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

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Masato Takimoto‡§, Takeshi Tomonaga‡§, Michael Maunus||, Mark Avigan‡, Henry Krutzhof‡, Gideon Dreyfuss‡, and David Levens**

From the ‡Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 and the §Howard Hughes Medical Institute and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6148

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 \textit{in vivo} and \textit{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). Derepression 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 (Cavaleri 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 \textit{in vivo} and \textit{in vitro} has been localized to a region 100–150 bp upstream of the human c-myc promoter P1. DNase I hypersensitivity of this region (termed site III; bySiebenlist 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 homopuridymine/homopyrimidine-like segment has been suggested to assume unusual DNA conformations, including single-stranded and triplex structures. Sensitivity of this region in naked DNA, as well as an RNase-sensitive factor recognizing this element has been reported (Boles and Hogan, 1987; Cooney et al., 1988; Davis et al., 1989). Similar long homopuridymine/homopyrimidine-like sequences occur infrequently elsewhere in the genome and have

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** This work represents equal contributions from the first two authors.

‡ Present address: Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan.

§ To whom correspondence should be addressed. Tel.: 301-496-2176; Fax: 301-402-0043.
been identified in the upstream sequences of several genes (Johnson et al., 1988; Gaullier 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 immunological 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 Δ56, which has a mouse fos promoter (from −56 to +109) and CAT gene, and plasmid SP6 mso109 were kindly donated by Dr. M. Z. Gilman (Gilman et al., 1986). Synthetic complementary wild-type and mutant CT oligonucleotides (44 bp, see Table 1 for sequence description below) were cloned into HindIII site of Δ56 to make Δ56CT and Δ56CT-mut, respectively. The wild-type CT oligonucleotides were cloned in both sense and antisense orientation relative to transcriptional start site. The wild-type and mutant CT oligonucleotides (template strand) are as follows: 5'-AGCTAGCTTCCACCTCCCTCACCCACCCACACCCACACCCACACCCACACCTCCCTCTGCACGACCGTCGCACTCGACGACGACGACGAG-3' (mutated residues are underlined). The sequences of other oligonucleotides used in this study are described in Table 1. Vectors expressing hnRNP protein K mRNA or anti-sense mRNA were constructed by inserting the EcoRI fragment encoding hnRNP protein K from pHK5 (the generous gift of W. Matthew Michael) into pcDNA1/AMV (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 × 10⁶ of HeLa cells were centrifuged and resuspended in 250 µl of DMEM, containing 10% FCS in a cuvette and incubated on ice for 10 min. Plasmid DNAs (10 µg) were added and incubated on ice for an additional 10 min before electrophoresis (200 V). After electrophoresis, the cells were incubated in DMEM containing 10% FCS for 30–48 h before harvest. Extract preparations and CAT assays were performed as described (Gelb et al., 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. (1983) was modified. 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)—EMSA 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) 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-volume columns of the same buffer, and eluted stepwise of buffer D containing 0.3, 0.5, 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% (v/v) trichloroacetic acid was added to a final concentration of 10%, and then the mixture was incubated at −20°C for 30 min and microcentrifuged at 4°C for 30 min. The supernatant was removed, the pellet was rinsed twice with cold acetone, air-dried, suspended in protein sample buffer (5.5% SDS, 4 M β-mercaptoethanol, pH 6.8, 0.5% SDS, 20% glycerol, 0.006% BPB), boiled, and loaded on 10% SDS-PAGE (acylamide/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 electrobotted to a nitrocellulose filter. The filter was processed for binding assay as described (Wiederecht et al., 1988).

Denaturation/Renaturation of Purified Protein—Slices containing separated protein fractions 1-6 in the SDS-proteins 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-paired proteins were resuspended in 20 mM HEPES, pH 7.5, 12.5 mM NaCl, 1 mM EDTA, 10% glyceral, 20% glycerol, 50 mM KCl, 0.1% Nonidet P-40, and 0.6 μg 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 (NNEGE) was performed as described by O’Farrell et al. (1977) using an amphotoline 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. The proteins were purified from HeLa cell nucleus 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 carboxymethylation, were sequenced according to the protocol of Stone et al. (1989). The resulting fragments were then separated by gradient elution on an Aquapore RP300 C₈ reverse-phase column: 0.1% (v/v) trifluoroacetic acid and 0–70% acetonitrile using a Dionex A1–490 BioLC system. After the digestion and purification, the amino acid sequences of peptides were determined by Microsequencing on a Procine Instruments 2600 off-line sequencer using standard program no. 1. Phenylthiohydantoin amino acid analysis 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, 7B10, was previously described (Pihol-Roma et al., 1989). A monoclonal anti-SV40 T-antigen antibody, PAB416, was purchased from Oncogene Science, Inc. (Manhasset, NY). A monoclonal anti-SV40 T-antigen antibody, PAb416, was purchased from Oncogene Science, Inc. (Manhasset, NY).

<table>
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<tr>
<th>Antibodies</th>
<th>Description</th>
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<tr>
<td>Anti-hnRNP K</td>
<td>Monoclonal antibody, 12G4 and 3C2</td>
</tr>
<tr>
<td>Anti-A1</td>
<td>Monoclonal antibody, 7B10</td>
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<tr>
<td>Anti-SV40 T-antigen</td>
<td>Monoclonal antibody, PAB416</td>
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renatured proteins from fractions 3 and 6 and 12 μl 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 μl of the supernatants were used for EMSA to check residual DNA binding activity.

UV-cross-linking/Immunoprecipitation—DNA-binding reactions, using 0.1 μl of the affinity-purified proteins and 32P-radiolabeled CT oligonucleotides, were performed as described above, except without BPO 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 Ltd., San Gabriel, CA), then 20 μl of buffer D containing 80 mm KCl, 1 mg/ml of BSA and 0.05% Tween 20 and 25 μl 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 μl 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 homopurine/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 Δ56 (Gilman et al., 1986). The resulting plasmid, Δ56CT, was used to assess CAT expression in vitro and to program transcription in vitro, as measured with RNase protection.

Introduction of Δ56CT into HeLa cells reproducibly supported a 4–10-fold greater CAT expression than Δ56 or Δ56CT-mut, with Δ56 harboring a mutated CT-element. The CT-element stimulated expression less effectively in the reverse orientation (Fig. 1A). Using Δ56CT and Δ56 as templates for transcription by HeLa cell nuclear extracts, stimulation by the c-myc CT-element was also observed (data not shown). Thus, this polydeoxypurimidine/polydeoxypurimine-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 oligodeoxyribonucleotides 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 oligodeoxyribonucleotides 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 32P double-stranded oligonucleotides.

TABLE I

c-myc DNA sequence and DNA sequences of oligonucleotides

c-myc−151 to +100 TCCCTCCCCA CCCCCCCC ACCTCTCCCA CCCCCCCC GACCTCCTCGAGCCCCTCTCTCCCTGG. 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).

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<td>AATTTCCCTCCCAACCTTCCCAACCTTCCCA</td>
</tr>
<tr>
<td>:CT-R</td>
<td>32</td>
<td>AGCTTGGGAGGCTGAGGCTGAGGAGGAG</td>
</tr>
<tr>
<td>Permuted CT</td>
<td>32</td>
<td>CACCTCCCTCCTCCCTCCCTCCCTCC</td>
</tr>
<tr>
<td>Nonspecific</td>
<td>34</td>
<td>CATCAAGGCTTGCAGTTAACCAAG</td>
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<tr>
<td>AP-1</td>
<td>22</td>
<td>CGAGAAAATAGTAGCTCAACAG</td>
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<tr>
<td>JC</td>
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<td>CCATCTGCCTCGAGGAG</td>
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<tr>
<td>HTLV1</td>
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A homopyrimidine/homopurine-like sequence of the upstream element (CT-element) of the human c-myc gene works as a positive transcriptional element on a heterologous promoter both in vivo and forms specific DNA-protein complexes, in vitro. Wild-type CT oligonucleotides were cloned into HindIII site of Δ56 to make a plasmid Δ56CT. Mutant CT oligonucleotides were also cloned into Δ56 to make Δ56CT-mut (map not shown). A, orientation-dependent and specific augmenting activity of CT-element in CAT assay. B, EMSA identifies distinct DNA-protein complexes with specificity for the CT-element. EMSAs, using HeLa cell nuclear extracts and radiolabeled CT oligonucleotides as probe, were performed as described under “Experimental Procedures.” Numbers indicate fold molar excess of oligonucleotide competitors compared with probe. I-IV and F indicate DNA-protein complexes and F-free probe, respectively. C, competition assay using DNA restriction fragments as competitors in EMSAs. Numbers indicate fold molar excess of competitor oligonucleotides relative to the probe is indicated.

To verify that these same polypeptides possessed appropriate specific binding activity, each polypeptide visualized by Coomassie Blue staining was individually electrophoresed from the gel and renatured. EMSAs on the renatured proteins revealed that the major bands migrating at 61 and 30-34 kDa possessed abundant binding activity to the CT-element (Fig. 2B). Other gel slices did not possess renaturable CT-element-specific DNA binding activity (data not shown).

The binding specificities and properties of the affinity-purified and the electrophoretically separated, denatured, and renatured proteins were closely examined. The affinity-puri-
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Identification of Purified Polypeptides as hnRNP Proteins—Because the 61-kDa protein showed CT-specific binding properties by both Southern 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 immuno-purified hnRNP proteins or with hnRNP proteins enriched on single-stranded DNA affinity columns, a much simpler pattern was observed. Most prominent among the polypeptides 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 Coomasie 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 sufficient 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.

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

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), hnRNP 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.
protein K is known to interact with polyribocytidilyic acid probe. The complexes observed behaved as sequence-specific DNA-protein interactions by all criteria. Because hnRNP protein K is known to interact with polyribocytidilyic 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 oligodeoxypyrimidine nucleotides 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 properties 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

![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 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.](image-url)
In Vitro Binding of hnRNP Protein K to the c-myc Promoter

**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 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 vivo 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 or 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. 5. Specific reduction of the CT binding activity of the affinity-purified proteins by single-stranded CT competitor oligonucleotides.](image)

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 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.
**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).
In Vitro Binding of hnRNP Protein K to the c-myc Promoter


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2A. Wolfe, personal communication.