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The gene-richest bands of human chromosomes replicate at the onset of the S-phase

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Dedicated to Dr. Susumu Ohno on the occasion of his 70th birthday.

Abstract. Previous investigations on the correlations between isochore organization and human chromosomal bands have identified three sets of R(everse) bands: H3$^+$, H3* and H3$^-$, endowed with large, moderate, and no detectable amounts of the gene-richest H3 isochores, respectively. In the present work we compared the replication timing of these three sets of bands and showed that the chromosomal bands containing H3 isochores replicate almost entirely (in the case of H3$^+$ bands) or largely (in the case of H3* bands) at the onset of S phase, whereas chromosomal bands not containing H3 isochores (H3$^-$ bands) replicate later. The existence, at a resolution of 400 bands per haploid genome, of at least three distinct subsets of R bands is, therefore, not only supported by their GC and gene concentration but also by their replication times.

The human genome is a mosaic of isochores, long DNA segments which are compositionally homogeneous and can be partitioned into five families, two GC-poor families (L1 and L2; collectively called L), representing about 62% of the genome, and three GC-rich families (H1, H2, and H3), representing about 22%, 9%, and 3–4% of the genome, respectively, the remaining 3–4% being formed by satellite and ribosomal DNAs (see Bernardi, 1995, for a recent review). Gene concentration parallels GC levels, being low in GC-poor isochores and increasingly higher in increasingly GC-rich isochores (Mouchiroud et al., 1991; Zoubak et al., 1996). Compositional mapping (Bernardi, 1989), i.e., mapping isochores on chromosomes, is therefore of interest, as it provides information not only on higher order structures but also on gene distribution in chromosomes.

The correlation between isochores and chromosomal bands has been investigated by "molecular" compositional mapping of specific chromosome arms or regions (Gardiner et al., 1990; Bettecken et al., 1992; Pilia et al., 1993; De Sario et al., 1996, 1997), as well as by "chromosomal" compositional mapping, viz., by in situ hybridization of compositional DNA fractions on human metaphase chromosomes (Saccone et al., 1992, 1993, 1996).

In the first investigation of the latter kind (Saccone et al., 1992), it was shown that the hybridization of single-copy sequences present in a DNA fraction derived from H3 isochores produced the highest concentration of signals on two largely coincident subsets of R(everse) bands, which correspond essentially to G(iemsa)-negative bands. These two subsets were (1) the T(elomeric) bands (Dutrillaux, 1973), which are the most heat-denaturation-resistant R bands, and (2) the chromomycin A3-positive, DAPI-negative bands (Ambros and Sumner, 1987), which are the GC-richest bands of human chromosomes (DAPI is 4,6-diamidino-2-phenylindole). Moreover, chromosomal localization of all the human isochore families (Saccone et al., 1993) established that (1) T bands contain not only H3 isochores, but also H2 and some H1 isochores; (2) R′ bands (i.e., R bands exclusive of T bands) are formed, on the average, to almost equal extents by H1 and L isochores, H2 and H3 isochores being only rarely present; and (3) G bands consist essentially of L isochores, H1 isochores being present at low levels. The GC-richest and gene-richest isochore family H3 hybridized strongly on 28 R bands (Saccone et al., 1992), and weakly on 31 additional R bands, whereas the remaining 140 or so R bands (400-band resolution) did not contain detectable H3

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isochores and seemed, moreover, to be characterized by GC levels very close, on average, to those of G bands (Saccone et al., 1996).

These results allowed the identification of three subsets of R bands, the H3$^+$, H3*, and H3$^-$ bands. In fact, the 28 H3$^+$ R bands and 31 H3* R bands are the only bands containing H3 isochores, which are characterized by some important features, such as the highest concentration of genes (Saccone et al., 1992) and CpG islands (Aïssani and Bernardi, 1991a, b) and a higher proneness to integration of most proviruses (see Bernardi, 1995). The very high concentration of genes, and of housekeeping genes in particular, in H3 isochores points to a very high level of transcription in H3 bands, which are also characterized by the highest recombination frequencies and by an "open" chromatin structure, as identified by the accessibility to DNases (Kerem et al., 1984) and by the scarcity or absence of histone H1, the acetylation of histones H3 and H4, and a wider nucleosome spacing (Tazi and Bird, 1990). As pointed out elsewhere (Saccone et al., 1996), H3$^+$ and H3* bands very largely coincide with gaps in the physical map of the human genome currently available.

The replication time of chromosomal bands has been intensively investigated. It has been shown that R bands replicate early in the S phase, whereas G bands replicate late (Dutrillaux et al., 1975, 1976; Biemont et al., 1978; Dutrillaux and Viegas-Péquinot, 1981; Camargo and Cervenka, 1982; Vogel et al., 1989; Drouin et al., 1991; Fetni et al., 1996; see also Drouin et al., 1994, for a recent review). A number of these results have been obtained by incorporating 5-bromodeoxyuridine (BrdU) into replicating DNA, which generates a G-band or an R-band pattern depending upon the period in S phase in which BrdU was incorporated and by the staining method used, such as FPG (fluorochrome-photolysis-Giemsa), acridine orange, or immunological staining with a BrdU antibody (Dutrillaux et al., 1976; Vogel et al., 1989; Drouin et al., 1990; Fetni et al., 1996).

Modifications of the above-mentioned procedures have demonstrated the existence of different times of replication among both R and G bands. In fact, four time-dependent groups of R bands (viz., bands visualized 30 min, 1 h, 2 h, and 3 h after the start of the S phase) were demonstrated for the first part of the S phase (Kim et al., 1975). In a more detailed investigation, replication times for 277 chromosomal bands were obtained by incorporating BrdU into DNA at different times before harvesting the cells (Dutrillaux et al., 1976). R bands and G bands showed no overlapping in replication times, and all chromosomal bands were classified into 18 replication groups (Dutrillaux et al., 1976). Classes I–IX comprised the totality of R bands (except for R bands belonging to the Y chromosome that replicate together with the G bands of class XI), whereas classes X–XVIII comprised the totality of G bands. The Xp21 band of the late-replicating X chromosome was placed in an additional class, XIX (Dutrillaux et al., 1976). No other subsequently published study has been performed at this level of resolution.

In the present analysis, we investigated the correlations between the distribution of H3 isochores in R bands and the replication time of the corresponding bands.

Materials and methods

DNA preparation

High-molecular-weight DNA was prepared from a human placenta and fractionated in a cesium sulfate/3,6-bis(acetato-mercurimethyl) 1,4-dioxane (BAMD) density gradient, as described by Cuny et al. (1981). Compositional DNA fractions were submitted to analytical centrifugation as described by Macaya et al. (1976). The profiles so obtained were published previously, and DNA from fraction 8, containing the H3 isochore family, was cloned in lambda phage (Saccone et al., 1996) and used in the present in situ hybridization experiments.

Chromosome preparation and in situ hybridization

Metaphase chromosomes were obtained by phytohemagglutinin-stimulated peripheral blood lymphocytes and prepared using standard cytogenetic procedures. In situ hybridization was performed according to a previous protocol (Saccone et al., 1996): 10 ng/µl aliquots of biotinylated DNA from the lambda-cloned fraction 8 (Saccone et al., 1996) were used in a hybridization mixture containing 500 ng/µl of human Cot-1 DNA (GIBCO BRL), 500 ng/µl of yeast tRNA, 500 ng/µl of salmon sperm DNA in 50% formamide, 2 × SSC, 10% dextran sulfate, and 50 mM sodium phosphate (pH 7.0). Chromosome pretreatment, hybridization, and detection were performed as described earlier (Saccone et al., 1993).

Results

In the present work, chromosomal bands belonging to replication classes I–IX (Dutrillaux et al., 1976), which include all R bands, were compared with our in situ hybridizations with DNA from H3 isochores. Figure 1 shows the replication class of each R band, as well as the H3 hybridization properties. A total of 177 R replication bands were scored; 34, 39, and 104 of them were classified as H3$^+$, H3*, and H3$^-$ bands, respectively (Fig. 1; the two R bands of the Y chromosome, belonging in class XI, were neglected in our analysis). It should be stressed that the differences in the number of bands classified as H3$^+$ and H3* bands compared to the number reported by Saccone et al. (1996) are due to the different levels of resolution between replication bands and H3 bands. Remarkably, the earliest replication bands are not telomeric, all class I bands being intercalary.

Figure 2 shows the distribution of the H3$^+$, H3*, H3$^-$, and R bands belonging to each replication class. This indicates that (1) H3$^+$ bands replicate prevalently in the very early part of the S phase; (2) H3* bands replicate mainly at an early stage, but also at a later time of replication; and (3) the large majority of H3$^-$ bands replicate late.

We also observed that the 30 R bands replicating in the first 30 min of the S phase (Kim et al., 1975) largely corresponded to the H3 bands and the early replication classes of Dutrillaux et al. (1976). More precisely, 14 of these bands were H3$^+$ bands and 10 were H3* bands. Out of the 30 bands, 20 coincided with classes I, II, and III of Dutrillaux (9, 8, and 3 bands corresponded to classes I, II, and III, respectively). In fact, only 1 of the 30 bands replicating very early in S phase belonged in class VII, the other 29 bands corresponding to classes that replicated earlier. Moreover, we compared our H3 isochores in situ hybridization with the very early replicating R bands of Fig. 4a of Vogel et al. (1989). In this case as well, the correspondence among many H3 bands and the early replicating R bands is clearly visible (Fig. 3).

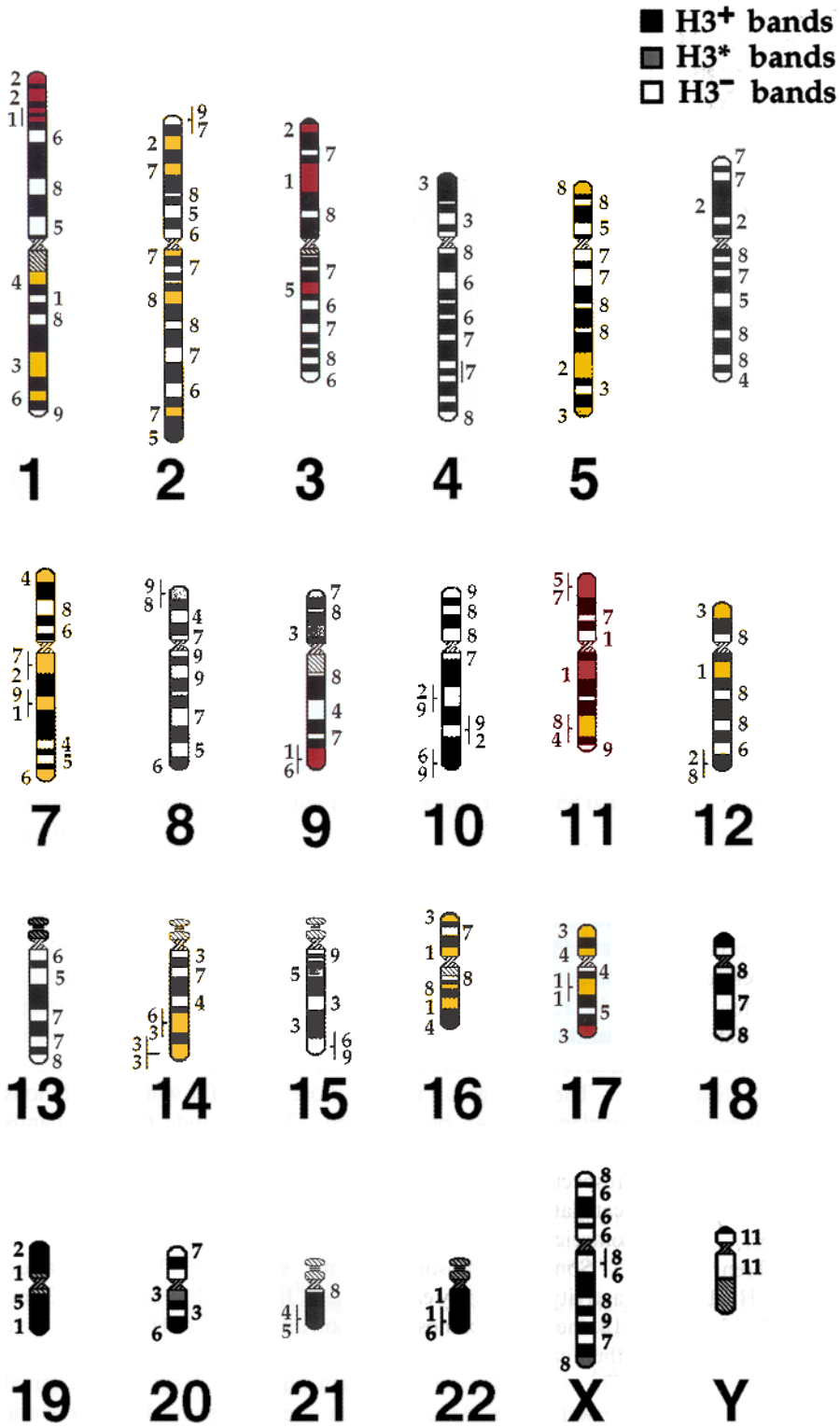

Fig. 1. G-banded karyotype showing the R bands identified by Saccone et al. (1996) as H3⁺ (red), H3* (yellow), and H3⁻ (white). G bands are black. The replication class of R bands of Dutrillaux et al. (1976) are indicated (by Arabic numerals) on the left (H3⁺ and H3* bands) and on the right (H3⁻ bands) of each chromosome. In some cases, the same R band corresponds to two different replication timings (see text).

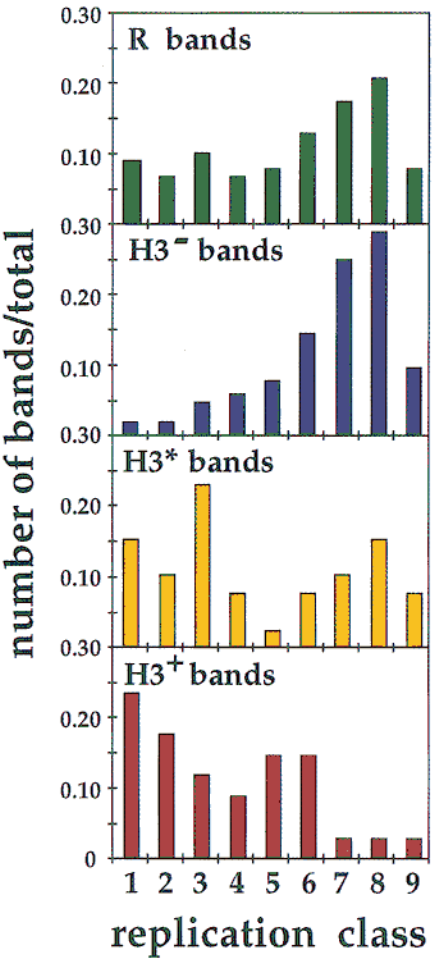

Fig. 2. Histograms showing the distribution of R bands and of H3⁻, H3*, and H3⁺ bands (Saccone et al., 1996) in each replication class (indicated by Arabic numerals) of Dutrillaux et al. (1976).

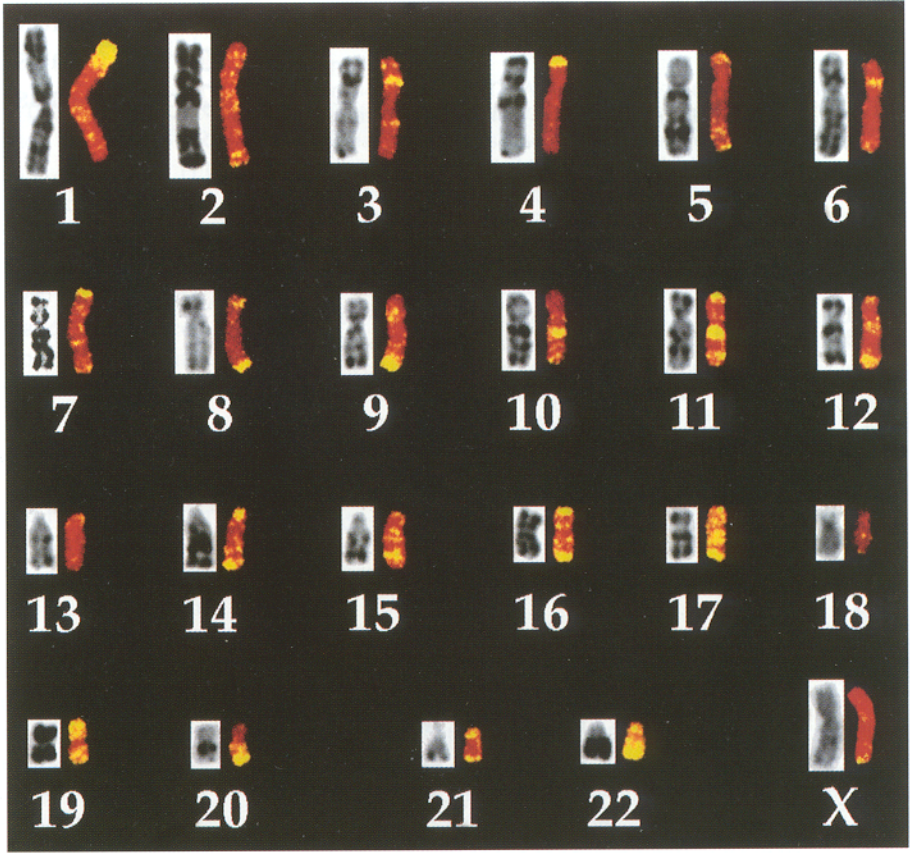

Fig. 3. Comparison of H3 isochore hybridization and very early replicating R bands. The right member of each pair of chromosomes shows the H3 isochore hybridization, whereas the left member shows the very early replicated R bands identified in Fig. 4a of Vogel et al. (1989). Biotinylated H3 isochores were detected with avidin-FITC, and chromosomes were stained with propidium iodide.

A more detailed analysis indicates that the histograms presented in Fig. 2 do not precisely reflect the real situation, because some chromosomal bands are not clearly defined, as shown in Fig. 1. In fact, while H3$^+$ and, to a lesser extent, H3* bands generally replicate early, and H3$^-$ bands replicate late, there are H3$^-$ bands that belong to an early replication class, as there are H3$^+$ and H3* bands that belong to late-replication classes. Moreover, there are H3$^+$ and H3* bands replicating at two different times, suggesting that these bands are formed by subbands with different features. A number of these inconsistencies can be explained about these bands, which can be pooled into three groups on the basis of their features.

The first group comprises H3 bands with two different replication times. At a higher resolution, each of the H3 bands 7q11.2, 7q22, 9q34, 10q22, 10q26, 11p15, 11q23, 12q24.3, 14q24, and 22q13 appear to be composed of (1) an R subband that contains no H3 isochores and belongs to a class replicating later than class VI and (2) an R subband which contains H3 isochores and belongs to a class replicating earlier than class IV (with the exception of the 10q26 and 11p15 subbands, which belong in classes VI and V, respectively). Thus classes VII, VIII, and IX, which were assigned to these H3 bands, could be reassigned to contiguous H3$^-$ subbands.

The second group comprises H3$^-$ bands belonging to a very early replication class (I, II, or III), which are located very near the H3$^+$ or H3* bands. These H3$^-$ bands are likely to contain H3 isochores that were not detected because, at the band resolution of the in situ hybridization, the fluorescence of the H3$^+$ or H3* band possibly coalesced with that of contiguous H3$^-$ bands. This group comprises bands 1q23, 5q33, 6p21.1, 10q24, 14q11.2, 15q22, and 20q13.1. In the case of 14q11.2, the neighboring nucleolar organizer gives a strong hybridization signal due to the rDNA present in the H3 isochore family.

The third group include those bands for which no obvious explanation can be proposed. These include the H3$^-$ bands 4p14 and 11p11.2, belonging to replication classes III and I, respectively, and the remaining H3$^+$ or H3* bands that replicate late, such as those belonging to chromosome 2 and the telomeric band 8p23.

Some chromosomes do not show any DNA replication activity in the very early S phase. Indeed, chromosomes 13 and 18, the only chromosomes showing no H3$^+$ or H3* bands, have their earliest replication bands in class V and VII, respectively. The same feature is present in some large chromosomal regions, such as the long arm of chromosome 4. As far as the long arms of chromosomes 2, 3, and 8 are concerned, although they contain H3$^+$ or H3* bands, they are predominantly composed of late-replicating DNA. Also, in the early replicating X chromosome, the earliest replicating band belongs in class VI. Finally, the Y chromosome is an exception, because its R bands replicate together with the G bands belonging to class XI.

Discussion

In the present work we compared the replication times of R bands and their hybridization with H3 isochore DNA. The results obtained showed that, in general, H3⁺ and H3* bands predominantly replicate at the onset of the S phase.

The finding that not all R bands start replication at the beginning of the S phase caused controversy in the past, and criticisms were raised about the reports on the existence of many replication time groups. These findings were attributed to the limited resolution of the techniques used and, in particular, to the fact that a critical number of BrdU substitutions is required for detection at the cytological level, this limitation being responsible for the appearance of bands at different times (Camargo and Cervenka, 1982). This interpretation must be rejected, however, because there is an unequivocal correlation among the R bands that replicate at the onset of S phase and the H3⁺ and H3* bands identified as GC-rich/gene-rich bands. Also, the previous results by Kim et al. (1975) showed that the majority of R bands replicating in the first 30 min and corresponding to replication classes I, II, and III coincide with H3⁺ and H3* bands. Moreover, when the highest sensitivity detection of the BrdU-substituted DNA obtained by immunological technique was applied, it was shown that not all the R bands, but only a subset of them, begin to replicate at the onset of the S phase (Vogel et al., 1989). When Fig. 4a from the latter paper was analyzed, it was clear that many H3⁺ and H3* bands are labeled, and some chromosomes could be easily identified (see Fig. 3).

It should be recalled that a replicon completes synthesis in 1–2 h and that a replication band is a cluster of replicons (Latt, 1975; Holmquist, 1988). Considering the length of the S phase (8–10 h for human lymphocytes; Drouin et al., 1994), subdivision of the S phase into a number of replication groups (chromosomal bands) can be easily understood.

This conclusion is also supported by other observations. Hydroxyurea, a synchronizing agent that blocks the cell cycle at the G_1/S transition (Lau and Arrighi, 1981), causes a block between the replication of the DNA belonging to classes III and IV (B. Dutrillaux, personal communication), indicating the possible existence of a T/R transition within the S phase. Also, the previous indication, that the order of replication during the S phase is T bands, followed by R bands, followed by G bands (Drouin et al., 1994) could be explained by the above findings. A further refinement of the results of Fig. 2 could be obtained when higher resolution data will become available.

Some recent results (Fukagawa et al., 1995) have shown that the isochore border between an L and a H2 isochore in the human major histocompatibility complex (MHC) corresponds to a sharp compositional transition between: (1) a 20-kb GC-poor segment made of long, interspersed repeated sequences (L1 sequences) associated with a 650-bp sequence homologous to the PAB1 motif described at the boundary of the pseudoautosomal region (Ellis and Goodfellow, 1989), and (2) a 30-kb GC-rich segment made up of short, interspersed (*AluI*) repeated sequences. Fukagawa et al. (1995) indicated that this transition region might correspond to a band boundary. Very interestingly, a 2-h difference in replication timing between the two sides of the transition was demonstrated (Tenzen et al., 1997). These results show that, at least in this single case studied, GC-poor isochores replicate later than GC-rich isochores and, moreover, that the border coincides with a boundary of both isochores and chromosomal bands. It has, however, been previously stressed that replication timing appears to be associated not with G/R banding, but with gene concentration and expression (Bernardi, 1989). Indeed, replication banding is also found in cold-blooded vertebrates that show no or poor G/R banding (Schmid and Guttenbach, 1988).

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