The Nucleolus, Chromosomes, and Visualization of Genetic Activity

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The Nucleolus and Ribosomal RNA Genes

By the time of Montgomery's classic paper on the nucleolus in 1898 (1), there were already 700 or so articles with observations on this nuclear organelle, beginning with a study by Fontana in 1781 entitled "Venom of Vipers." The early cyto logical emphasis on the nucleolus undoubtedly was due to the high visibility of the organelle in interphase nuclei of most cell types; however, the fact that nucleoli are directly involved with chromosomal activity was not demonstrated until Heitz (2) and McClintock (3) showed that nucleoli form during telophase at specific chromosome regions called "nucleolar organizers" (NOS) by McClintock. In 1940, Caspersson and Schultz (4), using ultraviolet (UV) absorption spectra, concluded that both nucleoli and cytoplasm of cells are generally rich in ribonucleic acid (RNA). Brachet (5) came to the same conclusion independently after discovering that RNase treatment of amphibian oocytes removed the basophilic components of both cytoplasm and nucleoli. Following these early observations, Caspersson and co-workers produced convincing evidence that a positive relationship exists between nucleolar size and levels of RNA and protein synthesis in cells (6).

In the early 1950s, Estable and Sotelo (7) used a silver-staining technique on a variety of cells, and suggested that during interphase, nucleoli contain a threadlike structure, termed the "nucleolonema," which associates with all of the chromosomes during mitosis and regroups at the NO during telophase, after which the nucleolus acts as a collecting site to accumulate additional material. Although the assignment of a hereditary continuity to the nucleolonema independent of chromosomes turned out to be incorrect, this provocative concept spurred interest by many investigators regarding the function of the nucleolus in cell metabolism. A comprehensive 1955 review by Vincent (8) provides a nice overview of earlier research on nucleoli.

Evidence for a Direct Relationship Between Cytoplasmic and Nucleolar RNA

Starting with Claude's "microsome" fraction in 1941 (9), techniques continued to be developed which allowed separation and biochemical analysis of different cellular fractions (reviewed in [10]). Cytoplasmic microosomal fractions were found to be rich in RNA and active in protein synthesis. When small ribonucleoprotein (RNP) particles were isolated from microsomal fractions treated with detergents (11), the RNP particles were found to contain essentially all of the RNA components and to be highly active in protein synthesis. Similar RNP particles already had been found in bacteria, and these were shown to contain two stable RNA molecules with sedimentation constants of 16S and 23S, which are complexed with a large number of proteins. Further studies using eukaryotic cells demonstrated that microsomal particles also contain two stable RNAs, but with somewhat higher S values of 18 and 28 (12). Porter (13), Sjöstrand and Hanzon (14), Palade (15), and Palade and Siekevitz (16), by using electron microscopy (EM), were the first to observe cytoplasmic granules in fixed cells and to correlate the morphology and chemistry of these granules. A similarity between such "Palade granules" with regard to size and composition and a granular component of the nucleolus was first noted by Porter (13) and later by Gall (17) and Swift (18). The first good indication that the nucleolus probably is involved in the production of the stable RNA components of the granular cytoplasmic "ribosomes" (a term introduced by Roberts in 1958 [19]) was provided by Woods and Taylor in 1959 (20). By use of autoradiography (ARG), these investigators showed that 3H-labeled cytidine first appears in nucleolar RNA of Vicia faba root tips, then in cytoplasmic ribosomal RNA (rRNA), and that pulse-labeled nucleolar RNA moves from the nucleolus to the cytoplasm in the presence of unlabeled medium. More rigorous proof of this relationship was given by Perry and co-workers (21, 22), who demonstrated that selective UV microbeam irradiation of HeLa cell nucleoli prevented the appearance of about two-thirds of newly synthesized RNA into the cytoplasm, relative to control cells. Additional evidence for a nucleolar origin of rRNA came from Edström and colleagues (23), who used ingenious microdissection and microelectrophoretic techniques in the analysis of starfish oocyte RNA. They showed that the base composition of nucleolar RNA, but not other nuclear RNA, is essentially the same as that of cytoplasmic RNA, the large majority of which is rRNA. Similar results were subsequently obtained by Edström and Beermann, (24) using Chironomus salivary glands, and by Edström and Gall (25), using amphibian oocytes. Complementing these studies on RNA, Birnstiel and co-workers (26) showed by amino acid analyses that the nucleolar...
proteins of pea seedlings are very similar to those of isolated cytoplasmic ribosomes.

Evidence for a Large Precursor to 18S and 28S Cytoplasmic Ribosomal RNA

Strong evidence that the 18S and 28S rRNAs are derived from larger nucleolar molecules was first provided by Perry (27). He used parallel ARG and sedimentation studies on control and actinomycin-D-treated mouse L cells, and found that rapidly labeled nucleolar RNA contains heterogeneous fast-sedimenting components, some of which sediment faster than the heaviest cytoplasmic rRNA. Scheerer et al. (28) next reported that the largest rRNA precursor molecule (pre-rRNA) in HeLa cell sediments at 45S and is cleaved to an intermediate 35S molecule in the derivation of the rRNAs. Precise details of the conversion of HeLa pre-rRNA into 18S and 28S rRNA were later given by Weinberg et al. (29), using very clean nucleolar fractions and acrylamide gel electrophoresis methods developed by Loening to accommodate RNA molecules as large as pre-rRNA. It was found that a single pre-rRNA molecule gives rise, through two intermediate RNA cleavage pathways, to one molecule each of 18S and 28S rRNA. In that the existence of the high molecular weights (mol wt) of pre-rRNA based on apparent sedimentation constants continued to be questioned, Granboulan and Scheerer (30), using Kleinschmidt’s protein film technique to visualize RNA molecules by EM, showed that there is a good correlation between the molecular-weight estimates by the two methods, and that the conversion of 45S pre-rRNA to rRNAs is the result of changes in lengths rather than configurations.

Evidence for Redundancy of 18S and 28S rRNA Cistrons

The first evidence that genomes of eukaryotic cells contain highly multiple sequences coding for cytoplasmic 18S and 28S rRNAs was provided by Chipchase and Birnstiel (31) who estimated from rRNA/DNA hybridization results that 0.3% of total pea-seedling DNA contains sequences homologous to rRNA. Also reported was the fact that nucleolar RNA competed with rRNA for such sequences. X-irradiation experiments done earlier by McClintock (3) and later by Beermann (32), showing that translocations involving partial NOs could function equally as well as intact NOs by morphological and growth criteria, had demonstrated that functional redundancy existed in NOs. This redundancy had now been given a molecular basis. Soon after the hybridization data from peas was obtained, McConkey and Hopkins (33) used similar methods to estimate that an average HeLa cell contains 400 28S rRNA cistrons and, more importantly, showed that rRNA sequences are enriched in nucleolar fractions.

Evidence for Localization of 18S and 28S rRNA Cistrons to NOs

The first highly suggestive evidence that all 18S and 28S rRNA cistrons are localized at the NO site of a specific chromosome was provided by Brown and Gurdon (34), using the Mendelian, anucleolate deletion mutant of Xenopus laevis first described by Elsdale et al. (35). In homozygous anucleolate tadpoles, the mutation prevents formation of normal nucleoli. These investigators showed that there also is no synthesis of 18S or 28S RNA or of higher molecular weight precursors, whereas 4S RNA and rapidly labeled heterogeneous nuclear RNA (hnRNA) are synthesized. These results indicated that the cistrons for 18S and 28S rRNA are under coordinate control and are located at a single chromosomal site, the NO. More definitive evidence that the rRNA cistrons are localized within NOs was soon provided by Ritossa and co-workers (36, 37), who used cytogenetically derived Drosophila stocks carrying from 1 to 4 NOs, and Birnstiel and colleagues (38, 39), who compared normal (2-NO) tadpoles with heterozygous (1-NO) and homozygous (0-NO) anucleolate Xenopus mutants. Both groups demonstrated by rRNA/DNA hybridization that the number of rRNA cistrons present in the various stocks is precisely correlated with the number of NOs present.

Isolation of Ribosomal DNA and the Arrangement of the 18S and 28S rRNA Cistrons

Birnstiel and co-workers (38, 39) predicted from the high guanosine-cytosine (G-C) content of rRNA that its complementary DNA sequences should have a higher G-C content (~63%) than that of total Xenopus DNA (~40%), and that this DNA should separate from bulk DNA in CsCl gradients because of the difference in buoyant density. These investigators showed that about 0.2% of the Xenopus genome separates on CsCl gradients as a high-density satellite that contains essentially all of the genomic DNA complementary to 18S and 28S rRNA. This marked the first isolation in pure form of DNA sequences of known function.

The question of whether 18S and 28S rRNA sequences are present in the NO in homogeneous contiguous blocks of one or the other or are strictly alternating, was then approached independently by Brown and Weber (40) and Birnstiel et al. (41). Their experiments were carried out by shearing high-density satellite DNA (rDNA) to progressively lower molecular weights, challenging the DNA with 18S and 28S rRNA, then determining the buoyant density of the hybrid molecules. Because of the difference in the G-C content of the two rRNAs, it could be determined that linkage between the two sequences was not disrupted until a DNA with close to 1.5 x 10^6 daltons was reached. When DNA with a molecular weight 0.5 x 10^6 daltons or lower was used, essentially no linkage between the two rRNA sequences was present. These results forced the conclusion that the two cistrons are strictly alternating and that their products are contained together within the 40S pre-rRNA molecule of amphibia.

5S rRNA

Using HeLa cells, Knight and Darnell (42) showed that, in addition to the 28S rRNA, there is one 5S RNA molecule per large ribosomal subunit. That this 5S rRNA becomes associated with nascent ribosomal particles in the nucleolus that contain the 32S precursor to 28S rRNA was demonstrated by Warner and Socciro (43). Brown and Weber (44) showed by RNA/DNA hybridization that 5S RNA genes (5S DNA) in Xenopus are not linked with rDNA, and Pardue et al. (45) subsequently demonstrated by recently innovated in situ hybridization techniques that the some 20,000 or so 5S rRNA genes are distributed among the ends of the long arms of probably all of the 18 chromosomes of X. laevis. A much more localized site was found by Frensky et al. (46) for the approximately 160 5S rRNA genes of D. melanogaster, in which the genes can be assigned to bands 56e-f on chromosome 2R. On the other hand, linkage between 5S DNA and rDNA was reported by Cockburn et al. (47) and Maizels (48) for Dicyostelium discoideum and by Maxam et al. (49) for Saccharomyces cerevisiae. In both of these primitive eukaryotes, the 5S genes
are present with their own promoters in the spacers between pre-rRNA genes. Because of this arrangement, it was proposed that these two primitive eukaryotes may represent an intermediate divergence from the bacterial organization in which 5S cistrons share promoters with the other rRNA cistrons (48).

A dual 5S rRNA system was reported by Wegnez et al. (50) and Ford and Southern (51) for X. laevis, in that somatic cells synthesize one type of 5S RNA whereas oocytes synthesize both the somatic type and several oocyte-specific types which differ slightly from one another in nucleotide sequence. The mechanism by which such differential regulation of oocyte-type 5S RNA synthesis is controlled remains obscure. The nucleotide sequence of the major oocyte 5S RNA (average repeat length, 720 base pairs [bp]) has been determined (52–54). The repeat unit consists of two regions: a G-C-rich region that contains both the 5S gene and a “pseudogene” sequence homologous to much of the 5S gene, and an A-T-rich region. The G-C-rich region is constant in size within families of 5S DNA repeats, whereas the A-T-rich region, which is composed of repeating, closely related 15-bp sequences, can vary considerably in length. The pseudogene is not transcribed, and may have arisen by gene duplication followed by mutational inactivation of one gene (52). The 5S DNA repeat unit of D. melanogaster, on the other hand, contains no pseudogene sequence and exhibits only slight heterogeneity in length of the A-T-rich spacer segment (55, 56).

Although 5S RNA is present in a 1:1 ratio with 28 rRNA in ribosomes, numerous studies, beginning with that of Perry and Kelley (57), have shown that 5S RNA synthesis is not coordinate with pre-rRNA production (see [58] for other references).

Amplification of Nucleolar Genes in Amphibia and Insects

Although chromosomal NOs are inherited as Mendelian units and there is only one to a few such loci, depending on the organism, rDNA has been shown to be preferentially amplified extrachromosomally in oocytes and oogonia of many animals, both invertebrate and vertebrate, and in the vegetative nuclei of some primitive eukaryotes (see review by Tobler (59)). The early cytological studies of this phenomenon, which, in many cases, results in the formation of highly multiple extrachromosomal nucleoli, were elegantly reviewed by Gall (60), and only a few of the early works pertinent to this chapter will be mentioned.

King (61), using a safranin-gentian-violet double-staining procedure, concluded that extrachromosomal chromatin becomes associated with the multiple nucleoli of Bufo oocytes after pachytene. Bauer (62) used the recently introduced Feulgen stain for DNA, and demonstrated that “Giardina’s body” in Dytiscus oocytes, as well as extrachromosomal bodies in oocytes of several other insect species, contains DNA. Brachet (5) next used this specific stain to show the presence of DNA in the multiple nucleoli of Rana oocytes. His work was followed quickly by a more extensive study of Bufo oocytes by Painter and Taylor (63), who independently confirmed Brachet’s observations and concluded that the extrachromosomal nucleoli are involved in the production of cytoplasmic RNA and that the extrachromosomal chromatin granules probably are equivalent to the NOs of somatic cells. After a significant interim, Kezer (64) and Miller (65, 66), in examining the circular nucleoli found in certain salamander oocytes, independently showed by enzymatic digestion experiments that the circular continuity of such nucleoli is maintained by DNA (Fig. 1). Considering evidence then becoming available regarding the function of somatic cell NOs in rRNA synthesis, these authors also concluded that extrachromosomal nucleoli probably are involved in rRNA synthesis. Similar conclusions regarding the probable role of extrachromosomal DNA in insect oocytes followed (see discussion in Gall [60]). Proof that the amplified DNA of amphibian oocytes is rDNA was independently shown by rDNA/DNA hybridization by Gall (67), using young Xenopus ovaries, and Brown and Dawid (68), using isolated oocyte nuclei of four amphibia. Macgregor (69) demonstrated by microspectrophotometry that the amount of extrachromosomal DNA per X. laevis oocyte is about 30 pg, or five times the total diploid genome. Evidence for amplified rDNA in insect oocytes was first presented for Dytiscid water beetles by Gall et al. (70) and for the cricket Acheta by Lima-de-Faria et al. (71). Gall and Rochaix (72) subsequently demonstrated that if not all, of the amplified rDNA of Dytiscid beetles is present in circular form (Fig. 2). The process of amplification in Xenopus oocytes begins before meiosis and is completed by the end of pachytene (73, 74). Brown and Blackler (75) presented evidence from reciprocal crosses between X. laevis and X. borealis (mulleri), in which only X. laevis rDNA is amplified in the oocytes, that rDNA amplification apparently proceeds by a chromosome.

**Figure 1** Phase contrast micrographs of circular nucleolar cores from a Triturus pyrogaster oocyte in the process of being cleaved by the action of pancreatic DNase, from Miller (66). Bar, 50 μm. × 250. All of the remaining figures are derived from electron micrographs.
FIGURE 2  A circular rDNA molecule isolated from a *Dytiscus* oocyte, showing transcriptional gradients of active rRNA genes separated by inactive spacer segments, from Trendelenburg (192). Circularity of such molecules was first demonstrated by Gall and Rochaix (72) by visualization of deproteinized rDNA molecules spread in a surface film. Bar, 1 μm. x 18,000.

copy mechanism rather than by germ-line transmission of episomal rDNA. Subsequent studies by Hourcade et al. (76) and Rochaix et al. (77) provided evidence that, after the presumptive chromosome copy event(s), the amplification process of *Xenopus* proceeds extrachromosomally by a rolling-circle mechanism (Fig. 3). To date, however, no definitive information regarding the molecular aspects of the initial events in rDNA amplification is known for either amphibia or insects.

**Ultrastructural Visualization of Nucleolar Function in Higher Eukaryotes**

Excluding vacuoles, nucleoli typically consist of two major ultrastructural components, one coarsely fibrous and one granular. The spatial relationships of the two components vary considerably depending on cell type, ranging from seemingly random interspersion to strict compartmentalization into a central or eccentric fibrillar core surrounded by a granular cortex (Fig. 4; for further examples, see Busch and Smetana [78]). In an early EM study of polytene chromosomes, Beer mann and Bahr (79) clearly showed that the central core region of the nucleolus is directly connected with the NO of the chromosome. Subsequently, EM-ARG studies by Granboulan and Granboulan (80), using tissue culture cells, and by Karasaki (81), using amphibian embryos, demonstrated that initial incorporation of RNA precursors occurs in the fibrous nucleolar component, and both concluded that the newly synthesized RNA appearing later in the granular component is derived from the fibrillar one. Similar results were obtained later by Macgregor (82) for amphibian oocyte nucleoli, the fibrillar core regions of which were already known to contain DNA.

By using newly devised spreading techniques for EM preparations, Miller and Beatty (83, 84) were able to visualize clearly the structure of dispersed core and cortex components of amphibian oocyte nucleoli. Analyses of EM-ARG and enzymatic digestion, combined with biochemical data from other sources, allowed the conclusion that the cores consist of single, circular deoxynucleoprotein (DNP) molecules of varying lengths that contain highly active, repetitive rRNA genes, each of which is separated from its neighboring genes by apparently inactive "spacer" segments of variable length (Fig. 5). The granular nucleolar component, which presumably

FIGURE 3  An extrachromosomal rDNA molecule isolated from a young *X. laevis* ovary, courtesy of A. H. Bakken (unpublished material). The silver grains indicate incorporation of 

[3H]thymidine in the "tail" extending from a small rolling circle. Bar, 1 μm. x 14,250.
contains the 30S RNA precursor to 28S rRNA, was found to consist of small granules fairly widely spaced on thin, but well-defined fibrils. The significance of the fibrillogranular network in the biogenesis of the large ribosomal subunit remains unknown.

Subsequent studies by Miller and Bakken (85) with HeLa cells, and Hamkalo et al. (86) on Drosophila embryos showed a basically similar organization of -spacer-gene-spacer-, with the length of the rRNA genes reflecting the different molecular weights of the pre-rRNA molecules in the three cell types. Similar techniques were used by Franke and co-workers who rapidly extended observations of active nucleolar genes to amplified rDNAs of Acheta (87) and Dytiscus (88) (Fig. 2). All of the rDNA repeats within one NO of higher eukaryotes appear to have the same transcriptional polarity, except for some infrequent observations of adjacent convergent or divergent gene polarity in amplified rDNA. Perhaps unsurprisingly, it could now be concluded that all higher eukaryotes probably have the same general morphological arrangement of active rDNA.

Chromatin spreading techniques have provided some information about the regulation of rRNA genes in several different cell types. McKnight and Miller (89) found that the maximal packing of RNA polymerases occurs on both newly activated and fully transcribed rRNA genes of Drosophila embryos, indicating that in this system the rate of transcription, rather than frequency of polymerase initiation, regulates pre-rRNA production on individual genes. On the other hand, modulation of RNA polymerase initiation appears to be involved in two other systems. Scheer et al. (90) observed that amplified rRNA genes of young oocytes of Triturus alpestris have reduced RNA polymerase packing ratios as compared with those of more mature oocytes, and Foe et al. (91) showed that newly activated rRNA genes of milkweed-bug embryos typically have quite low RNA polymerase densities compared with later stages. In addition, McKnight and Miller (89) found that the number of active rRNA genes increased as cellularization proceeds in Drosophila embryos, although no more than 50% of the rRNA genes ever appeared to be activated. A similar observation was reported earlier by Meyer and Hennig (92) for primary spermatocytes of Drosophila hydei.

Molecular Anatomy of rDNA Repeat Units of Higher Eukaryotes

In all cases in which rDNA of higher eukaryotes has been examined in detail, the rRNA genes have been found in tandem repeated units with each unit consisting of an rRNA gene and a nontranscribed spacer (NTS) segment. Each rRNA gene contains three cistrons coding for the 28S, 18S, and 5.8S rRNA. The 5.8S rRNA was first detected in HeLa cells by Pene et al. (93), who found it to be hydrogen-bonded to the 28S rRNA and presented evidence that the 5.8S molecule is derived from the same intermediate precursor molecule as the 28S rRNA. Subsequently, Speirs and Birnstiel (94) concluded from hybridization studies with X. laevis rDNA satellite that the 5.8S rDNA sequence is located between the 18S and 28S rDNA cistrons.

The question of transcriptional polarity within pre-rRNA molecules was a controversial subject for a number of years.
Experiments indicating an initiation-5'-18S-28S-3'-termination polarity included kinetics of rRNA labeling in *Euglena* (95), synthesis of *X. laevis* rRNA in vitro (96), and differential sensitivity of rRNAs upon inhibition of synthesis by 3'-deoxyadenosine (97) and UV irradiation (98). Results indicating an opposite polarity included identification of similar 5'-termini in 28S rRNA and pre-rRNA (99), kinetics of rRNA labeling in isolated nuclei from *Rana* (100), and secondary structure analysis of pre-rRNA and rRNAs after partial 3'-exonuclease digestion (101). More recently, results obtained by secondary structure analysis of nascent pre-rRNA compared with rRNAs and mature pre-rRNA (102), by new 3'-exonuclease experiments (103), and by restriction endonuclease analysis of repeating rDNA units with attached nascent pre-rRNA transcripts (104) have provided conclusive evidence of a 5'-18S-28S-3'-transcriptional polarity in Xenopus.

The average length of NTSs can be quite different, depending on the organism being examined. For example, the spacers in *Colymbetes* are about 15 kilobases (kb) long, whereas those in *Dytiscus* are about 45 kb long (72). Heterogeneity in NTS length has been detected in several organisms including mouse (105), *Drosophila* (106), and *X. laevis*, with the latter having NTS varying from about 11 kb to 22 kb or so in length (107). Reeder et al. (108) showed that the patterns of chromosomal NTS lengths of *Xenopus* are inherited in a Mendelian manner. Wellauer et al. (109) found that in some individual frogs repeat lengths rarely present in their chromosomal rDNA are amplified selectively, whereas others amplify their most abundant size classes, and that the preference for size-class amplification is inherited.

Wellauer et al. (107, 109) and Botchan et al. (110) studied the molecular basis for variable NTS length in *Xenopus* by heteroduplex mapping and restriction enzyme analysis of cloned rDNA. Their results indicated that such NTSs consisted of two conserved regions having no internal repetitions that alternate with two regions of variable length composed of short repetitive sequences. Somewhat later, Birnstiel and colleagues (111) reported the sequencing of essentially an entire cloned *Xenopus* NTS. Their data showed that this NTS is composed of four internally repetitive regions interdigitated with conserved nonrepetitive regions. High-sequence homology was found between a short segment immediately upstream from the pre-rRNA transcription initiation site and segments within the next two upstream nonrepetitive regions of the NTS. Similar high-sequence homology was demonstrated by Sollner-Webb and Reeder (112) who used a different cloned NTS. The arrangement of the high homology sequences within NTSs suggests that such sequences have been reduplicated and displaced upstream into *Xenopus* NTSs by saltation of repetitive region repeats during recent evolutionary time (111). As yet, however, there is no definitive evidence regarding the function of any portion of NTSs. Short transcription gradients occasionally are present on amplified rDNA spacers of *Xenopus* (113), and it is possible that these result from reduplicated and displaced promoters in the high homology regions which have remained functional (111, 112). It is typical, however, that no...
transcription is observed on NTSs, especially with regard to chromosomal rDNA. In contrast, McKnight et al. (114) have provided preliminary evidence from chromatin spreads of Drosophila embryos that NTSs may contain initiation sites for chromatin replication.

Another basis for length heterogeneity of rDNA repeats has been reported for D. melanogaster, in which a DNA segment that is not included in pre-rRNA is present in 60% of the rRNA gene sequences (106, 115, 116). The intervening sequences occur primarily in the NO of the X chromosome, and genes containing insertions appear to be randomly interspersed with genes without insertions. The insertions are located about two-thirds of the way into the 28S cistron, and range in length from 0.5 to 6.0 kb. Chooi (117) has reported the occurrence of a few longer-than-normal transcription units in spread NOs of D. melanogaster, suggesting that some insert-containing genes may be transcribed. Long and Dawid (118), however, used cloned insertion sequences, and have shown that the number of nuclear RNA molecules with insertion sequences is on the order of 10–20 per nucleus and, thus, cannot make any significant contribution to the production of 28S rRNA. Sequences homologous to the rDNA inserts and comprising some 0.2% of the haploid genome of D. melanogaster are present in chromatin outside the NOs (119).

Amplification of rDNA in Primitive Eukaryotes

In addition to that shown for amphibia and insects, extra-chromosomal amplification of rDNA has been documented for several primitive eukaryotes, including Tetrahymena pyriformis (120–122), Physarum polycephalum (123, 124), Paramecium tetraurelia (125), and several species of green algae (126–128). Restriction enzyme analysis and denaturation-renaturation studies showed that the free rDNA molecules of Tetrahymena (129, 130) and Physarum (123, 124) are large palindromes in which each molecule has two rRNA genes. The genes are separated by nontranscribed spacer regions and localized toward the ends of the molecules, with the 17S rRNA cistrons proximal to the 26S rRNA cistrons. Grainer and Ogle (131) showed that the rRNA genes on Physarum palindromes are transcribed divergently (Fig. 6), the polarity of the smaller and larger rRNA cistrons thus agreeing with that found previously in other eukaryotes (see previous section). Campbell et al. (132) found that the 26S rRNA cistron of Physarum contains two intervening sequences, in a manner somewhat analogous to Drosophila rDNA. In this case, however, it seems likely that the intervening sequences are usually transcribed, because they occur in at least 88% of the rRNA genes, and other data indicate that all of these genes are probably active in growing plasmodia.

Yao and Gall (133) have proposed a tentative model for the origin of extrachromosomal Tetrahymena palindromes that involves branch migration of the single rDNA unit integrated in the germline genome to form an extrachromosomal molecule, which unfolds into a linear palindrome by semiconservative replication. Such a mechanism would explain why the two sides of the palindrome are virtually identical and why there is no heterogeneity in the rDNA of Tetrahymena at the time of formation of the vegetative macronucleus.

In green algae and paramecia, the rDNA was found to exist not as palindromes, but in arrays of tandem repeats similar to that found in higher eukaryotes. Although, as discussed above, the rRNA genes in such arrays typically exhibit the same transcriptional polarity, a so-far unique arrangement has been reported by Berger et al. (134) for Acetabularia elegua in which rDNA repeats exhibit a strictly alternating polarity.

Chromosomes and Nonnucleolar RNA Synthesis

Through the years, many of the cytological studies of nonnucleolar RNA synthesis on eukaryotic chromosomes have focused on the so-called "giant chromosomes," primarily the diplotene-stage lampbrush chromosomes of amphibian oocytes and the polytene chromosomes of dipteran flies. The basic structural organization of these chromosomes is described by Gall in this volume, so only morphological and chemical aspects involving RNA synthesis will be considered here. Visualization of synthetic activity in the lampbrush-type loops found in primary spermatocytes of Drosophila, in embryos, and in certain miscellaneous cell types are also discussed.

Lampbrush Chromosomes of Amphibian Oocytes

Although lampbrush chromosomes have been observed in the oocytes of many vertebrate and invertebrate animals (135) and even in green algae (136), they attain their largest dimensions in the oocytes of amphibia. Although seen previously, the first extensive study of such chromosomes was done by Rückert in 1892 (137) on sectioned shark oocytes. It was not until 1940, after the Feulgen stain was introduced, that the DNA nature of the chromomeres forming the main axis of lampbrush chromosomes of Rana was demonstrated (5). In 1937 (see Duryee [138] and previous articles), Duryee made an important contribution toward the study of lampbrush chromosomes by showing that the germinal vesicles of amphibian oocytes can be isolated and their lampbrush chromosomes observed in the phase-contrast microscope in what appears to be essentially an in vivo condition. After earlier studies by Dodson (139), which indicated the presence of RNA in the lateral loops of lampbrush chromosomes, Gall (140), in a careful study of the lampbrush chromosomes of the newt, clearly demonstrated the presence of RNA in the Feulgen-negative lateral loops, which were presumed to be products synthesized or organized by the
Feulgen-positive chromomeres of the main axes. In this study, Gall introduced a very important optical innovation by using an inverted phase-contrast microscope and holey slides with coverslip bottoms, an arrangement which allows observation of undistorted chromosomes at the highest resolution provided by light microscopy. Although there had been several earlier EM studies, Gall (141) was the first investigator to demonstrate that lateral loops contain loosely associated granules some 300-400 Å in diameter. Both Callan and Gall (see references in [141]) had previously postulated from earlier EM studies that each lateral loop has a submicroscopic axis. That this is so was also clearly demonstrated by Gall (141), who used pepsin digestion of loop matrices after immobilizing lateral loops on support films. Soon thereafter, Lafontaine and Ris (142) observed lampbrush chromosomes of several amphibians after critical point-drying in carbon dioxide. The similar fibrillar nature of loops and chromomeres after such drying suggested to these investigators the possibility that the main axis or chromonema of each chromosome consists of a bundle of fibrils that may be continuous through chromomeres and loops, but that varies in composition within the two structures. Gall's earlier study, and subsequent studies by others, clearly showed that this was not so. Very shortly thereafter, the nature of the submicroscopic axes of lateral loops was nicely shown by Callan and Macgregor (143), who demonstrated that DNase breaks the continuity of both loops and main axes without disturbing the RNP matrix material associated with the loop fragments until the loop axes have been disintegrated.

The fact that RNA is being actively synthesized on lateral loops was demonstrated by Gall (144) and Gall and Callan (145) who autoradiographed isolated chromosomes after labeling them with tritiated RNA precursors. The association of newly synthesized protein with the RNA also was shown in the second study. Previously, Callan and Lloyd (146) had introduced the concept that the genetic information within lampbrush chromosome loops may be serially repeated along the loop axes. To avoid the problem of random mutations, it was proposed that a "master copy" would correct any sequence changes as the repeats along a loop spin out of its chromomere to be transcribed during early diplotene. This concept was reinforced by evidence from Gall's and Callan's study on RNA synthesis; they observed sequential labeling of one morphologically distinct loop and concluded that it probably was continuously being spun out of and back into its chromomere as oogenesis progressed. The so-called "Master-Slave" hypothesis was expanded upon by Callan in 1967 (147), and further evidence for loop-axis movement was provided by Snow and Callan in 1969 (148). Inherent in this concept are the assumptions that no genetic diversity exists within individual chromosomes and that RNA synthesized on such chromosomes would come from repetitive DNA sequences (see Macgregor [149] for discussion of this concept). Although this hypothesis stimulated considerable thought and research, it does not appear to be valid in view of later results which indicate that most of the template-RNA synthesized and stored during amphibian oogenesis is transcribed from unique or single-copy sequences (150, 151).

More definitive observations regarding the ultrastructural nature of the RNP molecules in loop matrices was next provided by Miller (152) and Miller and Beatty (153), who used newt oocytes and techniques designed to observe chromosomes free of nucleoplasm and to unwind the RNP fibrils attached to loop axes (Fig. 7). Their results demonstrated that the RNP fibrils of typical loops form gradients of fibrils of increasing lengths from the thin insertion end, with RNA polymerases quite closely spaced and extremely long RNA molecules being synthesized. Subsequently, the structural organization of loop RNA fixed under physiological conditions was reported by Mott and Callan (154), who found that nascent RNA transcripts and associated protein are arranged in linear arrays of 300 Å particles. Similar configurations were found in all loops, no matter what their gross morphology, but many loops had such strings of particles wound back on themselves to form dense aggregates some 2,000-3,000 Å or more wide. Malcolm and Sommerville (155) previously had isolated such particles and had shown the protein-to-RNA ratio to be at least 30:1. Scott and Sommerville (156) demonstrated by immunofluorescence techniques that some of the nonbasic proteins in lampbrush chromosomes are common to all loops, whereas others may be localized in specific groups of loops.

**Figure 7** A portion of a lampbrush chromosome loop at the thin, chromomeric insertion end where RNA synthesis is initiated, from Miller et al. (193). Preparation was isolated from an oocyte of *N. viridescens*. Bar, 1 μm. X 16,500.
Scheer and co-workers (157, 158) used chromatin-spreading techniques to expand greatly observations on the arrangement of transcriptional complexes in salamander oocytes and the green algae Acetabularia. In addition to loops that appear to be single transcription units, as inferred from single RNP fibril gradients, loops with multiple gradients of divergent, convergent, and/or similar polarities are sometimes observed. Estimates of the sizes of nascent RNA molecules range up to some 82 kb, based on lengths of transcriptional units, and similar sizes have been determined by sedimentation and gel electrophoretic analyses (157, 159).

The functional significance of the high levels of transcriptional activity on lampbrush chromosomes is not clear. Davidson and co-workers (160) estimated that about 2.2% of lampbrush-stage RNA in X. laevis is template RNA that is synthesized on about 2.7% of the genomic DNA. Subsequent studies by Sommerville and Malcolm (159) demonstrated that about 4% of the chromosomal DNA of Tribulus cristatus is transcribed during oogenesis. However, only some 0.05–0.1% of the RNA contains coding sequences; the remainder are noninformational repetitive sequences. Further studies, by Rosbash and colleagues (150, 151), show that the poly(A)-RNA molecules present in mature X. laevis oocytes contain some 20,000 different sequences that are transcribed almost entirely from single-copy DNA. The sedimentation profile of poly(A)-RNA from oocytes and X. laevis kidney-cell cultures were found to be similar. Whether loop transcription represents a relatively high activity on loci that are transcribed at much lower rates in somatic cells or rather represents transcription of larger segments of DNA than occurs in somatic cells remains to be determined.

Y Chromosome Lampbrush Loops in Drosophila Spermatocytes

The early genetic and light-microscope cytogenetic studies of Y-chromosome function in Drosophila spermatogenesis were reviewed in 1968 by Hess and Meyer (161). Emphasis was placed on the D. hydei subgroup, in which morphologically distinctive structures comparable to the loops of lampbrush chromosomes were found to be determined by a minimum of five Y-chromosome loci. The loop morphologies are species specific, and, as shown by deficiency-duplication studies, the loci are involved in postmeiotic sperm differentiation. After labeling with [3H]uridine, ARG demonstrates that RNA synthesis occurs on each of the loci, with some loci showing polarized labeling. A microspreading method for dispersing contents of primary spermatocyte nuclei as a surface film was used by Meyer and Hennig (162) and Hennig et al. (163) to observe structural aspects of these loci by EM. It was estimated that RNP molecules considerably longer than 10 µm are synthesized on some loops. Hennig (164) has more recently reviewed the state of knowledge about Y-chromosome loops, and has suggested that optional points for RNA polymerase initiation along a loop could account for the polarized incorporation that takes place on some of the loops after pulse-labeling with RNA precursors.

Polytene Chromosomes of Dipteran Flies

The occurrence, structure, and synthetic activities of polytene chromosomes have been the subject of a number of reviews (e.g., 165–167). The composition and function of “puffs,” which form by the unfolding of usually one chromosomal band and appear in the polytene chromosomes of many larval tissues of Dipteran flies, have received the most attention. This is especially true of the very large puffs, or Balbiani rings (BRs), found in the salivary glands of Chironomus species. Early light-microscope ARG by Pelling (168) and Rudkin and Woods (169) showed that such puffs are highly active in RNA synthesis. The early EM study by Beerman and Bahr (79) demonstrated that BRs consist of numerous branching filaments ~100 Å thick, with granules ~300 Å in diameter apparently attached to their ends. This study was extended later by Stevens and Swift (170), who provided EM evidence that the RNP products of BRs move into the cytoplasm through the pores of the nuclear envelope.

Because of the high lateral redundancy of polytene chromosomes, Swift (171) and, later, Gorovsky and Woodward (172), were able to show that there is no difference in the amount of histone in inactive and puffed loci. That nonhistone proteins become associated with RNA in puffs was demonstrated by Helmsing and Berendes (173), who also showed that some nonhistone protein will move into induced puffs even in the absence of RNA synthesis.

Grossbach (174) presented evidence that the BRs of Chironomus probably contain the genes for several secretory polypeptides. Because of this, and the fact that BRs and their associated RNAs can be isolated by microdissection techniques, the BRs, especially BR2, of C. tentans have been the subject of intensive investigation, and much of this work has been reviewed recently by Case and Daneholt (175). The primary transcripts of both BR1 and BR2 have sedimentation constants of 75S and are estimated to contain 37 kb. The 75S molecules of BR2 have been shown to be present in cytoplasmic polysomes and, thus, probably to code for one or more of the salivary secretion polypeptides. Recently, Lamb and Daneholt (176) were successful in employing chromatin-spreading techniques to visualize transcription units of chromosome 4 of C. tentans which contains the BRs. Highly active transcription units with a mean length of 7.7 µm are most often observed, and are presumed to be the units forming BR1 and BR2 which form the most conspicuous puffs.

Visualization of Nonnucleolar Transcription in Other Cell Types

After the observations on lampbrush chromosomes, the first clear visualization of the morphology of nonnucleolar or presumptive heterogeneous nuclear RNA (hnRNA) synthesis was reported by Miller and Bakken (85) for HeLa cells. RNP molecules were found to be attached to the genome at intervals and widely spaced, indicating that the initiation of transcription occurs infrequently on active loci in this differentiated tissue-culture cell. Miller and co-workers (86) next dispersed chromatin from 4- to 6-hour Drosophila embryos and found well-defined RNP fibric gradients, presumably reflecting the genetic activity involved in differentiation events that occur during that embryonic period. More precise quantitative studies of hnRNA synthesis in insect embryos were done by Laird and co-workers for Drosophila and Oncopeltus (91, 177, 178) and McKnight and Miller (89) for Drosophila. The latter authors compared transcription during the syncytial stage and early cellular blastoderm, and found that, whereas there is only a low level of template activity with a few short, dense, RNP fibric gradients present in the syncytial stage, a large new class of much longer gradients with generally intermediate polymerase densities appears at cellular blastoderm, again presum-
ably reflecting genetic activity involved in differentiation events. In all of the embryonic studies, a large variation in length and RNA-polymerase density was found among hnRNA transcription units. Estimates of the average size of hnRNA molecules synthesized on such units range from 10 to 18 kb. Similar studies subsequently were done by Busby and Bakken (179) on sea-urchin embryos. These investigators found that a large majority of active transcriptional units exhibited only a single nascent RNP fibril, and concluded that the polymerase density on single, versus multiple, fiber loci is caused by polymerase initiation frequency.

In their initial study of hnRNA synthesis in Drosophila, McKnight and Miller (89) noted that homologous, nascent, fiber arrays often could be identified on sister chromatids after chromatin replication in late S or G2 stage of early cellular blastoderm. Such arrays appeared to offer a unique opportunity to compare regulation of transcription on two copies of the same genetic locus, and a number of these were analyzed in a subsequent study (180). The results showed that, although size and polymerase density vary considerably among different loci, nascent fiber frequency and distribution is essentially the same for homologous pairs, indicating that sister chromatids inherit precisely similar transcriptional potentials. In addition, it was noted that different, but immediately adjacent, genetic units can differ in polarity and fiber frequency.

The first presumptive visualization of a specific structural gene was reported by McKnight et al. (181) for the silk fibroin gene of Bombyx mori (Fig. 8). The long, RNP-fibril gradients observed in this study were identified as active silk fibroin genes on the basis of gene size, the presence of such gradients only in the posterior portion of the silk gland where fibroin synthesis is localized, their single-copy nature, and high RNA-polymerase density, all of which can be correlated with known biochemical parameters of silk fibroin gene activity.

In Vivo and In Vitro X. laevis Oocyte Systems for Transcription of Specific DNAs

Except in cases where, predominately, only one to a few genes are expressed in a cell type, the analysis of transcription of a single gene is difficult, because its contribution to total RNA synthesis is small. The two, recently developed transcriptional systems discussed below, when combined with the availability of purified specific genes, offer the potential of overcoming such difficulties.

In Vivo Transcription of DNA Injected into Amphibian Oocyte Nuclei

The first report of transcription of DNA after microinjection was given by Mertz and Gurdon (182), who showed that RNA homologous to Simian Virus 40, as well as to several other foreign DNAs, is synthesized in oocyte nuclei. Very soon thereafter, Brown and Gurdon (183, 184) showed that, after microinjection, accurate transcription of both genomic and cloned Xenopus 5S rDNA takes place, and is sensitive to the α-amanitin concentration expected for RNA polymerase-III inhibition. As much as half of the RNA synthesized by an injected oocyte can be a result of injected SS DNA, although...
at low inputs it can be shown that the injected DNA is transcribed only about one-fifth as efficiently as the endogenous 5S DNA. After injection, the 5S DNA becomes complexed with a near-equal mass of protein, which may be important for accurate transcription. Telford et al. (185) injected a Xenopus DNA segment containing the structural gene for tRNA$_{\text{Met}}$ and only 22 base pairs to the 5' side of the gene. They found that mature tRNA$_{\text{Met}}$ was produced at a high rate from the injected fragment, and suggested the possibility that recognition between DNA and RNA polymerase III may be determined by the structural tRNA gene itself rather than 5' sequences outside of the gene. Grosschedl and Birnstiel (186) identified three regulatory segments in the prelude sequences of a sea urchin H2A histone gene by injection of cloned specific deletion mutants, and, in view of their results, speculated that eukaryotic promoters may have to be viewed as three-dimensional, rather than linear, chromosomal structures. The first visualization of transcription of injected DNA was reported by Trendelenburg et al. (187), who used circular amplified Dytiscus rDNA as a source of foreign DNA. The injected rDNA becomes complexed with protein, and apparently normal, as well as abnormal, transcriptional patterns are observed (Fig. 9). A high frequency of abnormally long RNP fibrils suggests that proper termination of nascent pre-rRNA molecules may not always occur. Subsequently, Trendelenburg and Gurdon (188) injected homologous cloned rDNA and found that accurate transcription takes place, with activated genes exhibiting the typically dense gradients of endogeneous rRNA genes. However, more than 90% of the injected DNA is assembled into inactive nucleosomal chromatin configurations, indicating that transcription is not regulated by the supply of RNA polymerase I but presumably by some limiting component which switches genes maximally on.

In Vitro Transcription of DNA in a Nuclear Extract from Oocytes

Brown and co-workers (189) recently demonstrated that cloned 5S genes are transcribed accurately after an initial 30' lag period when mixed with a supernatant fraction obtained from manually isolated, disrupted X. laevis oocyte nuclei. Although there is also significant transcription of the noncoding 5S strand, spacer, and plasmid DNA, up to 40% of the total RNA transcribed has been shown to be 5S RNA. Transcription involves RNA polymerase III, because this is the only active polymerase in this system. More recently, Brown and colleagues have shown by using deletion mutants that initiation of RNA polymerase III on 5S gene sequences can be maintained, as nucleotide pairs are sequentially removed from the 3' end of the gene until nucleotides between 50 and 55 are reached (190). Similarly, initiation can be maintained as nucleotide pairs are removed from the 5' end of the gene until between nucleotides 80 and 83 (as counted from the 3’ end of the gene) (191). These results demonstrate somewhat unexpectedly that the sequences responsible for proper initiation of RNA polymerase III are contained within the 33 nucleotides between nucleotides 50 and 83 of the gene itself.

Concluding Remarks

It has been possible, in a short review such as this, to list only some of the highlights of the discoveries by investigators studying the nucleolus and synthetic activities of chromosomes. Regrettfully, many observations of interest have had to be omitted. I have attempted to communicate some of the excitement generated by the increase in our knowledge regarding the function of the nucleolus and structural aspects of genetic transcription. Much of the progress in these areas, as in others, has been a result of the application of new techniques that have proved to be powerful probes in our attempts to understand the molecular basis of genetic activity. Much, much more remains to be discovered, but many tools are available and others will be forthcoming. Only the continued imagination and diligence of young scientists is required for further, exciting discoveries.

REFERENCES

1. Montgomery, T. H. 1898. J. Morphol. 15:266–260.
2. Heitz, E. 1931. Planis (Berl). 12:775–844.
3. McClintock, B. 1934. Z. Zellforsh. 21:294–328.
4. Caspersson, J., and J. Schultz. 1940. Proc. Natl. Acad Sci. U. S. A. 26:507-515.
5. Brachet, J. 1940. Arch. Biol. 51:151–165.
6. Caspersson, T. O. 1950. Cell Growth and Cell Function. W. W. Norton & Co., Inc., New York. 185.
7. Estable, C., and J. R. Sotelo. 1955. In Fine Structure of Cells. P. Noordhoff, N. V., Groningen. 170–190.
8. Vincent, W. S. 1955. Int. Rev. Cytol. 4:269-298.
9. Claude, A. 1941. Cold Spring Harbor Symp. Quant. Biol. 9:263–271.
10. Schneider, W. C., and G. H. Hogeboom. 1956. Annu. Rev. Biochem. 25:201–224.
11. Littlefield, J. W., E. B. Keller, J. Gross, and P. C. Zamecnik. 1955. J. Biol. Chem. 217:111–123.
12. Nomura, M., A. Tissieres, and P. Lengyel, editors. 1974. Ribosomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 930.
132. Campbell, G. R., V. C. Littau, P. W. Melera, V. G. Allfrey, and E. M. Johnson. 1979. Nucleic Acids Res. 6:1433-1447.
133. Yao, M.-C., and J. G. Gall. 1977. Cell. 12:121-132.
134. Berger, S., D. M. Zellmer, K. Kloppstech, G. Richter, W. L. Dillard, and H. G. Schweiger. 1978. Cell. Biol. Int. Repts. 3:291-50.
135. Davidson, E. H. 1976. Gene Activity in Early Development. Academic Press, Inc., New York. 452.
136. Spring, H., U. Scheer, W. W. Franke and M. F. Trendelenburg. 1975. Chromosoma (Berl.). 50:25-43.
137. Rücker, J. 1982. Anat. Anz. 7:107-158.
138. Durlee, W. R. 1950. Ann. N. Y. Acad. Sci. 50:921-953.
139. Dodson, E. O. 1948. Univ. Calif. Publ. Zool. 53:281-314.
140. Lamy, M., P. J. Ford, and J. O. Bishop. 1974. Proc. Natl. Acad. Sci. U. S. A. 71:119-128.
141. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
142. Hennig, W. 1974. Chromosoma (Berl.) 50:25-43.
143. Hennig, W., and W. H. Swift. 1962. In The Molecular Control of Cellular Activity. J. M. Allen, editor. McGraw-Hill, Inc., New York. 73-125.
144. Hennig, W. 1974. Chromosoma (Berl.) 50:25-43.
145. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
146. Hennig, W. 1974. Chromosoma (Berl.) 50:25-43.
147. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
148. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
149. Hennig, W. 1974. Chromosoma (Berl.) 50:25-43.
150. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
151. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
152. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
153. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
154. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
155. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
156. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
157. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
158. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.
159. Hennig, W., G. F. Meyer, I. Hennig, and O. Leoncini. 1974. Cold Spring Harbor Symp. Quant. Biol. 39:873-883.