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Chromosome Localization of Highly Repetitive Human DNA's and Amplified Ribosomal DNA with Restriction Enzymes

Abstract. *Restriction endonucleases cut and partially removed DNA throughout fixed air-dried human metaphase chromosomes. Some enzymes produced a G-banding pattern; some revealed the presence of multiple chromosome-specific classes of highly repetitive DNA in C-band heterochromatin. Enzymes that produced the informative C-band patterns had recognition sequences that were four or five, but not six, base pairs long and did not contain a cytosine-guanine doublet. In both rat and human chromosomes, regions containing amplified ribosomal RNA genes were specifically removed by the restriction endonuclease Msp I.*

The DNA in metaphase chromosomes fixed in methanol and acetic acid and immobilized on glass slides is susceptible to attack by nucleases. Deoxyribonuclease I and micrococcal nuclease cleaved such DNA into fragments just a few base pairs (bp) long (*1*); this extracted the DNA and abolished Giemsa staining (*2*). If the chromosomes were exposed to the GC-specific (G, guanine; C, cytosine) ligand chromomycin A3 prior to and during deoxyribonuclease digestion, the GC-rich DNA was protected while the remaining DNA was cut into tiny fragments and extracted, leaving an R-band pattern (*3*). Type II restriction endonucleases should provide more interesting probes because each enzyme cuts only within a specific recognition sequence 4 to 6 bp long, or longer, and produces longer fragments of DNA (*4*). Restriction endonuclease Hae III, which cuts at GGCC, produced a chromomere pattern of Giemsa staining on muntjac chromosomes fixed in methanol and acetic acid; Eco RI, which cuts in the much less abundant GAATTC (A, adenine; T, thymine) sequence, had no detectable effect on these chromosomes (*5*).

The present study was undertaken to characterize the effects of various restriction endonucleases on human chromosomes, especially on the highly repetitive, but complex and heterogeneous, human satellite DNA's (*6*), which are concentrated in the heterochromatic C-band regions (*7, 8*). In addition, we examined the effect of Msp I on the somewhat less repetitive amplified 18*S* plus 28*S* ribosomal RNA genes (rDNA), which contain abundant Msp I sites (*9*).

Digestion with Alu I, Hae III, or Mbo I, enzymes with recognition sequences 4 bp long, led to a marked decrease in Giemsa staining in localized regions of human chromosomes, producing G-band or modified C-band patterns (*10*) (Fig. 1). Four other enzymes that recognize 4-bp sequences, namely, Cfo I, Hha I, Hpa II, and Msp I, had no effect, probably because each contains in its recognition sequence the CG dinucleotide (*11*). Three enzymes with recognition sequences 5 bp long, Dde I, Eco RII, and Hinf I, also produced marked diminution of Giemsa staining and modified C-band patterns, whereas Ava II had no detectable effect. Enzymes with recognition sequences 6 bp long, or longer (*12*), generally produced very little effect beyond the G-band-like pattern sometimes present in the controls exposed to the reaction mixture minus the enzyme.

The chromosome banding patterns observed after restriction endonuclease digestion were highly specific for each enzyme (Fig. 1). Treatment with Alu I (Fig. 1a), Dde I, or Eco RII left intact the major C bands (those on chromosomes 1, 9, 16, and, in male cells, the Y). Treatment with Mbo I (Fig. 1b) removed most of the C bands on chromosomes 1, 16, and the Y (not illustrated), but not that on chromosome 9. These enzymes also had different effects on the minor C bands present on other chromosomes, which could be identified by the pale G bands that remained on most of them after enzyme treatment. For example, Eco RII had no effect on the C bands on chromosomes 11 and 12; Mbo I (Fig. 1b) removed the C band from 11 but not 12, and Alu I (Fig. 1a) or Dde I removed the C bands from both 11 and 12. Hae III abolished staining of the GC-rich R bands, producing a more pronounced G-band pattern than the other enzymes; it had no effect on the major C bands (Fig. 1c). Hinf I diminished staining of every chromosome region (Fig. 1d). It abolished Giemsa staining of all C bands except those on chromosomes 3 and 4 (identified by faint residual G-band patterns) and left large gaps in place of the major C bands. DNA in the entire human chromosome complement was available for reaction with restriction endonucleases, since Giemsa staining could be eliminated from every region of every chromosome. No variation in effect was observed between individuals in this small sample; differences between homologs (for example, the chromosomes 9 in Fig. 1, a to c) reflected differences in the amount of C-band material.

Sequential cutting with two enzymes sometimes yielded patterns not seen with either enzyme alone. For example, when Eco RI was followed by either Hae III or Hind III the staining of part of the C-band region of chromosome 1 was markedly diminished. Sequential cutting with Hae III and Msp I had no effect on the C bands of chromosomes 1, 16, and the Y, but removed part of the C band of chromosome 9. This effect on chromosome 9 did not occur when Hae III was followed by Hpa II, an indication that the internal cytosines in the CCGG recognition sites in the chromosome 9–specific sequences are methylated. Sequential digestion with Hinf I and Hae III virtually eliminated Giemsa staining from every chromosome arm and C band.

The banding patterns observed after treatment with restriction enzymes presumably reflected loss of DNA from the chromosomes, as shown earlier for micrococcal nuclease (*2*). There are many Alu I sites in human DNA, for example, and this enzyme produced a very large reduction in Giemsa staining everywhere except in the C bands (Fig. 1a). A similar reduction in staining after Alu I treatment was observed with both intercalating (acridine orange) and nonintercalating (Hoechst 33258) fluorochromes, yielding the same C-band pattern in each case. Our results suggest that fragments longer than 1 kilobase pair (kbp) were not extracted while those of about 100 bp or less could be. Double digests with Hae III and Msp I did not extract DNA from the distal segment of Yq, although they reduced the 2.1-kbp Y-specific DNA of this region to fragments of length 0.95 and 1.15 kbp (*13*). Hinf I, which cut satellite II, III, and IV DNA's into fragments usually no more than 80 bp long (*8, 14*), produced a very marked reduction of Giemsa staining of most C bands.

Digestion with Msp I did not produce a banding pattern on human chromosomes but did reduce the staining of a variant number 14 short arm (Fig. 1e) that contained amplified 18*S* plus 28*S* rDNA (*15*). Similar results were observed in rat hepatoma cells with amplified rDNA (*16*); only the amplified regions showed reduced staining after Msp I treatment (Fig. 1f). Hpa II did not have this effect, in keeping with the high degree of methylation of CCGG sites in the amplified rDNA (*9*). A cloned 5.7-kbp segment of the approximately 45 kbp human rRNA genes contained 30 CCGG sites fairly evenly spaced about 100 to 300 bp apart (*17*). The marked reduction of Giemsa staining we observed in regions containing amplified human or rat rDNA cut by Msp I suggests that the entire amplified region in both species contained comparable densities of CCGG sites, and that fragments of this size were removable from acid-fixed chromosomes.

Restriction enzyme digestion of fixed chromosomes, followed by simple Giemsa staining, provides an important adjunct to other methods for examining highly repetitive satellite DNA's. In situ hybridization of specific human satellite DNA's has been used for their chromosomal localization (*14, 18*), but this method has limitations because a number of these satellites cross-hybridize as a result of extensive homology (*6, 7, 18*). Southern blot hybridization of restriction endonuclease–digested DNA from interspecific hybrid cells has been used to

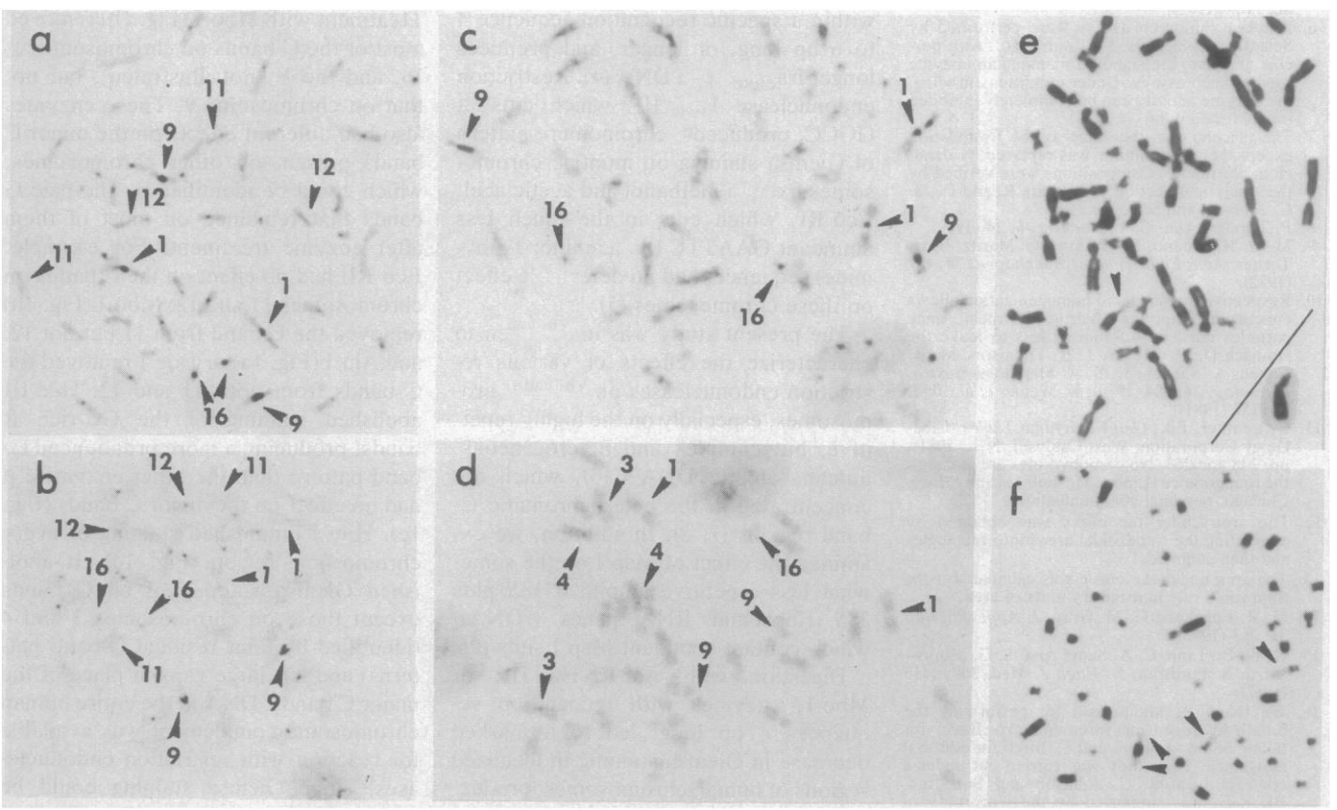

Fig. 1. Standard chromosome preparations fixed in methanol and acetic acid and air dried, then treated with a restriction enzyme and stained with Giemsa. (a to d) Cells from a single human female treated with (a) Alu I, (b) Mbo I, (c) Hae III, or (d) Hinf I. The staining of the chromosome arms is reduced by all four enzymes. Major C bands are present unchanged on chromosomes 1, 9, and 16 after Alu I or Hae III; present on chromosome 9 but markedly diminished on chromosomes 1 and 16 after Mbo I; and absent from chromosomes 1, 9, and 16 after Hinf I. Note the within-pair variation on chromosomes 9. Selected examples of differences in minor C bands: absent from chromosome 11 after Alu I or Mbo I, absent from chromosome 12 after Alu I but present after Mbo I, and present only on chromosomes 3 and 4 after Hinf I. (e) Human male cell treated with Msp I; arrow indicates reduced staining of short arm of chromosome 14 containing amplified rDNA. Inset: same chromosome without Msp I. (f) Part of a rat hepatoma cell treated with Msp I; arrows indicate reduced staining of regions containing amplified rDNA.

show that the distribution of some human satellite sequences is chromosome specific (*18*). However, the method we have used is simpler and can be used to screen much larger numbers of individuals and chromosomes.

Several lines of investigation are suggested by the results of this study. First, restriction enzymes might produce chromosome banding and permit identification of all of the individual chromosomes in the genomes of such diverse organisms as amphibia, fish, and plants lacking easily banded chromosomes. Second, the distribution of subclasses of satellite DNA could be studied in species other than the human. Mouse satellite DNA is more nearly homogeneous than human satellite DNA, but certain restriction enzyme sites are present in only a fraction of the repeating units (*19*). Such variant sequences could either be concentrated on one or a small number of chromosomes (similar to the human situation) or be present on every chromosome. Information of this kind is important for understanding the evolution of repetitive DNA's. Third, the finding that regions containing amplified rRNA genes in human and rat chromosomes can be revealed by specific cutting with the enzyme Msp I suggests that cutting with one or more restriction enzymes might reveal regions containing other amplified genes; for example, the well-known homogeneously staining regions (HSR's) and double minutes (DM's) seen in many cancers and in drug-resistant cell lines. Finally, the effects on chromosomes exposed to these enzymes before fixation remain to be explored.

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Differential Classical Conditioning of a Defensive Withdrawal Reflex in *Aplysia californica*

Abstract. *The defensive siphon and gill withdrawal reflex of* Aplysia *is a simple reflex mediated by a well-defined neural circuit. This reflex exhibits classical conditioning when a weak tactile stimulus to the siphon is used as a conditioned stimulus and a strong shock to the tail is used as an unconditioned stimulus. The siphon withdrawal component of this reflex can be differentially conditioned when stimuli applied to two different sites on the mantle skin (the mantle shelf and the siphon) are used as discriminative stimuli. The differential conditioning can be acquired in a single trial, is retained for more than 24 hours, and increases in strength with increased trials. Differential conditioning can also be produced within the field of innervation of a single cluster of sensory neurons (the LE cluster) since two separate sites on the siphon skin can serve as discriminative stimuli. The finding that two independent afferent inputs that activate a common set of interneurons and motor neurons can be differentially conditioned restricts the possible cellular loci involved in the associative learning.*

In classical conditioning, an animal learns to associate two stimuli by the specific temporal relationship between them. Conditioning is thus thought to represent a prototypical example of the learning of causal relationships by animals and humans. Although classical conditioning is well understood behaviorally (*1*), the cellular mechanisms that underlie conditioning are still unknown, in part because of the difficulty of cellular studies in vertebrates, where conditioning has predominantly been examined. A potentially important advance in the analysis of the cellular mechanisms of associative learning was therefore achieved when it was demonstrated that a variety of complex behaviors in higher invertebrates show classical conditioning (*2*), including cognitive features of learning once thought to be exclusively mammalian (*3, 4*). Classical conditioning was recently demonstrated in a simple reflex, the gill and siphon withdrawal reflex in *Aplysia* (*5*), which is mediated by a well-delineated neural circuit (*6*). We now report that the siphon withdrawal component of this reflex is capable of differential conditioning with discriminative stimuli applied to two different sites on the mantle skin—the mantle shelf and the siphon. The demonstration that differential conditioning can occur in an elementary withdrawal reflex mediated by a small number of cells indicates that this more advanced form of associative learning is not an exclusive feature of behaviors having complex neural circuitry. Moreover, the finding that two independent afferent inputs, each of which activates a common set of motor neurons and some common interneurons (*6*), can be differentially conditioned restricts the possible cellular loci for the associative changes. Finally, differential conditioning allows each animal to serve as its own control and thereby provides a useful behavioral tool for analyzing classical conditioning on a cellular level (*7*).

In a previous study of classical conditioning of the siphon and gill withdrawal reflex, Carew, Walters, and Kandel (*5*) used a light tactile stimulus to the siphon, which produces a weak siphon and gill withdrawal, as the conditioned stimulus (CS) and a strong electric shock to