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Heterogeneous Nuclear Ribonucleoprotein K Is a Transcription Factor

EMIL F. MICHELOTTI, GREGORY A. MICHELOTTI, ANDREW I. ARONSOHN, AND DAVID LEVENS*

Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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The CT element is a positively acting homopyrimidine tract upstream of the c-*myc* gene to which the well-characterized transcription factor Sp1 and heterogeneous nuclear ribonucleoprotein (hnRNP) K, a less well-characterized protein associated with hnRNP complexes, have previously been shown to bind. The present work demonstrates that both of these molecules contribute to CT element-activated transcription in vitro. The pyrimidine-rich strand of the CT element both bound to hnRNP K and competitively inhibited transcription in vitro, suggesting a role for hnRNP K in activating transcription through this single-stranded sequence. Direct addition of recombinant hnRNP K to reaction mixtures programmed with templates bearing single-stranded CT elements increased specific RNA synthesis. If hnRNP K is a transcription factor, then interactions with the RNA polymerase II transcription apparatus are predicted. Affinity columns charged with recombinant hnRNP K specifically bind a component(s) necessary for transcription activation. The depleted factors were biochemically complemented by a crude TFIID phosphocellulose fraction, indicating that hnRNP K might interact with the TATA-binding protein (TBP)–TBP-associated factor complex. Coimmunoprecipitation of a complex formed in vivo between hnRNP K and epitope-tagged TBP as well as binding in vitro between recombinant proteins demonstrated a protein-protein interaction between TBP and hnRNP K. Furthermore, when the two proteins were overexpressed in vivo, transcription from a CT element-dependent reporter was synergistically activated. These data indicate that hnRNP K binds to a specific *cis* element, interacts with the RNA polymerase II transcription machinery, and stimulates transcription and thus has all of the properties of a transcription factor.

Heterogeneous nuclear RNA (hnRNA) processing and the regulation of transcription initiation have been studied to date as distinct, unlinked physiological processes. Heterogeneous nuclear ribonucleoprotein particle (hnRNP) proteins are pre-mRNA-binding proteins thought to facilitate the various stages of mRNA biogenesis such as splicing and transport to the cytoplasm (17). Although overall cellular transcription initiation frequency should be coordinately regulated with transcript-binding capacity, links between these pathways have not been elucidated.

Monoclonal antibodies to several different hnRNP proteins immunoprecipitate a similar set of >20 proteins (55). hnRNP K is among this set and is the major cellular poly(rC)-binding protein (46). Two features of hnRNP K set it apart from other hnRNP proteins. First, nucleic acid binding is not mediated by an RNA-binding consensus sequence, as with most other hnRNP proteins (18), but by three repeats of a motif termed the KH[1] (K homology) domain (61, 62). Second, direct competition studies have revealed DNA to be the preferred ligand over RNA in vitro (68). Not surprisingly, therefore, hnRNP K has been repeatedly identified as a sequence-specific DNA-binding protein (23, 35, 52, 68). We identified hnRNP K as a potential modulator of *myc* transcription because of its ability to interact with a C-rich DNA sequence, which is termed the CT element, upstream of the c-*myc* gene (64). This region consists of four imperfect direct repeats of the sequence CCCTCCCCA. A fifth repeat is separated by a 9-bp spacer, downstream of the first four repeats, and further activates transcription in vivo (15). hnRNP K binds in a sequence-specific manner to the pyrimidine-rich single strand (64) but binds to duplex DNA efficiently only if the CT elements are presented to hnRNP K embedded in negatively supercoiled DNA. This binding requires at least two nonadjacent CT units (68). Although the biological processes which generate the DNA underwinding or melting necessary for hnRNP K to bind have yet to be identified (but see reference 44), the CT element has been shown to react with single-strand-specific chemical and enzymatic probes in vivo (49a).

Deletion analysis of the c-*myc* promoter has clearly shown the CT region to be a positive element (13, 32, 45) required for transcription from the minor c-*myc* start site P1 and augmenting expression from P2. In addition to hnRNP K, several different candidates have been proposed as the factor(s) that actually mediates CT activation, including nm23 (56), NSEP-1 (40), and Sp1 (15). Data directly linking most of these factors with specific CT-mediated activation are lacking. However, hnRNP K sense or antisense expression vectors have been shown to stimulate (by approximately three- to fivefold) or repress, respectively, expression from a cotransfected CT-dependent reporter (64). Although these transient transfection experiments suggest that hnRNP K participates in gene regulation, the nature of its role remains to be defined. Most simply, hnRNP K could be a transcription factor; however, a more complex mechanism, such as hnRNP K operating via an upstream DNA element to facilitate the assembly of hnRNP complexes necessary for mRNA processing and transport, could be imagined. What then is the role of hnRNP K in elevating CT-mediated expression? Is hnRNP K a transcription factor and an hnRNP protein? A series of experiments

* Corresponding author. Mailing address: Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Building 10, Room 2N105, Bethesda, MD 20892. Phone: (301) 496-2176. Fax: (301) 402-0043.

were performed to examine the interactions of hnRNP K with DNA and the transcription machinery.

MATERIALS AND METHODS

In vitro transcription and RNase protection assays. HeLa cells were grown in suspension in spinner-modified minimal essential medium supplemented with 10% fetal calf serum and nonessential amino acids to a density of approximately 10^6/ml. Nuclear extracts were prepared as previously described (16). In vitro transcription mixtures were performed in 50-μl volumes and contained 500 μM each ribonucleoside triphosphate, 50 mM NaCl, 1 mM dithiothreitol, 1.5 mM $MgCl_2$, 20 mM Tris (pH 8.0), 2 mM spermidine, and 100 ng of each template. The amounts of nuclear extract and column eluates used in the reactions are indicated in the figure legends in micrograms of protein. The reaction mixtures were incubated at room temperature for 1.5 to 2 h, the reactions were terminated by the addition of 50 μl of 10% sodium dodecyl sulfate (SDS), and the mixtures were phenol extracted and precipitated. In vitro-synthesized RNA was hybridized with ^{32}P-labeled antisense RNA in 30 μl of 0.5 M NaCl–20 mM Tris (pH 7.5)–1 mM EDTA at 65°C for 3 h. RNA probes were made according to the specifications of the manufacturer of SP6 RNA polymerase (Promega Biotec). RNase T_1 digestion was performed by the addition of 300 μl of 0.3 M NaCl–20 mM Tris (pH 7.5)–7% formamide–1,000 U of T_1 (BRL). After 1 h, digestion mixtures were extracted with a 1:1 mixture of phenol-chloroform and then ethanol precipitated. The products were separated on 6% denaturing polyacrylamide gels. For Sp1 immunodepletion, affinity-purified rabbit polyclonal antibodies (Santa Cruz Biochemicals) were titrated out to three times the minimum amount necessary to eliminate the Sp1 shift seen in gel shift experiments. Antigen-antibody complexes were removed with an excess of protein A-Sepharose (Pharmacia) blocked with bovine serum albumin. Recombinant hnRNP K was added to in vitro transcription reaction mixture as a preincubation with an appropriately tailed template on ice for 15 min. Although the crude extract contains hnRNP K, its ability to stimulate transcription under these conditions is apparently inhibited by competing factors capable of binding the ends of linear templates as well as the sequestration of the bulk of hnRNP K in hnRNP complexes. These reaction mixtures included 200 ng of nonspecific single-stranded oligonucleotide. Footprinting units used in in vitro transcription mixtures were as defined by the manufacturer (Promega Biotec). The sequence of the nonspecific oligonucleotide used in Fig. 3 as well as the negative control for the affinity column was AATTATGCAAAGCTTCATGGTGGATC.

Clones, constructs, and templates. pΔ56 (22, 27) was cleaved with *Xba*I and *Bam*HI, filled in with DNA polymerase I large fragment (Klenow), and religated to create psΔ56. pΔ56CT3 was cloned by restricting pΔ56 with *Hin*dIII, filling in with Klenow, and ligating in the presence of the double-stranded oligonucleotide CT3 (AATTCTCCTCCCCACCTTCCCCACCCTCCCCA; nontemplate top strand shown in all cases). psΔ56CT4 was cloned by restricting pΔ56 with *Hin*dIII and by cloning the oligonucleotide AGCTAGCTCCTCCCCACCTTC CCCACCCTCCCCACCCTCCCCAG. The complementary oligonucleotide was synthesized so that after annealing of the two oligonucleotides, each end of the hybrid had *Hin*dIII sticky ends (AGCT overhang). This was also the case in the cloning of pΔ56NF1 and pΔ56Sp1; pΔ56NF1 was cloned by restricting pΔ56 with *Hin*dIII and religating in the presence of an oligonucleotide consisting of four direct repeats of the sequence AGCT4(CCTTTGGCATGCTGCCAATATG). pSp1Δ56 was cloned by cleaving pΔ56 with *Hin*dIII and cloning the oligonucleotide AGCTAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGT CC. pgst-RNP K was created by PCR amplification of an hnRNP K cDNA (pHK5, a kind gift from G. Dreyfuss) with the following two oligonucleotides: TCA GATGAATTCATATGGAAACTGAACAGCCAGA-AGAAACCTTC and TA AAGCGAATTCTAAGAAAAACTTTCCAGAATACTGCTTCAC. PCR products were restricted with *Eco*RI, and the correct gel-purified band was cloned into *Eco*RI-restricted gex-2TK. pgst-TAT was a kind gift from F. Kashanchi and J. Brady. pgst-E1A and pgst-Rb were both kind gifts from J. Mellentin. pgst-PAC1 was a kind gift from I. Ward and K. Kelly. All clones were confirmed by direct DNA sequencing.

Preparation of tailed templates. The oligonucleotide AGCTGAATTCA GATCTCCC was phosphorylated and annealed to either the wild-type pyrimidine oligonucleotide (AGTCTCCTCCCCACCTTCCCCACCCTCCCCACCCT CCCCATGGGAGATCTGAATTC) or the mutant oligonucleotide (AGTCTC GTCGACACGTTCGACACGCTCGACACGCTCGACATGGGAGATCTGA ATTC). The duplex portion of these molecules was nonspecific. Twenty micrograms of oligonucleotides was incubated with either 20 μg of pΔ56 or 20 μg of P10 (AP1 site cloned into the *Sal*I site [22]) in 200 μl of *Hin*dIII reaction buffer at 37°C for 3 h with an excess of T4 DNA ligase and *Hin*dIII and 2 mM ATP. *Hin*dIII was added periodically to recut recircularized vector. The final products were gel purified. Separation of reaction products on a 4% polyacrylamide gel after digestion of final products with *Bam*HI confirmed that tailing reactions were essentially complete (simply from the increased size of the fragment from *Bam*HI to the end of linear molecule).

Chromatography. Oligonucleotides were coupled to CNBr-Sepharose CL4B (Pharmacia) according to the manufacturer's specifications at a concentration of 0.5 mg/ml. Glutathione *S*-transferase (GST) and GST-RNP K were coupled at approximately 1 mg/ml. Recombinant protein for this purpose was obtained as follows. The GST and GST-RNP K vectors were transformed into DH5α, which was then grown to an optical density at 595 nm of 0.5. Isopropyl-β-D-thiogalactopyranoside was added to a concentration of 0.2 mM, and bacteria were induced for 2 h. Recombinant protein was then purified (63), eluted, and dialyzed overnight against 10 mM phosphate (pH 7.5)–50 mM NaCl–0.05% Tween–20% glycerol. Phosphocellulose was preequilibrated according to the specifications of the manufacturer (Whatman). Because of contamination of the 0.5 M phosphocellulose eluate with low levels of TFIID, this fraction alone mediated low levels of regulated transcription and could not be directly assayed for complementation of the flowthrough of the GST-hnRNP K column (see Fig. 6). However, after inactivation of this contaminating TFIID with a mild heat treatment (47°C, 15 min) (49), the 0.5 M eluate was transcriptionally inactive and could not restore proper in vitro transcription regulation to the flowthrough of the GST-hnRNP K column (49). The columns were run in 20% glycerol–20 mM Tris (pH 8.0)–50 μM EDTA–50 μM $ZnCl_2$–0.05% Tween; the appropriate concentrations of NaCl, urea, and guanidinium-HCl used in the column elutions are indicated in the figure legends.

In vitro protein-protein interaction assays. ^{35}S-labeled in vitro-translated TATA-binding protein (TBP) and hnRNP K were synthesized according to the manufacturer's specifications (TBP, TnT rabbit reticulocyte lysate, hnRNP K, and TnT wheat germ extract were from Promega Biotec). Ten microliters of in vitro translation mix was incubated with 1.8×10^{-10} mol of recombinant fusion protein in a 110-μl final volume containing 0.2 M NaCl in the case of ^{35}S-TBP and 0.1 M NaCl in the case of ^{35}S-hnRNP K. The reaction mixtures also included 20 mM Tris (pH 8.0) and 0.05% Tween 20. After 3 h on ice, 25 μl of a 50% (vol/vol) slurry of glutathione beads (Sigma) in incubation buffer was added, and the samples were gently mixed for 15 min at 4°C. The samples were then washed three times with 1 ml of incubation buffer, and the beads were loaded onto a Laemmli gel. Samples incubated in the presence of DNase I and RNase A also included 1 mM $MgCl_2$.

Coimmunoprecipitation. A 500-μl volume of nuclear extract (approximately 6 mg/ml) was incubated with a 20-μl bed volume of protein A beads covalently coupled to appropriate antibody at 1 mg/ml (59). Antibodies noncovalently coupled were eluted with two washes with 4 M $MgCl_2$. The beads were then washed extensively with 20 mM Tris (pH 8.0)–90 mM NaCl–0.05 mM $ZnCl_2$–0.05 mM EDTA–0.1% Tween (binding buffer). Binding proceeded overnight at 4°C, after which the beads were washed eight times with 800 μl of binding buffer. Incubations were performed in the presence of 20 μg of RNase A and DNase I per ml. Epitope-tagged TBP HeLa cells were a kind gift from A. Berk. Cells were grown and nuclear extracts were prepared as described above.

Gel shifts and Western blots (immunoblots). One-nanogram oligonucleotides phosphorylated with [γ-^{32}P]ATP (10,000 cpm) were incubated with the indicated proteins in a final volume of 7 μl containing 50 to 70 mM NaCl, 20 mM Tris (pH 8.0), 0.25 mg of bovine serum albumin per ml, and 0.02% Tween 20. Binding reactions proceeded for 30 min on ice and were loaded on preelectrophoresed 4% nondenaturing polyacrylamide gels, which were then run at 15 to 20 V/cm, dried down, and exposed to autoradiography. The sequence of the hairpin oligonucleotide used as competitor was AGCTAGCGGCTGAGTCTCCTCCCCAC CTTCCCCACCCTCCCC-ACCCTCCCCATAAGCGCCAATTGTCTCGGCG CTTATGGGGAGGGTGGGGAGGGTGGGGAAGGTGGGGAGGAGAC TCAGCCG. Western blot analysis was as previously described (58).

Transfection and chloramphenicol acetyltransferase assays. HeLa cells were cultured in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum. A total of 8×10^6 cells were resuspended in 250 μl of phosphate-buffered saline on ice for 10 min with plasmid DNA. Electroporation was performed with Cellporator (Life Technologies, Inc.) at a setting of 180 V and 1,180 mF. After electroshock, the cells were incubated in ice for an additional 10 min. Transfected cells were then added to 15 ml of medium and the mixture was incubated for 15 to 18 h before harvesting for chloramphenicol acetyltransferase assays (28).

RESULTS

Sp1 alone is not sufficient for full activation of the CT element. To identify candidate transcription factors regulating the *c-myc* gene through the CT element, electrophoretic mobility shift assays (EMSAs) were performed with a radiolabeled probe consisting of the three 5′-most CT repeats. In agreement with previous observations (15), the only specific, exclusively double-stranded DNA-protein complex present was completely supershifted by antibodies to Sp1 (Fig. 1A, lanes 1 and 2). Subsequent experiments were designed to confirm that Sp1 stimulates in vitro transcription through the CT element and to determine whether Sp1 was solely responsible for this activation.

The abilities of nuclear extracts to support CT-activated in vitro transcription were compared before and after immunodepletion of Sp1. To monitor CT-dependent activation, the 5′-most three or four repeats of the CT element, as well as an

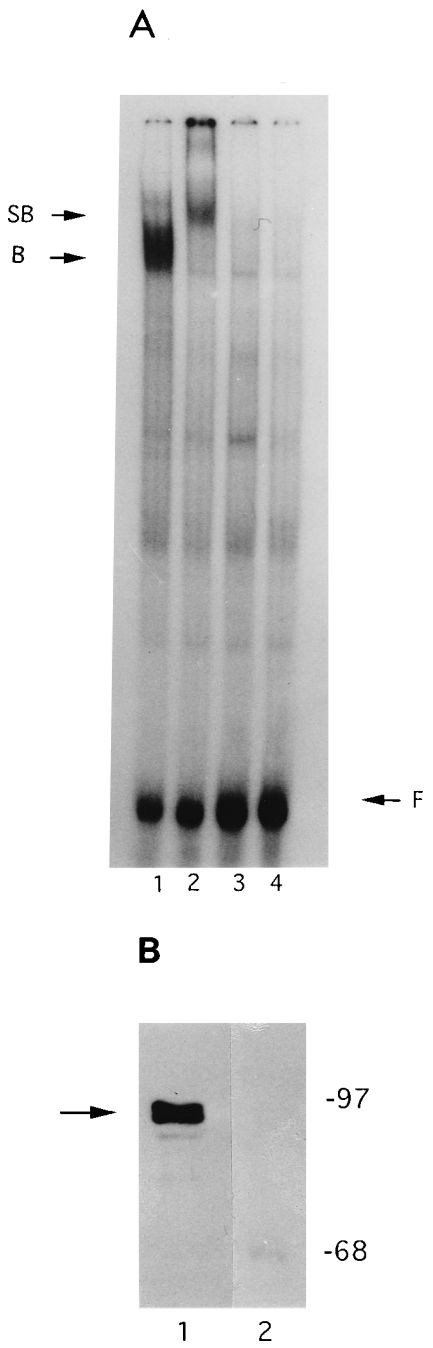

FIG. 1. Sp1 immunodepletion of a nuclear extract. (A) Nuclear extract (3 μg) was incubated with 1 ng of ^{32}P-labeled double-stranded CT4 oligonucleotide either without (lane 1) or with (lane 2) 0.25 μg of affinity-purified Sp1 antibody. The complexes were resolved by EMSAs. Extract immunodepleted of Sp1 with the antibody concentration described for lane 2 was likewise assayed either without (lane 3) or with (lane 4) an additional 0.25 μg of Sp1 antibody. F, free probe; B, Sp1-bound probe; SB, supershifted bound probe. (B) A 45-μg amount of protein from either intact nuclear extract (lane 1) or Sp1-depleted nuclear extract (lane 2) was fractionated on an SDS gel, transferred to nitrocellulose, and analyzed for Sp1 levels by Western blot analysis. Arrow, bands corresponding to Sp1.

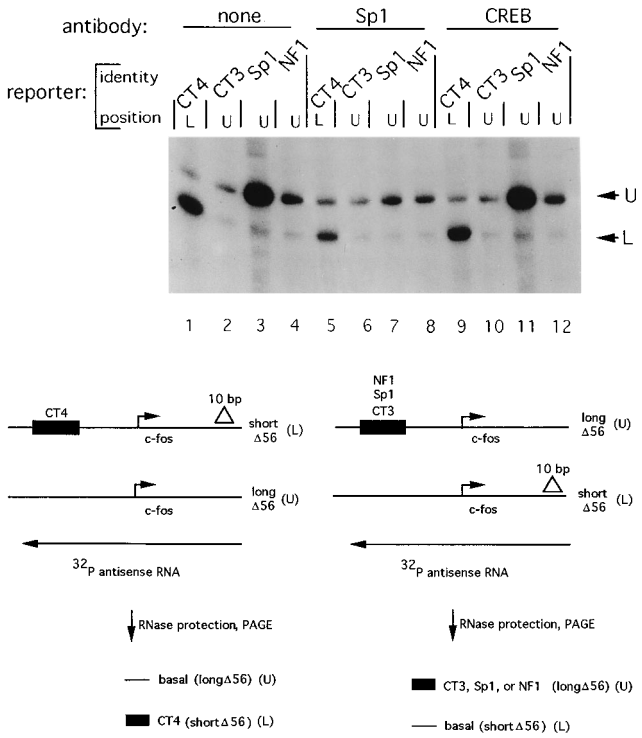

FIG. 2. Immunodepletion of Sp1 only partly eliminates CT-mediated transcription activation. The effects of Sp1 immunodepletion on in vitro transcription activation were assayed with pΔ56CT4 (lanes 1, 5, and 9), pΔ56CT3 (lanes 2, 6, and 10), pΔ56Sp1 (lanes 3, 7, and 11), or pΔ56NF1 (lanes 4, 8, and 12). Each reporter was transcribed with an equimolar amount of basal transcription internal control. Crude extract (60 μg) was used in lanes 1 to 4, Sp1-immunodepleted extract (60 μg) was used in lanes 5 to 8, and CREB-depleted extract (60 μg) was used in lanes 9 to 12. The reaction mixtures were assayed by RNase protection assays. The positions of the bands representing *cis* element-mediated transcriptional activation are indicated by U (upper) and L (lower) for each reaction (CT4-mediated transcription, lower; CT3-, Sp1-, and NF1-mediated transcription, all upper). Quantitation of the ratio of activated to basal transcription was determined by PhosphorImager analysis of each sample. Sp1 immunodepletion eliminated 57% of CT4 activation and 79% of Sp1 activation but only 18% of CT3 activation. A summary of the RNase protection procedure is shown at the bottom.

unrelated *cis* element (a tetramer of NF1/CTF sites), and a well-characterized trimer of Sp1 sites from the human immunodeficiency virus long terminal repeat (36) were each inserted upstream of the minimal mouse *fos* promoter (nucleotides −56 to +109) in pΔ56 (27). In the case of four CT repeats, the vector was a modified form of pΔ56 in which a 10-bp region of polylinker sequence immediately downstream of fos +109 was deleted (psΔ56). RNase protection assays of the transcripts directed by the deleted template produced a shorter specific product than those directed by the unmodified vector. To quantitate activation, transcription assay mixtures always included an equimolar mixture of basal promoter and *cis* element-bearing templates; the specific transcripts from these templates were distinguished by size after RNase protection of a single probe (see the bottom of Fig. 2).

The persistence of CT-mediated activated transcription despite Sp1 depletion would implicate other factors in CT regulation. To remove Sp1, nuclear extracts were incubated with affinity-purified anti-Sp1 antibodies, and then Sp1-antibody complexes were bound to protein A-Sepharose and removed centrifugally. The immunodepleted extract was devoid of Sp1 by EMSA and Western blot analyses (Fig. 1A and B). As a control, nuclear extract was similarly immunodepleted with affinity-purified anti-CREB antibodies. Activation through the human immunodeficiency virus-Sp1 sites was greatly diminished by the immunodepletion of Sp1 (Fig. 2, lanes 3 and 7).

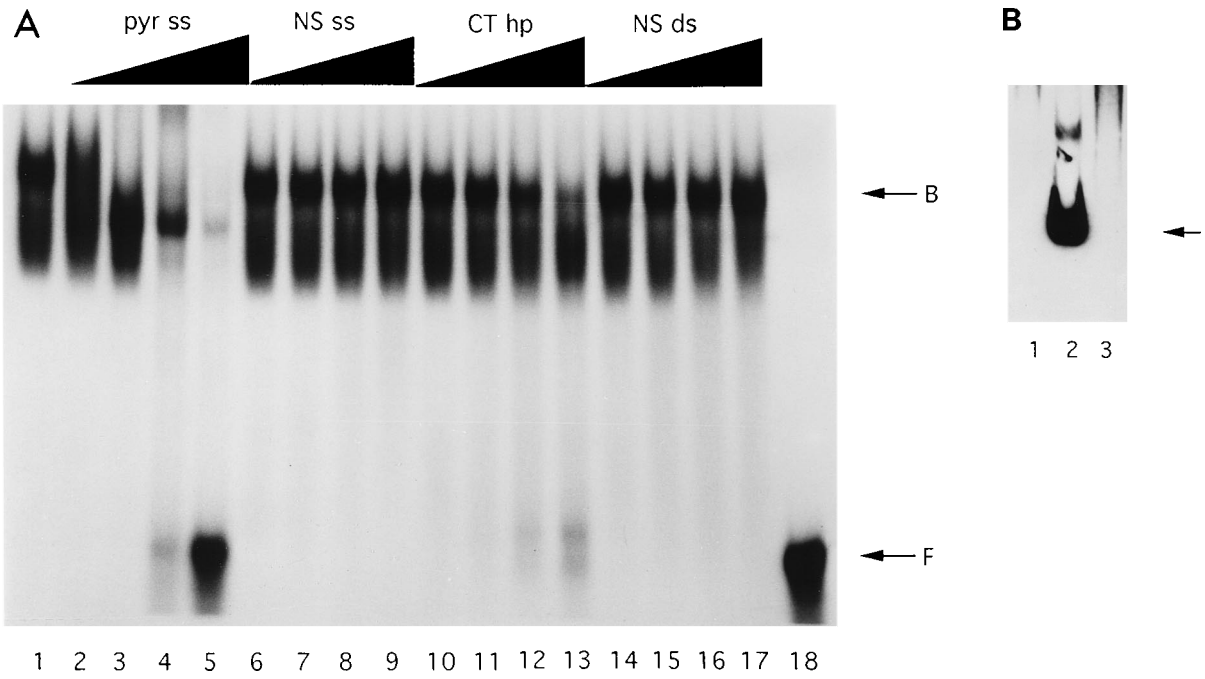

FIG. 3. hnRNP K has a higher affinity for the single-stranded pyrimidine oligonucleotide than for the double-stranded hairpin CT oligonucleotide. (A) HeLa cell nuclear extract (3 μg) incubated with 1 ng of ^{32}P-labeled single-stranded CT3 pyrimidine (pyr ss) oligonucleotide either alone (lane 1) or in the presence of 1, 5, 25, or 125 ng of single-stranded pyrimidine competitor (lanes 2 to 5) or nonspecific single-stranded (NS ss) competitor (lanes 6 to 9). To keep equimolar amounts of competitors, 2, 10, 50, and 250 ng of double-stranded hairpin (hp) CT competitor (lanes 10 to 13) or nonspecific double-stranded (NS ds) competitor (lanes 14 to 17) were used. Lane 18 shows probe alone as the control. Complexes were resolved on a 4% nondenaturing polyacrylamide gel. B, hnRNP K single-stranded shift, which was confirmed by Western blot analysis (see panel B). The two bands visualized with the pyrimidine single-stranded competitor (lanes 2 to 5) are probably due to the binding of more than one hnRNP K molecule per DNA molecule. F, free probe. (B) Extract (3 μg) incubated either alone (lane 1), with 250 ng of cold single-stranded pyrimidine CT3 competitor (lane 2), or with 500 ng of cold double-stranded CT hairpin competitor (lane 3). All samples contained 10 μg of poly(dI-dC). The complexes were resolved on a 4% nondenaturing polyacrylamide gel, transferred to nitrocellulose, and analyzed by Western blot analysis with a monoclonal antibody to hnRNP K. EMSAs with labeled single-stranded CT3 pyrimidine oligonucleotide were performed in parallel to verify which complexes contained hnRNP K (data not shown). Arrow, band reacting with hnRNP K antibody and comigrating with the major ^{32}P-labeled CT3 single-stranded pyrimidine shift.

The residual activity could be due to traces of Sp1, other Sp1 family members, or unrelated factors. As expected, NF1/CTF activity was unimpaired by Sp1 removal (Fig. 2, lanes 4 and 8), and CREB immunodepletion had no effect on any of the reporters (Fig. 2, lanes 9 to 12).

Surprisingly, depletion of Sp1 did not significantly impair transcriptional activation through CT3 (Fig. 2, lanes 2 and 6); therefore, other factors must contribute to CT3 *cis* element activity. Transcriptional activation of CT4 was partially reduced in Sp1-depleted extracts to intermediate levels (Fig. 2, lanes 1 and 5). These results indicate that although Sp1 is involved in transcription activation via the CT element, additional factors participate.

hnRNP K interacts preferentially with single-stranded CT elements. hnRNP K has been shown to transactivate the CT element in vivo and may be one of the factors, in addition to Sp1, that mediate the activity of the CT element in vitro. hnRNP K can bind to both the pyrimidine single strand and CT double-stranded DNA, suggesting that differences in binding specificity and the conformation of the *cis* element might help distinguish between utilization of hnRNP K and other factors. Therefore, experiments were performed to define better the CT element binding properties of hnRNP K. Figure 3A demonstrates that 25-fold more double-stranded than single-stranded CT DNA was required to obtain comparable competition of the complex produced with 3 μg of nuclear extract and 1 ng of pyrimidine single-stranded probe (compare Fig. 3A, lanes 2 to 5 and 10 to 13). To minimize the possibility that the low levels of competition seen with the CT double-stranded competitor were due to melting of the two strands, the oligonucleotide was synthesized as a hairpin with nonspecific nucleotides in the loop (Fig. 3A). Nonspecific single-stranded and double-stranded DNAs had no effect on the gel shift (Figure 3A, lanes 6 to 9 and 14 to 17). The presence of hnRNP K in a complex with the pyrimidine strand of the CT element as well as the lower-affinity interaction of hnRNP K with double-stranded compared with single-stranded DNA was confirmed by EMSA analysis of either extract alone, extract with single-stranded pyrimidine oligonucleotide, or extract with double-stranded CT hairpin oligonucleotide and then by Western blot analysis with a monoclonal antibody to hnRNP K (Fig. 3B). By this assay, the difference in affinities for double-stranded versus single-stranded CT DNA appeared to be even more than 25-fold, suggesting that the double-stranded complex may be unstable under EMSA conditions. From these results, we conclude that hnRNP K has at least a 25-fold greater preference for single-stranded versus double-stranded CT DNA. Therefore, excess single-stranded CT DNA would be expected to modify CT-driven transcription in vitro if hnRNP K were involved.

Sequence-specific, single-stranded binding proteins contribute to CT element activity. If hnRNP K stimulates transcription by binding one strand of the CT element, then competition with an excess of the pyrimidine oligonucleotide might reduce activation. Increasing amounts of either the pyrimidine strand or a nonspecific single-stranded oligonucleotide were added to in vitro transcription reaction mixtures programmed with a mixture of psΔ56CT4 and pΔ56 (the reporters used in

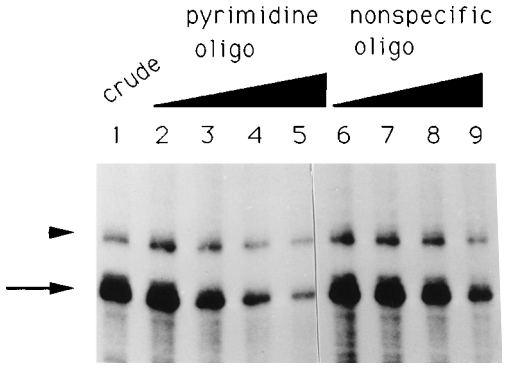

FIG. 4. Inhibition of CT-mediated transcription activation by the single-stranded pyrimidine-rich oligonucleotide (oligo). CT4+/− templates were transcribed in vitro with crude nuclear extract (60 μg [lane 1]) plus either 250 ng (lane 2), 500 ng (lane 3), 1 μg (lane 4), or 2 μg (lane 5) of pyrimidine-rich CT3 oligonucleotide or 250 ng (lane 6), 500 ng (lane 7), 1 μg (lane 8), or 2 μg (lane 9) of nonspecific oligonucleotide. Samples were assayed by RNase protection. Arrowhead, basal transcription; arrow, CT4-mediated transcription. CT-mediated activation was inhibited by 75% by the highest concentration of pyrimidine oligonucleotide but by only 10% by the highest concentration of nonspecific oligonucleotide.

Fig. 2, lanes 1, 5, and 9 [referred to hereafter as CT4+/−]) templates. CT-mediated activation (ratio of CT4+ to CT−) was reduced by 75% by the former oligonucleotide (Fig. 4, lanes 2 to 5) but only by 10% by the latter oligonucleotide (Fig. 4, lanes 6 to 9). The nonspecific inhibition of transcription seen at high oligonucleotide concentrations (Fig. 4, lanes 5 and 9) did not change the ratio of activated to basal transcription. Polynucleotides such as tRNA and poly(dI-dC) also nonspecifically inhibited transcription without changing the relative amounts of activated and basal transcription (data not shown).

The results presented above suggest that single-stranded CT element oligonucleotides can specifically titrate a factor necessary for CT element-mediated transcription activation and that anti-Sp1 antibodies can also interfere with this up-regulation. hnRNP K and Sp1 appear to be likely candidates responsible for CT element activity. With no indication of cooperativity between these proteins (49), the properties of the CT element seemed to be a composite of different populations of CT elements bound either by hnRNP K or by Sp1. The lack of synergy between Sp1 and hnRNP K was expected, considering the intrinsic incompatibility between their respective double- or single-stranded targets.

The unusual single-strand preference of hnRNP K was exploited to test whether it might activate transcription without the participation of Sp1. Linear templates bearing single-stranded tails of defined size and sequence were prepared. The tails were composed either of the pyrimidine strand of the CT element, which is competent to bind hnRNP K, or of mutated CT pyrimidine strand CT devoid of hnRNP K binding capacity. Neither template could bind Sp1. In the absence of added protein, the wild-type CT tail marginally stimulated transcription compared with the mutant tail (Fig. 5A, lanes 1 and 2). However, addition of recombinant GST-hnRNP K stimulated transcription most effectively from templates with wild-type but not mutant tails (Fig. 5A, lanes 3 to 6). Although transcription directed by the mutant tailed template was slightly stimulated, the wild-type template was reproducibly stimulated to a greater extent (3.1-fold stimulation by the wild type versus 1.2-fold stimulation by the mutant [compared with basal internal control] in this particular experiment). Significantly, addition of large quantities of GST alone had no effect on transcription. Since GST is an acidic protein with a pI and a percentage of acidic residues similar to those of hnRNP K, the observed stimulation could not be attributed simply to flooding the reactions with an acidic protein. These data indicate that the CT element can mediate transcriptional up-regulation by hnRNP K independently of input from Sp1.

The magnitude of the transcriptional up-regulation mediated by hnRNP K through the single-stranded tails, approximately threefold, approached that of the CT element-driven stimulation supported by hnRNP K in transfection experiments (64). Although the amino terminus of hnRNP K possesses transactivator function, as demonstrated in transfection experiments with a series of hnRNP K deletion mutants or with chimeras fusing segments of hnRNP K to the DNA binding domain of Gal-4, no portion of hnRNP K supported the dra-

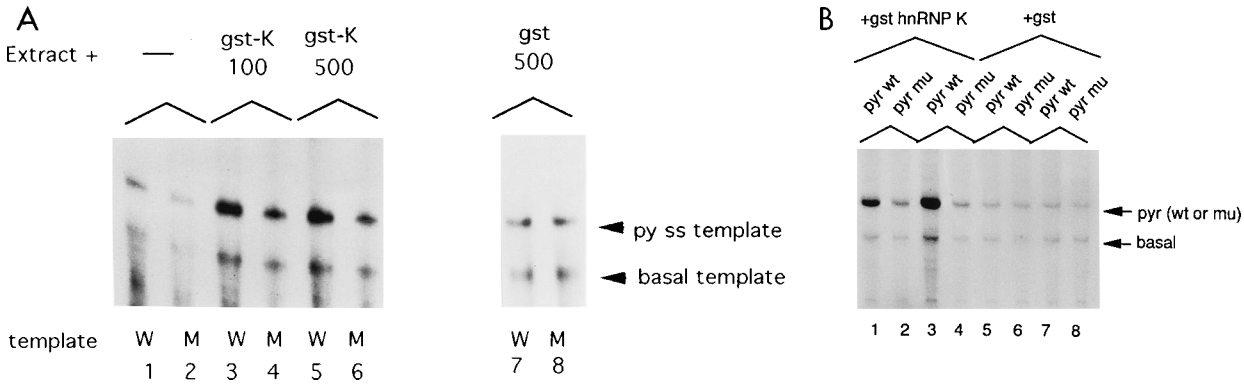

FIG. 5. hnRNP K stimulates in vitro transcription from templates containing its single-stranded pyrimidine-rich sequence. (A) Basal promoter. Templates containing either the wild-type (W) pyrimidine single-stranded (py ss) sequence (lanes 1, 3, 5, and 7) or a mutated (M) pyrimidine single-strand (lanes 2, 4, 6, and 8) were preincubated with either no protein (lanes 1 and 2), 100 ng of recombinant GST-hnRNP K (lanes 3 and 4), 500 ng of recombinant GST-hnRNP K (lanes 5 and 6), or 500 ng of GST alone (lanes 7 and 8). After addition of the remaining components and completion of the reactions, the products were analyzed by RNase protection on a 6% denaturing polyacrylamide gel. The products from the tailed template as well as a basal control template present in all reactions are indicated to the right. Transcription from the wild-type tailed template was 1.2-fold higher than that from the template with the mutant tailed oligonucleotide (relative to basal transcription [lanes 1 and 2]) but 3.1-fold higher when recombinant hnRNP K was included (lanes 3 to 6). Quantifications were done with the PhosphorImager system. (B) AP1 promoter in duplicate. Templates containing either wild-type (wt [lanes 1, 3, 5, and 7]), or mutated (mu [lanes 2, 4, 6, and 8]) single-stranded oligonucleotides tailed to an AP1-activated promoter were preincubated with either GST-hnRNP K (lanes 1 to 4) or GST alone (lanes 5 to 8) and were treated as described for panel A. Stimulation of transcription (normalized to basal promoter transcription) varied between 7- and 10-fold (lanes 1 and 3). pyr, pyrimidine.

matic transcriptional up-regulation typical of stronger transactivators such as the Gal-4 or adenovirus E1a proteins. Thus, it seemed likely that hnRNP K might act in conjunction with more traditional upstream activator proteins.

To ascertain whether hnRNP K might act concertedly with factors functioning through other upstream elements to stimulate transcription in vitro, wild-type or mutant pyrimidine strand tails were ligated to linear templates bearing three AP1 sites just upstream of the TATAA box, but otherwise identical to the tailed templates described above. The concentrations of the linear AP1 templates and the circular basal promoter internal control templates were adjusted to support equivalent levels of transcription in the absence of added recombinant protein. Recombinant hnRNP K specifically augmented transcription from the CT wild-type tailed templates by 7- to 10-fold (Fig. 5B, lanes 1 and 3) but had almost no effect when the reactions were programmed with templates ligated to the mutated pyrimidine strand oligonucleotide (Fig. 5B, lanes 2 and 4). In contrast, addition of GST did not stimulate transcription reactions, irrespectively of which oligonucleotide was ligated to the AP1-bearing template (Fig. 5B, lanes 5 to 8).

hnRNP K interacts with the basal transcription apparatus. hnRNP K might assist other factors to stimulate in vitro transcription by helping to recruit gene-specific or general components of the transcription machinery to promoters, thereby facilitating the assembly or stabilization of transcription complexes at transcription start sites. Such transcription assistance might employ hnRNP K as an architectural transcription factor which manipulates the template DNA conformation and trajectory so as to optimize the arrangement of other proteins with conventional activation domains to increase transcription. In this case, protein-protein interactions with specific or general factors are not predicted. Alternatively, hnRNP K might serve directly as an activator or coactivator by interacting with the transcription machinery.

To determine if hnRNP K could bind to components of the transcription apparatus in the absence of nucleic acid, nuclear extract was passed over either GST- or GST-hnRNP K-Sepharose columns. Prior to this affinity chromatography step, the bulk of the endogenous hnRNP K, which might otherwise compete with the immobilized recombinant protein, was removed from the nuclear extract by passage at 0.5 M NaCl, through a single-stranded CT element oligonucleotide affinity column. This treatment removed 90% of the endogenous hnRNP K; however, sufficient amounts of CT-specific and general transcription factors were unbound to allow both CT-stimulated and basal activity (data not shown; Fig. 6B, lanes 1 and 2). Since hnRNP K is an abundant protein, the residual 10% was adequate to support CT activation. This hnRNP K-diminished extract was passed once through a GST-hnRNP K-Sepharose column. The column was washed and then eluted with 0.5 M NaCl (subsequent elutions were designed to remove more tightly bound proteins). An activity required for CT-mediated transcriptional activation was indeed bound by the GST-hnRNP K column and depleted in the flowthrough (Fig. 6A, lanes 1 and 2). The 0.5 M NaCl eluate of the GST-hnRNP K column, which was inactive by itself (data not shown), biochemically complemented the depleted flowthrough (Fig. 6A, lanes 3 and 4), whereas subsequent elutions did not (Fig. 6A, lanes 5 to 8). As expected, nothing required for CT element activation bound to the control GST-Sepharose column (Fig. 6C).

An alternate means of purifying the CT element coactivators was sought in order to relate this transcriptional complementing activity to known factors. The fractionation of nuclear extract with phosphocellulose into a 0.1 M flowthrough and

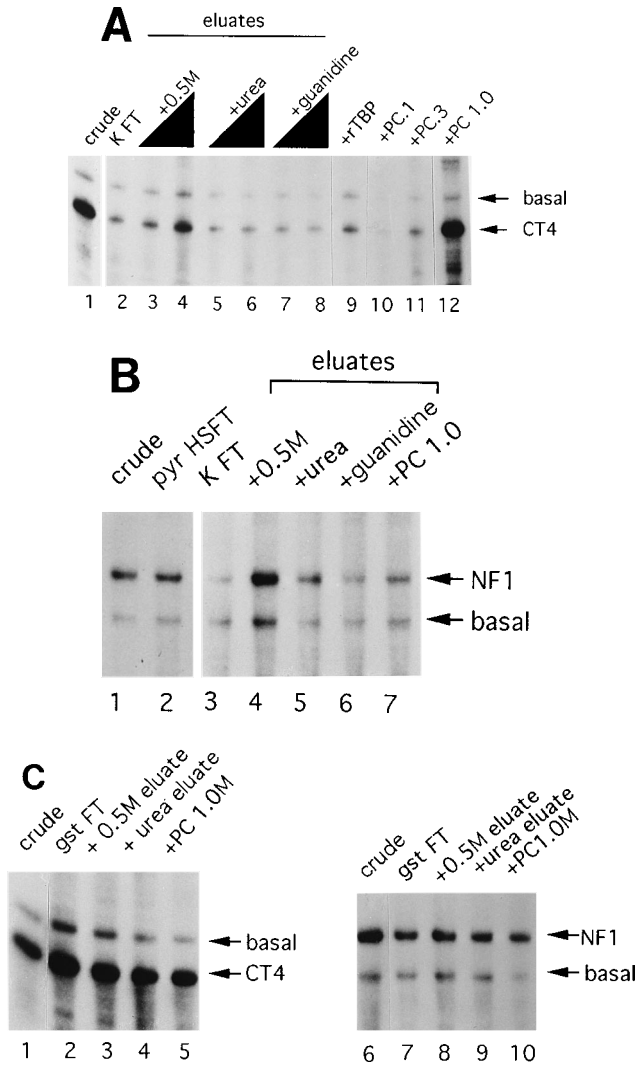

FIG. 6. hnRNP K binds to factors utilized in both CT- and NF1-mediated transactivation. Extract was fractionated over a GST-hnRNP K-Sepharose column at 0.1 M NaCl was washed, and was eluted with 0.5 M NaCl–0.5 M NaCl-4 M urea–5 M guanidine HCl. To independently derive fractions capable of restoring activity(ies) depleted by the GST-hnRNP K column, crude extract was also passed through a phosphocellulose column at 0.1 M NaCl, which was then step eluted with 0.3, 0.5, and 1.0 M NaCl. To ensure that any activity or activities removed by the GST-hnRNP K column were due to interactions with hnRNP K, extract was also passed through a GST column at 0.1 M NaCl and was step eluted with 0.5 M NaCl, 0.5 M NaCl plus 4 M urea, and 5 M guanidine HCl. (A) CT4+/− reporters were used in in vitro transcription reactions to assay 60 μg of nuclear extract (lane 1) and 60 μg of hnRNP K flowthrough (FT), either alone (lane 2) or supplemented with hnRNP K 0.5 M eluate (1.5 [lane 3] and 4.5 [lane 4] μg), hnRNP K 0.5 M NaCl plus 4 M urea eluate (0.3 [lane 5] and 0.9 [lane 6] μg), 5 M guanidine-HCl eluate (0.15 [lane 7] and 0.45 [lane 8] μg), rTBP (3 FPUs [lane 9]), phosphocellulose (PC) 0.1 M flowthrough (23 μg [lane 10]), phosphocellulose 0.3 M eluate (16.2 μg [lane 11]), or phosphocellulose 1.0 M eluate (5.4 μg [lane 12]). Bands corresponding to basal and CT4-mediated transcription are indicated to the right. (B) NF1+/− reporters were used in in vitro transcription reactions to assay crude extract (60 μg [lane 1]), pyrimidine high-salt flowthrough (HSFT; 60 μg [lane 2]), and the hnRNP K flowthrough (FT), either alone (60 μg [lane 3]) or supplemented with the hnRNP K 0.5 M eluate (4.5 μg [lane 4]), 0.5 M NaCl plus 4 M urea eluate (0.9 μg [lane 5]), 5 M guanidine-HCl eluate (0.45 μg [lane 6]), or phosphocellulose (PC) 1.0 M eluate (5.4 μg [lane 7]). Bands corresponding to basal and NF1-mediated transcription are indicated by arrows to the right. (C) CT4+/− (lanes 1 to 5) or NF1+/− (lanes 6 to 10) reporters were used to assay crude extract (60 μg [lanes 1 and 6]) and the flowthrough (FT) of the GST column either alone (60 μg [lanes 2 to 7]) or in combination with 0.5 M NaCl eluate (4.5 μg [lanes 3 and 8]), 0.5 M NaCl plus 4 M urea eluate (0.9 μg [lanes 4 and 9]), or phosphocellulose (PC) 1.0 M eluate (5.4 μg [lanes 5 and 10]). RNase-protected bands corresponding to activated and basal transcription are indicated to the right in both panels.

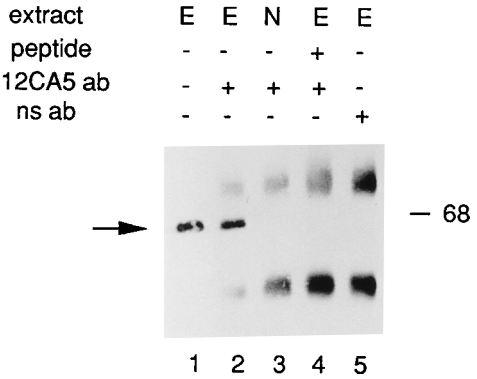

FIG. 7. hnRNP K and TBP form an in vivo complex. Nuclear extracts containing HA-tagged TBP (E) (lanes 2, 4, and 5) or normal (N) extracts (lane 3) were incubated with protein A beads bound to either the anti-HA monoclonal antibody (ab) 12CA5 (lanes 2 to 4) or nonspecific (ns) mouse immunoglobulin G (lane 5). The HA peptide either was (lane 4) or was not (lanes 1 to 3 and 5) included in the binding reaction mixture. After binding and extensive washing, the complexes were separated on an SDS-10% polyacrylamide gel, transferred to nitrocellulose, and analyzed by Western blot analysis with anti-hnRNP K antibodies. Lane 1, simply epitope-tagged extract alone (0.75 μg). Arrow, hnRNP K. Other bands are due to mouse antibody which leeched off the protein A beads and reacted with the secondary mouse antibodies during the Western blotting.

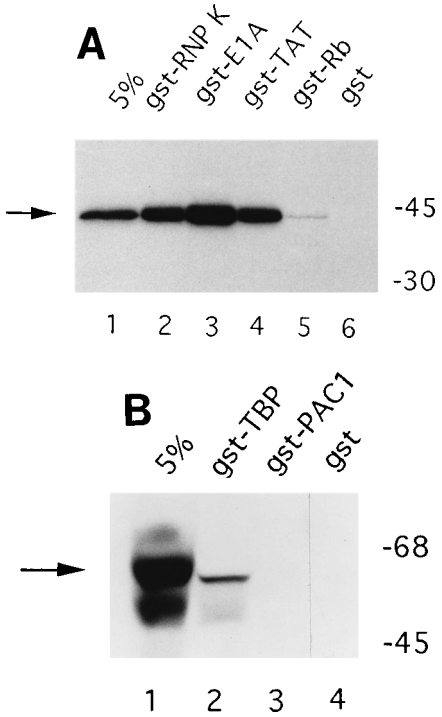

FIG. 8. hnRNP K binds directly to TBP. (A) In vitro-translated ^{35}S-TBP was incubated with either GST-hnRNP K, GST-E1A, GST-TAT, GST-Rb, or GST alone. TBP-recombinant fusion protein complexes were purified by the addition of glutathione beads followed by extensive washing. Complexes were separated on a 4 to 20% Laemmli gel and were quantitated by PhosphorImager analysis. Lane 1, 5% of the ^{35}S protein incubated with each of the recombinant fusion proteins. (B) In vitro-translated ^{35}S-RNP K was incubated with a molar excess of either GST-TBP, GST-PAC1, or GST alone. The samples were treated as described for panel A, resolved on a 10% SDS-Laemmli gel, and quantitated by PhosphorImager analysis. Lane 1, 5% of the ^{35}S protein added to each of the recombinant fusion proteins.

0.3, 0.5, and 1.0 M NaCl eluates was chosen because these fractions have provided a well-characterized starting point for the purification of basal transcription factors and coactivators (16). Surprisingly, only the phosphocellulose 1.0 M eluate, which was inactive by itself (data not shown), restored the regulation of the CT element seen with the crude nuclear extract (Fig. 6A, lane 12). Since this fraction is required for all activated RNA polymerase II transcription and has been shown to contain the TFIID complex, consisting of TBP and TBP-associated factor (TAF) complexes (20, 26, 57, 65), the flowthrough of the hnRNP K column was supplemented with recombinant TBP. However, this addition failed to restore CT regulation and only slightly stimulated general transcription (Fig. 6A, lane 9), suggesting that the hnRNP K column removed either the larger TBP-TAF complex or an unrelated factor(s) in the TFIID fraction (47). Other phosphocellulose fractions did not complement (Fig. 6A, lanes 10 and 11). Since the flowthrough of the hnRNP K column still supported basal transcription, a form of TBP (an obligatory component for almost all RNA polymerase II promoter activity) was not removed by affinity chromatography. We speculate that B-TFIID cannot bind to hnRNP K and hence was present in the flowthrough. B-TFIID is a TBP-containing complex, separated from TFIID on phosphocellulose, which sustains basal but not activated transcription by RNA polymerase II (48, 67). This inability of B-TFIID to associate with hnRNP K could be due to the absence or masking of the sites necessary to interact with hnRNP K.

If the hnRNP K column removed a general transcription component, for example a TBP-TAF complex, then activation mediated by many different *cis* elements should be impaired; the inability of the flowthrough of the GST-hnRNP K column to support NF1/CTF activation supported this idea (Fig. 6B, lanes 1 to 3). Addition of the 0.5 M NaCl eluate of the GST-hnRNP K column to the flowthrough also restored NF1/CTF-mediated activation (Fig. 6B, lane 4). It is noteworthy that the addition of the phosphocellulose 1.0 M eluate (crude TFIID) to the hnRNP K column flowthrough restored only CT-mediated transcription but not NF1/CTF-mediated transcription (Fig. 6B, lane 7). Thus, only some of the components necessary to coactivate NF1/CTF also bind to hnRNP K and are likely to participate in CT activation. Other components that are required by NF1/CTF do not participate in CT activation and chromatograph differently from the crude TFIID on phosphocellulose (see Discussion).

hnRNP K can interact with TFIID in vivo. Was the component which activated transcription from the CT element and which was retained on the hnRNP K affinity column a TBP-TAF complex or was it an unrelated factor in the crude TFIID fraction interacting with hnRNP K? To determine if hnRNP K and TBP associate in vivo, complexes containing epitope-tagged TBP were immunoprecipitated with anti-hemagglutinin (HA) or control antibodies and were analyzed by immunoblotting with anti-hnRNP K antibodies. This approach has been used to establish the association of TAFs with TBP (71). hnRNP K was clearly coprecipitated with the epitope-tagged TBP (Fig. 7). This association of hnRNP K with TBP was resistant to both DNase and RNase and hence cannot be attributed to nonspecific nucleic acid bridging. The converse experiment demonstrated that hnRNP K antibodies also immunoprecipitated TBP, although less efficiently (data not shown).

hnRNP K can interact with TBP in vitro. Because hnRNP K was coimmunoprecipated with TBP and the phosphocellulose TFIID fraction complemented the activity depleted by the hnRNP K column, the idea that hnRNP K might interact directly with a TBP-TAF complex seemed plausible. If this

hypothesis were correct, then hnRNP K could directly contact TBP, one or more TAFs, or a combination thereof. To test whether TBP and hnRNP K interact directly, ^{35}S-labeled TBP was translated in vitro and incubated in 0.2 M NaCl with an excess of either GST or GST-hnRNP K. Bound complexes were purified by the addition of glutathione beads and then by extensive washing. Thirty-five-fold more ^{35}S-TBP bound to GST-hnRNP K than to GST alone (Fig. 8A, lanes 2 and 6). The affinity of this interaction was therefore comparable to those of GST-E1A and GST-TAT with TBP. These two proteins have previously been shown to interact with TBP (38, 43) and in this experiment bound 73- and 40-fold more ^{35}S-TBP, respectively, than did GST alone (Fig. 8A, lanes 3, 4, and 6). GST-Rb was included as a negative control and bound only 3-fold more TBP than did GST (Fig. 8A, lane 5). The specificity of the hnRNP K-TBP interactions was confirmed by the reciprocal experiment in which in vitro-translated ^{35}S-hnRNP K bound to GST-TBP with 10-fold greater affinity than to GST alone or to GST-PAC1 (a nuclear tyrosine phosphatase) as controls (Fig. 8B). The interaction between hnRNP K and TBP was directly due to protein-protein recognition and was not a consequence of nucleic acid bridging between otherwise noninteracting nucleic acid binding proteins, since the inclusion of 50 μg of RNase A and DNase I per ml in the binding mixture did not alter these results (data not shown).

Transiently transfected TBP and hnRNP K costimulate CT element transcription in vivo. What are the functional consequences of the in vivo interaction between TBP and hnRNP K? hnRNP K and TBP are each present in several multicomponent complexes in vivo. Since few monomers of either molecule are likely to be available to associate in most cells, overexpression of both proteins together should enhance their direct or indirect association, thereby facilitating expression from a CT element-driven reporter plasmid. To test whether TBP and hnRNP K might synergize in vivo, both proteins were expressed singly or together, holding the total amount of DNA constant by including the appropriate vector. Conditions were selected to minimize the ability of hnRNP K or TBP, individually, to alter reporter expression. Whereas TBP and hnRNP K separately had marginal to no ability to augment the expression of a CT element-TATA box-driven CAT gene under the conditions employed, clear synergy was apparent when the proteins were coexpressed (Fig. 9A). Similar enhancement was not seen when the CT elements were mutated, attesting to the role of hnRNP K as a sequence-specific, DNA-binding transactivator (Fig. 9B). The identities of other transcriptional activators and coactivators influencing the TBP/hnRNP K transcription stimulation remain to be elucidated.

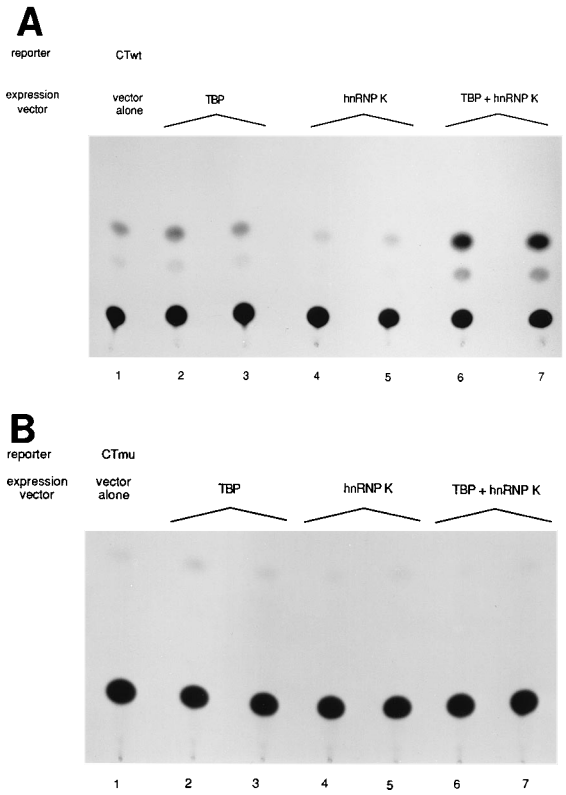

FIG. 9. hnRNP K and TBP synergize in vivo. HeLa cells were cotransfected with either vector alone, TBP expression vector, hnRNP K expression vector, or a combination of the two. Total DNA concentration was held constant at 28 mg in all cases by using the parental plasmid pcDNAI. A 10-μg amount of either CT wild-type (wt) reporter (A) or CT mutant (mu) reporter (B) was used in all cases. At 18 h posttransfection, cell extracts were prepared and chloramphenicol acetyltransferase assays were performed as described in Materials and Methods. TBP expression had no effect on expression; under these conditions, hnRNP K repressed expression to 60% of the levels of vector alone; TBP and hnRNP K together stimulated expression 2.9-fold above the level of vector alone and 4.8-fold above the level of hnRNP K alone. This is comparable to the synergy seen between TBP and other well-characterized TBP-binding proteins (7, 31, 39, 70).

DISCUSSION

hnRNP K is a transcription factor. Several lines of evidence indicate that hnRNP K functions as a transcription factor. (i) Addition of recombinant hnRNP K stimulates transcription in vitro through wild-type but not mutant single-stranded CT elements. This stimulation is augmented in the presence of other activating *cis* elements. (ii) Transfection of plasmids encoding hnRNP K mRNA in the sense or antisense orientation stimulates or represses, respectively, expression from a CT-dependent reporter (64). (iii) The single-stranded DNA-binding site of hnRNP K inhibits CT-mediated transcription. The pyrimidine strand of the CT element blocks in vitro transcription either when the element is added to reaction mixtures as a competitor or when it is used to deplete extracts of specific factors when immobilized on a column (data not shown). The amount of pyrimidine strand competitor required to inhibit transcription parallels levels that are necessary to eliminate hnRNP K binding to probe by EMSAs; this amount exceeds the level employed by others in an earlier study that found no effect of the pyrimidine oligonucleotide on c-*myc* in vitro transcription (12). (iv) hnRNP K immobilized on an affinity column interacts tightly with components of the transcription machinery. Addition of the high-salt eluate from this column restores regulation to the depleted flowthrough. (v) The flowthrough of the hnRNP K column is complemented either by the high-salt eluate of this column or with the phosphocellulose TFIID fraction previously shown to contain coactivators required for proper RNA polymerase II regulation (20, 64). (vi) hnRNP K is present in vivo in a complex with TBP. (vii) This in vivo association is functional, since overexpression of TBP and hnRNP K synergistically activates a wild-type but not mutated CT-dependent reporter. (viii) The idea that hnRNP K might function through a component of the TFIID complex is supported by the demonstration of a direct interaction with TBP. The affinity of the hnRNP K-TBP interaction is similar to those of previously characterized E1A-TBP (43) and TAT-TBP (38) interactions.

The interaction between TBP and hnRNP K is considerably weaker than is the interaction between TBP and the TAFs. In

general, upstream regulators of transcription interact weakly with the TAF-TBP complex. For multiple activators to coregulate transcription initiation through recruitment of the TBP-TAF complex, no single interaction should be too strong or it will act alone. If the extent of transcription activation by hnRNP K (approximately 7- to 10-fold) derives entirely from the contribution of its free energy of binding to the stabilization of a preinitiation complex, then only 1 to 1.5 kcal (ca. 4 to 8.4 kJ; the energy of a single hydrogen bond or hydrophobic interaction) is required. Thus, multipoint attachment through weak interactions integrates the input of several factors. If an abundant protein, such as hnRNP K, interacted too vigorously with the basal machinery, then it might prove a dangerous regulator of c-*myc*, where small fluctuations in transcription may have dramatic physiological consequences (60).

A growing list of proteins has been shown to bind to c-*myc* promoter sequences in single-stranded conformation. In addition to hnRNP K, these proteins include pur (−1700) (3), MSSP-1 (−2000) (51), far upstream binding protein (FBP) (−1525) (19), and NSEP-1 (−130) (40). Furthermore, the FBP binding region was shown to have single-stranded character in vivo, as assessed by $KMnO_4$ reactivity (19). Previous examinations of single-stranded DNA in vivo have been limited to the bubble caused by bound RNA polymerase II localized either at the start site because of an elongation arrest found in some genes (24, 41) or over a broader region downstream of the initiation site (50). This raises the question of whether regulation of the c-*myc* gene involves unique regulatory processes. Since the promoters of several other genes have been shown to contain binding sites for sequence-specific single-stranded DNA-binding proteins (1, 8, 25), homopyrimidine sequences (34), and regions of in vivo sensitivity to the single-strand-specific nuclease S1 (6, 8, 9, 10, 42), modulation of transcription initiation by single-stranded DNA-binding factors may be a more general phenomenon. Another KH family member, FBP, is a sequence-specific single-stranded DNA-binding protein that transactivates an element of the c-*myc* gene (2, 19).

Several factors may cooperatively or competitively mediate CT element activity. Is hnRNP K solely responsible for the activity of the CT element? In fact, hnRNP K may be only one in an ensemble of factors orchestrating CT element activity. The interaction of single-stranded binding factors with the CT element does not preclude a role for double-stranded binding proteins in a subpopulation of cells. For example, the data presented here indicate that Sp1 can account for 20 to 60% of the in vitro activity of the CT element. The possibility of an unusual role for Sp1 should not be overlooked; we have noted a low-affinity interaction of Sp1 with the purine strand of the CT element (49), and because Sp1 in crude extracts is part of a 500-kDa complex (5), its interaction with the CT element might require protein-protein interaction. Most likely, the relative abilities of hnRNP K and Sp1 to transactivate the CT element will be determined by interactions with factors bound to other *cis* elements of the c-*myc* gene.

If hnRNP K is an abundant hnRNP protein, can it also be a transcription factor? Since hnRNP K is found in hnRNPs prepared by a variety of methods (17), its assignment as an hnRNP protein is justified. Although most transcription factors are relatively rare proteins, hnRNP K is an abundant protein. Does this abundance mitigate a transcriptional regulatory role? Hardly; *Xenopus* TFIIIA, for example, is a well-characterized zinc finger protein unquestionably essential for transcription of the 5S rRNA gene (21) and occurring in a 7S complex with 5S rRNA (53). In stage III oocytes, TFIIIA constitutes approximately 10% of the cellular protein and is present in 10^{12} molecules per cell, mostly bound to RNA (4, 54). Similarly, if hnRNP K is to operate against a sink of binding sites on RNA as well as nonspecific sites on DNA, it must be an abundant protein.

If it is a transcription factor, might hnRNP K also be coupled to still other cellular processes through protein-protein interaction? Along these lines, hnRNP K has recently surfaced in two unexpected contexts. First, although hnRNP K is present throughout the cell cycle, one particular epitope is exposed only in G_1 and early S. In simian virus 40-transformed cells, however, this epitope is accessible throughout the cell cycle (14). Masking of this epitope, therefore, correlates with inhibition of cellular proliferation. Second, hnRNP K has been shown to bind tightly to the SH3 domain of c-*src* in vitro (66, 69) and to bind the SH3 domain of the *vav* protooncogene (33), both in vivo and in vitro. Considering the protein and nucleic acid contacts of hnRNP K, it appears to be more than an architectural component of hnRNP complexes.

hnRNP K interacts with a subpopulation of the basal machinery. Two points regarding the depletion of factors required for both CT and NF1 activation by the recombinant hnRNP K column merit discussion. First, since TBP is required for basal as well as activated transcription and since hnRNP K directly interacts with TBP, why then is not all transcription eliminated in the hnRNP K column flowthrough? In fact, we find residual basal transcription even after three consecutive batch absorptions of extract to immobilized hnRNP K (49). The most likely explanation is that in a subpopulation of TBP complexes, the region of TBP bound by hnRNP K is masked. These complexes are, therefore, refractory to depletion by hnRNP K. The heterogeneity of TBP complexes is supported by the existence of B-TFIID as well as by nonstoichiometric levels of some TAFs compared with TBP after immunoprecipitation of the TFIID fraction with anti-TBP antibodies (65). It should be noted that in addition to TBP-TAF complexes, additional components of the TFIID fraction that contribute to transcription activation have been characterized elsewhere (47). Additional interactions between hnRNP K and any of these factors cannot be excluded. Second, why is NF1 activation not restored to the hnRNP K column flowthrough by addition of the TFIID fraction, as is CT activation? The observation that NF1 activation can be partly restored to the flowthrough of the hnRNP K column by complementation with the phosphocellulose 0.3 M eluate (49) suggests that some of the coactivators necessary for NF1 transcription activation are biochemically separable from those mediating CT activation. The ability of B-TFIID to support activation mediated by a proline-rich activation domain such as that found in CTF-1 has not been evaluated (67).

Why single-stranded elements? What advantages are there to regulatory sequences being in a single-stranded conformation? First, single-stranded DNA possesses far greater torsional flexibility as well as modestly greater flexibility to bending than does B-form DNA (37). Thus, the introduction of a single-stranded bubble between the initiation site and a DNA-bound *trans* factor could reduce or eliminate potential barriers to upstream (or downstream) element-promoter interactions by facilitating DNA bending, thereby eliminating the need for correct phasing and mitigating against unfavorable DNA trajectories imposed by other factors. In this manner, the relative influences of different *cis* elements could be regulated. Another level of regulation would relate to the ability of single-stranded DNA upstream regions to accommodate the topological stress necessarily generated during transcription of constrained templates; for each helical turn unpaired, one negative supercoil is eliminated. If an unpaired *cis* element can bind a constellation of factors different from that of the same sequence in B-form DNA, then a single sequence could confer

two entirely different modes of regulation governed by the conformation.

The cooperative binding of proteins to tandemly repeated sequences, such as the CT element, is a well-described mechanism to render a gene sensitive to small fluctuations in factor concentration. With the additional cooperativity of binding potentially provided by interactions between factors bound to each of the single strands of the CT element, modulation of c-*myc* transcription could be made very sensitive to alterations in the levels of hnRNP K and any associated proteins. This could be extremely important for a gene such as c-*myc*, since decreases of as little as twofold have been shown to lengthen the G_1 phase of the cell cycle and decrease cell division rates (60).

What are the implications of the apparent multiple binding activities of hnRNP K? Clearly, although hnRNA processing, signal transduction, and transcription are separate processes, their regulation must be coordinated. Perhaps this is accomplished through the utilization of factors such as hnRNP K that modulate all of these processes, but at limiting concentrations. For example, excess hnRNA would cause a sequestration of hnRNP K in hnRNP complexes with a concomitant decrease in the transcription initiation rate of genes with hnRNP K binding sites. Since polypyrimidine tracts are found in the promoter regions of several growth control genes, a decrease in the pool of hnRNP K available for transcription could be expected to have global effects. It should be emphasized that the molecular mechanisms coordinating the macromolecular processes of RNA transcription, binding, splicing, transport, and translation have not yet been elucidated in eukaryotic systems. The characterization of feedback loops such as the negative effect of excess translationally competent ribosomes on rRNA transcription in *Escherichia coli* (11, 29, 30) predicts the existence of similar regulatory networks in eukaryotes.

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