
Modulation of hnRNP A1 protein gene expression by epidermal growth factor in Rat-1 cells

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ABSTRACT

The levels of mRNA encoding hnRNP core protein A1 have been compared in quiescent and proliferating Rat-1 embryonic fibroblasts. Northern blot hybridization analyses using probes made from an A1 cDNA clone, λ HDP-182, isolated by Cobianchi *et al.* (J. Biol. Chem. 261:3536-2543 (1986)) indicated that three sizes of poly A⁺ RNAs, 1.6, 2.0, & 4.0 kb, have extensive sequence homology. The levels of all three A1 RNA species are responsive to the proliferation state of the cells. Stimulation of quiescent Rat-1 cells with serum or epidermal growth factor resulted in a 2- to 5-fold increase in the levels of each of these three RNAs that was evident after 2 hours and reached a peak after about 8 hours. Addition of the protein synthesis inhibitor, cycloheximide, along with epidermal growth factor completely blocked the upsurge in A1 RNA levels. Thus, the A1 RNA species are not primary transcriptional targets of epidermal growth factor but do show an induction pattern similar to mRNAs encoding some glycolytic enzymes.

INTRODUCTION

In eukaryotes, a specific set of abundant proteins associate with heterogeneous nuclear RNA during transcription to form complexes referred to as hnRNP (1,2). Electron microscopic studies of chromatin spreads or nucleoplasm revealed that hnRNP has a beads-on-a-string appearance with smooth RNP fibrils connecting 20-30 nm RNP spheres (3). After mild RNase digestion, hnRNP "beads" may be isolated as 30 to 40 S particles with a uniform structure. The hnRNP particles are comprised of RNA about 700 bases long, wrapped around a core of protein (4,5,6). The hnRNP particle core proteins include several abundant proteins that range in size between 32,000 and 42,000 Daltons and display isoelectric point heterogeneity on 2-dimensional gels (2). Six proteins resolved by 1-dimensional SDS-polyacrylamide gels, termed A1, A2, B1, B2, C1, and C2 (1), comprise up to 90% of the total protein mass in hnRNP particles isolated from proliferating cells and have been found in the ratio 3A1:3A2:3C1:1B1:1B2:1C2 (5,6).

LeStourgeon *et al.* (7) first suggested that the relative level of A1 protein in hnRNP particles is dependent on the cellular growth rate. They found that nonhistone nuclear protein extracts of starved HeLa cells and of confluent mouse embryo fibroblasts contained about one-fifth as much A1 protein as extracts from the same cell lines undergoing exponential growth (7). In contrast, the levels of the other hnRNP core proteins did not display substantial differences. Supporting data had been published by Loeb *et al.* (8) who reported that a 31,000 Dalton protein, which is now believed to be the A1 protein, produced a strong band in

SDS-polyacrylamide gel electrophoresis analysis of non-histone nuclear extracts of rapidly growing teratocarcinoma cells, a faint band in slower growing L cell extracts, and no detectable band in normal liver extracts. In addition, Bravo and Celis (9) found a proliferation-sensitive protein by 2-dimensional electrophoresis of solubilized human and mouse cells that was later identified to be the hnRNP A1 protein (10). Rather than analyzing relatively crude nuclear or cellular extracts, de Koch *et al.* (11) carried their investigation one step farther and found that A1 protein was almost absent in 40 S hnRNP particles from resting bovine lymphocytes, but was abundant in particles from concanavalin A stimulated lymphocytes.

Cobianchi *et al.* (12) recently isolated and sequenced a full length cDNA clone, λ HDP-182, for a rat nucleic acid helix-destabilizing protein. Inspection of the amino acid sequence encoded by the open reading frame contained in this cDNA clone revealed that it exactly matched the amino acid sequence of the A1 protein (13). In this paper, we report the use of λ HDP-182 cDNA as a probe to study the expression of A1 mRNA in cultured embryonic rat fibroblast cells that have been forced into quiescence by serum-starvation and subsequently stimulated by the addition of media containing either serum or epidermal growth factor (EGF).

METHODS

Cell Culture

Rat-1 embryonic fibroblasts (14) were propagated at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (iron-supplemented, Hyclone) and equilibrated with 5% CO₂/95% air. Quiescent cultures were obtained by growing cells seeded at approximately 5 x 10³ cells/cm² for 5 days and then switching to fresh DMEM without serum for another 48 h. Stimulated cells were obtained by the addition of fresh DMEM containing either 10% iron-supplemented bovine calf serum or 5 ng/ml EGF (murine EGF α was a gift from B. Magun (15)).

Poly A+ RNA Isolation and Analysis

Poly A+ RNA was isolated from cell cultures by a modification of the procedure of Schwab *et al.* (16). Briefly, at the indicated times, the culture media was removed and the cells were washed with cold phosphate-buffered saline. The cells were scraped off the culture dish in phosphate-buffered saline, transferred to centrifuge tubes, and pelleted (500xg, 5 min). The cells were solubilized by the addition of lysis buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, and 1% SDS), briefly sonicated (2 15-sec bursts), and digested with 75 μ g/ml Proteinase K at 37 °C for at least 30 min. After adjusting the NaCl concentration to 0.4 mM by the addition of 5 M NaCl, the solution was heated at 95 °C for 2 min. and then quickly cooled to about 22 °C. Oligo dT-cellulose (Pharmacia, Type 7) was added, and the suspension was mixed overnight. The oligo dT-cellulose was washed with high-salt buffer (10 mM Tris-HCL, pH 7.4, 400 mM NaCl, 1 mM EDTA, and 0.2% SDS), transferred to a small column, and washed again with high-salt buffer followed by low-salt buffer (NaCl reduced to 100 mM). The bound poly A+ RNA was eluted with no-salt buffer (5 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% SDS) and precipitated at -70 °C after the addition of 1/9 vol 3 M Na acetate and 2.5 vol cold 95% ethanol. The RNA was collected by centrifugation at 60,000xg for 20 min, dried, and resuspended in water. For Northern blot analysis, the indicated amount of RNA was denatured with

formaldehyde and formamide, electrophoresed through a formaldehyde-containing 1.2% agarose gel, and blotted onto nitrocellulose as described in Maniatis *et al.* (17). Prehybridization incubations were performed in 50% formamide, 0.1% SDS, 50 $\mu\text{g/ml}$ poly A, 5x SSC (0.75 M NaCl, 0.075 M Na citrate), 1x Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), and 250 $\mu\text{g/ml}$ denatured salmon sperm DNA for 2-4 h at 43 °C. Hybridizations were performed overnight at 43 °C in prehybridization solution supplemented with the indicated ^{32}P -labeled probe. The blots were washed at 43 °C with 5x SSC and then exposed to Kodak XAR autoradiography film. Relative densities of the individual bands were obtained by scanning the film with a Hoefer densitometer and determining peak areas with the Bioquant morphometric analysis system.

^{32}P -labeled Probes

The A1 cDNA probe was prepared from gel-purified HDP-182A insert DNA (Figure 2) by random hexamer-primed incorporation of $\alpha\text{-}^{32}\text{P}$ -dCTP into DNA by the Klenow fragment of *E. coli* DNA polymerase I (18). The cyclophilin riboprobe was prepared from p1B15 DNA (a gift from J. Douglass) by transcription with SP6 RNA polymerase in the presence of $\alpha\text{-}^{32}\text{P}$ -CTP (19).

DNA Synthesis Assay

The time course of DNA replication induction by EGF was determined by monitoring the relative rate of ^3H -thymidine incorporation at various times after EGF stimulation.

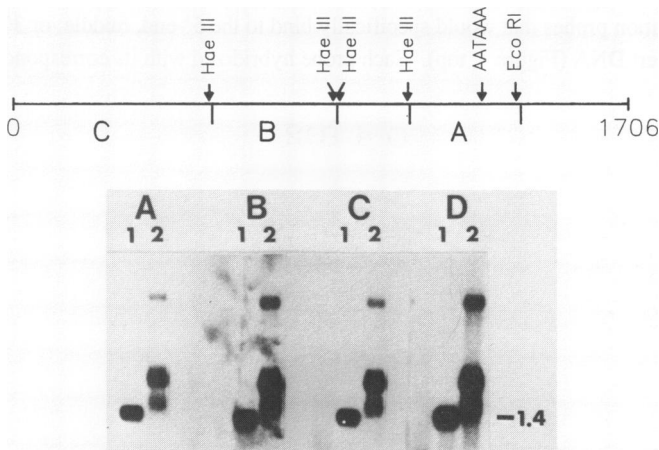


Figure 1. *TOP*: Restriction map of $\lambda\text{HDP-182}$ cDNA insert. The map is based on the sequence published by Cobianchi *et al.* (10). HDP-182A is the large Eco RI fragment from position 1 through 1397. The letters labeling the Hae III/Eco RI digest fragments correspond to the probe labels used in the bottom panel.

BOTTOM: Northern blot hybridization analysis of Rat-1 poly A+ RNAs with probes to different regions of the HDP-182A cDNA. Four sets of the samples indicated below were electrophoresed and blotted onto nitrocellulose. The membrane was cut into strips corresponding to the sample sets, and the nucleic acids hybridized to the indicated Hae III/Eco RI fragment probes (see map above). *Probes*: A - 3'-end; B - middle; C - 5'-end; D - complete HDP-182A cDNA. *Samples*: 1 - undigested HDP-182A cDNA; 2 - Rat-1 poly A+ RNA (6 μg).

Quiescent Rat-1 cells were prepared and stimulated (at 0 h) by the addition of 10 ng/ml EGF as described under cell culture. One hour before the indicated times, ^3H -thymidine (1 $\mu\text{Ci}/\text{ml}$, 20 Ci/mmole) was added to triplicate culture dishes. After a 1 h incubation period, the cells were washed with phosphate-buffered saline and the radioactivity precipitable with cold 10% trichloroacetic acid was measured for each culture dish.

RESULTS

Northern blot analysis of poly A+ RNA from exponentially growing Rat-1 embryonic fibroblasts (Figure 1, bottom) indicated that three sizes of RNA hybridize to the HDP-182A cDNA probe. The two strongest signals came from RNA species that are approximately 1.6 and 2.0 kb long. A weaker signal is seen for a band in the 4.0 kb region. It is not known yet if there are many fewer molecules of the 4.0 kb species or if it has a lower hybridization efficiency because of a slightly different nucleotide sequence. Cobianchi and Wilson (12) obtained similar results with poly A+ RNA isolated from rat brain and also pointed out that the 2.0 kb species is the appropriate size to have served as the template for the synthesis of the $\lambda\text{HDP-182 cDNA}$. Analogous RNA forms are found in murine cells as well. Northern blots of mouse AKR-2B embryonic fibroblast poly A+ RNA also showed strong hybridization to 1.6 and 2.0 kb species and perhaps a weak binding to a 4.0 kb species.

To determine the relationship between these 3 RNA species, we began to investigate the extent of structural homology between them. Three Hae III restriction fragments were used to make hybridization probes that would specifically bind to the 5'-end, middle, or 3'-end of the HDP-182A insert DNA (Figure 1, top). Each probe hybridized with its corresponding Hae III

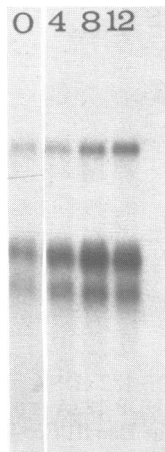


Figure 2. Elevation of A1 RNA levels after serum stimulation of quiescent cells. Rat-1 cells were forced into quiescence by incubation for 48 h in DMEM without serum. At 0 time, the medium was replaced with DMEM supplemented with 10% calf serum. After incubation for the indicated hours, cells were harvested and poly A+ RNA isolated. The RNA samples (9 μg) were analyzed by Northern blot hybridization with the complete HDP-182A cDNA probe.

fragment and did not cross-react with the other fragments. Each of these three probes bound to all 3 of the poly A+ RNA species (Figure 1, bottom). Therefore, these 3 RNA species share extensive structural homology and are not simply hybridizing to the HDP-182A probe through a small common segment.

To investigate the possibility that the 4.0 kb RNA species is a splicing intermediate for one or both of the smaller RNA forms, poly A+ RNA was extracted from nuclear and cytoplasmic fractions obtained from exponentially growing Rat-1 cells. All 3 RNA forms were detected in both the nuclear and cytoplasmic preparations. Therefore, the 4.0 kb RNA form does not appear to be a nuclear precursor for the two smaller RNAs.

The nucleotide sequence of λ HDP-182 cDNA includes a second consensus signal for polyadenylation, AATAAA, beginning at position 1301. Thus a plausible explanation for the 1.6 kb RNA species is that it results from the use of a secondary polyadenylation site. In support of this explanation, we have found that a 25-nucleotide probe corresponding to a region about 130 nucleotides 3' of this site (nucleotides 1439 to 1463) does not hybridize to the 1.6 kb RNA but does hybridize to both the 2.0 and 4.0 kb RNA forms).

Clearly, further analysis of additional cDNA clones is necessary to determine if the 3 RNA species are alternative processing products from one gene or are transcribed from closely related genes and to determine which one(s) of these RNA forms is(are) actually translated to produce the A1 protein. However, all of the available data suggest that the 3 RNA forms are interrelated, and we proceeded to investigate the dependence of the intracellular level of each RNA form on the proliferation state of the cell.

Rat-1 embryonic fibroblast cells can be forced into quiescence by serum-starvation. Addition of either serum or purified growth factors to the quiescent cells results in a cascade of events eventually leading to the initiation of DNA replication (20). To determine if the level of any of the three A1 hybridizing RNA species undergoes selective changes during induction of cellular proliferation, their expression was analyzed following serum stimulation of quiescent Rat-1 cells. As shown in Figure 2, the addition of media containing 10% bovine calf serum to cell cultures which had been deprived of serum for the previous 48 h resulted in an increase in the levels of all three A1-hybridizing RNA species. The maximum RNA levels were obtained approximately 8 h after stimulation and represented a 3- to 5-fold increase over basal levels.

To further characterize the nature of the induction of the A1-hybridizing RNA species, we switched to using a more clearly defined growth stimulator and began using EGF as the mitogen. The data in Figure 3 show that DNA synthesis, as measured by ^3H -thymidine incorporation, in serum-starved Rat-1 cells began about 8 h after EGF addition and reached a peak rate at about 18 hours. This result is in accord with previously published data using density labeling to determine the time course of DNA replication induced in Rat-1 cells by EGF (20). Northern blot analysis indicated that EGF stimulation of serum-starved cells caused a 2 to 4-fold increase over 8 hours in the abundance of each of the three species of A1-hybridizing RNA (Figure 4).

For comparison, the same blot was washed and reprobbed for cyclophilin mRNA with a ^{32}P -labeled riboprobe synthesized from the p1B15 plasmid (21). Cyclophilin is a cyclosporin A binding protein whose mRNA has an apparently ubiquitous tissue distribution and has been used

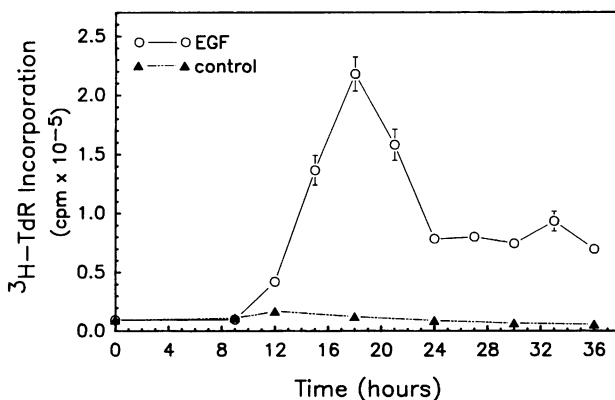


Figure 3. Rate of ³H-thymidine incorporation after stimulation of quiescent cells with EGF. At 0 time, serum-starved Rat-1 cells were stimulated by 10 ng/ml EGF. At the indicated times, the rate of ³H-thymidine incorporation was measured by pulse-labeling as described under Materials and Methods. The error bars indicate the standard deviation for the cases where the range was greater than the symbol size.

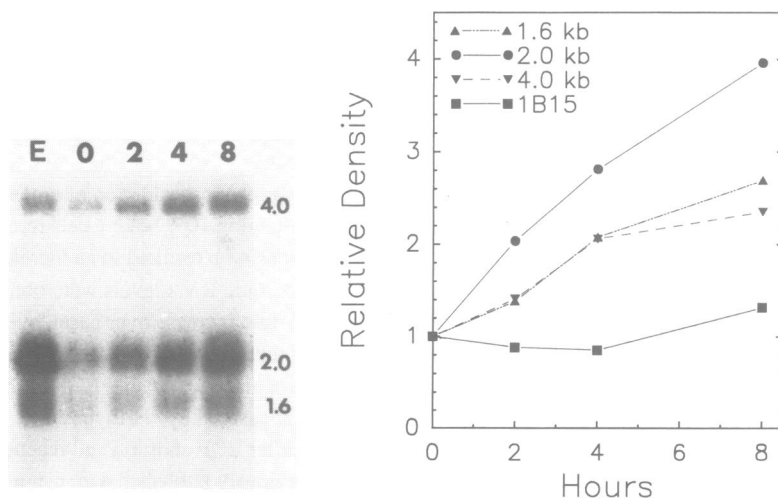


Figure 4. Elevation of A1 RNA levels in response to cell stimulation by EGF. Rat-1 cells were serum-starved for 48 h, and at 0 time, the culture medium was replaced with EGF (10 ng/ml) supplemented DMEM. Poly A+ RNA was isolated after incubation for the indicated hours and analyzed (7 μg) by Northern blot hybridization with the complete HDP-182A probe. E - poly A+ RNA from exponentially growing Rat-1 cells. The blot was washed, and the RNA hybridized to the 1B15 riboprobe. The values shown in the plot of relative densities were normalized to the 0 time sample for each RNA species.

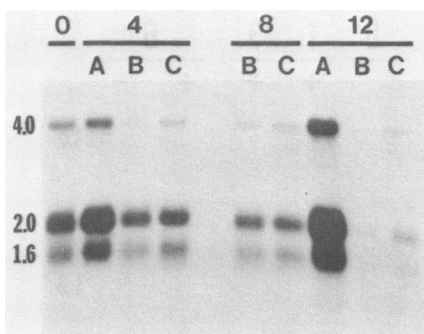


Figure 5. Cycloheximide blocks the EGF induced stimulation of A1 RNAs. This experiment was similar to that shown in Figure 4 except that some of the cultures received medium containing 25 $\mu\text{g/ml}$ cycloheximide along with or instead of the 10 ng/ml EGF. Lanes: A - EGF; B - cycloheximide; C - EGF + cycloheximide.

as a standard to monitor Northern blot analyses (21). As shown in Figure 4, the level of cyclophilin mRNA was relatively constant throughout the EGF induction period.

The effect of the protein synthesis inhibitor, cycloheximide, on the induction of the A1-hybridizing RNAs was determined for two reasons. The first was to determine if the EGF-mediated increase in A1-hybridizing RNA levels was a primary genetic response or required the prior synthesis of other gene products. The second was to learn if any of the A1-hybridizing RNAs should be grouped with the growth-related genes that have been found to be superinduced by treatment with growth factors in the presence of cycloheximide. These genes include *c-fos* (22), *actin* (23), and several genes with unknown products classified as "intermediate early genes" by Lau and Nathans (24). We measured RNA levels in serum-starved Rat-1 cells treated with EGF, cycloheximide, or both for periods up to 12 h. Northern blot analyses (Figure 5) indicated that addition of 25 $\mu\text{g/ml}$ cycloheximide together with the EGF completely blocked the stimulatory effects of EGF. Thus, EGF stimulation of the A1-hybridizing RNA species appears to require an intermediate protein synthesis step and does not fall into the class of genes which are superinduced by a combination of growth factors and cycloheximide.

The stability of the 3 A1-hybridizing RNAs was estimated by monitoring the RNA levels after addition of the transcription inhibitor, actinomycin D. For one set of experiments, Rat-1 cells were maintained in serum-free media for 48 h, and then fresh media with or without 3 $\mu\text{g/ml}$ actinomycin D was placed on the cells. This concentration of actinomycin was sufficient to inhibit ^3H -uridine incorporation by over 90% in exponentially growing Rat-1 cells. Even after 48 h of serum deprivation, none of the 3 A1-hybridizing RNAs had reached a steady-state level (Figure 6). All 3 RNA levels were decreasing with an apparent half-life of 5 to 10 h. Addition of actinomycin D caused the apparent half-lives to decrease to 2 to 3 h. In another set of experiments, the quiescent cells were stimulated by EGF for 8 hours before the actinomycin

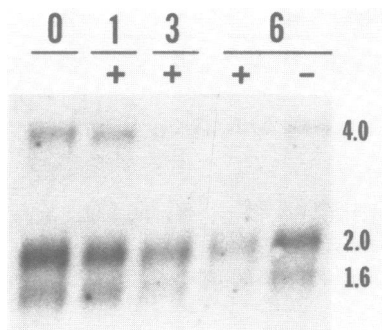


Figure 6. Stability of A1 RNA levels in serum-starved Rat-1 cells. After serum-starvation for 48 h, the culture medium was replaced by fresh DMEM with (+) or without (-) 3 µg/ml actinomycin D. After the indicated hours of incubation, poly A+ RNA was isolated and analyzed by Northern blot hybridization with the complete HDP-182A probe.

D was added. In this case, the apparent half-lives of the 1.6 and 2.0 kb RNA species in the presence of actinomycin D was 4 to 6 h (Figure 7), or about twice that of the unstimulated cells. In contrast, the level of the 4.0 kb species decreased rapidly after the addition of actinomycin D with an apparent half-life of less than 0.5 h. This marked reduction in the stability of the 4.0 kb species in EGF stimulated cells is the most dramatic difference in expression that we have observed among the 3 A1-hybridizing RNAs.

DISCUSSION

A cDNA clone encoding the hnRNP A1 core protein was found to hybridize to 3 species of poly A+ RNA from Rat-1 cells. This finding is in accord with similar results reported by Cobianchi and Wilson (12) for rat brain RNA. The relationship between these 3 RNA species is unclear. We have shown that they have a considerable region of sequence homology because probes specific for each end and the middle of the 1.4 kb HDP-182A cDNA insert each hybridize to all 3 RNA species. The 1.6 kb RNA species lacks sequences 3' to a putative polyadenylation signal and might result from use of an alternative polyadenylation site. Therefore, the three A1-hybridizing RNA species may derive from closely related genes or from alternative processing or transcription of one gene, but not from genes that only have a small stretch of homologous nucleotide sequence.

Other nuclear RNA-binding proteins have been shown to have multiple RNA species deriving from a single gene and in the same size classes as the A1-hybridizing RNAs (25). Swanson and coworkers have reported that the 1.4 and 1.9 kb mRNA species encoding the C1/C2 hnRNP core proteins in HeLa cells appear to derive from the same nascent transcripts by differential use of two polyadenylation signals (26). Another example is the hU1-70K protein which is a specific component of the human U1 snRNP and may facilitate the binding of the U1 snRNP to 5' splice sites in pre-mRNA. Spritz *et al.* (25) have shown that the hU1-70K protein is encoded by a single gene on chromosome 19. Cultured HepG2 hepatoma, 880 fibroblast,

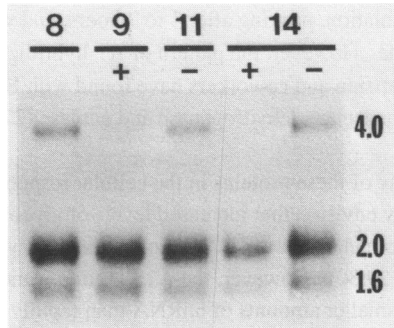


Figure 7. Stability of A1 RNA levels in EGF-stimulated Rat-1 cells. Serum-starved Rat-1 cells were incubated in fresh DMEM supplemented with 10 $\mu\text{g/ml}$ EGF (20 ml/dish) for 8 h. Then 30 μl of methanol with (+) or without (-) actinomycin D was added to each culture dish, and the incubation continued until the indicated hours. Poly A⁺ RNA was isolated and analyzed by Northern blot hybridization with the complete HDP-182A probe.

L144 lymphoblastoid, and HeLa cells contained two sizes of hU1-70K hybridizing RNA, 3.9 kb and 1.7 kb. The 1.7 kb species was at least 40-fold more abundant than the 3.9 kb species in all human cells assayed, and the 1.7 kb species was found to be colinear with the 3' half of the 3.9 kb RNA species. It has not yet been established if these two size classes of hU1-70K RNA originate from the use of alternative promoters or from different post-transcriptional processing of a single pre-mRNA. However, sequencing of different cDNA and genomic clones demonstrated that use of alternative splice sites and alternative polyadenylation sites does lead to multiple subspecies of hU1-70K mRNA within a given size class. Thus, there are several similarities between these results for the C1/C2 hnRNP mRNA and the hU1-70K mRNA and what we observe for A1-hybridizing RNAs. Further cloning and sequence analysis is required to see how far these similarities will persist.

Stimulation of serum-starved Rat-1 cells with serum or EGF caused an increase in the relative levels of all three A1-hybridizing RNA species. The stimulation was evident after 2 h and reached a peak at about 8 h. Northern blot analyses of the A1-hybridizing RNA levels after inhibition of transcription with actinomycin D indicated that the apparent half-lives of the 1.6 and 2.0 kb RNA species in EGF-stimulated cells were roughly twice that in quiescent cells. Thus, part of the increase in the levels of these two RNA species may be attributed to their increased stability. In contrast, the apparent half-life of the 4.0 kb RNA species was markedly decreased in EGF-stimulated cells. The observed increase in the intracellular level of the 4.0 kb RNA species must, therefore, ensue from an even more dramatic increase in genetic transcription. Confirmation of these conclusions will require the development of probes specific for each of the 3 A1-hybridizing RNAs that can be employed in more quantitative RNase protection and nuclear run-on assays.

The addition of cycloheximide along with the EGF blocked the stimulatory affect of EGF. Thus, the A1-hybridizing RNAs are clearly not a primary transcriptional target of the growth factor nor are these RNAs from the class of "intermediate early genes" (24) which

display a more transient stimulation, peaking after 1 to 2 hours, and which are superinduced in the presence of cycloheximide. However, the pattern of A1-hybridizing RNA induction is very similar to the results that Matrisian and coworkers have found with EGF induction in Rat-1 cells of glycolytic enzymes, such as lactate dehydrogenase and enolase (27), and of a secreted protease, transin (28).

The precise role of any of these proteins in the cellular response to growth factors has yet to be demonstrated. One may envision that increased levels of glycolytic enzymes are required to meet the metabolic needs of cells entering DNA replication and cytokinesis, and there is some data to support this position (29,30). However, the A1 situation is more obscure. Quiescent cells synthesize and process smaller amounts of hnRNA than rapidly proliferating cells, so one might expect that a quiescent cell would have fewer hnRNP particles and thus might need fewer molecules of A1 protein (and other hnRNP core proteins) per cell. Nevertheless, it is not clear why the composition of the hnRNP particles in quiescent cells would be changed to include fewer molecules of the A1 protein (11). Does the number of A1 protein molecules in an hnRNP particle influence the rate or efficiency of mature mRNA production? An answer to this question will come as the molecular functions of the hnRNP core proteins are uncovered.

NOTE: While this manuscript was in preparation Buvoli *et al.* (31) reported that the human A1 protein is encoded by 2 mRNA forms, 1.5 and 1.9 kb, which arise from alternative polyadenylation and that both A1 mRNAs are more abundant in actively proliferating human cells.

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