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Determination of Transgenic Loci by Expression FISH

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DNA targeting by homologous recombination in mouse embryonic stem (ES) cells has become a widely used method for manipulating the mouse genome and for studying the role of specific genes in mammalian development. For certain studies, it is necessary to target two or more DNA sequences residing on a particular chromosome. In these situations, it would be important to distinguish whether two sequential gene targeting events in the ES cells have occurred *in cis* or *in trans.* We report here a new application of fluorescence *in situ* hybridization to RNA molecules present at sites of transcription that allows the identification of *cis* and *trans* gene targeting events in ES cells. The method is based on detection of transcripts from commonly used selectable marker genes inserted during homologous recombination. Transcripts are detected in interphase nuclei, making the preparation of mitotic cells unnecessary and obviating the necessity for the more technically demanding DNA detection of genes. The method is applicable to any chromosomal locus, and compared with other methods (e.g., genetic linkage testing in chimeric mice), it will greatly shorten the time required for distinguishing *cis* and *trans* gene targeting events in ES cells. The method also may be useful for detecting changes in ploidy of individual chromosomes and loss of heterozygosity of genes in single cells in culture and also in animals, for example, during processes such as tumorigenesis. © 2001 Academic Press

INTRODUCTION

The development of techniques for manipulating the mouse genome by targeting specific DNA sequences in mouse embryonic stem (ES) cells, which can then be used to generate strains of mice, is advancing rapidly (Kuhn and Schwenk, 1997). Early applications of DNA targeting in ES cells were focused on inactivating single genes in mice (Shastry, 1998). Results from such studies suggested in many instances the existence of functional redundancy or compensation among members of gene families (Chambon, 1994; Erickson, 1993; Rudnicki and Jaenisch, 1995; Sirotkin *et al.,* 1995). To investigate further the role of such gene families in development or other processes, it becomes necessary to inactivate two or more genes in the family, often residing in a tightly linked segment of DNA at a particular chromosomal locus (Cocea *et al.,* 1999; van Ree *et al.,* 1995; Wang *et al.,* 1997). This can be accomplished by sequential gene inactivation in the ES cells, but if the genes are tightly linked, only targeting events that have occurred *in cis* are practical for generating compound mutant animals. Therefore it would be very useful to be able to distinguish between *cis* and *trans* gene targeting events in the ES cells.

An alternative approach for inactivating two or more genes in the ES cells is to produce a defined deletion in the ES cells encompassing the genes. The introduction of foreign site-specific recombination systems, especially the bacteriophage P1 Cre–*loxP* system (Sauer and Henderson, 1988), into mammalian cells has enabled the production of deletions and other types of chromosomal rearrangements in ES cells and mice (Zheng *et al.,* 2000). These types of genomic manipulations have numerous applications in mouse genetics, and they are expected to revolutionize genetic approaches to studying mouse biology. Use of the Cre–*loxP* system for manipulating large (>10 kb) segments of the ES cell genome usually requires integration of two (or more) *loxP* sites at defined positions in the genome by sequential gene targeting events. Once again, for this application, it is important to know whether the targeting events have occurred *in cis* or *in trans.*

The studies reported here were designed to determine whether fluorescence *in situ* hybridization (FISH) to nascent RNA transcripts could be used to identify *cis* and *trans* gene targeting events in ES cells. Because the selection of the targeted ES clones requires that they express the drug resistance markers present in the targeting vectors, we used the site of transcription of these genes to delineate their location and activity simultaneously. Our previous work (Femino *et al.,*

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1998) has demonstrated that oligonucleotide probes can be used with exquisite sensitivity to determine transcriptional activity of specific genes and that the intensity of signal provided by the natural amplification of sequences at the site of transcription provides a simple but effective strategy for localizing expressed genes.

MATERIALS AND METHODS

PCR analysis of mouse genotypes. To test the genotype of mice, PCR was performed on mouse tail DNA with the following primers: H1a null allele (H1a-Hygro): H1a 5′ sequence-specific primer (Pa3, 5′-GAACTGCAGTTGAGGCTAAGG-3′) and the PGK-Hygro gene-specific primer (PGK3, 5′-GAGGAGTAGAAGGTGGCGC-3′), predicted band size, 1.4 kb; H1t null allele (H1t-Neo): H1t 5′ sequence-specific primer (Pt1, 5′-GCCACATATGTAGAAGACCCC-3′) and the PGK-Neo gene specific primer (Pnf, 5′-TTTGAATGGAAGGATTG-GAG-3′), predicted band size, 660 bp; H1c null allele (H1c-Hygro): H1c 3′ sequence-specific primer (Pcr, 5′-GAGCATAGAAGCCAC-CTACAAG-3′) and the PGK-Hygro gene specific primer (pGK2, 5′-GCTGCTAAAGCGCATGCTCCA-3′), predicted band size, 400 bp; H1e null allele (H1e-Neo): H1e 5′ sequence-specific primer (Per, 5′-CCCTTAAAAGTTATCGACGTGG-3′) and the PGK-Neo gene-specific primer (Pnf, 5′-TTTGAATGGAAGGATTGGAG-3′); predicted band size, 650 bp.

Probe synthesis. Oligonucleotide probes consisting of 50 nucleotides each were designed complementary to five separate regions of hygromycin and neomycin mRNA, synthesized (Model 394, Applied Biosystems) to contain amino-modified thymidine (Glen Research), and then purified through an Oligonucleotide Purification Cartridge (Perkin–Elmer). The pure probes were then directly labeled with either Oregon green (Molecular Probes) or Cy3 (Amersham) fluorochromes in 0.1 M Na_2CO_3 (pH 9.0) or dimethyl sulfoxide overnight and then further purified through a Sephadex G-50 (Sigma) column.

Probes were as follows: HYGRO 1, 5′-CGGCGGGAGATGCAAT-AGGTCAGGCTCTCGCTGAATTCCCCAATGTCAAG-3′; HYGRO 2, 5′-TGCAGAACAGCGGGCAGTTCGGTTTCAGGCAGGTCTTGCAA-CGTGACACC-3′; HYGRO 3, 5′-CAACCACGGCCTCCAGAAGAA-GATGTTGGCGACCTCGTATTGGGAATCCC-3′; HYGRO 4, 5′-CCCTGCGCCCAAGCTGCATCATCGAAATTGCCGTCAAC-CAAGCTCTGATA-3′; HYGRO 5, 5′-TCGGTTTCCACTATCGGC-GAGTACTTCTACACAGCCATCGGTCCAGACGG-3′; NEO 1, 5′-GATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCT-CCACCCAAGCGG-3′; NEO 2, 5′-AGTCCCTTCCCGCTTCAGTGA-CAACGTCGAGCACAGCTGCGCAAGGAACG-3′; NEO 3, 5′-TCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTG-AGATGACAGGAG-3′; NEO 4, 5′-CAGCCCATTCGCCGCCAA-GCTCTTCAGCAATATCACGGGTAGCCAACGCT-3′; NEO 5, 5′-GAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGC-TGCGAATCGGGAG-3′.

In situ hybridization. ES cell lines were grown as described (Ramirez-Solis *et al.,* 1993) on coverslips coated with 0.5% gelatin. The cultures were coded by Y.F. and the *in situ* hybridization was carried out by S.A.B. The code was broken only after the image acquisition and analysis were completed. After the cultures had reached about 40% confluency, they were fixed in 4% paraformaldehyde/PBS for 30 min, washed with 1× PBS/5 mM $MgCl_2$ three times for 10 min each at room temperature, dehydrated, and stored overnight in 70% ethanol at 4°C. The cells were then extracted in acetone for 75 min at room temperature, returned to ethanol, rehydrated, and rinsed in 1× PBS/5 mM $MgCl_2$ twice at room temperature for 10 min. The cells were permeabilized with 0.5% Triton/PBS, rinsed with PBS/$MgCl_2$, and pretreated in 50% formamide/2× SSC for 10 min at room temperature. Coverslips were inverted (cell side down) onto parafilm-covered glass plates over 20 μl of hybridization mixture (20 ng probe mixture, 2.5 μg competitor DNA/tRNA, 20% DEPC water, 10% BSA (Roche Molecular Biochemicals), 10% vanadyl ribonucleoside complex (Gibco BRL), 10% 20× SSC, 50% formamide), sealed with parafilm, and incubated for 3 h at 37°C. Coverslips were washed twice in 50% formamide/2× SSC for 20 min at 37°C, followed by washing further for 10 min each (at room temperature) in 2× SSC, 1× SSC, and 1× PBS/5 mM $MgCl_2$. Nuclei were stained for 1 min using a 1:20,000 dilution of DAPI (Sigma) in 1× PBS and rinsed with PBS/$MgCl_2$ for 10 min. Coverslips were then mounted onto glass slides using an anti-fading mounting medium (100 mg *p*-phenylenediamine (Sigma), 10 ml of 10× PBS, 90 ml glycerol (pH 8.0)), sealed, and stored at −20°C. Further details of hybridization procedures are available at www.singerlab.org.

Image acquisition and analysis. Images were captured using Esprit software (LSR, Cambridge, UK) with an UltraPix FX1400 12-bit, cooled CCD camera (LSR) mounted on a BX60 fluorescence microscope (Olympus, Melville, NY) with a PlanApo 60X, 1.4 NA objective (Olympus) and HiQ bandpass filters (Chroma Technologies, Brattleboro, VT). Images were acquired in the DAPI, FITC, and Cy3 channels and then superimposed using Photoshop software (Adobe, San Jose, CA).

RESULTS AND DISCUSSION

The strategy for determining whether two sequential gene targeting events in ES cell clones have occurred *in cis* or *in trans* consisted of: (i) synthesizing two sets of antisense oligonucleotide probes to adjacent sequences in the RNA transcripts encoded by each of the two selectable marker genes (Neo and Hygro) that were inserted in the cells during gene targeting. Because the cells were selected to be doubly resistant for growth in G418 and hygromycin, both selectable marker genes must be expressed in the cells; (ii) conjugating different fluorochromes to specific sites on each set of oligonucleotide probes so that the transcripts from each selectable marker gene can be detected and distinguished from each other; (iii) hybridizing a mixture of the two sets of probes to a growing population of cells that were fixed in formaldehyde on coverslips; (iv) visualizing the transcription sites within nuclei of each clone and evaluating whether the signals colocalize (*cis* orientation) or do not colocalize (*trans* orientation).

To test this strategy, we utilized three mouse ES cell clones in which two different members of the H1 histone gene family residing on mouse chromosome 13 had been targeted sequentially by two independent homologous recombination events. Six of the seven known mouse H1 histone genes are located within a 650-kb segment of DNA in MMU13 A2-3 (Wang *et al.,* 1997). WTA-D18 and WTA-D32 are two ES clones in which the H1t and H1a histone genes have been targeted with vectors containing the PGK-Neo and the PGK-Hygro genes, respectively. The H1t and H1a genes are estimated to be separated by 50 kb (Wang *et al.,* 1997). The WTA-D18 and WTA-D32 clones were derived by two independent transfection experiments: (i) WW6 ES cells were transfected with an H1t targeting vector, clones were selected for resistance to G418, and clones that had undergone homologous recombination at the H1t locus were identified by Southern blotting; (ii) clone WT 12 from the first transfection was expanded and transfected with an H1a targeting vector, clones were selected for resistance to hygromycin,

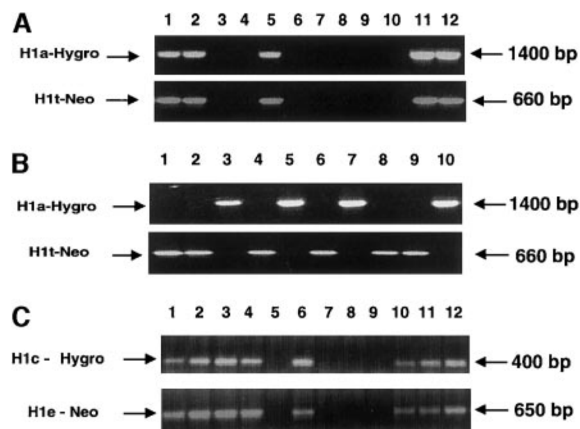

FIG. 1. Genotype analysis of progeny of chimeric mice. (**A**) Genotype analysis of agouti progeny of a WTA-D32-derived chimera. Mouse tail DNA was analyzed by PCR as described under Materials and Methods for the null H1a allele (H1a-Hygro) and the null H1t allele (H1t-Neo). (**B**) Genotype analysis of agouti progeny of a WTA-D18-derived chimera, utilizing the same analysis as in **A**). (**C**) Genotype analysis of agouti progeny of a WEC9.6-derived chimera. Mouse tail DNA was analyzed by PCR as described under Materials and Methods for the null H1c allele (H1c-Hygro) and the null H1e allele (H1e-Neo).

and clones that had undergone homologous recombination at the H1a locus were identified by Southern blotting. The results of these Southern blotting experiments showed that clones WTA-D18 and WTA-D32 had undergone homologous recombination at the H1t and H1a loci ((Lin *et al.,* 2000) and data not shown) and that these cell lines did not harbor additional copies of the targeting vector integrated at other sites.

We also wished to test whether our strategy for distinguishing *cis* vs *trans* gene targeting events could be applied to DNA sequences lying further apart on the chromosome than the approximately 50 kb separating the H1t and H1a genes. Therefore, we also used an ES clone, WEC 9.6, in which separate targeting constructs containing the PGK-Neo or PGK-Hygro genes were used to target sequentially the H1e and H1c genes, respectively, which are estimated to lie 200 kb apart on MMU13A2-3 (Wang *et al.,* 1997). Southern blotting experiments were used to show that clone WEC9.6 had undergone homologous recombination at the H1e and H1c loci (data not shown).

To ascertain whether the two gene targeting events that had occurred in these cell lines took place *in cis* or *in trans,* the cell lines were injected into mouse blastocysts, and chimeric mice were generated. The cell lines carry the dominant agouti coat color marker gene, and thus when these chimeras are mated to C57BL/6 mice, progeny from ES cell-derived germ cells will have an agouti coat color. Agouti progeny of such matings were genotyped for the different modified alleles by PCR utilizing primer pairs specific for the modified alleles (e.g., H1t-Neo and H1a-Hygro for WTA-D18 and WTA-D32; H1e-Neo and H1c-Hygro for WEC9.6). As shown in Fig. 1, the agouti progeny of both WTA-D32- and WEC9.6-derived chimeras contained either both or neither of the modified alleles (Fig. 1A, C). These results indicate that the modified alleles derived from these cell lines cosegregate and therefore that the two modified loci must be *in cis* in the ES cells. Conversely, agouti progeny of WTA-D18-derived chimeras contained only one or the other of the modified loci, indicating independent segregation of the modified alleles (Fig. 1B). Therefore, the modified alleles in line WTA-D18 must be *in trans.*

To determine whether RNA FISH could be used to distinguish the *cis* and *trans* configurations of the Neo and Hygro genes in the cell lines, we hybridized two sets of differently labeled, gene-specific probes to ES cells attached to coverslips. As shown in Fig. 2, in cell lines in which the Neo and Hygro genes are located *in cis* (WTA-D32 and WEC9.6), the two fluorescent signals colocalize. Colocalization of the two signals was consistently observed even in cell line WEC9.6, in which the two genes lie about 200 kb apart on chromosome 13. In contrast, the two signals were consistently

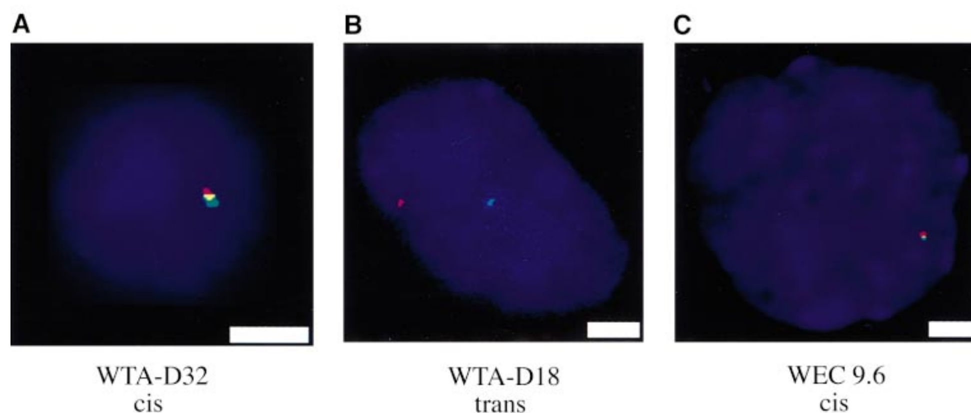

FIG. 2. RNA fluorescence *in situ* hybridization analysis of ES cells. Hybridization of fluorochrome-labeled oligonucleotide probes to transcription sites of "hygro" (red) and "neo" (green) selection markers. (**A, C**) Representative example of colocalization of the marker sites in the WTA-D32 and WEC9.6 ES cell clones indicating that the two gene targeting events are in a *cis* configuration. (**B**) Representative example of the independent positioning of the two marker signals in ES cell line WTA-D18 indicating that the two gene targeting events are in a *trans* configuration (scale bars, 3 μm).

found to be at random positions with respect to each other in cell line WTA-D18, in which the two genes are in the *trans* configuration. Because of the high intensity of signal at the transcription site, it was possible to remove spurious fluorescence or diffuse signal by establishing a threshold level for the images. Signal was verified by the single bright foci for each color present in most cells.

The method described here provides a rapid approach for determining the configuration of two or more gene targeting events in ES cells. The usual alternative approach of genetic linkage testing in chimeric mice requires several months for generation of the chimeras, sexual maturation, breeding, and analysis of progeny of the chimeras. Moreover, by providing such information about the modified ES cells prior to their injection into mouse blastocysts, the method allows for preselecting ES cell clones with a desired configuration. For example, certain types of genome modifications that utilize the Cre–*loxP* system require positioning of *loxP* sites *in cis.* These types of modifications include generating simple chromosomal deletions or inversions (Zheng *et al.,* 2000) as well as modifications designed to study the effect of distance between regulatory sequences and genes on gene expression. On the other hand, generating duplicated chromosomal segments and translocations requires *loxP* sites *in trans.*

The method also may be rather widely applicable for counting the number and allele composition of individual chromosomes in single cells. The approach is not limited to detecting transcripts of the selectable marker genes utilized here. Any expressed sequence for which a specific oligonucleotide probe can be designed can be detected. Furthermore, if probes can be designed to distinguish between different alleles of a gene, it becomes possible to count the number of allele-specific chromosomes in individual cells. For example, one important application could be to investigate loss of heterozygosity at tumor suppressor loci *in situ* in tumors during various stages of tumorigenesis.

The simplicity of the method is that it utilizes interphase nuclei rather than mitotic cells, making it especially attractive for applications in which obtaining metaphase spreads is cumbersome or impossible (e.g., nondividing cells). Therefore it also will be possible to apply such technology to tissue samples, allowing evaluation of the expression of individual, endogenous genes as well as transgenes. The principle of expression localization of genes not only provides a rapid and simple assessment of specific sequences, due to the amplification of signal by the nascent chains, but also provides information as to their level of expression. Such an approach could provide valuable information for screening specific sequences for their expression or for evaluating variables that influence their expression. An example of the latter would be the ability to evaluate drug resistance by expression levels.

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