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Clonal Analysis Using Recombinant DNA Probes from the X-Chromosome[1]

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ABSTRACT

It has been demonstrated that restriction fragment length polymorphisms of X-chromosome genes can be used in conjunction with methylation patterns to determine the clonal composition of human tumors. In this report, we show that several X-chromosome probes can be used for such analyses. In particular, probes derived from the hypoxanthine phosphoribosyltransferase gene and the phosphoglycerate kinase gene could be used for clonal analysis in over 50% of American females. The X-inactivation patterns observed with these probes were found to accurately reflect clonality in more than 95% of 92 tumors tested.

INTRODUCTION

Study of the clonal composition of human cell populations is important for a variety of investigations in cancer research. The fact that many human tumors are monoclonal (1, 2) provides strong supportive evidence for somatic mutation theories of carcinogenesis (3). Conversely, studies indicating that some tumors are polyclonal (4–9) are important for understanding the development of these neoplasms. Furthermore, clonal analysis can be used to study the stem cell nature and differentiation potential of acute and chronic nonlymphocytic leukemias (10–12), to help define the nature of proliferative diseases such as polycythemia vera (13), and to identify carriers of certain X-linked immune deficiency diseases associated with neoplasia (14–17).

Many of the clonal analyses noted above have been performed using the protein polymorphism exhibited by G6PD.[4] The analysis of G6PD isozymes has proven to be an extremely valuable research tool, but these studies have been limited by the fact that this polymorphism is found only in a small minority of the female population (2, 18). We have therefore sought to find more generally applicable methods for clonal analysis. In this regard, we have recently outlined a strategy using restriction fragment length polymorphisms and methylation patterns of X-chromosome genes for clonal analysis (19).

Three principles form the conceptual basis for this analysis: (*a*) A single X-chromosome is active in each somatic cell of an adult female. Inactivation of one X-chromosome occurs at an early stage of embryogenesis, and the pattern of X-chromosome inactivation in each progenitor cell is transmitted in a highly stable fashion to its progeny cells (18, 20). (*b*) Changes in the activity of many genes, including those of the X-chromosome, are accompanied by changes in the methylation of cytosine residues (21). These methylation differences can be monitored by restriction endonucleases that have the capacity to recognize methylated cytosine residues (22, 23). (*c*) RFLPs can be used to distinguish the maternal and paternal copies of X-chromosome genes from one another (24).

Based on these principles, the analysis requires the use of an X-chromosome gene and two restriction endonucleases. The first endonuclease is used to distinguish the maternal and paternal copies of the gene through an RFLP. Thus, clonal analysis can only be performed on the tissues of females who are heterozygous for RFLPs at particular X-chromosome genes. The second endonuclease recognizes differences in methylation patterns between the active and inactive copies of the X-chromosome gene. Because the X-inactivation process that occurs during embryogenesis is random, polyclonal female tissues develop from an approximately equal mixture of two types of precursor cells: some with the maternal X-chromosome active and others with the paternal X-chromosome active. Digestion of DNA from a polyclonal female tissue with a methyl-sensitive enzyme, such as *Hpa*II, will therefore alter the paternal and maternal alleles equivalently. In contrast, if the cell population arose from a single cell (monoclonal tumors are an example), then the maternal X-chromosome will be either active in all cells or inactive in all cells. Hence, the maternal allele will be cleaved by *Hpa*II in a different fashion than the paternal allele. This strategy was initially illustrated with the X-chromosome gene HPRT (19). In this report we refine the HPRT analysis and show that other X-chromosome gene sequences can be utilized for this purpose. Based on the data presented herein, clonal analysis can now be performed in over 50% of unselected American females.

MATERIALS AND METHODS

Sample Preparation

DNA was purified from human tumors using SDS-proteinase K digestion and phenol-chloroform extraction as previously described (25, 26). tRNA, used as carrier for ethanol precipitations, was purchased from Sigma (catalogue No. R0128; St. Louis, MO). To remove contaminating DNA from the tRNA, 80 mg tRNA was incubated with 750 U RNase-free DNase I in 10 ml D Buffer (10 mM $MgCl_2$, 50 mM Tris, pH 7.5) for 60 min at 37°C. After addition of EDTA to 20 mM, the tRNA was treated with SDS-proteinase K and extracted with phenol and chloroform. *Bst*XI and *Eco*0109 were purchased from New England Biolabs (Beverly, MA); all other restriction endonucleases were purchased from BRL (Bethesda, MD). *Bgl*II was used at 3–4 U/μg DNA, *Hpa*II at 5 U/μg DNA, and *Bst*XI at 8 U/μg DNA; all other enzymes were used at 10 U/μg of DNA, and all digestions were for 1.5–2 h. The multiple digestions were performed as follows.

***Bam*HI + *Pvu*II ± *Hha*I or *Hpa*II.** DNA (9–18 μg) was digested with *Bam*HI and *Pvu*II in a reaction volume of 0.4 ml in a buffer consisting of 200 μg/ml BSA, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris, pH

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[4] The abbreviations used are: G6PD, glucose-6-phosphate dehydrogenase; RFLP, restriction fragment length polymorphism; HPRT, hypoxanthine phosphoribosyltransferase; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TE, 3 mM Tris, 0.2 mM EDTA, pH 7.5; DTT, dithiothreitol; Hyb. Buf., 10% nonfat dried milk (28), 10% formamide, 1% SDS, heparin (0.5 mg/ml), salmon sperm DNA (0.2 mg/ml), 0.9 M NaCl, 2 mM EDTA, 0.05 M sodium phosphate, (pH 7.0).

8.0, for 90 min at 37°C. tRNA (25 μg) was added as carrier and the nucleic acids were precipitated by addition of 0.1 ml of 10 M ammonium acetate and 1 ml ethanol. After incubation on ice for 2 min, the precipitate was collected by centrifugation in a microfuge at 4°C for 12 min. The DNA pellet was washed with 70% ethanol, dried *in vacuo*, and dissolved in 0.31 ml TE. The volume was divided into three aliquots of 0.1 ml each and the aliquots were digested with either *Hpa*II or *Hha*I (in the manufacturers' recommended buffer) or left undigested. Each reaction was then precipitated with ethanol as described above before electrophoresis through a 1% agarose gel.

***Bst*XI + *Pst*I ± *Hpa*II.** DNA (6.5 μg) was digested with *Pst*I in a reaction volume of 0.2 ml in a buffer consisting of BSA (200 μg/ml), 50 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 8.0, for 90 min at 37°C. The volumes were adjusted to 0.3 ml by addition of 71 μl TE, 2 μl BSA (10 mg/ml), 23 μl of a buffer consisting of 1.5 M NaCl, 70 mM $MgCl_2$, 100 mM Tris, 15 mM DTT, pH 7.6, and then digested with *Bst*XI for 2 h at 55°C. Each reaction was then ethanol precipitated as described above. The DNA pellet was dissolved in 0.21 ml TE and the volume was divided into two aliquots of 0.1 ml each. One aliquot was digested with *Hpa*II and the other was left undigested. Each reaction was then ethanol precipitated as described above before electrophoresis through a 1.5% agarose gel.

***Bgl*I + *Bgl*II + *Eco*RI ± *Hpa*II.** DNA (6.5 μg) was digested with *Eco*RI and *Bgl*I in a reaction volume of 0.2 ml in a buffer consisting of BSA (200 μg/ml), 50 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 8.0, for 90 min at 37°C. The digestions were then heated to 70°C for 15 min to inactivate the enzymes. The volumes were adjusted to 0.350 ml by the addition of 117 μl TE, 3 μl BSA (10 mg/ml), and 25 μl of a buffer consisting of 100 mM Tris, 100 mM glycine, 100 mM $MgCl_2$, 1 M NaCl, 10 mM DTT, pH 9.5, and then digested with *Bgl*II for 90 min at 37°C. Each reaction was then ethanol precipitated, digested with *Hpa*II, and electrophoretically separated as described above.

***Eco*0109 ± *Hpa*II.** DNA (8–12 μg) was dissolved in a buffer consisting of BSA (200 μg/ml), 20 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4. Aliquots of 100 μl were either digested with *Hpa*II or left undigested. The volume of each aliquot was adjusted to 0.2 ml by the addition of 88 μl TE, 2 μl BSA (10 mg/ml), 10 μl of a buffer consisting of 500 mM Tris-HCl, 100 mM $MgCl_2$, 15 mM DTT, pH 8.0, and then digested with *Eco*0109 for 2 h at 37°C. Each reaction was then ethanol precipitated as described above before electrophoresis through a 3% agarose gel.

***Bgl*II ± *Hpa*II.** DNA (8–12 μg) was dissolved in a buffer consisting of BSA (200 μg/ml), 20 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4. Aliquots of 100 μl were either digested with *Hpa*II or left undigested. The volumes were adjusted to 0.4 ml by the addition of 152 μl TE, 8 μl BSA, 40 μl of a buffer consisting of 100 mM Tris, 100 mM glycine, 100 mM $MgCl_2$, 1 M NaCl, 15 mM DTT, pH 9.5, and then digested with *Bgl*II for 2 h at 37°C. Each reaction was then ethanol precipitated as described above before electrophoresis through a 1% agarose gel.

After electrophoresis, DNA was transferred to Nylon Plus membranes (Hoeffer, San Francisco, CA) in 0.4 M NaOH as described by Reed and Mann (27).

Hybridization

Filters (120 cm^2) were prehybridized for 0.5–3 h at 60°C in 10–14 ml of Hyb. Buf. Hybridization was performed at 60°C for 10–18 h in 4.5 ml Hyb. Buf. containing 5–15 × 10^6 dpm of probe/ml. Filters were washed at 65°C in 0.5 M NaCl, 1% SDS, 0.1 M Tris, pH 8.6, for 30 min, then twice in 0.1 × SSC, 0.1% SDS for 30 min each. (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, 1 mM Tris, pH 7.5). For the PGK probe, an additional wash at 68°C in 0.1 × SSC, 0.1% SDS, 0.1 mM Tris, pH 7.5, was sometimes performed to eliminate hybridization to PGK related genes (see text). Filters were exposed to Kodak XAR-5 film using Lightening-Plus Intensifying screens for 4–40 h at −70°. Autoradiographic signals were quantitated using a Hoeffer scanning densitometer.

Probes

The following plasmids were used to prepare probes: pPB 1.7 (29) contains a 1.7-kilobase *Bam*Hi/*Pst*I fragment from the 5′ region of the HPRT gene. pHPRT-600 contains a 600-base pair *Hpa*II subfragment from pPB 1.7 cloned into the *Acc*I site of pGEM-4 (Promega Biotech; Madison, WI). pHPRT-800 contains an 800-base pair *Hpa*II/*Pst*I subfragment of pPB 1.7 cloned into the *Acc*I/*Pst*I sites of pGEM-4 (see Fig. 1*A*). pSPT/PGK (30) contains an 800-base pair *Bam*HI/*Eco*RI fragment from the 5′ end of the PGK gene cloned into the *Bam*HI/*Eco*RI sites of pSP64 (Pharmacia). pXUT23-2.1 [designated DNA segment DxS16 (31)] contains a 2.1-kilobase *Bam*HI fragment from the X-chromosome cloned into pUC9. Probes consisted of the 600-base pair *Bam*HI/*Pst*I fragment from pHPRT-600, the 800-base pair *Bam*HI/*Pst*I fragment from pHPRT-800, the 800-base pair *Eco*RI/*Bam*HI fragment from pSPT/PGK and the 2.1-kilobase *Bam*HI fragment from pXUT 23–2.1. DNA fragments were purified from agarose gels by centrifugation(32), and labeled using the oligo-labeling method (33). Probes were ethanol precipitated prior to use. The HPRT probes were preannealed with normal human DNA to reduce hybridization to repeated sequences (34). The other probes were preannealed to salmon sperm DNA, which reduced background on the blots. Salmon sperm and human DNA were sheared by boiling in 0.4 M NaOH for 20 min, neutralized with Tris-HCl, ethanol precipitated, and dissolved in TE at 2 mg/ml. For preannealing, the probe, in 50 μl TE, was mixed with 50 μl 20 × SSC and 100 μl human or salmon sperm DNA, boiled for 10 min, incubated at 68°C for 20 min, then added to Hyb. Buf.

RESULTS

HPRT Gene. Clonal analyses of numerous normal and neoplastic tissues were performed using the pHPRT-800 probe described in "Materials and Methods." The restriction endonuclease conditions initially described (19) were modified by including digestion of samples with *Pvu*II. As shown in Fig. 1*A*, *Pvu*II cleaves the HPRT gene approximately 6 kilobases to the right of the polymorphic *Bam*HI site (35), thus rendering the two polymorphic *Bam*HI alleles closer in size. The two polymorphic fragments detected by the pHPRT probes in *Bam*HI-digested DNA are approximately 24 and 12 kilobases; the probe detects fragments of 18 and 12 kilobases in DNA digested with *Bam*HI and *Pvu*II. Because a small amount of DNA degradation is present in some neoplastic tissues, the reduction in the size of the larger allele allows this analysis to be performed in more cases. Also noted in Fig. 1*A* are the recognition sites for the methyl-sensitive enzymes *Hpa*II and *Hha*I in the relevant region of the HPRT gene.

The differential methylation of the HPRT gene in active and inactive X-chromosomes has been studied intensively (19, 36, 37).[5] *Hha*I site 1 is unmethylated in most active X-chromosomes and in some inactive X-chromosomes. *Hha*I sites 2 to 6 are each methylated in over 95% of active X-chromosomes, but at least one of *Hha*I sites 2 to 6 is unmethylated in over 90% of inactive X-chromosomes. Therefore, the differential methylation of *Hha*I sites in active *versus* inactive X-chromosomes is best seen by studying sites 2 to 6. *Hpa*II site 1 is unmethylated in active X-chromosomes and in some inactive X-chromosomes. *Hpa*II sites 2 to 9, however, are differentially methylated. In active HPRT alleles *Hpa*II sites 2 or 3 are unmethylated, while *Hpa*II sites 4 through 9 are methylated. There are two types of inactive HPRT alleles. In type I alleles, *Hpa*II sites 2 and 3 are methylated, while at least one of sites 4 to 9 is unmethylated. In type 2 alleles, *Hpa*II sites 2 to 9 are all methylated. In individual females, 60–100% of the inactive alleles are type 1, and the remainder are type 2.

Based on these data, the following results would be expected. *Bam*HI- and *Pvu*II-digestion of DNA from a female who is heterozygous for the polymorphic site should reveal fragments

[5] Unpublished results.

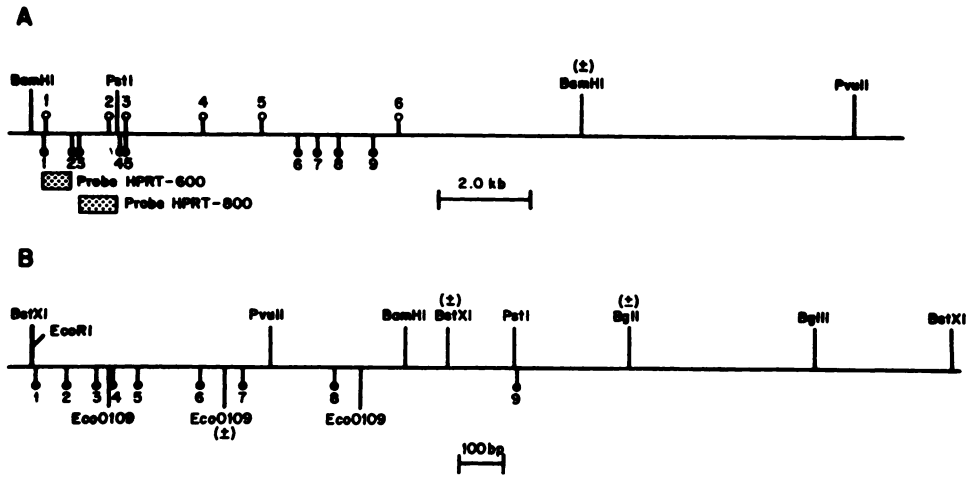

Fig. 1. *A*, restriction map of the 5′ region of the HPRT gene (35, 36). Transcription begins approximately 250 base pair to the left of the most 5′ *Bam*HI site shown and continues through the *Pvu*II site at the right. ●, nine *Hpa*II sites; ○, six *Hha*I sites; the *Bam*HI site (±) is polymorphic. *B*, restriction map of the 5′ region of the PGK gene (25). Transcription begins 72–81 base pair to the left of the *Pvu*II site and continues through the *Bst*XI site at the right. ●, nine *Hpa*II sites. The *Bgl*I, *Eco*0109, and *Bst*XI sites (±) are polymorphic. The plasmid pSPT-PGK contains the 800-base pair *Eco*RI/*Bam*HI fragment.

of 18 and 12 kilobases when hybridized to the HPRT-800 probe (Fig. 1*A*). Subsequent digestion of the DNA with *Hha*I should convert the *Bam*HI-*Pvu*II fragments to 17.8 and 11.8 kilobases if they were derived from the active X-chromosome. These fragments cannot be distinguished from the 18- and 12-kilobase *Bam*HI-*Pvu*II fragments under the usual conditions of electrophoresis. *Hha*I digestion of inactive X-chromosomes should convert the *Bam*HI-*Pvu*II fragments to fragments of less than 9 kilobases. *Hpa*II digestion of active X-chromosomes should convert the 18- and 12-kilobase *Bam*HI-*Pvu*II fragments to 17.2 and 11.2 kilobases, respectively. *Hpa*II digestion of type I inactive alleles should convert them to fragments of less than 8 kilobases. *Hpa*II digestion of type II inactive alleles would not affect the migration of the fragments, because *Hpa*II sites 2–9 are methylated.

These predictions were confirmed by analyses of normal tissues from 42 females heterozygous at the *Bam*HI site of the HPRT gene. After digestion with *Hpa*II or *Hha*I, the intensity of the 17.2–18-kilobase and 11.2–12-kilobase fragments both decreased by 30–70% in 41 of the 42 individuals examined. When normal tissues were digested with *Hpa*II, the 18- and 12-kilobase *Bam*HI-*Pvu*II fragments were converted to fragments of 17.2 and 11.2 kilobases (active alleles), to fragments of less than 8 kilobases (type I inactive alleles) or to fragments of unaltered mobility (type II inactive alleles). Examples are shown in Fig. 2, *1* and *2*. The relationship of the polymorphic fragments was reciprocal in that if the intensity of one allele decreased by more than 50%, the intensity of the other allele decreased by less than 50%. In several normal tissues of one individual, the intensity of the 18-kilobase fragment decreased by 85% while the intensity of the 12-kilobase fragment decreased by only 15% after *Hpa*II or *Hha*I digestion (data not shown). This unusual pattern was probably due to the random nature of the X-chromosome inactivation process (18, 20, 38).

Although the observed RFLP-methylation patterns at the HPRT gene in normal female tissues confirmed the predicted patterns, it was possible that neoplastic tissues would have abnormal methylation patterns that would complicate, and perhaps preclude, the use of the HPRT gene for clonal analysis in a large number of cases. Several human tumors have been shown to be aberrantly methylated, with some genes consistently hypomethylated (39, 40) and others hypermethylated in specific tumors (41). Therefore, to simplify the interpretation of results, we analyzed only tumors which were expected to be monoclonal based on previous studies. These tumor types included: acute and chronic nonlymphocytic leukemias, which have been shown to be monoclonal in a variety of studies (10, 11, 42); lymphocytic neoplasms, which have been shown to contain clonal rearrangements of immunoglobulin or T-cell receptor genes (43–45); uterine leiomyomas, a large number of which have been examined using G6PD isozyme analysis and have been shown to be monoclonal (1, 46); and miscellaneous solid tumors which exhibited monoclonal losses or gains of specific chromosomes (47–50).

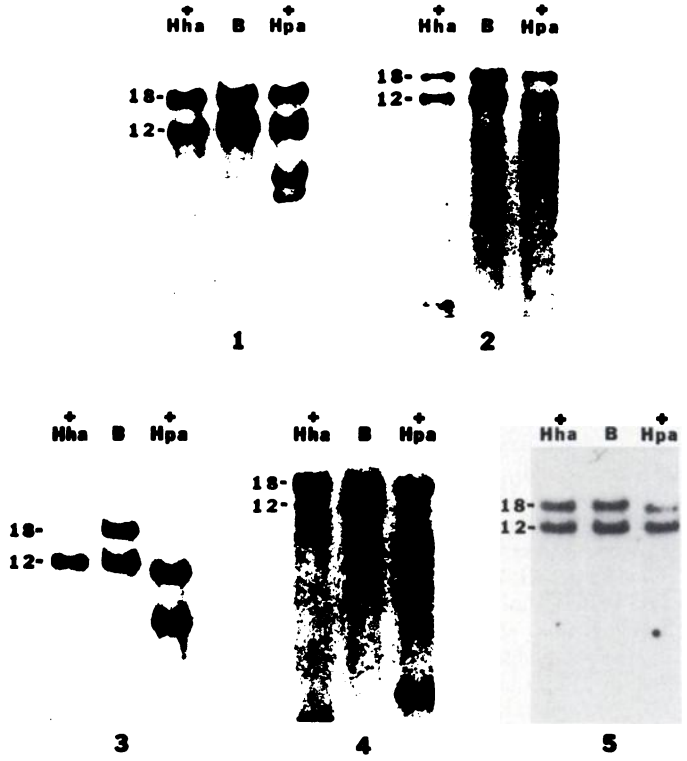

Fig. 2. Clonal analysis using HPRT-800 as probe. *Lanes marked B*, digested with *Bam*HI and *Pvu*II; *lanes marked +Hha or +Hpa*, also digested with *Hha*I or *Hpa*II, respectively; *1* and *2*, DNA from normal female tissues (colonic mucosa and normal lymphocytes respectively); *3–5*, DNA from neoplastic cells (chronic myelogenous leukemia cells in *3* and acute nonlymphocytic leukemia cells in *4* and *5*). *Left*, sizes (in kilobases) of the two polymorphic restriction fragments.

In monoclonal tumors, one of the two X-chromosomes should be active in each cell and the other should be inactive. Several practical considerations, however, affect the analysis of the clonal origin of tumors. Many tumor samples, especially solid tumors, are admixed with stromal elements (vascular and connective tissue, in addition to inflammatory cells) and these normal cells skew the RFLP-methylation pattern observed in the tumor sample toward those of the individual's normal polyclonal tissues. Second, there is some variability in DNA

loading, electrophoretic transfer, and hybridization efficiency. Based on these practical considerations, we considered a sample to exhibit a monoclonal pattern if the intensity of one allele decreased by over 80% and the intensity of the other allele decreased by less than 40% following *Hpa*II (or *Hha*I) digestion, Conversely, tissues were considered to exhibit a polyclonal pattern if both alleles decreased by 30–70% after digestion with the methyl-sensitive enzyme.

As noted above, examination of normal tissues from 41 of 42 females with the HPRT-800 probe revealed RFLP-methylation patterns consistent with the operational definition of a polyclonal pattern. In contrast, of 41 neoplastic tissues (each from a different individual) examined with the pHPRT-800 probe, 36 exhibited a monoclonal pattern with either *Hpa*II or *Hha*I (34 displayed monoclonal patterns with *Hpa*II and 30 displayed monoclonal patterns with *Hha*I). Examples of such monoclonal patterns are shown in Fig. 2, *3* and *4*. In the seven tumors that did not demonstrate a monoclonal pattern with *Hpa*II, one of the two alleles was cleaved by *Hpa*II to the size expected for an active allele (either 17.2 or 11.2 kilobases). The other allele was either partially or totally resistant to *Hpa*II digestion (and often to *Hha*I digestion as well). An example of such a tumor is shown in Fig. 2, *5*. RFLP-methylation patterns such as that seen in Fig. 2, *5*, most likely resulted from the proliferation of cells in which the inactive X-alleles were largely of the type II class (*i.e.*, *Hpa*II sites 2–9 methylated). The HPRT-800 probe could not be used to unambiguously determine the clonal origin of tumor cells in such cases, because it primarily detected differences in the methylation of *Hpa*II sites 4–9. However, we found that the methylation state of *Hpa*II sites 2 and 3 could be used to distinguish active and inactive X-chromosomes from one another, and thus determine clonal origin, in such cases.

The HPRT-600 probe (Fig. 1*A*) was useful for examining the methylation of *Hpa*II sites 2 and 3. Hybridization of the HPRT-600 probe to *Bam*HI- and *Pvu*II-digested DNA from a male whose X-chromosome lacked the polymorphic *Bam*HI site revealed an 18-kilobase fragment (Fig. 3, *1*). Subsequent digestion of the DNA with *Hpa*II produced a single hybridization fragment of 600 base pairs (Fig. 3, *1*), produced by cleavage at *Hpa*II site 2 or 3; *Hpa*II sites 2 and 3 were too close to one another to determine which one of them was unmethylated (see Fig. 1*A*). Similar results were seen when the DNA from cells of a male who had the polymorphic *Bam*HI site (*i.e.*, containing the 12-kilobase HPRT fragment) was studied (Fig. 3, *2*). Such results demonstrate that active X-chromosomes are unmethylated at *Hpa*II site 2 or 3. *Hpa*II digestion of DNA from the normal tissues of a female who was heterozygous for the polymorphic *Bam*HI site reduced the intensity of both the 18- and 12-kilobase fragments by approximately 80–90% (Fig. 3, *3*). The other fragments visible in Fig. 3, *3*, after *Hpa*II digestion were as follows: the 600-base pair fragment resulted from active X-alleles cleaved at *Hpa*II sites 2 or 3; the fragments between 0.6 and 12 kilobases resulted from cleavage of type I inactive alleles (*i.e.*, methylated at *Hpa*II sites 2 and 3 and unmethylated at one of *Hpa*II sites 4–9); the 18- and 12-kilobase fragments resistant to *Hpa*II digestion were derived from type II inactive alleles (*i.e.*, methylated at *Hpa*II sites 2–9). Graded digestion with increasing concentrations of *Hpa*II did not alter the patterns observed.

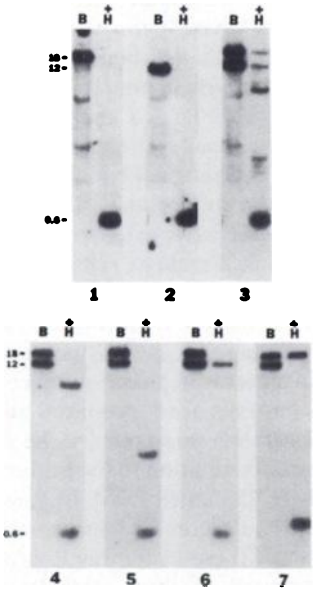

Fig. 3. Clonal analysis using HPRT-600 as probe. *DNA lanes marked B*, digested with *Bam*HI and *Pvu*II; *lanes marked +H*, additionally digested with *Hpa*II; *1* and *2*, DNA from normal male lymphocytes; *3*, DNA from normal female lymphocytes; *4–7*, DNA from the leukemia cells of four different patients with acute nonlymphocytic leukemia. Faint bands of 3 and 6 kilobases, representing HPRT-related genes, can sometimes be seen in the lanes marked B.

When DNA from tumors which displayed monoclonal patterns with the HPRT-800 probe were analyzed with the HPRT-600 probe, both the 18- and 12-kilobase fragments disappeared completely (Fig. 3, *4* and *5*). This was expected, as these tumors contained predominantly inactive alleles of type I, which were digested by *Hpa*II to fragments of less than 8 kilobases, while the active alleles in these tumors were cleaved to 0.6-kilobase fragments. Conversely, in tumors which contained a significant percentage of cells with type II inactive alleles, the 18- and 12-kilobase fragments were not digested equivalently by *Hpa*II. One allele (the active one) was digested completely by *Hpa*II, while the inactive type II allele was partially resistant to *Hpa*II digestion (Fig. 3, *6* and *7*). Note that monoclonal tumors such as those seen in Fig. 3, *6* and *7*, consisted of cells in which a significant percentage (30–80%), but not all cells, contained inactive alleles of the type II class.

In summary, monoclonal tumors could be divided into two types depending on the proportion of inactive type II alleles contained within their cells. Most tumors (34 of 41) contained predominantly type I inactive alleles and could be shown to be monoclonal using the HPRT-800 probe. The remaining seven tumors contained a significant portion of cells with type II inactive alleles, and these could be shown to be monoclonal with the HPRT-600 probe. Based on these results, we now perform clonal analysis by first examining DNA samples with the HPRT-800 probe. If a sample shows a high percentage of class II inactive alleles, we rehybridize the same blot with the HPRT-600 probe.

PGK Gene. The PGK gene, like the HPRT gene, is constitutively active and located on the long arm of the X-chromosome (51). It has previously been shown that eight *Hpa*II sites near the 5′ end of the gene are unmethylated in active X-chromosomes, but are methylated in inactive X-chromosome [*Hpa*II sites 1–8 in Fig. 1*B* (30)[6]]. In addition, we found one *Hpa*II site (Fig. 1*B*, *site 9*), which was unmethylated in 20–40% of inactive X-chromosomes. We tested 47 restriction endonucleases in an effort to find DNA polymorphisms in this region of the PGK gene. Through this screen, we identified a polymorphic *Bgl*I site located approximately 500 base pairs downstream from this differentially methylated region (Fig. 1*B*). 72

[6] A. M. Michelson and S. H. Orkin, unpublished results.

of 217 females (33%) were heterozygous for the *Bgl*I RFLP, producing alleles of 5 and 12 kilobases. For clonal analysis with the 800-base pair *Eco*RI-*Bam*HI PGK probe, samples were digested with *Bgl*I (to reveal the polymorphism) and *Bgl*II and *Eco*RI (to bracket the differentially methylated region; see Fig. 1*B*). One half of each DNA digest was then subsequently digested with *Hpa*II. Examples of the results obtained when normal tissues of males and females were digested with these enzymes are shown in Fig. 4, *1–3*. Two polymorphic alleles of 1.7 and 1.3 kilobases were identified. Digestion of active alleles from tissues of males with *Hpa*II completely eliminated the signal of either the 1.7-kilobase allele (Fig. 4, *1*) or the 1.3-kilobase allele (Fig. 4, *2*). *Hpa*II digestion of DNA from female tissues reduced the intensity of both polymorphic alleles by approximately 60–75%. This reduction in the intensity of each allele was due to: (*a*) cleavage of alleles from active X-chromosomes at the multiple unmethylated *Hpa*II sites; and (*b*) cleavage of 20–40% of inactive X-chromosome alleles at *Hpa*II site 9. In normal tissues from 36 of 40 females, the 1.7- and 1.3-kilobase fragments were both reduced in intensity by 30–70%, as predicted from the methylation differences noted above and the variations between individuals in the maternal:paternal X-chromosome inactivation ratio. In the normal tissue of two females, one of the alleles was digested by 80% and the other by 20%; in two additional females, both alleles were digested by 70–80%.

Tumors from 46 females who were heterozygous for the polymorphic *Bgl*I site were studied with the PGK probe. All but two of these tumors exhibited monoclonal RFLP-methylation patterns, as defined by the criteria outlined for the HPRT gene. Examples of the results obtained in the 46 tumors with monoclonal patterns are shown in Fig. 4, *4–7*. In the two tumors that did not show the expected monoclonal patterns, both alleles were completely digested by *Hpa*II (data not shown).

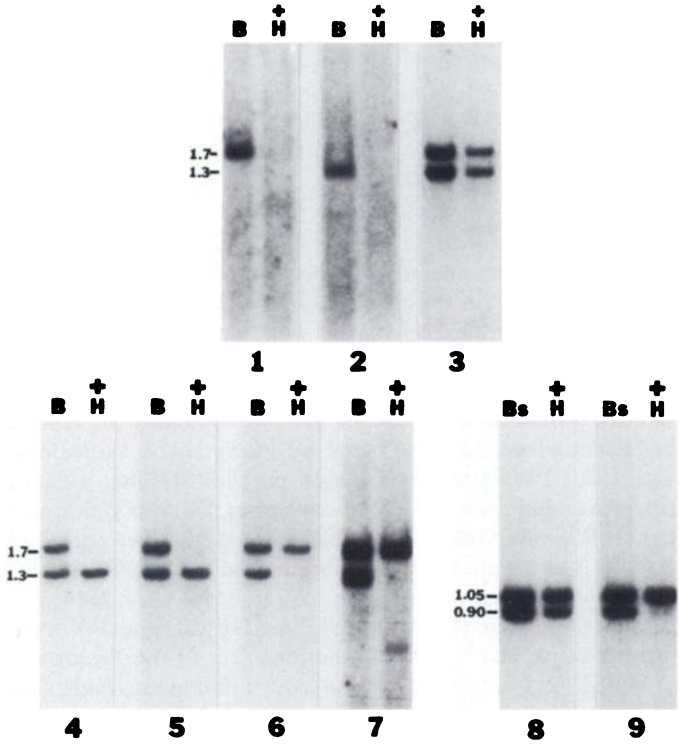

Fig. 4. Clonal analysis using the PGK probe for the *Bgl*I and *Bst*XI polymorphisms. *1–7: lanes marked B*, digested with *Bgl*I, *Eco*RI, and *Bgl*II; *lanes marked +H*, additionally digested with *Hpa*II; *1* and *2*, DNA from normal male lymphocytes; *3*, DNA from normal female lymphocytes; *4–7*, DNA from neoplastic cells (*4*, colorectal carcinoma; *5* and *6*, acute nonlymphocytic leukemia; *7*, acute lymphocytic leukemia). *8–9: DNA in lanes marked Bs*, digested with *Bst*XI and *Pst*I; *lanes marked +H*, additionally digested with *Hpa*II; *8*, DNA from normal female lymphocytes; *9*, DNA from acute nonlymphocytic leukemia cells. As noted in "Materials and Methods," stringent washing conditions were needed to eliminate hybridization of the PGK probe to PGK-related sequences contained in an *Eco*RI-*Bgl*II fragment of 1.7 kilobases (*1–7*). *Left*, sizes of the polymorphic restriction fragments.

Through our screen for RFLPs within the PGK gene, we also detected a *Bst*XI polymorphic site located approximately 250 base pairs downstream from the differentially methylated region (Fig. 1*B*). The *Bst*XI and *Bgl*I polymorphic sites appeared to be in strong linkage disequilibrium; twenty-nine females who were heterozygous for the *Bgl*I RFLP were all found to be heterozygous for the *Bst*XI RFLP. The *Bst*XI RFLP did not increase the percentage of females in whom we could perform clonal analysis. Nevertheless, the *Bst*XI RFLP was useful for clonal analysis for two reasons. First, the PGK-related genes did not contain *Bst*XI fragments of the same size as those from the PGK gene, thus eliminating the cross-hybridization problem noted in the legend to Fig. 4. Second, *Bst*XI digestion could be used in combination with *Pst*I digestion to eliminate *Hpa*II site 9 from the resulting fragments. Because *Hpa*II site 9 was unmethylated in some inactive alleles, the intensity of the inactive alleles was reduced by 20–40% after *Hpa*II digestion. From a practical standpoint, *Bgl*I was useful for screening for the RFLP (it is less expensive than *Bst*XI), but *Bst*XI was preferred for clonal analysis. Digestion of DNA from females who were heterozygous for the *Bgl*I RFLP with *Bst*XI and *Pst*I produced fragments of 1.05 and 0.9 kilobases. Examples of the RFLP-methylation patterns seen in polyclonal or monoclonal populations of cells from females heterozygous for the *Bst*XI polymorphism are shown in Fig. 4, *8* and *9*, respectively.

A polymorphic *Eco*0109 site was also identified in the differentially methylated region of the PGK gene (Fig. 1*B*). The *Eco*0109 RFLP was present in only seven of 137 females (5%) tested, but it appeared to be useful for clonal analysis when it was present. Several fragments were seen in *Eco*0109-digested DNA that was hybridized to the PGK probe; in addition to fragments of greater than 0.6 kilobase or less than 0.2 kilobase in size (some representing PGK-related genes), the probe hybridized to either a 544-base pair fragment and/or two fragments of 297 and 247 base pairs (Figs. 1*B* and 5). *Hpa*II digestion completely eliminated the 544-base pair fragment or 297- and 247-base pair fragments in active X-chromosome alleles (DNA from males; Fig. 5, *1* and *2*). As expected, *Hpa*II digestion reduced the intensity of these fragments by approximately 50% in DNA from normal female tissues (Fig. 5, *3*). In contrast, in DNA from monoclonal tumors, either the 297/247-base pair fragments were extensively digested by *Hpa*II while the 544-base pair fragment remained relatively spared (Fig. 5, *4* and *5*); or the converse pattern was observed (Fig. 5, *6* and *7*). Because of the low frequency of the *Eco*0109 polymorphism, we were unable to use this polymorphism to study RFLP-methylation patterns in a large number of tumors. However, monoclonal patterns were produced in all five tumors from females who were heterozygous for the *Eco*0109 RFLP.

pXUT23-2.1 Probe. In addition to the probes from the HPRT and PGK genes, over 25 cloned DNA fragments were used to search for methylation differences between active and inactive X-chromosomes. Of these additional probes, only one, pXUT23-2.1, was found to show methylation patterns that were relatively different between the active and inactive X-chromosomes. The pXUT23-2.1 plasmid contains sequences derived from the short arm of the X-chromosome; these sequences are not known to lie near a constitutively active gene

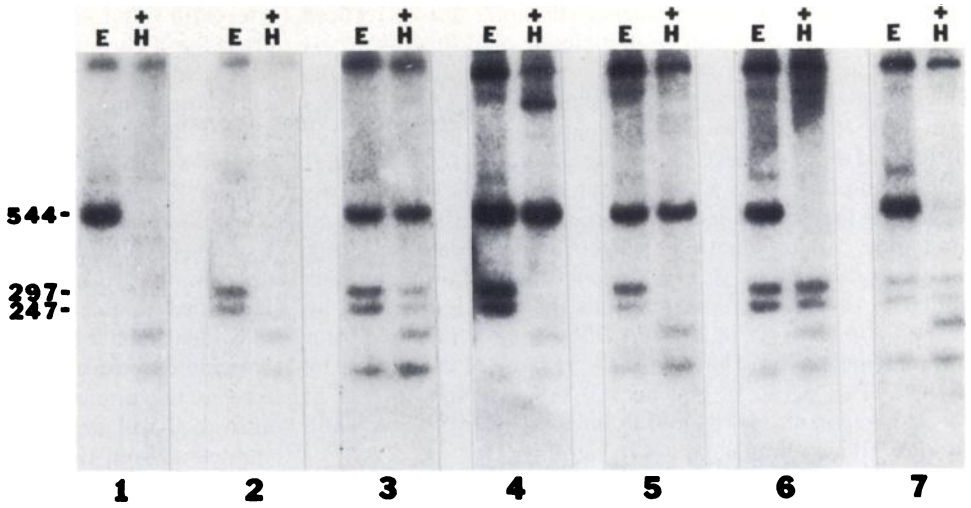

Fig. 5. Clonal analysis using the PGK probe for the *Eco*0109 polymorphism. DNA *lanes marked E*, digested with *Eco*0109; DNA *lanes marked +H*, additionally digested with *Hpa*II; *1* and *2*, DNA from normal male lymphocytes; *3*, DNA from normal female lymphocytes; *4–7*, DNA from acute nonlymphocytic leukemia cells of four different patients. *Left*, sizes of the restriction fragments.

(31). The pXUT23 probe detected a *Bgl*II RFLP with two alleles of 12.5 and 17.5 kilobases (31); 35 of 112 females tested (30%) were heterozygous for this polymorphism. *Hpa*II digestion of DNA from the active X-chromosome (male lymphocytes) reduced the intensity of the 17.5-kilobase fragment (Fig. 6, *1*) or 12.5-kilobase fragment (Fig. 6, *2*) by 80–90%, while the intensity of each of the *Bgl*II fragments was reduced by 60–80% when DNA from female lymphocytes was digested with *Hpa*II (Fig. 6, *3*). These results were consistent with the following model: the pXUT23 sequences on 80–90% of active X-chromosomes were unmethylated (*i.e.*, at least one unmethylated *Hpa*II site existed within the 12.5–17.5-kilobase *Bgl*II fragment), while 40–80% of inactive X-chromosomes contained methylated pXUT23 sequences (*i.e.*, all *Hpa*II sites within the 12.5–17.5-kilobase *Bgl*II fragments were methylated). Thus, although the methylation pattern of the pXUT23 sequences in most inactive X-chromosomes was different from that in most active X-chromosomes, the differential methylation of the two X-chromosome types was not as striking for pXUT23 as it was for the HPRT and PGK genes.

Despite the inability to distinguish all active from inactive X-chromosomes on the basis of their methylation patterns, the pXUT23 probe could be used for clonal analysis if the methylation pattern established on each X-chromosome had a high probability of being inherited in each progeny cell during the proliferation of a monoclonal neoplastic growth. Each *Hpa*II site within a pXUT23 allele in an individual cell is either methylated or is not methylated; the methylation patterns described above for active and inactive X-chromosomes apply only to the analysis of cell populations, not individual cells. Therefore, one would expect four types of female cells when classified according to the methylation status of their pXUT23-2.1 alleles. Type I cells [predicted to be 32% (80 × 40%, minimal) to 72% (90 × 80%, maximal) of the cellular population] will have the allele from the active X-chromosome unmethylated and the allele from the inactive X-chromosome methylated; Type II cells (2–12% of the cellular population) will have the allele from the active X-chromosome methylated and the allele from the inactive X-chromosome unmethylated; Type III cells (4–16% of the cellular population) will have alleles from both the active and inactive X-chromosomes fully methylated; and Type IV cells (16–54% of cellular population) will have the alleles from both the active and inactive X-chromosomes unmethylated. These expectations are not based on any mechanistic interpretations of methylation with regard to X-chromosome activity, but are simply based on empirical determinations of pXUT23 methylation patterns in cell populations from normal females.

If the methylation patterns in individual precursor cells were inherited during monoclonal neoplastic growth, then four classes of tumor cells would be defined with respect to methylation of the pXUT23 gene fragments. Type I and II cells would give rise to tumors with "monoclonal patterns" upon *Bgl*II/*Hpa*II digestion, *i.e.*, one allele would largely disappear upon *Hpa*II digestion while the other allele would be insensitive to *Hpa*II digestion. Type III and IV cells would also give rise to

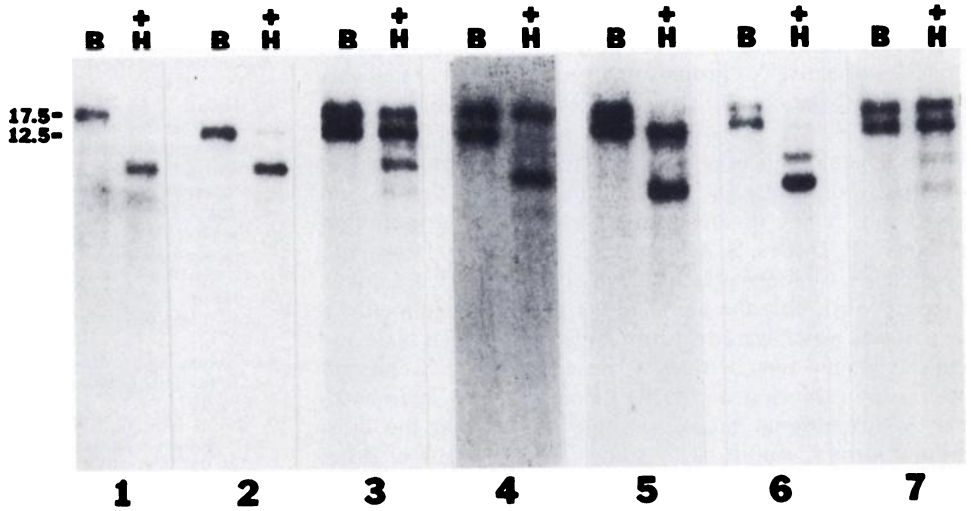

Fig. 6. Clonal analysis using the pXUT23 probe. *Lanes marked B*, digested with *Bgl*II; *lanes marked +H*, additionally digested with *Hpa*II; *1* and *2*, DNA from normal male lymphocytes; *3*, DNA from normal female lymphocytes; *4–7*, DNA from neoplastic cells. (*4* and *6*, acute nonlymphocytic leukemia; *5*, uterine leiomyoma; *7*, acute lymphocytic leukemia). *Left*, sizes of the polymorphic restriction fragments.

distinct patterns, since both alleles will either not be reduced by *Hpa*II digestion or will be completely abolished by *Hpa*II digestion, respectively. Analysis of tumors produced results which were consistent with these expectations. Of 29 tumors examined, 15 showed monoclonal patterns (as defined for the HPRT and PGK probes), suggesting outgrowth of Type I or II cells (examples in Fig. 6, *4–5*); four tumors showed patterns suggesting they originated from Type III cells (Fig. 6, *7*), and five tumors produced patterns suggesting their origin was from Type IV cells (Fig. 6, *6*). Finally, five tumors showed patterns similar to those seen in normal cells (both alleles digested by 60–80%). In contrast, in 23 of 24 DNA samples prepared from normal female tissues, both alleles were reduced in intensity by 50–80% (Fig. 6, *3*). In one normal tissue sample, the 17.5-kilobase fragment was reduced in intensity by over 95%, while the 12.5-kilobase fragment was reduced by 60%.

The results with the pXUT23-2.1 probe were obviously considerably more complex than those observed with the HPRT or PGK probes, and this probe could not be recommended for routine clonal analysis. However, these results are presented because they illustrate the problems and potential of performing clonal analyses with genes whose methylation patterns are not strictly dependent on X-chromosome activity. If the gene's methylation pattern established on each X-chromosome has a high probability of being inherited during cell division, then the inheritance of this methylation pattern can provide evidence for the clonal origin of a cell population.

DISCUSSION

The results of this study can be summarized as follows: Twenty-nine % of females were heterozygous at the *Bam*HI site of the HPRT gene, and monoclonal patterns were produced upon RFLP-methylation analysis in all 41 tumors studied if analyzed with both the HPRT-800 and HPRT-600 probes. Thirty-three % of females were heterozygous at the *Bgl*I site of the PGK gene; monoclonal patterns were produced upon RFLP-methylation analysis with the PGK probe in 96% of 46 tumors from females who were heterozygous for the polymorphic *Bgl*I site. Five % of females were heterozygous for the polymorphic *Eco*0109 site at the PGK gene, and monoclonal patterns were produced in all five tumors from *Eco*0109 heterozygotes. Of the 135 females studied for polymorphism at both the HPRT and PGK loci, 53% were heterozygous for at least one of the three polymorphisms.

It was noted above that the methylation patterns within the HPRT and PGK genes were more uniform than those seen with the pXUT23-2.1 probe, both in normal and neoplastic tissues. This is consistent with the hypothesis that methylation of the 5′ regions of active X-chromosome genes is tightly regulated, while the methylation of other sequences on the X-chromosome is not (30, 36, 37, 52–54). We have studied over 25 cloned DNA fragment sequences that detected restriction fragment length polymorphisms on the X-chromosome but that did not detect the 5′ region of constitutively active genes (kindly provided by K. E. Davies, S. A. Latt, J. C. Mandel, D. Drayna, L. M. Kunkel, S. Bhattacharya, R. L. Nussbaum, and H. J. Cooke). Of these probes, only the pXUT23-2.1 probe showed methylation patterns which were relatively different between male and female cells (and thus between active and inactive X-chromosomes). For extension of the RFLP-methylation analysis to other X-chromosome genes, it would appear that the most fruitful approach would be to study the 5′ regions of other constitutively active genes, rather than to attempt similar analyses with tissue-specific genes or randomly cloned sequences from the X-chromosome.

It is important to note that the ratio of active maternal X-chromosomes to active paternal X-chromosomes in female tissues has a normal distribution in the female population around a mean ratio of 50:50 (18). In the studies reported here, three of 81 females had X-inactivation ratios in excess of 80:20. This observation is consistent with previous studies of X-inactivation in females as assessed by G6PD isoenzyme analysis (18, 38, 46). To make conclusions about the clonality of any individual tumor, DNA isolated from normal cells of that individual should therefore be used as a control. Although it is preferable to use analogous normal cells from the same tissue type as the tumor being studied, any normal cells from the individual will probably suffice, as X-inactivation ratios are consistent from tissue to tissue within any individual female (18, 38).[5]

We have used the RFLP methylation strategy to successfully study normal and neoplastic cells from hematopoietic, digestive, and urogenital organs (12, 19)[5]. In several neuroblastomas, however, we found that the PGK gene (and less frequently, the HPRT gene) was unmethylated in both active and inactive alleles, thus precluding clonal analysis. It would be interesting in future studies to determine whether this unusual pattern of methylation is found in normal neural cells, or is limited to neoplastic neural cells.

Finally, the RFLP-methylation system for clonal analysis is subject to the same conceptual constraints posed by other methods of clonal analysis based on X-chromosome inactivation events (1, 2). Finding a monoclonal pattern in a tumor does not prove that the tumor originated from one cell, but only that one cell (or a small number of cells which fortuitously had the same X-chromosome inactivated) outgrew its companions during the process of neoplasia. Despite such limitations, clonal analysis has provided many useful insights into the neoplastic process as it occurs in humans, and the ability to perform these studies in additional patients should contribute to such studies in the future.

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REFERENCES

1. Linder, D., and Gartler, S. M. Glucose-6-phosphate dehydrogenase mosaicism: utilization as a cell marker in the study of leiomyomas. Science (Wash. DC), *150*: 67–69, 1965.
2. Fialkow, P. J. Clonal origin of human tumors. Biochim. Biophys. Acta, *458*: 283–321, 1976.
3. Knudson, A. G. Mutation and human cancer. Adv. Cancer Res., *17*: 317–352, 1973.
4. Gartler, S. M., Ziprkowski, L., and Krakowski, A. Glucose-6-phosphate dehydrogenase mosaicism as a tracer in the study of hereditary multiple trichoepithelioma. Am. J. Hum. Genet., *18*: 282–287, 1966.
5. Beutler, E., Collins, Z., and Irwin, L. E. Value of genetic variants of glucose-6-phosphate dehydrogenase in tracing the origin of malignant tumors. N. Engl. J. Med., *276*: 389–391, 1967.
6. Smith, J. W., Townsend, D. E., and Sparkes, R. S. Genetic variants of glucose-6-phosphate dehydrogenase in the study of carcinoma of the cervix. Cancer (Brussels), *28*: 529–532, 1971.
7. Fialkow, P. J., Sagabiel, R. W., Gartler, S. M., and Rimoin, D. L. Multiple cell origin of hereditary neurofibromas. N. Engl. J. Med., *284*: 298–300, 1971.
8. Fialkow, P. J., Jackson, C. E., Block, M. A., and Greenawald, K. A. Multicellular origin of parathyroid "adenomas." N. Engl. J. Med., *297*: 696–698, 1977.
9. Hsu, S. H., Luk, G. D., Krush, A. J., Hamilton, S. R., and Hoover, Jr., H. H. Multiclonal origin of polyps in Gardner syndrome. Science (Wash. DC), *221*: 951–953, 1983.
10. Fialkow, P. J., Jacobson, R. J., and Papayannopoulou, T. Chronic myelocytic

leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. Am. J. Med., *63*: 125–130, 1977.
11. Fialkow, P. J. Clonal evolution of human myeloid leukemias. *In*: J. M. Bishop, J. D. Rowley, and M. Greaves (eds.), Genes and Cancer, pp. 215–226. New York: Alan R. Liss, 1984.
12. Fearon, E. R., Burke, P. J., Schiffer, C. A., Zehnbauer, B. A., and Vogelstein, B. Differentiation of leukemia cells to polymorphonuclear leukocytes in patients with acute nonlymphocytic leukemia. N. Engl. J. Med., *315*: 15–24, 1986.
13. Adamson, J. W., Fialkow, P. J., Murphy, S., Prchal, J. F., and Steinmann, L. Polycythemia vera: stem-cell and probable clonal origin of the diseases. N. Engl. J. Med., *295*: 913–916, 1976.
14. Gealy, W. J., Dayer, J. M., and Harley, J. B. Allelic exclusion of glucose-6-phosphate dehydrogenase in platelets and T lymphocytes from a Wiskott-Aldrich syndrome carrier. Lancet, *1*: 63–65, 1980.
15. Prchal, J. T., Carroll, A. J., Prchal, J. F., Rist, W. M., Skalka, H. W., Gealy, W. J., Harley, J. B., and Malluh, A. Wiskott-Aldrich syndrome: cellular impairments and their implication for carrier detection. Blood, *56*: 1048–1054, 1980.
16. Conley, M. E., Brown, P., Pickard, A. R., Buckley, R. H., Miller, D. S., Raskind, W. H., Singer, J. W., and Fialkow, P. J. Expression of the gene defect in X-linked agammaglobulinemia. N. Engl. J. Med., *315*: 564–567, 1986.
17. Fearon, E. R., Winkelstein, J. A., Civin, C. I., Pardoll, D. M., and Vogelstein, B. Carrier detection in X-linked agammaglobulinemia by analysis of X-chromosome inactivation. N. Engl. J. Med., *316*: 427–431, 1987.
18. Gartler, S. M., and Riggs, A. D. Mammalian X-chromosome inactivation. Annu. Rev. Genet., *17*: 155–190, 1983.
19. Vogelstein, B., Fearon, E. R., Hamilton, S. R., and Feinberg, A. P. Use of restriction fragment length polymorphisms to determine the clonal origin of human tumors. Science (Wash. DC), *227*: 642–645, 1985.
20. Lyon, M. F. X-chromosome inactivation and developmental patterns in mammals. Biol. Rev. Camb. Philos. Soc., *47*: 1–35, 1972.
21. Cantoni, G. L., and Razin, A. Chemistry, biochemistry, and biology of DNA methylation. New York: Alan R. Liss, 1985.
22. Bird, A. P., and Southern, E. M. Use of restriction enzymes to study eukaryotic DNA methylation: I. The methylation pattern in ribosomal DNA from Xenopus laevis. J. Mol. Biol., *118*: 27–47, 1978.
23. Van Der Ploeg, L. H. T., and Flavell, R. A. DNA methylation in the human gamma delta beta-globin locus in erythroid and nonerythroid tissues. Cell, *19*: 947–958, 1980.
24. Botstein, D., White, R. L., Stolnick, M., and Davis, R. W. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet., *32*: 314–331, 1980.
25. Gross-Bellard, M., Oudet, P., and Chambon, P. Isolation of high-molecular-weight DNA from mammalian cells. Eur. J. Biochem., *36*: 32–38, 1973.
26. Goelz, S. E., Hamilton, S. R., and Vogelstein, B. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochem. Biophys. Res. Commun., *130*: 118–126, 1985.
27. Reed, K. C., and Mann, D. A. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res., *13*: 7207–7221, 1985.
28. Johnson, D. A., Gautsch, J. W., Sportsman, J. R., and Elder, J. H. Gene analysis techniques. Gene Anal. Tech., *1*: 308, 1984.
29. Jolly, D. J., Esty, A. C., Bernard, H. U., and Friedmann, T. Isolation of a genomic clone partially encoding human hypoxanthine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA, *79*: 5038–5041, 1982.
30. Keith, D. H., Singer-Sam, J., and Riggs, A. D. Active X-chromosome DNA is unmethylated at eight CCGG sites clustered in a guanine-plus-cytosine-rich island at the 5′ end of the gene for phosphoglycerate kinase. Mol. Cell Biol., *6*: 4122–4125, 1986.
31. Bakker, E., Hofker, M. H., Goor, N., Mandel, J. L., Wrogemann, K., Davies, K. E., Kunkel, L. M., Willard, H. F., Fenton, W. A., Sandkuyl, L., Majoor-Krakauer, D., Essen, A. J. V., Jahoda, M. G. J., Sachs, E. S., Van Ommen, G. J. B., and Pearson, P. L. Prenatal diagnosis and carrier detection of Duchenne muscular dystrophy with closely linked RFLPs. Lancet, *1*: 655–658, 1985.
32. Vogelstein, B. Rapid purification of DNA from agarose gels by centrifugation through a disposable plastic column. Anal. Biochem., *160*: 115–118, 1987.
33. Feinberg, A. P., and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem., *137*: 266–267, 1984.
34. Sealey, P. G., Whittaker, P. A., and Southern, R. M. Removal of repeated sequences from hybridization probes. Nucleic Acids Res., *13*: 1905–1922, 1985.
35. Nussbaum, R. L., Crowder, W., Nyhan, W. L., and Caskey, C. T. A three-allele restriction-fragment-length polymorphism at the hypoxanthine phosphoribosyltransferase locus in man. Proc. Natl. Acad. Sci. USA, *80*: 4035–4039, 1983.
36. Yen, P. H., Patel, P. Chinault, A. C., Mohandas, T., and Shapiro, L. J. Differential methylation of hypoxanthine phosphoribosyltransferase genes on active and inactive human X chromosomes. Proc. Natl. Acad. Sci. USA, *81*: 1759–1763, 1984.
37. Wolf, S. F., Jolly, D. J., Lunnen, K. D., Friedmann, T., and Migeon, B. R. Methylation of the hypoxanthine phosphoribosyltransferase locus on the human X-chromosome: implications for X-chromosome inactivation. Proc. Natl. Acad. Sci. USA, *81*: 2806–2810, 1984.
38. Fialkow, P. J. Primordial cell pool size and lineage relationships of five human cell types. Ann. Hum. Genet., *37*: 39–48, 1973.
39. Feinberg, A. P., and Vogelstein, B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature (Lond.), *301*: 89–92, 1983.
40. Goelz, S. E., Vogelstein, B., Hamilton, S. R., and Feinberg, A. P. Hypomethylation of DNA from benign and malignant human colon neoplasms. Science (Wash. DC), *228*: 187–190, 1985.
41. Baylin, S. B., Fearon, E. R., Vogelstein, B., deBustros, A., Sharkis, S. J., Burke, P. J., Stahl, S. P., and Nelkin, B. D. Blood, in press, 1987.
42. Rowley, J. D. The cytogenetics of acute leukaemia. Clin. Haematol., *7*: 385–406, 1978.
43. Korsmeyer, S. J., Arnold, A., Bakhski, A. Ravetch, J. V., Siebenlist, U., Hieter, P. A., Sharrow, S. O. LeBien, T. W., Kersey, J. H., Poplack, D. G., Leder, P., and Waldmann, T. A. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T-cell and B-cell precursor origins. J. Clin. Invest., *71*: 301–313, 1983.
44. Cleary, M. L. Chao, J., Warnke, R., and Sklar, J. Immunoglobulin gene rearrangement as a diagnostic criterion of B-cell lymphoma. Proc. Natl. Acad. Sci. USA, *81*: 593–597, 1984.
45. Minden, M. D., Toyonaga, B., Ha, K., Yanagi, Y., Chin, B., Gelfand, E., and Mak, T. Somatic rearrangement of T-cell antigen receptor gene in human T-cell malignancies. Proc. Natl. Acad. Sci. USA, *82*: 1224–1227, 1985.
46. Pearson, T. A., Dillman, J. M., Solez, K., and Heptinstall, R. H. Clonal markers in the study of the origin and growth of human atherosclerotic lesions. Circ. Res., *43*: 10–18, 1978.
47. Fearon, E. R., Vogelstein, B., and Feinberg, A. P. Somatic deletion and duplication of genes on chromosome 11 in Wilms' tumours. Nature (Lond.), *309*: 176–178, 1984.
48. Orkin, S. H., Goldman, D. S., and Sallan, S. E. Development of homozygosity for chromosome 11p markers in Wilms' tumour. Nature (Lond.), *309*: 172–174, 1984.
49. Koufos, A., Hansen, M. F., Campkin, B. C., Workman, M. C., Copeland, M. G., Jenkins, N. A., and Cavanee, W. K. Loss of alleles at loci on human chromosome 11 during genesis of Wilms' tumour. Nature (Lond.), *309*: 170–172, 1984.
50. Fearon, E. R., Feinberg, A. P., Hamilton, S. H., and Vogelstein, B. Loss of genes on the short arm of chromosome 11 in bladder cancer. Nature (Lond.), *318*: 377–380, 1985.
51. McKusick, V. A. Mendelian Inheritance in Man, 7th ed. Baltimore: Johns Hopkins University Press, 1986.
52. Wolfe, S. F., Dintzis, S., Toniolo, D., Persico, G., Lunnen, K. D., Akelman, J., and Migeon, B. R. Complete concordance between glucose-6-phosphate dehydrogenase activity and hypomethylation of 3′ CpG clusters: implication for X chromosome dosage compensation. Nucl. Acids Res., *12*: 9333–9348, 1984.
53. Wolfe, S. F., and Migeon, B. R. Studies of X chromosome DNA methylation in normal human cells. Nature (Lond.), *295*: 667–671, 1982.
54. Lindsay, S., Monk, M., Holliday, R., Huschtscha, L., Davies, K. E., Riggs, A. D., and Flavell, R. A. Differences in methylation on the active and inactive human X chromosomes. Ann. Hum. Genet., *49*: 115–127, 1985.