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Mammalian Recombination Hot Spot in a DNA Loop Anchorage Region: A Model for the Study of Common Fragile Sites

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Abstract We analyzed the replication pattern and the topological organization of a 200 kb long Chinese hamster polygenic locus, which spans the boundary of two isochores. One of them is G + C rich while the second one is highly A + T rich. Previous analysis of mutants amplified for this locus had identified, within the A + T rich isochore, a mitotic recombination hotspot and a replication origin separated by some 7 kb. The recombination hotspot exhibits structural features repeatedly observed at common fragile sites, including a typical enrichment in peaks of enhanced DNA helix flexibility. By studying the replication pattern of the same locus in the non-amplified CHO cells, we confirm here the localization of the replication origin and show that the mitotic recombination hotspot does not correspond to a replicon junction. This finding makes questionable current hypotheses correlating replication termination regions with recombination prone sequences. Using topoisomerase II-mediated DNA cleavage at matrix attachment sites, we identified a 40 kb-long DNA anchorage region extending all along a transcription unit nested within the A + T rich isochore. Both the recombination hotspot and the replication origin lie within this topoisomerase II sensitive region, which suggests that features essential for initiation of recombination and initiation of DNA replication cluster within DNA anchorage regions. Features common to this region and to common fragile sites are discussed. J. Cell. Biochem. Suppl. 36:170–178, 2001.

Recent evidence has demonstrated that DNA rearrangements occur non-randomly along the length of the chromosome in somatic cells [Henglein et al., 1989; Geng et al., 1993]. However, the nature of recombination prone regions remains obscure. At least some of the hotspots of illegitimate recombination seem to coincide with common fragile sites, which are chromosomal loci characterized by recurrent breaks or gaps on metaphase chromosomes from cells grown under stress conditions [Smith et al., 1998; Sutherland et al., 1998]. The coincident localization of common fragile sites and breakpoints of recurrent deletions, translocations, or inversions in tumor cells suggests that fragile site activation triggers at least some of these genetic alterations. In addition, fragile sites were recently shown to play a major role in gene amplification [Kuo et al., 1994; Coquelle et al., 1997, 1998], a mutation process specific to cancer cells [Tlsty et al., 1989; Wright et al., 1990; Livingstone et al., 1992; Yin et al., 1992]. Thus, identification of the mechanisms and sequences that contribute to fragility is of prime importance for understanding how chromosome rearrangements are generated in tumor cells.

A few common fragile sites have been cloned and sequenced, all of them are highly A + T rich and enriched in peaks of enhanced DNA helix flexibility [Mishmar et al., 1998, 1999; Mimori et al., 1999; Ried et al., 2000]. Putative motifs corresponding to DNA replication origins and matrix attachment regions have also been found nearby [Wang et al., 1997; Palin et al., 1998], but experimental evidence that such features are

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present at common fragile sites is still missing. Thus, the mechanisms responsible for fragility at these sites remain largely unknown. Since topoisomerase II, a genuine component of the nuclear matrix [Berrious et al., 1985], introduces double-strand breaks in DNA, hotspots of breakage and recombination may occur as a result of its activity [Gale and Osheroff, 1992]. Regions of loop anchorage to the nuclear matrix (LARs) may constitute such targets for illegitimate DNA recombination. Indeed, it was shown that LARs can be cleaved by topoisomerase II in vivo and in vitro [Vassetzky et al., 1989; Razin et al., 1993; Gromova et al., 1995], especially under conditions impairing its normal function [Maraschin et al., 1990; Shibuya et al., 1994]. In addition to LARs, the replication termination sites also constitute natural targets for topoisomerase II since it is the major eukaryotic enzyme able to resolve topological problems at these sites. Thus, it would be interesting to establish which, if any, of these two types of sequences co-maps with identified recombination hotspots and fragile sites.

Here we determine the pattern of replication and the localization of sequences hypersensitive to topoisomerase II within a 200 kb long domain of Chinese hamster chromosome I, bearing the gene coding for adenylate deaminase 2 (AMPD2). Previous analysis of several independent cell lines amplified for the AMPD2 gene has revealed the presence of a recombination hotspot about 30 kb upstream from this gene [Debatisse et al., 1986, 1992; Hyrien et al., 1987]. The studied domain contains at least four unrelated genes which encode respectively (listed according to their relative order) the αi3 and αt2 subunits of GTP-binding proteins (GNAI3 and GNAT2), the AMPD2 enzyme, and a glutathione S-transferase of the μ family (GSTM). The approximately 3 kb long recombination prone sequence lies within intron 7 of the highly A + T rich GNAI3 gene [Baron et al., 1996]. We have recently shown that peaks of enhanced flexibility are precisely co-localized with rearrangement sites identified within this hotspot, supporting the hypothesis that such DNA features mark the sites important for chromosome fragility [Toledo et al., 2000]. However, clusters of peaks of enhanced flexibility were also found in a region of the AMPD2 gene (unpublished results), which is not prone to breakage. This suggests that DNA flexibility is important, but not sufficient to make a locus fragile. Here we have identified DNA sequences interacting with the nuclear matrix by using the topoisomerase II-mediated DNA cleavage technique [Vassetzky et al., 1989; Razin et al., 1993]. The results indicate that the recombination hotspot is nested within a large DNA region of topoisomerase II hypersensitivity that does not co-map with a replication termination site. Hence, our data support the hypothesis [Felix, 1998; Razin et al., 1999] that some features found within DNA loop anchorage regions are involved in the initiation of illegitimate recombination and chromosome fragility.

RESULTS

Mapping of DNA Loop Anchorage Regions (LARs)

LARs were mapped using the topoisomerase II-mediated DNA loop excision protocol, a mapping approach that was described previously [Razin et al., 1993; Gromova et al., 1995; Iarovaia et al., 1996]. The procedure used here is the following: (1) CHO cell nuclei were extracted with high salt solutions, which do not impair nuclear matrix integrity or disrupt DNA loops, but still efficiently remove soluble proteins, including soluble topoisomerase II, (2) Extracted nuclei (nucleoids) were incubated in conditions allowing DNA cleavage by topoisomerase II. In these experimental conditions, cleavage occurs mainly at DNA sequences lying in close contact with the nuclear matrix since matrix bound enzyme is preferentially retained, (3) Accumulation of cleaved complexes was favored by addition of VM-26, a topoisomerase II inhibitor that selectively blocks the ligase activity of the enzyme, (4) Cleaved DNA was extensively deproteinized, then digested by SfiI, a rare cutting restriction enzyme. DNA fragments were finally separated by pulsed field electrophoresis (PFGE) and Southern analysis was carried out.

The locations of the four genes, the recombination hotspot mentioned previously, the SfiI sites and the two probes (I and II) used in this study are presented in Figure 1A. As expected, probe I detects a 120 kb long full-sized SfiI restriction fragment in each slot (Fig. 1B). In addition, a broad band corresponding to 30 to 70 kb long DNA fragments is present in slots 3, 4, 5, 6 but is weakly visible, if at all, in slot 1 and 2. Its intensity increases with increasing concentrations of VM26 (slots 1, 2, and 3), then reaches a maximum (slots 3, 4, 5, 6), indicating that this

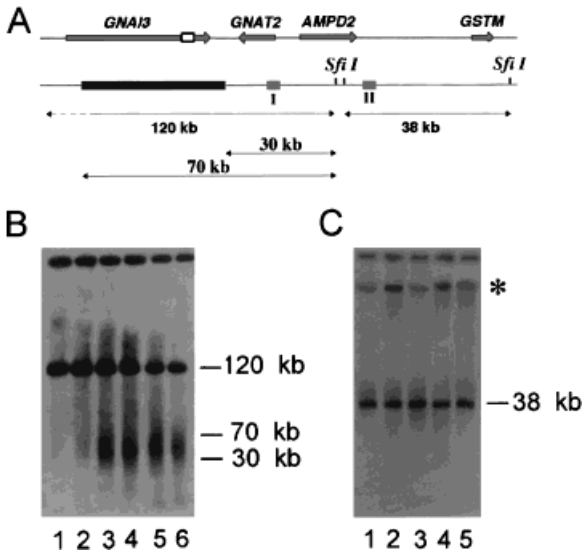

Fig. 1. Mapping of the DNA loop anchorage regions within the domain of interest. (**A**): Map of the domain. Identified genes are represented, the thick gray arrows show their sizes and transcription directions. Open rectangle within the GNAI3 gene: localization of a previously identified recombination hot spot. The localization of Sfi I restriction sites and hybridization probes (I and II, gray rectangles) are indicated. Double arrows point to fragments of interest, their size is indicated. The black rectangle represents the region hypersensitive to topoisomerase II cleavage. (**B**): Results of hybridization with probe I. Slots 1 to 6 were loaded with DNA samples from nucleoids treated with VM-26 (0, 10, 20, 40, 60, and 80 μg/ml respectively). (**C**): Results of hybridization with probe II. Slots 1 to 5 were respectively loaded with DNA from nucleoids treated with 0, 20, 40, 60, and 80 μg/ml of VM26. (*): compression zone.

smear corresponds to a population of fragments generated upon DNA cleavage by topoisomerase II. The probability of DNA cleavage by nuclear matrix-bound topoisomerase II seems to be rather constant along a 40 kb long zone since discrete bands were not clearly visible within the smear. We conclude that a site of preferential contact between DNA and nuclear matrix-bound topoisomerase II extends over a broad region overlapping most of the GNAI3 gene (Fig. 1A). On striking contrast, probe II has identified only full-sized (38 kb) Sfi I fragments (Fig. 1C). We conclude that this region does not contain a LAR.

Interestingly, the LAR region studied here was previously found to contain a cluster of MAR elements [Fernandez et al., 1997]. In the latter work, three other regions containing MAR elements were found, but none of them was identified as a LAR in the present study. One of them, the MAR located upstream to the GSTM gene, clearly does not participate in DNA loop anchorage to the nuclear matrix. The last two, which flank the AMPD2 gene, were too close to a Sfi I site or to the position of the probes used for end-labeling to be reliably studied in the present experiment. The identification of at least one region containing MAR elements, which lies outside a LAR, supports our previous conclusion that only a fraction of the MARs participate in DNA loop anchorage to the nuclear matrix [Iarovaia et al., 1996]. Moreover, this indicates that secondary binding (after high salt extraction) of MAR elements to the nuclear matrix does not affect the mapping of regions with genuine sensitivity to topoisomerase II.

Mapping of Replication Initiation and Termination Regions

The structural bases of chromosomal replication are better understood in the budding yeast *S. cerevisiae*. In these cells, available data indicate that replication termination occurs frequently throughout broad regions rather than at specific sites [Greenfeder and Newlon, 1992; Zhu et al., 1992]. These results encouraged us to determine whether the large topoisomerase II sensitive region identified here coincides with a DNA replication termination zone. Analysis of the polarity of leading (continuous) and lagging (discontinuous) strand synthesis, and identification of regions of transition from continuous to discontinuous DNA synthesis is a powerful experimental approach to study the organization of replication over long genomic domains. Indeed this method allows an efficient mapping of replication origins, from which the replication forks travel in opposite directions to the termination regions where they converge. Here we used a technique relying on the observation that inhibition of protein synthesis by emetine preferentially suppresses the synthesis of lagging strands in proliferating cells. In bromo-deoxyuridine (BrdU) containing medium, labeled nascent leading strands accumulate specifically. The polarity of DNA synthesis can be determined upon hybridization of strand-specific probes to nascent DNA [Handeli et al., 1989; Burhans et al., 1991] purified through immuno-precipitation with anti-BrdU antibodies.

Previous works have demonstrated that 20 nucleotides-long oligonucleotides can be used as strand-specific probes to assay the polarity of leading strand synthesis [Verbovaia and Razin, 1995, 1997]. Here we used 40 nucleotides-long probes in order to improve the specificity of

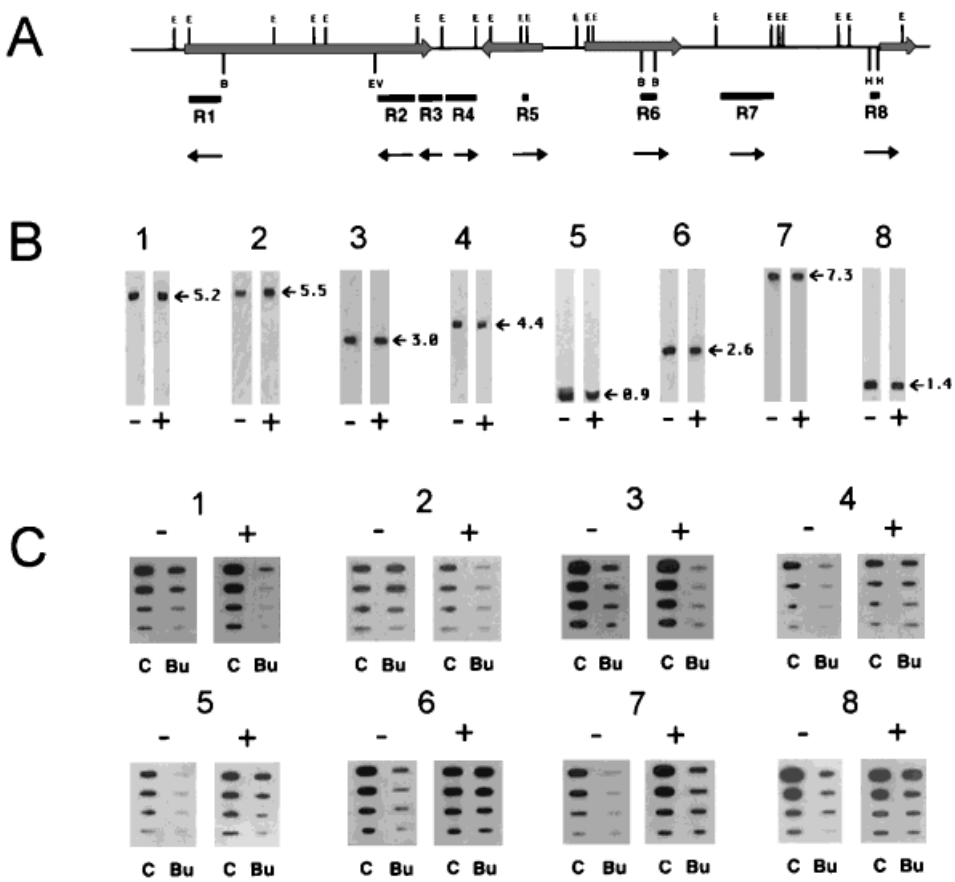

Fig. 2. Direction of replication fork progression. **(A)**: Eco RI (E) restriction map and localization of the genes (gray arrows, as in Fig. 1). Other sites of interest are represented; B: Bam HI, EV: Eco RV, H: Hind III. Restriction fragments (R1 to R8) expected to be detected by probes 1 to 8 are represented by thick lines beneath the map. Arrows represent the direction of replication fork progression as determined using each set of probes (see section C). **(B)** Results of hybridization of Southern blots with the different probes. Genomic DNA was digested by combinations of restriction enzymes giving rise to fragments R1 to R8. In each case, a single band of the expected size was detected. Arrows below the slots show the polarity of the 40 bp-long strand specific probes. **(C)**: Hybridization of the strand specific pairs of probes (1 to 8) with the dilution series of control (C) and nascent (Bu) DNA. Arrows above the slots show the polarity of the probes. Note that nascent DNA hybridizes preferentially with one of the two complementary probes of each pair.

hybridization. We have prepared 8 pairs (numbered 1 to 8) of ^{32}P-labeled strand-specific probes (Table I) distributed over the 200 kb long domain under study (Fig. 2A). In order to verify that all probes are specific and can be reliably used for hybridization in slot-blot experiments, each probe was first hybridized with Southern blots containing digested CHO cell DNA. Digestions were performed by EcoRI (for hybridization with probes 3, 4, 5, 7), by EcoRI and Bam HI (for hybridization with probes 1 and 6), by EcoRI and EcoRV (for hybridization with probe 2) or by Hind III (for hybridization with probe 8). In each experiment a single band having the size of the expected restriction fragment (R1 to R8) was observed (Fig. 2A, B). In order to study the replication organization of the domain, each set of probes was used to hybridize dilution series of control DNA and nascent DNA from CHO cells grown in the presence of emetine and BrdU (see Methods section for details). To distinguish the DNA strands, we arbitrarily designated by "minus strand" the one having the same polarity than the GNAI3, AMPD2 and GSTM mRNAs, while the "plus strand" corresponds to the antisense direction (Figs. 1A, 2A).

The results presented in Figure 2C show that at loci identified by probes 1 to 3, nascent DNA preferentially anneals to the "minus" strand probes, indicating that the leading strand has the "plus" polarity in this region. Thus, in this region, the replication forks travel from right to left relative to the map presented in Figure 2A. On the contrary, from position 4 to 8, nascent DNA anneals preferentially to the "plus" strand

TABLE I. Oligonucleotide Probes Used for Determination of the Replication Status

Name and direction of the probe	Nucleotide sequence of the oligonucleotides
1+	AGTAACGTGGATTGTCCAGGAACATAAAGGGGGAGGCGGGT
1−	ACCCGCCTCCCCCTTTATGTTCCTGGACAATCCACGTTACT
2+	AACCTAAACCAAAATGTCATCTGTTCAGAGTAAGGGGGTA
2−	ACCCCCTTACTCTGAACAGATGACATTTTGGTTTAGGTTT
3+	CCTTATCACACAGCCACTTACACACCTACCCCCCAAACAC
3−	GTGTTTGGGGGGTAGGTGTGTAAGTGGCTGTGTGATAAGG
4+	GTCGACGGATCCGAATTCGTCGTCTATAGCTCCATCCCTG
4−	ACGAATTCGGATCCGTCGACGTTGTGAGTTGCCGTGTGAG
5+	CTGGGGAGGATATAAGGGTCATAGGAAACCACCTGTATTT
5−	TACAGGTGGTTTCCTATGACCCTTATATCCTCCCCAGAAT
6+	CATACGTTTGTGCTGAGGCCGCACTGCGGGGAGGCTGGGC
6−	GCCCAGCCTCCCCGCAGTGCGGCCTCAGCACAAACGTATG
7+	CGGGGGTAGGTGTTCATTGTGGGGAGAAGCCATAAGGCCG
7−	CGGCCTTATGGCTTCTCCCCACAATGAACACCTACCCCCG
8+	CAGAAGGGGGGCATCGGGTCAGGGAGGGCTGGAACGCTAC
8−	GTAGCGTTCCAGCCCTCCCTGACCCGATGCCCCCCTTCTG

probes, which shows that this second region is replicated in the opposite direction with respect to the previous one (see the black arrows in Fig. 2A for a summary). Taking into consideration the direction of replication fork movement, which is identical to the direction of leading strand synthesis, our results indicate that a replication origin is located in between the positions of probes 3 and 4. In good agreement with observations made in other genomic domains [Gromova et al., 1995; Lagarkova et al., 1998], this replication origin is nested within a region of tight contact between DNA and the nuclear matrix.

If the topoisomerase II sensitive region lying over most of the GNAI3 gene was a DNA replication termination zone, it should give rise to a gradual shift in the abundance of nascent strands with "minus" to "plus" polarity. Since this was not observed and since no other switch in replication direction was observed within the studied domain, we conclude that it contains no other replication origin and no termination region.

DISCUSSION

In tumor cells, common fragile sites are involved in the generation of inter and intrachromosomal recombination events, sister chromatid exchanges, integration of various viruses or mobile sequences [Glover, 1998; Thorland et al., 2000] and gene amplification [Coquelle et al., 1997, 1998]. A few human aphidicolin sensitive common fragile sites are now cloned and sequenced. All of them were mapped within highly A + T rich regions and recently shown to be enriched in peaks of enhanced DNA helix flexibility [Mishmar et al., 1998, 1999; Mimori et al., 1999; Ried et al., 2000]. We identified an approximately 3 kb long sequence which was repeatedly rearranged during amplification of the AMPD2 locus [Debatisse et al., 1986; Hyrien et al., 1987]. This recombination hotspot lies within intron 7 of the highly A + T rich GNAI3 gene (> 60%) and peaks of enhanced flexibility were found precisely co-localized with sites of rearrangements in amplified cell lines [Toledo et al., 2000] and with sites of pseudogene insertion [Baron et al., 1994]. Thus, this region exhibits characteristic features of aphidicolin sensitive fragile sites. We directly checked this point and the results showed that aphidicolin induces breaks at low frequency within the GNAI3-GNAT2 region [Toledo et al., 2000]. Indeed, the susceptibility to damage of a given site could depend on the fragile site-inducing stress, tissue type, or other factors [Mimori et al., 1999]. Recently, using fiber FISH analysis with a probe overlapping the GNAI3-GNAT2 region, we obtained evidence that chromosomal breaks occur at very high frequency within the hotspot upon mechanical stress (Svetlova et al., manuscript in preparation). Thus, at least some properties identified at aphidicolin sensitive sites are not restricted to this category of sites, and the GNAI3 hotspot may constitute a valuable model for functional studies of common fragile sites.

The molecular bases leading to chromosome fragility are now partially elucidated in the case of the so-called rare chromosomal fragile sites, which all display microsatellite repeat expansion. The observation that all types of repeats found within these sites are able to form secondary structures gave some clue to the

mechanisms responsible for chromosome fragility [Sutherland et al., 1998]. Recurrent sequence motifs were also identified at common fragile sites, but how they contribute to fragility is essentially unknown. As mentioned above, all studied common fragile sites are highly A + T rich, which suggested that they could correspond to DNA replication origins and/or matrix attachment regions [Smith et al., 1998]. In the present work, using the topoisomerase II-mediated DNA loop excision protocol [Razin et al., 1993; Iarovaia et al., 1996], we have identified a 40 kb long region which encompasses most of the GNAI3 gene, a replication origin and the GNAI3 recombination hotspot. It suggests that this region is a LAR or a broad zone in which random replication termination occurs. We previously studied the replication pattern of the region surrounding the AMPD2 gene in amplified cell lines [Toledo et al., 1998, 1999], which facilitated the use of the 2D neutral-alkaline (2D NA) gel electrophoresis replicon mapping technique [Nawotka and Huberman, 1988]. We have identified a narrow zone of replication initiation in part of the GNAI3-GNAT2 intergenic region and confirmed its existence and position by using the competitive quantitative polymerase chain reaction developed by Giacca et al. [1994]. Here, we used a third technique to analyze the replication pattern of a 200 kb long sequence surrounding the AMPD2 gene in non-amplified CHO cells. By determining the polarity of leading strand synthesis, we identified a replication origin in the same position as in amplified cells. Moreover, we found that the 40 kb long topoisomerase II hypersensitive domain identified here does not correspond to a replication termination zone. Thus, the hotspot of recombination present within the GNAI3 gene, in fragment R2, coincides neither with a replication origin nor with a region of replicon junction. Hence, at least in this case, the hypothesis [Levac and Moss, 1996] that such hotspots result from topoisomerase II-mediated events occurring specifically at replication termination regions can be ruled out.

We have previously reported that DNA loops are attached to the nuclear matrix via several kb long DNA fragments [Razin et al., 1993; Gromova et al., 1995]. However, the size of the region identified in the present investigation is surprising. The existence of a 40 Kb long LAR can hardly be explained by a simple model postulating that the whole genome is organized in more or less regular loops, attached to the nuclear matrix via sequences that are much more shorter than the loops. Rather, since the GNAI3 gene belongs to an A + T rich isochore, our data suggest that the spatial organization of genes in A + T rich isochores significantly differs from others in the rest of the genome. In good agreement with this interpretation, Saitoh and Laemmli presented results suggesting that A + T rich genomic regions are organized into short loops while G + C rich regions are organized into long loops [Saitoh and Laemmli, 1994]. If a number of short loops are present within the GNAI3 gene, these loops will not be detected at the PFGE resolution level. The whole gene will be frequently cleaved by nuclear matrix-bound topoisomerase II, interacting with DNA at the bases of the short loops. This situation is formally indistinguishable from the existence of a single long LAR. In fact, the interactions of DNA with the nuclear matrix are not uniform along the whole length of the LARs, even within the relatively short LARs described previously [Razin et al., 1993; Gromova et al., 1995]. In other words, these LARs also are possibly composed of alternating attachment sites and short DNA loops.

Previous analysis has shown that the gene cluster under study overlaps an isochore boundary, the GNAI3 and GNAT2 genes being located within the A + T rich isochore, but only the GNAI3 gene is expressed in the cell type understudy [Fernandez et al., 1997]. The genes are not distributed at random among the different isochore families: gene concentration is low in A + T rich isochores, increases with increasing G + C richness, and reaches a maximum within the very G + C rich H3 isochores [Bernardi, 1995; Gardiner, 1996]. This is specially pronounced in the case of house-keeping genes, which are mostly confined to the H3 isochores. The special base composition of the house-keeping GNAI3 gene could require specific features to establish and/or to maintain a transcription competent chromatin state. The presence of a LAR extending all along the gene, or of a cluster of short loops, could be required in this case.

The finding that the recombination hotspot is nested within a gene organized as a large LAR or a group of short loops leaves open the possibility that topoisomerase II bound to the matrix contributes to DNA breakage. Alterna-

tively, it is also possible that chromatin features or DNA sequences specific to such spatially organized genes contribute to chromosomal instability. It should be noted that three out of four cloned human common fragile sites are composed of a few hotspots of breakage/recombination dispersed within large introns of A + T rich genes. Obviously, the mapping of LARs at these loci should establish if the topological characteristics of the GNAI3 gene represent a general property of common fragile sites.

MATERIALS AND METHODS

Cell Lines, Cell Culture

Chinese hamster CHO cells were grown in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum.

Mapping of DNA Loop Anchorage Regions

The LARs were mapped using the topoisomerase II DNA loop excision procedure, as previously described [Razin et al., 1993; Iarovaia et al., 1996]. In brief, the cells were embedded into agarose blocks, permeabilised by non-ionic detergent (NP-40), extracted with 2M NaCl, washed with the Topoisomerase II reaction buffer (20 mM Tris-HCl (pH 7,5), 50 mM KCl, 0.1 mM EDTA, and 10 mM $MgCl_2$) and treated with VM-26 (dimethylepipodophyllotoxinthenylidene-β-D-glucoside), an inhibitor of DNA topoisomerases II (Sandoz). After incubation with VM-26, the agarose blocks were digested by Proteinase K (0.5 mg/ml, Merck) for 36 h at 50°C with constant gentle agitation, in a buffer containing 1% SDS, 0,4 M EDTA (pH 8,0). The blocks were then washed 10 times (30 min each) in TE buffer and equilibrated in Sfi I buffer before digestion with Sfi I (100 U/ml) for 24 h at 50°C. The resulting DNA fragments were finally separated by pulsed field electrophoresis (PFGE) and Southern analysis was carried out in standard conditions.

Analysis of Replication Organization

To induce unbalanced synthesis of nascent DNA strands, exponentially growing cells were treated with emetine (2 mM), as previously described [Handeli et al., 1989; Burhans et al., 1991]. After 15 min of incubation, 5-bromo-2-deoxy-uridine (BrdU) (10 μg/ml) and ^{3}H-deoxycytidine (2 μCi/ml) were added. The cells were grown for 16 h in this medium, prior to DNA extraction. Nascent DNA chains were separated from the bulk of DNA by double immuno precipitation with BUdR-specific antibodies, as previously described [Vassilev and Russev, 1988]. Dilution series of total DNA and nascent DNA samples were immobilized on Biotrans Nylon membrane (ICN) using Slot Blot microfiltration unit (Hoefer Scientific Instruments). The filters were hybridized with 40 bp long oligonucleotides derived from the + and the − DNA strands. The probes were labelled with ^{32}P by polynucleotide kinase. Hybridization was carried out in a Rapid Hyb solution (Amersham) for 2 h at 42–48°C. After hybridization, the filters were washed one time in 5 × SSC-0.1% (w/v) SDS solution for 20 min at 42–55°C.

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