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Human Chromosomes: Analysis by Fluorescence *in situ* Hybridization

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I. Introduction

Research on human chromosomes has been ongoing for over a century. Until 1956, the number of human chromosomes was believed to be between 37 and 48. The correct number of chromosomes of 46 in a human somatic cell was finally determined by Tjio and Levan [18], using embryonic cultured cells. This result was rapidly confirmed by studies of testicular material [7]. These two articles stimulated a renewed interest in human cytogenetics. In the following years, cytogenetic study in the association of chromosomal abnormality with congenital disorders and malignant tumors, for example, Down's, Klineferter's and Turner's syndromes, and leukemias, has been developed. In the late 1960s and early 1970s new staining techniques were discovered that have made it possible for human cytogenetics not only to specify every chromosome but even to identify exactly parts of chromosomes that had been moved to unusual locations in the genome. This came about because the new techniques disclosed that each of the chromosomes possesses a unique banding pattern. A band is defined as part of a chromosome that is clearly distinguishable from its adjacent segments by appearing darker or lighter as a result of the new staining methods. These chromosome banding methods have been essential tools in the analysis of human genome, where the limitations of classical genetics have meant that the disciplines of cytogenetics and somatic cell genetics have been instrumental in localizing genes involved in inherited disease. Moreover, *in situ* hybridization is a strong tool to investigate and understand the molecular basis for chromosome banding itself as well as genomic organization [14, 19, 21]. Recent advances in these methodologies have for the first time enable us to see how the human genome is organized below the level for classical cytogenetics and visible chromosomes.

II. Chromosome Banding

In humans, a variety of treatments causes metaphase chromosomes to appear as a series of light and dark staining bands, such as the Giemsa (G) or Quinacrine (Q) bands, the Reverse (R) bands, and the Centromeric (C) bands. These bands are produced in metaphase chromosomes with fluorescence dyes, proteolytic digestion, or differential denaturing conditions. In G-banding, the chromosomes are subjected to controlled digestion with trypsin before Giemsa staining which reveals alternating positively (dark G-bands) and negatively staining regions (pale G-bands). The bands themselves may be a reflection of chromosome packaging. The banding patterns observed in a human cell depends on the degree of condensation of the chromosomes which in turn depends on the stage of cell division the cells have reached. In mid-prometaphase up to 2,000 light and dark bands can be seen along human chromosomes but by prometaphase only 850 are visible [11]. An identical banding pattern of Q-bands can be seen when the Giemsa stain is replaced with a fluorescent dye such as quinacrine which intercalates between bases of DNA.

Since the discovery of the differential stainings, it has become clear that the G/Q and R classes are associated with a broad range of inverse functional and biochemical attributes [3, 4, 10]. Although the average base composition of human genomic DNA is about 40% guanine plus cytosine (GC), the alternating pale and light bands are thought to reflect the compartmentalization of the human genome into isochores, defined chromosomal regions in which the base composition of the DNA is comparatively homogeneous but which is variable between isochores [2]. The G/Q bands are relatively rich in adenine plus thymine (AT), replicate their DNA late during the DNA synthetic period, condense early during mitosis, and reflect the meiotic chromomere pattern. In addition, they are thought to be relatively poor in expressed genes. In contrast to the G/Q bands, the R bands are relatively rich in GC, replicate their DNA early in the DNA synthetic period, and condense late in mitotic prophase. The R-G/Q band junctions, are the predominant exchange sites, including spontaneous translocations, spontaneous and induced sister chromatid exchange, and the chromosomal abnormalities seen after X-ray and chemical damages. They also include the hot spots for the occurrence of mitotic chiasmata. In addition, R bands seem to contain the majority of genes. R-banding is accomplished by heating the

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fixed chromosomes at 87.5 for 5–30 min in physiological saline. The AT-richer DNA presumably denatures faster, reducing its affinity for Giemsa stain. With increasing heat-treatment times, sets of originally dark R-bands gradually fade, leaving T-bands as an extremely heat-resistant subset of R-bands. T-bands, traditionally called telomeric bands, contain the extremely GC-rich components, which contain 65% of mapped genes [10]. The C-bands compose the centromeric regions of all chromosomes, the secondary constrictions of 1, 9, and 16, and the heterochromatic portion of the long arm of the Y. These are known to be the major locations of simple sequence, highly repetitive satellite DNAs, as summarized in Table 1.

III. *In situ* Hybridization

In situ hybridization is a powerful method to localize nucleic acid sequences in the cytoplasm, organelles, chromosomes or nuclei of biological materials. *In situ* hybridization differs from the analysis of nucleic acids by southern or northern hybridization in that the hybridization signals are localized *in situ* and not on a membrane sheet. Over the past few years *in situ* hybridization techniques have become increasingly popular and are being applied to a broad spectrum of interesting biological and clinical problems: (a) construction of physical maps of chromosomes, (b) analysis of chromosome structure and aberrations, (c) investigation of the structure, function and evolution of chromosomes and genomes, (d) determination of the spatial and temporal expression of genes, (e) identification and characterization of viruses, viral sequences and bacteria in tissues, (f) sex determination, (g) localization of transformation sequences and oncogenes, and (h) the analysis of neurotransmitter messages.

Recently, the sensitivity and resolution of *in situ* hybridization has been significantly increased by the development of fluorescence *in situ* hybridization (FISH) [13, 14, 19, 21]. In this technique, the DNA probe is labeled by addition of a reporter molecule. Following hybridization and washing to remove excess probe, the chromosome preparation is incubated in a solution containing fluorescently labeled affinity molecule which binds to the reporter on the hybridized probe. FISH has the advantage of providing rapid results which can be conveniently scored by eye using a fluorescence microscope. In metaphase spreads positive signals show as double spots, corresponding to probe hybridized to both sister chromatids. Using sophisticated equipment and reporter-binding molecules carrying different fluorophores, it is possible to map and order several DNA clones simultaneously. At present the maximum resolution of FISH on metaphase chromosomes is about 5–10 Mb. However, by using several differently labeled probes in interphase nuclei where the DNA is less condensed than metaphase chromosomes, much finer mapping is possible. Chromosomal locations cannot be identified in interphase preparations, but the relative order and spacing of different colored fluorescent probes can be established. Very recently, a technique for stretching chromatin DNA has been developed, which allows even greater resolution as fine as several kilo-bases [16]. Using interspersed repeated DNA sequences as probes, FISH showed that the LINES (long interspersed repeated sequences, e.g. L1 family) dominated in G/Q-bands and the SINES (short interspersed repeated sequences, e.g. *Alu* family) dominated in R-bands, *Alu* is 56% GC, and L1 is 58% AT, and each may comprise 13–18% of the total DNA in a chromosome band [12] (Table 1). Since the *Alu* family is preferentially located within R-bands, FISH with *Alu*-repeat family results in an R-banding-like staining pattern.

Gene mapping

The major applications of FISH that have received considerable attention of late are gene mapping and interphase cytogenetics, the former mostly because of the Human Genome Project and the latter because of its potential clinical utility. Chromosome assignment is not problematic as several conventional chromosome banding methods, for example, Q-banding, R-banding as well as hybridization banding [14] are fully compatible with probe detection by fluorescence. The chromosomal localization of DNA sequences can be directly determined with FISH. There is a fairly good correlation of hybridization efficiency and size of probe DNA sequences. To increase the intensity of the hybridization signal, long DNA probes are used, usually cosmid clones containing around 40 kb of insert or YAC clones which can be mapped with a resolution of less than 3 Mb on metaphase chromosomes. Because such large sequences will contain many interspersed

Table 1. *Major characteristics of chromosome bands*

	G/Q-band	R-band	T-band
AT/GC content	AT-rich	GC-rich	Very GC-rich
Timing of DNA replication	Late S phase	Early S phase	Early S phase
Repetitiveness of DNA	L1 (LINE) type	*Alu* (SINE) type	*Alu* type
Gene density	Low	High	Very high
Distribution of CpG-islands	Low	High	Very high
Compaction of chromatin	High density	Rough density	Rough density

repetitive DNA sequences, it is necessary to use competitive suppression hybridization. Before the main hybridization, the probe is mixed with an aqueous solution of unlabeled total genomic DNA. This saturates the repetitive elements in the probe, so that they no longer interfere with the specific *in situ* hybridization of the unique sequences. Hybridization signals from two or more probes can be spatially resolved on metaphase chromosomes when the probes are only several hundred kilobases apart, but a minimum of 0.5–1 Mbp separation is required to permit their physical order to be established [14].

To further improve the spatial resolution of gene mapping, co-hybridization studies with closely spaced probes from a single genomic region have been performed on interphase nuclei. Measurements of the distances between multiple pairs of probes after FISH to fixed flat nuclei demonstrate a fairly linear relationship between physical distance and genome order over the range from 10 kb up to about 1 Mbp. The combination of metaphase and interphase nuclear mapping, particularly using multiple probes simultaneously, offers the opportunity to physically order genomic DNA segments with a resolution presently achieved by gel electrophoretic methods and provides a new bridge to interrelate physical and genetic linkage information [16].

Progress in human gene mapping is reviewed at successive international Human Gene Workshops. Currently over 50,000 loci, of which only a minority are gene loci, had been mapped to specific chromosomal locations [6, 9, 15, 17].

Application to chromosome abnormality

A special application of FISH has been the use of a whole chromosome-specific DNA probe derived from a chromosome specific DNA library, to hybridize to many loci spanning a whole chromosome [13, 14, 19]. This causes whole chromosomes to fluoresce, resulting in chromosome painting. Painting of sub-chromosomal regions is also now possible, using DNA clones from a microdissected chromosomal region as a probe, and other chromosome specific probes, often clones of the alphoid DNA repeat family. Because of the high efficiency of FISH with these cloned probes or chromosome-specific DNAs, the direct quantitation of a specific gene, chromosome number or subregion can be readily detected in metaphase chromosomes as well as in interphase cells. Novel modalities for detecting multiple chromosomal translocations and other types of chromosome rearrangements in interphase cells of patient origin are also examined for clinical applications. These range from visualization of specific oncogene rearrangements in leukemias and lymphomas, sensitive detection of chromosome fragmentation due to radiation exposure and the identification of genomic DNA fragments that flank or span specific translocation break points that occur in certain types of human tumors [1, 5, 8].

IV. DNA Content of Human Chromosomes

The nuclear DNA content of individual human cells is determined by the number of nuclei and the number of chromosomes in the cell. The specialized germ cells, eggs and sperm cells, are haploid cells in which there is a single copy of the nuclear genome with the DNA being distributed between 23 chromosomes, comprising 22 autosomes and a single sex chromosome, X or Y. Fusion of a normal egg cell and sperm cell at conception generates a diploid zygote with two genome copies and 46 chromosomes, consisting of 23 pairs of homologous chromosomes, that is, a homologous pair of each of the 22 autosomes and two sex chromosomes which may be completely homologous (XX), of partially homologous (XY). Subsequent mitotic DNA duplication and cell division events during development and growth result in the great majority of somatic cells containing a single diploid in which there are additional rounds of chromosome duplica-

Table 2. *DNA content of human chromosomes*

Chromosome	Percentage of total length	Amount of DNA (Mb)	Chromosome	Percentage of total length	Amount of DNA (Mb)
1	8.3	250	13	3.6	110
2	7.9	240	14	3.5	105
3	6.4	190	15	3.3	100
4	6.1	180	16	2.8	85
5	5.8	175	17	2.7	80
6	5.5	165	18	2.5	75
7	5.1	155	19	2.3	70
8	4.5	135	20	2.1	65
9	4.4	130	21	1.8	55
10	4.4	130	22	1.9	60
11	4.4	130	X	4.7	140
12	4.1	120	Y	2.0	60

tion prior to cell division and cells which are multinucleated or which lack a nucleus.

The nuclear genome of a human haploid cell contains about 3×10^9 bp (3,000 Mb) of DNA and an average size chromosome has approximately 1.3×10^8 bp (130 Mb) of DNA but can vary between about 50 Mb and 250 Mb, as summarized in Table 2 [15, 17]. The DNA content of each chromosome is thought to consist of a single linear double-stranded DNA molecule which, if fully uncoiled, would be between 1.7 and 8.5 cm long. In the cell the structure of each chromosome is highly ordered and compaction of the chromosomal DNA is achieved by complexing with various DNA-binding proteins. The most fundamental unit of packaging is the nucleosome which consists of a central core complex of eight basic histone proteins around which a stretch of 146 bp of double-stranded DNA is coiled in 1.75 turns. Adjacent nucleosomes are connected by a short length of spacer DNA. The elementary fiber of linked nucleosomes is in turn coiled into a chromatin fiber of 30 nm diameter which can be resolved by electron microscopy. At the metaphase stage of cell division the chromosomes become even more condensed and can be resolved by optical microscopy as structures which are over 1 μm wide and range in length from 2 μm to 10 μm in chromosomes 22 and 1, respectively. Additionally, the G/Q positive and negative chromosomal regions differ in their predominant association with particular classes of interspersed repetitive DNA. At the resolution of approximately 400 band-stage, an average size band corresponds to approximately 6 Mb of DNA.

In the haploid nuclear genome the total number of genes is thought to be approximately 50,000–100,000. On this basis all nucleated cells have, on average, one gene per 30–60 kb, and about 2,000–4,000 per average chromosome. In a 400-band metaphase karyotype, one might anticipate about 100–200 genes on average per band. However, as noted above, average gene density is dependent on the base composition of the chromosomal region containing the gene and pale G-bands (R-bands) are relatively enriched in genes at the expense of dark G-bands [10].

The sex-averaged total genetic map distance for the 300 Mb genome is about 3700 cM. Thus, a genetic map distance of 1 cM, on average, corresponds approximately to a physical map distance of 0.8 Mb. However, the ratios of genetic and physical map distances on individual chromosomal segments often deviate considerably from this average figure due to non-random location of chiasmata. Chromosomal segments containing recombinational hotspots will show a high crossing-over frequency. There is a high recombinational frequency at telomeres, while recombination is suppressed near to centromeres and, to a lesser extent, in sub-telomeric regions.

V. Repeated Sequences in Chromosomes

Interspersed repetitive DNA sequences

The discovery of interspersed repeated DNA sequences similarly created a revolution in the understanding of eukaryotic genome organization. Interspersed

Table 3. *Major repetitive DNA classes*

Class	Size of repeat unit (bp)	Total number of copies of repeat units	Major chromosomal location(s)
Interspersed repeats			
Alu family	250	7×10^5	Euchromatin, G-positive bands
L1 (*Kpn*) family	1300	6×10^4	Euchromatin, G-negative (R-) bands
Tandem repeats			
Satellite DNA			
Simple sequence	5–25	?	Heterochromatin of 1q, 9q, 16q, Yq
Alpha	171	8×10^5	of centromeres
Beta	66	5×10^4	of 9, 13, 14, 15, 21, 22
Minisatellite DNA			
Telomeric family	6	$2–3 \times 10^4$	Telomeres
Hypervariable family	9–64	3×10^4	All chromosomes, often near telomeres
Microsatellite DNA			
$(A)_n/(T)_n$	1	10^7	All chromosomes
$(CA)_n/(TG)_n$	2	7×10^6	All chromosomes
$(CT)_n/(AG)_n$	2	3×10^6	All chromosomes

repeated sequences are now classified as SINEs or LINEs. The major human SINEs is the *Alu* DNA sequence family which is found in Old World primates and named for an *Alu*I restriction endonuclease site typical of the sequence. *Alu* units are found in nearly a million copies per haploid genome and can be found flanking genes, in introns, within satellite DNA, and in clusters with other interspersed repeated sequences. As already described above, FISH analysis demonstrated that these two classes of DNA sequences are clustered into different chromosome bands. In particular, the *Alu* sequences hybridize to regions of human chromosomes corresponding to G-negative bands (R bands), and L1 repeats tend to cluster in G-positive chromosome regions. This is consistent with the localization of these repeats within early and late replicating fractions of DNA, and within heavy and light isochores [2, 3, 12].

Tandemly repeated sequences

Although repeated sequences are a common feature of eukaryotic DNA, about 40% of the nuclear genome is composed of repetitive DNA in the human diploid genome. Repeated DNA sequences are composed of arrays of tandem repeats or of individual repeat units interspersed with other DNA sequences. The former category may be subdivided according to the average size of the arrays of tandem repeats into satellite DNA, minisatellite DNA and microsatellite DNA. These repeated sequences are organized in the genome tandemly or dispersely. In general, highly repetitive DNA is organized around centromeres and telomeres in the form of tandem repeats whereas moderately repetitive DNA is dispersed throughout the chromosome, as summarized in Table 3 [20].

Satellite DNA

Satellite DNA comprises large arrays of tandemly repeated DNA which usually fall within the 100 kb to several megabases range, and consists of simple or moderately complex repeat units. Repeated DNA of this type is not transcribed and accounts for the bulk of the heterochromatic regions of the genome. The base composition of such DNA regions is dictated by the base composition of their constituent short repeat units and may diverge substantially from the overall base composition of bulk cellular DNA. Certain satellite DNA species cannot be resolved by density-gradient centrifugation but can be identified following digestion with a restriction nuclease which typically has a single recognition site in the basic repeat unit. Alpha satellite or alphoid DNA constitutes the bulk of the centromeric heterochromatin on all of the chromosomes. It is characterized by tandem repeats of a basic mean length of 171 bp, although higher order units are also seen. The sequence divergence between individual members of the alphoid DNA family can be so high that it is possible to isolate repeat units which will hybridize under stringent conditions to specific chromosomes.

Presently, the extent to which satellite DNA can be considered junk DNA is not known. The centromeric DNA of human chromosomes largely consists of various families of satellite DNA, and no unique sequence has been found in a human centromere. Of these satellite DNA families, only the alpha satellite is known to be present on all chromosomes, although sequence divergence between the repeats has led to chromosome-specific subsets. Although the 171 kb repeat unit of alpha satellite DNA often contains a binding site for a specific centromere protein, PENP-B, there is presently no compelling evidence to suggest that centromere function depends on this association, or even on the presence of alpha satellite DNA.

Minisatellite DNA

Minisatellite DNA comprises a collection of moderately sized arrays of tandemly repeated DNA sequences which are dispersed throughout the nuclear genome. They include a family of hypervariable minisatellite DNA sequences which are organized in over 1,000 arrays of short (from 0.1 to 20 kb long) tandem repeats. The repeat unitis different hypervariable array vary considerably in size, but share a common core sequence, GGGCAGGAXC (where X=any nucleotide). Another major family of minisatellite DNA sequences is found at the telomeres. Telomeric DNA constitutes 10–15 kb of tandem hexanucleotide repeat units of TTAGGG, which are common in lower and higher eukaryotes.

Microsatellite DNA

Microsatellite DNA families include small arrays of tandem repeats which are simple in sequence (1–4 bp) and are interspersed throughout the genome. Arrays of CA and CT/AG repeats are very common, accounting for 0.5% and 0.2% of the genome respectively and are often highly polymorphic. Although the significance of microsatellite DNA is not known, it has generally been identified in intergenic DNA or within the introns of the genes.

VI. Evolutional Aspects of Chromosome Organization

The organization and banding patterns of human chromosomes are very similar to those of other primates. The major difference is that other primates have 23 different autosomes; two ancestral chromosomes appear to have fused in the human lineage but not in those leading to the other primates. Other minor differences are mostly accounted for by chromosome inversions and variations in constitutive heterochromatin; differences due to chromosome translocations are relatively rare. Mouse and human chromosomes would appear to have very different organizations; the mouse has 40 chromosomes with all acrocentrics whereas there are 46 chromosomes in humans

with mostly metacentrics and sub-metacentrics. However, comparison of high resolution human and mouse chromosome maps also shows considerable sharing of cytogenetic banding patterns over relatively small chromosomal regions. Small chromosomal regions appear to have been conserved, therefore, over comparatively long evolutionary time-scales.

In sex chromosomes, the X and Y chromosomes are very different in many aspects. The Y chromosome is much smaller than the X chromosome. However, homologous regions are found on both chromosomes, suggesting that they may have evolved from a single homologous pair. Of these homologous regions, a major region located on the distal ends of the short arms is the site of an obligate crossing-over during male meiosis and is thought to be required for correct meiotic segregation. As DNA sequences within this chromosomal segment do not show strict sex-linked inheritance, it is referred to as the pseudo-autosomal region.

The human X chromosome contains several thousand genes. In human male cells the X chromosome is genetically active, while in female cells only one of the two X chromosomes is active. The other replicates late in cell division, remains condensed throughout most of interphase and is cytogenetically visible as a densely staining heterochromatic feature as Barr body or sex chromatin mass. This X chromosome is thought to be mostly genetically inert as a consequence of X chromosome inactivation (Lyonization). X chromosome inactivation is thought to have evolved as a form of dosage compensation in cells with more than one X chromosome. Normal cellular processes include interactions between the products of active autosomal and X chromosome genes which depend on their relative dosage. However, in female cells XIST gene which is inducing X chromosome inactivation is only active on the inactive X chromosome.

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