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Rapid RFLP screening procedure identifies new polymorphisms at albumin and alcohol dehydrogenase loci

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Summary. A rapid screening procedure for restriction fragment length polymorphisms (RFLPs) is reported. DNA from ten individuals is pooled and compared to DNA isolated from a cell line containing a single chromosome 4. This single chromosome-containing line is an obligate hemizygote for chromosome 4 RFLPs so that only one band corresponding to a single allele will appear on a Southern blot. In the pooled DNA sample lane bands corresponding to both alleles will be seen. The technique allows for efficient detection of RFLPs with easier use of large numbers of enzymes. It provides estimates of allele frequencies and disequilibrium. New RFLPs for albumin and alcohol dehydrogenase detected with this technique are described.

Introduction

The use of restriction fragment length polymorphisms (RFLPs) has had a dramatic impact on the study of human genetics. RFLPs have wide use in the establishment of human linkage maps in both normal individuals (White et al. 1985) and in families with genetic disorders segregating such as Huntington disease (Gusella et al. 1983), polycystic kidney disease (Reeders et al. 1985), and cystic fibrosis (Tsui et al. 1985). Because of this widespread use, simplifications in the search strategies for RFLPs may provide significant benefits for researchers employing these markers. We report here a strategy for identifying RFLPs that is both rapid and cost effective. We have used it to identify new RFLPs at the human albumin and alcohol dehydrogenase loci on chromosome 4.

Materials and methods

DNA was prepared from whole blood by the method of Poncz et al. (1982). Five to 10 μg samples of DNA were digested with appropriate restriction enzymes (Bethesda Research Laboratories or New England Biolabs) and electrophoresed on 0.8%–1.0% agarose gels. Restriction enzymes used included AluI, ApaI, AvaI, AvaII, BamHI, BanII, BclI, BglI, BglII, BsmI, Bsp1286, BstE2, BstNI, BstXI, ClaI, DdeI, DraI, EcoRI, EcoRV, HaeIII, HincII, HindIII, HpaII, HphI, KpnI, MboI, MboII, MluI, MspI, NaeI, NciI, NdeI, NheI, NruI, NsiI, PvuII, PstI, RsaI, SacI, SacII, Sau3A, Sau96I, ScaI, StuI, TaqI, XbaI, XhoI, XmnI. Southern transfers of agarose gels were onto Zetabind (AMC) which were then dried, prehybridized, hybridized with ^{32}P-labeled probes, and washed according to manufacturers instructions. The albumin cDNA probe F47 was as described by Murray et al. (1983) and generously donated by Richard Lawn of Genentech, Inc. The alcohol dehydrogenase probe pADH74 was described by Smith et al. (1986). The probes were labeled by the random primer method as described by Feinberg and Vogelstein (1983, 1984) with ^{32}P-dCTP as a substrate.

The DNAs used in the pooled samples for the rapid screening method were from normal unrelated individuals in three groups as follows: 10 American Caucasoid individuals; 8 American Blacks plus 2 African Blacks; 10 Asians (5 of Japanese and 5 of Chinese descent). There were 20 individual chromosomes in each pooled sample. DNA from each individual in the pool was present in equimolar amounts. When potential RFLPs were identified, DNA from each individual in the pooled sample was digested and run separately. Family studies were performed to establish Mendelian segregation and conformance with Hardy-Weinberg equilibria was determined. DNA used in family studies was obtained from the Centre Etude du Polymorphisme Humaine (CEPH).

The procedures used to isolate interspecies cell hybrids between a temperature-sensitive Chinese hamster ovary (CHO) cell line and human leukocytes have been previously described (Dana and Wasmuth 1982). Cytogenetic and DNA probe analysis of the somatic cell hybrid HHW 416 were presented elsewhere (Carlock et al. 1986). HHW 416 contains only a single intact human chromosome 4 in a CHO background.

Results

Figure 1 shows the identification of polymorphisms at the human albumin locus on chromosome 4 (Harper and Dugaiczyk 1983) using pooled DNA samples to compare to DNA isolated from the cell line containing a single human chromosome 4. RFLPs shown in the pooled samples here detected with SacI have previously been shown to be present in high frequency in Caucasoid, Black, and Asian populations (Murray et al. 1984). Figure 2 shows a new Sau96I RFLP identified at the albumin locus with probe F47 using this method. This

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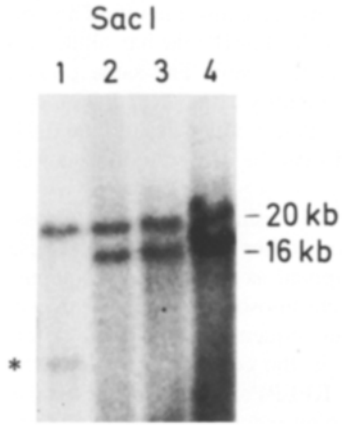

Fig. 1. Autoradiograph of a Southern blot hybridized with the albumin F47 probe as described in Materials and methods. *Lane 1* is a SacI digest of the DNA from the HHW 416 cell hybrid containing a single human chromosome 4 and showing only the 20 kb polymorphic allele. The *asterisk* indicates a crosshybridizing CHO DNA sequence. Pooled samples of DNA digested with SacI from 10 individuals from Caucasoid, Asian, and Black populations are shown in *lanes 2, 3,* and *4* respectively and demonstrate both the 20 kb and 16 kb alleles

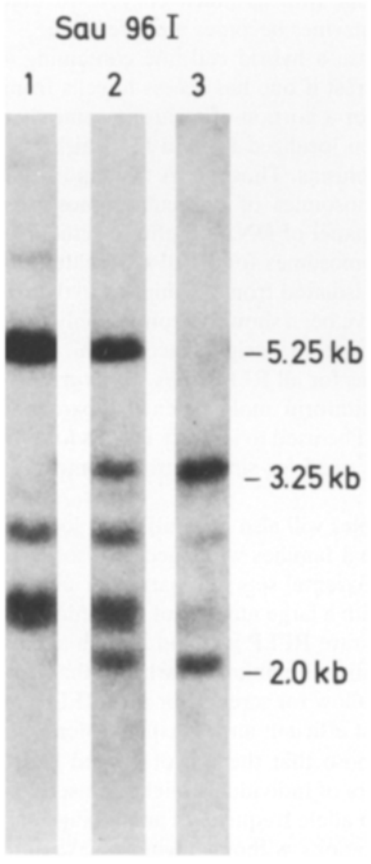

Fig. 2. Autoradiograph showing results of a Southern blot hybridization with the albumin F47 probe on Sau96I-digested DNA. DNA was run on a 1.0% agarose gel and transferred to Zetabind. *Lane 2* is DNA from an individual heterozygous for the Sau96I polymorphism showing the 5.25, 3.25, and 2.0 kb fragments. *Lane 1* is DNA from an individual homozygous for the 5.25 kb fragment and *lane 3* is DNA from an individual homozygous for the 3.25 and 2.0 kb fragments. This polymorphism was shown to segregate in a Mendelian fashion within families (data now shown)

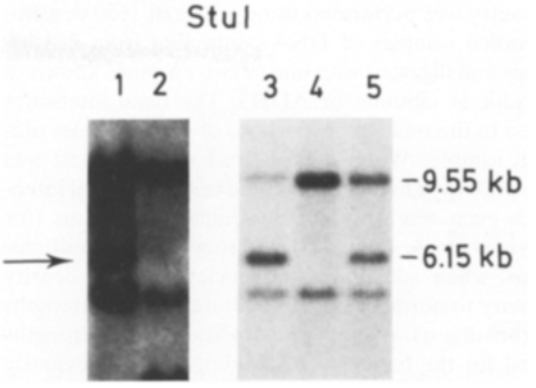

Fig. 3. Autoradiograph of StuI digested DNA on a Southern blot probed with pADH74 indicates a polymorphic band seen in *lane 1* (indicated by *arrow*) that is not present in *lane 2*. *Lane 1* contains DNA from a pooled sample of ten unrelated Caucasoid individuals digested with StuI. *Lane 2* contains DNA from the HHW 416 cell line containing a single human chromosome 4 digested with StuI. *Lanes 3* and *4* are DNA from homozygous individuals for the StuI polymorphism with fragments of 6.15 kb and 9.55 kb respectively. *Lane 5* is DNA from a heterozygous individual with both the 9.55 kb and 6.15 kb fragments. Constant bands of 9.6 kb and 5.0 kb are also seen. The constant 9.6 kb band is best seen in *lane 3*

Table 1. Allele frequency and linkage disequilibrium of ADH3 RFLPs. StuI RFLP described here. XbaI RFLP described in Smith et al. (1986). Observed distribution of + and − alleles for each with respect to the other in a Caucasoid population. Numbers are the observed chromosomal haplotypes of a specific type (for example 25 chromosomes had both the StuI+ allele and the XbaI+ allele). Fisher's probability of $P < 0.0001$ for random association of StuI and XbaI alleles suggests marked linkage disequilibrium with $\Delta = 0.61$. StuI+ allele has a frequency of 0.40 in this population

		XbaI	
		+	−
StuI	+	25	6
	−	9	38

RFLP is in tight linkage disequilibrium with previously described albumin RFLPs and thus does not provide any new information for linkage studies in the individuals studied to date in our laboratory (Ken Buetow, unpublished results).

Figure 3 shows a new polymorphism at the ADH3 locus on chromosome 4 (Smith et al. 1986) identified with the enzyme StuI on a screening Southern blot in lanes 1 and 2. Lanes 3, 4, and 5 show the alleles identified in the screening blot in single individuals. This polymorphism has a high frequency and is in linkage disequilibrium with a XbaI RFLP at the ADH3 locus reported elsewhere (Smith et al. 1986). Allele frequencies and disequilibrium values are shown in Table 1. Mendelian segregation has been observed in three families (data not shown).

For the albumin and ADH3 RFLPs, all enzymes used in the screening blots were also studied by screening 10 unrelated individuals in separate lanes. This included 30 different enzymes for albumin and 22 for ADH3. In no case was an RFLP not seen in the pooled samples identified in the individual lanes screened. The known RFLPs for albumin and the new RFLPs for albumin and ADH3 reported here were all identified in the pooled DNA screening procedure. Thus, no common RFLPs were missed by this technique.

Densitometry was performed using a Biorad 1650 densitometer on pooled samples of DNA containing from 2 to 40 chromosomes and digested with one or two enzymes known to be polymorphic at albumin or ADH3. The band intensities corresponded to the relative proportions of + or − alleles present in each sample. When a StuI/XbaI double digest was probed with ADH3 individual alleles did not have equal intensity of bands even when present in equimolar amounts (for example as in a single individual heterozygous for both enzymes). Thus, when calculating frequencies based on density it was necessary to normalize for the different signal strengths that arose from digests. A similar inequality of signal strengths was observed for the bands corresponding to the previously reported albumin EcoRV RFLP when double digests of SacI and EcoRV were performed.

Discussion

We have used DNA isolated from a cell line containing a single human chromosome 4 as a comparison lane against pooled samples of DNA from 10 unrelated individuals to search for RFLPs. Since the DNA isolated from a cell line retaining only a single human chromosome 4 is an obligate hemizygote, only a single allele can be present in this sample for any chromosome 4 RFLP. If a second allele is present in pooled DNA run in an adjacent lane, this will be seen as a band on a Southern blot not present in the DNA from the hemizygous cell line. This approach combines previous suggestions to use DNA from a male against a panel of DNA from females to enhance X-chromosome RFLP detection (Aldridge et al. 1984) and the use of pooled DNA samples to detect RFLPs in linkage disequilibrium in normal and insulin-dependent diabetic populations (Arnheim et al. 1985).

Several types of erroneous results may be produced by this method. First, the hemizygous cell hybrids must contain a single chromosome with a single clonal origin. A mixture of cells, containing chromosomes of more than one origin, are no more useful than analyzing DNA from a single individual. Even small amounts of contamination may seriously interfere with appropriate interpretation of screening blots. For analyzing our single chromosome hybrids we have overexposed our autoradiographs and detected no evidence of secondary bands, using probe and enzyme combinations that produce known polymorphic alleles. A second potential source of error would be partial digests of the pooled DNA samples. We only use highly purified DNAs that are known to completely digest with the appropriate restriction enzyme. In most cases digestions proceed for at least 12 hours with a two-fold enzyme excess. Since all potential RFLPs identified in screening need to be validated with family studies and Hardy-Weinberg calculations, this source of error is eventually revealed.

A third confounding variable is that approximately equal amounts of DNA from each individual should be present in the pooled sample. In our hands, signals of 5% intensity (representing one variant chromosome out of 20) can be detected. If by chance the DNA from a single variant individual in the pooled sample is present in disproportionately lower amounts, an RFLP in a single chromosome might be missed. Since RFLPs with a frequency of less than 5% are not as useful, we feel that 10 individuals is a reasonable number to use if looking for RFLPs to use in linkage studies (Skolnick and White 1983).

Using these screening blots we have also identified an epidermal growth factor RFLP with HincII that has allele frequencies of 0.4/0.6 (Murray et al. 1986). The same pooled DNA screening blots failed to identify a SacI RFLP with epidermal growth factor that has allele frequencies of 0.06/0.94, demonstrating that rare RFLPs can be missed by this as well as other screening procedures.

A fourth source of error is in using a probe that cross-hybridizes with sequences from other chromosomes. These cross-hybridizing bands may appear as new alleles in comparing pooled samples to single chromosome samples. This will occur when the crosshybridizing sequence is found on a different chromosome not present in the cell line containing the single chromosome for which RFLPs are sought. These false RFLPs will be eliminated when one separates the pooled samples into individual lanes and sees that each has a constant band in a defined position. In addition, this band would not segregate with the particular chromosome under study in a panel of somatic cell hybrids.

There are significant benefits from using the procedure. First, it makes effective use of blot space and DNA. We formerly used 10 individuals in 10 lanes in our screening panels. We now use two lanes − one for hemizygous DNA and one for pooled DNA from 10 individuals. Thus, we use one-fifth as much blotting space and one-tenth as much DNA in the initial screen. It also uses one-fifth as much enzyme so that screening with expensive enzymes becomes more feasible.

It is not necessary to use a hybrid cell line containing a single chromosome of interest if one has access to cells from an individual monosomic for a portion of a chromosome that a particular probe has been localized to, and for which one wishes to identify polymorphisms. Thus, DNA from individuals who have partial monosomies of particular autosomes could be used to create a panel of DNAs useful in screening various subregions of chromosomes for RFLPs. An alternative would be to use DNA isolated from a complete hydatidiform mole. Such tumors have been shown to contain only a set of chromosomes of haploid paternal origin (Jacobs et al. 1980) and so would be hemizygous for all RFLP sites. Blots created using DNA from a hydatidiform mole when compared to pooled DNA samples could be used to identify RFLPs for any chromosome obviating the need for single chromosome-containing cell lines.

The use of pooled samples will also have utility in looking for rare RFLPs in individual families with specific disorders. Using pooled DNA from parental sets in a particular family, one could rapidly screen with a large number of restriction enzymes. Once an otherwise rare RFLP is found in such a family, that RFLP has great utility as a linkage marker in that particular pedigree. This will allow for screens for rare RFLPs in interesting families in a cost efficient and specific fashion.

Finally, we would propose that the use of pooled DNA samples from large numbers of individuals might be useful in providing estimates of both allele frequencies and linkage disequilibrium in such populations without having to examine each individual separately. Since signal strength of the bands is proportional to allele frequencies, the screen provides an estimate of frequencies so that apparently more common RFLPs may be pursued first. This requires densitometer determinations of band intensities in the pooled samples and so is subject to the limitations of densitometry but does provide useful approximations for many purposes. To determine the allele frequencies, only a restriction enzyme digest of the pooled

DNA would be needed. To measure pairwise linkage disequilibrium, one would do a double digest using two polymorphic enzymes at a given locus. Four possible band sizes representing the four possible two allele/two enzyme haplotypes would then be studied by densitometer and the observed proportions compared to the expected proportions to determine if there is random association of alleles at the two loci. As noted above, it may first be necessary to normalize band intensities as equimolar amounts of different alleles may have different hybridization signals. The advantages here could be enormous in that even several thousand individuals (if mixed in equimolar amounts) could be studied in a single lane providing enormous savings of DNA, money, and time while providing reasonable assessment of frequencies and disequilibrium in very large population samples.

In summary, we report a rapid screening procedure for RFLPs using pooled DNA samples and DNA prepared from a hybrid cell line hemizygous for a human autosome. Two new RFLPs are reported and the utility of the procedure for several other applications discussed.

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