Polyadenylic Acid Content and Electrophoretic Behavior of
in Vitro Released RNA’s in Chemical Carcinogenesis

Edward A. Smuckler and R. Marlene Kopfitz

The Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, University of Washington, Seattle, Washington 98195

SUMMARY

Cytoplasmic RNA from control and thioacetamide-intoxicated rat livers was compared with regard to components separated by polyacrylamide electrophoresis and for the proportion containing polyadenylic acid segments. A further comparison was made with the RNA’s released from rat liver nuclei in vitro. Exposure in vivo to thioacetamide was associated with more cytoplasmic RNA’s with migrations of 9 to 16 S and with both an acute and prolonged increase in the relative quantity of polyadenylic acid-containing polyribonucleotides. Incubation of control nuclei in vitro was associated with a leakage of approximately 1 to 2% of nuclear RNA with a major migration band at 9 S. Addition of adenosine 5’-triphosphate increased the transport 20-fold, and 9 and 16 S species were transported as well as a 4 S group. Following exposure to thioacetamide, an enhanced “leakiness” released 9 S RNA. Addition of adenosine 5’-triphosphate doubled the quantity of released RNA, which consisted of 9 and 4 S species. Both leaked and adenosine 5’-triphosphate-transported RNA’s contained polyadenylic acid segments in roughly 20% of the macromolecules. These studies extend the observation of less stringent control of RNA release and transport in carcino genesis and suggest the potential usefulness of the in vitro release system for assaying cellular regulatory phenomena.

INTRODUCTION

Epigenetic regulation in eukaryotic cells involves the processing of RNA within the nucleus and the transport of selected RNA’s from the nucleus to the cytoplasm (4, 25, 28). Several of these released polyribonucleotides appear to have structural roles, as rRNA, while others appear to have functional significance, as m- and tRNA’s. These latter macromolecules may regulate cell activity by their presence, absence, or relative quantity. Different spectra of these RNA’s are released during differentiation and regeneration (2, 3, 21). Several lines of evidence point to altered RNA release in neoplastic transformation (5, 22, 23). All 3 processes, differentiation, regeneration, and neoplasia, represent altered phenotypic expression. The selection of specific RNA for transport may underlie expression of these differing phenotypes. The presence of nuclear restricted RNA’s leaking to the cytoplasm provides a biochemical basis for the more markedly altered phenotypic expression in neoplasia (22, 23) and focuses upon altered RNA transport as a potentially significant mechanism in the process of transformation.

Altered release and transport of RNA during carcinogen exposure has been shown both in vivo and in vitro (20, 23), in the former by competition hybridization experiments and in the latter by detection of altered release of RNA from nuclei to surrogate cytoplasm (24). The following experiments were designed to assess the similarities of in vivo and in vitro release of RNA following exposure to a chemical carcinogen and to measure the electrophoretic behavior and poly(A)2 acid content of the RNA released under these circumstances.

MATERIALS AND METHODS

Animal Manipulation. Pathogen-free male Sprague-Dawley rats were obtained from the Charles River Breeding Laboratory, Wilmington, Mass., or from the Tyler Laboratories, Seattle, Wash. The animals were maintained in wire-bottomed cages in our animal quarters for at least 3 days prior to experimentation, were fed Purina laboratory chow, and were provided food and water ad libitum. Following a 16-hr fast, the animals were weighed and given water or thioacetamide dissolved in water at a dosage rate of 5 or 20 mg/100 g body weight. The water or thioacetamide solution was administered by stomach tube without anesthesia.

RNA Labeling. Prelabeling of liver by [3H]orotate was accomplished by either of 2 procedures. For pulse labeling, animals were given a single i.v. administration of 3 μCi of [3H]orotic acid per 100 g body weight, (specific activity, 50 mCi/mmole) via the tail vein 90 min prior to sacrifice. For saturation labeling (see below), the same total quantity of isotope was injected i.p. in 4 divided doses at 12-hr intervals, the last dose given 12 hr prior to sacrifice.

RNA Isolation. Cytoplasmic RNA was prepared from fasting animals by a modification of described methods (5, 20, 24). The rats were sacrificed by exsanguination by heart puncture while under ether anesthesia. The livers were excised and immediately minced in 2 volumes of ice-cold 0.25 M sucrose solution containing 5 mM MgCl2, 25 mM NaCl, 50 mM Tris (pH 7.4), and 5 mM mercaptoethanol. The livers were disintegrated in Teflon-glass homogenizers rotating at 1,000 rpm and the resultant brei was centrifuged at 6,000 x g for 10 min. The supernatant fluid was carefully decanted and centrifuged again at 6,000 x g for 10 min and harvested

1 These studies were supported in part by USPHS Grants CA 13900 and GM 13540.
2 The abbreviations used are: poly(A), polyadenylic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; poly(U), polyuridylic acid; poly(dT), polydeoxythymidylic acid.
by aspiration from pelleted debris. The final suspension was made 0.5% SDS and an equal volume of water-saturated phenol was added. The mixture was shaken at 60° for 15 min following which the suspension was chilled and centrifuged at 12,000 x g for 10 min. The lower phenolic layer was aspirated and discarded and the interface and aqueous layers were reextracted with an equal volume of hot phenol. Two volumes of absolute ethanol were added to the water fraction, and the RNA was permitted to precipitate for 2 to 16 hr at −20°. The precipitated RNA was harvested by centrifugation at 24,000 x g for 10 min, the supernatant was carefully removed, and the RNA was suspended in 20 ml of 0.01 M LiCl-0.01 M acetic acid (pH 5.1) and made 0.5% SDS. An equal volume of water-saturated prewarmed phenol was added, and the resultant mixture was heated to 60° and shaken for 10 min. The phenolic mixture was chilled on ice and centrifuged for 10 min at 12,000 x g, and the aqueous phase was carefully collected. The RNA was reprecipitated following the addition of 2 volumes of cold alcohol and recovered by centrifugation. The RNA pellet was solubilized and reprecipitated as described. The yield of RNA (as TCA-precipitable material) was 84.7 ± 9.2% of total precipitable RNA in the 6,000 x g supernatant fluid and was not different comparing treated and control animals. The RNA was suspended in water or in 300 mM LiCl-0.1 mM EDTA-0.1% SDS-10 mM Tris (pH 7.6), depending on whether RNA was to be subjected to electrophoresis or poly(U)-Sepharose chromatography.

Electrophoretic Analysis of RNA. Gel electrophoresis was carried out by modification of Loening's technique (14); 2.5% polyacrylamide gels prepared with 0.1% SDS were run at 5 ma/10-cm gel column for 3 hr. Routinely, between 1.0 and 1.2 A260 units were applied to the gels. The buffer was recirculated; no external cooling was necessary to maintain a temperature of 24°. The resultant gels were scanned at 260 nm in a Gilford spectrophotometer equipped with the gel scanner and Servograph recorder. Reference RNA's include 28 and 18 S obtained from rat liver ribosomes and 4 S from rat liver 105,000 x g supernatant. These RNA's were run contemporaneously as markers. Molecular weight and sedimentation coefficients of unknowns were calculated by plotting the migration versus the log of the molecular weight (19). "Melting" of the RNA prior to electrophoresis is described in "Results."

Isolation of Poly(A)-containing RNA. Poly(U)-Sepharose was prepared as previously described (27). The RNA was melted at 60° for 10 min and then brought to room temperature. Either 5 or 10 absorbance units of RNA were utilized per ml of bed volume of the column gel. The RNA was applied to the column and washed into the column with 300 mM LiCl-0.1 mM EDTA-0.1% SDS-10 mM Tris (pH 7.6), and the eluate was followed spectrophotometrically at 260 nm. The RNA retained by the poly(U)-Sepharose column was then released with 70% formamide containing 1 mM EDTA (pH 7.6), and the eluates from the column were assayed both for radioactivity and for absorbance. The amount of radioactivity eluted with formamide was calculated as the percentage of the total RNA recovered from the column and as the percentage of poly(A)-containing RNA. Recoveries of RNA applied to these columns routinely exceeded 95%.

A series of preliminary experiments were carried out using poly(U)-Sepharose, cellulose (12), poly(dt)-cellulose (1), and Millipore filters (13) for separation of poly(A)-containing RNA. Prelabeled rat liver RNA and poly(A) was separated and quantified comparing these several procedures. The recovery of RNA and the separation of poly(A)-containing RNA from the bulk RNA was measured over a wide concentration range from 1 to 20 absorbance units (260 nm) of RNA per 0.25-ml column bed volume.

RESULTS

Electrophoretic Analysis of Cytoplasmic RNA following In Vivo Carcinogen Exposure. The yield of RNA separated from the 6000 x g supernatant was 84.7 ± 9.2% of the total TCA-precipitable pool. The yield figure was calculated on the basis of recovery of TCA-precipitable radioactivity. No differences were encountered comparing material isolated from controls versus the carcinogen-treated rat livers. These RNA's were subjected to electrophoretic separation on polyacrylamide gels (Chart 1). Control liver cytoplasm showed distinct 28, 18, and 4 S peaks. In addition, a reproducible minor component with a migration equivalent to 22 S was noted between the 28 and 18 S species. Five and 9 S peaks were also identified, as well as a poorly defined broad band from 9 to 16 S. The RNA isolated from thioacetamide-treated animals showed a 28, 18, and 4 S band similar to the controls. The 22 S and 9 and 5 S peaks were also noted. The 9 to 16 S broad band was less marked, but a particularly prominent 35 S band with a molecular weight equivalent to 2.2 x 106 daltons was noted.

The possibility that the macromolecules with migration greater than 28 S were an aggregate was tested by melting the RNA's prior to electrophoresis. A series of experiments testing time and temperature effects on the hypochromicity of the isolated RNA and the preservation of 18 and 28 S migration bands were carried out. We found that heating these RNA's for 10 min at 60° in 0.1% SDS-containing buffer provided a most pronounced hypochromic shift (30%) with a most significant preservation of the 28 and 18 S structures, assayed electrophoretically. No differences in the hypochromic shift were noted comparing RNA isolated from control or thioacetamide-treated animals. Electrophoresis of these macromolecules following melting showed several
described in "Materials and Methods." RNA, 1.0 to 1.2 A_260, was subjected to electrophoretic separation, and the resultant gels were monitored by scanning at 260 nm. The direction of migration is from right to left. In all experiments 28, 18, and 4 S isolated from rat liver were separated under identical conditions and utilized as markers; the cytoplasmic RNA from control animals shows a prominent 28 and 18 S peak and a significant 4 S BAND. Five and 9 S material is also noted. RNA separated from thioacetamide-exposed animals shows more prominent 5 and 9 S peaks and a distinctly prominent 32 S peak.

Animals shows more prominent 5 and 9 S peaks and a distinctly prominent 32 S peak. This correlates with increased poly(A)-containing RNA species with similar migrations identified below. No differences in RNA recovery following heating were noted, and 96% of the polyribonucleotides from both control and thioacetamide-exposed animals were recovered. These results suggest the presence of more RNA with a size range from 9 to 16 S in the cytoplasm of treated animals and also show the appearance of a material that migrates more rapidly than 28 S rRNA, in addition to the 4, 5, 18, and 28 S material usually described. Furthermore, the altered RNA pattern occurs by 16 hr following exposure, is present with both 5- and 20-mg dose ranges, and is present for at least 40 hr. During this time period, the livers of the lower dose animals show only few scattered inflammatory cells and few, if any, dying hepatocytes. The large dose produces much greater cell injury and more inflammation but no difference in gel patterns. These findings suggest that neither dying cells nor the inflammatory exudate is the basis for the observed changes. The appearance of these smaller RNA's might also arise either as breakdown products of rRNA or by a premature release of fragments of preribosomal RNA. Neither possibility can be discounted with certainty, but several pieces of evidence point more to an augmented altered nuclear release. The evidence for altered nuclear permeability, the appearance of nuclear restricted RNA detected by hybridization techniques, and the augmented poly(A)-containing RNA species seen at this time all point to a potential nuclear source for this material. Furthermore, sizing of poly(A)-containing RNA's from whole cytoplasm also fits in this size range. Admittedly, the potential for this material to represent ribosomal breakdown must be considered, but the presence of these poly(A)-containing RNA's in both dose ranges and at times of reduction of RNA formation makes this seem unlikely. Addition of thioacetamide to isolated control nuclei does not change the release pattern, nor does it alter electrophoretic behavior of the RNA. This observation underscores the requirement for metabolic interaction with the carcinogen to result in these changes.

Electrophoretic Behavior of RNA Released by Nuclei in Vitro. Isolated nuclei incubated in vitro with ATP release a species of RNA with messenger-like activity (9-11, 15, 17, 18). We have shown that transport in vitro from nuclei of the carcinogen-treated animals to a surrogate cytoplasm was less restrictive than in controls (24). The RNA released by control nuclei and nuclei from livers exposed to a carcinogen in vivo has not previously been critically analyzed. We made use of the superior resolution of gel electrophoresis to determine the size of released RNA's.

A comparison was made of RNA's released from nuclei in vitro with and without the addition of ATP (Charts 3 and 4). Nuclei isolated from both control and thioacetamide-exposed livers were used. The RNA released from control rat liver nuclei in the absence of ATP constituted only 1 to 2% of the total prelabeled nRNA pool. Small 28 and 18 S RNA fractions with a major 9 S band appeared (Chart 3). A smaller 5 S peak was evident, and a particularly small quantity of 4 S could also be observed. The addition of ATP to the in vitro system markedly enhanced RNA release to 20 to 30% of the prelabeled nRNA, a 10- to 20-fold increased yield over...
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Chart 3. Polyacrylamide gel electrophoresis of RNA released in vitro from control nuclei. In vitro release in the presence and absence of ATP was carried out, the RNA extracted from the supernatant fluid by the method described, and this RNA subjected to electrophoretic separation. Polyribonucleotides released without ATP consist of a major RNA band at 9 S with a small band at 16 S. There is virtually no 4 S released. Addition of ATP increases total release 20-fold and, concomitantly, there is a significant increase in 16 and 4 S RNA and a lesser increase in the 9 S species.

Chart 4. Polyacrylamide gel electrophoresis of RNA released in vitro from thioacetamide-treated rat liver nuclei. Isolation and electrophoresis of in vitro-released RNA's was carried out as indicated in Chart 3. In the absence of ATP, a prominent 9 S peak is present. In contrast to the control preparation, there are less of the heavier RNA's. Addition of ATP results mainly in an increase in the 9 S peak with smaller quantities of heavier RNA and noticeably less 16 S RNA than in controls. Some 4 S RNA was also transported.

The energy-independent RNA transfer (11). There is also significant change in the electrophoretic behavior of material thus isolated. The most prominent bands that appeared were both a 16 and 9 S RNA; a profound increase in the amount of 4 S RNA was also noted.

Similar experiments were carried out with nuclei from thioacetamide-exposed livers (Chart 4). Compared to controls, these nuclei released a 5- to 10-fold increase in the amount of prelabeled RNA in the absence of ATP, 10% versus 1 to 2%. This material was subjected to polyacrylamide separation. A uniquely sharp band at 9 S, not accompanied by discernible 28, 18, 16, or 4 S RNA's, appeared in bathing fluid. The addition of ATP increased the levels of RNA released to those of controls (20 to 30%), representing only a 2-fold increase. Once more, there was a prominent 9 S band but, in these instances, some 4 S material appeared as well. The most striking finding was the release of a 9 S material from both treated and control nuclei in the absence of an energy source (ATP) and the relatively larger release following thioacetamide exposure. A 2nd change was the enhanced release of 4 S RNA in the presence of ATP from both the control and treated nuclei. One significant difference in the treated and thioacetamide nuclei was the absence of a 16 S species in the latter.

These results clearly point to an altered capacity for nuclear restriction and transport of RNA in vitro by nuclei of animals exposed to carcinogens (5, 22, 23). The size of the released RNA is similar to the increased RNA seen in in vitro experiments. The results also suggest that ATP plays a role in the release of RNA from the nuclei and that the energy-dependent transport process selects species of RNA. In the presence of ATP, control and treated nuclei show a marked increase of RNA with size consistent with a messenger species. In addition, 4 S RNA was also transported. This altered transport, seen in vivo and in vitro, confirms and extends the identification of differences in cytoplasmic RNA's in carcinogen-exposed animals.

Poly(A) Content of Cytoplasmic- and in Vitro-Released RNA's. The altered phenotypic expression predicated upon a decrease in nuclear restriction of RNA requires that at least some of the released species have messenger-like functions. The size range of some of the polyribonucleotides released in vivo and in vitro is consistent with that of a messenger species (8). Indirect assay of messenger-like species and a means of enriching the quantity of these RNA's involve the isolation of poly(A)-containing macromolecules (28).

A series of experiments was carried out to test for the most effective means of recovery and quantitation of both authentic poly(A) and poly(A)-containing rat liver RNA (7). These preliminary experiments seemed essential, because it was important to attempt quantitation over potentially broad and unknown concentration ranges. RNA was labeled with orotate and isolated from control rat livers (28). Both authentic poly(A), obtained from Miles Laboratories, Kankakee, Ill., and rat liver RNA labeled with orotate were separated by poly(U)-Sepharose, Millipore filter techniques, poly(dT)-cellulose, and cellulose chromatography. We found that the poly(U)-Sepharose procedure provided the greatest reproducibility over the broadest range of RNA's. Our uncertainty concerning the relative quantities of poly(A)-containing RNA's to be isolated from the different animals directed us to this most consistent means of separation, i.e., poly(U)-Sepharose. A Millipore filter worked equally well at lower concentration ranges but appeared to become saturated at higher RNA concentrations. Poly(dT)-cellulose or cellulose bound poly(A)-RNA in an inverse concentration-dependent fashion and seemed less reproducible.

A 2nd series of experiments was undertaken to follow the
labeling pattern of poly(A) in isolated RNA. We sought the differences in specific activity and percentage of poly(A)-containing RNA determined on the basis of RNA recovery. Attempts at direct assay by absorbance at 260 nm of the relative quantity of poly(A) and non-poly(A)-containing RNA bound were difficult and, because of the presence of formamide in the eluting buffer, not reproducible. Removal of the formamide introduced a 2nd extraction with its attendant modifications. Instead, we sought to quantitate the poly(A)-containing RNA indirectly on the basis of radioactivity derived from orotate. Since orotate is converted to UMP, its incorporation should not be so different in poly(A) and non-poly(A)-containing RNA's, and the major selection of RNA species labeled should relate to the duration of labeling. The shorter time should preferentially label more "messenger-like" species whereas longer labeling should equally label all RNA's (28).

Two types of experiments were undertaken: those in which RNA was isolated following a short pulse of orotate (90 min), and a 2nd series in which orotate was administered at 12-hr intervals for 2 days by i.p. placement. It was found that the specific activity for the poly(A)-containing RNA was relatively constant during a 48-hr period of exposure by the i.p. route (Chart 5). In addition, there was an increase in the specific activity of total cytoplasmic RNA, which approached that of the poly(A)-containing material. Under these circumstances, we felt that the short pulse exposure would preferentially label the rapidly labeling RNA consistent with messenger-like species. Saturation labeling would label all RNA's produced during a 48-hr period and could be used as a relative assay of the total amount of poly(A)-containing RNA expressed as radioactivity. Under these circumstances, the percentage of poly(A)-containing RNA assayed by radioactivity was approximately 2.5% of the total cytoplasmic pool.

Utilizing both pulse and saturation labeling, we followed the apparent percentage of poly(A)-containing RNA in cytoplasm of thioacetamide-intoxicated animals. Chart 6 shows that within the 12- to 24-hr period following thioacetamide intoxication, there is a prominent increase in the release of "rapidly labeled" poly(A)-containing RNA species from the nucleus to the cytoplasm. This percentage must represent a minimum figure, since RNA formation is reduced during this time period. By 24 hr the percentage of rapidly labeled poly(A)-containing RNA species has fallen, but, in fact, release does not fall to control levels until 72 hr. This suggests that one of the acute effects of thioacetamide is to cause a sudden and pronounced change in the release of RNA species from the nucleus to the cytoplasm. In order to determine whether or not a significant change occurs in the total quantity of poly(A)-containing RNA species released to the cytoplasm, a saturation labeling experiment was undertaken. The same figure (Chart 6) shows that by 16 hr there is a statistically significant increase in the amount of poly(A)-containing RNA species present in the cytoplasm. This elevation of poly(A)-containing RNA was still present at 72 hr, and its mean value was statistically different from controls (but at p < 0.05), suggesting that there are indeed increased quantities of poly(A)-containing RNA species released into the cytoplasm following thioacetamide intoxication.

Nuclei of rats fed thioacetamide 16 hr previously were subjected to similar experiments in which the nuclei were
prelabeled for short time periods and incubated in vitro for release as previously described; the RNA was separated subsequently on poly(U)-Sepharose. We found the quantity of poly(A)-containing RNA's increased slightly in control preparations incubated with the ATP (Table 1). The mean values are not statistically different, but the variances are large and in each experimental pair the ATP-released RNA contained more poly(A) macromolecules. The quantity of poly(A)-containing RNA seemed greater in both carcinogen-exposed nuclei with and without ATP. Once more, all experiments with treated nuclei were higher than controls, but the variance from experiment to experiment was large. These results suggested an enhanced messenger-like release but with very small differences. These materials were subjected to polyacrylamide electrophoresis for further assay.

The poly(A)-containing RNA bound to and eluted from the

### Table 1

**Comparison of poly(A) content of in vitro-released RNA's from control and 16-hr thioacetamide-exposed rats**

<table>
<thead>
<tr>
<th>% poly(A)-RNA released in vitro</th>
<th>Without ATP</th>
<th>With ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (4)*</td>
<td>17.5 ± 3.8*</td>
<td>19.7 ± 2.1</td>
</tr>
<tr>
<td>Thioacetamide (4)</td>
<td>20.5 ± 3.7</td>
<td>25.1 ± 3.8</td>
</tr>
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* Numbers in parentheses, number of separate experiments carried out.

* Mean ± 1 S.D.

...poly(U)-Sepharose column was prepared for gel electrophoresis. Preparations from carcinogen-exposed animals were compared to controls. Cytoplasmic RNA's not retained on poly(U)-Sepharose consisted of 28 and 18 S and a large quantity of 4 S RNA. On the other hand, the RNA bound to the poly(U)-Sepharose and eluted with formamide (the poly(A)-containing RNA) consisted, in large part, of 9 to 16 S RNA from both treated and control species. A similar analysis of RNA's released from nuclei in vitro was carried out. The poly(U)-Sepharose-retained RNA (poly(A)-containing RNA) from control and treated animals was very similar. A major component was the 9 S RNA both in control and especially, in treated animals released with and without ATP (Charts 7 and 8).

### DISCUSSION

Posttranscriptional regulatory control may involve the selection of specific RNA species for transport from the nucleus to the cytoplasm in eukaryotes (28). Isolated rat liver nuclei resuspended in surrogate cytoplasm release RNA upon addition of nucleotide triphosphates (9-11, 15-18, 24). We have confirmed and extended previous observations concerning this energy-dependent transport process and, more specifically, have described the electrophoretic mobility and the poly(A) content of the RNA released from control nuclei in the presence and absence of ATP (11, 16, 18). It has been suggested that released RNA's have messenger-like properties. Ishikawa et al. (9, 10) describes an informosome-like structure with size, composition, and function consistent with this concept. Our results are also consistent with this notion; i.e., the RNA released, both in the presence...
and absence of ATP, has a mobility consistent with messenger-like species, 9 to 16 S (8). In addition, the poly(A) content of the material released in vitro is significantly higher than that of liver cytoplasm. The differences in our results and those produced by Schumm and Webb (18) may be related to our use of rat liver RNase inhibitor. These observations suggest that at least a portion of in vitro release consists of messenger-like RNA. Our results also show that not all released RNA is messenger like. A surprising finding was the enhanced release of material with a 4 S migration. This smaller-molecular-weight material seems not to be a degradation product since careful attention is paid to RNase contamination; a RNase inhibitor is added to the incubation, and similar release of 4 S material does not occur in the absence of added ATP, even with nuclei from carcinogen-treated animals. In this group a significant proportion of larger species (greater than 9 S) of prelabeled RNA is indeed released without ATP, but no 4 S RNA appears. Direct analysis of acceptor RNA activity to confirm this point has still to be undertaken. These findings point clearly to an energy-dependent release of 4 S RNA, and this represents a further indication of RNA selection in in vitro release.

There is a similarity in electrophoretic behavior between the RNA released in vivo and the RNA isolated in vitro. This is also true when one compares the poly(A)-containing RNA species selected from cytoplasmic RNA by poly(U)-Sepharose chromatography to the material released from nuclei. It would appear that the in vitro model does, in some aspects, resemble the in vivo nuclear release of RNA. A fruitful comparison of the effects of carcinogen treatment on RNA-released material, both in vivo and in vitro, can be carried out. There is clearly an enhanced release of material with a size and poly(A)content consistent with those of mRNA from carcinogen-exposed nuclei in the absence of added ATP. Not only is there an increased quantity of this material released in the absence of the nucleotide triphosphate, but there is actually a further transport in the presence of an ATP system. These findings are consistent with previous studies done by competition hybridization indicating a leakiness of liver subjected to carcinogen treatment in vivo.

The observations that addition of thioacetamide to nuclei during incubation is not associated with release, that there is a time delay before release is observed in vivo, and that the release seems independent of the presence of inflammatory cells or cell injury suggest that this change is referable to the action of this carcinogen on the liver. The use of a single dose of thioacetamide has not been shown to be associated with aberrant programming of ribosomal maturatation. Furthermore, the brief period of exposure before experimentation suggests that the potential effect of altered programming must be small. Also, the size of the rRNA species involved is different from the macromolecules we describe. The appearance of transformed cells occurs temporally remote from these changes induced by the carcinogen (6), but with feeding the altered release phenomenon it is persistent in the liver and it is present in the resulting tumor cell population. This suggests that the alteration in nuclear restriction may be necessary but not sufficient for malignant transformation to occur. In skin there is a 2-stage mechanism for carcinogenesis (26). The application of the carcinogen results in initiation of certain cell populations. Subsequently, a promoter is necessary to elicit the carcinogenic transformation. It may be that a similar 2-stage system exists within the liver and that the changes observed regarding nuclear release are related to an initiation rather than to a promotional event. This suggests that a subsequent alteration takes place, which allows the appearance of the transformed phenotype. Consistent with this concept is the fact that there is a persistence of nuclear leakiness up to and including the time of transformation of rat liver cells by thioacetamide and several other carcinogens. This leakiness occurs early in the injury and is maintained beyond the temporal appearance of transformed cells. The specific relevance of the altered nuclear restriction to the process of transformation requires more extensive analysis. The altered release of putative messengers that may code for different proteins and hence for different functions can be tested by assay of their products in vitro.

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