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Specific Binding of Heterogeneous Ribonucleoprotein Particle Protein K to the Human *c-myc* Promoter, *in Vitro**

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A homopurine/homopyrimidine-like sequence is found 100–150 base pairs upstream of the human *c-myc* promoter P1. This element, termed the CT-element, has been shown to augment expression from P1, and it serves as a positive transcriptional element when coupled to a heterologous promoter *in vivo* and *in vitro*. Synthetic oligonucleotides comprising this element were used to form DNA-protein complexes in electrophoretic mobility shift assays. By using conventional and affinity methods, 61- and 34-kDa proteins were shown to be associated with these complexes. Amino acid sequence analysis and immunological methods have identified these proteins as heterogeneous ribonucleoprotein particle (hnRNP) proteins K and A1. Surprisingly, hnRNP protein K binds to the pyrimidine-rich strand of the CT-element in a sequence-specific manner as well as to the double-stranded molecule. Cotransfection of vectors encoding hnRNP protein K in the sense or anti-sense orientations with reporter plasmids driven by wild-type or mutant CT-elements demonstrates that hnRNP protein K augments gene expression in a cis-element-dependent manner. Taken together, these results suggest that hnRNP protein K may play a role in the transcriptional regulation of the human *c-myc* gene.

The *c-myc* protooncogene product encodes a sequence-specific DNA-binding protein that plays a central role in the regulation of cell growth and differentiation (Blackwell *et al.*, 1990; Prendergast and Ziff, 1991; Blackwood and Eisenman, 1991). Deregulation of *c-myc* expression by chromosomal translocation, retroviral insertion, gene amplification, and point mutation can cause immortalization and transformation of normal cells (for review, see Bishop, 1987; Cole, 1986; Cory, 1986; Kelly and Siebenlist, 1986; Spencer and Groudine, 1991). Enforced expression of *c-myc* can drive a cell into S phase, resulting in DNA synthesis (Cavalieri and Goldfarb, 1987). Reflecting its central role in the control of growth and

differentiation, *c-myc* synthesis is regulated at multiple levels including transcription (both initiation and elongation), mRNA stability, and translation. The stability of the *c-myc* protein is also regulated (see reviews, and Bentley and Groudine, 1986). Transcription of the *c-myc* gene is initiated from two major promoters, P1 and P2, and it can be up-regulated by mitogenic agents, such as growth factors, and down-regulated by differentiation-inducing agents. A host of pharmacological and physiological agents have been reported to modify *c-myc* expression (Spencer and Groudine, 1991). A composite of positive- and negative-acting cis-elements located upstream and downstream of the transcription start sites control *c-myc* expression, and numerous trans-factors have been reported to interact with these elements (Hay *et al.*, 1987; Lipp *et al.*, 1987; Zajac-Kaye *et al.*, 1988b). These trans-factors are the targets of numerous signal transduction pathways and confer upon the *c-myc* gene a system for monitoring and integrating diverse intracellular and extracellular stimuli, thereby coordinating *c-myc* expression with other cellular processes (Zajac-Kaye *et al.*, 1988a, 1988b; Takimoto *et al.*, 1989; Hay *et al.*, 1989; Avigan *et al.*, 1990; Hiebert *et al.*, 1989; Thalmeier *et al.*, 1989).

An element that augments *c-myc* expression both *in vivo* and *in vitro* has been localized to a region 100–150 bp¹ upstream of the human *c-myc* promoter P1. DNase I hypersensitivity of this region (termed site III₁ by Siebenlist *et al.*, 1984) suggests regulation of the relevant trans-factors. A sensitive exonuclease assay as well as EMSAs have identified factors in HeLa and HL-60 cells with binding sites in this region (Hay *et al.*, 1987; Siebenlist *et al.*, 1984; Davis *et al.*, 1989; Postel *et al.*, 1989). The DNA sequence encompassing these binding sites contains four repeats of a 9-bp sequence (the CT-element) that is either identical to CCCTCCCCA or differing by only a single pyrimidine substitution. A fifth copy is identical in seven of nine positions (Hay *et al.*, 1987). Spanning 50 bp, this homopyrimidine/homopurine-like segment has been suggested to assume unusual DNA conformations, including single-stranded and triplex structures. S1 sensitivity of this region in naked DNA, as well as an RNase-sensitive factor recognizing this element have been reported (Boles and Hogan, 1987; Cooney *et al.*, 1988; Davis *et al.*, 1989). Similar long homopyrimidine/homopurine-like sequences occur infrequently elsewhere in the genome and have

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¹ The abbreviations used are: bp, base pair(s); EMSA, electrophoretic mobility shift assay; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; CAT, chloramphenicol acetyltransferase; BPB, bromophenol blue; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; NEPHGE, nonequilibrium pH gradient polyacrylamide gel electrophoresis; hnRNP, heterogeneous ribonucleoprotein particle.

been identified in the upstream sequences of several genes (Johnson *et al.*, 1988; Gaillard *et al.*, 1988; Hoffman *et al.*, 1990; Wilkison *et al.*, 1990).

In this report, we show that the CT-element forms multiple, specific DNA-protein complexes with HeLa cell nuclear extracts. Purification of the protein components of one of the complexes followed by biochemical and immunochemical analyses identified two major proteins with specificity for the CT-element as hnRNP proteins. These observations suggest a potential link between RNP assembly, RNA processing, and the transcriptional regulation of *c-myc* expression.

EXPERIMENTAL PROCEDURES

Cells and Media—HeLa cells were grown in DMEM containing 10% FCS and nonessential amino acids (Life Technologies Inc.) were supplemented in the media.

Plasmid DNAs and Oligonucleotides—Plasmid $\Delta 56$, which has a mouse *fos* promoter (from -56 to +109) and CAT gene, and plasmid SP6 mfos109 were kindly donated by Dr. M. Z. Gilman (Gilman *et al.*, 1986). Synthetic complementary wild-type and mutant CT oligonucleotides (44 bp), with sequences described below, were cloned into *Hind*III site of $\Delta 56$ to make $\Delta 56$ CT and $\Delta 56$ CT-mut, respectively. The wild-type CT oligonucleotides were cloned in both sense and antisense orientation relative to transcriptional start site. The sequence of the wild-type and mutant CT oligonucleotides (template strand) are as follows: 5'-AGCTAGCTCTCCCCACCTTCCCCACCTCCCC ACCCTCCCCAG-3' (wild-type), 5'-AGCTAGCTCGTCGACAGTTCGACACGCTCGACACGCTCGACAG-3' (mutant) (mutated residues are underlined). The sequences of other oligonucleotides used in this study are described in Table I. Vectors expressing hnRNP protein K mRNA or anti-sense mRNA were constructed by inserting the *Eco*RI fragment encoding hnRNP protein K from pHK5 (the generous gift of W. Matthew Michael) into pcDNA1/AMP (Invitrogen) in both orientations relative to the cytomegalovirus immediate early enhancer and promoter.

CAT Assays—Transfections of plasmid DNAs into HeLa cell lines were performed by electroporation. Briefly, 5×10^6 of HeLa cells were centrifuged and resuspended in 250 μ l of DMEM, containing 10% FCS in a cuvette and incubated on ice for 10 min. Plasmid DNAs (10 μ g) were added and incubated on ice for an additional 10 min before electroschock (200 V). After electroschock, the cells were incubated in DMEM containing 10% FCS for 30–48 h before harvest. Extract preparations and CAT assays were performed as reported (Gorman, 1985).

Preparation of Nuclear Extracts—HeLa cell nuclear extracts were prepared by the procedure of Dignam *et al.* (1983), with some modifications as previously described (Takimoto *et al.*, 1989). To make extracts for *in vitro* transcription reaction, the procedure of Dignam *et al.* was used without modification. The protein concentration of the prepared extracts was approximately 8 mg/ml. Protein concentrations were determined by Bio-Rad Protein assay kit.

Electrophoretic Mobility Shift Assay (EMSA)—EMSAs were performed as previously described (Takimoto *et al.*, 1989). Probe DNAs, annealed oligonucleotides, or restriction fragment, were always radiolabeled by *E. coli* DNA polymerase I large fragment (Klenow fragment) enzyme. When the affinity-purified and denatured/renatured proteins were used, bovine serum albumin (BSA: Fraction V, Sigma) and Tween 20 were included in binding reactions to final concentrations of 1 mg/ml and 0.05%, respectively. Oligonucleotide competitors are listed in Table I.

Purification of CT-element-binding Proteins—Preparation of crude HeLa cell nuclear extracts was described above. The crude extracts were fractionated by a cation chromatography on Bio-Rex 70 (Bio-Rad) with some modifications as previously described (Takimoto *et al.*, 1989). Extracts were diluted with buffer D to a final concentration of 50 mM KCl, loaded onto a Bio-Rex 70 column equilibrated with the same buffer (approximately 1 ml of resin was used for every 10–20 mg of nuclear protein), the flowthrough was collected, the column was washed with 3–5 column volumes of buffer D containing 50 mM KCl, and finally the column was eluted with a linear gradient from 50–800 mM KCl. Collected fractions were subjected to EMSA using radiolabeled CT oligonucleotides as probe. Appropriate fractions were pooled and loaded onto a hydroxylapatite column, which was equilibrated with buffer D containing 50 mM NaCl. For hydroxylapatite

chromatography, buffer D was supplemented with 0.2 mM $MgCl_2$. Approximately 1 g of dry powder of hydroxylapatite (Bio-Rad) was used for every 45 mg of nuclear protein. After washing the column with 2–3 column volumes of buffer D containing 50 mM NaCl, the column was eluted stepwise with buffer D containing 50, 100, and 300 mM sodium phosphate buffer, pH 7.5, respectively, at room temperature. The fractions containing binding activity were pooled and subjected to chromatography on a CT-oligonucleotide affinity column prepared according to Kadonaga and Tijan (1986). The active fractions from the hydroxylapatite chromatography were loaded onto the affinity column (2 ml) equilibrated with buffer D containing 50 mM sodium phosphate, pH 7.5, washed with 15-column volumes of the same buffer, and eluted stepwise of buffer D containing 0.3, 0.6, 1, and 2 M of NaCl, respectively. The fractions with binding activity were pooled, dialyzed to 50 mM NaCl, and subjected to a second cycle of oligonucleotide affinity chromatography. The affinity-purified proteins were diluted with deionized water to a final glycerol concentration of 15% (v/v); 100% (w/v) trichloroacetic acid was added to a final concentration of 10%, and then the mixture was incubated at $-20^\circ C$ for 30 min and microcentrifuged at $4^\circ C$ for 30 min. The supernatant was removed, the pellet was rinsed twice with cold acetone, air-dried, suspended in protein sample buffer (1.5% SDS, 4 M β -mercaptoethanol, 58 mM Tris, pH 6.8, 1.2% glycerol, 0.006% BPB), boiled, and loaded on 10% SDS-PAGE (acrylamide/bis (37.5:1)). The separated proteins in the protein gel were fixed with 10% methanol/10% acetic acid and stained with Coomassie Brilliant Blue.

Southwestern Blotting Assay—Affinity-purified proteins were loaded onto 10% SDS-PAGE as described above. The separated proteins were electroblotted to a nitrocellulose filter. The filter was processed for binding assay as described (Wiederrecht *et al.*, 1988).

Denaturation/Renaturation of Purified Protein—Slices containing separated protein fractions 1–6 in the SDS-protein gel were cut out from the gel. The proteins were electroeluted in 50 mM ammonium bicarbonate and 0.1% SDS and lyophilized. SDS was removed by ion-pair extraction as described (Henderson *et al.*, 1979). The ion pair-extracted proteins were resuspended in 20 mM HEPES, pH 7.5, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 100 mM KCl, 0.1% Nonidet P-40, and 6 M guanidine-HCl and incubated for 30 min at room temperature. Portions of each fraction were renatured by dilution (50-fold) with buffer D containing 40 mM KCl, 1 mg/ml BSA, 0.05% Tween 20 and incubation for 20 min to 3 h at room temperature. DNA binding activity was checked by EMSA.

Two-dimensional Gel Electrophoresis—Two-dimensional nonequilibrium pH gradient polyacrylamide gel electrophoresis (NEPHGE) was performed as described by O'Farrell *et al.* (1977) using an ampholine gradient of pH 3–10 separated for 4 h at 400 V in the first dimension. Proteins were separated by SDS-PAGE in the second dimension and visualized by silver staining. hnRNP proteins were purified from HeLa cell nucleoplasm by single-stranded DNA chromatography as previously described (Matunis *et al.*, 1992).

Amino Acid Sequence Analysis of the Purified Proteins—Electroeluted proteins were sequentially digested with cyanogen bromide and, following reduction and pyridylethylation, with trypsin, according to the protocol of Stone *et al.* (1989). The resulting fragments were then separated by gradient elution on an Aquapore RP300 C-8 reverse-phase column: 0.1% (v/v) trifluoroacetic acid and 0–70% acetonitrile using a Dionex A1-450 BioLC system. After the digestion and the purification, the amino acid sequences of peptides were determined on a Porton Instruments 2020 off-line sequencer using standard program no. 1. Phenylthiohydantoin amino acid analysis of sequencer runs was performed on a Beckman System Gold HPLC using a modified sodium acetate gradient program and a Hewlett-Packard C-18 column.

Antibodies—Monoclonal anti-hnRNP K antibodies, 12G4 and 3C2, were described by Matunis *et al.* (1992). A monoclonal anti-hnRNP A1 antibody, 4B10, was previously described (Pinol-Roma *et al.*, 1989). A monoclonal anti-SV40 T-antigen antibody, PAB416, was purchased from Oncogene Science, Inc. (Manhasset, NY).

Absorption of DNA Binding Activities by Anti-hnRNP Protein Antibodies—One or 2 μ l of monoclonal antibodies were incubated with 25 μ l (packed volume) of Protein A-Sepharose CL-4B (Pharmacia LKB) in a total reaction volume of 50 μ l by addition of binding solution at room temperature for 1 h. The binding solution was composed of buffer D containing 80 mM KCl, 1 mg/ml of BSA, and 0.05% Tween 20. After incubation, the antibody-bound Protein A-Sepharose was washed three times with phosphate-buffered saline containing 0.1% BSA and 0.05% Tween 20. Three μ l of denatured/

renatured proteins from fractions 3 and 6 and 12 μ l of the binding solution were mixed with the antibody-bound Protein A-Sepharose and incubated with mild vortexing at 4°C for 80 min. After incubation, the reaction mixtures were microcentrifuged briefly, and 10 μ l of the supernatants were used for EMSA to check residual DNA binding activity.

UV-cross-linking/Immunoprecipitation—DNA-binding reactions, using 0.1 μ l of the affinity-purified proteins and 32 P-radiolabeled CT oligonucleotides, were performed as described above, except without BPB dye. For the first 5 min, the binding reactions were performed without UV irradiation. For the next 20 min, the binding reactions were irradiated from above with Mineral light lamp (UVP Inc., San Gabriel, CA), then 20 μ l of buffer D containing 80 mM KCl, 1 mg/ml of BSA and 0.05% Tween 20 and 25 μ l of packed volume of the antibody-bound Protein A-Sepharose was added, mixed with gentle vortexing at 4°C overnight, centrifuged briefly, and the pellets washed three times with phosphate-buffered saline containing 0.2% Tween 20. The cross-linked DNA-protein complexes were eluted from the Protein A-Sepharose beads in 40 μ l of protein sample buffer and analyzed on 10% SDS-PAGE.

RESULTS

The CT-sequence: a Positive cis-Element in Vivo and in Vitro—A positive transcriptional element with a homopyrimidine/homopurine-like character occurring 100–150 bp upstream of the human c-myc promoter P1 has been reported by several groups. This element, composed of five repeats, has been reported to be necessary for P1 expression and to augment P2 expression *in vivo*, and to increase P2-driven expression *in vitro* (Hay *et al.*, 1987; Lipp *et al.*, 1987; Postel *et al.*, 1989). Because the multiple cis-elements upstream and downstream of c-myc promoters P1 and P2 may interact in a complex combinatorial manner (Zajac-Kaye *et al.*, 1988a, 1988b; Takimoto *et al.*, 1989; Hay *et al.*, 1989), we sought to demonstrate whether the CT-element alone could stimulate expression from a heterologous promoter both *in vivo* and *in vitro*. Synthetic oligonucleotides containing the CT-element were cloned adjacent to the minimal c-fos promoter driving the CAT gene in the vector Δ 56 (Gilman *et al.*, 1986). The resulting plasmid, Δ 56CT, was used to assess CAT expression *in vivo* and to program transcription *in vitro*, as measured with RNase protection.

Introduction of Δ 56CT into HeLa cells reproducibly supported a 4–10-fold greater CAT expression than Δ 56 or Δ 56CT-mut, with Δ 56 harboring a mutated CT-element. The CT-element stimulated expression less effectively in the reverse orientation (Fig. 1A). Using Δ 56CT and Δ 56 as templates for transcription by HeLa cell nuclear extracts, stimulation by the c-myc CT-element was also observed (data not shown). Thus, this polydeoxypyrimidine/polydeoxypurine-like element serves as a positive element both *in vivo* and *in*

vitro on a heterologous promoter as well as on the c-myc promoters.

Formation of Multiple DNA-Protein Complexes with the CT-element—Previous studies using a sensitive exonuclease assay demonstrated a cluster of binding sites coinciding exactly with the repeated sequence comprising the CT-element (Hay *et al.*, 1987). To analyze the factors specific for this element more completely, EMSAs were performed using HeLa cell nuclear extracts, radiolabeled CT oligonucleotides, and a variety of DNA and oligonucleotide competitors. Four distinct DNA-protein complexes were observed. Complexes I, II, and IV were specifically eliminated by CT oligodeoxynucleotides but not by nonspecific oligonucleotides (Fig. 1B). The specificities of the DNA-protein complexes were also verified using restriction fragments derived from plasmid DNAs. Only a fragment possessing the CT-element eliminated complexes I, II, and IV (Fig. 1C). The minor complex III displayed reduced specificity compared with the other complexes. The lack of competition with a variety of restriction fragments and double-stranded oligodeoxynucleotides with sequences comprised of regulatory regions of viruses and binding sites of known factors failed to identify any of the complexes with well-characterized transcription factors. Thus multiple specific complexes of unknown composition form on the CT-element.

Purification of the Proteins Generating One of DNA-Protein Complexes—Due to the multimeric nature of the CT-element and to the observation that a single repeat was ineffective as a probe or as a competitor in binding assays (data not shown), the complex pattern observed on EMSA may be attributed to multimeric binding of a single factor or binding of multiple distinct factors. To resolve this question and to better define the nature of the factors binding to the CT-element, HeLa cell nuclear extracts were fractionated according to the purification scheme described under "Experimental Procedures." Early in the fractionation, the binding activities producing the major complexes separated from each other, indicating that distinct factors (or combinations thereof) were associated with each band. Complex IV, the fastest migrating species, showed the most abundant binding activity and was therefore considered a factor likely to bind to the CT-repeated element either individually or in combination with other components.

After performing multiple chromatographic steps, including conventional and affinity methods, major polypeptides of 61 and 30–34 kDa were present following SDS-PAGE and staining with Coomassie Blue. In addition, several minor bands were noted (Fig. 2A). The affinity-purified proteins, separated by SDS-PAGE, were electroblotted onto nitrocellulose, renatured, and incubated with 32 P double-stranded oligonucleo-

TABLE I
c-myc DNA sequence and DNA sequences of oligonucleotides

c-myc -151 to -100 TCCTCCCCA CCTTCCCCA CCCTCCCCA CCCTCCCCA TAAGCG CCCTCCCGG. Only the template strands are shown except wild-type CT oligonucleotides, in which CT-Y and CT-R indicate the pyrimidine and purine strand, respectively. Sequences of nonspecific, JC, and AP-1 oligonucleotides derive from a negative element located upstream, first intron of human c-myc gene and from long term repeat of Gibbon ape leukemia virus, respectively (Takimoto *et al.*, 1989; Zajac-Kaye *et al.*, 1988b; Quinn *et al.*, 1987). SP-1 and HTLV1 oligonucleotides are gifts of Dr. U. Hansen and J. Brady, respectively (Jeang *et al.*, 1988).

Oligonucleotide	Length	DNA sequence (5' → 3')
Wild-type CT:CT-Y	32	AATTCTCTCTCCACCTTCCCAACCTCTCCCA
:CT-R	32	AGCTTGGGGAGGGTGGGGAAGGTGGGGAGGAG
Permuted CT	32	CCATTCCCACTCTCCACCTCTCTCCACCTCC
Nonspecific	34	GATCAAGCCTGCGATGATTATACTACAGGAAG
AP-1	22	CGAGAAATAGATGAGTCAACAG
JC	22	CCAGAGTAGTTATGGTAACCTG
SP-1	23	CCATGGGCGGAGTTAGGGCGGGA
HTLV1	25	GATCAAGGCTCTGACGTCTCCCCC

fied material generated two CT-specific complexes as well as a more slowly migrating nonspecific complex (Fig. 2B). The binding of the renatured protein to the CT-element was eliminated by excess CT-element containing DNA, but not by unrelated sequences. In addition restriction fragments of c-myc encompassing the CT-element, but not heterologous restriction fragments, competed specific binding of the 61-kDa protein (data not shown). Consistent with their sizes, the specific complex containing the 30–34-kDa protein (fraction 6) migrated faster than that containing the 61-kDa species (fraction 3). All binding assays contained poly(dI-dC), and the double-stranded oligonucleotide probes were end-labeled by filling in 3' recessed ends with DNA polymerase in a template-dependent manner.

Identification of Purified Polypeptides as hnRNP Proteins—Because the 61-kDa protein showed CT-specific binding properties by both Southwestern blot analysis and by EMSA using renatured protein, this polypeptide was subjected to cleavage with CNBr and digested with trypsin. The resulting peptides were separated with HPLC, and a partial sequence was determined by automated Edman degradation. Sequence analysis and computer homology revealed identity between five of seven peptides and predicted trypsin peptides of the hnRNP protein K (Matunis *et al.*, 1992). Because of the existence of altered spliced forms of hnRNP protein K in RNA and the possibility that additional isoforms differing in primary sequence exist, the remaining two peptides cannot yet be ascribed either to hnRNP protein K or to another polypeptide present in the binding fraction.

The same treatment of the 30–34-kDa protein yielded several peptides that were analyzed for sequence. Segments of hnRNP proteins A1 and B1 were identified by searching the sequences determined against a protein database (Table II). Sequences from other hnRNP proteins were not identified; reinspection of the SDS-polyacrylamide gels from which hnRNP proteins K, A1, and B1 were excised showed no major bands compatible with other hnRNP proteins.

Selected Isoforms of Specific hnRNP Proteins Enriched by CT-element Chromatography—To confirm that the purification scheme did not enrich for bulk hnRNP complexes and to better visualize the polypeptides present in the purified preparations, samples were subjected to two-dimensional electrophoresis following conventional chromatography and two cycles of affinity chromatography. Compared with immunopurified hnRNP proteins or with hnRNP proteins enriched on single-stranded DNA affinity columns, a much simpler pattern was observed. Most prominent among the polypep-

tides visualized with silver stain was a protein migrating in both dimensions identically with one member of the hnRNP protein K family. An unidentified 58-kDa species (p58) appeared relatively less abundant in Coomassie Blue-stained SDS-polyacrylamide gels. Specific binding activity, assayed by EMSA, could not be renatured from the 58-kDa protein. In addition, lesser amounts of the closely related A1, A2, and B2 hnRNP proteins were present. Although hnRNP K is comprised of multiple isoforms (Matunis *et al.*, 1992), only a single species was observed. Thus, the purification scheme was sufficiently selective to discriminate between closely related forms of the same protein (Fig. 3A).

Because hnRNP proteins were unexpectedly enriched in fractions selected for CT-element binding, it was important to confirm that these same proteins possessed specific DNA binding activity. Monoclonal antibodies against hnRNP proteins K and A1 were used in two ways to relate these proteins immunologically with CT-element binding activity. First, the renatured 61-kDa protein (fraction 3), which yielded multiple peptides with sequences contained in the hnRNP protein K open reading frame, was incubated with Protein A-Sepharose beads charged with different monoclonal antibodies. The beads with specifically bound material were removed, and the unbound fraction was assayed for CT-element binding. As shown in Fig. 3B, Protein A beads that were loaded with anti-hnRNP protein K (either of two different monoclonal antibodies) removed virtually all of the 61-kDa CT-element binding activity of fraction 3. In contrast, antibodies against SV40 T-antigen or anti-hnRNP protein A1 left the CT-element binding activity undiminished. This experiment demonstrated immunological cross-reactivity between hnRNP protein K and CT-element binding activity (Fig. 3B, left). Repeating this set of experiments using the SDS-PAGE-separated, -electroeluted, and -renatured 30–34-kDa protein (fraction 6), which yielded peptides comprised of hnRNP protein A1 sequence, revealed immunocross-reactivity between the CT-element-binding protein and hnRNP protein A1. A monoclonal anti-hnRNP protein A1 caused a modest decrease in CT-element complex formation, whereas anti-hnRNP protein K (two different monoclonal antibodies) and anti-SV40 T-antigen removed no detectable activity (Fig. 3B, right).

A second method for directly detecting the interaction of hnRNP proteins with the CT-element is to UV cross-link-labeled DNA to the protein, immunoprecipitate with appropriate antibodies, separate the precipitated protein by SDS-polyacrylamide gel electrophoresis, and display the cross-linked material by autoradiography. After this scheme, ³²P-labeled CT-element adducts with both hnRNP proteins K and A1 were precipitated with appropriate monoclonal antibodies but not with anti-SV40 T-antigen monoclonal antibodies (Fig. 3C). The small signal detected with anti-hnRNP protein K antibodies may be attributed to inefficient cross-linking of DNA to this molecule, as has been previously observed.²

As hnRNP proteins possess broadly selective binding properties for polyribo- and deoxyribonucleotides, it was important to determine if the sequence specificity and/or particular or structural recognition properties could restrict the DNA binding specificity of hnRNP proteins K and/or A1 sufficiently to warrant consideration of a role for these molecules in regulatory processes, such as transcription.

hnRNP Protein K: a Sequence-specific DNA-binding Protein—Throughout the purification and identification of the proteins interacting with the CT-element, specificity was

TABLE II

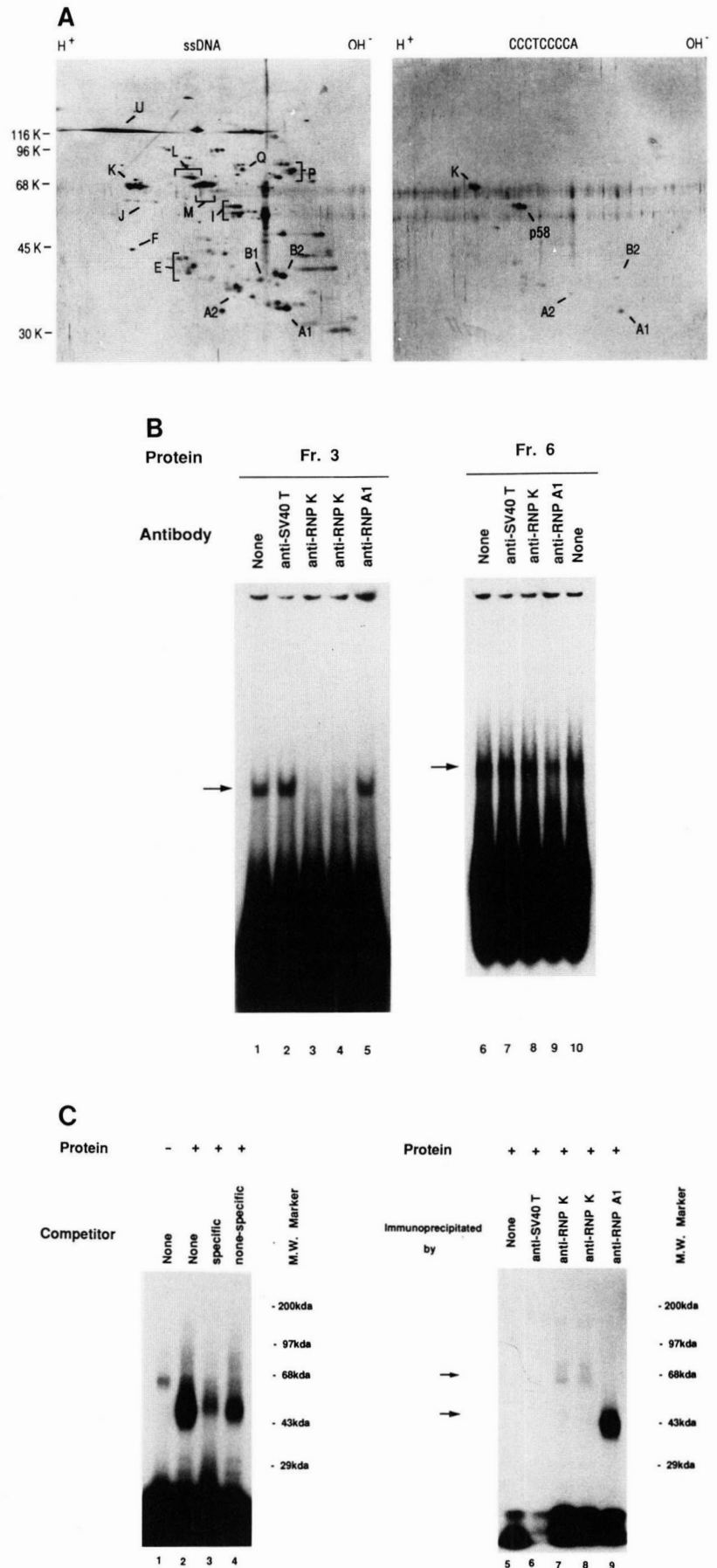
Amino acid sequences of HPLC separated peptides of the proteins in fractions 3 and 6

Peptides were generated from the proteins in fractions 3 (61 kDa) and 6 (30–34 kDa) as described under "Experimental Procedures."

Protein	Amino acid sequence	Identical/hnRNP protein
61 kDa (in fraction 3)	ILLQS	K (residues 47–51)
	AQPDNPFYDET	K (residues 222–232)
	TDYNASVSVP	K (residues 70–79)
	AYEPQGGGSYD	K (residues 360–370)
	GSYDGLGGPI	K (residues 378–387)
	IKEEP TEEKPPS	
30–34 kDa (in fraction 6)	GGGGYGG	A1 (residues 233–240)
	SSGPYGGG	A1 (residues 285–292)
	NQGGY	A1 (residues 301–305)
	RDPASK	B1 (residues 54–59)
	GGGGNFGPG	B1 (residues 206–214)

² G. Dreyfuss, personal communication.

FIG. 3. hnRNP protein K binds the CT-element. **A**, two-dimensional gel analysis of the purified proteins from CT oligonucleotide affinity chromatography: CT-binding proteins (right) or hnRNP proteins (left) were purified by affinity chromatography and resolved by two-dimensional gel electrophoresis (NEPHGE in the first dimension, SDS-PAGE in the second dimension). Proteins were visualized by silver staining. As indicated, the CT-binding proteins consist largely of hnRNP K (the more basic isoform), an unidentified protein (p58), hnRNPs A1, A2, and B2 were also identified. The molecular mass of protein standards is indicated on the left. **B**, absorption of the binding activities of the denatured/renatured proteins by anti-hnRNP protein antibodies: the denatured/renatured proteins in fraction 3 (61 kDa) and 6 (30–34 kDa) were incubated with monoclonal antibodies, which were prebound to Protein A-Sepharose CL-4B, centrifuged, and the supernatants assayed for DNA binding with radiolabeled CT oligonucleotides as described under "Experimental Procedures." Arrows indicate the specific DNA-protein complexes. The anti-hnRNP protein K antibodies used were 12G4 in lanes 3 and 8, and 3C2 in lane 4. **C**, UV-cross-linking of the affinity-purified proteins to the CT-element and immunoprecipitation of the DNA-protein complexes by anti-hnRNP protein antibodies. **Left**, UV-cross-linking of the affinity-purified proteins to the CT-element. Binding reactions, with or without competitor oligonucleotides, and UV-cross-linking were performed and analyzed by SDS-PAGE as described under "Experimental Procedures." Seventy-five-fold molar excesses of specific (wild-type CT) and nonspecific oligonucleotides were used as competitors in lanes 3 and 4, respectively. **Right**, immunoprecipitation of the UV-cross-linked DNA-protein complexes by anti-hnRNP protein antibodies. The UV-cross-linked radiolabeled CT oligonucleotides and affinity-purified proteins were immunoprecipitated with anti-hnRNP protein antibodies and control anti-SV40 large T antibody, and analyzed by SDS-PAGE as described under "Experimental Procedures." Arrows indicate the precipitated DNA-protein complexes. The anti-RNP protein K antibodies 12G4 and 3C2 were used in lanes 7 and 8, respectively.



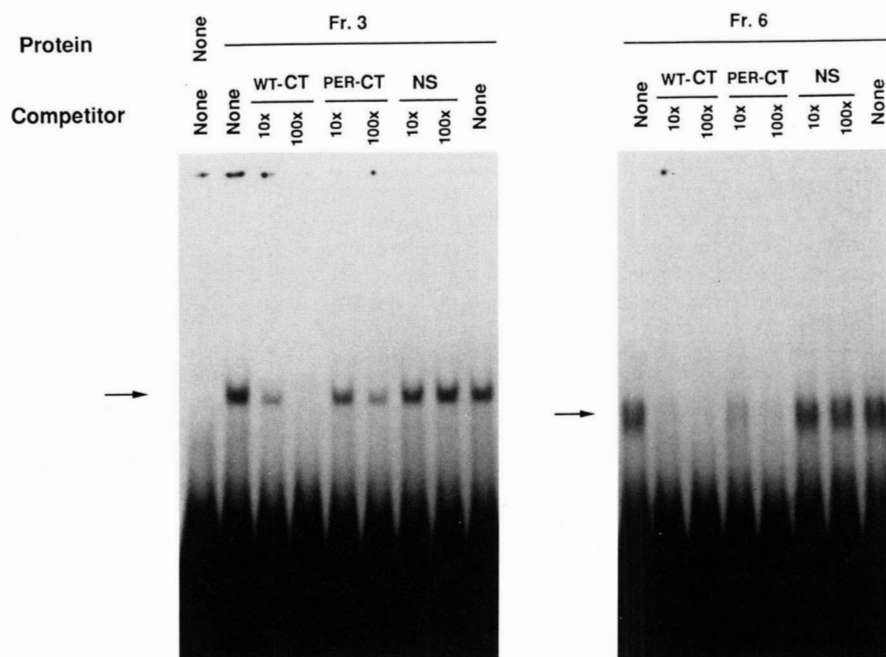
monitored frequently by binding assays performed in the presence of a variety of competitor nucleic acids or by using alternate sequences that were unrelated to the CT-element as probe. The complexes observed behaved as sequence-specific DNA-protein interactions by all criteria. Because hnRNP protein K is known to interact with polyribocytidylic acid (Swanson and Dreyfuss, 1988), we permuted the repeating unit of the CT-element, still retaining the pyrimidine/purine strand bias, to generate the binding substrate "permuted CT" (Table I). Due to the highly skewed base composition of the CT-element, each permuted repeating unit still bears 6/9-bp homology with the natural c-myc sequence (7/9 conservation if C-T substitutions are allowed, as is suggested by the sequence of the natural element). After careful quantitation, wild-type CT, permuted CT, and an irrelevant sequence displaying no purine-pyrimidine strand bias, were compared for their ability to compete for wild-type CT probe binding to gel-purified, renatured hnRNP protein K. As shown in Fig. 4, whereas a 100-fold excess of the wild-type CT virtually eliminated hnRNP protein K complex formation with the probe, the permuted CT was greatly reduced in its ability to displace the probe; the nonspecific competitor had no effect upon the extent of binding. Therefore, hnRNP protein K sees some feature of the probe, determined by primary sequence and not merely by base composition. In the same experiment, hnRNP protein A1 displayed a lesser ability to discriminate between the wild-type and permuted CT-elements, which indicates that the degree of selectivity displayed by hnRNP protein K is not a general property of hnRNP proteins (Fig. 4).

Because hnRNP proteins can interact with single-stranded nucleic acid (Dreyfuss, 1986; Bandziulis *et al.*, 1989; Kenan *et al.*, 1991), the single strand binding properties of the affinity-purified and renatured hnRNP K and A1 proteins were explored. Although the oligodeoxyribonucleotides used as probes in EMSAs were carefully checked to confirm that minimal unannealed sequences were present and that the probe was labeled by extending a 3' recessed end with DNA polymerase in a template-dependent, and hence double-strand-dependent, manner, the possibility remained that hnRNP K and/or hnRNP A1 could form a stable complex with single-stranded DNA. Some hnRNP proteins have helix-destabilizing prop-

erties that could allow hnRNP protein K or A1 to insinuate itself into the double-stranded probe and form a stable complex with one strand (Herrick and Alberts, 1976). Alternatively, if the double-stranded probe were in a sufficiently rapid equilibrium with the individual strands, then hnRNP proteins could trap the single-stranded intermediates, shifting the equilibrium to generate even more single-stranded material. This latter possibility is mitigated against by the high melting temperature of the G-C-rich probe; spontaneous melting of the probe should not be a rapid event at room temperature. To assess the ability of hnRNP proteins K and A1 to interact with single-stranded sequences, double-stranded ³²P-labeled CT-element was incubated with the specific binding fractions eluted from the affinity column in the presence of various single- or double-stranded deoxyribonucleotides as competitors. First, a nonspecific oligonucleotide of similar length, when included in either single- or double-stranded form, failed to disturb the CT-probe-protein complexes for either hnRNP protein K or A1; only a slowly migrating nonspecific complex was eliminated. In contrast, the CT-sequence competed effectively either as double-stranded DNA or with the pyrimidine-rich strand alone; the purine-rich strand also reduced binding significantly but less effectively than the pyrimidine-rich strand. Therefore, hnRNP proteins K and A1 form stable complexes preferentially with the oligodeoxypyrimidine strand (Fig. 5). Denaturing a restriction fragment of c-myc encompassing four CT-element repeats allowed specific complex formation with hnRNP protein K; whereas homologous sequence eliminated binding, inclusion of a variety of unrelated single- and double-stranded sequences either as oligonucleotides or as native or denatured restriction fragments all failed to eliminate binding of hnRNP protein K (data not shown).

hnRNP Protein K Facilitates the Activity of the CT-element—Is binding of hnRNP protein K to the CT-element biologically significant? If so then maneuvers which modify hnRNP protein K levels *in vivo* might be expected to alter the efficacy of the CT-element as a positive cis-element. hnRNP protein K was cloned behind the powerful cytomegalovirus immediate early enhancer and promoter in the sense and anti-sense orientations. The resulting plasmids were in-

FIG. 4. Reduced binding activity of permuted CT oligonucleotides to hnRNP proteins K and A1 compared with wild-type CT oligonucleotides. The denatured/renatured proteins in fractions 3 and 6 were mixed with radiolabeled CT oligonucleotides, with or without competitor oligonucleotides, as described under "Experimental Procedures." WT-CT, per-CT, and NS indicate wild-type CT, permuted-CT, and nonspecific oligonucleotides, respectively. The molar excess of competitor oligonucleotides relative to the probe is indicated. The oligonucleotides are described in Table I. Arrows indicate specific DNA-protein complexes.



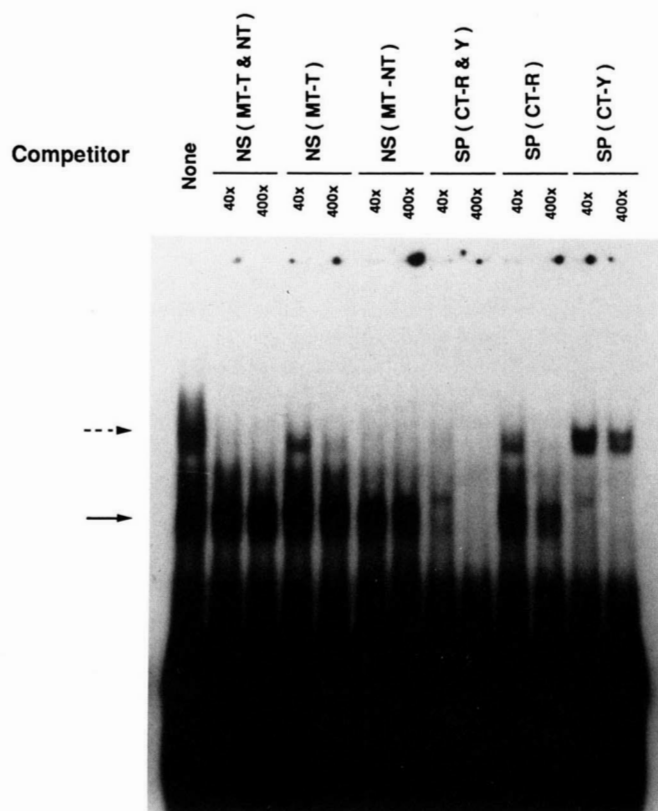


FIG. 5. Specific reduction of the CT binding activity of the affinity-purified proteins by single-stranded CT competitor oligonucleotides. The affinity-purified proteins and the radiolabeled CT oligonucleotide were mixed with or without competitor oligonucleotides, as described under "Experimental Procedures." SP and NS represent specific (wildtype CT) and nonspecific oligonucleotides, respectively. MT-T and MT-NT are the template and non-template strands of an irrelevant competitor oligonucleotide. CT-R and CT-Y indicate the purine-rich and pyrimidine-rich strands of the CT-element, respectively. CT-R and Y and MT-T and NT indicate annealed double-stranded oligonucleotides. Sequences of the oligonucleotides are described in Table I. The molar excess of competitor oligonucleotides compared with the probe is indicated. The *thick arrow* indicates specific DNA-protein complexes, while the *dotted arrow* indicates nonspecific ones.

troduced by electroporation into Hela cells along with the wild-type or mutant CT-elements driving the fos minimal promoter coupled to the chloramphenicol acetyltransferase gene as a reporter. In a dose-dependent manner, the hnRNP protein K expression vector was able to augment CT-mediated expression approximately 3-fold (Fig. 6, A and B). The hnRNP protein K anti-sense expression vector virtually abolished the positive action of the CT-element (Fig. 6, A and B). In contrast, only marginal effects of the sense and anti-sense vectors were noted on the fos promoter driven by mutant CT-elements (C) or three tandem AP1-sites (data not shown). It is important to note that all three reporter plasmids encode identical CAT mRNAs; therefore the alterations of CAT activity seen upon cotransfection with sense or anti-sense hnRNP protein K expression vectors must reflect differential sensitivity of wild-type CT, mutant CT, or AP1 upstream elements to modulation of hnRNP protein K levels. The simplest interpretation of these experiments requires that hnRNP protein K participates in the activity of the CT-element, *in vivo*.

DISCUSSION

hnRNP protein K has been identified as a component of hnRNP complexes that assemble on hnRNA concurrent with

transcription (Swanson and Dreyfuss, 1988; Piñol-Roma *et al.*, 1989). Although transcription, RNA processing, and RNA transport are distinct processes, and it would seem reasonable that regulatory mechanisms exist to coordinate these processes. For energetic and physiologic reasons, interlinks between major processes in macromolecular synthesis often serve as control points to integrate and coordinate biochemical reactions.

Is there any reason to expect that hnRNP protein K may play dual roles, one hnRNA processing and/or transport and a second in transcription? How are the biochemical properties of hnRNP protein K reconciled with these processes? Although hnRNP protein K binds to both RNA and DNA with high affinity, it possesses several features that distinguish this molecule from other well-characterized RNA-binding proteins. Foremost among these properties is the sequence-specific DNA binding activity reported here. This activity is distinct from the general nucleic acid binding properties displayed by many other hnRNP proteins and almost certainly requires a distinct mechanism for interacting with nucleic acid. hnRNP protein K lacks an RNA-binding motif present in many RNA-binding proteins (Matunis *et al.*, 1992; Bandziulis *et al.*, 1989; Kenan *et al.*, 1991).

hnRNP protein K possesses sequence-specific binding activity, interacting with both single and double-stranded DNA, which comprises a positive cis-element for the human c-myc gene both *in vivo* and *in vitro*. Although the possibility that the activity represents an *in vitro* phenomenon unrelated to the trans-factor, which drives the CT-element *in vivo*, evidence suggests that the interaction of hnRNP protein K with the c-myc promoter cannot be similarly described as an arcane artifact. First, in experiments in which reporter plasmids with wild-type CT, mutant CT, or AP1-elements driving CAT are cotransfected with sense or anti-sense hnRNP protein K expression vectors, the results are exactly as predicted for a *bona fide* specific regulatory protein. Second, the interaction displays many layers of specificity: (i) the DNA binding is sequence-specific; heterologous sequences do not bind with hnRNP protein K, and mutant CT-elements are greatly compromised in their ability to interact with hnRNP protein K. These same mutations abolish CT-element activation *in vivo*. (ii) Only hnRNP protein K (and to a lesser extent hnRNP protein A1) survive the purification scheme described under "Experimental Procedures." Although other hnRNP proteins interact tightly with single-stranded nucleic acid (Dreyfuss, 1986; Bandziulis *et al.*, 1989; Kenan *et al.*, 1991), they are not found or are greatly diminished in fractions enriched for hnRNP protein K. (iii) Only one of several isoforms of hnRNP protein K is selected by the purification scheme, indicating an additional degree of specificity in the hnRNP protein K-DNA interaction. The exact relationship of the isoform purified with the rest of the hnRNP protein K family is not known because the biochemical basis of the different isoforms of hnRNP protein K has not been fully elucidated although small differences in cDNA sequence, suggestive of alternate mRNA splicing, have been identified (Matunis *et al.*, 1992; Burd *et al.*, 1989). In addition, posttranslational modifications may occur. Identification of the differences between the sequence-specific binding form of hnRNP protein K and other forms may prove useful in defining the DNA-binding domain of this molecule. Can a molecule such as hnRNP protein K affect a gene-specific role in transcriptional regulation, or does its abundance relegate it to a more general role? Pyrimidine-rich sequences bearing some similarity to the CT-element occur in the regulatory regions of a variety of genes, indicating that hnRNP protein K is unlikely to be

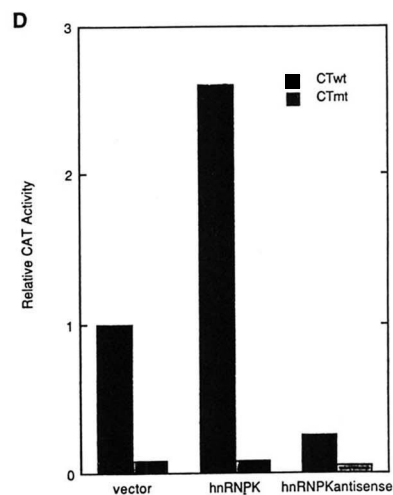
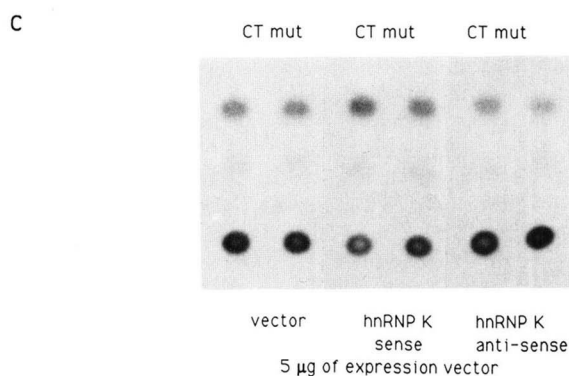
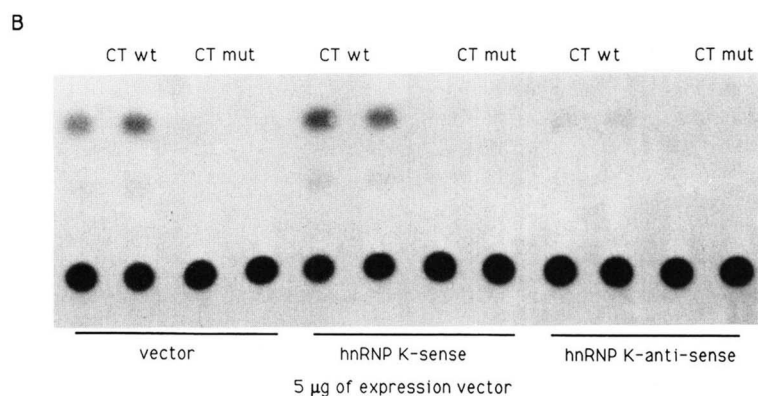
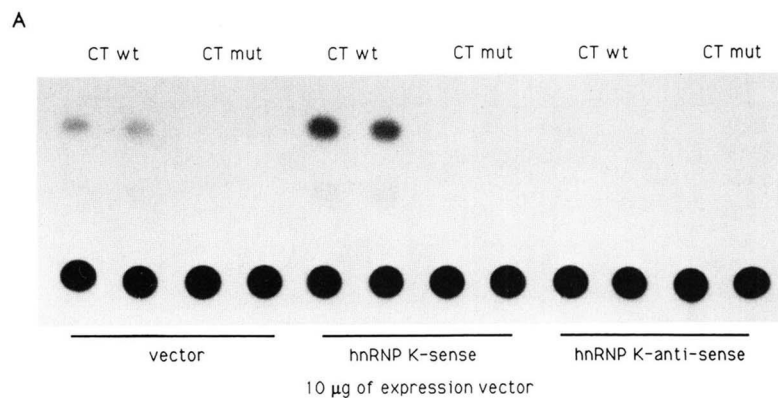


FIG. 6. hnRNP protein K facilitates CT-element mediated expression, *in vivo*. Reporter plasmids expressing CAT from the murine *c-fos* minimal promoter driven by wild-type or mutant CT-elements were cotransfected with a cytomegalovirus enhancer driving sense or anti-sense hnRNP cDNA expression; alternatively the vector alone was transfected. *A*, 10 µg of the indicated expression vector and 10 µg of the indicated CAT reporter plasmid were cotransfected by electroporation as described under "Experimental Procedures." *B*, 5 µg of the indicated expression vector and 10 µg of the indicated CAT reporter plasmid were cotransfected as above. *C*, to confirm that hnRNP protein K expression did not perturb the basal promoter activity of the CT-mutant reporter, the same extracts as shown in *B* were reassayed using 20-fold more protein; only marginal differences were noted upon cotransfection with the vector alone as well as the sense and anti-sense hnRNP protein K expression plasmids. *D*, liquid scintillation quantitation of [¹⁴C]chloramphenicol acetylation resulting from CAT activity directed by CT-wild-type or CT-mutant reporter plasmids (10 µg) cotransfected with the indicated expression vector (10 µg).

dedicated only to the control of *c-myc* (Johnson *et al.*, 1988; Gaillard *et al.*, 1988; Hoffman *et al.*, 1990; Wilkison *et al.*, 1990).

The biochemical properties of hnRNP protein K are consistent with a homeostatic mechanism that coordinates cell growth with global transcription of RNA polymerase II genes. The majority of hnRNP protein K most likely is associated nonspecifically to intranuclear nucleic acid. hnRNP protein K must partition between RNA, nonspecific DNA-binding sites, and any specific DNA-binding sites, with the distribution being determined largely by the relative equilibrium constants and the abundance of these various sites. In an actively growing cell, many tens of thousands of nascent transcripts and hnRNA molecules are each associated with multiple hnRNP complexes containing hnRNP protein K. Against this sink of transcriptionally irrelevant sites, positive cis-elements, such as the CT-element of the *c-myc* gene, must compete if hnRNP protein K is to play a relevant role. Clearly, such a competition will be shifted by the overall number of nascent transcripts and the amount of hnRNA in a cell. The more transcriptionally active a cell, the more hnRNP protein K would partition from positive cis-elements to RNA. If these cis-elements drive genes such as *c-myc*, *c-ras*, and the EGF-receptor, which facilitate growth, then this titration may result in decreased cell growth and, consequently, a lesser amount of global transcription. If the pool of nascent transcripts and hnRNA were to decrease, more hnRNP protein K would be available to interact with *c-myc* and similarly regulated genes, which would act to increase growth rate and, presumably, transcription.

The ability of a protein to interact with both RNA and DNA is not unique to hnRNP protein K. In *Xenopus laevis*, the RNA polymerase III transcription factor TFIIIA activates both 5 S rRNA transcription and binds 5 S rRNA to form a 7 S particle (Honda and Roeder, 1980; Pelham and Brown, 1980). TFIIIA is extremely abundant in oocytes, but little of this important regulatory molecule is found free. Given hnRNP protein K's affinity for nucleic acid, as for TFIIIA, it is unlikely to be present in an unbound state. Similarly, the *X. laevis* transcription factor FRG Y2 has been shown to be a major mRNA-binding protein that forms a complex with maternal mRNA and influences nuclear cytoplasmic transport of mRNA in oocytes.³ In addition, a protein with hnRNP protein-like features has been shown to interact with regulatory regions of the transferrin promoter (Brunel *et al.*, 1991). Thus, the cross-utilization of transcription factors and RNA-binding proteins may prove a general phenomenon and serve to coordinate the transcriptional and posttranscriptional processes required to generate mature RNA molecules.

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³ A. Wolfe, personal communication.

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