

Coordinated replication timing of monoallelically expressed genes along human autosomes

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A number of genes in the mammalian genome are expressed from only one of two alleles in either an imprinted or random manner. Those belonging to the random class include X-linked genes subject to X inactivation, as well as a number of autosomal genes, including odorant receptors, immunoglobulins, T-cell receptors, interleukins, natural killer-cell receptors and pheromone receptors. Monoallelically expressed genes display the unusual property of asynchronous replication and for those genes whose transcription is randomly monoallelic, the asynchronous replication is also random. In mice, recent work has shown that the random asynchronous replication of distributed autosomal genes is coordinated at the whole chromosome level, indicative of chromosome-pair non-equivalence. Here, we show that autosome-pair non-equivalence is present in human cells, and demonstrate its ability to cross the centromere. Additionally, by examining the replication of these genes in a number of human trisomies, we consistently find one allele replicating early and the other two alleles replicating late, similar to previous observations in X trisomies.

INTRODUCTION

The differential treatment of two sequence-identical alleles is a hallmark of random X inactivation. Established at the time of implantation, X inactivation represents a random choice made at the whole chromosome level, with half of the cells inactivating the paternal X and half the cells inactivating the maternal X (1). X inactivation extends its influence across the centromere. In addition, X inactivation exhibits the so-called ' $n - 1$ rule', as a single X chromosome is chosen to be active regardless of the number of other copies present (2). One of the earliest observable differences between the two X chromosomes is a difference in their respective replication timing (3). While most human genes are biallelically transcribed and have both alleles replicated synchronously during a specific portion of S-phase (4), genes on the inactive X replicate later in S-phase than their active counterparts. This asynchronous replication, initially thought of as unique to X-linked genes, has since emerged as a property shared by all monoallelically expressed genes (5–8). While asynchronous replication and monoallelic expression probably represent different manifestations of a shared epigenetic mark, one important feature of asynchronous replication is that it can be observed in all cell types. For instance, odorant receptors are only expressed in specific, post-mitotic olfactory neurons, yet the DNA encoding these genes replicates asynchronously in fibroblasts,

lymphoblasts and all other cell types examined. Similarly, genes like the X-linked opsins undergo X inactivation early in development such that the inactive allele replicates later in all differentiated cell types.

The chromosome-wide nature of X inactivation was first revealed by early cytological observations and was subsequently confirmed by molecular analyses. In mice, we have studied a number of autosomal loci and shown that their asynchronous replication is also coordinated, rendering the alleles of all the randomly monoallelically expressed genes, scattered across the chosen chromosome, earlier replicating than the alleles on the homologous chromosome (8). Here, we have asked whether this process is conserved within the mammalian lineage and extend our analyses to examine autosome-pair non-equivalence in a number of human trisomies.

RESULTS

Asynchronous replication in humans

To test whether asynchronous replication is coordinated in humans, we first demonstrated that the human homologs of several monoallelically expressed mouse genes replicate asynchronously in human cells. Asynchronous replication is established early in development before tissue-specific transcription is

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established, thus making it possible to study the phenomenon in a number of cell types in which the genes are not expressed, including fibroblasts and lymphoblasts (6,8,9). Loci can be identified as replicating either synchronously or asynchronously using a fluorescence *in situ* hybridization (FISH) assay of replication timing (10). FISH analysis of interphase nuclei pulse-labeled with BrdU allows selective examination of cells in S-phase. Using a probe to a particular chromosomal site, some cells display two single hybridization dots, indicating that neither allele has replicated (an SS pattern), while cells of a second class display two double dots, indicating that both alleles have replicated (a DD pattern). A third class has cells with one single dot and one double dot indicating replication of only one of the two alleles (an SD pattern). Asynchronously replicating genes show the SD pattern in S-phase cells 30–40% of the time; this is higher than what is observed for synchronously replicating genes, which typically present this pattern in only 10–20% of S-phase cells.

The FISH assay we use is an accurate indicator of asynchronous replication; it has been corroborated by direct measurements of asynchronous replication using a number of S-phase fractionation methods (11,12). Recently, we confirmed that this was the case for odorant receptor genes (8). Note that, while the FISH assay we use detects asynchronous replication, the fraction of cells with a visible doublet signal for a given allele may be influenced by differences in sister chromatid cohesion, especially when different FISH protocols are used (13). These different protocols, known as three-dimensional FISH, utilize substantially different cell-fixation and denaturation conditions in order to visualize cohesion. By contrast, the FISH conditions we use are designed to minimize the detection of differences in sister chromatid cohesion (see Discussion). Irrespective of the relative contributions of replication asynchrony and sister chromatid cohesion to the doublet FISH signal, it represents an interesting epigenetic mark that distinguishes between the two alleles of monoallelically expressed genes.

Using the FISH assay, we confirmed our expectation that a number of odorant receptor genes, interleukin genes and the κ immunoglobulin gene (*IGK*), as well as two X-linked genes, all replicate asynchronously in human cells (Table 1, Fig. 1A). In order to determine whether the asynchronous replication we observed for human odorant receptors was random, we obtained a nonclonal cell line that is heterozygous for a specific deletion on the same arm of chromosome 2 as the odorant receptor, *OR6B3*. This deletion served as a mark for one of the two alleles, as we performed two-color FISH using a BAC mapped within this deletion labeled with FluorX (green) in concert with a Cy3-labeled (red) *OR6B3* probe. We scored 35 cells which displayed a single–double FISH pattern, and observed 17 nuclei in which the deletion was linked to the early allele, and 18 nuclei in which the early allele of *OR6B3* resided on the intact copy of chromosome 2 (Fig. 1B). These results confirmed our expectation that the asynchronous replication of human odorant receptors is random.

Coordination of asynchronous replication in human cells

Asynchronously replicating genes are scattered throughout the human genome, with synchronously replicating genes, which comprise the bulk of genes, interspersed between them. The

Table 1. FISH analysis of a number of human genes

Probe	SD (%)
<i>IGK</i>	42
<i>IL1F9</i>	41
<i>IL5</i>	42
<i>IL12B</i>	37
<i>IL16</i>	42
<i>IL17B</i>	33
<i>OR2BH1P</i>	36
<i>OR1J4</i>	45
<i>OR2AT4</i>	41
<i>OR4F15</i>	43
<i>OR4X2</i>	41
<i>OR5AH1P</i>	38
<i>OR6B3</i>	44
<i>OR7D2</i>	37
<i>OR10A3</i>	41
<i>OR10B1P</i>	43
<i>OR13C4</i>	37
<i>PPEF1</i>	42
<i>DMD</i>	47
<i>C40</i>	17
<i>LARP</i>	22
<i>C9orf43</i>	17

Loci can be identified as replicating either synchronously or asynchronously using a FISH assay of replication timing. In the assay, asynchronously replicating genes show the single-dot double-dot (SD) pattern in S-phase cells 30–40% of the time; this is higher than what is observed for synchronously replicating genes, which typically present this pattern in only 10–20% of S-phase cells.

random choice of which allele to replicate early could be made at the level of the individual locus, or the individual chromosome (as for X-linked genes). We sought to determine the level of this choice for a number of human loci. The level of coordination of two distant genes on a particular chromosome was examined by using two-color FISH analysis and scoring cells which displayed a single dot–double dot (SD) signal for both genes (8). This type of pattern can be found if the two genes replicate in an overlapping portion of S-phase. If the two genes are coordinated, the double dots for both genes should reside on the same chromosome (either maternal or paternal) and thus will be near each other in the nucleus. If the two genes are not coordinated, then the double dots for both genes should be on the same chromosome only 50% of the time. Note that the two-color FISH assay depends on the physical proximity of two linked loci within the nucleus. When probes are greater than 50 Mb apart, the feasibility of the assay begins to diminish, as signal coming from the paternal allele of one gene may be closest to the maternal allele of the other gene. When possible, we examined the coordination of genes which were roughly 10–30 Mb apart. This distance ensures that the two genes are in different replication domains, but are close enough to display nearby FISH signals within each nucleus.

Using this two color approach, chromosome-level coordination was analyzed on four autosomes and the X chromosome for comparison. Two X-linked genes that are located 13.8 Mb apart, dystrophin (*DMD*) and a serine/threonine phosphatase, *PPEF1*, reveal coordination (31 of 35 cells; Fig. 2A). The fact that the assay did not show coordination in all 35 cells counted

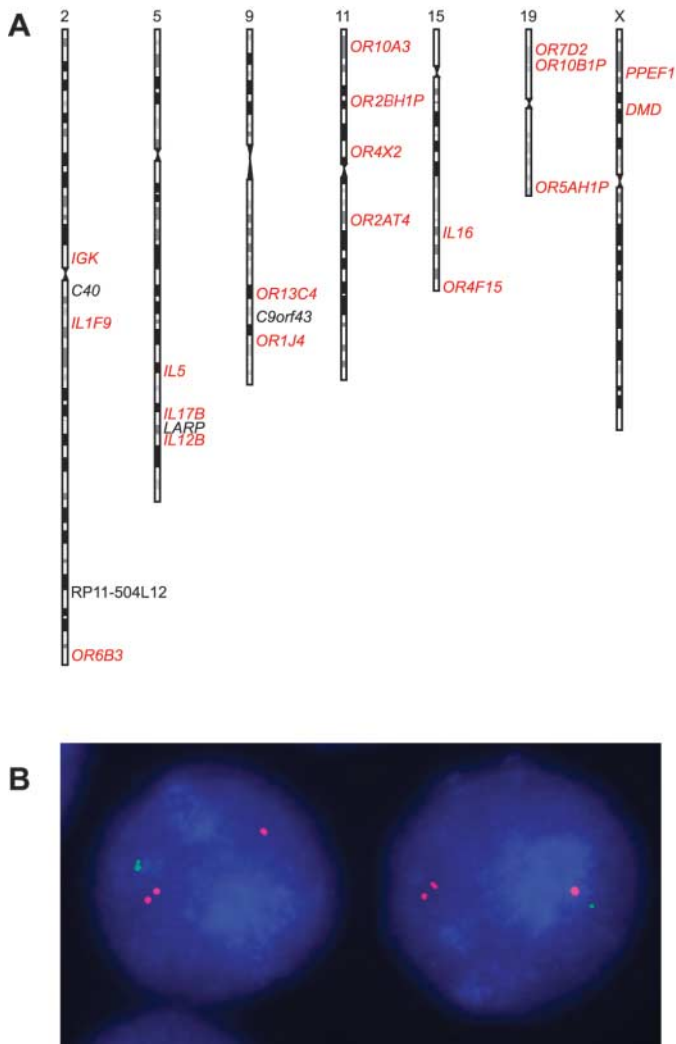


Figure 1. Examining asynchronous replication in human cells. (A) The set of genes analyzed in this study. Asynchronously replicating genes are represented in red, and synchronously replicating genes are represented in black. Synchronously replicating genes (black) located in between asynchronous loci demonstrate that the flanking asynchronous genes reside in different replication domains. The location of the BAC, RP11-504L12, is also given. (B) A probe for RP11-504L12 (green) was used to mark one of the two copies of chromosome 2 in lymphoblasts with a chromosome 2 deletion (pter>q34::q36>qter). By analyzing these cells with two-color FISH, we examined whether the asynchronous replication of the nearby *OR6B3* (red) odorant receptor was random. Two neighboring cells have replicated a different allele early. In 35 cells counted, 18 nuclei replicated the intact chromosome 2 early (as did the left cell shown here), whereas 17 first replicated the allele linked to the deletion (as did the right cell), indicating that the asynchronous replication of this odorant receptor is random.

suggests that, while the assay is robust ($P < 0.001$ for a deviation from 50% in the above example), it does not allow visualization of the coordination in all cells examined. We next analyzed *OR13C4* and *OR1J4*, two odorant receptors located 17.8 Mb apart on chromosome 9, and observed evidence of coordination (30/34 cells, $P < 0.001$; Fig. 2B). These analyses were extended to other chromosomes, between *IL17B* and *IL12B*, located in two different interleukin clusters 9.9 Mb apart on chromosome 5 (30/36 cells, $P < 0.001$; Fig. 2C), as well as between an odorant receptor, *OR4F15*, and an interleukin,

IL16, 21.5 Mb apart on chromosome 15 (28/35 cells, $P < 0.001$; Fig. 2D). Together with our earlier results in mice (8), these analyses suggest that chromosome-pair non-equivalence may be a general feature of mammalian chromosomes.

X inactivation is a chromosome-wide process. Not only do genes on both arms of the X chromosome replicate asynchronously, but the direction of this asynchrony is also fixed, such that in any given cell either all the X-linked genes replicate their maternal allele early or all replicate their paternal allele early. While the current mechanistic understanding of X inactivation helps explain why this is the case, this characteristic of X-linked genes can be observed without any assumptions as to how the process occurs. This led us to determine whether the effects of autosomal coordination can also be observed for genes on opposite sides of the centromeric boundary. If such were the case, one would expect the asynchronous replication of two genes on opposite arms of an autosome to be coordinated. While this question could not be asked in the mouse where all chromosomes are telocentric, human chromosomes have two arms of varying lengths. Although several human chromosomes possess asynchronously replicating genes on both sides of the centromere, the requirements of our assay constrained our analysis to those loci which are less than 50 Mb apart, but on opposite arms of the chromosome. We identified chromosome 2 as being ideally suited for this analysis, as it contains two genes, *IGK* and the interleukin *IL1F9*, which are located on different arms of chromosome 2, yet only 22.2 Mb apart. The synchronously replicating gene, *C40*, resides between these two genes (Table 1, Fig. 1A), indicating that *IGK* and *IL1F9* are part of different replication domains, rather than belonging to one large domain spanning the centromere. Similar to the analyses with probes on the same side of a centromere, we observed coordination in 32/39 cells ($P < 0.001$; Fig. 2E), demonstrating that coordination extends beyond the centromere and most likely reflects a chromosome-wide choice whose underlying mechanisms are not impeded by centromeric structure. In addition to these two genes on chromosome 2, we also examined two odorant receptors on opposite arms of chromosome 11, *OR4X2* and *OR2AT4*, which are located 26.3 Mb apart. These loci were also coordinated (26/32 cells, $P < 0.001$; Fig. 2F), despite their location on opposite sides of the centromere. While in the case of X inactivation, the spreading of the *XIST* RNA across the centromere is thought to mediate the coordination of silencing and replication timing differences on the two arms, the mechanism allowing the coordination of autosomal genes on opposite sides of the centromere remains to be determined.

The observation of coordination between a number of linked pairs of autosomal genes suggested that the asynchronous replication of those genes was subject to chromosome-wide coordination. However, the possibility still remained that coordination in humans was not chromosome-wide, but rather existed in large subdomains of chromosomes. In order to confirm that the coordination observed between pairs of asynchronously replicating genes scattered on human autosomes was indeed chromosome-wide, we next sought to expand our observations to other genes located on the same chromosomes. This approach was based on the understanding that, if gene A is coordinated with gene B, and gene B is coordinated with gene C, then gene A, by extension, is

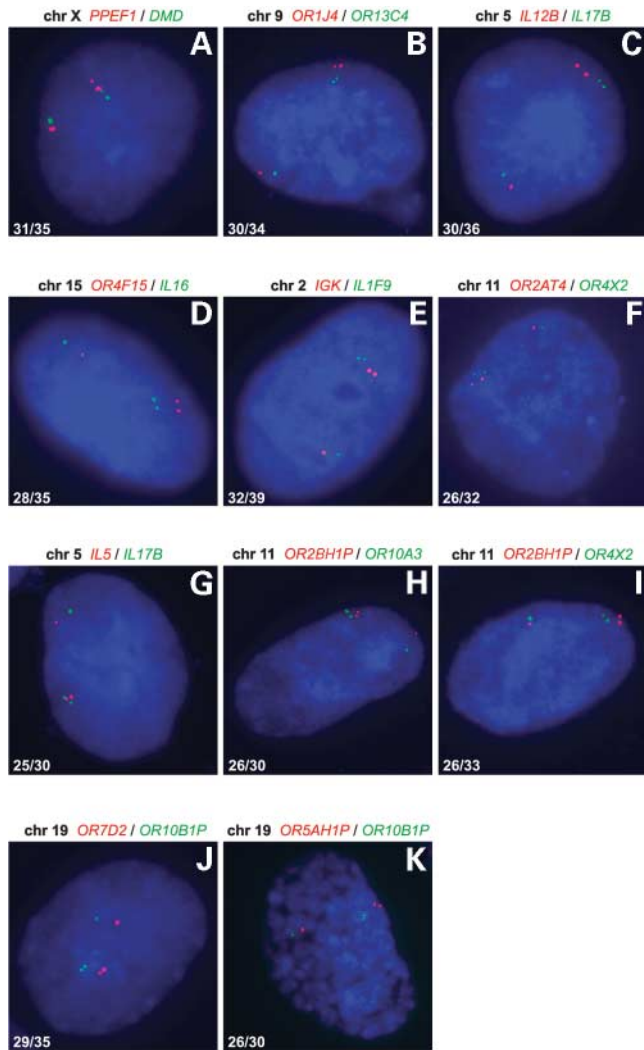


Figure 2. Coordination of asynchronous replication for individual human chromosome pairs. Two-color FISH analysis was performed on an apparently normal human 46,XX primary fibroblast population (WI-38). DAPI staining of nuclei (blue) is visible, and individual loci are visualized with 10 kb PCR products labeled with either Cy3 (red) or FluorX (green). The fraction of cells displaying the coordinated pattern is indicated for each probe in the bottom left of each panel. (A) Analysis of the X chromosome as a control. Two X-linked genes, dystrophin (*DMD*, green) and *PPEF1* (red). In 31/35 cells counted, the double-dot signals for each gene were on the same chromosome, indicating coordination of these two distant loci, consistent with uniform late replication of loci on the inactive X chromosome. (B) Two odorant receptors on chromosome 9, *OR13C4* (green) and *OR1J4* (red) show a similar coordinated pattern of replication (30/34 cells counted). (C) Two interleukins on chromosome 5, *IL17B* (green) and *IL12B* (red) (30/36 cells showed the coordinated pattern). (D) An OR and an interleukin on chromosome 15, *OR4F15* (red) and *IL16* (green) (28/35 cells showed the coordinated pattern). (E) *IGK* (red) and *IL1F9* (green), two asynchronous genes that reside on opposite arms of chromosome 2, represent the first demonstration that autosomal coordination can cross a centromere (32/39 cells counted). (F) Likewise, two odorant receptors on opposite arms of chromosome 11, *OR2AT4* (red) and *OR4X2* (green) are coordinated (26/32 cells). (G) In addition to *IL12B*, *IL17B* (green) is also coordinated with *IL5* (red) (25/30 coordinated) indicating that all three of these loci are coordinated. (H) On chromosome 11, *OR2BH1P* (red) and *OR10A3* are coordinated (26/30 cells). (I) *OR2BH1P* is also coordinated with *OR4X2* (green) (26/33 cells). (J) On chromosome 19, *OR7D2* is coordinated with the cluster containing *OR10B1P* (29/35 cells). (K) Since *OR10B1P* (green) is also coordinated with *OR5AH1P* (red) (26/30 cells), all three of these loci, covering most of chromosome 19, are coordinated.

coordinated with gene C. We examined *IL5* and *IL17B* on chromosome 5 and observed evidence of coordination (25/30 cells, $P < 0.001$; Fig. 2G). Taken together with our observations of coordination between *IL12B* and *IL17B*, these results indicate that all three of these loci, residing over 26.8 Mb of chromosome 5, are coordinated with each other. Likewise, we extended our analysis of chromosome 11 to include the coordination of *OR10A3* and *OR2BH1P* (26/30 cells, $P < 0.001$; Fig. 2H). *OR2BH1P* and *OR4X2* are also coordinated (26/33 cells, $P < 0.001$; Fig. 2I). Since *OR4X2* and *OR2AT4* are also coordinated with one another, the asynchronous replication of all four of these loci on chromosome 11 is coordinated, covering most of 11p and 66.6 Mb in total. While the distribution of randomly asynchronously replicating genes on particular autosomes limits the contiguous expanses which can be examined by our assay, we identified three asynchronously replicating odorant receptor loci on chromosome 19 for analysis: *OR7D2*, *OR10B1P* and *OR5AH1P*. These three loci on chromosome 19 extend to both arms and together cover 52.8 Mb or 83% of this chromosome. We observed coordination of *OR7D2* with *OR10B1P* (29/35 cells, $P < 0.001$; Fig. 2J) and *OR10B1P* with *OR5AH1P* (26/30 cells, $P < 0.001$; Fig. 2K). These results provide further support that random asynchronous replication is coordinated across entire chromosomes in humans.

Asynchronous replication in trisomies

Classical studies of X inactivation indicate that, regardless of the number of X chromosomes present, in otherwise diploid cells there is always one active X chromosome with every other copy becoming inactivated ('the $n - 1$ rule' of X inactivation) (2). With respect to replication timing, when there are more than two X chromosomes, one X replicates early in S-phase (the active X), while the remaining (inactive) X chromosomes replicate late. We used FISH to determine whether such a replication pattern could be observed for autosomal genes. The number of single dots in any given nucleus corresponds to the number of unreplicated alleles, whereas the number of double dots corresponds to the number of replicated alleles. Thus, if one allele replicates much earlier than the other two, a population of S-phase cells should contain more cells which exhibit a pattern with two single-dots and one double-dot (SSD) than those cells which exhibit one single-dot and two double-dots (SDD).

We studied trisomies of chromosomes 2, 9, 15 and X. Chromosome 21, while also commonly trisomic, does not provide a source of monoallelically expressed genes to use in these analyses. (All odorant receptor genes on chromosome 21 are products of recent duplications with copies present on multiple chromosomes, and no other monoallelically expressed genes are present on chromosome 21.) Since the number of X chromosomes inactivated in a cell is sensitive to the complement of other chromosomes present, trisomic cell lines were carefully selected whose only apparent aberration was the addition of a complete, extra copy of one chromosome. The first cell line examined was a primary fibroblast line trisomic for chromosome 2 but otherwise diploid. The *IGK* locus, located at 2p11.2, was analyzed, revealing four patterns of BrdU-positive cells (Fig. 3A, Table 2). Nuclei with either three

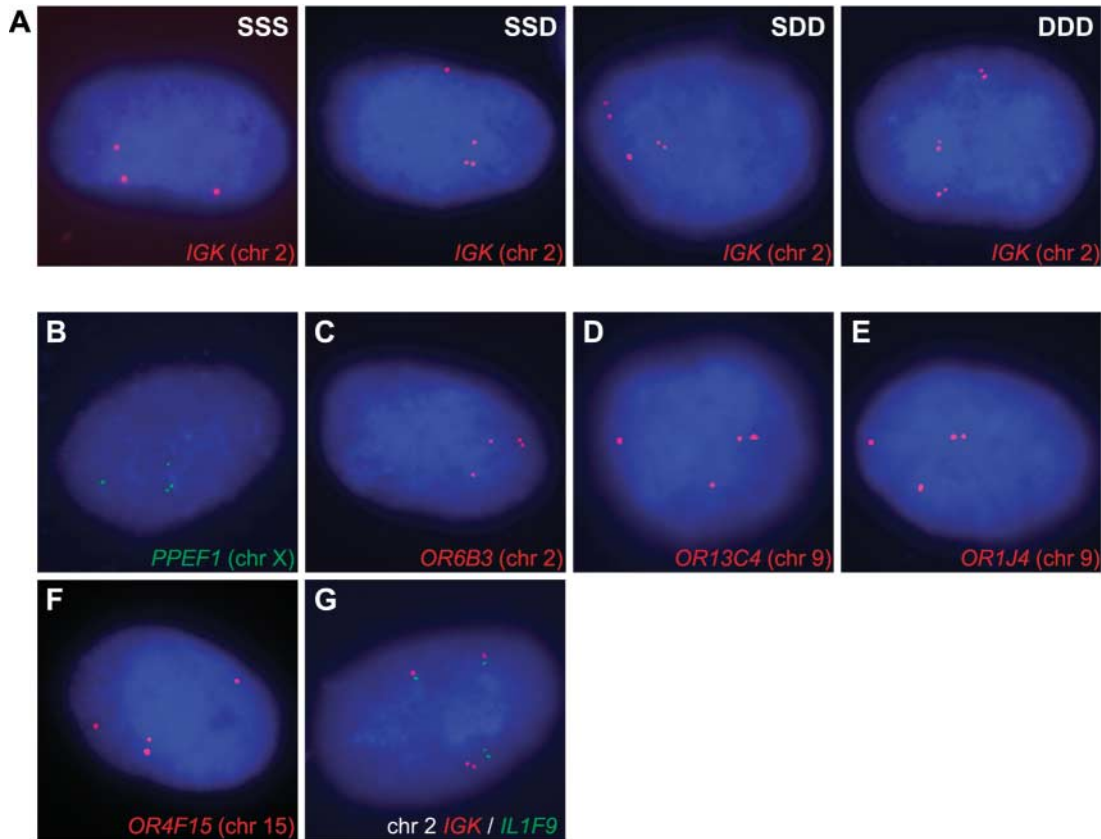


Figure 3. FISH analysis of trisomic human cell lines. (A–F) Examples of FISH analysis for six asynchronously replicating genes, in the context of four trisomic human cell lines. When three alleles of an asynchronously replicating gene are present, two intermediate points in S-phase are observable, the SSD pattern, in which one allele has replicated, and the SDD pattern, in which two (of the three) alleles have replicated. (A) The four cells shown represent the four types of cells observed examining the *IGK* gene (red) in a cell line trisomic for chromosome 2. *IGK* replicates one allele early and two late as evidenced by the large excess of SSD when compared with SDD nuclei. (B) The gene dystrophin (green), in an XXX cell line also replicates one allele early and two late, as expected based on the ‘ $n - 1$ rule’ of X inactivation. (C) A similar pattern was observed for an odorant receptor, *OR6B3* on the distal end of chromosome 2. Likewise, one allele replicates early and two replicate late for two odorant receptors on chromosome 9, (D) *OR13C4* and (E) *OR1J4*, as well as an odorant receptor on chromosome 15, (F) *OR4F15*. (G) Two-color FISH shows coordination between *IGK* (red) and *IL1F9* (green) in trisomy 2 cells; in cells that were SSD for both genes, 17/22 show coordination, with the double-dot signal for each gene on the same chromosome. Thus, the choice of one early allele is a chromosome-wide decision.

Table 2. An autosomal equivalent of the ‘ $n - 1$ rule’ of X inactivation

Probe (trisomy)	SSS (%)	SSD (%)	SDD (%)	DDD (%)
<i>PPEF1</i> (chromosome X)	45	41	11	3
<i>IGK</i> (chromosome 2)	36	42	9	13
<i>OR6B3</i> (chromosome 2)	48.5	36	9	6.5
<i>OR13C4</i> (chromosome 9)	44	38	9	9
<i>OR1J4</i> (chromosome 9)	44	38	10	8
<i>OR4F15</i> (chromosome 15)	37	37	10	16
<i>C40</i> (chromosome 2)	42	19	17	22

When three alleles of an asynchronously replicating gene are present, one allele replicates early in S-phase and two replicate late, as evidenced by the large excess of SSD when compared with SDD nuclei. The X-linked loci *PPEF1* and a synchronously replicating gene, *C40*, were also examined for comparison. At least 100 BrdU-positive cells were counted for each percentage given.

single-dots (SSS) or three double-dots (DDD), respectively, represented cells in which either none or all of the alleles of *IGK* had replicated. In addition, two intermediate replication states, in which either only one (SSD) or two (SDD) alleles had replicated were also present within the population of cells

progressing through S-phase. These cells were highly enriched for the SSD pattern, suggesting that, in the trisomic state, one allele of *IGK* replicates early and two replicate late. These results were almost indistinguishable from our analysis of an X-linked gene, dystrophin (*DMD*) in an XXX cell line. As expected, the SSD pattern represented the predominant intermediate in S-phase, indicating that in these cells, one X chromosome replicated early and two alleles replicated late (Fig. 3B, Table 2). A control analysis of a synchronously replicating gene, *C40*, revealed that the two intermediate classes of cells (SSD and SDD) were equally represented in S-phase (Table 2). We next sought to determine how widespread this phenomenon was amongst the randomly asynchronously replicating autosomal genes.

When the analyses of the trisomy 2 cells used to study *IGK* were extended to an additional locus, an odorant receptor located at 2q37.3 (*OR6B3*), many more SSD than SDD cells were again observed, suggesting that this odorant receptor also replicates one allele early and two alleles late (Fig. 3C, Table 2). The SSD pattern also predominated for two chromosome 9 odorant receptor clusters (*OR1J4* and *OR13C4*) in

trisomy 9 cells (Fig. 3D and E, Table 2), as well as an odorant receptor (*OR4F15*) in the context of a chromosome 15 trisomy (Fig. 3F, Table 2). Thus, when autosomal asynchronously replicating genes are present in the trisomic state, one allele replicates relatively early in S-phase, and the other two alleles replicate late, similar to what is observed for X-linked genes. Note that, in the case of the X chromosome, these observations can be extended to XXXX, XXXXX and XXXXXX individuals, which led to the formulation of the ' $n - 1$ rule', yet no analogous autosomal aneuploidies exist which would allow for such an extension to the autosomes.

To address the issue of chromosome-wide coordination in the trisomic state, we used two-color FISH to simultaneously explore the replication status of two discrete trisomic loci. Examining the *IGK* locus and *IL1F9* in the context of the previously described chromosome 2 trisomy, we asked if the same chromosome was early replicating for both genes. In 17/22 SSD cells examined ($P < 0.001$), *IGK* and *IL1F9* replicated the same allele first, suggesting that the choice of which allele replicates early is a well-regulated, coordinated choice. This suggests that the choice of only one early allele in the trisomic state is a chromosome-wide choice, with cells selecting one early chromosome such that asynchronously replicating genes on the remaining homologous chromosomes replicate later in S-phase.

DISCUSSION

Our previous studies of autosomes in mice have shown that random asynchronous replication is coordinated at the level of chromosomes, suggesting chromosome-pair non-equivalence for the autosomes. Genes subject to this coordination include the odorant receptors, immunoglobulins, T-cell receptors, pheromone receptors and interleukins. The regulation of individual members of these families is critical in specifying the identity of the distinct cells within a tissue type. In this current investigation, evidence has been provided that random asynchronous replication is coordinated in human cells. In addition, by examining human chromosomes, coordination has been observed between genes on opposite sides of the centromere, further supporting the idea of a chromosome-wide phenomenon. Additionally, based on the analysis of several different human trisomies, the data demonstrate that autosome-pair non-equivalence must provide a means by which only one copy of each chromosome replicates its asynchronously replicating genes early in S-phase. For the X chromosome, an unknown mechanism prevents stable *XIST* expression (and subsequent inactivation) on only one chromosome, regardless of the number of X chromosomes present.

In this investigation, we have used a fluorescence *in situ* hybridization assay to examine the random asynchronous replication of a number of human genes. In the FISH assay of replication timing, a single dot is interpreted as an unreplicated locus whereas a double dot is interpreted as DNA which has replicated. However, in order for a replicated segment of DNA to appear as a double dot in the nucleus, not only must the locus replicate, but the two pieces of DNA must also separate from one another sufficiently to give two FISH signals. For this reason, it has long been proposed that differences in sister

chromatid cohesion might affect the assay's ability to reliably measure replication timing, despite corroboration of the assay with direct measurements of DNA replication by this lab and others (8,11,12).

Recent work by Azuara *et al.* (13) has demonstrated that sister chromatid cohesion can be observed through fluorescence *in situ* hybridization methods, known as three-dimensional FISH; however the conditions under which such detection is done should not be confused with the methods utilized here and by others in the field to measure replication timing. Specifically, under three-dimensional FISH, cells are subjected to fixation conditions (paraformaldehyde) designed to optimize the preservation of nuclear proteins and architecture. The preservation of nuclear structure, while typically a desirable aim, is likely to interfere with the measurement of replication timing due precisely to the architecture it maintains. Such an interpretation is supported by the observation by Azuara *et al.* (13) that the FISH-based assay of replication timing not only gives different results than three-dimensional FISH, but it also more closely reflects results they obtained from direct measurements of replication timing. Thus, the most precise measurements of DNA replication using fluorescence *in situ* hybridization are likely to be made under conditions in which the minimal amount of structure is maintained that might interfere with the separation of replicated sister chromatids.

The coordination of random asynchronous replication along human autosomes suggests the intriguing possibility that chromosome-pair non-equivalence, rather than being limited to X inactivation, could be a fundamental property of mammalian chromosomes. The autosomal genes affected by this phenomenon belong to a number of different gene families, each of which probably makes use of asynchronous replication (or the underlying epigenetic mark it reflects) in the complex gene regulation that characterizes these families. For instance, in the case of the immunoglobulin genes, it has been shown that the early replicating allele is preferentially rearranged (11). While these genes all depend on monoallelic expression for their proper function, it is difficult to understand the reasons behind any sort of chromosome-wide process related to this expression. Indeed, many of the genes belonging to these families do not share overlapping patterns of expression. Similarly, many of the X-linked genes which are inactivated along one of two chromosomes in females are expressed in different tissues. The functional relevance of this inactivation is that only one of two alleles is expressed, not that the different genes subject to such inactivation are silenced in a chromosome-wide manner. The chromosome-wide nature of X inactivation can be regarded as a consequence of the mechanism behind mammalian dosage compensation rather than a requirement of the latter. Likewise, the chromosome-wide nature of autosomal 'inactivation' may merely reflect the mechanisms utilized by this system to arrive at random monoallelic expression rather than some underlying requirement for chromosome-wide regulation.

It is interesting to consider whether X inactivation and autosome-pair non-equivalence might have arisen from a common ancestral process which rendered the two copies of a chromosome pair different from one another. Perhaps X inactivation is an adaptation of general chromosome-pair non-equivalence, with much more extensive events occurring further downstream in the development of an inactive X than in

the creation of a coordinated autosome pair. Consistent with this notion is recent work which suggests that the non-random distribution of LINE-1 elements hypothesized to play a role in X inactivation (14) may also extend to monoallelically expressed genes on the autosomes (15). Despite an increasingly robust understanding of the manifestations of X-chromosome inactivation, many of its earliest events remain unknown. It is possible that X inactivation and autosome-pair non-equivalence take advantage of similar mechanisms to achieve similar ends; that a series of epigenetic modifications result in the differential treatment of two chromosomes which at one point in development were equal.

MATERIALS AND METHODS

Cell culture

The primary human fibroblast cell line, WI-38 (American Type Culture Collection), was used to determine whether loci were either asynchronously replicating or synchronously replicating as well as in subsequent coordination analyses. For the trisomic studies, early passage, primary fibroblasts, GM04626 (47,XXX), GM10401 (47,XX,+2), GM09286 (47,XY,+9) and GM03184 (47,XY,+15) were purchased from the NIGMS Human Genetic Cell Repository (Corriell), as were lymphoblasts, GM10918, with a specific chromosome 2 deletion [46,XX,del(2)(pter>q34::q36>qter)] near *OR6B3*. Cells were maintained under standard conditions, fed 24 h prior to harvest, and were pulse-labeled with BrdU for 35–45 min prior to fixation in 3:1 methanol–acetic acid as previously described (8).

FISH

FISH analysis was performed as previously described (8), with an adaptation of using large PCR products as probes. BACs were obtained from BAC PAC Resources and served as templates in the following 10 kb long-range PCRs: the constant region of *IGK* (RP11–344F17), *IL1F9* (RP11–261F13), *OR6B3* (RP11–98P19), *IL17B* (RP11–92I17), *IL12B* (RP11–117N12), *OR13C4* (RP11–317C20), *OR1J4* (RP11–345A24), *IL16* (RP11–350O20), *OR4F15* (RP11–259N2), *IL5* (RP11–17K19), *OR10A3* (RP11–1105A14), *OR2BH1P* (RP11–62M5), *OR4X2* (RP11–111N23), *OR2AT4* (RP11–158C6), *OR7D2* (RP11–1114G15), *OR10B1P* (RP11–1109J16), *OR5AH1P* (RP11–381F14), *PPEF1* (RP11–42E12), *DMD* (RP11–318G17) and *C9ORF43* (RP11–10I9). Primers supplied by Integrated DNA Technologies (sequences available upon request) were designed to flank each gene's coding region (except the *IL5* probe, which used a PCR product from 9 kb upstream of the interleukin's coding region), producing a 9000–11 000 bp product using the Advantage 2 PCR system (BD Biosciences Clontech). Products were purified using the Wizard PCR Preps DNA Purification System (Promega). Aliquots of 5 μ l (one-tenth of the PCR reaction) were direct-labeled with either Cy3-dCTP or FluorX-dCTP using a Nick Translation kit according to the manufacturer's instructions (Amersham Biosciences). Labeled probes were purified using G-50 Sephadex columns (Roche) and precipitated with 30 mg human cot-1 DNA and 70 mg salmon sperm DNA (Invitrogen), washed in 75% ethanol

followed by 100% ethanol and were resuspended in 100 μ l hybridization buffer (50% formamide, 10% dextran sulfate, 1 \times SSC). A 10 μ l aliquot of each probe was prehybridized (90°C for 5 min, followed by 10 min at 37°C) and then hybridized overnight with cells dropped on poly-L-lysine slides. Subsequent washes and antibody detection of BrdU were also as previously described. As mentioned in the results, under these conditions, the FISH assay has been shown to corroborate with direct measurements of asynchronous replication using a number of S-phase fractionation methods (11,12) and recently we confirmed this was the case for odorant receptor genes in mice (8). In this study, the FISH assay was used because the application of S-phase fractionation methods to the study of randomly asynchronously replicating genes in human cells is complicated by a number of factors. Unlike imprinted genes, the study of randomly asynchronously replicating genes requires the generation of a clonal population of cells, yet human EBV-transformed lymphoblasts are much more difficult to subclone and subsequently maintain in culture than Abelson Murine Leukemia virus transformed mouse lymphocytes. Moreover, even S-phase fractionation studies of imprinted genes (whose study does not require the generation of clonal cell lines) have not been as robust as similar studies of mouse genes; this is probably due to differences in S-phase fractionation accuracy.

P-values

P-values were calculated based on a binomial probability distribution.

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