DEVELOPMENTAL GENETICS

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Foreword

The Developmental Genetics seminar, held in Aspen July 6–11, 1975, was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Grant #5-TO1-GM-02084-05.

The seminar was divided into two main areas: (1) chromatin structure, organization and expression, and (2) diagnosis and treatment of developmental abnormalities. Although departments of Genetics have grown tremendously in the past 20 years, two developments are presently taking place. Through the use of banding technics, fluorescent probes and molecular and cellular hybridization, new knowledge concerning chromosome structure is being revealed and extensive gene mapping of autosomes as well as chromosomes is now a reality. In the first section of this monograph, various probes, experimental models and current advances are explained in detail.

Although genetic counseling services have been available in major medical centers for several years, the magnitude of the clinical problem is only now being appreciated. Early diagnosis (e.g., amniocentesis) and the complexity of the counseling problem in relation to the patient, his siblings, parents and relatives is of increasing importance and is emphasized in the second section.

Donald West King

内容提要

发育遗传学

本书是《病理生物学进展》丛书第3卷，包括两部分内容，①染色质的结构、组成及表现；②发育异常的诊断与治疗。共包括11篇论文：①染色体的结构，②染色体的结构亚单位和功能亚单位，③转录和染色质亚单位结构，④作为染色体结构和功能研究手段的真核体 DNA 节段的纯系繁殖，⑤染色体结构的免疫化学探讨，⑥哺乳类动物基因组的细胞化学分析，⑦哺乳类动物的细胞调节，⑧人类疾病的生化遗传，⑨遗传疾病的妊娠诊断，⑩—⑪遗传学咨询。

CONTENTS

Introduction

The chromosomes which are present in the nuclei of eukaryotes have long been known to carry the genetic information. However, how these structures store, replicate, and transmit this information remains unclear. It is the purpose of this monograph (1) to explore the structural and biochemical relationships of chromosomes, (2) to indicate some of the current problems in cytogenetic and chromatin research, and (3) to show how technical advances in cytogenetics can bring the areas of molecular biology, cytology, and clinical genetics closer together. It will become evident from the papers which are included that certain probes can be made once we understand something of chromosomal structure, and that such probes are useful tools for dissecting the mechanisms of human genetic disease. An issue crucial to the definition of eukaryotic gene expression is the structure of the genetic material itself. What is the nature of the components of eukaryotic chromosomes and what are the interactions which may establish this structure?

The initial chapters of this monograph will consider these problems.

For some time it has been known that the chromosomes consist of both nucleic acids and protein. More recently, the genetic component has been identified with DNA and, as discussed by Ris, one of the most remarkable features of the complex eukaryotic DNA is the enormous amount which is contained in the nucleus of each cell. Therefore, within the cell, DNA must be compressed many times in length. This enormous compression of the DNA raises several topological problems. How is the DNA physically arranged within a chromosome, and how much precision is there in its organization? Is there a general structure for most chromosome regions, or do particular sequences of DNA always interact with specific proteins? How does this structure change during the cell cycle?

With the advent of such technics as (1) high voltage electron microscopy (see Ris), (2) viscoelastic retardation measurements (see Taylor), (3) restriction endonucleases (see Axel, Thomas), and (4) recombinant DNA molecules (see Thomas), how the DNA component of the chromosome is organized is just beginning to be understood. The DNA of eykaryotic chromosomes exists in a highly folded condition, largely as a consequence of its interactions with histone proteins [3,4,6]. Numerous models of this folded state have been presented, based upon data from low angle X-ray diffraction studies and from electron microscopy. The models which appear to be favored currently consist of superhelical coiling of a fundamental nucleohistone strand [1]. Evidence has been presented [5] that chromatin fibers consist of chains of spheroid particles about 70Å in diameter and nu bodies, which contain DNA folded by association with a small number of histone

molecules. Spread chromatin fibers from isolated eukaryotic nuclei reveal linear arrays of nu bodies about 70Å in diameter connected by thin filaments about 15Å wide. The model of beads on a string has been attractive to many investigators because it correlates well with varying approaches to chromatin structure and at the same time provides an explanation for how DNA condenses in order to fit into the cell nucleus. When a fragment of DNA is tied up in a bead, it is about one-sixth the length it would be if it were extended. It is believed that DNA wraps around the outside of a globular cluster of histone and nonspecific proteins that could be folded when the chromatin is inside a cell nucleus (see Ris, Axel). Viscoelastic retardation measurements by Taylor have provided more evidence to support the concept that each chromosome consists of a single long polymer of DNA. Taylor has suggested that the genes may be bracketed by special sites which might allow discrete excision.

One perplexing issue in using isolated chromatin is whether factors governing the expression of genes *in vivo* are retained in an *in vitro* chromatin preparation. Using increasing concentrations of staphylococcus nuclease as a probe, the interphase eukaryotic chromosome has been shown to be composed of regularly repeating nucleoprotein subunits, each of which are 185 base pairs of DNA, 8–10 histone molecules and varying amounts of nonhistone protein. The determining factor in the production of DNA fragments lies in the structure of the histones and how they attach to DNA in native chromatin structure. This problem is explored in the chapter by Axel on the transcription and structure of chromatin.

The chapter by Thomas indicates how it is possible to examine a portion of the genome by obtaining a single DNA segment and replicating it many times in a suitable biologic vehicle, such as a bacterial plasmid. Each time the plasmid replicates, the foreign chromosomal segment replicates along with it. After amplification, the chromosomal segment can be recovered, and, by using a number of molecular probes and/or restriction endonucleases to break and join DNA fragments, specific portions of the genome can be examined. These cloning technics make it possible to obtain chromosomal segments comparable in size to viral DNAs. By obtaining DNA and RNA sequence data on these segments, eventually a map will be made which can be related to the classic genetic map.

Cytologic studies of chromosomes at a level of organization above the molecular and biophysical one have largely been confined to observations during cell division, since individual chromosomes are most distinctive during metaphase and anaphase, when they are condensed and stain intensely with basic dies.

As discussed in the sections by Miller and Hsu, many kinds of banding patterns are now recognizable in the mitotic chromosome, and these pat-

terns are related to the underlying molecular structure as explored in the earlier sections of this monograph.

As explained in the section by Hsu, it is possible to differentiate heterochromatin on a cytochemical basis, using the affinity of DNA for different fluorochromes. At the present time, sequences of DNA are classified by their degree of repetition in the haploid genome. The unique sequences (present in only one copy) are presumed to include the genes. The highly repetitive fraction (known to be associated with constitutive heterochromatin) often forms a satellite and appears to have a structural role, since it is not transcribed and is located in centromeric heterochromatin. (Some of these structural relationships are referred to in the initial section by Ris.)

C-banding technics, together with *in situ* DNA/RNA hybridization, allow identification of chromosome regions that are largely genetically inert (see Hsu). Using banding technics such as Giemsa and quinacrine as well as antibodies to AT and GC-rich regions, it has become possible during the past few years to identify chromosomes with similar morphology, localize repetitive DNA sequences, investigate the organization of the chromosome, and estimate genome size on the basis of amount of heterochromatin. Molecular cytogenetic advances allowing the recognition of chromosomes of similar morphology have allowed for more exact studies on human genetic disease, as is illustrated in the last sections of this monograph.

There have been many significant advances in human genetics since the twin method was first developed by Galton in 1876 [2] and Garrod in the early 20th century focused on "in-born errors of metabolism." The historical aspects of advances in biochemical aspects of human genetics are summarized by Seegmiller. Seegmiller also discusses the pathogenetic mechanisms by which a genetic abnormality in the biosynthetic pathways may lead to human disease. Although human genetic analysis is hampered by long generation time, small families, and random mating, the possibilities of prenatal diagnosis by amniocentesis, as discussed by Hirschhorn, provide the framework within which to view the role of genetics in medicine, since both biochemical and cytogenetic studies may be performed on the cells thus obtained.

These studies are performed on cells during amniocentesis and then grown *in vitro*. The kinds of somatic cell genetic studies discussed in the chapter by Puck hold promise for providing a reasonably accurate method for identifying a particular cell product and for locating the chromosome(s) on which its associated genetic loci are located.

All human disease may be thought of as resulting from the interplay of both genetic and nongenetic factors. In some of the rarer biochemical disorders, the genetic basis for disease is evident, but in others, other factors, such as environmental ones, must also be considered.

The precise mode of inheritance, gene frequency, mutation rate and dynamics in the population are discussed (Robinson and Lubs). Common multifactorial disorders are being examined to see if there is a significant factor in the etiology and pathogenesis of the given disorder. Some of the approaches used are: (1) to determine whether there is a predisposition of cases among relatives of persons with a given disorder, (2) to use twin studies to provide specific evidence for genetic etiology, and (3) to compare frequencies of a given disorder within different ethnic groups, blood groups, and disease associations.

The newer biochemical and cytogenetic technics discussed in the last half of the monograph enable one to intensively screen the population for genetic disorders and form the rational basis of informed genetic counseling both before and after marriage. The general public is largely unaware of the existence of genetic counseling, and intensive education directed at high school, college, and adult education groups is required. Only in this way will people be helped to make responsible decisions about reproduction and ultimately diminish "society's burden of chronic disease," which is far more expensive than genetic counseling. Changing patterns in genetic counseling are being developed to enable larger number of families to be counseled regardless of social class and cost. Funding for these programs will have to come from state and federal funds (see Lubs and Lubs).

The Editors

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Chromosome Structure

Hans Ris, Ph.D

CHROMOSOMES: LIGHT MICROSCOPIC OBSERVATIONS

In eukaryotes, the nuclear DNA, the major genetic system of the cell, is subdivided into a specific number of characteristic segments. In association with nuclear proteins, these DNA segments are folded into compact structures called chromosomes. Light microscopic studies have shown that chromosomes undergo complex structural changes during the cell cycle: During mitosis they become maximally condensed, while in interphase they are for the most part unraveled. Some regions which remain condensed during interphase are distinguished as *heterochromatin*, in contrast to the unraveled *euchromatin*. Certain chromosome regions are always condensed (*constitutive heterochromatin*), while others may be condensed in some cells and unraveled in others (*facultative heterochromatin*). This metaphase condensation appears to be regular, i.e., mitotic chromosomes have the appearance of a spring coil (Fig. 1). A thread about 200 nm thick, the *chromonema*, is arranged in a helical coil. In early prophase one finds a large number of *gyres* which progressively increase in diameter and decrease in number as the chromosomes thicken. In metaphase, each chromosome has a characteristic number of gyres, though variations may occur in different tissues under different environmental conditions. The direction of the helix may be both left and right handed in the same chromosome arm. In some organisms this helical structure is visible in living cells [1] while in others it becomes obvious only after specific treatments, such as with hypotonic salt solutions [16]. One or several short regions of metaphase chromosomes are never coiled and appear as constrictions, e.g., centromere region (primary constriction), next to the nucleolar organizer and certain other regions (secondary constriction). In these regions, the chromonema runs parallel to the chromosome axis for a short distance. Primary and secondary constrictions were the first markers which cytogeneticists used to recognize individual mammalian chromosomes.

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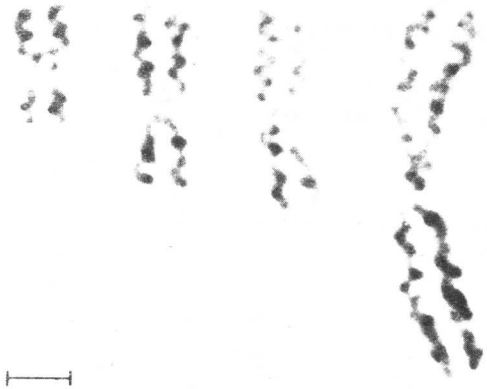

FIG. 1. Metaphase chromosomes from human leukocytes treated with special hypotonic salt solution to reveal spiral structure. (*From* Ohnuki Y: Chromosoma 25: 402, 1968; reproduced with permission of the author.)

More recently, other technics have been developed which demonstrate previously unknown structural and chemical differences. One of these technics is known as banding, and imparts to the chromosomes a characteristic striped or cross-banded appearance. For example, the fluorescent dye quinacrine reveals regions with AT rich DNA (Q bands) [28]. Giemsa staining after certain pretreatments produces bands which in general correspond to the quinacrine bands (G bands). Another Giemsa method restricts the staining to certain types of heterochromatin often associated with centromeres (C banding). Fluorochrome labeled antinucleoside antibodies have been used to indicate AT rich versus GC rich regions [15]. The relationship of these "bands" to the underlying chemical and structural differences along the chromosome is not yet understood but these methods have been of tremendous value in distinguishing the chromosomes of a species, establishing karyotypes and mapping chromosome alterations in humans and other organisms [8]. (See also the chapters by Hsu and Miller.)

In species with large chromosomes, another way of identifying the individual chromosome has long been known. In prophase of mitosis and meiosis the chromosome is divided into a series of larger or smaller condensed elements known as *chromomeres*. In early prophase of meiosis of the lily, about 5000 chromomeres can be distinguished. In mitotic prophase there are fewer, suggesting that these might be aggregates of smaller units. In polytene chromosomes of Diptera (e.g., *Drosophila*) the chromomeres of the many parallel chromatids that are in register form discs or bands (Figs. 2 a,b). (These chromomere bands must not be confused with the entirely different bands of metaphase chromosomes.) The chromomeres and bands of Diptera are of special interest because cytogenetic work has indicated that each chromomere is the structural equivalent of a genetic-functional unit or *cistron* [9]. It is not known whether the meiotic prophase

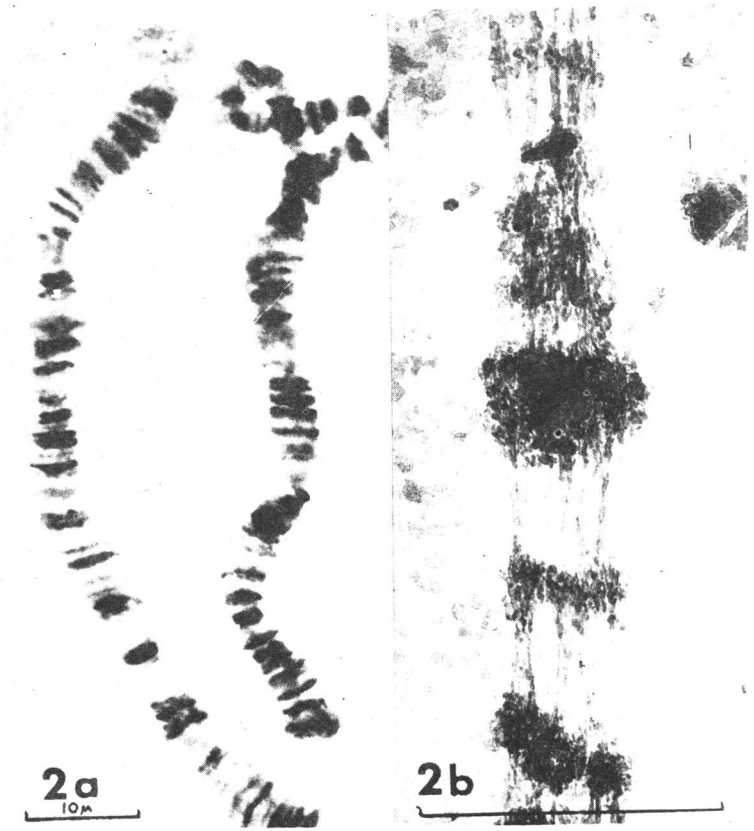

FIG. 2a. Polytene chromosomes from salivary gland of Drosophila virilis (Feulgen squash). The bands, which correspond to specific gene loci, are formed by lateral association of chromomeres in this multistranded chromosome.

FIG. 2b. Electron micrograph of a polytene chromosome from fat body of Drosophila melanogaster. Each chromatid consists of a 20 nm nucleohistone fiber which is highly folded in the chromomere (band) region. (Fixed in formalin-acetic acid, squashed in 50% acetic acid, critical point dried, photographed with the AEI EM-7 1 MeV Electron microscope at the University of Wisconsin.)

chromomeres correspond to the interphase chromomeres of the polytene chromosomes. While the prophase "chromomere patterns" of chromosomes are a constant feature for a given cell type or stage in the cell cycle and can serve for mapping specific chromosomes, it is not necessarily the same in all cell types. For instance in certain plants, prophase chromomere number may vary from one tissue to another [14]. Thus light microscopic studies have established that chromosomes are the constant and persisting units of the genome (corresponding to the linkage groups of genetic analysis). During nuclear division, they condense into helically

coiled compact structures, while in interphase they are generally unraveled. Patterns of differential condensation are frequently found along the chromosome fiber from the richly detailed banding pattern of dipteran polytene chromosomes and of meiotic prophase to the coarser patterns seen in mitotic prophase and in specifically treated metaphase chromosomes.

CHROMOSOMES: ELECTRON MICROSCOPIC OBSERVATIONS

The conventional 100 KV electron microscope is limited to specimens less than 100 nm thick (mainly because chromatic aberration and multiple scattering cause a rapid decrease in resolution in thicker specimens). This has severely limited analysis of chromosome organization with the EM. The million volt electron microscope has improved this situation because a resolution of 2–3 nm can be obtained with either isolated chromosomes or thick sections containing entire metaphase chromosomes (Figs. 3 a,b). The

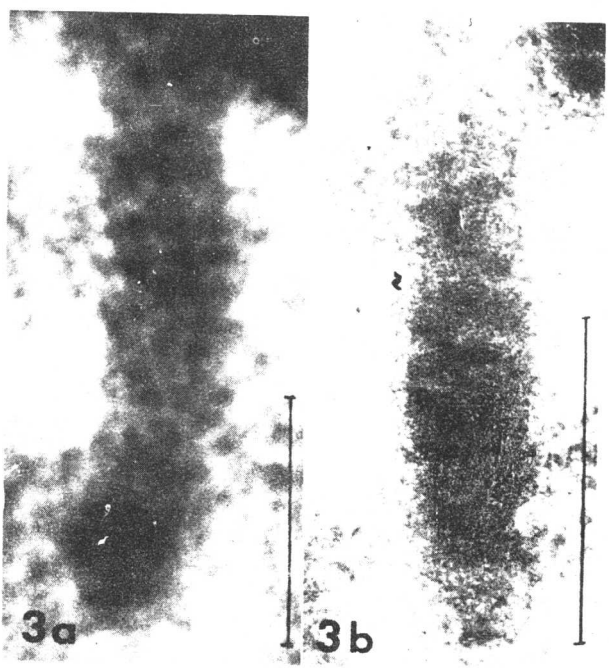

FIG. 3. Chromosome of Dinoflagellate (Cryptothecodinium cohnii). One micron section. (a) Photographed at 80 kV with Siemens Elmiskop I. (b) photographed at 1000 kV, with 1.5 MeV microscope in Toulouse, France. The 2 nm thick DNA filaments in this chromosome are resolved in the high voltage electron microscope, but not in the conventional electron microscope.

present limitation lies in the intrinsic complexity of three-dimensional chromosome structure. The use of stereoscopic electron micrographs has a definite advantage, but computer aided reconstruction may be necessary for three dimensional analysis. Another limitation of conventional electron microscopy is the need for specimen dehydration. It is known from X-ray diffraction studies that this changes the 11 nm periodic repeating structure present in chromosome material. Thus the use of hydrated or frozen specimens is required to preserve some aspects of chromosome organization. The high voltage electron microscope provides better resolution with both of these technics and their application to chromosomes is now being pursued at the High Voltage EM laboratory in Madison.

Despite these limitations, the 100 KV conventional electron microscope has made many important contributions to our knowledge of chromosome structure [22,23]. These are briefly summarized below.

Individual chromosomes can usually not be recognized except during mitosis. The term *chromatin* is therefore used to describe chromosomal material in general. Chromatin organization differs according to functional state. *Active chromatin* refers to chromosome regions which are actively being transcribed and *inactive chromatin* to those which are turned off. In most nuclei, 80% or more of the chromatin is inactive.

1. *Inactive Chromatin.* In almost all eukaryotes studied so far (exceptions are some primitive eukaryotes, such as Dinoflagellates and Radiolaria), inactive chromatin is composed of fibers around 20 nm thick, called the *native fiber* (Fig. 4). It can be made visible by freeze fracturing (Fig. 5) [21] by spreading chromatin on water or other low ionic strength media and fixation of nuclei with aldehydes in nonchelating buffers. This fiber often shows side branches of variable lengths. Treatment with chelating agents (EDTA, EGTA, sodium citrate, and commonly used buffers, such as phosphate buffer, Tris-HCl, veronalacetate, cacodylate) changes this native fiber into a knobby 10 nm fiber. Digestion with pronase demonstrates that this 10 nm fiber contains a single DNA double helix; I therefore call it the *unit fiber* (Fig. 4). Since biochemical studies on chromatin always use chelating agents to solubilize chromatin, the unit fiber is the constituent of chromatin studied by biochemists. In either sections or after critical point drying, the unit fiber has a relatively uniform knobby appearance. Recently a different structure has been described following air drying from a detergent (Photo-flo). In contrast to this uniform appearance, the unit fiber may appear as a string of beads 8–11 nm in diameter (Fig. 6) [17,18,29]. I shall return to this seeming discrepancy in relation to recent biochemical studies on nucleohistones. Using potassium pyroantinomate-osmium tetroxide, which forms a specific precipitate with cations, one can show that whereas the native fiber contains demonstrable cations (probably both Ca^{++} and Mg^{++}), the 10 nm fiber does not [22]. It is not yet clear how the native

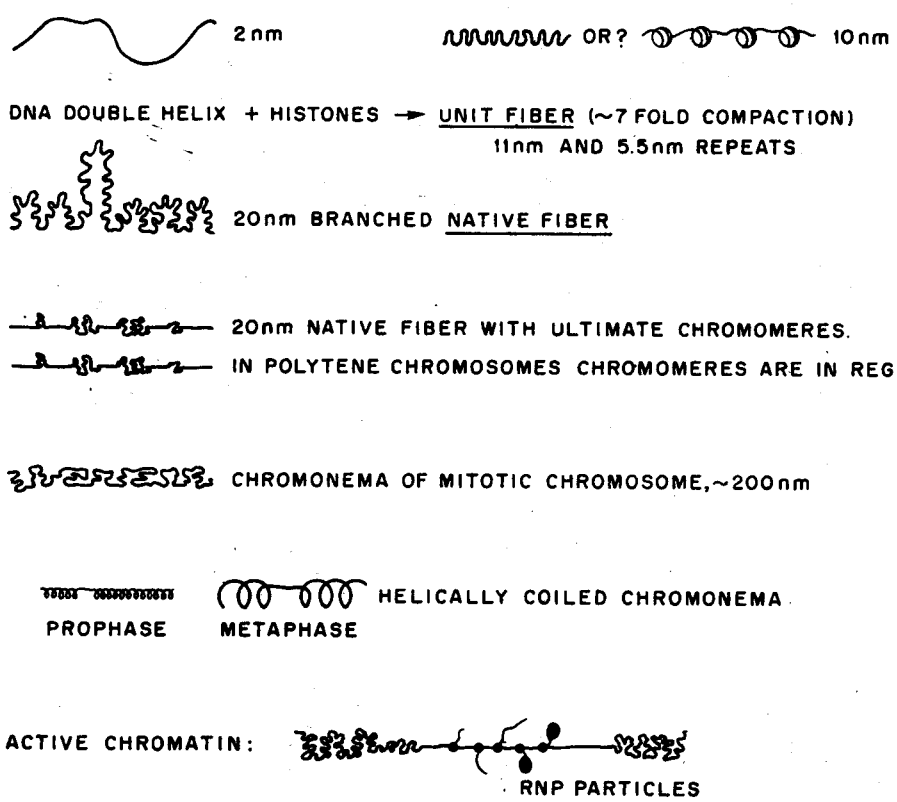

FIG. 4. The various levels of organization in chromosomes

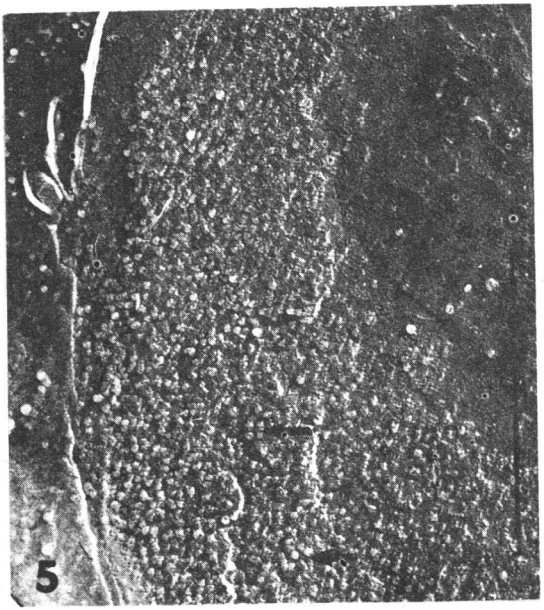

FIG. 5. Freeze-etching of nucleus in spleen of salamander. The chromatin shows cross fractures through the 20nm native fibers (arrows).

fiber is formed from the unit fiber, but cations seem to be involved. Fixing after brief treatment with citrate indicates that the side branches are loops of the native fiber folding back on itself (Fig. 7). Supercoiling of the unit fiber may also be involved, but such coiling has not been seen so far in the native fiber. Twisting a flexible thread, such as a thick rubber band, produces supercoiling as well as side branches and thus might model the origin of the native fiber.

This 20 nm thick native fiber constitutes all inactive chromatin in somatic nuclei whether it is highly condensed as heterochromatin or dispersed as euchromatin. It also forms both the bands and interbands of polytene chromosomes. It is the only constituent of mitotic chromosomes. The first step in understanding the structure of chromomeres, heterochromatin, and mitotic chromosomes is to describe how the native fiber is arranged to form these structures.

2. *Active Chromatin.* O. Miller [15] and co-workers [7] have developed a method by which active regions are spread out on electron microscope grids so that the chromatin fiber and the transcripts still attached to it are clearly seen in electron micrographs. The unit fiber is definitely thinner in active chromatin than in inactive chromatin. We assume that those factors that turn on transcription and unravel the native fiber also cause a change in the DNA-histone binding, which leads to an unraveling of the DNA and