Xba I (sites indicated by lowercase letters in the primers) and cloned into the Bam HI and Xba I sites of the vector pSK–. Plasmid pTNF-α1281-1350 contained the seven AUUUA motifs of the TNF-α ARE (bases 1281 to 1350 of X02611). This was constructed using similar methods. Correct sequences of these plasmids were confirmed by dideoxy sequencing (Amersham). For radiolabeling of the RNA transcripts with [α-^{32}P]uridine triphosphate (800 Ci/mmol), plasmid TNF-α1197-1350 was linearized with Xba I and used as the template in the Riboprobe in vitro transcription system (Promega) protocol. The resulting product was precipitated with ammonium acetate and ethanol.

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28. Confluent dishes were washed three times with cysteine-free medium supplemented with fetal calf serum (10%). Cells were stimulated for 4 hours in the same medium with control conditions, LPS (1 μg/ml), or TNF-α (10 ng/ml). For the last 3 hours of the incubation, [^{35}S]cysteine (200 μCi/ml; NEN Life Sciences, Boston) was added to the cultures. Cells were washed twice with ice-cold PBS, scraped into 10 ml of PBS, and pelleted by centrifugation (1000*g* for 5 min at 4°C). Cells were then resuspended in 600 μl of lysis buffer [50 mM tris-HCl (pH 7.5), 50 mM NaCl, 3 mM $MgCl_2$, 5% (v/v) glycerol, 0.5% (v/v) NP-40, 0.02% (w/v) sodium azide, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (20 μg/ml), and leupeptin (8 μg/ml)], incubated on ice for 20 min, and lysed by passage five times through a 28-gauge needle attached to a 1-ml syringe with no dead space (Becton Dickinson). The nuclear pellet (after centrifugation at 1000*g* for 5 min at 4°C) was washed once in ice-cold wash buffer [10 mM tris-HCl (pH 7.5), 15 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, and 5% glycerol], centrifuged at 1000*g* for 5 min at 4°C, and then resuspended and sonicated in the same volume of lysis buffer used initially to lyse the cells. The cytosolic fraction (supernatant) was clarified by centrifugation at 45,000*g* for 30 min at 4°C, using a tabletop ultracentrifuge (Beckman TL-100, rotor TLA.45). This method results in separation of cytosol and nuclear fractions, as assessed by protein immunoblotting with an antibody to SP1 as described (*18*). Cytosolic extracts matched by trichloroacetic acid–precipitable radioactivity and equivalent volumes of nuclear extracts were incubated with preimmune rabbit serum (1:100 dilution, 1 hour at 4°C) and protein A–Sepharose (1 hour at 4°C), and then incubated overnight at 4°C in the presence of either preimmune serum (1:100) or a 1:100 dilution of a polyclonal rabbit antibody to mouse TTP (*18, 19*). Immune complexes were recovered by centrifugation after the addition of protein A–Sepharose, washed three times with wash buffer [50 mM tris-HCl (pH 8.3), 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40], resuspended in 100 μl of SDS sample buffer [P. J. Blackshear, *Methods Enzymol.* **104**, 237 (1984)], and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (9% gel). For autoradiography, gels were fixed and treated with Autofluor (National Diagnostics, Atlanta).

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Genetic Dissection of a Mammalian Replicator in the Human β-Globin Locus

Mirit I. Aladjem, Luo Wei Rodewald, John L. Kolman, Geoffrey M. Wahl*

The timing and localization of DNA replication initiation in mammalian cells are heritable traits, but it is not known whether initiation requires specific DNA sequences. A site-specific recombination strategy was used to show that DNA sequences previously identified as replication initiation sites could initiate replication when transferred to new chromosomal locations. An 8-kilobase DNA sequence encompassing the origin of DNA replication in the human β-globin locus initiated replication in the simian genome. Specific deletions within the globin origin did not initiate replication in these chromosomal sites. These data suggest that initiation of DNA replication in mammalian cells requires specific sequence information and extend the replicon hypothesis to higher eukaryotes.

The decision to initiate DNA replication is crucial to normal progression through the cell cycle (*1*). The replicon model (*2*) proposed that cells regulate DNA replication by means of a bipartite control mechanism comprising a trans-acting "initiator" that interacts with a cis-acting DNA element called the replicator. The replicon model has been validated, with some modifications, in bacteria (*3*), prokaryotic and eukaryotic DNA viruses (*4*), and yeast (*5*). Identification of replicators in these systems was expedited by their ability to function in extrachromosomal plasmids (*6*). In mammalian cells, initiation can be localized to specific chromosomal regions by biochemical methods, but the chromosomal sites identified in this manner as initiation regions (IRs), origins or origins of bidirectional replication (OBRs), could not, by themselves, support replication of plasmids (*7*). This may be due to elimination of acentric extrachromosomal DNA (*8*), failure to license transfected DNA for initiation before it integrates (*9*), or greater complexity of mammalian replicators, exceeding the cloning capacity of the vectors used (*7*). Alternatively, replication initiation may be determined by a specific nuclear structure established within a defined cell cycle interval rather than by DNA sequence (*10*).

We developed a genetic system to identify mammalian replicators on the basis of intrachromosomal initiation. Candidate replication origins that are included within lambda phage and cosmid clones can initiate DNA replication when transfected into mammalian cells and integrated into random sites (*7*), indicating that some specific DNA sequences or structures may act as replicators in ectopic chromosomal locations. If sequence-specific replicators exist, such sequences should initiate DNA replication when transferred to defined chromosomal sites that do not otherwise exhibit origin activity. Furthermore, if short, specific sequences direct initiation, then it should be possible to produce nonfunctional replicators by mutation or deletion. Because chromosome structure is an important component of replicator activity (*10–12*), wild-type and variant replicators must be analyzed at the same chromosomal location.

Our approach (Fig. 1A) uses the site-specific recombinases FLP from yeast and Cre from bacteriophage P1 to target replicators to unique chromosomal locations containing the small target sequences recognized by these proteins (*13*). We analyzed the human β-globin (hβG) IR using this approach because previous studies showed that cells with a naturally occurring deletion of the IR do not initiate replication within the >70-kb globin locus (*14*). Because initiation also requires the locus control region (LCR), located ~50 kb from the IR (*11*), we used the dual recombination system to create isogenic variants containing the IR with or without a 5′ mini-LCR (Fig. 1A).

We placed an 8-kb region from the hβG locus that encompasses the IR in ectopic sites in the simian (*Cercopithecus aethiops*) genome (Fig. 1A). A polymerase chain reaction (PCR)–based nascent strand abundance assay (Fig. 1B) (*15*) revealed that short, newly replicated nascent strands were generated from the β-globin IR in both sites (Figs. 2A and 3) (*16*). Such short strands are produced from regions within which DNA replication initiates (*11*). By contrast, short nascent strands were not generated at detectable levels from the β-galactosidase (β-Gal) gene sequences located 5 kb 3′ of the IR (Figs. 2A and 3). Globin sequences were also present in preparations of small nascent strands ob-

Gene Expression Laboratory, The Salk Institute, San Diego, CA 92037, USA.

*To whom correspondence should be addressed at Gene Expression Laboratory, The Salk Institute, 10010 North Torrey Pines Road, San Diego, CA 92037, USA.

tained from cells in which the transferred IR was inserted into a second ectopic location (*16*).

The globin locus replicates in early S phase in erythroid cells, in part because of an open chromatin structure mediated by the LCR (*17*, *18*). Replication occurs late in S phase in other somatic tissues. We determined whether the LCR altered replication timing of the ectopic IR by estimating the abundance of globin sequences in nascent DNA from cells collected at various intervals of S phase (*19*). PCR reactions included competitors as mock targets for amplification, which allowed us to compare the relative representation of DNA sequences in fractions isolated at various times in S phase (Fig. 2B) (*20*). This analysis showed that sequences within the transferred IR are more abundant in the early S phase fractions, suggesting that the globin sequences replicated early during S phase. Replication timing was not affected by the LCR. Thus, the transferred 8-kb IR can act as a portable replicator that does not require the LCR for initiation in some genomic locations.

The presence of IR sequences in small nascent strands suggested that DNA replication can initiate within the transferred IR. We delimited the site of initiation by using two analyses that estimated the relative abundance of nascent replicated DNA at different locations within the IR. First, we isolated short (1 to 2 kb) nascent strands and determined the relative abundance of globin sequences using multiple primer pairs arrayed across the transferred 8-kb IR. All primer pairs were equally efficient in amplifying genomic DNA (Fig. 3, A and B). If initiation occurred from a single site within the IR, then short nascent strands would be abundant only in the vicinity of this site, whereas sequences farther from the initiation site would be rare (Fig. 1). Some short strands might derive from DNA degraded during nascent strand isolation, but the amplification efficiency of such broken strands should provide a uniform background as it is not expected to depend on the distance of the primers from replication origins. Indeed, short nascent strands were not seen in the β-Gal region, located 5 kb 3′ to the IR. The highest abundance of short strands was detected with the hβG primers that are located near the biochemically defined origin (*11*) (Fig. 3, A and B). We also reproducibly observed short nascent strands in the βivs region, located less than 1 kb 3′ to hβG, although they were less abundant than those at hβG (Fig. 3, A and B).

In the second strategy, we isolated nascent strands 0.5 to 8.0 kb in size and determined the abundance of globin sequences within them. Because the abundance of origin sequences in nascent strands was greater by at least two orders of magnitude than the abundance of nonorigin sequences in similar preparations (Fig. 3A), we used a semiquantitative approach to measure the representation of DNA sequences in the size-fractionated samples. Within an initiation region, it is predicted that sequences near an initiation site should be represented in short and long nascent strands, whereas sequences far from an initiation region should be present in long, but not short, nascent strands. Small, size-fractionated nascent strands were amplified in the presence of constant concentrations of competitor DNA to estimate the relative abundance of IR sequences within the preparation. The hβG sequences were abundant in short and long nascent strands; βivs sequences were more abundant in long nascent strands, although some short strands were also observed (Fig. 3B). β-Gal sequences were below the threshold of detection. This is similar to the pattern of nascent strands observed in the native hβG locus (*11*) and when the IR was integrated into a second, independent genomic site in simian cells (*16*). Similar nascent strand length patterns have been reported to be produced in yeast (*21*, *22*) and in Chinese hamster cells (*23*) at loci where several adjacent initiation sites fire with different efficiencies. The data from the two types of nascent strand analysis described above are consistent with the interpretation that replication initiates preferentially at a site near hβG and that other sites within the IR may initiate at a lower frequency.

In sum, our results demonstrate that DNA replication initiates within the IR when it is located in two different chromosomal locations in monkey cells. Replication initiates at,

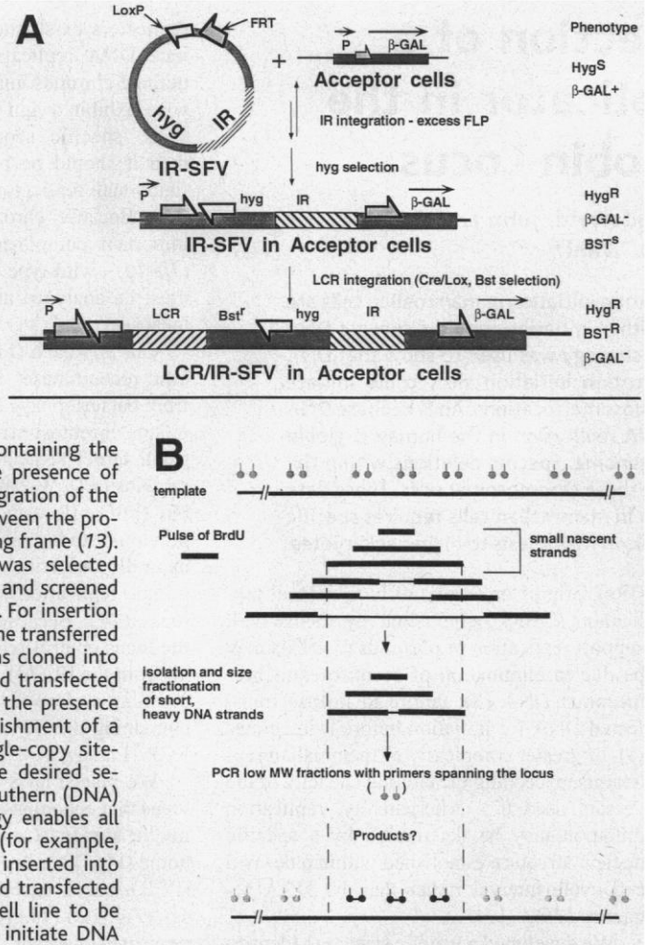

Fig. 1. (**A**) Design of the site-specific integrations into unique genomic sites. Two CV-1–derived cell lines, each containing an independent integration of a β-Gal cassette interrupted by a FLP recombination target (FRT), were used as acceptor cell lines. Putative replicators were cloned into a shuttle vector, SFV, containing an identical FRT and LoxP site, the target for the Cre recombinase (*13*). Transfection of the SFV vector, containing the putative replicator, in the presence of excess FLP resulted in integration of the vector at the FRT site between the promoter and the open reading frame (*13*). Site-specific integration was selected for hygromycin resistance and screened by negative β-Gal staining. For insertion of LCR sequences 5′ into the transferred origin, a mini-LCR (*31*) was cloned into the SFV shuttle vector and transfected into the acceptor lines in the presence of excess Cre. The establishment of a cell line containing a single-copy site-specific integration of the desired sequence was verified by Southern (DNA) blot analysis. This strategy enables all variants of the replicator (for example, deletions and mutations) inserted into the SFV shuttle vector and transfected into the same acceptor cell line to be assayed for the ability to initiate DNA replication in an identical genetic background and chromosomal environment. Selective markers: hyg, hygromycin resistance; Bst^r, blasticidin resistance. (**B**) An assay for initiation of DNA replication. Initiation of DNA replication from the integration site was tested by the nascent strands abundance assay (*40*). Cells are pulse-labeled with BrdU, lysed gently, and small DNA strands isolated after fractionation through a sucrose gradient. Newly replicated DNA strands are isolated on the basis of selective incorporation of BrdU. The strands are separated by size by alkaline gel electrophoresis and then amplified by PCR to detect specific DNA fragments in newly replicated strands (*11*, *15*, *40*). Competitors were included in these reactions to verify that PCR products were produced at similar efficiencies in different fractions. The use of specific PCR primers (*15*) allowed us to distinguish between the transported human globin sequences and the host simian globin sequences.

or very close to, the same site or sites used in the native human locus. Although the dependence of initiation on the LCR was not observed upon relocation of the IR, the similarity between replication patterns in the native and relocated sequences suggests that the human IR functions as a genetic replicator in human and simian cells. Thus, features that may be critical for initiation, such as primary sequence, local chromatin structure, or ability to form vital protein complexes, seem to be conserved among primates.

Replicators analyzed in bacteria (*3*), yeast (*5*), and viruses (*4*) have a modular organization comprising sequences within which initiation occurs and auxiliary elements that affect initiation efficiency. We tested a set of deletion mutants of the globin IR to determine if it has a similar organization (Fig. 4). Replication initiated when constructs contained a 1.5-kb deletion of the 5′ end (Fig. 4A, IR-PH) or an ~4 kb deletion of the 3′ end (Fig. 4A, IR-HS) of the 8-kb IR. However, deletion of both distal sequences reduced initiation to a level no longer detectable by the assay used (Fig. 4A, IR-PS). Deletion of the "core" region of ~2.6 kb from the 8-kb IR (IR-HP/SH) also abolished initiation (Fig. 4B). The shortest fragment containing a 5′ deletion that was able to initiate was located between positions 61,110 and 65,288 (Fig. 4A, IR-PN). Nascent strands from cells harboring the PN fragment were also analyzed for initiation competence by means of a recently developed method to identify RNA-terminated nascent strands on the basis of λ exonuclease sensitivity (*23*, *24*). The results confirmed that cells harboring the PN fragment initiated replication within the transferred globin sequence (*16*). These data are consistent with the existence of a 5′ fragment (59,589 to 61,110) and a 3′ fragment (63,754 to 65,288) that contain auxiliary elements surrounding a "core" replicator region (61,110 to 63,754). Thus, the initiation of DNA replication in ectopic locations requires specific sequences and can be achieved by various combinations of auxiliary sequences complementing an essential core.

Our data support a model in which a DNA sequence, such as the IR, specifies where replication can initiate in the chromosomes of mammalian somatic cells. The data are difficult to reconcile with an alternate hypothesis that any sequence of sufficient size will enable initiation of DNA replication (*25*) because such a model predicts that DNA fragments of similar length should confer initiation at similar efficiency regardless of sequence. The two auxiliary sequences in the β-globin IR may be redundant, as are the auxiliary sequences in replicators in *Saccharomyces cerevisiae* (*22*) and *Schizosaccharomyces pombe* (*21*). These auxiliary elements may serve to facilitate double-strand DNA unwinding or to recruit proteins involved in the preinitiation complex (*26*, *27*).

Replication in the native β-globin locus initiates within the IR but requires the LCR (*11*), which itself has no origin activity but is involved in determining tissue and developmental specific chromatin structure, replication timing, and gene expression (*28*). Similar dependence of replication on distant elements has been suggested for the Chinese hamster DHFR locus (*29*, *30*). Our results indicate that replication at an ectopic location does not require the LCR. LCR involvement in replication at the original locus is correlated with its ability to decondense chromatin (*11*), suggesting that specialized chromatin structures may be required to allow the binding of initiator to replicator. In the original locus, the LCR alleviates a repressing effect of chromatin on transcription (*31*, *32*). In the ectopic locations, this function may be subserved by other cis-acting sequences. Because our FLP recombination target (FRT) integration sites were selected for the expression of antibiotic resistance markers, it is reasonable to assume that these sites are in open chromatin. Thus, replicator activity in mammalian cells may be defined in terms of the elements needed to confer initiation of DNA replication within a particular chromosomal context.

Initiation of DNA replication involves the binding of initiators to replicators, licensing the DNA for replication, and providing access to the polymerization machinery (*33*). The first step in DNA replication in viruses consists of the binding of viral replicators to viral factors that recruit or act as helicases (*34*). Replication from the *Escherichia coli* chromosomal replicator, oriC, initiates through a similar mechanism (*3*, *33*). In yeast, replicators [that is, ARS (autonomously replicating sequences) elements] bind a multiprotein complex, ORC, which is activated by recruiting MCM proteins and accessory factors before DNA replication (*26*, *35*). Activation of

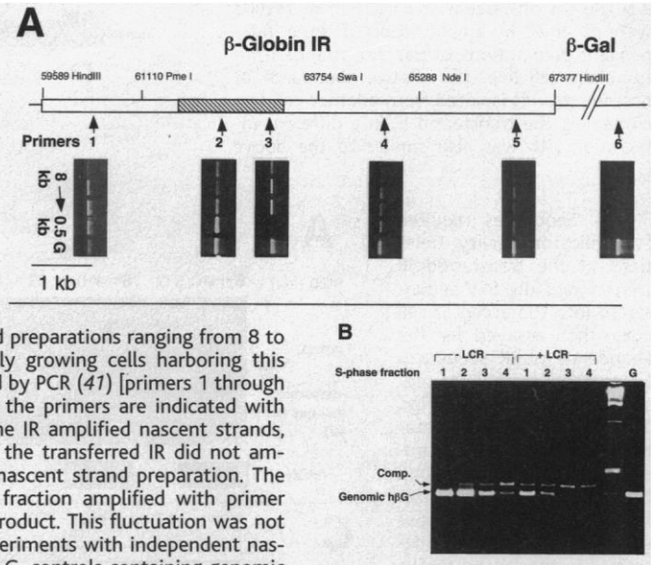

Fig. 2. (**A**) Initiation of DNA replication from the transferred IR. The first substrate integrated into the acceptor cell lines consisted of an 8-kb Hind III fragment from position 59,589 to position 67,377 of the hβG locus (GenBank accession number J00179). The abundance of origin-specific DNA sequences in nascent strand preparations ranging from 8 to 0.5 kb from exponentially growing cells harboring this fragment was determined by PCR (*41*) [primers 1 through 6 (*15*)]. The positions of the primers are indicated with arrows. Primers within the IR amplified nascent strands, whereas primers outside the transferred IR did not amplify products from the nascent strand preparation. The smallest nascent strand fraction amplified with primer pair 3 showed less PCR product. This fluctuation was not observed in repeated experiments with independent nascent strand preparations. G, controls containing genomic DNA. The hatched bar marks the position of the biochemically defined β-globin origin as determined by replication fork direction analysis (*11*). (**B**) Replication timing of the transferred IR is independent of the LCR. Exponentially growing cells harboring the transferred IR, with or without the LCR, were labeled with BrdU for 90 min, separated into distinct S phase fractions (from early S to late S) with a fluorescence-activated cell sorter, and then tested by PCR to detect specific DNA fragments in BrdU-substituted DNA (*19*). By contrast to the experiments shown in (A), this analysis detects all the genomic DNA replicated during the pulse period, not only short nascent strands. Comparison of strand abundance in different DNA preparations required that the DNA quantities were first normalized by PCR with the use of a primer pair corresponding to simian mitochondrial sequences (*15*, *42*). To estimate the relative abundance of specific DNAs in the nascent-strand preparation, and to control for variable efficiency of PCR from different primer pairs, PCR reactions to detect β-globin sequences in the S phase fractions included a standard DNA fragment that competed with the genomic PCR product in the manner described by Giacca *et al.* (*43*). The mimic competitors were amplified from genomic DNA with primer 2, cloned, and added at 0.1 pg per reaction. The upper PCR fragment in each reaction is the product of competitor amplification; the lower PCR fragment is the product of amplification of the genomic DNA. Lanes 1 to 4, S phase fractions from early S to late S; G, total genomic DNA. This analysis revealed that replication of the transferred IR initiated early during S phase regardless of the presence of the LCR. The hβG primer pair (*15*) was used in the analysis shown, but other primers (βivs and β-Gal) were used to amplify the S phase fraction with similar results. The results shown are from a single experiment in which S phase fractions were concomitantly isolated from both cell lines. Similar results were obtained by amplification of three independent preparations of S phase fractions.

ORC coordinates the timing of DNA replication within the yeast cell cycle (*26*). ORC homologs and MCM proteins, which are part of the licensing process, have been identified in metazoans (*36*, *37*). Our results suggest that in mammalian cells, initiation of DNA replication also requires specific replicators. By analogy with the systems described above, initiation is likely to be mediated by

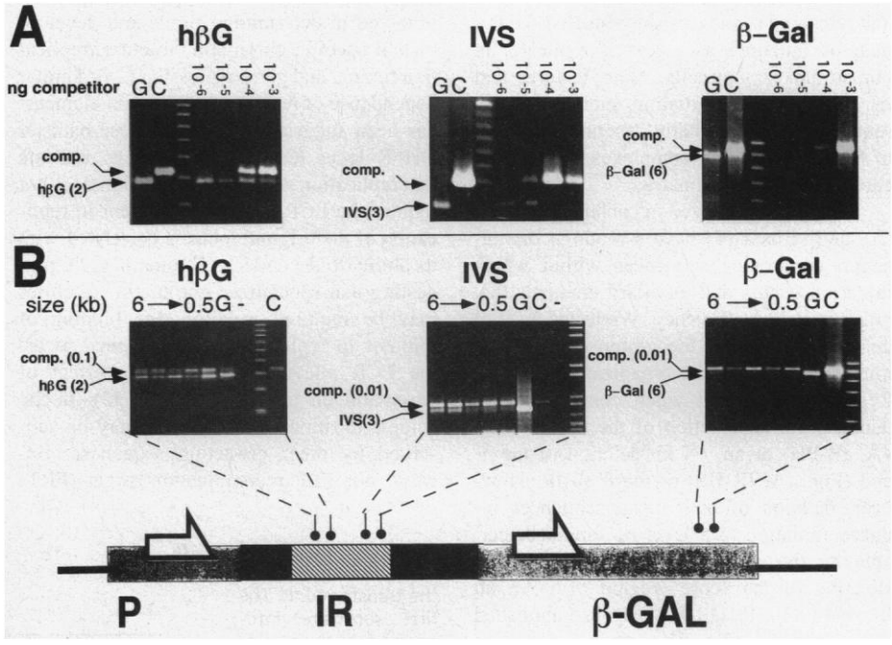

Fig. 3. Analysis of initiation within the transferred IR. (**A**) Short nascent strands (1 to 2 kb, 2% of the nascent strands prepared from 5×10^7 cells) were amplified by two PCR primer pairs within the transferred IR and one PCR primer pair 3′ to the IR in the presence of increasing competitor concentration (ranging from 0.001 to 1.0 pg). G, genomic DNA; C, competitor. Hatched bar, position of the β-globin origin as in Fig. 2. Nascent strands were most abundant in the hβG region but were also present in the βivs region. (**B**) Nascent strands varying in size from 0.5 to 8 kb (*15*) were separated by alkaline gel electrophoresis. DNA was isolated from gel fractions and amplified in the presence of 0.1 pg of competitor for the hβG primer, and 0.01 pg of competitor for the βivs and the β-Gal primers. The hβG and the ivs primers amplified products of all sizes within the range tested, but hβG sequences were most abundant in the shorter strands. G, genomic DNA (in the presence of competitor); C, competitor only. The results shown are from a single set of nascent strands. Similar results were obtained by amplification of three independent preparations of nascent strands from the same cell line. The relative abundance of nascent strands isolated from another cell line containing the transferred IR in a different integration site was also similar to the above (*16*).

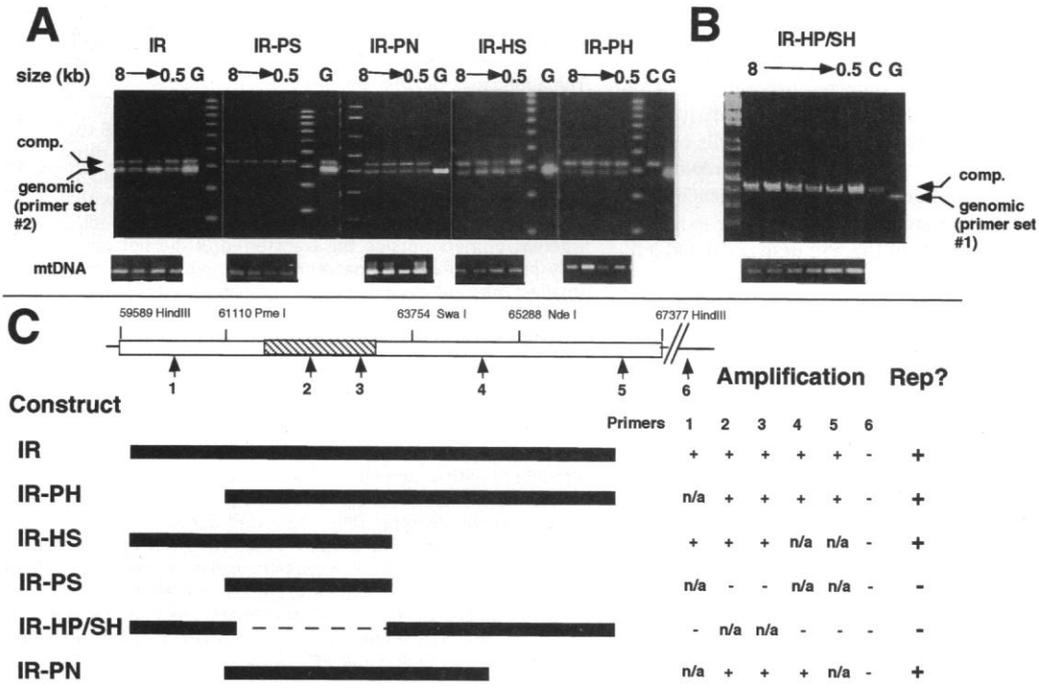

Fig. 4. Sequences required for replicator activity. Deletions of the transferred IR were cloned into SFV and inserted into the acceptor cell lines, then assayed for the abundance of IR sequences in nascent strands as described in the legend to Figs. 1 and 3B. (**A**) A representative analysis with hβG primers (0.1 pg per reaction). Nascent strands were size-fractionated on an agarose gel and analyzed as described in the legend to Fig. 3B. (**B**) An analysis of nascent strands from clone IR-HP/SH, in which the sequences amplified with the hβG primers were deleted, with 0.1 pg of primer pair 1. Nascent strands were size-fractionated on an agarose gel and analyzed as described in the legend to Fig. 3B. (**C**) A summary of nascent-strand abundance assays. Fragments containing the core region (position 61,100 to position 63,754) in conjunction with either 3′ or 5′ auxiliary sequences triggered initiation of DNA replication, whereas the core fragment alone or the auxiliary sequences without the core were not sufficient to allow initiation. The core region encompasses the biochemically defined β-globin origin (*11*, *14*) (hatched bar). Sequences from position 61,110 to 65,288 or sequences from position 59,589 to position 63,754 were minimally required for initiation in the two ectopic positions analyzed. The letters in the IR designations indicate the restriction fragment or fragments included in the clone (for example, IR-PH consists of the Pme I site at position 61,110 to the Hind III site at position 67,377). All the IR fragments described above were inserted into the same chromosomal sites, and at least two sets of nascent strands were analyzed for each fragment. Similar results were obtained when the abundance of nascent strands in a second site was analyzed (*16*).

sequence-specific interactions with ORC-like initiators. In prokaryotes, yeast, viruses, and now perhaps mammals, the proteins involved in the initiation process, and the cell cycle control of initiation, may be better understood through analysis of replicator structure (*22*, *26*, *38*, *39*).

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41. All PCR reactions were performed in 25 μl with an initial denaturation of 2 min at 94°C, 35 cycles of 45 s denaturation at 94°C, 45 s annealing at 60°C, and 45 s extension at 72°C. Final extension (7 min) was performed at 72°C. All the reactions were performed with Taq polymerase (Boehringer Mannheim), and the salt conditions were optimized for each primer pair with the Invitrogen PCR optimizer kit. Competitor concentrations were determined by reading the absorbency of the cloned fragments at 260 nm. Because the transferred IR was inserted in simian cells, all the primer pairs were assayed for selective amplification of human, but not simian, DNA before use in this assay. PCR reactions with primers only, or with genomic or competitor DNA with no primers, yielded no product.
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BRCA1 Required for Transcription-Coupled Repair of Oxidative DNA Damage

Lori C. Gowen, Anna V. Avrutskaya, Anne M. Latour, Beverly H. Koller, Steven A. Leadon*

The breast and ovarian cancer susceptibility gene *BRCA1* encodes a zinc finger protein of unknown function. Association of the BRCA1 protein with the DNA repair protein Rad51 and changes in the phosphorylation and cellular localization of the protein after exposure to DNA-damaging agents are consistent with a role for BRCA1 in DNA repair. Here, it is shown that mouse embryonic stem cells deficient in BRCA1 are defective in the ability to carry out transcription-coupled repair of oxidative DNA damage, and are hypersensitive to ionizing radiation and hydrogen peroxide. These results suggest that BRCA1 participates, directly or indirectly, in transcription-coupled repair of oxidative DNA damage.

An elaborate array of DNA repair systems has evolved in the cell to maintain the integrity of the genetic material. The removal of many types of DNA damage occurs by transcription-coupled repair (TCR), a process in which damage is repaired more rapidly in transcriptionally active DNA than in the genome as a whole (*1*–*5*). This rapid repair is attributable to a faster repair of lesions in the transcribed strand (TS) than in the nontran-