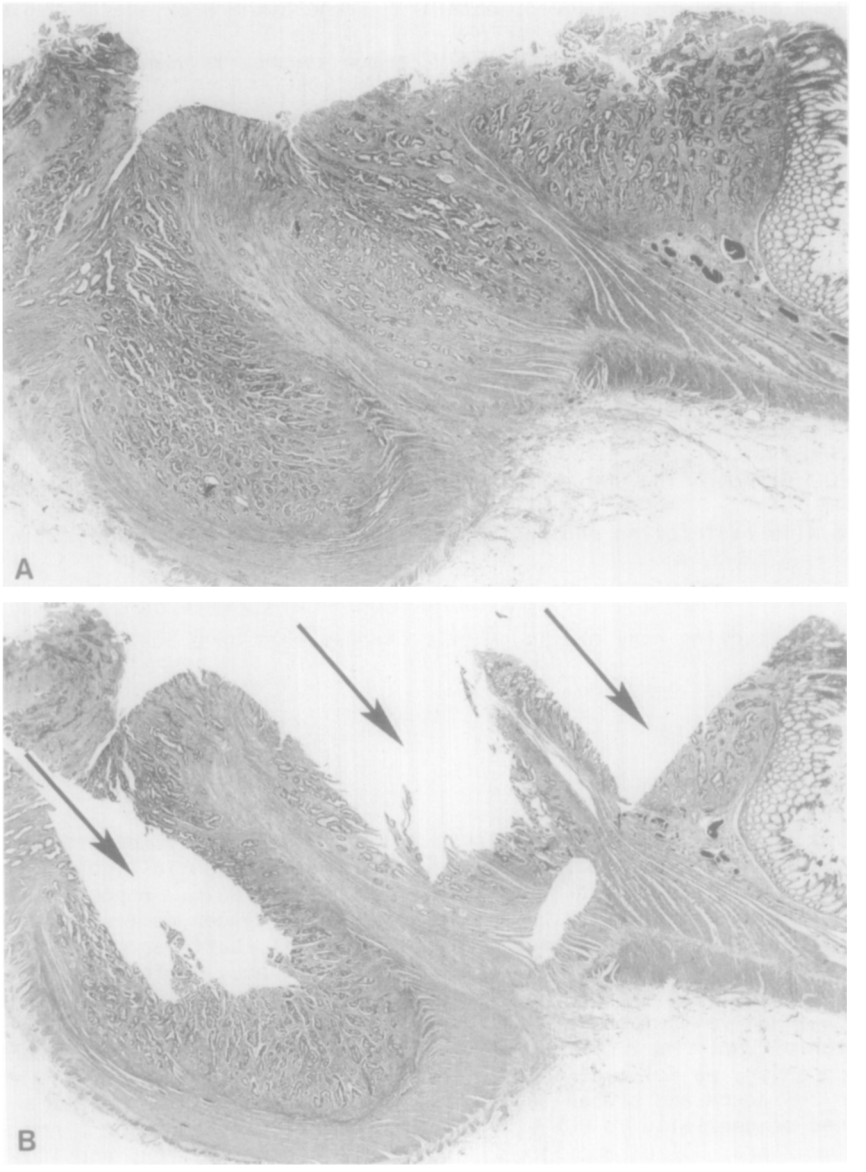

Figure 1: Illustration of tissue removal from paraffin block of colorectoal carcinoma from the Surgical Pathology files.

A. Histopathologic section from paraffin block before removal of portions for DNA extraction. Islands of tumor as well as non-tumorous tissue surrounding the tumor islands are evident (H&E, 7X).
B. Histopathologic section from same block as in A after removal of tissue for DNA extraction. Areas from which tumorous tissue was selectively removed are indicated by arrows (H&E, 7X).

ug/ml proteinase K (11) (BRL, Bethesda, Maryland). For embedded samples, it was important to cut away as much of the paraffin as possible before weighing and to mince the tissue extremely finely. (The minced pieces were less then 0.5 mm in any dimension). For samples less than 50 mg, the tissues were

suspended in 5 ml of TE9 and for samples of 50-500 mg, 10 ml of solution were used. The suspended tissue was vortexed at high speed for 2-3 min and incubated for 24 hrs at 48°C. The sample was then vortexed for 2-3 min and additional proteinase K and SDS were added, to final concentrations of 1 mg/ml and 2%, respectively. The mixture was incubated for another 40 hrs and the sample was again vortexed for 2-3 min. By this time most of the sample had dissolved. The solution was forced through an 18 gauge needle 3 times using finger pressure. Nucleic acid was then extracted three times using one volume of phenol-chloroform (consisting of the organic phase of a mixture of 3 parts phenol, 2 parts TE9, and 4 parts chloroform) and then extracted once with 1 volume of chloroform. Ammonium acetate (0.33 volumes of a 10 M solution) was added to each sample and the DNA precipitated by addition of 2.5 volumes of cold ethanol. The DNA was incubated at -70° for at least 2 hrs and centrifuged for 1 hr at 9000 x g. The pellet was washed with 70% ethanol, dried thoroughly and resuspended in 3 mM Tris, 0.2 mM EDTA, pH 7.2 to a concentration of 200-1000 ug/ml. DNA concentrations were determined by the diphenylamine method (12).

Gel Electrophoresis and Transfer to Nitrocellulose

Samples were cleaved with restriction endonucleases (BRL, Bethesda, Maryland) with the buffers recommended by the supplier, using 10 units of enzyme per microgram of DNA for 16 hrs at 37°. Samples were analyzed on agarose gels as previously described (13). Before transfer to nitrocellulose, the gels were stained in 250 ml of ethidium bromide (2 mg/ml for 5 min), and photographed. Gels were prepared for transfer to nitrocellulose by sequential depurination in 12 mM HCl (500 ml, 30 min; a modification of ref. 14), denaturation in 0.5 M NaOH, 1 M NaCl (500 ml, 30 min) and neutralization in 3 M NACl, 1 M Tris, pH 7.2 (500 ml, 45 min). The gels were then transferred to nitrocellulose essentially as described by Southern (15), using 3 M NaCl, 0.12 M sodium citrate, 20 mM Tris, pH 7.4. The filters were baked at 80° for 4 hrs without vacuum.

Hybridization

Prehybridization and hybridization of Southern blots were performed at 60°C in 10% formamide, 10% dextran sulfate, 0.2% bovine serum albumin, 0.2% polyvinyl pyrrolidone, 0.2% Ficoll (16), 0.4 mM EDTA, 0.72 M NaCl, 40 mM sodium phosphate, pH 7.0. The probes were labeled by oligolabelling (17). Prehybridization and hybridization were carried out in Seal N Save plastic bags (Sears) using 10 ml of prehybridization solution, and 7 ml of hybridization solution containing the probe. After hybridization for 18 hrs, the filters were removed and washed as previously described (18), except that the first three washes were each for 30 min and the fourth and fifth washes were in 45 mM NaCl, 1.8 mM sodium citrate, 0.3 mM Tris, pH 7.4, 0.3% SDS for 45 min each. The filters were exposed to XAR-5 Kodak film for 16 hrs at -80° using intensifying screens (19).

RESULTS AND DISCUSSION: Initially, it was anticipated that formaldehyde fixation would be the critical process to overcome if usable DNA was to be obtained from fixed and embedded tissue. Starting with tissue fixed in buffered formaldehyde (but not embedded), many parameters were tested. Use of ionic detergents, relatively high pH, high concentrations of a protease, and long periods of digestion with the protease, were found to be critical for success. The length of time in fixative (overnight to several weeks) did not seem to influence the results. Details of the method finally devised are given in the Materials and Methods section.

The procedure devised for extracting DNA from fixed tissues could be successfully used to extract DNA from embedded tissues. Cutting away most of the excess paraffin surrounding the embedded tissue, and subsequent fine mincing of the embedded tissue itself, were adequate to allow proteinase K and SDS diffusion into the tissue. Completely removing the paraffin (by heating the sample or dissolving the paraffin in chlorform or xylene) did not appreciably improve the yield or the quality of DNA obtained. DNA could also be extracted from frozen tissues embedded in O.C.T. compound (Miles Scientific Product No. 4583) using the procedure described here.

Signficant yields of DNA were obtained from formaldehyde fixed and paraffin embedded tissue; the yields ranged from 0.5-3 ug/mg of embedded tissue compared to 1-3 ug/mg of wet, unfixed tissue. The variation in yield was partially due to different cellular densities within the tissues and to the difficulty in ascertaining how much residual paraffin contributed to the measured weight.

The fixation and embedding procedures in and of themselves caused relatively little degradation of DNA. When fresh tissue, which had been frozen immediately after surgical removal, was fixed or was fixed and embedded, the extracted DNA was relatively large (Fig. 2A). This DNA could be cleaved with restriction endonucleases to a limit digest, could be transferred to nitrocellulose and could hybridize with labeled DNA probes (i.e., could be used for Southern blots). As seen in Figure 2B, the hybridization signals obtained using DNA from fixed and embedded tissues were quite comparable to those obtained with DNA from unfixed tissues.

Embedded pieces of colon tumors, which had been accumulated over the past 5 years, were obtained from the pathology archives. When the DNA was extracted and analyzed on agarose gels, it was found that its size ranged from 100 base pairs (bp) to over 10,000 bp, with the bulk between 100 and 1500 bp. Representative samples are shown in Figure 3A. The variation in the size of DNA from such samples was at least in part due to: (1) variable lengths of time between surgical removal of the tissue and formaldehyde fixation by the

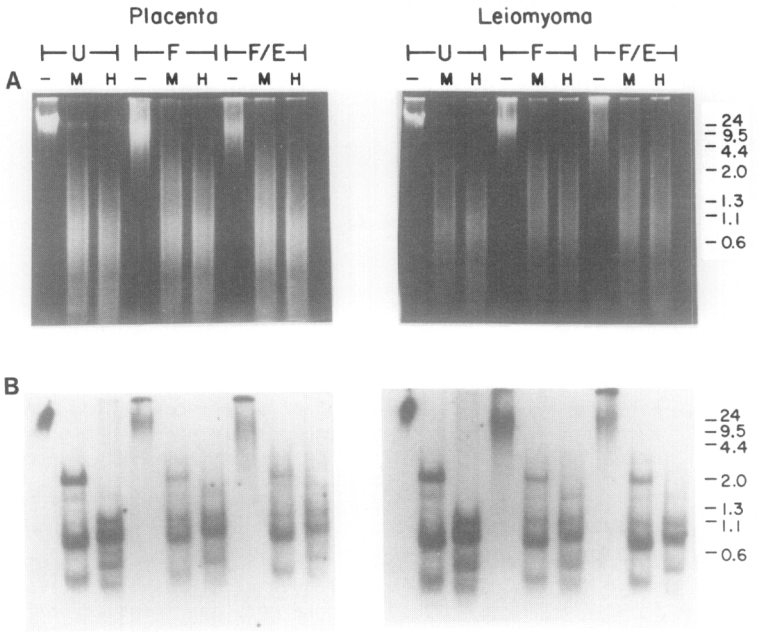

Figure 2: Analysis of DNA from tissues which had been fixed or fixed and embedded.

A. Ethidium bromide stained 1.5% agarose gels containing DNA from unfixed (U), fixed (F) and fixed and embedded (F/E) tissue. DNA prepared as described in Methods was either undigested (-) or digested with either Mbo I (M) or HinfI (H). 3 ug of DNA are contained in each lane.

B. Autoradiographs of Southern hybridizations of the gels shown in (A). The Southern blots were hybridized with a probe to the gamma-crystallin gene [a 3.4 kb HindIII genomic fragment, derived from the plasmid p5G1, prepared as described previously (17)]. Molecular weight markers, given in kilobase pairs, were lambda DNA cleaved with HindIII and ØX DNA cleaved with HaeIII.

pathologist (during which time nucleases act freely); (2) variable levels of nucleases in different tumors, which could act either before or after fixation; and (3) length of storage in paraffin block form; although there was no absolute distinction in size between DNA prepared from samples maintained in paraffin for varying periods, DNA prepared from surgical samples embedded in 1982-1984 was often larger than DNA prepared from samples embedded in 1979-1981.

DNA prepared from most of the colonic carcinoma samples could be successfully used for hybridization experiments. Examples of Southern blots from the 5 DNA samples shown in Fig. 3A are exhibited in Fig. 3B. Several points were noted in examining such blots. (1) Signal intensity from DNA

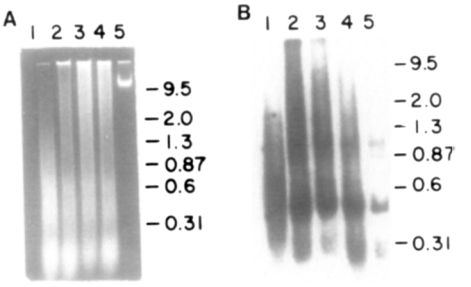

Figure 3: Analysis of DNA from colon tumors obtained from the pathology archives.

A. Ethidium bromide stained 2% agarose gels containing DNA extracted from colon tumors embedded in paraffin blocks. 1 = colon tumor from 1980, 2 = colon tumor from 1983, 3 = colon tumor from 1983, 4 = colon tumor from 1983, 5 = fresh (unfixed) placenta as a control. Three micrograms of DNA are contained in each lane.

B. Autoradiographs of Southern hybridizations of the samples shown in (A). The DNA was cleaved with Hinf I, fractionated by electrophoresis on 2% agarose gels and transferred to nitrocellulose paper. Lanes 1-4 contained 12 ug of DNA and lane 5 contained 5 ug of DNA. The Southern blots were hybridized with a probe for beta-chorionic gonadotropin [a 0.6 Hind III cDNA fragment, derived from the plasmid pPE-B, prepared as described previously (16,22)]. Molecular weight markers, given in kilobase pairs, were lambda DNA cleaved with Hind III and ΦX DNA cleaved with Hae III.

prepared from the embedded colon tissues varied from 10-80% of that obtained from an equal amount of standard DNA when the restriction fragments analyzed were 300-500 bp in length. The signal intensity was directly related to the initial size of the DNA, with DNA of the smallest size yielding the least intense signals. DNA from embedded samples could also be successfully used to perform dot-blots; intensities of the resultant signals ranged from 15-95% of that obtained from standard DNA controls (data not shown). (2) Background hybridization ("noise") was greater with DNA prepared from embedded tissues than from standard DNA preparations. The noise was probably due to small DNA fragments which did not contain two restriction endonuclease recognition sites within the gene chosen for analysis. This noise was more of a problem with DNA of small size. (3) Because of the increased noise and decreased signal obtained with DNA of small size, approximately 25% (7 of 30 examined) of DNA samples from the embedded colon cancer tissues were not suitable for Southern blots. Fig. 3B, lane 1, exemplifies a DNA sample in which the noise obscured

the signal. (4) The electrophoretic mobility of some restriction fragments was slightly decreased compared to DNA from unfixed tissues.

DNA prepared from fixed and embedded tissue could also be cloned using plasmid vectors. In an initial pilot experiment, when MboI cleaved DNA from fixed embedded placental tissue was cloned into the BamHI site of pBR322, 28 x 10^3 colonies per ug were obtained, compared with 30 x 10^3 colonies obtained per ug of DNA from the same tissue before fixation. The transfectants were all tetracycline sensitive and ampicillin resistant and most contained inserts of the expected size.

In conclusion, it is shown that DNA can, indeed, be extracted from tissues prepared by routine histopathologic procedures. The DNA from most samples, though not intact, can be used for standard hybridization experiments as long as the fragments to be detected are relatively small. Hence, the DNA from fixed and embedded tissue samples could be used to identify the presence of viral genes or amplified oncogenes in human tumors. Other analyses that require only small fragments of DNA, (e.g., the analysis of mutations through genomic sequencing [20] or through restriction site alterations [21]) should also be possible.

ACKNOWLEDGEMENTS: We thank S.L. Smith for preparing the manuscript and Annette Folgueras for technical assistance. We also thank M. Breitman and L.-C. Tsui for the gamma-crystallin probe and J. Fiddler for the beta-chrionic gonadtropin probe. This work was supported by the Clayton Fund, Training Grant CA 09071 (SG), and grants GM 31676 and CA 35494 from the NIH, DHHS.

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