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Isolation of anonymous DNA sequences from within a submicroscopic X chromosomal deletion in a patient with choroideremia, deafness, and mental retardation

(retinal dystrophy/gene mapping/phenol-enhanced reassociation)

ROBERT L. NUSSBAUM*†‡§, JOHN G. LESKO*, RICHARD ALAN LEWIS¶‖, SUSAN A. LEDBETTER¶, AND DAVID H. LEDBETTER¶

*Howard Hughes Medical Institute, †Department of Human Genetics, and ‡Department of Pediatrics, University of Pennsylvania, Philadelphia, PA 19104-6072; and ¶Institute for Molecular Genetics, and ‖Cullen Eye Institute, Baylor College of Medicine, Houston, TX 77030

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ABSTRACT **Choroideremia, an X-chromosome linked retinal dystrophy of unknown pathogenesis, causes progressive nightblindness and eventual central blindness in affected males by the third to fourth decade of life. Choroideremia has been mapped to Xq13–21 by tight linkage to restriction fragment length polymorphism loci. We have recently identified two families in which choroideremia is inherited with mental retardation and deafness. In family XL-62, an interstitial deletion in Xq21 is visible by cytogenetic analysis and two linked anonymous DNA markers, DXYS1 and DXS72, are deleted. In the second family, XL-45, an interstitial deletion was suspected on phenotypic grounds but could not be confirmed by high-resolution cytogenetic analysis. We used phenol-enhanced reassociation of 48,XXXX DNA in competition with excess XL-45 DNA to generate a library of cloned DNA enriched for sequences that might be deleted in XL-45. Two of the first 83 sequences characterized from the library were found to be deleted in probands from family XL-45 as well as from family XL-62. Isolation of these sequences proves that XL-45 does contain a submicroscopic deletion and provides a starting point for identifying overlapping genomic sequences that span the XL-45 deletion. Each overlapping sequence will be studied to identify exons from the choroideremia locus.**

Choroideremia (McK30310) is an X-chromosome linked retinal dystrophy in which affected males suffer progressive nightblindness and visual field constriction, leading to eventual blindness by the third to fourth decade of life (1, 2). Carriers can be reliably detected by characteristic patchy changes in the retinal pigmented epithelium that reflect random X-chromosome inactivation (1–3). Prenatal diagnosis for choroideremia is not available. The gene involved has not been identified, and the molecular pathogenesis of the disease is totally unknown.

Subregional localization of the choroideremia locus to the region Xq13–21 on the X chromosome has been accomplished in two ways. First, tight linkage in families has been found between choroideremia and restriction fragment length polymorphisms (RFLPs) located in the region Xq13–21 (4–6). Second, the disease has been reported as part of a chromosome deletion syndrome in two unrelated mentally retarded males with interstitial deletions affecting the region Xq13–21.3 that were presumed to include the choroideremia gene (7, 8).

In this report, we describe genetic and molecular studies in probands from two unrelated families in which choroideremia, mental retardation, and deafness were inherited concordantly in an X-linked manner. In probands from one of these families, XL-62, an interstitial deletion within Xq21 was found cytogenetically and confirmed by Southern blot analysis of the probands' DNA by demonstrating the deletion of two RFLP loci, DXYS1 and DXS72, known to be tightly linked to choroideremia. In the second family, XL-45, an unequivocal interstitial deletion could not be demonstrated cytogenetically and no linked markers were found to be deleted (*see below*). However, similarities in phenotype between the affected males in the two families led us to hypothesize that the probands from the second family, XL-45, also had an interstitial deletion, albeit a much smaller one, responsible for the phenotype. To test this hypothesis, we used a phenol-enhanced reassociation technique (PERT) (9, 10), to enrich for DNA deleted in XL-45. With this approach, we have identified two DNA sequences that are deleted in the XL-45 probands, who lack an obvious cytogenetic deletion, as well as in the XL-62 probands, who do show a visible chromosomal deletion at Xq21. These probes are the first step toward isolating overlapping DNA sequences that span the submicroscopic deletion in XL-45, with the ultimate aim of identifying and isolating the choroideremia gene.

FAMILY STUDIES

Clinical information for family XL-45 has been published (11); there are three probands (two brothers and their maternal uncle) with choroideremia, mental retardation, and deafness. Female carriers had typical retinal changes indicative of the choroideremia carrier state. The two probands in family XL-62 (first cousins through their mothers) have a syndrome of choroideremia, short stature, mental retardation, and deafness similar to the probands in XL-45 (unpublished data). All family members underwent confirmatory ophthalmological examination and blood was obtained both for DNA and establishment of lymphoblastoid cell lines.

MATERIALS AND METHODS

DNA was extracted from peripheral leukocytes as described (12). For Southern blotting, 10 μg of DNA was digested with appropriate restriction enzymes for 6–16 hr, separated by electrophoresis, and blotted to nitrocellulose membranes (13). Radioactive probes were prepared by random oligonucleotide priming (14). Plasmid p36B-2, an anonymous sequence that detects restriction polymorphism DXS10 and is

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Abbreviation: PERT, phenol-enhanced reassociation technique.

§To whom reprint requests should be addressed at: Howard Hughes Medical Institute, University of Pennsylvania School of Medicine /6072, 37th and Hamilton Walk, Philadelphia, PA 19104-6072.

known to map in Xq26, was used as a control for showing X chromosomal dosage (15).

Routine and high-resolution chromosome analyses were performed on phytohemagglutinin-stimulated blood cultures of affected males and carrier females from each of the two families. For high-resolution studies, lymphocytes were synchronized by the method of Yunis (16). Trypsin G-banding analysis was used for all karyotype analyses.

For *in situ* hybridization studies, a human male lymphoblastoid cell line with a normal karyotype was synchronized by blocking with bromodeoxyuridine (100 μg/ml) for 18 hr. Cells were released by washing twice in Hanks' balanced salt solution, followed by addition of 10 μM thymidine 6 hr prior to cytogenetic harvest by conventional methods. Hybridization to metaphase chromosomes was carried out by the method of Harper and Saunders (17). Probe was labeled by random oligonucleotide priming (14) to a specific activity of 10^8 cpm/μg. Slides were dipped in Kodak NTB2 emulsion and exposed at 4°C for 8–14 days. After standard development, slides were banded by the sodium borate method of Cannizzarro *et al.* (18).

For PERT enrichment, DNA from a lymphoblastoid line from one of the XL-45 probands was digested to completion with *Hae* III (6 units/μg) overnight and dephosphorylated for 1 hr with calf intestinal alkaline phosphatase (0.1 unit/μg). DNA from a 48,XXXX line GM1416 (Institute for Medical Research, Camden, NJ) was cut to completion with *Hae* III, ligated to a 50-fold molar excess of *Eco*RI linkers with T4 DNA ligase, digested with *Eco*RI, separated from digested linker fragments by electrophoresis, and extracted from low-melting agarose.

The DNA from XL-45 (200 μg) was mixed with the GM1416 DNA in 0.1 M Tris·HCl (pH 8) and boiled for 10 min. The solution of denatured DNA was then immediately made 13% (vol/vol) phenol and 1.75 M NaSCN in a final vol of 1.05 ml. The tube was placed on a Vortex at top speed for 40 hr at 20°C. The supernatant was removed, extracted three times with chloroform, dialyzed against 1000 vol of 10 mM Tris·HCl/1 mM EDTA, pH 8, and ethanol precipitated. The precipitated DNA was redissolved and ligated with T4 DNA ligase overnight at 13°C to 1 μg of dephosphorylated λgt10 arms (Vector Laboratories, Burlingame, CA) in a vol of 30 μl and packged *in vitro*. Aliquots of packaged phage were plated on host strains C600 and C600(hfl).

Each phage was characterized by isolating DNA prepared from plate lysates made from a single plaque (19). Phage DNA was digested with *Eco*RI, the ends were labeled with [^{32}P]dATP using the Klenow fragment of *Escherichia coli* DNA polymerase, and the insert fragment was separated by gel electrophoresis and identified by autoradiography of the gel. This method of detecting inserts was very sensitive and required <50 ng of purified phage DNA. When an insert was identified, 0.5–1 μg of phage DNA was digested with *Hin*dIII and the fragment between 6.5 and 9.3 kilobases (kb), which contains the *Eco*RI insertion site, was isolated from low-melting agarose after gel electrophoresis. The *Hin*dIII fragment containing the insert was labeled with [^{32}P]dCTP to >10^9 cpm/μg by random oligomer priming and used to probe Southern blots of DNA from GM1416 and the probands from both the XL-45 and the XL-62 families.

RESULTS

High-Resolution Analysis of the X Chromosome in XL-45 and XL-62 Families. Both routine and high-resolution chromosome analysis of X chromosomes in family XL-62 showed an obvious and consistent deletion, which reduced the size of band q21 ≈50% in males and in one X chromosome homolog of carrier females (Fig. 1 *a* and *b*). Resolution was not adequate to determine whether the deletion was in the proximal (q21.1) or distal (q21.3) region of band q21, but it did not extend into q13 or q22. Karyotype analysis in family XL-45 was more difficult to interpret. In female carriers, a small difference in the size of the q21 band was suspected in some cells but not in others (Fig. 1 *c* and *d*). The size variation was considered to be within the range of contraction variation between two homologs, so that we could not discriminate confidently between a small deletion of q21 and normal contraction variation.

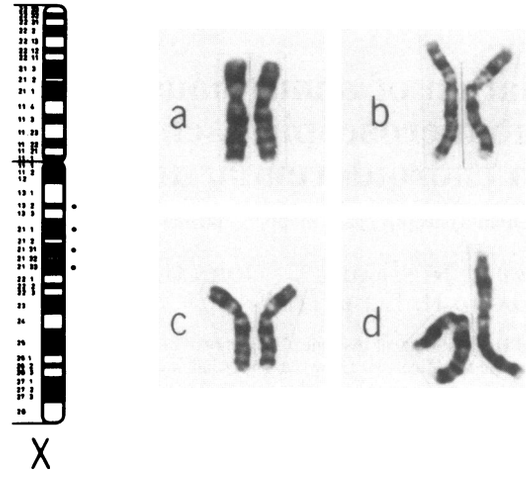

FIG. 1. Routine and high-resolution chromosome analysis of families XL-62 (*a* and *b*) and XL-45 (*c* and *d*). An idiogram of the X chromosome at approximately the 850-band stage of contraction (20) is shown. Dots have been placed at the G-positive subbands q13.2, q21.1, q21.3, and q21.33 as a reference to the region of interest. On the upper left (*a*) is a pair of metaphase X chromosomes from a carrier female in family XL-62, showing an obvious difference in the size of band q21 in the normal (*Left*) and deleted (*Right*) homolog. This difference is also apparent in the prometaphase pair from the same individual (*b*), although it is not clear which of the q21 subbands is involved in the deletion. On the lower left (*c*) is a metaphase pair from a carrier female in family XL-45, showing no obvious difference in the size of the q21 bands. A longer prometaphase pair (*d*) from the same carrier is suggestive of a small deletion in the homolog on the right, although this could be accounted for by differential contraction.

Probe Analysis of the Xq21 Region in XL-45 and XL-62 Families. DNA from the probands from both families was analyzed initially by Southern blotting with five probes known to be tightly linked to choroideremia: DXYS1, DXS72, DXYS12, DXS3, and PGK (5). Loci DXYS1 and DXS72 were found to be deleted in the XL-62 family; none of these loci was found deleted in probands from XL-45 (data not shown).

Isolation of DNA from Within the XL-45 Deletion. Phenol-enhanced reassociation of 48,XXXX DNA containing *Eco*RI ends with a 200-fold excess of blunt-end dephosphorylated DNA from XL-45 yielded a library of 1500 λgt10 clones on the C600 (hfl) host. A total of 145 phage have been analyzed to date (Table 1). Of these, 62 (43%) were found not to contain

Table 1. Characterization of PERT library

	Number	%
Phage studied	145	—
Phage with inserts	83	57
Inserts characterized	74	—
Repetitive	19	26
Autosomal unique	45	61
X-linked unique	8*	10
Deleted in XL-45	2	3

*Including one sequence, λJL77, that is deleted in XL-62 but not in XL-45.

inserts in the *Eco*RI cloning sites despite selection in C600 (hfl) against nonrecombinant phage retaining an intact *c*II gene (21). However, phage without inserts that grew on the selective host were found to have lost the *Eco*RI site, suggesting that such phage had escaped selection through deletion, rather than insertion, at the cloning site within the *c*II gene.

The remaining 83 phage (57%) contained inserts ranging in size from 100 base pairs (bp) to 2.1 kb (average, 800 bp). To date, 74 inserts have been characterized by using them as probes for Southern blots of XL-45, XL-62, and 48,XXXX DNA digested with *Eco*RI. An example of these Southern blot analyses, performed on three identically prepared filters, is shown in Fig. 2. In all three filters, lane 1 contains DNA from the 48,XXXX cell line, lane 2 contains DNA from an XL-45 proband, and lane 3 contains DNA from an XL-62 proband. Phage λJL74 (filter A) gave a signal of equal intensity in all three lanes, phage λJL137 (filter B) showed greater intensity with the 48,XXXX line than with the two males from XL-45 and XL-62, and λJL68, carrying a 1.2-kb insert (filter C), was deleted in the probands from both the XL-45 and XL-62 families.

The results of characterizing these 74 inserts are summarized in Table 1. Probes made from 19 (26%) of these inserts yielded a smear in Southern blots of human DNA and are probably repetitive in nature. An additional 45 (61%) appeared to be autosomal single copy sequences, as judged by the presence of one or two bands of equal intensity in autoradiographs of Southern blots of DNA from the 48,XXXX line and the probands in families XL-45 and XL-62. Eight inserts (10%) appeared to be X-linked since they gave greater intensity in the 48,XXXX line than in the male probands of either the XL-45 or XL-62 families; of these X-linked phage, one, λJL77, was found serendipitously to be deleted in XL-62 but not in the XL-45 DNA used to make the PERT library. Finally, 2 of 74 phage (3%), λJL68 and λJL8, contained inserts that were deleted in both families.

To confirm that λJL68 and λJL8 mapped to the appropriate Xq13–21 region of the X chromosome, we carried out chromosomal and subregional localization of the sequences. First, both sequences were shown to be on the X chromosome. In Fig. 3, pJL68, a plasmid carrying the λJL68 insert, was used as a probe in a Southern blot against equal amounts of DNA from an XL-45 proband (lane 1) and from individuals with one (lane 2), two (lane 3), and four (lane 4) X chromosomes. No signal was seen with pJL68 in lane 1 containing DNA from the XL-45 deletion, confirming that it was indeed

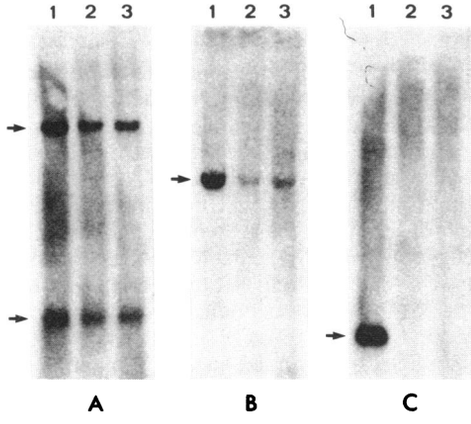

FIG. 2. Autoradiographs of three Southern blots of *Eco*RI-digested DNAs from the 48,XXXX cell line (lane 1), a proband from XL-45 (lane 2), and a proband from XL-62 (lane 3). Filter A was probed with λJL74, filter B was probed with λJL137, and filter C was probed with λJL68. Arrows point to the hybridizing fragments seen with each of the three probes.

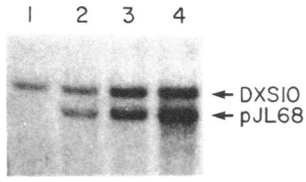

FIG. 3. Autoradiograph of a Southern blot of *Pvu* II-digested DNA from an XL-45 proband (lane 1), control male (lane 2), control female (lane 3), and the 48,XXXX individual (lane 4). The filter was probed with pJL68 and DXS10 sequentially. Arrows point to the *Pvu* II fragments found to hybridize with each probe.

deleted in this proband. The pJL68 sequence also showed proportionally increasing signal intensity in lanes 2–4 with the increasing number of X chromosomes. Another X-linked locus, DXS10, located well outside the region of deletion, served as a control and showed equal hybridization in the XL-45 proband and a normal male and the equivalent increase in signal intensity with increasing number of X chromosomes. The same filter was washed free of probe and reprobed with λJL8: λJL8 was also shown to be deleted in XL-45 and gave a similar increase in signal intensity with increasing number of X chromosomes (data not shown). λJL68 and λJL8 were also mapped to the X chromosome with a somatic cell hybrid. Both sequences hybridized on Southern blot to DNA from a human–Chinese hamster hybrid, 4.12 (22), containing a single human X as its only human chromosome and not to its Chinese hamster parent or to a 6-thioguanine-resistant derivative that had lost its human X chromosome (data not shown).

Although both λJL68 and λJL8 were shown to be located on the X chromosome and were clearly deleted in Southern blots of DNA from probands from XL-62 with the visible Xq21 deletion, we sought to confirm independently that the sequences were derived from Xq21 using *in situ* hybridization to a normal X chromosome. The results for λJL68 are shown in Fig. 4. Since dosage and somatic cell hybrid data had unambiguously assigned λJL68 to the X chromosome, only grains on the X chromosome were scored for their specific band localization. The overall background frequency of grains on chromosomes was approximately 1 per cell. Of 34 cells analyzed with a grain localized to the X chromosome, 20 (58%) were located at proximal Xq21. Thus, λJL68 was mapped to Xq21, the region of the choroideremia locus as previously determined by both linkage in families and deletion mapping in patients.

The λJL68 and λJL8 sequences have been used to probe Southern blots of DNA from a panel of 16 choroideremia patients from unrelated families in whom the retinal dystro-

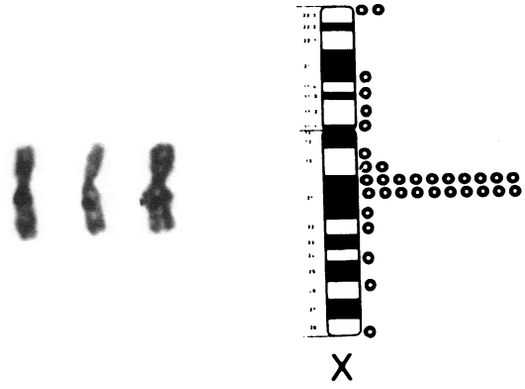

FIG. 4. G-banded X chromosomes from three different cells showing grains localized to q21. A total of 34 grains are plotted on an idiogram of the X chromosome at approximately the 400-band state of resolution (23).

phy is an isolated disease without extraocular findings such as mental retardation or deafness. No Southern blot alterations have been detected (data not shown).

DISCUSSION

Recent advances in human disease gene mapping have evolved from the development and application of human RFLP linkage maps in families segregating for hereditary diseases (24). For X-linked choroideremia, linkage analysis places the disease locus in the region of Xq13–21. In our linkage studies, the closest markers—DXYS1, DXS72, and PGK—have recombination frequencies with choroideremia between 0% and 2%, with upper 90% confidence limits of 9–11%. Such linkage data are sufficiently accurate for medical genetic applications such as disease classification, heterozygote detection, and prenatal diagnosis. From regional localization, identification of the defective gene constitutes the next step in the molecular characterization of this retinal disease. However, since a crude comparison between recombination frequency and physical distance suggests that 1% recombination equals ≈1000 kb (25), these linked markers may be as far as 10,000 kb from the choroideremia locus and therefore may be difficult to use for isolation of the choroideremia gene.

An alternative method of mapping disease genes uses chromosomal abonormalities such as interstitial deletions and X/autosome translocations in females. Interstitial chromosomal deletions can produce disease syndromes consisting of a constellation of single gene defects due to deletion of neighboring loci (24, 26). Cytogenetic localization of the deletion then provides subregional localization of the disease loci, which is independent of linkage analysis. For example, study of a male with Duchenne muscular dystrophy, mental retardation, and chronic granulomatous disease with an interstitial Xp21 deletion (27) provided not only an alternative method for mapping these disease loci independent of linkage, but also provided a means of isolating DNA sequences extremely close to or within the genes of interest. These DNA sequences were then used to identify messenger RNAs with a high likelihood of being transcribed from the genetic loci for both Duchenne muscular dystrophy and chronic granulomatous disease (20, 28).

Family XL-62, with an interstitial deletion at Xq21 confirmed by both cytogenetic analysis and Southern blotting, showed a unique and rare phenotype of choroideremia, mental retardation, and deafness and thus provided strong but circumstantial evidence for the presence of a deletion in family XL-45 with a similar phenotype but no obvious deletion. If this smaller putative deletion could be confirmed in the XL-45 family, it would define even more narrowly the region of the chromosome in which the choroideremia gene resides. Therefore, we sought to isolate DNA from within a putative XL-45 deletion using a modification of a technique exploited by Kunkel *et al.* (9) to isolate DNA from within a cytogenetically detectable deletion causing Duchenne muscular dystrophy. An excess of DNA from the deletion patient is rendered unligatable, is denatured, and is then mixed with single-stranded DNA from a 48,XXXX cell line containing ends that can be ligated into the restriction site of a cloning vector. Reannealing results in a heterogeneous mixture of DNA sequences in which a double-stranded fragment of DNA with two ligatable ends can only arise if two complementary single strands from the 48,XXXX DNA reanneal. Sequences in the 48,XXXX DNA that are missing in the deletion patient's DNA have a much greater chance of forming double-stranded DNA with two ligatable ends than do those sequences present in the deletion patient's DNA since these other sequences are more likely to form double-stranded molecules without ligatable ends. As a result, enrichment for sequences in the 48,XXXX cell line that are absent in the deletion patient occurs. Double-stranded DNA fragments with two ligatable ends constitute a small fraction of the total DNA in the reannealing reaction. We chose, therefore, to use *in vitro* packaging with a phage vector because of the selectivity of *in vitro* packaging mixtures for DNA sequences of proper length, when flanked with cos sites, even in the presence of an overwhelming excess of unpackageable DNA.

Phenol-enhanced reassociation will result in enrichment for single-copy sequences deleted in XL-45 only if the reannealing is complete enough to reconstitute double-stranded single-copy DNA molecules with ligatable ends. Theoretical calculations (see *Appendix*) predict that, with complete reannealing, 60% of the sequences with two ligatable ends would be autosomal unique, 13% would be X-linked, and 27% would be repeated. Incomplete reannealing would result in overrepresentation of repeated sequences in the PERT library. The close correspondence between these theoretical values and the observed frequencies in Table 1 demonstrates that the reassociation protocol did allow nearly complete reannealing of unique sequences.

The exact size of the deletion in the XL-45 probands is unknown. If, however, the 3×10^6 kb in the haploid human genome (23) are assumed to be distributed uniformly among ≈800 chromosome bands in a high-resolution karyotype analysis (29), each band should contain ≈2500 kb. Any deletion that reduces a band by >50% should be visible cytogenetically, and thus we estimate that the XL-45 deletion should be <1250 kb.

The size of the XL-45 deletion constitutes an upper bound for the region of the X chromosome in which we will be searching for the choroideremia gene. Further studies may allow us to limit the search even more by taking advantage of an increasing number of patients with choroideremia and X chromosomal deletions (refs. 7 and 8; unpublished data). We know that λJL68, λJL8, and the choroideremia locus are all deleted in both the XL-45 and XL-62 families, and thus all three loci must reside in the region of overlap between these two deletions. We do not know at this time whether the XL-45 deletion overlaps in part with the XL-62 deletion or is contained completely within it. Additional studies will determine whether a region of overlap between these two deletions will narrow the region for the choroideremia gene still further. Regions of overlap with additional deletions in patients with choroideremia may contribute further to defining the region in which the choroideremia gene is located.

APPENDIX

Following the PERT reaction, sequences with two ligatable ends will fall into four classes: repeated sequences, autosomal unique sequences, nondeleted unique X-linked sequences, and unique sequences deleted from XL-45. The fraction of ligatable DNA from each DNA class following the PERT reannealing reaction will depend on (*i*) the relative abundance of each of these types of sequences in the 48,XXXX genome and (*ii*) the depletion factor resulting from annealing of strands without ligatable ends to strands with ligatable ends. Single-copy sequences constitute ≈70% of the haploid genome of 3×10^6 kb (23), of which 5%, or 3.5% of the total genome, are X-linked and the remaining 66.5% are autosomal. Repeated sequences constitute 30% of the total. The fraction of the genome deleted in XL-45, *d*, is unknown but small, certainly <0.1%. Depletion factors depend on the relative amounts of DNA from the deletion and 48,XXXX cell lines added to the reannealing mixture. Since the ratio of deletion DNA to 48,XXXX DNA was 200:1 in the PERT reannealing reaction, the depletion factor for all repeated sequences and all autosomal unique sequences is 1/200. For

nondeleted X-linked sequences, the depletion factor is 4/200 or 1/50. Only for unique sequences from within the deletion is there no depletion due to annealing of nonligatable sequences—i.e., the depletion factor is 1. The fraction of the library, therefore, expected to contain autosomal unique sequences is approximated by

$$(1/200) \times 66.5\%/\{[(1/200) \times 66.5\%] + [(1/200) \times 30\%] + [(1/50) \times 3.5\%] + [1 \times d]\},$$

or ≈60% for small values of d. Similarly, 27% would be repeat sequences in nature and 13% would be X-linked.

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