INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or “target” for pages apparently lacking from the document photographed is “Missing Page(s)”. If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in “sectioning” the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from “photographs” if essential to the understanding of the dissertation. Silver prints of “photographs” may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106
RACEVSKIS, Janis, 1943-  
CYTOPLASMIC CONTROL OF RIBOSOME BIOGENESIS.  
The Ohio State University, Ph.D., 1974  
Chemistry, biological  

University Microfilms, A XEROX Company, Ann Arbor, Michigan
CYTOPLASMIC CONTROL OF RIBOSOME BIOGENESIS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Janis Racevskis, B.S.

The Ohio State University

1974

Reading Committee:
Dr. T.E. Webb
Dr. F.A. Kruger
Dr. J.A. Merola
Dr. J.S. Rieske

Approved By

Advisor

Department of Physiological Chemistry
ACKNOWLEDGMENTS

I wish to express my gratitude to my advisor, Dr. T. E. Webb, whose guidance made this work possible.

I am also deeply indebted to Dr. L. C. Yu for her collaboration and assistance in the initial stages of this investigation.

I thank Dr. D. J. McNamara for his assistance in the in vitro synthesis studies and for many helpful discussions.

I am grateful to Dr. D.E. Schumm for performing the competition hybridization studies and also for many helpful discussions.

Dr. Harold P. Morris of Howard University supplied us with rats bearing Morris hepatomas 5123 D and 7800.

Thanks are due to Ethel-Marie LeVasseur for her skillful typing of this Dissertation.

I am grateful to the Procter and Gamble Company for awarding me a fellowship.
VITA

June 17, 1943 . . . . Born - Latvia

1966 . . . . . . . . B.S., Chemistry, City College of New York

1967 - 1968 . . . . Lab. Technician, Montefiore Hospital, Bronx, N.Y.

1968 - 1970 . . . . Research Assistant, New York University Medical School, Department of Psychiatry, New York, N.Y.

1968 - 1970 . . . . Master's program, Biochemistry, Hunter College, City University of New York, N.Y.

1970 - 1974 . . . . Doctoral program, Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio.

PUBLICATIONS


PUBLICATIONS (CONT'D)


FIELDS OF STUDY

Major Field: Biochemistry

Controls of RNA synthesis, processing and transport in normal and neoplastic mammalian cells.

Professor Thomas E. Webb
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>I. INTRODUCTION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Discovery of Ribosomes and Elucidation of their Cellular Function</td>
<td>1</td>
</tr>
<tr>
<td>Ribosome Structure</td>
<td>5</td>
</tr>
<tr>
<td>Ribosomal RNA Genes in the Nucleolus of Eukaryotes</td>
<td>7</td>
</tr>
<tr>
<td>1. Amplification of Ribosomal RNA Genes</td>
<td>10</td>
</tr>
<tr>
<td>Control of Ribosomal RNA Synthesis in Procaryotes</td>
<td>12</td>
</tr>
<tr>
<td>DNA Dependent RNA Polymerases</td>
<td>15</td>
</tr>
<tr>
<td>Processing of Ribosomal RNA</td>
<td>19</td>
</tr>
<tr>
<td>2. Methylation of Ribosomal RNA</td>
<td>23</td>
</tr>
<tr>
<td>Ribosomal Proteins</td>
<td>25</td>
</tr>
<tr>
<td>The Nucleases</td>
<td>27</td>
</tr>
<tr>
<td>3. 5S Ribosomal RNA</td>
<td>30</td>
</tr>
<tr>
<td>Nuclear rRNA Degradation, the Wastage Phenomenon</td>
<td>31</td>
</tr>
<tr>
<td>The Ribosome Cycle</td>
<td>36</td>
</tr>
<tr>
<td>Size of Monomer Pool and Ribosome Production</td>
<td>38</td>
</tr>
<tr>
<td>Cytoplasmic Turnover of Ribosomes</td>
<td>39</td>
</tr>
<tr>
<td>Nucleo-cytoplasmic Transport</td>
<td>42</td>
</tr>
<tr>
<td>The Nuclear Membrane</td>
<td>43</td>
</tr>
<tr>
<td>Nucleo-cytoplasmic Controls</td>
<td>45</td>
</tr>
<tr>
<td>The Cell-free System</td>
<td>46</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (CONT'D)

II. EXPERIMENTAL PROCEDURES

<table>
<thead>
<tr>
<th>Material/Method</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials</td>
<td>47</td>
</tr>
<tr>
<td>Animals</td>
<td>48</td>
</tr>
<tr>
<td>Methods</td>
<td></td>
</tr>
<tr>
<td>Preparation of Ribosomes</td>
<td>49</td>
</tr>
<tr>
<td>Preparation of the Cytosol Fraction</td>
<td>50</td>
</tr>
<tr>
<td>Preparation of Ribosomal Proteins</td>
<td>50</td>
</tr>
<tr>
<td>Determination of Protein Concentration</td>
<td>51</td>
</tr>
<tr>
<td>Isolation of Nuclei</td>
<td>51</td>
</tr>
<tr>
<td>Fractionation of Cytosol</td>
<td></td>
</tr>
<tr>
<td>Ammonium Sulfate Precipitation</td>
<td>52</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>52</td>
</tr>
<tr>
<td>Diaflow Filtration</td>
<td>53</td>
</tr>
<tr>
<td>The Cell-free System</td>
<td>53</td>
</tr>
<tr>
<td>Detection of Released RNA</td>
<td>54</td>
</tr>
<tr>
<td>Extraction of RNA</td>
<td>54</td>
</tr>
<tr>
<td>Sucrose Density Gradients</td>
<td>55</td>
</tr>
<tr>
<td>Competition - Hybridization</td>
<td>56</td>
</tr>
<tr>
<td>Preparation of DNA</td>
<td>57</td>
</tr>
<tr>
<td>Preparation of DNA bound to Filters</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>vi</td>
</tr>
</tbody>
</table>
### TABLE OF CONTENTS (CONT'D)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>II. EXPERIMENTAL PROCEDURES</strong></td>
<td></td>
</tr>
<tr>
<td>Specific Radioactivity of Soluble Proteins</td>
<td>58</td>
</tr>
<tr>
<td>Analysis of the Size Distribution of Ribosomal Components</td>
<td>59</td>
</tr>
<tr>
<td>In-vitro Synthesis of RNA</td>
<td>60</td>
</tr>
<tr>
<td>Polyacrylamide Disc-gel Electrophoresis</td>
<td>60</td>
</tr>
<tr>
<td><strong>III. RESULTS</strong></td>
<td></td>
</tr>
<tr>
<td>Ribosomal RNA release from Isolated Nuclei</td>
<td>62</td>
</tr>
<tr>
<td>Transport of Ribosomal Subunits</td>
<td>67</td>
</tr>
<tr>
<td>Contribution of Extranuclear Ribosomes</td>
<td>68</td>
</tr>
<tr>
<td>Energy Requirement</td>
<td>72</td>
</tr>
<tr>
<td>Nature of Material Transported</td>
<td>80</td>
</tr>
<tr>
<td>Competition-hybridization Studies</td>
<td>83</td>
</tr>
<tr>
<td>Differences in the Profiles of RNA Transformed by Resting and Regenerating Rat Liver Nuclei</td>
<td>86</td>
</tr>
<tr>
<td>Requirement for Nondialyzable Factors and Polyamines</td>
<td>87</td>
</tr>
<tr>
<td>The Nuclear Stabilizing Effect of Cytosol</td>
<td>92</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (CONT’D)

III. RESULTS

<table>
<thead>
<tr>
<th>Characterization of the Cytosol</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration Dependence</td>
<td>96</td>
</tr>
<tr>
<td>DEAE-cellulose Fractionation of Cytosol</td>
<td>98</td>
</tr>
<tr>
<td>Ammonium Sulfate Fractionation</td>
<td>100</td>
</tr>
<tr>
<td>Diaflow Filtration</td>
<td>100</td>
</tr>
<tr>
<td>Protein Nature of the Cytoplasmic Factors</td>
<td>102</td>
</tr>
<tr>
<td>Dependence of In-vitro Nuclear RNA Processing on Cytosol and Energy</td>
<td>104</td>
</tr>
<tr>
<td>The Effect of Camptothecin on rRNA Processing and Transport</td>
<td>107</td>
</tr>
<tr>
<td>Studies on Pactamycin Inhibition of Protein Synthesis</td>
<td>111</td>
</tr>
<tr>
<td>In-vitro Synthesis</td>
<td>112</td>
</tr>
<tr>
<td>Cytoplasmic Turnover of Ribosomal RNA</td>
<td>117</td>
</tr>
</tbody>
</table>

IV. DISCUSSION

<table>
<thead>
<tr>
<th>List of References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Page</td>
</tr>
<tr>
<td>121</td>
</tr>
<tr>
<td>127</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. COMPOSITION OF THE COMPLETE IN VITRO INCUBATION MEDIUM</td>
<td>64</td>
</tr>
<tr>
<td>2. EFFECT OF ACTINOMYCIN D AND RIBONUCLEASE INHIBITORS ON RNA RELEASE</td>
<td>66</td>
</tr>
<tr>
<td>3. SPECIFIC ACTIVITIES OF RIBOSOMAL RNA ISOLATED FROM THE CYTOPLASM OR RELEASED FROM NUCLEI OF LABELED LIVER CELLS</td>
<td>71</td>
</tr>
<tr>
<td>4. ENERGY DEPENDENCE OF THE RNA RELEASE</td>
<td>79</td>
</tr>
<tr>
<td>5. REQUIREMENTS FOR NON-DIALYZABLE FACTORS IN THE CYTOSOL AND POLYAMINES</td>
<td>91</td>
</tr>
<tr>
<td>6. AMMONIUM SULFATE SALT FRACTIONATION OF NORMAL LIVER CYTOSOL</td>
<td>101</td>
</tr>
<tr>
<td>7. HEAT LABILITY OF TRANSPORT FACTORS IN THE CYTOSOL</td>
<td>103</td>
</tr>
<tr>
<td>8. COMPOSITION OF THE IN-VITRO SYNTHESIZING MEDIUM</td>
<td>115</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Radioactivity and Absorbancy Profiles of Nucleoprotein Components released to the complete Medium at 36° C during a 12 min. Incubation</td>
<td>69</td>
</tr>
<tr>
<td>2. Kinetics of the Release of Labeled RNA from Prelabeled Nuclei in the Complete Medium at 0° or 36° C</td>
<td>74</td>
</tr>
<tr>
<td>3. Effect of Preincubating Regenerating Liver Nuclei in the Absence of an Energy Source on Subsequent RNA Transport</td>
<td>77</td>
</tr>
<tr>
<td>4. Radioactivity Profiles of the RNA Released to the Complete Medium from Nuclei Pre-labeled 2 hours In-Vivo</td>
<td>81</td>
</tr>
<tr>
<td>5. The Distribution of Radioactivity in the Residual RNA in Nuclei Following Incubation for 15 min. at 36° or 0° C</td>
<td>82</td>
</tr>
<tr>
<td>6. Radioactivity Profiles of RNA Released to the Complete Medium from Regenerating Liver Nuclei Prelabeled 2 hours In-Vivo</td>
<td>88</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>7.</td>
<td>Radioactivity Profiles of RNA Released to the Complete Medium from Resting Liver Nuclei Pre-labeled 2 hours <em>In-Vivo</em></td>
</tr>
<tr>
<td>8.</td>
<td>The Time Course of Release of Labeled RNA from Nuclei of Normal Liver, to Medium Containing zero, 2.5 or 19.6 mg of Cytosol proteins per ml of Reaction Mixture</td>
</tr>
<tr>
<td>9.</td>
<td>Time Course of the Release of Labeled RNA from Resting Liver Nuclei to Medium Containing Freshly Prepared or Stored Cytosol</td>
</tr>
<tr>
<td>10.</td>
<td>The Release of Labeled RNA from Prelabeled Nuclei of Normal Liver as a Function of the Concentration of Homologous Cytosol</td>
</tr>
<tr>
<td>11.</td>
<td>Relative Size Distribution of the RNA Released from Regenerating Liver Nuclei to Medium Containing the Cytosol Fraction which Adsorb and which do not Adsorb to DEAE-cellulose</td>
</tr>
<tr>
<td>12.</td>
<td>Radioactivity Profiles of the Residual RNA in the Nuclei following Incubation for 10 min. with and without ATP</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>13.</td>
<td>Radioactivity Profiles of the Residual RNA in the Nuclei following Incubation for 10 min. in varying concentrations of Cytosol Protein</td>
</tr>
<tr>
<td>14.</td>
<td>Radioactivity Profiles of the Residual RNA in the Nuclei of a camptothecin treated rat, following Incubation</td>
</tr>
<tr>
<td>15.</td>
<td>Size Distribution Profiles of the Ribosomal Components in the Supernatant prepared from the Liver of a Pactamycin treated rat</td>
</tr>
<tr>
<td>16.</td>
<td>Radioactivity Profiles of Newly Synthesized RNA extracted from Nuclei Incubated for 30 min. in different Synthesizing Mediums</td>
</tr>
<tr>
<td>17.</td>
<td>Decay of Specific Radioactivity of Resting Liver Ribosomal RNA</td>
</tr>
<tr>
<td>18.</td>
<td>Decay of Specific Radioactivity of Ribosomal RNA from Morris Hepatoma 5123 D Tumors</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.V.</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>S</td>
<td>Svedberg unit</td>
</tr>
<tr>
<td>A</td>
<td>Angstrom unit</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>c</td>
<td>curie</td>
</tr>
<tr>
<td>uc</td>
<td>microcurie</td>
</tr>
<tr>
<td>Kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>gm</td>
<td>gram</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ug</td>
<td>microgram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CTP</td>
<td>cytosine triphosphate</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS (CONT'D)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>S 2</td>
<td>post-mitochondrial supernatant</td>
</tr>
<tr>
<td>S 3</td>
<td>post-microsomal supernatant</td>
</tr>
<tr>
<td>x g</td>
<td>times gravity</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloro-acetic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>S.A.</td>
<td>specific activity</td>
</tr>
<tr>
<td>R.T.</td>
<td>room temperature</td>
</tr>
<tr>
<td>a.v.</td>
<td>average velocity</td>
</tr>
<tr>
<td>e.u.</td>
<td>enzyme units</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium chloride-sodium citrate buffer</td>
</tr>
<tr>
<td>TKM</td>
<td>tris, KCl, MgCl₂ buffer</td>
</tr>
</tbody>
</table>
The Discovery of Ribosomes and Elucidation of Their Cellular Function

The first hint for the role of ribonucleic acids in cells, came from the histochemical studies over three decades ago, of Brachet (1), and the ultraviolet spectrophotometric observations of Caspersson (2). Using these independent techniques, both researchers surveyed a number of cell types for RNA, and found that a high concentration of "pentose nucleic acid" was characteristic of rapidly growing or secretory cells which had a high rate of protein synthesis.

Isolated ribosomes were probably first observed by the virologist Wyckoff in 1937 (3) who was studying extracts of virus infected tissues by ultracentrifugation and ultraviolet absorption optics. In a preparation derived from extracts of equine encephalomyelitis virus-infected tissues, he observed that in addition to a sharp 245-S boundary which was assumed to represent the virus, there was another U.V. absorbing boundary moving about one-third as fast. We now
know that the latter components represented eukaryotic ribosomes sedimenting at their typical coefficient of about 80S.

Since ribosomes are smaller than the limits of resolution of the light microscope, they were not visualized until the advent of electron microscopy. Pigmented heavy particles from *Streptococcus pyogenes*, with diameters around 210Å were described by Sevag et al. in 1941 (4). Later, the presence of rounded particles of approximately 180 Å diameter, in electron micrographs of extracts of human and chick embryonic brain, were reported by Taylor et al. (5).

Among the many early tissue fractionation studies reported, the ones carried out by Claude (6) are particularly outstanding. Working with guinea pig liver homogenates, he separated them into nuclear, mitochondrial, and microsomal fractions. He coined the term microsome, and separated and identified microsomal RNA. He described microsomes as "complex structures consisting mostly of phospholipids and nucleoproteins of the ribose type."

The ubiquitous nature of ribosomes became apparent as they continued to be identified in an increasing number of various tissues. Progress in the study of mammalian microsomes and ribosomes was greatly accelerated in 1948, with the description by Hogeboom et al. (7) of a method for fractionating rat liver homogenates in 0.88 or 0.25 M
sucrose. Unlike the salt media, which was widely employed at the time, sucrose medium did not cause the aggregation of cellular components, so that more clear-cut separations of the subcellular components could be achieved.

Isotopic tracers, especially the radioactive ones which became available after World War II, have proven to be the most valuable tools available to the biochemist. Their use made possible the elucidation of cellular processes which to that time seemed impossible. In vivo incorporation studies using glycine labeled with the stable isotope $^{15}\text{N}$, (8) showed that the liver microsomes from newly hatched chicks were more highly labeled than any other fraction. Similar findings were subsequently reported for subcellular fractions of rat liver in cell-free systems in which the much more sensitive $^{14}\text{C}$ labeled amino acid labeling technique was used (9). When rat liver microsomes prelabeled in vivo were disintegrated by the anionic detergent sodium deoxycholate (10), the deoxycholate insoluble fraction, shown by ultracentrifugal analysis and electron microscopy to consist mainly of ribosomes, contained most of the radioactivity.

The existence and involvement of multiple ribosomal structures in protein biosynthesis was inferred from ultracentrifugal studies of rat liver microsomes (11), which showed a number of discrete boundaries moving ahead of the
single (80S) ribosome. Direct confirmation of the existence of multiple ribosome structures was provided by electron micrographs of whole cells (12) which depicted linear clusters of ribosomes held together by a thread-like strand, now known to be messenger RNA. This linear cluster of ribosomes held together by a messenger RNA molecule, is the active unit of protein biosynthesis, and is called a polyribosome. Evidence for this role of the polyribosome came from studies on the rabbit reticulocyte system (13), in which it was discovered that incorporation of radioactively labeled leucine occurred only in association with polyribosomes, whereas single ribosomes from the same population were inactive.

Despite the fact that our understanding of the function of the ribosome is barely ten years old, a great deal has been learned about the structure of ribosomes, their synthesis and their role in the protein synthesizing machinery of the cell; yet a great deal more remains to be worked out. The aspect of ribosomes which at present is least understood, namely the area of controls of ribosome production, is the subject of the work reported in this thesis. It is known that ribosome production fluctuates in response to the needs of the cell and that a certain level must be met before mitosis can occur. In order to achieve an understanding of the controls governing proliferating,
and non-proliferating cells, the mechanisms regulating ribosome production must be understood, for ribosome biogenesis and cell proliferation are highly integrated processes (14).

Subsequent sections of the "Introduction" will deal with all aspects of ribosome production, especially in mammalian cells, with special emphasis on those aspects involving regulatory mechanisms.

Ribosome Structure

Ribosomes occur in all living cells and consist of two subunits, a large and a small one, both containing RNA and protein. The complete ribosome, the subunits, and the RNA molecules within the subunits, are often operationally defined by their sedimentation coefficient or $S$ values.

The sedimentation coefficients reported for ribosomes from various species of microorganisms, plants, and animals, indicate that ribosomes fall into two distinct classes (15). Ribosomes from bacteria, blue-green algae, mitochondria, and chloroplasts have an $S$ value of about 70, whereas cytoplasmic ribosomes from green plants, animals, yeast, and some fungi have a sedimentation coefficient of approximately 80S. The $S$ values in the 70S and 80S ribosomal classes show a rather wide range, i.e., from 67S to 73S and from 77S to 81S, respectively (16).

The larger subunit of a mammalian ribosome with an $S$
value of 60, contains three RNA molecules with the following specifications: a 28S species (M.W. \(1.8 \times 10^6\) Daltons) of about 5000 nucleotides; a 5S species (\(3.2 \times 10^4\) Daltons) of 120 nucleotides; and a 7S of some 150 nucleotides (\(4 \times 10^4\) Daltons) (17). The smaller subunit, with a sedimentation coefficient of 40S, contains but one RNA molecule, an 18S species (molecular weight \(0.7 \times 10^6\) Daltons) of about 2,000 nucleotides.

Associated with the RNA molecules, forming the complete ribosomal subunit, are a large number of proteins. Some 70 different protein species have been resolved from rat liver ribosomes by means of two-dimensional polyacrylamide gel electrophoresis (18); about 40 of these are from the larger and 30 from the smaller subunit. No agreement has yet been reached as to the exact number of proteins in the mammalian ribosome, because current isolation techniques do not rule out contamination of the ribosomes by non-ribosomal proteins, or the loss of loosely bound true ribosomal proteins. Furthermore, as will be discussed in a subsequent section, ribosomes and ribosomal subunits cycle to and from the polyribosomes and there is the association and dissociation of certain protein factors at different stages of this cycle.
DNA:RNA hybridization studies reveal that the genes coding for ribosomal RNA (rRNA), with the exception of the 5S rRNA, are situated on the nucleolar organizer regions of the chromosomes; the nucleoli are formed by segments of DNA containing rRNA genes associated with specific proteins (19, 20). The 5S RNA species alone is coded for by extranucleolar genes of even greater multiplicity than those for 7, 18 and 28S rRNA (21).

The number of nucleoli per somatic cell varies greatly even within a species, but generally there are between one and ten (22). The number per somatic cell is probably the same as the number of nucleolar organizer regions within the genome. For reasons unknown, the nucleolar organizers in the human cell are located on five pairs of chromosomes instead of a single pair as in all other species studied to date (23, 24). RNA: DNA hybridization studies indicate that ribosomal RNA genes (rDNA) make up only a small fraction of the total DNA of the nucleolar organizer, i.e., 0.075% in mouse tissues (25), and 0.20% in rat tissues (26). The lower percentage of rDNA in the mouse cell might be due to the fact that much of the nucleolar DNA from mouse liver consists of light satellite DNA (27), whereas none is found in rat liver nucleoli.
The rRNA genes within the nucleolar organizer are present in tandem duplicate form, and in all eukaryotes examined, at least 100 to 1000 copies are present per diploid genome. Some estimates of the number of rRNA genes per diploid cell, obtained from saturation hybridization experiments between purified rRNA and cellular DNA, are as follows: rat liver - 330 (39), Neurospora - 100 (29), human spleen - 320 (30), He La cell (heteroploid) - 1100 (31), and Xenopus Laevis - 900 (32).

The number of extranucleolar genes coding for the 5S rRNA is one to several orders of magnitude higher than that for the high molecular weight rRNA (21). Reasons for this nonequivalence is at present open to speculation. Perhaps this difference in quantity, and location in the genome could be related to some other function of the 5S species besides or in addition to that of a structural RNA component of ribosomes, i.e., it may have a regulatory function.

Tandem nucleolar rRNA genes have been identified in electron micrographs (33), and it has been observed that they are interspersed with long spacer regions of unknown function (34). Insofar as the rDNA makes up but a small part of the nucleolus organizer region (see above), it is of interest that recent genetic studies on Drosophila (35) indicate that genes for ribosomal proteins are found clustered in the vicinity of the rDNA.
The multiplicity of rRNA genes may satisfy quantitatively a template requirement sufficient to maintain the rate of rRNA synthesis required for cell growth or differentiation (36). The minimum number of ribosomal genes required to maintain the growth of He La cells has been calculated as follows. Based on an average rRNA content of 24 pg per cell (19), an estimate of about 1100 45S genes per cell (31), a transcription time of 2.3 minutes for the synthesis of one 45S molecule (37), and a maximum number of 100 RNA polymerase molecules transcribing simultaneously on each gene, (as suggested by the electron micrographs of the amphibian oocyte rRNA genes (38) ) it can be estimated that it would be necessary for a minimum 100 ribosomal RNA genes to be transcribed continuously at a maximum rate during the 24 hour doubling time to produce the average cell complement of rRNA.

Alterations in rRNA genes are implied by studies which show differences in base sequences of mature rRNA of tumor and rat liver cells (39, 40). Tumor and fetal liver cells of the mouse contain fewer nucleoli than do adult mouse liver cells (41). The reduction in the number of nucleoli in the tumor cells might be due to fusion of chromosomes containing nucleolar organizer (25), for even though the individual nucleoli have an increased complement of rDNA, the total nuclear rDNA remains constant.
A loss or rRNA genes (42) has been implicated in the reduction of function in non-replenishing tissues during aging. The studies showed a selective loss of genes coding for rRNA in the DNA from brains of aging beagles. Johnson and Strehler postulated that this loss occurred as a result of the natural process of repair of single strand scissions in the DNA and that rRNA genes were particularly susceptible to inaccurate repair because of their ability to form stable intrastrand duplexes (hairpin loops), which result in improper positioning of complementary bases.

The DNA which codes for rRNA has a higher specific gravity and melting point than the average DNA of a cell due to its higher G-C content, which makes it separable from the bulk of the DNA by physical means such as equilibrium centrifugation in CsCl and differential melting or hybridization (43). Another manifestation of this unique base composition is the very high affinity of the antibiotic Actinomycin D for rDNA (44); low dosages of actinomycin D have been used extensively to selectively inhibit the transcription of rDNA, while permitting the transcription of mRNA.

Amplification of Ribosomal RNA Genes

One of the most dramatic controls over ribosome biosynthesis is observed during amphibian oogenesis. In developing amphibian oocytes, massive ribosomal RNA synthesis
takes place in preparation for the needs of early embryo development. In the frog, for example, all the ribosomes present up to the stage of gastrulation are synthesized before fertilization in the oocytes (45).

It can be seen morphologically that the oocytes contain large multiple nucleoli (46, 47), that rapidly incorporate isotopes into ribosomal precursor RNA and eventually into ribosomes. These multiple nucleoli, in contrast to the two nucleoli of somatic cells, are located in the nucleoplasm unattached to the chromosomes (48, 49).

It has been demonstrated by hybridization techniques that the oocytes contain a vastly increased amount of ribosomal DNA. In the case of Xenopus laevis oocytes (50, 51), for example, there is about a thousandfold amplification of the ribosomal RNA genes. The diploid Xenopus somatic cell contains about 900 copies of the rRNA genes (32, 52).

Thus the demand for more ribosome formation during amphibian oogenesis is met by a well-controlled and selective DNA synthesis which provides additional templates for rRNA synthesis. After the oocytes reach the stage of mature eggs, ribosome formation ceases and there is no formation of 45S precursor RNA. This condition exists even after fertilization until the gastrula stage (45, 53). These observations seem to indicate a complete repression of rRNA genes.
The mechanism of this selective gene amplification has been intensively studied and the most convincing mechanistic model proposed to date is that of the rolling circle intermediate (54). Differential amplification of the genes for rRNA has also been demonstrated in a variety of other amphibian and insect organisms (48, 55).

One reason why so much interest has been shown in the rRNA gene amplification phenomenon is the possibility that a similar phenomenon might be operating during the differentiation of cells of higher organisms to produce large quantities of a single or a few types of proteins. So far, however, (56) this expectation has not been born out.

In conclusion, the selective amplification of rRNA genes and their subsequent repression or degradation seems to be a mechanism of rRNA production control at the transcriptional and DNA replicative level, typical of oocytes and embryos possessing small numbers of chromosomes.

Control of Ribosomal RNA Synthesis in Procaryotes

When E. Coli is deprived of a required amino acid, there is an abrupt cessation of stable RNA (mainly ribosomal RNA) accumulation (57). This phenomenon is known as the "stringent RNA control response." The actual control mechanism that is triggered by amino-acid starvation in E. coli is amenable to study because it is abolished by mutation of a single locus, the "rel" gene (58).
A possible clue as to the identity of modulators of this response comes from observations that normal rel+ (stringent) but not mutant rel- (relaxed) strains of E. coli accumulate one or sometimes two unusual guanine nucleotides simultaneously with the onset of the stringent response (59). These nucleotides have been identified as guanosine 5' - diphosphate 2' or 3' diphosphate (ppGpp) and the analogous guanosine pentaphosphate pppGpp, referred to as magic spots I and II respectively in some of the earlier literature (60).

The actual trigger for the stringent response appears to be depletion of one or more of the aminoacyl-tRNA pools, rather than the intracellular concentration of the amino acids per se, for if one of the aminoacyl-tRNA synthetases is inactivated, the stringent response is elicited, even in the presence of abundant free amino acids (61). Further studies on this control mechanism showed that the signal for making the guanine nucleotides is the presence of an uncharged tRNA in the ribosomal acceptor site (62), and that the synthesis and accumulation of these compounds is stimulated by a ribosome bound factor specified by the "normal," or "rel" allele (63). The guanine nucleotides are not accumulated if the ribosomes are actively engaged in protein synthesis (62).

By the use of in-vitro synthesizing systems, it has been shown that ppGpp inhibits ribosomal RNA synthesis.
Specifically, it preferentially inhibits \( \psi \) (psi) factor stimulated ribosomal RNA synthesis (64). Psi (65) is a protein which \textit{in-vitro} can direct RNA polymerase to transcribe the genes of \textit{E. coli} which code for ribosomal RNA. Psi acts on the complete RNA polymerase enzyme (core enzyme and sigma factor) and it is this interaction that seems to be affected by ppGpp (66). This control of stable RNA synthesis by psi factor and by ppGpp, a metabolic product of the ribosome, demonstrates the coupling between protein synthesis and the transcription of RNA species which will become stable parts of the protein synthesizing machinery of the cell. Under conditions other than amino acid starvation, which also depress RNA synthesis, there is seen a similar negative correlation between ppGpp levels and stable RNA synthesis (67). The accumulation of ppGpp in response to amino acid starvation is seen in such divergent procaryotic organisms as \textit{Bacillus subtilis} and \textit{E. Coli} (68).

Attempts to detect these regulatory guanosine nucleotides in various eukaryotic cell cultures such as: 3T3 cells (69), \textit{Neurospora crassa} (70), \textit{Ankistrodesmus braunii} (71), undergoing responses analogous to the \textit{E. coli} stringent response, have proven negative. Thus the unusual guanosine nucleotides do \textbf{not} appear to be involved in the regulation of ribosome formation in eukaryocytes.
DNA Dependent RNA Polymerases

Bacterial DNA-dependent RNA polymerase is easily solubilized and purified from bacterial sources, and for this reason its structure and role in the regulation of transcription is quite well understood (72). Unlike the prokaryotic RNA polymerase, the eukaryotic enzyme is tightly bound to a complex consisting of DNA, histones, acidic nuclear proteins, non-histone protein and RNA. Consequently, until quite recently, studies on RNA synthesis in the mammalian cell have been confined to the use of whole nuclei or an "aggregate enzyme", which is a crude, insoluble preparation obtained from nuclei by precipitation with KCl (73).

Several investigators were however, successful in obtaining RNA polymerase in good yields with high degrees of purity by extraction with high ionic strength buffers (74). Chromatography of the solubilized enzyme on DEAE-Sephadex revealed multiple forms of RNA polymerase (75). This multiplicity of nuclear DNA-dependent RNA polymerases has been observed in all eukaryote cells studied, but there is still some disagreement as to the exact number and their localization. Confusion also exists about the nomenclature of the various RNA polymerases, although most investigators use the one adopted by Roeder and Rutter (75); these authors referred to the enzymes by Roman numerals, in order of their elution from the DEAE-Sephadex column between 0.10 and 0.37M
salt. The catalytic activities of the three enzymes isolated from rat liver nuclei (74), were differentially affected by divalent metal ions, and ionic strength (i.e., ammonium sulfate). Polymerase I had maximal activity at low ionic strength, in the presence of Mg$$^{++}$$, and was localized exclusively in the nucleolus. Polymerase II had maximal activity at a higher ionic strength, in the presence of Mn$$^{++}$$ and was localized in the nucleoplasm. The third RNA polymerase (III) is a minor component detected in the nucleoplasm. From these observations, Roeder and Rutter postulated that Polymerases I and II are involved in the synthesis of rRNA in the nucleolus and the synthesis of DNA-like RNA in the nucleoplasm respectively (75). This hypothesis has been confirmed by other workers (76, 77). Polymerases I and II are the major RNA polymerases detected in most tissues examined, although more than just these two (perhaps as many as 5) distinct forms of RNA polymerase have been separated by column chromatography (78).

In vitro, the activities of the two main RNA polymerases can be distinguished by the use of the fungal toxin, α-amanitin, which inhibits II but not I (79). The inhibition of RNA polymerase under conditions of high ionic strength (Mn$$^{++}$$ and ammonium sulfate) by α-amanitin (80) was first observed in isolated nuclei. Subsequent studies (76, 81), demonstrated that the toxin selectively inhibits the
nucleoplasmic enzymes (RNA polymerase II) \textit{in-vitro} at a concentration as low as 0.03 ug/ml (3 \times 10^{-8} \text{M}) whereas the nucleolar enzymes are not affected even at much higher doses. Unlike most inhibitors of RNA synthesis (82), a-amanitin inhibits RNA synthesis by binding to RNA polymerase (83) rather than to the DNA template. Studies on the stoichiometry of the reaction indicate that one molecule of a-amanitin inhibits one molecule of enzyme II (84). This selectivity of a-amanitin raised hopes that the drug could be used \textit{in vivo} to study the turnover rate of rRNA in higher animals and the transcriptional control of biochemical processes. Contrary to expectations however, both the synthesis of ribosomal as well as nonribosomal RNA is significantly inhibited within an hour of a-amanitin treatment (85,86,87).

In an effort to explain this apparent inconsistency between the \textit{in vivo} and \textit{in vitro} action of a-amanitin, it was hypothesized that the continuous synthesis of DNA-like RNA is necessary for the unimpaired functioning of the nucleolus. Evidence supportive of this theory comes from experiments of Sekeris and Schmid (88), which provided data suggesting that the effect of a-amanitin on nucleolar RNA synthesis is exerted either by the inhibition of the synthesis of some DNA-like RNA which is necessary for rRNA synthesis, or by inhibition of transcription of the messenger RNA(s) of some essential, short-lived, nucleolar proteins. Consistent with
this theory is the observation that protein synthesis is necessary for the continuous synthesis of ribosomal RNA (89, 90).

Numerous studies suggest a role for RNA polymerase I, which may actually be two closely related enzymes (91, 92), in the regulation of ribosomal RNA synthesis. One of the most pronounced, and earliest responses to estrogen stimulation of uterine cells (93), or glucocorticoid stimulation of liver cells (94, 95), is a stimulation of ribosomal RNA synthesis. On the basis of their findings on the glucocorticoid-stimulated rat liver system, Yu and Feigelson (96, 97) propose that the stimulation of rat hepatic ribosomal RNA synthesis by cortisone is not due to the derepression of ribosomal RNA genes per se, nor to an allosteric activation of pre-existing ribosomal RNA polymerase, but rather is a consequence of increased synthesis of essential protein components of the polymerases or polypeptide factors required for transcription. It must be kept in mind that various inhibitors of transcription and protein synthesis were used in these studies, and that interpretations based on such experiments are equivocal (98). A more convincing proof of de novo RNA polymerase synthesis would be measurement of the actual amount of the enzyme by immunoprecipitation; however, this latter technique requires a highly
purified enzyme for the preparation of an authentic anti-serum.

A possible role of RNA polymerase in cellular regulation is suggested by the finding of greatly increased polymerase activities in a minimal-deviation rat hepatoma cell line (99). Regulation of nucleolar RNA polymerase activity by availability of amino acids in the culture media of Ehrlich ascites tumor cells has been reported (100). Factors which stimulate RNA polymerase I have been isolated from rat liver nucleoli (101), and Novikoff ascitis cells (92).

Processing of Ribosomal RNA

The 28S and 18S ribosomal RNA of eukaryotic cells are transcribed in the nucleolus as a single large precursor molecule (102), which then undergoes various maturation steps. Recent evidence (103) suggests that this is also the case in prokaryotes.

In all eukaryotic tissues examined to date (104), the primary transcription product of the ribosomal RNA gene by nucleolar RNA polymerase, is a polynucleotide of higher molecular weight than the sum of the molecular weights of the two mature ribosomal RNA species. This initial transcription product has been termed ribosomal precursor RNA and is commonly referred to by its sedimentation coefficient
Characterization of the precursor rRNA became possible as methods were developed for the extraction of rapidly labeled nuclear RNAs in an undegraded form (105). Comparisons between various eukaryotic organisms show that while the size of rRNA (106) has been strongly conserved through evolution, the size of the precursor has not. In general it appears that the more primitive eukaryotes have precursor molecules of lower molecular weight than do the higher eukaryotes such as birds and mammals (107). There are some reports that even within the tissues of a single species, there is some heterogeneity in the high molecular weight precursor RNA (108).

Most of the extensive studies of the processing of precursor molecules involved cultured cells, especially HeLa cells, whose RNA can be radioactively labeled in a relatively simple medium of defined composition. From these, and studies in various other cell types, the following maturation pathway has emerged (109): the 45S transcription product is converted by a series of stepwise cleavages and methylation reactions first to a 41S RNA, which is then converted to a 32S RNA; which matures into the ribosomal 28S RNA and a 20S RNA which matures to the 18S ribosomal RNA.

The 7S RNA which is found hydrogen bonded to the 28S RNA in the large ribosomal subunit (110), is a third cleavage product of the 45S rRNA precursor molecule.
The processing scheme for HeLa cell ribosomal precursor RNA according to Weinberg & Penman (109).
The important experimental approaches employed to elucidate the detailed steps in the processing of precursor RNA include:

(1) Treatment of cells, in which the RNA is pulse-labeled, with actinomycin-D (111) which stops further RNA synthesis but allows the processing of most species of pre-labeled RNA to continue unaffected, then analyzing the nuclear RNA profiles at various time periods after the pulse-label.

(2) Following label in RNA after labeling of cells with methyl-labeled methionine (37) which mainly labels the rRNA precursor over heterogeneous nuclear RNA and hence reduces the background against which synthesis and processing of 45S RNA is followed. (A very recent report (112) provides evidence that mRNA and HnRNA of mouse L cells are also methylated. The proportion of methyl groups however, is much lower than in ribosomal 45S RNA, too low to have affected the results of the earlier experiments which were based on the assumption that methylations occurred exclusively on ribosomal and transfer RNA).

(3) Infection of HeLa cells with poliovirus which causes an accumulation of intermediates normally present only in trace amounts (113).

All of these studies produced convincing, but nevertheless circumstantial evidence for the various steps in
the maturation pathway. Direct proof of the maturation pathway was provided by:

(1) An analysis of the "fingerprints" of nuclease digests of the various RNA species in the pathway (114), which established a direct product-precursor relationship between all the species.

(2) Linear measurements of RNA molecules by electron microscopy (115), revealed that ribosomal RNA displayed a characteristic secondary structure, involving hairpin loops reproducibly located along the length of each molecule. By following these structures through the various intermediate RNA species, the previously deduced pathway of rRNA processing was further confirmed. In addition, the location and orientation of the 18S and 28S rRNA molecules within the precursor 45S molecule was established, supporting the earlier scheme (109) proposed on the basis of labeling kinetics experiments. According to this scheme, 18S RNA is proximal to the 3' end of the molecule and is separated from 28S rRNA by a spacer RNA.

Methylation of Ribosomal RNA

Ribosomal RNA isolated from HeLa cells was found to contain methyl groups (116) both attached to nucleic acid bases and to the 2' OH of the ribose of rRNA. Using methyl-labeled methionine as a precursor for these methyl groups,
it was shown that the first molecules in the pathway to ribosomes which become labeled were the 45S rRNA (117). Furthermore, all the incorporated methyl groups in 45S rRNA were conserved during further RNA processing (118).

Later detailed studies showed that simultaneously with transcription, the 45S RNA undergoes methylation at some 110 points (119) within its ribosomal sequences. All except for five or six, are ribose methylations. Later, during maturation, six base methylations occur on 18S RNA, one of these after 18S leaves the nucleolus, and one on 28S RNA (119, 120).

The fact that all methylated sequences from the 45S precursor are conserved in the mature ribosomal RNA species, suggests that they may play a role in conferring protection to those sequences from the action of nucleases. This conclusion is supported by the observation that during methionine starvation of HeLa cells (121), the production of ribosomes is completely abolished, although 45S precursor RNA continues to be synthesized. A failure to methylate ribosomal precursor RNA is induced by foot-and-mouth disease virus (122) with a subsequent inhibition of new ribosome formation. Finally, the important role of methylation in the production of ribosomes is exemplified by the finding that the anti-tumor activity of agents such as polyinosinate (123) is attributable to their action as specific inhibitors
of ribosomal RNA methylases.

**Ribosomal Proteins**

While the 45S precursor molecule is being synthesized, it becomes associated with protein. This process has been visualized directly by electron microscopy (38) in the case of the transcription of *Xenopus* rDNA. In some earlier studies, ribonucleoprotein particles with an appearance similar to cytoplasmic ribosomes were observed in the nucleolus by electron microscopy (124). Convincing evidence, that ribosomal precursor molecules undergo maturation while in the form of ribonucleoprotein particles, was provided by the isolation of two types of nascent ribonucleoprotein particles with sedimentation coefficients of about 80S and 55S from HeLa and L cell nucleoli (125, 126). The 55S particles account for most of the nucleolar particles and are made up of 32S RNA, 5S RNA, and most of the proteins found on the cytoplasmic large ribosomal subunit. The 80S particles contain mostly 45S ribosomal precursor RNA as well as 5S RNA and proteins similar to the cytoplasmic ribosomes (127). Based on observations from pulse chase experiments, with radioactive amino acids, and other data (125), it is inferred that the nucleolar ribonucleoprotein particles are direct precursors to the cytoplasmic ribosomes.

Even though the newly synthesized RNA associates with
ribosomal proteins, concurrent protein synthesis is not essential for the production of ribosomes (128). This observation is interpreted to mean that a pool of ribosomal proteins pre-exists in the HeLa cell, which is sufficient to support new ribosome formation for a considerable period of time, although at a reduced rate, in the absence of ongoing protein synthesis. A similar finding has been reported for the rat liver system (129) although in the case of regenerating rat liver, the available protein pool is much more limiting. Free ribosomal protein may constitute as much as 17% of the total soluble protein (130), indicating that in the resting rat liver there are, on the average, ten times as many ribosomal proteins in the soluble form as are constituents of ribosomes.

The finished ribosomal particles appear in the cytoplasm with only the structural ribosomal proteins, leaving behind the true nucleolar proteins which are then recycled (131).

The high degree of nucleocytoplasmic interaction that is involved in the production of ribosomes, is illustrated by the observation that the nucleolus only contains about 1% of the protein of the cell, yet in growing cells, some 5% of the cell's total protein must pass through it in the form of ribosomal proteins (which are synthesized in the cytoplasm) (131).
The Nucleases

The sites at which ribosomal RNA molecules are cleaved during processing must be highly specific. The factors which might be involved in conferring specificity to the cleavage or degradation sites are: (1) the secondary structure of the rRNA molecule, (2) methyl groups, (3) specific base sequences and (4) attachment of specific ribosomal or pre-ribosomal proteins. Though there is as yet little definitive information about the enzymes responsible for rRNA processing, some clues as to their possible nature have been provided by studies in cell-free systems consisting of isolated nucleoli (132, 133). In these experiments, isolated nucleoli of mouse L Cells, in which the rRNA was prelabeled, were incubated in vitro under conditions which permitted limited nuclease activity. Nonspecific degradative reactions were sufficiently controlled to allow the conversion of the precursor 45S RNA into large segments resembling the naturally occurring rRNA intermediates and products. Based on the results of an analysis of these products and the Mg\(^{++}\) dependence of the processing, it was postulated (133) that the 45S RNA undergoes an initial cleavage by an endonuclease followed by exonucleolytic trimmings of the subsequent precursors to the intermediates and end products. The Mg\(^{++}\) dependence and production of 5'‐mononucleotides indicated that the nuclear exoribonuclease
was probably the same as the one described in earlier studies by Sporn and co-workers (134, 135). Over one-half of the 45S precursor is not conserved in the processing.

Experiments carried out with highly purified preparations of the nuclear exoribonuclease and mouse L cell pre-rRNA components (136) give further support to the proposed mechanism and indicate that the enzyme is specific for the 3'-OH methyl-deficient segments. The results indicate that there are methyl deficient and thus presumably non-conserved regions at the 3'-OH ends of both the 45S and 32S molecules which are hydrolyzed by the exonuclease. Based on this data, Perry proposes the following map of the arrangement of the 28S, 18S and non-conserved RNA segments within the 45S molecule:

\[
\begin{array}{c}
28S \\
5' \quad \rightarrow \quad \leftarrow \quad 3' \\
\end{array}
\]

\[
\begin{array}{c}
18S \\
\downarrow \\
\text{Initial endonuclease cleavage}
\end{array}
\]

\[
\begin{array}{c}
\quad \leftarrow \quad \uparrow \\
\text{Subsequent hydrolysis by exonuclease}
\end{array}
\]

( Perry & Kelley (136) )
This arrangement is in agreement with the sequence studies of Choi and Busch (137), which indicate that in the rat the 45, 32 and 28S components have identical 5' terminal sequences. The arrangement observed in electron microscope studies of secondary structure maps of HeLa cell ribosomal precursor RNA's (115) is also in complete agreement with this scheme.

The first single cleavage of precursor rRNA from sea urchin eggs (138) can be made by pancreatic RNase. This suggests that there is only one site susceptible to generalized RNase activity, which in turn is determined by the secondary structure of the molecule and the specific attachment of proteins.

The purine and pyrimidine analogs, such as Toyo-camycin, Tubercidin, 6-thioguanosine, 5-fluoroorotic acid, 5-fluorouridine, 5-azacytidine and 8-azaguanine which disrupt normal cellular processes through incorporation into various RNA species, are widely used for their cytotoxic and antitumor activities (139, 140, 141). On the basis of observations on the effects of these analogs in cultured Novikoff hepatoma cells, it was proposed that all these cancerostatic drugs shared a common site of action (142), namely the interference with normal processing of pre-ribosomal RNA.

In cells treated with the drugs, the initial
endonucleolytic step proceeds normally, while subsequent exonucleolytic trimming steps are inhibited. One can speculate that by their presence in the RNA precursor molecules, these nucleoside analogs alter the secondary structure of the molecule and prevent the proper attachment of the specific proteins, such that the preribosomal RNA processing enzymes cannot act properly.

**5S rRNA**

Eukaryotic cytoplasmic ribosomes contain four molecules of RNA of which three, i.e., the 7, 18, and 28S rRNA of mammalian cells are derived from a 45S rRNA precursor produced in the nucleolus; the fourth species is 5S rRNA which is also produced in the nucleus but is of extranucleolar origin (143). Fractionation of HeLa cell chromosomes further shows that 18 and 28S rRNA genes are found on small chromosomes, while the 5S genes are present on several different sized chromosomes (144). The 5S RNA is synthesized by an α-amanitin-resistant, nucleoplasmic polymerase which is probably RNA polymerase III (145).

The 5S RNA appears to become associated with the nucleolar 80S ribonucleoprotein particle which contains the 45S precursor, and which is the common precursor to both ribosomal subunits (146). After processing of the 45S precursor, the 5S RNA is transported to the cytoplasm as part
of the completed large (60S) ribosomal subunit, with which it remains stably associated. Kinetic studies on the synthesis and utilization of the 5S species in a number of cell types, indicate that about four to five times more 5S RNA is synthesized, as will be needed for use in ribosome assembly (147). The purpose of this overproduction, and the fate of the unused 5S RNA is at present unknown.

**Nuclear rRNA Degradation, the "Wastage" Phenomenon**

It has been well established that a much accelerated accumulation of ribosomes takes place (14, 148, 149) in cells entering the proliferative phase (during $G_1$ phase of the cell cycle). The mechanism of this increased production has been investigated by several laboratories using various systems. One, which has been studied extensively is the phytohaemagglutinin stimulated human peripheral blood lymphocyte in culture. When human lymphocytes are incubated in *vitro* they remain metabolically active synthesizing ribosomes and protein continuously. However, there is no net accumulation of ribosomes, nor is there DNA synthesis leading to mitosis unless they are stimulated to divide (150, 151).

In these resting (nongrowing) human lymphocytes, about one-half of the newly synthesized 18S rRNA and 28S rRNA molecules are randomly degraded in the nucleus (152). This nuclear degradation of newly synthesized rRNA was
referred to as the "wastage phenomenon" (152). However, upon stimulation of the lymphocytes to proliferate with phytohaemagglutinin (PHA), a nonspecific mitogen for thymus derived lymphocytes, the nuclear degradation of rRNA was observed to diminish, with a concomitant increase of rRNA accumulation in the cytoplasm. These findings suggest that the reversible control of ribosomal RNA degradation may serve to regulate lymphocyte growth. It must be emphasized that reversion of rRNA wastage is not the sole mechanism by which growing lymphocytes increase cytoplasmic ribosome concentrations. The rate of synthesis of 45S precursor rRNA is also increased 2-4 fold following PHA stimulation, and there is a simultaneous increase in the activity of the appropriate DNA dependent RNA polymerase, i.e., RNA polymerase I (153). There also appears to be an accelerated rate of processing in proliferating lymphocytes, since the appearance of labeled species in the 32S and 18S pools is accelerated (154).

A clue to the manner by which rRNA wastage is controlled was provided by the observation that inhibition of protein synthesis by cycloheximide results in a virtual complete degradation of the newly synthesized 18S RNA (155). Similar findings have been reported in other eukaryotic cell systems (90). In other words, continuous protein synthesis is necessary for the survival of newly synthesized rRNA.
This requirement for new protein production is restricted to the period of synthesis of the 45S precursor, as demonstrated by pulse-chase studies in combination with various inhibitors (155). Thus, the newly-synthesized proteins, which combine with the 45S rRNA precursor, impart protection from later degradation to the appropriate regions of the cleavage products. According to Cooper's hypothesis (156), the degree of rRNA survival in lymphocytes is determined by the ongoing rate of synthesis of this ribosomal protective protein(s) and PHA stimulation causes a rapid increase in production of the protein(s), resulting in decreased rRNA wastage.

A case where such control mechanisms may be impaired, is in lymphocytes from patients with chronic lymphocytic leukemia (CLL). Such cells are characterized by a delayed or poor response to PHA stimulation (157). When compared with cultures of normal lymphocytes, the CLL cell cultures showed a similar prompt rise in the rate of 45S rRNA synthesis upon addition of PHA (158). However, failure to conserve 18S rRNA subunits persisted i.e., the normal rapid reversal of rRNA wastage did not occur. These observations suggest that the sluggish growth response of CLL cells might be due to a defect in the post-transcriptional mechanism regulating rRNA wastage.

As in most new areas of research, some controversy
exists as to the interpretation of the kinetic data relative to post-transcriptional regulation of ribosome formation. For example, studies on the synthesis and accumulation of rRNA during contact inhibition of growth, during exponential growth and during serum activation of growth of chick skin fibroblasts in culture, suggest (159) that the regulation of rRNA accumulation and cellular growth are highly integrated in these cells, and that rRNA accumulation is controlled mostly at the level of transcription of rRNA precursor. Emerson proposes that the main control is at the transcriptional level and that the synthesis of rRNA precursor is regulated by a mechanism which controls the length of time required for transcription of the precursor. There are many obstacles to clear interpretation of these kinetic labeling studies, among them: rate of uptake of labeled precursors, the uniformity of specific-activity of the nucleotide pools during the experiment, the relation between these and cell growth stage, and nonspecific artifacts of the various inhibitors employed.

In an attempt to measure the rate of RNA synthesis under conditions not affected by the cell-membrane permeability barrier and the slowly equilibrating cell-nucleotide pools, "ghost monolayers" were prepared with detergent from mouse-fibroblast line 3T6 (160). At various times after
stimulation of resting cultures of 3T6 with serum containing fibroblast growth factors, "ghost monolayers" were prepared and the rate of RNA synthesis measured as incorporation of labeled ribonucleoside triphosphates. An increase in the rate of ribosomal RNA synthesis was observed within 10 minutes after stimulation (l61). RNA synthesis continued to increase until the beginning of DNA synthesis. The authors report that no changes in turnover rate of the synthesized rRNA were observed in the ghost monolayers prepared from both resting and serum-stimulated cultures. However, they base their conclusion on the effect of a 15' chase with unlabeled UTP, which is not a sufficiently long period of time (156) for detecting nuclear degradation of newly synthesized 28S and 18S rRNA. Furthermore, the effect that a drastic procedure such as detergent treatment might have on posttranscriptional controls and nucleo-cytoplasmic interactions, is obvious.

All these studies confirm the observation that ribosomal RNA synthesis and cell proliferation are tightly coupled, and that numerous points of control are possible. The experimental difficulties encountered by researchers using whole cells to study controls of rRNA synthesis prompted the development of the cell-free system which formed the basis for the work reported in this thesis.
The Ribosome Cycle

Ribosomes consist of two unequal subunits which are transported from nucleus to cytoplasm as individual subunits (162), and there undergo cyclic dissociation and association during protein synthesis. The initiation of protein synthesis in eukaryotes involves the formation of an initiation complex made up of the small 40S ribosomal subunits (163), an initiator transfer RNA (Met-tRNA<sub>f</sub>) (164), at least three protein initiation factors (165, 166), an energy source (GTP) (167), and messenger RNA. The 60S ribosomal subunit attaches itself to this "initiation complex" forming the functional 80S monosome; a number of the complete ribosomes attached to the messenger RNA make up the working unit of protein synthesis - the polyribosome (168). Upon completing translation, the ribosomes dissociate into subunits (169), capable of entering a new cycle. However, single 80S ribosomes are present in the cytoplasm of all cells examined; these monomeric ribosomes do not carry nascent polypeptide chains, nor do they readily enter polyribosomes (170). It has been found that these single ribosomes must dissociate into subunits before entry into polyribosomes (171). This work is supported by the isolation of a factor that promotes the dissociation of ribosomes into ribosomal subunits (172, 173), and consequently stimulates protein synthesis in the rabbit reticulocyte.
lysate system.

All these studies are consistent with the hypothesis that ribosomes are released at chain termination as separate subunits which may either become "native" subunits (those which appear as free subunits in cell extracts, as contrasted to those which are coupled to form ribosomes) and recycle or may recombine to form monomers. In this scheme, the subunits are direct intermediates in protein synthesis and the monomers are inactive products of a side reaction and most probably constitute a reserve pool of ribosomal subunits. Some recent work by Henshaw et al. (174) provides evidence for a mechanism regulating the level of monomers versus subunits. It was found that the 40S subunit is modified, at or soon after release at chain termination, by association with a protein which reduces its affinity for the 60S subunit and allows it to exist as a free or "native" subunit. They put forth the hypothesis that the size of the subunit pool is determined by the supply of protein factors (which is limited), and that monomers accumulate when this supply is all bound to subunits. Further studies on these proteins (175) suggest that they may also be initiation factors, promoting initiation complex formation, thereby also promoting subunit re-entry into polyribosomes. Thus it is proposed (176) that the 40S subunit-associated protein factors function stoichiometrically and cyclically,
regulating the size of the subunit and indirectly the size of the monomer pool; as components of the subunits, they are involved in the catalysis of chain initiation in protein synthesis.

Size of Monomer Pool and Ribosome Production

Following partial hepatectomy in the rat, there is increased accumulation of cytoplasmic ribosomes (129), and an increase in protein synthesis followed by DNA replication and mitosis (177). The increase in protein synthesis is characterized by a shift in the polyribosome size distribution towards the heavier aggregates (178), and is accompanied by a drastic reduction in the size of the monomer pool. Thus, during regeneration the normally inactive monomers are recruited into functionally active polyribosomes.

Based on these observations, Rizzo and Webb (129) postulated that the depletion of the cytoplasmic monomer pool during liver regeneration and the concomitant increased production of ribosomes might be related events, and that the monomers might be involved in some regulatory mechanism of ribosome formation. More extensive studies on the size distribution of the cytoplasmic polyribosomes and monomers, and the rate of labeling of these components at various times during regeneration (129), showed a linear negative correlation between the size of the monomer pool and the rate of accumulation of ribosomes. Further support for this
hypothesis was provided by studies in the 42 hour regenerating liver, using inhibitors of protein biosynthesis such as puromycin, which catalyzes the breakdown of polyribosomes and consequently increases the size of the monomer pool (179), and cycloheximide which preserves the polyribosome structure. Puromycin, in contrast to cycloheximide, treatment of rats resulted in a markedly decreased rate of labeling of the cytoplasmic ribosomes with orotic acid $^{6\text{14}}$C in regenerating liver; again showing the negative correlation between size of monomer pool and rate of cytoplasmic ribosome formation, since the former drug causes polyribosome breakdown while the latter preserves polyribosome structure. These inhibitors of protein synthesis showed a similar effect in resting liver as well. (140).

Cytoplasmic Turnover of Ribosomes

Any study of the regulatory mechanisms of ribosome formation must also take into account their cytoplasmic degradation as another controlling factor. Numerous studies on the turnover rate of rat liver ribosomes, suggest that the half-life of rat liver ribosomal RNA is about five days (180, 181, 182). The approach most often used in such experiments is to label the ribosomes with a single injection of orotic acid $^{6\text{14}}$C and then measure the decline in specific activity of the ribosomes or the purified ribosomal 28S and 18S RNA's
over a period of time.

There seems to be an increased rate of degradation of rat liver ribosomes in response to fasting (180). This increased rate of catabolism is of a transitory nature, lasting only approximately 2 days on a protein deficient diet (183). This increased rate of degradation, rather than a reduction in the rate of synthesis of rRNA accounts for the rapid loss of liver RNA during the first 48 hours of feeding a protein-free diet. There is an eventual depression of rRNA synthesis during fasting, but only after extensive breakdown of liver polyribosomes and loss of RNA has occurred (184). Incidentally, this finding is consistent with the theory of Rizzo and Webb (129) that an increase in the size of the monomer pool, such as would occur after degradation of polyribosomes, will result in a decreased production of ribosomes.

Fasting also brings about a degradation of hepatic rough endoplasmic reticulum (185), which results in an increase in the amount of membrane free ribosomes. On the basis of observations that membrane-free (i.e., not attached to endoplasmic reticulum) ribosomes, unlike the membrane-associated particles are very susceptible to enzymatic degradation (186), Enwonwu et al. proposed that the increased degradative rate, observed after fasting, is due to this increase in membrane-free ribosomes rather than the
activation of nucleases (184).

The mechanism by which cytoplasmic ribosomes are degraded is still not well understood. In their study of rRNA turnover, Hirsch and Hiatt (180) also measured ribosomal protein turnover using labeled arginine. It was observed that the ribosomal protein had the same half-life as the rRNA, and thus suggested that the ribosomes were degraded as a unit. However, recent more extensive studies on the turnover of ribosomal proteins (130), contradict this conclusion; rather they indicate that ribosomal proteins have heterogeneous turnover rates. According to the latter study it appears that ribosomes are in a continual state of flux, in which most protein constituents of the ribosome exchange with similar ribosomal proteins in the cytoplasm.

The degradation of ribosomal RNA is presumably mediated by the cytoplasmic ribonucleases which are present in both the free form and bound to an inhibitor protein (187). This inhibitor-bound ribonuclease activity can be released in-vitro by substances that react with -SH groups, such as p-chloromercuribenzoate (188). It has been postulated that the "free RNase - inhibitor bound RNase" system regulates cytoplasmic RNA degradation rate (189). Support for this hypothesis comes from studies on compensatory renal growth in the rat (187), where an increase in RNase inhibitor is observed before an increase in RNA synthesis.
**Nucleo-Cytoplasmic Transport**

One of the essential features which distinguishes the higher organisms (eukaryotes) from the prokaryotes (bacteria and blue-green algae) is the presence of a double membranous envelope enclosing the nuclear material. Since the production of ribosomes in eukaryotic cells involves the synthesis of a precursor ribosomal RNA in the nucleolus which then undergoes various maturation steps to the finished ribosomal subunits which in turn are exported to the cytoplasm, a potential regulatory site in this pathway is at the nuclear envelope. Indeed there is increasing evidence that the processing, selective transport, and nuclear restriction of both ribosomal RNA and messenger RNA from the nucleus to the cytoplasm play a significant role in the regulation of cell growth.

For example, work with the PHA stimulated lymphocyte system (156) as discussed in a previous section, indicated that much of the potential ribosomal RNA is ordinarily degraded in the nucleus of resting cells. However, when the cells are stimulated to proliferate with PHA, this nuclear degradation or "wastage" is greatly reduced and most of the rRNA synthesized is efficiently transported to the cytoplasm as ribosomal subunits.

Hydrocortisone treatment of rats 19 hours after partial hepatectomy results in a temporal inhibition of RNA
synthesis (190), yet ribosome production is enhanced (191). Analysis of the rates of synthesis and processing of the rRNA in the nucleus and of the accumulation of the products in the cytoplasm of hydrocortisone-treated and control rats (192), suggested that the increase in the hydrocortisone-treated rats was due to increased transport to the cytoplasm. It was inferred that, as in the case of the lymphocyte, a portion of the nuclear rRNA in the resting liver of the untreated rats remained in the nucleus and was degraded. Induction of liver regeneration by partial hepatectomy, leads to increased transcription of 45S and decreased wastage of 18S and 28S RNA (129, 89).

Nucleic acid hybridization techniques, capable of discerning RNA's of differing base sequences, have provided convincing evidence that a great deal of the RNA transcribed in eukaryotic cells is restricted to the nucleus and is never transported to the cytoplasm (193, 194). Such a situation obviously requires post-transcriptional controls operating within the nucleus, or at the nuclear membrane, regulating both qualitatively and quantitatively, the flow of ribonucleoproteins to the cytoplasm.

The Nuclear Membrane

Numerous electron microscope studies on the nuclear envelope indicate that this cellular component has the same
fundamental structure in all eukaryotic cells (195). It consists of two concentric 70-80 Å thick membranes separated by a 200-700 Å wide perinuclear space. At intervals along the surface of the envelope, the two membranes are joined together to form a circular pore of approximately 300 to 1000 Å diameter. The nuclear pores are not simply unobstructed holes in the envelope for they have associated protein, extending into both nuclear and cytoplasmic compartment in a cylindrical, tube-like fashion. A pore and its associated protein (annular material) are termed a "pore complex" (196).

Several lines of evidence support the concept that the annular material restricts the permeability of the nuclear pore-complex and regulates the nucleocytoplasmic RNA-efflux (197). Studies with *Xenopus* oocytes indicate that there occur marked differences in the ribosomal RNA "nuclear pore flow rates" from nucleoplasm to cytoplasm (198) at various stages during *Xenopus* oogenesis. Isolated pore complexes from oocytes reveal association with ribosomal and other species of RNA. Curiously, the RNA in the pore complex has a higher molecular weight than that in ribosomes, suggesting that some final maturational cleavage step must take place before it can be released to the cytoplasm. In accord with this interpretation is the observation that
no mature rRNA species can be found in the nucleus of the amphibian oocytes (199).

**Nucleo-Cytoplasmic Controls**

That cytoplasmic factors can control nuclear genetic expression has been dramatically demonstrated by nuclear transplantation (200, 201) and cell fusion (202) experiments. Gurdon (201) was able to demonstrate that a nucleus isolated from a frog intestinal epithelial cell, when transplanted into an enucleated egg cell, resulted in the normal development of the ovum to a fertile adult frog. The inescapable conclusion to be drawn from this phenomenon is that the egg cytoplasm is able to reprogram gene expression of a differentiated cell nucleus and that in addition, the genome of all somatic cells is the same in an organism. McKinnell (200) obtained essentially the same results (i.e. development to a tadpole stage) by using nuclei from Lucké tumor cells, derived from frog kidney, thereby providing the even more dramatic conclusion that tumor cell nuclei may have the capacity for normal growth, if properly regulated.

The cell fusion experiments of Harris (202) demonstrated that an inactive, completely repressed nucleus such as the hen erythrocyte nucleus can be activated by exposure to the cytoplasm of a proliferating cell such as the HeLa
cell. An accumulation of host cell specific proteins by the reactivated hen erythrocyte nucleus has recently been demonstrated (203).

The Cell-Free System

To make possible the direct analysis of the nucleocytoplasmic controls of RNA production, cell-free systems approximating the in vivo environment, capable of inducing detectable RNA transport from isolated nuclei, were developed in this lab (204, 205). The approach used for studying controls of ribosomal RNA production in this system, consists of isolating rat liver nuclei prelabeled for two hours in vivo with orotic acid $^{14}$C, incubating them in a fortified medium containing dialyzed whole, or fractionated cytosol, then analyzing the transported RNA and comparing it to the remaining labeled nuclear RNA species (204). This comparison between transported and restricted RNA species is done both quantitatively and qualitatively by analyzing each purified RNA fraction on sucrose density gradients.

It was found that the release of preformed ribosomal subunits from the isolated nuclei was dependent upon the presence of non-dialyzable factors in the cytosol, showed an absolute requirement for energy and was temperature dependent (204).
CHAPTER II

EXPERIMENTAL PROCEDURES

Materials

Orotic acid-6-\(^{14}\)C hydrate (Specific Activity: 57.7 mc/mM), methionine-methyl-\(^{14}\)C (S.A. = 15 mc/mM), l-leucine-1-\(^{14}\)C (S.A. = 20 mc/mM), and thymidine-methyl-\(^{3}\)H (S.A. = 15 c/mM) were purchased from the New England Nuclear Corp., Boston. Uridine-5-\(^{3}\)H-5' triphosphate (S.A. = 12.87 c/mM) was obtained from ICN, Irvine Cal.

Actinomycin D was obtained from Calbiochem, Los Angeles. Cycloheximide and puromycin were purchased from the Nutritional Biochemicals Corp., Cleveland. DEAE Cellulose (medium mesh. 0.87 meq/mg capacity), pyruvate kinase, and a-amanitin were purchased from Sigma Chemical Co., St. Louis. Ribonuclease-free sucrose was obtained from Schwarz/Mann Research Laboratories, New York. Camptothecin was kindly provided by Dr. H. B. Wood, Jr., of the National Cancer Institute. Pactamycin was a gift from the Upjohn Co., Kalamazoo. Low-molecular weight yeast RNA was obtained from General Biochemicals Corp., New York. The \(\alpha, \beta\)-methylene and the \(\beta, \gamma\)-methylene analogs of ATP were purchased from Miles
Laboratories, Inc., Kankakee, Illinois. The nitrocellulose B-6 filters used in the competition-hybridization experiments were obtained from Schleicher and Schuell Co., Kenne, New Hampshire. Diaflow ultrafilters were purchased from Amicon, Lexington, Mass. All reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad, Richmond, Cal. The following products were employed for the liquid scintillation counting procedures: Hyamine 10X (Packard Instrument Co., Chicago), Aquasol (New England Nuclear, Boston), Scintosol-Complete, Unisol, and Unisol-Complement (Isolab Inc., Akron).

Drugs and labeled compounds were dissolved in 0.9% NaCl and administered in small volumes (0.2 - 0.8 ml).

Animals

Male Sprague-Dawley rats, weighing 250-300 g. were maintained on Purina chow and water available ad libitum. All animals were fasted overnight in wire bottom cages prior to the start of the experiment. Lighting was mechanically controlled from 6 a.m. to 6 p.m.

Hepatic regeneration was induced by the surgical removal (partial hepatectomy) of two-thirds of the liver (the median and left lateral lobes) under light ether anesthesia according to the method of Higgins and Anderson (206). All experiments were performed with the 19 hour post-operative
regenerating liver, since it has been determined (129) that maximal accumulation of cytoplasmic ribosomes occurs at this stage of liver regeneration.

The transplanted Morris hepatomas 5123-D and 7800 (207) were received from Howard University, Washington, D.C., as i.m. implants in the hind legs of Buffalo strain rats. The livers or tumors were removed under light ether anaesthesia, and, unless otherwise stated, all injections were via the intraperitoneal route.

Methods

Preparation of Ribosomes

Prior to removal under light ether anaesthesia, the livers were perfused via the portal vein with 10 ml of cold 0.25 M sucrose in TKM buffer (50 mM tris - (hydroxymethyl) - aminomethane, 25 mM KCl, and 5 mM, MgCl₂, pH 7.5). The livers and tumors were chilled after excision; the latter were dissected free of blood clots and of any fibro-fatty tissue. The tissue was weighed, then homogenized in 0.25 M sucrose in TKM (2 ml/gm tissue) using a Potter-Elvehjem type homogenizer fitted with a teflon pestle with a 0.02 mm clearance and rotated at 670 r.p.m. A previous study (129), had established that under such conditions, cell breakage is consistently 80%. The homogenate was centrifuged at 13,000 xg for 10 minutes, and the resulting supernatant fraction (the
post-mitochondrial supernatant) was withdrawn and centri-
fuged at 105,000 xg for 60 minutes, pelleting the ribosomes
and leaving the post-microsomal supernatant.

**Preparation of the Cytosol Fraction**

The post-microsomal supernatant fraction was dialyzed
against TKM buffer pH 7.5 overnight at 4°C, then for an addi-
tional 3 hours against fresh dialyzing medium. Before use
in the incubation medium, the dialyzed cytosol was cleared of
precipitated proteins by a 15-minute centrifugation at 13,000
xg.

**Preparation of Ribosomal Proteins**

A pelleted microsome fraction, prepared as in the
procedure described above, was suspended in 50 mM EDTA, 160
mM NaCl and 10 mM tris, pH 7.4, then centrifuged at 13,000 xg
for 15 minutes. The resultant supernatant was removed and
centrifuged at 105,000 xg for 3 hours to obtain a pellet of
purified ribosomal subunits.

The ribosomal protein was extracted by suspending the
ribosomal subunit pellet in LiCl according to the method of
Hamilton and Ruth (208). After letting the suspension stand
at 4°C for 2 days, it was centrifuged at 105,000 xg for 60
minutes to sediment the RNA. The ribosomal protein contain-
ing supernatant was dialyzed against 6 M urea in 0.03 M Na
acetate pH 5.6 overnight, followed by extensive dialysis in distilled water.

**Determination of Protein Concentration**

The protein concentration in the cytosol or fractions thereof, was estimated using the biuret reagent (209).

**Isolation of Nuclei**

The nuclei were prepared using procedures described by Muramatsu and Busch (210). Prior to removal, the livers were perfused with 10 ml 0.25 M Sucrose containing 3.3 mM calcium acetate. The livers were then suspended and finely minced with scissors in 2.4 M sucrose (15 ml/gm tissue) containing 2.5 mM MgCl$_2$ and 1.0 mM Ca Acetate, and homogenized with 4 strokes of a teflon pestle with a clearance of 0.02 mm rotating at 800 r.p.m. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 40,000 xg for 1 hour. The sides of the tube were cleaned of cellular debris, and the nuclear pellet was resuspended in 1.0 M sucrose (1.5 ml/g. liver) containing 1.0mM calcium acetate, and centrifuged at 2000 xg for 5 minutes to give a pellet of purified nuclei.

The purity of the nuclei, prepared using the procedure outlined above, has been confirmed by electron microscopy (192).
Fractionation of Cytosol

Ammonium Sulfate Precipitation

Salt fractionation of the cytosol was performed in the cold by the sequential addition with stirring of 114 gm/l (0-20% saturation), 262 gm/l (20-60% saturation) and 269 gm/l (60-95% saturation) of ammonium sulfate. After each addition the precipitated proteins were removed by centrifuging for 15 minutes at 13,000 xg, then resuspending them in TKM buffer and dialyzing as for the unfractionated cytosol.

DEAE-Cellulose

Fractionation of cytosol with DEAE-cellulose was performed by stepwise chromatography through a DEAE-cellulose (medium mesh, 0.87 meg/mg capacity) column (1.5 cm x 12 cm) equilibrated with TKM buffer, pH 8.5 by loading with 15 ml. of cytosol at pH 8.5. The non-adsorbing proteins were eluted with TKM buffer, pH 8.5, then the adsorbed proteins were eluted with 0.6M KCl in TKM buffer pH 7.5; both fractions were dialyzed overnight at 4o against TKM buffer, pH 7.5, before assay. The pH of the eluting solutions appears to be somewhat critical as variations of this pH caused marked changes in the relative transport capacities of the protein fractions in several experiments.
Diaflow Filtration

Diaflow filtration is a technique whereby fractionation of the cytosol is achieved by forcing it, with air pressure, through membrane filters of uniform pore size able to retain or filter macromolecules, depending on their molecular size. Whole, dialyzed cytosol was passed through filters able to retain molecules larger than M.W. 10,000, 30,000 and 50,000. The procedure was done at 4° C, with stirring at 25-30 p.s.i.

The Cell-Free System

The freshly purified nuclei were incubated (4 x 10^6 nuclei/ml) at 0° (control), or at 30° in 5 ml. of a "complete medium" containing the following components: 50 mM Tris buffer (pH 7.8), 2.5 mM MgCl₂, 25 mM KCl, 0.5 mM CaCl₂, 0.3 mM MnCl₂, 2.5 mM Na₂HPO₄, 5 mM NaCl, 5.0 mM spermidine, 2.5 mM dithiothreitol, 2.0 mM ATP, 2.5 mM phosphoenol pyruvate and 6.4 units of pyruvate kinase per ml. The medium also contained 0.7 volumes of dialyzed cytosol (or fractions thereof), and 0.5 mg of low molecular weight yeast RNA per ml in a final volume of 6.0 ml.

In order to determine the concentration of nuclei in the incubation medium and confirm the presence or absence of nuclear lysis, nuclear counts were made, after various periods
of incubation, in a Neubauer counting chamber under a light microscope.

Detection of Released RNA

After the incubation of the nuclei in the complete or modified medium on a shaker bath, and chilling the samples, the suspensions were centrifuged at 1000 xg for 10 minutes to remove the nuclei. In some experiments 0.4 ml. aliquots of the nuclei-freed incubation medium were treated with cold 5% trichloroacetic acid to precipitate the RNA as an acid-insoluble fraction; when nuclear counts were desired the nuclei were treated similarly.

Extraction of RNA

The RNA was purified from 6 ml. of the incubation medium (freed of nuclei), from the nuclei removed from 6 ml of the medium, or from ribosomal pellets by the hot phenol method of Muramatsu and Busch (210). The nuclei and ribosomal pellets were homogenized for 1 minute at 4° C in 0.14 M NaCl - 0.05 M sodium acetate buffer, pH 5.1, containing 0.5% sodium dodecyl sulphate (1.5 ml/g of original liver). An equal volume of redistilled phenol, containing 0.1% 8-hydroxyquinoline and saturated with the acetate buffer, was added to the latter suspension or to the incubation medium, and the RNA was deproteinized by shaking the emulsion at 65°
for 10 minutes, followed by a 15 minute shaking at 25° C. The phases were separated by centrifugation at 10,000 xg for 5 minutes, the phenol phase was removed and the aqueous phase and interphase were reextracted with an equal volume of fresh phenol for 10 minutes at 25° C. Phases were again separated and the aqueous top layer was carefully removed and re-extracted with an equal volume of phenol. After phase separation, the aqueous layer was removed and mixed in with 2.5 volumes of 95% ethanol containing 2% potassium acetate, and stored overnight at -15° C to precipitate the RNA. The RNA was pelleted from the ethanol by centrifugation at 10,000 xg. for 20 minutes at -10° C. The RNA pellet was taken up in NaCl - Na Acetate buffer and again mixed in with 2.5 volumes ethanol-potassium acetate and precipitated by storage for 2 hours at -15° C. The purified RNA was collected by centrifugation, then dissolved in a small volume of 0.02 M sodium acetate pH 5.1, for further analysis.

According to Muramatsu and Busch (210), the above procedure extracts over 75% of the RNA from the nuclear preparation with less than 3% DNA contamination.

Sucrose Density Gradients

Aliquots (0.8 ml) of the purified RNA in 0.02 M sodium acetate (pH 5.1) were layered over linear 26 or 28 ml
(10% to 30%) sucrose gradients containing 0.1 M NaCl, 0.02 M sodium acetate, 0.001 M EDTA (pH 5.1). The various RNA species were separated by centrifuging the gradient for 16 hours at 51,000 xg, average, in an SW 25.1 rotor of a Beckman ultracentrifuge. Ribonuclease-free sucrose was used in the preparation of the sucrose gradients. The effluent from the bottom of the tube was monitored at 260 nm in a recording spectrophotometer and collected in one ml fractions in liquid scintillation vials. Radioactivity was determined by adding 0.5 ml of water, 10 ml of scintosol, and counting in a Packard Tricarb scintillation counter at an efficiency of 80%.

**Competition – Hybridization**

The procedure of Gillespie and Spiegelman (211) was used, except that the incubations between RNA and DNA were carried out for 18 hours at 65° C on B-6 filters (Schleicher and Schuell Co.,) in 4X (four fold concentrated) SSC (SSC = 0.15 M NaCl, 0.01 M Na citrate, pH 7.0). The filter bound DNA, along with blank filters, were incubated 18 hours at 65° with either 2 ml of 4X SSC or this buffer containing cold competitor RNA (The competitor unlabeled ribosomal or transfer RNAs were prepared by hot phenol extraction of the purified ribosomes, or the post-microsomal supernatant from rat liver). The filters were rinsed twice in 2X SSC and then treated 45 minutes to 1 hour with RNase (50 µg/ml) at 25° C
in 2X SSC, followed by twice rinsing in 2X SSC in a batch and then individually with 5 ml of 2X SSC per side of filter. The filters were then placed in clean vials for hybridization and incubation as above, with the labeled RNA of unknown composition. Following incubation, the filters were treated with RNase and washed as above, then dissolved in scintillant for counting.

**Preparation of DNA**

Rat liver DNA for the competition-hybridization experiments was purified by the method of Ono et al. (212). The rat liver was homogenized in 10 volumes of 0.35 M sucrose containing 0.05 M tris, 0.025 M KCl, 0.005 M Mg acetate (pH 7.6) with 6 strokes of a teflon pestle with 0.02 mm clearance. The homogenate was filtered through 8 layers of gauze, then centrifuged at 10,000 xg for 15 minutes. The pellet was suspended in 27% sucrose containing 0.1 M EDTA, 0.05 M tris (pH 8.0), 0.2% SDS, then heated at 60° for 10 minutes. The pH was lowered to 7.2, RNase was added (50 μg/ml) and the mixture incubated at 37° for 1 hour, after which, 100 μg/ml pronase was added and the suspension incubated for another 3 hours at 37°. The DNA was extracted with phenol, then equilibrated three times at 25° C with 0.01 M tris pH 8.5 containing 2% SDS. After addition of 2 volumes of 95% ethanol, the
DNA was spooled and dissolved in 50 ml 1X SSC, then stored at 4° with a small amount of chloroform.

**Preparation of DNA Bound to Filters**

The stored DNA was dialyzed 24 hours against 1 X SSC, then sheared by forcing through a 20 gauge syringe needle several times. The dialyzed solution was diluted to 0.1 X SSC (5 ml to 50 ml), heated at 100° for 15 - 20 minutes, then poured into 50 ml of ice cold 8 X SSC, with stirring. One ml of this suspension was slowly passed through B-6 filters which had been soaked in 4 X SSC. The O.D. at 260 nm of the DNA solution was checked before and after passage through the filters, in order to determine the amount of DNA retained in the filter. The filters were washed with 5 ml 4 X SSC, dried overnight at 25° C, then heated at 80° for 2 hours in the oven, and stored at R.T. in a closed container.

**Specific Radioactivity of Soluble Proteins**

The postmitochondrial supernatant, (S-2) prepared as described above, was diluted with TKM buffer (1 in 10 v/v), and the post-microsomal supernatant (S-3) prepared by centrifugation of the latter for 1 hour at 105,000 xg. 2.5 ml of 1.0 N NaOH was added to an equal volume of S-3 to hydrolyze any radioactive aminoacyl - t RNA present. The samples were allowed to stand at room temp. for 30 minutes before neutralization and precipitation with 1 ml of 4N TCA. The
precipitate was collected by centrifugation, then extracted consecutively with 95% ethanol and ether: ethanol (1:1) for 15 minutes at 40° C. The residue was dissolved in Unisol and counted. An equivalent sample was used for protein determination by the Biuret method (209).

Analysis of the Size Distribution of Ribosomal Components

A 4 gm piece of perfused liver was minced in 0.25 M sucrose TKM (2 volumes/gm) and homogenized by 20 slow strokes of the loose fitting pestle (0.04 mm clearance). The homogenate was centrifuged at 13,000 xg for 10 minutes, then the resulting supernatant treated with 0.7% sodium deoxycholate, diluted with an equal volume of TKM buffer, and layered over 30 ml. 10 - 35% sucrose gradients. The latter were centrifuged in an SW 25.1 rotor of a Beckman L-2 ultracentrifuge at 63,000 xg. a.v. for 4.5 hours at 0° - 2° C. Following centrifugation, a continuous recording of the size distribution of the separated components was obtained by monitoring the effluent as it flowed from the bottom of the gradient tube, at 260 nm in a Beckman DB spectrophotometer, equipped with a flow cell and recorder. A drop counter activated an event marker on the recorder, which registered each ml of effluent passing through the flow cell.
In-Vitro Synthesis of RNA

Isolated nuclei, were incubated in a medium containing dialyzed cytosol, 50 mM tris (pH 7.5), 2.5 mM MgCl₂, 2.0 mM dithiothreitol (DTT), 0.5 mM MnCl₂, 0.5 mM CaCl₂, 10 mM NaCl, 1.0 mM Na₂HPO₄, 5mM spermidine, 500 μg/ml yeast RNA, 0.25 mM L-Cysteine, 2.5 mM phosphoenol pyruvate (PEP), 35 enzyme units pyruvate kinase, 0.8 mM GTP, 0.8 mM CTP, 2.0 mM ATP, 0.2 mM UTP (4.0 μCi ³H UTP). The incubations were carried out at 30° for 30 minutes, in 2 ml volumes consisting of 1 ml cytosol, 0.4 ml nuclei in 1.0 M sucrose containing 1.0 mM Ca acetate, and 0.6 ml containing the cofactors, salts and ³HUTP. After incubation, the nuclei were pelleted by centrifugation at 5,000 xg for 10 min. and washed with 1.0 M sucrose -1.0 mM Ca acetate. The RNA was extracted by the hot phenol method, and analyzed either on sucrose gradients, or by disc gel electrophoresis.

Polyacrylamide Disc-Gel Electrophoresis

Purified RNA was taken up in 10% sucrose in gel buffer (0.04 M tris (pH 7.5), 0.02 M Na acetate, 2 mM EDTA, 0.2% SDS). An aliquot (50 - 100 ul) was layered on top of the gel, and electrophoresis was carried out at 5 mA/gel until the tracking dye (0.002% bromophenol blue in the RNA sample) reached the bottom of the gel. The gels were composed of 2.6% acrylamide, 0.5% agarose and 0.2% SDS and
were made up according to the method of Summers (213). The gels were sliced by a manual gel slicer, then the 2 mm slices were solubilized in 0.5 ml Unisol, and counted in Unisol-Complement. Prior to slicing, the gels were also scanned at 260 nm in a gel scanner.
CHAPTER III
RESULTS

Ribosomal RNA Release from Isolated Nuclei

An earlier report (214) describes how rat liver cell nuclei, prelabeled in vivo, release detectable amounts of RNA during the in vitro incubation of the homogenate. At that time there was little appreciation of the post-transcriptional regulation of RNA formation, and the system was not studied further. These present studies were undertaken in order to test the feasibility of developing a controlled artificial medium in which purified nuclei would exhibit RNA transport and be responsive to various manipulations of this medium. Such a system would be invaluable for studying the post-transcriptional regulation of ribosome formation and in particular, nucleocytoplasmic controls; the study of which is severely restricted in the intact cell. Indeed, preliminary studies indicated that nuclei, purified by the method of Muramatsu and Busch (210), released detectable amounts of RNA, when incubated in the appropriate medium.

The initial experiments, which were carried out with assistance from Dr. L. C. Yu in this laboratory, were done
using nuclei purified from the 19-hour regenerating liver of 250 g Sprague-Dawley rats. Subsequent studies involved in addition, the resting liver nuclei i.e., nuclei from normal adult rat liver, and nuclei obtained from Morris Hepatomas 5123-D and 7800. The 19-hour regenerating liver was chosen as the source of nuclei for the initial experiments because the incorporation of labeled precursors into ribosomal RNA is maximal during this period of regeneration (192, 129). The nuclei were prelabeled in vivo with orotic acid-6-\(^{14}\)C for 2 hours, since as noted in the introduction, the processing time of rRNA is about 45 minutes, and a 2-hour labeling will essentially saturate intranuclear pools with ribosomal precursors, thereby facilitating the study of the processing and nucleocytoplasmic transport of RNA in vitro. Examination of the purified nuclei by electron microscopy (89) reveals that they are well preserved and essentially free of cytoplasmic contamination, including endoplasmic reticulum.

The freshly purified nuclei were incubated in a medium of the following composition; as shown in Table 1.

It was found that dialyzing the supernatant was necessary in order to prevent clumping of the nuclei during the incubation (204). Nuclear counts were made under the light microscope in a Spencer Bright-Line counting chamber with Neubauer rulings, in order to detect any lysis after
TABLE 1

COMPOSITION OF THE COMPLETE IN VITRO INCUBATION MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7 volume dialyzed postmicrosomal supernatant (cytosol)</td>
<td></td>
</tr>
<tr>
<td>Tris buffer (pH 7.8)</td>
<td>50 mM</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.25 M</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>25.0 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>Spermidine</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>Yeast RNA</td>
<td>0.5 mg/ml</td>
</tr>
<tr>
<td>ribosomal protein</td>
<td>5.0 µg/ml</td>
</tr>
<tr>
<td>dithiothreitol</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>ATP</td>
<td>2.0 mM</td>
</tr>
<tr>
<td>Phosphoenol pyruvate</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Pyruvate Kinase</td>
<td>6.4 e.u./ml</td>
</tr>
</tbody>
</table>
incubation. No lysis was detected after incubation in complete medium for up to 40 minutes using this technique.

The release of RNA from the isolated nuclei is shown to be temperature dependent as indicated in Table 2. We see that after incubation at 36° C for 15 minutes, there is released to the medium 3.7% of the nuclear labeled RNA, whereas at 0°, only 1.7% of the counts are released. Furthermore, the increased release at 36° is made up of mostly acid insoluble counts, i.e., large molecular weight RNA, not breakdown products or labeled nucleotides. However, under these particular assay conditions (15 minute incubation at 36°), the release of RNA seems to be unaffected by the presence of Actinomycin D in the medium, which should block all DNA-dependent ribosomal RNA synthesis. This observation argues for the independence of the RNA release from ongoing RNA synthesis i.e., the two processes are not tightly coupled. It is expected that RNA synthesis would be minimal in this system since CTP, UTP and GTP are not added to it.

Low-molecular weight yeast RNA, which has been shown to be an effective inhibitor of RNase activity (215), is also a necessary component of the medium in order to prevent RNA degradation. The yeast RNA is not an actual inhibitor of RNase, but rather, acts as a competitive inhibitor, tying up RNase molecules and thus preventing significant degrad-
TABLE 2
EFFECT OF ACTINOMYCIN D AND RIBONUCLEASE INHIBITORS ON RNA RELEASE

The nuclei were isolated from the 19 hour regenerating liver 2 hours after the administration of 200 μCi/kg of orotic acid-6-¹⁴C. The purified nuclei were incubated for 15 minutes at 0°C, or 36°C. Aliquots of the nuclei-freed medium (1000 xg supernatant) were assayed for total or TCA-insoluble radioactivity. The assay medium was modified as indicated.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Total cpm/ml or 4x10⁶ nuclei</th>
<th>% nuclear cpm</th>
<th>Acid Insoluble cpm/ml or 4x10⁶ nuclei</th>
<th>%total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete medium at 36°C</td>
<td>4,320</td>
<td>3.7</td>
<td>2,880</td>
<td>66.8</td>
</tr>
<tr>
<td>at 0°C rather than 36°C</td>
<td>2,165</td>
<td>1.7</td>
<td>1,240</td>
<td>57.1</td>
</tr>
<tr>
<td>plus actinomycin D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 μg/ml</td>
<td>4,250</td>
<td>3.8</td>
<td>2,950</td>
<td>69.0</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>4,300</td>
<td>3.4</td>
<td>2,680</td>
<td>62.2</td>
</tr>
<tr>
<td>minus yeast RNA</td>
<td>12,365</td>
<td>10.2</td>
<td>2,670</td>
<td>31.0</td>
</tr>
<tr>
<td>minus yeast RNA plus PVS</td>
<td>15,650</td>
<td>12.9</td>
<td>3,950</td>
<td>25.1</td>
</tr>
</tbody>
</table>
ation of the transported RNAs. The data presented in Table 2 shows that when yeast RNA is omitted from the incubation medium, there is a greatly increased release of radioactivity from the nuclei; however, this release is due to acid soluble counts i.e., degradation products. The natural RNase inhibitor found in the rat liver cytosol (216) is obviously not sufficient by itself to prevent RNase degradation in this system. Another RNase inhibitor, polyvinyl sulfonic acid (132), cannot be substituted for the yeast RNA, for it causes significant lysis of the nuclei. A concentration of 500 μg of yeast RNA per ml was found to inhibit effectively RNA degradation as estimated by recovery of TCA insoluble counts from the medium following removal of nuclei.

Despite the maximal suppression of RNase activity, about a third of the label released during the 15 minute incubation is TCA soluble. Part of this TCA-soluble radioactivity probably represents normal breakdown products of nuclear RNA processing. Experiments outlined below confirm that nuclear RNA processing is an actively ongoing process during such an incubation.

Transport of Ribosomal Subunits

In order to demonstrate that the 18S and 28S ribosomal RNAs are transported to the medium as constituents of
40S and 60S ribosomal subunits, respectively, the incubation medium (freed of nuclei by low-speed centrifugation), was layered directly on density gradients. As shown in Figure 1, the radioactivity profile of the released components coincides with the UV absorbance pattern of added (unlabeled) ribosomal subunits. In experiments where the nuclei were prelabeled for only 30 minutes in vivo (too short a time for incorporation of label into completed ribosomal subunits), the labeled ribonucleoproteins sedimented exclusively in the 40S region (217), i.e., in the informosome (218) region of the gradient. The RNA sedimenting in the 60S and 40S regions of the gradient, the latter including some mRNA in the form of informosomes, accounted for 70% of the acid-precipitable RNA released after a 15 minute incubation at 36°.

**Contribution of Extranuclear Ribosomes**

In order to ensure that nuclear RNA was actually released from the isolated nuclei, the following experiments were performed to determine what if any was the contribution of the release of labeled extranuclear ribosomes to the apparent transport. Extranuclear ribosomes are those that are on the fragments of the endoplasmic reticulum that are still attached to the isolated nuclei (it is known that the outer nuclear membrane is continuous with the endoplasmic reticulum (195), and those nonattached cytoplasmic ribosomes, or subunits that are cytoplasmic in origin and which are not
Figure 1 - Radioactivity and absorbancy (260 nm) profiles of nucleoprotein components released to the complete medium from 2 hour pre-labeled nuclei during a 12 minute incubation at 36°C. Further details are given in Materials and Methods.
procedure methods employed. As shown in Table 3, the specific radioactivity of the 18S and 28S RNA released from nuclei, prelabeled in vivo for 2 hours with orotic acid-6-\textsuperscript{14}C, is 10 times higher than that of the cytoplasmic ribosomes of the same cells from which the nuclei were isolated. By comparison, the specific radioactivity of the 18S and 28S RNA released from nuclei, prepared after a 5-day in vivo labeling, is essentially the same as that of the cytoplasmic ribosomes from the same cells. The actual labeling was accomplished by the administration 17 hours postoperatively of 100 \textsuperscript{14}C uCl/250 g orotic acid-6-\textsuperscript{14}C and sacrificing the animal five days later. The nuclear pool of labeled rRNA precursors is essentially depleted after this period, hence any radioactivity associated with these nuclei would have to be contributed by cytoplasmic contaminants of high specific radioactivity. The presence of some cytoplasmic contaminants in the standard purified nuclear preparation is observed in the controls held at 0\textdegree or, as will be detailed in experiments described below, incubations at 30\textdegree and 36\textdegree without an added energy source. When the nuclei from the five day labeled cells are incubated in the complete medium at 36\textdegree for 15 minutes, there is no significant release of RNA over and above that detected in the 0\textdegree control.

It is known that the 60S subunits of the membrane bound ribosomes are very tightly bound to the cytoplasmic
TABLE 3

SPECIFIC ACTIVITIES OF RIBOSOMAL RNA ISOLATED FROM THE CYTOPLASM OR RELEASED FROM NUCLEI OF LABELED LIVER CELLS

Nuclei were prepared from the regenerating rat liver either 2 hours or 5 days after administering 50 μCi or 100 μCi respectively, of orotic acid-6-14C to 250 gm rats 17 hours post-operative. The ribosomal RNA was extracted from the post-mitochondrial supernatant (9000 xg for 10 min.) prepared from 1 gm of liver. The nuclei prepared from the residual liver was incubated in the complete medium at 36°C for 15 minutes (cf. Table 1). The phenol purified RNA from the ribosomes and medium (in amounts detectable by U.V. absorption) was separated on density gradients as for Chart 2. The specific radioactivities were estimated from the absorbancy and radioactivity profiles in the region of the peaks of radioactivity in the 18S and 28S RNA species.

<table>
<thead>
<tr>
<th>Duration of labeling in vivo</th>
<th>Specific Radioactivity (cpm/A260)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytoplasmic Ribosomes</td>
</tr>
<tr>
<td></td>
<td>18S</td>
</tr>
<tr>
<td>2 hours</td>
<td>15,300</td>
</tr>
<tr>
<td>5 days</td>
<td>13,070</td>
</tr>
</tbody>
</table>
membrane (219). From this we might deduce that most of the radioactive rRNA recovered from the incubation medium, following incubation of the nuclei at 0°, is contributed by free cytoplasmic ribosomes which contaminate the nuclear preparation, and not by ribosomes attached to the outer nuclear membrane. In conclusion, the above results indicate that most of the labeled 28S and 18S rRNA recovered from the medium after incubation at 36° of isolated nuclei prelabeled in vivo for 2 hours, is predominantly of nuclear and not cytoplasmic origin.

Energy Requirement

A time course study of the release of RNA by purified nuclei (Figure 2) indicates that there is an initial burst of transport in the first five minutes of incubation in the complete medium at 36°. There follows a sharp decline in the rate of release, the release eventually stopping within 30 minutes of the start of the incubation. A control experiment in which ATP and phosphoenolpyruvate are omitted shows almost no RNA release, demonstrating the energy-dependence of this system. This RNA release must be an active catalytic process, since incubation in the complete medium at 0° results in no RNA release. There is a detectable amount of radioactive RNA present in both the control
Figure 2 - Kinetics of the release of labeled RNA from pre-labeled nuclei in the complete medium at 0°C or 36°C. The data represent the percentage of nuclear radioactivity (zero time) released in an acid precipitable form to the complete medium (●); the complete medium, with the further addition of 2.5 mM PEP at 15 minutes (○); the complete medium with the further addition of 2.5 mM PEP at each 5 minute (cf arrows) interval (■); the complete medium incubated at 0°C, (■); and the complete medium minus ATP and PEP (▲). Note that the release at 0°C, or at 36°C without an added energy source does not increase with time, indicating that this radioactive RNA is present as a contaminant in the nuclear preparation.
RNA RELEASED (% OF NUCLEAR cpm)

DURATION OF INCUBATION (MIN)
held at 0° C and in the incubation mixture minus energy. However, this radioactivity, which is present in the freshly prepared nuclei, does not increase with time indicating that it is a contaminant in the nuclear preparation. This contaminant was subtracted as background in all subsequent experiments.

The decline in transport seen in Figure 2 after 5 minutes incubation at 36° (a subsequent study, Figure 9), carried out at 30° with measurements at one minute intervals, indicates that there is a considerable leveling off after 4 minutes incubation) is brought about by the depletion of the energy supply. This depletion is due to both the energy requiring catalytic transport process and the nonspecific action of phosphatases present in the cytosol. The RNA release can be reactivated with the addition of 2.5 mM phosphoenolpyruvate after 15 minutes of incubation. Maximal release of RNA is achieved if PEP is added at 5 minute intervals to the incubation mixture. This maximal release of RNA (32% of nuclear label in 45 minutes) displays a biphasic curve. A curve of this nature is expected since initially, the transported ribosomal subunits are those which were completed in vivo; subsequent transport depends upon processing of the rRNA precursor in vitro. The in vivo processing of the 45S precursor to the completed ribosomal subunits is
known to be achieved in approximately 30 minutes (192). Further experiments on the energy dependence of the release of RNA, outlined below, confirmed and extended these findings.

All later studies, except where indicated, were carried out at 30° instead of 36° in order to slow down the release of RNA and to render the effects of other factors more readily observable. In addition, the duration of most of the later incubations was 10 minutes at 30° with just one initial addition of energy. This was done in order to standardize the assay conditions, and make possible comparisons between separate experiments.

One of the latter experiments was based on an approach similar to that used to demonstrate the ATP-dependence of the release of viral RNA from isolated nuclei (220). According to this procedure one aliquot of the nuclei is incubated at 30° for 10 minutes in the absence of ATP and the kinase system, while the other aliquot is held at 0°. After the preincubation of one aliquot, energy is added to both, which are then incubated at 30° for 10 minutes. As shown in Figure 3 there is no release of labeled RNA in the absence of ATP. Furthermore, there is no appreciable difference in the rate of release of labeled RNA from the control nuclei and from those preincubated at 30° in the absence of energy.
Figure 3 - Effect of preincubating regenerating liver nuclei in the absence of an energy source on subsequent RNA transport. ATP was added either at the beginning of the incubation at 30° (■) or after a 10 minute preincubation at 30° (●). The duration of the incubation in the presence of ATP was 10 minutes.
These results suggest that the intranuclear RNA processing is energy-dependent, or that transport is necessary for processing to proceed. This experiment also demonstrates the intactness and stability of the isolated nuclear system.

The results of a more detailed study of the actual ATP requirements of the system are listed in Table 4. This experiment was actually done to rule out the possibility that the ATP was merely playing some chelating role, thereby causing for example the opening of nuclear pores, in this system, rather than providing actual energy for an active catalytic process. The results do indeed indicate that most of the RNA transport is dependent upon the hydrolysis of the high-energy beta, gamma-pyrophosphate bond of ATP. Phosphoenolpyruvate (2.5 mM) and PEP Kinase, in this system, regenerate sufficient ATP to maintain maximal transport for at least 10 minutes. Without phosphoenolpyruvate, the ATP is exhausted within 2.5 minutes at 36° and transport is greatly reduced. No significant difference is obtained whether the initial concentration of ATP is 2 mM or 3 mM. Both GTP and alpha, beta-methylene-ATP are seen to be equally as efficient as ATP. However substitution of ATP with beta, gamma-methylene ATP, or AMP in this system, reduces transport to 15% of the control. Since the beta, gamma bond in beta, gamma methylene
TABLE 4
ENERGY DEPENDENCE OF THE RNA RELEASE

Nuclei, isolated from the 19 hour regenerating liver following a 2 hour pre-labeling in vivo with orotic acid \(-6^{14}\text{C}\) (200 \(\mu\text{Ci/kg}\)) were incubated in the cell-free system for 5 minutes at 36\(^\circ\text{C}\). Aliquots of the nuclei-freed medium (1000 xg supernatant) were assayed for acid-insoluble radioactivity; the latter is corrected for the radioactive RNA released at 0\(^\circ\text{C}\). The complete incubation medium was modified as indicated.

<table>
<thead>
<tr>
<th>Energy Source</th>
<th>Acid-insoluble Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm/ml or (4\times10^6) nuclei</td>
<td>% of control</td>
</tr>
<tr>
<td>1. 2.5 mM PEP + 2 mM ATP</td>
<td>1,062</td>
</tr>
<tr>
<td>2. 2.0 mM ATP</td>
<td>552</td>
</tr>
<tr>
<td>3. 3.0 mM ATP</td>
<td>568</td>
</tr>
<tr>
<td>4. 2.0 mM GTP</td>
<td>541</td>
</tr>
<tr>
<td>5. 2.0 mM AMP</td>
<td>170</td>
</tr>
<tr>
<td>6. 2.0 mM alpha-beta-methylene ATP</td>
<td>520</td>
</tr>
<tr>
<td>7. 2.0 mM alpha-gamma-methylene ATP</td>
<td>158</td>
</tr>
<tr>
<td>8. No potential energy source</td>
<td>0</td>
</tr>
</tbody>
</table>
ATP is resistant to hydrolysis, like AMP it cannot furnish energy. The 15% background transport in both instances might be due to some physical effect on the nuclear pores.

**Nature of Material Transported**

Sucrose density gradient profiles of the RNA extracted from the incubation medium by the use of phenol (210, 192) show that a major portion of the transported RNA is ribosomal. About 80% of the labeled RNA is recovered following the phenol extraction. Figures 4 and 5 show typical radioactivity profiles of the size distribution of the RNA released to the medium from regenerating liver nuclei, as well as the size distribution of the nuclear RNA before and after incubation in vitro at 36° for 15 minutes (UV absorbance profiles are not shown, for the amount of RNA extracted and separated on the gradient is below the level of detection at 260 nm). The sedimentation constants indicated in Figures 4 and 5 were established using UV detectable amounts of extracted cytoplasmic, or nuclear RNA, separated on identical gradients. The values obtained are in agreement with those of previous studies (129).

There is a small release of 18S and 28S RNA, presumably as 40S and 60S subunits, in addition to the release of 4 and 7 S species by the nuclei in complete medium held at 0°.
Figure 4 - Radioactivity profiles of the RNA released to the complete medium from nuclei pre-labeled 2 hours in vivo.
Figure 5 - The distribution of radioactivity in the residual RNA in these nuclei following incubation for 15 minutes at 36°C or 0°C. The RNA purified from the medium following removal of the nuclei, or from the nuclei, was separated on 10-30% linear gradients (cf. Materials and Methods).
Upon raising the temperature to $36^\circ$, there is a three fold increase in labeled RNA release during the 15 minute incubation. Quantitation of the areas under the radioactivity profiles of Figure 4 shows that the 18S and 28S fractions account for approximately 65% of the released counts. The ratio of the 28S to 18S RNA (Figure 4), corrected for the 0° control, and the ratio of the 60S to 40S subunits (Figure 1), are both approximately 3.0 instead of the expected 2.0, due to differences in the pool size of these components in the nucleus (221).

In similar studies carried out by Schumm et al (217) designed to study the release of messenger RNA from isolated nuclei, an in vivo prelabeling period of 30 minutes or less was used. Sucrose gradients of the presumed mRNA revealed that it sediments predominantly in the lighter (10S to 14S) regions of the gradient. In vivo studies (222) also demonstrate that the mRNA appearing in the cytoplasm of rat liver cells within 1 hour of the administration of labeled precursor, sediments mostly in the 12S region of the gradient with only slight overlap in the 18S and 28S regions.

**Competition-Hybridization Studies**

The identity of the transported RNA was also tested using competition hybridization techniques, whereby one can
measure qualitative differences of various RNA species. The estimation of the % ribosomal RNA in a particular RNA preparation was done as follows:

Purified DNA is attached to special nitrocellulose filters. Two of these filters, with bound DNA, are incubated with purified unlabeled ribosomal RNA. Segments of the immobilized DNA that have a complementary sequence to the rRNAs (rRNA genes), will hybridize to the rRNAs in the incubation, forming stable, RNase resistant double stranded regions. After incubation, the filters are treated with RNase and washed in specific buffers. The radioactively labeled RNA preparation that is being studied, is then incubated with three separate sets of filters: blank filters, to test for nonspecific attachment of RNA to the nitrocellulose filter (background); filters with bound DNA that have not been incubated with anything; and the filters with bound DNA that were incubated with the unlabeled (competitor) ribosomal RNA. After incubation the filters are treated with RNase, washed with buffers and then dissolved in scintillant for counting.

The counts attached to the DNA that was not previously incubated with competitor RNA, represent the total radioactivity of the RNA in the sample. The labeled RNA that hybridized to the DNA that was incubated previously with
competitor cold rRNA, represents all labeled RNA species in
the sample other than ribosomal RNA. Thus the difference
between the two values represents the fraction of the sample
made up by ribosomal RNA. The detailed experimental pro-
cedure is outlined in the methods section.

RNA extracted and purified from the medium following
a 15 minute incubation, that hybridized with rat liver DNA
on filters (65 µg DNA per B-6 filter) showed over 60% loss of
binding in the presence of 5 or 10 mg. of competitor rRNA.
In comparable studies on mRNA release by Schumm et al.(217),
the hybridization of only 10% of the released labeled RNA to
DNA was inhibited by saturating amounts of rRNA if the nuclei
were prelabeled in vivo for only 30 minutes with orotic acid
-6-14C. Upon longer incubation of the nuclei (up to 30 min-
utes) with extra additions of energy, it was found that the
proportion of ribosomal RNA, of the total released RNA, in-
creased up to 70% of the total. This might be the result of
a more rapid decrease in the transport of nonribosomal RNA
and a reflection of the greater reserve of rRNA precursors
in the nucleus.

It has been determined that the nuclear heterogeneous
RNA (precursor of mRNA) is more rapidly labeled than is the
rRNA precursor (223). This anomalous labeling will result in
different specific activities of the mRNA and rRNA species,
hence our estimate of the actual rRNA transported is probably low.

**Differences in the Profiles of RNA transported by**

**Resting and Regenerating Rat Liver Nuclei**

A density gradient profile of the transported (cytoplasmic) RNA shows three prominent peaks. The two heaviest peaks are 28S and 18S rRNA; the lighter peak is known to contain mRNA, tRNA and 5S and 7S rRNA (205). The gradient was standardized as outlined in Methods. After comparing numerous profiles from both regenerating and resting liver nuclei, a pattern becomes evident. The profile from regenerating liver nuclei (Figure 6) always shows a greater amount of the two rRNA peaks in relation to the "light" peak. In addition, there is often discernible a small shoulder on the lighter side of the 18S peak which might be some mRNA species (This interpretation is supported by the findings of Schumm *et al.* (217). In the profile of resting nuclei (Figure 7), both ribosomal peaks are considerably smaller in relation to the light peak.

Incubating both types of nuclei in either homologous cytosol, or in cytosol from Hepatomas 5123-D or 7800, does not result in any greatly noticeable differences in profiles. Other studies by Schumm *et al.* (217, 224) show, however, that there are pronounced differences in the types of mRNA trans-
ported in response to various supernatants, as measured by hybridization - competition techniques.

Requirements for Nondialyzable Factors and Polyamines

Omitting cytosol from the incubation medium decreases markedly the release of RNA during a 12 minute incubation of 36° as seen in Table 5. The release of RNA that does occur in the absence of cytosol is probably due to nuclear lysis since a decrease occurs in the number of nuclei observed under the light microscope. Later experiments confirm that the release of RNA is indeed caused by lysis in the absence of cytosol, for the released RNA increased very sharply with increasing incubation time. The cytosol requirement is specific and not merely a nonspecific protein or osmotic effect, for substitution of bovine serum albumin, as shown in Table 5, does not prevent the lysis of the nuclei. It has been reported earlier (225) that spermine and spermidine are necessary for maintaining nuclei integrity. And indeed we have found lysis of the nuclei occurs in the absence of spermine or spermidine. However, spermidine is the polyamine employed in this system since 5 mM spermine inhibits not only nuclear lysis but also the release of nuclear RNA. Omitting the energy source prevents nuclear lysis and RNA release in the absence of cytosol.

The observations may be summarized as follows: Both spermidine and cytosol are required for maintaining the
Figure 6 - Radioactivity profiles of RNA released to the complete medium from regenerating liver nuclei prelabeled 2 hours \textit{in vivo} with orotic acid-\textsuperscript{6-\text{14}C}. The RNA was separated on 10 to 30% linear sucrose gradients.
Figure 7 - Radioactivity profiles of RNA released to the complete medium from resting liver nuclei prelabeled 2 hours in vivo with orotic acid-6-$^{14}$C. The RNA was separated on 10 to 30% linear sucrose gradients.
TABLE 5
REQUIREMENTS FOR NON-DIALYZABLE FACTORS IN THE CYTOSOL AND POLYAMINES

The acid-insoluble cpm released from the 2 hour pre-labeled nuclei incubated for 12 minutes at 36°C have been corrected for the cpm present in the controls similarly incubated at 0°C. Bovine serum albumin and spermine were each added to the medium in a final concentration of 10 mg/ml and 5 mM respectively. In those experiments where energy (i.e. 20 mM ATP and 2.5 mM PEP) was omitted from the incubation medium UDP was added to a final concentration of 5 mM. The nuclear counts were reproducible to within 10 percent. The standard errors are recorded for the acid-insoluble cpm released per ml of incubation mixture (based on 3 experiments).
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incubation Medium</th>
<th>Radioactivity in Released RNA</th>
<th>% of Control</th>
<th>% of Nuclear Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm/ml or 4x10^6 nuclei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Complete (control)</td>
<td>1, 383 ± 171</td>
<td>100</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2. (1) Minus cytosol</td>
<td>663 ± 48</td>
<td>48</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>3. (2) Plus albumin</td>
<td>1, 569 ± 199</td>
<td>113</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>4. (1) Minus spermidine</td>
<td>3, 758 ± 213</td>
<td>272</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>5. (2) Minus spermidine</td>
<td>4, 147 ± 150</td>
<td>300</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>6. (4) Plus spermine</td>
<td>327 ± 20</td>
<td>24</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>7. (5) Plus spermine</td>
<td>128 ± 25</td>
<td>9</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>8. (1) Plus spermine</td>
<td>216 ± 5</td>
<td>16</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>9. (1) Minus energy, plus UDP</td>
<td>430 ± 15</td>
<td>33</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>10. (9) Minus supernatant</td>
<td>38 ± 8</td>
<td>3</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>11. (10) Minus spermidine</td>
<td>2, 196 ± 200</td>
<td>157</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>12. (9) Minus spermidine</td>
<td>2, 690 ± 210</td>
<td>193</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

*Nuclear counts were precluded since the nuclei aggregated under these conditions.*
integrity of the nuclei, and both energy and cytosol are obligatory for the transport of RNA from intact nuclei. The observed lysis in the absence of cytosol is enhanced by the presence of an energy source (ATP and PEP).

The Nuclear Stabilizing Effect of Cytosol

As discussed above, it has been found that cytosol is necessary for both maintaining the integrity of the isolated nuclei, and promoting release of nuclear RNA. In order to detect the presence of possible factors in the cytosol which promote ribosome transport, the effect of reducing the cytosol protein concentration in the incubation medium on RNA transport was studied. The results of an early study of this phenomenon are depicted in Figure 8. It is a time course study of the release of rRNA from isolated prelabeled (2 hours, orotic acid-6-\(^{14}\)C) resting liver nuclei, in response to 2 different cytosol concentrations and omission of cytosol. We see that at the higher cytosol concentration (19.6 mg. protein/ml) a typical release curve, similar to the one in Figure 2, is obtained with a plateauing after 5 minutes incubation. At the low cytosol concentration (2.5 mg. protein/ml) there is a greatly decreased release of RNA. The complete omission of cytosol causes an exponential release of RNA after 5 minutes incubation which is the result
of nuclear lysis. Extensive studies determined that the reduction of the protein concentration in the incubation medium below 2.5 mg/ml resulted in increasing amounts of nuclear lysis.

It must be noted that the cytosols used in the experiments shown in Figure 8 had been dialyzed against distilled water and had subsequently been stored frozen. Later investigations revealed that such a treatment resulted in some loss of the cytosol activity that facilitates release of RNA from the nuclei. Such a loss in activity is especially evident when diluted cytosol fractions are studied.

In Figure 9 we see the results of a study comparing the effects of freshly prepared cytosol dialyzed against TKM buffer, with cytosol dialyzed against H₂O and subjected to freeze-thawing. A considerable difference in activity is seen between the two cytosols, at low protein concentration, the freshly prepared cytosol releasing considerably more RNA. The nuclei were maintained intact in both preparations at cytosol concentrations of about 2.5 mg/ml or higher.

Thus there appears to be macromolecules present in the cytosol which promote RNA transport and which are subject to inactivation during dialysis against water and freeze-thawing. The macromolecular factors which facilitate RNA
Figure 8 - The time course of release of labeled RNA from nuclei prepared from normal liver to medium containing zero (X), 2.45 (■), or 19.6 (●) mg of cytosol protein per ml. of reaction mixture. Each incubation mixture contained approximately $10^6$ nuclei/ml, which in turn contained 30,000 acid precipitable counts.
Figure 9 - Time course of the release of labeled RNA from nuclei prepared from normal liver to medium containing 2.45 (●) or 19.6 (○) mg per ml of reaction mixture of cytosol protein, after dialysis of the latter against water and storage at −15° for 24 hours or (2) 2.45 (▲) or 19.6 (△) mg per ml of reaction mixture of cytosol protein after dialysis against water and storage at −15° for 7 days. Each incubation medium contained approximately $10^6$ nuclei per ml, which in turn contained 30,000 acid precipitable counts.
transport seem to be limiting in such preparations at the lower protein concentrations. The results of these experiments not only provide evidence for cytosol-facilitated transport, but also differentiate between the nuclear stabilizing and transport factors.

Incidentally, the kinetics of RNA release were determined at one minute intervals for the first 5 minutes, for the high concentration freshly prepared cytosol as shown in Figure 9. We see that the slope of the release curve decreases somewhat throughout the first 5 minutes of incubation, but noticeably so after the 4 minute point.

**Characterization of the Cytosol Concentration Dependence**

The release of RNA from prelabeled nuclei of resting liver as a function of the concentration of resting liver cytosol in the incubation medium follows the curve shown in Figure 10. The incubation was carried out at 30°C for 6 minutes, and each point represents the labeled RNA transported from 10^6 nuclei. The resulting curve is essentially sigmoidal in nature. Such a relationship might be expected on the basis of a feedback control mechanism. Because of the observed sigmoidal relationship, further experiments of the transport factors were performed at cytosol protein concentrations falling on the linear portion of the curve.
Figure 10 - The release of labeled RNA from prelabeled nuclei of normal liver as a function of the concentration of homologous cytosol. Each 6 minute assay contained $10^6$ nuclei per ml which in turn contained 11,000 acid precipitable counts.
DEAE Cellulose Fractionation of Cytosol

Among the techniques used in trying to fractionate and purify the transport factor(s), DEAE-Cellulose column chromatography was most successful. Undialyzed, freshly prepared regenerating liver cytosol was passed through a DEAE-Cellulose column, pre-equilibrated to pH 8.5 at 4°C. At this pH almost half of the proteins in the cytosol fraction bind to the column, while the more basic ones pass through. The adsorbed proteins were eluted in a stepwise fashion with 0.6 M KCl pH 7.5. Both fractions were extensively dialyzed against TKM buffer to remove salts, before assay. The RNA transported from regenerating liver nuclei in the presence of each fraction was extracted with phenol and analyzed on sucrose gradients. As can be seen from Figure 11, the fraction which adsorbed to DEAE-Cellulose shows the greatest stimulatory activity for the release of 18S and 28S ribosomal RNA, more specifically, some 4 times more than the non-adsorbing fraction. However, both fractions induce the release of approximately equal amounts of the lighter (4 - 10 S) RNA species. Whether the light 4 - 10 S RNA species differ in response to each cytosol fraction has not been determined. It is of interest that in similar studies on the mRNA release (224), it has been observed that under similar experimental conditions most of the enhancement for
Figure 11 - Relative size distribution of the RNA released from regenerating liver nuclei to medium containing the cytosol fractions which adsorb (●) and which do not adsorb (○) to DEAE-cellulose. Both fractions were added to the assay mixture in a concentration equivalent to 12.0 mg per ml of original homologous cytosol. The incubation were carried out for 10 minutes.
mRNA transport is concentrated in the non-adsorbing cytosol fraction, in contrast to the adsorbing fraction tested in the present study.

**Ammonium Sulfate Fractionation**

The factors which facilitate ribosomal RNA release from isolated nuclei were also partially purified by ammonium sulfate fractionation, details for which are given in the methods section. Table 6 shows that most of the RNA transport factor activity is located in the protein fraction that precipitates between 20 and 60% ammonium sulfate saturation.

**Diaflow Filtration**

Diaflow filtration is a simple method for separating macromolecules according to molecular size. In this technique, the mixture to be fractionated is forced through a filter of uniform pore size by air pressure; molecules too large to pass through the specific filter are concentrated, while smaller ones are removed in the effluent. Three types of filters, able to retain molecules larger than 10,000, 30,000 and 50,000 approximate molecular weight, were used.

The results obtained with this approach, however, were variable and difficult to interpret. A possible explanation for the difficulties encountered, is that the method probably separated and concentrated cytoplasmic rat liver
### TABLE 6

SALT FRACTIONATION OF NORMAL LIVER CYTOSOL

<table>
<thead>
<tr>
<th>(NH₄)₂SO₄ Fraction (% Saturation)</th>
<th>Total Units of Transport Activity (cpm transported/mg protein X % total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 - 10S</td>
</tr>
<tr>
<td>Unfractionated cytosol</td>
<td>100</td>
</tr>
<tr>
<td>0-20%</td>
<td>11</td>
</tr>
<tr>
<td>20-60%</td>
<td>75</td>
</tr>
<tr>
<td>60-95%</td>
<td>14</td>
</tr>
</tbody>
</table>
ribonuclease (molecular weight less than 30,000 (226)), and ribonuclease inhibitor (M.W. 50,000 (227)), along with the sought after cytoplasmic factors in the various fractions.

Protein Nature of the Cytoplasmic Factors

The foregoing results, particularly those based on the transporting activity of the cytosol fractions, suggest that the release of ribosomal subunits from the nucleus is dependent upon positive feedback by macromolecules (probably proteins) in the cytosol. That these macromolecular components of the cytosol are protein in nature is indicated by their heat lability. As shown in Table 7, a 25 to 30% loss of transport activity follows the heating of cytosol from resting liver, or Hepatoma 5123-D for 10 minutes at 50°C at pH 7.5. Heating the cytosol at higher temperatures or longer periods of time was not feasible, for it resulted in the loss of the cytosol's ability to maintain the isolated nuclei intact.

These macromolecules are not, however, ribosomal proteins because in experiments where ribosomal proteins (isolated from purified ribosomes) were added to the incubation medium, there was observed either no effect or an actual slight inhibition of transport. These results were observed
TABLE 7
HEAT LABILITY OF TRANSPORT FACTORS<sup>a</sup>
IN THE CYTOSOL

<table>
<thead>
<tr>
<th>Source of cytosol</th>
<th>Transport Activity&lt;sup&gt;b&lt;/sup&gt; after heating for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero min.</td>
</tr>
<tr>
<td>1. Normal liver</td>
<td>384 ± 25</td>
</tr>
<tr>
<td>2. Hepatoma 5123D</td>
<td>388 ± 28</td>
</tr>
</tbody>
</table>

<sup>a</sup>The protein concentration of the cytosol from normal liver and from Hepatoma 5123D was adjusted to 20 mg/ml and triplicate samples of each were heated at 50° for 10 minutes, then centrifuged. The supernatants from the latter and corresponding aliquots of the unheated cytosol were tested for transport activity in an assay containing prelabeled nuclei from normal liver.

<sup>b</sup>The activity is recorded as cpm transported in RNA to 0.4 ml aliquots of incubation medium during a 10 minute incubation at 30°.
in various systems, including those containing the adsorbing and nonadsorbing DEAE-cellulose fractions. In systems where the cytosol in the complete medium is substituted by 50 μg ribosomal proteins/ml, the RNA release is the result of nuclear lysis.

Dependence of In-Vitro Nuclear RNA Processing on Cytosol and Energy

An earlier experiment described above (Figure 5), indicated that the processing of nuclear rRNA species takes place during a 15 minute incubation at 36°. It was of interest to study this process more closely and to determine the effect on RNA transport of the various components of the system; in particular the energy and cytosol components. Isolated nuclei from regenerating liver were incubated in homologous supernatant for 10 minutes at 0°, or 30° with ATP, and at 30° without ATP. The nuclei were isolated from the incubation mixtures and their RNA extracted by hot phenol, then fractionated on sucrose gradients. Figure 12 shows the profiles of the nuclear RNAs from each of the three incubation mixtures. It can be seen that there is significant processing of the 45S ribosomal precursor at 30° even in the absence of energy, although this processing is much less than in the presence of ATP. Since there is considerable release
Figure 12 - Radioactivity profiles of the residual RNA in the nuclei following incubation for 10 minutes with ATP at 0° (■), without ATP at 30° (○) and with ATP at 30° (▲). Nuclei, prelabeled in vivo for 2 hours with orotic acid-6-\textsuperscript{14}C (15 μCi/250 gm body weight) were purified from 3.5 gm of regenerating liver, then divided into 3 equal aliquots for incubation in complete medium, or the latter minus ATP. The nuclear RNA was analyzed on 28 ml (10-30%) sucrose gradients.
Figure 13 - Radioactivity profiles of the residual RNA in the nuclei following incubation for 10 minutes in complete medium (12 mg of cytosol protein per ml) at 0° (■) or 30° (△) or at 30° in medium containing a suboptimal (2.4 mg/ml) amount of cytosol protein (○). Prelabeling, incubations and size analysis of the residual nuclear RNA was the same as that for Fig. 12 except that 26 ml (10-30%) sucrose gradients were used.
of rRNA in the presence, but not in the absence of ATP, it is not presently clear whether the increase in processing is due to a direct action of ATP at the site of processing, or is an indirect result of a coupling between processing and transport.

A similar study of the effect of cytosol on nuclear RNA processing is shown in Figure 13. Since the complete omission of cytosol will result in nuclear lysis, optimum levels of cytosol protein concentration were compared to suboptimal levels (12 mg protein/ml v.s. 2.5 mg protein/ml respectively) as described above in Figures 9 and 10. After a 10 minute incubation at 30°, the nuclei from the optimal system had a markedly greater processing of the 45S precursor and a shift of the major nuclear precursor RNA to lighter RNA species which represent terminal ribosomal products. Since about 6% of total nuclear counts are transported in the optimal system, versus 2% in the suboptimal system, as in the case of ATP enhancement of processing, it cannot be concluded with any certainty whether the cytosol factors act directly on processing, or just act on transport, which in turn is coupled to processing.

The Effect of Camptothecin on rRNA Processing and Transport

Recent studies have shown that the plant alkaloid, camptothecin, inhibits the synthesis of RNA and DNA in
cultured mammalian cells (228). One study (229) also suggested that camptothecin blocks a specific step in the processing of ribosomal precursor RNA in HeLa cells, allowing the conversion of 45S RNA to 32S RNA, but inhibiting the conversion of 32S RNA to 28S RNA. If the latter is indeed true, its mode of action would be amenable to study in our cell-free system.

Isolated prelabeled nuclei from regenerating liver were therefore preincubated without energy for 5 minutes at 30° in the standard medium, minus energy, but in the presence of camptothecin (20 μg/6 ml incubation volume). After the preincubation, energy was added and the incubation carried out for 10 minutes along with preincubated controls. Analysis of the transported and nuclear RNA species, however, did not reveal any detectable effect of camptothecin. However, as shown in Figure 14, injection of camptothecin into a rat (3.5 mg/Kg) 15 minutes prior to a 2 hour in vivo labeling with orotic acid-6-14C, resulted in a greatly reduced incorporation of label into the nuclear RNA, in comparison to an untreated control. However, there does not seem to be any aberrant processing of ribosomal precursor RNA during the 10 minute incubation at 30°, when compared to a profile of nuclear RNA from the nuclei of the untreated rat. These observations agree with the findings that camptothecin
Figure 14 - Radioactivity profiles of the residual RNA in the nuclei of a camptothecin treated rat, following incubation for 10 minutes at 0° (●), and 30° (○). RNA profiles of nuclei from untreated rat, following incubation for 10 minutes at 0° (●), and 30° (○). Nuclei were prelabeled in vivo for 2 hours with orotic acid-6-¹⁴C (15 uc/250 gm body weight).
inhibits the synthesis of RNA, but do not support the proposal (229) that the alkaloid interferes with rRNA processing.

Studies on Pactamycin Inhibition of Protein Synthesis

Pactamycin is an antibiotic which inhibits protein synthesis in both bacterial and mammalian systems; it appears to exert its main effect by inhibiting the initiation step of protein synthesis (230), i.e., the stepwise addition of the 40S and 60S ribosomal subunits to the 5'-end of the mRNA. It seemed worthwhile to investigate the unique mode of action of this antibiotic as a follow up to the studies of Rizzo & Webb (129, 140) which showed that inhibitors of protein biosynthesis which maintained the structure of the polyribosome and therefore the normal size of the monomer pool (i.e. anisomycin and cycloheximide) did not affect the transport of ribosomal subunits to the cytoplasm while inhibitors which caused the conversion of polyribosomes to monomers (i.e. purcmycin, sparsomycin and 8-azaguainine) drastically inhibited this transport. The relative size of the 40S and 60S ribosomal subunit pools was not measurably affected by either class of inhibitor.

Treatment of a rat with as little as 0.5 mg/Kg of pactamycin for 60 minutes, resulted in almost complete inhibition of protein synthesis as determined by L-leucine-14C
incorporation into cytosol protein (cf. Methods section under "Specific radioactivity of soluble protein"). According to this procedure, the rat was injected with 20 μc labeled leucine 50 minutes after the pactamycin injection, in order to determine the rate of ongoing protein synthesis, then sacrificed 10 minutes later.

In order to study the effect of pactamycin on polyribosome and monomer pools, an analysis of the size distribution of ribosomal components was carried out on pactamycin treated rat liver, as outlined under Methods. As shown in Figure 15, no dramatic difference is apparent between the ribosomal components in the liver of the pactamycin-treated rat and the control. If anything, the monomer peak from the pactamycin sample is smaller than the control, although a slight decrease in the polyribosome pool is also observed. These apparently opposing changes may indicate that there is an increase in the subunit pools, which is not resolved by the present sucrose density gradient technique, but a phenomenon which would also be in agreement with the mechanism of action proposed by Cohen et al. (230). Since the monomer pool was not affected, it was not considered relevant to continue the study further.

In-Vitro Synthesis

The success of the in vitro system for studying the
Figure 15 - Size distribution profiles of the polyribosomes and the relative size of the monomer pool in the deoxycholate-treated supernatant prepared from the liver of a rat which was treated for 1 hour with 2 mg/Kg of pactamycin (---), and a control rat (---). The monomer peak is located between fractions 20 and 25.
transport of RNA encouraged us to attempt to adopt the basic system to the study of the in vitro synthesis of ribosomal RNA in isolated nuclei. The composition of a successful modified system, developed in cooperation with Dr. D. J. McNamara in this laboratory, is outlined in Table 9. The assay for rRNA synthesis in isolated nuclei consisted of incubating the nuclei for 30 minutes at 30° in the media described in Table 9. It will be noted that the media contains salts, cofactors, cytosol, nucleotide triphosphate precursors, an energy regenerating system and tritium labeled UTP. After the incubation, the nuclei were extracted by the hot phenol method, and the size distribution of the purified RNA was analyzed by means of disc-gel electrophoresis. The profiles of radioactively labeled nuclear RNA, synthesized under 3 different conditions, are shown in Figure 16. In the presence of alpha-amanitin, a potent inhibitor of nucleoplasmic RNA synthesis (79), there is a decrease of label incorporation into the heavy RNA species. Most striking and relevant, is the observation that in the absence of cytosol, almost no synthesis of the heavier RNA species takes place, indicating that as in the case of nucleocytoplasmic transport of ribonucleoprotein, cytoplasmic factors play a regulatory role in RNA synthesis as well.

While this study was in progress, a number of recent reports described similar observations; e.g., RNA synthesis
### TABLE 8

**COMPOSITION OF THE IN-VITRO SYNTHESIZING MEDIUM**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzed cytosol</td>
<td></td>
</tr>
<tr>
<td>Tris buffer (pH 7.5)</td>
<td>50 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>DTT</td>
<td>2.0 mM</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 mM</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>Spermidine</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>Yeast RNA</td>
<td>500 µg/ml</td>
</tr>
<tr>
<td>L. Cysteine</td>
<td>0.25 mM</td>
</tr>
<tr>
<td>PEP</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>35 e.u./ml</td>
</tr>
<tr>
<td>GTP</td>
<td>0.8 mM</td>
</tr>
<tr>
<td>CTP</td>
<td>0.8 mM</td>
</tr>
<tr>
<td>ATP</td>
<td>2.0 mM</td>
</tr>
<tr>
<td>UTP</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>³HUTP</td>
<td>4.0 uc/ml</td>
</tr>
</tbody>
</table>
Figure 16 - Radioactivity profiles of newly synthesized RNA, extracted from nuclei incubated for 30 minutes in the presence of cytosol (■), with cytosol and alpha-amanitin (▲), and without cytosol (○). Labeling was achieved with 10 uc\(^3\)HUTP/2ml incubation mix. The RNA was analyzed by means of polyacrylamide - agarose disc - gel electrophoresis in 2.7% gels.
is stimulated in isolated mouse brain nuclei by a brain cytosol factor (231), and phosphorylated non-histone chromatin protein stimulate RNA synthesis in a rat liver cell-free system (232).

Cytoplasmic Turnover of Ribosomal RNA

The regenerating liver of the young adult rat is characterized by an early increase in ribosome formation (233) which is maximal (e.g. a four-fold increase) by the time nuclear DNA synthesis is initiated at approximately 19 hours post-operatively (177); nuclear DNA synthesis and mitosis subsequently peak at 24 hours and 31 hours, respectively (177). Despite the fact that ribosomes constitute approximately 85% of the RNA in eukaryocytes (234) and evidence (235) that resting (non-proliferating) cells contain fewer ribosomes than proliferating cells, there is little information available concerning the degree of integration between ribosome formation and cellular DNA synthesis.

The steady state concentration of ribosomes is a function not only of synthesis but also degradation of rRNA. Studies were therefore carried out on the cytoplasmic turnover rate of ribosomal RNA in three different tissues: resting rat liver, regenerating rat liver, and Hepatoma 5123-D, in order to determine whether this parameter varied
Figure 17 - Decay of specific radioactivity of resting liver ribosomal RNA following an initial labeling with 5 uc orotic acid-6-$^{14}$C/250 gm body weight.
Figure 18 - Decay of specific radioactivity of ribosomal RNA from Morris Hepatoma 5123-D tumors, following an initial labeling with 5 uc orotic acid-6-\textsuperscript{14}C/250 gm body weight.
with the growth potential of the tissues. The experimental approach used for the studies, was to label the ribosomal RNA with a single injection of orotic acid-6-\textsuperscript{14}C and then to measure the decline in specific activity (S.A. = radioactivity/Absorbance at 260 nm) of the purified ribosomal RNA over a period of about 10 days. One can determine the half-life of the rRNA from a plot of \( \log_{10} \) specific activity v.s. time. Figures 17 and 18 show two such specific activity plots of rRNA in resting rat liver and Hepatoma 5123-D, respectively. The half-life of rRNA in both tissues is observed to be the same, i.e., about 5.5 days, which is in good agreement with that reported for resting rat liver by other investigators (180, 181, 182). It was not feasible to measure the rate of cytoplasmic rRNA turnover in regenerating liver by this approach due to the complication that increased rRNA synthesis is taking place, thus decreasing the specific activity of the cytoplasmic rRNA. In any case it is concluded that the increased potential for cellular proliferation in the hepatoma cell is not due to a decrease in the turnover rate of the ribosomes.
The studies reported in this dissertation were undertaken in order to gain a further understanding of the processes involved in the regulation of ribosome formation in eukaryotic cells, using the rat liver as a test system. Specifically, these studies were directed at shedding some light on the often overlooked regulatory site at the level of post-transcriptional nuclear processing and nucleocytoplasmic transport.

The various RNA species of the eukaryotic cell are transcribed in the nucleus, from where they are then transported to the cytoplasm to play their role in the protein synthesizing systems of the cell. However, there is mounting evidence, from numerous studies, which indicates that the nucleo-cytoplasmic transport of both ribosomal RNA (152, 192) and messenger RNA (193, 194) is selective, and that a significant fraction of the transcribed RNA never leaves the nucleus, but is degraded. Furthermore, a loss of this selectivity (nuclear restriction) has been implicated in the process of neoplastic transformation (236, 237). A loss of
the nuclear restriction of messenger RNA has been observed to develop as a result of exposure of cells to carcinogens (238, 239). In spite of these dramatic findings however, relatively little work has been done to elucidate these post-transcriptional controls of RNA production. One possible reason why this area has been somewhat overlooked is the fact that our understanding of most of the basic biochemical processes such as replication, transcription and translation has been derived from work in bacterial systems, and as a result there perhaps is a lack of appreciation for such phenomena as nuclear restriction and post-transcriptional controls, which are obviously unique to eukaryotic cells. More important perhaps are the experimental difficulties encountered in trying to study these controls in eukaryotic cells. To circumvent these difficulties, as was discussed in the introduction, cell-free systems were developed (204, 205) which permitted direct observation of nucleocytoplasmic interactions and controls of RNA transport from the nucleus.

It was found that the release of ribosomal subunits from isolated nuclei is regulated by a positive feed-back mechanism involving non-dialyzable factors (macromolecules) in the cytoplasm. This release also had an absolute requirement for energy and was predictably temperature dependent.
The energy in this system was provided by added ATP and an ATP regenerating system: phosphoenol pyruvate and pyruvate kinase. That the ATP satisfied an actual energy requirement rather than exerting some secondary role such as chelation of cations or direct physical effects on nuclear pores, was established by demonstrating the inability of non-hydrolyzable methylene analogs of ATP to substitute for ATP.

Incubation of the nuclei in the complete medium at 30° for 10 minutes in the absence of ATP and the kinase system resulted in no release of ribosomal subunits. Upon addition of energy to the preincubated sample, a normal release of RNA is observed. This experiment suggests that intranuclear RNA processing is energy dependent or dependent upon ongoing transport; incidentally, the experiment also provides a functional test for the preservation of the nuclei in this system.

The macromolecular cytoplasmic factors involved in the release of ribosomal RNA adsorb to DEAE-cellulose at pH 8.5, precipitate within the range of 20% and 60% saturation with ammonium sulfate and are heat labile. Cytosol was also shown to be necessary for maintaining nuclear integrity; incubation of the nuclei in cytosol concentrations of less than 2.5 mg protein/ml resulted in nuclear lysis.
The cytosol activity regulating release of rRNA was distinguished from that responsible for the preservation of the nuclei by its greater lability upon storage, freeze thawing and dialysis in water. The sigmoidal relationship observed between ribosome transport and the concentration of the cytosol factors added to the incubation medium, is precisely what one would predict, should the cytosol factor act in a positive feedback manner.

Related studies on messenger RNA transport (205, 217, 224) also indicate that protein components in the cytosol are necessary for the release of messenger RNA from the nucleus in the form of informosomes. As opposed to the ribosomal release factors, these regulatory components do not adsorb to the DEAE-cellulose column at pH 7.5. Also the concentration-dependence curve for the messenger transport factors appears to be linear (224), the linearity being a consequence of the presence of both positive and negative feedback components.

The presence of cytoplasmic factors and energy, also appear to be necessary for the in vitro intranuclear processing of the 45S ribosomal precursor RNA, as determined from a comparison of the nuclear RNA profiles after various incubation conditions. That the in vitro processing of precursor rRNA indeed occurs, was also demonstrated in the mRNA transport system (205). In the latter studies, a thirty
minute *in vivo* prelabeling period is used, which is too short for the incorporation of radioactive uridine into completed ribosomal subunits. After prolonged *in vitro* incubation (over 30 minutes) however, label begins to appear in the released ribosomal subunits, indicating that intranuclear maturation must have occurred *in vitro*. The studies on the remaining nuclear RNA showed that after incubating nuclei (prelabeled *in vivo*, 2 hours) in the complete medium at 30° for 10 minutes, there was a marked shift of the major nuclear precursor RNA to lighter RNA species as compared to samples that were incubated without energy or with limiting amounts of cytosol factors (i.e., 2.5 mg protein/ml cytosol concentration). However, on the basis of these observations, it cannot be concluded with certainty, whether the ATP and cytosol factors act directly at the site of processing, or just affect transport, which in turn is coupled to processing.

Incubations in the presence of Actinomycin-D, which blocks all nucleolar transcription, established that ongoing rRNA synthesis is not necessary for *in vitro* transport and processing to take place. One would not expect much synthesis in this system, anyway, since the requisite nucleotide precursors are not present. However, preliminary studies, with a system that does support *in vitro* synthesis
of RNA in isolated nuclei, indicate that, just as in the case of transport and processing, cytoplasmic factors stimulate in vitro synthesis as well.

The cytoplasmic regulation of ribosome formation was studied in resting and regenerating rat liver and in the transplantable Morris Hepatomas, 5123-D and 7800; however no qualitative differences, such as those seen in the regulation of informosome transport (217, 224), were observed.

The stimulatory action of these cytoplasmic positive feed-back modulators could not be substituted for by purified ribosomal protein. The identity of the cytoplasmic factors and their relationship to the inactive ribosomal monomer pool, the size of which was observed to vary inversely with the rate of ribosome formation (129, 140), and to other components such as the ribosomal dissociation factors (173), or initiating factors and 40S subunit associated proteins (174), remains to be established. The stimulatory factor(s) is probably identical to that detected indirectly in the intact cell through the use of inhibitors of protein synthesis (156).

It seems probable that the controls of ribosome formation at the nuclear transcriptional and post-transcriptional levels are coupled in some way with the fluctuating cytoplasmic requirement for new ribosomal subunits to be incorporated into the cell's protein synthesizing machinery.
LIST OF REFERENCES

110. Pene, J.E., Knight, E., Jr., and Darnell, J.E., Jr., J. Mol. Biol., 33, 609 (1968).
120. Zimmerman, E.F., Biochemistry, 7, 3156 (1968).


193. Shearer, R.W., and McCarthy, B.J., Biochemistry, 6, 283 (1967).