ROLE OF IONS IN ACTIVATION OF HUMAN LYMPHOCYTES

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ABSTRACT

The role of extracellular ions in activation of PHA stimulated lymphocytes was examined. Cells were cultured in media containing varying levels of K^+, Mg^{++}, and Ca^{++}. Lymphocyte transformation was monitored by measuring nuclear diameter, and by evaluating the area of nucleolus which reacted with silver nitrate.

Decreasing extracellular K^+ from normal levels (5.0 mM) to 14% of normal (0.7 mM), and decreasing extracellular Mg^{++} from normal levels (1.0 mM) to 14% of normal (0.14 mM), did not affect nuclear diameter or silver nitrate reactivity of PHA stimulated lymphocytes, nor did it affect the number of AgNORs in metaphase cells.

Chelation of extracellular Ca^{++} with EGTA, during the first 24 hours post stimulation, completely inhibited the increases in silver reactivity, nuclear diameter, and cell division associated with PHA stimulation. Addition of EGTA, 48 hours post PHA, did not inhibit lymphocyte stimulation. Inhibitory effects of EGTA were completely reversed if $CaCl_2$ was added to the medium within 24 hours of PHA stimulation. By 48 hours, the effects were irreversible. Therefore, Ca^{++} is essential for activation of genes coding for proliferation of lymphocytes *in vitro*.

Increasing extracellular Ca^{++} (to 7.8 mM) and/or calmodulin, did not significantly activate the proliferative genes of lymphocytes cultured without PHA.

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INTRODUCTION

I. REGULATION OF GENE ACTIVITY

Only a fraction (2-15%) of the genetic potential of a cell is expressed (Grouse et al., 1972). The fraction expressed fluctuates in response to physiological signals. Thus cells having the same genetic potential may be differentially activated to fulfill different, specialized functions. The mechanics of regulating gene activity is not only of fundamental importance to understanding how normal cells function, but also is paramount to understanding the autonomous proliferation of neoplastic cells, which are not controlled by normal cell regulatory mechanisms.

Little is known of the mechanisms involved in regulation of eukaryote gene expression, though various models have been proposed. Some are patterned after bacterial models, postulating highly specific repressor, derepressor and inducer molecules that interact with specific genes. Others involve the binding of hormone complexes either to chromatin acceptor sites or to cell membrane receptors. These stimulate intracellular activation or inactivation of a biochemical reaction affecting gene expression. The regulation of the highly reiterative rDNA is likely to be different from that of unique sequence DNA.

II. APPROACH TO STUDY

Human lymphocytes were stimulated by phytohemagglutinin (PHA) to reenter the cell cycle. Reentry involves turning on of those repressed genes which code for the proliferogenic components. Stimulation of lymphocyte activity is morphologically characterized by an increase in nuclear and nucleolar size, and by an increase in the ability of the nucleolus to reduce colourless silver nitrate ($AgNO_3$) to black metalic silver (Schwarzacher et al., 1978; Arrighi et al., 1980). These morphological changes are directly related to stimulation of rRNA genes.

The role of ions in regulation of lymphocyte activation was investigated by altering the extracellular concentrations of K^+, Mg^{++} and Ca^{++}. Activity of rRNA genes was monitored by measuring the diameter of the nucleus and the silver reactivity of the nucleolus.

Examination of the model system utilized, and of theories of regulation of rDNA activity will aid in understanding the role of ions in regulating rDNA activity.

LITERATURE REVIEW

I. ACTIVATION OF LYMPHOCYTES WITH PHYTOHEMAGGLUTININ (PHA)

The small peripheral blood lymphocyte was considered to be a fully differentiated cell, incapable of division. Nowell (1960) found that PHA, a mucoprotein extract of the bean plant, Phaseolus vulgaris, caused a high percentage of lymphocytes to undergo mitosis. It is a potent mitogen, stimulating 80 to 90% of small lymphocytes to reenter the cell cycle (Douglas, 1972). Cultured lymphocytes remain in a non-growing state unless exposed to a mitogen which induces cells to transform by enlarging, producing RNA, replicating DNA and undergoing mitosis.

Transformation induced by PHA is initiated by an interaction with membrane receptors (Kornfeld and Kornfeld, 1970). Soon after, membrane-bound adenylate cyclase rapidly increases in activity resulting in an increase in the two primary intracellular regulators, cyclic adenosine 3', 5'-monophosphate (cAMP) (Smith et al., 1971), and Ca^{++} (Whitney, 1972).

After PHA treatment a sequence of morphological changes occurs in human lymphocytes. Nucleus and total cell size, cytoplasmic basophilia and number of vacuoles progressively increase. Mitotic cells are first observed at about 40 hours after the addition of the mitogen, and the number observed peaks at about 72 hours (Ling, 1968).

After as little as 4 hours of exposure to PHA, the nucleolus may begin to enlarge (Douglas, 1972) and the silver reactivity of the nucleolus increases (Arrighi et al., 1980), probably due to the stimulation of nucleolar rRNA synthesis. At about 48-60 hours there are ribosomal aggregates in the cytoplasm and increased amounts of rough endoplasmic reticulum, golgi apparatus and lysosomes. Mitochondria often are swollen and show loss of cristae (Douglas, 1972).

PHA stimulates protein synthesis, initially by increasing the efficiency of pre-existing ribosomes and by preventing wastage, and later by utilizing newly synthesized ribosomes. Prior to PHA, about 30% of ribosomes are engaged in protein synthesis. Soon afterwards about 70% are engaged (Cooper, 1972). Approximately half of rRNA of unstimulated lymphocytes is degraded. This wastage is virtually eliminated after PHA treatment (Cooper and Gibson, 1971).

Waste control may be a mechanism for cells to quickly shift from the resting state to rapid growth upon the appropriate stimulus (Cooper, 1972). Chronic lymphatic leukemia cells are characterized by their inability to reverse the wastage of ribosomes upon stimulation (Rubin, 1971). rRNA production increases as in normal cells but the onset of blastogenesis is delayed.

The increase in efficiency of pre-existing ribosomes and control of wastage is not adequate for normal cell growth and division. Blockage

of rRNA synthesis prohibits DNA synthesis and thus cell proliferation (Kay et al., 1969). In a PHA stimulated nucleolus there is a drastic increase in polymerase A activity (Cooke and Brown, 1973) accompanied by a 10-to 50-fold increase in newly synthesized rRNA (Cooper, 1972) which peaks at around 18 hours (Purtell and Anthony, 1975).

PHA does not stimulate all lymphocytes. Approximately 10 to 20% of lymphocytes are not transformed. Purtell and Anthony (1975) found two rRNA processing pathways in guinea pig lymphocytes, only one of which was stimulated by PHA. This may be explained either by the existence of two major subpopulations of cells, only one of which is preferentially stimulated by PHA, or by all lymphocytes having the potential to use both pathways.

It is not known how PHA activates the genetic components necessary for proliferation. It is known that rRNA synthesis is essential for DNA synthesis and thus for cell proliferation. Regulation of rRNA synthesis may be exercised during transcription, maturation or degradation.

II. RIBOSOMAL RNA (rRNA) PRODUCTION AND PROCESSING

A. General

At least 80% of total cellular RNA is rRNA (Darnell, 1968). Genes coding for 18S and 28S rRNA are located in the nucleolar organizing regions (NORs) of human acrocentric chromosomes (Henderson et al., 1972). During interphase rRNA is transcribed in the nucleolus and modified to produce the precursor molecules of ribosomes. In the cytoplasm, ribosomes translate messenger RNA (mRNA) to produce protein. The activity of the ribosomal cistrons might be controlled at any stage from transcription into RNA to the translation of active proteins.

B. Ribosomal DNA

It has been demonstrated by in situ hybridization that the chromosomal location of genes coding for 18S and 28S rRNA are NORs (Henderson et al., 1972). The NORs of humans are situated at the secondary constrictions of the short arms of the five pairs of acrocentric chromosomes (13, 14, 15, 21 and 22) (Ohno et al., 1961). DNA clusters coding for 5S rRNA are scattered outside the NOR, primarily on the larger chromosomes.

rDNA is highly repetitive. At least 500 sets of genes per haploid

genome, encode for human 18S and 28S rRNA (Jeanteur and Attardi, 1969). Some 2000 genes per haploid genome encode for 5S rRNA (Hatlen and Attardi, 1971). This high degree of repetition allows for rapid production of ribosomes. The capacity for ribosome production in hepatocytes was estimated to be 15- to 50-fold greater than the consumption of ribosomes (Hadjiolov and Nikolaev, 1976). If rRNA is maximally loaded with RNA polymerase, as found by Spring et al. (1974), the control of rRNA processing is critical.

The rDNA is organized into transcriptional units, separated from adjacent regions by non-transcribed spacers (Fig. 1). The transcriptional units are highly reiterative, containing up to several hundred rDNA copies in a cluster. Units from different species vary in length, primarily due to the presence of transcribed spacers. Spacers are segments of gene elements which do not relate to any cytoplasmic products. The evolutionary trend is for transcribed units to increase in size, primarily due to an increase in length of the transcribed spacer units. The lengths of the 18S and 28S RNA sequences has been highly conserved during evolution, indicating it must be under stronger genetic pressure than the rest of the transcribed unit.

Sequences of nucleotides in rDNA are not random. Up to 78% of the base pairs are guanine and cytosine (Hadjiolov and Nikolaev, 1976). Strong conservation of 18S and 28S rDNA nucleotide sequences occurs throughout a wide range of eukaryotes (Evans, 1977).

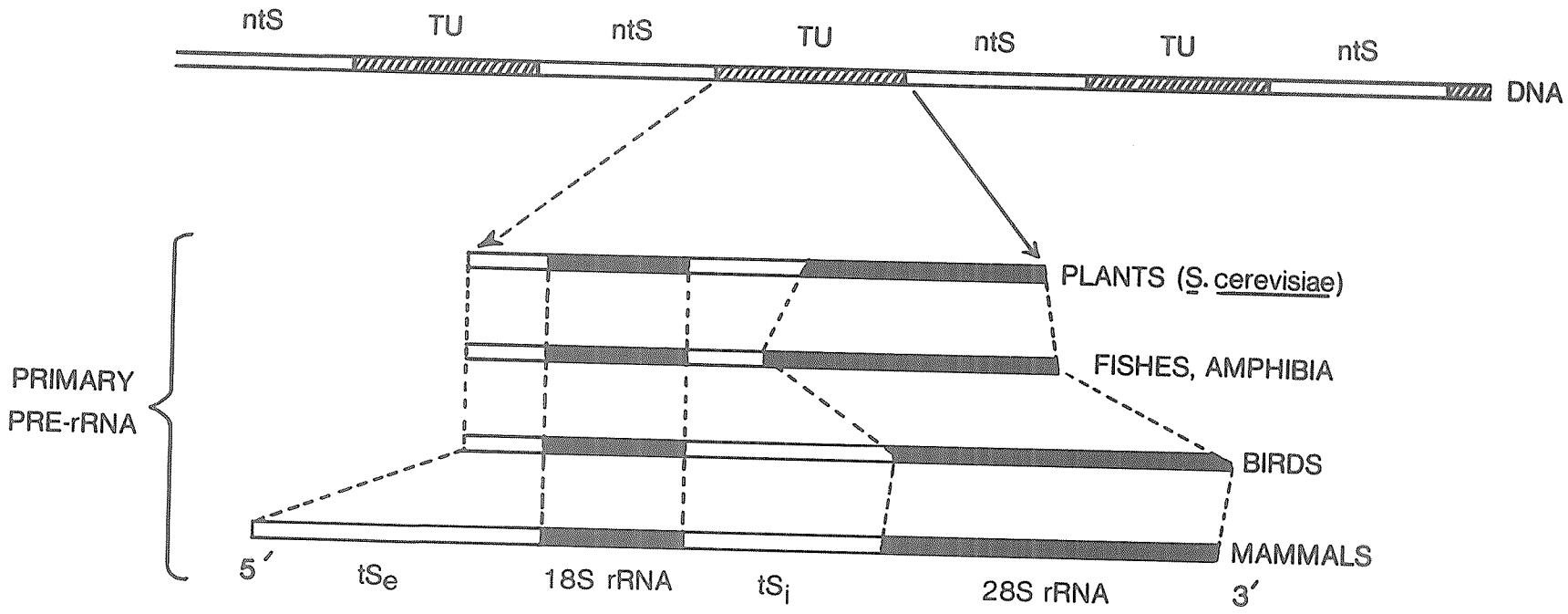


FIG.1 ARRANGEMENT OF SPECIFIC SEQUENCES IN rRNA GENES AND PRIMARY PRE-rRNA OF EUKARYOTES. TU= TRANSCRIPTIONAL UNITS ntS= NON TRANSCRIBED SPACER.

tSe = EXTERNAL TRANSCRIBED SPACER

tS_i = INTERNAL TRANSCRIBED SPACER

(FROM HADJIOLOV,1977)

Spacer sequences differ widely, even between closely related species. In Xenopus laevis and Xenopus mulleri hybrid cells, rRNA of X. laevis is preferentially transcribed (Honjo and Reeder, 1973). Their 18S and 28S sequences are indistinguishable by molecular hybridization. Spacer units are of the same length, but their G-C content has diverged enough to allow separation with cesium chloride (Miller and Hamkalo, 1972), and to be distinguished by molecular hybridization (Honjo and Reeder, 1973). Spacer sequences may play an important role in regulation of transcription or processing.

Silver nitrate is histochemically reduced to form black spheres at the NORs (AgNORs). All NORs do not react. In humans a characteristic number of AgNORs occur, with a mode of 8 per cell (Ray and Pearson, 1979). As RNase and DNase do not eliminate silver reduction, while pretreatment with trypsin or pronase does, it has been concluded that an acid protein is responsible for the silver nitrate reaction (Schwarzacher et al., 1978). Studies of human-mouse somatic cell hybrids showed that the silver reaction detects sites of rRNA activity, rather than the amount of rRNA or DNA (Miller et al., 1976 a & b).

C. Nucleolus

1. Introduction

The nucleolus is the established site of pre-rRNA synthesis and maturation. This involvement in the production of the protein synthesizing machinery of the cell gives the nucleolus a prominent position in cell metabolism.

Nucleolar structure and function is dynamic. It changes with cell cycle variations (Grant, 1972; Grummt et al., 1977), with diurnal rhythms (Glasser and Spelsberg, 1972), and with cell differentiation (Grummt, 1978). The size and activity levels of the nucleolus increase after stimulation with mitogens such as PHA (Schwarzacher et al., 1978), with hormones or serum, and after fusion of cells to form hybrid heterokaryons (Ringertz et al., 1971). A prominent nucleolus characterizes cells engaged in intensive protein synthesis.

Nucleolar hypertrophy is striking in most malignant and virus infected cells (Bernhard, 1966). The increase in size is usually accompanied by structural variations. Inhibition of rRNA synthesis with low doses of Actinomycin D (AMD), reduces the size of nucleolus and has a cytostatic effect on a variety of tumors. Control of nucleolar activity is central to a cell's ability to respond normally to physiological stimuli.

2. Structure and function

The nucleolus was discovered by Fontana in 1781 (Ghosh, 1976). Ruzicka (1891) noted silver impregnation in nucleoli. Association of the secondary constriction regions of the chromosome with the nucleolus was pointed out by Heitz (1931). He termed the regions sine acid thymonucleinico, or SAT regions. Estable and Sotelo (1951) described two components, a filamentous nucleonoma and a structureless pars amorpha, within the nucleolus. Lettré and Siebs (1954) reported the presence of functionally active DNA within the nucleolar filament.

With the advent of the electron microscope, morphological descriptions were refined. The nucleolus is known to be composed of three components: chromatin, the fibrillar component and the granular component (Fig. 2). Approximately 85 to 90% of nucleolar chromatin is located at the periphery of the nucleolus. The remaining 10 to 15% is located within the nucleolus. Only 0.2 to 1% of intranuclear DNA consists of rRNA genes.

Pulse labelling with ^{3}H-uridine and autoradiography demonstrate that the fibrillar component consists of 80S ribonucleoprotein (RNP), which contains 45S pre-rRNA (Karasaki, 1965). The granular component consists of RNP particles containing primarily 28S rRNA and is derived from the fibrillar component (Recher et al., 1971). The relative size of the fibrillar and granular areas is constant for a given cell type (Hardin et al., 1970), implying that maturation is relatively slow

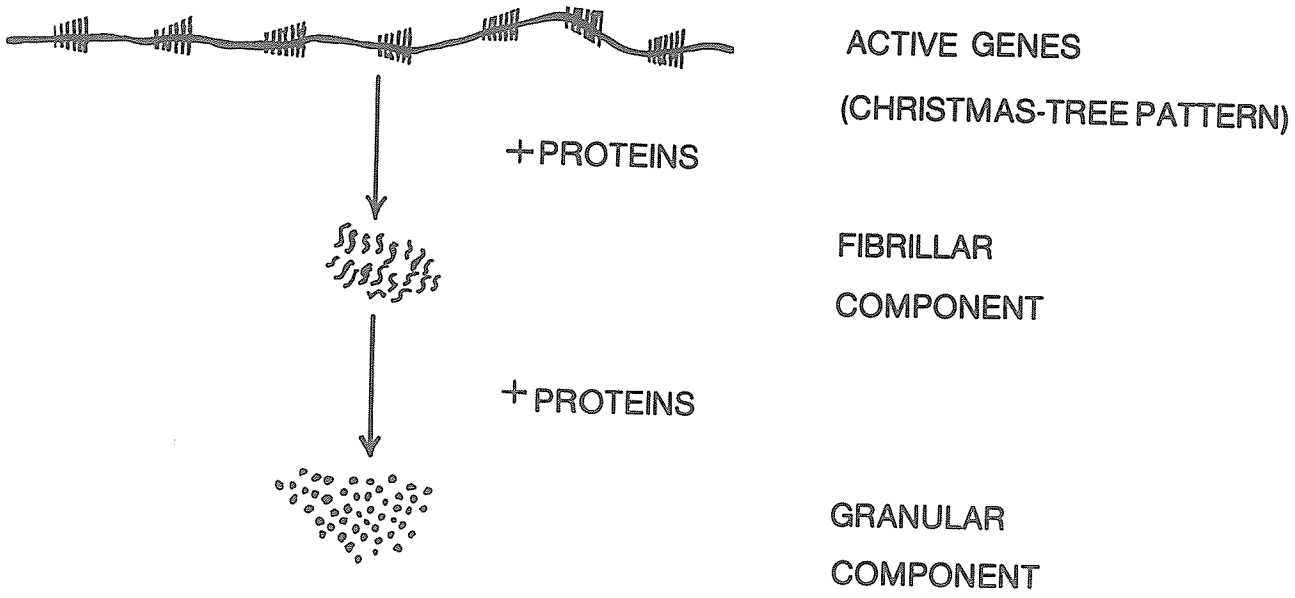
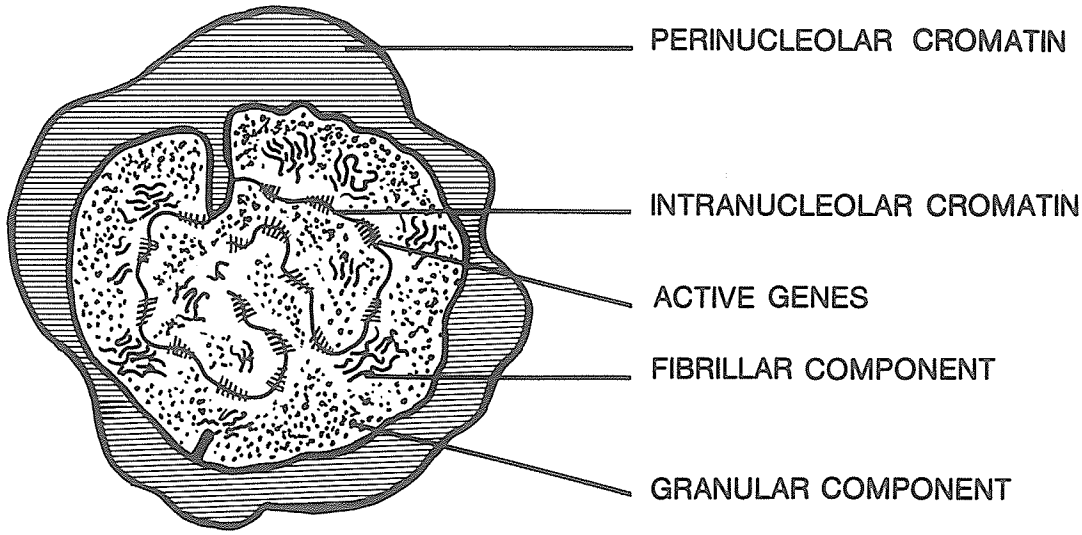


FIG.2 SCHEMATIC PRESENTATION OF NUCLEOLUS.
(FROM HADJIOLOV AND NIKOLAEV, 1976)



and subject to precise regulation.

Nucleoli appear during telophase and increase in size as the cell produces more RNA. Chromatin from the NORs uncoils and is dispersed within the nucleolus. During prophase the nucleolus disintegrates. If disintegration is late, there is insufficient time for complete condensation of chromatin and the NOR of the metaphase chromosome is usually large (Ghosh, 1976).

Solitary nucleolar DNA fibrils may be visualized after implementing the spreading techniques of Miller and Hamkalo (1972) (Fig.2). The axial fibril of DNA is coated at regular intervals with gradients of short to long matrix fibres, perpendicular to the axis. All units have the same polarity of gradients and are separated by matrix-free segments. This is the classic "Christmas-tree" pattern of active rRNA genes (Miller and Hamkalo, 1972). DNA intervals with matrix fibres are transcribed units and intervals without fibres are non-transcribed spacer units.

Transcribed rRNA units undergo a complex maturation process during which transcribed spacer units are eliminated and numerous RNA-protein interactions occur. Heavy rRNA, sedimenting at 45S, is transcribed at the NOR and interacts with proteins to form the fibrillar component of the nucleolus. This is subsequently degraded via intermediate steps to produce the granular component containing 28S and 18S rRNA.

3. Regulation of rRNA production and maturation

The capacity for rRNA production is genetically determined. It has been estimated that this capacity is 15- to 50-fold higher than the consumption of ribosomes (Hadjiolov and Nikolaev, 1976). Critical sites for control of rRNA production in the nucleolus are during transcription, maturation and transportation.

Transcription may be limited by the availability of purine nucleotides or by polymerase A concentrations or activity. A close parallel between the intracellular level of ATP and GTP and the amount of RNA polymerase active in transcription has been reported (Grummt, 1978). As ATP and GTP levels are related to many cellular reactions, the parallel may not be related to the availability of nucleotide pools.

Changes in nucleolar RNA synthesis were correlated with changes in RNA polymerase A activity during transcription (Cooke and Brown, 1973; Grummt, 1978). In contrast, Roeder et al. (1970) found no correlation between levels of enzyme activity and amount or type of RNA synthesis. Spring et al. (1974) estimated that rDNA is maximally loaded with RNA polymerase, suggesting that post-transcriptional control mechanisms are critical.

Maturation may be controlled at one or more of the RNA-protein interactions. In a given cell type one major pre-RNA maturation pathway occurs, but the RNA cleavage sites may be flexible, resulting in other

maturation pathways, involving different pre-rRNA molecules. PHA preferentially stimulates one of two maturation paths in lymphocytes (Purtell and Anthony, 1975). This channelling may be a critical site for qualitative and quantitative regulation of RNP.

Maturation pathways may be influenced by the secondary structure of RNP (Hadjiolov and Nikolaev, 1976). Secondary structure consists of numerous hairpin loops which correspond to G-C rick areas of the transcribed spacer units. Loops could physically direct protein interactions and, as even closely related species have great variability in spacer sequences and secondary structure, cause inter-species and possibly intraspecies variability in ribosome structure.

Inspite of the fact that equal amounts of 28S and 18S RNA are produced, the bulk of the granular component of the nucleolus is 28S rRNA. This discrepancy suggests either a great difference in maturation time and/or in speed of migration to the cytoplasm.

The migration of RNP from the nucleolus to the cytoplasm is poorly characterized, though the passage of RNP through the nuclear pore complex has been observed (Bernhard, 1971). In heterokaryons, rRNA passage was inhibited until a nucleolus was present and synthesizing RNA. Since the migration of both rRNA and tRNA to the cytoplasm was inhibited after irradiation of the nucleolus (Sidebottom and Harris, 1969), the nucleolus may control the passage of all RNA to the cyto-plasm.

The synthesis of DNA, often triggered by physiological stimuli, may be coordinated in the nucleolus. Blockage of rRNA synthesis with low doses of AMD, prohibit DNA synthesis by indirectly regulating DNA polymerase production (Cooper, 1972). The period of greatest sensitivity is early during activation, long before DNA polymerase synthesis begins.

D. Ribosomes

Ribosomes are large complex organelles, which probably change in shape and protein content at different stages of protein synthesis (Brimacombe, 1976). Each ribosome consists of a large and a small RNP subunit. In eukaryotes, the smaller subunit contains 20 to 30 proteins, dependent on the species, and a 18S rRNA molecule. Larger subunits contain 30 to 40 proteins, one 28S RNA, and one 5S RNA molecule. Few of the proteins are present in multiple copies.

In every cell, ribosomes translate mRNA to produce proteins. The concept that an excess of stable ribosomes is involved in translating a fluctuating level of mRNA is oversimplified. In eukaryotes, the control of ribosome biogenesis is more rapid and versatile than mRNA or tRNA formation. Serum growth stimulation of contact inhibited mouse fibroblast resulted, within 2 hours, in a 2-to 3-fold increase in protein synthesis and formation of new ribosomes, while mRNA synthesis increased only 20 to 30% (Rudland et al., 1975). During metaphase, protein synthesis decreases to 20-40% that of interphase. This cannot be explained by a decrease in mRNA, but is associated with a rapid disappearance of polyribosomes. Synthesis of new RNA is not necessary for protein synthesis to increase in interphase (Hodge et al., 1969). Changes in cell biosynthetic pathways often result in old ribosomes being degraded and new ribosomes being formed (Grasso and Woodard, 1966; Cocucci and Sussman, 1970). This suggests that

ribosomes are heterogeneous and that special classes of ribosomes may fulfill different functions.

Crick (1968) suggested that the primitive apparatus for protein synthesis might have originally consisted only of RNA, and that the addition of proteins during evolution refined the machinery. No essential differences have been found in the basic functions of ribosomes, however the greater complexity of architecture of eukaryote ribosomes supports the idea that the realization of genetic information may be at the ribosomal level.

Existing ribosomes respond to requirements for increased protein synthesis by increasing their efficiency and by decreasing ribosome degradation. Approximately 70% of resting lymphocytes are not engaged with mRNA in protein synthesis. After PHA addition, only 30% are not engaged (Cooper, 1972). The degradation of significant portions of rRNA is reversed after PHA stimulation of normal lymphocytes (Cooper and Gibson, 1971). Wastage of rRNA may be a mechanism by which resting cells may quickly shift to rapid growth.

The increased utilization of pre-existing ribosomes is inadequate for normal cell growth and mitosis. Blockage of rRNA synthesis prohibits DNA synthesis and thus cell division.

E. Silver Nitrate Reaction

1. History

The reaction of the nucleolus to silver nitrate was observed almost 100 years ago by Ruzika (1891). Cajal (1910) (Schwarzacher et al., 1978) described the silver stained granula of the nucleolus. Estable and Sotelo (1951) described a nucleonema of silver positive fibrils. Lettré and Siebs (1954) and Lettré et al. (1966) described this network as silver positive granules around a backbone of the chromosomal NORs. This view was confirmed with electron microscope observations (Schwarzacher et al., 1978). Cellular reactions with silver nitrate have recently gained interest because it was found that NORs react (Goodpasture and Bloom, 1975).

2. Location and molecular basis of reaction

Colourless silver nitrate is differentially reduced at the chromosomal locations of rDNA to form a black precipitate. In maximally activated cells of a given type, the degree of silver nitrate precipitation is characteristic of an individual chromosome, and this characteristic is an inherited property (Markovic et al., 1978; Mikelsaar et al., 1977). Silver nitrate reactivity is associated with actively transcribing rDNA cistrons in the fibrillar region of the nucleolus. As it is not located in the granular component, the reactive