ELECTRONMICROSCOPIC STUDIES OF ACTIVE GENES

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SUMMARY

Structural aspects of ribosomal and non-ribosomal RNA transcription are compared and contrasted in a representative prokaryote (Escherichia coli) and in several diverse eukaryotes (developing amphibian oocytes, Drosophila melanogaster embryos, HeLa cells, and yeast). The conformations of these active loci are described in relation to genetic and biochemical studies of the activity of the two major classes of genes.

1. INTRODUCTION

A combination of rapid isolation procedures and simple preparative techniques for electron microscopy has made it possible to identify active genes (regions of the genome that are being transcribed) in both prokaryotes and eukaryotes (for review see MILLER and HAMKALO 1972, HAMKALO and MILLER 1973). This approach provides the opportunity to compare the ultrastructure of active genes coding for both stable and rapidly turning over RNA's in various cell type under different growth conditions and during differential gene activity.

2. PREPARATIVE PROCEDURES

Although each system requires somewhat different isolation

techniques, the basic procedure for preparation of material for electron microscopy remains the same and has been published in detail (MILLER and BAKKEN 1972). Briefly, lysed cells or nuclear contents are deposited on a hydrophilic carbon-coated electron microscope grid by low speed centrifugation (~2350 x g) through a cushion of 0.1 M sucrose-10% formalin in a chamber that fits into a swinging bucket rotor. After rinsing the grid in an aqueous solution of low surface tension (0.4% Kodak Photoflo), it is air-dried, positively stained by immersion for 30 seconds in ethanolic phosphotungstic acid (PTA), rinsed in 95% ethanol, and air-dried. Use of acidic PTA provides preferential staining of proteins (SILVERMAN and GLICK 1969). This technique appears to be non-destructive in the sense that chromatin active in RNA systhesis at the time of isolation retains nascent RNA's and the deoxyribonucleoprotein (DNP) fiber does not appear to be sheared noticably (i.e., few ends are seen). Specific isolation procedures are found in the legends of the plates on page 100.

3. RIBOSOMAL RNA GENES - PROKARYOTIC

Eighty-five percent of cellular RNA in rapidly-growing *Escherichia coli* cultures is 16S, 23S, and 5S ribosomal RNA (rRNA) (SPIEGELMAN and YANKOFSKY 1965). Since there are only about six gene copies for each rRNA species per bacterial chromosome (PURDOM et al. 1970), it has been calculated that each gene must be transcribed simultaneously by many closely-spaced RNA polymerases (BREMER and YUAN 1968, MANOR et al. 1969, WINSLOW and LAZZARINI 1969). Biochemical and genetic evidence suggest that the rRNA cistrons are arranged as essentially contiguous 3 gene units with the order 16S-23S-5S (DOOLITTLE and PACE 1971, COLLI et al. 1971). The nascent rRNA chains, unlike messenger RNA's (mRNA), are associated with proteins (MANGIAROTTI et al. 1968) existing as ribonucleoprotein (RNP) fibers. This information, combined with the sum of the molecular weights of the rRNA's, allows one to predict a structural configuration of active bacterial rRNA genes: segments of the genome about 1.7 μm in length associated with 60-80 closely-spaced RNP fibrils that are arranged as short-to-long fibril gradients.

Plate 1 shows such a region from the contents of an osmotically-shocked *E. coli* cell. In addition to the fact that the structure of such regions agrees well with that predicted above, as will be discussed below, the disappearance of the fibril-containing matrices at restrictive growth temperature in a temperature-sensitive mutant that fails to synthesize rRNA at 42° (A.G. Atherly, personal communication) is evidence that these chromosomal segments are ribosomal RNA genes. Each matrix unit measures about 1.3 μm and is composed of 60-70 RNP fibrils that make up two short-to-long fibril gradients, the first about one-half the length of the second, in agreement with the

difference in molecular weights of the two large rRNA's. The fact that these regions are shorter than predicted may result from an alteration of the DNA from B conformation at sites of high transcriptive activity. Identical active segments have also been observed in preparations from rapidly-growing *Salmonella typhimurium* and *Bacillus subtilis* cultures (HAMKALO, unpublished data).

The existence of two fibril gradients at rRNA synthesis sites is consistent with the lack of a large precursor for the two molecules, unlike the siutation in higher cells. However, the pattern of rRNA fibril gradient disappearance after addition of rifampin, which inhibits initiation but not elongation of RNA chains, suggests that RNA polymerase can initiate only at the beginning of the 16S cistron; thus mature 16S rRNA is liberated from the DNA while the enzyme molecule that has completed synthesizing it continues to transcribe distal to this site. The same conclusion was reached from a biochemical analysis of rifampicin readout (PATO and von MEYENBERG 1970).

We are not yet able to estimate the minimum distance between two adjacent rRNA segments although one frequently sees polyribosomes, attached to the genome at varying distances, bracketing rRNA segments. That is, rRNA loci are not very closely-clustered on the bacterial chromosome and the space between adjacent segments is transcribable.

4. RIBOSOMAL RNA GENES - EUKARYOTIC

The ultrastructure of active ribosomal precursor RNA (rpRNA) genes in several eukaryotes provides evidence for conservation in the arrangement of these redundant genes in species as diverse as amphibians, *Drosophila* humans, and yeast (HAMKALO et al. 1973 a, b).

MILLER and BEATTY (1969 a, b) first identified active rpRNA genes in nuclear material of developing amphibian oocytes. There is a high selective amplification of genes coding for rpRNA in these cells (BROWN and DAWID 1969) and rDNA transcription accounts for nearly all of the nuclear RNA synthesis. Ribosomal RNA genes are located in the central core regions of the hundreds of extrachromosomal nucleoli that are free in the nucleoplasm of each oocyte (EVANS and BIRNSTIEL 1968). Plate 2a shows a portion of an unwound nucleolar core from a *Triturus viridescens* oocyte preparation.

Identification of the components seen in Figure 2a is based on enzymatic digestions and predictions of structure based on biochemical and autoradiographic data. The axial fiber is a deoxyribonucleoprotein (DNP) complex and the attached lateral fibers that make up short-to-long fibril gradients are RNP. Each gradient is separated from its neighbors by matrix-

free DNP, designated spacer. Autoradiographic experiments provide evidence that these spacers are not transcribed (MILLER and BEATTY 1969 b). The length of each matrix unit is about 2.3μm only slightly shorter than the length of an rpRNA gene based on a molecular weight of 2.5 x 10^6 for the amphibian 40S precursor molecule (LOENING et al. 1969). Mature rpRNP's are about 1/10 the length they would be if the RNA were fully extended; this suggests a tenfold foreshortening of the RNA within an RNP fiber. These observations led to the conclusion that each fibril gradient is an active coding for rpRNA and that these genes are visible because 80-100 RNA chains are being transcribed simultaneously from each locus (MILLER and BEATTY 1969 a, b). In fact, each nascent RNP is attached to the DNP axis by a granule about 125Å in diameter, likely to be RNA polymerase (Figure 2a).

Although the spacer regions between adjacent rpRNP genes are not transcribed they may well posses an important nucleolar function because of their universal occurrence in the ribosomal DNA of all eukaryotes studied (see below). Another conserved component whose function is presently unknown is the granule at the free end of nascent RNP's.

We can use the oocyte nucleolar gene structure as a basis of comparison with putative active rpRNA genes in several other eukaryotic cell types. Nucleoli become visible in *Drosophila melanogaster* embryos after the syncytial nuclei become surrounded by plasma membranes (SONNENBLICK 1965). Electron microscopic analysis of disrupted embryos at this stage show active regions of chromatin that exhibit structural similarities to amphibian rpRNA genes (Figure 2b). Again the exceedingly high rate of transcription initiation generates distinct fibril gradients. Based on a precursor molecular weight of 2.85 x 10^6 (PERRY et al. 1970) a *Drosophila* rpRNA gene in B conformation would measure about 2.8μm each fibril matrix in Figure 2b measures about 2.65μ. Adjacent rpRNA genes are again separated by apparently untranscribed spacer which average about 0.4μm. The dense granules on the free ends of nascent rpRNP's in *Drosophila* appear to be analagous to those seen in amphibian preparations. Similar structure have been identified in nuclear contents of *Drosophila* tissue culture cells (CHOOI and LAIRD, personal communication) and spermatocytes (HENNIG et al. 1973).

Active putative rpRNA genes are also visible in the dispersed contents of lysed cultured mammalian cells. Plate 2c shows several such regions from a HeLa cell nucleus. Although a tremendous amount of chromatin is released from these nuclei, the exceedingly high transcription initiation frequency of these chromosome segments permits putative identification of these genes. Compared to active rpRNA genes of amphibian or *Drosophila* material, the major structural difference is the length of the fibril-containing matrix. Matrices in HeLa are about 3.8μm and the rpRNA molecule has a molecular weight of about 4 X 10^6 (WEINBERG et al. 1967); again high transcriptive activity on a restricted length of chromatin could alter the DNA conformation so as to shorten it from B conformation length.

The large amount of chromatin in the background of these preparations makes it difficult to follow individual DNP strands for any great distance and hence to definitively resolve spacer segments. In favorable preparations, however, it is possible to identify untranscribed spacer DNP between adjacent rpRNA loci. Once again, granules are evident at the free ends of nascent RNP's. There is little apparent evolution of the structural arrangement of the rpRNA genes in higher eukaryotes. Notable differences are those of matrix unit length, which is correlated with changes in precursor molecular weight, and spacer length.

Genetic (FINKELSTEIN et al. 1972, CRYER et al. 1974) and biochemical (CRAMER et al. 1972) data on the location and arrangement of yeast rDNA (γDNA) suggest that the ultrastructure of rpRNA genes in lower eukaryotes may be quite similar to that of more advanced forms. Yeast chromatin released after osmotic shock of protoplasts and nuclei exhibit regions similar in conformation to partially unwound nucleoli (Plate 2d). We are now attempting to optimize dispersal conditions in order to study the ultrastructure of these regions relative to the higher cell types already described. However, putative yeast nucleolar genes appear to be composed of closely-spaced fibrils with dense granules on their free ends.

5. STRUCTURAL GENES - PROKARYOTIC

Genes that do not code for stable RNA species (rRNA's, tRNA's) have been designated structural genes because the products of their transcription messenger RNA's (mRNA) code for proteins. In prokaryotes, transcription of mRNA and its subsequent translation into proteins by ribosomes are closely-coupled processes (STENT 1964). This arrangement is demonstrated clearly in Plate 3. Nascent mRNA chains are attached to the genome and coated by ribosomes that generate short-to-long polyribosome gradients, indicating the direction of transcription of this chromosome segment. Approximately 1.3 μm of DNA lies between the site of attachment of a single ribosome to that of the longest polyribosome. Since lactose operon DNA, which codes for a polycistronic messenger that is translated into three proteins, is approximately 1.4μ (SHAPIRO et al. 1969), Plate 3 must illustrate an active polycistronic operon. Small granules (~80Å in diameter) appear to be attached to the DNA at the site of attachment of each polyribosome; these granules are probably RNA polymerases that were transcribing these genes at the time of isolation.

There is evidence for mRNA degradation in the 5'- to 3'-direction (KUWANO et al. 1969, MORIKAWA and IMAMOTO 1969, MORSE et al. 1969); assuming degradation and transcription occur simultaneously, then the free ends of nascent mRNA's should be sites of nucleolytic attack. This seems likely from our

observations since we do not see free polyribosomes in typical bacterial preparations. The fact that active structural genes exhibit fairly regular short-to-long polyribosome gradients argues against random degradation of messengers. These questions can be answered unambiguously by studying a specific active structural locus of known length.

Although segments of the bacterial genome that are active in RNA synthesis are recognized readily in the extruded contents of shocked cells, they represent a small fraction of the chromosomal material. This means that at any instant very few genes are being transcribed, in agreement with conclusions based on RNA-DNA hybridization experiments (KENNEL 1968).

6. STRUCTURAL GENES - EUKARYOTIC

Unlike the uniformly high transcription initiation frequency of rpRNA genes in the eukaryotes described above, comparisons of the ultrastructure of regions active in non-ribosomal RNA synthesis reveal dramatic differences in initiation frequency. Active amphibian oocyte chromosomes are termed lampbrush chromosomes because of their configuration as seen by light microscopy. Each member of a paired bivalent is seen as a series of Feulgen-positive chromomeres from which pairs of lateral loops extend. Autoradiographic experiments show that the loops are active in RNA synthesis and the thin-to-thick matrices, characteristic of lampbrush loops, are composed of RNA and protein (GALL and CALLAN 1962). Figure 4a is an electron micrograph of a portion of such a lateral loop. The fact that nascent RNP's are very closely-spaced indicates a transcription initiation frequency as high as that of rpRNA genes (Figure 2a). The noticeable difference between these sites and active rpRNA genes is the length to which RNP fibers grow, a reflection of the size of the transcription unit. If one assumes that a typical lateral loop measures 50μm (GALL 1956), and that each loop possesses a single initiation site, then nascent non-ribosomal RNP's should grow to many times the length of mature rpRNP's. In fact, nascent RNP's measuring 20μm have been observed in amphibian oocyte preparations. Unfortunately, virtually nothing is known of the genetic information content of these giant transcripts.

Active segments of chromatin from *Drosophila* embryos (Figure 4b) are quite different in structure from those of amphibian oocytes. One obvious difference is in the number of nascent RNP's per length of DNA. The existence of measurable spaces between adjacent RNP's indicates relatively infrequent initiation of transcription. The segment shown in Figure 4b is somewhat more active than those typically seen since a short-to-long RNP fibril gradient is discernable. The fact that much of the DNP in *Drosophila* embryos does not have nascent RNP's attached is evidence for a lower level of transcriptive activity

in these cells as compared to oocytes. Nevertheless, when one compares the level of activity in these cells with that of HeLa cells in culture (Plate 4c), *Drosophila* embryos possess a larger amount of active chromatin than do the relatively quiescent human cells. Most of the HeLa chromatin is free of attached fibers and active regions show few distantly-spaced RNP's. RNP fibrils also are seen free in the nucleoplasm of HeLa; presumably these molecules are in transit from the site of their transcription to cytoplasmic ribosomes.

The general features of non-ribosomal transcription in eukaryotes other than amphibian oocytes (i.e., low levels of transcription initiation and much genetically silent chromatin) are also seen in yeast nuclear preparations (Plate 4d).

CONCLUSION

It has been possible to survey structural aspects of the transcription of ribosomal and non-ribosomal genome segments in prokaryotic and eukaryotic cells. Future progress in an electron microscopic approach to problems of molecular genetics will depend upon technical innovations that allow one to identify specific genes in order to directly analyze their arrangement on chromosomes and the regulation of their transcription by a variety of controlling factors.

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SEE PLATES ON PAGES 100, 101 and 102.

Plate 1. Active ribosomal RNA gene segment of an *Escherichia coli* chromosome. Logarithmically-growing cells were treated with T4 lysozyme and osmotically shocked by dilution into cold distilled water prior to preparation for electron microscopy as described in the Procedures section.

Plate 2. (opposite page) Active eukaryotic putative ribosomal precursor RNA gene
a) Manually-isolated nuclear contents from *Triturus viridescens* (the spotted newt of North America) were allowed to disperse for 10 min. in distilled water before the standard electron microscopy preparation.
b) Four- to six-hour *Drosophila melanogaster* embryos were immersed in cold 0.1% *JOY* detergent (Proctor and Gamble); chorions were removed; contents were pressed from the vitelline membrane and allowed to disperse for 10 min. before fixation and deposition on electron microscope grids.
c) HeLa cells from a rapidly-growing culture were lysed by 30 sec. treatment with cold 0.33% *JOY* prior to standard preparation.
d) Log phase yeast cells were converted to protoplasts by glusalase (Endo Labs) treatment followed by incubation for 2-3 hours in growth medium plus 0.1 M sorbitol prior to osmotic shock.

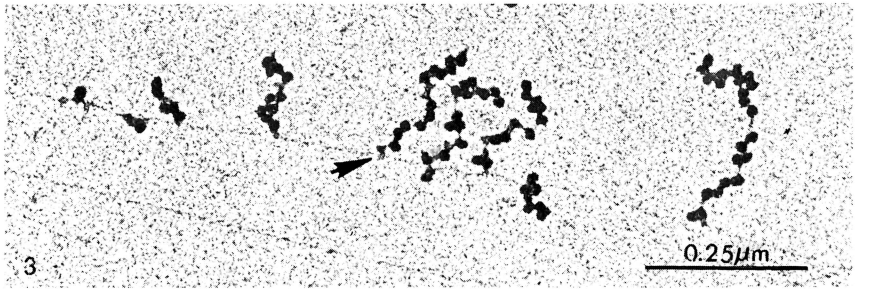
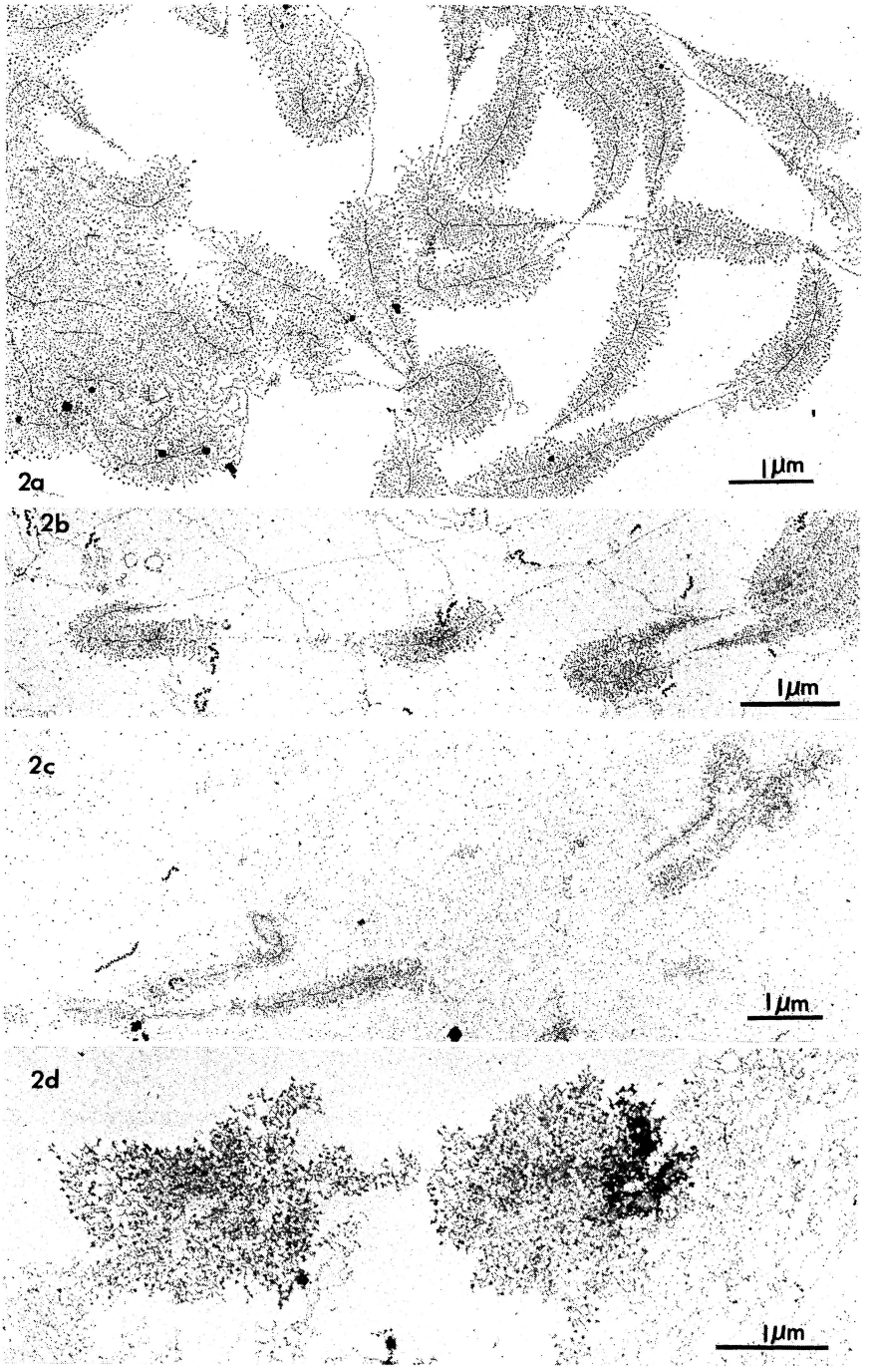


Plate 3. Active polycistronic operon from *E. coli*. Arrow indicates the location of a presumptive RNA polymerase molecule.

Plate 4. Active eukaryotic non-ribosomal chromatin (see page 102).
a) A portion of a *Triturus viridescens* oocyte lampbrush chromosome loop near the initiation site for transcription.
b) *Drosophila melanogaster* embryo nuclear material.
c) Active HeLa chromatin.
d) Active yeast chromatin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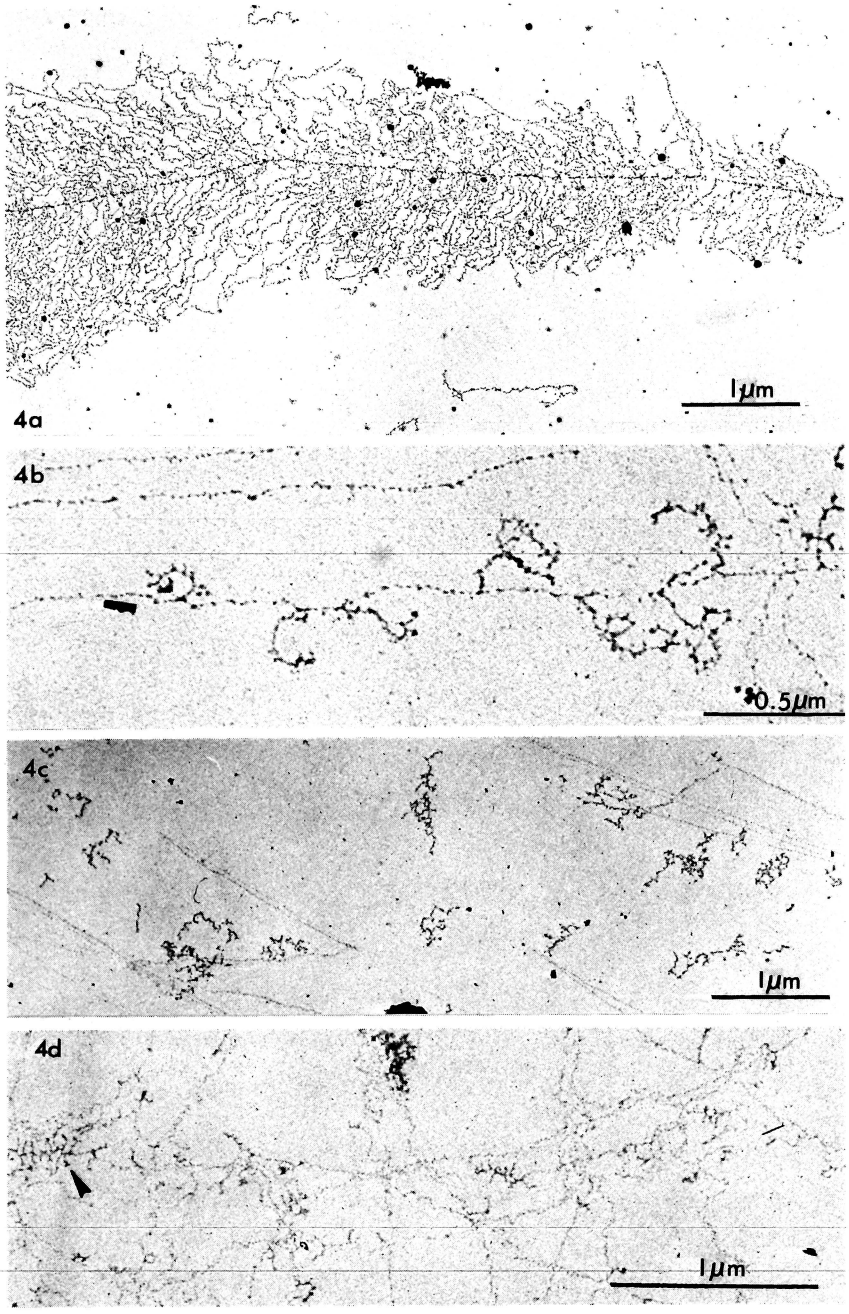
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