Patterns in the Genome

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Abstract: Our genome is not randomly organised, with respect to both the linear organisation of the DNA sequence and to the spatial organisation of chromosomes. Here I discuss how these patterns of sequence organisation were first discovered by molecular biologists and how they relate to the patterns revealed decades earlier by cytogeneticists and manifest as chromosome bands.

As a postdoc in the late 1980s, I was trying to track down the gene responsible for a human genetic disease. It is hard to imagine now but, at that time, there was no human genome sequence, there was not even a map of the human genome. You had to construct your own maps using the laborious methods of chromosome walking and jumping using combinations of cosmid and YAC libraries screened with specific probes, and long-range restriction maps made using rare-cutter enzymes and pulsed field gel electrophoresis. These are methods now largely consigned to the dustbin of history – although pulsed field gels are still useful for sizing chromosomes that are up to a few Mb in size (e.g. from yeasts).

I was rather proud of my map – a total of about 7Mb in size. So where were the genes located? The assumption was that the disease I was interested in was caused by a mutation in a protein coding gene – almost no attention was paid to the non-coding genome in those days. One of the few ways to identify genes in long stretches of human DNA was to look for the unusual sequence content found at the 5' end of a large proportion (70%) of human genes – regions where there is no depletion of CpG relative to GpC, and where CpGs are unmethylated. These are CpG islands or CGIs (Deaton and Bird, 2011). I had noticed a strange pattern to my map – all the CGis were squashed down one end of it and I had no idea why.

My story might have ended there if it were not for the serendipity arising from institute building renovations. I never did manage to clone that gene I was after, but during the laboratory refurbishments I was relocated temporarily to the cytogenetics department of the institute I worked in – at that time called the MRC Clinical and Population Cytogenetics Unit (CAPCU; https://www.ed.ac.uk/mrc-human-genetics-unit/about/mrc-hgu-history). For a young gun-slinger of molecular biology who could clone, map and resolve DNA fragments of the human genome 100s of kilobases in length, looking down the light microscope at chromosomes seemed a deeply uncool and primitive activity. However, once I got the chance to see banded human metaphase chromosomes with my own eyes I was hooked by their beautiful structures, and, somewhere in my brain a connection was made between the pattern I had in my small genome map, and the transverse patterns of chromosome bands manifest in

plain sight along the length of the human genome by chromosome banding techniques (Craig and Bickmore, 1993).

At that time, journal clubs were a very important activity in the institute. If it was your turn to do journal club you retired to the library for a week with a bunch of acetates, photocopies and bound volumes of old journals and researched the background to your chosen paper carefully. John Evans, the Director of CAPCU - who himself had contributed to the development of early chromosome banding techniques, suggested to me that I might be interested in presenting a paper by Julie Korenberg and Mary Rykowski (Korenberg and Rykowski, 1988) that used the emerging technique of fluorescence in situ hybridisation (FISH). They had cleverly combined FISH with new developments in charge-coupled device (CCD) camera technology to investigate the genomic distribution of different classes of sequence across the human genome using metaphase chromosomes spread on glass slides as a visual readout of relative position in the genome. This suggestion from Joh Evans and reading the work of Korenberg and Rykowski proved to be a pivotal moment that has influenced my entire independent research career.

The Korenberg and Rykowski experiment

Approximately one-third of the human genome is composed of interspersed repeated sequences that fall into two main families – short - and long – interspersed repeat elements (abbreviated as SINEs and LINEs, respectively). The most abundant SINEs in the human genome are Alu elements - dimers of 7SL RNA-derived sequences (Kojima, 2018). The non-LTR retrotransposon L1 is the predominant LINE. By hybridising biotin-labelled probes detecting the consensus Alu and L1 repeats to human metaphase chromosomes stained with 4,6-diamidino-2-phenyl indole (DAPI), and detecting the hybridisation signals with streptavidin conjugated to Texas Red, Korenberg and Rykowski were able to see that Alus and L1s were non-uniformly distributed along the chromosomes, and hence along the genome sequence. Moreoever, their hybridisation patterns corresponded with classical chromosome bands - Alus were concentrated in the so-called Reverse or R-bands, and L1s in the alternating Giemsa or G-bands (Korenberg and Rykowski, 1988). Good examples include the high concentration of Alus at the distal tip of the short arm of chromosome 1 (1p34-p36) and the middle of the short arm of chromosome 6 (6p21) and the depletion of L1s from these same regions (Figure 1). These regions are called T-bands, which are the most intensely stained and most GC-rich fraction of R bands (Craig and Bickmore, 1993).

This paper revealed that there is a non-random distribution of DNA sequence (in this case interspersed repeats) along the human genome and that this relates to the visible compartmentalisation of chromosomes in the form of mitotic chromosome bands. It also exemplified the compelling nature of the visual image and showed that through the use of CCD cameras, which provide a linear measure of signal intensity over a large dynamic range, imaging with fluorescent light could be quantitative.

Visualising the distribution of genes along the human genome

Inspired by the Korenberg and Rykowski experiment, and given that the genome map I had been making had shown an apparently uneven distribution of CGIs, myself and my first PhD student – Jeff Craig – decided to see if we could take a similar approach to ask how genes are distributed across the human genome. As a probe to detect human genes, we used the small restriction fragments liberated from the human genome by the CpG methylation sensitive enzyme HpaII (CCGG). These HpaII tiny fragments (HTFs) originate mainly from CGIs (Bickmore and Bird, 1992) at the promoter of approximately 70% of human genes. Hybridising this fraction of the genome to metaphase chromosomes together with a probe (late replicating DNA) for the inactive gene-poor portion of the genome dramatically revealed the concentration of human genes - or at least those associated with CGIs - in specific chromosomes bands, particularly in T-bands (Craig and Bickmore, 1994) (Figure 2). As with the Alu hybridisation pattern (Figure 1), a high density of CGIs is seen on the distal tip of the short arm of chromosome 1 and the middle of the short arm of chromosome 6. Other striking features of the the hybridisation patterns that we saw is the high density of CGIs on human chromosome 19 and the paucity of them on human chromosome 18. This eventually led us to investigate the organisation of these two chromosomes in the nucleus and the discovery that human chromosomes are non-randomly organised in the nucleus and have a radial organisation with the mouse gene poor chromatin localised toward the nuclear periphery (Croft et al., 1999; Boyle et al., 2011).

By digesting the human genome with restriction enzymes that cleave, on average, once per CGI we also used pulsed field gel electrophoresis to isolated fractions of the human genome with ever decreasing CGI density, where inter-island distances were 15-100, 100-500, 500-1000, 1000-6000kb. The hybridisation pattern to metaphase chromosomes rom the fraction with the shortest inter-CGI distances (15-100kb) was very similar to that of the HTFs – i.e. concentrated in T-bands. Fractions with inter-island CGI distances of 100-500kb highlighted the other R-bands. G-bands were lit up by fractions of the human genome with extremely low

CGI density (>1Mb between CGIs). There is a similar organisation on rodent chromosomes (Cross et al., 1997). We therefore concluded that mammalian genomes have a non-random organisation, with genes concentrated together into specific regions of chromosomes that are manifest as T- and R-bands. This organisation was later borne out when the sequencing of the human genome was finally completed (Lander et al., 2001).

The function of this level of linear genome organisation remains unclear. Whilst, there may be a functional advantage to keep broadly and highly expressed genes clustered together in chromosomal domains (R-bands) (Sproul et al., 2005), gene 'deserts' may be the location of complex regulatory landscapes for genes with intricate roles and patterns of expression during development, and populated by multiple enhancers – non-coding regulatory elements (Salzburger et al., 2009).

Investigating interphase chromosome structure using visual assays

The striking hybridisation patterns of repeated sequences and genes along chromosomes demonstrated that human mitotic chromosomes spread on a glass slide could be an ordered visual readout of primary genome sequence organisation – a kind of 'poor man's microarray. Mitotic chromosome spreads can also report on functional aspects of genome organisation that occur during the preceding interphase. The most striking example of this is replication banding, revealed by the incorporation of thymidine analogues – most typically 5-bromo-2'-deoxyuridine (BrdU) – into the genome during defined periods of S-phase (Dutrillaux et al., 1976; Vogel et al., 1989). The period of S-phase is normally controlled using drugs that interfere with S-phase progression. Detection of the thymidine analogue on the metaphase chromosomes formed after S phase showed that the human genome sequence is organised into domains (bands) that replicate at defined periods of S phase. Domains that correspond to genomic regions with analogue incorporation during the early period of S phase (early replicating) were shown to correspond to R-bands and later replicating bands correspond to G-bands (Figure 2). Moreover, T-bands replicate on average earlier than ordinary R-bands (Drouin et al., 1994).

Immunofluorescence with antibodies detecting histone post-translational modifications has also been used to explore the distribution of different epigenetic states along metaphase chromosomes revealing, perhaps unexpectedly given their high gene-density, that R-bands are more enriched in acetylated histones than G bands (Jeppesen and Turner, 1993) and that other specific modifications of histone H3 found at the promotes of active genes (H3K9ac, H3K27ac, H3K4me3) in the interphase genome are also quantitatively enriched at R-bands

during mitosis (Terrenoire et al., 2010). Thus, despite the general cessation of mRNA transcription during mitosis, aspects of the epigenome associated with active genes are inherited to daughter nuclei through mitotic chromosomes. Contemporary research is now extending this concept of mitotic inheritance to binding of transcription factors (Festuccia et al., 2019) and the core transcriptional machinery itself (Teves et al., 2018).

Developing this idea further, we went on to use hybridisation to metaphase chromosome spreads to begin investigating different aspects of the structural organisation of the interphase genome. We first tried to analyse how different parts of the human genome may attach to various substructures within the nucleus. Such structures are defined by different biochemical extraction methods and termed the nuclear matrix, nuclear scaffold and nuclear skeleton. Typically, <10% of genomic DNA remains attached to these sub-structures after extraction (Craig et al., 1997). Regions of the genome corresponding to G-bands appeared to have more frequent attachments to the nuclear matrix (remaining after high salt extraction) and the nuclear scaffold – a residual structure after extraction of nuclei with the anionic detergent lithium diiodosalicylate. This result is consistent with the suggestion that sites of attachment to a nuclear and mitotic chromosome scaffold would be most frequent in G-bands (Saitoh and Laemmli, 1994). In contrast, genomic regions corresponding to R-bands had more attachments to the nuclear skeleton, the substructure remaining inside nuclei after electroelution of unattached sequences (Craig et al., 1997). Nuclear skeleton preparation methods were designed to preserve more functional aspects of interphase function (transcription and replication) by avoiding the use of high salt or detergents that might cause protein aggregation (Jackson et al., 1988). Therefore, our finding of nucleoskeleton associations being more frequent in the gene-rich DNA/R-band fraction of the genome is consistent with a link between the aspect of nuclear organisation probed by this method and gene regulation.

A lot of the controversy that surrounded the field of nuclear organisation in the 1980s and 1990s arose from the fact that no one really knew what the various methods used did to native chromatin structure and nuclear architecture, nor which method – if any – might give the most physiologically relevant result. In an attempt to apply a more biophysical approach to higher-order interphase chromatin organisation, and to examine chromatin fibres per se, rather than their association with nuclear substructures, we used micrococcal nuclease (MNase) digestion and sucrose gradient sedimentation to separate fractions of the human genome with more open or more compact chromatin fibres. Chromatin sedimenting fast for its mass has a compact hydrodynamic shape, and when hybridised to metaphase chromosomes was shown to originate from the gene-poor (G-band) fraction of the genome (Gilbert et al., 2004). The

slow sedimenting most 'open' chromatin fractions originated from the genomic regions corresponding to the most gene-rich T-bands – see chromosome 1 in Figure 3.

From chromosomes to microarrays

The experiments described above give a consistent picture of the human genome as organised into distinctive compartments with genes most concentrated together into domains that replicate early in S phase and packaged into chromatin fibres with an open disordered structure. These are manifest on metaphase chromosomes as T- and R-bands. The other euchromatic fraction of the genome (G-bands) has a very low gene density, replicates in the second half of S phase and is packaged into compact chromatin fibres. However, this broad compartmentalisation is very coarse-grained due to the limited resolution (5-10Mb) of metaphase chromosomes.

Progress toward higher resolution analysis came with the advent of genomic microarrays. These were initially often composed of arrayed BACs or PACs tiled across the genome and had limited resolution (Mb) (Ishkanian et al., 2004; Lodén and van Steensel., 2005; Woodfine et al., 2005), but the spotting of oligonucleotides at high density allows for higher-resolution interrogation of specific genomic regions. Early applications of this approach were the determination of replication timing domains (Repli-chip), allowing for the borders between early and later replicating domains to be demarcated in a way that was not possible by hybridisation to metaphase chromosomes (Ryba et al., 2011), and study of the distribution of specific histone modifications across the genome purified by chromatin immunoprecipitation (Kondo et al., 2004). We used this approach to improve the resolution of our mapping of open and closed chromatin fibres in the human genome (Gilbert et al., 2004). A related approach (Weil et al., 2004) assessed the genomic distribution of chromatin compaction states from the differential solubility of MNase digested chromatin to $MgCl_2$ and KCl.

Bas van Steensel and colleagues devised a new method based on targeted adenine methylation of DNA - DAMID - to determine domains of the genome that come into close contact with a protein of the nuclear lamina – Lamin B1. Originally applied to study of the Drosophila genome, they went on to perfom DAMID for regions of the human genome associated with Lamin B1, hybridising the resulting DNA adenine methylated by a LaminB1-Dam fusion protein compared with that methylated by free Dam, to high-density microarrays across the entire human genome (Guelen et al., 2008). This revealed 1,000 to 1,500 large (on average 0.5 Mb) domains of gene-poor, CGI-poor, inactive, late replicating chromatin that preferentially associate with lamin B1 and that are often seen by FISH to locate at the nuclear periphery – i.e. they have the characteristics of G-bands.

Where are we today

Fast forward to the modern day and we are, of course, in the world of high-throughput sequencing which can be applied to any genome-wide assay of chromosome organisation and function whose output is a sequenceable library of DNA fragments. This has opened the door to a plethora of high-throughput genome-wide assays of genome organisation, whose resolution is potentially very high, dependent on the details of the assay and the depth of the sequencing. Many of these assays - e.g. DAMID - were developments of techniques originally designed to be read-out on microarrays, but are now being assayed by high-throughput sequencing and are even being applied to study genome organisation in single cells (Kind et al., 2015). High-resolution analysis of replication timing by sequencing (Repli-seq) (Marchal et al., 2018) has allowed identification of domains of the genome replicating at defined periods of S phase – of approximately the same size as LADs. Moreover, as for DAMID-seq, high sequencing depths have facilitated a precise mapping of the transition regions between domains.

The most notable new technology that has opened our eyes to the compartmentalisation of the mammalian genome is Hi-C. Two levels of compartmentalisation are generally considered – the division of the genome into A and B compartments, that tend to cluster with in the nucleus – A with A and B with B. These categories seem to generally correspond to early replicating T/R bands (A compartment) and late replicating G bands (B compartment) (Lieberman-Aiden et al., 2009; Ryba et al., 2010; Kalhor et al., 2011). Very high resolution Hi-C also revealed the organisation of the mammalian and Drosophila genomes into self-interacting domains – topologically associating domains (TADs) (Dixon et al., 2012; Sexton et al., 2012; Eagen et al., 2015). In mammals, very elegant mechanistic experiments and modelling have shown that TADs are formed by a process of loop extrusion by the cohesin complex, interrupted by particular orientations of CTCF sites (Nuebler et al., 2018). In Drosophila, the formation of TAD boundaries is less well understood (Ramirez et al., 2018). There are at least 2000 TADs in the mammalian genome, though its hard to give a precise number because of the presence of sub-TADs within TADs. However, there is a very good correspondence between TADs, LADs and replication domains, with the transition points between replication domains aligning well with TAD boundaries (Pope et al., 2014). So a set of specific structural and functional properties (gene density, replication timing, lamin-association and intra-and inter-TAD interactions) seem to co-segreate with each other across the genome and with the properties of different metaphase chromosome bands. So are they one and the same thing, are we – using the powerful tool box of modern molecular biology – just rediscovering

chromosome bands. Without very high-resolution mapping by FISH to metaphase chromosomes it is hard to be sure, but certainly the overall number of TADs/replication domains/LADs (one to two thousand) in the human genome is very close to the number of chromosome bands that have been seen on early prometaphase chromosomes (Yunis, 1981; Drouin and Richer, 1989) (Figure 4).

Similarly, there is a remarkable correspondence between TADs and TAD boundaries and the bands and inter-bands seen on Drosophila polytene chromosomes (Eagen et al., 2015)., and indeed a correspondence to chromomeres - locally coiled chromatin domains that had been seen decades ago by microscopy on mitotic and meiotic prophase chromosomes (Macgregor, 2012; Eagen, 2018) and which have also now been revealed in the interphase nucleus of diploid cells using FISH and the latest super-resolution optical imaging methods (Szabo et al., 2018).

So maybe there is nothing really new in biology and we are often just rediscovering principles and phenomena that were first revealed many decades before the advent of the latest molecular and cellular technologies. However, we now have the ability to reveal and resolve these structures at unprecedented levels of resolution, in different cells at different stages of development or in disease states. Moreover we have the tools to begin manipulating these levels of genome organisation. That surely must remain the grand challenge for genome organisation – what do all of these domains, compartments and structures mean for genome functions.

Footnote: This article is dedicated to the memory of Herbert Macgregor who sadly died in 2018. Herbert truly appreciated the beauty of chromosome structure and he tirelessly dedicated his time and effort into promoting the field of Chromosome Biology. He founded the journal *Chromosome Research* in 1992, he edited it for 20 years and I had the pleasure of serving alongside him on the Editorial board of that journal.

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Figure Legends

Figure 1. **FISH reveals the distribution of Alu and L1 repeats on human chromosomes 1 and 6**. A) Left; Alu hybridisation signal (white) for human chromosome 1 (top) and 6 (bottom). Right; Ideogram of chromosomes with T bands (the most extreme R-bands) in red,

R-bands in white and G-bands in black. B) Left; L1 hybridisation signal (white). DAPI-stained chromosomes are shown on the left (R-bands are pale). Adapted from Korenberg and Rykowski (1988).

Figure 2. **FISH reveals the distribution of CpG islands across the human genome**. For each metaphase chromosome, the hybridisation signal from CpG islands (red) is shown on the left of each pair. DAPI stained chromosomes are on the left. Late replicating G bands are shown in green. Modified from Craig and Bickmore (1994).

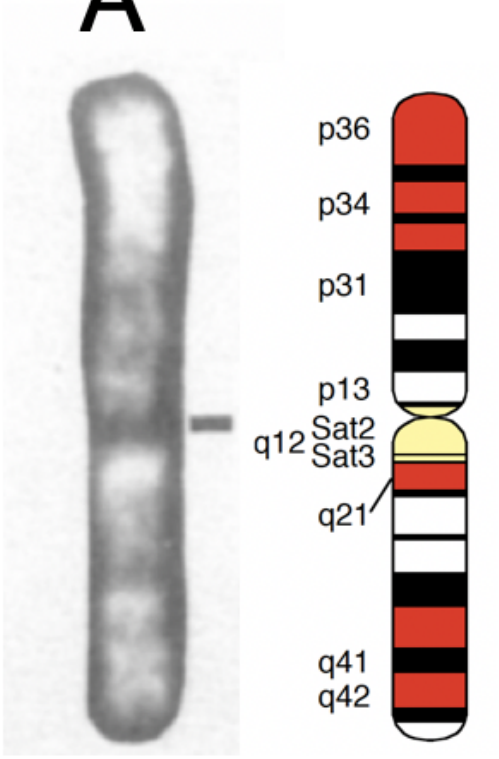
Figure 3. **FISH reveals the distribution of the open chromatin fibres on human chromosome 1.** A) Left; Hybridisation signal (green) from the open chromatin fraction on a DAPI stained human chromosome 1. Right; Ideogram of chromosome 1 with T bands in red, R-bands in white and G-bands in black. Adapted from Gilbert et al. (2004).

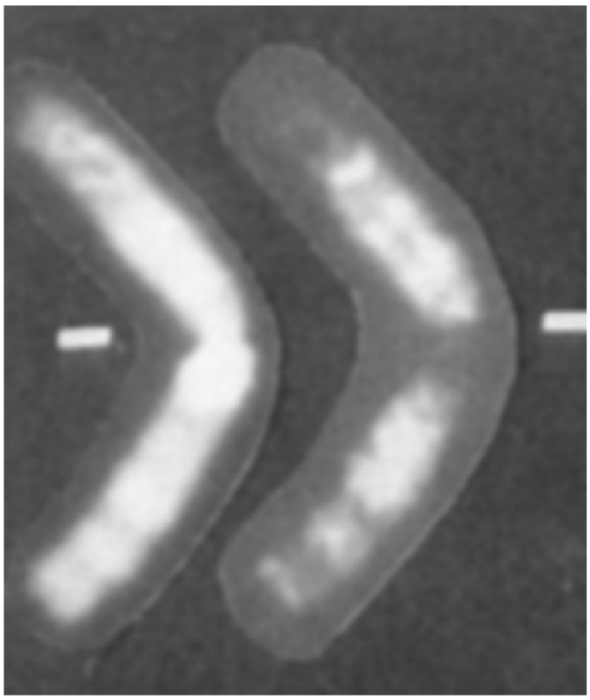
Figure 4. **High resolution human chromosome bands.** Ideograms of G- and R-banded human chromosome 11 at increasing levels of resolution. Up to 2000 G-bands have been recorded and 1,250 R-bands.

A

B

1





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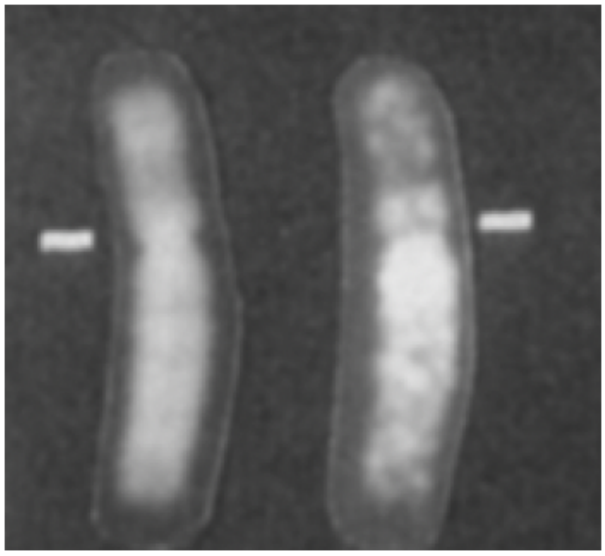

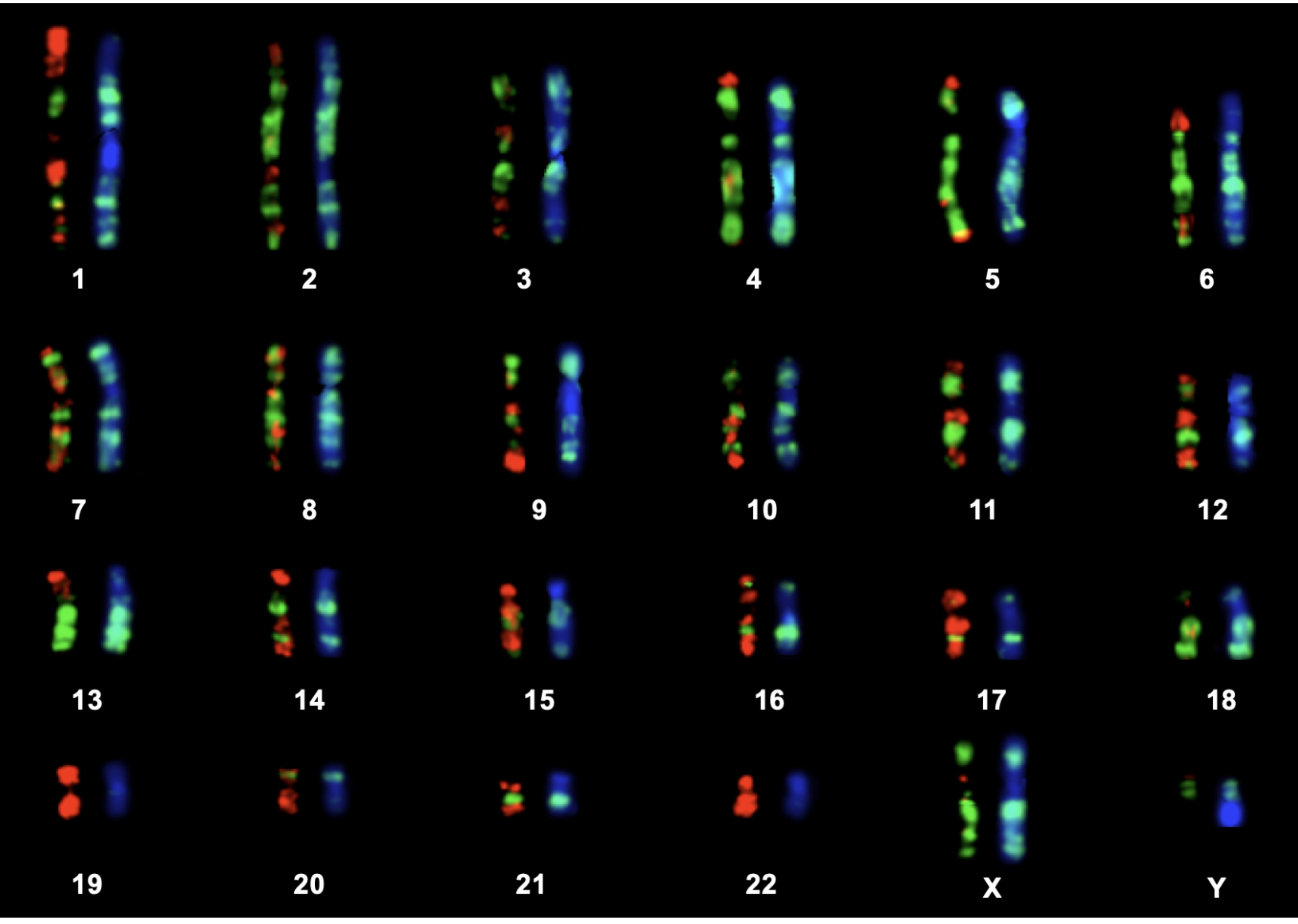
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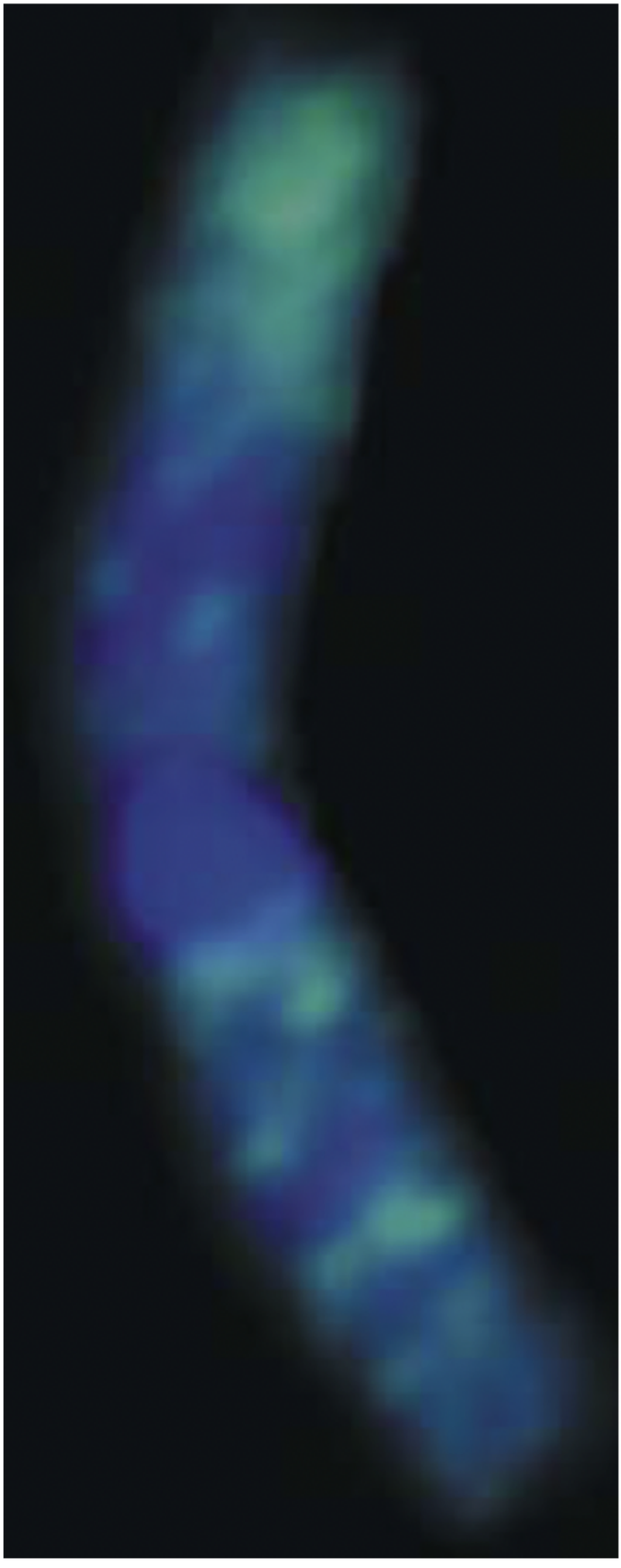

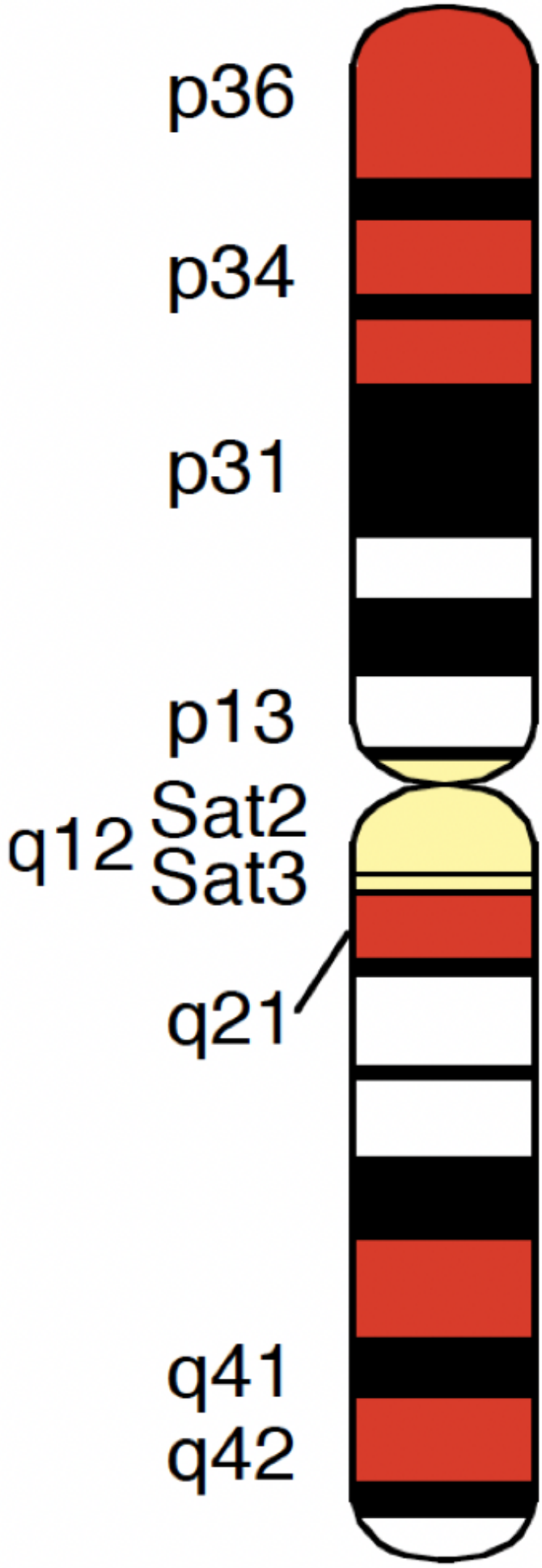
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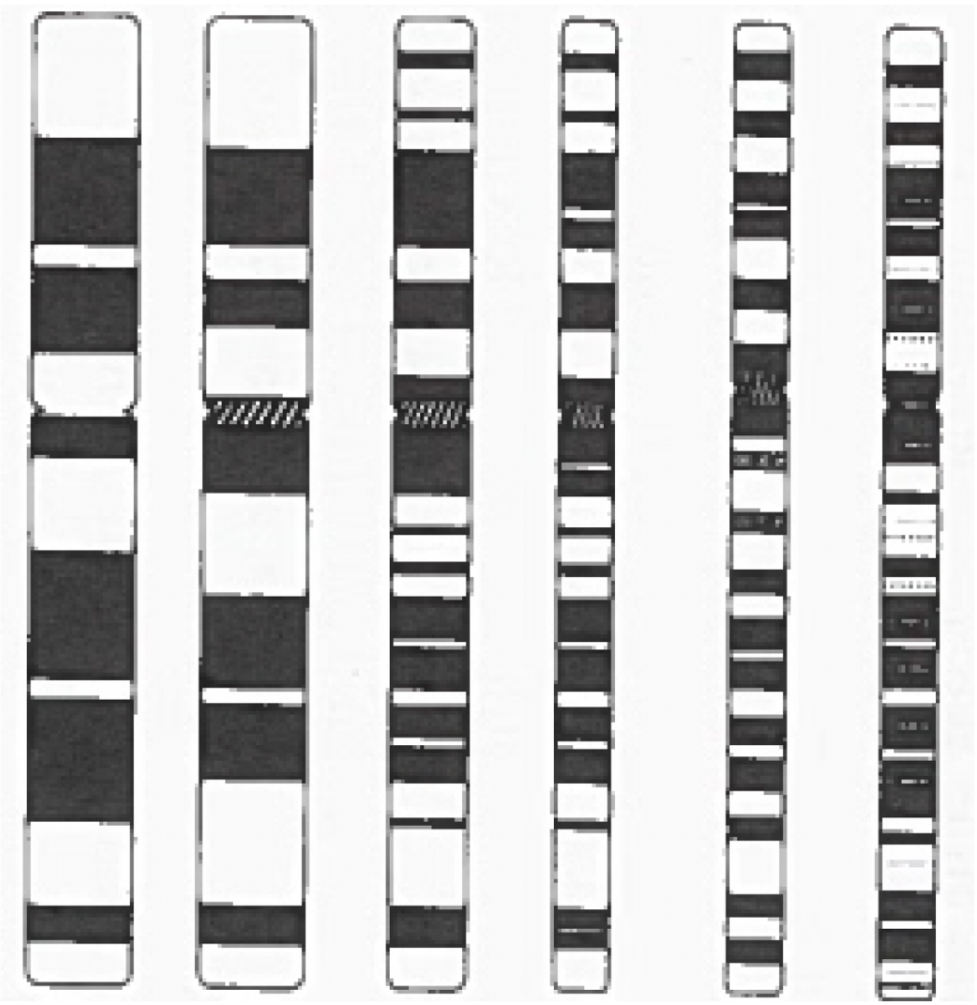
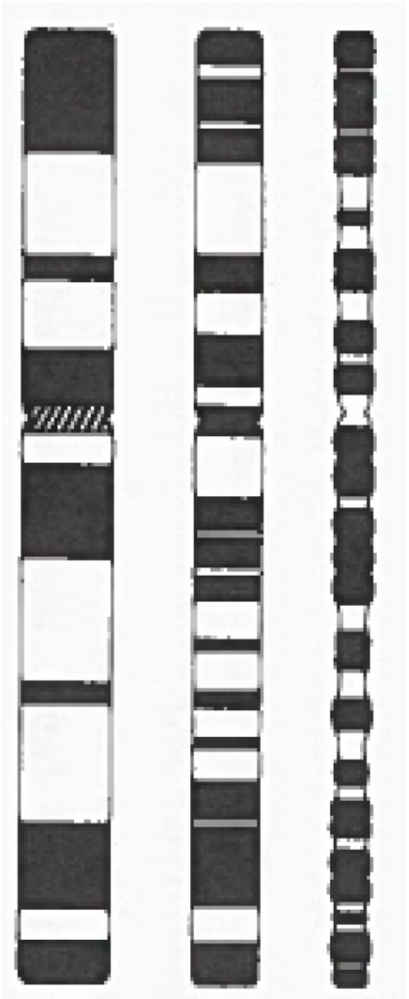


350 400 550 850 850 2000

G bands



350 550 1250

R bands