Chromosomal Fragile Sites: Molecular Test of the Delayed-replication Model

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Variations in chromosomal morphology and stability have long attracted the attention of geneticists and cytologists. Such variations have proved useful in addressing significant biological questions (Sutton 1903; Creighton and McClintock 1931). One interesting morphological variation results from mutant or normal alleles that lead to failure of a specific chromosomal site to condense in mitotic chromosomes. Breakage of chromosomes occurs more frequently at these sites, leading to what are called "weak points" (Kaufmann 1939) and "fragile sites" (see Sutherland and Hecht 1985). Darlington and La Cour (1940) observed that poorly condensed sites also could be induced in chromosomes of several species of *Trillium* and other plants by cold temperature. They suggested that such sites are "under-charged with nucleic acid," and that it would be useful to look for abnormalities in nucleic acid and in "timing and regularity of gene division" (Darlington and La Cour 1940).

An inherited human disease, fragile X syndrome, has been genetically linked to such a fragile site (Lubs 1969). This syndrome, which represents one of the most common causes of inherited mental retardation (see Brown 1990), has aided our understanding of the genetic and epigenetic bases of fragile sites. We review here molecular data from fragile X syndrome that substantiate the suggestions of Darlington and La Cour.

We (Laird et al. 1987) and other investigators (Taylor and Hagerman 1983; Gollin et al. 1985; Sutherland et al. 1985; Nussbaum and Ledbetter 1986; Sutherland and Baker 1986) have applied the concept of DNA replication timing to the fragile site, FRAXA, that is associated with the fragile X mutation. In particular, the timing during the cell cycle of sensitivity to inducers of the fragile X site suggested to us that replication of fragile site DNA is delayed in mutant cells, extending well into what is usually referred to as the G_2 part of the cell cycle (Fig. 1). Results of cytogenetic tests of our model were consistent with the prediction of delayed replication (Yu et al. 1990; Webb 1992). These cytogenetic data are most easily understood if the region influenced by the mutation at FRAXA is extensive.

Molecular characterization of breakpoints at FRAXA, and of a candidate gene *FMR1* containing an expanded CGG repeat (Kremer et al. 1991; Oberlé et al. 1991; Verkerk et al. 1991), provided molecular tools to test in more detail the delayed-replication model of fragile sites. Five specific predictions of Figure 1 are (1) DNA representing the normal allele at a fragile site replicates late in S phase; (2) replication of mutant fragile-site DNA is delayed relative to normal-allele DNA; (3) mutant fragile-site DNA replicates during the part of the cell cycle normally referred to as G_2; (4)

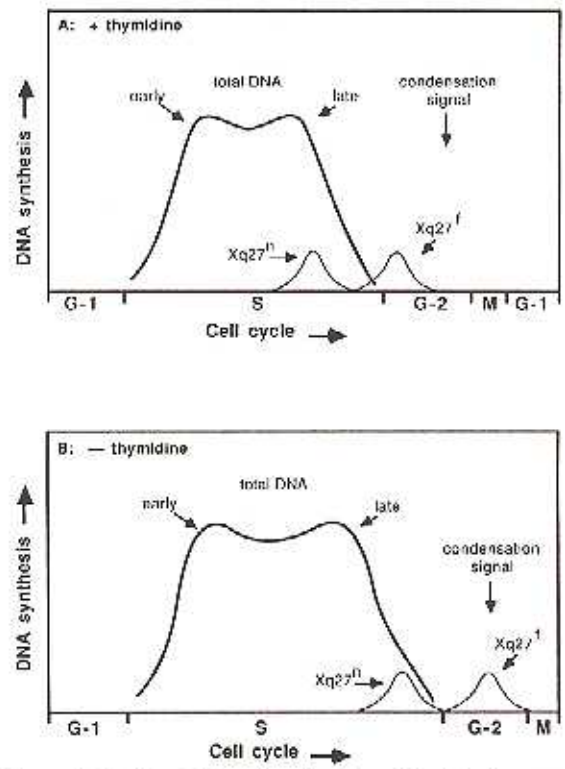

Figure 1. Model of delayed replication of fragile site DNA. The proposed timing of DNA synthesis during the cell cycle for "bulk DNA" and fragile site DNA is indicated. Normal (+) alleles are distinguished from mutant (f) alleles at fragile sites (fs). Fragile site induction by thymidine deprivation is proposed to accentuate the replicative delay, extending it even further into what is normally referred to as G_2. The hypothetical "condensation signal" indicates the timing during the cell cycle before which replication of a sequence must occur in order for normal chromosome condensation to occur at that site in preparation for mitosis. (Reprinted, with permission, from Laird et al. 1987.)

the mutation is *cis*-acting in that it does not affect replication of distant sequences; (5) interfering with DNA replication to induce fragile sites further delays replication of mutant fragile-site DNA.

We have successfully tested the first four of these predictions for FRAXA and have found them to be correct (Hansen et al. 1993). The region of delayed replication in cells from affected males is extensive, as expected from the cytogenetic results. In all cell lines from affected males that we have examined, more than 150 kb of DNA shows delayed replication relative to that in cells from normal males (Fig. 2). The timing of replication of two other X-linked genes, factor IX and phosphoglycerate kinase, was not affected by the fragile X mutation. The large region of DNA over which delayed replication is observed raises the possibility that other genes are included in this region and perhaps altered in transcriptional state.

One of the interesting aspects of fragile X syndrome is that it behaves genetically as a disorder of genomic imprinting, leading to predictions of gene silencing (Laird 1987), with the mechanism of silencing likely to be hypermethylation (Laird 1987; Laird et al. 1987). For the fragile X candidate gene *FMR1*, these predictions have been confirmed (Dietrich et al. 1991; Heitz et al. 1991; Oberlé et al. 1991; Pieretti et al. 1991). Moreover, the predicted pattern of methylation is similar to that observed for normal alleles of *FMR1* when on the inactive X chromosome in female cells (Bell et al. 1991; Heitz et al. 1991; Hansen et al. 1992; Hornstra et al. 1993), as predicted by the imprinting model (Laird 1987). Other fragile sites have genetic segregation properties that are also consistent with imprinting (Samadder et al. 1990), although fewer genetic data are available for these fragile sites than for FRAXA.

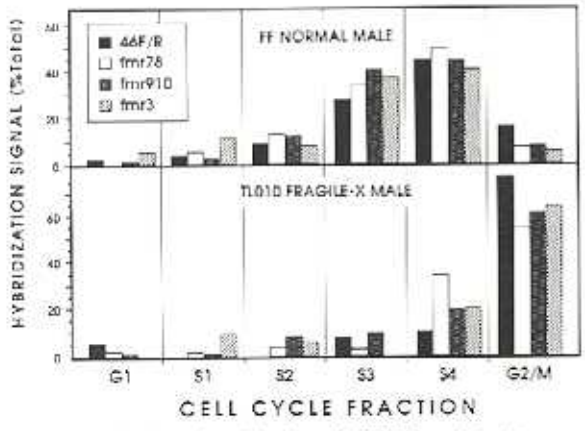

Figure 2. Timing of replication of *FMR1* and flanking sequences in cells from normal (*top*) and affected (*bottom*) males. These experiments relied on pulse labeling of replicating DNA by 5-bromodeoxyuridine (BrdU), followed by cell sorting based on total DNA content. Newly replicated DNA was purified from fractionated cells by precipitation with anti-BrdU antibody; PCR was used to amplify specific DNA fragments; primer set 46F/R amplifies sequences about 150 kb 5' of *FMR1*; primer sets fmr78 and fmr910 amplify sequences just 5' and 3', respectively, of the CGG repeat of *FMR1*; primer set fmr3' amplifies sequences about 33 kb 3' to the CGG repeat. (Adapted, with permission, from Hansen et al. 1993 [copyright held by Cell Press].)

To assess whether or not the replication delay is correlated with the hypermethylation of the CpG island of *FMR1*, we have compared the replication timing of the alleles of FRAXA from affected males with that of alleles from normal males and from nonpenetrant transmitting males. Males in this latter group have CGG expansions to between 55 and 200 repeats, which are termed primary expansion alleles (Hansen et al. 1992) or premutations (Pembrey et al. 1985; Oberlé et al. 1991). The CpG island of *FMR1* in such males is usually not hypermethylated. Alleles from nonpenetrant transmitting males and from normal males exhibit similar replication profiles. Our initial results with a cell line containing an intermediate-sized expansion of CGG repeats (~ 200) within an unmethylated CpG island indicated no replication delay of *FMR1* (Hansen et al. 1993). These results suggest that hypermethylation of the CpG island at *FMR1* rather than the CGG expansion may be intimately involved in the delayed replication. Experiments are in progress using a larger, unmethylated expansion of the CGG repeat in order to resolve this issue.

Tools are thus available to determine if the replication of DNA from other fragile sites is similarly influenced by mutant alleles (Knight et al. 1993). It may also be useful to return to a more general question: How is the degree of chromosome condensation affected locally by the timing of DNA replication? (see Fig. 1) (Darlington and La Cour 1940; Sperling and Rao 1974).

Fragile X syndrome thus joins a member of the deletion thalassemias $(\gamma\delta\beta)^0$ as syndromes with a molecular phenotype of delayed DNA replication (Forrester et al. 1990). The existence of such syndromes raises the possibility that altered cell cycle control is involved in the disease phenotype. Do cells with abnormally delayed replication have a normal or extended S phase? Do they begin G_2 functions while still continuing replicative DNA synthesis? An altered cell cycle control could have clinical significance that extends beyond the inhibition of transcription of genes within the region.

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