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Activating transcription from single stranded DNA

(heterogeneous nuclear ribonucleoprotein K/CT element/c-*myc*/DNA supercoiling/gene regulation)

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ABSTRACT **Sequence specific regulators of eukaryotic gene expression, axiomatically, act through double stranded DNA targets. Proteins that recognize DNA cis-elements as single strands but for which compelling evidence has been lacking to indicate *in vivo* involvement in transcription are orphaned in this scheme. We sought to determine whether sequence specific single strand binding proteins can find their cognate elements and modify transcription *in vivo* by studying heterogeneous nuclear ribonucleoprotein K (hnRNP K), which binds the single stranded sequence (CCCTCCCCA; CT-element) of the human c-*myc* gene *in vitro*. To monitor its DNA binding *in vivo*, the ability of hnRNP K to activate a reporter gene was amplified by fusion with the VP16 transactivation domain. This chimeric protein was found to transactivate circular but not linear CT-element driven reporters, suggesting that hnRNP K recognizes a single strand region generated by negative supercoiling in circular plasmid. When CT-elements were engineered to overlap with lexA operators, addition of lexA protein, either *in vivo* or *in vitro*, abrogated hnRNP K binding most likely by preventing single strand formation. These results not only reveal hnRNP K to be a single strand DNA binding protein *in vivo*, but demonstrate how a segment of DNA may modify the transcriptional activity of an adjacent gene through the interconversion of duplex and single strands.**

Most gene specific regulation is conferred through the action of sequence specific DNA-binding transcription factors and their cofactors. Although most proteins studied until now recognize double stranded DNA cis-elements, single stranded regulatory sequences have been proposed based on theoretical considerations (1) and indications of single strandedness have been detected with chemical and enzymatic probes in or near the regulatory regions of a variety of genes (2–4). Despite identification of a growing number of proteins that bind sequence specifically to single stranded DNA *in vitro* (5–13), the biochemical basis or biological relevance of these proteins to the putative melted sites has not been determined. One such protein is heterogeneous nuclear ribonucleoprotein K (hnRNP K), which binds to a sequence found upstream of the human c-*myc* gene (14). Binding of hnRNP K is single strand specific and requires at least two nonadjacent repeats of a cytidine-rich nonanucleotide sequence, designated the CT-element (15). Originally identified as a component of hnRNP particles (16, 17), hnRNP K, belying its name, in fact binds more tightly to DNA than to RNA (15). Expression of reporter genes with upstream CT-elements is increased modestly (3- to 10-fold) both *in vivo* and *in vitro* by hnRNP K (15, 18). As hnRNP K also interacts biochemically with *src* (19) and TATA-binding protein (18), it may prove to be a multifunctional regulatory protein. This study uses hnRNP K as a prototype to demonstrate gene activation through single stranded cis-elements *in vivo* and reveals that the transition between single and double strands provides a regulatory device that allows a single DNA segment to bind different factors and confer alternative properties onto a nearby promoter.

MATERIALS AND METHODS

Plasmid Construction. G4/VP16 was created by inserting the transactivation domain of VP16(409–490) (the kind gift of T. Kristie, National Institute of Allergy and Infectious Diseases) between the *Bam*HI and *Xba*I sites of pSG424, a vector expressing GAL4(1–147) (20). G4/K/VP16 was created by inserting hnRNP K cDNA(15) into the *Eco*RI site of G4/VP16. The reporter plasmid (GAL4)5/E1b contained the adenovirus E1bTATA promoter driven by five GAL4 sites coupled to the chloramphenicol acetyltransferase (CAT) gene as described (21). CT4, NF1, or CT-lexA-CT/E1b was created by replacing the GAL4 sites with: '5-AGCTAGCTCCTCCCCACCTTCCCCACCCTCCCCACCCTCCCCA; '5-GGCCGCCCTTTGGCATGCTGCCAATATGCCTTTGGCATGCTGCCAATATGCATATTGGCAGCATGCCAAAGGCATATTGGCAGCATGCCAAAGGT; or '5-AGCTTCCCTCCCCACTGTATGTACATACAGCTGTATGTACATACAGCCCTCCCCAG (template strand), respectively. The lexA expression plasmid was created by inserting lexA(1–202) from pBXL1 (the kind gift of Vicky Seyfert) into *Hin*dIII-*Eco*RI site of pcDNAI/Amp (Invitrogen), National Cancer Institute.

Protein Expression. Recombinant proteins, glutathione *S*-transferase (GST) and GST-hnRNP K, were purified from extracts of *Escherichia coli* transformed with the pGEX-2TK and pGEX-hnRNP K by glutathione-agarose affinity chromatography. Fusion proteins were eluted with 10 mM glutathione and checked for purity, correct size, and concentration with SDS/PAGE. Recombinant lexA protein was kindly donated by D. P. Shepley and J. Little (both from University of Arizona).

Cell Culture, Transfection, and CAT Assays. HeLa and COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. HeLa (7×10^6 cells) and COS (5×10^6 cells) cells were resuspended in 250 μl of either phosphate-buffered saline or DMEM, respectively, and incubated on ice for 10 min with plasmid DNA. Electroporation was performed with a Cell-porator (Life Technologies) at 1180 μF at 180 V for HeLa cells and 230 V for COS cells; after electroshock, the cells were incubated on ice for an additional 10 min. Transfected cells were harvested at the indicated time for CAT assays.

Potassium Permanganate ($KMnO_4$) Treatment and Ligation-Mediated Polymerase Chain Reaction (LM-PCR). $KMnO_4$ treatment after LM-PCR was performed as described (22, 23). Briefly, plasmid was incubated with the appropriate

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Abbreviations: hnRNP K, heterogeneous nuclear ribonucleoprotein K; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; LM-PCR, ligation-mediated polymerase chain reaction.

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recombinant protein in 60 μl of binding buffer [20 mM Hepes, pH 8.0/5% glycerol/1 mg/ml bovine serum albumin (BSA)/1 mM DTT/10 mM NaCl] for 15 min at room temperature. $KMnO_4$ was added for 30 sec on ice to a final concentration of 25 mM and reaction was stopped by addition of an equal volume of stop buffer (50 mM EDTA/0.4 M 2-mercaptoethanol). Modified DNA was cleaved in 10% piperidine at 90°C for 30 min and purified DNA was resuspended in distilled water and 100 pg were used in LM-PCR reactions as described (24). The primer sets used for LM-PCR are: 5′-GCTTCCTTAGCTCCTGAAAAT; 5′-GAAAATCTCGCCAAGCTGATGAATTCG; and 5′-TCTCGCCAAGCTGATGAATTCGAGCTCGGTA.

Electrophoretic Mobility-Shift Assay. The indicated amount of recombinant proteins and 1 ng of labeled probe were incubated in 15 μl of binding buffer {20 mM Hepes, pH 8.0/5% glycerol/1 mg/ml BSA/1 mM DTT/20 mM NaCl/5 μg/ml poly[d(I-C)]} for 20 min. at room temperature and the protein-DNA complexes were resolved by electrophoresis on 4% acrylamide gel. The probe is '5-AGCTTCCCTCCCCACTGTATGTACATACAGCTGTATGTACATACAGCCCTCCCCAGTCGA (top strand); and '5-TCGACTCTACTCATCTGTATGTACATACAGCTGTATGTACATACAGACTACTCAGAAGCT (bottom strand).

RESULTS

Converting hnRNP K from a weak into a strong transactivator would expand the dynamic range of expression stimulated on binding to CT-elements *in vivo*. Therefore the powerful VP16 transactivation domain (VP16 AD) was fused directly to the C terminus of hnRNP K (Fig. 1*A*). If this fusion protein bound specifically to the CT-element *in vivo*, then it should prove to be a powerful transactivator through this cis-element. However, if hnRNP K could not bind to CT-elements, then this chimeric molecule should not transactivate a CT-driven reporter. To verify the integrity of the activation domain in this fusion of hnRNP K with the VP16 AD, the well-characterized GAL4 DNA binding domain was transferred to the N terminus of hnRNP K, thus yielding a tripartite protein (G4/K/VP16) predicted to activate gene expression either through duplex GAL4 upstream activator sequences or CT-elements, if single stranded. Three reporter plasmids encoding identical transcribed sequences, but differing only in the upstream cis-elements used to activate CAT gene transcription from the adenovirus E1b promoter, were used to monitor transactivation by G4/K/VP16, by G4/VP16, or by endogenous transcription factors. In HeLa cells, G4/K/VP16 activated expression from four tandem CT-elements (Fig. 1*B*, lane 2), but G4/VP16 did not (lane 1). Transfected alone, the CT4/E1b reporter was minimally active in these cells. Expression of the identical CAT gene, driven by endogenous NF1 through the NF1 site in NF1/E1b, was equivalent when either G4/VP16 or G4/K/VP16 was cotransfected (lanes 3 and 4), thus proving that activation of CT4/E1b by the latter was not due to enhanced posttranscriptional processing or increased mRNA stability mediated by hnRNP K binding to RNA. If hnRNP K acted posttranscriptionally, then G4/K/VP16, but not G4/VP16, should increase CAT expression irrespective of the particular cis-element activating transcription. Thus, to exert its influence in this system, hnRNP K requires the appropriate upstream sequence.

If hnRNP K is sequence and single strand specific and if G4/K/VP16 binds to CT4/E1b through hnRNP K to activate transcription, then the four CT-elements in this reporter must possess some single strand character. Negative supercoils, either resident in plasmid preparations or driven by transcription, provide one source of energy for focal strand separation (25, 26); this notion was supported by the report that hnRNP K melts and binds the CT-region (five tandem CT-elements) of the human c-*myc* gene only with negatively supercoiled, but not linear DNA *in vitro* (23). Accordingly, CT4/E1b was modified with the single strand selective agent $KMnO_4$ in the presence and absence of recombinant protein to determine if hnRNP K melted the CT4/E1b reporter plasmid (Fig. 2*A*). GST-hnRNP K clearly induced $KMnO_4$ hypersensitivity within CT-elements, but only if the target DNA was negatively supercoiled (lane 3) and not linear (lane 4), consistent with the hypothesis that hnRNP K requires at least transient single-strand character. If the interaction of G4/K/VP16 with CT4/E1b *in vivo* is biochemically equivalent with the binding of GST-hnRNP K to CT-elements *in vitro*, then CAT expression should be dependent on reporter topology. To test whether reporter topology indeed governed G4/K/VP16 activation, negatively supercoiled or linearized CT4/E1b was cotransfected with G4/K/VP16 (Fig. 2*B*). As predicted, activation through the CT-element was completely abrogated by linearization (compare lanes 2 and 4) of CT4/E1b at the *Nde*I site

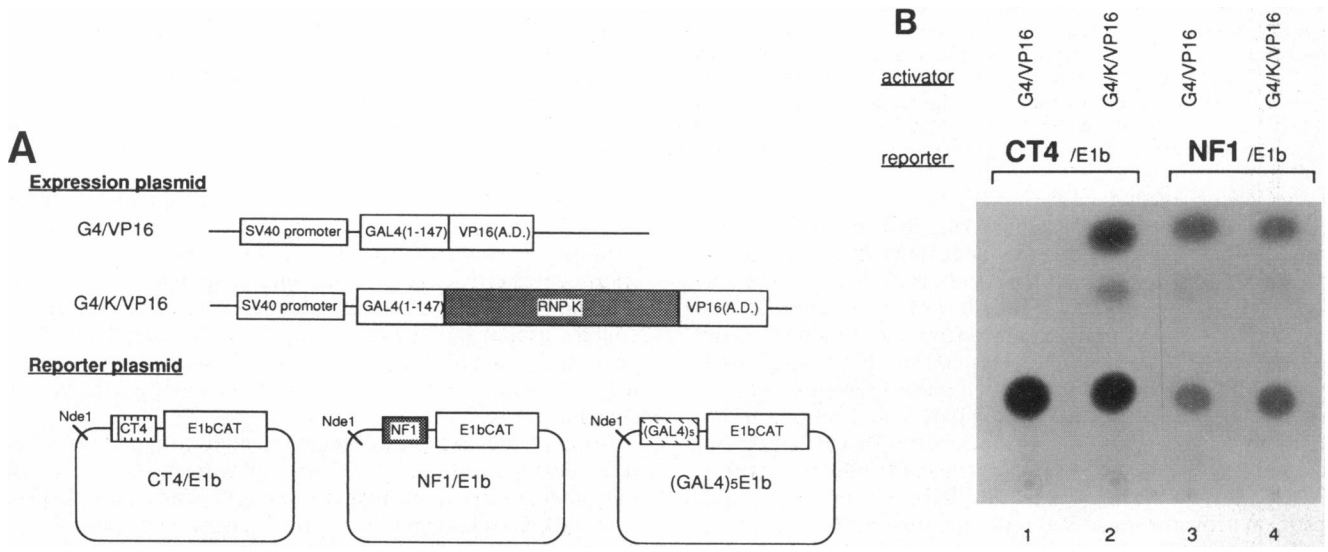

FIG. 1. G4/K/VP16 stimulates expression from CT-element but not NF1-driven reporters. (*A*) Diagrams of the expression and reporter plasmids used in Figs. 1 and 2 are shown. *Nde*I was used to linearize the reporters. (*B*) Expression plasmid (1 pmol) of either G4/VP16 or G4/K/VP16 was transfected with 10 μg of either CT4-driven (CT4/E1b; lanes 1 and 2) or NF1-driven (NF1/E1b; lanes 3 and 4) reporters, expressing CAT from the adenovirus E1b promoter in HeLa cells. Cells were harvested at 20 hr after transfection and assayed for CAT.

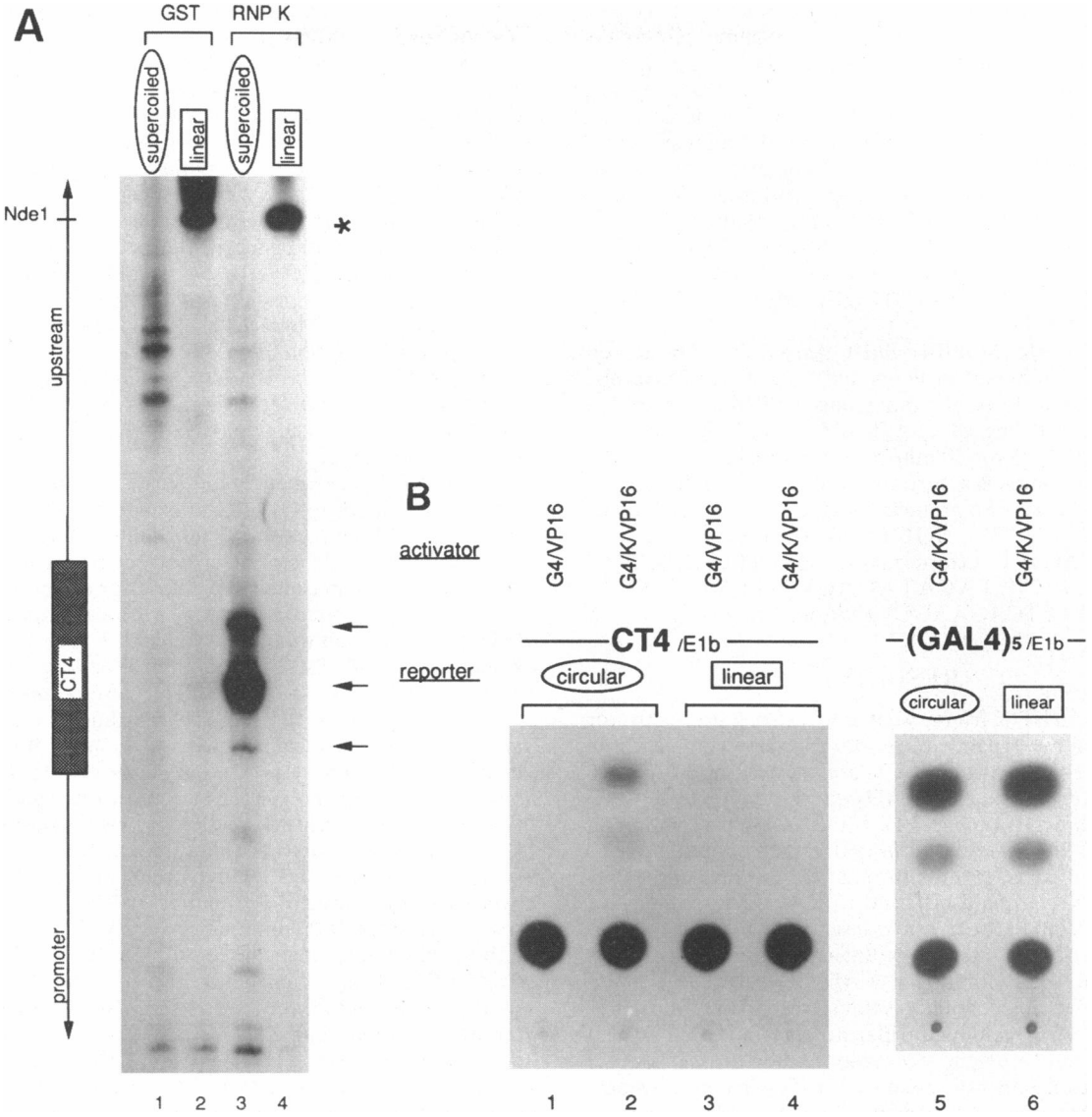

FIG. 2. hnRNP K binds to and transactivates from CT-elements in supercoiled but not linear DNA. (*A*) GST (20 pmol) (lanes 1 and 2) or recombinant hnRNP K (20 pmol) (lanes 3 and 4) were incubated with 10 ng (3.8 fmol) of either supercoiled (lanes 1 and 3) or *Nde*I linearized (lanes 2 and 4) CT4/E1b plasmid. Samples were treated with KMnO4 and the top strand was analyzed by LM-PCR. Arrows indicate hypersensitive sites within CT-element found only on supercoiled but not linear DNA. *, The site of *Nde*I cleavage of linear DNA. (*B*) G4/VP16 (1 pmol) or G4/K/VP16 (1 pmol) was transfected with 10 μg of supercoiled (lanes 1 and 2) or linear CT4/E1b (lanes 3 and 4) into HeLa cells. Supercoiled or linear E1b CAT reporter with five GAL4 binding sites(GAL4)5/E1b) was also transfected with G4/K/VP16 (lanes 5 and 6). CT4/E1b and (GAL4)5/E1b were linearized at *Nde*I site in the vector located 83-bp and 99-bp upstream of the CT-element or GAL4 binding sites, respectively. Cells were harvested at 18 hr after transfection and CAT assays were performed.

in the vector located 83-bp upstream of the CT-element. Similar results after linearization with *Bgl*I, cleaving ≈1-kb upstream in the vector, demonstrated that altered topology and not local perturbation of DNA ends is likely to account for G4/K/VP16 inactivity (data not shown). This topology dependence might have derived simply from different processing of transfected linear versus circular plasmids. For example, the expression difference between supercoiled or linear CT4/E1b might reflect plasmid stability, compartmentalization, or accessibility to the transcription machinery. To exclude these possibilities, G4/K/VP16 was transfected with a reporter (GAL4)5/E1b differing from CT4/E1b only by five GAL4 upstream activator sequences replacing the four CT-elements. Equivalent strong activation was supported whether or not (GAL4)5/E1b was linearized (compare lanes 5 and 6), thus demonstrating that activation by G4/K/VP16 was sensitive to template topology only when binding to DNA through hnRNP K but not through the GAL4 DNA binding domain. This result also verified equal availability of linear or circular reporter plasmids for expression. Southern blot analysis of Hirt supernatants prepared from transfected cells (27) confirmed equal transfection efficiency of linear and supercoiled DNA (data not shown). These blots also demonstrated that the supercoiled plasmids were progressively relaxed during the first day posttransfection as reported (28), which necessitated harvesting cells within 18 hr after transfection for the experiments described here.

If CT-elements must be single stranded to bind hnRNP K, then locking the CT-region into duplex form should abolish this binding. Due to its high G-C content, single strandedness within the CT-element is likely to be generated by extension of loops arising within more easily melted adjacent sequences (29). Therefore, stabilization of the immediately adjacent double stranded conformation should restrict access to hnRNP K. Such stabilization of duplex might be achieved by using a well-characterized protein that recognizes B-DNA as an ad-

jacent clamp to prevent the cognate target sequence from melting. We previously characterized the DNA sequence required for the binding of hnRNP K and found that at least two CT-elements (CCCTCCCCA) separated by a spacer are necessary (ref. 15; the exact distance between the CT-elements is not important because there is no helical phasing in single strand DNA). Accordingly, a CAT reporter driven by two CT-elements separated by 34 base pairs comprising two lexA binding sites (CT-lexA-CT/E1b) was constructed to determine whether or not lexA protein could interfere either with hnRNP K binding *in vitro* or with G4/K/VP16-mediated activation *in vivo*. First, supercoiled CT-lexA-CT/E1b was incubated *in vitro* with GST-hnRNP K and with or without increasing concentrations of lexA. Then unpaired nucleotides were reacted with $KMnO_4$, cleaved with piperidine, and mapped by LM-PCR (Fig. 3*A*). Alone, GST-hnRNP K induced clear hypersensitive bands within each CT-element, especially in the upstream repeat (lane 3). When increasing levels of lexA protein were included in the binding reactions with hnRNP K, the upstream hypersensitive site was abolished and the downstream site diminished (lanes 4 and 5). Binding of lexA protein to these lexA operators does not unwind DNA and hence cannot be visualized with this single strand specific, chemical modification. Instead, lexA binding was confirmed by dimethyl sulfate footprinting (data not shown). These results indicate that lexA interferes with hnRNP K binding to CT-lexA-CT/E1b *in vitro*. To test if this same interference occurred *in vivo*, G4/K/VP16 and CT-lexA-CT/E1b were cotransfected into COS cells with or without a plasmid expressing the lexA protein, and transactivation by G4/K/VP16 was monitored (Fig. 3*B*). COS cells were chosen for this experiment to maximize synthesis of lexA protein. Indeed, enhanced CAT expression by G4/K/VP16 (lane 2) was reduced to basal levels by coexpression of lexA (lanes 3 and 4), demonstrating the predicted interference

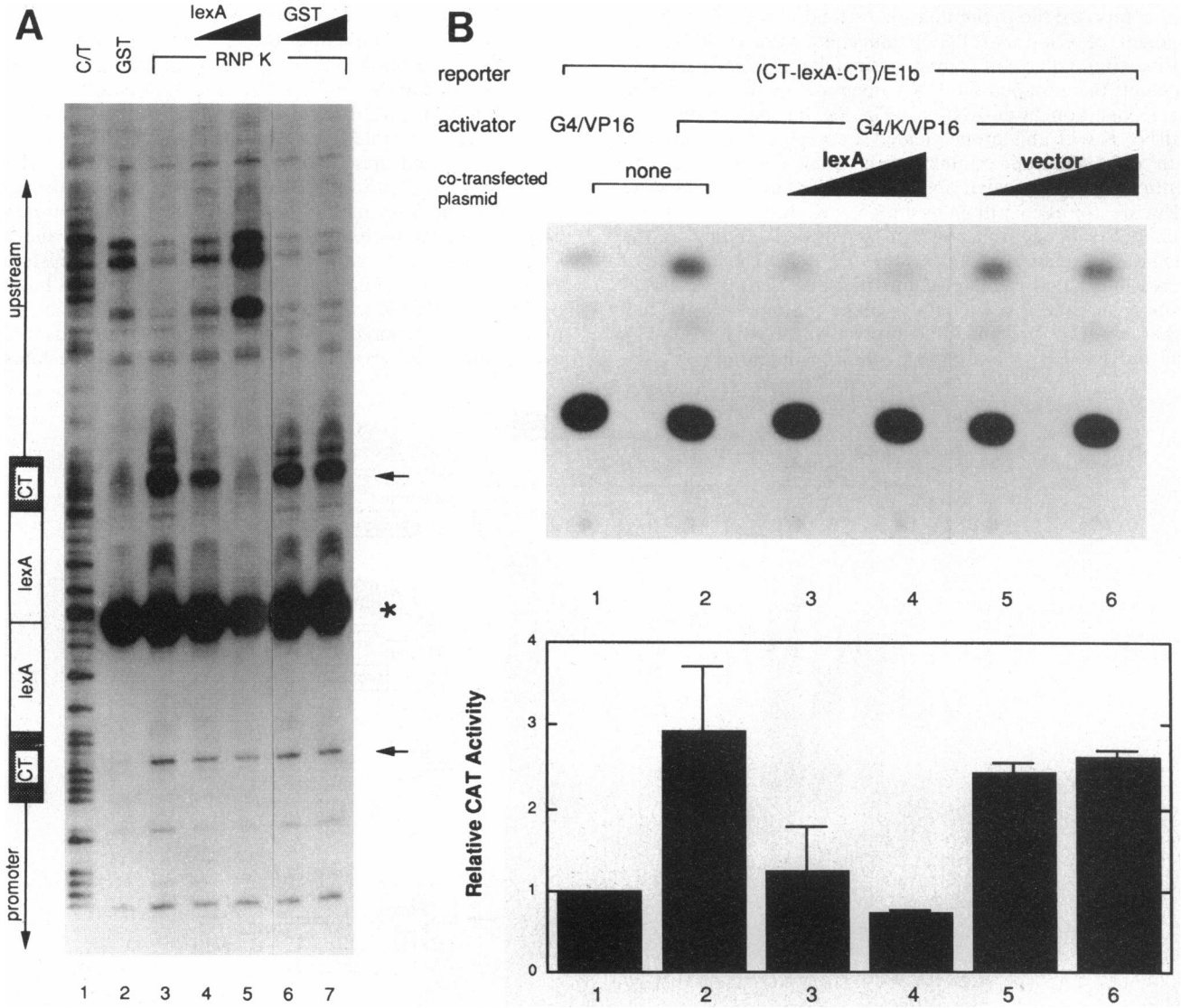

FIG. 3. The binding and transactivation of hnRNP K is abolished when lexA binds to operators separating CT-elements. (*A*) GST (20 pmol) (lane 2) or recombinant hnRNP K (20 pmol) (lanes 3–7) were incubated with 10 ng of E1bCAT containing two CT repeats (CCCTCCCCA) that were separated by two lexA binding sites (CT-lexA-CT/E1b). Either recombinant lexA (4 or 20 pmol) (lanes 4 and 5) or GST (4 or 20 pmol) (lanes 6 and 7) was coincubated with hnRNP K and treated with $KMnO_4$; the top strand was analyzed by LM-PCR as described in Fig. 2. Lane 1, pyrimidine ladder. Arrows indicate hypersensitive sites within CT-element eliminated (top arrow) or reduced (bottom arrow) by the addition of lexA. *, A strong $KMnO_4$ hypersensitive site at the junction of the two lexA operators, even in the absence of protein, possibly due to DNA bending. (*B*) G4/VP16 (0.04 pmol) (lane 1) or G4/K/VP16 (0.04 pmol) (lanes 2–6) was transfected with 10 μg of CT-lexA-CT/E1b into COS7 cells. Either lexA expression plasmid (0.04 and 0.2 pmol) (lanes 3 and 4) or expression vector (pcDNAI/Amp; 0.04 and 0.2 pmol) (lanes 5 and 6) was cotransfected, respectively. The cells were incubated for 60 hr before harvesting for CAT assays. The histogram below shows a comparison of CAT activity in three separate transfections.

between the two proteins *in vivo*. It should be noted that COS cells support a relatively higher level of basal expression from CT-lexA-CT/E1b than do HeLa cells, most likely reflecting Sp1 or an Sp1-like activity operating through the CT-elements. Sp1 was shown to bind and act weakly through CT-elements (30), and expression driven by Sp1 sites from simple promoters is upregulated by the simian virus 40 T-antigen, which is present in COS but not in HeLa cells (31–33).

Two different mechanisms might explain how lexA interfered with hnRNP K binding to the CT-element *in vitro* and blocked G4/K/VP16 activation *in vivo*. Most simply, lexA might sterically hinder the interaction of hnRNP K with DNA. Alternatively, lexA might clamp both strands together, raising the energy needed to separate strands. If the latter mechanism holds, enforcing single stranded DNA within each CT-element would be expected to bypass the lexA-dependent impediment to hnRNP K binding. To test this prediction, an oligonucleotide probe was designed for eletrophoretic mobility-shift assay that comprised the pyrimidine-rich strand of the CT-lexA-CT segment of CT-lexA-CT/E1b annealed with a ^{32}P-labeled purine-strand partner having mismatches within each CT-element; this propped the DNA open and made it accessible for recognition by hnRNP K (Fig. 4). Incubation of GST-hnRNP K with this probe yielded a complex easily detected with electrophoretic mobility-shift assay (lanes 6 and 7), confirming that the small single strand loops in the probe were adequate for recognition by hnRNP K. Incubation of this dumbbell shaped probe with lexA verified binding of the bacterial repressor to its operator (lanes 2–5). The addition of increasing amounts of lexA to mixtures of GST-hnRNP K and probe generated a new complex migrating more slowly than either lexA-probe or hnRNP K-probe complexes (lanes 8–11). The hnRNP K-probe complex could be quantitatively converted into the lexA-hnRNP K-probe complex. The ability of lexA to add to the hnRNP K-probe complex and produce a supershift proved that the arrangement of CT-elements and lexA operators did not preclude simultaneous protein DNA interactions. No such supershift would be predicted if binding of lexA to the probe sterically precluded binding of hnRNP K. Although this result does not exclude a steric contribution to the interference of lexA with binding of hnRNP K *in vivo* and *in vitro*, it suggests that lexA's ability to remove hnRNP K from supercoiled DNA most likely derives from suppression of single stranded character in the adjacent CT-elements.

DISCUSSION

In these experiments, fusion of hnRNP K and the potent transactivation domain of VP16 has been exploited to prove that hnRNP K is a DNA-binding protein *in vivo*. But what is the physiological role of native hnRNP K after binding to DNA *in vivo*? Is there any biochemical function for a transcription factor lacking a dominating effector domain? First, the N terminus of hnRNP K does possesses weak activating properties when fused to the GAL4 DNA-binding domain alone (15), and full-length hnRNP K, itself, can augment *in vitro* RNA synthesis up to 10-fold from an appropriate template that has been tailed with single stranded CT-elements (18). These results indicate that hnRNP K might directly adjust the level of expression from nearby promoters operating as a weak, conventional transcription factor. On the other hand, another single strand DNA binding protein, called the far upstream element (FUSE) binding protein (FBP), which shares homology with hnRNP K in its nucleic acid binding domain (both proteins use K homology or KH motifs) and also binds to the c-*myc* gene, possesses a far more potent transactivation domain

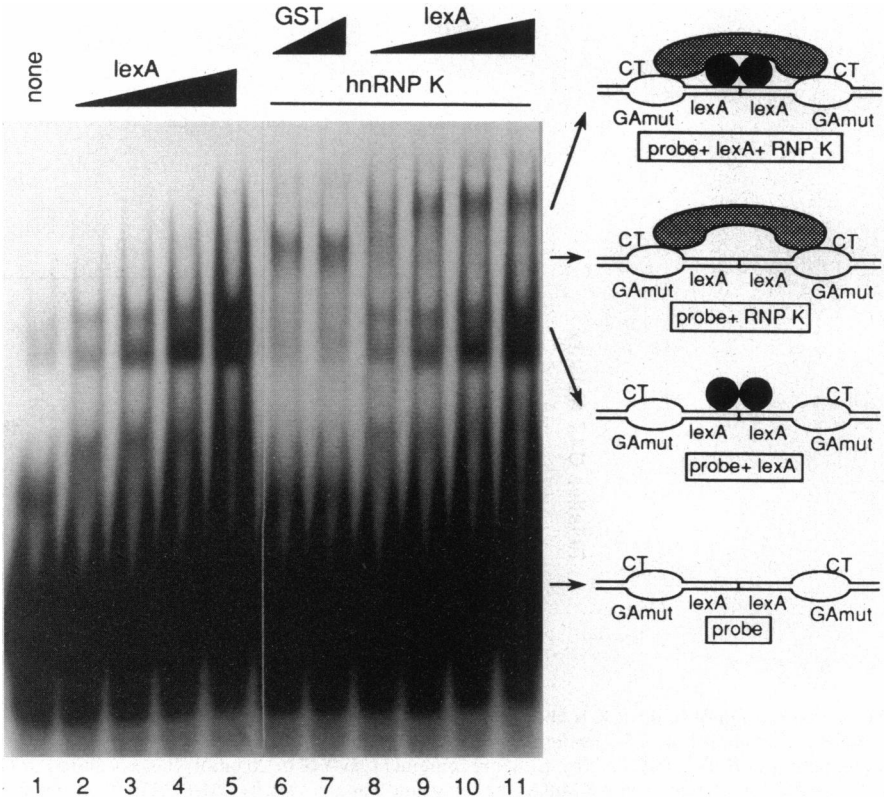

FIG. 4. Interference between hnRNP K and lexA is due to DNA conformation change and not steric hindrance. Recombinant lexA (10, 50, 200, and 600 ng) (lanes 2–5) or recombinant hnRNP K (10 ng) with either 200 or 600 ng of GST (lanes 6 and 7) or 10, 50, 200, and 600 ng of recombinant lexA (lanes 8–11) were incubated with 1 ng of ^{32}P labeled probe and electrophoretic mobility-shift assay was performed. Lane 1, probe alone. Arrows indicate the position and identity of bands formed by the migration of the probe, alone, or of the three different DNA-protein complexes illustrated.

(22, 34). Such fine and coarse tuning of expression might be important for a gene such as c-*myc*. Second, another possible role for hnRNP K, not incompatible with the first, would be to facilitate transcription indirectly. For example, by creating a single stranded bubble, hnRNP K could function as an architectural transcription factor by making DNA more flexible and thus allowing other upstream DNA-binding factors to align more easily with the basal machinery. As little as three unpaired base pairs reduces the torsional rigidity of a DNA double helix by several hundred-fold, virtually relieving any helical phase dependence for ligating small DNA fragments into circles (35). Accordingly, it is possible that hnRNP K or another single-strand DNA-binding protein may create a single stranded hinge facilitating the interaction of elements and factors that might otherwise be energetically disfavored.

The ability of a single segment of DNA to interact specifically with single strand- or duplex-dependent factors evokes the notion of regulating gene expression through a DNA conformational switch modulated by protein binding. These studies have demonstrated how duplex requiring lexA interferes with hnRNP K binding to adjacent single strands. In fact, some of the native CT-elements of the human c-*myc* gene that bind hnRNP K have also been shown to bind Sp1 (30). The balance between the inputs of hnRNP K and Sp1 onto c-*myc* expression may be adjusted, at least conceptually through many mechanisms. First, the relative levels of hnRNP K and Sp1 will, of course, influence which factor predominates (as with lexA and G4/K/VP16). This regulatory device is found whenever two factors recognize overlapping sequence and is not unique to hnRNP K. However, the use of one or the other factor, either as a prop to separate strands or as a duplex clamp, enlarges the segment of DNA that can be governed by a single bound protein. Second, parameters that locally alter the melting temperature (T_m) of the relevant sequence might dramatically influence the relative input of each factor. For example, control of superhelical density through transcription, matrix attachment, topoisomerase activity, and nucleosome placement and removal might directly or indirectly modify the proclivity of strands to separate. Together with promoter dependent sensitivity of transcription initiation to superhelical density through basal factor use (36, 37), the output of a particular gene might be rendered highly susceptible to topological and conformational constraints. The range of physiological properties and the degree of control conferred by a given regulatory sequence can be expanded by modulating both DNA conformation and the array of factors available to interact with a given sequence.

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