Heterogeneous Nuclear Ribonucleoproteins as Regulators of Gene Expression Through Interactions With the Human Thymidine Kinase Promoter

Julie S. Lau,[1] **Peter Baumeister,**[1] **Edmund Kim,**[1] **Binayak Roy,**[1] **Tsai-Yuan Hsieh,**[2] **Michael Lai,**[2] **and Amy S. Lee**[1]*

[1]Department of Biochemistry and Molecular Biology and the USC/Norris Comprehensive Cancer Center, Keck School of Medicine of the University of Southern California, Los Angeles, California 90089-9176
[2]Howard Hughes Medical Institute and Department of Molecular Microbiology and Immunology, Keck School of Medicine of the University of Southern California, Los Angeles, California 90089-9176

Abstract   In search for nuclear proteins that interact with the human thymidine kinase (htk) promoter, we discovered that p37AUF, a hnRNP C-like protein, and hnRNP A1, both members of the heterogeneous ribonucleoprotein family, can bind with high affinity to an ATTT sequence motif contained within the cell cycle regulatory unit (CCRU). We report here that over-expression of p37AUF stimulates gene expression mediated by the htk promoter in a promoter-sequence specific manner, whereas hnRNP A1 suppresses it. Both recombinant p37AUF and hnRNP A1 can bind the htk CCRU, suggesting that their binding to the DNA target does not require additional cellular components. We further discovered that hnRNP K is a potent suppressor of htk mediated gene activity. However, its mechanism of action is mediated through protein-protein interaction, since hnRNP K itself cannot bind the htk CCRU but can competitively inhibit the binding of other hnRNPs. The binding site for the hnRNPs on the htk CCRU is not required for S-phase induction of the htk promoter. However, in stable but not transient transfectants, the mutation of the hnRNP binding site results in 5- to 10-fold reduction of htk mediated gene activity in synchronized and exponentially growing cells. Collectively, these findings support emerging evidence that hnRNPs, in addition to their traditional role in RNA biogenesis, could be regulators of gene expression through direct DNA binding or interaction with other proteins. J. Cell. Biochem. 79:395–406, 2000.   © 2000 Wiley-Liss, Inc.

Key words: heterogeneous nuclear riboproteins; human thymidine kinase promoter; gene regulation

INTRODUCTION

The thymidine kinase gene encodes an enzyme of the pyrimidine salvage pathway which catalyzes the phosphorylation of thymidine to form thymidine 5′-monophosphate. The expression of the thymidine kinase gene is regulated at multiple levels; the relative contribution of each level of control is highly dependent on the growth state and the type of cell [Lipson et al., 1989; Naeve et al., 1991]. Previously, it has been demonstrated that a 70 base pair (bp) fragment of the human thymidine kinase (htk) promoter spanning -130 to -64 is sufficient to confer G1/S regulation to a heterologous promoter in synchronized Chinese hamster fibroblast cells [Kim et al., 1988]. Within this region, a 25 bp sequence spanning -109 to -84 (termed htk CCRU below) is required for the S-phase induction activity [Li et al., 1993].

Using electrophoretic mobility gel shift assays (EMSAs) and nuclear extracts from Chinese hamster ovary (CHO) cells, a predominant complex (termed complex I) was formed with the htk CCRU throughout the cell cycle [Li et al., 1993]. Although the htk CCRU contains GC-rich sequence motifs and has been shown to be the target for cyclin A, p107 and cdk2 during S-phase, E2F/DP is not a component of complex I by a multitude of biochemical criteria [Li et al., 1993; Kim et al., 1996]. Fur-

ther affinity purification of DNA binding components of complex I revealed that a series of protein bands of approximately 40 kDa, which bind with high affinity to the htk CCRU, are immunologically distinct from E2F1, E2F-2, E2F-3 and E2F-4.

In this study, we report the purification and identification of the family of 40 kDa proteins which formed the most abundant protein complex with the htk CCRU. Peptide sequencing demonstrates that these proteins are identical to p37AUF, a hnRNP C-like protein, and hnRNP A1, which are members of the adenosine-uridine-rich RNA binding factors [Nakamaki et al., 1995]. Since the htk CCRU contains CT rich sequences similar to the putative binding site for hnRNP K, we further tested whether the htk CCRU is a novel interactive target for hnRNP K. As heterogeneous nuclear ribonucleoproteins, the primary function of p37AUF, hnRNP A1 and hnRNP K originally has been ascribed to stabilizing RNA and assisting in the processing and transport of cellular RNA [Dreyfuss et al., 1993]. However, emerging evidence suggests that some hnRNPs, in particular, hnRNP K, can also function as transcription factors with both activating as well as repressing activities [Michelotti et al., 1996; Miau et al., 1998]. It has also been reported that p37AUF, also known as E2BP, can stimulate the hepatitis B virus enhancer promoter activity [Tay et al., 1992]. Recently, hnRNA A1 has been shown to bind to the transcription-regulatory region of mouse hepatitis virus RNA [Li et al., 1997]. As single-strand DNA binding proteins in vivo, it has been suggested that these proteins could serve unique functions in transcriptional control of specific genes at the level of chromosomal configuration [Tomonaga and Levens, 1996].

Here we showed that p37AUF is a component of complex I and can stimulate gene expression mediated by the htk promoter through interaction with an ATTT sequence motif in the htk CCRU. HnRNP A1 is also a component of complex I and binds to the same sequence motif as p37AUF. In contrast, overexpression of hnRNP A1 suppresses reporter gene activity mediated by the htk promoter. Both recombinant p37AUF and hnRNP A1 can bind the htk CCRU, suggesting that their binding to the DNA target does not require additional cellular components. We further discovered that hnRNP K is a potent suppressor of htk directed gene activity, however, its mechanism appears to be mediated through protein-protein interaction. Further analysis of the functional contribution of the hnRNP binding site within the htk CCRU reveals that it is not required for the S-phase induction of the htk promoter activity. However, in stable but not transient transfectants, mutation of the hnRNP binding site results in 5- to 10-fold reduction of the htk promoter activity in synchronized and exponentially growing cells. Our results support emerging evidence that hnRNPs, in addition to their traditional role in RNA biogenesis, could be regulators of gene expression through direct DNA binding or protein-protein interaction.

MATERIALS AND METHODS
Protein Sequencing

The protein bands from preparative SDS-polyacrylamide gels were excised following Coomassie Blue staining. Partial protein sequencing was performed after tryptic digestion and HPLC separation by mass spectrophotometry by W.M. Keck Biomedical Mass Spectrometry Laboratory of the University of Virginia Biomedical Research Facility (Charlottesville, VA).

Cell Culture

The Chinese hamster fibroblast K12 cell line is maintained in DMEM supplemented with 10% bovine calf serum and 1% PSN antibiotics. For synchronization of stable transfectants, subconfluent cell cultures were washed with PBS and grown for 48 to 72 h in DMEM containing 0.5% bovine calf serum and 0.5% PSN to arrest the cells in G0 phase. S-phase cells were harvested after 16 to 18 h following serum release. Progression of the synchronized cells through the cell cycle was determined by DNA synthesis as measured by [^{3}H]-thymidine kinase incorporation [Kim et al., 1988].

Plasmids

The construction of phtk(474R)CAT and phtkLS(-112/-98)CAT has been previously described [Kim et al., 1996]. For transfections, the β-gal reporter plasmids used were driven either by the RSV or CMV promoters. The E2BP expression plasmid (gift of Dr. E.-C. Ren, National University of Singapore) has been previously described [Tay et al., 1992]. The expression vectors for hnRNP A1 and hnRNP K

contained the respective cDNA driven by the CMV promoter [Hsieh et al., 1998].

Transfections and CAT Assays

Transient transfections were carried out on exponentially growing K12 cells using the calcium phosphate method. For the transfections analyzing the effect of p37AUF/E2BP, each transfection contained 5 μg of the CAT reporter plasmid, 3 μg of CMV β-gal, and 20 μg of the E2BP or pSV2neo expression vector. For transfections analyzing the effect of hnRNPs, 5 μg of the CAT reporter plasmid, 3 μg of RSV β-gal, and 5 μg of hnRNP A1, hnRNP K, or the empty CMV vector was used. Conditions for cotransfection with the hepatitis C core protein expression vector has been described [Hsieh et al., 1998]. Transfection efficiency was measured with the β-gal assay. Extracts with equal β-gal activity were assayed for CAT activity and quantitated using the AMBIS radioanalytic imaging system. Each transfection was repeated two to four times.

To generate stable transfectants, 7 μg of phtk(474R)CAT or phtkLS(-112/-98)CAT and 3 μg of pSV2neo plasmids were complexed with the Superfect Transfection reagent (Qiagen, Ventura, CA) and added to subconfluent K12 for 3 h. After recovery for 48 h, cells expressing neo were selected in DMEM containing 800 μg/ml G418 for two weeks. Resistant colonies were pooled and expanded for analysis. The equivalence of CAT plasmids integrated into the respective pools was established by PCR of the genomic DNA extracted from the pooled transfectants followed by Southern blot analysis with the CAT coding sequence.

Recombinant Proteins

The his-tagged p37AUF was a gift from G. Brewer (Bowman Gray School of Medicine, NC). The hnRNPA1 and K cDNAs were cloned into the pGEX 4T-1 expression vector and then bacterially expressed in *E. coli* cells [Hsieh et al., 1998]. Protein expression was induced with IPTG, at a final concentration of 0.2 mM, for 5 h at 37°C. The lysed bacteria were sonicated and then a 50% slurry of Glu-Agarose was added to the supernatant. After 4 h of rotation, the beads were collected and washed with PBS. The proteins were repeatedly eluted from the beads with 50 mM Tris-Cl (pH 8.0)/5 mM reduced glutathione. Collected fractions were analyzed by SDS-PAGE.

Electrophoretic Mobility Shift Assays

Probes were prepared by annealing oligonucleotides and labeling the duplex with [α-^{32}P]dATP and [α-^{32}P]dCTP with the Klenow enzyme. The labeled probe was purified with an ammonium acetate/ethanol precipitation and resuspended with TE (10 mM Tris, pH 8.0/1 mM EDTA).

For the analysis of the htk complexes formed with CHO NE, 2 ng of probe were added to 0.5 μg of extract with 100 ng of poly(dl · dC) and 4 to 10 μg of BSA. Reactions were carried out for 20 min on ice in a total volume of 20 μl containing 12 mM HEPES (pH 7.9), 60 mM KCl, 5 mM MgCl$_2$, 0.5 mM DTT, 0.5 mM EDTA, and 6% glycerol. Competitor binding site oligonucleotides were added to the reactions at the concentrations indicated in the figure legends. For antibody reactivity, 1 μl of rabbit anti-p37 AUF and rabbit preimmune serum (gifts from G. Brewer) were added after allowing the reaction to proceed 10 min. Complexes were resolved on 4 to 7% native polyacrylamide gels (29:1, acrylamide:bisacrylamide) electrophoresed in 1× TBE either at 200 V for 2.5 h with cooling or 150 V for 4 h at 4°C.

For the analysis of the complexes formed with recombinant hnRNPs, 2 ng of probe were added to 30 to 60 ng of protein with 100 ng of poly(dl · dC) and 4 μg of BSA. Reactions were carried out identical to those formed by the CCRU and CHO NE. For antibody reactivity, 5 μl of chicken anti-hnRNP A1 or chicken preimmune serum were added after allowing the reaction to proceed 10 min. Complexes were resolved on 4–6% native polyacrylamide gels (29:1, acrylamide:bisacrylamide) electrophoresed in 1× TBE at 200 V for 2.5 h with cooling.

For the analysis of recombinant hnRNP protein interactions using EMSA, following quantitation of the GST protein concentrations by Coomassie Blue staining, each pair of proteins at the indicated amount was incubated for 20 min at 20°C. The proteins were then added to a reaction mixture containing 3 ng of probe, 100 ng of poly(dl · dC) and 4 μg of BSA. Reactions were carried out for 10 min at room temperature in a total volume of 20 ml containing 12 nM HEPES (pH 7.9), 60 mM KCl, 10 mM MgCl$_2$, 0.5 mM DTT, 0.5 mM EDTA and 6% glycerol. The complexes were resolved on 8% native polyacrylamide gels and electrophoresed at 150 V for 3.5 h at 4°C.

TABLE I. Sequences of Synthetic Oligonucleotide[a]

Oligonucleotide	Sequence
	C/T
wt (-110/-84)	aagctTGCGGCCAAATCTCCCGCCAGGTCAGC
	ACGCCGGTTTAGAGGGCGGTCCAGTCG
	A/T G/C
LS (-97/-84)	aagctTGCGGCCAAATCTaaataactagtcta
	ACGCCGGTTTAGAtttattgatcagatttcga
LS (-109/-98)	aagctTaatactagtgagCCCGCCAGGTCAGC
	AttatgatcactcGGGCGGTCCAGTCGttcga
LS (-103/-101)	aagctTGCGGCCgtgTCTCCCGCCAGGTCAGC
	ACGCCGGcacAGAGGGCGGTCCAGTCG
(CT)$_4$ oligo	aagctTCTCCTCCCCACCTTCCCCACCCTCCCCACCCTCCCCATAAGC
	ttcgaAGAGGAGGGGTGGAAGGGGTGGGAGGGGTGGGAGGGGTATTCG
Random #1	ctcgaTGTATGCATCTGATTACTATCGACTAGCTTGCTACG
	ACATACGTAGACTAATGATAGCTGATCGAACGATGCcagctg

[a]The CT, AT and GC rich motifs are underlined in the wt (-110/-84). The bold italic print indicates mutated bases substituted in the wild-type site. The lowercase letters at the end of the oligomer indicate polylinker DNA sequence.

RESULTS

Identification of p37AUF and hnRNP A1 as Major Nuclear Proteins that Bind the htk CCRU

Towards identifying the nuclear proteins that bind to the htk CCRU, nuclear extracts from exponentially growing CHO cells were first fractionated on Bio-Rex70. Fractions containing complex I binding activity were further subjected to DNA affinity column chromatography. The affinity column was designed by coupling a short reannealed oligomer spanning only the functional site between -103 to -84 of the htk promoter (Table I) to CNBr-activated Sepharose 4B. The majority of the DNA binding activity eluted between 0.35 and 0.4 M KCl and correlated directly with the intensity of staining of a doublet protein species migrating at around 40 kDa, and another set of bands at 35 and 30 kDa [Kim et al., 1997]. To identify the proteins, preparative SDS-polyacrylamide gels were prepared. The protein bands were excised, and subjected to amino acid sequence analysis using mass spectrophotometry. For each protein band, 6 to 8 peptide sequences were generated. The 40 kDa doublet bands were matched to p37AUF/E2BP (Fig. 1A), and the peptides from the 35 to 30 kDa bands were matched to hnRNP A1 (Fig. 1B).

Binding of p37AUF to the ATTT Motif of the htk CCRU

To confirm that p37AUF is a component of the htk complex I, EMSAs were performed using the htk CCRU (spanning -110/-84) as probe and nuclear extracts from exponentially growing CHO cells. As shown in Figure 2, complex I was competed efficiently by molar excess of the wild-type htk CCRU (Lane 2), but not by a random oligomer (Lane 5). Examination of the htk CCRU revealed that an AT-rich motif was immediately 5′ adjacent to a GC-rich motif previously shown to be critical for the S-phase induction of the htk promoter [Li et al., 1993] (Table I). Mutation of the AT-rich motif in LS (-109/-98) resulted in weaker competition for the complex I (Fig. 2, Lane 4) than mutation of the GC-rich motif in LS(-97/-84) (Lane 3). Thus, complex I appeared to prefer binding to the AT-rich region of the htk CCRU. Using an antibody against p37AUF, the upper band of complex I (complex IA) was eliminated, and the appearance of a new supershifted band was noted (Fig. 2, Lane 6), whereas the preimmune serum indicated no effect (Lane 7). These results indicated that p37AUF is a component of complex I.

To confirm independently that p37AUF can bind htk CCRU and to test whether this binding is dependent on interaction of other cellular components, his-tagged p37AUF was used in the EMSA with htk CCRU as probe. As shown in Figure 2, recombinant p37AUF readily formed a complex with the htk CCRU (Fig. 2, Lane 9) and the complex exhibited identical properties as complex IA in competition assays (Lanes 10 to 13) and reactivity with the p37AUF antibody (Lanes 14 and 15). The rea-

A p37AUF

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1          11         21         31         41         51         61         71
MSEEQFGGTG RRHANGGGRR SAGDEEGAMV AATQGAAAAR EADAGPGAEP RLEAPKGSAE SEGAKIDASK NEEDEGKMFI

81         91         101        111        121        131        141        151
GGLSWDTTKK DLKDYFSKFG EVVDCTLKLD PITGRSRGFG FVLFKESESV DKVMDQKEHK LNGKVIDPKR AKAMKTKEPV

161        171        181        191        201        211        221        231
KKIFVGGLSP DTPEEKIREY FGGFGEVESI ELPMDNKTNK RRGFCFITFK EEEPVKKIME KKYHNVGLSK CEIKVAMSKE

241        251        261        271        281
QYQQQQQWGS RGGFAGRARG EFRNSSEAGE GLELPPNSIH CWQLSV
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B hnRNP A1

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1          11         21         31         41         51         61         71
MSKSESPKEP EQLRKLFIGG LSFETTDESL RSHFEQWGTL TDCVVMRDPN TKRSRGFGFV TYATVEEVDA AMNARPHKVD

81         91         101        111        121        131        141        151
GRVVEPKRAV SREDSORPGA HLTVKKIFVG GIKEDTEEHH LRDYFEQYGK IEVIEIMTDR GSGKKRGFAF VTFDDHDSVD

161        171        181        191        201        211        221        231
KIVIQKYHTV NGHNCEVRKA LSKQEMASAS SSQRGRSGSG NFGGGRGGGF GGNDNFGRGG NFSGRGGFGG SRGGGGYGGS

241        251        261        271        281        291        301        311
GDGYNGFGND GSNFGGGGSY NDFGNYNNQS SNFGPMKGGN FGGRSSGPYG GGGQYFAKPR NQGGYGGSSS SSSYGSGRRF
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Fig. 1. Alignment of peptide sequence derived from htk complex I with hnRNP. (**A**) The amino acid sequence of human p37AUF [Zhang et al., 1993] and (**B**) of human hnRNP A1 (Genepept. r95 Accession #X79536) are shown. The matching peptides are underlined.

son for the slower electrophoretic mobility of the his-tagged p37AUF complex is unclear and could be due to self oligomerization of the recombinant protein.

Since p37AUF is known to be a single-strand RNA binding protein with preference for AU-rich sequence, we tested whether it exhibits affinity for the htk CCRU in its single-stranded form. For this purpose, EMSAs were performed using his-tagged p37AUF and htk CCRU as probe. Although the complex formed could be competed away by the upper and lower strand of the htk CCRU, we observed that the lower strand, bearing the ATTT sequence, was a highly efficient competitor for the p37AUF complex as compared to the upper strand (Fig. 3, Lanes 2 to 4). Specific mutation of the AT motif eliminated its ability to compete for p37AUF binding (Fig. 3, Lane 5). Collectively, these results indicate that p37AUF can bind to double-stranded htk CCRU as well as the lower strand of htk CCRU containing an ATTT motif, and that this binding can occur independent of other cellular components.

Sequence-Specific Activation of htk Promoter Mediated Gene Expression by p37AUF

To test the effect of p37AUF on htk promoter mediated gene activity, a eukaryotic expres-

sion vector of p37AUF/E2BP was used to co-transfect hamster fibroblast K12 cells with phtk(474R)CAT. The latter plasmid contains 441 bp of the htk promoter sequence driving the expression of the reporter CAT gene. Our results showed that in these transient assays, p37AUF/E2BP was able to activate htk promoter mediated gene expression by about 2.5-fold (Fig. 4A). This stimulatory effect is promoter-sequence specific since mutation of the putative p37AUF binding AT-rich sequence within the htk promoter [phtkLS(-112/98)CAT] eliminated the stimulatory effect of p37AUF (Fig. 4B).

Binding of hnRNP A1 to the ATTT Motif of the htk CCRU

To determine whether hnRNP A1 can bind to htk CCRU, the binding properties of complex I were compared to that of GST-hnRNP A1 in EMSAs using htk CCRU as probe. As shown in Figure 5B, recombinant hnRNP A1 formed a strong complex with the htk CCRU (Lane 2). Thus, as in the case of p37AUF, it can bind the htk CCRU independent of other cellular components. Furthermore, the competition profile of the GST-hnRNP A1 complex with wild-type and mutated forms of the htk CCRU was identical to that of complex I formed with CHO nuclear extracts (Fig. 5, A and B). The results
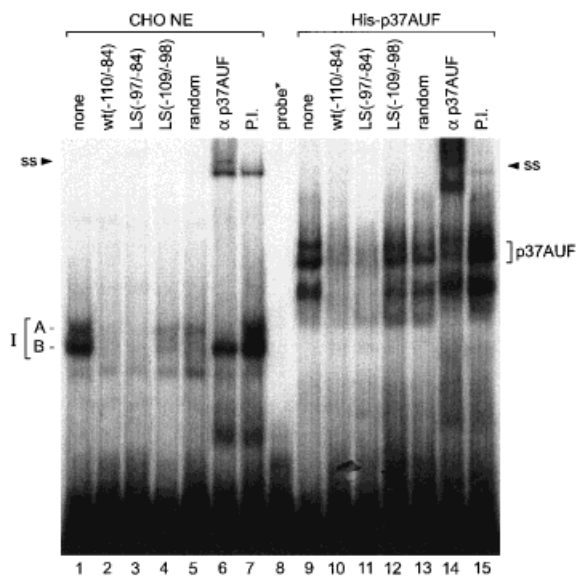
Fig. 2. Interaction of p37AUF with the htk CCRU. The probe used in the gel mobility shift assay (EMSA) was radiolabeled htk CCRU [wt(-110/-84)]. Reactions were either performed with nuclear extracts (NE) from CHO cells (Lanes 1 to 7) or with his-tagged p37AUF (Lanes 9 to 15). Free probe was applied in lane 8. The reactions contained either no competitor (Lanes 1 and 9), 10-fold molar excess of the oligomers (Table 1) as indicated on top (Lanes 2 to 5, 10 to 13), rabbit polyclonal anti-p37AUF antibody (Lanes 6 and 14), or the rabbit preimmune serum (Lanes 7 and 15). The reactions were applied onto a 4% native polyacrylamide gel and ran for 2.5 h at 200 V. The positions of the htk complex I, with an upper (**A**) and lower (**B**) band, the recombinant p37AUF complex, and the supershifted band (ss) are indicated.

Fig. 3. Binding of recombinant p37AUF to single-stranded htk CCRU. The EMSA was performed using radiolabeled htk CCRU [wt(-110/-84)] as probe. In lane 1, only free probe was applied; lane 2, no competitor was added; from lanes 3 to 5, 10-fold molar excess of the upper strand (U) of wt(-110/-84), the lower strand (L) of wt(-110/-84), or the lower strand (L) of LS(-103/-101) was added respectively. The reactions were applied onto a 6% native polyacrylamide gel and ran for 2.5 h at 200 V.

showed that hnRNP A1 can bind the double-stranded form of the wild-type but not the AT-mutated form of the htk CCRU (Fig. 5B, Lanes 2 to 4). It also exhibits affinity for the lower strand of the htk CCRU, and mutation of the ATTT motif in the LS(-103/-101) oligomer destroyed the binding affinity (Fig. 5B, Lanes 5 and 6).

To confirm that hnRNP A1 is a component of complex I, antibody against hnRNP A1 was used in EMSAs to determine whether it could disrupt the formation of complex I. The potency and specificity of the hnRNP A1 antibody was first tested on the GST-hnRNP A1 complex. As shown in Figure 6A, the anti-hnRNP A1 antibody, while relatively diluted in titer (data not shown), was able to inhibit the formation of the GST-hnRNP A1 complex. In addition, a new supershifted band was observed (Lanes 3 and 4). This new band was absent in reactions when preimmune serum was used (Lanes 5 and 6). Similarly, the formation of complex I was partially suppressed and a supershifted band was formed when the anti-hnRNP A1 an-
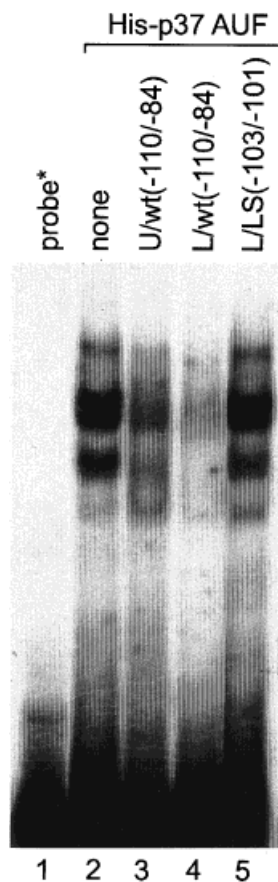
tibody was added to the EMSAs using CHO nuclear extracts (Fig. 6B). Therefore, hnRNP A1 is a component of complex I in addition to p37AUF.

Sequence-Specific Suppression of htk Promoter Mediated Gene Activity by hnRNP A1

To test the effect of hnRNP A1 on the htk promoter mediated gene activity, co-transfection studies were performed using a eukaryotic expression vector of hnRNP A1 and phtk(474R)CAT as the reporter gene. In multiple independent transfection experiments, over-expression of hnRNP A1 resulted in a 4-fold reduction of the htk promoter activity (Fig. 7A). This suppressive effect was mediated through the AT-rich sequence since mutation of this sequence in phtk(-112/-98)CAT abolished the response (Fig. 7C). Fur-
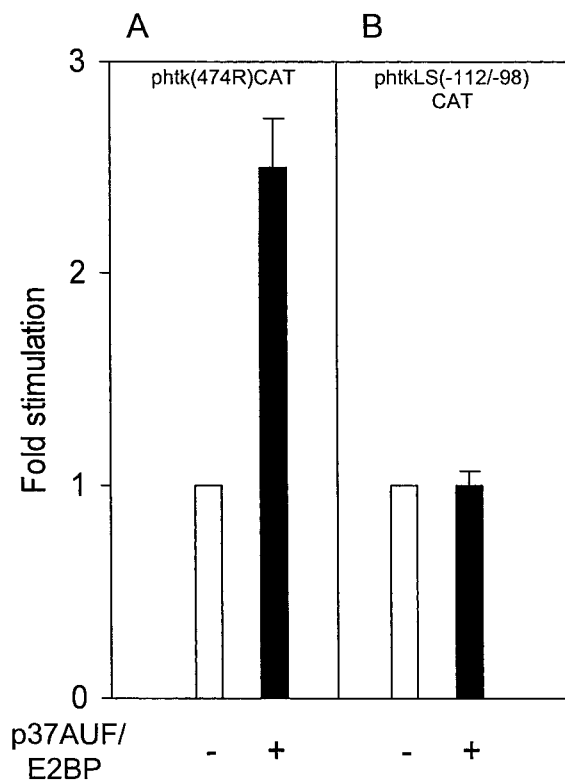
Fig. 4. Stimulation of htk promoter mediated gene activity by p37AUF. (**A**) Five μg of phtk(474R)CAT reporter gene were co-transfected with 20 μg of either empty pSV2 vector (−), or pSV2 driven expression plasmid for p37AUF/E2BP (+) into K12 cells, in the presence of 3 μg of CMV-driven β-galactosidase gene serving as the transfection efficiency control. (**B**) Five μg of phtkLS(-112/-98)CAT were used as the reporter gene. The CAT activities were determined after transfection efficiencies were normalized by β-galactosidase activity. The level obtained with the empty pSV2 vector was set as one. The fold stimulation of promoter activity with standard deviations is shown.

Fig. 5. Binding of hnRNP A1 to the htk CCRU. Radiolabeled wt(-110/-84) was used as probe in the EMSAs. In panel (**A**), CHO nuclear extract was used. The reactions contained either no competitor (Lane 1); 10-fold molar excess of double-stranded wt(-110/-84) (Lane 2); double-stranded LS(-103/-101) (Lane 3); the lower strand (L) of wt(-110/-84) (Lane 4); or the lower strand (L) of LS(-103/-101) (Lane 5). Panel (**B**) is the same as (A) except that GST-hnRNP A1 was used. The reactions were applied onto a 6% native polyacrylamide gel and ran for 2.5 h at 200 V. The positions of complex I A and B and the recombinant hnRNP A1 complex are indicated.

thermore, to determine whether this inhibition is dependent on the supercoiled topology of the reporter plasmid, the co-transfection experiments were repeated with linearized phtk(474R)CAT. The results, as shown in Figure 7B, showed that hnRNP A1 can suppress htk promoter mediated gene activity when the reporter plasmid was either in supercoiled or linearized form, suggesting that in these assays DNA configuration is not critical.

Lack of Physical Interaction of hnRNP K with the htk CCRU

The DNA binding site for hnRNP K has been identified as single-stranded CT elements [Michelotti et al., 1996]. Examination of the htk CCRU sequence revealed the presence of a CTCCC sequence motif at the junction of the AT
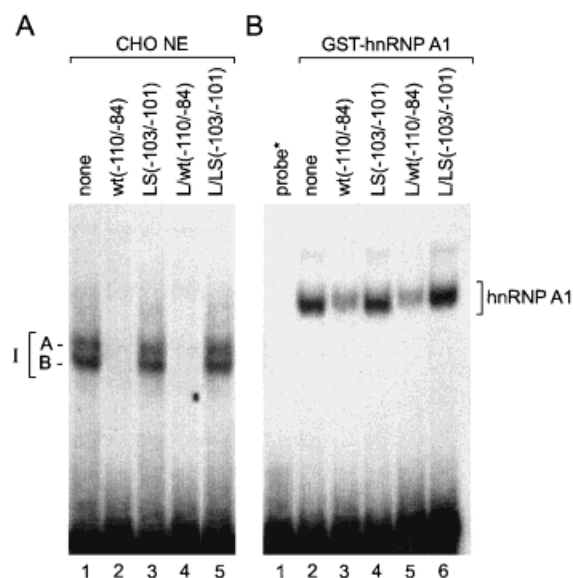
and GC motifs (Table 1). Thus, although we could not detect hnRNP K binding to the htk CCRU through affinity chromatography of HeLa nuclear extract, we tested whether recombinant hnRNP K could bind the htk CCRU. In EMSAs using GST-hnRNP K and a probe consisting of CT tandem repeats known to bind hnRNP K [Michelotti et al., 1996; Hsieh et al., 1998], we detected a strong binding complex (Fig. 8A, Lane 2) which could be specifically competed away with the CT but not a random oligomer (Fig. 8A, Lanes 3 and 4). However, in EMSAs performed with the htk CCRU probe, no binding was observed (Fig. 8B, Lanes 1 and 2). Therefore, by the criteria of chromatography purification and recombinant protein binding in vitro, hnRNP K binding to the htk CCRU was undetectable.

Strong Suppression of htk Promoter Mediated Gene Activity by hnRNP K

Despite the lack of physical interaction between hnRNP K and the htk promoter, its overexpression resulted in about 6-fold reduction in the htk promoter mediated gene activity (Fig. 9A). As in the case of hnRNP A1, this suppres-
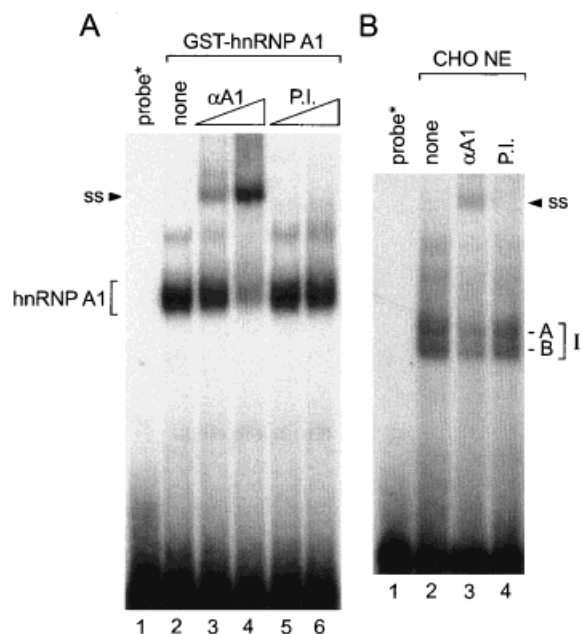
Fig. 6. Immuno-reactivity of complex I with anti-hnRNP A1 antibody. The probe used in the EMSAs was radiolabeled wt(-110/-84). (**A**) GST-hnRNP A1 was used. In lane 1, free probe was applied; lane 2, no antibody was added; lanes 3 and 4, increasing amounts (1 or 3 μl) of chicken polyclonal hnRNP A1 (αA1) was added respectively; lanes 5 and 6, chicken preimmune serum (PI) (1 or 3 μl) was added respectively. The reactions were applied onto a 6% native polyacrylamide gel and ran for 2.5 h at 200 V. (**B**) CHO nuclear extract was used with 10 μg of BSA. In lane 1, free probe was applied; lane 2, no antibody was added; lanes 3 and 4, 5 μl of anti-hnRNP A1 antibody or the preimmune serum were added respectively. The reactions were resolved on a 7% native polyacrylamide gel and ran at 150 V for 4 h at 4°C. The positions of the recombinant hnRNP A1 complex, the htk complex I with upper A and lower B band, and the supershifted band (ss) are indicated.

Fig. 7. Sequence-specific suppression of htk promoter mediated gene activity by hnRNP A1. (**A**) Five μg of phtk(474R)CAT reporter gene were co-transfected with 5 μg of either empty pCMV vector (−), or pCMV driven expression plasmid for hnRNP A1 (+) into K12 cells, in the presence of 3 μg of RSV-driven β-galactosidase gene serving as the transfection efficiency control. (**B**) Linearized ptk(474R)CAT was used as the reporter gene, and (**C**) phtkLS(-112/-98)CAT was used as the reporter gene. The CAT activities were determined after the transfection efficiencies were normalized by β-galactosidase activity. The level obtained with the empty pCMV vector was set as 100%. The relative promoter activities with standard deviations are shown.

sive effect was independent of the topology of the reporter gene (Fig. 9B). Thus, supercoiled and linearized phtk(474R)CAT were suppressed to the same magnitude when co-transfected with hnRNP K1. In contrast, when phtkLS(-112/-98)CAT was used as a reporter, the suppression was reduced to about 2.5-fold (Fig. 9C). This result shows that despite the lack of direct binding of hnRNP K to the htk CCRU itself the full suppressive effect of hnRNP K requires the integrity of the sequence spanning -110/-98. In agreement with previous observations [Hsieh et al., 1998], titration of hnRNP K through co-expression of its interactive protein, the hepatitis C virus core protein, resulted in partial reversion of the suppression of the htk promoter, whereas ex-
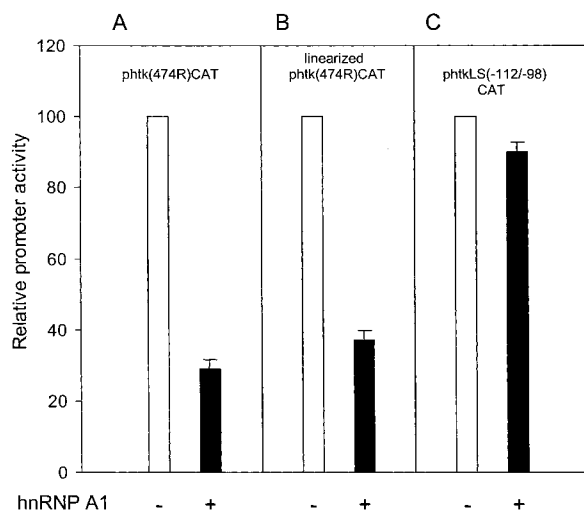
pression of the hepatitis C virus core protein alone had no effect (Fig. 9D).

Competitive Inhibition of hnRNP Binding to the htk CCRU by hnRNP K

To examine the mechanism whereby hnRNP K can affect htk promoter activity, EMSAs were used to investigate whether hnRNP K can affect binding of p37AUF and hnRNP A1 to the htk CCRU. GST-hnRNP K added in increasing amounts to the his-tagged p37 AUF caused a significant decrease in its complex formation with the htk CCRU (Fig. 10A, Lanes 2 to 4) when compared to p37AUF alone (Fig. 10A, Lane 1) or when GST was added (Fig. 10A, Lanes 5 to 7). Similarly, GST-hnRNP K also inhibited binding of GST-hnRNP A1 to the htk CCRU in EMSAs performed under identical parameters (Fig. 10B, Lanes 1 to 7). Furthermore, using CHO nuclear extract in EMSAs and radiolabeled htk CCRU as probe, increasing amounts of GST-hnRNP K but not GST caused a decrease in the formation of both complex 1A and 1B (Fig. 10C, Lanes 1 to 7). These
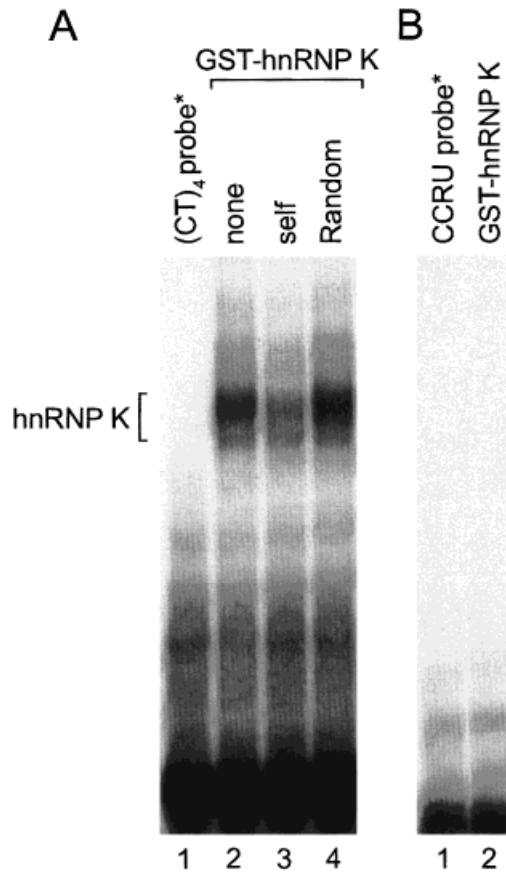
Fig. 8. Lack of binding of recombinant hnRNP K to the htk CCRU. GST-hnRNP K was used in both EMSAs. (**A**) The probe used was the $(CT)_4$ oligomer. In lane 1, free probe was applied; lane 2, no competitor; lane 3, 10-fold molar excess of the $(CT)_4$ oligomer; lane 4, 10-fold molar excess of the random oligomer. (**B**) The probe used was the wt(-110/-84). In lane 1, free probe was applied and in lane 2, mixed with GST-hnRNP K. The reactions were applied onto a 6% native polyacrylamide gel and run for 2.5 h at 200 V. The position of the recombinant hnRNP K complex is indicated.

Fig. 9. HnRNP K as potent suppressor of htk promoter mediated gene activity. In (**A**) and (**D**), 5 μg of phtk(474R)CAT reporter gene were co-transfected with 5 μg of either empty pCMV vector (−), or pCMV driven expression plasmid for hnRNP K (+) into K12 cells, in the presence of 3 μg of RSV-driven β-galactosidase gene serving as the transfection efficiency control. (**B**) Linearized ptk(474R)CAT was used as the reporter gene. (**C**) phtkLS(-112/-98)CAT was used as the reporter gene. In (D) 5 μg of an expression vector for the hepatitis C virus core protein (HCV core) were also included in the transfection mixture where indicated. The CAT activities were determined after the transfection efficiencies were normalized by β-galactosidase activity. The level obtained with the empty pCMV vector was set as 100%. The relative promoter activities with standard deviations are shown.

results show the ability of hnRNP K to inhibit binding of both His-p37 AUF and GST-hnRNP to the htk CCRU and may be a factor in its ability to suppress the htk promoter activity.

Functional Contribution of the hnRNP Binding Site to the htk Promoter Activity In Vivo

To examine effect of mutation of the hnRNP binding site in vivo, stable transfectants of phtk(474R)CAT and phtk(-112/-98)CAT were established. The amount of CAT plasmids integrated into each pool of cells was determined to be similar (data not shown). The cells were synchronized in G_0 or S-phase, and the CAT activities were determined. For comparison,
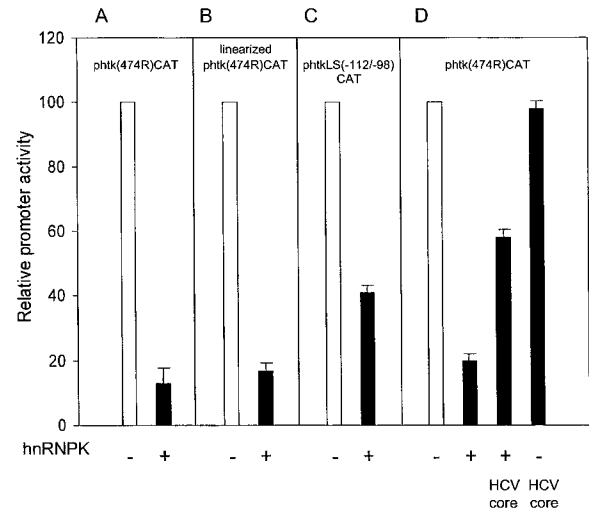
cell extracts were also prepared from exponentially growing cells and the CAT activity determined. We observed that in both G_0 and S-phase cells, the CAT activity of phtk(-112/-98)CAT transfectants was about 5-fold lower than the wild-type phtk(474R)CAT transfectants (Fig. 11A). Nonetheless, the 5-fold increase in the htk promoter activity in S-phase cells as compared to G_0 cells was intact with the mutant promoter, suggesting that the sequence between -112 and -98 is not required for G_1/S induction. In exponentially growing cells, the CAT activity of phtk(-112/-98)CAT was about 10-fold lower than that of phtk(474R)CAT (Fig. 11A). In agreement, through immunostaining, the amount of CAT protein expression was considerably less in the phtk(-112/-98)CAT stable transfectants compared to phtk(474R)CAT (data not shown). We noted that the 5- to 10-fold reduction in htk promoter activity through mutation of (-112/-98) was observed in stable transfectants but not in the same cells transiently transfected with the reporter genes
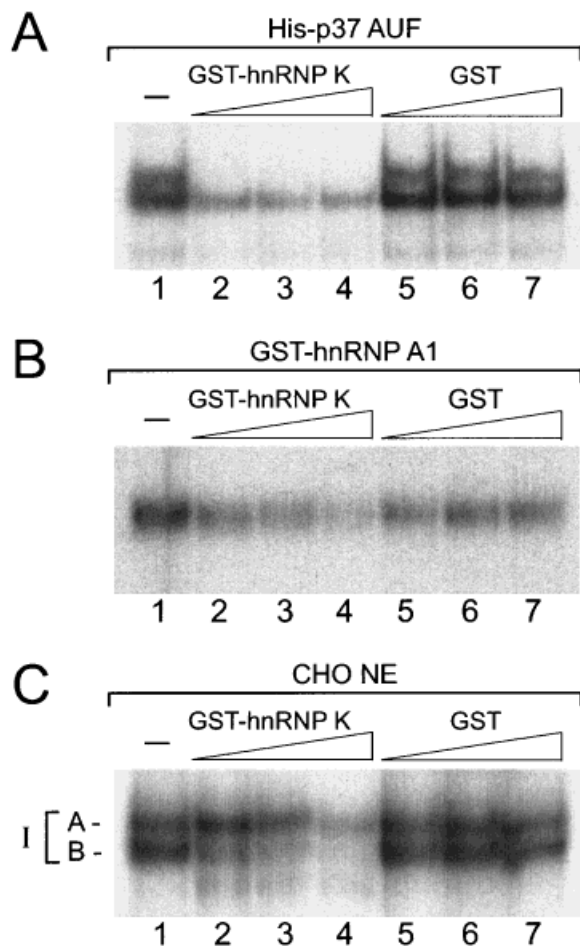
A

His-p37 AUF

B

GST-hnRNP A1

C

CHO NE

Fig. 10. Inhibition of hnRNP binding to the htk CCRU by hnRNP K. The probe used in the EMSAs was radiolabeled wt(-110/-84). (**A**) His-p37 AUF was added alone (Lane 1); or preincubated with increasing amounts (1, 2 and 5 μl) of either GST-hnRNP K (Lanes 2 to 4) or GST (Lanes 5 to 7). (**B**) GST-hnRNP A1 was added alone (Lane 1) or preincubated with increasing amounts (1, 2 and 5 μl) of either GST-hnRNP K (Lanes 2 to 4) or GST (Lanes 5 to 7). (**C**) Nuclear extract prepared from CHO cells (CHO NE) was added alone (Lane 1) or preincubated with increasing amounts (1, 2 and 5 μl) of either GST-hnRNP K (Lanes 2 to 4) or GST (Lanes 5 to 7). The positions of the htk complex I with band A (upper) and B (lower) are indicated.

Fig. 11. In vivo function of the htk promoter sequence spanning (-112/-98). (**A**) Pooled stable transfectants of phtk(474R)CAT or phtk(-112/-98)CAT were synchronized at G0 by serum deprivation, or at S-phase following serum release. Whole cell extracts were prepared from the synchronized cells as well as cells growing exponentially (exp). Equal amounts of protein from each cell extract were assayed for CAT activity. (**B**) K12 cells were transiently transfected with either phtk(474R)CAT or phtk(-112/-98)CAT. Whole cell extracts were prepared from exponentially growing cells. After normalization of transfection efficiency through the measurement of the co-transfected β-gal activity, the CAT activities were determined. The relative CAT activities with standard deviations are shown.

(Fig. 11B). Thus, chromosomal integration is important for the manifestation of the effect of the promoter mutation.

DISCUSSION

In our search for the transcription factors that bind the htk CCRU, we uncovered nuclear ribonucleoprotein family members capable of binding with high affinity to the htk promoter by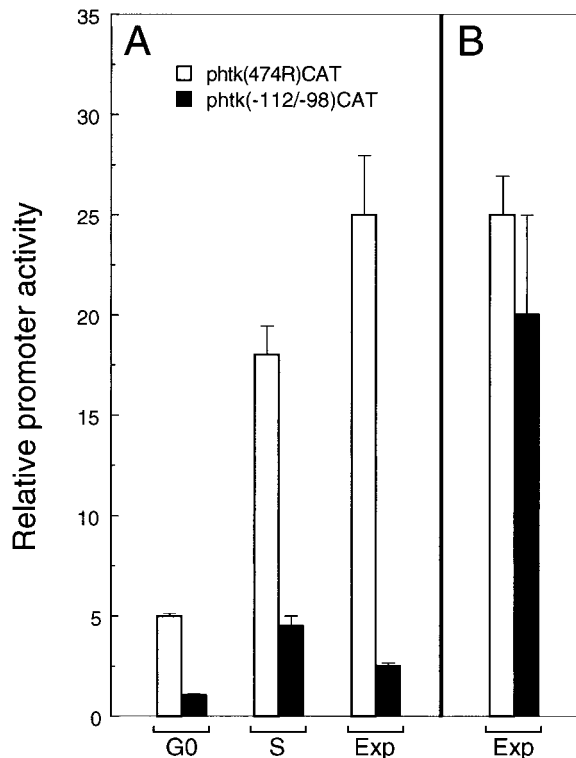 interacting with an ATTT motif. This motif is directly adjacent to the GC-rich motif shown to be critical for the S-phase induction of the htk promoter. Historically, hnRNPs are associated with facilitating mRNA splicing and transport. Recent evidence suggest that they can serve as transcription factors and are themselves differentially phosphorylated during the cell cycle [Tay et al., 1992; Pinol-Roma and Dreyfuss, 1993]. In the case of hnRNP K, a proven single-strand DNA binding protein which recognizes CT elements on the c-myc promoter, it stimulates transcription through interconversion of duplex and single-strands and through protein-protein interaction with the TATA binding protein [Michelotti et al., 1996]. In another study, hnRNP K was shown

to be a repressor of the C/EBP β-mediated activation of the agp gene [Miau et al., 1998]. However, hnRNP K cannot bind to the promoter region of the agp gene, nor does it interfere with the binding of c/EBP β to its cognate DNA site. Thus, the repressive mechanism is postulated to be acting through protein-protein interaction.

In this report, we discovered several new properties of p37AUF, hnRNP A1 and hnRNP K that may explain their mode of action as regulators of gene activity. First, in the case of p37AUF and hnRNP A1, we found that they can bind to double as well as single-stranded DNA with high affinity. Their binding exhibits sequence specificity. As they are known to recognize AUUU sequence in their RNA targets, the interactive site in the htk CCRU contains the corresponding ATTT motif.

We found efficient binding of p37AUF and hnRNP A1 on DNA does not require the interaction of other cellular components to the htk CCRU. The fact that recombinant p37AUF and hnRNP A1 prepared from bacterial extracts can bind DNA efficiently suggests that post-translational modification such as phosphorylation may not be critical for the protein to bind. It is possible that subsequent phosphorylation or other post-translational modification may negatively affect their ability to bind DNA. Since hnRNPs are known to be differentially phosphorylated during the cell cycle, their ability to regulate cell cycle dependent genes could be linked to such modification. Nonetheless, in the case of the htk promoter, we detected constant complex I binding to the promoter during the cell cycle after serum release [Li et al., 1993]. Thus, if there are changes, they would be subtle and perhaps affect the composition of the complex rather than the formation of the complex.

Here we provide new evidence that p37AUF/E2BP can act as an activator in the context of a cellular promoter directed gene expression in a non-hepatic cellular environment. This work extends the earlier observation that p37AUF/E2BP can stimulate gene activity directed by the hepatitis virus enhancer [Tay et al., 1992]. The transactivation of the htk promoter by p37AUF, although modest in magnitude, is promoter-sequence specific. Further, this stimulatory effect was not observed with the CMV promoter nor with β-galactosidase as the reporter gene (our unpublished results). It is an-

ticipated that in different cell lines, the effectiveness of transfected, exogenous p37AUF to act as an activator will depend on the endogenous level of the protein and the availability of other co-activators which may or may not be rate-limiting.

We discovered that hnRNP A1 can act as an effective repressor of htk promoter mediated gene activity. While this repression is dependent on the integrity of the ATTT sequence motif within the htk promoter, it is independent of the supercoiled topology of the DNA target. These results suggest that other cellular promoters bearing these sequence motifs are potential targets of hnRNP A1 binding and regulation.

Over-expression of hnRNP K results in potent inhibition of htk promoter mediated gene activity, however, we could not detect hnRNP K binding to its putative CTCCC binding site within the htk CCRU. Survey of the remainder of the promoter did not reveal any similar CT motifs. Therefore, the action of hnRNP K is likely to involve protein-protein interaction. However, since mutation of the AT-rich motif resulted in partial reversion of the repression, it appears that the hnRNP K inhibitory mechanism involves a downstream component which interacts with the AT motif. In support, we show that hnRNP K can inhibit the ability of both p37AUF and hnRNP A1 to bind to the htk CCRU in vitro. Our observation that hnRNP K can compete away other hnRNP binding to the htk CCRU is likely due to protein-protein interaction among the hnRNPs. Thus, hnRNP proteins are known to have a modular structure, and some of the glycine-rich domains may mediate protein-protein interaction [Dreyfuss et al., 1993]. This allows them the opportunity to form homodimers or trimers, facilitate or interfere with the oligomerization of other hnRNPs or cellular proteins, such as components of the transcription machinery. This can provide an explanation for the indirect effect of hnRNP K on the htk promoter despite its inability to bind directly to the promoter sequence.

Lastly, while the exact physiological role played by the positive and negative acting hnRNPs on the htk promoter requires further analysis, our results support accumulating evidence that traditional RNA binding proteins could modulate gene activity either by direct DNA binding or interaction with other proteins [Tay et al., 1992; Michelotti et al., 1996; Miau

et al., 1998]. While the exact mechanism for their regulatory function remains to be discovered, the specific effects of the hnRNPs on the htk promoter is dependent on their DNA binding site on the promoter. We further discovered that the hnRNP binding site within the htk CCRU is important in maintaining high level htk promoter activity only in the context of stable transfectants when the reporter genes are integrated into the chromosome. This intriguing observation is consistent with the hypothesis that these single-strand DNA binding proteins could serve unique functions such as maintaining the transcribability of the gene as cells go through one cell cycle to the next [Michelotti et al., 1996; Tomonaga and Levens, 1996]. Our studies open up the possibility that hnRNPs, acting in concert with other chromosomal components, serve as regulators of gene expression through interaction with cellular promoter elements.

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