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INTRODUCTION

The level of a messenger RNA depends not only on its rates of synthesis, processing, and transport, but also its rate of turnover. The turnover rate of a mRNA can, in turn, determine its lifetime as a template for protein synthesis. It is particularly important to understand how the levels of mRNAs encoding oncoproteins and cell cycle proteins are regulated because sustained synthesis of these gene products can favor cell growth rather than differentiation, a hallmark of the neoplastic phenotype. Many cell cycle and proto-oncogene mRNAs exhibit extremely short half-lives. Their decay is controlled in part by A+U-rich elements (AREs) located in the 3'-untranslated region. Moreover, the half-lives of their mRNAs are frequently subject to regulatory control. This control is exerted via a balance between the action of at least two ARE-binding proteins, AUF1 and HuR. AUF1 targets the degradation of ARE-mRNAs like the *c-myc* proto-oncogene and the cell cycle regulator cyclin D1. By contrast, HuR promotes stabilization of ARE-mRNAs. *c-myc* and cyclin D1 are particularly important in this regard, since both can play a causative role in mammary tumorigenesis. Our central hypothesis is that AUF1 may act as a novel tumor suppressor by limiting expression of genes that promote cell growth. On the other hand, HuR may act as a novel oncoprotein by stabilizing those mRNAs. Our approach is to alter the expression of AUF1 or HuR in human breast carcinoma cells and examine the resulting effects on cell growth and tumorigenesis in a nude mouse model. Specifically, we have two goals: (1) to examine the effects of AUF1 and HuR expression levels on gene expression in cultured cells; and (2) to assess the roles of AUF1 and HuR in cell growth and tumorigenesis *in vivo*. We plan to introduce into human breast carcinoma cells expression vectors that will permit either overexpression or knocked-down expression of AUF1 or HuR. We will classify clones by their expression levels of AUF1 or HuR (e.g. low, medium, or high). These will then be profiled for the resulting effects on gene expression using a novel DNA microarray strategy. Genes involved in the cell cycle, metastasis and invasion, and angiogenesis will be of particular interest, as many of these contain AREs. Cell clones will also be tested for both their growth phenotypes and their ability to induce tumors as xenographs on nude mice. Our prediction is that tipping the balance to high levels of AUF1 expression will reduce the expression of ARE-mRNAs important for the cell cycle, invasion, metastasis, and angiogenesis. This will likely lower the efficacy of engineered cell lines to induce tumors. By contrast, tipping the balance to high levels of HuR expression will likely increase expression of these genes and lead to increased tumorigenic efficiency.

BODY

The approved SOW is as follows:

Task 1. To examine the effects of AUF1 and HuR expression levels on gene expression in cultured breast carcinoma cells (Months 1-24):

- a. Construct Tet-Off plasmids for overexpression of AUF1 and HuR (Months 1-4).
- b. Construct plasmids for RNA interference (RNAi)-based knockdown of AUF1 and HuR (Months 1-4).
- c. Transfect plasmids into breast carcinoma Tet-Off cells and select individual clones (Months 4-24).
- d. Characterize clones for AUF1 and HuR expression levels to define those with low, medium and high levels of AUF1/HuR expression (Months 6-24).
- e. Using RNA from engineered cell lines, perform DNA microarray analyses of genes affected by altered AUF1/HuR expression, in particular, those involved in the cell cycle, invasion, metastasis, and angiogenesis (Months 6-24).
- f. Identify those transcripts that are direct binding targets of AUF1 and HuR (Months 6-24).

Task 2. To assess the roles of AUF1/HuR in cell growth and tumorigenesis (Months 6-36):

- a. Characterize the growth phenotypes of engineered cell lines obtained from Task 1d (Months 6-24).
- b. Characterize the cell cycle distribution of these cell lines by flow cytometry (Months 6-24).
- c. Introduce clones onto nude mice and score tumor formation as a function of AUF1/HuR expression levels and growth/cell cycle phenotypes (Months 6-36).

We are currently in the midst of Task 1-a, -b, and -c. We have prepared a tet-regulated expression construct for p37AUF1. Figure 1 contains a map of this plasmid. Construction of tet-regulated p40AUF1 and HuR is in progress. For Task 1-b, we have completed preparation of short hairpin RNA (shRNA) vectors for AUF1 and HuR. We accomplished this by annealing oligos containing sequence of the respective mRNAs, a short hairpin, and sequence antisense to the 5' portion of the oligo (Figure 2). We then cloned these into a pSilencer vector (Figure 2). We then sequenced across the length of the inserted oligos to insure fidelity. Figure 3 depicts the sequencing waveform for the HuR shRNA vector and Figure 4 depicts the sequencing waveform for the AUF1 shRNA vector. Specifically, the nucleotides between positions 30 and 99 are the shRNA-encoding bases in Figures 3 and 4. The AUF1 shRNA will knockdown expression of all four AUF1 isoforms. We plan to create vectors for isoform-specific knockdown of AUF1 as well, for comparison. Transfection of the vectors we already have has begun for Task 1-c. We have had a problem, however, in that MCF7-Tet Off cells are quite difficult to transfect. We are trying a new transfection method using Fugene (Roche), which is supposed to be useful for cell lines difficult to transfect. If Fugene reagent does not solve our problem, we will turn to a lentiviral vector system recently shown to permit virtually 100% introduction of shRNA RNAs into cells (J. Virol. 2003 77: 8957-8951). In any event, once we solve this problem, we can begin Task 1-d.

KEY RESEARCH ACCOMPLISHMENTS

- Preparation of pTRE-p37AUF1, an expression construct for overexpression of p37AUF1 in MCF7-Tet Off breast carcinoma cells (Figure 1)
- Preparation of expression constructs for inducible shRNA formation in MCF7-Tet Off cells (Figures 2-4). These will permit tetracycline-induced knockdown of AUF1 and HuR.

REPORTABLE OUTCOMES

We are in the process of building the tools that will allow us to reengineer expression of the key ARE-binding proteins AUF1 and HuR in human breast carcinoma cells. We have prepared three vectors so far. Our feeling is that preparation of vectors does not, per se, constitute a reportable outcome (i.e., a peer-reviewed publication).

CONCLUSIONS

Our central hypothesis is that AUF1 may act as a novel tumor suppressor by limiting expression of genes that promote cell growth. On the other hand, HuR may act as a novel oncoprotein by stabilizing those mRNAs. Our approach is to alter the expression of AUF1 or HuR in human breast carcinoma cells and examine the resulting effects on cell growth and tumorigenesis in a nude mouse model. Specifically, our work has two phases: (I) examining the effects of AUF1 and HuR expression levels on gene expression in cultured cells; and (II) assessing the roles of AUF1 and HuR in cell growth and tumorigenesis *in vivo*. We are currently focused on Phase I. This phase requires us to prepare expression vectors that will permit either overexpression or knocked-down expression of these two proteins. We have about half the required vectors completed. In the meantime, we have begun transfecting these into breast carcinoma cells for stable selection. We have experienced some transfection difficulties, but these are not insurmountable, since we can utilize a lentiviral vector system if need be. These tools (vectors and cell lines) will allow us to address our novel, central hypothesis stated above.

Why is this important? The discovery of AREs in 1986 and the subsequent identification of the ARE-binding proteins that control ARE-mRNA decay have led to the realization that many genes that play active roles in oncogenesis are regulated via their AREs. What has unfortunately received scant attention is the idea that the regulators of these important ARE-mRNAs might themselves possess oncogenic or tumor suppressor activities. Our work is designed to address this idea. Two potential contributions of our studies are: (1) They could have a major impact on our thinking about the pathways by which normal cells become transformed. This is particularly true in breast cancer, since *c-myc* and cyclin D1, two AUF1-regulated mRNAs, are frequently overexpressed. (2) Our studies could spur development of a new generation of pharmaceuticals designed to target an ARE-binding protein such as AUF1 or HuR. This strategy would permit control of large networks of ARE-mRNAs by simply acting on a single target ARE-binding protein.

REFERENCES

None

APPENDICES

Four figures depicting the results of our vector constructions are enclosed as pages 8-11, respectively.

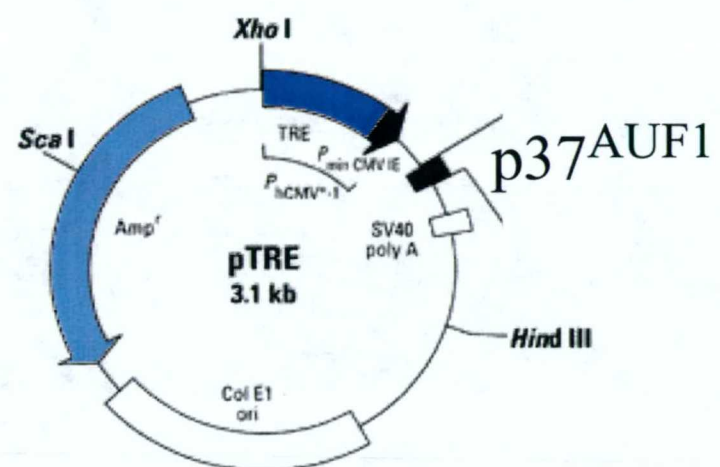
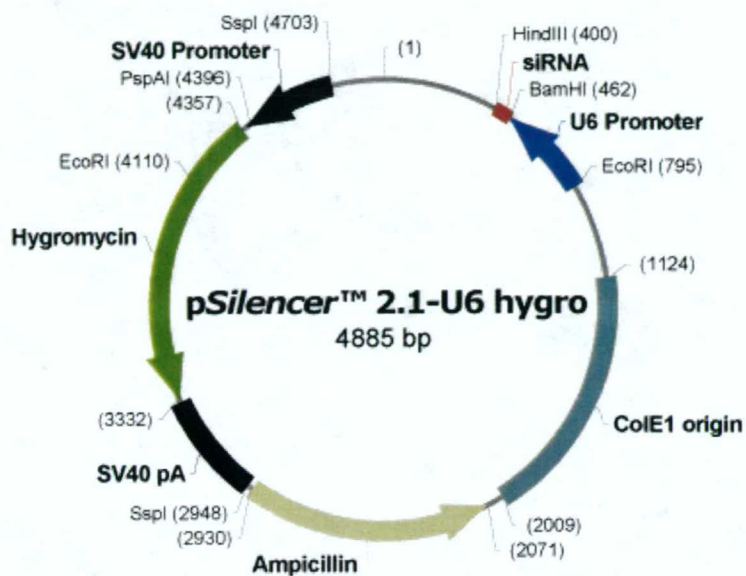


Figure 1

Figure 2



shHuR

BamHI Sense loop Antisense

5'-GATCCCGCACGCTGAACGGCTTGAGGT**TCAAGAG**ACCTCAAGCCGTTTCAGCGTGT TTTTGGAAA-3'

GGCGTGCGACTTGCCGAACCTCCA**AGTTCTC**TGGAGTTCGGCAAGTCGCACAAAAAACCTTT**TTCGA**-5'

HindIII

shAUF1

BamHI Sense loop Antisense

5'-GATCCCGTTGTAGACTGCACTCTGAT**TCAAGAG**ATCAGAGTGCAGTCTACAAC TTTTGGAAA-3'

3'-GGCAACATCTGACGTGAGACTA**AGTTCTC**TAGTCTCACGTCAGATGTTGAAAAAACCTTT**TTCGA**-5'

HindIII

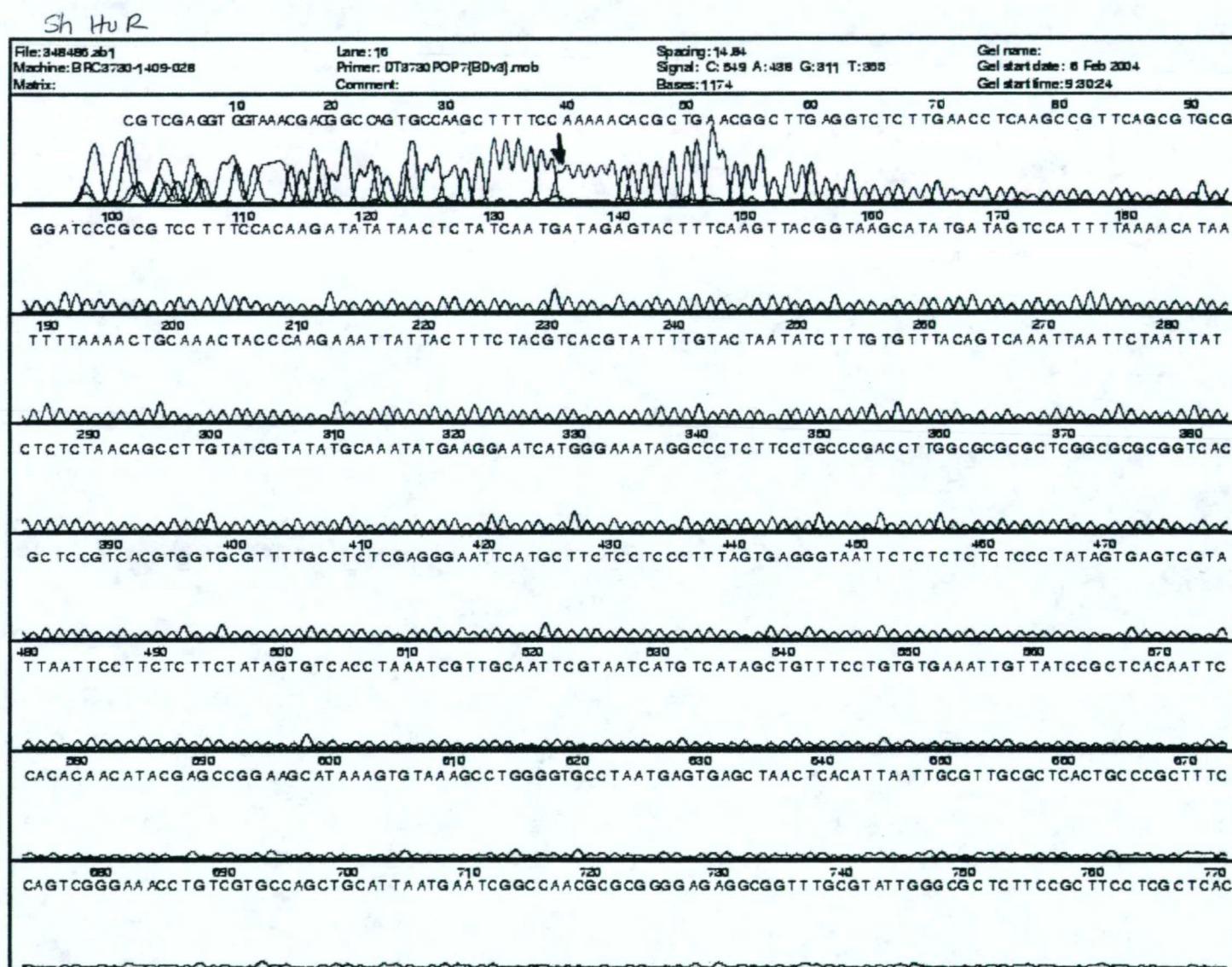


Figure 3

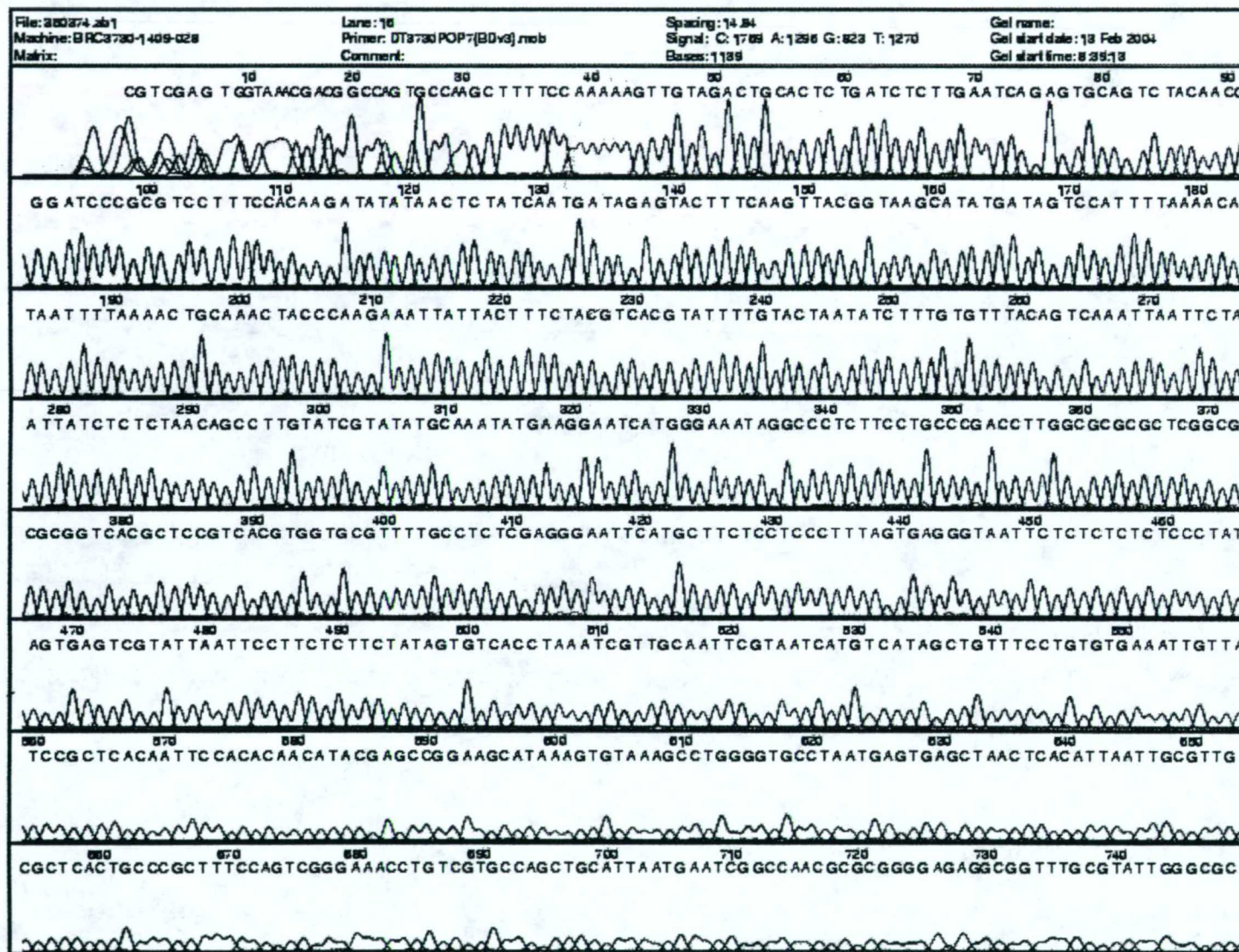


Figure 4