Comparison of RNA Metabolism in G1-arrested and Stimulated Nontransformed and Chemically Transformed Mouse Embryo Cells in Culture

Harold L. Moses, David J. Wells, Douglas E. Swartzendruber, and Michael J. Getz

ABSTRACT

RNA metabolism and content in AKR-2B cells has been studied extensively in the G0/G1-arrested state and following the stimulation to proliferate. The purpose of the present study was to examine selected parameters of RNA metabolism in a chemically transformed derivative of the AKR-2B cell, AKR-MCA, at a G1 arrest point and following the stimulation to undergo DNA synthesis. Flow microfluorometric analysis indicates that the two cell lines have a comparable degree of growth arrest while autoradiographic data suggest a greater degree of growth arrest for the AKR-MCA cells. There is a similar magnitude of response following growth stimulation. Relative to the resting AKR-2B cells, the transformed AKR-MCA cells have higher resting levels of the following: (a) endogenous RNA polymerase II activity; (b) quantity of RNA polymerase II as determined by [3H]amanitin binding; (c) rate of accumulation of poly(A)- RNA; (d) rate of accumulation of poly(A)+ RNA; and (e) polysome content. Following the stimulation to proliferate, there is less of an increase in these parameters in the AKR-MCA cells than in the AKR-2B cells so that in late G1 (6 to 8 hr following stimulation) the levels are roughly equivalent in the two cell lines. The MCA cells were found to be insensitive to inhibition of stimulation of DNA synthesis by a-amanitin (an inhibitor of RNA polymerases II and III) and by 5-fluorouridine (an inhibitor of ribosomal RNA processing) at the same concentrations which cause complete inhibition of stimulation of DNA synthesis in the AKR-2B cells. In addition, a-amanitin and 5-fluorouridine were found to inhibit stimulation of DNA synthesis in the nontransformed C3H/10T½ cells but were not inhibitory for the chemically transformed derivative, C3H/MCA-58. These data provide further evidence that the growth-arrested chemically transformed cells are in a different metabolic state and are perhaps at a different arrest point in G1 than the quiescent nontransformed cells. The alterations in the chemically transformed cells which render them insensitive to inhibition of DNA synthesis stimulation by two compounds which are specific for inhibition of two separate steps in RNA metabolism remain to be established.

INTRODUCTION

It is well known that G1-arrested nontransformed cells have low levels of RNA and protein synthesis [reviewed by Pardee et al. (29) and Prescott (31)]. When stimulated to proliferate, there is a complex sequence of events which occurs during the prereplication interval between the stimulation to proliferate and the onset of DNA synthesis. This complex set of events has been termed the pleiotropic response (36). However, those specific events in this pleiotropic response which are necessary for the subsequent onset of DNA synthesis have not been completely defined. It has been reported that mouse 3T3 and 3T6 cells that are stimulated to proliferate have 2 to 4 times more cytoplasmic mRNA than do quiescent cells (21-23). The increased mRNA content of proliferating cells was thought not to be the result of increased transcription since there was no observed change in the rate of synthesis of hnRNA (25). The mRNA stability was reported not to be increased (1), and it has been suggested that increased efficiency of a posttranscriptional step could be responsible for the increased mRNA (21-23).

Studies from our laboratory on the AKR-2B cells have shown an increase in the endogenous DNA-dependent RNA polymerase activity (synthesizing hnRNA) (4) and in the quantity of RNA polymerase II as determined by [3H]amanitin-binding assays (43) to accompany the increase in the rate of accumulation and content of polysomal poly(A)+ RNA (4). An approximately 14-fold increase in the rate of accumulation of rRNA has also been shown to occur during stimulation of proliferation in nongrowing AKR-2B cells (43). Additional studies using metabolic inhibitors suggest that ribosome availability is a limiting factor in the subsequent synthesis of proteins required for DNA synthesis and cell division in these cells (43). The inhibitors used were a-amanitin, a specific inhibitor of RNA polymerase II at low concentrations and an inhibitor of RNA polymerase III at high concentrations (37, 42), and 5-FU, a specific inhibitor of rRNA processing (5, 22, 28). It was shown that a-amanitin in addition to inhibiting hnRNA synthesis also inhibited 45S preribosomal RNA synthesis and accumulation of poly(A)+ rRNA (43). The accumulated data on these cells indicate that an increased rate of structural gene transcription is necessary for stimulation of DNA synthesis (4, 15, 43). In addition, the data suggest that at least one gene which becomes more transcriptionally active in serum-stimulated cells encodes a protein factor which is necessary for an increase in the rate of ribosomal gene transcription and a subsequent increase in the number of cytoplasmic ribosomes.

We have previously compared selected aspects of mRNA metabolism in chemically transformed derivatives of both AKR-2B and C3H/10T½ cells (16, 17). In a comparison of rapidly growing, mid-log-phase cells, extensive sequence homology was observed in the populations of polysome-associated, poly(A)-containing mRNA of AKR-2B cells and a chemically

---

1 This investigation was supported by Grant CA 16816 from the National Cancer Institute, Department of Health, Education, and Welfare, and by the United States Department of Energy.

2 To whom requests for reprints should be addressed.

Received January 26, 1979; accepted August 6, 1979.

4516 CANCER RESEARCH VOL. 39

---

The abbreviations used are: mRNA, messenger RNA; hnRNA, heterogeneous RNA; 5-FU, 5-fluorouridine; MuLV, murine leukemia virus; FMF, flow microfluorometry; MCA, methylcholanthrene; rRNA, ribosomal RNA.
transformed derivative (AKR-MCA). The frequency distributions of mRNA sequences were largely indistinguishable in both cell lines, with the only identifiable exception being a large increase in the frequency of AKR-murine leukemia virus-related mRNA (17). A similar increase was not observed in a chemically transformed derivative of the C3H/10T½ cells (C3H/MCA-58), suggesting that the increase in MuLV mRNA observed in AKR-MCA cells is not directly related to the maintenance of the transformed phenotype (18). In addition, these previous studies suggested that qualitative changes in structural gene expression which are associated with chemically induced transformation in these cells are relatively few and may not be apparent in nonsynchronous populations of rapidly dividing cells.

Subsequent studies (26) have suggested that the alterations in RNA metabolism which are associated with the chemically transformed phenotype in these cells may be manifested in a cell cycle-specific fashion and may be related to the regulation of cell proliferation. The chemically transformed cell lines derived from the AKR-2B and C3H/10T½ cell lines have been shown to grow arrest in G1 when allowed to grow to saturation density in medium containing relatively high serum (10%) (26). The mechanism of growth arrest in the chemically transformed cells appears to be different from that of the nontransformed cells. The chemically transformed cells growth arrest due to depletion of amino acids and glucose from the chemically defined medium (26). In this state, the cells are unresponsive to serum and growth factors. On the other hand, AKR-2B and C3H/10T½ cells, like other nontransformed cells in culture (39, 41), growth arrest in the G0/G1 phase of the cell cycle due to depletion of serum growth factors (26). In this state, these cells can be stimulated to reenter the cell cycle by various growth factors and other mitogenic substances including serum, epidermal growth factor, fibroblast growth factor, and the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (26).

The purpose of the present study was to examine RNA metabolism in the chemically transformed AKR-MCA cells at quiescence and following the stimulation to proliferate. In addition, the effects of a-amanitin and 5-FU on stimulation of DNA synthesis in AKR-MCA cells as well as the nontransformed C3H/10T½ and the chemically transformed C3H/MCA-58 were determined. The data further substantiate the hypothesis that the chemically transformed cells are in a different metabolic state than are the nontransformed cells when growth arrested in G1.

MATERIALS AND METHODS

Cell Culture. The characteristics of the 4 cell lines used in this study have been described previously (16, 17, 32, 34). The AKR-MCA and C3H/MCA-58 cells are transformed as indicated by growth morphology, growth in soft agar, and tumorigenicity in nude mice (16, 17, 32). The AKR-2B and C3H/10T½ cells have a nontransformed morphology, do not grow in soft agar, and do not form tumors in nude mice (16, 17, 32, 34). The AKR-2B and AKR-MCA cells are grown in McCoy's Medium 5a supplemented with 10% fetal bovine serum (Reheis Chemical Company, Phoenix, Ariz.) without antibiotics. The C3H/10T½ and C3H/MCA-58 cells are grown in Eagle's basal medium also supplemented with 10% fetal bovine serum and without antibiotics. Experiments are carried out both in 490-sq cm plastic roller bottles (Corning Glass Works, Corning, N. Y.) or Falcon No. 3002 Petri dishes with a 21-sq cm growth surface (Falcon Labware Division, Oxnard, Calif.). All experiments are carried out within 10 passages of the frozen stocks from which cells are recovered periodically. These stock cells have been shown to be free of Mycoplasma contamination by culture methods, by scanning and transmission electron microscopy, and by staining with the Hoechst No. 33258 stain (9). Cells are examined after Hoechst No. 33258 staining monthly to ensure that they have remained free of Mycoplasma.

All of the cell lines are growth arrested by allowing them to grow to saturation density in medium with 10% fetal bovine serum and are stimulated to proliferate by changing to fresh medium containing 10% fetal bovine serum. The nongrowing state of the cells and the response to stimulation are verified in all experiments by assaying for [3H]thymidine incorporation as described below.

α-Amanitin or 5-FU is dissolved in sterile distilled water and added directly to the culture medium to give the concentrations indicated in “Results.”

Assay for DNA Synthesis. Cells are pulsed for 60 min with 1.0 μCi [methyl-3H]thymidine (6.7 μCi/mmol; New England Nuclear, Boston, Mass.) per ml of medium, and the incorporation into acid-precipitable material is determined as previously described (4, 15). DNA is determined by the diphenylamine reaction of Burton (7), and the results are expressed as cpm [3H]TMP incorporated μg DNA/hr. Autoradiography is performed as described previously (4).

Flow Microfluorometric Analysis. The methods for cell preparation for FMF and a detailed description of the instrumenta are available. Cells were fixed in 70% ethanol for at least 24 hr. They were then pelleted (500 x g for 5 min) and resuspended at a concentration of approximately 100,000 cells/ml in the mithramycin stain [mithramycin (100 μg/ml); (Pfizer Co., Groton, Conn.) in 0.15 M NaCl with 15 mM MgCl2]. The FMF-derived DNA distributions were analyzed according to the methods of Dean and Jett (13) and Jett (20).

Endogenous DNA-dependent RNA Polymerase II Activity. Nuclei from cells grown in roller bottles are prepared as previously described (4, 43) and suspended in storage buffer [0.05 mM Tris (pH 7.9), 0.1 mM EDTA, 1.0 mM dithiothreitol, human γ globulin (400 μg/ml), and 25% glycerol, ν/ν] and stored at −70° until used. The assay for endogenous DNA-dependent RNA polymerase II activity is as previously described (4, 43).

[3H]Amanitin-binding Assay for RNA Polymerase II. Suspensions of whole cells are sonicated for 10 sec (Branson 250 sonifier step-down microprobe, Power Setting 3) in storage buffer containing 0.3 m ammonium sulfate. Binding of [3H]-O-methyl-demethyl-γ-amanitin (specific activity, 2.4 Ci/mmol) (a generous gift of H. Faulstich, Heidelberg, Germany) was measured in the disrupted cells (20 to 30 μg of DNA per assay) by the ammonium sulfate precipitation technique described by Cochet-Meilhac et al. (10, 11). Under the assay conditions, the [3H]Amanitin had a specific activity of 2.48 x 10⁶ cpm/μg. Results are calculated as μg of RNA polymerase II per mg DNA assuming that the molecular weight of the RNA polymerase II is 5.5 x 10⁶ and assuming a stoichiometric ratio of 1:1 for amanitin binding to polymerase (10, 11). Using 4 x 10⁻¹⁰ μg of [3H]Amanitin per assay, the assay was linear at least up to 40 μg DNA added as a total cell homogenate.
Rate of Accumulation of Poly(A)+ and Poly(A)− RNA. For determination of the rate of accumulation of polysomal RNA before and after growth stimulation, RNA is labeled for 60 min with [5-3H]uridine at a concentration of 1 μCi/ml prior to harvest. Unlabeled uridine is added to give a final concentration of 5.4 x 10⁻⁶ M. This concentration has previously been shown to saturate internal precursor pools and to maintain these pools at a constant specific activity for at least 120 to 180 min (4). Polysomes are isolated from Nonidet P-40-lysed cells, and polysomal RNA is extracted and separated by 2 cycles of oligo(dT)-cellulose chromatography as previously described (15).

Preparation of Polysomes and Analysis of Polysome Profiles. Cells are pretreated with cycloheximide (1 μg/ml) for 30 min prior to harvest and are lysed by the Nonidet P-40 method (15). After centrifugation at slow speed to obtain a crude nuclear pellet, the supernatant is adjusted to 0.25 M sucrose and centrifuged at 10,000 rpm for 10 min in the Beckman JS-13 rotor. The resulting postmitochondrial supernatant is adjusted to 1% in Triton X-100, layered over a 5-ml pad of 2 M sucrose in lysing buffer (15), and centrifuged at 55,000 rpm at 4° for 2.5 hr in the Beckman 60 Ti rotor. The polysome pellet is dissolved in buffer consisting of 25 mM Tris (pH 7.6):40 mM KCl:7.5 mM MgCl₂ containing 1 mM dithiothreitol and centrifuged on linear 10 to 30% sucrose gradients. The polysome profiles are obtained by monitoring absorbance at 254 nm.

RESULTS

Relative Degree of Growth Arrest and Restimulation in AKR-2B and AKR-MCA Cells. In a previous study, we presented autoradiographic data obtained following a 1-hr pulse with [3H]thymidine which showed that both the AKR-2B cells and the AKR-MCA cells had approximately 1% of the nuclei labeled in the resting state (26). Following stimulation, 61% of the AKR-2B cells and 58% of the AKR-MCA cells were labeled. This indicated that there was a comparable degree of growth arrest and restimulation in the 2 cell lines. Because of the importance of this question in the interpretation of comparative data concerning RNA metabolism in the 2 cell lines, additional studies were performed. Autoradiography was performed following a 24-hr exposure of the quiescent cells to [3H]thymidine. Under these circumstances, the AKR-2B cells showed 4.8 ± 0.6% (S.D.) labeled nuclei while the AKR-MCA cells had 0.9 ± 0.4% labeling.

FMF analysis of mithramycin-stained cells was also performed, and the results are shown in Chart 1. Similar profiles were obtained for both cell lines in the resting state. Analysis of the data as described previously (13, 20) indicate that 79% of the AKR-2B cells and 85% of the AKR-MCA cells are in G₁, with 14 and 11%, respectively, being in the S phase. Autoradiography of replicate dishes following a 1-hr [3H]thymidine pulse showed 2.0 and 0.1% labeling for the 2B and MCA cells, respectively. There are at least 2 possible explanations for this discrepancy between the FMF and autoradiographic data. One is that the FMF analysis gives an overestimate of cells in S due to the presence of debris. The second is that a small percentage of the cells growth arrest during the S phase so that they have a DNA content typical of S, but are not incorporating [3H]thymidine. Both factors probably contribute to the discrep-
The percentage of cells in G2 plus M (7% for the AKR-2B cells and 4% for the AKR-MCA cells) may be a slight overestimate due to coincident passage of 2 cells through the laser beam.

The AKR-2B cells were analyzed by FMF at 20 hr following stimulation, and the AKR-MCA cells were analyzed at 18 hr since previous studies have shown peaks of $[^3H]$thymidine incorporation at these times (26). The FMF profiles of stimulated AKR-2B and AKR-MCA cells are somewhat different (Chart 1). The AKR-2B cells show an estimated 60% of cells in S while the MCA cells have 73% in S.

RNA Polymerase II Activities and Quantities in Quiescent and Stimulated AKR-2B and AKR-MCA Cells. Previous studies have shown that nongrowing AKR-2B cells have low levels of RNA polymerase II activity compared to growing cells and that there is an increase early following the stimulation to proliferate (4). To determine whether the transformed AKR-MCA cells have a similar response, both AKR-2B and AKR-MCA cells handled concurrently were growth arrested as previously described (26), and the levels of activity and quantity of RNA polymerase II were determined. As can be seen in Table 1, the resting AKR-MCA cells have a mean level of RNA polymerase II activity over 100% greater than that of the resting AKR-2B cells. The quantity of polymerase II as determined by $[^3H]$-amanitin-binding assay was approximately 50% greater in the resting AKR-MCA cells as compared to the resting AKR-2B cells.

To determine the changes in RNA polymerase II in AKR-MCA cells following the stimulation to proliferate, the experiment shown in Chart 2 was performed. The $[^3H]$-amanitin-binding assay for quantitation of RNA polymerase II was used because of its greater reproducibility in our hands. Quiescent AKR-2B cells stimulated to proliferate by the addition of epidermal growth factor (10 ng/ml) were utilized for comparison. Stimulation by changing to fresh medium with 10% serum gives a similar response in these cells (43). The resting MCA cells were stimulated by changing to fresh medium. In this particular experiment, the difference between the resting levels of polymerase II in the 2 cell lines was approximately 30%. In the AKR-2B cells, there was a 60% increase in RNA polymerase II by 4 hr followed by a slight decline to 8 hr. The quantity of RNA polymerase II in the AKR-MCA cells showed less increase following the stimulation to proliferate so that 4 to 8 hr following stimulation the levels of polymerase II in the 2 cell lines are far more similar than in the resting state.

Rate of Accumulation of Polysomal Poly(A)$^+$ and Poly(A)$^-$ RNA in Growth-stimulated AKR-2B and AKR-MCA Cells. Previous studies have shown that resting AKR-2B cells maintain minimal levels of polysomal poly(A)$^+$ and poly(A)$^-$ RNA which increase markedly following a stimulus to proliferate (4, 15). In general, the rate of accumulation of poly(A)$^+$ RNA (as measured by $[^3H]$uridine incorporation) reflects the general level of RNA polymerase II activity and amounts (4), although quantitative differences exist. Since AKR-MCA cells exhibit higher resting levels of RNA polymerase II than do AKR-2B cells, it was of interest to determine whether this difference was also reflected in the levels of poly(A)$^+$ mRNA. In 3 separate experiments, resting AKR-MCA cells exhibited consistently higher rates (average, 11-fold) of incorporation of $[^3H]$uridine into poly(A)$^+$ mRNA than did resting AKR-2B cells. Since these determinations were performed under conditions designed to obviate possible differences in the precursor nucleotide pools (4, 15), it is likely that much of this difference reflects an actual difference in the rate of accumulation of polysomal poly(A)$^+$ mRNA. In these same experiments, resting AKR-MCA cells exhibited an average 8-fold higher rate of incorporation of $[^3H]$uridine into polysomal poly(A)$^-$ RNA than did the AKR-2B cells.

Chart 2 illustrates the results of one of these experiments in which the rate of accumulation of $[^3H]$uridine into poly(A)$^+$ and poly(A)$^-$ RNA was measured in AKR-2B and AKR-MCA cells in both the resting state and following the stimulus to proliferate. As illustrated, AKR-2B cells exhibit the previously observed difference in the rates of accumulation upon serum stimulation. AKR-MCA cells, however, exhibit similar rates of accumulation in both the resting and stimulated states. While a large difference exists between resting populations of the 2 cell types, this difference becomes progressively less as the cells traverse the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>RNA polymerase II in quiescent AKR-2B and AKR-MCA cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
<td>Enzyme activity (pmol $[^3H]$UMP/mg DNA/min)</td>
</tr>
<tr>
<td></td>
<td>AKR-2B</td>
<td>47.2 ± 5.8$^a$ (7)$^b$</td>
</tr>
<tr>
<td></td>
<td>AKR-MCA</td>
<td>113.2 ± 6.8 (3)$^c$</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.D.

$^b$ Numbers in parentheses, number of separate experiments, each performed in triplicate.

$^c$ p for comparison of AKR-2B and AKR-MCA values are <0.005 as determined by Student's t test for both activity and quantity of enzyme.
prereplicative interval. This is chiefly due to little or no changes in the rates of accumulation in AKR-MCA cells while these rates are increasing markedly in the AKR-2B cells.

While the interpretation of the data illustrated in Chart 3 depends upon assumptions regarding the specific activity of internal nucleotide pools, optical measurements of polyribosomes and their profiles do not. Determination of the relative quantity of polyribosomes per cell was performed by measuring absorbance at 260 nm of the polysome preparations from a known quantity of cells prior to centrifugation to obtain the profiles shown in Chart 4. These data indicated that the resting AKR-MCA cells have an approximately 2.9-fold greater quantity of polysomes than do the resting AKR-2B cells. Chart 4 illustrates that polysomes isolated from resting AKR-2B cells consist predominately of monosomes with a few high-molecular-weight polysomes. Six hr following serum stimulation, these same cells exhibit a large increase in the ratio of polysomes to monosomes which is consistent with an increase in the rates of mRNA accumulation and protein synthesis. In contrast, resting AKR-MCA cells exhibit a considerably higher ratio of polysomes to monosomes in the resting state which increases only minimally by 6 hr following stimulation. Interestingly, the serum-stimulated AKR-2B cells appear to contain significantly more disomes and trisomes than do the AKR-MCA cells, but this is difficult to evaluate quantitatively.

**Effect of α-Amanitin on Stimulation of DNA Synthesis in Resting AKR-MCA Cells.** In previous studies, we have shown that α-amanitin at concentrations of 6 µg/ml or higher completely inhibits stimulation of DNA synthesis in the AKR-2B cells (43). The data presented in Table 1 and Charts 2 to 4 indicate that the AKR-MCA cells are in a different metabolic state when growth arrested where they maintain higher levels of mRNA than are the resting AKR-2B cells. To determine whether the AKR-MCA cells are sensitive to α-amanitin, the following experiment was performed. Growth-arrested AKR-MCA cells were stimulated in the presence of varying concentrations of α-amanitin as shown in Chart 5. No significant inhibition of stimulation of DNA synthesis was observed with concentrations of α-amanitin up to 25 µg/ml.

Since the AKR-MCA cells have a shorter prereplicative interval than do the AKR-2B cells (26), the explanation for lack of sensitivity of the AKR-MCA cells could be an inadequate time for the α-amanitin to exert its effect prior to the onset of DNA synthesis in these cells. To test this possibility, α-amanitin was added at various times prior to stimulation as shown in Chart 6. The data indicate that there is no significant inhibition even if α-amanitin is added up to 8 hr before stimulation. Therefore, the length of time between addition of α-amanitin and the onset of DNA synthesis cannot explain the different sensitivity of the AKR-2B and AKR-MCA cells to α-amanitin.

**Chart 3.** Rate of accumulation of poly(A)* and poly(A)* polysomal RNA in AKR-2B (open columns) and AKR-MCA (stippled columns) cells following the stimulation to proliferate. Cells in roller bottles were growth arrested and stimulated to proliferate at zero time. Cells were harvested in the resting state (zero hr) or at the times indicated after stimulation following a 1-hr pulse with [3H]uridine. Polysomes were isolated and the polysomal RNA extracted and separated by oligo(dT)-cellulose chromatography.

**Chart 4.** Polysome profiles of resting and stimulated AKR-2B and AKR-MCA cells. Polysomes were isolated, and the profiles were determined as described in "Materials and Methods.** A, AKR-2B cells in the resting state; B, AKR-2B cells 6 hr following the stimulation to proliferate; C, AKR-MCA cells in the resting state; D, AKR-MCA cells 6 hr following the stimulation to proliferate.
Another possible explanation for this lack of sensitivity of the AKR-MCA cells to α-amanitin is that these cells have a mutation making them resistant to α-amanitin as has been reported with a number of other cell lines (6, 18, 24). To test this possibility, cloning assays were performed in the presence of varying concentrations of α-amanitin as shown in Chart 7. Identical studies were performed on the AKR-2B cells for comparison. As can be seen in Chart 7, there is some difference in the cloning efficiency of the 2 cell lines at the 0.5 and 1.0 μg/ml concentration of α-amanitin. However, concentrations of α-amanitin of 3 μg/ml or higher completely inhibit growth in both cell lines, indicating the AKR-MCA cells are not α-amanitin-resistant mutants. Additional data which support the contention that these cells are not α-amanitin-resistant mutants are that endogenous RNA polymerase II activity in isolated AKR-MCA nuclei is inhibited by α-amanitin (1 μg/ml) (data not shown).

Effect of 5-FU on Stimulation of DNA Synthesis in AKR-MCA Cells. In previous studies, we have shown that the stimulation of DNA synthesis in AKR-2B cells is completely inhibited by 5-FU at concentrations of 0.5 μg/ml or higher (43). These and other data indicate that ribosome availability is a limiting factor in the subsequent synthesis of proteins required for DNA synthesis and cell division in these cells. To determine whether ribosome availability is a limiting factor in the stimulation of DNA synthesis in AKR-MCA cells, the effect of 5-FU on stimulation of DNA synthesis was determined (Chart 8). 5-FU was found not to inhibit stimulation of DNA synthesis in the AKR-MCA cells, even at concentrations 20-fold higher than that found to be inhibitory in the AKR-2B cells. In separate experiments, the effects of 5-FU on polysomal RNA accumulation in AKR-MCA cells was determined. Table 2 illustrates that 4 hr following stimulation in the presence of 5-FU (5 μg/ml), the rate of accumulation of poly(A)− polysomal RNA was inhibited >70% relative to control cells stimulated in the absence of the drug while poly(A)− RNA accumulation was relatively unaffected. Sucrose gradient analysis (data not shown) of the poly(A)− RNA which continues to accumulate in the 5-FU-treated cells revealed a sedimentation profile identical to that of poly(A)+ mRNA with no discernible 18 or 28S rRNA. This fraction of nonribosomal poly(A)− RNA will be the subject of a future communication. The present data, however, illustrate that 5-FU virtually completely inhibits the accumulation of newly synthesized rRNA in serum-stimulated AKR-MCA cells. This is an identical result to that obtained previously for AKR-2B cells (43) and suggests that the very different effects of 5-FU on serum-stimulated DNA synthesis in these 2 cell types are not
H. L. Moses et al.

Chart 7. Effect of α-amanitin on plating efficiency of AKR-2B (○) and AKR-MCA (●) cells. Two hundred cells from each cell line were plated in 100-mm Petri dishes in the presence of varying concentrations of α-amanitin as indicated. After one week, the cells were fixed and stained with Giemsa, and the number of colonies per dish was counted. The data are expressed relative to the plating efficiency obtained for each cell line without α-amanitin.

due to differences in the degree of inhibition of rRNA accumulation. The data, therefore, suggest that the AKR-MCA cells have sufficient ribosomes in the resting state for the synthesis of proteins required for DNA synthesis.

Effect of α-Amanitin and 5-FU on Stimulation of DNA Synthesis in C3H/10T½ and C3H/MCA-58 Cells. The non-transformed C3H/10T½ cells and the chemically transformed derivative C3H/MCA-58 have been previously shown to be similar to the AKR cells with respect to their mechanisms of growth arrest (26). The C3H/10T½ cells growth arrest due to depletion of serum growth factors, while the transformed C3H/MCA-58 cells growth arrest due to depletion of low-molecular-weight nutrients from the medium (26). Therefore, the experiment shown in Chart 9 was performed to see if the C3H cells had a similar response to inhibition of DNA synthesis by α-amanitin and 5-FU. As can be seen, stimulation of DNA synthesis in the C3H/10T½ cells is completely inhibited by α-amanitin (7 μg/ml) and 5-FU (1.0 μg/ml). The chemically transformed C3H/MCA-58 cells are not inhibited by these 2 compounds at these same concentrations (Chart 9).

DISCUSSION

Previous studies have shown that the nontransformed (AKR-2B and C3H/10T½) and the chemically transformed (AKR-
Comparison of RNA Metabolism

MCA and C3H/MCA-58) cell lines when allowed to reach saturation density in medium containing 10% fetal calf serum growth arrest due to different mechanisms. The nontransformed cells growth arrest due to depletion of essential growth factors while the transformed cells growth arrest due to depletion of low-molecular-weight nutrients from the medium (amino acids and glucose) (26). In this paper, we provide further evidence that the transformed and nontransformed cells are in a different metabolic state when growth arrested, perhaps at different points in the G1 phase of the cell cycle. Relative to the resting AKR-2B cells, the transformed AKR-MCA cells have higher resting levels of the following parameters of RNA metabolism: (a) endogenous RNA polymerase II activity; (b) quantity of RNA polymerase II as determined by [3H]amanitin binding; (c) rate of accumulation of poly(A)* RNA; (d) rate of accumulation of poly(A)* RNA; and (e) polysome content. Previous studies from this laboratory have also shown that the quantity of poly(A)-containing RNA is higher in resting AKR-MCA cells than in resting AKR-2B cells (17). Following the stimulation to proliferate, there is a greater increase in the various parameters of RNA metabolism in the nontransformed AKR-2B cells so that in late G1, 6 to 8 hr following stimulation) the levels are roughly equivalent in the 2 cell lines. In addition, we have shown that the AKR-2B cells become insensitive to inhibition of DNA synthesis by α-amanitin and 5-FU in late G1, 2 to 4 hr prior to the beginning of S phase (43). The transformed cell lines are insensitive to the inhibitory effects of these 2 compounds even if added at the time of stimulation. These data suggest that the chemically transformed cells may grow arrest at a later point in the G1 phase of the cell cycle, past the α-amanitin and 5-FU-sensitive step exhibited by the arrested nontransformed cells. The shorter prereplication interval previously observed in the chemically transformed cells is also consistent with this hypothesis (26).

The FMF and autoradiographic data indicate that the differences in RNA metabolism observed in the AKR-2B and AKRMCA cells cannot be accounted for by differences in the degree of growth arrest and restimulation between the 2 cell lines. If any differences in the degree of G1 arrest do exist, the AKR-MCA cells have greater growth arrest than do the AKR-2B cells. Therefore, the higher levels of RNA metabolism in the AKR-MCA cells is not due to fewer of these cells being G1 arrested. The 2 cell lines also have a comparable magnitude of response following stimulation with any differences being in favor of the AKR-MCA cells showing a slightly greater response. Hence, the smaller increase in RNA metabolism in the AKR-MCA cells following stimulation cannot be accounted for by a lower growth response in these cells relative to the AKR-2B cells. Another question which must be addressed is whether the different levels of RNA metabolism and content observed in the AKR-MCA cells reflect changes in the general population or in only a few cells. The best evidence that the changes observed reflect those in the general population is that α-amanitin and 5-FU are completely inhibitory for the nontransformed cells and cause no detectable inhibition in the transformed cells. This strongly suggests that all responding cells in the transformed population are in the same metabolic state.

Previous studies on the mechanism of inhibition of stimulation of DNA synthesis in the AKR-2B cells by α-amanitin and 5-FU indicate that both compounds may act through a common mechanism (43). α-Amanitin was shown to inhibit synthesis of 45S rRNA (43), and it is known that 5-FU is specific for inhibition of 45S rRNA processing (5, 22, 28). This suggests that the AKR-2B cells do not have sufficient ribosomes for synthesis of proteins necessary for DNA replication. The reason for the insensitivity of the chemically transformed cells to inhibition of stimulation of DNA synthesis by these 2 metabolic inhibitors is not known. The most straightforward interpretation of the data is that the transformed cells have high levels of macromolecular synthesis and content in the resting state so that they maintain a higher level of RNA and/or proteins necessary for RNA synthesis. However, Connan and Rabotti (12) have reported that α-amanitin does not inhibit transcription of hnRNA, rRNA precursors, or low-molecular-weight RNA's in Rous sarcoma virus-transformed chicken fibroblasts at the same concentrations which cause complete inhibition of synthesis of these RNA's in the nontransformed chicken fibroblasts. In agreement with these results, preliminary experiments in our laboratory have indicated that α-amanitin has little effect upon the accumulation of labeled polyosomal poly(A)* RNA or upon polyribosome profiles at exposure periods of up to 8 hr in stimulated AKR-MCA cells. This is in contrast to its effects upon these parameters in AKR-2B cells (43) and suggests that a reduced permeability to α-amanitin may partially explain the lack of the effect of this drug upon the stimulation of DNA synthesis in AKR-MCA cells. This reduced permeability, however, does not appear to extend to uridine (Chart 3) or its analogue, 5-FU.

The reason for the lack of sensitivity of chemically transformed cells to inhibition of DNA synthesis by 5-FU appears to be more straightforward since the data of Table 2 indicate that this drug is indeed taken up and has the expected effect upon RNA processing. Electron microscopic studies (17) and quantitation of polyribosomes indicate that the resting transformed cells maintain larger quantities of ribosomes in the resting state than do the nontransformed cells. It would therefore seem that the transformed cells have sufficient ribosomes for synthesis of proteins necessary for DNA synthesis so that the inhibition of ribosome processing has no effect.

Some of the findings described in this paper to some extent appear similar to those described by Baserga et al. (2) and Rovera and Baserga (33). Resting spontaneously transformed 3T6 cells were reported to be stimulated by medium without serum. Both the 3T6 cells and 2RA cells (SV40 virus-transformed WI-38 cells) did not show an early increase in chromatin template activity following stimulation while G0-arrested nontransformed WI-38 cells did show an early (1 hr after stimulation) increase. It was concluded that the 3T6 and 2RA cells were G0 arrested while the nontransformed cells were arrested in G0. However, since FMF studies were not performed, it is not clear whether the 2RA cells were G0 arrested or merely stationary at various stages of the cell cycle. It is now generally accepted that DNA virus-transformed cells usually do not arrest in G0, but are distributed around the cell cycle (14). In addition, it appears that the chromatin template activity as measured by this group reflects nucleolar changes rather than nucleoplasmic changes (3). Thus, the lack of an increase in chromatin template activity in the transformed 3T6 (2, 33) cells may reflect the same basic alteration as the observations in our

*H. L. Moses, D. J. Wells, and M. J. Getz, unpublished observations.
study that there is a minimal increase in the rate of accumulation of poly(A)− RNA and an insensitivity to inhibition of DNA synthesis by 5-FU in the chemically transformed cells.

Several other drugs have been shown to inhibit growth in nontransformed cells while not inhibiting transformed cells. These include cytochalasin B (27), caffeine (30), streptovitacin A (30), asparaginase (8), puromycin aminonucleoside (38), and agents which raise intracellular cyclic adenosine 3′:5′-monophosphate levels (35). The present report of 2 additional compounds which selectively inhibit nontransformed cells is of importance in that these 2 compounds have known relatively specific actions on different aspects of RNA metabolism. They could prove to be highly useful in elucidating basic differences between chemically transformed cells and their nontransformed counterparts.

ACKNOWLEDGMENTS

The authors wish to thank Earl L. Branum, Jacqueline A. Proper, Mary E. Volkenant, and Gayle R. Travis for excellent technical assistance, Dr. H. Faulstich for supplying the [3H]amanitin, Nathan Belcher for supplying the mithramycin, Volkenant, and Gayle R. Travis for excellent technical assistance. Dr. H. Faulstich

REFERENCES


Comparison of RNA Metabolism in G₁-arrested and Stimulated Nontransformed and Chemically Transformed Mouse Embryo Cells in Culture

Harold L. Moses, David J. Wells, Douglas E. Swartzendruber, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/39/11/4516

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.