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Molecular analysis of sister chromatid recombination in mammalian cells

Nadine Puget¹, Melodie Knowlton, and Ralph Scully*

Beth Israel Deaconess Medical Center and Harvard Medical School, 330 Brookline Ave, HIM-923, Boston, MA 02215, USA

Abstract

Sister chromatid recombination (SCR) is a potentially error-free pathway for the repair of double-strand breaks arising during replication and is thought to be important for the prevention of genomic instability and cancer. Analysis of sister chromatid recombination at a molecular level has been limited by the difficulty of selecting specifically for these events. To overcome this, we have developed a novel “nested intron” reporter that allows the positive selection in mammalian cells of “long tract” gene conversion events arising between sister chromatids. We show that these events arise spontaneously in cycling cells and are strongly induced by a site-specific double-strand break (DSB) caused by the restriction endonuclease, I-SceI. Notably, some I-SceI-induced sister chromatid recombination events entailed multiple rounds of gene amplification within the reporter, with the generation of a concatemer of amplified gene segments. Thus, there is an intimate relationship between sister chromatid recombination control and certain types of gene amplification.

Dysregulated sister chromatid recombination may contribute to cancer progression, in part, by promoting gene amplification.

Keywords

Sister chromatid; Homologous recombination; Long tract gene conversion; Gene amplification; I-SceI

1. Introduction

Double strand breaks (DSB) arising during the S or G2 phases of the cell cycle can be repaired by sister chromatid recombination (SCR), a homologous recombination pathway that uses the neighboring intact sister chromatid as a template for repair [1–4]. A major physiological trigger to SCR during replication is thought to be the stalling of a DNA polymerase complex on abnormal DNA structure [5–10]. Because of the sequence identity of the two sister chromatids, SCR could be mutationally silent and error-free. Current models propose a hierarchical relationship between SCR and error-prone mechanisms of double-strand break repair (DSBR), including non-homologous endjoining (NHEJ) and single strand annealing [11–13]. If SCR is not used, a DSB arising at a stalled fork may recombine or rejoin with a heterologous locus resulting in a chromosomal translocation or deletion. SCR is therefore an important mechanism for preventing genomic instability.

Since SCR is likely to occur at sites of DNA polymerase stalling, it is notable that several recombination proteins accumulate at sites of replication arrest in mammalian cells. These include BRCA1, BARD1, Rad51 [14], BRCA2/FANCD1 [15] and the product of the Bloom's syndrome gene, *BLM* [16]. This suggests a role for these gene products in SCR [7]. Consistent with this, primary cells lacking *BRCA1*, *BRCA2/FANCD1*, other Fanconi anemia genes or *BLM* develop spontaneous chromosome aberrations with predominant "chromatid-type" errors—lesions that reflect a failure of recombination during replication [7,17–20]. Other cancer genes potentially involved in SCR include *H2AX* [21–23], *ATR* [24], the ataxia telangiectasia gene, *ATM*, the Nijmegen breakage syndrome gene, *NBS1*, and the *MRE11* gene [25–29]. S phase checkpoint proteins that function with ATR might also regulate SCR [30–32]. Thus, a number of distinct protein complexes appear to cooperate to control SCR, and the failure of this control seems to be a potent trigger to cancer.

SCR in mammalian cells has been studied predominantly by use of the "sister chromatid exchange" (SCE) assay. This allows the cytological identification of crossover events between sister chromatids, but does not provide a molecular picture of the repair event. Further, crossover events represent a minority of recombination events in somatic cells [2,33,34]. Although many ways exist to quantify homologous recombination in somatic cells [11,35–37], few address specifically the SCR pathway. A major reason for this is the difficulty of selecting for some SCR outcomes to the exclusion of other recombination pathways. Two previous molecular studies of SCR used random screening of clones to identify a number of I-SceI restriction endonuclease-induced SCR events in which "long tract gene conversion" (LTGC) between sister chromatids produced a characteristic expansion of the recombination reporter [2,4]. This approach was of limited power, since it employed Southern blot *screening* of clones, not *selection* of clones in which SCR had occurred. Spontaneously arising SCR events could not be detected by this method.

In an effort to improve our understanding of SCR control, we have developed a novel recombination reporter, termed a "nested intron" reporter, which allows the positive, specific selection of SCR events in mammalian cells. We have used this to analyze spontaneously arising SCR/LTGC events in mammalian cells, as well as I-SceI/DSB-induced events. We find that the molecular outcomes of SCR, both spontaneous and I-SceI-induced, vary qualitatively on a clone-to-clone basis. We report here a novel outcome of SCR, characterized by multiple rounds of gene amplification within the "nested intron" reporter.

2. Materials and methods

2.1. Plasmid construction

The "nested intron" recombination reporter HRsub (Fig. 1) was constructed in several steps using PCR and standard cloning procedures; the resulting construct was confirmed correct by DNA sequencing. Modified *gfp* copies were produced by PCR from the enhanced green fluorescent gene (*GFP*, Clontech). The donor *gfp* copy (first repeat) has no promoter and lacks the first 12 amino acids of the ORF. The recipient *gfp* copy (second repeat) is driven by the human CMV promoter, but is inactivated by an insertion of the 18 bp I-SceI recognition site 5'-TAGGGATAACAGGGTAAT-3', replacing 4 bp in the middle of the natural *GFP* sequence and creating a premature stop codon (underlined). The blasticidin-*S*-deaminase gene (*BsdR*) expression cassette was constructed by PCR from the pUB6/V5-HisC plasmid (*BsdR* cDNA; Invitrogen) and from the pSG5 vector (rabbit β -globin intron II; Stratagene). The SV40 early promoter driving *BsdR* expression was amplified from pUB6/V5-HisC and the BGH polyadenylation signal was amplified from vector pcDNA3 (Invitrogen). The HRsub reporter was assembled in pBlue-script II SK(–) (Stratagene), then subcloned into a modified version of the pPUR vector (Clontech), pPURO', in which the promoter driving expression of the puromycin resistance gene was replaced by the PGK promoter from pMSCVpuro (Clontech).

and the polyadenylation signal replaced by a PCR fragment amplified from pEGFP-N3 (Clontech) containing the HSV TK polyA. Test constructs described in Fig. 2 were produced from the same PCR fragments and similar procedures, and were subcloned into vector pcDNA3 (Invitrogen). The I-SceI expression vector, pcDNA3 β mycNLS-I-SceI, is a modified version of pCMV-I-SceI [38] containing the I-SceI coding sequence fused at its 5' end to a triple myc tag and a nuclear localization signal, in vector pcDNA3 β [39].

2.2. PCR analysis

Genomic DNA (gDNA) was extracted from $\sim 5 \times 10^6$ cells using the Puregene DNA Isolation Kit (Gentra Systems). PCR analysis of BsdR+ clones was carried out using 200 ng of gDNA and primers F (*Bsd* gene-mut-Fw 5'-atggccaagccattgtctcaa) and R (*Bsd* gene-Rev 5'-tagccctccacacataacc) to amplify a 2.6 kb fragment from *BsdR* exon A to *BsdR* exon B (Expand Long Template PCR System, Roche). The PCR products were digested with I-SceI (New England Biolabs) and analyzed by 1% agarose gel electrophoresis (Fig. 3).

2.3. Southern analysis

Southern blotting analysis was performed using 5 μ g of gDNA as described previously [40]. A 0.7 kb PCR fragment containing the complete *GFP* coding sequence was used as a probe (Random-primed DNA labeling kit; Roche). This product was amplified from pcDNA3-wt*GFP* (Fig. 2) using primers *EGFP* gene-Fw 5'-atggtgagcaaggcgagg and *EGFP* gene-Rev 5'-ctttactgtacagctcgtcc.

2.4. Northern analysis

Total RNA was prepared from 2×10^6 to 5×10^6 electroporated cells, selected in either G418 (400 μ g/ml) or blasticidin (5 μ g/ml), by using the RNeasy Mini kit (Qiagen). Northern hybridization was performed with a 0.4 kb PCR fragment probe containing the complete *BsdR* coding sequence. This product was amplified from pcDNA3-wt*BsdR* (see Fig. 2) using primers F and R (Fig. 4) and labeled using the BrightStar Psoralen-Biotin labeling kit (Ambion). Detection was performed using the BrightStar Biodetect kit (Ambion).

2.5. Cell culture and transfections

Cell lines were cultivated in DMEM medium supplemented with 10% Fetal Clone I (Hyclone) at 37 °C and 6% CO₂. To generate stable cell lines, 6×10^6 U2OS cells were electroporated with 1 μ g of linearized plasmid DNA in a 0.4 cm-electrode gap cuvette (BioRad Gene Pulser; 960 μ F/250 V). The constructs were linearized with AflIII prior to electroporation. To construct the U2OS reporter cell lines #18 and #24, puromycin (2 μ g/ml) was added to the medium 48 h after electroporation. After 2 weeks under continuous selection, puromycin-resistant colonies were isolated and screened by Southern-blotting with the *GFP* probe. The U2OS Clones #18 and #24 were determined to contain a single, complete, randomly integrated copy of the 7.8 kb HRsub reporter. Seven different restriction enzymes were used for Southern analysis: *Bam*HI, *Eco*RI, *Pst*I, *Eco*RV, *Hind*III, *Spe*I and *Sma*I (data not shown). In subsequent transfections (0.5 – 1.0×10^6 cells) were transfected with 25 μ g of pcDNA3 β mycNLS-I-SceI, using a modified calcium phosphate-mediated transfection procedure.

2.6. Recombination assays

Recombination was induced by transient transfection of cells with pcDNA3 β mycNLS-I-SceI. To assay *GFP* expression, cells were analyzed 4–5 days post-transfection using a Becton–Dickinson FACScan on a green (FL1) versus red (FL2) fluorescence plot with 25% FL2–FL1 compensation to visualize GFP+ cells [37]. Transfection efficiency was measured by flow cytometry for a calcium phosphate-mediated co-transfection of pcDNA3 β mycNLS-I-SceI with pcDNA3-wt*GFP* (ratio 10:1), the percentage of green cells representing in this case the

percentage of transfected cells. On average, the transfection efficiency of I-SceI or control plasmid was >75%. To assay SCR events, 24 h post-transfection, cells were trypsinized, counted and replated at 50,000, 100,000 and 200,000 per 10 cm dish (duplicates). Blasticidin (5 µg/ml, Invitrogen) was added to the medium 4 days after plating. After 2 weeks under continuous selection, BsdR⁺ colonies were either stained and counted to determine frequencies, or isolated and expanded for molecular analysis of genomic DNA. Staining of colonies was performed using fixation in 10% methanol/10% acetic acid, followed by incubation in 0.4% crystal violet/20% ethanol. Viability and plating efficiency were determined by seeding 200 cells per 6 cm dish (quadruplicates) into non-selective medium, incubation for 2 weeks and colony staining. On average, the plating efficiency of each clone was ~30–40%.

3. Results

3.1. Strategy for molecular analysis of sister chromatid recombination

We based the “nested intron” reporter on a standard recombination reporter containing tandem mutant copies of the gene encoding enhanced green fluorescent protein (*GFP*, Fig. 1). The first *GFP* copy is truncated at the 5'-end; the second is full-length, with an interruption by an 18 bp sequence recognized by the rare-cutting restriction endonuclease, I-SceI [38]. Because of the orientation of the *GFP* direct repeats, homologous recombination between these two copies, but not single strand annealing, could generate a wild-type *GFP* gene. “Short tract” gene conversion (STGC) could arise from either inter-chromatid recombination (SCR) or intrachromatid recombination (Fig. 1A). LTGC or crossing over between sister chromatids could each generate an expansion of the reporter resulting in triplication of the *GFP* copies (Fig. 1B; [2]). Since LTGC and crossing over are both SCR events, “*GFP* triplication” is diagnostic of SCR. In yeast and mammals, crossing over is suppressed in somatic cells [2,33, 41]. Hence, LTGC is the major pathway leading to “*GFP* triplication” and we have accordingly termed this outcome “SCR/LTGC”. It should be noted that a small proportion of “*GFP*” triplication events might in fact represent crossing over associated with SCR.

In the “*GFP* triplication” outcome, the region between the *GFP* copies (indicated by a red star, Fig. 1B) is duplicated. To select for this duplication event, we inserted into this region an expression cassette containing two artificial exons (A and B) of the blasticidin-*S*-deaminase gene (*BsdR*; Fig. 1C). *BsdR* exons A and B were placed “head-to-toe”, so that wild-type *BsdR* is not expressed when the reporter is in its original, unarranged form. Intron sequences were placed to create a splice donor site 3' of exon A and a splice acceptor site 5' of exon B. An SV40 promoter was inserted 5' of exon A and a polyadenylation signal 3' of exon B (Fig. 1C). Cells containing the unarranged reporter or the products of STGC are sensitive to blasticidin (BsdR⁻) since the two *BsdR* exons are not properly orientated with respect to each other. In contrast, an LTGC or cross over event could duplicate the *BsdR* cassette, resulting in the positioning of one *BsdR* exon A in a correct orientation with respect to exon B of the neighboring, duplicated *BsdR* cassette (Fig. 1D). The corresponding transcript (initiated at promoter α , Fig. 1D) could then be spliced to generate a wild-type *BsdR* mRNA, making the cell blasticidin resistant (BsdR⁺). The *BsdR* intron would contain a “nested” wild-type *GFP* gene (Fig. 1D). Through analysis of a series of test constructs mimicking the “nested intron” rearrangement (Fig. 2 and Table 1), we found that optimal expression of *BsdR* was obtained when the *BsdR* and *GFP* genes were transcribed in similar orientations, and we designed the final reporter, termed “HR sub”, accordingly (Fig. 1C). Other test constructs revealed no detectable deleterious effect of the *GFP* polyadenylation signal upon expression of *BsdR* (data not shown). This is consistent with in vitro data showing that a polyadenylation signal encountered within an intron is suppressed pending completion of the splicing event [42,43].

3.2. Frequencies of spontaneous and DSB-induced gene conversion events in mammalian cells

We electroporated the recombination-proficient human osteosarcoma cell line, U2OS, with the linearized HR sub reporter and used Southern blotting and PCR to identify two clones, #18 and #24, out of a total of ~60 independent clones examined, which contained only one full-length copy of the reporter (data not shown, see also Figs. 5 and 6). We used flow cytometry for GFP to quantify gene conversion in the reporter, in each of the clones. In addition, we quantified SCR/LTGC by counting BsdR+ colonies after selection of cells in blasticidin. In the same assays, parental U2OS cells, lacking any recombination reporter, generated no GFP+ and no BsdR+ events.

Passage of Clones #18 and #24 in tissue culture revealed low frequencies of spontaneously arising GFP+ cells at $\sim 45 \times 10^{-6}$ (Clone #18) and $\sim 30 \times 10^{-6}$ (Clone #24, Fig. 3A) and of BsdR+ cells at $\sim 32 \times 10^{-6}$ (Clone #18) and $\sim 3 \times 10^{-6}$ (Clone #24, Fig. 3B). Thus, some spontaneous recombination events at this locus (as measured by the generation of a GFP+ cell) occurred by a SCR/LTGC mechanism (giving rise to a BsdR+ cell). However, as discussed in detail below, the majority (>95%) of *spontaneous* recombination events produced BsdR+ clones that were GFP-. Because of this, it is difficult to estimate accurately the ratio of spontaneous SCR/LTGC:GC events. These measurements of spontaneous recombination represent *cumulative frequencies* of stochastic events—parameters only indirectly related to the actual spontaneous recombination *rates* at the loci in question. The measured *frequencies* of spontaneous GFP+ events varied ~2-fold between U2OS Clones #18 and #24. For each of U2OS #18 and #24, the frequencies of spontaneous BsdR+ events varied up to 10-fold. This wider variability is probably a reflection of the very low actual numbers of BsdR+ colonies counted in such assays (typically, between 3 and 30 per million cells).

The endonuclease I-SceI can be used to generate a site-specific DSB within the recombination reporter and thereby promote gene conversion at the reporter locus. Transfection of an I-SceI-encoding plasmid into Clones #18 or #24 strongly induced both GFP+ and BsdR+ events (Fig. 3A and B). To obtain accurate values, the frequencies noted here have been corrected for transfection efficiency and (for BsdR+ events) plating efficiency of the cells (see Section 2). More than 95% of *I-SceI-induced* BsdR+ colonies were found to be GFP+ (as predicted for the SCR/LTGC outcome; Fig. 1D). This high percentage of GFP+ clones is to be expected, since the DSB induced by I-SceI in the second copy of the *GFP* gene is repaired using the wild-type sequence of the first *GFP* copy on the sister chromatid. Results of four independent experiments using U2OS Clone #18 are summarized in Table 2. In these experiments, on average ~7.0% of I-SceI-transfected cells became GFP+ and ~1.1% of transfected cells became BsdR+. In one experiment using U2OS Clone #24, ~3.8% of I-SceI-transfected cells became GFP+ and ~1.3% became BsdR+. Thus, ~16% (Clone #18) and ~35% (Clone #24) of I-SceI-induced GFP+ cells were also BsdR+. These results suggest that between 1/6 and 1/3 of all *I-SceI-induced* gene conversion events that lead to the formation of wtGFP engage a SCR/LTGC mechanism. In summary, therefore, by examining two independent U2OS clones carrying single copies of randomly integrated “nested intron” reporters, we found a 2-fold variation in I-SceI-induced GC events (measured as GFP+), and a 2-fold variation in the ratio of I-SceI-induced SCR/LTGC:GC events (measured as BsdR+:GFP+).

To confirm that the expected splicing events had occurred within the *BsdR* transcript in BsdR+ cells, we analyzed Northern blots from pooled I-SceI-induced BsdR+ colonies, using a *BsdR* probe. This revealed a processed *BsdR* transcript that co-migrated with wild-type *BsdR* mRNA (pcDNA3-wt*BsdR*; Fig. 2B and Fig. 3C), as well as with the processed *BsdR* mRNA expressed from a series of test constructs containing appropriately orientated *BsdR* exons A and B separated either by a simple intron (p Test BsdR A+B; Fig. 2C and Fig. 3C) or by an intron containing a functional expressed *GFP* gene (pTest SAMOR; Fig. 2D and Fig. 3C). The

insertion of a simple, intact intron within the *BsdR* gene dramatically increased expression of *BsdR* (compare constructs depicted in Fig. 2B and C; *BsdR* expression levels shown in Fig. 3C). The presence of a functional *GFP* gene within the “nested intron” reduced expression of the *BsdR* gene (compare constructs depicted in Fig. 2C and D; *BsdR* expression levels shown in Fig. 3C). The cause of this “knock-down” effect is not known, but may reflect promoter interference within the “nested intron” construct (pTest SAMOR; Fig. 2D).

3.3. Analysis of long tract gene conversion products

To determine the mechanism(s) responsible for generating BsdR⁺ events, we prepared genomic DNA from 88 individual BsdR⁺ clones (27 spontaneously arising, 61 I-SceI-induced), and analyzed the recombination breakpoint within the “nested” intron by PCR, using a forward primer specific for *BsdR* exon A and a reverse primer specific for *BsdR* exon B (Fig. 4). By this arrangement, only those clones that contained the duplicated *BsdR* cassette should generate a PCR product; this was in fact the case (Fig. 4 and data not shown). If the breakpoint leading to duplication of the *BsdR* cassette were homologous (reflecting recombination between *GFP* repeats), the PCR product generated across the “nested” intron should be 2.6 kb (Fig. 4A). Conversely, a non-homologous mechanism of *BsdR* cassette duplication might generate PCR products across the “nested” intron of sizes other than 2.6 kb. We found that *all* PCR products analyzed from 88 distinct BsdR⁺ clones (whether spontaneously arising or I-SceI-induced) were 2.6 kb in size (Fig. 4B and data not shown), consistent with a mechanism of *BsdR* cassette duplication caused by homologous recombination between two *GFP* repeats.

Surprisingly, all but one *spontaneously arising* BsdR⁺ clones (26/27; 96%) were GFP[−] and gave rise to PCR products that contained an intact I-SceI site (Fig. 4B, S1 and S2). The one remaining BsdR⁺ clone was GFP⁺ and gave rise to a PCR product lacking an I-SceI site (data not shown). Sequencing of a number of PCR products from different BsdR⁺ GFP[−] clones showed that the *GFP* copy “nested” within the wt *BsdR* intron was identical in sequence to the I-SceI-containing *GFP* copy of the original, unrearranged reporter (data not shown). This indicates that, in these clones, the site for recombination within the I-SceI-containing *GFP* copy lay 3′ of the I-SceI restriction site, thus preserving the I-SceI site in the final recombination product and leading to a GFP[−] clone. The fact that there was a bias in favor of GFP[−] status for spontaneously-arising BsdR⁺ events implies that the preferred site for *spontaneous* recombination within the I-SceI-containing *GFP* copy lies 3′ of the I-SceI site. The reasons for this observed preference are unclear. However, this might be a reflection of the available sequence homology either side of the I-SceI site, the 5′ homology being 353 bp and the 3′ homology 569 bp. In studies of spontaneous recombination between episomal plasmids, the efficiency of recombination was found to increase as a function of available sequence homology [44]. The shortest tract of homology sufficient to support recombination between episomal plasmids was found to be ~200 bp [45]. Perhaps the 5′ tract of 353 bp is less efficient in supporting recombination than the more extensive 3′ tract of 569 bp.

In the analysis of *I-SceI-induced* BsdR⁺ clones, PCR products derived from GFP⁺ clones were found to lack an I-SceI site (Fig. 4B, I1 and I2). We also analyzed the rare GFP[−] BsdR⁺ clones present in I-SceI-transfected cultures. All but one PCR product from these GFP[−] clones were found to contain an intact I-SceI site, suggesting that the restriction site had not been cut by I-SceI. These clones therefore represent “background” *spontaneously arising* SCR/LTG events, more than 95% of which would be expected to retain an I-SceI site and be GFP[−] (as noted above). One exception to this pattern was a single BsdR⁺ GFP[−] clone, derived from an I-SceI-transfected cell culture, which generated a PCR product of apparently unaltered size (2.6 kb) that paradoxically *lacked* an I-SceI site. Sequencing of the “nested” *GFP* copy of this clone revealed an 8 bp deletion within the I-SceI site, indicating that I-SceI had cut this site and suggesting that the DSB was subsequently repaired by NHEJ. It is possible that this represents

a novel, NHEJ-mediated fusion event between sister chromatids. A more parsimonious interpretation is that it represents non-homologous end-joining of a cleaved I-SceI site, occurring in a *spontaneously arising* BsdR⁺ GFP⁻ cell that was already present at the time of I-SceI transfection. Given the existence of “background” spontaneously arising BsdR⁺ GFP⁻ clones in I-SceI transfected cultures, the preferential retention of an intact I-SceI site in the “nested” GFP copy in such clones and the high efficiency of end-joining in mammalian cells, the latter interpretation seems more likely.

3.4. Structure of the long gene conversion tracts

To determine the rearrangements associated with BsdR⁺ status, we first analyzed the structure of the reporter locus in BsdR⁺ colonies, using Southern blotting with GFP as a probe (Fig. 5A). The entire reporter can be retrieved as one GFP-hybridizing fragment in genomic DNA digested with *Pst*I. In this case, the unrearranged reporter locus of the parental Clones #18 and #24 produced a GFP-hybridizing fragment of 5.9 kb (Fig. 5A; Fig. 5B and C, parental lane “P”). Conversely, any expansion in the size of the reporter should cause an increase in the size of the *Pst*I restriction fragment. The predicted size of this fragment in the “GFP triplication” outcome is 9.1 kb (Fig. 5A). The *BsdR* cassette contains a *Hind*III site (Fig. 5A). By digesting genomic DNA with *Hind*III, we therefore expected to separate the two GFP copies of the original unrearranged reporter into two GFP-hybridizing fragments—one fragment of 3.05 kb and one larger fragment, the size of the latter depending upon the location of the nearest *Hind*III site on the chromosome 3' of the reporter locus. This was the case (Fig. 5D and E, parental lane “P”). The larger fragment varied between Clones #18 and #24, since the reporters were randomly integrated. If a clone had undergone “GFP triplication”, this would entail duplication of the *BsdR* cassette and should therefore introduce a second *Hind*III site within the reporter, resulting in the separation of all three GFP copies onto different sized genomic DNA fragments (Fig. 5A). Digestion of genomic DNA with *Hind*III should now generate a new GFP-hybridizing fragment of 3.2 kb in addition to the pre-existing 3.05 kb and larger fragments.

We first examined the structure of the reporter in *spontaneously arising* BsdR⁺ clones. In both Clone #18 and Clone #24, the majority of BsdR⁺ clones examined revealed a Southern-blot GFP hybridization pattern consistent with “GFP triplication” (Fig. 5B–E). Although most of these BsdR⁺ clones contained the expected three GFP copies, a minority revealed aberrant SCR/LTGC events (Fig. 5B and D, Clones #2 and #4), in which only two GFP copies were detected in the expanded reporter. PCR analysis of the “nested intron” of these clones, using the above-noted *BsdR* primers (Fig. 4), revealed the expected 2.6 kb PCR product, indicating that a SCR/LTGC event had indeed occurred. Repair events of this type must have entailed a gene conversion tract sufficiently long (>1.03 kb) to duplicate *BsdR* exon B, but not long enough to triplicate the GFP genes (i.e., <3.22 kb).

Southern analysis of some *spontaneously arising* BsdR⁺ clones in U2OS #24 cells revealed a retained, apparently un-rearranged reporter locus in addition to the expanded reporter locus (Fig. 5C and E, #3, #5 and #6). These hybridization patterns may reflect non-dysjunction of sister chromatids occurring in the same cell cycle as the spontaneous SCR/LTGC event. The significance of these observations is unclear at present.

Next, we analyzed the structure of the rearranged reporter in *I-SceI-induced* BsdR⁺ GFP⁺ colonies by Southern blotting, with use of a GFP probe (Fig. 6). For all 61 clones examined, the *Pst*I digest revealed a GFP-hybridizing Southern blot fragment larger than the 5.9 kb of the original parental reporter locus. As was noted for spontaneously arising SCR/LTGC events, most I-SceI-induced BsdR⁺ clones examined revealed “GFP triplication” within the reporter. However, as was noted in spontaneously arising BsdR⁺ clones, some I-SceI-induced clones contained rearrangements suggestive of early termination of the “long tract” (Fig. 6A and C,

Clone #5; Fig. 6B and D, Clones #3, 4, 5, 7). In total, 23/27 (85%) I-SceI-induced BsdR+ GFP+ clones of U2OS #18 revealed the typical “GFP triplication” outcome (see Fig. 1D), and 4/27 (15%) revealed aberrant patterns of LTGC/SCR. Equivalent frequencies for U2OS #24 were 28/34 (82%) and 6/34 (18%), respectively.

Southern analysis of some *I-SceI-induced BsdR+* colonies revealed two copies of the reporter—both the “GFP triplication” outcome and the parental reporter (data not shown). Invariably, a proportion of cells in these colonies were GFP[−]. Recloning of these colonies, initially in the absence and later in the presence of blasticidin, followed by assessment of GFP status and Southern analysis, revealed two distinct types of sub-clone. The first was GFP⁺ BsdR⁺, and contained only the “GFP triplication” reporter structure. The second was GFP[−] BsdR[−], and contained only the unrearranged parental reporter structure (data not shown). The existence of these colonies of mixed reporter structure, and the separation of reporter types by recloning, was also noted by Johnson and Jasin [2]. Perhaps the initial survival in blasticidin of those cells that contained the parental reporter structure (and were therefore *genetically BsdR*[−]) reflects the retention of wild-type *BsdR* mRNA by both daughter cells at the mitosis following the recombination event.

3.5. Identification of multiple rounds of gene amplification mediated by SCR

Surprisingly, a small proportion (~3%) of *I-SceI-induced BsdR+ GFP+* clones (1/27 for U2OS #18; 1/34 for U2OS #24) revealed unexpected gene amplification events within the reporter. In Southern blots of these clones, the *GFP* probe hybridized intensely with only one greatly expanded fragment of *Pst*I-digested genomic DNA (Fig. 6A, U2OS #18 lane 4; Fig. 6B, U2OS #24 lane 6). In the same clones, the *Hind*III digest, which cuts within the *BsdR* cassette, revealed an amplified fragment of 3.2 kb (Fig. 6C, U2OS #18 lane 4; Fig. 5D, U2OS #24 lane 6)—the size predicted for SCR-mediated duplication of the *BsdR* cassette (Fig. 5A). In one clone (U2OS #18, Fig. 6A and C, lane 4), densitometric analysis suggested that the *BsdR* cassette had been quadruplicated (Fig. 6E); in another (U2OS #24, Fig. 6B and D, lane 6), it had been triplicated. PCR performed on genomic DNA from these clones, using primers spanning the “nested” intron (Fig. 4A), revealed a 2.6 kb product lacking an I-SceI site in each case. These rearrangements therefore arose from multiple rounds of gene amplification within the reporter, mediated by homologous recombination between *GFP* repeats of two sister chromatids.

4. Discussion

This work describes a novel molecular analysis of sister chromatid recombination in mammalian cells. By developing a “nested intron” reporter for the positive selection of “long tract” gene conversion events between sister chromatids, we have made quantitative analysis of this pathway technically straightforward. This has allowed us to extend the analysis of SCR beyond the scope of previous work, which relied upon random screening of clones to identify and analyze a small number of I-SceI-induced SCR events [2]. Here, we have identified *spontaneously arising* SCR/LTGC events for the first time, proving that this pathway can be engaged during normal cell growth and that SCR/LTGC is not unique to I-SceI-mediated DSBs. We have also analyzed *I-SceI-induced* SCR/LTGC events in detail. This has produced the surprising finding that SCR can initiate gene amplification and mediate the subsequent concatemerization of the amplified locus.

Johnson and Jasin [2] estimated the frequency of I-SceI-induced SCR/LTGC events in the Chinese hamster cell lines V79 and AA8 as between 16 and 33% of all measurable I-SceI-induced recombination events. Using the human osteosarcoma cell line, U2OS, we estimate this ratio to be ~16 and ~35% for two distinct reporter lines with different (random) sites of integration. Despite the fact that we used a cell line different to those used by Johnson and Jasin and that the reporters were integrated at random loci, our estimate of this ratio agrees

closely with that made by Johnson and Jasin. The “nested intron” reporter provides a means to study the genetic basis of the “choice” between short and long tract gene conversion pathways. Notably, U2OS cells are hypertriploid and, being a cancer cell line, have numerous genetic alterations. To study the genetic control of SCR, it may be advantageous in the future to study SCR “nested intron” reporters in genetically defined cells. Our current work suggests that the reporter functions in a similar fashion in at least one other cell type (N.P., unpublished observations).

It is not clear to what extent spontaneous SCR will resemble I-SceI-induced LTGC events. I-SceI could induce SCR at any cell cycle stage subsequent to replication of the reporter locus, including the G2 phase of the cell cycle. Even during S phase, DSBs induced by I-SceI need not be generated at or close to the replication fork. In contrast, spontaneously arising SCR in somatic cells may be triggered predominantly by DNA polymerase stalling, which would restrict the initiation of SCR to stalled replication forks in S phase. Further, although replication-related DSBs can arise in pathological situations [27,46–48], it is not clear that DSBs are necessary physiological triggers to SCR. Potentially, single-stranded DNA at stalled forks might stimulate SCR [49–51].

Whether spontaneously arising or I-SceI-induced, SCR/LTGC generates gene conversion tracts of variable length. In the I-SceI-induced SCR/LTGC events reported here, a majority (82–85%) of the tracts entailed triplication of the *GFP* copies. Other tracts terminated earlier, indicating some heterogeneity in the control of termination of the long gene conversion tract. Conceivably, factors that influence heteroduplex stability, branch migration or Holliday junction resolution might influence the extent of LTGC. Candidate regulators of these processes include, at a minimum, the Rad51 paralogs XRCC3 and Rad51C [52,53], BLM [54] and potentially mismatch repair genes. Short gene conversion tracts in mammalian cells commonly extend less than 100 bp [35,55]. In contrast, “*GFP* triplication” implies a gene conversion tract of at least 3.22 kb. These long tracts presumably require a negotiation between the branch migration process and chromatin neighboring the DSB. Notably, chromatin is modified extensively around DSBs, with the phosphorylation of the C terminal tail of histone variant, H2AX, and recruitment of associated proteins such as 53BP1, MDC1 and BRCA1/BARD1 [56–58]. In yeast, SIR silencing proteins relocalize to chromatin near a DSB [59,60]. Perhaps one function of these chromatin responses is to control resolution of the gene conversion tract. If so, “long tract” gene conversion might represent a failure of this regulation, perhaps loosely analogous to break-induced replication in yeast [61]. The “*GFP* triplication” outcome specifies a minimum gene conversion tract length (3.22 kb) but not a maximum tract length. Some “*GFP* triplication” events may therefore entail gene conversion tracts longer than 3.22 kb.

A surprising feature of some SCR events described here is the amplification and concatemerization of the *BsdR* cassette within the reporter. These events were rare, constituting only ~3% of all I-SceI-induced *BsdR*⁺ events. Analysis of the amplified segment showed that it had been generated by homologous recombination between *GFP* repeats. Possibly, these events reflect repeated DSB induction by I-SceI and repeated engagement of an SCR pathway (whether LTGC or crossing over). However, each clone containing concatemers of *BsdR* cassettes revealed only one expansion form, rather than a mixture of intermediate forms containing fewer expansions. This raises the possibility that these amplification outcomes arose from single recombination “events”, rather than by repetitive breakage induced by I-SceI. Whatever the mechanisms involved, these findings indicate that SCR can both initiate gene amplification and mediate the further concatemerization of the amplified locus.

Rare examples of gene amplification have been reported in the normal development of certain model organisms, such as “magnification” in *Drosophila* rDNA genes [62,63]. Gene

amplification can also arise pathologically in oncogene loci of cancer cells. Early gene amplification is *intrachromosomal*, indicating that dysregulated sister chromatid interactions initiate gene amplification [64]. Interestingly, mismatch repair genes have been implicated in suppressing gene amplification [65], a finding consistent with the role for recombination identified here.

The boundaries of some oncogene amplicons have been found to coincide with chromosomal “fragile sites” [46,66]—loci that are prone to persistent breakage in the presence of agents that arrest DNA synthesis. Fragile site expression is controlled by the S phase signaling kinase, Atr, a key mediator of the cell’s response to replication arrest [67]. Together, these observations suggest that fragile site breakage is initiated at or close to stalled replication forks. If so, fragile site expression and subsequent gene amplification might result from dysregulated SCR control. “Nested intron”—type reporters will serve as useful tools to test this hypothesis, and to determine the specific roles of cancer genes such as *BRCA1*, *BRCA2*, Fanconi anemia genes and *BLM* in this process.

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Fig. 1.

Construction of the sister chromatid recombination reporter. Open boxes: mutant *gfp* genes; green boxes: wt*GFP*. Black arrow: human CMV promoter; black boxes: SV40 polyadenylation signals for *GFP* genes. Double red lines identify the restriction site cut by I-SceI. (A) Repair of an I-SceI-induced DSB by STGC, whether inter- or intra-chromatid, generates wt*GFP* but does not duplicate the intervening sequence between the two *GFP* copies (marked by red star). (B) Repair of the I-SceI-induced DSB by LTGC between sister chromatids, or by crossing over (not shown) can generate “*GFP* triplication”. The middle *GFP* gene becomes wild-type and the red star is duplicated. Depending on the length of the gene conversion tract, other LTGC products are also possible (see text). (C) Schematic of the *BsdR* cassette, inserted at the site of

the red star, and of the final reporter, “HRsub”. Artificial *BsdR* exons A and B are orange; fragments of the rabbit β -globin intron II are grey. Each intron-exon boundary constitutes a consensus splicing site, as shown. The SV40 early promoter (black arrow) is located 5' of exon A and the BGH polyadenylation signal (black box/pA) is 3' of exon B. The murine PGK promoter (black arrow) drives expression of the puromycin resistance gene, *PuroR* (blue box), for selection of cells having integrated the HRsub reporter. An HSV TK polyadenylation signal is 3' of *PuroR*. (D) Selection for LTGC events. In the “*GFP* triplication” outcome, the *BsdR* cassette is duplicated. Promoter α (SV40) drives expression of wt*BsdR*, and promoter β (CMV) drives expression of wt*GFP*. Expected RNA transcripts are shown.

Fig. 2.

Test constructs mimicking the “nested intron” rearrangement. See Fig. 1 legend for key to gene/intron fragments and other elements. (A) Plasmid encoding a wtGFP gene (β : CMV promoter). (B) Plasmid encoding a wtBsdR gene (α : SV40 promoter). (C) Test construct with artificially split exons A and B of the BsdR gene, separated by the intact rabbit β -globin intron II. (D) Test construct “SAMOR” contains a wtGFP gene “nested” within the BsdR intron, both GFP and BsdR genes being transcribed in the same orientation. (E) Test construct “INVOR” contains a wtGFP gene “nested” within the BsdR intron, but with GFP gene transcription in the opposite orientation to that of the BsdR gene. See Table 1 and text for more details.

Fig. 3.

Induction of GFP+ cells and BsdR+ colonies by I-SceI. (A) FACS analysis after transfection of recombination reporter Clone U2OS #24 with control plasmid or with I-SceI plasmid. FL1-H, green fluorescence; FL2-H, red fluorescence. GFP+ cells lie above the diagonal, percentages as indicated. (B) Crystal violet staining of BsdR+ colonies after transfection of U2OS Clone #24 with control plasmid or with I-SceI plasmid. For each transfection, 1.2×10^6 cells were cultured in blasticidin for 2 weeks before staining. (C) Northern blot analysis of *BsdR* transcripts. RNA from U2OS cells transfected with a series of test constructs (lanes 1–7) or from pooled I-SceI-induced BsdR+ colonies of reporter Clone #24 (lane 8). The cells were selected either in G418 (resistance given by the plasmid backbone for the test constructs) or in Blasticidin. *Top panel*: Northern blot probed with *BsdR* cDNA (400 bp) reveals a ~1 kb mRNA in all cell lines expressing *BsdR* (lanes 2–7). *BsdR* mRNA abundance is greatly increased by the presence of the β -globin intron between *BsdR* exons A and B (pTest-BsdR A+B, lanes 3 and 6; see Fig. 2C) but this effect is reduced when the wtGFP gene is “nested” within the intron (pTest-SAMOR, lanes 4 and 7; see Fig. 2D). Pooled I-SceI-induced BsdR+ colonies from U2OS Clone #24 contain a processed *BsdR* transcript (lane 8). *Bottom*: 18S and 28S rRNA from the same gel revealed by ethidium bromide staining.

Fig. 4.

PCR analysis of the reporter locus in BsdR⁺ colonies. (A) Scheme for PCR analysis, showing parental reporter (*top*) and “GFP triplication” outcome (*bottom*). Duplication of the *BsdR* cassette in the rearranged reporter allows a 2.6 kb fragment to be amplified from genomic DNA of BsdR⁺ clones with the indicated primers F and R (short black arrows). The presence of an I-SceI site in the PCR product allows I-SceI digestion to generate two smaller fragments, as indicated. (B) Representative results of the PCR analysis in Clone #24. *P*: genomic DNA from parental #24 clone does not give rise to a PCR product, since the *BsdR* cassette is not duplicated; *S1*, *S2*: 2.6 kb PCR products from spontaneously arising GFP[−] BsdR⁺ colonies contain an I-SceI site; *I1*, *I2*: 2.6 kb PCR products from I-SceI-induced GFP⁺ BsdR⁺ colonies lack an I-SceI site; *M*: 1 kb ladder molecular weight marker.

Fig. 5.

Southern blot analysis of spontaneously arising BsdR⁺ colonies. (A) *PstI* and *HindIII* maps of the reporter, restriction fragment sizes indicated. Figure shows the parental reporter (*top*) and the predicted rearrangement in the “GFP triplication” outcome (*bottom*). (B–E) Southern blotting performed on U2OS Clones #18 (B, D) and #24 (C, E), using *PstI* (B, C) or *HindIII* (D, E) digested genomic DNA. The probe was the 700 bp *GFP* cDNA. P: parental Clone #18 or #24 indicates the unrearranged reporter; 1–7: for each clone, #18 and #24, seven spontaneously-arising BsdR⁺ colonies are shown.

Fig. 6. Southern blot analysis of I-SceI-induced BsdR⁺ colonies. (A–D) Southern blotting was performed on U2OS reporter Clones #18 (A, C) and #24 (B, D) using *Pst*I (A, B) or *Hind*III (C, D) digested genomic DNA. The probe was the 700 bp *GFP* cDNA. See restriction maps in Fig. 5A. *P*: parental Clone #18 or #24 indicates the unrearranged reporter; 1–7: representative rearrangements of the reporter resulting from sister chromatid recombination events. Lanes 1–3 for Clone #18 and lanes 1–2 for Clone #24 show the most frequent LTGC products (“*GFP* triplication”). Lanes 4–6 for Clone #18 and lanes 3–7 for Clone #24 show aberrant patterns of expansion (see text). Note the large intensely hybridizing *Pst*I fragment in Panel A (Clone #18), lane 4, and the correspondingly increased intensity of the 3.2 kb band in Panel C (Clone #18),

lane 4. (E) Schematic of the gene amplification event in Clone #18, lane 4 (Panels A and C)
—not to scale. Stars indicate positions of *BsdR* cassettes.

Table 1

Analysis in U2OS cells of a series of test constructs

Plasmid transfected	GFP+ cells (%)	BsdR+ colonies
(1) pcDNA3	<0.1%	–
(2) pcDNA3–wt <i>GFP</i>	61%	–
(3) pcDNA3–wt <i>BsdR</i>	<0.1%	+++
(4) pTest–SAMOR	56%	+++
(5) pTest–INVOR	60%	±

U2OS cells were transfected in parallel with each of the plasmids indicated. Cells were analysed by flow cytometry (for GFP status) and by colony formation in blasticidin. The constructs mimicking the “inverted exon” rearrangement (lines 4 and 5) are described in Fig. 2. The control plasmid used is the empty vector (line 1). The percentages of GFP+ cells and the frequency of BsdR+ colonies are indicated. (+++): Large number of colonies; (±): scanty colony growth; (–): no colony growth.

Table 2

Frequencies of gene conversion events in U2OS Clone #18

	Experiment				Average
	#1	#2	#3	#4	
Spontaneous GC					
% GFP+ cells ^a	0.027	0.013	0.023	0.017	0.020 ± 0.006
(FACS analysis)	(2.5 × 10 ⁴)	(25 × 10 ⁴)	(50 × 10 ⁴)	(50 × 10 ⁴)	
% BsdR+ cells ^b	0.007	0.005	0.007	0.004	0.006 ± 0.002
(Blasticidin selection)	(40 × 10 ⁴)	(40 × 10 ⁴)	(70 × 10 ⁴)	(32 × 10 ⁴)	
% BsdR+/GFP+ ^c	25.00	40.18	31.25	23.03	29.86 ± 7.72
DSB-induced GC ^d					
% GFP+ cells ^a	6.22	5.36	9.74	6.55	6.97 ± 1.91
(FACS analysis)	(2.5 × 10 ⁴)	(25 × 10 ⁴)	(50 × 10 ⁴)	(50 × 10 ⁴)	
% BsdR+ cells ^b	1.16	1.06	1.37	0.77	1.09 ± 0.25
(Blasticidin selection)	(20 × 10 ⁴)	(40 × 10 ⁴)	(30 × 10 ⁴)	(32 × 10 ⁴)	
% BsdR+/GFP+ ^c	18.69	19.83	14.02	11.73	16.07 ± 3.83

Spontaneous or I-SceI-induced events were measured after transfection with control plasmid or with I-SceI plasmid, respectively. In each experiment, the percentages reflect the number of GFP+ cells or BsdR+ colonies divided by the total number of cells analysed by FACS or plated for selection in blasticidin (indicated by values in parentheses). Data shown are corrected for transfection and plating efficiencies. The average values and standard errors between four independent experiments are shown.

^aGFP+ cells represent short tract gene conversions (STGC) plus long tract gene conversions (LTGC).

^bBsdR+ cells represent SCR/LTGC events.

^cBsdR+/GFP+ represents the ratio of LTGC events to all GFP+ events.

^dI-SceI-induced gene conversion frequencies were corrected by subtraction of equivalent spontaneous frequencies.