CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

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BACKGROUND OF THE INVENTION

The problem with humans and other mammals, at least from a genetic diagnostic perspective, is that they are diploid. Mutations in one allele, such as those responsible for all dominantly inherited syndromes, are always accompanied by the wild-type sequence of the second allele. Though many powerful techniques for genetic diagnosis have been developed over the past decade, all are compromised by the presence of diploidy in the template. For example, the presence of a wild-type band of the same electrophoretic mobility as a mutant band can complicate interpretation of sequencing ladders, especially when the mutant band is of lower intensity. Deletions of a segment of DNA are even more problematic, as in such cases only the wild-type allele is amplified and analyzed by standard techniques. These issues present difficulties for the diagnosis of monogenic diseases and are even more problematic for multigenic diseases, where causative mutations can occur in any of several different genes. Such multigenism is the rule rather than the exception for common predisposition syndromes, such as those associated with breast and colon cancer, blindness, and hematologic, neurological, and cardiovascular diseases. The sensitivity of genetic diagnostics for these diseases is currently suboptimal, with 30% to 70% of

cases refractory to genetic analysis.

There is a need in the art for simply separating and analyzing individual alleles from human and other mammalian cells.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for detecting mutations in a gene of interest on a human or other mammalian chromosome.

It is another object of the invention to provide a method for making test cells suitable for sensitive genetic testing.

It is yet another object of the invention to provide a population of fused cell hybrids which are useful for genetic analysis.

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment a method of detecting mutations in a gene of interest of a human or other mammal is provided. Cells of a human or other mammal are fused to rodent cell recipients to form human-rodent or other mammal-rodent cell hybrids. Fused cell hybrids are selected by selecting for a first marker contained on a rodent chromosome and for a second marker contained on a first human or other mammalian chromosome, forming a population of fused cell hybrids. A subset of hybrids are detected among the population of fused cell hybrids. The hybrids are haploid for a second human other mammalian chromosome which is not the same chromosome as the first human or other mammalian chromosome and which was not selected. The subset of hybrids are tested to detect a gene, an mRNA product of said gene, or a protein product of said gene. The gene resides on the second human or other mammalian chromosome. Diminished amounts of the mRNA or protein product or altered properties of the gene, mRNA, or protein product indicate the presence of a mutation in the gene in the human or other mammal.

According to another embodiment, a method is disclosed which provides test cells for genetic testing. The test cells are haploid for human or other mammalian genes. Cells of a human or other mammal are fused to transformed, diploid, rodent cell recipients to form human-rodent or other mammal-rodent cell hybrids. Fused cell hybrids are selected by selecting for a marker on each of a first human or other mammalian chromosome and a rodent chromosome, forming a population of cells

which stably maintain one or more human or other mammalian chromosomes in the absence of selection for the human or other mammalian chromosomes. Cells which are haploid for a second human or other mammalian chromosome which is distinct from the first human or other mammlian chromosome are detected among the population of cells; the second human or other mammalian chromosome was not selected.

Also provided by the present invention is a population of rodent-human or rodent-other mammalian hybrid cells wherein each homolog of at least 2 human or other mammalian autosomes is present in haploid form in at least one out of one hundred of the cells.

The present invention thus provides the art with a method which can be used to increase the sensitivity and effectiveness of various diagnostic and analytic methods by providing hybrid cells to analyze which are haploid for one or more genes of interest. The human or other mammalian chromosome content of the hybrid cells is stable and uniform.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Strategy for hybrid generation. The recipient mouse cell line E2 was fused with human lymphocytes and clones were subsequently selected with HAT plus geneticin, which kill unfused E2 cells and lymphocytes, respectively. All clones contained a human X chromosome responsible for growth in HAT. Clones were genotyped to determine which human chromosomes were retained. Chromosomes marked "A" and "B" represent the two homologs of a representative human chromosome. The average proportion of clones which retained neither, both, or either of the six chromosome homologs analyzed is indicated (see text). Mutational analysis was carried out on nucleic acids of clones which retained single alleles of the genes to be tested.

Fig. 2. Allelic status and gene expression in hybrids. **(Fig. 2A)** Polymorphic markers from the indicated chromosomes were used to determine the genotype of the indicated hybrids. "Donor" denotes the human lymphocytes used for fusion with the mouse recipient cells. **(Fig. 2B)** cDNA of E2 and four hybrids were used as templates to

amplify *hMSH2*, *hMSH6*, *hMLH1*, *hTGF β-RII*, *hPMS1*, *hPMS2*, and *APC* sequences. The results were concordant with the genotypes observed in (**Fig. 2A**), in that hybrids 5 - 7 retained at least one allele of each of the chromosomes containing the tested genes, while hybrid 8 contained alleles of chromosomes 3, 5, and 7 but not of chromosome 2 (containing the *hMSH2, hPMS1, and hMSH6* genes).

Fig. 3. Mutational analysis of an HNPCC patient refractory to standard genetic diagnosis. Nucleic acids from the indicated hybrids were tested for retention of chromosomes 2 and 3 using polymorphic markers (**Fig. 3A**) and for expression of *hMSH2* and *hMLH1* genes on chromosomes 2 and 3, respectively (**Fig. 3B**). Hybrids 1, 2, 3, and 6 contained allele A from chromosome 2 and did not express hMSH2 transcripts, while hybrids 4 and 5 contained the B allele and expressed hMSH2. hMLH1 expression served as a control for the integrity of the cDNA. (**Fig. 3C**) Sequences representing the indicated exons of *hMSH2* were amplified from the indicated hybrids. Exons 1- 6 were not present in the hybrids containing allele A, but exons 7 - 16 were present in hybrids containing either allele.

Fig. 4. Mutational analysis of Warthin family G. (**Fig. 4A**) Sequence analysis of RT-PCR products from hMSH2 transcripts of hybrid 1, containing the mutant allele of a Warthin family G patient, illustrates a 24 bp insertion (underlined; antisense primer used for sequencing). The wild-type sequence was found in hybrid 3, containing the wt allele. RT-PCR analysis of transcripts from lymphoid cells of the patient showed that the mutant transcript was expressed at significantly lower levels than the wild-type sequence. Sequence analysis of the genomic DNA of the same hybrids (**Fig. 4B**) showed that the insertion was due to a A to C mutation (antisense sequence, indicated in bold and underlined) at the splice acceptor site of exon 4, resulting in the use of a cryptic splice site 24 bp upstream. The signal of the mutant C is not as strong as the wild-type A in the donor's DNA. Such non-equivalence is not unusual in sequencing templates from diploid cells, and can result in difficulties in interpretation of the chromatograms. (**Fig. 4C**) Extracts from hybrids 1 and 5, carrying the mutant allele of chromosome 2, were devoid of hMSH2 protein, while extracts of hybrids 2 and 3, carrying the wt allele, contained hMSH2 protein. Hybrid 4 did not contain either allele of chromosome 2. Hybrids 1, 3, 4, and 5 each carried at least one allele of chromosome

3 and all synthesized hMLH1 protein. α-tubulin served as a protein loading control. Immunoblots with antibodies to the indicated proteins are shown.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

We have devised a strategy for generating hybrids containing any desired human or other mammal's chromosome using a single fusion and selection condition. Importantly and unexpectedly, the human or other mamalian chromosomes in these hybrids were stable, and they expressed human or other mammalian genes at levels sufficient for detailed analysis. The approach is based on the principle that fusion between human or other mammal and rodent cells creates hybrid cells that contain the full rodent genomic complement but only a portion of the human or other mammalian chromosomes. In the past, selection for retention of a specific human or other mammalian chromosome (by complementation of an auxotrophic rodent cell, for example) has allowed the isolation of hybrids containing a desired chromosome (7, 8). Though such fusions have proven useful for a variety of purposes (8, 9), their utility has been limited by the availability of appropriate rodent recipients for many chromosomes and by the inefficiencies and variation of the fusion and selection conditions. For the analysis of multigenic diseases, it would be necessary to perform a separate fusion and selection for each chromosome.

The stability of the human or other mammalian chromosomes in the hybrids of the present invention was surprising. Though the human genetic constitution of radiation hybrids is relatively stable, this stability has been presumed to be due to the integration of small pieces of human DNA into rodent chromosomes following irradiation of the donor cells. The human chromosomes in whole cell fusions have been believed to be unstable unless continuous selection pressure for individual chromosomes was exerted. The reasons for the stability in our experiments is unclear, but may be related to the diploid nature of the rodent partner. Such diploidy reflects a chromosome stability that is unusual among transformed rodent cells. Previous experiments have indeed shown that chromosomally stable human cells retain all chromosomes upon fusion with other chromosomally stable human cells, unlike the situation when one of the two partners is chromosomally unstable.

The diploid, rodent recipient cells of the present invention provide useful

reagents for the facile creation of cells with functionally haploid human genomes. Nucleic acids or proteins from these hybrids can be used as reagents for any standard mutational assay. As mutational assays are constantly being improved and automated (1), the value of the hybrid-generated materials correspondingly increases. It may soon become possible, in fact, to examine the sequence of entire genes (promoters and introns in addition to exons). Nucleic acid templates generated from single alleles are clearly superior for such analyses, as the homogeneous nature of the templates dramatically enhances the signal to noise ratio of virtually any diagnostic assay. We therefore envision that this approach can be productively applied to a wide variety of research and clinical problems.

Genes of interest are typically those which have been found to be involved in inherited diseases. These include genes involved in colon cancer, breast cancer, Li-Fraumeni disease, cystic fibrosis, neurofibromatosis type 2, von Hippel-Lindau disease, as well as others. The identified genes include *APC*, *merlin*, *CF*, *VHL*, *hMSH2*, *p53*, *hPMS2*, *hMLH1*, *BRAC1*, as well as others. Mutations which can be identified at the protein level include those in sequences that regulate transcription or translation, nonsense mutations, splice site alterations, translocations, deletions, and insertions, or any other changes that result in substantial reduction of the full-length protein. Other subtler mutations can be detected at the nucleic acid level, such as by sequencing of RT-PCR products.

Cells of the human which may be used in fusions are any which can be readily fused to rodent cells. Peripheral blood lymphocytes (PBL) which are readily available clinical specimens are good fusion partners, with or without prior mitogenetic stimulation, whether used fresh or stored for over one year at -80° C. Since inherited mutations are the subject of the present method, any cells of the human body can be used, since all such cells contain essentially the same genetic complement. Cells of other mammals which can be used include in particular those of cats, dogs, cows, sheep, goats, horses, chimpanzees, baboons, and hogs. More generically, the cells of the other mammals can be selected from the ruminants, primates, carnivora, lagomorpha, and perissodactyla. Typically the other mammalian cell fusion partner is not a rodent cell.

Rodent cell recipients for fusion are preferably diploid, more preferably

oncogene-transformed, and even more preferably have microsatellite instability due to a defect in a mismatch repair gene. Selection of particular clones which grow robustly, are stably diploid, and fuse at a high rate is well within the skill of the ordinary artisan. The rodent cells may be, for example, from mice, rats, guinea pigs, or hamsters.

Fusion of cells according to the present invention can be accomplished according to any means known in the art. Known techniques for inducing fusion include polyethylene glycol-mediated fusion, Sendai virus-mediated fusion, and electro-fusion. Cells can desirably be mixed at a ratio of between 10:1 and 1:10 human to rodent. Clones of fused cells generally become visible after about two to three weeks of growth.

Fused hybrid cells can be selected using any markers which result in a positively selectable phenotype. These include antibiotic resistance genes, toxic metabolite resistance genes, prototrophic markers, etc. The surprising advantage of the present invention is that a single marker on a single human or other mammalian chromosome can be used in the selection, and that stable hybrids containing more than just the single, selected human or other mammalian chromosome result. Thus markers on other chromosomes can be analyzed even when the chromosomes on which the markers reside were not selected.

Fused hybrid cells can be analyzed to determine that they do in fact carry a human or other mammalian (non-rodent) chromosome which carries a gene of interest. Hybrid cells which have either of the two relevant human or other mammalian chromosomes can be distinguished from each other as well as from hybrids which contain both of the two human or other mammalian chromosomes. See **Fig. 1.** While any means known in the art for identifying the human or other mammalian chromosomes can be used, a facile analysis can be performed by assessing microsatellite markers on the human or other mammalian chromosome. Other linked polymorphic markers can be used to identify a desired human or other mammalian chromosome in the hybrids.

Once hybrid cells are isolated which contain one copy of a human or other mammalian gene of interest from a human or other mammal who is being tested, mutation analysis can be performed on the hybrid cells. The genes can be tested directly for mutations, or alternatively the mRNA or protein products of the genes can

be tested. Mutations that result in reduced expression of the full-length gene product should be detectable by Western blotting using appropriate antibodies. Tests which rely on the function of the protein encoded by the gene of interest and enzyme assays can also be performed to detect mutations. Other immunological techniques can also be employed, as are known in the art.

If an immunological method is used to detect the protein product of the gene of interest in the hybrids, it is desirable that antibodies be used that do not cross-react with rodent proteins. Alternatively, the rodent genes which are homologous to the gene of interest can be inactivated by mutation to simplify the analysis of protein products. Such mutations can be achieved by targeted mutagenesis methods, as is well known in the art.

Functional tests can also be used to assess the normalcy of each allelic product. For example, if one inserted an expression construct comprising a β-galactosidase gene downstream from a p53 transcriptional activation site, into a rodent-human hybrid cell that contained human chromosome 17 but no endogenous p53, then one could detect mutations of the p53 on the human chromosome 17 by staining clones with X-gal. Other enzymatic or functional assays can be designed specifically tailored to the gene of interest.

Any method of detecting mutations at the DNA or RNA level as are known in the art may be employed. These include without limitation, sequencing, allele-specific PCR, allele-specific hybridization, microarrays, DGGE, and automated sequencing.

It is a possibility that expression of the gene of interest might be inhibited in the hybrid cell environment. In order for the loss of expression of a gene of interest in the hybrid cells to be meaningfully interpreted as indicating a mutation in the human or other mammal, one must confirm that the gene of interest, when wild-type, is expressed in rodent-human or other mammal hybrid cells. This confirmation need not be done for each patient, but can be done once when the assay is being established.

When the assay of the present invention indicates that a mutation exists in the gene of interest, other family members can be tested to ascertain whether they too carry the mutation. Alternatively, the other family members can be tested to see if they carry the same chromosome as the affected family member. This can be determined by testing for a haplotype, *i.e.*, a set of distinctive markers which are found on the

chromosome carrying the mutation in the affected family member. Determination of a haplotype is a by-product of performing the assay of the invention on the first family member. When the hybrid cells are tested to confirm the presence of the relevant chromosome in the hybrid, for example by use of microsatellite markers, a distinctive marker set will be identified, which can then be used as a haplotype.

Mixed populations of hybrid cells made by the fusion process of the present invention may contain hybrid cells which are haploid for a number of different human or other mammalian chromosomes. Typically each homolog of at least 2, at least 5, at least 10, at least 15, at least 20, or even 22 human or other mammalian autosomes will be present in the population in a haploid condition in at least one out of one hundred, seventy-five, fifty, thirty or twenty-eight of the cells. Thus a high proportion of the cells contain multiple human or other mammalian chromosomes, and a relatively small number of cells must be tested to find cells harboring a single copy of a non-selected chromosome.

Populations of cells resulting from a single hybrid are uniform and homogeneous due to the high stability of the human or other mammalian chromosomes in the hybrid cells of the invention. Thus at least 75%, 80%, 85%, 90%, 95%, 97%, 99%, or 100% of the cells in the population resulting from a single hybrid cell contain the same complement of human or other mammalian chromosomes.

The following examples provide experimental details which demonstrate one of many ways to carry out the invention. The invention is not limited to the particular methods of cells employed in the examples. The claims and the specification as a whole provide the measure of the invention.

Examples

Example 1

An outline of the approach is presented in Fig. 1. The rodent fusion partner was a line derived from mouse embryonic fibroblasts transformed with ras and adenovirus E1A oncogenes. HPRT-deficient subclones of this line were generated, and one subclone (E2) was chosen for further experimentation based on its robust growth characteristics, maintenance of diploidy, and fusion efficiency (10). Human lymphocytes cells were mixed with E2 cells at an optimum ratio and electrofused, and hybrids selected in geneticin (to kill unfused human cells) and HAT (to kill unfused E2

cells) (11). Colonies appearing after two weeks of growth were expanded and RNA and DNA prepared for analysis. From a single fusion experiment, an average of 36 hybrid clones were obtained (range of 17 to 80 in five different individuals).

All hybrids contained the human X chromosome, as this chromosome contains the HPRT gene allowing growth in HAT. To determine whether other human chromosomes were present in the hybrids, polymorphic microsatellite markers (12) were used as probes in PCR-based assays (Fig. 2A). We focused on the chromosome arms (2p, 2q, 3p, 5q, 7q, and 16q) known to contain colorectal cancer (CRC) predisposition genes. One copy of each of these chromosome arms was present in a significant fraction of the hybrid clones. For example, of 476 hybrids derived from 14 individuals and examined for chromosome 3, 136 hybrids contained neither donor chromosome, 211 hybrids contained both donor chromosomes, 60 hybrids contained one parent's chromosome, and 69 hybrids contained the other parent's chromosome. Similar retention frequencies were found for all six chromosome arms analyzed. Testing of markers from both arms of a single chromosome showed that whole chromosomes, rather than chromosome fragments, were generally retained in the hybrids. This result was confirmed with fluorescence in situ hybridization (FISH) on metaphase spreads from the hybrids, which indicated the presence of 11 ±3 human chromosomes in each hybrid cell. Calculations based on the genotypic data indicated that the analysis of 25 hybrids would ensure a 95% probability of identifying at least one hybrid containing the maternal allele and one hybrid containing the paternal allele of a single chromosome under study. Moreover, it would require only 45 hybrids to similarly ensure that each allele of all 22 autosomes was present and separated from its homolog in at least one hybrid (13).

Example 2

Two other features of the hybrids were essential for the analyses described below. First, the human chromosome complements of the hybrids were remarkably stable. Polymorphic marker analysis in ten hybrids revealed identical patterns of retention after growth for 90 (30 passages) generations after initial genotyping. Second, those hybrids containing the relevant chromosome expressed every human gene assessed, including all known colorectal cancer susceptibility genes (the *hMSH2*

and *hMSH6* genes on chromosome 2p, the *hPMS1* gene on chromosome 2q, the *TGF-β Receptor Type II* gene and *hMLH1* gene on chromosome 3p, the *APC* gene on chromosome 5q, the *hPMS2* gene on chromosome 7q, and the *E-cadherin* gene on chromosome 16q; representative examples in Fig. 2B) (14).

Example 3

Having established the stability and expression patterns of CRC-predisposition genes in these hybrids, we used this "conversion" approach to investigate ten patients who had proven refractory to standard genetic diagnostic techniques. Each of these patients had a significant family history of colorectal cancer and evidence of mismatch repair deficiency in their tumors, yet sequencing of the entire coding sequence of each known MMR gene had failed to reveal mutations. Indeed, these and similar studies have prompted the speculation that other major HNPCC genes must exist. (25-34) Hybrids were generated from lymphocytes of each patient, and at least one hybrid containing the maternal allele and one hybrid containing the paternal allele of each MMR gene was isolated. Analysis of the nucleic acids from these hybrids revealed specific mutations in all ten patients (Table 1). In every case, an abnormality was found in a single allele of either *hMSH2* or *hMLH1*. The nature of the abnormalities revealed why they had not been detected with the standard methods previously used for their analysis. Three cases were due to large deletions, encompassing six or seven exons. When corresponding nucleic acids from the cells of such patients are evaluated by any PCR-based method, only the wild type sequences from the unaffected parent would be amplified, leading to the false impression of normalcy (for example, case #1 in Figure 3). Though Southern blotting can reveal deletions of one or a few exons in MMR, larger deletions are refractory to such blotting methods. In three cases (#4, 6, and 9), no transcript was generated from one allele, though the sequences of all exons and intron-exon borders from this allele were normal. Presumably, mutations deep within an intron or within the promoter of the gene were responsible. The absence of transcripts from one specific allele of these three patients was confirmed in at least three other converted hybrids from each patient. In four other cases, point mutations were found (Table 1). These mutations were not detected in the original sequence analyses because the signals from the mutant allele were not as robust as those from the wild type. Such asymmetry can be caused by instability of mutant transcripts due to

nonsense mediated decay (36-38), or to nucleotide preferences of the polymerases in specific sequence contexts, and represents a common problem for both manual and automated sequencing methods (39). The conversion approach eliminates these problems because only one sequence can possibly be present at each position. A good example of this was provided by Warthin G (17). The mutation in this prototype kindred was an A to C transversion at a splice site. The signal from the mutant "C" in the sequencing ladder was not as intense as the wild type "A" (Fig. 4b). This mutation led to the use of a cryptic splice site 24 bp upstream of exon 4, and an under-represented transcript with a 24 base insertion (Fig. 4a). To demonstrate that this mutation had an effect at the protein level, we analyzed the hybrids by immunoblotting with specific antibodies (19). The hybrids containing the mutant allele did not make detectable levels of human hMSH2 protein, though they did synthesize normal levels of a control protein (Fig. 4C).

The results described above demonstrate that individual alleles of human chromosomes can be readily isolated upon fusion to mouse cells.

HNPCC provides a cogent demonstration of the power of the conversion approach because it is a common genetic disease that has been widely studied. In the last three years, for example, extensive analyses of the major MMR genes have been performed in 303 HNPCC kindreds from nine cohorts distributed throughout the world (25-34). Based on the fraction of such patients with characteristic microsatellite instability in their cancers (30-34), it can be estimated that 239 (78%) of the kindreds had germ-line mutations of mismatch repair genes. Yet MMR gene mutations were identified in only 127 (42%) of these 239 kindreds (25-34). Our cohort was similar, in that it was derived from a total of 25 kindreds, 22 of whom had tumors with microsatellite instability and presumptive MMR gene mutations. Of these 22, our initial analyses revealed mutations in only 12 (54%) (ref. 14 and unpublished data). Mutations of the other ten patients were only revealed upon conversion analysis, which thereby increased the sensitivity from 54% to 100%. The conclusion that virtually all cases of HNPCC associated with MSI are due to germline mutations of known MMR genes is consistent with recent immunohistochemical data demonstrating the absence of either MSH2 or MLH1 protein staining in the cancers from the great majority of HNPCC patients (40, 41). A corollary of these results is that the search for new human

MMR genes should not based on the premise that a large fraction of HNPCC cases will prove attributable to such unknown genes.

The system described above can be applied to other genetic diseases in a straight forward manner. It should be emphasized that this approach is not a substitute for the many powerful methods currently available to search for specific mutations. Rather, conversion can be used to maximize the sensitivity of existing techniques. Converted nucleic acids provide the preferred substrates for such methods because of the higher signal to noise attainable and the inability of the wild type allele to mask or confound detection of the mutant allele. As DNA-based mutational assays are improved in the future, and progressive incorporate microarrays and other automatable features (42-44), the value of conversion-generated nucleic acids will correspondingly increase, significantly enhancing the effectiveness of genetic tests for hereditary disease.

Methods

Cell culture

Mouse embryonic fibroblasts were derived from MSH2-deficient mice (46) and transformed with adenovirus E1A and RAS oncogenes. HPRT-deficient subclones were selected by growing the fibroblasts in 10 μM 2-amino-6-mercaptopurine. Clones were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS and 10 μM 2-amino-6-mercaptopurine.

Cell fusion and the generation of hybrids

The patients were from kindreds with HNPCC as defined by the Amsterdam criteria (44); in no case was linkage analysis feasible due to the lack of a sufficient number of affected individuals. Microsatellite instability (MSI) in the cancers from these patients was determined through the markers recommended in ref. 45. 3×10^6 E2 cells and 12×10^6 lymphocytes cells were mixed, washed, and centrifuged twice in fusion medium (0.25 M D-sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% Bovine Serum Albumin (BSA), pH 7) and resuspended in 640 μl fusion medium. The solution was pipetted into a cuvette (BTX cuvette electrode 470; BTX, San Diego). Cells were fused using a BTX ElectroCell Manipulator, model ECM200. The settings that yielded the greatest number of hybrids were: 30V (AC) for 22 seconds, followed by three 300V (DC) pulses of 15 μsec each. The cells from one

fusion were plated into three 48-well plates (Costar) in DMEM supplemented with 10% FCS. After 24 hours, the medium was replaced by DMEM supplemented with 10% FCS, 0.5 mg/ml geneticin and 1 x HAT (Life Technologies, Gaithersburg, MD). The medium was changed after a week. Hybrid clones became visible two weeks after fusion and were expanded for another week prior to genotyping. From a single fusion, an average of 23 +/- 15 hybrid clones were obtained. The lymphocytes used for the experiments described here were derived from Epstein-Barr Virus infection of peripheral blood leukocytes, but it was found that freshly drawn lymphocytes could also be successfully fused and analyzed using identical methods.

Genotyping

Genotyping was performed as described (12). PCR products were separated on 6% denaturing gels and visualized by autoradiography. The microsatellite markers used were D2S1788 and D2S1360, D2S1384, D3S2406, D7S1824, and D16S3095, from chromosome 2p, 2q, 3p, 5q, 7q and 16q, respectively. Fluorescence in situ hybridization was performed as described previously (21).

PCR and sequencing

Polyadenylated RNA was purified and RT-PCR performed as described previously. Sequencing was performed using ABI Big Dye terminators and an ABI 377 automated sequencer. All primers used for amplification and sequencing will be made available through an internet site.

Statistical analysis

the number of hybrids containing none, both or a single allele of each chromosome tested were consistent with a multinomial distribution. Monte Carlo simulations were used to estimate the number of hybrids required to generate mono-allelic hybrids containing specific numbers of each chromosomes.

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10. E2 cells were derived from mouse embryonic fibroblasts derived from MSH2-deficient mice (generously provided by T. Mak) and transformed with adenovirus *E1A* and *RAS* oncogenes. HPRT-deficient subclones were selected by growing the fibroblasts in 10 μM 2-amino-6-mercaptopurine. Clones were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS and 10 μM 2-amino-6-mercaptopurine.

11. 3×10^6 lymphocytes cells were mixed, washed, and centrifuged twice in fusion

[1]All references are explicitly incorporated by reference.

medium (0.25 M D-sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% Bovine Serum Albumin (BSA), pH 7) and resuspended in 640 µl fusion medium. The solution was pipetted into a cuvette (BTX cuvette electrode 470; BTX, San Diego). Cells were fused using a BTX Electro Cell Manipulator, model ECM200. The settings that yielded the greatest number of hybrids were: 30V (AC) for 22 seconds, followed by three 300V (DC) pulses of 15 µsec each. The cells from one fusion were plated into three 48-well plates (Costar) in DMEM supplemented with 10% FCS. After 24 hours, the medium was replaced by DMEM supplemented with 10% FCS, 0.5 mg/ml geneticin and 1 x HAT (Life Technologies, Gaithersberg, MD). The medium was changed after a week. Hybrid clones became visible two weeks after fusion and were expanded for another week prior to genotyping. The lymphocytes used for the experiments described here were derived from Epstein-Barr virus infection of peripheral blood leukocytes, but we found that freshly drawn lymphocytes could also be successfuly fused and analyzed using identical methods

12. Genotyping was performed as described in F.S. Leach et al., *Cell* 75, 1215 (1993). PCR products were separated on 6% denaturing gels and visualized by autoradiography. The microsatellite markers used were D2S1788, D2S13360, D3S2406, D7S1824, and D16S3095, from chromosomes 2p, 2q, 3p, 5q, and 16q, respectively.

13. The numbers of hybrids containing none, both, or a single allele of each chromosome tested were consistent with a multinomial distribution. Monte Carlo simulations were used to estimate the numbers of hybrids required to generate mono-allelic hybrids containing specific numbers of chromosomes.

14. Polyadenylated RNA was purified and RT-PCR performed as described in B. Liu et al., *Nat Medicine* **2**, 169 (1996).

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18. Sequencing was performed using ABI Big Dye terminators and an ABI 377 automated sequencer. All primers used for amplification and sequencing will be made available through a *Science* internet site.

19. Cytoplasmic extracts of cells hybrids were separated by electrophoresis through SDS-polyacrylamide gels and immunoblotted with antibodies specific for human hMSH2 (#NA26, Calboiochem), human hMLH1 (#13271A, Pharmingen), or β-Tubulin (#N357, Amersham).

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CLAIMS

1. A method of detecting mutations in a gene of interest of a non-rodent mammal, comprising the steps of:

fusing cells of a non-rodent mammal to rodent cell recipients to form non-rodent mammal-rodent cell hybrids;

selecting for fused cell hybrids by selecting for a first marker contained on a rodent chromosome and for a second marker contained on a first non-rodent mammalian chromosome, to form a population of fused cell hybrids;

detecting among the population of fused cell hybrids a subset of hybrids which are haploid for a second non-rodent mammalian chromosome which is not the same chromosome as the first non-rodent mammalian chromosome and which was not selected;

analyzing said subset of hybrids to detect a mutation in an mRNA product of a gene or in said gene, wherein the gene resides on the second non-rodent mammalian chromosome.

2. The method of claim 1 wherein the rodent cell is diploid.

3. The method of claim 1 wherein the rodent cell recipients are deficient in mismatch repair.

4. The method of claim 1 wherein the rodent cell recipient is geneticin resistant.

5. The method of claim 1 wherein the rodent cell recipients are transformed with an oncogene.

6. The method of claim 5 wherein the oncogene is *ras*.

7. The method of claim 5 wherein the oncogene is *E1A*.

8. The method of claim 1 wherein the rodent cell recipients are transformed with *ras* and *E1A* oncogenes.

9. The method of claim 1 wherein the rodent cell recipients are *MSH2*$^-$.

10. The method of claim 1 wherein the rodent cell recipient is diploid, and contains both a selectable marker and a counterselectable marker.

11. The method of claim 10 wherein the counterselectable marker is HPRT deficiency.

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12. The method of claim 1 further comprising the step of:

detecting among the population of fused cell hybrids a third non-rodent mammalian chromosome, wherein the first, second, and third non-rodent mammalian chromosomes are distinct, and neither the second nor the third non-rodent mammal chromsomes were selected.

13. The method of claim 1 further comprising the step of:

detecting among the population of fused cell hybrids a third and fourth non-rodent mammalian chromosome, wherein the first through fourth non-rodent mammalian chromosomes are distinct, and neither the second through fourth non-rodent mammal chromsomes were selected.

14. The method of claim 12 further comprising the step of testing said subset of hybrids to detect an mRNA product of a second gene or a protein product of said second gene, wherein the second gene resides on the third non-rodent mammalian chromosome, wherein diminished amounts of said mRNA or protein product or altered properties of said mRNA or protein product from said second gene indicate the presence of a mutation in the second gene in the non-rodent mammal.

15. The method of claim 13 testing said subset of hybrids to detect an mRNA product of a second and third gene or a protein product of said second and third gene, wherein the second and third genes reside on the third and fourth non-rodent mammalian chromosomes, wherein diminished amounts of said mRNA or protein product or altered properties of said mRNA or protein product of said third or fourth genes indicate the presence of a mutation in third or fourth genes in the non-rodent mammal.

16. The method of claim 1 wherein the cells of the non-rodent mammal are lymphocytes.

17. The method of claim 1 wherein the step of detecting a subset of hybrids which are haploid for the second non-rodent mammalian chromosome is accomplished by identifying the presence of a microsatellite marker on the second non-rodent mammalian chromosome.

18. The method of claim 1 wherein the step of analyzing to detect a mutation is performed by a technique selected from the group consisting of: nucleic acid sequencing, allele-specific PCR, allele-specific hybridization, hybridization to a

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microarray, discontinuous gradient gel electrohoresis (DGGE), and automated nucleic acid sequencing.

19. A method of providing test cells for genetic testing, wherein said test cells are haploid for genes of a non-rodent mammal, comprising the steps of:

fusing cells of a non-rodent mammal to transformed, diploid, rodent cell recipients to form non-rodent mammal-rodent cell hybrids;

selecting for fused cell hybrids by selecting for a marker on each of a first non-rodent mammalian chromosome and a rodent chromosome, whereby a population of cells are formed which stably maintain one or more non-rodent mammalian chromosomes in the absence of selection for the non-rodent mammalian chromosomes;

detecting among the population of cells those cells which are haploid for a second and third non-rodent mammalian chromosome which are distinct from the first non-rodent mammalian chromosome and which were not selected.

20. The method of claim 19 wherein the rodent cell recipient is geneticin resistant.

21. The method of claim 19 wherein the rodent cell recipients are transformed with an oncogene.

22. The method of claim 21 wherein the oncogene is *ras*.

23. The method of claim 21 wherein the oncogene is *E1A*.

24. The method of claim 19 wherein the rodent cell recipients are transformed with *ras* and *E1A* oncogenes.

25. The method of claim 19 wherein the rodent cell recipients are deficient in mismatch repair.

26. The method of claim 19 wherein the rodent cell recipient contains a counterselectable marker.

27. The method of claim 26 wherein the counterselectable marker is HPRT deficiency.

28. The method of claim 19 wherein the cells of the non-rodent mammal are lymphocytes.

29. The method of claim 19 wherein the step of detecting cells which are haploid for said second and third non-rodent mammalian chromosomes is performed



by identifying the presence of a microsatellite marker on said second and third non-rodent mammalian chromosomes.

30. The method of claim 19 further comprising the step of:

detecting among the population of cells, cells haploid for a fourth non-rodent mammalian chromosome which is distinct from the first, second, and third non-rodent mammalian chromosomes and which was not selected.

31. The method of claim 19 futher comprising the step of:

detecting among the population of cells, cells haploid for a fourth, fifth, and sixth non-rodent mammalian chromosome, wherein said fourth through sixth non-rodent mammalian chromosomes are distinct from the first, second and third non-rodent mammalian chromosomes and were not selected.

32. The method of claim 19 further comprising:

testing nucleic acids of a cell haploid for the third non-rodent mammalian chromosome for a mutation in a gene on the third non-rodent mammalian chromosome.

33. The method of claim 19 further comprising:

testing proteins of a cell haploid for the second and third non-rodent mammalian chromosome for a mutation in a gene on each of the second and third non-rodent mammalian chromosomes.

34. The method of claim 1 wherein mRNA of a cell in the subset of hybrids is tested for a mutation in a gene on the second non-rodent mammalian chromosome.

35. The method of claim 1 wherein proteins of a cell in the subset of hybrids are tested for a mutation in a gene on the second non-rodent mammalian chromosome.

36. A population of rodent-non-rodent mammal hybrid cells wherein each homolog of at least 2 non-rodent mammal autosomes is present in haploid form in at least one out of one hundred of the cells.

37. The population of claim 36 wherein each homolog of at least 5 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

38. The population of claim 36 wherein each homolog of at least 5 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

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39. The population of claim 36 wherein each homolog of at least 5 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

40. The population of claim 36 wherein each homolog of at least 10 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

41. The population of claim 36 wherein each homolog of at least 10 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

42. The population of claim 36 wherein each homolog of at least 10 non-rodent mammal autosomes is present in haploid form in at least one out of a thirty of the cells.

43. The population of claim 36 wherein each homolog of at least 15 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

44. The population of claim 36 wherein each homolog of at least 15 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

45. The population of claim 36 wherein each homolog of at least 15 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

46. The population of claim 36 wherein each homolog of at least 20 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

47. The population of claim 36 wherein each homolog of at least 20 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

48. The population of claim 36 wherein each homolog of at least 20 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

49. The population of claim 36 wherein each homolog of at least 22 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred

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of the cells.

50. The population of claim 36 wherein each homolog of at least 22 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

51. The population of claim 36 wherein each homolog of at least 22 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

52. A population of rodent-non-rodent mammal hybrid cells which stably maintain their non-rodent mammalian chromosome content such that at least 95% of the hybrid cells contain the same non-rodent mammalian chromosomes.

53. The population of rodent-non-rodent mammal hybrid cells of claim 52 whererein at least 97% of the hybrid cells contain the same non-rodent mammalian chromosomes.

54. The population of rodent-non-rodent mammal hybrid cells of claim 52 whererein at least 99% of the hybrid cells contain the same non-rodent mammalian chromosomes.

55. The method of claim 1 wherein the non-rodent mammal is a human.

56. The method of claim 1 wherein the non-rodent mammal is selected from the group consisting of goats, sheep, horse, cows, pigs, hogs, cats, and dogs.

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