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Ectopic Gene Targeting Exhibits a Bimodal Distribution of Integration in Murine Cells, Indicating that Both Intra- and Interchromosomal Sites Are Accessible to the Targeting Vector

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Ectopic gene targeting is an alternative outcome of the gene targeting process in which the targeting vector acquires sequences from the genomic target but proceeds to integrate elsewhere in the genome. Using two-color fluorescent in situ hybridization analysis, we have determined the integration sites of the gene targeting vector with respect to the target locus in a murine fibroblast line (LTA). We found that for ectopic gene targeting the distribution of integration sites was bimodal, being either within 3 Mb of the target or on chromosomes distinct from the chromosome carrying the target locus. Inter- and intrachromosomal sites appeared to be equally accessible to the targeting vector, with site-specific variations. Interestingly, interphase analysis indicated that vector sequences which had integrated ectopically in chromosomes other than the target colocalized with the target locus at a significant frequency compared to that of colocalization to random unlinked loci. We propose that ectopic gene targeting could be used to determine which chromosomal domains within the genome are accessible to a given genetic locus. Thus, recombination access mapping may present a new paradigm for the analysis of DNA accessibility and interaction within the genome.

Ectopic gene targeting is a process by which an extrachromosomal molecule (recipient) obtains a DNA sequence from a target locus via one-end invasion and gene conversion followed by release of the recipient molecule and integration, complete with the newly acquired sequence from the target locus, elsewhere in the genome. Such events were first observed in gene targeting experiments involving the adenine phosphoribosyltransferase (APRT) locus in CHO cells (1) and in experiments involving retroviral transfection of rat cells (9). Consequently, a model for the mechanism of ectopic gene targeting has been proposed (4; reviewed in reference 3). Instances of ectopic gene targeting and/or ectopic gene conversion have been seen in *Drosophila* (*roo* element [23] and *p* and *hobo* elements [reviewed in reference (22)]), plants (25, 26, 33), yeast (between dispersed repeated genes [10, 16, 17, 21, 26] or Ty 1 repeat elements [19]), fungi (in *Ustilago maydis* [11]), chickens (immunoglobulin rearrangement [reviewed in references (27) and (31)]), rabbits (generation of antibody repertoire [reviewed in reference (18)]), mice (germ line ectopic gene conversion in spermatids [24] and gene conversion between Line-1 elements [4]), and humans (pseudoautosomal region on X and Y chromosomes [38] and gene conversion between Line-1 elements [32]).

Although the phenomenon of ectopic gene targeting is well documented, the question of where the recipient molecule integrates, with respect to the target locus, has not been answered. It is apparent from Southern analysis that the recipient integrates in most cases at a site distinct from the target, but Southern analysis does not permit the determination of the relative position of the ectopic sites with regard to the target locus. Fluorescent in situ hybridization (FISH) analysis, which can be used to identify the genomic location of distinct DNA sequences with a general resolution of ~100 kb at interphase and ~2 to 3 Mb at metaphase (37), is a unique tool for analysis of DNA sequences with respect to their chromosomal positions and with respect to each other.

We have developed an assay to study ectopic gene targeting that uses two vectors, a target and a recipient vector which can recombine to produce a functional gene (*neo*$^+$) via the one-end invasion mechanism of recombination. A murine fibroblast line (LTA) was transfected with the target vector. Three distinct clones containing the target vector integrated into their genomes were then transfected with the recipient vector. Ectopic gene targeting events, which are characterized by the acquisition of sequences from the target by the recipient vector and then its integration into the genome, were selected by G418 resistance. The distribution of ectopic gene targeting events in relation to the target locus was determined by two-color FISH. The results indicated that the distribution of ectopic gene targeting events is bimodal. Ectopic integration of the recipient vector occurred either close to the target locus (<2 to 3 Mb) or in chromosomes altogether different from the target chromosome. In contrast, illegitimate integration showed no bias for any single chromosome or chromosomal location at megabase resolution. A corollary to these observations is that both inter- and intrachromosomal DNA interactions appear to occur during ectopic gene targeting. We propose that the assay we have devised, which we call recombination access mapping (RAM), could be used to determine

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which chromosomal domains within the genome are accessible to a given genetic locus.

MATERIALS AND METHODS

Vector construction and preparation. Plasmids pA1059λTk (target) and pB115AdHyg (recipient) were derived from pMC1neopA (Stratagene). Briefly, 3′ deletion of the *Ngo*MI/*Bam*HI fragment or 5′ deletion of the *Eag*I/*Nde*I fragment of the neomycin resistance gene was followed by introduction of a *Not*I restriction site into the *Nde*I or *Aat*II restriction site (to allow linearization of the vector). Next a herpes simplex virus thymidine kinase (HSV-TK) cassette (from pAGO) and a hygromycin resistance (*hyg*) cassette (from p3′SS; Stratagene) were cloned into the multicloning site 3′ of the truncated neomycin resistance gene. Finally, a 16-kb λ virus sequence (Gibco BRL) or adenoviral sequence (Gibco BRL), both lacking a *Not*I restriction site, was cloned into the vectors between the neomycin resistance cassette and either the HSV-TK or hygromycin resistance cassette, respectively. Due to the large size of the vector, subsequent subcloning to prepare transfection quantities of DNA was carried out with SURE cells (Stratagene), and a normal alkaline lysis miniprep procedure followed by G-50 column chromatography was used to purify the DNA. Vector DNA was linearized by *Not*I and subjected to phenol-chloroform extraction and ethanol precipitation before being resuspended in 1× Tris-EDTA for storage at −20°C.

Cell culture and transfection of LTA murine fibroblasts. LTA murine fibroblasts (TK^-, $APRT^-$) were cultured at 37°C and 5% CO_2 in complete medium (DMEM–F-12 medium supplemented with 10% fetal bovine serum). Cells were split the day before transfection and plated either at 5×10^5 to 1×10^6 cells ($CaPO_4$) or at ~60% confluence (electroporation). Mother cell lines were produced by electroporation of LTA cells. Briefly, cells were trypsinized and concentrated by centrifugation and then resuspended in 1 ml of complete medium at room temperature. Upon being counted, cells were diluted to 2.5×10^6 to 5×10^6 per ml with complete medium and 400 μl of cell suspension was electroporated (300 V, 900 μF) with a Gene Zapper 450/2500 apparatus (IBI) in the presence of 1 to 2 μg of target plasmid linearized at the *Not*I site. Cells were selected for integration of the plasmid in hypoxanthine-aminopterin-thymidine (HAT) medium. Positive clones were subcloned by using glass cloning rings and expanded in culture for no more than five passages before being stored in liquid nitrogen. Genomic DNA was digested with restriction endonucleases, and Southern analysis was carried out to determine the number of integrations. Cells exhibiting a simple hybridization pattern of integrated plasmid were then subjected to $CaPO_4$ transfection. Cells were fed 2 h prior to transfection with 10 ml of fresh medium. Approximately 10 μg of DNA was coprecipitated with $CaPO_4$, and the precipitate was left on the cells for 4 h followed by 3 min of dimethyl sulfoxide shock (10% dimethyl sulfoxide in complete medium) or 16 h without shock. Cells were washed with phosphate-buffered saline twice and fed with 10 ml of complete medium. Thirty-six hours after transfection, 400 μg of G418 (Gibco BRL) per ml was added to the medium. G418-resistant colonies were picked as described for resistance to HAT medium above. In addition, colonies were also subjected to hygromycin (Gibco BRL) at 250 μg/ml to determine resistance to the antibiotic.

Harvest of cells for FISH. LTA fibroblasts were grown to near 95% confluence before being trypsinized and replated at one-half to one-fourth original confluence. Depending on the growth characteristics of each clone, harvest of cells began at 20 to 22 h after trypsinization by the addition of 2 drops of Colcemid per 5 ml. Cells were incubated at 37°C and 5% CO_2 for 2 h in the presence of Colcemid, after which the cells were trypsinized and collected by centrifugation in 15-ml Falcon tubes. Cells were then subjected to hypotonic shock by the addition of 10 ml of KCl (0.07 M; Sigma, St. Louis, Mo.) for 20 min at 37°C. Cells were then centrifuged again before fixation in 10 ml of ice-cold Carnoy I (3 parts MeOH and 1 part acetic acid). Fixation was repeated three or four times, and cells were dropped on frozen slides (Fisher). Slides were cured for 24 h at room temperature before being frozen at −20°C.

Southern analysis. Genomic DNA was prepared as described above and digested with restriction endonucleases. Digested DNA was electrophoresed on 0.7% agarose gels (Agarose-NA; Pharmacia Biotech) and transferred to nylon membranes (Hybond N; Amersham). Hybridization was carried out with a radiolabelled probe in 0.5 M sodium phosphate (pH 7.2)–7% sodium dodecyl sulfate (SDS)–1 mM EDTA for 16 to 24 h at 65°C. The blot was washed with several changes of 40 mM sodium phosphate (pH 7.2)–0.1% SDS at 65°C and autoradiographed at −80°C for 3 to 7 days.

FISH. FISH analysis was carried out as previously described (20). Briefly, after RNase treatment, chromosomes were denatured in 70% formamide in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 70°C for 2.5 min. Hybridization was performed overnight at 37°C in 50% formamide–10% dextran sulfate–2× SSC–0.1% SDS–1× Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin [pH 7])–1 mg of denatured sonicated salmon sperm DNA per ml. Probes were denatured for 10 min at 95°C in the same medium. The probe concentrations were 4 to 5 ng of digoxigenin-labelled adenovirus type 2 DNA per μl and/or 5 to 6 ng of biotin-labelled λ virus DNA per μl in a volume of 20 μl per slide. Rinses were performed at 37°C for 2 min, twice in 50% formamide in 2× SSC followed by twice in 2× SSC. In addition, high-stringency washes were performed at 42°C for 15 min, once in 50% formamide in 2× SSC followed by a single rinse in 2× SSC for 8 min at 37°C. Probes were generated by using the Bionick labelling system (Gibco BRL) for the biotin-labelled probe or the Nick translation kit (with addition of digoxigenin-11–dUTP; Boehringer Mannheim) for digoxigenin labelling.

Fluorescence detection and image acquisition. After hybridization, the slides were incubated for 45 min at 37°C with rabbit antibiotin (Enzo), 4.6 μg/ml, in PBT {PBS (0.2 N NaH_2PO_4, 0.2 N Na_2HPO_4, 0.15 M NaCl [pH 7.3]) containing 0.15% bovine serum albumin and 0.1% Tween 20}. After two rinses in PBT at room temperature for 5 min, incubation was continued for 45 min in the presence of 10 μg of biotinylated anti-rabbit goat antibody (Gibco BRL) per ml. After incubation, slides were rinsed again as described before addition of fluorescein-streptavidin conjugate (Gibco BRL) at 8 μg/ml for a final incubation for 45 min. During double detection, the incubations were continued with anti-digoxigenin mouse antibodies (1 μg/ml), anti-mouse sheep Fab fragment (14 μg/ml) and rhodamine-conjugated sheep antidigoxigenin antibody (20 μg/ml) (Boehringer Mannheim). Counterstaining of DNA with propidium iodide (as described in reference 20) or 4′,6-diamidino-2-phenylindole (DAPI) was carried out before visualizing slides in the presence of 10 to 15 μl of antifade solution. Antifade solution contained *p*-phenylenediamine (Sigma) and a mixture (1 mg/ml) of glycerol and phosphate-buffered saline, 9:1 (vol/vol), adjusted to pH 9 with NaOH. Slides were visualized on a fluorescence microscope (Aristopan; Leitz) without a signal amplification system. Red, blue, and green fluorescence was observed by viewing through a triple-band-pass filter (Omega Optical Inc., Brattleboro, Vt.). Images were captured by using a charge-coupled device camera (Xybion Electronic Systems) and MacProbe version 2.5 on a Quadra 840av Macintosh computer. Color balance adjustments and file conversion were accomplished with Adobe Photoshop version 2.5.1. Although images were captured electronically for publication, signals could easily be seen through the microscope, and slide film was taken to attest this fact.

RESULTS

Our approach was to use ectopic gene targeting to analyze chromatin accessibility and DNA interaction in vivo. To do so we have used FISH analysis to determine the integration pattern of an exogenous vector in three distinct cell lines, each containing a target vector. These cell lines were derived by electroporation of the murine fibroblast line LTA (TK^- $APRT^-$) with the target vector A1059λtk in a linear form (Fig. 1A). The target vector contains 16 kb of lambda sequence (used for FISH analysis) flanked by a 3′ truncated *neo* gene (used for gene targeting) and an HSV-TK gene which was used for selection. Three TK^+ cell lines were chosen and were designated A6 and A14, containing a single target vector, and A1, which contains two copies of the target vector (arranged head to tail). Each of the three lines was then subjected to $CaPO_4$ transfection with a recipient vector, B115Adhyg (Fig. 1A), containing a 5′ truncated *neo* cassette with 600 bp of perfect homology with the 3′ truncated neomycin resistance gene of the target plasmid. Like the target vector, the recipient vector contains unique sequences for FISH analysis (16 kb of adenovirus type 2 DNA) and a selection gene (for hygromycin). Homologous recombination between the overlapping *neo* sequences in the two vectors will produce a functional *neo* gene which can be used for clonal selection in the presence of G418. The recipient vector was linearized via a *Not*I site, directly adjacent to the region of homology, to favor ectopic gene targeting events by leaving only one end of the recipient vector homologous to the target.

The usual gene targeting process involves invasion of the target by two homologous ends of the exogenous vector. Ectopic gene targeting involves invasion of the target sequence by only one homologous end of the exogenous vector, which then primes DNA synthesis leading to gene conversion (Fig. 1B). At this point, there can be two outcomes. The recipient vector can form a homologous junction with the target at the target site, while the other end of the recipient vector forms an illegitimate junction at or near the target site (Fig. 1B, panel i). In this case the target site is modified. The other possibility is that the homologous end of the recipient vector is released and the recipient vector integrates elsewhere in the genome (Fig. 1B, panel ii). In this case the target site is unchanged. Thus, by

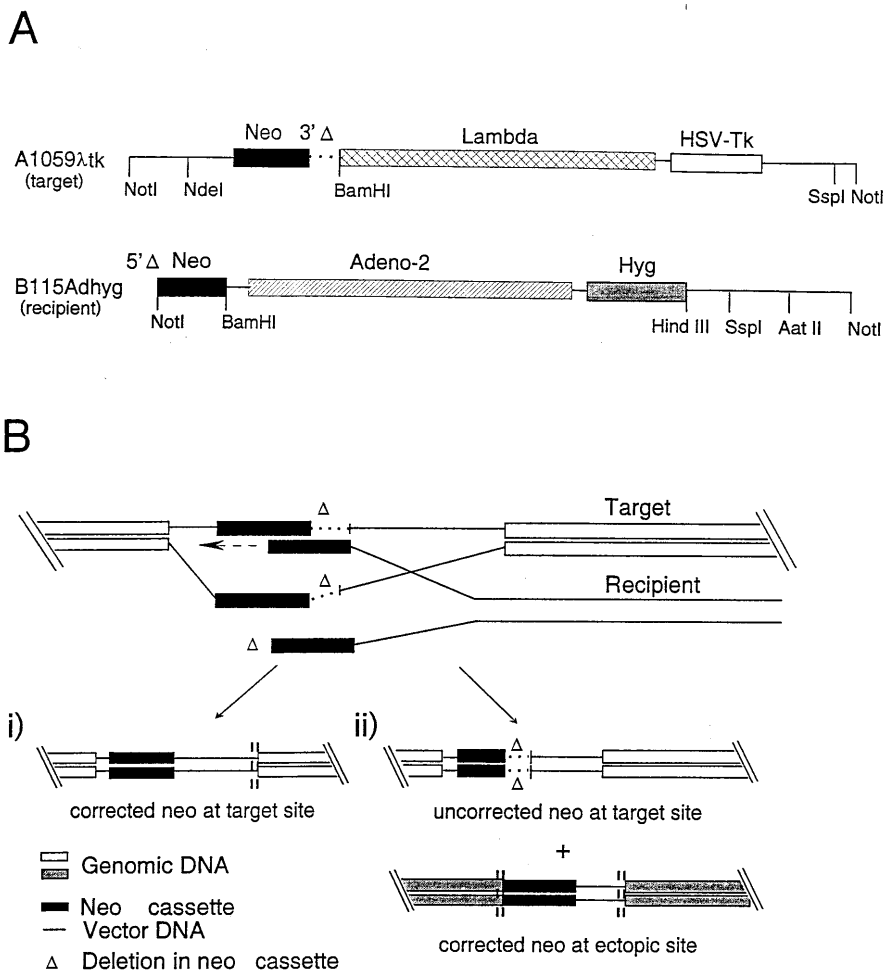

FIG. 1. (A) Two plasmid-based vectors are used in RAM. The vectors are depicted in linear form after digestion via *Not*I prior to transfection. The vector A1059λtk (target) contains a 3′ truncated *neo* cassette. Vector B115Adhyg (recipient) contains a 5′ truncated *neo* cassette. Both vectors contain selection markers for illegitimate integration (HSV-TK and *hyg*, respectively) and specific DNA for detection via FISH analysis (lambda DNA and adenovirus type 2 DNA, respectively). (B) The mechanism of ectopic gene targeting leading to reconstruction of a functional *neo* gene between an integrated copy of the target vector and an extrachromosomal recipient molecule is shown. A simplified version of the vectors from panel A is depicted for clarity. One-end invasion of the target locus by the homologous 3′ end of the recipient molecule leads to the formation of a D loop. The invading 3′ end of the recipient primes DNA synthesis, leading to gene conversion and extension of the D loop. Resolution by nicking of the D loop results in noncrossover and crossover products, the latter involving integration within the target locus (panel i). Alternatively, resolution can occur via unwinding of the newly synthesized strand and release of the recipient molecule or its displacement due to branch migration. In the case of a noncrossover event, the recipient molecule may integrate illegitimately in a different locus, leaving the target locus unchanged (panel ii) (3). Illegitimate junctions are depicted by double horizontal dashed lines, where recipient DNA is joined to chromosomal DNA in the absence of homology.

determining where the released recipient molecule integrates, one can determine what other areas (domains) of the genome were accessible to the target site at the time of the recombination event. This was accomplished by FISH analysis with probes specific for the target and recipient vector, which enabled the direct analysis of the distribution of integrated recipient DNA with respect to the target.

Characterization of mother clones A1, A6, and A14. Mother cell lines A1, A6, and A14 were subjected to Southern analysis to determine the copy number and structure of the integrated target sequences (Fig. 2). By probing against the neomycin resistance gene, a diagnostic band(s) was produced for the target locus. Two diagnostic bands are apparent for A1 at 5.6 kb (intervector band) and 8 kb (junction band), indicating the integration of two target vectors in a head-to-tail configuration (Fig. 2A and E). A6 and A14 exhibit single bands of 5 and 3.8 kb, respectively, which indicates a single integrated copy of the target for each cell line (data for A6 in Fig. 2B and E and for A14 in Fig. 2C and E).

Mother cell lines A1, A6, and A14 were then subjected to single-color FISH to determine the location of the target vector. The location of the target locus in these cell lines is shown in Fig. 3. Clone A1, which has integrated two copies (arranged head to tail in tandem) of the target vector, contains the integrated sequences in a single site between the two centromeres of a dicentric chromosome (Fig. 3A). A6 contains a single integration of the target vector in the short satellite arm of an acrocentric chromosome (Fig. 3F), and A14 contains a single integration of the target vector in the midarm of a large metacentric chromosome (Fig. 3I). Shortly after isolation of the A6 cell line, there occurred a rearrangement such that now one-third of the cells contain the target sequence in a meta centric chromosome; however, the target sequences remain unmodified.

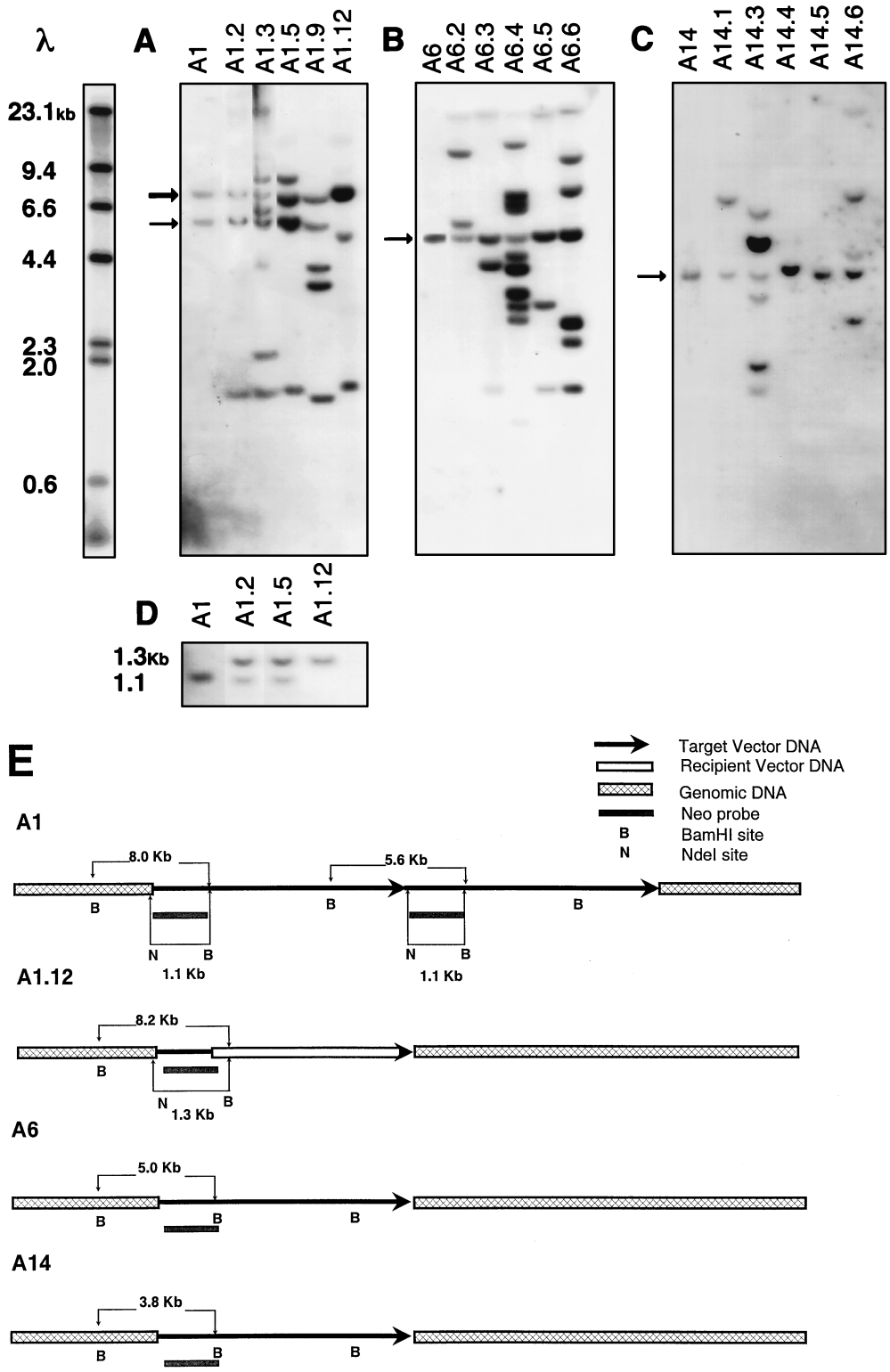

FIG. 2. Southern analysis of mother clones A1, A6, and A14 and selected daughter clones. Genomic DNA was isolated and digested before electrophoresis and capillary transfer to nylon membranes as described in Materials and Methods. DNA was digested with *Bam*HI either alone (A to C) or in combination with *Nde*I (D), and blots were probed with a *neo* probe that lacked the promoter sequence (*Eag*I/*Hin*cII fragment of pMC1neopA). *Hin*dIII-digested lambda DNA was used as a marker of molecular size. Arrangements of vector sequences in the genome, as well as restriction enzyme sites, are also indicated (E). Two specific bands appear for mother clone A1 (data for A1 in panels A and E). The two bands indicate that two target vectors are arranged head to tail and that the 5.6-kb band represents an intervector band and the 8-kb band spans the junction of one vector with genomic sequences (data for A1 in panel E). These bands are maintained in four daughter clones (A1.2, A1.3, A1.5, and A1.9), indicating that the target locus has remained intact in these clones. A1.12 is an exception in which the junction band has increased in size to approximately 8.2 kb, suggesting reconstruction of the full-length *neo* gene. Digestion with *Nde*I in combination with *Bam*HI should produce a 1.1-kb band

These cell lines were then transfected with the recipient vector. Selection for ectopic and illegitimate integration events was carried out in medium supplemented with G418 or hygromycin, respectively. Resistant clones were counted and the frequencies of homologous (ectopic gene targeting) and illegitimate integration were determined. The frequencies of illegitimate integration in the three cell lines were similar, with a mean frequency of 5.6×10^{-3}. Although A1 had twice the copy number of A6 or A14, the rates of homologous recombination for all three clones were quite similar, with an average frequency of 2.0×10^{-6}. This agrees with previous reports that demonstrated that copy number does not affect the frequencies of homologous targeting significantly in mammalian cells (36, 39).

Ectopic gene targeting exhibits a bimodal distribution. Cell lines A1, A6, and A14 were transfected with the recipient vector, and targeted events ($G418^r$ clones) were selected to be analyzed by FISH. $G418^r$ clones were expanded with no more than five passages before cryopreservation and genomic-DNA extraction. Of the five daughter cell lines analyzed for A1, all but one (data for A1.12 in Fig. 2A) contained the two characteristic bands representing the target locus (i.e., the 5.6- and 8.0-kb bands). The loss of the 5.6-kb target band and an apparent shift of the 8.0-kb band, coupled with sensitivity to HAT medium (i.e., TK^-) and FISH analysis (Fig. 3E), indicate that daughter clone A1.12 contains a crossover event. This event most likely involved the deletion of the 3′ region beyond the neomycin resistance gene of the first integrated copy of the target vector along with the entire second copy of the target vector. Replacing these sequences is the 3′ region of the recipient containing the adenovirus type 2 DNA and the hygromycin resistance gene (data for A1.12 in Fig. 2E). Consequently, both the lambda viral DNA and TK genes of both copies of the target have been deleted, resulting in a loss of the green fluorescent signal representing the target locus, which is replaced by the red signal of the recipient (Fig. 3E). It is more apparent in Fig. 2D that A1.12 contains a crossover event at the target locus, as the *neo* 1.1-kb band disappears and is replaced by a 1.3-kb band representing a full-length neomycin resistance gene (clones A1.2 and A1.5, which are ectopic events, are shown for comparison).

Overall, it is important to note that of more than 30 $G418^r$ daughter clones tested by Southern analysis for five different mother clones, only 2 were scored for the loss of the target locus (data not shown). Four of the five daughter clones analyzed for A1 contain bona fide ectopic integrations (i.e., the target locus is intact in each of them). Two of them, A1.2 and A1.5, have the recipient integrated close to the target (Fig. 3B and C, respectively) and retain the two characteristic target bands as shown in Fig. 2A. As well, in Fig. 2D the 1.1-kb band of the target locus is present as expected for both A1.2 and A1.5. The two fluorescent signals can be resolved as two closely spaced but distinct spots at metaphase; thus, we can set the limit on the distance between the target and recipient in these clones at less than 2 or 3 Mb (37). Clones A1.9 and A1.3, on the other hand, had integrated the recipient vector in a chromosome other than the target (midarm of a small acrocentric chromosome [Fig. 3D] and midarm of a metacentric chromosome [not shown], respectively).

All five of the daughter clones derived from A6, in contrast, were the result of ectopic integration that occurred in chromosomes other than the target. A single band of 5.0 kb representing the target locus is maintained in all five daughter clones, indicating an intact target locus (Fig. 2B). Two daughter clones of A6, A6.2 and A6.3, are shown in Fig. 3G and H, respectively. Daughter clones of A14 contain the single diagnostic band of 3.8 kb, which indicates that the target locus is intact in all five daughter clones. An intermediate distribution of ectopic events is seen, with one clone exhibiting ectopic integration less than 2 to 3 Mb from the target, A14.4 (Fig. 3J), and four others exhibiting integration of the recipient DNA molecule in chromosomes other than the one containing the target. The diagnostic band for the target locus is very intense and slightly shifted for A14.4. This shift was suggestive of a crossover event, and Southern analysis with an alternate digestion (as in Fig. 2D) indicated that the target locus had been converted (appearance of a 1.3-kb band and loss of a 1.1-kb band [data not shown]), yet FISH analysis indicated that both target and recipient sequences were present (i.e., as separate spots at mitosis, approximately 2 to 3 Mb from each other [Fig. 3J]). A crossover would be expected to produce a single red spot, as in A1.12, with a loss of the green signal representing the target locus, or an intermediate white color, which would indicate juxtaposed sequences less than 100 kb apart. Therefore, A14.4 most likely does not involve a crossover event and represents a rare ectopic event involving an as-yet-undefined mechanism. Figure 3K shows colony A14.6, an example of one of the distant ectopic integration events with an apparent duplication of the target chromosome (most likely explained by nondisjunction).

No insertion-type events were observed in our experiments. Such events were not likely to occur, since the recipient vector was linearized prior to transfection and only one end of the gene targeting vector is homologous to the target.

Thus, it appears that we have two distinct types of ectopic integration events: those events which are close (<3 Mb) to the target (close) and those that occur in other chromosomes which do not contain the target locus (far). We did not see integration of the recipient vector on the same chromosome at distances greater than 3 Mb from the target. This certainly does not imply that such events do not occur, but it does indicate that such integration events are not more likely than ones on distinct chromosomes.

Pooled FISH analysis of ectopic gene targeting events. Pooled FISH analysis of an additional 24 $G418^r$ clones, i.e., 12 clones for A1 and 6 clones for both A6 and A14, was carried out to discern trends in the preferences for certain chromosome morphologies for far integrations. In general, far ectopic integration events occurred in morphologically different chromosomes at multiple sites in the daughter clones of the A1 and

for the target locus or a 1.3-kb band for a corrected *neo* gene (D). In the case of A1.2 and A1.5, the target locus remains uncorrected, i.e., the 1.1-kb band is maintained, while the recipient is corrected, producing a 1.3-kb band. Clone A1.12, on the other hand, exhibits only a 1.3-kb band, indicating that the target locus has been corrected. Such a pattern is indicative of a crossover event in A1.12 which has replaced downstream sequences from the *neo* gene of one vector (including the *neo* gene and lambda sequences of both target vectors) with sequences from the recipient (data for A1.12 in panel E). (B) The digestion profiles of A6 and five daughter clones are shown. The lane containing A6 shows a single target band of 5.0 kb which is maintained in all five daughter clones (lanes containing A6.2 through to A6.6) and represents a single integration of the target locus (data for A6 in panel E). (C) The digestion profiles of A14 and five daughter clones are shown (C). A single 3.8-kb band appears for A14, which indicates a single integration of the target locus in these clones (data for A14 in panel E). This band is maintained in all daughter clones except for A14.4, in which the target band is shifted slightly to approximately 4.0 kb (data for A14.4 in panel C). Again, this indicates that the target locus had been corrected. Indeed, double digestion with *Nde*I and *Bam*HI gives the same profile as seen for A1.12 (data not shown). All other bands represent integrations of the recipient vector.

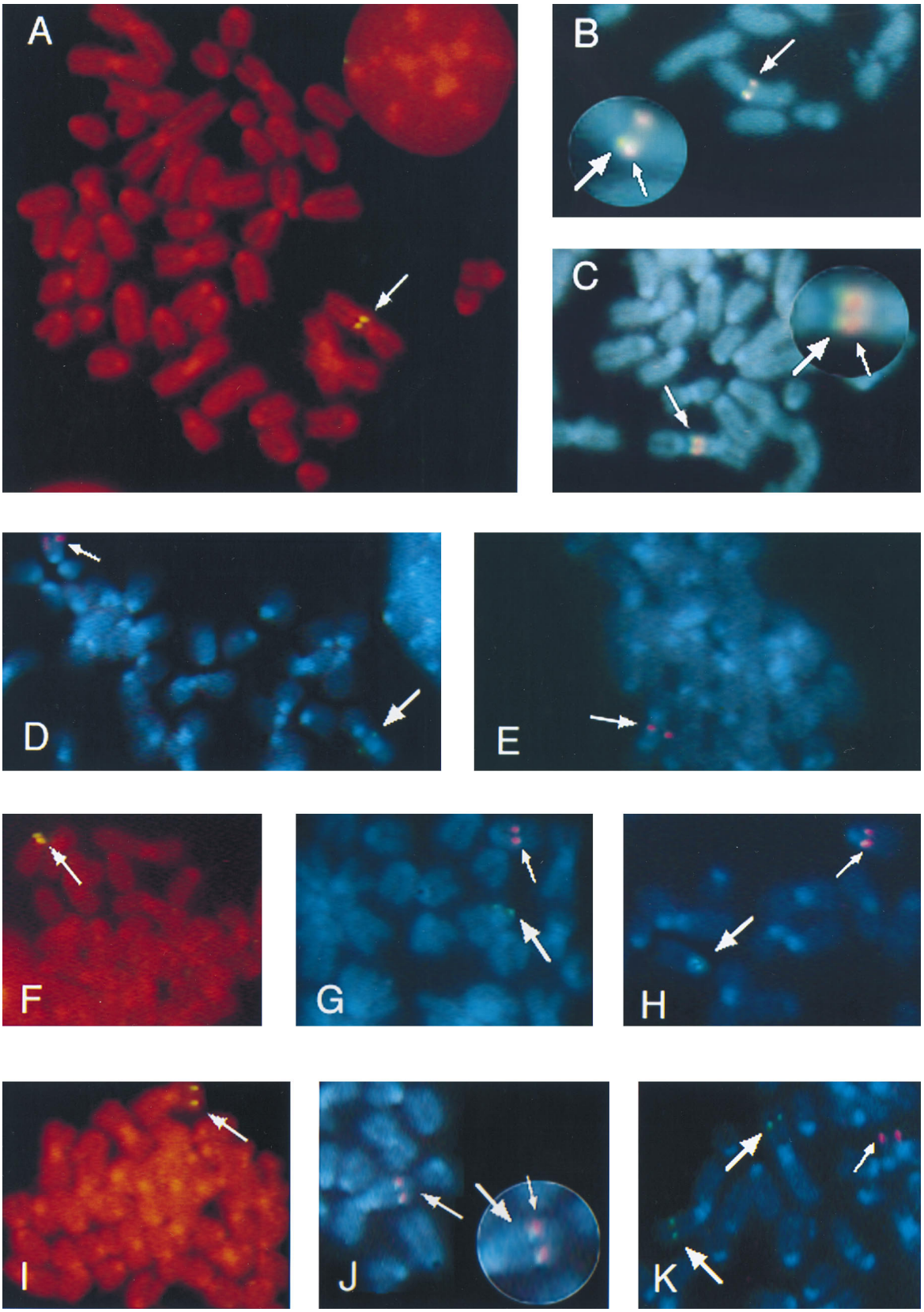

FIG. 3. FISH analysis of mother clones A1, A6, and A14 and selected daughter clones. Cell cultures were prepared for in situ hybridization, and FISH analysis was carried out as described in Materials and Methods. DNA was counterstained with either propidium iodine (red) (A, F, and I) or DAPI (blue) (B to E, G, H, J, and K). Target sequences appear as green signals on a blue background or yellow on a red background. Recipient sequences appear as red signals against a blue background. A full complement of chromosomes from mother clone A1 (A) in which two copies of the target vector have been integrated into a dicentric chromosome between

two centromeres is shown (arrow). Daughter clones A1.2 (B) and A1.5 (C) have integrated the recipient (small arrows) close to the target locus (large arrows). Both the green and red signals can be seen separately rather than as a single white or yellow dot, suggesting that the recipient and target sequences are more than 100 kb but not more than 2 to 3 Mb apart (37). In clone A1.9 (D), the recipient sequences have integrated in the midarm of an acrocentric chromosome (small arrow) while the target locus has remained intact in the dicentric chromosome (large arrow). Clone A1.12 (E) shows a crossover event in which the target sequences have been completely replaced by the recipient sequences at the target locus (arrow). In mother clone A6 (F), a single target vector has integrated into small satellite arms of an acrocentric chromosome (arrow). In both clones A6.2 and A6.3, the recipient sequences have integrated in the midarm of acrocentric chromosomes (small arrows in panels G and H, respectively), leaving the target locus intact in the original acrocentric chromosome (large arrows). There is a single integration of a target vector in the midarm of a metacentric chromosome in mother clone A14 (I) (arrow). In clone A14.4 (J), the recipient sequences (small arrow) have integrated into the same metacentric chromosome within 2 to 3 Mb of the target locus (large arrow). This is interesting, as Southern analysis indicates a crossover event which should have resulted in an intermediate white signal (juxtaposition of recipient and target DNA) or a loss altogether of the green signal (i.e., the target locus), as in A1.12 (E). Thus, clone A14.4 may represent a rare event in which a crossover occurred but the recipient was still able to integrate ectopically. Such a "broken arrow" suggests the commitment of both ends of the recipient molecule at the time of the recombination event rather than sequential participation of each end in recombination. In clone A14.6 (K), recipient sequences have integrated into the telomere of an acrocentric chromosome (small arrow) and the metacentric chromosome containing the target vector (large arrows) has been duplicated, most likely by a nondisjunction event.

A14 mother cell lines, whereas A6 contains a large number of ectopic integrations in the midarms of acrocentric chromosomes. A summary of all 39 clones analyzed by FISH analysis, singularly or in pools, is shown in Table 1.

A1 daughter clones showed a striking number of close ectopic events within 2 to 3 Mb of the target (14 of 17 clones), whereas A14 and A6 showed much smaller numbers of close events (2 of 11 and 1 of 11 clones, respectively). Two of the close events for A1 were single crossover events, and one event for A14 had an apparent crossover coupled with ectopic integration, which brings the total number to 3 of 39 clones (8%). For A6, 7 of 11 clones (63.6%) were scored for the appearance of the recipient signal in the midarm of an acrocentric chromosome. A14 did not show any bias for any one chromosome morphology or position, and due to the few number of far ectopic events, it was not possible to discern a trend for such events for A1. Although superficially it appears that A6 has a bias for acrocentric/midarm localization of ectopic integration, it must be mentioned that there are three times more acrocentrics in the karyotype than metacentrics, and thus, a frequency of ~64% does not indicate a statistically significant correlation for a specific chromosome morphology or position. What our analysis does indicate is that by using a pooling approach to FISH analysis one can produce a relatively large data set for analyzing the distribution of ectopic gene targeting at a given locus. Our observations also lead us to suggest that far ectopic integrations can occur in more than one chromosome for a given locus. It remains to be determined if far events are random or if they occur in specific chromosomes.

Analysis of the illegitimate integration patterns in mother clones A1, A6, and A14. The large number of close events in A1 versus A6 or A14 raises a question of bias for integration near the target in clone A1. Thus, we compared the patterns of illegitimate integration events in clones A1, A6, and A14 via FISH analysis, which enabled the direct visualization of the distribution of illegitimate integration events at megabase resolution.

Approximately 220 and 250 hygromycin-resistant clones were pooled for cell lines A1 and A6, respectively, and subjected to two-color FISH analysis. Approximately 3,000 hygromycin-resistant clones were pooled for A14 to determine if the number of clones pooled for A1 and A6 could produce a representative distribution of illegitimate events. The distribution of illegitimate integration events in relation to the target locus was scored in 200 mitoses for relative position of the fluorescent signal on a chromosome (centromeric, telomeric, or midarm) as well as the morphology of the chromosome (acrocentric, dicentric, or metacentric) containing the signal. The resulting distributions of illegitimate events for all three clones were similar, without any evidence of a bias toward integration near the target site in clone A1 (data not shown). This indicates that illegitimate integration in these clones cannot account for any differences in the pattern of ectopic gene targeting.

Interphase analysis of far ectopic events. Interphase FISH analysis was conducted for nine clones (two derived from A1, four derived from A6, and three derived from A14) which contained far ectopic integration events on chromosomes distinct from the target. For each of the nine clones, distances between target (green) and recipient (red) sequences were measured to determine the frequency of colocalization of signals. Similar measurements were made for pools of clones containing illegitimate integrations of the recipient, derived from cell lines A1 and A6, to provide a random, or "unlinked," distribution for colocalization of red and green signals. In addition, two clones from A1 and one clone from A14, containing close ectopic integrations, were used as a "linked" control for colocalization of FISH signals.

Of a total of 247 nuclei observed for the nine far ectopic clones, 35 (~14%) exhibited coincident or nearly coincident red and green signals. In contrast, a pool of ~440 clones containing random illegitimate integration events (unlinked loci) did not exhibit colocalization of red and green signals in 69 nuclei observed. The close (linked) control exhibited 63 coincident or nearly coincident FISH signals in all 63 nuclei observed. The distribution of intersignal distances for both the random (unlinked) control and the pool of far ectopic events approximated a normal distribution, with the exception of a significant deviation ($P < 0.0001$) for the number of colocalizations for the far ectopic events (Fig. 4, bin 0). These results suggest that during interphase the site of integration of the recipient vector in far ectopic clones is found close to the target locus in a significant number of nuclei.

DISCUSSION

Ectopic gene targeting exhibits a bimodal distribution in murine fibroblasts. The recipient DNA molecule may integrate nonrandomly within 3 Mb of the target or may integrate in other chromosomes, perhaps randomly. If indeed far events are random, then integration in the target chromosome at distances beyond 3 Mb from the target locus may also occur. In our study, no far ectopic integrations were seen on the same chromosome as the target locus; nonetheless, we cannot statistically exclude such events. A bimodal distribution was observed for 39 ectopic gene targeting events analyzed for three independent target loci (either separately or in pools). In contrast, illegitimate integration of the recipient vector in the same clones did not indicate any bias for the target site or any other chromosomal site. Interestingly, the frequency at which we observed ectopic gene targeting (2×10^{-6}) is also similar to the frequency of traditional gene targeting. This similarity in

TABLE 1. Summary of FISH analysis of G418^r daughter clones

Mother line	No. of clones[a]											
			With integration events on:									
	Total[b]	With crossover[c]	Same chromosome	Different chromosomes[d]								
				Total	Acrocentric				Metacentric			
					Total	T	C	M	Total	T	C	M
A1	17	2	14	3	1	0	0	1	2	1	0	1
A6	11	0	1	10	7	0	0	7	3	1	1	1
A14	11	1	2	9	7	1	2	4	2	1	0	1
Total	39	3	17	22	15	0	2	5	7	1	1	2

[a] Includes all daughter clones analyzed by pool or independently.
[b] In the case of pooled clones, 10^5 cells were pooled for each clone and the resulting pool of cells was passaged twice before harvest for FISH analysis. One hundred mitoses were counted and scored for chromosome morphology and location of the fluorescent signal (red) for the recipient in relation to the target locus (green). The resulting numbers of mitoses were converted to numbers of clones by dividing the number of each morphology type by 4.2, as on average one would expect to see 4.2 mitoses for each of the 24 clones in 100 mitoses.
[c] Crossovers where scored when no target (green) fluorescent signal was present but had been replaced at the target locus by the recipient fluorescent signal (red).
[d] C, centromere; M, midarm; T, telomere.

frequencies has been observed previously by other groups (1, 2, 5). Lastly, our experiments were carried out on unsynchronized cell populations such that the effects of the cell cycle cannot be addressed directly. Nevertheless, the results were highly reproducible between independent experiments.

The results we obtained in our study, combined with recently published results, lead us to suggest a model for genomic domain interactions that takes into account the observed bimodal distribution of ectopic gene targeting. FISH analysis of the integration pattern of illegitimate events indicates that there is no evident bias with respect to chromosomal location. This is the case despite the presence of the homologous genomic target. Thus, it appears that homology per se does not act as a determining factor in the localization of the integration site. Yet when a double strand break (DSB) is introduced into genomic homologous sequences, integration occurs highly preferentially at the site of the break by homologous recombination (34). From these observations it seems that the location of the integration site is first and foremost determined by the occurrence of a DSB in genomic DNA and that if this DSB occurs at or near homologous sequences, then the integration will most likely involve homologous recombination. These considerations could explain the ectopic gene targeting events that occur close to the target. How, then, can one explain the far events of ectopic gene targeting? According to the above reasoning, this would mean that the DSB occurred in a domain that, although situated in a distinct chromosome, was close to the domain containing the homologous target at the time of the ectopic gene targeting event. In support of a close association of far ectopic sites and the target locus at the time of recombination, we have observed a significant number of colocalizing recipient and target signals by FISH in interphase nuclei of nine separate far ectopic clones produced from mother clones A1, A6, and A14. In contrast, no colocalizations were observed for pools of random illegitimate events, which exhibited a normal distribution of intersignal distances between recipient and target sequences at interphase. A DSB proximity model, presented in Fig. 5, summarizes our hypothesis.

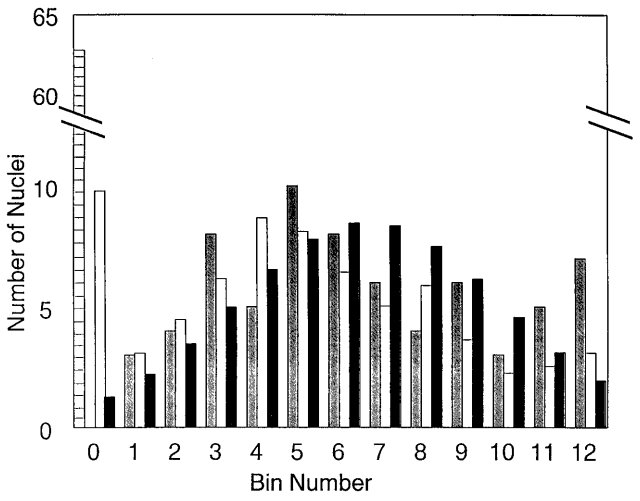

FIG. 4. The distribution of intersignal distances was determined for target (green) and recipient (red) FISH signals in interphase nuclei. Photographic slides of nuclei were projected at a distance of 3 m onto a screen, and distances (in centimeters) between red and green signals were measured. The intersignal distances were pooled in bins of 0 to 12 cm (average diameter of an interphase nuclei) in 1-cm intervals. Shown is a histogram of the number of nuclei in each bin for random, unlinked sequences (grey bars) (n = 69; data from two independent pools of ~220 clones), linked sequences (striped bars) (n = 63; pooled data for 3 independent clones), and far ectopic events (white bars) (n = 247; pooled data for 9 independent clones). Bin 0 represents colocalization or near colocalization of red and green signals. All values have been normalized for 63 nuclei. Black bars represent the normal distributions expected from the mean (6.37 cm) and standard deviation (3.27 cm) of the observed random, unlinked intersignal distances. The far ectopic events follow a normal distribution for bins 1 to 12, but the observed number of colocalizations (i.e., ~10 nuclei in bin 0) deviates significantly from the expected number (~1), with a P value of less than 0.0001. (Note that although data were pooled for several clones to increase the total number of nuclei observed, no significant deviations were seen between independent clones with regard to the distribution of intersignal distances.)

An obvious alternative to this model would be that gene conversion between the target and the exogenous vector occurs first, followed by release of the vector and random integration in the genome via illegitimate recombination. The frequencies for each of these events do not support this hypothesis. We have measured gene conversion between an exogenous vector and a genomic homologous target by our assay and found it to be $<10^{-6}$ (data not shown). Since the frequency of illegitimate integration in our assay is 6×10^{-3}, then the frequency of ectopic gene targeting, if it occurred in these two successive steps, should be the product of the frequencies of each step, or 10^{-8} to 10^{-9}. This is at least 2 orders of magnitude lower than what we saw for the frequency of ectopic gene targeting. Of

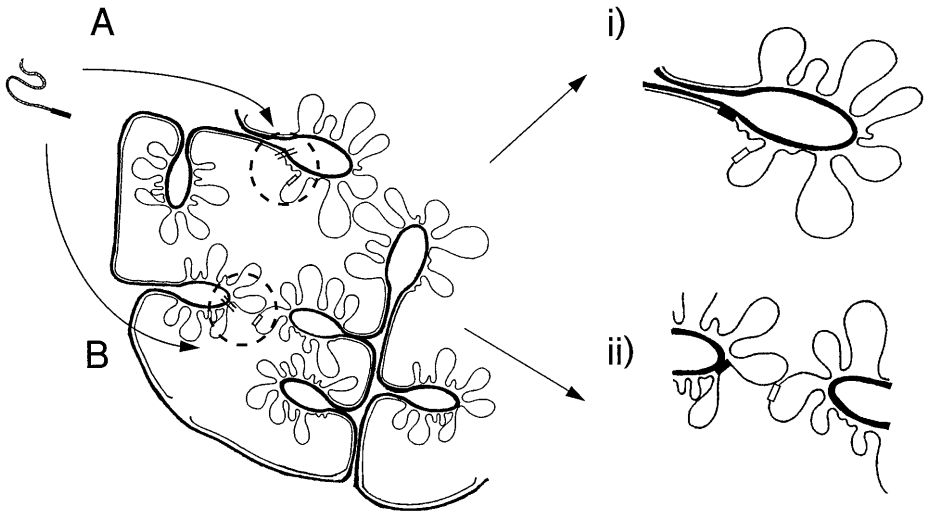

FIG. 5. The DSB proximity model is based on the ability of a DSB to enhance recombination in the local domain in which it has occurred. Heavy lines indicate the nuclear matrix/lamina, and finer lines indicate chromosomal DNA. Note that the break in chromosomal DNA at the lamina delineates the end of one chromosome and the beginning of another. When a DSB (double slanted lines on chromatin loops) occurs, the cell cycle arrests and the DNA at the site of the break becomes associated with DNA repair proteins which may reside on the nuclear matrix as "repair factories." This association alters the accessibility of the domain of chromatin in which the DSB has occurred (indicated by a circle of dashed lines) such that recombination over this domain is enhanced. An incoming linear DNA molecule can mimic a DSB and may therefore be targeted to repair factories at the nuclear matrix, much like genomic DSBs. Accordingly, if linear recipient DNA (black rectangle) is used by the cell to repair a genomic DSB in the same domain of accessibility as the target locus (white rectangle), one-end invasion of the target locus and gene conversion would reconstruct a functional *neo*$^+$ gene (A). Resolution of the event by unwinding would lead to two fluorescent spots adjacent to each other, the distance between them being within a local domain of chromatin accessibility of less than 2 to 3 Mb (panel i). Crossover would lead to deletion of intervening sequences between the target locus and the site of integration of the recipient (with only one fluorescent spot for the recipient present). Alternatively, far ectopic events would involve interaction of chromatin domains of two separate chromosomes (B). A linear recipient molecule used to repair a DSB on one chromosome would be able to interact with the target locus on a different chromosome if the domains containing the target locus and DSB are close to each other (most likely by association with the nuclear matrix). One-end invasion and gene conversion at the target locus would lead to reconstruction of the *neo*$^+$ gene. Release and unwinding of recipient would leave the target locus and chromosome unrearranged, with the recipient integrating in the chromosome with the DSB (panel ii). Resolution by crossover with the target locus would lead to translocation between the two chromosomes. (Note that the method of representation of chromosomes has been adapted from reference 28.)

course, we cannot exclude the possibility that, after the gene conversion step, the exogenous vector becomes highly potentiated for integration. Taking this possibility into account, we would still have to suggest that the site of integration was near the target site, as depicted in Fig. 5, to explain the observed bimodal distribution, which was obviously not random.

In support of the concept that integration is driven by a DSB, we and others have observed that illegitimate integration of an exogenous vector occurs at only one site greater than 90% of the time (8, 12, 32). Furthermore, when there is a gene targeting event, rarely is there also in the same cell a separate illegitimate integration event, even though the latter occurs usually a thousand times more frequently than the former (29). Thus, this suggests that in most of the cells where an integration event (homologous or illegitimate) occurs, there is only one genomic site available for integration. Recently, it has been shown that as few as one DSB can cause p53-dependent cell cycle arrest in human embryonic fibroblasts (14). $CaPO_4$ treatment of cells alone can also induce p53 cell cycle arrest (30). Cell cycle arrest triggered by $CaPO_4$ and/or DNA damage may therefore provide a means of limiting the number of DSBs that can accumulate during a given cell cycle, thus limiting the potential number of sites of integration of exogenous DNA.

The number and types of close (targeted or crossover) events and far ectopic gene targeting events were distinct for all three loci. These trends were even more evident upon pooled analysis of an additional 24 G418^r clones from A1 (12 clones), A6 (6 clones), and A14 (6 clones). Clone A1 had the highest number of close events and the least far ectopic integrations, followed by A14 and then A6 (with the most far events and only one close event). Only clone A6 showed a strong preference for a specific chromosome location and morphology, where ~64% of ectopic integrations occurred in the midarms of acrocentric chromosomes. A1 and A14 showed weaker trends for specific chromosomes, but this may only have been due to the reduced number of distant ectopic integrations seen for these clones. Since the distribution of close and far events differs between loci, this suggests a site-specific effect on ectopic gene targeting.

It is apparent that chromatin within the nucleus is organized in a coherent manner such that gene sequences may be accessed at certain points in the cell cycle for replication and transcription. Compartmentalization seems to occur for these processes (e.g., transcription factories [6]) and replication factories [13, 15]), the factors they require (e.g., splicing factors [35]), and the chromatin involved (chromosome territories [7]). We suggest that perhaps transcription and replication factories may be able to organize DNA domains from the same chromosome or from distinct chromosomes in such a way that accessibility of one domain to another may be enhanced. Using this line of reasoning, it may not be at all surprising that domains on distinct chromosomes have access to each other.

Ectopic gene conversion occurs naturally among nonallelic sequences in many organisms, and the mechanism of ectopic gene targeting also seems to be conserved across phyla. In the present work, we have demonstrated that ectopic gene targeting exhibits a bimodal distribution of integration in murine cells. This indicates that both intra- and interchromosomal sites are accessible to the targeting vector. Thus, we propose that the assay we have used to analyze ectopic gene targeting,

designated RAM, could be used to determine which chromosomal domains within the genome are accessible to given genetic locus.

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REFERENCES

1. **Adair, G. M., R. S. Nairn, J. H. Wilson, M. M. Seidman, K. A. Brotherman, C. MacKinnon, and J. B. Scheerer.** 1989. Targeted homologous recombination at the endogenous adenine phosphoribosyltransferase locus in Chinese hamster cells. Proc. Natl. Acad. Sci. USA **86:**4574–4578.
2. **Aratani, Y., R. Okazaki, and H. Koyama.** 1992. End extension repair of introduced targeting vectors mediated by homologous recombination in mammalian cells. Nucleic Acids Res. **20:**4795–4801.
3. **Belmaaza, A., and P. Chartrand.** 1994. One-sided invasion events in homologous recombination at double-strand breaks. Mutat. Res. **314:**199–208.
4. **Belmaaza, A., J. C. Wallenburg, S. Brouillette, N. Gusew, and P. Chartrand.** 1990. Genetic exchange between endogenous and exogenous LINE-1 repetitive elements in mouse cells. Nucleic Acids Res. **18:**6385–6391.
5. **Berinstein, N., N. Pennell, C. A. Ottaway, and M. J. Shulman.** 1992. Gene replacement with one-sided homologous recombination. Mol. Cell. Biol. **12:**360–367.
6. **Cook, P. R.** 1995. A chromomeric model for nuclear and chromosome structure. J. Cell Sci. **108:**2927–2935.
7. **Cremer, T., et al.** 1993. Role of chromosome territories in the functional compartmentalization of the cell nucleus. Cold Spring Harbor Symp. Quant. Biol. **58:**777–792.
8. **Deshayes, A., L. Herrera-Estrella, and M. Caboche.** 1985. Liposome-mediated transformation of tobacco mesophyll protoplasts by an Escherichia coli plasmid. EMBO J. **4:**2731–2737.
9. **Ellis, J., and A. Bernstein.** 1989. Gene targeting with retroviral vectors: recombination by gene conversion into regions of nonhomology. Mol. Cell. Biol. **9:**1621–1627.
10. **Ernst, J. F., J. W. Stewart, and F. Sherman.** 1981. The cyc1-11 mutation in yeast reverts by recombination with a nonallelic gene: composite genes determining the isocytochromes c. Proc. Natl. Acad. Sci. USA **78:**6334–6338.
11. **Ferguson, D. O., and W. K. Holloman.** 1996. Recombinational repair of gaps in DNA is asymmetric in Ustilago maydis and can be explained by a migrating D-loop model. Proc. Natl. Acad. Sci. USA **93:**5419–5424.
12. **Folger, K. R., E. A. Wong, G. Wahl, and M. R. Capecchi.** 1982. Patterns of integration of DNA microinjected into cultured mammalian cells: evidence for homologous recombination between injected plasmid DNA molecules. Mol. Cell. Biol. **2:**1372–1387.
13. **Hozak, P., A. B. Hassan, D. A. Jackson, and P. R. Cook.** 1993. Visualization of replication factories attached to nucleoskeleton. Cell **73:**361–373.
14. **Huang, L. C., K. C. Clarkin, and G. M. Wahl.** 1996. Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest. Proc. Natl. Acad. Sci. USA **93:**4827–4832.
15. **Jackson, D. A.** 1990. Nuclear organization: uniting replication foci, chromatin domains and chromosome structure. Bioessays **12:**587–591.
16. **Jinks-Robertson, S., and T. D. Petes.** 1986. Chromosomal translocations generated by high-frequency meiotic recombination between repeated yeast genes. Genetics **114:**731–752.
17. **Jinks-Robertson, S., and T. D. Petes.** 1985. High-frequency meiotic gene conversion between repeated genes on nonhomologous chromosomes in yeast. Proc. Natl. Acad. Sci. USA **82:**3350–3354.
18. **Knight, K. L., and M. A. Crane.** 1994. Generating the antibody repertoire in rabbit. Adv. Immunol. **56:**179–218.
19. **Kupiec, M., and T. D. Petes.** 1988. Allelic and ectopic recombination between Ty elements in yeast. Genetics **119:**549–559.
20. **Lemieux, N., B. Dutrillaux, and E. Viegas-Pequignot.** 1992. A simple method for simultaneous R- or G-banding and fluorescence in situ hybridization of small single-copy genes. Cytogenet. Cell Genet. **59:**311–312.
21. **Lichten, M., R. H. Borts, and J. E. Haber.** 1987. Meiotic gene conversion and crossing over between dispersed homologous sequences occurs frequently in Saccharomyces cerevisiae. Genetics **115:**233–246.
22. **Lim, J. K., and M. J. Simmons.** 1994. Gross chromosome rearrangements mediated by transposable elements in Drosophila melanogaster. Bioessays **16:**269–275.
23. **Montgomery, E. A., S. M. Huang, C. H. Langley, and B. H. Judd.** 1991. Chromosome rearrangement by ectopic recombination in Drosophila melanogaster: genome structure and evolution. Genetics **129:**1085–1098.
24. **Murti, J. R., M. Bumbulis, and J. C. Schimenti.** 1994. Gene conversion between unlinked sequences in the germline of mice. Genetics **137:**837–843.
25. **Offringa, R., M. E. Franke-van Dijk, M. J. De Groot, and P. J. van den Elzen.** 1993. Nonreciprocal homologous recombination between Agrobacterium transferred DNA and a plant chromosomal locus. Proc. Natl. Acad. Sci. USA **90:**7346–7350.
26. **Puchta, H., B. Dujon, and B. Hohn.** 1996. Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. Proc. Natl. Acad. Sci. USA **93:**5055–5060.
27. **Ratcliffe, M. J., and K. A. Jacobsen.** 1994. Rearrangement of immunoglobulin genes in chicken B cell development. Semin. Immunol. **6:**175–184.
28. **Razin, S. V., and I. I. Gromova.** 1995. The channels model of nuclear matrix structure. Bioessays **17:**443–450.
29. **Reid, L. H., E. G. Shesely, H. S. Kim, and O. Smithies.** 1991. Cotransformation and gene targeting in mouse embryonic stem cells. Mol. Cell. Biol. **11:**2769–2777.
30. **Renzing, J., and D. P. Lane.** 1995. p53-dependent growth arrest following calcium phosphate-mediated transfection of murine fibroblasts. Oncogene **10:**1865–1868.
31. **Reynaud, C. A., B. Bertocci, A. Dahan, and J. C. Weill.** 1994. Formation of the chicken B-cell repertoire: ontogenesis, regulation of Ig gene rearrangement, and diversification by gene conversion. Adv. Immunol. **57:**353–378.
32. **Richard, M., A. Belmaaza, N. Gusew, J. C. Wallenburg, and P. Chartrand.** 1994. Integration of a vector containing a repetitive LINE-1 element in the human genome. Mol. Cell. Biol. **14:**6689–6695.
33. **Risseeuw, E., R. Offringa, M. E. Franke-van Dijk, and P. J. Hooykaas.** 1995. Targeted recombination in plants using Agrobacterium coincides with additional rearrangement at the target locus. Plant J. **7:**109–119.
34. **Rouet, P., F. Smih, and M. Jasin.** 1994. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol. Cell. Biol. **14:**8096–8106.
35. **Spector, D. L., R. T. O'Keefe, and L. F. Jimenez-Garcia.** 1993. Dynamics of transcription and pre-mRNA splicing within the mammalian cell nucleus. Cold Spring Harbor Symp. Quant. Biol. **58:**799–805.
36. **Thomas, K. R., K. R. Folger, and M. R. Capecchi.** 1986. High frequency targeting of genes to specific sites in the mammalian genome. Cell **44:**419–428.
37. **Trask, B. J.** 1991. Fluorescence in situ hybridization: applications in cytogenetics and gene mapping. Trends Genet. **7:**149–154.
38. **Weil, D., I. Wang, A. Dietrich, A. Poustka, J. Weissenbach, and C. Petit.** 1994. Highly homologous loci on the X and Y chromosomes are hot-spots for ectopic recombinations leading to XX maleness. Nat. Genet. **7:**414–419.
39. **Zheng, H., and J. H. Wilson.** 1990. Gene targeting in normal and amplified cell lines. Nature **344:**170–173.