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Genetics

Molecular hybridization under conditions of high stringency permits cloned DNA segments containing reiterated DNA sequences to be assigned to specific chromosomal locations

(nucleic acid hybridization/repetitive sequence DNA/genetic fine structure)

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ABSTRACT **Identifying the specific DNA sequences involved in the chromosomal abnormalities in developmental and neoplastic diseases may be essential to understanding the molecular biology of these disorders. The use of recombinant DNA techniques in conjunction with rodent–human hybrid cells makes it possible to assign chromosomal locations to specific DNA sequences. However, the ubiquitous presence of reiterated DNA species often complicates the application of straightforward molecular hybridization. To accelerate the mapping of cloned sequences to specific chromosomal locations, we investigated the possibility that cloned sequences containing reiterated DNA might be used without isolating unique sequences. By varying conditions of hybridization (specifically temperature) and using restricted DNA samples from human genomic DNA, Chinese hamster ovary–human chromosome 11 hybrids, and non-chromosome 11 hybrids, we have been able to assign cloned DNA sequences containing reiterated sequences to their chromosome of origin. By hybridization under the high-stringency condition of 55°C, specific banding was produced with both human genomic DNA and the human-chromosome-containing hybrid from which the probe was prepared. Furthermore, using a panel of chromosome 11 deletion mutants, we have been able to assign a cloned sequence to a specific chromosomal location. We believe that this approach will accelerate gene mapping procedures and facilitate identification of DNA sequences involved in chromosomal abnormalities.**

The mammalian genome contains large numbers of repetitive DNA sequences widely distributed among the chromosomes. Their function is largely unknown (1–3). These elements often complicate straightforward applications of molecular hybridization techniques for genetic analysis, but they have facilitated identification of cloned DNA segments containing human DNA prepared from human–rodent hybrids and thus have made possible the preparation of chromosome-specific libraries from these hybrids (4). In addition, low copy number reiterated DNA has recently been used in conjunction with deletion mutants of human chromosomes in human–Chinese hamster ovary (CHO) hybrids as a marker for specific chromosomal segments (5).

Repetitive sequence DNA constitutes 20–30% of the human genome (2). Some highly reiterated species appear to be localized in chromosomal centromeres and telomeres; others appear to be interspersed among specific sequences throughout the genome (6). At least some reiterated DNA is transcribed and some of these reiterated transcripts, for example, members of the *Alu* family, may appear in the cytoplasmic RNA pool (7). However, when specific genetic sequences are to be mapped, the need to prepare probes free of repetitive DNA can be onerous. This is particularly true when specific DNA sequences are to be related to structural chromosomal defects that are associated with human disease. Thus, it would be desirable to be able to assign cloned DNA sequences that contain reiterated elements to specific chromosomal locations directly. Although some repetitive mammalian DNA segments are sufficiently homologous to readily hybridize to each other under standard conditions, their exact nucleotide sequences have been shown in some cases not to be identical (8). Therefore, it seemed desirable to investigate the specificity of molecular hybridization reactions between recombinant molecules containing reiterated elements and genomic DNA by increasing the stringency of the annealing conditions. Under these circumstances, probes containing repetitive sequences, which are readily secured, might behave like more specific reagents and might be valuable in identifying specific human chromosomes or chromosomal regions. The present experiments show the feasibility of this approach.

METHODS

Hybridizations were carried out with probes containing reiterated human DNA against restricted genomic DNA from human fibroblasts and human–CHO hybrid cells.

DNA was purified from cultured normal human fibroblasts and from CHO–human somatic cell hybrids (9). Sample DNA was restricted to completion with either *Bam*HI or *Eco*RI using conditions recommended by the supplier (New England BioLabs). Complete restriction was obtained by using a 5- to 10-fold excess of endonuclease and by incubating for 3–6 hr at 37°C. Activity of the enzymes was assayed by digestion of λ phage DNA in parallel and evaluating the molecular weight of restriction fragments on ethidium bromide-stained agarose gel.

Five to ten micrograms of restricted cellular DNA was loaded on a 0.75% agarose gel at 3 V/cm for 12–16 hr in 1× Tris acetate buffer. Fragments were transferred to nitrocellulose paper by the method of Southern (10). Nitrocellulose filters were then baked at 80°C for 2–3 hr.

Nitrocellulose filters were prehybridized for 12 hr in a Seal-a-Meal bag with 0.066 ml of prehybridization buffer per cm^2 of nitrocellulose filter. Prehybridization buffer consisted of 50% formamide (vol/vol)/5× Denhardt's solution (11)/0.05 M sodium phosphate, pH 6.5/500 μg of salmon sperm DNA per ml/5× standard saline citrate. Prehybridization buffer was removed and hybridization buffer consisting of 50% formamide (vol/vol)/10% dextran sulfate/1× Denhardt's solution/20 mM sodium phosphate, pH 6.5/5× standard saline citrate/200 μg of salmon sperm DNA per ml of

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Abbreviation: kb, kilobase(s).

hybridization buffer was added. [^{32}P]CTP nick-translated probe was denatured by heating at 95°C for 5 min, cooled rapidly, and added to this mixture (1×10^6 dpm per ml of hybridization buffer). The Seal-a-Meal bag was sealed with care to exclude all bubbles and was incubated at various temperatures for 18–24 hr. Nitrocellulose filters were then washed four times (5 min each time) with 2× standard saline citrate/1% $NaDodSO_4$ at room temperature. The filters were then washed for 15 min at 50–55°C with 0.1× standard saline citrate. Filters were autoradiographed with Dupont Cronex X-ray film and Dupont Lightning Plus intensifying screens at −70°C.

CHO–Human Somatic Cell Hybrids. All hybrids were the kind gift of Carol Jones and have been described (12–14). Briefly, cell hybrids were generated by fusion of various auxotrophic mutants of CHO-K1 cells with human fibroblasts or lymphocytes. The human chromosome 11 hybrid, J1 (12) is the parent cell line for a series of deletion mutants, each generated by treatment of J1 with chromosomal breaking agents and selected by growth in antisera specific to human cell surface antigens and complement. Each deletion mutant has been defined by cytogenetic analysis, LDH isoenzyme analysis, and acid phosphatase determination (13, 14). The human chromosome-21- and chromosome-12-containing hybrids (E9A and E4E, respectively) were similarly prepared by fusion of CHO-K1 cells with human lymphocytes and have been evaluated by cytogenetic and biochemical analysis (15, 16).

Probe Preparation. DNA probes specific to human chromosome 11 were prepared from human–hamster hybrid J1-11, which contains the short arm, centromere, and a small portion of the long arm of human chromosome 11 as its only identifiable nonhamster genetic material (4). Partial *Eco*RI digests of the hybrid cell DNA were cloned into the vector Charon 4A. Recombinant clones containing human DNA were identified by hybridization with nick-translated HeLa cell DNA. Selected clones were amplified in liquid culture in LE392 according to National Institutes of Health recombinant DNA guidelines. DNA was purified by the method of Varsanyi-Breiner (17). A restriction map was prepared for each probe used in hybridization and only probes that were clearly different were used. Probes 29 and 30 (HP29 and HP30) were clearly distinct and were chosen for additional investigation. The human DNA insert in HP29 was ≈15 kilobases (kb) with only one *Eco*RI restriction fragment. The human insert in HP30 showed two human restriction fragments at 9.6 and 5 kb for an insert size of 14.6 kb. To verify that each probe used contained reiterated DNA, hybridization was carried out against the cloned *Alu* sequence BLUR 8 (the gift of Warren Jelinek) (18). Blots of restricted probes were hybridized against whole human DNA as well as BLUR 8 sequence at 42°C. The single *Eco*RI fragment of HP29 hybridized with both BLUR 8 sequence and HeLa cell DNA. Both the 9.6- and 5-kb restriction fragments of HP30 hybridized with BLUR 8 sequence and HeLa cell DNA. Only probes containing human DNA inserts that hybridized with both HeLa cell DNA and BLUR 8 sequences were used. Recombinant DNA probes were nick-translated to 5×10^7 to 1×10^8 dpm/μg with [^{32}P]CTP by the method of Rigby *et al.* (19).

RESULTS

Temperature was chosen as the initial variable with which to increase stringency. Nick-translated recombinant probe (HP29) was hybridized against Southern blots of restricted whole human DNA and restricted CHO–human chromosome 11 hybrid DNA at 42, 45, 50, 55, 60, and 65°C. Fig. 1 compares hybridization at 42°C and at 55°C. At 42°C, intense nonspecific hybridization occurs, resulting in continuous deposition of radioactivity. At 55°C, reproducible banding occurs at identical molecular weights with both hybrid and total human DNA. Because the hybrid used contained chromosome 11 as its only identifiable human genetic material, the results suggest (*i*) that these DNA segments are present on human chromosome 11, an observation consistent with their mode of isolation, and (*ii*) that, if they are present on other human chromosomes, their restriction patterns are highly conserved.

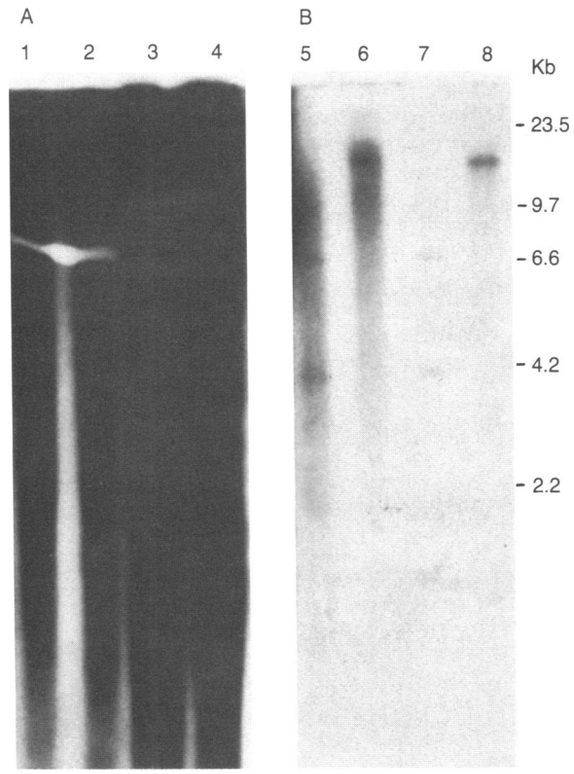

FIG. 1. Hybridization of human chromosome 11-derived recombinant probe containing reiterated DNA at 42°C (*A*) and 55°C (*B*). Exposure time, 72 hr and 14 days, respectively. Lanes: 1 and 8, *Eco*RI digests of hybrid J1-11 DNA (a CHO hybrid containing part of human chromosome 11 as its only identifiable human genetic material); 2 and 7, *Bam*HI digests of hybrid J1-11 DNA; 3 and 6, *Eco*RI digests of human DNA; 4 and 5, *Bam*HI digests of human DNA. Digests were done using 10 μg of DNA per lane. At 55°C, identical banding occurs with digested human and hybrid DNA with two different endonucleases.

To test whether this banding was indeed chromosome specific, recombinant probes were hybridized against Southern blots of restricted genomic DNA from the following human–hamster hybrid cells: J1-11, short arm, centromere, and small portion of the long arm of chromosome 11 (4); E4E, chromosome 12 (16); and E9A, chromosome 21 (15). As shown in Fig. 2 there was specific hybridization only with hybrid J1-11, which contains human chromosome 11 with a terminal deletion of the long arm. These experiments support the hypothesis that specific binding at 55°C between probe and genomic DNA is specific for a locus on the fragment of chromosome 11 retained in the J1-11 hybrid and that no homologous sequences to HP29 detectable by the method exist on chromosomes 12 or 21.

Although unlikely, the possibility remained that binding occurred between families of reiterated DNA that are unique to each chromosome but common to each probe. Such a situation would lead to distinct banding but no discernible difference among probes. To evaluate this possibility, total human genomic DNA and human chromosome 11-derived recombinant probes HP29 and HP30 were restricted with *Eco*RI, re-

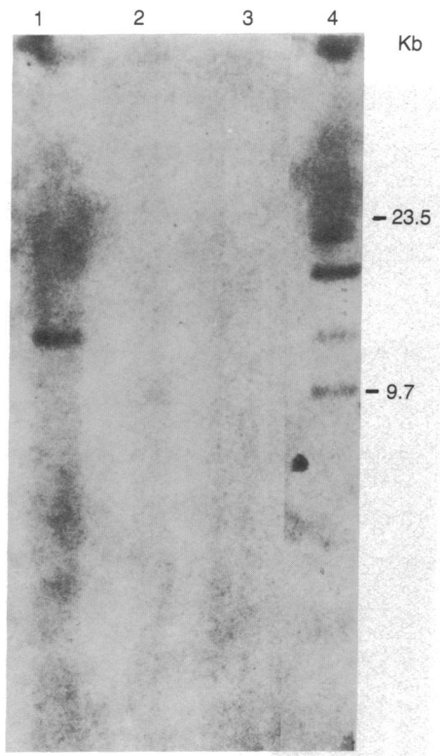

FIG. 2. Hybridization of chromosome 11-derived recombinant probe HP29 at 55°C with *Eco*RI-digested DNA from CHO hybrids containing three different human chromosomal elements. Lanes: 1, J1-11 (CHO hybrid containing fragment of human chromosome 11); 2, E4E (CHO hybrid containing human chromosome 12); 3, E9A (CHO hybrid containing human chromosome 21); 4, HP29 (molecular hybridization with its own *Eco*RI digest). Ten micrograms of hybrid DNA and 2 ng of HP29 were loaded onto gels. Exposure time, 14 days. Specific molecular hybridization with J1-11 occurs at the same molecular weight as the internal standard HP29. There is no specific molecular hybridization with non-chromosome 11-containing hybrids.

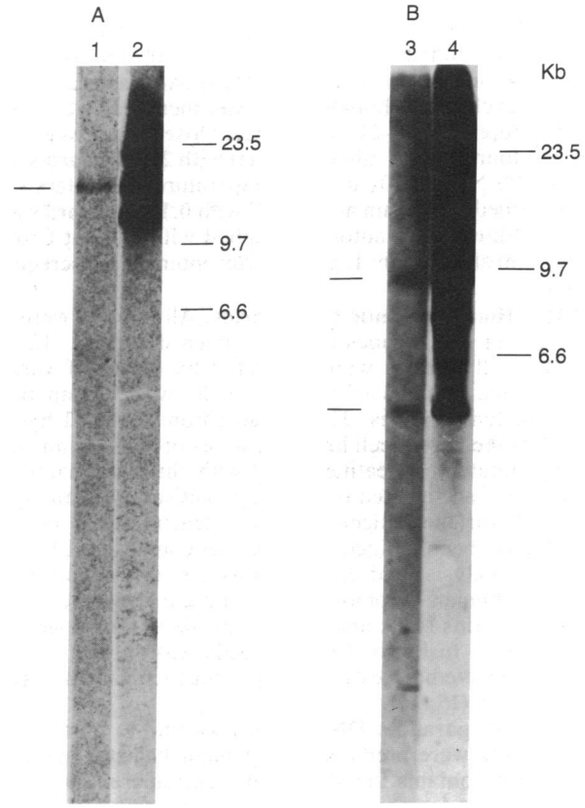

FIG. 3. Molecular hybridization of human chromosome 11-derived recombinant probe with *Eco*RI-restricted genomic DNA using *Eco*RI-restricted probe as a parallel standard in each case. Lanes 2 and 4, *Eco*RI digests of probes HP29 (*A*) and HP30 (*B*) (5.0 ng). Lanes 1 and 3, *Eco*RI digests of human genomic DNA (5 μg). Exposure time, 14 days. Specific hybridization with genomic DNA is shown by one or, in the case of HP30, two prominent bands. This hybridization occurs at the same molecular weight as hybridization with human insert contained within the probe.

solved in alternating parallel lanes, and blotted. Each strip of nitrocellulose paper contained restricted total human genomic DNA and a restricted probe (Fig. 3). Each strip was then probed with the same recombinant probe it contained. Specific binding was observed at identical molecular weights in both probe and genomic DNA, and binding occurred at a different molecular weight for each probe. This suggests that the probes recognize different nucleotide sequences and that specific binding occurs with DNA from the locus of origin of the probe.

These findings suggested that recombinant probes containing reiterated sequences could be assigned chromosomal locations by carrying out hybridization at 55°C with restricted DNA from a panel of single human chromosome deletion mutant hybrids. DNA from a panel of human chromosome 11 deletion mutant hybrids (4) was kindly provided by Carol Jones. The chromosome 11 fragments present in each hybrid

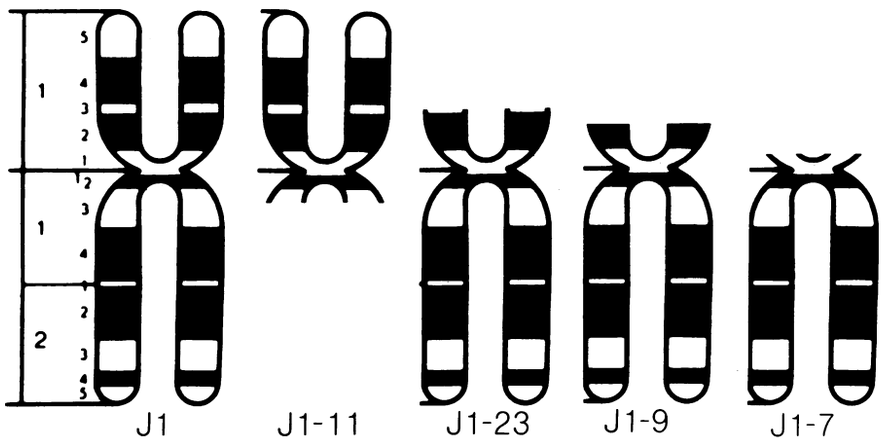

FIG. 4. Schematic representation of the fragments of chromosome 11 that are present in the deletion mutant panel. J1 is the parent hybrid.

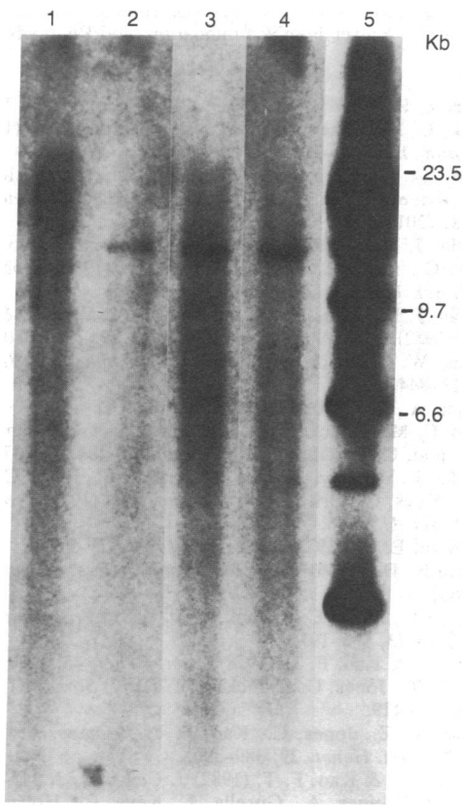

FIG. 5. Molecular hybridization of recombinant probe HP29 with *Eco*RI-digested DNA from a deletion mutant panel. Ten micrograms of DNA from each mutant cell was loaded onto the gel. Exposure time, 14 days. Lane 5 is molecular weight standard. There is specific hybridization with J1-9 (lane 2), J1-11 (lane 3), and J1-23 (lane 4) but not with J1-7 (lane 1). Thus, the probe is localized to the p12 region of chromosome 11.

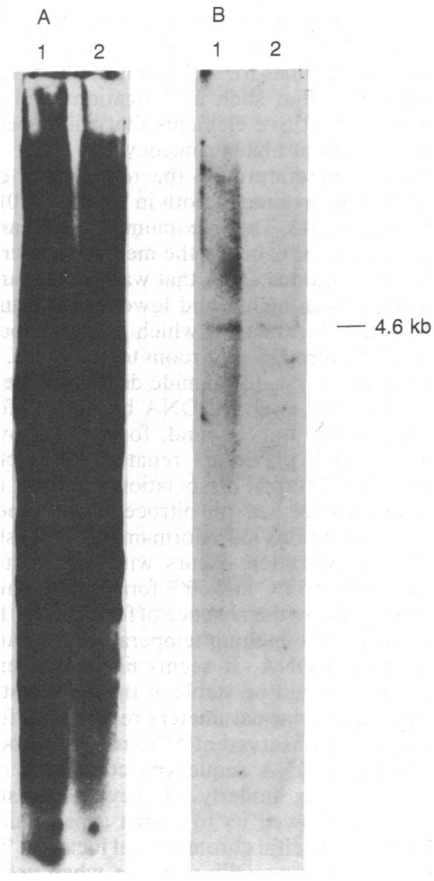

FIG. 6. Hybridization of cloned *Alu* sequence at 42°C (*A*) and 55°C (*B*). Ten micrograms of human fibroblast genomic DNA (lanes 1) and human chromosome 11-containing CHO cell hybrid DNA (lanes 2) digested with *Eco*RI. The 42°C autoradiograph was exposed for 24 hr and shows relatively uniform deposition of radioactivity. The 55°C autoradiograph was exposed for 14 days and shows little nonspecific hybridization. The observed bands are at a molecular weight consistent with contamination by plasmid DNA.

are depicted schematically in Fig. 4. DNA samples from each hybrid were restricted with *Eco*RI, resolved on an agarose gel, and blotted. These blots were then hybridized with one nick-translated recombinant probe. Fig. 5 shows specific hybridization with hybrids J1-11, J1-9, and J1-23 but not with J1-7. These findings allow us to assign the probe HP29 to the p12 band on chromosome 11.

To determine whether hybridization at high temperature improved specificity when a reiterated sequence was used as a probe, the cloned *Alu* sequence BLUR 8 (18) was hybridized at 42 and 55°C with Southern blots of total human genomic and human chromosome 11-containing CHO hybrid cell DNA. Intense nonspecific hybridization was obtained at 42°C as shown by the 24-hr autoradiograph in Fig. 6. However, when hybridization was carried out at 55°C (2 wk autoradiograph), one band, thought to represent contaminating plasmid DNA, was observed but there was not significant background hybridization. These results suggest that high-temperature hybridization significantly improves specificity even when a repetitive well-conserved sequence such as *Alu* is used as a probe.

DISCUSSION

Generally, specific mapping information cannot be obtained when cloned DNA sequences containing highly reiterated DNA are hybridized to genomic DNA. Although the probes we used contained highly reiterated sequences and showed intense nonspecific hybridization at 42°C, hybridization at 55°C resulted in distinct bands. These bands were of a specific and reproducible molecular weight for each probe. In addition, they occurred at identical molecular weights when hybridizations were carried out against human cellular DNA and DNA from a hybrid cell containing chromosome 11 as its only recognizable human genetic material. Furthermore, by using parallel standards of *Eco*RI-restricted recombinant probe, we have shown with each probe that banding occurs at the same molecular weight as banding in *Eco*RI-restricted cellular DNA. These findings support the hypothesis that specific molecular hybridization occurs at the locus of origin of the probe. Finally, by high-stringency hybridization of one probe with cellular DNA from a deletion mutant panel of the chromosome of its origin, we have been able to produce observations consistent with its regional mapping to a particular area of the chromosome.

The general applicability of this approach remains to be resolved. Additional probes, both for the chromosome studied, number 11, and for other chromosomes, must be investigated. The finding that the probes investigated produced only a few bands, while the *Alu* family sequence BLUR 8 produced no specific hybridization under the same conditions, also is unexplained. One reasonable speculation is that the cloned *Alu* sequence BLUR 8 is sufficiently divergent from consensus *Alu* sequence to preclude significant hybridization at high temperature. We wish to emphasize that we used *Alu* sequence to verify that our probes contained repetitive DNA sequence. Probes were not selected on the basis of containing *Alu* alone, and we make no conclusion that the

banding results obtained were directly related to *Alu*. Clearly, the observations must be expanded to characterize the repetitive sequence families for which this method is applicable. It is anticipated that such investigations will identify DNA inserts with repetitive elements that will appear to be highly repetitive even at high stringency.

The effects of temperature on the reannealing of DNA have previously been evaluated both in solution (20) and on nitrocellulose paper (21). The maximum rate of reannealing was found to occur ≈20°C below the melting temperature of the native double-stranded DNA that was being reannealed. Rate decreased at both higher and lower temperatures (20, 21). The addition of formamide, which in high enough concentration can denature DNA at room temperature, appears to have two effects. First, formamide decreases the melting temperature of double-stranded DNA by ≈0.7°C for every 1% formamide in solution. Second, formamide lowers the temperature at which maximum renaturation occurs to a similar degree (21). Thermal dissociation curves of mammalian DNA duplexes formed on nitrocellulose paper under high-stringency conditions (50% formamide, 45°C) show that more specific hybridization occurs with higher stringency (21). Hybridization at 55°C and 50% formamide is analogous to hybridizing at 90°C in the absence of formamide. This temperature is close to the melting temperature for native double-stranded human DNA. It seems likely that only well-matched duplexes would be stable at this temperature.

The detailed molecular parameters responsible for the increase in specificity observed at 55°C remain to be worked out, and all cloned DNA sequences containing reiterated DNA may not behave similarly. However, high-stringency hybridization has allowed us to assign a particular cloned DNA segment to a specific chromosomal location. This technique appears to be especially valuable when used in conjunction with a deletion mutant panel and may allow more rapid identification of deleted or translocated DNA segments associated with human disease.

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1. Davidson, E. H. & Britten, R. J. (1979) *Science* **204,** 1052.
2. Houck, C. M., Rinehart, F. PL. & Schmid, C. W. (1979) *J. Mol. Biol.* **132,** 289–306.
3. Kreyev, A. S., Kramerov, A. A., Skryabin, K. G., Ryskov, A. P., Bayev, A. A. & Georgiev, G. P. (1980) *Nucleic Acids Res.* **8,** 1201–1215.
4. Gusella, J. F., Keys, C., Varsanyi-Breiner, A., Kao, F. T., Jones, C., Puck, T. T. & Housman, D. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2829–2833.
5. Gusella, J. F., Jones, C., Kao, F. T., Housman, D. & Puck, T. T. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7804–7808.
6. Jelinek, W. R. & Schmid, C. W. (1982) *Annu. Rev. Biochem.* **51,** 813–844.
7. Weiner, A. M. (1980) *Cell* **22,** 209–218.
8. Rubin, C. M., Houck, C. M., Deininger, P. L., Friedmann, T. & Schmid, C. W. (1980) *Nature (London)* **284,** 372–374.
9. Gusella, J., Varsanyi-Breiner, A., Kao, F. T., Jones, C., Puck, T. T., Keys, C., Orkin, S. & Housman, D. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5239–5243.
10. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
11. Denhardt, D. (1966) *Biochem. Biophys. Res. Commun.* **23,** 641–652.
12. Kao, F. T., Jones, C. & Puck, T. T. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 193–197.
13. Jones, C. & Kao, F. T. (1978) *Hum. Genet.* **45,** 1–10.
14. Kao, F. T., Jones, C. & Puck, T. T. (1977) *Somatic Cell Genet.* **3,** 421–429.
15. Moore, E. E., Jones, C., Kao, F. T. & Oates, A. C. (1977) *Am. J. Hum. Genet.* **29,** 389–396.
16. Law, M. L. & Kao, F. T. (1982) *J. Cell Sci.* **53,** 245–254.
17. Varsanyi-Breiner, A., Gusella, J., Keys, C., Housman, D., Sullivan, D., Brisson, N. & Verma, D. P. L. (1979) *Gene* **7,** 317–334.
18. Jelinek, W. R., Toomey, T. P., Leinwand, L., Duncan, C. H., Biro, P. A., Choudary, P. V., Weissman, S. M., Rubin, C. M., Houck, C. M., Deininger, P. L. & Schmid, C. W. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1398–1402.
19. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berev, P. (1977) *J. Mol. Biol.* **113,** 237–251.
20. Wetmur, J. G. & Davidson, N. (1968) *J. Mol. Biol.* **31,** 349–370.
21. McConaughy, B. L., Laird, C. D. & McCarthy, B. J. (1969) *Biochemistry* **8,** 3289–3295.