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Chromosomal Localization of Mouse Satellite DNA

Abstract. *Hybridization of radioactive nucleic acids with the DNA of cytological preparations shows that the sequences of mouse satellite DNA are located in the centromeric heterochromatin of the mouse chromosomes. Other types of heterochromatin in the cytological preparations do not contain satellite DNA.*

One characteristic which distinguishes the DNA of higher organisms from that of bacteria is the presence of families of repeated nucleotide sequences. These repeated sequences are found in multiplicities ranging from 10^2 to 10^6 per genome (*1*), but very little is known about their function or their organization within the chromosomal complement. Recently a technique which makes possible the cytological localization of specific nucleotide sequences has been developed (*2*). This localization is accomplished by hybridizing the DNA of cytological preparations with radioactive nucleic acid. The regions in the preparation to which the radioactive nucleic acid has bound are then detected by autoradiography. Such a technique permits a direct investigation of the distribution of families of repeated sequences within the genome.

Perhaps the most thoroughly studied fraction of repetitive DNA, with the exception of the sequences coding for ribosomal RNA, is the mouse satellite DNA. Therefore we chose mouse satellite DNA for our first investigations of the cytological localization of multiply repeated DNA sequences. We show that this fraction is located in the centromeric heterochromatin of the mouse chromosomes, a fact which we have briefly reported (*3*).

Mouse satellite DNA forms a band slightly separated from the main peak when mouse DNA is spun to equilibrium in a CsCl density gradient (*4*). It makes up about 10 percent of the total DNA, regardless of the tissue from which the DNA has been prepared, and is found in about the same proportion in tissue culture lines (*5*). From renaturation kinetics it has been estimated that mouse satellite DNA consists of approximately 10^6 copies per genome of a sequence some 400 nucleotide pairs in length (*6*). It is possible that the copies are not all identical. However, the rapid reassociation seen after denaturation indicates a high degree of homogeneity (*6*). Although the sequences of the mouse satellite make up 10 percent of the mouse DNA they do not seem to code for a corresponding fraction of the RNA in the tissues which have been studied. Flamm, Walker, and McCallum (*7*) were unable to detect any hybridization of satellite DNA to RNA from mouse liver, spleen, or kidney. Recently Harel *et al.* (*8*) have reported that rapidly labeled RNA from some of the same tissues did bind to satellite DNA. The coding properties of this fraction require further study.

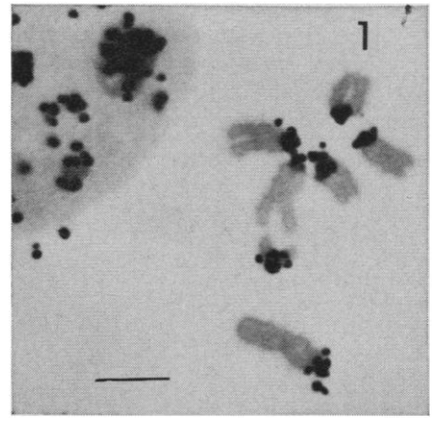

Fig. 1. Autoradiograph of a mouse tissue culture preparation after cytological hybridization with radioactive RNA copied in vitro from mouse satellite DNA. The RNA has bound to the centromeric heterochromatin of the chromosomes and to the chromocenters of the interphase nucleus on the left. The DNA of this preparation was denatured in situ by treatment with 0.07*N* NaOH. The slide was then incubated with radioactive RNA for 10 hours at 66°C. The preparation was treated with ribonuclease to remove RNA that was not specifically bound and then coated with autoradiographic emulsion. The RNA had a calculated specific activity of 7×10^7 disintegrations min^{-1} μg^{-1}. Slide stained with Giemsa. Exposure, 5 days; × 2000; scale length, 5 μm.

Our hybridization experiments on the localization of mouse satellite DNA have been done in two ways. First, we have applied fractions of radioactive mouse DNA to cytological preparations of mouse tissue culture cells (*9*). The radioactive DNA was extracted from tissue cultures of the mouse A9 line grown in medium containing [^{3}H]thymidine (*3*). Satellite DNA was separated from the rest of the mouse DNA by silver-ion–cesium sulfate (*10*) density gradient centrifugation. The DNA was denatured with heat before use in the hybridization reaction. This DNA had a specific activity of 200,000 cpm μg^{-1} as determined by spotting known amounts of the DNA on a nitrocellulose filter and counting the filter in toluene fluor in a scintillation counter. In the second type of experiment we applied radioactive RNA, transcribed in vitro from mouse DNA, to cytological preparations of both mouse testis and mouse tissue culture (*2*). Mouse liver DNA was fractionated by silver-ion–cesium sulfate centrifugation. The satellite DNA and the main peak DNA were transcribed separately with *Escherichia coli* RNA polymerase (*11*) and tritiated ribonucleotide triphosphates. This complementary RNA had a calculated specific activity of 7×10^7 disintegration $min^{-1} \mu g^{-1}$. In all experiments the DNA of the cytological preparations was denatured by treatment with NaOH before hybridization. Autoradiographs were exposed for several days when hybridization was done with complementary RNA and for several months when hybridization was done with radioactive DNA.

The normal mouse chromosomal complement consists of 20 pairs of telocentric or acrocentric chromosomes (*12*). Each chromosome has a region next to the centromere which can be identified as heterochromatin by its staining properties. In some mouse tissue culture lines a few of the chromosomes are metacentric and have presumably arisen by fusion of two of the chromosomes of the normal com-

plement. There is heterochromatin adjacent to the centromere on both arms of these chromosomes. All of our preparations are stained with Giemsa after the development of the autoradiograph. We find that with this stain the centromeric heterochromatin on chromosomes that have been treated with NaOH stains more densely than the rest of the chromosome.

We obtained similar results from both the DNA-DNA and the DNA-RNA hybridization experiments. Satellite DNA and its complementary RNA bound only to the centromeric heterochromatin of the chromosomes (*13*). Only one chromosome in our preparations appeared consistently unlabeled. Because the unlabeled chromosome was small and more heterochromatic than the other chromosomes in the testis preparations, we believe that it is the Y chromosome. In parallel experiments radioactive DNA from the main band and complementary RNA hybridized with many regions distributed over the entire chromosomal complement, an indication that the euchromatic regions of the chromosomes had been denatured and were capable of binding complementary nucleic acid sequences.

Localized binding of satellite DNA was evident throughout the cell cycle in both mitosis and meiosis. The binding was in the centromeric heterochromatin in all of the stages in which the chromosomes are condensed. In other stages the positions of the centromeres could be followed because of the localization of the satellite binding. In interphase nuclei, satellite DNA bound preferentially to the chromocenters, indicating that the majority of the centromeric regions are associated in these deeply staining chromatin blocks. In spermatids the centromeric regions were concentrated in a single mass in the central region. This mass also differs from the rest of the nuclear DNA in its binding of Giemsa stain.

The Sertoli cells of the testis contain what has been described as a compound nucleolus, consisting of an acidophilic body and one or more basophilic bodies. The basophilic bodies bound satellite DNA, indicating that in these cells the centromeres are closely associated into small groups near the nucleolus.

In the mouse both the X and the Y chromosomes show heterochromatization at various times, yet the binding of mouse satellite DNA indicates that satellite sequences are present only in the centromeric regions of the X. Figure 4 shows a pachytene spermatocyte. The heterochromatic X and Y make up the "sex vesicle" (*14*). Ribonucleic acid copied from the mouse satellite DNA is bound to the centromeric heterochromatin of the autosomes and to the tip of the sex vesicle (arrow in Fig. 4).

Our studies confirm and extend previous work on the localization of mouse satellite DNA. Maio and Schildkraut (*15*) separated isolated metaphase chromosomes from mouse L cells into several size classes by sedimenting the chromosomes through sucrose density gradients. They found that each size class contained approximately the same proportion of satellite DNA as did the entire genome, which suggests that satellite DNA makes up a constant proportion of the DNA of the individual chromosomes. Furthermore, although 70 percent of the DNA could be extracted from the chromosomes with 2*M* NaCl, the satellite DNA remained bound to the insoluble pro-

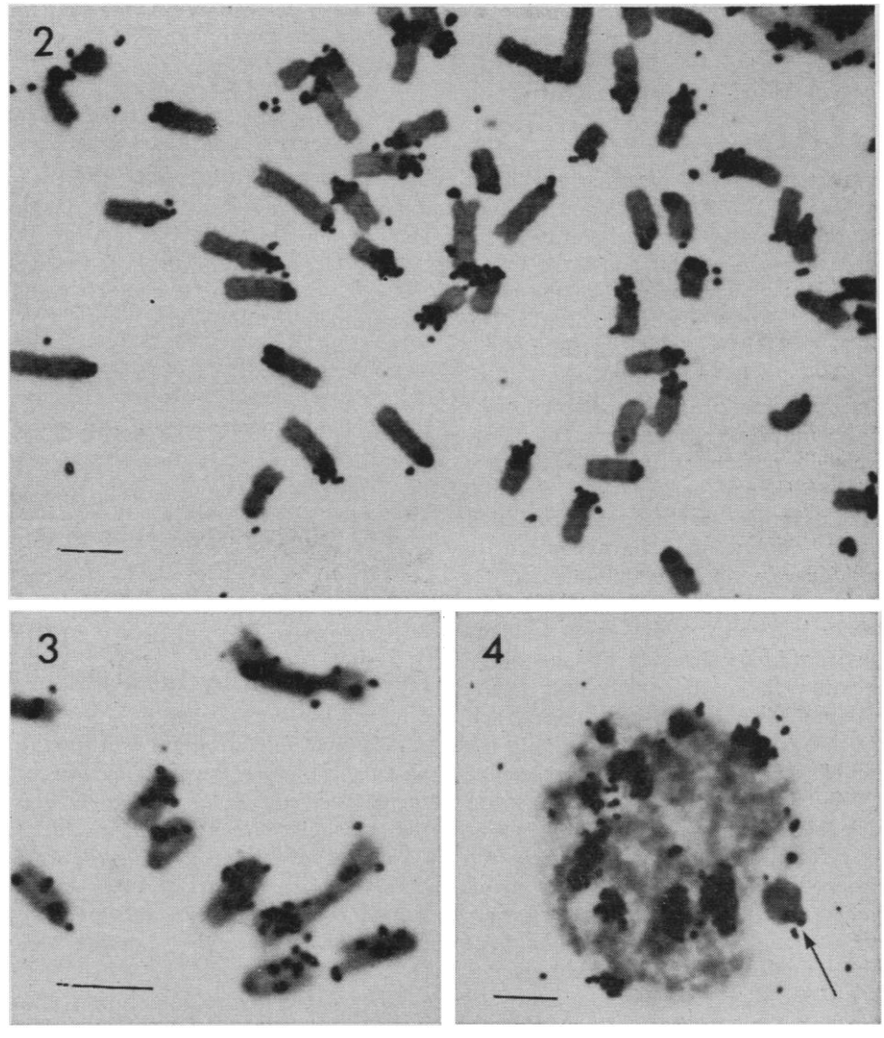

Fig. 2. Autoradiograph of a tissue culture preparation similar to that in Fig. 1. In these heteroploid tissue cultures the number of grains per chromosome is variable but no chromosome is consistently unlabeled. In testis squashes (not shown here) the Y chromosome appears to be unlabeled. Exposure, 5 days; × 1750; scale length, 5 μm. Fig. 3. Autoradiograph of a mouse tissue culture preparation after cytological hybridization with radioactive RNA copied in vitro from mouse main peak DNA. This RNA has bound to regions of the chromosome other than the centromeric heterochromatin. The conditions of denaturation and hybridization are the same as for Fig. 1. Exposure, 23 days; × 2700; scale length, 5 μm. Fig. 4. Autoradiograph of a mouse pachytene spermatocyte hybridized with radioactive RNA copied in vitro from mouse satellite DNA. The densely stained heterochromatic regions of the autosomes are heavily labeled but the sex vesicle, containing the heterochromatic X and Y chromosomes, shows only four grains and these are localized over the tip (arrow). The conditions of denaturation and hybridization are the same as for Fig. 1. Exposure, 10 days; × 1700; scale length, 5 μm.

tein matrix of the chromosomes, an indication that the protein associations of satellite DNA differ from those of main-band DNA. Yasmineh and Yunis (*16*) found that most of the DNA extracted from isolated heterochromatin of the mouse liver and brain cells was satellite DNA. The euchromatin prepared in these same experiments contained only DNA of main-band density.

Our results clearly demonstrate that heterochromatin is not a single category. It has been recognized for some time that certain chromosome regions maintain their heterochromatic character at all times. This type of heterochromatin has been termed constitutive and is usually considered genetically inert. Other chromosome regions or entire chromosomes, such as the mammalian X (*17*), become heterochromatic only in some particular stages or tissues. The second type of heterochromatin has been termed facultative or functional and is often associated with a turning-off of the genes on the chromosomes involved. The regions adjacent to the centromere are usually thought of as constitutive heterochromatin. This heterochromatin contains a specific type of DNA which is not present in the facultative heterochromatin of the sex chromosomes. It has also been reported that mouse chromosomes have constitutive heterochromatin at the telomeres (*18*). If these regions do contain satellite DNA sequences, these sequences are too few to be detected on our slides.

Although we have localized mouse satellite DNA in the centromeric heterochromatin, this localization does not establish a function for either satellite DNA or heterochromatin. It seems that this function is one which is necessary to the chromosome since the proportion of satellite DNA is maintained in established mouse cell lines even though the chromosomes have undergone other morphological change. We have found the same pattern of satellite hybridization in all of the mouse cell lines which we have studied. In this respect it becomes important to establish whether the apparent lack of hybridization at the Y centromere indicates a quantitative or qualitative difference from the other chromosomes. It is possible that centromeric heterochromatin might bind the proteins of the spindle microtubules during mitosis. We have investigated the binding of purified microtubular subunits to mouse satellite DNA in vitro (*19*) but our preliminary experiments showed no binding under the conditions used in our assay (*20*).

Centromeric heterochromatin has been described in the chromosomes of many animals and plants. It seems possible, therefore, that the centromeres are regularly adjacent to regions of repetitive DNA. Experiments in this laboratory have shown that the fly *Rhynchosciara hollaenderi* has a satellite of repetitive DNA. This satellite, like that of the mouse, has been localized in the centromeric heterochromatin by cytological hybridization (*21*). Cytological hybridization has also shown the presence of repetitive DNA at the centromeres in *Triturus viridescens* (*22*) and *Drosophila melanogaster* (*23*). It now seems important to determine whether noncentromeric heterochromatin consists of repetitive DNA with nucleotide sequences different from those of centromeric heterochromatin (*24*).

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25. Supported by PHS grants GM 12427 and GM 397. We thank Mrs. C. Barney for technical assistance. This work will be submitted by M.L.P. in partial fulfillment of requirements for the doctoral degree at Yale University.

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Motoneuron Morphology and Synaptic Contacts: Determination by Intracellular Dye Injection

Abstract. *The structure and dendritic connections of an identified crustacean motoneuron were analyzed by intracellular injection of dye. Some processes of the neuron end in the ganglionic neuropil, but most terminate on axons which pass through the ganglion in specific, identifiable tracts. The former processes are ipsilateral to the soma, while the latter, as well as their connections, display bilateral symmetry. Structural and functional evidence suggests that the demonstrated contacts are synaptic junctions, and that the approach can therefore be used to study patterns of synaptic organization in complex neural networks.*

One goal of neurophysiologists is to associate the functional properties of specific nerve networks with the underlying and presumably causal structural "circuitry." Toward this end Stretton and Kravitz (*1*) recently developed a technique for determining the geometry and positions of single neurons and their dendritic fields. A single nerve cell is injected with the fluorescent dye Procion Yellow, which becomes distributed throughout the cell and remains confined within it during subsequent histological procedures. When viewed with a fluorescence microscope, the dye-filled branches of the neuron appear brilliant yellow against a deep green background of uninjected tissue. This technique has been used, for example, in the study of the morphology and spatial relationships of the giant fibers and fast flexor motoneurons in the crayfish abdomen (*2*).

I have applied the dye injection technique to motoneurons supplying relatively complex locomotory appendages, the abdominal swimmerets of the lobster *Homarus americanus*. The mo-