Contributions of Electron Microscopic Spreading Preparations ("Miller Spreads") to the Analysis of Chromosome Structure

U. SCHEER [1]

1 Introduction

Before introduction of the chromatin spreading technique by Miller and Beatty in 1969 (Miller and Beatty 1969 a–d), relatively limited information was available concerning the submicroscopic organization of chromosomes in mitosis and interphase. Thus, at the end of the 1960s the general picture that emerged from inspection of ultrathin sections of cell nuclei, isolated chromatin, and of whole mount preparations was that mitotic chromosomes and interphase chromatin consisted of a complex meshwork of irregularly sized and knobby fibers with diameters ranging from 20 to 30 nm (for reviews see Ris 1969; DuPraw 1970; Solari 1974; Ris and Korenberg 1979; Hozier 1979). Although the internal organization of chromatin fibers was particularly suitable for study by surface spreading on an air-water interface, originally introduced by Gall (1963), neither a discrete subunit organization was recognized nor could the "thick" chromatin fibers be reproducibly unfolded into thinner fibers which were believed to be transcriptionally active (Gall 1966). Hence, electron microscopic methods were unavailable to study features of chromosomes during interphase, i.e., their functional subdivision into transcriptionally active and inactive domains.

A technical breakthrough came with the chromatin spreading technique developed by O. Miller and co-workers (Miller and Beatty 1969 a–d; Miller and Hamkalo 1972; Miller and Bakken 1972; Hamkalo and Miller 1973; Hamkalo et al. 1973). This spreading procedure was designed to unfold chromatin gently, but extensively by exposure to a medium of very low ionic strength at slightly alkaline pH and to avoid mechanical stress due to surface tensions. The "Miller spreading", as the preparation is often referred to, unraveled chromosomes and interphase chromatin to the first level of the nucleoprotein organization and permitted visualization of transcript fibrils which remained associated with the extended chromatin filaments. In fact, the first papers published by Miller and Beatty (1969 a–d) described the morphological features of transcriptionally active genes while only later, in 1973, Olins and Olins and independently Woodcock drew attention to repeating globular subunit structures present in transcriptionally inactive chromatin fibers prepared from a variety of cells according to the Miller procedure (Olins and Olins 1973; Woodcock 1973).

[1] Institute of Zoology I, University of Würzburg, Röntgenring 10, 8700 Würzburg, FRG.

It is obvious that one essential step of the Miller spreading technique is the extensive unfolding of chromatin by hypotonic media in order to disrupt all higher levels of chromosomal arrangements as they exist in the living cell. Although modifications of the Miller procedure (e.g., inclusion of salt in the dispersal medium, very brief exposure to low salt medium) have also been successfully applied to the study of higher order, supranucleosomal packaging of chromatin and to identify intermediate, transitory states during experimentally induced relaxation (see, e.g., Hozier 1979; Zentgraf et al. 1980a, b, 1981; Hamkalo et al. 1981; Labhart et al. 1982), such aspects will not be considered in the present chapter. Rather, this overview focuses on molecular aspects of chromatin organization with special emphasis on structural modifications that are concerned with transcription. Furthermore, more recent developments of the Miller chromatin spreading method will be discussed which combine immunolocalization and in situ hybridization techniques. Such applications of the chromatin spreading method are of great potential value for identification of DNA- and RNA-associated proteins and the genetic content of specific transcription units.

2 The Nucleosome Chain:
The Elementary Structural Component of Chromosomes

When mitotic chromosomes or interphase chromatin preparations are unfolded by exposure to a buffer of very low ionic strength (e.g., 0.1 mM Na-borate buffer, pH 8–9) and centrifuged onto an electron microscopic grid essentially as described in detail by Miller and Hamkalo (1972), Miller and Bakken (1972), and Hamkalo and Miller (1973), the bulk of the transcriptionally inactive chromatin displays the characteristic linear arrays of closely packed particles, the nucleosomes, connected by thin filaments representing linker DNA (Fig. 1a, b). Such extended beaded chains were first described by Olins and Olins (1973, 1974) and Woodcock (1973; Woodcock et al. 1976); the term nucleosome was introduced by Oudet et al. (1975). The beaded chromatin fiber or primary nucleofilament organization has been found in all eukaryotic species so far studied with the notable exception of some dinoflagellates which do not contain histones (Herzog and Soyer 1981). Thus, chromatin of plant and animal species is morphologically indistinguishable in Miller spreads (e.g., chromatin from human HeLa cells and amphibian oocytes; Fig. 1a, b; amphibian and avian erythrocytes, *Drosophila* polytene chromosomes, *Chlamydomonas:* Woodcock et al. 1976; rat and chicken: Olins et al. 1975; Zentgraf et al. 1980a; sea urchin sperm: Zentgraf et al. 1980b; *Physarum polycephalum:* Scheer et al. 1981; *Dytiscus marginalis:* Scheer and Zentgraf 1978; *Drosophila melanogaster:* McKnight and Miller 1976; *Oncopeltus fasciatus:* Foe 1978; *Cyclops furciper:* Beermann 1984; *Zea mays:* Zentgraf et al. 1981; Greimers and Deltour 1981; *Allium cepa* and *Sambucus nigra:* Lutz and Nagl 1980; cultured mammalian cells of various origin: Howze et al. 1976; Franke et al. 1978; Hamkalo et al. 1981; Labhart et al. 1982).

The packing of DNA into a repeating subunit structure has also been established by biochemical and physicochemical methods and it is fascinating to follow
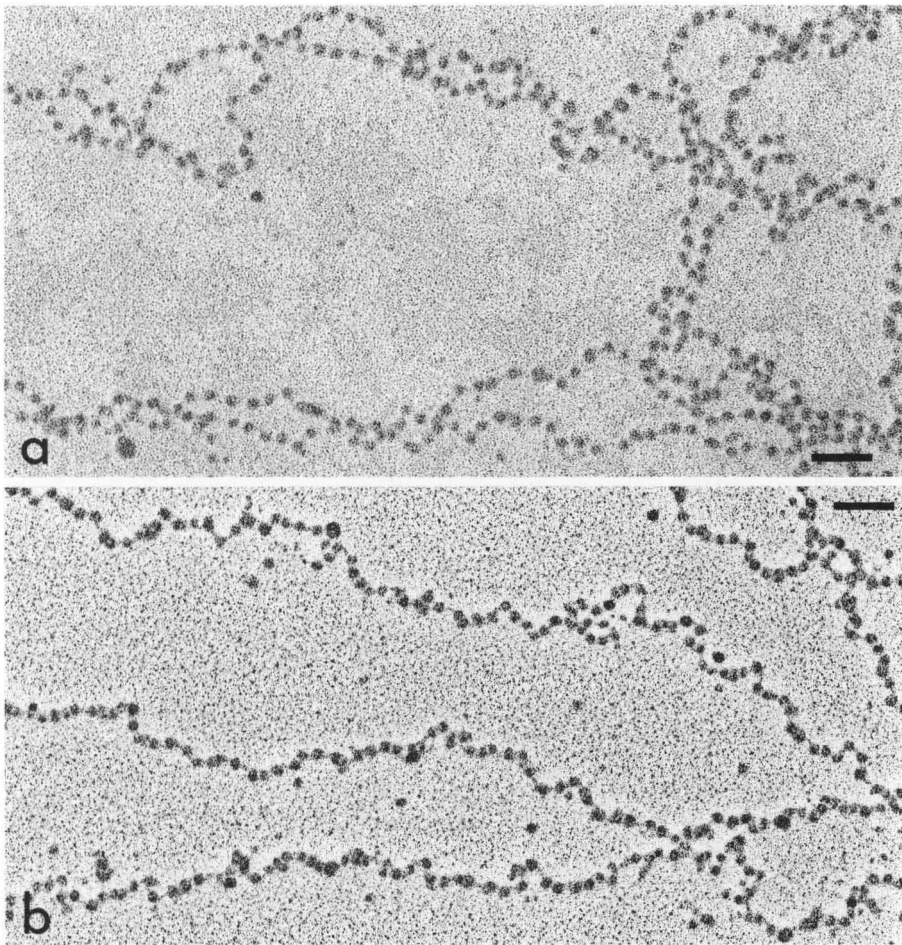
Fig. 1 a, b. Electron microscopic appearance of transcriptionally inactive chromatin in Miller spreads. Chromatin from human HeLa cells (**a**) and oocytes of the salamander *Pleurodeles waltlii* (**b**) was unfolded by exposure to very low salt conditions prior to centrifugation onto electron microscopic grids. The chromatin fibers are composed of linear arrays of globular subunits, the nucleosomes. $\times 80\,000$; *bars* $= 0.1$ µm

how the data, obtained by various methods, were eventually integrated into the nucleosome model (for reviews see McGhee and Felsenfeld 1980; Igo-Kemenes et al. 1982; Kornberg 1977). Since each nucleosome contains about 200 base pairs (bp) of DNA (with an extended length of the B-form of 200×3.4 Å $= 680$ Å), the coiling of the DNA around the nucleosome core with a diameter of approximately 110 Å results in an apparent foreshortening of the DNA by a factor of about 6. In Miller spreads nucleosomes are usually somewhat distantly spaced (Fig. 1 a, b) so that the apparent contraction ratio of DNA is only about two to three.

3 Visualization of Transcribing rRNA Genes

The amplified ribosomal RNA (rRNA) genes of amphibian oocytes were the first genes visualized by electron microscopy (Miller and Beatty 1969 a–d; Figs. 2 and 3). Subsequently numerous investigators employed the Miller technique to
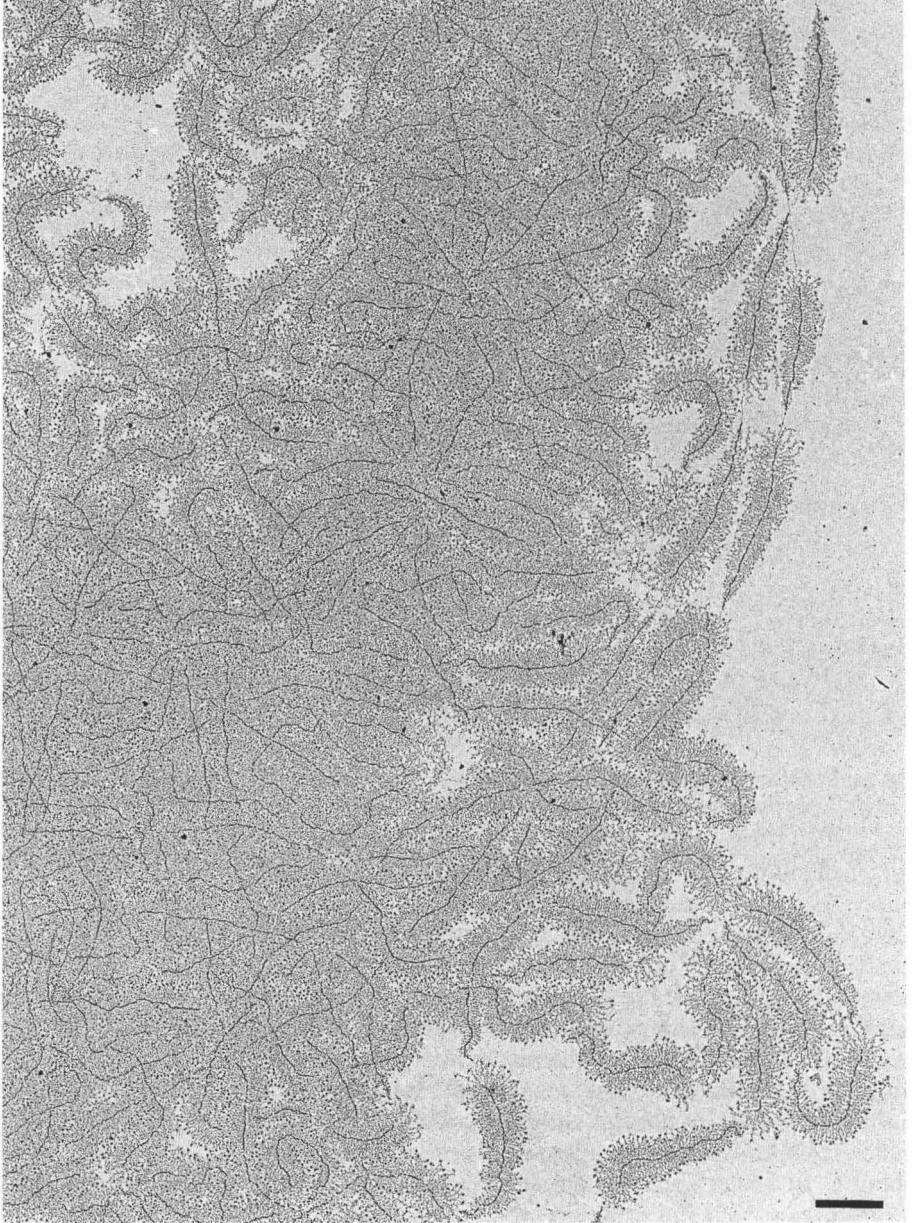

Fig. 2. Survey electron micrograph of spread nucleolar chromatin from a mid-sized *Pleurodeles* oocyte. This oogenic stage is characterized by intense synthesis of rRNA. × 9000; *bar* = 1 μm
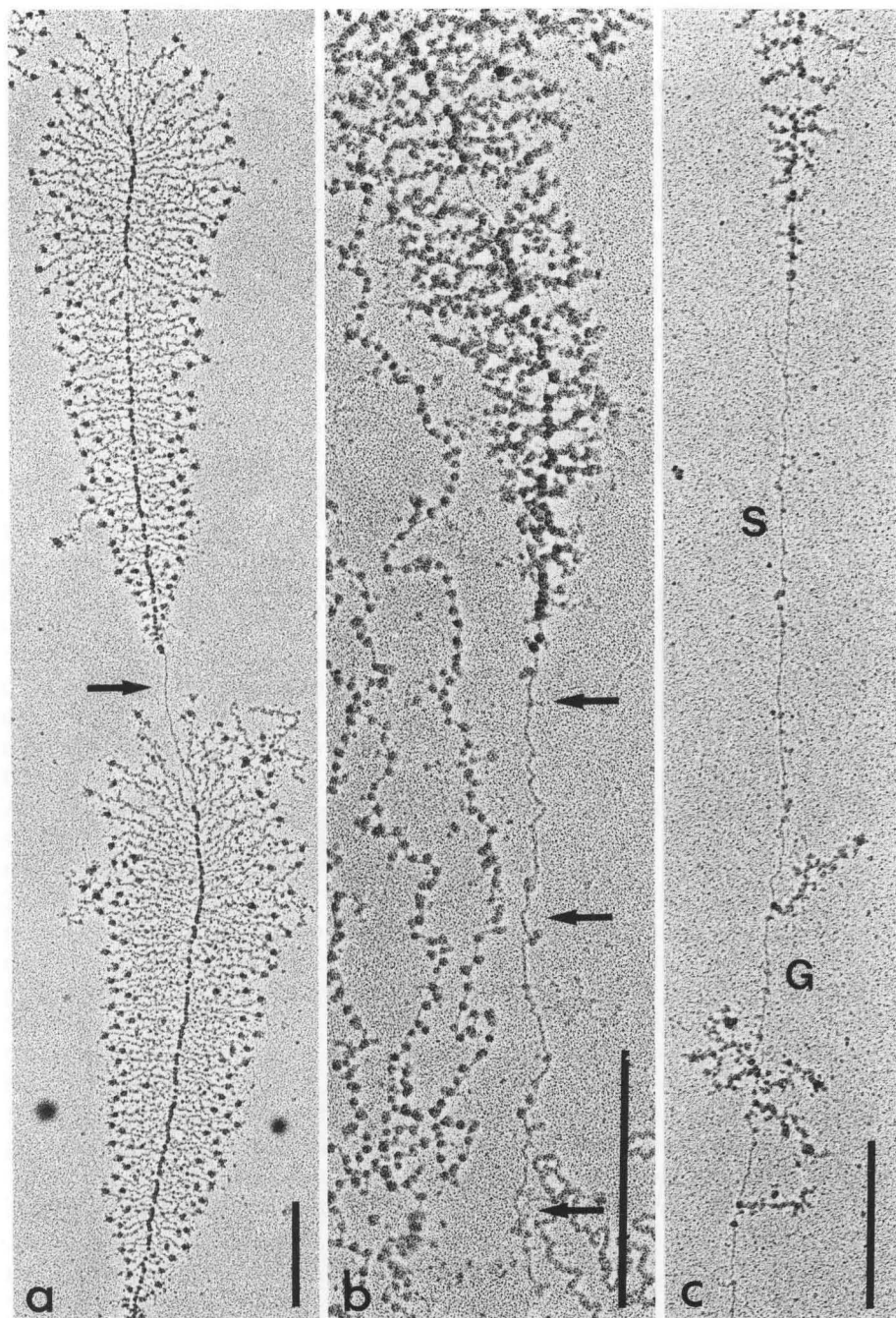
Fig. 3a–c. Structural details of transcribed rRNA genes as seen in Miller spreads. **a** Two tandemly arranged rRNA genes from a *Pleurodeles* oocyte. Note the high packing density of the RNA polymerases and the smooth configuration of the spacer region (*arrow*). **b** Higher magnification of the spacer region (*arrows*) and the proximal portion of an rRNA gene from an oocyte of *Triturus cristatus*. The spacer chromatin is clearly different from adjacent inactive chromatin fibers which reveal the characteristic nucleosome arrangement. **c** The smooth, nonnucleosomal chromatin conformation is maintained in the spacer (*S*) and the gene (*G*) regions in stages of reduced transcriptional activity (spread preparation from a mature oocyte of *Triturus cristatus*).
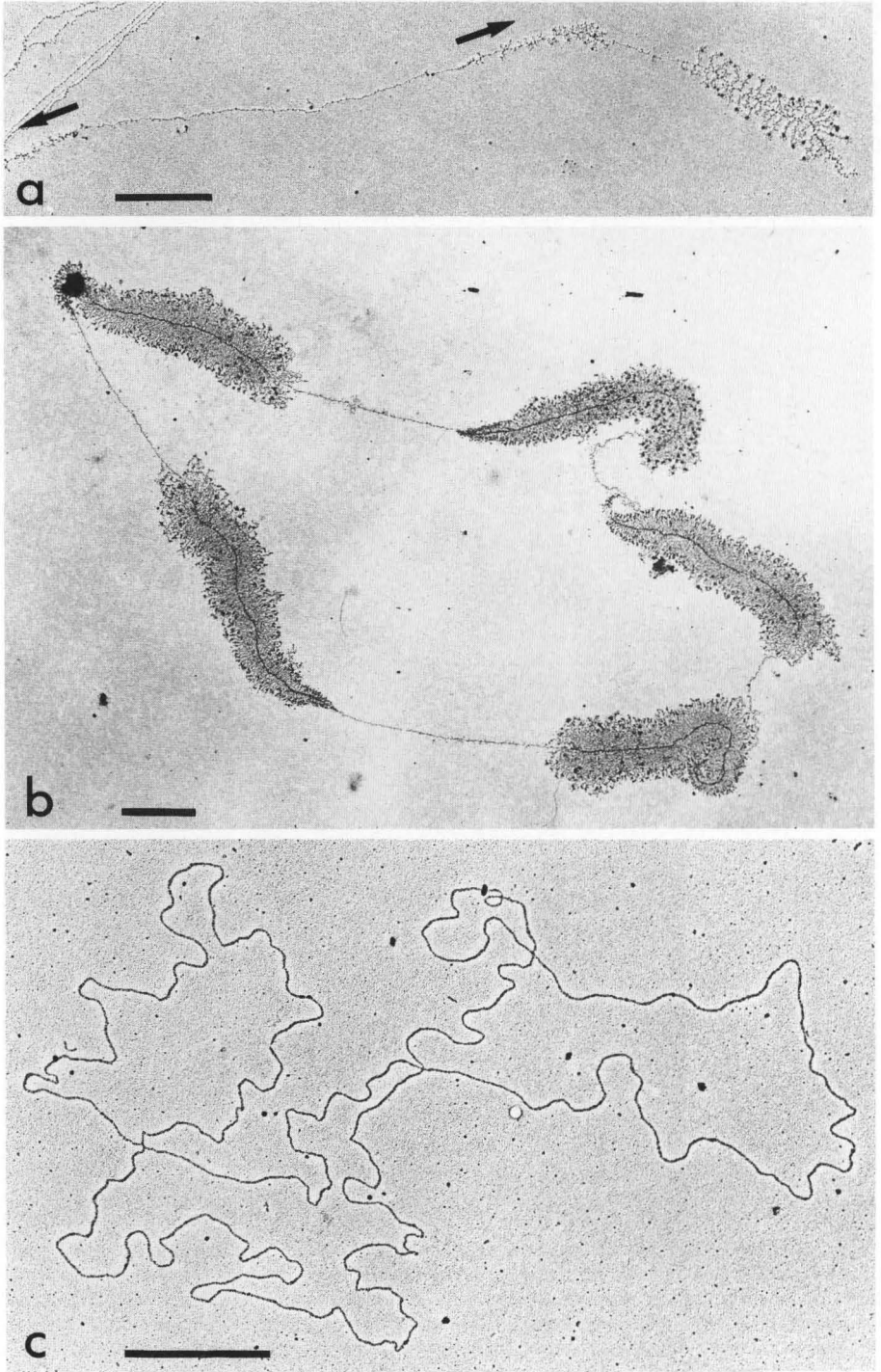**a** ×28 000; **b** ×70 000; **c** ×45 000; *bars* = 0.5 μm

Fig. 4 a–c

analyze transcribing rRNA genes from a variety of plant and animal cells (for reviews see Miller and Hamkalo 1972; Miller and Bakken 1972; Hamkalo and Miller 1973; Hamkalo et al. 1973; Chooi 1976; Franke and Scheer 1978; Franke et al. 1979; McKnight et al. 1979; Miller 1981, 1984; Scheer and Zentgraf 1982; Berger and Schweiger 1982; Puvion-Dutilleul 1983; Trendelenburg 1983). Figures 2 to 4 illustrate the appearance of rRNA genes from amphibian and insect oocytes and from the slime mold, *Physarum polycephalum*. Since the rDNA of *Xenopus laevis* has been completely sequenced, the ribosomal transcription units of this species can be correlated precisely with specific DNA sequences (e.g., Sollner-Webb et al. 1982; Bakken et al. 1982).

In spite of extensive interspecies differences in the lengths of transcription units, the interspersed nontranscribed spacers and the resulting repeat units (for a compilation of quantitative data see Franke et al. 1979), several common characteristic features of rRNA genes emerged from these studies.

1. The chromatin axis of active rRNA genes is usually densely covered by RNA polymerase I-containing particles from which the transcript fibrils extend laterally. The lateral fibrils, containing nascent pre-rRNA chains, gradually increase in length in the direction of transcription so that the characteristic "Christmas trees" are formed (Figs. 2 and 3a). The free ends of the transcripts are often accentuated by a terminal knob. In most transcription units the lateral fibrils increase in length as a function of the distance from the transcription initiation site, although the length increment seems to be lower or almost zero for the more distal transcripts (Franke et al. 1976a; Laird et al. 1976; Puvion-Dutilleul et al. 1977; Angelier et al. 1979). In *Dictyostelium,* the fibril length gradient is interrupted, suggesting that processing of the nascent pre-rRNA takes place at a specific period during transcription (Grainger and Maizels 1980).

2. In most species, multiple rRNA genes are arranged in tandem with identical polarities along a given chromatin strand and are separated from each other by nontranscribed spacers (Fig. 3a). It should be noted, however, that at least in amphibians, the so-called nontranscribed spacer might, in fact, be transcribed. Spacer transcripts arranged in defined transcription units are not infrequently found in Miller spreads of *Xenopus laevis* oocytes and seem to originate from the reduplicated promotor-like sequences in the spacer DNA (cf. Trendelenburg 1981; Morgan et al. 1983). There are, however, notable exceptions to the familiar gene-spacer-gene pattern. The rRNA genes of certain unicellular green algae are tightly clustered without detectable spacers or are arranged with alternating polarities along the chromatin axis (Berger and Schweiger 1982). Opposite polarities

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Fig. 4. a Linear nucleolar chromatin strand from the slime mold *Physarum polycephalum* with two rRNA genes at either end in opposite polarities (the direction of transcription is indicated by the *arrows;* only the proximal part of the left transcription unit is shown). The intergenic nontranscribed region is compacted into nucleosomes. In such Miller spreads the intergene region has an average length of 4.4 μm as compared to the extended length of the corresponding DNA (B-form) of 8.5 μm (cf. Seebeck and Braun 1982). **b** Nucleolar chromatin ring with five repeating units from an oocyte of the water beetle, *Dytiscus marginalis*. The contour length of the ring shown is 35 μm. **c** rDNA circle isolated from *Dytiscus* nucleolar chromatin with a contour length of 34.3 μm (cytochrome c-surface spreading). Molecules of this size class represent 5-gene rings.
a × 13 000; **b** × 9800; **c** × 19 800; *bars* = 1 μm

of adjacent rRNA genes are also present in the amplified nucleolar chromatin of *Physarum, Dictyostelium,* and *Tetrahymena* which form linear strands with two rRNA genes at either end in a palindromic arrangement (Fig. 4 a; cf. Grainger and Ogle 1978; Grainger and Maizels 1980; Seebeck and Braun 1982; Vavra et al. 1982; Engberg 1985).

3. Transcribed nucleolar chromatin, as well as the intervening spacers are devoid of nucleosomes. The absence of nucleosomes is compatible with the full extension of the rDNA to the length of its B-form in Miller spreads. This has been shown by the congruence of the lengths of rDNA repeating units at the DNA and chromatin level (Trendelenburg et al. 1976; Scheer et al. 1977; Reeder et al. 1978) and by estimation of the DNA length contained in ribosomal transcription units by gel electrophoretic determinations of the molecular weight of the pre-rRNAs (e.g., Meyer and Hennig 1974; Scheer et al. 1977; Spring et al. 1976).

In Miller spreads the chromatin structure of active rDNA is readily distinguished from inactive chromatin. Providing that the transcripts are more distantly spaced, thus allowing visualization of the underlying chromatin axis, active rDNA has a thin, nonbeaded conformation (Fig. 3 b, c; see also Franke et al. 1976 b, 1978; Scheer 1978; Foe 1978; Busby and Bakken 1980; Labhart and Koller 1982; Scheer and Zentgraf 1982; Puvion-Dutilleul 1983). A similarly extended, nonnucleosomal organization has also been found for the nontranscribed spacer of several species (Fig. 3 a–c; Trendelenburg et al. 1976; Scheer et al. 1977; Scheer 1980; Scheer and Zentgraf 1982; Labhart and Koller 1982). That the nonnucleosomal conformation of nucleolar chromatin is maintained even when transcriptional activity is reduced suggests that the altered chromatin conformation is due to an intrinsic property of rDNA chromatin and not the transcription process per se (Fig. 3 c; Foe 1978; Scheer 1978). Only in stages of complete inactivation does rDNA-containing chromatin assume the characteristic nucleosomal conformation (see next section). The most compelling evidence for a stable extension of the DNA of both the gene and the spacer of transcribing nucleolar chromatin is found in studies of the circular rRNA genes of *Dytiscus* oocytes (Fig. 4 b; see also Trendelenburg et al. 1976). These circles contain one or more repeating units and, therefore, fall into discrete size classes. The rDNA can be isolated from the chromatin rings and surface spread by the cytochrome c-method (Fig. 4 c). The identical contour lengths of both the chromatin rings and the corresponding rDNA circles indicate that the rDNA is fully extended in the chromatin (see also Trendelenburg et al. 1976; Scheer and Zentgraf 1978).

The central nontranscribed chromatin regions separating the palindromically arranged rRNA genes of *Physarum, Dictyostelium,* and *Tetrahymena* are exceptional in that they reveal a bead-like pattern, indicative of a nucleosomal organization (Fig. 4 a). In fact, the DNA of this intergene region is compacted by a factor of about 2 relative to the length of the corresponding DNA (Fig. 4 a; see also Grainger and Ogle 1978) and reveals the characteristic canonical nucleosome repeat after nuclease digestion (Borchsenius et al. 1981; Prior et al. 1983; Palen and Cech 1984).

3.1 rRNA Genes in Different States of Activity

Ultrastructural studies of several biological systems have provided considerable insight into the regulation of rRNA synthesis. During insect embryogenesis and amphibian oogenesis, the rate of rRNA accumulation is extensively modulated (Foe 1978; McKnight and Miller 1976; Scheer et al. 1976a). Using these systems, it is possible to examine rRNA genes by the Miller spreading technique in states of increasing and decreasing rRNA synthesis. Such studies have shown that rRNA synthesis is regulated at the level of transcription since reduced rRNA synthesis is correlated with a decreased number of transcripts per gene (Fig. 3c) and/ or a decreased number of active genes. Furthermore, it has been demonstrated that rRNA gene activation proceeds via a two-step mechanism. First, there is a structural transition of rDNA chromatin from a beaded to a smooth form, which is then followed by transcription (Foe 1978; Scheer 1978). Each rRNA gene appears to be regulated individually since adjacent genes reveal variable degrees of transcript densities. The same pattern of chromatin reorganization is seen after inactivation of rRNA genes, both in natural stages of inactivation (e.g., Trendelenburg and McKinnell 1979) or after inhibition of transcription by drugs, such as actinomycin D (Scheer 1978; Franke et al. 1979). Long-term inactivated nucleolar chromatin is structurally indistinguishable from the bulk chromatin. Not only is it condensed into nucleosomes, but it is also organized into thick, i.e., higher order, fibrils (Scheer and Zentgraf 1978).

4 Visualization of Transcriptionally Active Nonnucleolar Genes

The electron microscopic chromatin spreading method has also provided new approaches to analyze the structural organization of transcriptionally active nonnucleolar genes and, in a few cases, to identify the transcription units of specific protein coding genes. The first nonnucleolar genes to be visualized in the electron microscope were the transcriptional units of the lateral loops of lampbrush chromosomes from amphibian oocytes (Miller and Beatty 1969d). Amphibian lampbrush chromosomes are especially well suited for such studies since their numerous lateral loops represent sites of intense transcriptional activities. As shown originally by Miller and co-workers and subsequently by other authors, loop transcription units are characterized by a high packing density of RNA polymerases (of type II, see Bona et al. 1981) and by nascent RNP transcripts reaching considerable lengths (Miller and Beatty 1969d; Miller and Hamkalo 1972; Miller et al. 1972; Miller and Bakken 1972; Miller 1981; Angelier and Lacroix 1975; Franke et al. 1976b; Scheer et al. 1976b, 1979a; Hill 1979; Hill and McGregor 1980).

The light microscopic appearance of isolated lampbrush chromosomes from a salamander oocyte is presented in Fig. 5a. Usually one loop represents a single transcription unit. Occasionally, however, a single loop contains more than one
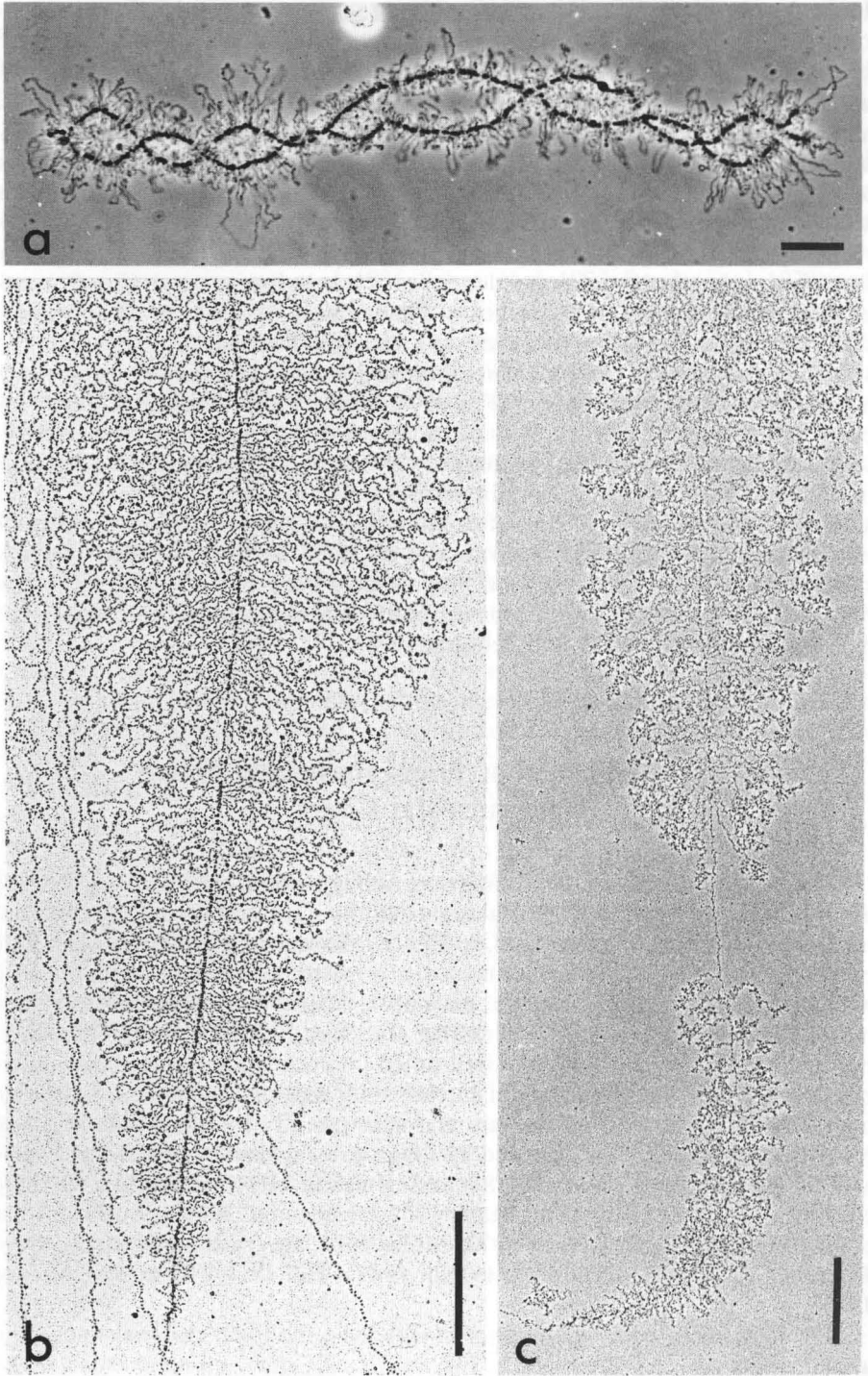
Fig. 5 a–c

transcription unit arranged with identical, opposite, or alternating polarities (Scheer et al. 1976 b). Often the transcriptional polarity is recognized by the increase in thickness of the loop matrix between both ends of a loop (for a detailed account of lampbrush chromosome features see Callan 1982). In spread preparations transcriptional units of the lampbrush chromosomes (or fragments therefrom) are identified by their large size, the close spacing of transcripts, and the
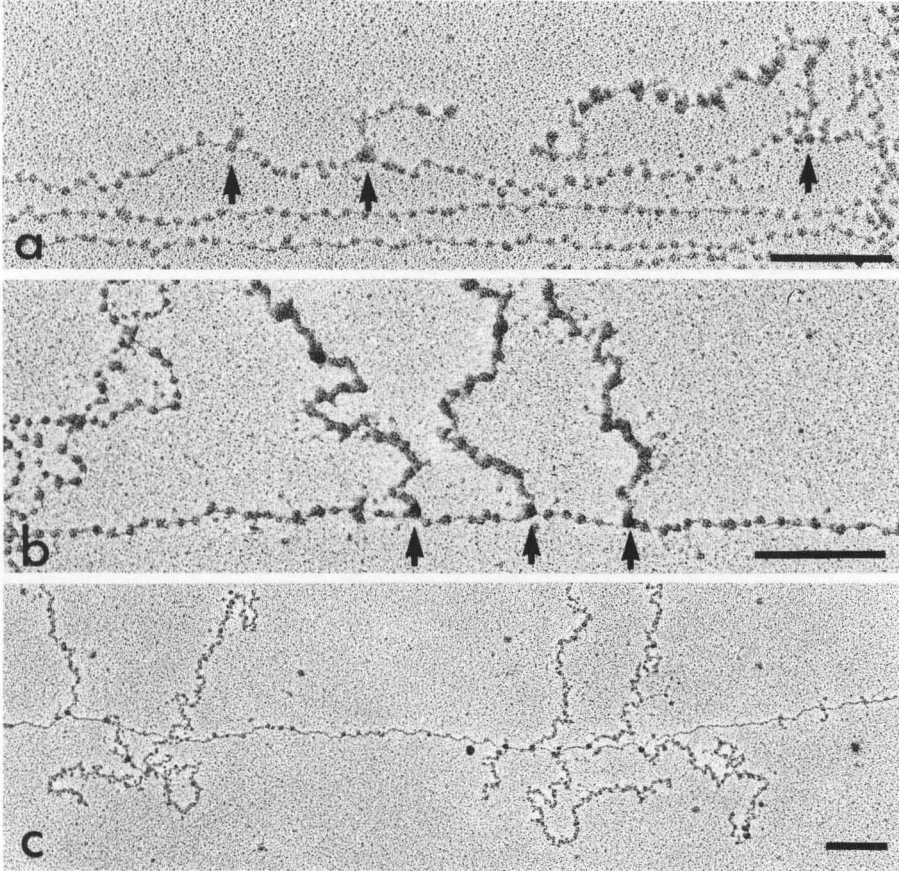

Fig. 6 a–c. Nucleosomal organization of moderately transcribed nonnucleolar chromatin from cultured *Xenopus laevis* kidney cells (**a**) and oocytes of *Pleurodeles waltlii* (**b**). The attachment sites of the transcripts to the chromatin axis are denoted by *arrows*. Inclusion of the anionic detergent Sarkosyl in the spreading medium releases the histones from the DNA and destroys the beaded organization (**c**). The transcripts remain stably bound to the DNA under such conditions.
a × 80 000; **b** × 85 000; **c** × 40 000; *bars* = 0.2 μm

Fig. 5. a Light microscopic appearance of a lampbrush chromosome bivalent from a *Pleurodeles waltlii* oocyte. The lateral loops represent loci of intense transcriptional activity. **b–c** Miller spreads of lampbrush chromosomes from *Pleurodeles waltlii* oocytes. Note the different morphological aspects of the nascent transcripts in **b** and **c**. **a** × 430; **b** × 20 000; **c** × 12 000; *bars* = 20 μm (**a**) and 1 μm (**b, c**)
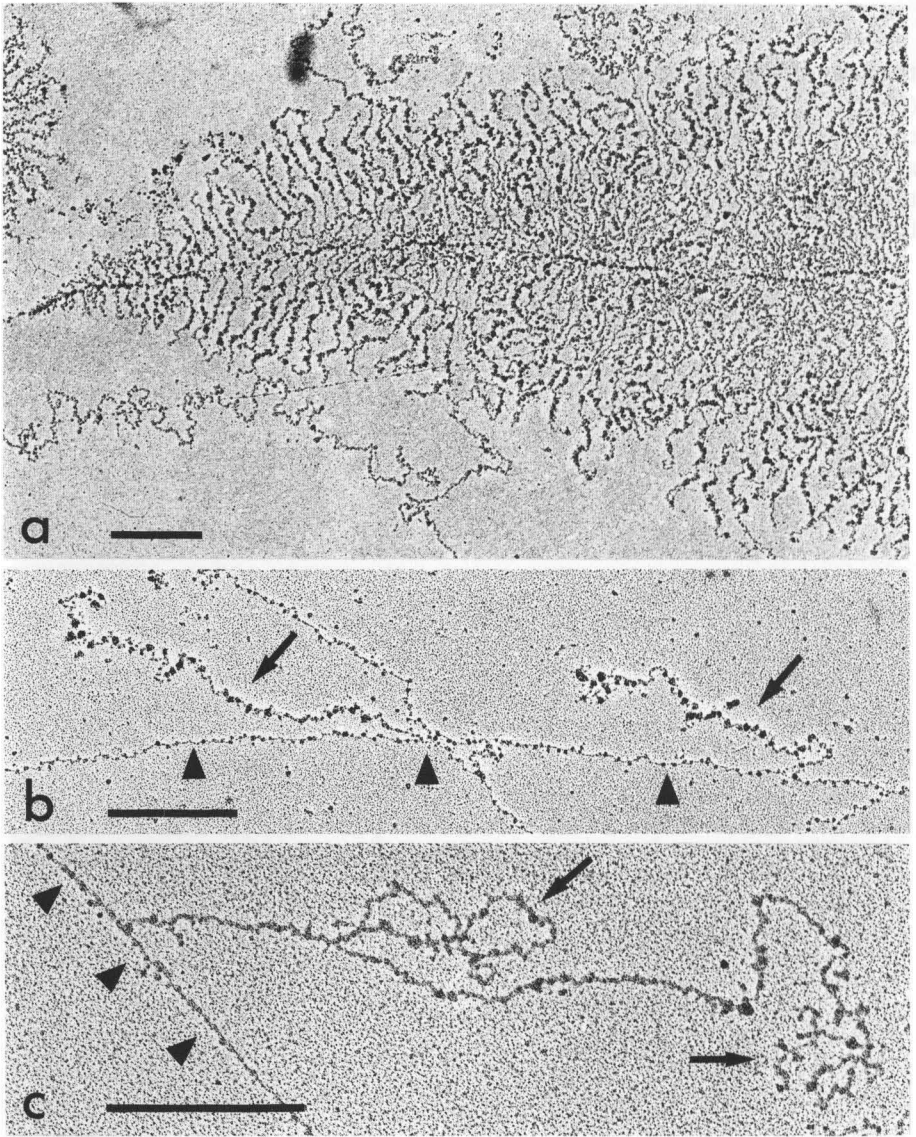
Fig. 7 a–c. Various aspects of transcript organization in Miller spreads of lampbrush chromosomes of *Triturus cristatus* (**a**) and cultured kidney cells of *Xenopus laevis* (**b, c**). Each transcript of the transcription unit shown in (**a**) reveals a subterminal thickening at corresponding positions. The two distantly spaced transcripts in **b** (*arrows*) are condensed into arrays of globular particles. Secondary structures characteristic of nonnucleolar transcripts (ring- and bushlike formations) are shown in **c** (*arrows*). The chromatin axis is indicated by *arrowheads* in **b** and **c**. **a** × 24 000; **b** × 34 000; **c** 54 000; *bars* = 0.5 μm

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Fig. 8 a, b. Part of a lampbrush loop transcription unit from a *Triturus helveticus* oocyte after spreading for electron microscopy according to the Miller procedure. Each lateral fibril carries a conspicuous "loop-stem" structure at corresponding positions (*arrows* in **b**). A transcript fibril from the terminal region of the same transcription unit is shown in (**a**). In addition to the loop-stem structure indicated by the *arrow* a second fold-back structure of the nascent RNA is visible (*arrowhead*). Note that the double-stranded RNA "stem" structures are almost devoid of a protein coat. × 60 000; *bars* = 0.2 μm
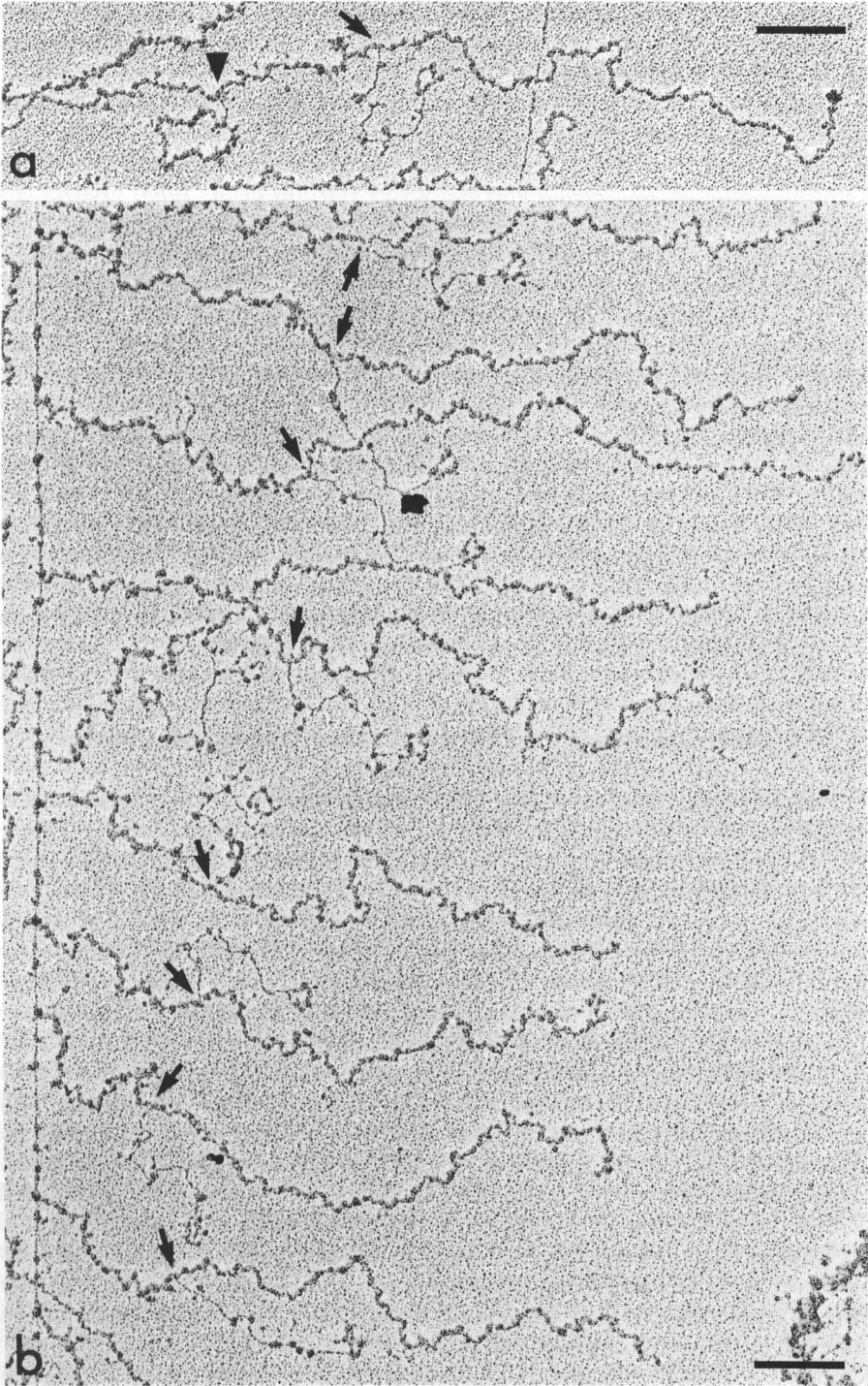
Fig. 8a, b

extensive secondary structures of the transcript fibrils which often give rise to complex bush- and ring-like configurations (Figs. 5 b, c, 7 a, 8 a, b; for reviews see Scheer et al. 1979 a; Sommerville 1981).

Similar high packing densities of nascent transcripts are also found in lampbrush chromosomes of the unicellular green algae, *Acetabularia* (Spring et al. 1975; Franke et al. 1976 b; Scheer et al. 1979 a), in certain lampbrush loops of the Y-chromosome of *Drosophila* spermatocytes (Grond et al. 1983; Glätzer and Meyer 1981), in autosomal chromosomes of *Drosophila* primary spermatocytes (Glätzer 1975), in silk fibroin genes of *Bombyx mori* (McKnight et al. 1976), in chorion protein genes of *Drosophila* (Osheim and Miller 1983; Osheim et al. 1985), and in Balbiani ring genes of polytene chromosomes from *Chironomus tentans* salivary glands (Lamb and Daneholt 1979; Widmer et al. 1984). Usually, however, nonnucleolar transcription units of a variety of cell types and species are characterized by relatively few transcript fibrils, with extended unoccupied chromatin regions, indicative of a low level of ongoing transcriptional activity (Figs. 6 a, 7 b, c; see also Miller and Bakken 1972; Hamkalo and Miller 1973; Hamkalo et al. 1973; Kierszenbaum and Tres 1975; Laird and Choii 1976; Laird et al. 1976; Foe et al. 1976; Amabis and Nair 1976; Oda et al. 1977; Villard and Fakan 1978; McKnight et al. 1979; Harper and Puvion-Dutilleul 1979; Busby and Bakken 1979, 1980; Hughes et al. 1979; Cotton et al. 1980; Petrov et al. 1980; Beyer et al. 1980, 1981; Scheer et al. 1981; Greimers and Deltour 1981; Bachvarova et al. 1982; Rattner et al. 1982; Puvion-Dutilleul 1983).

4.1 Chromatin Structure in Different States of Transcriptional Activity

Intensely transcribed nonnucleolar chromatin regions with maximum packing densities of RNA polymerases are devoid of nucleosome-sized particles (Franke et al. 1976 b). Furthermore, the DNA appears to be extended to almost the length of the B-conformation. This has been shown by a comparison of the length of the transcription units of the highly active silk fibroin genes of *Bombyx mori* and the chorion protein genes of *Drosophila melanogaster* follicle cells with the corresponding DNA (McKnight et al. 1976; Osheim et al. 1985). However, in stages of reduced activity and more distantly spaces transcripts, the chromatin axis not engaged in transcription generally assumes a beaded, nucleosomal configuration. The rapid reformation of nucleosomes after the passage of a transcriptional event is seen in lampbrush chromosomes in stages of experimentally induced or naturally occurring reduced transcriptional activity (Fig. 6 b, c; see also Angelier and Lacroix 1975; Scheer 1978; Bona et al. 1981), as well as in transcription units of somatic cells (Figs. 6 a, 7 b, c; see also Kierszenbaum and Tres 1975; Foe et al. 1976; Laird and Choii 1976; Laird et al. 1976; Oda et al. 1977; McKnight et al. 1979; Villard and Fakan 1978; Busby and Bakken 1979, 1980; Hughes et al. 1979; Cotton et al. 1980; Petrov et al. 1980; Greimers and Deltour 1981). Thus, in contrast to transcribing rDNA chromatin, nonnucleolar chromatin appears to assume the nucleosomal organization relatively fast, provided that the RNA polymerases are sufficiently apart to allow reformation of nucleosomes (for identification of nucleosomes in Miller spreads see below).

4.2 Ultrastructure of Nascent RNP Transcripts

The specific low salt chromatin spreading conditions induce the nascent RNP fibrils to unravel from a condensed into a largely extended state. This is best illustrated by comparing the in situ conformation of nascent RNP transcripts of amphibian lampbrush chromosomes with their appearance in spread preparations. When lampbrush chromosomes are isolated in the presence of physiological salt concentrations followed by fixation and flat embedding for electron microscopy, ultrathin sections reveal that the majority of the transcripts are arranged in linear arrays consisting of 25 to 40 nm globular subunits (Mott and Callan 1975; Spring and Franke 1981; N'Da et al. 1986). In contrast, in Miller spreads much thinner fibrils of the transcripts predominate (Figs. 5 b, c, 6 b, c) which apparently are derived by relaxation of the higher order globular packaging structures (see also Sommerville 1981; Scheer and Dabauvalle 1985). An ordered packaging of the basic 10 nm transcript fibril into a RNP particle of defined morphology and size has also been described for the transcripts of the Balbiani ring genes in *Chironomus tentans* (Skoglund et al. 1983). It should be noted that the extended transcripts as seen in Miller spreads are much thicker than naked RNA, indicating that the nascent RNA chains become coated with proteins immediately following their synthesis.

Not all RNP transcripts unravel into extended fibrils when spread for electron microscopy. Some retain extensive secondary structures and/or display a distinctly beaded morphology due to the presence of 20 nm particles. Examples are presented in Figs. 5 c, 6 a, 7 a–c, and 8 a, b, both from amphibian lampbrush chromosomes and cultured *Xenopus laevis* kidney cells (for further examples see, e.g., Kierszenbaum and Tres 1975; Hughes et al. 1979; Beyer et al. 1980, 1981; Greimers and Deltour 1981; Osheim et al. 1985). A remarkably high degree of secondary structure occurs along the transcripts of the Y chromosome in spermatocytes of *Drosophila hydei*. The transcripts possess a folding pattern that is characteristic for a specific transcription unit (Glätzer 1975; Glätzer and Meyer 1981; Grond et al. 1983; de Loos et al. 1984; Hennig 1985).

To what extent intramolecular base pairing of the nascent RNA chain or protein-protein interactions are involved in establishing the secondary structures of transcript fibrils seen in Miller spreads is not known (for discussion see Sommerville 1981). Several authors have analyzed the distribution of secondary structures, such as loop formations, bush-like ramifications, or RNP particles, in the multiple transcripts of a given transcription unit. The conclusion stemming from such studies is that secondary structures are not randomly distributed. Rather, they occur at specific sites indicating a relationship to specific RNA sequences (e.g., Glätzer 1980; Beyer et al. 1980; Hennig 1985; Osheim and Beyer 1985).

Two transcription units of *Pleurodeles* lampbrush chromosomes with different types of transcript organization are shown in Fig. 5 b and c. The transcripts are either fully extended (Fig. 5 b) or display, specifically at their termini, a particulate morphology and are folded up into complex bush-like structures (Fig. 5 c). Another example is shown in Fig. 7 a where the subterminal region of each consecutive transcript carries an array of closely spaced particles at corresponding positions.

A clear example that the RNA of nascent transcripts can form intramolecular duplex structures, probably by a fold-back mechanism and base pairing of inverted repeat sequences, is provided in Fig. 8. Two different loop-stem structures are recognized, a large loop with a short stem and a small loop with a long stem (Fig. 8 a). The loops presumably contain single-stranded RNA, whereas the stems are formed by duplex regions of the RNA (cf. Sommerville 1981). It is noteworthy that the loops display the same ultrastructural morphology as the principal RNP fibril. However, the stem connecting the loop to the transcript fibril is very thin and is apparently deficient of proteins. This finding is in agreement with biochemical studies showing that double-stranded RNA regions of hnRNA, in comparison with single-stranded regions, are relatively devoid of proteins in living cells (Calvet and Pederson 1979). The loop-stem structures form at corresponding positions of the multiple transcripts shown in Fig. 8 b. The micrograph depicts only a relatively small area close to the transcription initiation site where the second loop has not yet been formed. It is obvious that the distance between the hairpin structures and the anchoring sites of the fibrils into the chromatin axis increase as a function of the growth of the transcripts. A quantitative analysis of the total transcription unit with 48 traceable fibrils is presented in Fig. 9.

Whether the foldback structures of the type depicted in Fig. 8 have a function in the process of RNA sequence excision is presently not known. However, RNA particles frequently seen on transcripts of a given gene, at sequence-specific sites, might be functionally involved in splicing reactions by bringing together two splice junctions with the intron transcript looping out (Osheim et al. 1985; Osheim
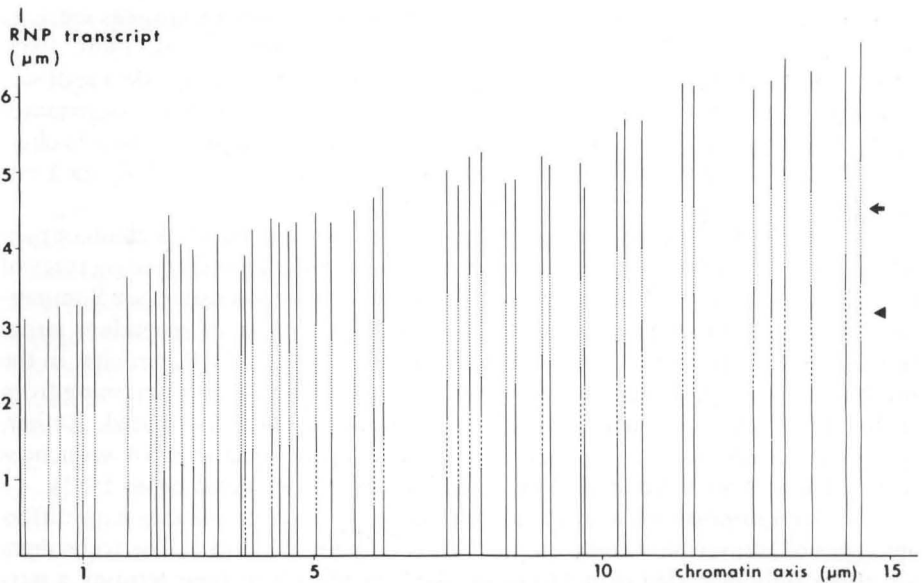

Fig. 9. Quantitative evaluation of 48 traceable transcripts from the transcription unit shown in Fig. 8. Each transcript is represented in its linearized form, i.e., loop-stem structures are linearized and indicated as *dotted lines* (at the *arrow* and *arrowhead;* the corresponding structures are shown in Fig. 8 a). The graphic representation clearly demonstrates that the hairpin structures are related to specific sequences of the nascent RNA chains

and Beyer 1985). Such splicing reactions might well occur during transcription. In fact, numerous nonnucleolar transcription units from a variety of species analyzed by the chromatin spreading method reveal interruptions in the RNP fibril length gradients. This indicates that cleavage of transcripts occurs during transcription (e.g., Laird and Choii 1976; Scheer et al. 1979 a; Beyer et al. 1981). In addition, a considerable proportion of the RNA contained in terminal transcript fibrils of amphibian lampbrush chromosomes apparently does not consist of contiguous chains, but of nicked molecules (Scheer and Sommerville 1982).

5 Application of Localization Techniques to Miller Spreads

An advantage of the chromatin spreading technique is that it is potentially capable of elucidating the ultrastructure and biochemical composition of transcription units of defined genes in various states of activity. So far only a few protein-coding genes have been identified in Miller spreads (silk fibroin genes of *Bombyx mori:* McKnight et al. 1976; Balbiani ring genes of salivary glands of *Chironomus tentans:* Lamb and Daneholt 1979; and chorion protein genes of *Drosophila melanogaster* follicle cells: Osheim and Miller 1983; Osheim et al. 1985). With the availability of specific nucleic acid probes it should be feasible, by using modified protocols for in situ hybridization, to correlate transcription units in Miller spreads with specific genes. In this way one may analyze the distribution of specific proteins within the transcription unit, in the flanking regions, and along the nascent RNP transcripts by immunochemical approaches.

5.1 In Situ Hybridization of Nucleic Acids

Transcription units with numerous attached lateral fibrils represent a favorable situation for in situ hybridization of DNA or RNA probes to the nascent RNA chains because of the multiplication of the DNA coding sequence at the RNA level. Hybridization of nucleic acid probes to nascent RNA has already been used, at the light microscopic level, to localize histone genes to specific loops of amphibian lampbrush chromosomes (Old et al. 1977; Callan 1982; Gall et al. 1981; Diaz et al. 1981; Diaz and Gall 1985).

Similar procedures may also be adapted to investigations at the electron microscopic level. Visualization of the hybridized DNA or RNA probe could be accomplished by autoradiographic or nonradioactive methods, such as biotinylation or chemical modification of the probe which are then detected by specific antibodies (e.g., Fostel et al. 1984; Landegent et al. 1985).

When recombinant plasmids containing rDNA sequences of *Xenopus laevis* are hybridized to Miller spreads of amphibian oocyte nuclei, the DNA probe binds selectively to the pre-rRNA of the lateral fibrils of the rRNA genes (for examples see Scheer and Zentgraf 1982). We are confident that further refinements of this approach will permit a better ultrastructural preservation of the transcrip-

tion units and a higher hybridization efficiency so that protein coding genes can be identified as well.

An additional approach to identify transcribed genes in the electron microscope is based on restriction endonuclease cleavage prior to chromatin spreading in order to produce identifiable fragments of transcription units (e.g., Reeder et al. 1976; Pruitt and Reeder 1984).

5.2 Biochemical and Immunological Techniques

It is known that transcriptional complexes are extremely stable to high salt concentrations and ionic detergents, i.e., conditions which lead to the dissociation of the majority of chromatin proteins including histones (for details see Scheer 1978). This selective stability of transcriptional complexes has been exploited to demonstrate that the beaded morphology of a chromatin fiber is dependent on the presence of histones and does not reflect, e.g., arrays of template-bound RNA polymerases without nascent transcripts. When chromatin is spread in the presence of the anionic detergent Sarkosyl or the commercial dishware detergent "Joy" (originally introduced by Miller and Bakken 1972) at concentrations which release most chromatin proteins from the DNA, the beaded organization of chromatin is no longer visible (Scheer 1978). Figure 6b and c illustrate the effect of Sarkosyl on the ultrastructure of lampbrush chromosome loops transcribed with moderate efficiency. Without detergent the chromatin axis displays a beaded morphology (Fig. 6b); addition of Sarkosyl results in the disappearance of the beads while the transcripts remain attached to the smoothly contoured chromatin fiber (Fig. 6c), indicating that the beads in fact represent nucleosomes.

A direct approach to localize specific proteins in Miller spreads is based on the use of immunolocalization techniques. As shown by several authors, antibodies to core histones react specifically with nucleosomal beads of inactive chromatin (e.g., Bustin et al. 1976; Ghose et al. 1983). Antibodies to the core histones H2B and H3 also bind to the axis of nonnucleolar genes which are sparsely covered with transcripts (McKnight et al. 1978). The identification of histones by this immuno-electron microscopic approach in conjunction with the beaded morphology of the chromatin regions between distantly spaced transcripts clearly indicates that a transient nucleosomal packaging of the chromatin template takes place between successive transcriptional events.

Whether histones remain associated also with intensely transcribed chromatin regions, such as the lampbrush chromosome loops or nucleolar chromatin, is as yet an open question (for discussion see Mathis et al. 1980; Karpov et al. 1984; Sargan and Butterworth 1985). Indirect evidence based on the effect of histone antibodies microinjected into nuclei of living amphibian oocytes suggests that some histones are present on the heavily transcribed lampbrush loops. However, these experiments did not allow quantitative estimates and also did not permit the precise localization of histones (Scheer et al. 1979b; Scheer 1986).

Immuno-electron microscopy has also been used to study, at a very high level of resolution, the distribution of nonhistone proteins in chromatin. T-antigen has been mapped to a specific region of SV40 minichromosomes, the nucleosome-

and histone-free gap containing the origin of replication and promotor elements (Harper et al. 1984; Weiss et al. 1985).

Do specific ribosomal proteins bind to nascent pre-rRNA of rRNA genes? This question has been studied by Choii and Leiby (1981) by combining Miller spreads with immunolocalization techniques. They used two different protocols for binding of the antibodies. Either the antibodies were added directly to the lysed *Drosophila* embryonic cells followed by centrifugation of the chromatin onto the electron microscopic grid or rRNA genes were first immobilized on the grid followed by incubation with the antibodies. Their results show that individual ribosomal proteins bind to specific sequences of the nascent pre-rRNA. In this connection it should be emphasized that not all ribosomal proteins are involved in the early steps of ribosomal assembly, but become integrated into preribosomal particles only at more advanced stages of maturation (Hügle et al. 1985).

Another approach to localize nucleolus-specific proteins in Miller spreads was taken by Angelier et al. (1982). These authors adapted the silver staining method to spread preparations of rRNA genes of amphibian oocytes. They showed that silver staining proteins were preferentially associated with the transcribed rDNA and/or with the basal portions of the nascent RNP transcripts.

In conclusion, the tools and the probes necessary for identification and analysis of transcription units at the molecular level are presently available. Their application, in conjunction with the Miller chromatin spreading technique, should lead to a more detailed understanding of the conformational and compositional differences between active and inactive chromatin and the complex processes involved in generation and maturation of RNP transcripts.

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