Random hexamer-primed, double-stranded cDNA was prepared from 2 μg of poly(A)$^+$ RNA (Superscript Plasmid System, Gibco BRL) as suggested by the manufacturer, except that cDNAs were ligated to linkers containing Not I sites, digested with Not I, and were size-selected by agarose gel electrophoresis. Fragments ≥1.5 kb were ligated to Not I–digested dephosphorylated pVP16 (36), the resulting ligation products electroporated into *Escherichia coli* strain DH-10B (Gibco BRL), 2 × 10^6 transformants plated at 10^5 colonies per 15-cm dish, and plasmid DNA prepared from the bacterial colonies (Qiagen Plasmid Maxi Kit). Analysis of an arbitrarily chosen population of plasmids indicated that ≥98% were recombinant, and inserts ranged from 1.5 to 4 kb.

20. The L40 reporter yeast strain expressing the LexA-PER bait hybrid was transformed with the *Drosophila* head cDNA library (at efficiencies ≥10^4 per microgram of plasmid DNA) as described [D. Gietz, A. St. Jean, R. A. Woods, R. H. Shiestl, *Nucleic Acids Res.* **20**, 1425 (1992); J. Hill, K. A. Ian, G. Donald, D. E. Griffiths, *ibid.* **19**, 5791 (1991)]. For solid-phase amplification of the library, ~10^7 transformants were plated at 10^5 colonies per 15-cm plate on TULL , a standard medium lacking tryptophan and leucine (to select for the bait and library plasmids, respectively) and uracil and lysine (to maintain the genomic loci at which the reporter genes were integrated) (*36*). After 60 hours of growth at 30°C, colonies were removed from the plates with a sterile scraper, resuspended in 500 ml of sterile water, pelleted at 4000*g* for 10 min, and resuspended in 50 ml of sterile water. After thorough mixing, 20 μl of the suspension was diluted into 2 ml of liquid TULL medium, and the transformants were grown at 30°C to one doubling [final absorbance at 600 nm (A_{600}) of 0.8], pelleted, washed with sterile water, and a total of 2 × 10^7 transformants were spread onto 20 plates containing synthetic medium as above, but lacking histidine (THULL; to select additionally for activation of the *HIS3* reporter gene). This 10-fold over-screen of the cDNA library was performed to maximize the chance of detecting very rare clones. Plates were incubated at 30°C, and 360 His$^+$ colonies were patched to fresh THULL plates after 48 to 72 hours of growth for retesting of the His$^+$ phenotype and for β-galactosidase assays, which were performed by a filter-lift method [L. Breeden and K. Nasmyth, *Cold Spring Harbor Symp. Quant. Biol.* **50**, 643 (1985)].
21. Inocula from His$^+$ β-Gal$^+$ yeast colonies were grown in 2 ml of liquid THULL medium (*20*) for 24 to 48 hours at 30°C, and plasmid DNA was recovered after glass bead lysis of the cells [C. S. Hoffman and F. Winston, *Gene* **57**, 267 (1987)]. Plasmid DNA was electroporated into *E. coli* strain MC1066 [M. I. Chiu, H. Katz, V. Berlin, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12574 (1994)], which is auxotrophic for Trp and Leu, deficiencies that are complemented by the *Saccharomyces cerevisiae TRP1* and *LEU2* markers, respectively. For selective recovery of VP16–cDNA library plasmids (*LEU2* marker) rather than LexA-PER bait plasmid (*TRP1* marker), bacterial transformants were plated onto minimal medium plates (plus carbenicillin, tryptophan, and uracil) lacking leucine. Transformants were then checked for the absence of bait plasmid by demonstrating failure to grow on minimal medium plates (plus carbenicillin, leucine, and uracil) lacking tryptophan. Across different experiments, 1 to 5% of Leu$^+$ transformants were found to carry both plasmids; demonstration of a Trp$^-$ phenotype before plasmid purification is thus important for the reliability of subsequent specificity tests, which could be confounded by the presence of the original bait plasmid.
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23. The consistent differences in PER-dependent β-galactosidase activity produced by the six clones could result from differences in the affinity of their respective protein products for PER, but these differences could just as well result from other factors, such as differential hybrid protein stability or variability of transactivation activity in different VP16 hybrid proteins.
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25. K. Chua and C. J. Weitz, unpublished observations.
26. TIM fragments labeled with [^{35}S]methionine were synthesized in vitro by coupled transcription-translation (TNT Lysate System, Promega). GST or GST-PER fusion proteins were produced in *E. coli* with the pGEX vector (Pharmacia) and purified on glutathione-agarose beads. For in vitro binding reactions, glutathione-agarose beads with coupled GST or GST-PER fusion protein (50 μl, hydrated bead volume) were incubated at room temperature for 30 min with labeled TIM (1.5 × 10^5 cpm, Cerenkov) in binding buffer [200 μl (final volume) of 20 mM Hepes (pH 7.4), 100 mM KCl, 5 mM EDTA, 5 mM EGTA, 10% (v/v) glycerol, 5% (w/v) bovine serum albumin (BSA), 0.4% NP-40, and 1 mM dithiothreitol]. The beads were then washed at room temperature twice in binding buffer and twice in binding buffer lacking BSA (1 ml per wash). Beads were resuspended in an equal volume of 2× Laemmli buffer, incubated at 100°C for 5 min, and the entire sample was analyzed on 6% (Fig. 3C) or 7% (Fig. 3D) SDS-PAGE.
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28. Each yeast transformant was patched to different plates (one to be grown at 22°C, and the other 37°C) containing TULL medium (*20*) supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Sigma) to a final concentration of 100 μg/ml (added to TULL medium as a 1000× stock in *N,N*-dimethylformamide). The transformants shown in Fig. 4A were grown as follows: TIM1: (22°C) for 6 days; TIM1 (37°C) for 5 days; TIM2 and TIM4: (22°C) for 1.5 days; TIM2 and TIM4 (37°C); for 1 day.
29. For each experiment, single TIM1,PER (wild-type) and TIM1,PER^L transformants were processed in parallel. Each transformant yeast colony was resuspended in 6 ml of TULL medium (*20*). The suspension was divided into three 2-ml cultures, which were then grown at 22°, 28°, or 35°C, respectively, for 24 hours. Cultures were diluted 100-fold with fresh TULL medium, and 2 ml of the diluted culture was grown (at the same temperature as the initial incubation) to a final A_{600} of 0.8 to 1.2. Extraction of β-galactosidase from yeast and assays of β-galactosidase activity were performed as described [F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1988)]. Specific β-galactosidase activity was calculated as the change in A_{420} per hour per milligram of protein. Within each independent experiment, specific β-galactosidase activities were normalized to that of the TIM1,PER (wild-type) control culture grown at 22°C.
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37. Supported by NSF grant IBN-9421475 (C.J.W.) and a McKnight Scholars Award (C.J.W.), the Howard Hughes Medical Institute (M.P.M. and M.W.Y.), the NSF Science and Technology Center for Biological Timing (L.S. and M.W.Y.), the NSF (A.S.), and an American Cancer Society Junior Faculty Research Award (A.S.). M.W.Y. is an investigator in the Howard Hughes Medical Institute. We thank D. Shore, S. Hollenberg, A. Vojtek, J. Cooper, P. Bartel, J. Hall, V. Berlin, and I. Chiu for protocols, plasmids, and strains; P. Chambon and S. Reppert for antibodies to VP16 and PER, respectively; and M. Morris, K. Chua, R. MacKinnon, and J. Cohen for critical comments on the manuscript.

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Participation of the Human β-Globin Locus Control Region in Initiation of DNA Replication

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The human β-globin locus control region (LCR) controls the transcription, chromatin structure, and replication timing of the entire locus. DNA replication was found to initiate in a transcription-independent manner within a region located 50 kilobases downstream of the LCR in human, mouse, and chicken cells containing the entire human β-globin locus. However, DNA replication did not initiate within a deletion mutant locus lacking the sequences that encompass the LCR. This mutant locus replicated in the 3′ to 5′ direction. Thus, interactions between distantly separated sequences can be required for replication initiation, and factors mediating this interaction appear to be conserved in evolution.

The human β-globin locus consists of five linked genes, ε, γ_A, γ_G, δ, and β, which exhibit erythroid-specific, developmentally regulated expression (*1*). Replication of this locus initiates from a region 5′ to the β-globin gene regardless of expression or replication timing (*2*). Gene expression, chromatin structure (*3*, *4*, *5*), and replication timing (*6*) are regulated by the LCR, an upstream element that contains five deoxyribonuclease (DNase) I hypersensitive sites (HSs) (*1*, *7*). The regulatory importance of the LCR in the native chromosomal context was established in naturally occurring deletions encompassing the LCR, the smallest of which (35 kb) deletes HSs 2 to 5 and 20 to 25 kb 5′ to HS5 (Hispanic thalassemia). Absence of this region prevents β-globin expression in erythroid cells, shifts replication timing from early to late S

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phase, and results in the entire locus being DNase I resistant. This influence is conserved in mouse-human somatic cell hybrids containing either normal or thalassemic human chromosomes (6). Here we address the importance of LCR sequences, as defined by the Hispanic thalassemia, in the initiation of DNA replication.

In somatic cell hybrids, we found that replication originates from the same initiation region (IR) 5′ to the β-globin gene regardless of gene expression. Bidirectional initiation from a fixed site generates leading strands that are complementary to the 5′-3′ template strand on one side of the IR, and to the 3′-5′ template on the other side (8, 9). We identified a leading strand template switch (Fig. 1) (*10*) in three cell lines: in NMEL, a murine erythroleukemia cell line containing a normal human chromosome 11 (*4*, *5*); in NMEL HR9, an NMEL clone containing a targeted insertion into the LCR between HS1 and HS2 (*11*); and in DT40 HR9, a chicken B cell hybrid containing the HR9 chromosome (*12*–*14*). The HR9 insertion suppresses transcription of the β-globin gene (*11*). The leading strand switch was mapped 5′ to the β-globin gene (units 61 through 64) in these hybrid cell lines, as well as in human erythroid (K562 and HEL-R) and nonerythroid (293) cells. Thus, the IR is recognized in murine and avian nuclear environments regardless of the expression or of the identity of the expressed gene (K562 cells express γ and ε, and NMEL cells express β).

Leading strands from TMEL, a hybrid cell line containing a human chromosome 11 from a Hispanic thalassemia patient (6), displayed no strand switch at the 5′ β-globin IR, even though the IR is located more than 50 kb away from the deletion boundary (Fig. 1). Instead, the locus replicated apparently from a downstream initiation site. These results strongly indicate that sequences within or adjacent to the LCR are necessary for the activation of the β-globin IR.

A second, independent strategy that measures the abundance and sizes of nascent DNA strands (*15*) was used to confirm the findings of the leading strand analysis. DNA probes hybridizing to short nascent strands indicate that the probe recognizes a region close to an IR; probes hybridizing to only long nascent strands recognize sequences that are more distant but within 20 kb of an IR; and probes that recognize sequences more than 20 kb away from an IR will not hybridize to nascent strands (*16*). Analyses of NMEL cells (*15*, *17*) showed that two probes flanking the β-globin IR hybridized to short nascent strands (Fig. 2A). In contrast, similar analyses of TMEL cells showed that the same probes hybridized only to long nascent strands (Fig. 2A). A probe from the ε-globin region, >20 kb 5′ to the IR, did not hybridize to NMEL- or TMEL-derived nascent strands; thus, DNA replication did not initiate near the ε-proximal sequence (*16*).

A polymerase chain reaction (PCR) amplification step was introduced into the nascent strand procedure to increase the sensitivity and to enable simultaneous analysis of multiple probes (Fig. 2, B and C) (*18*). PCR primers (*19*) between map units 59.9 (Fig. 2B) and 65.5 (Fig. 2C) detected long and short nascent strands from NMEL cells, but only long strands (>9 kb) from TMEL cells. Primers homologous to sequences residing further 5′ in the globin locus, map units 33.3 (Fig. 2B), 45.8 (Fig. 2C), and 50.6 (*20*), yielded no products from either cell line, as expected (*16*). Finally, primers located ~7 kb 3′ to the IR detected only longer strands in both cell lines (Fig. 2B). In NMEL cells, these strands may originate from the 5′ IR or from another 3′ IR. In TMEL cells, in which the 5′ IR does not initiate, this result indicates that initiation occurs 3′ to the β-globin locus, in agreement with the leading strand data (Fig. 1).

The direction of DNA replication within the β-globin locus in NMEL and TMEL cells was further analyzed by fluorescent in situ hybridization (FISH) (*2*, *21*). A cosmid probe will reveal a single hybridization dot per nucleus before DNA replication, whereas two hybridization dots will appear after replication of the target sequence (Fig. 3). When two cosmid probes labeled with different reagents are hybridized simultaneously, replication order is determined by the proportion of nuclei displaying two dots with one probe and one dot with the other probe (*2*). Table 1 shows that the IR replicated before the 5′ ε-globin region in both NMEL and TMEL cells. In NMEL cells, the 5′ ε region replicated before the hereditary persistence of fetal hemoglobin (HPFH) region, 70 kb 3′ to the IR probe, whereas in TMEL cells the HPFH region replicated before the 5′ ε region. Thus, the replication fork moved in the 3′ to 5′ direction in

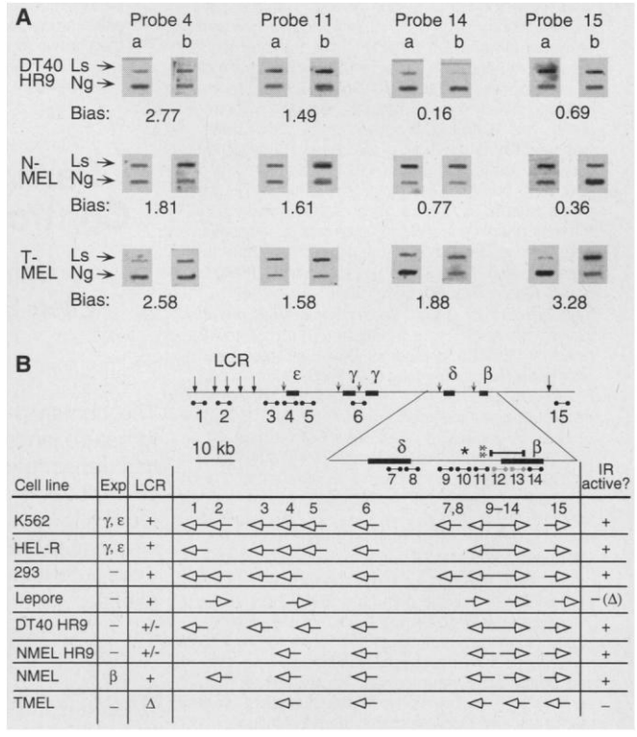


Fig. 1. Leading strand analysis of replication in the β-globin locus. (**A**) Leading strands (Ls) and normal genomic (Ng) DNA were immobilized on slot blots and hybridized to strand-specific RNA probes (*8*, *9*). Within the β-globin locus, DNA strands traveling from the LCR (5′) toward the β-globin gene (3′) are designated "top" strands, whereas the opposite strands are designated the "bottom" strands. Blots labeled "a" were hybridized to RNA probes transcribed from the top strand (probe a), and blots labeled "b" were hybridized to RNA probes transcribed from the bottom strand (probe b). The bias ratio was calculated as (Ls/Ng for probe b)/(Ls/Ng for probe a). Bias ratios larger than 1 imply that the top strand is the template for leading strands; therefore, the leading strands travel from the β-globin gene toward the LCR. Bias ratios smaller than 1 indicate that the bottom strand is the template for leading strands traveling in the opposite direction. A strand switch was observed between probes 11 and 14 in NMEL and DT40 HR9 cells, but no switch was observed in TMEL cells. (**B**) Summary of leading strand analyses. Probes (*36*) are represented in the map as short lines delimited by circles. Long vertical arrows indicate developmentally stable HSs, and short vertical arrows indicate tissue-specific HSs. Map units are in kilobases and refer to the globin GeneBank sequence J00179. A single star indicates the origin consensus sequence (*25*) at map unit 58.5 and two stars, the PUR protein binding site (*37*) at map unit 60.9. Probes within the IR, indicated by the line delimited by boxes (map units 61.2 to 62.5), are gray. Horizontal arrows represent the direction of leading strands synthesis as determined from hybridization bias ratios (*10*). A bias of more than 1.6 for a specific direction, if consistent between experiments, was considered sufficient for assigning a direction to leading strand synthesis. Within the origin region, biases were typically less than 1.5, and the inferred direction varied between experiments. The data for the Lepore deletion is quoted from (*2*) for comparison. Exp, expressed genes; delta (Δ) indicates a deletion.

TMEL cells, confirming the results obtained with the leading strand assay (Fig. 1).

In contrast to the 3′ to 5′ replication observed in TMEL cells, the β-globin locus replicates from the 5′ to 3′ direction in cells from Lepore hemaglobinopathy patients, in which an 8-kb fragment encompassing the entire IR is deleted (*2*). This difference may be explained by a cryptic IR that is active in the Lepore cells and deleted in TMEL cells. Alternatively, this result may reveal suppression of a 3′ IR by sequences that are removed by the Hispanic deletion. Limiting initiation to one IR may prevent interference between two closely spaced IRs, as reported for adjacent origins in budding yeast (*22*).

Our results implicate that some vertebrate IRs are recognized by the replication machinery of other vertebrates. This result is consistent with the conserved initiation within a Syrian hamster CAD IR transfected into Chinese hamster cells (*23*), as well as with the apparent recognition of the Chinese hamster dihydrofolate reductase (DHFR) ori-β by a replication-competent protein extract from *Xenopus* oocytes (*24*). Interestingly, the β-globin locus harbors a degenerate consensus sequence shared by initiation sites in various metazoans (*25*) at map unit 58, slightly 5′ to the mapped IR (Fig. 1B).

The lack of IR function in TMEL hybrids suggests that the sequences encompassing the LCR, located 40 to 60 kb 5′ to the IR, are necessary for origin activation. A similar interaction may occur in the Chinese hamster DHFR locus in which a deletion that abolishes transcription cannot initiate replication from the distant ori-β (*26*). Importantly, the β-globin IR is functional

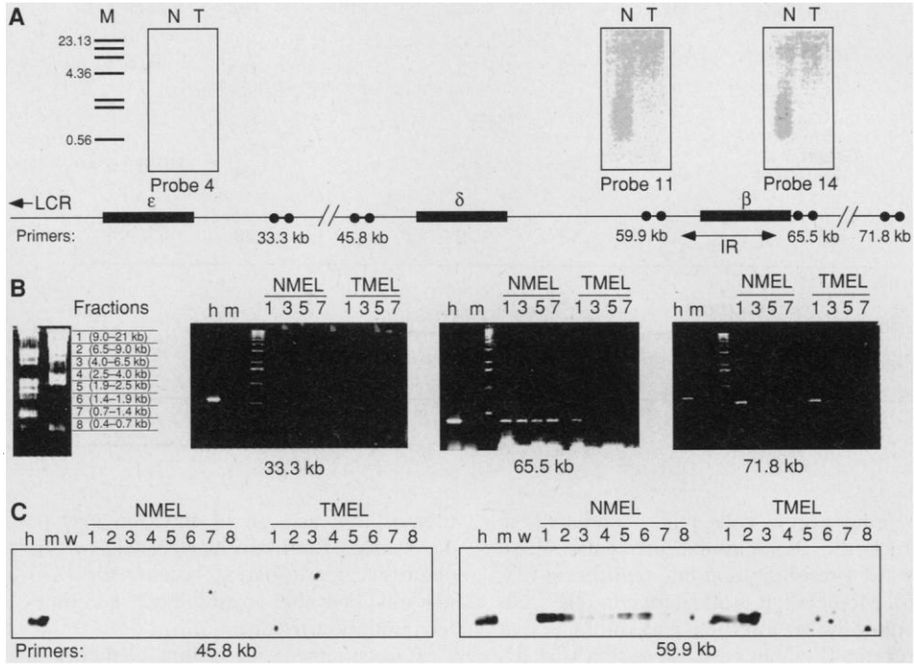


Fig. 2. Nascent strand abundance analysis. (**A**) Southern (DNA) blot analysis of nascent strand DNA (*15*) from NMEL cells (lanes N) and TMEL (lanes T). Probe numbers (*36*) are assigned as in Fig. 1. Molecular size markers (M), which were run simultaneously with the nascent strands, are from a lambda Hind III digest (23.13, 9.42, 6.56, 4.36, 2.32, 2.03, and 0.56 kb). The 3′-β and 5′-β probes hybridize to short nascent strands from NMEL but not from TMEL cells, whereas the 3′-ε probe failed to hybridize to nascent strands. All the probes hybridized equally well to restriction digests of genomic DNA on Southern blots (*20*). (**B**) PCR analysis of nascent strands (*18*) with the primer pairs (*19*) indicated in (A) (bold lines delineated by circles, numbered according to map position). The molecular size markers and sizes for each fraction are shown in the leftmost panel. Lanes h, human DNA; lanes m, mouse DNA; numbered lanes correspond to fractions from the alkaline gel on the left (every odd fraction from the gel was analyzed). Primer pairs 33.3 (left), 45.8, and 50.6 (*20*) did not yield PCR products from nascent strands [see (*16*)]. Primer pairs 65.5 (middle), 59.9, and 61.9 (*20*) yielded products from all of the NMEL fractions and only from the longest strands from TMEL cells. Primer pair 71.8 (far right) yielded products only from long strands in both cell lines. (**C**) Analysis of nascent strands within the linear range of PCR amplification. Products were transferred to Southern blots and hybridized to digoxygenin-labeled oligonucleotides complementary to sequences located between the PCR primers (*18*). Lanes are as in (B); lane w, water. There was no detectable signal in similar blots with PCR products obtained with primer pairs from positions 50.6 and 33.3 (*20*).

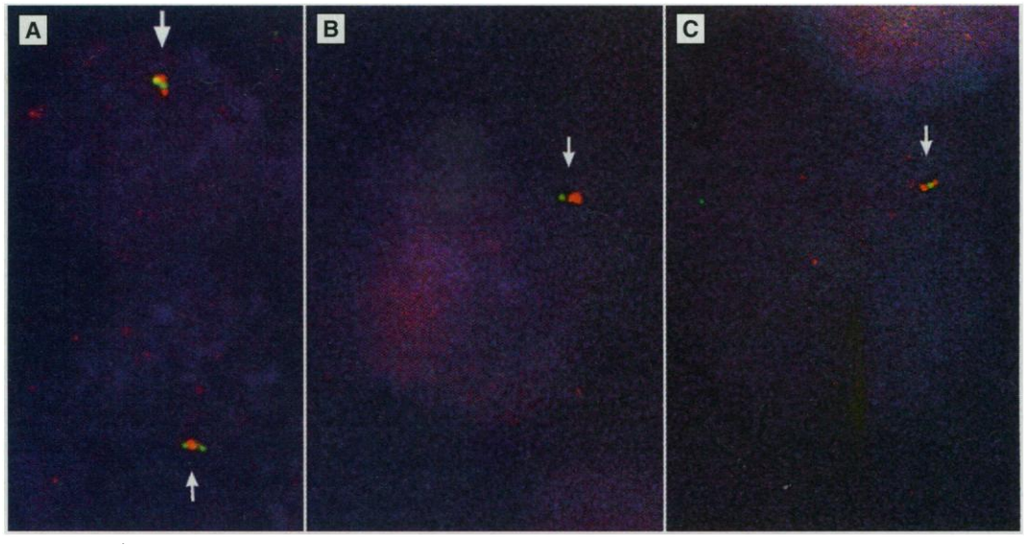
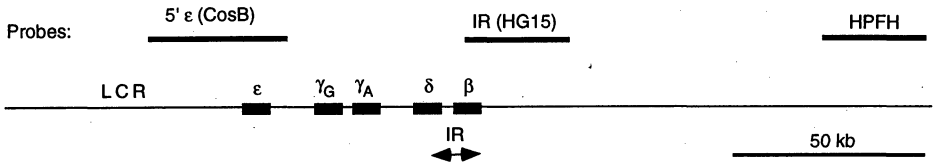


Fig. 3. In situ hybridization analysis of replication direction in the β-globin gene locus. In situ hybridizations were performed with two probes labeled with different reagents: CosB was detected as green and HPFH was detected as red (*38*). (**A**) NMEL cells. The top nucleus (wide arrow) shows a double green/double red (DG/DR) pattern, and the bottom nucleus (narrow arrow) scores double green/single red (DG/SR). (**B**) A TMEL cell showing a single red/single green (SR/SG) pattern. (**C**) A TMEL cell showing a double red/single green (DR/SG) pattern.

Table 1. Summary of in situ hybridization mapping analysis. Nuclei were hybridized to three different probes throughout the β-globin locus (*38*). The percentage of cells hybridizing with the different probes is shown for NMEL and TMEL cells. Only cells where both signals could be visualized were scored. No cells scored DG/DR in TMEL cells. The IR region replicates before the 5′ region in both cell lines. The 5′ ε-globin region replicates before the HPFH region in NMEL cells, whereas the HPFH region replicates before the 5′ ε region in TMEL cells. S/S, cells in which both probes hybridized to single dots; DR/SG, double red/single green cells; DG/SR double green/single red cells; and DG/DR, cells in which both probes hybridized to two dots. A small fraction of nuclei show an opposite pattern of hybridization in each case, which has been reported by others (*2*).

Probe labels	Cells hybridizing to probes (%) S/S	DR/SG	DG/SR	DG/DR	Total cells	Replication order
			NMEL			
Green 5′ ε red HPFH	60	1	23	16	142	5′ ε, HPFH
Green 5′ ε red IR	75	18	5	3	114	IR, 5′ ε
			TMEL			
Green 5′ ε red HPFH	84	14	2	—	44	HPFH, 5′ ε
Green 5′ ε red IR	80	18	2	—	49	IR, 5′ ε



in nonerythroid cells [Fig. 1 and (*3*)], in which the β-globin–like genes are silent, DNase I resistant, and late replicating (*27, 28*). Moreover, it is also active in HR9 cells containing an insertion into the LCR that prevents β-globin gene expression (Fig. 1). Because subsets of LCR hypersensitive sites are present in HR9 (*11*) and nonerythroid cells (*4, 5, 28*), factors involved in the formation of hypersensitive sites may be necessary for origin activation.

Interactions between enhancers and replication origins are necessary for the initiation of replication in viral systems (*29–31*). This interaction may be mediated through chromatin structure modifications that relieve nucleosome-dependent repression (*29, 30*). Enhancers also influence the time at which gene expression is induced during development (*30*) and the coincident conversion of DNA replication from random to preferred initiation regions (*32*). The LCR may provide an example of a developmental-specific regulator (*4, 5, 33*) that confines initiation to the intergenic region in the β-globin locus. Thus, the lack of initiation in TMEL cells may be due to the absence of the sequences from the TMEL chromosome at the time the IR is established during development. Because the IR functions but transcription of the β-globin gene is abolished in HR9 cells, the effect of the LCR on transcription and on IR function may be dissociable. Alternatively, HR9 cells may be permissive for initiation because the LCR was modified after it established a competent initiation region.

A requirement for distant elements to initiate mammalian DNA replication may explain, in part, why small cloned fragments consisting solely of IRs typically fail to establish autonomous replication (*9, 34*) but can function within large yeast artificial chromosomes (*35*), episomes (*23*), or after integration into the genome (*8, 23*). Clearly, an important area for future investigation will be to determine the sequences in the LCR or adjacent regions that contribute to replication initiation and the sites within the IR that interact with those sequences.

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10. For the leading strand assay we used cells treated with emetine in the presence of bromodeoxyuridine (BrdU) and fluorodeoxyuridine (FrdU) for 24 hours. Emetine preferentially inhibits the synthesis of lagging strands, resulting in enrichment for leading strands. BrdU-substituted DNA was separated from template DNA by two rounds of alkaline Cs_2SO_4 gradients. Next, 6 μg of purified leading strands were blotted on each slot blot and hybridized to single-stranded RNA probes. Probes (*36*) are fragments originating from the human β-globin locus that are devoid of repetitive sequences and were cloned into pBlueScript KSII to facilitate transcription of opposite strands. ^{32}P-labeled single-stranded RNA probes were generated by linearization of the plasmids with the appropriate restriction enzyme and transcription with either T3 or T7 RNA polymerase using standard methodology. For mouse cell hybrids, the hybridization conditions developed by Wahl *et al.* (*39*) were used to minimize background from mouse DNA. Only probes 2, 3, 8, 9, 11, 12, 14, and 15 were used because of unacceptably high background hybridization of the other probes to mouse DNA (*20*). The bias ratios between leading strands and normal genomic DNA were calculated for each blot with ImageQuant software, and then the total bias for each probe was calculated as described in Fig. 1. Although the magnitude of the bias ratios were variable between experiments (between 1.6 and 3.5 upstream of the IR and between 0.7 and 0.3 downstream of the IR), the direction of the bias was identical in three to seven repetitions for each probe when independent DNA preparations were used. In general, probes that are farther from the IR yield higher biases. We localized the IR to 1.3 kb, but further refinement was not possible because probes within this region produced inconsistent and very low strand bias ratios. Attempts to enhance bias ratios by changing the hybridization conditions, labeling the leading strands for shorter periods (up to 14 hours), or hybridizing only the heavier part of the "heavy" fraction did not significantly alter the bias ratios.
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12. DT40 HR9 was created by microcell fusion (*13*) between DT40, a chicken B cell line (*14*), and MEL HR9 (*11*).
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16. In this assay, only nascent strands that are larger than 20 kb are isolated and analyzed. Assuming equal fork movement, probes not recognizing nascent strands isolated in this assay are likely to be more than 10 kb away from the nearest initiation site. This distance could vary if fork progression is not uniform and nascent strands are located asymmetrically relative to their initiation site.
17. For the nascent strand assay, DNA from asynchronous replicating cells was labeled with [^{14}C]thymidine for 24 hours, then pulse-labeled for 20 min with BrdU and [^{3}H]deoxycytidine (*15*). Nuclei from the labeled cells were then collected, lysed, and the shorter DNA strands were isolated from an alkaline sucrose gradient. Fully substituted short nascent strands were purified by two rounds of alkaline CsCl gradient centrifugation, size fractionated by alkaline agarose gel electrophoresis, transferred to a Hybond-N membrane, and hybridized consecutively to probes labeled by random priming.
18. The following modifications of the nascent strand analysis were implemented for PCR analysis. Alkaline agarose gel electrophoresis was carried out with low–melting point agarose (SeaPlaque; FMC, Rock-

land, ME), and the region containing DNA strands sized 0.5 to 25 kb was sliced into 10 fractions. Gel slices were melted with Gelase (Epicentre) to release the DNA. DNA was collected by ethanol precipitation and then amplified by PCR. PCR was performed in saturating conditions (35 thermocycles), or during exponential amplification (28 cycles). The PCR products were analyzed by gel electrophoresis and visualized by ethidium bromide (EtBr) staining after saturation PCR. The PCR products derived from the exponential range of amplification that did not display visible bands on EtBr-stained agarose gels were visualized by transferring them to Hybond-N membranes and hybridizing them to digoxygenin-labeled oligonucleotide probes derived from a region within the expected amplification products. Hybridization signals were detected by chemiluminescence according to the manufacturer's instructions (Boehringer-Mannheim). To ensure specific reaction in the cell hybrids, we optimized the PCR conditions for every primer pair to yield a single fragment with human (K562) DNA and no signal with mouse (MEL) cell DNA. Primers were selected for efficient amplification of total human chromosomal DNA and lack of amplification of mouse DNA. Amplification of nascent strands from both cell lines with mouse β-globin primers yielded similar products, indicating that nascent strand size and integrity were similar between preparations. The above modifications proved essential for analyzing initiation in the β-globin locus as numerous attempts to use previously published PCR-based methods to detect initiation in the β-globin locus were unsuccessful (*20*).

19. Primer pairs used were as follows: position 33.3, upper primer, AACAAAAGCAAAACCAAACC, and lower primer, GTATGTAGGCACCCGATGAT; position 45.8, upper primer AAGGGCCTAGCTTGGACTCA, and lower primer GCAAATATCTAAGGGTAAAG; position 50.6, upper primer, TGAGAAATAAATGTGAAAGC, and lower primer, TGTATGTAAGGAGGATGAGC; position 59.8, upper primer, CCTGAGGAGAAGTCTGCCGT, and lower primer, CAGTGCAGCTCACTCAGTGT; position 61.9, upper primer, GGAAGGGGAGAAGTAACAGGGT, and lower primer, AAGGGCCTAGCTTGGACTCA; position 65.6, upper primer, TATCTTCATTTTCCCCTTCC, and lower primer, GAGTCTCATGCTGTCACCTG; position 71.8, upper primer, TTGACAAACCTGAGGGAAAC, and lower primer, TGATGGCTAGTGATGATGAG.
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36. Probes 1 through 15 are as follows: probe 1, 500–base pair (bp) Eco RI fragment from map unit 4 to 5; probe 2, 1400-bp Xba I fragment from map unit 8.8 to 10.1; probe 3, 450-bp Hind III–Xba I fragment from map unit 10.1 to 10.5; probe 4, 1300-bp Bam HI–Hind III fragment from map unit 12.4 to 13.7; probe 5, 700-bp Bam HI fragment from map unit 19.3 to 20; probe 6, 1200-bp Eco RI fragment from map unit 42 to 44; probe 7, 2300-bp Eco RI fragment from map unit 54 to 56; probe 8, 1800-bp Eco RI fragment from map unit 56 to 57.8; probe 9, 800-bp Bam HI fragment from map unit 59.7 to 60.5; probe 10, 650-bp Hpa I fragment from map unit 60.5 to 61.3; probe 11, 550-bp Hpa I–Sna BI fragment from map unit 61.2 to 61.8; probe 12, 750-bp Sna BI–Bam HI fragment from map unit 61.8 to 62.6; probe 13, 900-bp Bam HI–Eco RI fragment from map unit 62.2 to 63.5; probe 14, 1800-bp Eco RI–Bgl II fragment from map unit 65.5 to 67.3; probe 15, 900-bp Hind II–Eco RI fragment from map unit 80. It should be noted that most of these probes are different from the probes used by Kitsberg *et al.* (*2*). All cloned fragments used in this work were tested for the absence of hybridization to repetitive sequences on Southern blots of total genomic DNA.
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38. Cos B included the ε and 5′ ε (5′ ε) region, cos HG15 consisted of β and 3′ β sequences (IR), and the HPFH probe was derived from a series of pooled plasmids from the HPFH region. The HPFH probe was labeled with biotin and detected with avidin–Texas Red after amplification. The cosB and HG15 probes were labeled with digoxigenin (DIG) or biotin and detected with fluorescein isothiocyanate (FITC)–conjugated antibody to DIG or by avidin–Texas Red. Nuclei were counter-stained with 4′,6′-diamidino-2-phenylindole, scored under a double band pass filter, and photographed.
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Mutations in the Dystrophin-Associated Protein γ-Sarcoglycan in Chromosome 13 Muscular Dystrophy

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Severe childhood autosomal recessive muscular dystrophy (SCARMD) is a progressive muscle-wasting disorder common in North Africa that segregates with microsatellite markers at chromosome 13q12. Here, it is shown that a mutation in the gene encoding the 35-kilodalton dystrophin-associated glycoprotein, γ-sarcoglycan, is likely to be the primary genetic defect in this disorder. The human γ-sarcoglycan gene was mapped to chromosome 13q12, and deletions that alter its reading frame were identified in three families and one of four sporadic cases of SCARMD. These mutations not only affect γ-sarcoglycan but also disrupt the integrity of the entire sarcoglycan complex.

The muscular dystrophies are genetically heterogeneous (*1*). X-linked recessive muscular dystrophy, or Duchenne muscular dystrophy (DMD), is the most common form and arises from mutations in the dystrophin gene (*2*). Autosomal inheritance is present in a significant percentage of muscular dystrophy cases (*1*, *3*). In North Africa, the incidence of SCARMD [OMIM 253700 (*1*)], also referred to as limb girdle muscular dystrophy (LGMD) 2C (*4*), accounts for 10 to 50% of the total muscular dystrophy cases (*5*, *6*). The early age of onset and severity of the clinical course seen in North Africa are features also seen in DMD (*6*). Segregation of the SCARMD phenotype with chromosome 13 markers was first documented in a number of Tunisian kindreds (*7*). Recently, strong linkage disequilibrium with the marker D13S232 was documented for eight Tunisian families and one Egyptian family, sug-

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