PROGRESS REPORT

MASTER

October 1, 1978 - September 30, 1979

U.S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

CONTRACT NO. EY-76-S-02-3335

A 502-76880 3335

MOLECULAR EVENTS BASIC TO CELLULAR RADIATION RESPONSE

DISCLAIMER

This book was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

to

Radiology Research Laboratory

Massachusetts General Hospital

Boston, Massachusetts

from October 1, 1978 to May 31, 1979

and

Division of Nuclear Medicine

Beth Israel Hospital

Boston, Massachusetts

from June 1, 1979 to September 30, 1979

Principal Investigator

G.M. Kolodny

G.M. Kolodny, M.D.

Director, Division of Nuclear Medicine

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

PLAN OF REPORT

	<u>Page</u>
I. Introduction	-2-
II. Report	-11-
Albumin Synthesis in Cultured 3T3 Cells Induced by Mouse Liver RNA	
III. Time and Effort	-21-
IV. Publications and Reports	-22-

I. Introduction

The effects of x-irradiation on whole animals and their multicellular tissues are manifold and complex. To better understand these effects, much of modern radiobiology has been concerned with understanding radiation effects at the less complex cellular and subcellular levels. A clearer knowledge of the mechanisms of action at these more fundamental levels will help to explain the diverse effects seen at the multicellular level. Studies of cellular and subcellular radiation effects have at least in part been motivated by the desire and the necessity to reduce patient radiation effect in diagnostic radiology and nuclear medicine and increase the tumoricidal effect in therapeutic radiation and medicine.

Significant doses of x-irradiation at the cellular level lead ultimately to either malignant transformation or cell death. In both cases, x-irradiation usually produces these effects by acting upon the sequence of mechanisms responsible for cell division. Clearly then, it would help to understand how normal cells initiate and control their division process in order to discover how normal cells change in these regards with x-irradiation and neoplasia.

A. Laboratory Historical Background

1. Mitotic Apparatus

Efforts to understand the biochemical mechanisms of cell division were begun in our laboratory in 1964. At that time, we undertook a study on the mitotic apparatus of the dividing sea urchin egg. We derived optical rotatory dispersion data for the dissolved apparatus and found that these data were close to those for the muscle protein actin. This suggested the possibility of an active contractile process for chromosome separation (1).

2. Patterns of Protein Synthesis During Cell Cycle

Several lines of evidence (2) suggested that the mitotic apparatus was not

synthesized de novo at mitosis but was assembled from subunits formed earlier in the cell cycle, presumably in the G_2 phase. Moreover, a very large proportion of the total cell protein at mitosis was thought to be mitotic apparatus protein. We therefore compared the pattern of protein synthesis during G_2 with the cell cycle as a whole in an attempt to differentiate one predominant band of protein synthesized during G_2 which might represent the mitotic apparatus protein. Proteins labeled with one isotopic precursor (H^3 leucine) during G_2 were mixed with proteins labeled with another isotopic precursor (C^{14} leucine) during the entire cell cycle and separated on polyacrylamide gels. We compared the H^3/C^{14} ratio down the length of the gels to find one band showing a marked increase that could be attributed to mitotic apparatus protein. Instead of one unique band, we found a very complex array of differences in the pattern of protein synthesis in G_2 as compared to the rest of the cell cycle (3,4). Changes in patterns of protein synthesis form the biochemical basis for differentiation and are presumed to represent changing patterns of gene transcription. Our results describing changes in the pattern of protein synthesis during the cell cycle suggest that not only during development but also during a single cell cycle gene transcription may show variations in activation and repression.

3. Contact Inhibition

These changes in the pattern of gene expression leading up to cell division are apparently initiated in the G_1 phase of the cell cycle, prior to the DNA synthetic period. Once cells commit themselves to DNA synthesis they almost always go on to divide (5). For this reason, our efforts focused on cells which ordinarily remain in a resting state (G_1 or G_0) until stimulated to divide. By understanding the mechanisms underlying this stimulation we hoped to gain some background to the problem of initiation of cell division.

Untransformed cells grown in tissue culture multiply until they cover their

growth surface (become confluent), whereupon their mitotic rate either drops to zero or decreases markedly. At this time, the cells are said to show "contact inhibition" (6,7). When more growth area is made available by trypsinizing and reseeding into new larger tissue culture flasks, the cells once again start dividing rapidly until they again become confluent. At confluence, the cells in the "monolayer culture" are in the G_1 phase of the cell cycle (8), as are most resting mammalian cells (5). Whether or not the in vivo condition is duplicated in tissue culture, contact inhibition does represent an entry into understanding cellular mechanisms that lead to cell division.

Investigations on intracellular changes following release of contact inhibition had been conducted on cells released from contact inhibition by reseeding cells in a larger growth area after trypsinization, by added fresh serum (9,10) or by transforming these cells (11-13). These methods of releasing contact inhibition possess some disadvantages for the interpretation of experimental results.

Increasing the growth area along a border of the culture leads to stimulation of cell division in only a very small and insignificant fraction of cells immediately adjacent to the border. Cells within the center of the culture are not released from contact inhibition (14).

We developed (15) methods for releasing contact inhibition of division by increasing the area of growth of a confluent culture in a fairly uniform manner throughout the culture. Cells are seeded into culture dishes in the presence of small glass beads. The cells multiply between the beads until confluent. Contact inhibition is removed by discarding the beads, leaving behind numerous spaces until they are again confluent. Using this method, we were able to describe the changes in protein, and RNA synthesis that occur after the release of contact inhibition. We were also able to show by comparison of two different mouse fibroblast lines that apparently not all resting cells are blocked in the same location within the G_1 phase (16).

4. Cell to Cell Communication

Elucidation of the earliest events following the release of contact inhibition requires knowledge of how cells in confluence signal their neighbors to stop dividing, and how they interrupt that signal when the cells have their growth area increased and are no longer confluent. We hoped that an understanding of this process of cell to cell communication would lead to an approach to unraveling the intracellular control mechanisms responsible for switching on the processes of cell division in resting cells.

We wished first to develop a method for studying the problem of transfer of macromolecules between cells. The method had to allow for biochemical examination of the transferred macromolecules. The desired method would also have to be able to recognize transfer between genetically and phenotypically similar cells.

A method (17) was developed to culture two populations of cells together in tissue culture and then separate the two populations. One population was then examined for labeled macromolecules which had passed over from the other. One group of cells was grown in the presence of tantalum particles (2 μ avg. diam.). These tantalum-containing heavy cells were added to fresh cells and the two groups of cells cultured together. After 5-20 hours, during which time the tantalum-containing cells and fresh cells became confluent (i.e., in intimate contact with one another), the culture was trypsinized and the cells put on 0-17% ficoll gradients and centrifuged. The heavy tantalum-containing cells passed to the bottom while the light cells floated upon the ficoll. The light cells were removed and the labeled macromolecules transferred from the heavy donor cells were examined.

Using this method, we were able to demonstrate transfer of RNA between cells (18). By several different methods, we established that high molecular weight RNA was transferred but not precursors or degradation products. Moreover, trivial explanations of our results such as presence of donor cells or donor cell fragments with the recipient cell fraction were ruled out. However, no unique RNA species

that could be related to maintenance of the contact inhibited state could be identified.

This same method also showed transfer of RNA between transformed cells (19), and intercellular protein transfer with transfer into cell nuclei (20). Interestingly, of all the nuclear protein fractions the histones showed the greatest degree of transfer.

Because of this finding of a relatively greater transfer of histones, we investigated the effects of histones on growing and confluent cell cultures. Some of the major histone fractions were found to increase cell number at confluence and to cause disruption of the normal ordered appearance of cultures at confluence (21). It is not clear whether this was an intracellular effect or was due merely to alternations caused in the cell membranes.

5. Regulation of Eukaryotic Gene Expression

At this point in our work, it seemed to us that the problem of cell division and its initiation was bound up with the general biological problem of regulation of gene expression. For if cell division is initiated early in the cell cycle and if the cell cycle is accompanied by and presumably caused by changes in gene expression, then the initiating trigger for cell division is probably found in the activation of one or more specific genes.

Although bacterial systems have mechanisms for gene control which have been well documented, those for eukaryotic cells appear to be more complicated. Such cells are probably controlled by different or additional genetic regulatory mechanisms to those described for prokaryotes. Because of the enormous number of genes to be controlled within a eukaryotic cell, it would seem that only RNA or protein would have the necessary specificity to control the specific gene transcription. RNA can use its well documented property of hybridization to uniquely determine its interaction with genomic DNA.

Because of the ability of RNA to hybridize with DNA, we have investigated the possible role of RNA as an inhibitor of gene transcription. If RNA were acting to repress gene transcription, then the more highly differentiated a cell, the more repressor RNA it would have. We have therefore examined a fully differentiated cell, which shows no genetic transcription, the chick erythrocyte, for the presence of DNA-associated RNA. Using in vitro labeling procedures, we found less than one ribonucleotide in association with 10^5 deoxyribonucleotides of the avian erythrocyte DNA, which is far too little to possibly repress all the genes within a fully mature avian erythrocyte (22).

Although segments of RNA may not act as genetic repressors, it is still possible that such segments could exercise genetic control as activators of transcription as proposed in several hypotheses (23-25). However, none of these hypotheses propose a source for their activator RNA. We have therefore put forward a new hypothesis for gene regulation by activator segments of RNA which does postulate a source for this RNA (26,27). We propose that segments of RNA derived from breakdown of high molecular weight RNA act as primer for RNA synthesis by forming the 5' ends of new RNA molecules. Since we first proposed the "primer hypothesis" (28), two other laboratories (29,30) have proposed particular variations of the primer hypothesis.

a. RNA Turnover

In studies on growing and confluent 3T3 cells (31), we found that ribosomal RNA does not show appreciable turnover in growing cells but does show turnover when the cells become confluent. We found a single component decay curve if the cells were labeled after confluence was attained. However, if the confluent cells were prelabeled when subconfluent, the decay curve had two components. We have also examined the methylation pattern of 18s and 28s rRNA. These studies also showed that in confluent cells, 18s rRNA was not turning over as rapidly as the 28s

rRNA and that variations in the ratio of base to ribose methylation occurred during the turnover of 18s RNA, but not 28s RNA. These results are consistent with the above model, since RNA lies at the 5' end of the 45s precursor molecule. When cells are in a steady state condition merely turning over their ribosomal RNA, the 5' end of the ribosomal precursor is reutilized and is therefore not labeled. Turnover of label therefore involves only the nonconserved portion of the precursor. If, however, the cells are labeled while growing, there will be turnover at confluence of two labeled components; the short lived nonconserved RNA and the longer-lived primer RNA.

The "Primer Hypothesis" (26,29,30) for regulation of eukaryotic gene transcription suggests that oligonucleotide degradation products of high molecular weight RNA prime the transcription of new RNA and thus become incorporated into newly synthesized RNA. To test the primer hypothesis, we sought evidence for en bloc incorporation of oligonucleotides into newly synthesized RNA (32).

Labeled low molecular weight RNA oligonucleotides were incubated with HeLa cells in the presence of a large excess of unlabeled mononucleotides to limit the incorporation of labeled mononucleotide breakdown products. The cellular RNA was then extracted and examined for radioactive label in high molecular weight RNA.

After incubation of HeLa cells with ^3H uridine labeled oligonucleotides, the cellular RNA was extracted with phenol. After sedimentation on sucrose gradients, the degraded RNA was virtually all low molecular weight RNA with only background radioactivity in the 18s and 28s region. The RNA extracted from cells incubated with degraded RNA was also sedimented on sucrose gradients and showed a radioactivity peak sedimenting at about 18s. This peak had a higher molecular weight than the added degraded RNA and indicated incorporation of label from the added oligonucleotide degradation products.

Radioactivity in the 18s peak after incubation with radioactive oligonucleotides

was not due to incorporation of labeled mononucleotides produced by complete degradation of the added oligonucleotides. Both 18s and 28s RNA are synthesized as part of a single 45s precursor. If labeled mononucleotides were used in the synthesis of 18s RNA, they would be expected in 28s RNA as well. The absence of significant label in 28s RNA argues against the radioactivity in the 18s peak being due to mononucleotide incorporation.

To further rule out mononucleotide reincorporation as being responsible for the 18s radioactivity seen or some anomaly in RNA metabolism, cells were incubated in 1 uCi ^3H -uridine, about twice the radioactivity in the added degraded RNA in the above experiments, and the same amount of unlabeled uridine as in the previous experiments. In this case, as expected, both 18s and 28s RNA are labeled with the proper ratio of 18s radioactivity to 28s radioactivity. Moreover, despite using twice as much mononucleotide radioactivity in the control experiment as oligonucleotide radioactivity in the original experiment, there was still less radioactivity noted in the 18s peak in the control experiment. Therefore, even if all the oligonucleotides had broken down to mononucleotides, there still would not have been sufficient radioactivity to account for that seen in the 18s peak in the oligonucleotide incorporation experiments. The excess of unlabeled uridine in the incubation media severely limited, therefore, the incorporation of mononucleotides. The 18s peak in radioactivity seen was derived primarily from the reincorporation of labeled oligonucleotides.

Control experiments using denaturing conditions and actinomycin established that the radioactivity seen in the 18s peak was not due to non-specific association of labeled oligonucleotides. The 18s peak in radioactivity was also alkali labile indicating that it was neither a DNA nor a protein contaminant. Experiments with ^3H guanosine labeled oligonucleotide gave similar results to those obtained with ^3H uridine labeled oligonucleotides.

These results (32) were strong evidence for en bloc incorporation of oligonucleotides into newly synthesized RNA --- a central prediction of the Primer Hypothesis.

II. Report

Albumin Synthesis in Cultured 3T3 Cells Induced by Mouse Liver RNA

The available evidence suggests that all cells within an organism have the same DNA content. The specialized nature of different cells is thought to arise from differential gene transcription in different cells. The primer hypothesis predicts that introduction into a cell of the primer for a particular mRNA should result in the production of that mRNA in any cell of the organism. As one step in testing the primer hypothesis, one would like to add primer oligonucleotides from one specialized cell type to a different cell type and prime the production of a messenger RNA found in the first cell type but not in the second.

The detection of synthesized mRNA is usually more difficult than detection of the protein specified by that mRNA. Gurdon (33) and his associates have shown that after introduction into eggs of a specific mRNA, the eggs will produce the protein specified by that mRNA. Anderson (34) has passed rabbit globin mRNA into CHO cells using erythrocyte ghosts and detected production of a putative rabbit globin in these cells. It would appear, therefore, if one were to activate synthesis of a particular mRNA within a cell, one might be able to detect its protein product perhaps as well as the mRNA itself.

Liver cells produce relatively large amounts of albumin. Fibroblasts, however, produce no detectable albumin (35,36). Peterson and Weiss (37), however, have been able to demonstrate induction of mouse albumin production in hybrids from rat hepatoma cells and 3T3 mouse fibroblasts. Others (38) have shown that murine hepatoma-human leukocyte cell hybrids will produce human albumin. In these experiments, some factor from the hepatoma cells presumably interacted with the albumin gene in the fibroblasts to cause production of that gene product.

The Primer Hypothesis would predict that primer RNA was the factor responsible for the induction of albumin synthesis in the above experiments. It would also

predict that if mouse liver RNA were introduced into 3T3 mouse fibroblasts, these cells might produce albumin in a fashion similar to the hybrids used in the above experiments. Several studies (39) have shown that RNA in media can be taken up by cultured cells. In some cases, this uptake can be potentiated by the use of DEAE dextran, protamine (40) or polylysine (41).

In experiments during the past year, we have incubated mouse 3T3 fibroblasts with mouse liver RNA. Both cells and media were then examined for the presence of mouse albumin using immunologic techniques.

Incubation of 3T3 Cells with Mouse Liver RNA

Mouse 3T3 cells were incubated for 48 hours with mouse liver RNA in the presence of polylysine. The media was removed, concentrated and counterimmuno-electrophoresed (CIEP) against mouse albumin antisera. The stained CIEP plates demonstrated a single precipitin line in the same location as that produced by mouse albumin controls (Figure 1).

Control studies were done to insure that no contaminating albumin was present in the hot phenol extracted liver RNA used in the incubation with 3T3 cells. Mouse liver RNA alone counterimmuno-electrophoresed against mouse albumin antisera gave no precipitin line (Figure 2). Moreover, control incubations using mouse liver RNA and polylysine in identical culture dishes in the absence of cells also did not give a CIEP precipitin line. Additional controls were done to insure that mouse albumin or a media component immunologically similar to albumin did not adhere to the naked culture dish surface in these latter control experiments, resulting in an absent CIEP band. Media containing albumin was incubated with naked dishes and gave precipitin lines on CIEP identical to similar amounts of albumin run directly on CIEP. These control studies indicate that the RNA used did not contain contaminating albumin.

1	O MAA
2	O MAA
3	O MAA

7 | Figure 1: Media from 3T3 Cultures Incubated with Liver RNA Counterimmuno-electrophoresed Against Mouse Albumin Antisera

Confluent 3T3 cells were incubated for 48 hours with one mg. mouse liver RNA complexed with 0.25 mg. polylysine in serum free media. The media was then dialyzed, lyophilized and counterimmunoelectrophoresed (CIEP) against goat anti mouse albumin. The stained CIEP plate shows an immunoprecipitant line at the same location as control albumin. MAA--Mouse albumin antisera in cathode wells. Well 1 - 200 mg. albumin. Well 2 - media from culture incubated with intact liver RNA. Well 3 - media from culture incubated with degraded RNA.

Figure 2: Mouse Liver RNA Controls After CIEP Showing No Albumin Contamination of Mouse Liver RNA

MAA--mouse albumin antisera. Well 1 - 200 mg. mouse albumin. Well 2 - 1 mg. mouse liver RNA. Well 3 - 1 mg. mouse liver RNA and 0.25 mg. polylysine incubated 48 hours in serum free media in culture dish without cells. Media centrifuged and supernatant dialyzed, lyophilized and CIEP. Well 4 - 200 mg. albumin incubated in serum free media in culture dishes without cells. CIEP shows identical precipitin line to control albumin in well 1.

Media from cultures incubated with liver RNA, polylysine and ^{14}C leucine were subjected to CIEP. Autoradiograms of the stained CIEP plates showed a single radioactive band in the same location as the stained band indicating that the precipitin line was composed of newly synthesized protein immunologically similar to mouse albumin.

To rule out a nonspecific effect of RNA on 3T3 cells which may have caused production of one or more compounds immunologically similar to albumin, cultures were incubated with RNA from yeast, E. Coli and rat liver. No precipitin line was observed when media from these incubations was run on CIEP using mouse albumin antisera (Figure 3).

Monospecificity of Antibody

In an Ouchterlony system the mouse antisera gave only a single precipitin line. However, the monospecificity of the mouse albumin antisera was also demonstrated by CIEP. The antisera was counterimmunoelectrophoresed against mouse serum. Only a single precipitin line resulted in the same location as albumin controls. This line was cut out and electrophoresed on polyacrylamide gels (Figure 4). The stained gels showed the antibody bands and only a single other band in the same location as albumin on the control gel indicating the monospecificity of the antisera using CIEP.

We have also determined the purity of the mouse albumin control preparation by CIEP of the albumin against mouse serum antisera. This demonstrated a single band in contrast to many bands seen when the antisera was electrophoresed against mouse sera. When this band was cut out and run on polyacrylamide gels, it ran identically with a mouse albumin CIEP band formed with mouse albumin antisera (Figures 4 and 5).

Cell cultures were washed prior to incubation in serum free media. However, traces of calf serum containing bovine albumin may still have been present in the incubation media. However, CIEP of bovine albumin against mouse serum albumin

Figure 3: Absence of Albumin Synthesis in Mouse Cells Incubated with Rat Liver RNA

Confluent cell cultures were incubated 48 hours with 1 mg. rat liver RNA and 0.25 mg. polylysine in serum free media. The media was dialyzed, lyophilized and run on CIEP. No precipitin bands were visible with goat anti mouse albumin antisera or goat anti rat albumin antisera. MAA--mouse albumin antisera. RAA--Rat albumin antisera. Well 1 and 4 - 200 mg. mouse albumin, Well 2 and 3 - dialyzed lyophilized media.

Figure 4: Monospecificity of the Goat Antimouse Albumin Antisera

Antisera CIEP against mouse albumin gave a single line which was cut out and electrophoresed on a 7.5% polyacrylamide gel. I--5 μ g albumin, II--10 μ l mouse serum CIEP against antisera. Precipitin line cut out and electrophoresed on gel. Gel shows single gel band in same location as mouse albumin. III--5 μ g albumin CIEP against goat anti mouse albumin antisera showed single line (see Figure 4). Line cut out and electrophoresed on gel. Gel shows single band in same location as mouse albumin. A - albumin band, B and C - antibody bands, D - bromphenol blue dye marker.

Figure 5: Monospecificity of Mouse Albumin Antisera

Mouse albumin and mouse serum CIEP against goat anti mouse serum antisera and goat anti mouse albumin antisera demonstrating the monospecificity of the mouse albumin antisera. MAA--mouse albumin antisera. MSA--mouse serum antisera. Well 1 and 3 - mouse albumin, Well 2,4,5 - mouse serum.

produced no precipitin lines or evidence of cross reactivity.

Albumin mRNA Newly Transcribed

These results provide strong evidence for the induction of albumin synthesis in 3T3 cells by mouse liver RNA. This induction may have been caused by the translation of added albumin mRNA or by the transcription of new albumin mRNA specified by the 3T3 genome and its subsequent translation.

To explore the possibility of new mRNA transcription, the added RNA was degraded so that it was smaller in size than albumin mRNA. Added liver RNA was degraded prior to extraction. This degraded RNA was then incubated with 3T3 cells in the presence of polylysine. This RNA was sedimented on a 15-30% sucrose gradient and as in previously reported experiments (44), all of the degraded RNA was less than 5s in size and therefore far smaller than the 18-22s size of intact albumin mRNA (57).

Media from incubation with degraded RNA was subjected to CIEP and also produced an immunoprecipitin band identical to control albumin which could be seen both on the stained and radioautographed plates. The band produced in several experiments was at least of the same intensity as the band produced by undegraded RNA and often was more intense. The added small molecular weight liver RNA could be acting as primers for new albumin mRNA synthesis.

Albumin mRNA contains a poly A segment at its 3' end (58). The poly A containing fraction of liver RNA was retained on poly U sepharose columns and the remainder of the RNA incubated with 3T3 cells. A characteristic precipitin line was found on CIEP of the media from these incubations. These results indicate that intact albumin mRNA is not necessary to induce synthesis of protein immunologically similar to albumin in 3T3 cells.

Cells were also incubated in the presence of liver RNA, polylysine and 15µg/ml actinomycin. No precipitin line was observed, suggesting again that new RNA syn-

thesis and not merely mRNA translation was occurring during the induction of albumin synthesis in the 3T3 cells.

Gel Electrophoresis of Newly Synthesized Albumin

Media from 3T3 cell cultures incubated with liver cell RNA contain a newly synthesized product which on CIEP produces an immunoprecipitin band with mono-specific mouse albumin antisera which is in the same position as control mouse albumin. To further characterize this immunoprecipitable product and compare it with known mouse albumin, the CIEP immunoprecipitin lines produced from incubation media containing ^3H leucine label were electrophoresed on polyacrylamide gels with unlabeled mouse albumin marker. After electrophoresis, the gels were stained, destained and then sliced. Radioactivity in the slices was determined and is plotted in Figure 6.

A single large peak of radioactivity was found on the gels which had migrated approximately 2 mm further than the stained albumin band. It was thought possible that this peak represented synthesized albumin which had been slightly degraded during procedures prior to polyacrylamide electrophoresis. To control for this, unlabeled mouse albumin was added to cultures incubating with liver RNA, polylysine and ^3H leucine. The concentrated media was subjected to CIEP and the immunoprecipitant run on polyacrylamide gel electrophoresis. Again, the radioactive band ran about 2 mm ahead of the stained albumin band produced by the albumin added to the cultures. This data would suggest that the albumin synthesized in vitro contained less than the complete polypeptide chain of in vivo mouse albumin.

Other Considerations

Mouse fibroblasts (3T3) incubated with mouse liver RNA synthesize and secrete into their media a protein which is immunologically similar to mouse albumin.

Figure 6: Polyacrylamide Gel Electrophoresis of Radioactive Albumin Synthesized in 5T3 Cells

Confluent cell cultures were incubated 48 hours with 1 mg. mouse liver RNA and 0.25 mg. polylysine in serum free media containing 125 μ Ci/ml ^{3} H leucine and 5 μ g carrier non radioactive albumin. Media was concentrated and counterimmuno-electrophoresed against mouse albumin antisera. Precipitin line formed was cut out and electrophoresed on 7.5% polyacrylamide gels. Gel was stained, destained and sliced. Two mm gel slices dissolved in H₂O₂ and then ^{3} H radioactivity counted. Peak in radioactivity runs slightly (~ 2 mm) faster than carrier albumin.

When this protein is coelectrophoresed with albumin standard on polyacrylamide gels, it is found to run slightly ahead of albumin. It could represent a protein immunologically similar but distinct from albumin and very close to albumin in size, which is produced and released into the media by 3T3 cells after incubation in liver RNA. However, in view of the demonstrated monospecificity of the antisera, it would appear more likely that the protein induced represents albumin which lacks a short segment of its polypeptide chain. Studies are now underway, which are to be reported separately, to analyze the peptides produced by protease digestion of the labeled protein which reacts with mouse albumin antisera and to compare these peptides with peptides generated from mouse albumin. Such studies will be of help in distinguishing between these two possibilities.

For these experiments, RNA has been incubated in the presence of polylysine. Polylysine at the concentrations used is highly toxic to the cells. When RNA is combined with polylysine of MW~13,000 at the concentrations used in these experiments, a fine granular precipitate results, caused by the highly positively charged polylysine interacting with the highly negatively charged RNA. Phase microscopic examination of the cultures after 24 hours of incubation demonstrates a fine reticular pattern overlying the cells. When polylysine at ~3000MW is used, only a fine light precipitate is seen. Ratios of RNA:polylysine less than those used results in a decreasing quantity of precipitate and increased cell toxicity. Increasing this ratio also decreased the amount of precipitate but does not affect cell toxicity. The maximum synthesis of albumin like protein occurs at the ratio of maximum precipitation. Presumably the cells phagocytize the precipitated RNA-polylysine complex and the greater the amount of precipitate, the greater the amount of incorporated RNA.

The quality of phenol used to extract the RNA from the liver, free of contaminating proteins, was found to be critical in obtaining the most active preparations

of liver RNA. The pH of liquefied phenol was found to vary from vendor to vendor presumably because of stabilizers now added by commercial vendors. Phenol was used that had no added stabilizers and that was fully equilibrated with extraction buffer.

Incubations of RNA and 3T3 cells were done in serum free media. If the media contained 1% serum all of the cells were dead within 24 hours. This effect was demonstrated only with mouse liver RNA. This did not appear to be due to a non-specific effect of added RNA since it was not seen with yeast RNA, or E. Coli RNA and polylysine or in cells incubated in serum with liver RNA in the absence of polylysine.

Since added liver RNA appears to induce a hepatic cell function of albumin synthesis in 3T3 cells, it was thought possible that morphologic changes in 3T3 cells might be induced by liver RNA as well. However, no change was observed in the microscopic appearance of the 3T3 cells after incubation with liver RNA either in confluent or growing cells.

The Primer Hypothesis for eukaryotic gene regulation proposes that small molecular weight RNA acts as primer for new RNA synthesis by hybridizing with DNA and there initiating the transcription of a new RNA chain. The experiments reported here indicate that small molecular weight RNA will induce the production of new proteins. These results are consistent with the Primer Hypothesis, and are the first reported demonstration that RNA can be taken up from the media by cells in culture and can induce in vitro the production of differentiated cell products.

REFERENCES

1. Kolodny, G.M. and Roslansky, J.R.: Optical Rotatory Dispersion of Mitotic Apparatus Isolated from Dividing Eggs of Strongylocentrotus drobachiensis, J. of Mol. Biol., 15: 381, 1966.
2. Mazia, D., Ann. N.Y. Acad. Sci. 90: 455 (1960).
3. Kolodny, G.M. and Gross, P.R.: Changes in Patterns of Protein Synthesis During the Mammalian Cell Cycle. Exp. Cell Res. 56: 117 (1969).
4. Kolodny, G.M.: Patterns of Protein Synthesis in the HeLa Cancer Cell, Am. J. Roentgen., Rad. Ther. and Nuc. Med., 105: 746 (1969).
5. Quastler, H.: Ann. N.Y. Acad. Sci. 90: 580 (1960).
6. Abercrombie, M.: Cold Sprng. Hbr. Symp. Quant. Biol, 27: 427 (1962).
7. Golde, A.: Virology 16: 9 (1962).
8. Nilausen, K. and Green, H.: Exp. Cell Res. 40: 166 (1965).
9. Todaro, G.J. et al: J. Cell Physiol. 66: 325 (1965).
10. Yoshikura, H. and Hirokawa, Y.: Exp. Cell Res. 52: 439 (1968).
11. Kara, J. and Weil, R.: Specific Activation of the DNA-Synthesizing Apparatus in Contact Inhibited Mouse Kidney Cells by Polyoma Virus, Proc. Nat. Acad. Sci., 57: 63 (1967).
12. Dulbecco, R.: Persp. Biol. Med. 9: 298 (1966).
13. Sachs, L.: Curr. Topics Dev. Biol. 2, 129 (1967).
14. Fisher, H.W. and Yeh, J.: Contact Inhibition in Colony Formation, Science 155: 581 (1967).
15. Kolodny, G.M. in "Tissue Culture Methods and Applications" ed. P.F. Kruse and M.D. Patterson, Academic Press, N.Y., p. 226 (1973).
16. Kolodny, G.M. and Gross, P.R.: A Simple Mechanical Method for the Efficient Release of Contact Inhibition, Exp. Cell Res., 57: 423 (1969).
17. Kolodny, G.M., in "Cell Communication" ed. R.P. Cox, Wiley-Interscience, N.Y. p. 97 (1974).
18. Kolodny, G.M.: Evidence for Transfer of Macromolecular RNA Between Mammalian Cells in Culture, Exp. Cell Res., 65: 313 (1971).
19. Kolodny, G.M.: Cell to Cell Transfer of RNA into Transformed Cells, J. Cell Phys., 79: 147 (1972).

20. Kolodny, G.M.: Transfer of Cytoplasmic and Nuclear Proteins between Cells in Culture. *J. Mol. Biol.* 78: 197 (1973).
21. Kolodny, G.M.: Histone Mediated Changes in Morphology and Confluent Density of Cultured Cells, *J. Cell Phys.*, 81: 223 (1973).
22. Kolodny, G.M. and Rosenthal, L.J.: Absence of DNA Associated RNA in the Fully Repressed Nucleus of the Chicken Erythrocyte. *Exp. Cell Res.* 83: 429 (1973).
23. Georgiev, G.P.: On the Structural Organization of Operon and the Regulation of RNA Synthesis in Animal Cells. *J. Theoret. Biol.* 25: 473 (1969).
24. Britten, R.J. and Davidson, E.H.: Gene Regulation for Higher Cells; A Theory. *Science* 165: (1969).
25. Frenster, J.H.: A Model of Specific De-repression Within Interphase Chromatin. *Nature* 206: 1269 (1965).
26. Kolodny, G.M.: The Regulation of Gene Expression in Eukaryotic Cells. *Medical Hypotheses* 1: 15 (1975).
27. Kolodny, G.M.: A New Hypothesis for the Regulation of Gene Expression in "Extracellular Matrix Influences on Gene Expression" ed. H.D. Slavkin and R.C. Gruelich. Acad. Press, New York, p. 75 (1975).
28. Kolodny, G.M.: Regulation of Gene Expression in Eukaryotic Cells. *J. Cell Biol.* 59: 175a (1973).
29. Robertson, H.D. and Dickson, E.: RNA Processing and the Control of Gene Expression. *Brookhaven Symp. Biol.* 26: 240 (1974).
30. Brawerman, G.: A Model for the Control of Transcription During Development. *Cancer Res.* 36: 4278 (1976).
31. Kolodny, G.M.: Turnover of Ribosomal RNA Species in Mouse Fibroblasts in Culture. *Exp. Cell Res.*, 91: 101 (1975).
32. Kolodny, G.M.: Evidence for En Bloc Incorporation of Exogenous Oligonucleotides into HeLa Cell RNA. *Nuc. Acids Res.* 4: 271 (1977).
33. Gurdon, J.B., Woodland, H.R. and Lingrel, J.B.: The Translation of Mammalian Globin mRNA Injected into Fertilized Eggs of *Xenopus laevis*. *Devel. Biol.* 39: 125 (1974).
34. Anderson, W.F. and Krueger, L.J.: Microinjection and Translation of Rabbit Globin mRNA in CHO Cells. *J. Cell Biol.* 70: 185a (1976).
35. Alpern, M. and Rubin, H.: Proteins Released from Chick Embryo Fibroblasts in Culture. *Exp. Cell Res.* 60: 86 (1970).
36. Kaighn, M.E. and Prince, M.M.: Production of Albumin and other Serum Proteins by Clonal Cultures of Normal Human Liver. *Proc. Nat. Acad. Sci.* 68: 2396 (1971).

37. Peterson, J.A. and Weiss, M.C.: Expression of Differentiated Functions in Hepatoma Cell Hybrids: Induction of Mouse Albumin Production in Rat Hepatoma - Mouse Fibroblast Hybrids. Proc. Nat. Acad. Sci. 69: 571 (1972).
38. Darlington, G.J., Gernhard, H.P. and Ruddle, F.H.: Human Serum Albumin Phenotype Activation in Mouse Hepatoma - Human Leukocyte Cell Hybrids. Science 185: 859 (1974).
39. Sakai, T.T. and Cohen, S. Uptake and Aminoacylation of Exogenous Escherichia Coli tRNA by Mouse Fibroblasts. BBRC 78: 539 (1977).
40. Amos, H. and Kearns, K.: Influence of Bacterial Ribonucleic Acid on Animal Cells in Culture. Exp. Cell Res. 32: 14 (1963).
41. Mayhew, E. and Juliano, R.: Interaction of Polynucleotides with Cultured Mammalian Cells. Exp. Cell Res. 77: 409 (1973).
42. Taylor, J.M. and Tse, T.P.H.: Isolation of Rat Liver Albumin Messenger RNA. J. Biol. Chem. 251: 7461 (1976).
43. Taylor, J.M. and Schimke, R.T.: Synthesis of Rat Liver Albumin in a Rabbit Reticulocyte Cell-Free Protein-Synthesizing System. J. Biol. Chem. 248: 7661 (1973).

III. Time and Effort

During the past year, the principal investigator has spent about 70% of his time on the projects outlined in this progress report. He will continue to spend about 50% of his time and effort on these projects during the remainder of the current term of the agreement.

IV. Publications and Reports

Since the previous progress report, the following publications reporting on the work described in this progress report and previous reports are now in press:

Book:

Eukaryotic Gene Expression; Editor, G.M. Kolodny, Uniscience Series, CRC Press, Cleveland, Ohio (1979).

Toombs, B.W. and Kolodny, G.M. In Vitro Synergistic Effects of Radiation and Ultrasound, Radiology (1979).

The following manuscript based on the work described in this and previous progress reports has been submitted for publication:

Kolodny, G.M.: Albumin Synthesis in Cultured 3T3 Cells Induced by Mouse Liver RNA.