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Quantitative hybridization to genomic DNA fractionated by pulsed-field gel electrophoresis

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ABSTRACT

Hybridization to genomic DNA fractionated by CHEF electrophoresis can vary >100-fold if the DNA is acid depurinated prior to Southern blotting. The level of hybridization is high or low depending on whether the molecule being analyzed migrates at a size coincident with or different from the size of the majority of genomic DNA in the sample, respectively. Techniques that avoid acid depurination including in-gel hybridizations and UV irradiation of DNA prior to blotting provide more accurate quantitative results. CHEF analysis of DNA molecules containing repetitive satellite sequences is particularly prone to this effect.

Pulsed-field gel electrophoresis (PFGE; 1) in conjunction with Southern-blot hybridization is routinely used to look at large DNA molecules in genome mapping experiments and investigations of chromosome structure (reviewed in 2). Large DNA molecules are often acid depurinated prior to Southern blotting to reduce their size and improve transfer out of the gel during blotting (3). Although acid depurination has been shown to cause a 3–5-fold reduction in hybridization to PFGE-fractionated DNA (4), it remains a commonly used technique. In fact, acid depurination of DNA prior to Southern blotting is still recommended, and even considered essential, by some current PFGE protocols (5,6). We have discovered, however, that acid depurination of PFGE-fractionated DNA can have a more profound effect on Southern-blot hybridization levels than was previously appreciated and can significantly compromise quantitative analysis even preventing detection of specific molecules present in samples of genomic DNA. Specifically, we found that hybridization was highest when the molecule being analyzed migrated at a size coincident with the size of the majority of genomic DNA in the sample and lowest when no other DNA migrated at the same size or position in the lane of the pulsed-field gel. Normally, the presence and size distribution of the bulk of the genomic DNA in a sample is of little consequence when analyzing Southern-blot hybridizations, but we found that contour-clamped homogeneous electric field (CHEF)–PFGE (7) can cause this non-hybridizing 'background' DNA to significantly affect hybridization to specific molecules. The effect is illustrated in Figure 1. An equal molar amount of large and small cloned DNA molecules were mixed with *Drosophila* genomic DNA of either large or small size thus placing the cloned DNAs either within or outside background DNA on the gel. Picogram amounts of cloned DNA were used to reflect typical amounts of unique-sequence molecules present in samples of genomic DNA. The DNA was fractionated by CHEF–PFGE and analyzed by Southern-blot hybridization in which the DNA was acid depurinated prior to blotting. Hybridization signals to the cloned DNAs were low or undetectable if no genomic DNA was added to the sample (Fig. 1A, lanes 4 and 10). In contrast, when genomic DNA was added, 100-fold increases in hybridization were observed, but only when the cloned molecules were located within background DNA present in the lane of the gel. For example, hybridization to the 166 kb T4 DNA increased 100-fold, but only when the genomic DNA was of a similarly large size, while hybridization to the 4.1 kb T4 DNA increased 20-fold, but only when the genomic DNA was of small size (Fig. 1A, lanes 4–6). Thus the size distribution of non-hybridizing background DNA on an acid-treated CHEF gel can, in the same sample, both permit detection of one size molecule while preventing the detection of another size molecule of equal abundance. Fractionation by CHEF electrophoresis was clearly responsible for this acid-dependent sensitivity to background DNA since hybridization to a 4.5 kb molecule was sensitive to background DNA only after CHEF but not conventional electrophoresis (Fig. 1B). Whether other PFGE formats might also cause acid-dependent sensitivity to background DNA is not known, although field-inversion gel electrophoresis has been shown to reduce overall Southern-blot hybridization levels after acid depurination (4). The effects illustrated in Figure 1 were not a peculiarity of using *Drosophila* as the source of genomic DNA since the same results were obtained using human DNA from HeLa cells (data not shown). Finally, no other changes to the Southern blotting protocol were found, including transfer under alkaline conditions, that could prevent or reverse the effect acid treatment had on hybridization to CHEF-fractionated DNA (data not shown).

Hybridization techniques that do not involve acid depurination of DNA provide more accurate quantitative analysis of CHEF-fractionated DNA. Two such techniques, in-gel hybridization in which hybridization is done directly to dried-down agarose gels without blotting (8,9) and using exposure to UV light instead of

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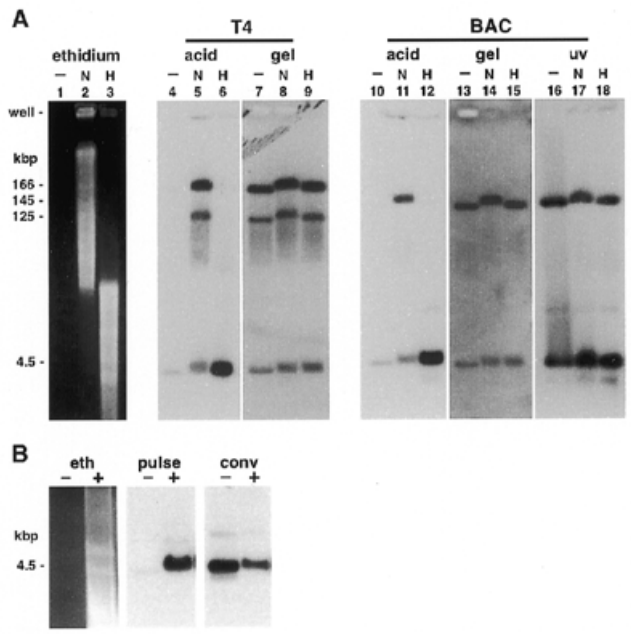

Figure 1. Characterizing the effects of CHEF electrophoresis on Southern-blot hybridizations. (**A**) Samples of cloned DNA alone (–) or mixed with fly genomic DNA digested with the restriction enzymes *Not*I (N) or *Hin*dIII (H) were fractionated by CHEF electrophoresis. The pattern of ethidium bromide staining is shown (ethidium). After electrophoresis the DNA was analyzed by three different methods: Southern-blot hybridization after acid depurination of the DNA (acid), in-gel hybridization (gel) and Southern-blot hybridization after UV nicking of the DNA (uv). T4 DNA samples included an equimolar mixture of T4 alc7tdΔi phage DNA (166 and 125 kb bands) and linearized plasmid pSU8-dtΔi containing T4 sequences (4.5 kb band). BAC DNA samples included an equimolar mixture of linearized BAC clone 57-4/O derived from mouse chromosome 10 (145 kb band) and linearized plasmid BAC4/O-5′ containing sequences from BAC 57-4/O (4.1 kb band). Fly genomic DNA isolated from adult ovaries was prepared in agarose inserts and restriction digested as described previously (15). Agarose inserts containing fly DNA were melted at 68°C for 5 min, mixed with T4 or BAC DNA, and loaded onto agarose gels. Gels were 0.8% SeaKem GTG agarose (FMC) in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) and were 0.73 cm thick. DNA was fractionated by CHEF electrophoresis at 14°C and 6 V/cm for 10 h with a 10 s switch time. The following protocol was used when the DNA was acid depurinated and then Southern blotted: DNA was stained with 0.5 μg/ml ethidium bromide, photographed, acid depurinated 15 min in 0.25 N HCl at 23°C, denatured 30 min in 0.4 N NaOH/0.8 M NaCl, neutralized 2 × 10 min in 0.025 M $NaHPO_4$, and blotted for 16–24 h onto GeneScreen (New England Nuclear) by ascending capillary transfer using 5× SSPE (50 mM NaH_2PO_4, 75 mM NaCl, 5 mM EDTA). After blotting, the membrane was rinsed for 5 min in 5× SSPE and the DNA crosslinked to the membrane by exposure to UV light. The following protocol was used when the DNA was nicked by exposure to UV light and then Southern blotted: DNA was stained with 1 μg/ml ethidium bromide, exposed to 120 mJ/cm^2 of 254 nM UV light (Stratagene Spectrolinker), then denatured, neutralized, and blotted as described above. In-gel hybridizations were done as described previously (9). All hybridizations to Southern blots and dried gels were done using the procedures of Church and Gilbert (16). The probes used were a 1.8 kb restriction fragment from T4 subclone pSU8-dtΔi and a 0.65 kb restriction fragment from BAC subclone BAC4/O-5′. Probes were labeled with ^{32}P by random priming. After hybridization, blots and gels were exposed to Kodak XAR film to generate autoradiograms and to stored-phosphor imaging plates for collection of quantitative data (Molecular Dynamics). Exposure times for the autoradiograms shown were: acid, 20 h; gel, 6.5 h; uv, 95 h. (**B**) Samples of linearized plasmid BAC4/O-5′ loaded alone (–) or mixed with fly genomic DNA digested with *Hin*dIII (+) were fractionated by CHEF electrophoresis at 14°C and 6 V/cm for 3 h with a 10 s switch time (pulse) or by conventional electrophoresis (conv). The pattern of ethidium bromide staining is shown (eth). After electrophoresis, the DNA was acid depurinated and analyzed by Southern-blot hybridization using the methods described above. Exposure time for the autoradiograms shown was 21 h.

acid depurination to reduce the size of the DNA prior to Southern blotting (10) were used to analyze samples like those described above. Both techniques revealed molecules undetected on acid-treated gels and provided more accurate measurements of the relative abundances of the cloned DNA molecules of differing size without interference from background DNA (Fig. 1A).

It is known that PFGE induces conformational changes in DNA as well as the agarose matrix that are distinct from changes observed during conventional electrophoresis (11–14). Perhaps such PFGE-dependent conformational changes in combination with acid depurination causes DNA entrapment within agarose gels inhibiting DNA transfer during blotting and lowering subsequent levels of hybridization. The presence of background DNA in the gel may increase hybridization by acting as a non-specific blocking agent allowing specific DNA molecules to transfer more efficiently. Attempts to confirm this hypothesis by hybridizing to gels after they were Southern blotted and demonstrating the quantitative retention of DNA in the gel, however, were inconclusive because variable levels of residual hybridization were observed for all DNA molecules regardless of their hybridization intensity after Southern blotting (data not shown). Thus, we cannot exclude the possibility that DNAs transfer normally out of CHEF gels but for some reason are unable to be subsequently detected.

The effect of CHEF electrophoresis on Southern-blot hybridization is of particular significance when studying repetitive sequences in samples of genomic DNA. Repetitive sequences, such as satellite repeats within heterochromatic DNA, are often devoid of sites for restriction enzymes that otherwise cut frequently in the genome. For this reason, restriction fragments containing repetitive sequences are often much larger than the majority of restriction fragments from the rest of the genome and migrate well above the position where the bulk of background genomic DNA migrates during electrophoresis. The level of hybridization to such repeat-containing molecules is therefore sensitive to background DNA if fractionated by CHEF electrophoresis and subject to acid depurination prior to Southern blotting. An example is illustrated in Figure 2.

Genomic DNA was isolated from *Drosophila* ovaries and then restriction digested, fractionated by CHEF electrophoresis and analyzed using the same Southern blotting and in-gel hybridization techniques described above. The probe hybridized to two different molecules, an 85 kb molecule containing satellite sequences that migrated above most background DNA and a 10 kb molecule containing unique sequences that migrated within background DNA (Fig. 2). Hybridization to the 85 kb molecule is predicted to be half the level of hybridization to the 10 kb molecule since the fly's genotype includes one copy of the larger DNA and two copies of the smaller DNA. Consistent with the analysis illustrated in Figure 1, hybridization to the 85 kb molecule was 23-fold lower than expected when the DNA was acid depurinated prior to Southern blotting but was at predicted levels when the DNA was assayed by in-gel hybridization and only 2–3-fold lower than expected when the DNA was assayed after UV nicking (Fig. 2; see also 9). The distribution of background DNA in these samples, which would normally be inconsequential when quantitating Southern-blot hybridizations after conventional electrophoresis, caused a significant artifactual reduction in hybridization to the 85 kb molecule as a consequence of CHEF electrophoresis and acid depurination prior to Southern blotting. Analysis of these DNAs by in-gel hybridization or by

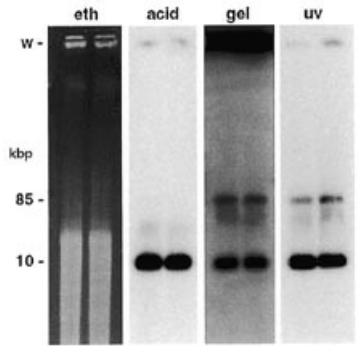

Figure 2. Analysis of restriction fragments containing *Drosophila* satellite repeats. Agarose-imbedded ovary DNA from adult flies of genotype *X/X*; *Dp(1;f)1187* was isolated in agarose inserts as described previously (15). Duplicate samples of DNA were digested with *Hin*dIII and fractionated by CHEF electrophoresis at 14°C and 6 V/cm for 9 h with a 10 s switch time. The pattern of ethidium bromide staining is shown (eth). The DNA was analyzed using the same Southern-blot (acid, uv) and in-gel (gel) hybridization methods described for Figure 1A. The probe was a 2.6 kb restriction fragment isolated from plasmid pBSsc^{101}XH3.7 (9) and labeled with ^{32}P by random priming. With this probe, DNA molecules of 85 and 10 kb are detected in flies of this genotype (9). Exposure times for the autoradiograms shown were: acid and uv, 73 h; gel, 35 h. The heterogeneous population of molecules smaller than 85 kb detected on these gels is created during the process of polyploidization that occurs in the follicle and nurse cells of the *Drosophila* ovary (17).

use of UV nicking provided more accurate quantitative results. Similar observations have been made for the analysis of *Drosophila* genomic DNAs up to 4000 kb in size and containing a variety of repetitive sequences (unpublished observations).

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