Detection of mutations and DNA polymorphisms using whole genome Southern Cross hybridization

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ABSTRACT

We report a general method for the detection of restriction fragment length alterations associated with mutations or polymorphisms using whole genomic DNA rather than specific cloned DNA probes. We utilized a modified Southern Cross hybridization to display the hybridization pattern of all size-separated restriction fragments from wild-type *Caenorhabditis elegans* to all the corresponding fragments in a particular mutant strain and in a distinct *C. elegans* variety. In this analysis, almost all homologous restriction fragments are the same size in both strains and result in an intense diagonal of hybridization, whereas homologous fragments that differ in size between the two strains generate an off-diagonal spot. To attenuate the contribution of repeated sequences in the genome to spurious off-diagonal spots, restriction fragments from each genome were partially resected with a 3' or 5' exonuclease and not denatured, so that only the DNA sequences at the ends of these fragments could hybridize. Off-diagonal hybridization spots were detected at the expected locations when genomic DNA from wild-type was compared to an *unc-54* mutant strain containing a 1.5 kb deletion or to a *C. elegans* variety that contains transposon insertions. We suggest that this modified Southern Cross hybridization technique could be used to identify restriction fragment length alterations associated with mutations or genome rearrangements in organisms with DNA complexities as large as 10^8 base pairs and, using rare-cutting enzymes and pulse-field gel electrophoresis, perhaps as large as mammalian genomes. This information could be used to clone fragments associated with such DNA alterations.

INTRODUCTION

In a variety of organisms, many genes have been identified only by mutation. The analysis both of genetic diseases and of induced mutations in laboratory organisms requires the molecular cloning of such genes. We have been interested in cloning genes defined

only by mutations in the nematode *Caenorhabditis elegans* (1,2). Conventional approaches for cloning genes from higher organisms, such as cDNA cloning, are not appropriate for genes that have been identified only genetically as there is no hint of the identity or, in some cases, even the existence of a corresponding protein product. To date, such mutationally-defined genes have been cloned using two techniques that demand either powerful genetic screens or fine-structure genetic mapping. First, transposons have been used to clone genes by isolating rare insertion mutations in the gene of interest and using the cloned transposon as a probe to isolate the target gene (3,4). Second, restriction fragment length polymorphisms (RFLPs) genetically-linked to genes of interest have been identified and chromosomal walking used to isolate such genes (2,5). We discuss here a third approach, using a modified genomic Southern Cross hybridization protocol (6). This protocol is general, does not demand the sophisticated genetic tools available for, e.g. *Drosophila* or *C. elegans*, and could be useful for cloning mutationally-defined genes from more genetically intractable organisms and cell lines.

MATERIALS AND METHODS

The Southern Cross hybridization protocol is outlined in Figure 1b. Genomic DNA was isolated from *C. elegans* as described by Emmons et al. (7). About 500 μ g of wild-type *C. elegans* genomic DNA was digested with *Bam*HI in $1\times$ medium salt buffer (8). After digestion, the DNAs were extracted with phenol and precipitated with ethanol. These DNAs were partially digested with exonuclease III (New England Biolabs) in $1\times$ medium salt buffer (8) using 1.3 units per μ g of DNA for 5 minutes at room temperature (9). The reaction was quenched by phenol extraction or by adding 0.1 volumes of 0.1M EDTA pH 8.0. (In pilot experiments, the extent of 3' digestion was quantitated by mixing the digested genomic DNA with *Sau*3A-digested pBR322 DNA that had been 5'-end labeled, and after exonuclease digestion, the size decrease of the denatured labeled DNA strands was determined on a DNA sequencing gel. We found that exonuclease III in these buffers resects at 10 to 30 bases per minute per unit of exonuclease III for each μ g of input DNA.) The volume of the exonuclease III-digested DNA was

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adjusted to 400 μ l, 80 μ l of 25% Ficoll, 0.01% xylene cyanol and 0.01% bromphenol blue was added, and the mixture was loaded into a single 20 cm slot on a 20 cm by 25 cm 0.8% agarose gel. 0.5 μ g of *Hind*III-digested λ DNA was added to one end of the slot for size markers, and the DNAs were electrophoretically separated for 12 to 16 hours at 5V/cm. The gel was stained with 0.5 μ g/ml ethidium bromide and photographed. DNA was transferred without denaturation to Gene Screen (Dupont, New England Nuclear) by electroblotting in 0.5 $\times$ TBE (8) at 5V/cm for 12 hours. After transfer, the DNA was cross-linked to the membrane by UV irradiation (10). This membrane could be dried, but not baked.

To label DNA, about 2 μ g *Bam*HI-digested *C. elegans* genomic DNA was treated with 1 unit of T4 DNA polymerase (New England Biolabs) without dNTPs for 30 minutes at 37° in 1 $\times$ medium salt buffer (8). Under these conditions, the 3'-exonuclease activity of T4 DNA polymerase removed about 200 to 400 bases (the extent of 3' exonuclease digestion was quantitated on a DNA sequencing gel using labeled *Sau*3A-digested pBR322 DNA, as described above). 100 picomoles of 5000 Ci/mmol 32 P-dATP at 0.3 μ M (New England Nuclear, Dupont), unlabeled dGTP, dCTP, and dTTP at 1mM, and 5 units of Klenow fragment of DNA polymerase I were added and incubation was continued for 30 minutes at 37°. Klenow fragment was probably not necessary, given that T4 DNA polymerase was also present, but it was added to ensure complete filling in of all ends. This DNA was extracted with and separated from unincorporated dNTPs by ethanol precipitation. About 5 $\times$ 10⁸ cpm were incorporated. This labeled DNA was mixed with 500 μ g of unlabeled *Bam*HI-digested DNA from the wild-type (N2) or Bergerac (RW7000) strain that had also been filled in using the Klenow fragment plus unlabeled dNTPs. This unlabeled DNA served as competitor for all restriction fragments that were unchanged between the two genomic DNAs compared in the cross hybridization (see Results for further explanation). About 500 μ g of this mixed DNA sample was partially digested with 24 units of the 5' exonuclease T7gp6 for 5 minutes at 37° in 50 mM Tris pH 8, 20 mM KCl, 1 mM DTT, and 5 mM MgCl₂ (11, enzyme from M. Cusick and C. Richardson, Boston, MA). The reaction was quenched with phenol or addition of 0.1 volume 0.1M EDTA pH 8. (In pilot experiments, *Sau*3A-digested, 3'-end-labeled pBR322 DNA was mixed with the genomic DNAs before the T7gp6 digestion, so that the extent of 5'-resection could be quantitated on a DNA sequencing gel. Under these conditions, the T7gp6 resected about 2 bases per minute per unit for 500 μ g of genomic DNA in the reaction. λ exonuclease, another 5' exonuclease, was found to be unsatisfactory, because it is a processive enzyme and did not resect all restriction fragments as synchronously as did T7gp6, a distributive enzyme (9, 11)). The mixture of labeled and unlabeled 5'-resected DNAs were suspended in 400 μ l 10 mM Tris pH 8, 1 mM EDTA pH 8.0, and 80 μ l of 25% Ficoll, 0.01% xylene cyanol, 0.01% bromphenol blue was added, and the DNA was loaded onto a single 20 cm slot of a large 20 cm by 25 cm 0.8% agarose gel. The gel was run at 5 V/cm for 12 to 16 hours and the DNAs were transferred without denaturation to Gene Screen by electroblotting in 0.5 $\times$ TBE for 12 hours at 5V/cm. The membrane with the attached restriction fragments could be stored for at least one day in 0.5 $\times$ TBE without significant loss of bound counts until the Southern Cross hybridization assembly was constructed.

To construct the hybridization assembly, the labeled membrane equilibrated in 0.5 $\times$ TBE was laid onto a glass DNA sequencing

plate and all bubbles were rolled out with a 10 ml pipette. A blank piece of Gene Screen also equilibrated in 0.5 $\times$ TBE was laid over the membrane with the labeled DNA fragments and all bubbles removed as above. The membrane(s) containing the unlabeled exonuclease III-resected DNA was prehybridized for 4 to 12 hours at 65° in 25% Dextran sulphate, 4 $\times$ SSPE, 10 $\times$ Denhardt's solution, 0.1% SDS, 0.1% sodium pyrophosphate, 10% Formamide, 200 μ g calf thymus DNA (pH 7.8). (The high concentration of dextran sulphate was used to accelerate the rate of hybridization (12), because the actual concentration of particular DNA fragments at each point of intersection in a Southern Cross hybridization was estimated to be about 10⁴ lower than in traditional Southern hybridization with specific cloned DNA probes.) This membrane was laid over the blank membrane and oriented perpendicular to the array of the labeled DNA bands on the other membrane. Up to 10 other membranes containing unlabeled DNAs could also be overlaid similarly. The stack was then 'rolled' with a 10 ml pipette to extrude all excess hybridization buffer from between the membranes. Finally, a piece of Whatman 540 paper soaked in the same hybridization/prehybridization buffer was laid onto the stack, and another glass DNA sequencing plate was placed on top. The assembly was clamped together and incubated without any agitation at 65° for 72 hours. The stack was disassembled and the unlabeled membranes were washed in 3 liters 2 $\times$ SSPE, 1% SDS at 65° with multiple changes for 1 hour and 6 liters of 0.2 $\times$ SSPE, 1% SDS at 65° with multiple changes for 2 hours. Autoradiography was done at -70° with an intensifying screen.

RESULTS

Rationale

Figure 1a shows schematically how Southern Cross hybridization (6) can detect a single restriction fragment length difference between two otherwise identical genomes. In short, DNA from one genome is digested with a restriction enzyme, labeled, separated electrophoretically on an agarose gel with a single wide lane, denatured, and transferred reversibly to a membrane. DNA from the other genome is digested with the same restriction enzyme, separated electrophoretically, denatured, and transferred irreversibly to a second membrane. These two membranes are then stacked upon on another with the arrays of fragments oriented perpendicularly so that every restriction fragment from one genome intersects every fragment from the other genome, and hybridization is allowed to proceed *in situ*. During the incubation, the labeled DNA dissociates from the membrane and diffuses the short distance to the unlabeled bound DNA; if these DNAs are homologous they hybridize. Autoradiography of the target membrane after washing reveals that almost all fragments are of equivalent size in the two strains and hybridize along a diagonal line; however, any restriction fragment length difference between mutant and wild type will appear as an asymmetric off-diagonal spot.

Repeated DNA sequence elements in genomes make this simple approach useless for higher eukaryotes: for example, a twenty-fold [n-fold] repetitive element would reveal 380 [n(n-1)] symmetrical off-diagonal spots on such a denatured Southern Cross hybridization due to *inter se* hybridization of repeated elements in the genome. These off-diagonal spots would confound the detection of asymmetric off-diagonal spots caused by mutation. We therefore modified the Southern Cross hybridization protocol of Potter and Dressler (6) so that repeated elements in the genome were less likely to be single-stranded during the

Figure 1: a. Detection using Southern Cross hybridization of a 1.5 kb deletion in a hypothetical genome with four restriction fragments. A Southern hybridization membrane with bands of labeled restriction fragments from the mutant genome extending across the full width of the membrane is laid perpendicular to a similar membrane to which bands of unlabeled restriction fragments from the wild-type genome have been crosslinked. The labeled fragments are allowed to diffuse and hybridize to the unlabeled fragments that are homologous. Shown is the hypothetical pattern of hybridization to this unlabeled membrane. Particular restriction fragments from the wild-type genome (electrophoretically separated top to bottom and shown by the thick horizontal lines) hybridize to the corresponding labeled restriction fragments from the mutant genome (electrophoretically separated left to right and shown by the thin vertical lines): an off-diagonal spot at (3 kb in wild type, 1.5 kb in mutant) is observed along with the diagonal spots of (4 kb, 4 kb), (2 kb, 2 kb), and (1 kb, 1 kb) unchanged in these strains.

b. Diagram of the steps used to prepare partially resected labeled and unlabeled restriction fragments for Southern Cross hybridization. Preparation of the partially single-stranded unlabeled restriction fragments is shown in the left panel. Preparation of the partially single-stranded labeled restriction fragments is shown in the right panel. Labeled regions are shown as thick lines and unlabeled regions as thin lines. 3' ends are denoted by an arrow→. 5' ends are denoted by the symbol 5'. See Materials and Methods for details.

hybridization and therefore less likely to hybridize to otherwise non-homologous fragments and generate spurious off-diagonal signals. By partially digesting restriction fragments with strand-specific exonucleases and never denaturing these DNAs during the processing of the Southern transfers, only about 200 bases at each end of every genomic restriction fragment was single-stranded and thus available for hybridization in a Southern Cross experiment (Figure 1b). Such a procedure decreased the background of inter-repeated sequence hybridization because of the lower probability of exposing a repeated element to hybridization when only the ends of restriction fragments are single-stranded (assuming the restriction enzyme used does not cut in the repeat). For example, if the weight-average size of restriction fragments is 20 kb and the single-stranded ends are each 200 bases long, a particular repeated sequence has a probability of $(0.2 \text{ kb} + 0.2 \text{ kb})/20 \text{ kb} = 0.02$ of being exposed on any one restriction fragment, and a probability of $(0.02)^2 = 0.0004$ of being exposed on two distinct restriction fragments that would hybridize to generate two off-diagonal spots. Thus, the twenty-fold repeat family described above would most likely generate no off-diagonal spots using this modified protocol (average number of off-diagonal spots would be $0.0004 \times 380 = 0.15$).

Detecting a deletion in the *C. elegans unc-54* gene

To test this approach, we performed this modified Southern Cross hybridization protocol to compare two genomes with one known restriction fragment difference. The *C. elegans* mutant strain

Figure 2: The 1.5 kb deletion in the myosin gene used to test the Southern Cross hybridization protocol. Autoradiograph of Southern hybridization of ³²P-cloned *unc-54* myosin heavy chain gene λ23-10 (13) to BamHI-digested wild-type DNA in lane 1 and to BamHI-digested *unc-54(r241)* strain DNA in lane 2. The 5.5 kb BamHI fragment in the wild type (lane 1) is replaced by a 4.0 kb BamHI fragment in the *unc-54(r241)* mutant strain (lane 2). Other hybridization bands of 4.8 kb and 2.8 kb are the flanking BamHI fragments homologous to the probe.

TR287 (genotype *unc-54(r241)*) contains a 1.5 kb partial deletion in the myosin heavy chain gene, resulting in a 4.0 kb rather than 5.5 kb *Bam*HI fragment (Figure 2)(P. Anderson, personal communication). This difference between the wild type and TR287 genomes should be visible if all restriction fragments are compared. We attempted to visualize this restriction fragment length alteration in a Southern Cross hybridization performed as described in Figure 1b. About 500 μ g DNA from wild-type *C. elegans* strain N2 was digested to completion with *Bam*HI. This DNA was then partially digested with the 3' exonuclease Exonuclease III under conditions that resect only about 200–400 bases from all restriction fragments (9, see Materials and Methods). This unlabeled DNA was single-stranded only at the 5' ends of each *Bam*HI fragment; the majority of the genome remained double-stranded and therefore was not hybridizable. This DNA was loaded onto an agarose gel with a single 20 cm slot, and the resulting restriction fragments were electrophoretically separated and transferred without denaturation by electroblotting to a nylon membrane. The partially single-stranded restriction fragments were irreversibly bound to this membrane with UV light (10). This membrane was prehybridized for 4 to 16 hours in a modified hybridization/prehybridization buffer (see Materials and Methods).

The radioactive probe was made from genomic DNA isolated from the *unc-54(r241)* deletion mutant strain TR287. About 2 μ g of this DNA was digested with the restriction enzyme *Bam*HI, and all resulting restriction fragments were 32 P-labeled at their 3' ends to high specific activity using T4 DNA polymerase and the Klenow fragment of DNA polymerase I (see Materials and Methods). This labeled DNA was then mixed with 500 μ g (250-fold excess) of unlabeled, *Bam*HI-digested wild-type N2 DNA, and both DNAs were partially resected about 200 bases at their 5' ends using the 5' exonuclease T7 gene product 6 (T7gp6) (11). Thus only the 200 bases at each 3' end of these restriction fragments were single-stranded and hybridizable. This set of single-stranded restriction fragment ends was the complement of the set constructed using *Bam*HI and exonuclease III on the unlabeled DNA. The labeled DNA was loaded onto an agarose gel with a single 20 cm slot, electrophoretically separated, and transferred without denaturation onto a membrane by electroblotting. This DNA was not UV-crosslinked to the membrane. This membrane was not treated with any prehybridization solution and was not allowed to dry before assembling the Southern Cross hybridization.

The majority of labeled DNA fragments from the *unc-54(r241)* genome migrated coincidentally with the homologous and 250x more abundant unlabeled fragment from the wild-type genome; only those labeled fragments that are different sizes in the mutant and wild-type, i.e. the fragment containing the 1.5 kb *unc-54* deletion mutation, should migrate separately. Thus, after electrophoresis the specific activity of each labeled restriction fragment that is the same size in the two strains was 250-fold less than that of any fragment with a size difference. This competitor DNA attenuated the intense diagonal and remaining spurious signals due to repeated elements by about a factor of about 250, but did not decrease the specific activity of any restriction fragment that differs in size between the wild-type and mutant genomes.

The membrane bearing the 32 P-labeled *unc-54(r241)* strain genomic DNA was then laid onto the prehybridized unlabeled membrane with the bands on one membrane oriented perpendicularly to the bands on the other membrane. This

assembly was incubated at 65° in a modified DNA hybridization buffer (see Materials and Methods) to allow DNA:DNA hybridization of these very dilute fragments to proceed *in situ* with no circulation of this buffer. After 72 hours of diffusion and hybridization, the stack of membranes was disassembled and the membranes were washed extensively under moderately stringent conditions (see Materials and Methods).

After autoradiography, a weak but reproducible off-diagonal spot was detected in the position predicted for the mutation, i.e. at 5.5 kb in the wild-type dimension and at 4.0 kb in the myosin mutant dimension (5.5 kb, 4.0 kb) (Figure 3a). The identity of this off-diagonal spot was confirmed by reprobing the same membrane with 32 P-labeled cloned myosin gene probe (λ 23-10)(13) and observing that the 5.5 kb band of hybridization intersected the off-diagonal spot (see Figure 3b). The only other off-diagonal spots on the autoradiograph were symmetric about the diagonal and are probably associated with the 1.05 kb 5S gene tandem array (14): these spots are symmetric about the bright (1.05 kb, 1.05 kb) spot where the multiple 5S gene restriction fragments comigrate and hybridize. Most but not all of the 5S genes contain a *Bam*HI site, thus accounting for the observed weaker hybridization of 1.05 kb to 2.1 kb and larger fragments, and even weaker hybridization of 2.1 kb to 3.15 kb fragments, etc. which could generate symmetrical off-diagonal spots observed at multiples of 1.05 kb.

Evidence confirming that a 4.0 kb *Bam*HI fragment from the myosin mutant strain TR287 hybridized to a 5.5 kb *Bam*HI fragment from the wild-type *unc-54* gene was obtained from the experiment shown in Figure 3c. In this experiment, 1 μ g of a λ -phage containing the wild-type myosin gene, phage λ 23-10, was added to 100 μ g wild-type DNA in the unlabeled dimension, and Southern Cross hybridized to labeled *unc-54(r241)* DNA as above. In this experiment, the off-diagonal spot at the expected location is more easily observed because the myosin gene was 'doped' to about 20 times the level of other unique gene sequences in the unlabeled genomic DNA.

Detecting polymorphisms between the *C. elegans* Bristol and Bergerac strains

A second experiment to detect known restriction fragment length differences using genomic DNAs involved a Southern Cross hybridization of the genomic DNAs from two interbreeding varieties of *C. elegans*. These two varieties, called Bristol and Bergerac, differ from each other at about 400 loci due to the presence in the Bergerac strain of about 400 randomly dispersed insertions of the 1.6 kb transposon Tc1 and in the Bristol strain of only 30 Tc1 copies (7). Thus by comparing these strains, we expected to observe a series of off-diagonal spots along a second diagonal (with coordinates n kb in Bristol and n + 1.6 kb in Bergerac) displaced by 1.6 kb in the Bergerac dimension from the main diagonal (co-ordinates n kb in Bristol, n kb in Bergerac). About 2 μ g of genomic DNA from the Bristol strain TR287 was *Bam*HI-digested and 3'-end-labeled as above. This labeled DNA was mixed with 500 μ g of *Bam*HI-digested genomic DNA isolated from the Bergerac variety RW7000 (15), and the mixture of DNAs was partially resected with the 5' exonuclease T7gp6, as described above. The partially single-stranded restriction fragments were separated by electrophoresis and transferred reversibly to a membrane as above. About 500 μ g of Bergerac genomic DNA was *Bam*HI-digested, partially resected with exonuclease III, and the resulting fragments were electrophoretically separated, transferred and UV-crosslinked to

Figure 3a: Autoradiograph of a Southern Cross hybridization of ^{32}P -labeled, *Bam*HI-digested, partially 5'-resected DNA isolated from the *unc-54(r241)* strain TR287, containing a 1.5 kb deletion in the *unc-54* gene (electrophoretically separated and oriented left to right as shown), to *Bam*HI-digested, partially 3'-resected wild-type strain N2 DNA (electrophoretically separated top to bottom as shown). The intense diagonal corresponds to all restriction fragments that are equivalent in the two strains. The asymmetric off-diagonal spot due to the myosin deletion (5.5 kb in the wild type, 4.0 kb in *unc-54(r241)*) is marked by the arrow. Two weak symmetric off-diagonal spots at multiples of 1.05 kb are probably caused by sequence divergence in a subset of the tandemly repeated 5S gene sequences (see text) (14). **Figure 3b:** Autoradiograph of the same membrane shown in Figure 3a subsequently hybridized to ^{32}P -labeled *unc-54* myosin heavy chain gene clone λ 23-10 (13). The hybridization band at 5.5 kb (marked by arrow) in the wild-type DNA dimension (electrophoretically separated top to bottom as shown) intersects the off-diagonal spot at (5.5 kb in wild type, 4.0 kb in *unc-54(r241)*) shown in Figure 3a. Other hybridization bands of 4.8 kb and 2.8 kb correspond to adjacent *Bam*HI fragments homologous to the 15 kb cloned segment in λ 23-10. The hybridization bands to the far right correspond to λ DNA digested with *Hind*III size markers that hybridized to the vector sequences in λ 23-10. **Figure 3c:** Autoradiograph of a membrane containing *Bam*HI-digested, partially 3'-resected wild-type DNA 'doped' with 20-fold molar excess of *Bam*HI-digested, partially 3'-resected myosin clone λ 23-10 DNA (13) Southern Cross hybridized to labeled *unc-54(r241)* mutant DNA as above. The off-diagonal spot at 5.5 kb in the wild type and 4.0 kb in the *unc-54(r241)* strain is denoted with an arrow. Because the probe DNA was more extensively resected with T7gp6 in this experiment (data not shown), the diagonal and the off-diagonal spot are broadened in the T7gp6-digested labeled DNA dimension only (shown left to right).

a membrane. These two membranes were stacked onto each other perpendicularly as above and allowed to hybridize for 72 hours.

As shown in Figure 4, the expected second diagonal is visible. However, this diagonal does not appear to be composed of 400 spots, but rather of considerably fewer (approximately 25 spots). One plausible reason that all dimorphic Tc1 elements were not

visible in this experiment is that 250-fold excess unlabeled Bergerac competitor DNA was mixed with the labeled Bristol DNA before electrophoresis. Many individual Tc1-containing restriction fragments in Bergerac have been shown to somatically excise the inserted Tc1 element (7, G. Ruvkun, unpublished observations) to the extent that about 10 to 30% of each of these

Figure 4: Autoradiograph of a Southern Cross hybridization of ^{32}P -labeled *Bam*HI-digested, partially 5'-resected DNA isolated from the Bristol strain TR287 (electrophoretically separated left to right as shown) to *Bam*HI-digested, partially 3'-resected DNA isolated from the Bergerac strain (RW7000) (electrophoretically separated top to bottom as shown). The intense diagonal at n kb, n kb corresponds to all restriction fragments that are equivalent in the two strains. The second diagonal at $n + 1.6$ kb in Bergerac and n kb in Bristol is marked with arrows and identifies multiple polymorphic restriction fragments containing the transposon Tc1 in Bergerac but not Bristol. In addition to the $(n + 1.6$ kb, n kb) displaced diagonal, other asymmetric off-diagonal spots are visible. Because the Bergerac strain is highly polymorphic relative to the Bristol strain (2), these additional off-diagonal spots could correspond to other non-Tc1-associated polymorphisms. In one case, shown with open arrows, two off-diagonal spots at 2.0 kb and 0.8 kb in the Bergerac dimension both appear to be detected by a 2.8 kb restriction fragment in the Bristol dimension; the spots form a vertical line, and the sizes of the two spots in Bergerac add up to the size of the detecting Bristol restriction fragment. This pattern is expected for restriction site variation.

fragments in Bergerac genomic DNA have lost the Tc1 and will therefore comigrate with the corresponding 1.6 kb smaller Bristol fragments. Thus, on the labeled membrane, the labeled Bristol DNA fragments containing no Tc1 insertions comigrated during electrophoresis with up to 75-fold excess unlabeled Bergerac-derived DNA fragments for those Tc1 dimorphic sites that show a high level of excision. This excess unlabeled DNA decreased the specific activity of those Tc1-dimorphic restriction fragments. Those off-diagonal spots we observe in this experiment may be unique Tc1 dimorphic sites that do not somatically excise (or do so at low frequencies). A second possible reason for detecting so few spots on the $(n + 1.6$ kb, n kb) diagonal may be that under the conditions of this experiment, only those off-diagonal spots generated by comigrating Tc1-dimorphic restriction fragments could be detected. We believe that this alternative is unlikely, because the conditions under which this experiment was done were very similar to those that detected the *unc-54* mutation above, so that all 400 spots should have been detected, and because the pattern of spots on the $n + 1.6$ kb, n kb diagonal

does not correspond to the pattern of comigrating Tc1 hybridization bands on a Southern blot of *Bam*HI-digested Bergerac DNA (data not shown). Other spots marked by arrows on the autoradiograph may reveal additional non-Tc1 associated polymorphisms between Bristol and Bergerac strains (see Figure 4).

DISCUSSION

We have shown here that using a modified Southern Cross hybridization protocol, particular deletion and insertion mutations can be detected in *C. elegans* without using a cloned DNA probe. While repeated sequences are present in the *C. elegans* genome (7), they did not contribute to a high background of symmetrical off-diagonal spots presumably because they were not exposed for hybridization by the partial exonuclease treatments. The genome of *C. elegans* is about 10^8 bp (A. Coulson and J. Sulston, personal communication) and 34% G+C (16), so that restriction digestion with *Bam*HI generates about 10^4 different fragments. If only one of these fragments is affected in a mutant strain, then the potential signal to be detected in a Southern Cross hybridization is only one part in 10^4 of the input label. Since in a typical experiment the total incorporation is about 5×10^8 cpm, so that about 5×10^4 cpm is incorporated into any one fragment. These counts are distributed over a 20 cm long band due to the wide slot on the agarose gel. Since each band on a gel is about 1 mm across, only 0.5%, or 250 cpm, of the band can potentially hybridize to an intersecting target sequence. The signal in the myosin deletion vs. wild-type off-diagonal spot in Figure 3a was about 25 cpm, or about 10% of this possible signal. The efficiency of signal detection is a function of the concentrations, diffusion constants, and relative amounts of the homologous DNA fragments. We used a 250-fold excess of the unlabeled DNA on the target membrane over the labeled DNA on the source membrane to drive the hybridization and decrease the time necessary to complete hybridization by a factor of 250 relative to that if equal concentrations of probe and target had been used. Even so, the efficiency of hybridization was only 10%. A higher concentration of dextran sulphate or phenol-enhanced hybridization (17) could be used to increase this efficiency.

The off-diagonal signal was about twice background. The background is presumably due to radiolysis of the labeled DNA fragments during electrophoresis that distributes ^{32}P -oligonucleotides throughout the gel and membrane. If ^{35}S -labeled dNTPs were used, such strand scissions due to radiolysis would not occur, and the background might decrease. While one could lower the amount of DNA on the unlabeled membrane to decrease the background, this modification would probably also cause a proportionate decrease in the rate of hybridization of the signal spot.

For organisms with a DNA complexity equal to or less than that of *C. elegans*, e.g. yeast, fungi, bacteria, *Arabidopsis* etc., the modified Southern Cross hybridization protocol should be useful in identifying mutation-associated DNA alterations that change the size of unknown restriction fragments. Because X-ray and gamma-ray induced mutations are likely to cause a change in the size of a restriction fragment (2,4), such alleles would be appropriate to screen in this way. This protocol would be especially useful for detecting mutations in genetically intractable organisms and cell cultures. In addition, genomic DNAs from different tissues, cell types, or physiological states could be compared using Southern Cross hybridization to detect genome

rearrangements that occur during development or as a function of physiological state (6,18,19,20,21).

The exonuclease resection and non-denaturing membrane transfer techniques we describe here can also be used in a conventional Southern hybridization experiment to expose only the ends of DNA fragments to hybridization, and thus reduce the effects of repetitive element hybridization. Thus if both the DNA probe and the genomic DNA on a Southern blot are partially exonuclease resected after digestion with the same restriction endonuclease, the genomic DNA fragment containing single-stranded sequences homologous to the single-stranded sequences in the probe will be detected but only a small percentage of the rest of the genome would be available for hybridization. In this way, large DNA fragments could be used to search for RFLP loci in for example mouse or humans without the usual worry about repetitive sequences causing spurious hybridization bands.

If human or mouse DNA were analyzed using the Southern Cross hybridization protocol described here to detect mutations or genome rearrangements, the ratio of potential signal in one particular restriction fragment to all other labeled fragments would be about one part in 3×10^5 for most 6 bp recognition site enzymes. This signal could not be detected unless additional techniques were developed to decrease the background hybridization we consistently have observed. Also, the presence of extremely repetitive interspersed elements (Alu or Kpn repeats (22)) in mammalian DNAs might generate many spurious off-diagonal spots that would interfere with the detection of mutation-associated or rearrangement-associated off-diagonal spots, even using our exonuclease resection techniques. However using restriction enzymes with 8 bp recognition sites, pulsed field gel electrophoresis (23) to separate these much larger DNA fragments, and the exonuclease resection techniques described here, it may be possible to use the Southern Cross hybridization protocol to detect some mutations in mammalian genomes. The number of fragments generated in such an experiment would be 16-fold fewer and those fragments generated 16-fold longer, so that a repetitive element would be 256-fold less likely than in the case of 6 bp recognition site enzymes to be exposed on both labeled and unlabeled DNA fragments after partial exonuclease resection. While the use of larger fragments would leave most small deletions undetected, translocations and large deletions should be detectable, even in a heterozygous state. Such gross chromosome aberrations have been seen in many tumors (24), and the restriction fragments that contain the breakpoints of these aberrations could be detected by comparing tumor DNA to non-tumor DNA in a Southern Cross hybridization. Such breakpoints could identify oncogenes.

Southern Cross hybridization analysis of genomic DNAs from non-inbred organisms such as humans would be complicated by the many genetic polymorphisms between individuals and even within a normally multiply heterozygous outbred individual. Thus many DNA fragments would be polymorphic and generate off-diagonal spots in a Southern Cross hybridization. In the case of analyzing human tumor DNAs, adding excess unlabeled non-tumor competitor DNA to labeled tumor DNA in the labeled dimension would decrease the signals from the polymorphism-associated off-diagonal spots relative to the mutation-associated spots. Furthermore, if large DNA fragments generated by 8 bp recognition site restriction enzymes were compared between, for example, human tumors and normal non-tumorous tissue from the same individual, it is likely that much less such spurious

polymorphism due to heterozygosity would be detected; most point mutations and small insertions and deletions would not detectably change these large fragments whereas chromosome aberrations would. Similarly, DNA alterations associated with other genetic diseases could be detected by Southern Cross hybridization of DNAs isolated from affected and unaffected individuals.

One could use the information derived from a Southern Cross hybridization experiment to clone the restriction fragment(s) altered by the mutation. For example, if the coordinates of an off-diagonal spot reveal that the gene of interest is on a 6 kb *Bam*HI fragment in the wild type and on a 2 kb *Bam*HI fragment in the mutant, a library could be constructed from the wild-type DNA 6 kb *Bam*HI fragment size fraction and screened with labeled 2 kb *Bam*HI fragment size fraction from the mutant strain. (The exonuclease resection technique could be used to make a partially single-stranded DNA probe to decrease the probability of hybridizing to repeated elements.) Positive-hybridizing clones would be expected to include a 6 kb restriction fragment that hybridizes to a 2 Kb *Bam*HI fragment in mutant genomic DNA and a 6 Kb *Bam*HI fragment in wild-type genomic DNA.

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