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Human chromosomal banding by *in situ* hybridization of isochores

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Abstract. Further to the classical methods that involve different chromosome treatments followed by staining, *in situ* hybridization of isochores represents a novel approach to chromosomal banding. Isochores are long compositionally homogeneous DNA segments that, in the human genome, belong to five families, two GC-poor families (L1 and L2) representing 30% and 33% of the genome, respectively, and three GC-rich families (H1, H2 and H3) representing 24%, 7.5% and 4–5% of the genome, respectively. Gene concentration increases with increasing GC levels, reaching an up to 20-fold higher level in H3 compared to L1 isochores. *In situ* hybridization of DNA from different isochore families on metaphase chromosomes allow to distinguish different sets of Giemsa and Reverse bands. In addition, it also provides information on the chromosomal distribution of genes.

Key words: *Gene distribution*, Human genome, Metaphase chromosomes, *Replication timing*

Introduction

Human chromosomes are cytogenetically identified not only on the basis of their relative size and centromere positions but also by different staining properties of chromosomal regions, namely by chromosomal bands. Many methods can be used for chromosomal banding, the most widely known being the G(iemsa) and the R(everse) banding, which involve different chromosome treatments (trypsin digestion or heat denaturation), followed by Giemsa staining.

Bands corresponding to G and R bands can also be obtained by different methods, exploiting the different replication timing of DNA of each set of bands. This is the so-called *dynamic banding*, in contrast with the above G and R *structural banding*. In fact, R and G bands replicate in the early and late S phase, respectively [9, 10, 18, 22, 23, 26, 40, see also 19 for a review]. Using 5-bromodeoxyuridine (BrdU) incorporation into replicating DNA produces G or R bands, depending upon the S phase period in which BrdU is incorporated and the staining methods used, such as FPG (Fluorochrome-Photolysis-Giemsa), acridine orange, or immunological staining involving BrdU-antibody [17, 23, 26, 40]. Modifications of the above procedures demonstrated the existence of different times of replication among both R and G bands. In fact, four replication time dependent groups of R bands (i.e., bands visualized after 30 min, 1, 2 and 3 hours after the start of the S phase) were demonstrated for the first part of the S phase [31]. Moreover, in a more detailed investigation, replication times for 277 chromosomal bands were obtained, leading to the identification of 18 different replication groups [23].

Further to the above classical methods, different approaches to chromosome banding were developed using *in situ* hybridization. In fact, the improvement of this technique, together with the isolation and/or cloning of many different DNA sequences, allowed obtaining banding patterns that are based on the chromosomal distribution of the DNA sequence(s) used as probe. For instance, G and R banding can be obtained by hybridization with LINE and SINE sequences, respectively [32]. *In situ* hybridization with DNAs from different isochore families as probes [35, 36] allows the identification of chromosomal regions characterized by different compositional properties (see following section). Thus, *in situ* hybridization of DNA from different isochore families, that was originally used to study the gene distribution on human metaphase chromosomes, is a novel method to obtain two new different sets of bands, the H3 and the L1 bands, so called on the basis of the hybridized isochore [25, 37]. Moreover, an R banding could be also obtained by hybridizing a mixture of GC-rich and GC-richest isochores [36].

The organization of the human genome

The isochores

Vertebrate genomes are made up of long DNA segments (several hundred kb in size), the isochores, which are compositionally homogeneous, and which can be obtained by preparative ultracentrifugation in

Cs_2SO_4/BAMD density gradients (BAMD is 3,6-bis(acetato-mercurimethyl)-1,4-dioxane) (Figure 1A). Isochores belong to a small number of families (from two, in cold-blooded vertebrates, to six, in birds) that are characterized by different GC levels. The GC level range covered by isochores is very broad in warm-blooded and very narrow in cold-blooded vertebrates [6]. The most extensively studied human genome is formed by isochores that belong to five families (see Figure 1B): two GC-poor families (L1 and L2) representing about 30% and 33% of the genome, and three GC-rich families (H1, H2 and H3)

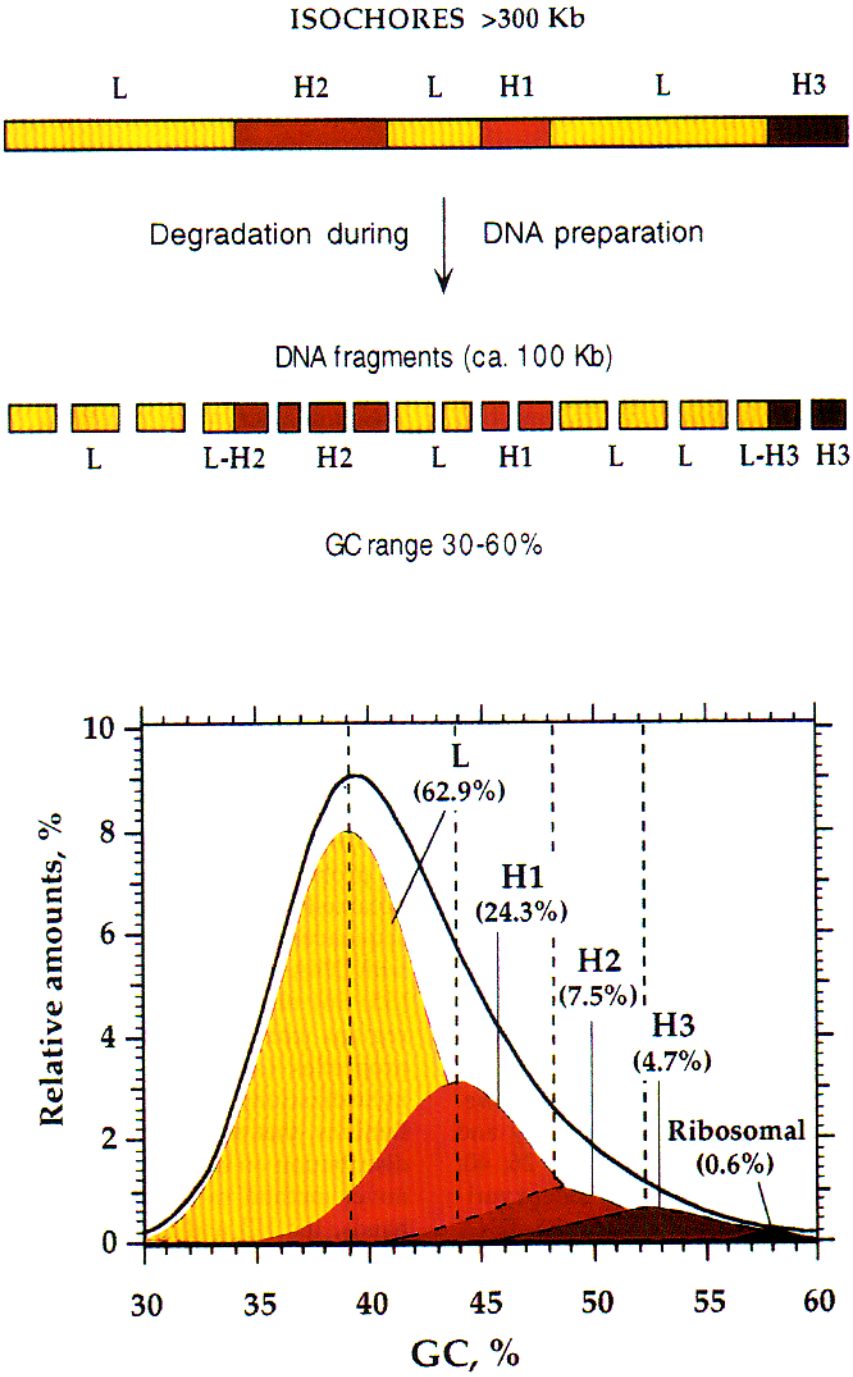

Figure 1. **A.** *Scheme of the isochore organization of the human genome.* This genome, which is typical of the genome of most mammals, is a mosaic of large (>300 kb, on average) DNA segments, the isochores, which are compositionally homogeneous (above a size of 3 kb) and can be partitioned into a smaller number of families, 'light' or GC-poor (L1 and L2; collectively indicated as L), and 'heavy' or GC-rich (H1, H2 and H3). Isochores are degraded during routine DNA preparations to fragments of approximately 100 kb in size. The GC-range of the isochores from the human genome is 30–60% (From [5]). **B.** *The CsCl profile of human DNA* is resolved into its major DNA components, namely the families of DNA fragments derived from isochore families L (i.e., L1 + L2), H1, H2, H3. Modal GC levels of isochore families are indicated on the abscissa (broken vertical lines). The relative amounts of major DNA components are indicated. Satellite DNAs are not represented (From [41]).

representing about 24%, 7.5% and 4–5% of the genome, respectively (see [5, 6] for reviews).

Isochore families differ not only in GC levels but also in gene concentration, which increases with increasing GC levels of isochores. Indeed, gene concentration parallels GC levels, being low in GC-poor isochores and increasingly high in increasingly GC-rich isochores [5, 7, 33, 41] The compositional correlations between GC_3 (GC level of the third codon positions) and isochore GC [11] allowed positioning the human coding sequence histogram relative to the CsCl profile of human DNA (Figure 2). This profile indicates that gene concentration is low in GC-poor isochores, increases with increasing GC in isochore families H1 and H2, and reaches a maximum in isochore family H3, which exhibits up to a 20-fold higher gene concentration compared to GC-poor isochores. This value may still be an underestimate, because of the presence of ribosomal DNA in the buoyant density range of H3 isochores (see Figure 1B), and because housekeeping genes, which seem to be preferentially located in H3 isochores, are currently underrepresented in gene banks.

The H3 isochore family is endowed not only with the highest GC level and the highest gene concentration, but also with other important features, such as the earliest replication timing, open chromatin structure, and the highest transcriptional and recombinogenic activities.

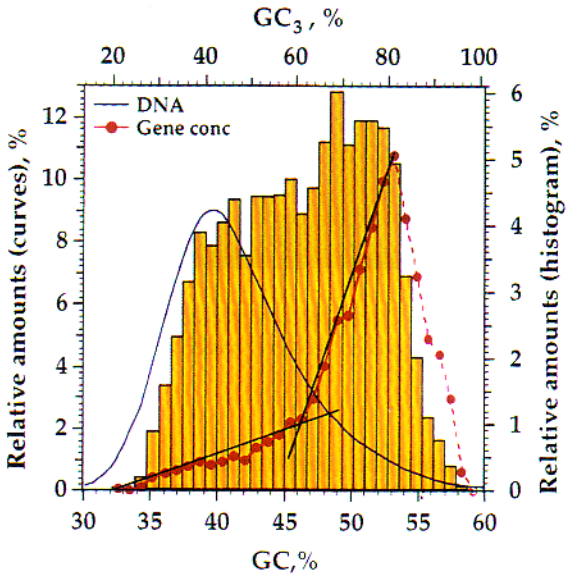

Figure 2. *Profile of gene concentration* (red dots) in the human genome, as obtained by dividing the relative numbers of genes in each 2% GC_3 interval of the histogram of gene distribution (yellow bars) by the corresponding relative amounts of DNA deduced from the CsCl profile (blue line). The apparent decrease in the concentration of protein-encoding genes for very high GC values (broken line) is due to the presence of ribosomal DNA in that region. The last concentration values are uncertain because they correspond to very low amounts of DNA (From [41]).

Correlation between isochores and chromosomal bands

The correlation between isochores and chromosomal bands has been investigated by 'chromosomal compositional mapping', namely by *in situ* hybridization of compositional DNA fractions on metaphase chromosomes [35–38] and, at a higher resolution, by 'molecular compositional mapping' [4] of specific chromosome arms or regions [8, 16, 28, 34] Both approaches provide information not only on higher order DNA structures, but also on gene distribution in chromosomes, and, more generally, on all those features that are correlated with the GC levels and gene concentration.

The GC-richest bands

Some years ago, we used the *in situ* suppression hybridization (Figure 3) of compositional DNA fractions on human metaphase chromosomes [35, 36], showing that the hybridization of a DNA fraction derived from the H3 isochore family produced the highest concentration of signals on a small number of R bands [35]. The set of bands identified by H3 isochore painting were largely coincident with two other previously defined group of bands, the T bands described by Dutrillaux [21], which are the most heat-denaturation-resistant R bands and which are located prevalently on telomeric positions, and the chromomycin A3-positive/DAPI-negative bands described by Ambros and Sumner [3], which are the GC-richest bands of human chromosomes (Figure 4). It was so established that T bands are enriched in GC-rich, gene-rich 'unique' sequences, the contribution of repetitive DNA sequences being suppressed by competition with excess of unlabeled repetitive DNA sequences (see Figure 3). Moreover, a minor concentration of signals on other R bands were also detected (see Figure 2 in [35]), leading to the identification, at a resolution of 400 bands per haploid genome, of three subsets of R bands that we called $H3^+$, H3* and $H3^-$ bands on the basis of their high, medium or undetectable levels of H3 isochores [37, 39].

The previous finding that the highest concentration of CpG islands are in the H3 isochore family [1, 2], indicated that the above bands, should also be enriched in CpG islands, a finding that was subsequently directly shown by hybridization of CpG island DNA [13]. It should be noted that the hybridization of fragments enriched in CpG islands did not lead to pictures clear enough to identify chromosomal bands. This identification could only be done by comparison with $H3^+$ bands.

The existence of three distinct sets of R bands was also further supported (i) by the different compositional features of genes located in them; (ii) by the very low gene density of chromosomes 13 and 18, in which all R bands are $H3^-$ bands; (iii) by the com-

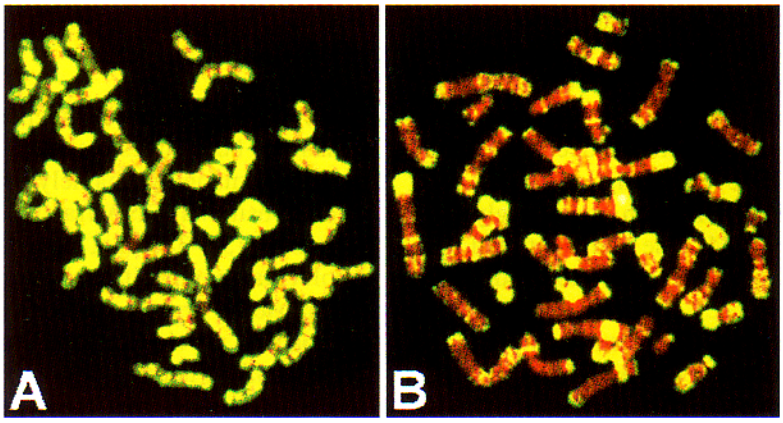

Figure 3. In situ *suppression hybridization with the isochores*. Human metaphases hybridized with the GC-rich isochores without (left) or with (right) 50 times excess of unlabeled shared Cot1 DNA. The probe was biotin-labeled by nick translation and hybridization was detected by avidin-fitc (yellow-green signals). Chromosomes were stained with propidium iodide (red).

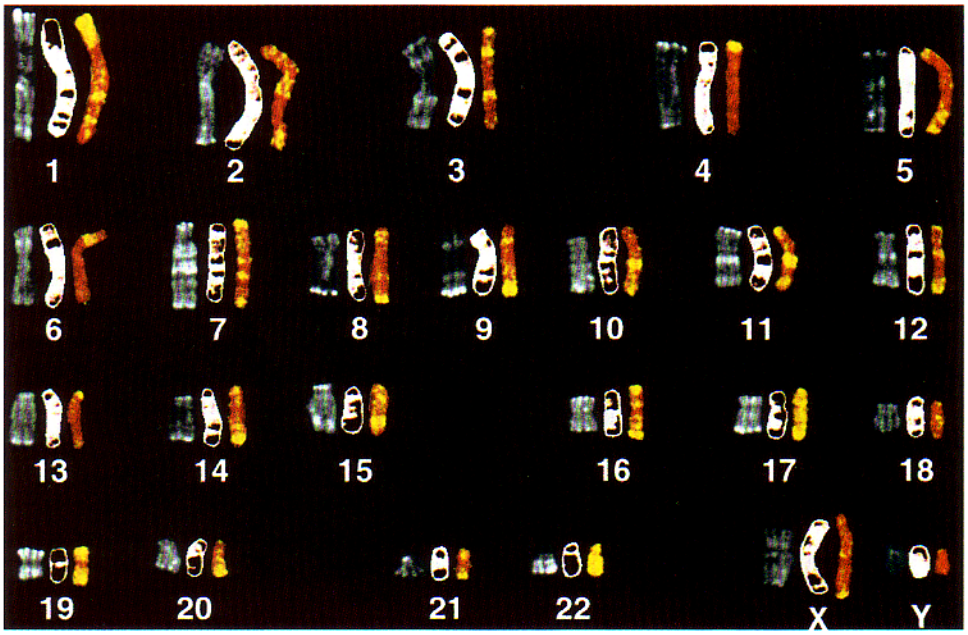

Figure 4. *Human chromosomes showing T, CMA3+/DAPI–, and H3 banding*. Comparative representation of the three coincident sets of bands: T banding (left), chromomycin A3 positive – DAPI negative (center); banding with the H3 isochores (modified from [3, 21, 37]).

positional map of a H3* band, Xq28; (iv) by the overwhelming presence of GC-rich and GC-poor long (>50 kb) sequences in $H3^+$/H3* and in $H3^-$/G bands, respectively; and (v) by the large degree of coincidence of $H3^+$ and H3* bands with CpG island-positive bands [13, 37]. Moreover, the chromosomal bands that contain H3 isochores replicate mainly (in the case of $H3^+$ bands), or largely (in the case of H3* bands) at the onset of S phase [25], whereas chromosomal bands containing no detectable H3 isochores ($H3^-$ bands) replicate later, and G bands replicate even later (Figure 5). Expectedly, CpG islands, which are most abundant in H3 isochores [1, 2, 30] were found to replicate coordinately early in S phase [15]. Interestingly, the R bands not containing H3 isochores seem to be characterized by GC levels very close, on the average, to those of G bands [37].

The GC-poorest bands

G bands, routinely shown as dark bands in ideograms, are composed, in fact, by four classes characterized at a 850-band resolution by different degrees of darkness [27]. For the sake of simplicity, we will call here the G bands endowed by the four level of grey as G1, G2, G3, and G4 bands, from the more pale grey to the black, respectively. The GC-poorest L1 isochores, hybridize on a subset of the G bands (Figure 6), which we called L1 bands, and that correspond to the G3 and G4 bands of Francke [27]. In contrast, L1 DNA is almost absent in the large

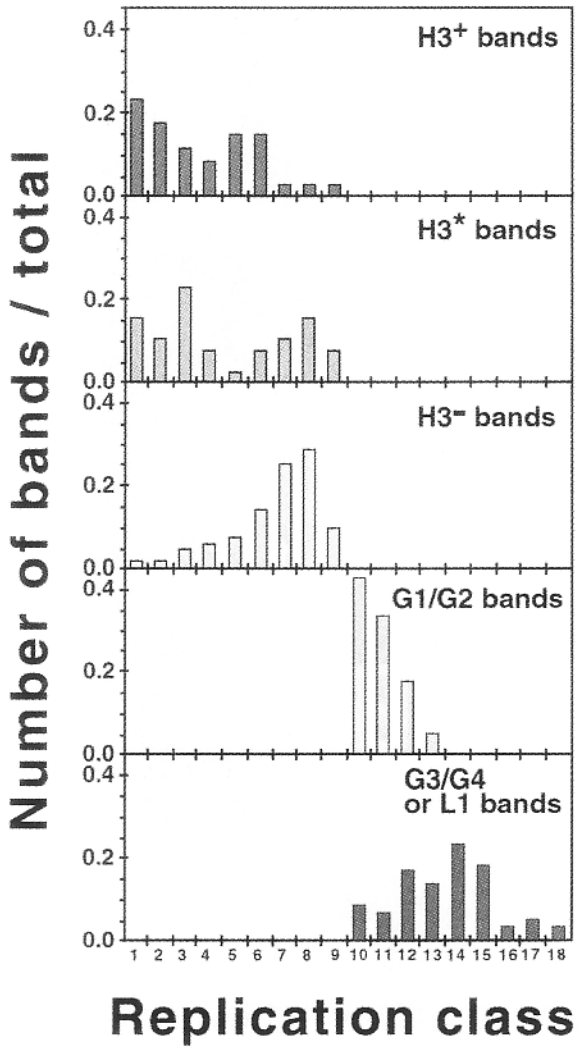

Figure 5. *Histograms with the replication timing of the chromosomal bands.* Histograms showing the human chromosomal bands as distributed in the replication classes described by [23] and [9] (Modified from [24, 25]).

majority of the H3$^+$ and H3* bands. This means that L1 isochores are absent where H3 isochores are present and *viceversa*. Thus, the banding pattern obtained with the H3 and the L1 isochores identifies chromosomal regions with the contrasting features of these two isochore families.

The intermediate bands

The same clear banding pattern was not obtained with the L2 and H1 isochore families, since these two isochore families are practically present on all the remaining G and R bands, namely on those bands not containing L1 and H3 isochores. However, the L2 and H1 isochores are more abundant in G1/G2 and H3$^-$ bands, respectively [25]. This indicates a very close GC level between these two sets of bands, a remarkable observation considering that these sets are cytogenetically seen as G or R bands, respectively.

The classification of chromosomal bands

The classical viewpoint, which has prevailed for the past thirty years, of two sets of chromosomal bands, G and R, needs to be modified. This is clearly the case for R bands, which comprise a very distinct set of bands endowed with the highest GC (and gene) levels, the H3$^+$ or T bands [3, 4, 21, 28, 35]. The H3 bands, namely those bands largely composed of H3 isochores, are characterized by the specific properties of this isochore family. For this reason, these bands could be distinguished from the rest of R bands. A further subdivision could be done among the H3 bands, between those with a very high levels of H3 isochores (the H3$^+$ bands) and the other ones with a low level of these isochores (H3*). In this way, the R bands could be considered as composed by three different sets of bands having very different GC levels from H3$^+$ to H3$^-$ bands. Moreover, the three sets of R bands identified on the basis of the relative amount of the H3 isochores were directly related to another crucial parameter, namely gene concentration.

The same observation could be done for the G bands, generally considered as very homogeneous as far as their GC levels are concerned. They are in fact composed by two very different sets of bands. One is very GC-poor, whereas the other one shows a relatively higher GC content, if we consider that the former contains the highest concentration of the GC-poorest isochore family and the latter contain both L2 and H1 isochores, with L2 isochores more abundant compared to H1 isochores [25]. Also the H3$^-$ bands are generally composed by L2 and H1 isochores but, in this case, L2 isochores are under-represented relative to the H1 isochores [25]. Thus, the G bands (more precisely the G1/G2 bands), share a DNA composition similar to the H3$^-$ bands, but the former seem to be still GC-poorer, on the average, relative to the latter. However, we observed that this is not a general rule in the human genome. In fact, as observed on chromosome 21, which is almost completely sequenced [29] and where the correlation between GC level and chromosomal bands could be easily established (Saccone et al., in preparation), there are G bands (G2 21q11.2 and 21q21.2) characterized by a GC level higher than that of R bands (H3$^-$ 21q22.12 and 21q22.2). Expectedly, also the gene concentration parallels the GC level of the above G3/G4 (our L1 bands) and G1/G2 bands, reaching a very low density in the former.

Thus, H3$^-$ and G1/G2 bands, in spite of their close composition belong into two cytogenetically very distinct classes of bands, R and G [25, 29]. This suggested that the basic difference between G and R bands is not simply due to differences in base composition, contrary to a long-standing explanation (see [12]), but, in addition, the regional GC level is important to produce a G or an R band, as demonstrated

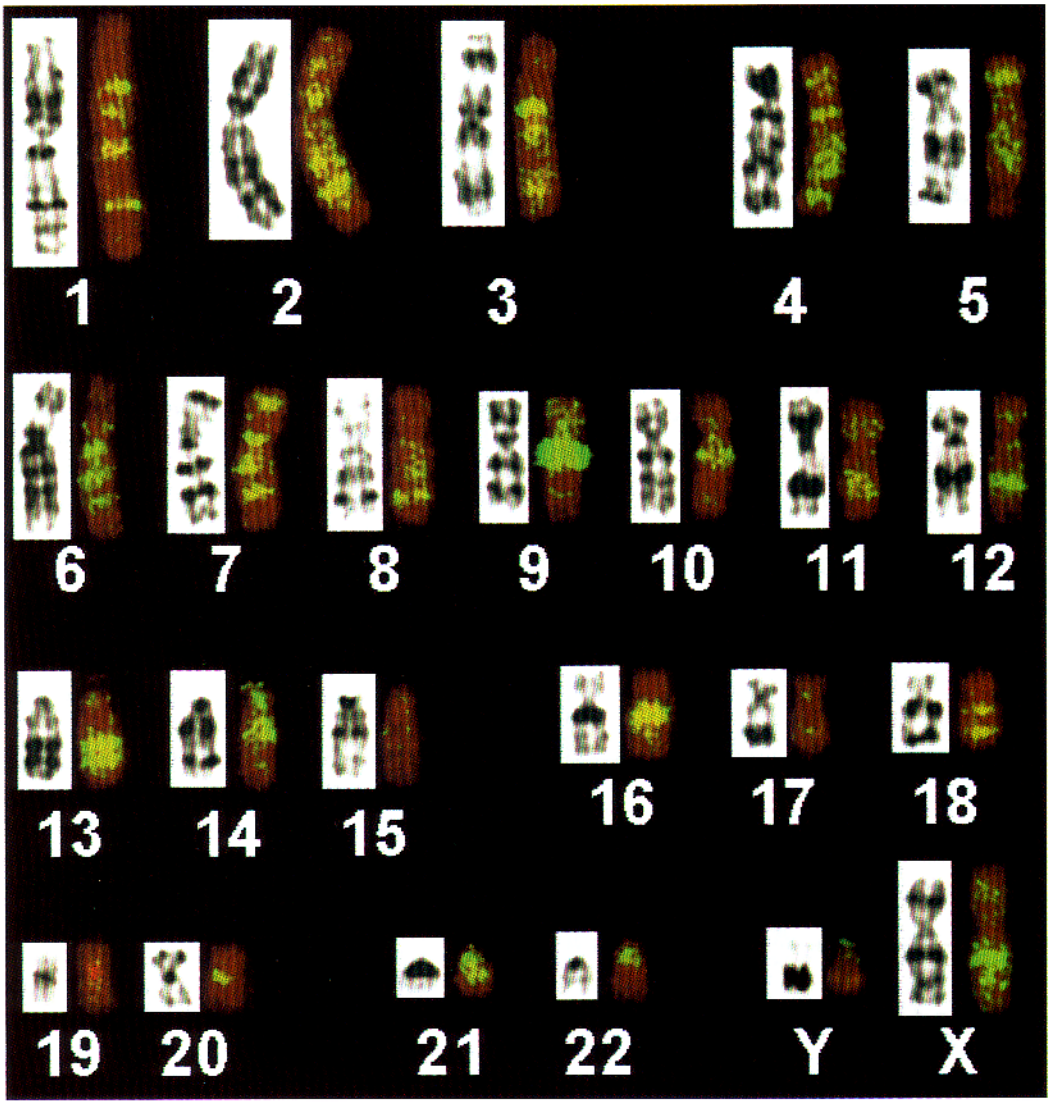

Figure 6. *Painting with L1 isochore DNAs.* The left and right member of each chromosome pair show the GTG bands and the L1 isochore hybridization, respectively. Biotinylated L1 isochores were detected with avidin-FITC. Chromosomes were stained with propidium iodide (From [25]).

by bands on chromosomes 21 and 22. An alternative interpretation of the differences between G and R bands is that G bands are compositionally more homogeneous, endowed with a closer chromatin structure and with a higher DNA packing than $H3^-$ bands [4].

In conclusion, considering the DNA composition, we have identified two very different chromosomal compartments the GC-richest and the GC-poorest. Interestingly, the GC-richest bands are preferentially located on telomeric regions and the GC-poorest bands in internal regions of chromosomes (Figure 7). Moreover, the contiguity between GC-poorest and GC-richest bands is very rare. This particular distribution of the GC-richest and the GC-poorest DNA is probably relevant as far as the evolution of chromosome structure is concerned (paper in preparation). As expected, these two compartments have contrasting features since the former replicates earlier, is gene-richer, is composed of open chromatin and is more recombinogenic and transcriptionally active compared to the other. Between these two compartments, there are the other bands endowed with an intermediate GC level. In this latter case, a defined chromosomal region will be cytogenetically a G or an R band (more precisely, a G1/G2 or an $H3^-$ band) not only on the basis of their GC level, but also of the GC level of the adjacent regions (Saccone et al., paper in preparation).

Chromosomal painting with the isochores: conclusions

The *in situ* hybridization of DNA fractionats belonging to the GC-poorest and the GC-richest isochore families, previously used to identify the chromosomal regions endowed by different gene concentrations, is a novel approach to obtain specific band patterns (see Figures 4 and 6). In fact, when

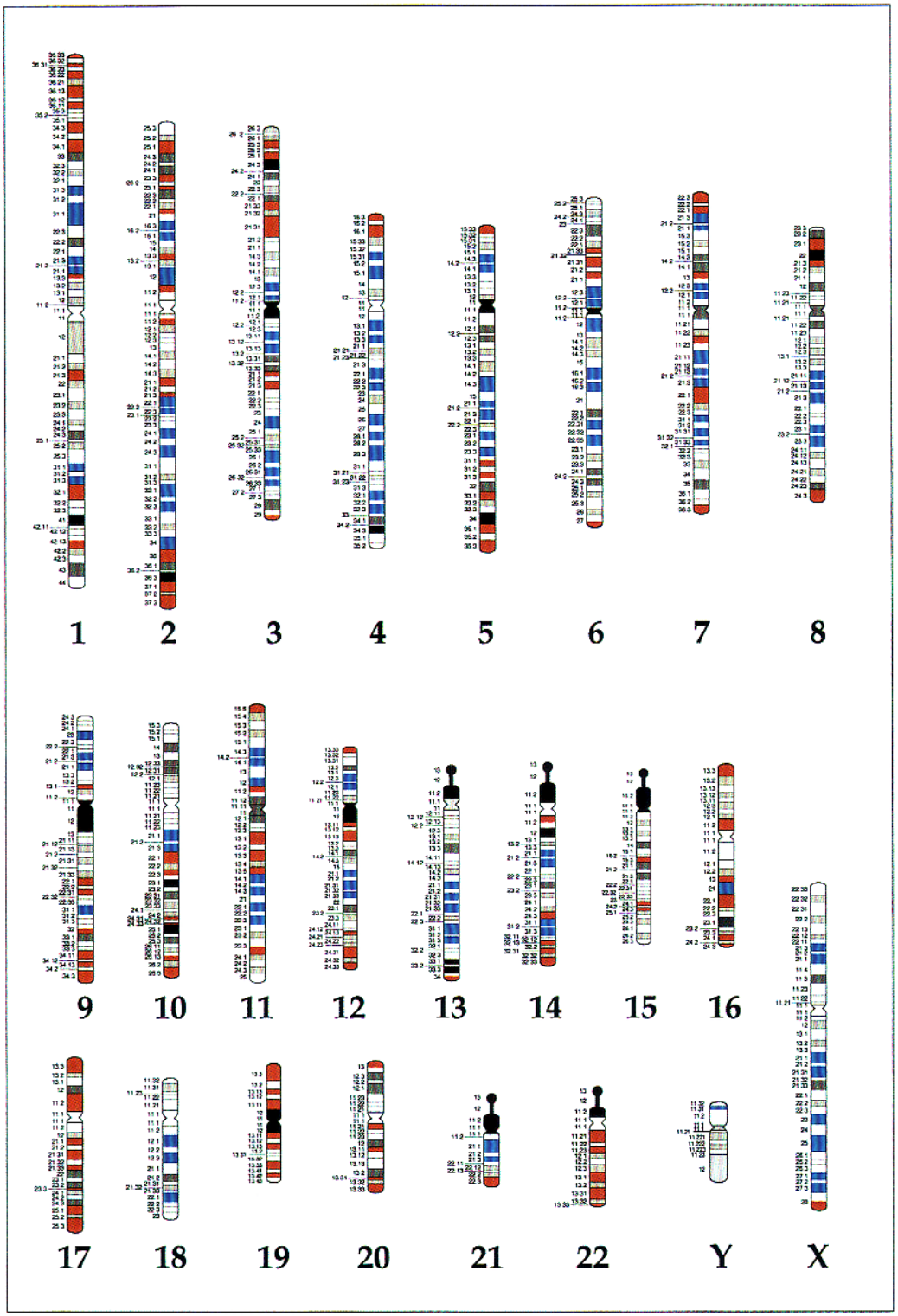

Figure 7. *Human chromosome ideograms showing the H3 and L1 bands.* Human karyotype at a resolution of 850 bands per haploid genome showing the chromosomal bands containing the GC-poorest (blue bands) and the GC-richest isochores (red bands). The grey scale of the G bands is according to [27]. The GC-richest isochore bands are from [39] (Modified from [25]).

DNA from the GC-richest isochores is used as a probe, a H3 banding is obtained. Instead, to obtain the entire set of the R bands, it is necessary to hybridize a mixture of H3, H2, and some H1 isochores (see Figure 3 in 36). In this way the hybridization covers all the $H3^+$ bands and the remaining R bands, where the H2 and the H1 isochores are also located.

In the case of the hybridization with the GC-poorest DNA, namely the L1 isochores, a subset of G bands could be easily identified and more precisely those G bands characterized by the highest level of

grey, as indicated by Francke (1994). At the moment, it was not possible to obtain the total pattern of G bands using isochore hybridization (except by difference from the R bands), the isochores present in the G1/G2 bands being also present, even if to a lower extent, in the H3 bands. A finer DNA fractionation that will lead to a better separation of the L2 and the H1 isochores could be the way to highlight the intermediate bands, namely the G1/G2 and the H3 bands.

Methods

DNA preparation

Isochore DNAs are prepared by fractionating high molecular weight DNA extracted from human placenta. Genomic DNA was ultracentrifuged in a Cs_2SO_4/BAMD preparative density gradient [14]. BAMD is 3,6-bis(acetato-mercurimethyl) 1,4-dioxane) and is subdivided in a number of fractions (usually 10–12). These fractions are analyzed in analytical CsCl gradients to assess the isochores they contain. Aliquots of the above fractions were generally labeled by nick translation and used for *in situ* hybridization.

Chromosome preparation and in situ *hybridization*

In situ hybridization with isochore DNA is essentially performed as a normal chromosome painting. Chromosomes are generally prepared by standard cytogenetic techniques and treated, before hybridization, with RNase, pepsin, and formaldehyde, to enhance accessibility of the probe, and to have a good signal to noise ratio. A classical chromosome painting with isochore DNA, that lead to good quality chromosomal bandings, is made with 200 ng of a compositionally fractionated DNA supplemented with 50 times excess of unlabeled Cot1 DNA to avoid unspecific signals due to the repetitive sequences present in the probe. Hybridization is made using a mixture of 2× SSC, 10% dextran sulphate, 50 mM phosphate buffer at pH 7.0, at 37 °C for at least 16 hours in a humidified box. The post-hybridization washes are generally done in 0.1× SSC at 60 °C (three times for a total of 30 min). Then, an incubation of the slides with blocking reagents (non-fat dry milk, bovine serum albumin, etc.) is necessary before the incubations with conjugated avidin or specific antibody (in the case of indirect detection of the hybridization signals), to reduce the unspecific binding of the fluorescent reagents. Chromosomes could be stained with propidium iodide or DAPI, according to the fluorochrome used to detect the hybridization.

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