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Factors affecting the timing and imprinting of replication on a mammalian chromosome

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SUMMARY

Fluorescence in situ hybridisation has been used to follow replication of the short arm of human chromosome 11 using chromosome anomalies to distinguish the maternally- and paternally-derived homologues. The temporal difference in replication timing within and between chromosomes has been estimated by combining S phase detection with dual colour fluorescence in situ hybridisation. Proximal regions of 11p, including the *WT1* gene, tend to replicate earlier on the maternally-derived chromosome than on the paternally-derived homologue. More distal parts of 11p (including the *IGF2* gene) have the opposite imprint. The average difference in replication timing between homologous loci in the population of cells is small compared to the differences between loci along a single chromosome. The imprint is not strictly adhered to since many nuclei have hybridisation patterns opposite to the trend within the population. The nature of the imprinting signal has been investigated. Absolute replication time, but not the imprint, was affected by azacytidine, an inhibitor of DNA methylation. The replication imprint was modified by treatments that inhibit histone deacetylation. We suggest that replication imprinting reflects differences in chromatin structure between homologues.

Key words: fluorescence in situ hybridisation, histone acetylation, human chromosome 11, imprinting, replication

INTRODUCTION

Fluorescence in situ hybridisation (FISH) can efficiently detect chromosomal DNA sequences. Before chromatid replication, FISH signals usually appear as single dots. After replication, in G_2 nuclei and metaphase chromosomes doubled dots are produced. Hence it was reasoned (Selig et al., 1992) that FISH could assay DNA replication. Whilst we show that singlet and doublet FISH signals are not always indicative of unreplicated and replicated loci, respectively, results obtained with FISH generally appear to agree with those obtained by other methods (Kitsberg et al., 1993b).

The advantage of a FISH-based replication assay is that large chromosomal regions can be analysed within the context of individual nuclei. In this way, Kitsberg et al. (1993a) found that several regions of the human and mouse genomes appeared to show asynchronous replication between homologues, influenced by the parental origin of the chromosomes - a form of imprinting. The paradigm for asynchronous replication of homologues is the late replicating inactive X of mammals (Taylor, 1960). Replication banding of autosomes does not consistently reveal gross differences between the replication of other homologues (Lemieux et al., 1990). However, direct cytogenetic observation and FISH analysis of human chromosomes 15 (Izumikawa et al., 1991; Knoll et al., 1994) have detected regions of asynchronous replication that are parent of origin dependent, though mosaicism of this imprint was also found.

The Prader-Willi and Angelmann syndromes (PWS/AS) show that there are functional differences between chromosome 15 homologues. The other region of the human genome where imprinting has been implicated in genetic disorders is the short arm of chromosome 11. Familial Beckwith-Wiedemann syndrome (BWS), a somatic overgrowth syndrome with predisposition to mesodermally-derived embryonal neoplasms including Wilms' tumour, is linked to 11p15.5 - the location of the *IGF2* and *H19* genes that are known to be transcriptionally imprinted. BWS-associated duplications and uniparental disomies involve the paternally-derived chromosome whereas balanced chromosome anomalies are maternally transmitted from normal mothers (Mannens et al., 1994) and those with breakpoints distant from *IGF2* may alter the imprint of this region. More proximally (at 11p13) the *WT1* gene is involved in Wilms' tumour and the WAGR (Wilms', anirida, genitourinary anomalies and mental retardation) syndrome (Hastie, 1993). *WT1* expression may be imprinted in some instances (Jinno et al., 1994). Additionally, maternal allele loss at 11p15 is seen in some Wilms' tumours and loss of *IGF2* and *H19* imprinting has been reported in some Wilms' tumours and in BWS (Ogawa et al., 1993; Rainer et al., 1993; Weksberg et al., 1993).

We have examined replication timing of human 11p13-p15 using WAGR-associated chromosome anomalies to assign parental origin to FISH signals in both cultured cell lines and peripheral blood lymphocytes. We do observe imprinted effects on replication, with a transition from a proximal region (11p12-p13) where the maternally-derived chromosome tends to replicate before the paternally-derived one, to a distal region

(11p14-p15.5) where this situation is reversed. For the first time, we have combined FISH analysis at multiple loci with simultaneous detection of S phase nuclei, so that we can estimate the average differences in replication timing between homologous loci and between different loci along the same chromosome. The overall imprint we measure is not very large or strictly adhered to; however, stringent statistical examination indicates it is significant. To understand the mechanism that imposes these differences we investigate whether the replication imprint can be modulated by agents that affect DNA methylation or histone acetylation.

MATERIALS AND METHODS

Cell culture and preparation of nuclei

Most chromosome deletions have been described previously (Fantes et al., 1995). Peripheral blood lymphocytes were obtained from VAGA and her daughter JUEV, stimulated with phytohaemaglutinin and sampled at 64 hours. Lymphoblastoid cell lines were grown in RPMI, 10% foetal calf serum and ascertained as mycoplasma free. Cells were fed every two days to maintain asynchrony.

To identify S phase cells 10^{-4} M bromodeoxyuridine (BrdU) was added 90 minutes before harvest. Washed cells were swollen in hypotonic in the presence of 0.1 μg/ml colcemid, to help flatten the nuclei so that FISH signals could be collected without too much adjustment of focus. Nuclei were fixed in 3:1 methanol:acetic acid and stored in the dark at −20°C. The proportion of nuclei in S was assessed with anti-BrdU-FITC.

For topoisomerase II inhibition cells were treated with 40 μm amsacrine, or etoposide (in DMSO) for 3 hours before adding BrdU. Control cells had an equivalent level of DMSO added. To examine the effects of chromatin modification, cells were treated for 16 hours with either 10^{-8} M 5-azacytidine, 1 mM sodium butyrate or 33 nM Trichostatin A prior to BrdU addition.

Cosmid probes for chromosome 11

The cosmids used are entered in GDB and have been described previously (Fantes et al., 1995). They are listed here with their corresponding locus number or gene name: c65-6/13 (D11S104), c1-11-464 (D11S676), c1-11-458 (D11S458), c20/18 (D11S377), cH11148 (D11S2134), cB2.1 (*WT1*), cp60 (D11S324), cA08102 (D11S323) cFAT5 (*PAX6*), cA04160 (D11S317), cH0229 (D11S21) and cE048 (TPH). cINS/IGF2 (a gift from M. Mannens, Amsterdam) contains the *INS* and *TH* genes and the 5′ end of *IGF2* up to exon 9. Cosmid DNAs were nick translated with biotin-16-dUTP or digoxigenin-11-dUTP. 50 ng of each cosmid were applied per slide together with 3 μg of human CotI DNA (GibcoBRL).

FISH

FISH was as described by Fantes et al. (1995). After hybridisation the washed slides were incubated, for 30 minutes each at 37°C with: anti-digoxigenin-FITC (made in sheep) and avidin-Texas Red, anti-sheep-FITC (rabbit), biotinylated anti-avidin (goat) together with anti-BrdU (mouse, BCL) and finally with avidin-Texas Red and anti-mouse-AMCA. Unless otherwise stated, all antibodies were from VectorDCS. Between each incubation the slides were washed in 4× SSC, 0.1% Tween-20 for 3× 2 minutes. The slides were mounted in Vectashield, without a DNA counterstain.

Slide scoring

Slides were examined with an oil ×100 objective on a Zeiss Axioplan fluorescence microscope fitted with a Pinkel #1 filter set and automated filter wheel changer, or on a Zeiss Axioskop with a standard Zeiss filter set for DAPI, FITC and Texas Red emissions. Results obtained on either system, from the same slide, were similar. We considered it important to eliminate scoring and ascertainment bias as much as possible. Slides were scanned using AMCA fluorescence to systematically identify S phase nuclei. As each S phase nucleus was encountered the signal from the non-deleted locus was recorded, only then was the signal from the probe deleted on one homologue examined to assign parental origin to the signals. FISH patterns in >200 consecutive S phase nuclei were scored for each slide. Data were acquired by one of us (W.A.B.) and subject to statistical analysis by A.D.C. without prior knowledge of the experimental details for each dataset (Carothers and Bickmore, 1995). Selected images were captured with a Photometrics CCD camera coupled to Digital Scientific (Cambridge) software. To correlate nuclear area with FISH signal, slides were examined on a Zeiss AxioHome and the nuclear area calculated with Immediate Morphometry software.

RESULTS

Parent of origin of 11p chromosome deletions

The chromosome deletions centre around 11p13 since they derive from individuals with either WAGR syndrome or isolated cases of Wilms' tumour or aniridia. As the behaviour of DNA in the immediate vicinity of breakpoints may be affected by changes in chromosome context, we used nested sets of deletions that extend from the centromere proximal part of 11p13 through to p14, and that range in size from 0.5-7 Mb

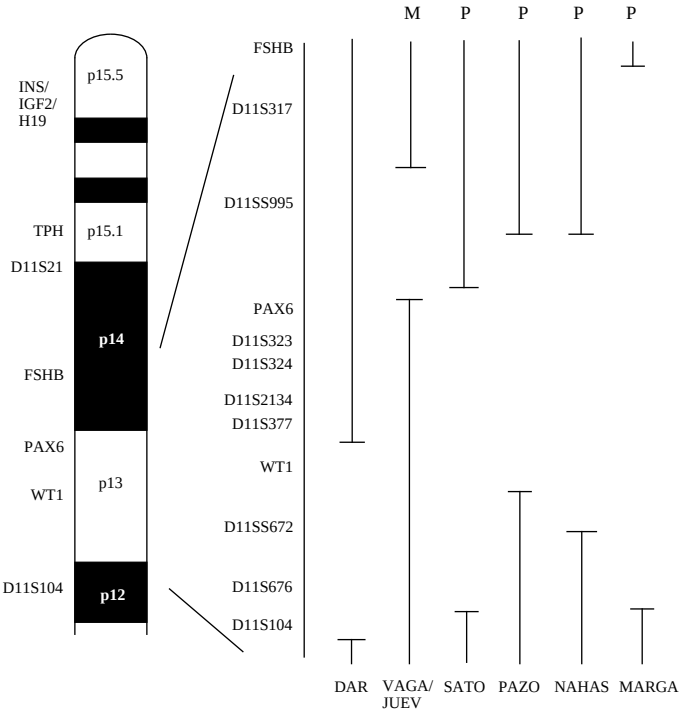


Fig. 1. Extent and parental origin of 11p deletions. G band ideogram (R bands white, G bands black) of distal human chromosome 11p is shown on the left with the location of key genes and loci used. The expanded segment shows the physical order of other loci whose replication has also been studied. On the right, the extent and parent of origin (M, maternal; P, paternal) of deletions is shown. The origin of DAR could not be established as no parental DNA was available.

(Fantes et al., 1995). The parental origin of the deletions was established by genotyping deletion individuals and their normal parents with microsatellites within and outside of the deletions. In accordance with other studies de novo deletions had arisen on the paternally derived chromosome (Huff et al., 1990). The exception was VAGA who had a maternal aniridia-associated deletion which she has passed on to her daughter JUEV. The origin of the DAR deletion could not be determined (Fig. 1).

S phase FISH patterns on each homologue

We used fluorescence-based detection of S phase (BrdU-positive) nuclei simultaneously with FISH from two loci, one present on both homologues and the other deleted from one chromosome. Since the biotin and digoxigenin-labelled cosmids were detected with Texas Red or FITC conjugates, respectively, and BrdU incorporation was assayed with AMCA, there was no fluorescence channel available for a DNA counterstain. Hence, nuclei not in S were detected only by their FISH signals (Fig. 2).

Slide scoring was done systematically and in such a way as to avoid bias in the assignment of chromosomal origin to signals. FISH patterns in nuclei at all stages of S from asynchronous cultures were scored, so the frequency of each pattern should reflect the time of replication. We have used statistical models to estimate the difference in replication timing between homologues at various loci, as well as between loci on the same chromosome (Carothers and Bickmore, 1995). Replication of a DNA sequence in asynchronous cultures is represented by a sigmoid-type response curve, with replication of the sequence occurring at time T in most cells, and earlier or later than this time in a small proportion of cells. The curve is represented by:

$$Pr\,(T \leq t) = \frac{1}{1 + e^{-4\alpha(t-\beta)}}$$

A standard model was fitted in which all loci were considered to complete their replication across the population of nuclei at the same rate (α). The free parameters were the replication time of each locus relative to the duration of S (β), where a low value (approaching 0) represents early replication, and a value approaching 1 late replication. For most datasets the goodness-of-fit of this model was close to the best possible. In a few cases a better fit was obtained where the rate of replication of each locus was allowed to vary, however, this did not affect the conclusions about β.

Using two probes, one of which is hemizygously deleted, there are eight possible combinations of S phase FISH signal. These are shown in Fig. 3, where replication at *INS*/*IGF2* (green) and *PAX6* (red) has been examined in the line PAZO with a paternally-derived deletion encompassing *PAX6*. The predominant hybridisation patterns are those where: no duplication is seen (35% of S phase nuclei - Fig. 3A), where only the paternal copy of *INS*/*IGF2* is duplicated (12% - Fig. 3B), where both copies of *INS*/*IGF2* are duplicated (13% - Fig. 3C) and finally, where all loci assayed are duplicated (13% - Fig. 3D). Nuclei with the remaining four patterns of signal are seen at a lower frequency (Fig. 3E-H). The relative proportions of each pattern suggested that the paternal copy of *INS*/*IGF2* replicates before the maternal copy. Statistical analysis (Table 1) confirms this, indicating that the paternal *INS*/*IGF2* genes replicate on average 0.07 of S before the maternally derived copies. On the maternal homologue the *INS*/*IGF2* genes are replicated almost 0.10 of S before *PAX6* (Table 1). The progression of nuclei through S is accompanied by qualitative changes in BrdU staining from an early diffuse pattern (Fig. 3A,B) to the more punctate patterns characteristic of the latter stages of S (Fig. 3C,D), and also by an increase in nuclear size. The area of BrdU +ve nuclei, with singlet, asynchronously duplicated, and fully duplicated FISH signals at *INS*/*IGF2* was measured. Additionally, where through autofluorescence the outline of G_1 and G_2 nuclei were visible their areas were also estimated though we cannot be sure that an unbiased set of these nuclei were selected. Although within each class there is a broad spread of values, the mean and modal nuclear area (and presumably nuclear volume) increased as cells pass from G_1 to S phase FISH patterns and continue to increase through progressive stages of replication (Fig. 4) so that by the time both copies of *INS*/*IGF2* are duplicated nuclei are threefold larger in area (mean nuclear area ($\bar{x}$) = 495 μm^2) than those in G_1 ($\bar{x}$ = 163 μm^2). G_2 nuclei were smaller than those in late S, reflecting increasing levels of chromatin condensation as cells approach mitosis. This analysis is a further validation of the use of FISH to assay DNA replication.

Replication at six other loci were examined in PAZO. All 11p14 and p15 loci studied had a tendency for the paternal locus to be earlier replicating than the maternally-derived one. For more proximal loci in 11p12 and p13 the situation was

Table 1. Replication analysis of PAZO nuclei

Locus	No. nuclei	S phase nuclei with doublets at M	P	D (*PAX6*)	βM	βP	βD	βM-βP	βM-βD
INS/*IGF2*	314	124 (39%)	142 (46%)	100 (31%)	0.629	0.559	0.726	+ 0.070 (±0.040)	–0.097* (±0.041)
TPH	229	137 (59%)	142 (62%)	86 (37%)	0.397	0.372	0.645	+ 0.025 (±0.041)	–0.249*** (±0.043)
D11S21	359	188 (53%)	206 (57%)	173 (48%)-*WT1*	0.474	0.407	0.528	+ 0.067 (±0.043)	–0.055 (±0.043)
D11S317	332	99 (31%)	126 (38%)	155 (47%)	0.758	0.650	0.540	+ 0.108* (±0.043)	+ 0.218*** (±0.044)
D11S672	327	210 (65%)	198 (61%)	119 (37%)	0.309	0.360	0.685	–0.051 (±0.047)	–0.376*** (±0.054)
D11S676	272	191 (71%)	173 (64%)	109 (40%)	0.251	0.337	0.623	–0.086 (±0.045)	–0.373*** (±0.050)
D11S104	320	101 (32%)	79 (25%)	123 (39%)	0.738	0.837	0.646	–0.100* (±0.046)	+ 0.091* (±0.044)

The number and (percentage) of S phase nuclei duplicated at M (maternal copy of the indicated locus), P (paternal), and D (the locus deleted on the paternal 11) are shown. D was PAX6 except where D11S21 was examined, where D was WT1. Statistical analysis of the datasets is shown. βM, βP and βD are the relative replication times of M, P and D, respectively, across the population of cells (0 and 1 are replication at the start and end of S, respectively). The difference in replication timing between homologous loci is βM-βP (standard error in brackets). A negative integer is earlier replication of the maternal locus, a positive value paternal-early replication. The difference in timing between M and D on the normal chromosome is βM-βD. Asterisks indicate P values: none, P>0.05; *P=0.01-0.05; **P=0.001-0.01; ***P<0.001.

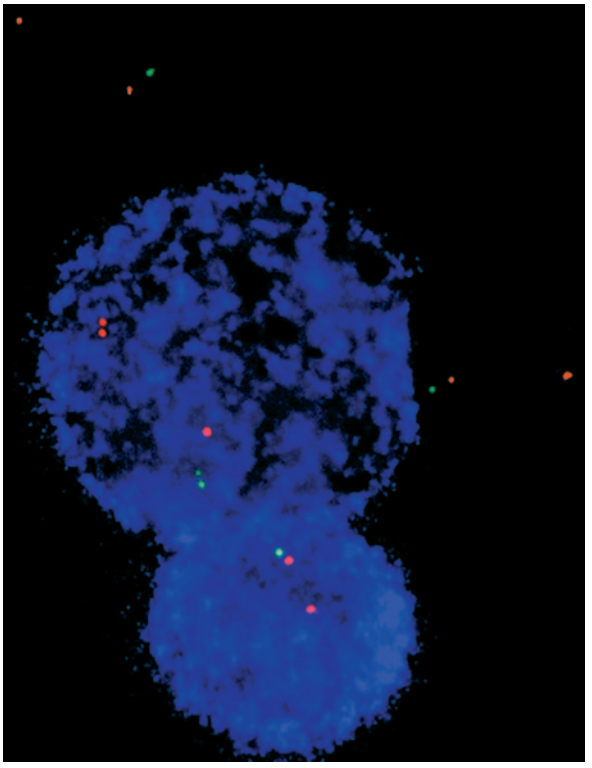


Fig. 2. Detection of S phase nuclei with multicolour FISH. Four nuclei from an asynchronous SATO culture are shown. The central and bottom nuclei are in S phase as evidenced by their AMCA fluorescence (blue). FISH was with biotinylated *INS*/*IGF2* (red) and digoxigenin-labelled *PAX6* (green) that is deleted from the paternally-derived chromosome. The G_1 nuclei at the top and right are BrdU −ve and only their FISH signals can be seen. The bottom S phase nucleus has not replicated any of the *INS*/*IGF2* or *PAX6* copies. The larger central nucleus has punctate BrdU staining characteristic of later stages of S and has replicated the sole (maternal) copy of *PAX6* (doublet green signal) and the paternal copy of *INS*/*IGF2* (solitary red doublet) but not the maternal copy of *INS*/*IGF2* (singlet red signal in close proximity to the *PAX* signal).

reversed, with the maternally derived loci being replicated earlier. Within this one cell line the differences in relative replication time between homologues were small compared to the standard error calculated on this estimate (Table 1 and Fig. 5). Hence, no statistical confidence could be placed on any apparent replication imprint by examining loci in the one cell line. Therefore we examined these and additional 11p loci in five other independent cell lines (Fig. 1). The collated data from all six lines now reveal statistically meaningful differences in the average replication time of loci on homologous chromosomes (Fig. 6). From 11p14-p15.5 the paternal chromosome 11 is replicated earlier than the maternally derived homologue on average, whilst at 11p13-p12 it is the maternally-derived 11 that is earlier replicating.

Between *WT1* and D11S317, loci appear to act in different ways in different lines and adjacent loci on the same chromosome showed opposing patterns of imprint (Fig. 6). This may reflect the fact that the deletion breakpoints are in this part of the chromosome and might disrupt normal patterns of DNA replication (Fig. 1).

Are doublet FISH signals always indicative of chromosome replication?

In all analyses we found many S phase nuclei with FISH patterns indicative of asynchronous replication in a direction opposite to the trend in the overall population. Can these be ascribed to technical problems of the assay, or do they really reflect a mosaic or variegating imprint? Failure to detect chromatid duplication on a homologue can result from poor hybridisation efficiency or the deposition of duplicated chromatids on top of one another on the slide. In addition to DNA replication, resolution of duplicated molecules by, for example topoisomerase II, may be needed in order to visualise doublet signals. However, the topoisomerase II inhibitors amsacrine and etoposide did not significantly affect apparent replication times assayed by FISH (data not shown).

Conversely, doublet FISH signal can arise in the absence of replication. On metaphase chromosomes, where chromatid

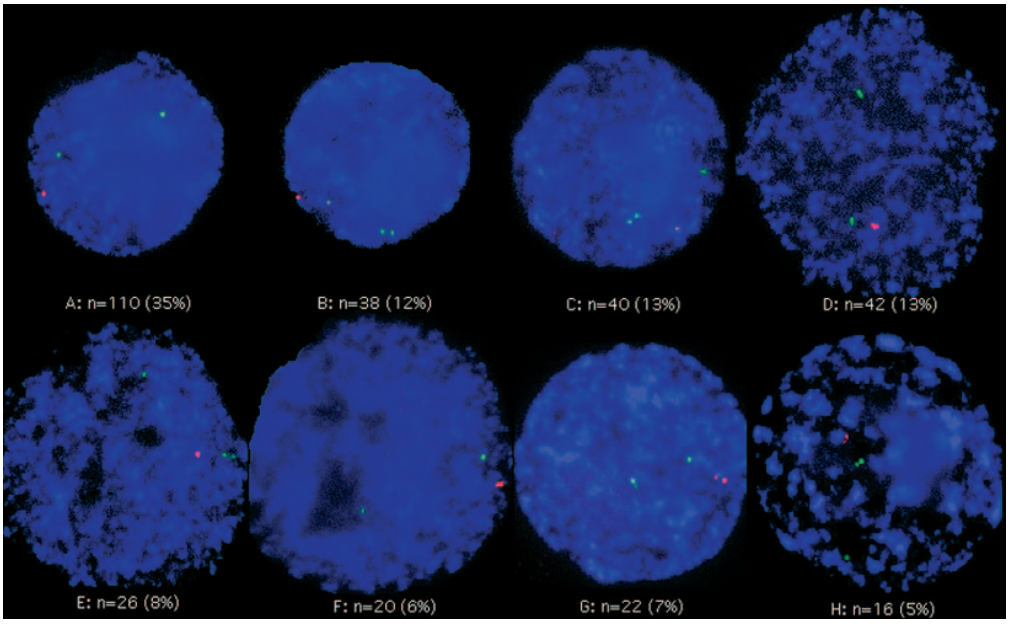


Fig. 3. Patterns of FISH signal at *INS*/*IGF2* and *PAX6* in S phase PAZO nuclei. S phase PAZO nuclei detected with AMCA (blue). Hybridisation of *INS*/*IGF2* was detected with FITC (green), and *PAX6* with Texas Red (red). The number and, in brackets, the % of S phase nuclei seen for each of the eight possible hybridisation patterns is shown.

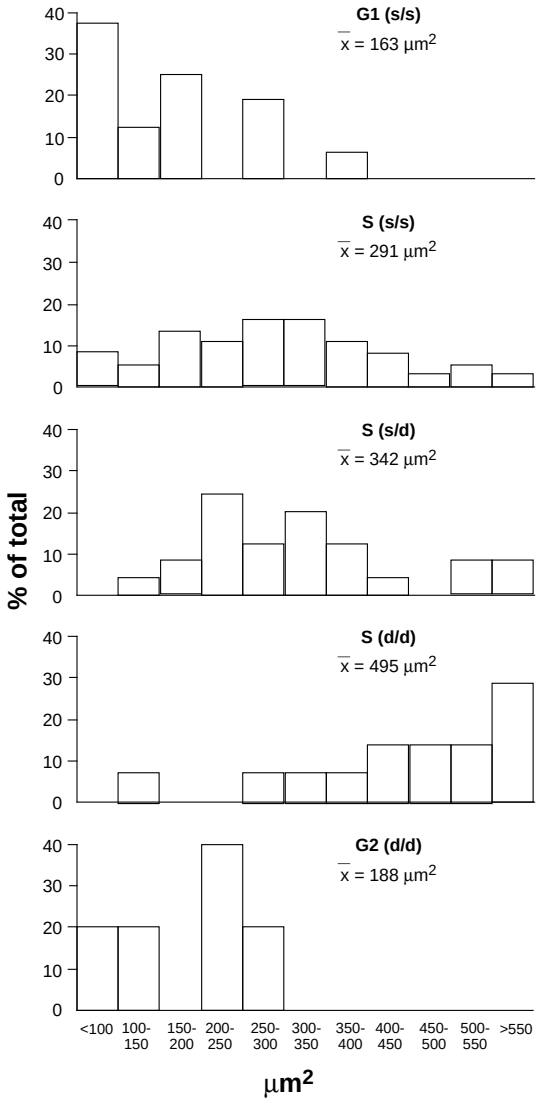


Fig. 4. Correlation of nuclear size with DNA replication. The histograms show the proportion (*y* axis) of PAZO nuclei in G_1 (singlet FISH signal and no BrdU staining), G_2 (BrdU– and doublet FISH signals) and various stages of S (BrdU +ve and combinations of singlet and doublet FISH signals) with areas falling into the denoted size classes (*x* axis in μm^2). FISH was with *INS*/*IGF2*. The area of each nucleus was calculated using Immediate Morphometry software on a Zeiss AxioHome. For G_1 nuclei the mean nuclear area ($\bar{x}$) was 163 μm^2 (σ=85, *n*=16). For G_2 $\bar{x}$ = 188 μm^2 (σ=76, *n*=5). For S phase nuclei with both *INS*/*IGF2* loci unreplicated (s/s) $\bar{x}$ = 291 μm^2 (σ=128, *n*=37). Where *INS*/*IGF2* is replicated asynchronously (s/*d*) $\bar{x}$ = 342 μm^2 (σ=188, *n*=25) and when both copies of *INS*/*IGF2* are replicated in S $\bar{x}$ = 495 μm^2 (σ=160, *n*=14).

number can be independently assessed, hybridisation efficiencies of >95% were routinely achieved but 8% of the individual chromatids apparently had doublet FISH signal on close examination. This might result from separation of denatured chromatin strands or from chromatin decondensation (Houseal and Klinger, 1994). FISH patterns suggestive of S phase were scored in both BrdU +ve and –ve PAZO nuclei. At the maternal and paternal D11S676 loci the percentage of nuclei with doublet signal were 70% (191/272) and 64% (173/272), respectively, in the BrdU+ population (reflecting the earlier replication of the maternal allele), and 14% (34/241) and 16% (39/241), respectively, in BrdU– nuclei (excluding nuclei that appear to be in G_2 i.e. duplicated signal at all loci examined). Some replication may have occurred before cytologically detectable amounts of BrdU had been incorporated, but the relative replication time calculated for this locus (Fig. 5 and Table 1) and the fact that 19% (51/272) of the BrdU+ nuclei show no duplication at either D11S672 allele, suggests that this is unlikely. Hence some doublet FISH signal is being scored in the absence of DNA replication. Using FISH in the absence of a BrdU incorporation assay may therefore overestimate the extent of replication. We conclude that doublet FISH signal is usually, but not always, indicative of DNA replication and can be used to assay replication as long as due care is given to the limitations of the technique and the collection and interpretation of the data.

Relative timing of DNA replication along 11p

We assessed the difference in replication timing not only between homologous loci but also between different loci along a single chromosome. Fig. 5 shows the relative replication timing (β) of loci along the maternal and paternal PAZO chromosomes. Calculated temporal separation between loci on the same chromosome was generally larger than that between homologous loci. Hence, in a single cell line the replication order between loci could often be established with a high degree of statistical certainty (Table 1), whereas assessing the average asynchrony between homologues with confidence often required analysis of the same locus in several cell lines (Figs 5, 6). In PAZO the latest replication (highest values of β) was found at D11S104, and the maternal copy of D11S317. The earliest replication was at the maternal D11S676 and D11S672 loci (Fig. 5).

Levels of histone acetylation but not DNA methylation modulate replication imprinting

What is the nature of the imprint that underlies replication asynchrony and how might it be modified? Chromosome imprints that manifest as allelic differences in gene expression are modified during development and pathogenesis (Efstratiadis, 1994) and with age (Mannens et al., 1994). The molecular basis for imprinting may be at the level of DNA methylation, or chromatin structure. DNA methylation certainly plays some role in imprinting (Li et al., 1993). Culturing cells in 5-azacytidine reduces DNA methylation, and the deacetylase inhibitors sodium butyrate and Trichostatin A can be used to increase levels of histone acetylation (Yoshida et al., 1990). We analysed the effects of these agents on DNA replication.

Replication of the *INS*/*IGF2* genes and the D11S104 locus was examined in three lymphoblastoid cell lines (PAZO, SATO and MARGA) and in MARGA cells that had been cultured in either 5-azacytidine, sodium butyrate or Trichostatin A. In all lines cultured under normal conditions the average replication time of the paternal *INS*/*IGF2* preceded that of the maternal copy and conversely the maternal D11S104 locus replicated before the paternal locus. Treating MARGA with either 5-azacytidine, sodium butyrate or trichostatin A advanced the apparent time of replication (β) of both loci compared to controls, but in different ways. Azacytidine

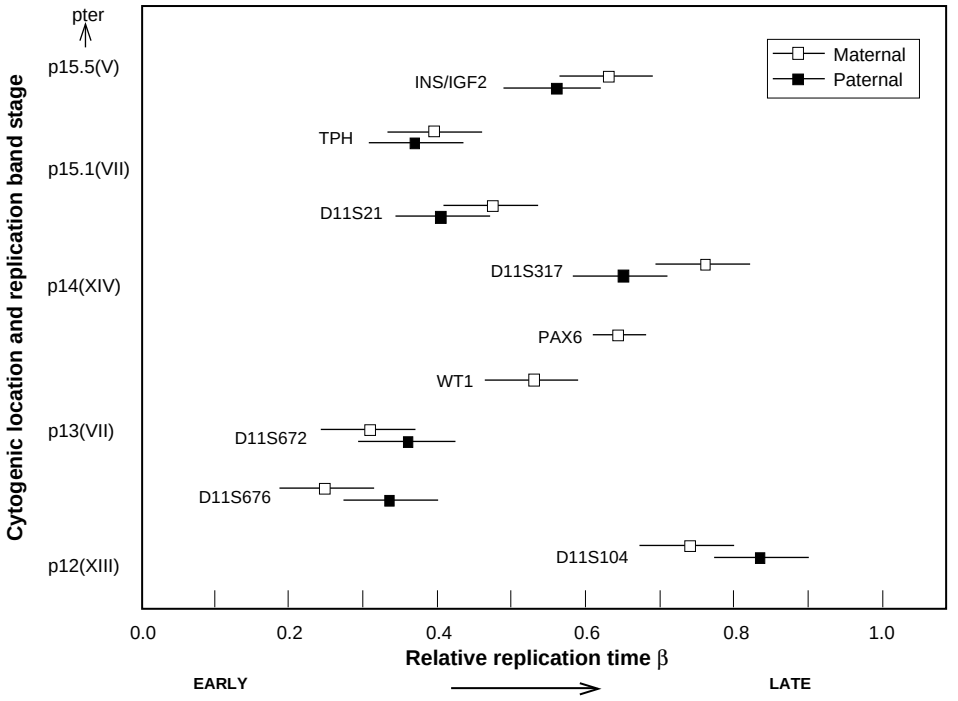
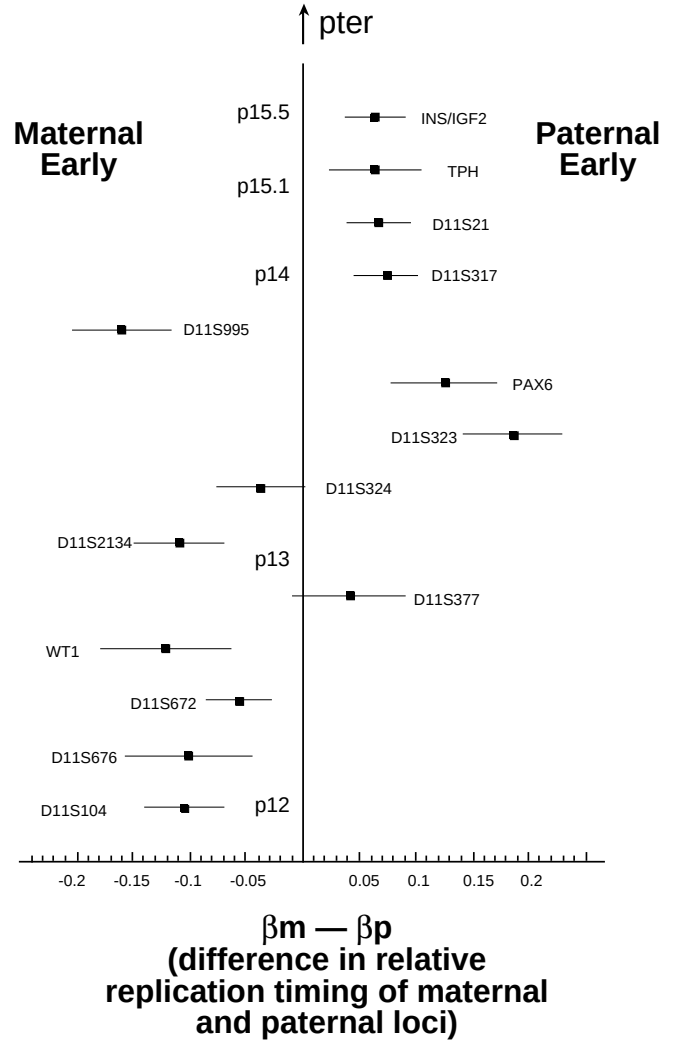


Fig. 5. Replication map of chromosome 11p. Temporal map showing the order of replication of 11p loci on maternal and paternal PAZO chromosomes from data in Table 1. The *x* axis shows the relative replication time (β). Early replicating loci lie toward the left. On the vertical axis the cytogenetic location of loci and the corresponding replication band stage (Dutrillaux et al., 1976) are shown. For each locus the box represents the mean value of β, with the bars showing one standard error. β for *PAX6* has been calculated by averaging the values for this locus (Table 1). Open and filled boxes signify the maternal and paternal copies of each locus, respectively. The paternal PAZO chromosome 11 carries a deletion that encompasses *PAX6* and *WT1*.



advanced the replication time of homologous loci similarly so that the overall imprinting was maintained (Fig. 7). Examination of non-S phase nuclei attributed some of this effect to chromatin decondensation. By contrast, sodium butyrate or trichostatin A advanced the relative replication time on the later replicating alleles (paternal D11S104 and maternal *INS*/*IGF2*) to a larger extent than on the earlier replicating alleles. Therefore, although there was still a proportion of asynchronously replicating nuclei in the culture, the overall direction of the replication imprint was abolished (Fig. 7). A similar effect was not seen in non-S phase nuclei.

DISCUSSION

As eukaryotic replication is influenced by chromatin structure, gene expression and chromosomal context it is not surprising that regions of the genome, particularly those with known transcriptional differences between homologues, have allelic differences in DNA replication time. This has been examined by FISH in previous studies (Kitsberg et al., 1993a; Knoll et al., 1994; Boggs and Chinault, 1994), but without statistical analysis so that the actual differences in the average replica-

Fig. 6. Replication imprint map of human 11p. Differences in the relative replication timing (βm-βp) of maternally- (m) and paternally- (p) derived 11p loci compiled from data obtained using all the cell lines of Fig. 1. Negative values of βm-βp signify earlier replication of the maternal allele, positive values earlier replication of the paternally-derived locus. The boxes show the mean value of (βm-βp) with the line showing one standard error about that mean. The vertical axis indicates the cytogenetic location of each locus. The percentage of nuclei showing asynchronous replication at each locus were: *INS*/*IGF2* 31%, TPH 35%, D11S21 34%, D11S317 35%, D11S995 33%, *PAX6* 30%, D11S323 32%, D11S324 30%, D11S2134 28%, D11S377 36%, *WT1* 25%, D11S672 28%, D11S676 30%, D11S104 23%.

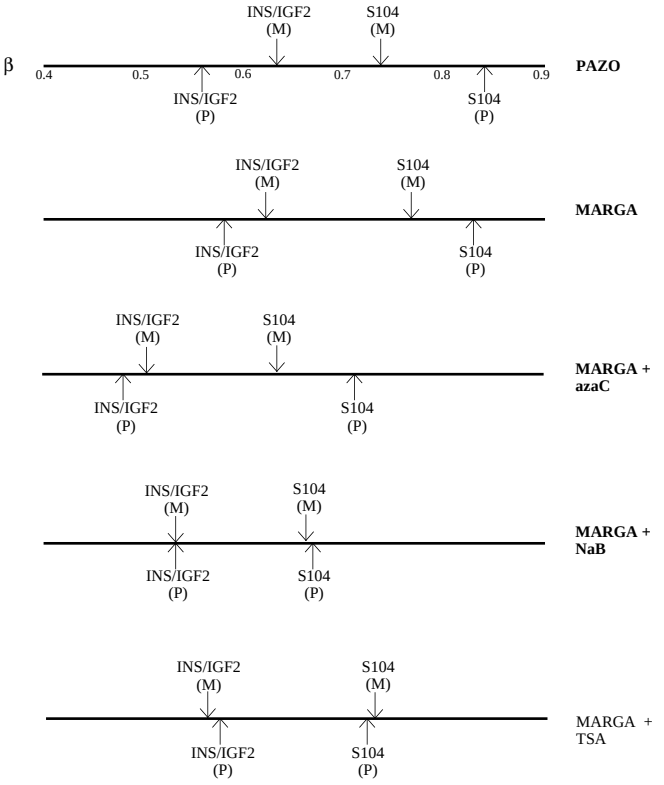


Fig. 7. Effects of azacytidine, sodium butyrate and Trichostatin A on DNA replication. The relative replication times β for maternal and paternal D11S104 and *INS*/*IGF2* loci are shown in lymphoblastoid cell lines PAZO and MARGA together with the results obtained from MARGA cells treated with 5-azacytidine (azaC), sodium butyrate (NaB) or Trichostatin A (TSA). In the control cultures and azacytidine treated cells the maternal D11S104 and paternal *INS*/*IGF2* loci are the earlier replicating alleles. However, in cells treated with either of the histone deacetylase inhibitiors sodium butyrate or trichostatin A both D11S104 and *INS*/*IGF2* have lost their overall replication imprint.

tion time of homologues could not be determined within a population of cells. We have quantitated replication asynchrony on different parts of human chromosome 11 by systematically examining large numbers of S phase nuclei, and subjecting the data to statistical analysis.

The differences we find in relative replication timing between loci along a chromosome are generally larger than those between homologous loci (Figs 5, 6). The replication time of a locus is consistent between cells of a similar type and in agreement with that expected from the eighteen stages of S phase defined cytogenetically (Dutrillaux et al., 1976). 11p12 and p14 (the locations of D11S104 and D11S317, respectively) replicate at late S stages XIII and XIV, whilst p13 (the location of D11S676 and D11S672) replicates at the earlier stage VII. The only locus that did not fit this correlation was *INS*/*IGF2* (Fig. 5), which being in 11p15.5 was expected to be early replicating (stage V). However, an isolated locus may replicate at a time uncharacteristic of the whole chromosomal domain, dependent on the expression state of nearby genes (Dhar et al., 1989).

In the line DAR, adjacent loci D11S377 and D11S2134 are approximately 50 kb apart, and D11S377 lies close to the deletion endpoint on the presumptive paternally-derived chromosome (Fantes et al., 1995) (Fig. 1). On the normal chromosome the β values for these loci (0.491 and 0.534) suggests that they are replicated only 4% of S phase apart consistent with estimates of eukaryotic replication fork rates. On the DAR deletion-bearing homologue, this temporal separation is increased fivefold. It is likely that the deletion breakpoint has disrupted the usual pattern of replication as has been seen close to deletion breakpoints at the β-globin complex (Kitsberg et al., 1993b).

In the proximal part of 11p maternally-derived loci, including *WT1*, tend to replicate first. In more distal regions (11p15) the situation is reversed so that the paternally-derived loci (including *IGF2*) are earlier replicating on average (Fig. 6). A similar switch in the direction of the replication imprint occurs on human chromosome 15 (Knoll et al., 1994). Our results confirm the direction but not the apparent magnitude of the imprint at *IGF2* determined previously (Kitsberg et al., 1993a). We scored many nuclei with FISH patterns indicative of asynchronous replication in an opposite direction to the overall trend. Kitsberg et al. (1993a) reported that 80% of human nuclei with singlet/doublet *IGF2* FISH signal (a third of nuclei) had the doublet associated with the paternally-derived homologue. This suggests that the *IGF2* alleles replicate several hours apart from each other. In our analyses we found that although 28-33% of S phase nuclei had singlet/doublet *IGF2* FISH patterns, only 54-59% of these had the doublet on the paternal homologue. We calculate the mean separation between the replication of the alleles (Fig. 6) as 5% of S.

Imprinting is commonly taken to mean monoallelic patterns of gene expression, however, the term was first applied to aspects of chromosome behaviour such as differential heterochromatinisation and replication behaviour in insects. The paradigm for asynchronous replication of homologues in mammals is X inactivation. Late replication is a common feature of the inactive X in all mammals including the egg-laying monotremes (Graves, 1987). Control of replication timing has been proposed as a mechanism for gene regulation and self-perpetuating inherited chromatin structure (reviewed by Riggs and Pfeifer, 1992) and hence imprinting. Indeed X inactivation, and the accompanying late replication, is imprinted in marsupials and in mammalian extraembryonic tissues. However, this imprint is either erased or not recognised in the embryo proper of eutherian mammals, where inactivation is random.

Although genes with imprinted patterns of expression reside in regions of imprinted replication there is not a simple relationship between earlier replication and transcriptional potential. The physically linked *H19* and *IGF2* genes are both replicated earlier on the paternal homologue (Kitsberg et al., 1993a), but at the transcriptional level they are imprinted in opposite directions. Additionally, genes (such as PAX6) with biallelic expression can still reside in regions of replication asynchrony (Fig. 6). The recent report that *WT1* may be monoallelically expressed (from the maternally-derived chromosome) in some tissues of some individuals (Jinno et al., 1994) is interesting in light of our observation that *WT1* is replicated earlier on the maternally-derived chromosome 11 than the paternal copy (Fig. 6).

Why is the overall level of replication imprint we measure

small, and its direction not strictly adhered to by all cells in a population? Loss of monoallelic gene expression occurs during development and pathogenesis (Efstratiadis, 1994) and imprinted methylation differences for *INS*/*IGF2* can also disappear with age (Mannens et al., 1994). Biallelic *IGF2* or *H19* expression and co-expression of these genes occurs in a variety of adult tissues, BWS fibroblasts, Wilm's tumours, rhabdomyosarcomas and androgenetic trophoblasts (Weksberg et al., 1994; Rainer et al., 1993; Ogawa et al., 1993; Zhang et al., 1993; Mutter et al, 1993). In light of this, perhaps it is not surprising that the replication imprint we detect on human chromosome 11p (including the *IGF2* and *WT1* genes), in nuclei from adult peripheral lymphocytes and EBV transformed cell lines, is mosaic and not very distinct.

Are replication asynchrony and transcriptional imprinting related and if so, what is the mechanism that underlies them? DNA methylation is involved in transcriptional control, X inactivation and imprinting. Although methylation clearly has some role in imprinting (Li et al., 1993) and X inactivation, it is unlikely to be involved in establishing the repressed, late replicating state since X inactivation in marsupials and mammalian extraembryonic tissues occurs without methylation of CpG islands, and most methylation differences between imprinted alleles occur postzygotically (Reik and Allen, 1994). 5-azacytidine progressively demethylates DNA by inhibiting maintenance methylase. This can both reactivate silent genes on the inactive X and also advance its replication time so that the two Xs appear cytogenetically to replicate in synchrony (Jablonka et al., 1985). We found that 5-azacytidine did not alter the replication imprint since replication of both early and late replicating alleles was similarly advanced. In contrast, histone deacetylase inhibitors had a more potent effect on later replicating loci, whichever homologue they were on, so that the replication imprint across the population of cells was abolished (Fig. 7). Sodium butyrate and trichostatin A increase levels of histone acetylation by inhibiting deacetylation (Jeppesen and Turner, 1993; Yoshida et al., 1990). We suggest that histone acetylation may be part of the mark distinguishing homologues that contributes to their replication at different times. The differences in H4 acetylation between the two mammalian X chromosomes (very low levels of acetylation on the inactive X), and the high levels on the hyperactive X of *Drosophila* males (Jeppesen and Turner, 1993), illustrates the involvement of histone acetylation in chromosome marking. Histone acetylation is also important in silencing and heterochromatin formation in yeast (Braunstein et al., 1993) and in another phenomenon that involves determination of a heterochromatin-like state with parallels to imprinting - position effect variegation (PEV). Indeed, several genetic modifiers of PEV in *Drosophila* are subject to parental origin effects (i.e. imprinting) including *Suvar (2)1* a suppresser of PEV that causes hyperacetylation of H4 (Dorn et al., 1986; Szabad et al., 1988; Reuter and Spierer, 1992). Moreover, sodium butyrate itself suppresses PEV (Mottus et al., 1980). Although we found that sodium butyrate removed the overall replication imprint at *INS*/*IGF2*, it does not activate transcription of the maternal *Igf2* allele in the mouse, whereas azacytidine does - the reverse of the situation described here (Eversole-Cire et al., 1993). This might indicate fundamental differences in the mechanisms determining transcriptional and replication imprinting or might reflect the different cell types and assays used.

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