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Temporal Order of DNA Replication

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THE REPLICATION CLOCK

In every organism, DNA replication takes place in an ordered physical and temporal fashion. Although this process is primarily necessary to carry out the task of copying the genome, it may also indirectly play a role in the regulation of gene expression. In *Escherichia coli*, for example, the entire 4-Mb circular chromosome undergoes bidirectional DNA synthesis that is initiated from a single origin. It takes at least 40 minutes to copy the complete bacterial genome, and as a result, genes located near the origin are replicated early in the cell cycle whereas those positioned at the opposite end of the chromosome are synthesized late in the cycle. One direct consequence of this temporal organization is that in rapidly dividing cells, the early-replicating genes themselves accumulate differentially in high copy number (von Meyenburg and Hansen 1987), and this most certainly has profound effects on the overall expression pattern of the organism.

Compared to prokaryotes, eukaryotic organisms contain a much larger genome. Although the total DNA is divided into separate chromosome molecules, each of these entities in itself carries a considerable amount of genetic information, and DNA synthesis is accomplished through the action of multiple bidirectional origins. If all of these replication units were activated simultaneously, the entire process of replication could be completed very quickly. However, replication starts are actually distributed in a programmed manner throughout the length of S phase, and, as a consequence, each origin is turned on at a specific time. In the yeast *Saccharomyces cerevisiae*, DNA located near telomeres, for example, undergoes replication late in S phase, whereas sequences associated with centromeres are early replicating (Reynolds et al. 1989). Other regions of the genome are replicated at various fixed times within the 30-minute S phase.

DNA Replication in Eukaryotic Cells

The process of DNA replication in animal cells takes place over a longer period of time, an average of 8–10 hours. Despite the complexity of these genomes (3000 Mb), a number of related methods have been developed to pinpoint the replication time of individual genes. Basically, replicating DNA is first labeled in vivo with bromodeoxyuridine (BrdU) and then purified by gradient centrifugation from cells at various stages of S phase. This fractionation is accomplished either by prior synchronization (Goldman et al. 1984; Schmidt and Migeon 1990) or by cell-sorting techniques (Braunstein et al. 1982; Gilbert 1986), and the resultant newly synthesized DNA samples are then subjected to blot hybridization with specific probes. Early-replicating genes hybridize to fractions from the beginning of S phase, whereas late-replicating sequences only hybridize with BrdU DNA from cells in the later fractions of S. By increasing the number of interval fractions in S phase, one can obtain better cell-cycle resolution of replication timing. Using this methodology, a large number of specific genes from various organisms have been analyzed, and in general, the results confirm the idea that replication proceeds progressively in an ordered programmed manner throughout S phase (for review, see Holmquist 1987).

REPLICATION TIMING AND GENE EXPRESSION

Although the biological function of replication timing control in the cell cycle is not yet clear, studies in yeast and in animal cells have revealed a striking association between replication timing and gene expression. In *S. cerevisiae*, for example, the unexpressed *HML* and *HMR* loci are located in subtelomeric regions that replicate late in S phase. When, as part of the normal process of cell-type-specific switching, they are copied to the *MAT* locus in an early-replicating region of the same chromosome, the promoters are activated (Reynolds et al. 1989). That replication timing is involved in this control mechanism is suggested by the observation that positional silencing at *HMR* requires an autonomously replicating sequence (ARS) element, and mutations in the ARS that abolish origin function also relieve transcriptional silencing (for review, see Laurenson and Rine 1992). In higher organisms, the general relationship between transcription and replication is even more straightforward. Constitutively transcribed housekeeping genes undergo relatively early DNA replication (some time during the first half of S) in a variety of cell types. In contrast, many tissue-specific genes are late-replicating (the last 50% of S) in their repressed state, but are early-replicating in cells in which they are expressed (for review, see Holmquist 1987). It thus appears that in

Table 1 Replication times for genes in vertebrate cells

Gene	Replication time
Housekeeping	
HGRPT	E
APRT	E
CAD	E
DHER	E
arginine succinate synthetase	E
G6PD	E
β-actin	E
β-tubulins	E
metallothionein-1	E
PDG X linked	E
Tyr aminotransferase	E
c-ras	E
c-myc	E
all histones tested	E
Xenopus somatic 5s	E
Tissue-specific	
apolipoprotein	E
placental lactogen	E
α_1-antitrypsin	L
β-casein	L
Phe hydroxylase	L
α-globin	E
fibronectin	L
β-globin	L*
immunoglobulin V_H	L*
Xenopus oocyte 5s	L*
cystic fibrosis	L*

Data on replication timing were either derived from standard cell-culture procedures (adapted from Holmquist 1987) or obtained by FISH analysis (e.g., cystic fibrosis genes). It has already been demonstrated that these two different techniques yield similar results (Selig et al. 1992). As shown, all housekeeping genes replicate in the first half of S phase (E), whereas many tissue-specific genes replicate in the last half (L) of S in cell types that do not express the gene. In some cases, these same gene sequences were found to replicate early in expressing cells (*). This table only includes representative examples and is not exhaustive.

these organisms, replication timing is a developmentally regulated process that is closely associated with gene expression (Table 1).

Unlike some organisms, such as *Xenopus* or *Drosophila*, that are characterized by extremely short replication cycles during early-cleavage stages of development, in mammals, programmed, cell-cycle-controlled replication is present even at the earliest cell divisions (Takagi 1974). To

obtain a better picture of how the replication timing pattern of the genome is established during development, replication was analyzed in F9 teratocarcinoma cells (S. Selig, unpubl.). These studies suggest that tissue-specific genes, such as β-globin or albumin, are initially late-replicating in cells of the early embryo prior to organogenesis. It is likely that these same genes then remain late-replicating in most cell types, but shift to early replication as a part of the differentiation process that brings about gene activation. In parallel, housekeeping genes remain early-replicating and active throughout development. Almost all studies on replication timing have been carried out in rapidly growing tissue-culture cells, and much work must still be done to evaluate this process in vivo.

Tissue-specific genes, which are initially late-replicating in the early embryo, undergo conversion to early replication in specific cell types. However, other DNA sequences in the genome have an opposite pattern and become late-replicating in a developmentally regulated manner. Genes on the X chromosome, for example, shift dramatically from early to late replication in association with the inactivation process that occurs at about the time of implantation in female eutherans (Takagi 1974). At the same stage in development, centromeric mouse satellite sequences also undergo a shift to a later time of replication (Selig et al. 1988). Although the role of replication timing in this case is not known, this change probably reflects the overall stage-specific structural alterations that take place on these chromosomal regions.

REPLICATION TIME ZONES IN MAMMALIAN CELLS

Since in each cell type, individual sectors of DNA are programmed to undergo replication at specific times in S phase, the genome must be organized into replication time units. By following BrdU labeling, these can be visualized cytogenetically as an alternating pattern of early- and late-replicating regions on each chromosome (Fig. 1). In this procedure, nonsynchronized cell cultures are incubated with BrdU for fixed times, and the resulting incorporated label is then detected in metaphase chromosomes. After 4 hours of labeling, for example, all of the BrdU visualized in metaphase represents sequences that replicate late in S phase just prior to condensation in G_2 (~2 hours), and this produces a striking pattern of highly ordered late-replication bands on each chromosome. Labeling with BrdU for longer intervals reveals a continuous series of chromosomal bands that replicate in a programmed manner throughout S (Latt 1973; Wolf and Perry 1974; Vogel et al. 1986). The very existence of this visible banding pattern immediately

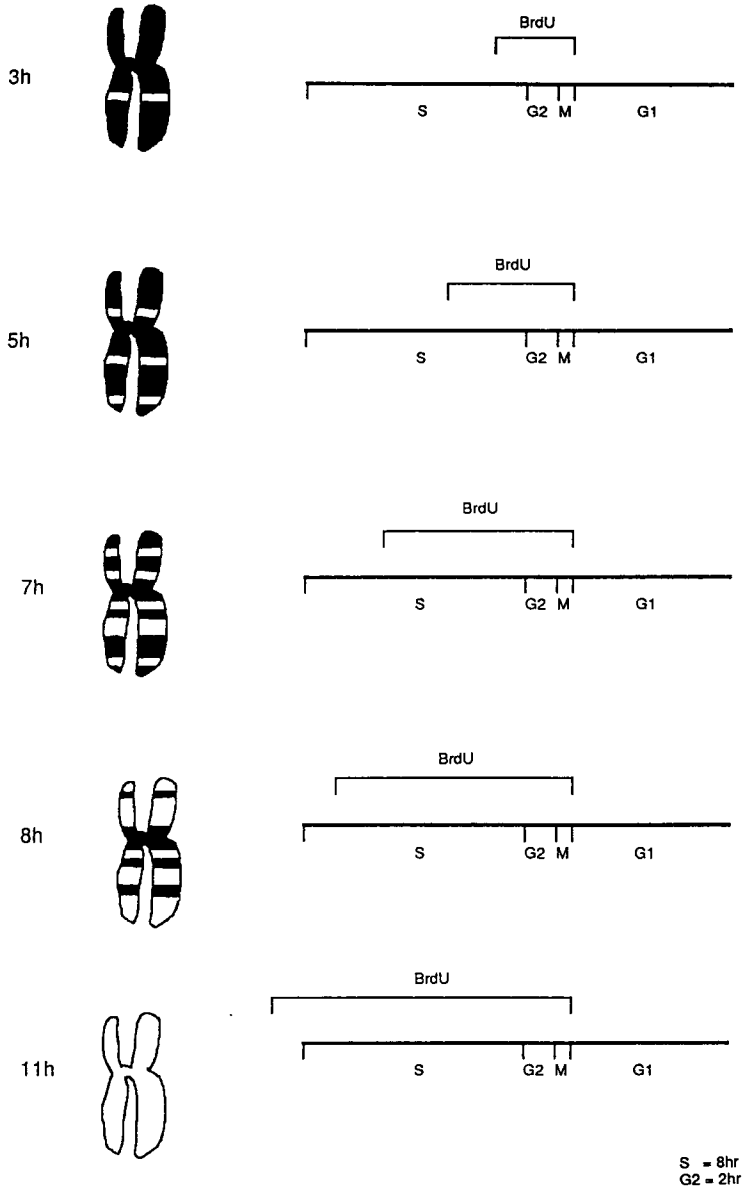

Figure 1 Replication banding. Nonsynchronous cells are cultured in the presence of BrdU for various fixed times, and nuclei are then prepared and stained (see Drouin et al. 1990). The locations of BrdU incorporation are visualized as light (quenched) bands. Since chromosomes are only observed in metaphase cells (M), the 3-hr incubation labels only those regions that replicate in the last hour of S, and the 5-hr incubation labels those that replicate in the last 3 hr of S. After 11 hr, the entire chromosome is labeled with BrdU.

suggests that DNA replication timing units are not only quite large, but also spatially well delineated. Replication bands are an integral part of the basic chromosome unit structure, and these same early-replicating DNA regions appear to comap quite precisely with Giemsa-stained light G bands (Hand 1978). Using DNase I sensitivity as a molecular probe for genome accessibility, it has been possible to map "active" regions (D bands) on metaphase chromosomes, and these also correlate with early-replicating time zones (Kerem et al. 1984). The fact that these banding patterns do not vary appreciably from cell type to cell type suggests that the early-replicating active regions may represent genomic loci rich in housekeeping genes. Taken together, these morphological observations indicate that the entire genome may be subdivided into a series of basic modules which represent units of common structure, function, and replication timing.

Original estimates for the size of each replication band suggest that these domains may cover 5–10 Mb and contain multiple replicons. High-resolution mapping of both replication bands (Drouin et al. 1990) and G bands (Yunis 1981) on extended pro-metaphase chromosomes, however, has delineated at least 2000 individual stripes, suggesting that each chromosomal unit is actually much smaller, containing, on average, 1.5 Mb of DNA (Kitsberg et al. 1991). Due to their relatively large size, most of these replication domains have not yet been mapped at the molecular level, but the availability of extensive cloned contigs, as well as recent advances in technology, should now make it possible to examine these units in detail.

The replication time of a genomic DNA fragment can be measured in almost any cell type by using fluorescence in situ hybridization (FISH) to interphase nuclei. In this method, unreplicated DNA is visualized as a single hybridization dot, and replicated DNA appears as a double dot. In a nonsynchronized population of dividing cells, a high percentage of nuclei with double hybridization signals indicates that this particular gene replicates relatively early in S phase, whereas a low count is obtained for gene sequences that replicate late in the cycle (Selig et al. 1992). Furthermore, by labeling cells with BrdU prior to harvesting, it becomes possible to specifically identify nuclei in S phase and thus to disregard single-dot nuclei from G_1 and double-dot nuclei from G_2 (Kitsberg et al. 1993b). This method is particularly accurate for measuring the replication time of one zone relative to another. In this case, each probe is labeled with a different fluorescent dye, making it possible to detect nuclei where one gene has already replicated while the other is still uncopied in the same cell (Selig et al. 1992). Using this approach,

replication time zones can probably be resolved within minutes on the cell-cycle clock.

A 1.5-Mb region of DNA containing the cystic fibrosis (CF) gene has been studied using this method. By assaying a contiguous set of phage and cosmid clones in fibroblasts or lymphocytes, cells that do not express this gene, one observes two distinct, but internally uniform, replication time bands: an early/middle zone located 5′ to the gene and an extensive late zone that includes the 250-kb domain that carries the CF coding sequence. In the intestinal epithelial-derived cell line, Caco-2, in which the CF gene is transcriptionally active, the basic replication structure is quite similar, except for a distinct region of 500–700 kb around the CF gene that replicates very early (Selig et al. 1992). Thus, in this region, replication timing is organized into defined spatial domains and is developmentally regulated (Fig. 2). The human β-globin gene region has also been mapped for replication timing at the molecular level. In this case, as well, a relatively large domain containing the β-like genes is late-replicating in a number of different cell types, but early-replicating in erythroleukemia cells (Epner et al. 1988; Hatton et al. 1988). Because only 250 kb of sequence surrounding these genes has been analyzed, it has not been possible to map the full extent and outer boundaries of this time zone.

REPLICON STRUCTURE

Since animal cell replicons are, on average, 50–300 kb in length (Hand 1978), it is likely that a typical replication band is made up of multiple adjacent replicons that are coordinately regulated to undergo replication at the same time in S. This was originally derived from autoradiograph studies of DNA synthesis, which clearly reveal replicating regions containing strings of multiple pulse-labeled origins (Edenberg and Huberman 1975), and this observation has now been confirmed at the molecular level. The most clear-cut example of this phenomenon is in the mouse major histocompatibility complex (MHC) locus, where a large region of more than 2 Mb contains at least 5–6 distinct replicons that all replicate fairly synchronously as a single time zone (Spack et al. 1992). Several well-mapped replication origins near the DHFR gene also appear to fire in a coordinated manner, all at the beginning of S phase (Ma et al. 1990).

Several lines of evidence indicate that the activation time of any particular origin is not an intrinsic property of the origin sequence itself. The chromosome V origin, ARS501 in yeast, is activated in the second half of S phase and is responsible for the late replication of a 66-kb domain

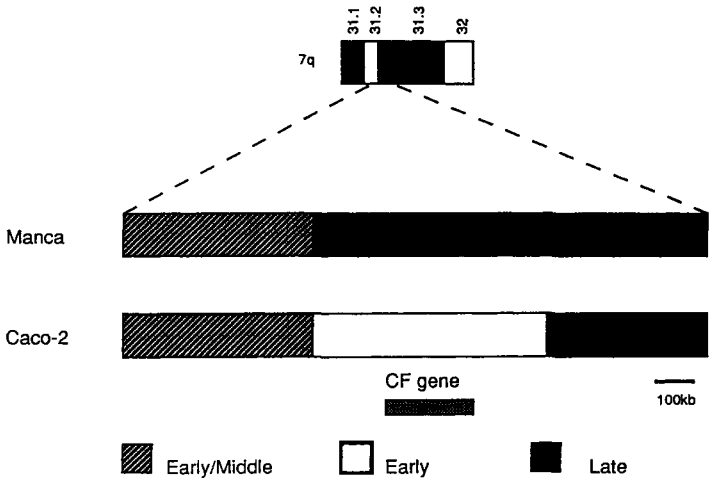

Figure 2 Replication time zones in the cystic fibrosis gene domain. A large number of plasmid, phage, and cosmid clones covering a 1.5-Mb region on human chromosome 7 were analyzed for replication timing in two cell types: Manca lymphocytes, which do not express the CF gene, and Caco-2 cells, which do (see Selig et al. 1992). This map corresponds to chromosome region 7q31.

that includes the right telomere (Ferguson et al. 1991). The time of replication of this origin is regulated by position, since late activation requires the presence of a telomere nearby, and when this origin is transplanted to another site, it replicates early. These observations show that the time at which an origin is activated can depend on its chromosomal context (Ferguson and Fangman 1992). Origins in animal cells apparently behave in a similar manner. In the human β-globin gene domain, mapping has demonstrated that a large region of at least 100 kb is part of a single replicon, with an origin located slightly 5′ to the β-globin gene sequence. Interestingly, this same site serves as the origin both in lymphocytes, where replication occurs late in S, and in erythroleukemia cells, where replication is early (Kitsberg et al. 1993a). This represents clear-cut evidence that the origin sequence serves as a topographical marker for the site of initiating DNA synthesis, whereas temporal activation is controlled by chromosomal context in *cis* and most likely by protein factors in *trans*.

REGULATION OF REPLICATION TIMING

Several experiments indicate that long-range *cis*-acting elements must play a role in the control of replication timing. In animal cells this effect

is most obvious in regions that have undergone chromosomal rearrangement. The Xic region on the inactive X, for example, probably plays an important role in controlling replication timing, since its translocation to another chromosome causes adjacent sequences to become late-replicating and, in many cases, inactive (see, e.g., Disteche et al. 1979). Significant changes in replication timing also occur during the normal process of human immunoglobulin heavy chain (IgH) rearrangement in the B-cell lineage. In most somatic cells, the heavy-chain constant region is embedded within a large early-replicating domain (Brown et al. 1987), whereas distal variable sequences replicate late in S. When rearrangement takes place, however, the juxtaposed variable gene falls under control of the constant domain and assumes an early replication time (Calza et al. 1984). This shift could be part of the variable gene promoter activation process, which is ultimately responsible for antibody production in B cells. Similar changes in replication timing also occur at the site of the IgC_H-*myc* translocation in MPC11 plasmacytoma cells (Calza et al. 1984) or at other translocated loci in human leukemias (Karube and Watanabe 1988).

Genomic mapping studies in yeast have succeeded in identifying and characterizing defined *cis*-acting sequences that control replication timing. A telomere itself, for example, can induce late replication on origins located within approximately 30 kb. This effect is undoubtedly mediated through the telomeric DNA sequence itself, since small $C_{1\text{-}3}A$ tracts also shift replication timing when placed close to an origin (Ferguson and Fangman 1992). It is likely that *cis*-acting sequences in other parts of the genome can also direct the initiation time of DNA synthesis, and an element of this nature has recently been mapped near the late-replicating KEX2 ARS on yeast chromosome XIV (B.J. Brewer et al., unpubl.).

Replication timing in animal cells is also under the control of *cis*-acting sequences, and there is now good evidence for a defined replication timing regulatory element associated with the locus control region (LCR) in the human β-globin gene domain. This enhancer-like sequence, located about 20 kb upstream of the ε gene, probably plays a major role in the regulation of genes in this cluster, since patients carrying a deletion of this control region (Hispanic thalassemia) do not make any β-globin transcripts (Driscoll et al. 1989). In normal human lymphocytes, this locus is in a repressed configuration, but fusion to mouse erythroleukemia cells induces a striking activation of the domain and a concomitant shift to early replication. In contrast, the β-globin domain from Hispanic thalassemia lymphocytes fails to undergo transcriptional

activation and, at the same time, remains late-replicating. It thus appears that seqences associated with, or near, the globin LCR are probably involved in replication timing control (Forrester et al. 1990).

Indirectly, these fusion studies also suggest that the donor erythroleukemia cells contain a set of protein factors which control the replication timing of the globin domain in *trans*, perhaps through specific interactions with LCR elements. Experiments in culture have shown that treatment of cells with the demethylating agent, 5-azacytidine (5azaC), dramatically shifts the replication time of both the inactive X chromosome (Shafer and Priest 1984; Jablonka et al. 1985) and mouse satellite sequences (Selig et al. 1988) from late to early in the cycle. The highly coordinated and quantum nature of this response suggests that this drug does not act independently on each of the multiple chromosomal regions themselves. Rather, 5azaC mediates the changes in replication timing either through an effect on a master *cis*-acting control element or by inducing *trans*-acting protein factors. This latter possibility is supported by cell fusion experiments. Thus, for example, when embryonic carcinoma cells are fused with female-derived thymocytes, this brings about a dramatic early shift in X-chromosome replication timing (Takagi et al. 1983). The abnormal early replication time of trisomic chromosomal region 15E seen in cases of T-cell leukemia represents another case where protein factors in the nucleus must play some role in the control of the replication clock (Somssich et al. 1984). These studies, taken together, thus support a model whereby replication timing is regulated through *cis* elements that are recognized by and interact with specific *trans*-acting factors.

ALLELE-SPECIFIC GENE EXPRESSION

Transcriptional regulation is thought to be carried out in a large part through the interaction of regulatory sequences with combinations of multiple *trans*-acting factors in the nucleus. In parallel, *cis*-acting effects, such as chromatin structure or DNA methylation, also modulate gene expression by affecting the accessibility of gene regions to these same factors. The importance of *cis*-acting mechanisms is nowhere more obvious than in cases where only one allele is expressed in a given nucleus. Thus, for example, genes on both X chromosomes in female cells are potentially exposed to the same *trans*-acting factors in the nucleus, yet structural differences render one X inaccessible and therefore excluded from the normal transcriptional machinery. DNA methylation represents one important mode for mediating allelic selection. Methyl moieties are known

to inhibit transcription both by altering specific protein-DNA interactions and by modulating general chromatin structure (Eden and Cedar 1994). When only one allele is modified, this leads to selective inhibition.

A major role of DNA methylation on the X chromosome is to maintain the differential expression pattern from generation to generation (Mohandas et al. 1981). Once established, methyl moieties are conservatively preserved in daughter cells due to the action of a maintenance methylase that copies the modification pattern of the previous generation (Cedar 1988). Another parameter that differentiates between the active and inactive X is their time of replication in S phase; this may represent yet another *cis*-acting structural marker for distinguishing between two otherwise equivalent alleles in the same cell. Assuming that replication timing is set by cell-cycle-controlled factors, it is easy to see that this parameter, much like methylation, can be propagated from generation to generation and can thus be used to maintain allelic identity.

Replication timing may also be involved in the allele-specific regulation of imprinted genes. A number of these genes have now been identified and characterized in the mouse. Insulin-like growth factor 2 (*Igf2*), U2af-binding protein-related sequence, and small nuclear ribonucleoprotein polypeptide n (*Snrpn*), for example, are expressed exclusively from the paternal allele, whereas insulin-like growth factor 2 receptor (*Igf2r*) and *H19* are transcribed only from their maternal copy in somatic cells (for review, see Razin and Cedar 1994; see also Villar and Pedersen 1994). Imprinting has also been observed for some of these same genes in humans. The Prader-Willi and Angelman syndromes (PWS/AS), for example, come about as a result of either uniparental disomy or monoparental deletions on chromosome 15, and it is likely that imprinting of *SNRPN* or other gene sequences located in this region may explain the variety of symptoms seen in these diseases (Nicholls 1993).

IMPRINTED REPLICATION TIME ZONES

Much like the genes on the X chromosome, these sequences have a genomic structure that distinguishes between the two parental alleles. Thus, as might be expected, almost all of the loci that have been analyzed show differential methylation (Razin and Cedar 1994). In situ hybridization has been employed to evaluate the replication timing properties of these genes. This method is especially appropriate for detecting asynchronous replication. In any cell population, FISH analysis generally reveals mainly nuclei with either two single or two double hybridization dots, indicating that both homologs of most genes replicate

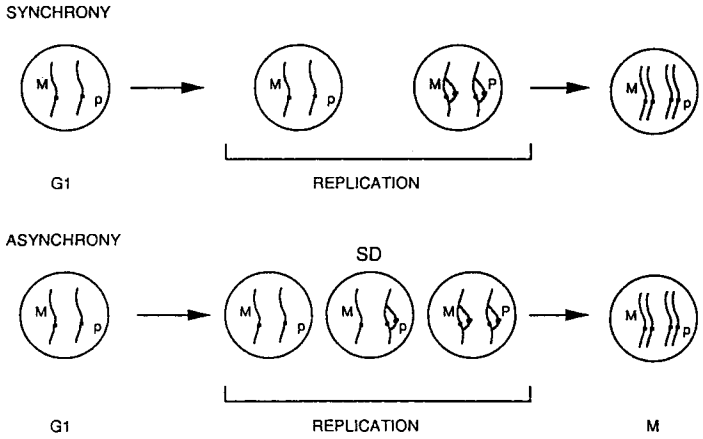

Figure 3 Allele-specific replication timing. By using FISH analysis, all gene regions are unreplicated on both alleles in G_1 and therefore show two single hybridization dots. For most sequences, both homologs replicate relatively synchronously, so nuclei in S phase either have two single dots or two double dots. For imprinted genes, each allele replicates at a different time in S, so a high percentage of nuclei show both an unreplicated (single dot) and a replicated (double dot) allele. At metaphase, all of the DNA has already replicated.

synchronously during the cell cycle. In contrast, for imprinted genes, a large number (~30%) of nuclei contain one replicated and one unreplicated gene (Fig. 3), suggesting that these alleles undergo DNA synthesis asynchronously in the cell cycle (Kitsberg et al. 1993b; Knoll et al. 1994). It appears from these studies that many imprinted genes are imbedded within large differentially replicating domains which, in some cases, can even be visualized by standard replication banding techniques (Izumikawa et al. 1991). By using polymorphic markers to identify each parental allele individually, it has been shown that most of these regions replicate coordinately in an allele-specific manner, with the paternal allele generally replicating earlier in the cell cycle (Kitsberg et al. 1993b). As a result of this regional pattern, the active allele of some imprinted genes actually replicates late in the S phase. This clearly represents an exception to the general correlation between early replication timing and gene expression and suggests that this standard structure/function relationship may be altered for imprinted genes.

REGIONAL REGULATION

The finding of domain-wide replication-timing control strongly suggests that imprinting itself may be regulated at the regional level. This idea is

supported by the observation that imprinted genes are clustered. Experiments with mono-parental disomic mice initially indicated the existence of a limited number of relatively well defined chromosomal regions that may be involved in the generation of parent-of-origin defects during development (for review, see Cattanach 1986), and more recently, genomic mapping has shown that imprinted genes are indeed clustered. *Igf2* and *H19*, for example, are positioned within 90 kb of each other and are imprinted in both mouse and man (Razin and Cedar 1994). Furthermore, the nearby insulin gene has been found to be imprinted in extraembryonic tissues (Giddings et al. 1994), and *Mash-2*, a newly identified paternally transcribed gene, is located in the same chromosomal region about 500 kb upstream of the *Igf2* locus (Guillemot et al. 1995).

Even more striking is the PWS region on human chromosome 15, where at least four individual genes are transcribed exclusively from the paternal allele (Sutcliffe et al. 1994; Wevrick et al. 1994). Further evidence suggesting regional control at this locus comes from cases of PWS carrying small deletions around the *Snrpn* gene on the paternal chromosome (Buiting et al. 1995). Although these mutations are relatively small, several nearby intact imprinted genes on this chromosome become transcriptionally inactive (Sutcliffe et al. 1994), selective adjacent sites adopt a maternal pattern of methylation (Reis et al. 1994), and for at least one case, an entire 600-kb domain undergoes a dramatic change in replication timing on the paternal allele (Gunaratne et al. 1995). It is thus likely that these individuals have a defect in *cis*-acting DNA sequences directly involved in the regionwide regulation of imprinting. In general, these imprinted domains include gene sequences that are not necessarily expressed in an allele-specific manner. It thus seems likely that the regulation of imprinting is a complicated process that utilizes multiple control mechanisms and most certainly involves both local and regional effectors of expression.

The organization and control of asynchronous replication, both on the X chromosome and in imprinted domains, are not well understood. Although the two X chromosomes in female cells generally replicate asynchronously (Takagi 1974), the inactive X still retains a detailed multireplication-band structure, with each individual domain replicating at a fixed time in S, usually later than the corresponding region on the active X (Wahrman et al. 1983). Because of this structure, most genes on the active X replicate in the first half of S, but for some genes, despite their asynchrony, both alleles actually replicate in late S phase (Hansen et al. 1993; Boggs and Chinault 1994). Whereas replication timing on the X chromosome appears to be coordinated, individual replication bands

may also be regulated autonomously. In the fragile X syndrome, for example, a single large domain of over 150 kb is shifted to a very late replication time while the rest of the chromosome replicates with normal timing properties (Hansen et al. 1993). Overall replication timing asynchrony on the X is most likely centrally regulated by *cis*-acting sequences at *Xic*, a locus that may itself be essential for the inactivation process (Brown et al. 1991b). Further studies have highlighted the possible role of the *Xist* gene which maps to this region and codes for an untranslated nuclear RNA species transcribed exclusively from the inactive chromosome (Brown et al. 1991a, 1992). Interestingly, this gene is embedded in a unique domain that replicates asynchronously, but unlike the rest of the chomosome, is copied earlier on the inactive X (Boggs and Chinault 1994; Torchia et al. 1994). Recent studies have shown that this gene is not required for the maintenance of X inactivation, making it unlikely that it itself plays a role in the control of replication timing (Brown and Willard 1994).

Complex patterns of replication time zones may also be characteristic of imprinted regions. Both in the Igf2r and Igf2 domains, asynchronous replication appears to be uniform and coordinated in such a way that large regions on each parental chromosome replicate together (Kitsberg et al. 1993b). In contrast, it is likely that the asynchronously replicating 3- to 4-Mb PWS/AS domain is actually made up of smaller individual subunits, each subject to its own replication timing control. Most DNA from this region clearly replicates with the paternal allele early, but it has also been demonstrated that sequences near the GABA receptor genes replicate maternally early (LaSalle and Lalande 1995), and at least one probe near the P locus replicates asynchronously, but without any allele specificity (Knoll et al. 1994).

ALLELIC EXCLUSION

The most striking aspect of asynchronous replication timing is its consistent association with the phenomenon of domain-wide allelic inactivation. Olfactory receptor gene loci may represent yet another example of this unique type of regulatory structure. The sense of smell in a number of different organisms is mediated through olfactory neurons in the nasal organ (Buck and Axel 1991). Each of these neurons evidently expresses one, or a small number, out of a repertoire of about 1000 odorant receptor genes (Vassar et al. 1993). The problem of distinguishing which receptors have been activated therefore reduces to a problem of identifying which neurons have been activated. In such a model, perception of an

odor encountered by the organism would result from the identification of the subset of cells responsive to that odorant.

Recent evidence suggests that in an individual neuron the receptor gene is expressed from only one of the two parental alleles (Chess et al. 1994). In some cells it is the paternal gene which is active, whereas in others the maternal gene undergoes transcription. Since olfactory receptor genes are clustered at a number of different chromosomal sites, it has been suggested that a single gene in an array is turned on stochastically by means of a *cis*-acting locus control element. If this is the case, there must be a mechanism of allelic exclusion to prevent the second allele from activating another gene. Although there is, as yet, no direct experimental proof for this model, it has been shown that these loci indeed replicate asynchronously (Table 2), and this suggests indirectly that only one allele in each cell may be in an active conformation. Unlike imprinted gene domains, the replication pattern of the olfactory receptor loci is not allele-specific, and this is in keeping with the observation that either allele for any given receptor gene can be active. The asynchronous pattern is probably generated in the early embryo, since it has been observed in a variety of different cell types and in F9 teratocarcinoma cells (Chess et al. 1994). In a sense, this structure would thus represent a form of autosomal allelic exclusion analogous to X-chromosome inactivation. There may very well be additional regions of the genome that behave in this manner, and the replication assay provides a convenient method for detecting these.

REGULATION OF GENE EXPRESSION

Although there is a correlation between replication timing and gene expression, the cause and effect relationship between these phenomena has not yet been explained. One possibility is that shifts in replication timing are secondary to changes which occur in gene expression or chromatin structure. Since replication time zones are so large, however, this model would predict that a local modulation in the transcription of one particular gene could bring about a domain-wide shift in replication timing. It is much more likely that replication time is controlled independently by long-range *cis*-acting elements (e.g., X-inactivation center or LCRs) and the resulting domain-wide structure then influences specific genes at the local level.

Studies on *Xenopus* 5S RNA genes provide a good model for understanding how replication timing may modulate transcription. Two different sequence types for this gene are carried in the genome; one pro-

Table 2 Synchronous and asynchronous replication timing

Locus	Single-double (%)
Control loci	
c-*myc*	9
H-*ras*-1	12
IgH	10
albumin	9
p53	12
C48	9
Pfk-L	11
Imprinted gene	
Igf2	23
Igf2R	35
H19	30
Snrpn	34
Olfactory receptors	
102	34
107	35
I7	38
I28	38
Y22(MEL)	32
Y22(F9)	39
Y22(PEF)	31

A number of gene regions were tested for replication timing using FISH analysis and the number of nuclei showing asynchronous replication (see Fig. 3) was scored (% single-double). Most loci replicate relatively synchronously with approximately 10% single-double nuclei. Imprinted genes and olfactory receptor genes have a high percentage of nuclei showing asynchronous replication. In the case of imprinted genes, replication is allele-specific (Kitsberg et al. 1993b), whereas for olfactory receptor genes either allele can replicate early (Chess et al. 1994). Olfactory receptor gene replication was analyzed in mouse erythroleukemia cells (MEL), embryonal carcinoma (F9), or primary embryonic fibroblasts (PEF).

vides the major transcript in the oocyte and another is expressed mainly in somatic tissues. In the oocyte, high concentrations of transcription factors activate both gene types equally, but since the oocyte 5S RNA genes are more numerous, this species provides the overwhelming majority of RNA molecules. In somatic cells, on the other hand, the oocyte genes are in a repressed chromatin state which prevents their transcription (Wolffe and Brown 1988).

In contrast to the somatic 5S RNA genes, the oocyte genes replicate late in the cell cycle (Gilbert 1986). It has been suggested that this may put them at a disadvantage for binding the critical transcription factors in somatic cells, and through this simple mechanism, they would be maintained in an inactive state even following replication. The feasibility of this mechanism is, of course, based on the assumption that certain

protein factors can only interact with the gene while it is undergoing replication and the basic chromatin structure is thus disrupted. In keeping with this prediction, transcription factors indeed stimulate oocyte 5S RNA synthesis, but only after histone H1 is removed from the chromatin (Wolffe and Brown 1988). Replication timing appears to be an important part of this model, since even the oocyte 5S RNA gene is activated in a cell line carrying a translocation that forces this sequence to undergo early replication (Guinta et al. 1986). There is no question that the control of somatic and ooctye 5S RNA gene expression is both complex and multifaceted, but it certainly appears that replication timing is an important element in this process.

Direct evidence for an effect of replication timing on gene expression has been obtained from an analysis of the *URA3* gene in the yeast *S. cerevisiae*. At its normal locus in the genome, this gene is constitutively expressed at a basal level, but it can be induced to higher levels of expression by the binding of the *trans*-activator PPR1 to the gene regulatory domain (Roy et al. 1990). When *URA3* is located in a late-replicating telomeric region, however, its basal level of expression is markedly repressed and, as a result, the cells become phenotypically ura3$^-$, unless they are rescued by the presence of *PPR1*. In an elegant series of experiments, the *PPR1* gene was put under the regulation of a galactose-inducible promoter. Cells were then arrested at the G_1/S interphase in the absence of *PPR1* expression, a condition that prevents *URA3* transcription. Surprisingly, the induction of *PPR1* did not bring about *URA3* activation, and only when the cells were allowed to continue through the later part of the cell cycle did *URA3* become transcriptionally competent (Aparicio and Gottschling 1994). These studies clearly show that the induction of this gene is dependent on cell-cycle events and strongly suggest that replication is required for the activation process.

Two models have evolved for the effect of chromatin structure on gene regulation. In one case, chromatin prevents *trans*-activating proteins from gaining access to the DNA, thus keeping the gene irreversibly repressed. However, during DNA replication the chromatin structure of the gene is perturbed, and activation factors have the opportunity to gain access and establish transcription before reassembly of the chromatin is completed. In an alternate model, the *trans*-acting proteins can induce gene transcription at any time in a replication-independent manner, effectively disrupting the repressive nature of the chromatin. Examples of both forms of regulation have been observed (Felsenfeld 1992).

Assuming that at least some genes require a replication event in order to set up an active transcription complex, the time at which replication

takes place could play a big role in determining the fate of each particular sequence. Since dynamic changes take place during the cell cycle, the levels of pertinent factors may fluctuate and thus be present in the nucleus only during specific intervals of S. In this way, the activation of a target gene may only be possible if it is replicated in this specified "window of opportunity." Replication at an alternate time in S could lead to an inactive configuration. E2F proteins may represent an example of this type of transcription factor, since their availability is carefully monitored in a cell-cycle-dependent manner (Shirodkar et al. 1992).

CONCLUSIONS

DNA replication takes place in an ordered and programmed manner during S phase. In animal cells, the genome is divided into distinct Mb-size time zones whose replication timing is probably controlled by *trans*-acting factors and central *cis*-acting elements. Housekeeping genes generally replicate during the first half of S phase, whereas many tissue-specific genes are late-replicating, yet developmentally regulated to undergo early replication in their tissue of expression. Replication time is apparently associated with chromosome structure, but it is not yet clear whether it is a consequence of chromatin interactions or whether it actually plays a role in the regulation of gene expression. Alternate replication time structures can also serve to distinguish between two allelic regions in the same nucleus, as is observed for imprinted genes, for the X chromosome in female cells, and in cases of allelic exclusion.

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REFERENCES

Aparicio, O.M. and D.E. Gottschling. 1994. Overcoming telomeric silencing: A *trans*-activator competes to establish gene expression in a cell cycle-dependent way. *Genes Dev.* **8:** 1133–1146.

Boggs, B.A. and A.C. Chinault. 1994. Analysis of replication timing properties of human X-chromosomal loci by fluorescence in situ hybridization. *Proc. Natl. Acad. Sci.* **91:** 6083–6087.

Braunstein, D., D. Schulze, T. DelGiudice, A. Furst, and C.L. Schildkraut. 1982. The temporal order of replication of murine immunoglobulin heavy chain constant region

sequences corresponds to their linear order in the genome. *Nucleic Acids Res.* **10:** 6887–6902.

Brown, C.J. and H.F. Willard. 1994. The human X-inactivation centre is not required for maintenance of X-chromosome inactivation. *Nature* **368:** 154–156.

Brown, C.J., A. Ballabio, J.L. Rupert, R.G. Lafreniere, M. Groumpe, R. Tonlorenzi, and H.F. Willard. 1991a. A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* **349:** 38–44.

Brown, C.J., B.D. Hendrich, J.L. Rupert, R.G. Lafreniere, Y. Xing, J. Lawrence, and H.F. Willard. 1992. The human Xist gene: Analysis of 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell* **71:** 527–542.

Brown, C.J., R.G. Lafreniere, V.E. Powers, G. Sebastio, A. Ballabio, A.L. Pettigrew, D.H. Ledbetter, E. Levy, I.W. Craig, and H.F. Willard. 1991b. Localization of the X inactivation centre on the human X chromosome in Xq13. *Nature* **349:** 82–84.

Brown, E.H., M.A. Iqbal, S. Stuart, K.S. Hatton, J. Valinsky, and C.L. Schildkraut. 1987. Rate of replication of the murine immunoglobulin heavy-chain locus: Evidence that the region is part of a single replicon. *Mol. Cell. Biol.* **7:** 450–457.

Buck, L. and R. Axel. 1991. A novel multigene family may encode odorant receptors: A molecular basis for odor recognition. *Cell* **65:** 175–187.

Buiting, K., S. Saitoh, S. Gross, B. Dittrich, S. Schwartz, R.D. Nicholls, and B. Horsthemke. 1995. Inherited microdeletions in the Angelman and Prader-Willi syndromes define an imprinting centre on human chromosome 15. *Nat. Genet.* **9:** 395–400.

Calza, R.E., L.A. Eckardt, T. DelGiudice, and C.L. Schildkraut. 1984. Changes in gene position are accompanied by a change in time of replication. *Cell* **36:** 689–696.

Cattanach, B.M. 1986. Parental origin effects in mice. *J. Embryol. Exp. Morphol.* (suppl.) **97:** 137–150.

Cedar, H. 1988. DNA methylation and gene activity. *Cell* **53:** 3–4.

Chess, A., I. Simon, H. Cedar, and R. Axel. 1994. Allelic inactivation regulates olfactory receptor gene expression. *Cell* **78:** 823–834.

Disteche, C.M., E.M. Eicher, and S.A. Latt. 1979. Late replication in an X-autosome translocation in the mouse: Correlation with genetic inactivation and evidence for selective effects during embryogenesis. *Proc. Natl. Acad. Sci.* **76:** 5234–5238.

Driscoll, M.C., C.S. Dobkin, and B.P. Alter. 1989. γδβ-Thalassemia due to a de novo mutation deleting the 5′ β-globin gene activation-region hypersensitive sites. *Proc. Natl. Acad. Sci.* **86:** 7470–7474.

Drouin, R., N. Lemieux, and C.-L. Richer. 1990. Analysis of DNA replication during S phase by means of dynamic chromosome banding at high resolution. *Chromosoma* **99:** 273–280.

Eden, S. and H. Cedar. 1994. Role of DNA methylation in the regulation of transcription. *Curr. Opin. Genet. Dev.* **4:** 255–259.

Edenberg, H.J. and J.A. Huberman. 1975. Eukaryotic chromosome replication. *Annu. Rev. Genet.* **9:** 245–285.

Epner, E., W.C. Forrester, and M. Groudine. 1988. Asynchronous DNA replication within the human β-globin gene locus. *Proc. Natl. Acad. Sci.* **85:** 8081–8085.

Felsenfeld, G. 1992. Chromatin as an essential part of the transcriptional mechanism. *Nature* **355:** 219–224.

Ferguson, B.M. and W.L. Fangman. 1992. A position effect on the time of replication origin activation in yeast. *Cell* **68:** 333–339.

Ferguson, B.M., B.J. Brewer, A.E. Reynolds, and W.L. Fangman. 1991. A yeast origin of replication is activated late in S phase. *Cell* **65:** 507–515.

Forrester, W.C., E. Epner, M.C. Driscoll, T. Enver, M. Brice, T. Papayannopoulou, and M. Groudine. 1990. A deletion of the human β-globin locus activation region causes a major alteration in chromatin structure and replication across the entire β-globin locus. *Genes Dev.* **4:** 1637–1649.

Giddings, S.J., C.D. King, K.W. Harman, J.F. Flood, and L.R. Carnaghi. 1994. Allele specific inactivation of insulin 1 and 2, in the mouse yolk sac, indicates imprinting. *Nat. Genet.* **6:** 310–313.

Gilbert, D.M. 1986. Temporal order of replication of *Xenopus laevis* 5S ribosomal RNA genes in somatic cells. *Proc. Natl. Acad. Sci.* **83:** 2924–2928.

Goldman, M.A., G.P. Holmquist, L.A. Caston, and A. Nag. 1984. Replication timing of genes and middle repetitive sequences. *Science* **224:** 686–692.

Guillemot, F., T. Caspary, S.M. Tilghman, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, D.J. Anderson, A.L. Joyner, J. Rossand, and A. Nagy. 1995. Genomic imprinting of *Mas2*, a mouse gene required for trophoblast development. *Nat. Genet.* **9:** 235–242.

Guinta, D.R., J. Yun Tso, S. Narayanswami, B.A. Hamkalo, and L.J. Korn. 1986. Early replication and expression of oocyte-type 5S RNA genes in a *Xenopus* somatic cell line carrying a translocation. *Proc. Natl. Acad. Sci.* **83:** 5150–5154.

Gunaratne, P.H., M. Nakao, D.H. Ledbetter, J.S. Sutcliffe, and A.C. Chinault. 1995. Tissue-specific and allele-specific replication timing control in the imprinted human Prader-Willi syndrome region. *Genes Dev.* **9:** 808–920.

Hand, R. 1978. Eucaryotic DNA: Organization of the genome for replication. *Cell* **15:** 317–325.

Hansen, R.S., T.K. Canfield, M.M. Lamb, S.M. Gartier, and C.D. Laird. 1993. Association of fragile X. syndrome with delayed replication of the *FMR1* gene. *Cell* **73:** 1403–1409.

Hatton, K.A., V. Dhar, E.H. Brown, M.A. Iqbal, S. Stuart, V.T. Didamo, and C.L. Schildkraut. 1988. Replication program of active and inactive multigene families in mammalian cells. *Mol. Cell. Biol.* **8:** 2149–2158.

Holmquist, G.P. 1987. Role of replication time in the control of tissue-specific gene expression. *J. Hum. Genet.* **40:** 151–173.

Izumikawa, Y., K. Naritomi, and K. Hirayama. 1991. Replication asynchrony between homologs 15q11.2: Cytogenetic evidence for genomic imprinting. *Hum. Genet.* **87:** 1–5.

Jablonka, E., R. Goitein, M. Marcus, and H. Cedar. 1985. DNA hypomethylation causes an increase in DNase-I sensitivity and an advance in the time of replication of the entire inactive X chromosome. *Chromosoma* **93:** 152–156.

Karube, T. and S. Watanabe. 1988. Analysis of the chromosomal DNA replication pattern using the bromodeoxyuridine labeling method. *Cancer Res.* **48:** 219–222.

Kerem, B.-S., R. Goitein, G. Diamond, H. Cedar, and M. Marcus. 1984. Mapping of DNAase I sensitive regions of mitotic chromosomes. *Cell* **38:** 493–499.

Kitsberg, D., S. Selig, and H. Cedar. 1991. Chromosome structure and eukaryotic gene organization. *Curr. Opin. Genet. Dev.* **1:** 534–537.

Kitsberg, D., S. Selig, I. Keshet, and H. Cedar. 1993a. Replication structure of the human β-globin gene domain. *Nature* **366:** 588–590.

Kitsberg, D., S. Selig, M. Brandeis, I. Simon, I. Keshet, D.J. Driscoll, R.D. Nicholls, and H. Cedar. 1993b. Allele-specific replication timing of imprinted gene regions. *Nature*

364: 459–463.

Knoll, J.H.M., S.-D. Cheng, and M. Lalande. 1994. Allele specificity of DNA replication timing in the Angelman/Prader-Willi syndrome imprinted chromosomal region. *Nat. Genet.* **6:** 41–46.

LaSalle, J.M. and M. Lalande. 1995. Domain organization of allele-specific replication within the GABRB3 gene cluster requires a biparental 15q11-13 contribution. *Nat. Genet.* **9:** 386–394.

Latt, S.A. 1973. Microfluorometric detection of deoxyribonucleic acid replication in human metaphase chromosomes. *Proc. Natl. Acad. Sci.* **70:** 3395–3399.

Laurenson, P. and J. Rine. 1992. Silencers, silencing, and heritable transcriptional states. *Microbiol. Rev.* **56:** 543–560.

Ma, C., T.H. Leu, and J.L. Hamlin. 1990. Multiple origins of replication in the dihydrofolate reductase amplicons of a methotrexate-resistant Chinese hamster cell line. *Mol. Cell. Biol.* **10:** 1338–1346.

Mohandas, T., R.S. Sparker, and L.J. Shapiro. 1981. Reactivation of an inactive human X chromosome: Evidence for X inactivation by DNA methylation. *Science* **211:** 393–396.

Nicholls, R.D. 1993. Genomic imprinting and candidate genes in the Prader-Willi and Angelman syndromes. *Curr. Opin. Genet. Dev.* **3:** 445–456.

Razin, A. and H. Cedar. 1994. DNA methylation and genomic imprinting. *Cell* **77:** 473–476.

Reis, A., B. Dittrich, V. Greger, K. Buiting, M. Lalande, G. Gillessen-Kaesbach, M. Anvret, and B. Horsthemke. 1994. Imprinting mutations suggested by abnormal DNA methylation patterns in familial Angelman and Prader-Willi syndromes. *Am. J. Hum. Genet.* **54:** 741–747.

Reynolds, A.E., R.M. McCarroll, C.S. Newlon, and W.L. Fangman. 1989. Time of replication of ARS elements along yeast chromosome III. *Mol. Cell. Biol.* **9:** 4488–4494.

Roy, A., F. Exinger, and R. Losson. 1990. *cis-* and *trans*-acting regulatory elements of the yeast URA 3 promoter. *Mol. Cell. Biol.* **10:** 5257–5270.

Schmidt, M. and B.R. Migeon. 1990. Asynchronous replication of homologous loci on human active and inactive X chromosome. *Proc. Natl. Acad. Sci.* **87:** 3685–3689.

Selig, S., K. Okumura, D.C. Ward, and H. Cedar. 1992. Delineation of DNA replication time zones by fluorescence in situ hybridization. *EMBO J.* **11:** 1217–1225.

Selig, S., M. Ariel, R. Goitein, M. Marcus, and H. Cedar. 1988. Regulation of mouse satellite DNA replication time. *EMBO J.* **7:** 419–426.

Shafer, D.A. and J.H. Priest. 1984. Reversal of DNA methylation with 5-aza-C alters chromosome replication patterns in human lymphocyte and fibroblast cultures. *Am. J. Hum. Genet.* **36:** 534–545.

Shirodkar, S., M. Ewen, J.A. DeCaprio, J. Morgan, D.M. Livingston, and T. Chittenden. 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. *Cell* **68:** 157–166.

Somssich, I.E., J. Spira, H. Hameister, and G. Klein. 1984. Correlation between tumorigenicity and banding pattern of chromosome 15 in murine T-cell leukemia cells. *Chromosoma* **91:** 39–45.

Spack, E.G., E.D. Lewis, B. Paradowski, R.T. Schimke, and P.P. Jones 1992. Temporal order of DNA replication in the H-2 major histocompatibility complex of the mouse. *Mol. Cell. Biol.* **12:** 5174–5188.

Sutcliffe, J.S., M. Nakao, S. Christian, K.H. Orstavik, N. Tommerup, D.H. Ledbetter, and

A.L. Beaudet. 1994. Deletions of a differentially methylated CpG island at the SNRPN gene define a putative imprinting control region. *Nat. Genet.* **8:** 52–58.

Takagi, N. 1974. Differentiation of X chromosomes in early female mouse embryos. *Exp. Cell Res.* **86:** 127–135.

Takagi, N., M.A. Yoshida, O. Sugawara, and M. Sasaki. 1983. Reversal of X-inactivation in female mouse somatic cells hybridized with murine teratocarcinoma stem cells in vitro. *Cell* **34:** 1053–1062.

Torchia, B.S., L.M. Call, and B.R. Migeon. 1994. DNA replication analysis of FMR1, XIST, and factor 8C loci by FISH shows nontranscribed X-linked genes replicate late. *Am. J. Hum. Genet.* **55:** 96–104.

Vassar, R., J. Ngai, and R. Axel. 1993. Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. *Cell* **74:** 309–318.

Villar, A.J. and R.A. Pedersen. 1994. Parental imprinting of the Mas protooncogene in mouse. *Nat. Genet.* **8:** 373–379.

Vogel, W., M. Autenrieth, and G. Speit. 1986. Detection of bromodeoxyuridine-incorporation in mammalian chromosomes by a bromodeoxyuridine-antibody. I. Demonstration of replication patterns. *Hum. Genet.* **72:** 129–132.

von Meyenburg, K. and F.G. Hansen. 1987. Regulation of Chromosome Replication. In Escherichia coli *and* Salmonella typhimurium: *Cellular and molecular biology* (ed. F.C. Neidhardt), pp. 1555–1577. American Society for Microbiology, Washington, D.C.

Wahrman, J., C. Richler, E. Neufeld, and A. Friedman. 1983. The origin of multiple sex chromosomes in the gerbil *Gerbillus gerbillus* (Rodentia: Gerbillinae). *Cytogenet. Cell Genet.* **35:** 161–180.

Wevrick, R., J.A. Kerns, and U. Francke. 1994. Identification of a novel paternally expressed gene in the Prader-Willi syndrome region. *Hum. Mol. Genet.* **3:** 1877–1882.

Wolf, S. and P. Perry. 1974. Differential staining of sister chromatids and the study of sister chromatid exchanges without autoradiography. *Chromosoma* **48:** 341–353.

Wolffe, A.P. and D.D. Brown. 1988. Developmental regulation of two 5S ribosomal RNA genes. *Science* **241:** 1626–1632.

Yunis, J.J. 1981. Mid prophase human chromosomes. The attainment of 2000 bands. *Hum. Genet.* **56:** 293–298.