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EVOLUTIONARY ASPECTS

Mammalian chromosomes can be defined by three structural classes visualized as the G/Q-bands, the R-bands, and the C-bands, which are associated with different functional and biochemical attributes. This chromosomal prepattern has developed during vertebrate evolution and determines the kind and quality of banding which can be obtained from different vertebrate taxa (Holmquist, 1989). The vertebrate genome is organized in very long DNA sequences of homologue base composition called isochores (Bernardi, 1989). During evolution, mammals and birds have separated from reptiles and lower vertebrates with regard to distribution and base composition of the isochores. This difference, which is reflected in the chromosomal prepattern, indicates that isochores are evolutionary units of the vertebrate genome (Bernardi, 1989). In mammals and birds, the genome can be divided into two subgenomes, the first one, *the paleogenome*, has not changed during vertebrate evolution. It is composed of late replicating dG–dC-poor isochores. It contains the long interspersed repeated DNA-sequences (LINES) and the tissue-specific genes (Holmquist, 1989). It corresponds to the late replicated G-band chromatin of early-condensating chromomeres and is subjected to lyonization during embryonic development (Holmquist, 1989). The second one, *the neogenome*, has changed from the ancestral early replicating dG–dC-poor isochores of late-condensating interchromomeres into the early-replicating compositionally heterogeneous dG–dC-rich isochores of late-condensating interchromomeric chromatin. This subgenome contains the short interspersed repeated DNA-sequences (SINES) and the housekeeping genes (Holmquist, 1989). It corresponds to the early replicated R-band chromatin and is not subjected to any kind of inactivation during embryonic development except for the late X-inactivation.

REPLICATION PATTERNS

DNA in mammalian chromosomes replicates in a segmented and coordinated fashion, the S phase is bimodal with a distinct reduction of DNA synthesis in

mid S phase, giving two temporal classes of replicons (Holmquist *et al*, 1982). The reduction in DNA synthesis corresponds to the period when the replication complex shifts from early to late replicons (Reddy and Pardee, 1980). Clusters of early and late replicons alternate and create replication patterns (Hand, 1978). The clusters may contain up to 100 individual replicons giving a DNA content which corresponds to a chromosomal band (Hand, 1978). In premature chromosome condensation (PCC) experiments, Lau and Arrighi (1981) demonstrated that S phase PCC fragments were condensed, non-replicated, chromosomal segments separated by replicating ones. Electron microscopy examinations of chromatin have revealed alternating patterns of loops and extended fibers. Each structure accounts for about half the total mass of DNA. If these structures represent the ultimate subdivision, the mammalian genome may contain 8 000 bands, each with about 410 kilobases of DNA per band (Yunis and Bahr, 1979). The number of bands demonstrated in mammalian mitotic karyotypes is, however, much lower. For G-bands it amounts to about 1 700 bands per haploid set (Yunis, 1981), and the RBG patterns are now demonstrated at the 1 250 band stage (Drouin *et al*, 1988).

PREPARATION TECHNIQUES

The substrate for chromosome preparation is a single cell suspension which contains a sufficient amount of dividing cells. The procedures involve either direct preparations from proliferating tissues or establishment of cultures with proliferating potential *in vitro*. However, to produce chromosomal slides suitable for induction of high-resolution banding, a series of preparational procedures must be performed in an ordered and sequential manner. The number of bands which on the average can be obtained per metaphase cell depends upon the cell type, the culture, the preparation and the banding technique employed. For high-resolution banding studies, the procedures most often involve synchronization, spindle inhibition, hypotonic treatment, fixation, production of air-dried slides, and differential staining with Giemsa or fluorescent dyes. Synchronization with S phase inhibitors provides mitotic waves at known times after block release and options for precise BUdR-substitution in one of the subgenomes. In connection with synchronization, the use of spindle inhibitors can be limited or omitted without unacceptable reduction of mitotic indices. Drouin *et al* (1988) questioned the reason for using spindle inhibitors in association with high-resolution banding. They observed that exposure to colcemid reduced the absolute number of mitotic cells which, after harvesting and appropriate staining, displayed more than 650 bands per haploid set. However, in an exponentially growing cell culture, interphase cells continue to enter mitosis, so the total number of metaphases with long chromosomes suitable for banding does not necessarily change (Bosman *et al*, 1975; Rønne, 1985), and there is at present no evidence that colcemid has a direct effect on chromosome structure. Hypotonic solution induces swelling of animal cells, but chromosome contraction and morphology are influenced by the concentration and the type of salt used. If the spindle is depolymerized due to the presence of a spindle inhibitor, the chromosomes are free to swim around after the nuclear membrane has broken down at prometaphase. To equilibrate inside and outside salt concentrations, the cell will actively absorb water and start to swell in a hypotonic medium. This treatment will also free

the chromosomes of the ribonucleoprotein complex which surrounds them. Several different hypotonic solutions have been used in conjunction with high-resolution banding in order to increase band number, however, the 0.075 M KCl solution has, in most studies, proven sufficient for the purpose. Fixation with methanol–acetic acid denatures and precipitates proteins under acid conditions. The fixative penetrates into the cells rapidly, coagulates nuclear proteins, preserves the chromosome morphology and strips cytoplasmic proteins from the cells. It has been suggested (Summer *et al*, 1973) that almost all the histones are removed from the chromosomes by the fixation. However, later observations (Burkholder and Duczek, 1982) have demonstrated that a substantial part of the histones is preserved during fixation. Methanol–acetic acid fixation does enhance the effect of Giemsa-staining and appears to induce conformational changes in H1 (Barnett *et al*, 1980). According to van Duijn *et al* (1985), the fixation causes an irreversible denaturation of the core- and H1-histones. H1 may loose its capacity to seal the DNA coils, so in a neutral buffer the negatively charged DNA helix will be attracted by the positively charged polyarginine sequences of the core histones. This causes the DNA coils to wind around the denatured core with a smaller pitch. Such processes may explain the collapses seen in SSC-treated but unstained chromosomes (Harrison *et al*, 1983).

POSTFIXATION STAINING

The substrate for staining procedures is the methanol–acetic acid-fixed chromosomes prepared on clean glass slides by air-drying. Staining procedures which provide a uniform unbanded appearance of the chromosomes are normally called conventional staining. Although several dyes can be used for permanent conventional staining of chromosomes, the most often used one is the Giemsa mixture. Because of continued oxidation of the dye components, the exact composition of Giemsa cannot be determined, but two dyes only, the cationic azure B and the anionic eosin Y are required for the deep purple staining of chromatin and chromosomes. This color cannot be obtained by using azure B or eosin Y alone. The Giemsa complex has one negative and two positive charges. The positively charged part interacts with the negatively charged DNA, while the negatively charged part of the complex may interact with the positively charged histones (van Duijn *et al*, 1985). Since the Giemsa complex has no specificity for any particular base in the DNA, a near-neutral Giemsa solution stains the chromosomes uniformly purple if banding methods are not employed. In methanol–acetic acid-fixed chromatin, about 20% of the DNA's potential binding sites are involved in protein interactions and therefore inaccessible to the Giemsa dye. When the fixed chromatin is prepared for postfixation GTG-banding, an additional 30% of the binding sites become inaccessible. The Giemsa complex may then compete for binding sites during the staining procedure and change chromatin structure by removing or displacing proteins in the G-band DNA, while R-band proteins are bound too tightly to the DNA to allow displacement and dye precipitation. The findings that high-mobility group I (HMG-I) is located to the G/Q-bands (Disney *et al*, 1989) are interesting in light of GTG-band induction. The brief trypsin digestion used in GTG-band induction may preferentially remove HMG-I and thereby provide an easy access for the Giemsa complex to the G-band DNA. Trypsin is also known to digest histones. Since H1 is

situated outside the nucleosome core, it is more exposed to trypsin during GTG-banding than the core histones. The trypsin treatment may dissociate H1 from the fixed nucleosomes and thereby facilitate charge rearrangements and tight linkage between dG–dC-rich DNA and the arginine-rich core histones, which may prevent the insertion of the dye complex into the negative G-bands. After hot saline treatment of chromosome slides, the centers of the chromosomes collapse leaving a rim of material around their perimeters (Harrison *et al*, 1983). After Giemsa-staining, transversely swollen ridges appear in G-band-positive areas. According to van Duijn *et al* (1985), these observations indicate that the SSC-treatment rearranges core histones. The polyarginine-rich core has a strong affinity for the dG–dC-rich R-band DNA, which may explain the swelling differences between negative and positive G-bands. Postfixation R-band induction involves incubation of chromosome slides in hot saline. This induces substantial chromatin aggregation, probably irreversible denaturation of non-histone proteins and quantitative reduction of H1 (Burkholder and Duczek, 1982). Chromatin aggregation takes place in the positive R-bands, so alterations in protein–DNA interactions due to differential denaturation may explain the variation in staining intensity along the chromosomes. The extraction of proteins is minimal during RH-banding and the bands appear to be affected by the pH and the temperature of the surrounding medium. By varying these factors, G-banding will replace R-banding or *vice versa* (Eiberg, 1973), indicating similarities between the mechanisms of the two banding techniques. Thus, the banding technique, the pH of the surrounding medium, the dye concentration and dye affinity for accessible binding sites are interrelated parameters, all of which influence the equilibrium between dye and proteins. Changes in any of these parameters will change the equilibrium and, consequently, alter chromosome morphology with regard to banding structure and chromosome swelling (Rønne *et al*, in preparation).

PREFIXATION STAINING

Replication patterns visualized after fluorescence plus Giemsa (FPG)-staining (Perry and Wolff, 1974) resemble postfixation-induced banding. With BUdR incorporation into early replicated DNA, the patterns correspond to G-banding, whereas the replication patterns seen after BUdR incorporation into late replicons resemble RH-banding. The effect of FPG-staining may be due to DNA loss after UV photolysis of BUdR-substituted DNA (Webber *et al*, 1981), BUdR concentration, the template used (Jack *et al*, 1989), and preferential breakage of disulfide bonds (Buys and Stienstra, 1980). BUdR-substituted DNA binds chromosomal proteins more tightly (Burkholder and Duczek, 1982) and UV irradiation acts differently on proteins in BUdR-substituted and unsubstituted chromatids (Buys and Stienstra, 1980). Jack *et al* (1989) suggested that the tighter binding of proteins to BUdR-substituted DNA may increase the formation of protein–protein DNA crosslinks after UV irradiation, which may exclude the Giemsa-binding sites between the histone core and DNA in a manner similar to that described for postfixation G-banding. Distamycin A, netropsin and Hoechst 33258 are ligands with preferential non-intercalative binding to dA–dT-rich DNA. Although *in vitro* exposure of mammalian cells to these agents seems to have similar effects on chromosome structure, the biochemical actions of distamycin A and netropsin are quite different from that of Hoechst 33258

(Krey, 1980). Combined *in vitro* exposure of mammalian cell cultures to BUdR and one of the three ligands in late S and G_2 phases results in BUdR substitution of late replicated DNA and a corresponding pattern of decreased chromosome contraction which can be observed after both traditional and modified Giemsa-staining (Goyanes and Mendez, 1981; Rønne, 1985). After FPG-staining, the BUdR-substituted segments appear light, the unsubstituted segments appear dark. The decreased contraction in the substituted segments enhances band resolution and band contrast (Rønne, 1985). The consequences of *in vitro* exposure to distamycin A, netropsin and Hoechst 33258 may not be explained only by their interactions with DNA, but also by the presence of HMG-I in the G/Q-band chromatin. According to Disney *et al* (1989), it is likely that the dA–dT-binding ligands in question compete with HMG-I for binding sites in the negative R-bands with inhibition of chromosome contraction and reduced accessibility for the Giemsa complex to potential dye-binding sites as a consequence. The high affinity for BUdR-substituted DNA displayed by these ligands and the FPG-staining subsequently employed in the RBG-procedure then enhances the differences between R- and G/Q-band regions.

PRESENT ASPECTS

The high-resolution banding techniques provide the possibilities to detect chromosome breaks and rearrangements even within major bands. The developments in culture and banding techniques have been extremely fast; ten years after the Paris conference in 1971, Yunis (1981) published the haploid human karyotype at the 1 700-band stage; the number of described bands had increased by about a factor of 5. For other mammals, the development was slower, but recently good results have narrowed the gap between human and animal cytogenetics. The increase of bands in a given karyotype is, however, not an aim by itself, but a research tool, so the value of a high band level is reduced if band quality is sacrificed in the process. When high-resolution banding is combined with other chromosomal techniques, specific sites on the chromosomes can be detected and precisely localized. High-resolution banding techniques have been extremely valuable, especially for the localization of single-copy genes and specific breakpoints. Banding based on BUdR-substitution in one of the subgenomes followed by FPG-staining is highly resistant to the hybridization procedures (Zabel *et al*, 1983), and the uniform mitotic population of lymphocytes prepared for subsequent RBG-banding after double synchronization (Rønne, 1985), seems to be an excellent substrate for *in situ* hybridization of single-copy genes (Rønne *et al*, 1990). Inhibitors of dihydrofolate reductase and thymidylate synthetase have been used to unmask fragile sites (Tommerup *et al*, 1981). In the concentration range from 10^{-7} to 10^{-6} M, methotrexate, 5-fluoro-2′-deoxyuridine and fluorouracil also act as reversible S phase inhibitors, so with the traditional synchronization techniques some fragile sites may be unmasked as a side effect of the culture and banding procedures. The banding patterns obtained after differential staining of chromosomes are not only a result of the method employed, but also a consequence of isochore organization and chromosomal prepattern. The marked differences in banding potential between different cell systems obtained from the same individuals indicate, however, that other factors influence chromosome mor-

phology and banding potential, but the exact molecular mechanisms remain to be identified.

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