

trans-Activation by the hnRNP K Protein Involves an Increase in RNA Synthesis from the Reporter Genes*

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The function of many of the pre-mRNA-binding proteins in mRNA biogenesis is unclear. We have analyzed the biochemical function of the hnRNP K protein by using a mouse cDNA clone. A previous study indicated that the expression of hnRNP K activates *c-myc* promoter in transient transfection assays. We show that the expression of hnRNP K results in a *trans*-activation of a variety of RNA polymerase II promoters. The *trans*-activation function depends on the sequences of hnRNP K that are also necessary for RNA binding. However, the RNA binding motifs are not sufficient for *trans*-activation. We could identify a mutant that bound RNA *in vitro* but was impaired in its ability to *trans*-activate the reporter genes. The *trans*-activation was not a result of the stabilization of the reporter mRNA, because hnRNP K increased the steady-state level of the reporter mRNA without altering its decay rate. By doing nuclear run-on assays, we provide evidence that the hnRNP K protein *trans*-activates the reporter genes by increasing the level of transcription.

In eukaryotic cells, elongating precursors of mRNA are packaged with pre-mRNA-binding proteins to form the heterogeneous nuclear ribonucleoprotein complexes called hnRNPs. The pre-mRNA-binding proteins are believed to be involved in the maturation of the precursor mRNA. At least 20 pre-mRNA-binding proteins or hnRNP proteins (A through U) have been identified (Barnett *et al.*, 1989; Burd and Dreyfuss, 1994a; Conway *et al.*, 1988; Dreyfuss *et al.*, 1993). However, the precise function performed by each of these hnRNP proteins in mRNA biogenesis has remained elusive. The hnRNP A1 protein has been studied in greater detail. It was shown that hnRNP A1 preferentially bound to sequences that resemble the sequences found in the splice sites (Burd and Dreyfuss, 1994b). Moreover, the purified hnRNP A1 protein has been shown to be involved in selecting the 5' splice site (Ge and Manley, 1990; Mayeda and Krainer, 1992). The A1 protein also shuttles between nucleus and cytoplasm; thus, a role in the export of mRNA is not unlikely (Pinol-Roma and Dreyfuss, 1992). hnRNP C has also been implicated in splicing. Antibodies raised against hnRNP C were shown to inhibit splicing *in vitro* (Choi *et al.*, 1986).

The hnRNP K protein has drawn attention because of its KH motif, which is also found in the protein encoded by the FMR1

gene (which is involved in fragile X syndrome) (Ashly *et al.*, 1993; Siomi *et al.*, 1993b, 1994). The KH motif is an evolutionarily conserved RNA binding motif found in several other RNA-binding proteins, including the archeobacterial ribosomal S3 protein and the meiosis-specific splicing factor MER1 (Siomi *et al.*, 1993a). More recently, it has been shown that a sequence-specific single-stranded DNA-binding protein FBP, which stimulates transcription of the *c-myc* gene, possesses KH motifs within a region that is important for the DNA binding (Duncan *et al.*, 1994). Besides the KH motif, the hnRNP K protein contains an arginine/glycine-rich region with several copies of the RGG box, which is found in other RNA-binding proteins (Burd and Dreyfuss, 1994a; Dreyfuss *et al.*, 1993).

In vitro, the hnRNP K protein binds with an unusually high affinity to poly(rC) or poly(dC) (Matunis *et al.*, 1992). Although the significance of this high affinity binding to poly(rC) and poly(dC) is not quite clear, it has been shown that the hnRNP K protein can bind to a C-rich sequence (the CT element) in the *c-myc* promoter and stimulate transcription from that promoter (Takimoto *et al.*, 1993; Tomonaga and Levens, 1995). Besides an effect on the *c-myc* promoter, hnRNP K has also been implicated in transformation. Dejgaard *et al.* (1994) identified four splice variants of hnRNP K and showed that the levels of these polypeptides were up-regulated in SV40-transformed cells.

We investigated the RNA binding properties of hnRNP K using natural RNA sequences as substrate.¹ Results of these studies indicated that the hnRNP K protein possesses selective RNA binding activities. This RNA binding activity depends upon the KH domains as well as the arginine/glycine-rich regions.¹ Here, we show that the expression of the hnRNP K protein *trans*-activates expression from reporter genes with a variety of RNA polymerase II promoters. The *trans*-activation is not specific for the CT element found in the *c-myc* promoter. The stimulation of the reporter gene expression depends on the sequences that are also necessary for RNA binding by the hnRNP K protein. However, RNA binding alone does not account for the *trans*-activation function of the hnRNP K protein. Finally, the *trans*-activation by the hnRNP K protein involves an increase in RNA synthesis from the reporter gene. The hnRNP K protein increases the steady-state level of the reporter mRNA without altering its decay rate.

MATERIALS AND METHODS

Expression Plasmids and Mutants—The pGEX clones of hnRNP K and its mutants will be described elsewhere.¹ Eukaryotic expression plasmids of hnRNP K protein and its mutants were constructed by cloning the polymerase chain reaction fragments into *EcoRI/SalI* sites of CMVHA-poly(A) vector. CMVHA-poly(A) was constructed by introducing nucleotide sequences corresponding to HA tag downstream of the CMV promoter (Pani *et al.*, 1992). The hnRNP K cDNA clones were introduced in frame with HA.

RNA Binding and Gel Retardation Assay—*In vitro* transcribed and

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¹ M.-H. Lee, S. Mori, and P. Raychaudhuri, manuscript submitted.

polyacrylamide-urea gel purified α - 32 P-labeled RNAs were heated to 95 °C for 5 min and subsequently incubated in ice for 5 min. RNA probes (20,000 cpm/0.5–1.0 ng) were incubated with 20–30 ng of GST-hnRNP K or the mutants in a total volume of 30 μ l containing 20 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 10 μ M $ZnCl_2$, 0.02% Nonidet P-40, 70 mM NH_4Cl , and 1 μ g of yeast tRNA for 20 min at room temperature. Equal aliquots of the incubation mixtures were analyzed by gel retardation assays (Scherly *et al.*, 1989; Ray *et al.*, 1992).

RNA Probe—The pGEM clone of +8 fos-CAT² was constructed by cloning the *Pst*I/*Pvu*II fragment of pFC(–58)fos-CAT into *Pst*I and *Hinc*II sites of pGEM3Zf(+). *Bam*HI-linearized pGEM clone of +8 fos-CAT was used to generate an RNA probe of 202 nucleotides. This probe contains 32 nucleotides from the human *c-FOS* mRNA (between +8 and +40) and 170 nucleotides from the CAT mRNA (+1 to +170). The RNA probe was synthesized by *in vitro* transcription using SP6 RNA polymerase in the presence of NTPs and [α - 32 P]UTP. The full-length RNA was purified by polyacrylamide-urea gel.

Antibodies—A synthetic peptide corresponding to the sequence between residues 218 and 232 was used to raise rabbit antiserum. The peptide was coupled to keyhole limpet hemacyanin by using an additional cysteine residue at the C terminus. The peptide antibody was purified by peptide affinity chromatography. The antibody specifically recognizes hnRNP K protein in Western blot assays of crude extracts. The antibody purification and Western blots were performed following procedures described by Harlow and Lane (1988).

Reporter CAT Gene Constructs—The CAT gene constructs pFC(–58)/CRE, pFC(–58)/AP1, pFC(–58)/NF-IL6, pFC(–58)/GRE, and pFC(–58)/E2F were obtained by cloning oligonucleotides at position –58 of the human *c-FOS* promoter-CAT construct. Each construct contained *c-FOS* promoter sequences corresponding to –58 and +42 upstream of the CAT gene. AGCTGTGACGTTTGTGACGTTTGTAG was used as CRE-oligo; AGCTCTGCGTCAGTGCGTCAG was used as AP1 oligo; AGCTATTAGGACATATTAGGACAT was used as the NF-IL6 oligo; AGCTTTAGTGTTCATTTTCCTATGTTCTTTTGAAT was used as the GRE oligo; and the sequences between –30 and –70 of the adenovirus E2 promoter was used as the E2F oligo.

Transfections and CAT Assays—DNA transfections into NIH 3T3 cells and CAT assays were performed following previously described procedures (Arroyo and Raychaudhuri, 1992).

RNAse Protection Assay—Total cellular RNA from transfected cells was isolated following a procedure described before (Chomczynski and Sacchi, 1987). The RNA preparations were treated with RNase-free DNase I (final concentration, 667 units/ml; Boehringer Mannheim) for 5 min at 37 °C. For CAT mRNA, 25 μ g of RNA from each of the samples was analyzed. To obtain an antisense CAT-specific probe, a DNA fragment between *Hind*III and *Pvu*II from the CAT gene construct pFC(–58)/E2F was subcloned into the *Hind*III/*Sma*I sites of pGEM3Zf(+). The plasmid was linearized at the *Hind*III site and transcribed by using T7 RNA polymerase in the presence of ribonucleotide triphosphates and [α - 32 P]UTP. To probe for the GAPDH RNA, a rat GAPDH cDNA (gift of R. Costa, Department of Biochemistry, University of Illinois at Chicago) cloned into a pGEM vector was used to generate antisense RNA probe; and 5 μ g of total RNA from each of the samples was analyzed. The hybridization was carried out for 14–16 h at 60 °C in 10 μ l of a solution containing 80% formamide, 40 mM PIPES (pH 7.6), 0.4 M NaCl, and 0.5 mM EDTA. The reaction mixtures were then digested with 26 units/ml of RNase T2 for 1 h at 32 °C (Adami and Babiss, 1990; Hart *et al.*, 1985). The protected fragments were analyzed by 4% polyacrylamide-urea gels followed by autoradiography.

Nuclear Run-on Assays—Nuclear run-on assays with transfected cell nuclei were carried out essentially following a previously described protocol (Greenberg and Ziff, 1984). Briefly, cells were harvested 36 h after transfection and washed three times with ice-cold phosphate-buffered saline. The cell pellets were resuspended in Nonidet P-40 lysis buffer, which contained 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% (v/v) Nonidet P-40. After an incubation on ice for 2 min, the nuclei were pelleted by centrifugation at 650 \times g for 5 min. The supernatants were removed, and the nuclei were resuspended in buffer (200 μ l) containing 50 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA. 200 μ l of the reaction buffer was added to the resuspended nuclei, and reaction was allowed to continue for 30 min at 30 °C. The reaction buffer contained 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM each of ATP, CTP, and GTP, and 200 μ Ci

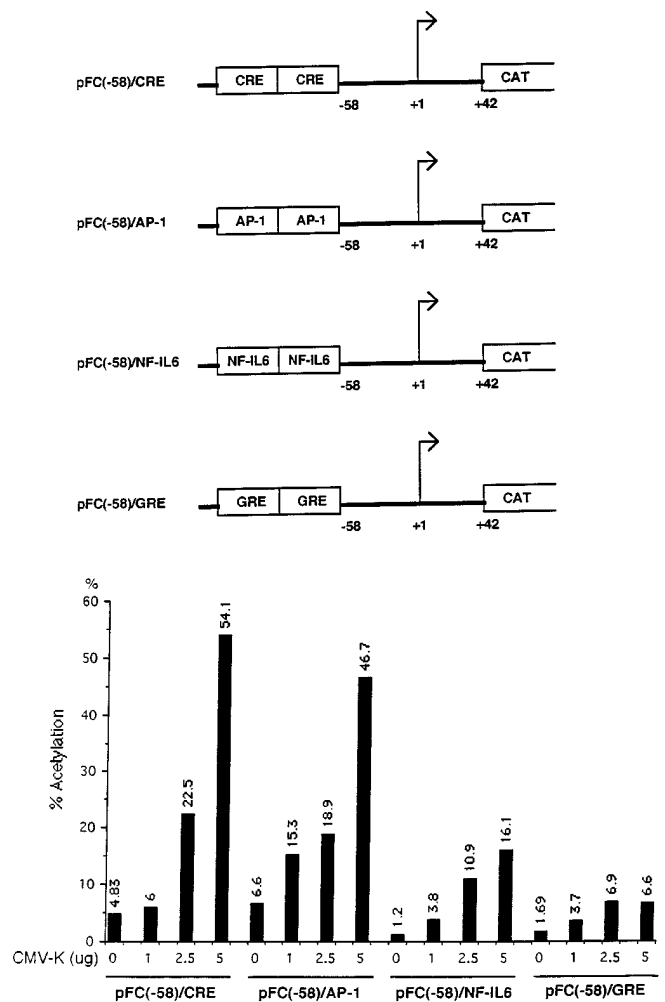


FIG. 1. Activation of the CAT gene expression by the hnRNP K protein. Schematic diagrams of the plasmids containing reporter CAT gene with various promoter sequences are shown in the upper panel (also see "Materials and Methods"). NIH 3T3 cells were cotransfected with the reporter CAT gene constructs (5 μ g) and the indicated amounts of the hnRNP K expressing plasmid (CMV-K). The transfections and the CAT assays were carried out as described under "Materials and Methods." The percentage of conversion (% acetylation) is shown. The number above each bar represents the actual percentage of acetylation.

of [α - 32 P]UTP. The 32 P-labeled RNA was isolated following the previously described procedure (Greenberg and Ziff (1984) and references therein). The labeled RNA was used to hybridize with specific probes that were immobilized onto a nitrocellulose membrane by using a slot blot apparatus (Schleicher & Schuell). For CAT mRNA, a 250 nucleotide cDNA fragment (0.5 μ g) corresponding to the 5' end of the CAT gene; and for rRNA, a 800-nucleotide cDNA fragment (0.5 μ g) corresponding to the mouse 18 S rRNA was used. The blots did not contain any plasmid sequences.

RESULTS

Effects of hnRNP K on the Expression of Reporter Genes—The hnRNP K protein was shown to stimulate CAT gene expression from the *c-myc* promoter, and this stimulation was correlated with the binding of the hnRNP K protein to the CT element in the *c-myc* promoter (Takimoto *et al.*, 1993; Tomonaga and Levins, 1995). To investigate the trans-activation function of the hnRNP K protein, we carried out transient transfection experiments using several chimeric reporter gene constructs. We observed that the mouse hnRNP K protein could stimulate reporter gene expression from a variety of promoters irrespective of the CT element. The reporter constructs containing two copies of the CRE site, the AP1 site, the NF-IL6 site, or the GRE site upstream of the basal *c-FOS* promoter were cotrans-

² The abbreviations used are: CAT, chloramphenicol acetyltransferase; PIPES, 1,4-piperazinediethanesulfonic acid; CMV, cytomegalovirus.

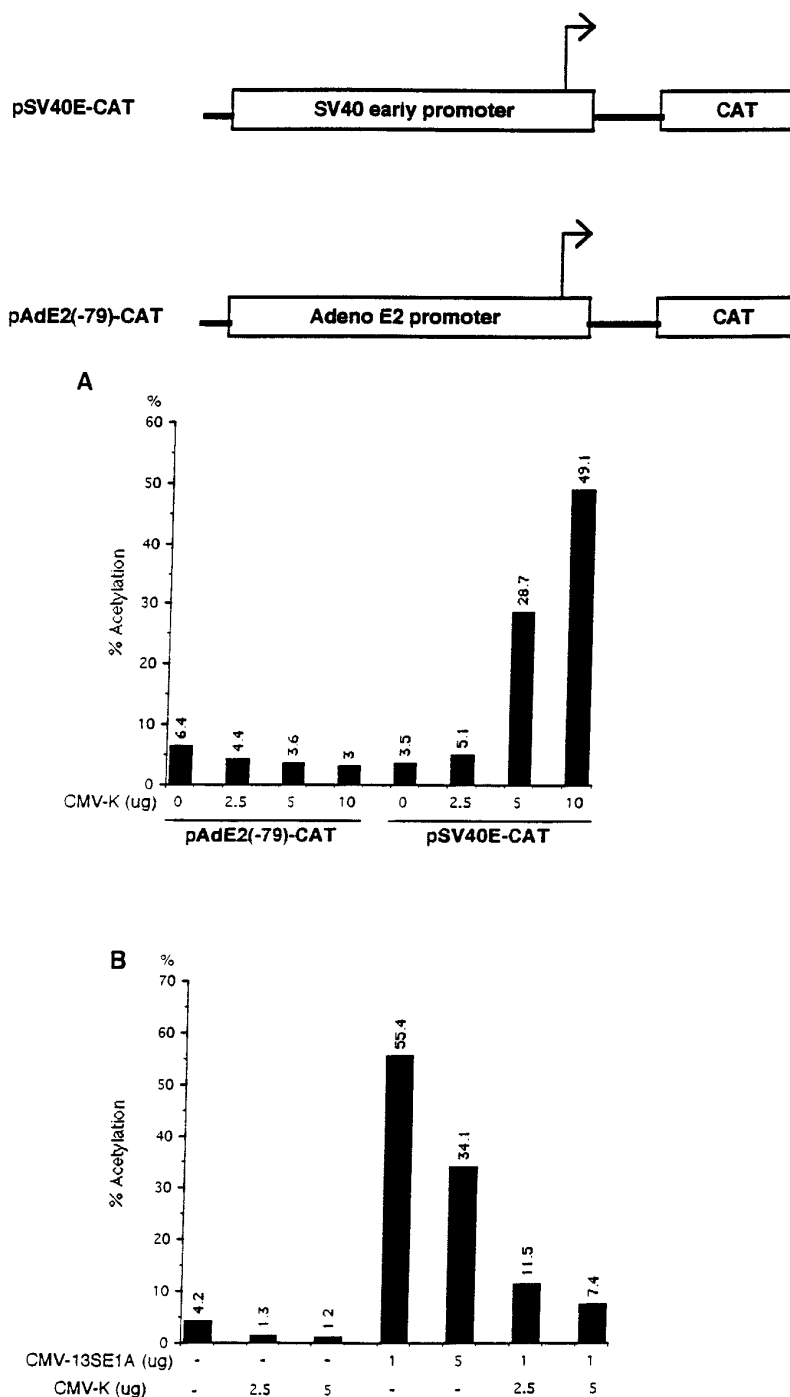


FIG. 2. *trans*-Activation by hnRNP K depends upon promoter structure. A, CAT gene reporter constructs containing adenovirus E2 promoter (E2 CAT) or SV40 early promoter were transfected into NIH 3T3 cells along with the indicated amounts of the hnRNP K-expressing plasmid. The transfection and CAT gene assays were performed as described before (Arroyo and Raychaudhuri, 1992). The number above each bar represents the actual percentage of acetylation. B, adenovirus E2 promoter construct (E2 CAT) was transfected into NIH 3T3 cells along with the E1A (12S)-expressing plasmid in the presence or the absence of the indicated amount of the hnRNP K-expressing plasmid. The CAT gene activities are shown.

fected into NIH 3T3 cells along with a mouse hnRNP K-expressing plasmid. The coexpression of the hnRNP K protein resulted in a stimulation of the CAT gene expression from the reporter gene constructs (Fig. 1).

The hnRNP K-mediated stimulation of the CAT activity was dependent on the structure of the promoter. For example, we observed that the adenovirus E2 promoter and the SV40 early promoter elicited an opposite response by the coexpression of hnRNP K. The adenovirus E2 promoter consistently expressed the reporter CAT gene at a level similar to what was observed from the SV40 early promoter in NIH 3T3 cells (Fig. 2). Expression of the K protein resulted in a modest repression of CAT activity from the E2 promoter (Fig. 2, A and B). The repression was also detected in the presence of E1A, which is known to activate this promoter (Fig. 2B). The SV40 early promoter, on the other hand, exhibited a high level of stimula-

tion by the cotransfection of the K-expressing plasmid (Fig. 2A).

So far we have not been able to identify a promoter-reporter construct whose activity is not altered by the coexpression of the hnRNP K protein. Majority of the promoters tested were *trans*-activated by the coexpression of hnRNP K. Because of this promiscuity of hnRNP K effect, we were not able to use an internal control for transfection efficiencies. However, all of the transfection experiments presented above and below were repeated several times, and they very accurately reflect the average.

trans-Activation Function of the hnRNP K Protein Depends upon the Sequences That Are Also Necessary for RNA Binding—hnRNP K was identified as a pre-mRNA-binding protein. We did not detect a direct interaction of hnRNP K with the promoters used in the *trans*-activation assays. However, we

could easily detect a specific interaction of the hnRNP K protein with a 202-nucleotide RNA probe derived from one of the reporter genes used in this study (Fig. 3). The RNA probe

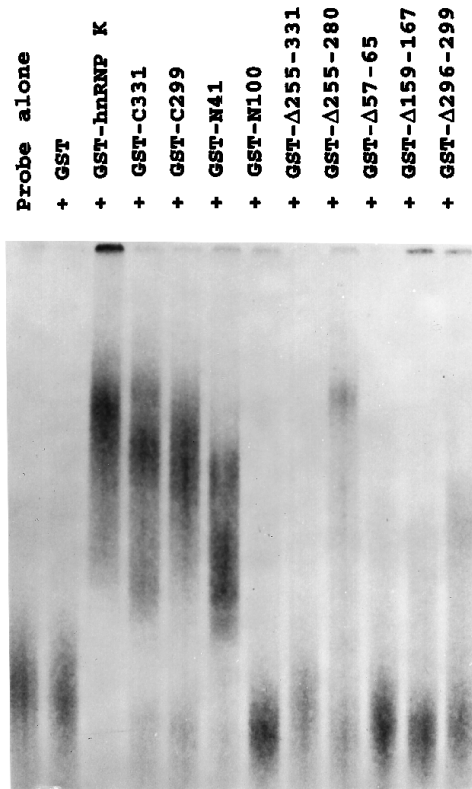


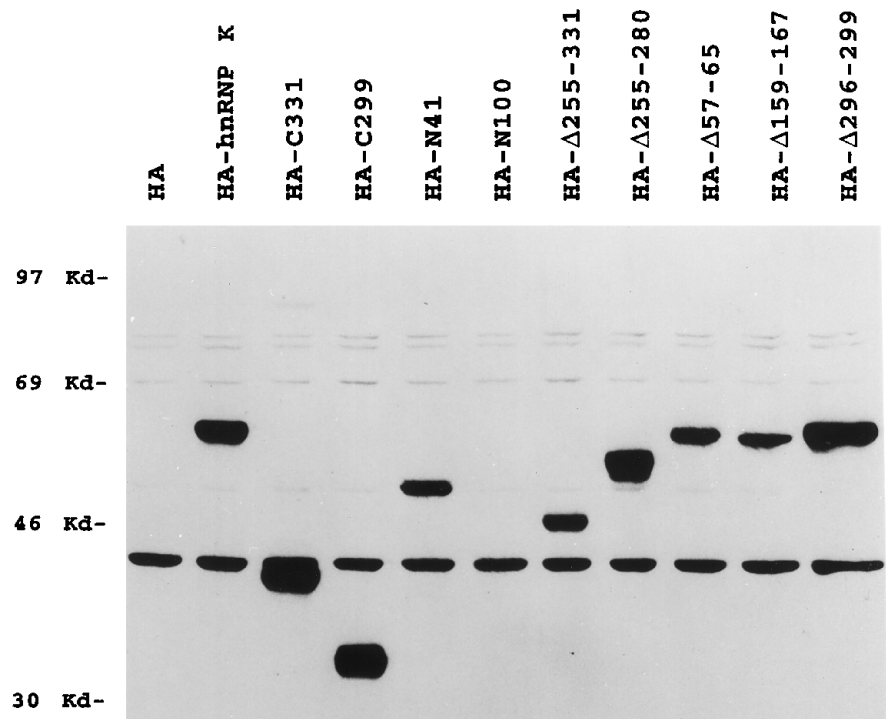
FIG. 3. hnRNP K binds to the reporter mRNA depending upon the KH motifs and the RGG box. 30 ng of GST-hnRNP K or the indicated mutants were incubated with α - 32 P-labeled RNA probe containing 32 nucleotides of the *c-FOS* mRNA and 170 nucleotides of CAT mRNA (see "Materials and Methods") in the presence of 1 μ g of tRNA as described under "Materials and Methods." After 20 min of incubation at room temperature, aliquots (7 μ l) were analyzed by gel retardation assay as described under "Materials and Methods."

(fos-CAT) contained 32 nucleotides of the *c-FOS* mRNA and 170 nucleotides of CAT mRNA. The hnRNP K protein interacted with this RNA probe depending upon the KH motifs as well as the arginine/glycine-rich region (Fig. 3). Mutations that altered the KH1 and KH2 domains (N100, Δ 57–65, and Δ 159–167) significantly reduced the RNA binding ability of the hnRNP K protein. In addition, mutations that altered the arginine/glycine-rich region (Δ 256–331, Δ 256–280, and Δ 296–299) also reduced in binding to the fos-CAT probe.

To analyze the mutants in *trans*-activation assays, an eukaryotic expression vector containing the CMV promoter was employed. The mutants were subcloned into this vector in-frame with a flu virus epitope (HA tag) in the N terminus. The HA tag allowed us to detect the proteins expressed from the transfected genes without interference from the endogenous hnRNP K protein. To look at the expression and localization of the mutants, nuclear extracts of the transfected cells were analyzed in Western blot assays. The blots were probed with a monoclonal antibody against the HA tag. As shown in Fig. 4, the mutants produced expected size polypeptides, and they were detected in the nuclear extracts of the transfected cells. We were consistently unable to detect the polypeptide corresponding to the mutant N100 in the nuclear extracts of the transfected cells.

To identify the region of hnRNP K protein involved in the *trans*-activation function, the mutants described in Fig. 4 were analyzed in cotransfection assays. The mutants were transfected into NIH 3T3 cells along with the CRE sites containing reporter construct. The results of these transfection experiments are summarized in Fig. 5. An average of six independent experiments is shown. A mutant that lacked the N-terminal amino acid residues up to 41 (N41) was active in *trans*-activation of the reporter gene. However, a complete deletion of the first KH domain (N100) or a small internal deletions within the core consensus region of the first KH domain (Δ 57–65) or a small internal deletion within the second KH domain (Δ 159–167) resulted in a significant impairment of the *trans*-activation function. The mutants harboring C-terminal deletions up to amino acids 360 and 331, which removed the third KH domain but left the RGG clusters intact, still exhibited signif-

FIG. 4. Expression of the hnRNP K mutants in the transfected cells. The hnRNP K or its mutants were cloned into CMV-HA tag-poly(A) plasmid as described under "Materials and Methods." 5 μ g of HA tag-hnRNP K or the mutants (see Fig. 5) was transfected into NIH 3T3 cells. Nuclear extract was prepared from the transfected cells. 30 μ g of nuclear extracts was separated by SDS-polyacrylamide gels and transferred to the nitrocellulose membrane. The nitrocellulose blot was probed with a monoclonal antibody against HA tag (12CA5; Boehringer Mannheim). The blot was developed by ECL.



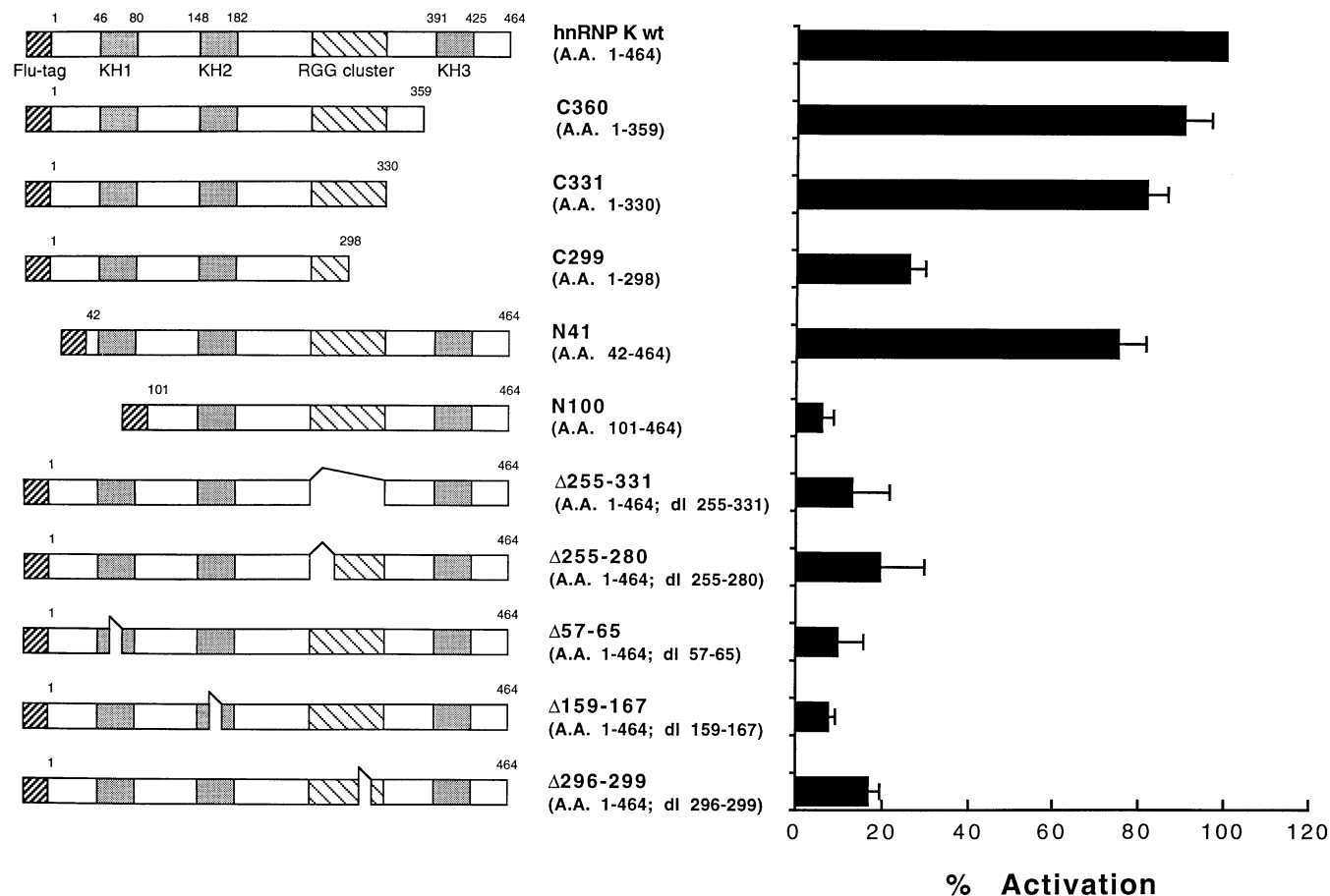


FIG. 5. **trans-Activation of the reporter genes depends upon sequences that are also necessary for RNA binding.** A schematic diagram of the HA tag hnRNP K or its mutants is shown in the left panel. The plasmid pFC(-58)/CRE-CAT (5 μ g) was used as a reporter construct. 5 μ g of the plasmid that expressed the wild type hnRNP K or the indicated mutants was transfected into NIH 3T3 cells along with the reporter gene. The CAT assays were performed as described under "Materials and Methods." The percentage of activation of wild type is shown. An average of at least six independent experiments is shown.

icant *trans*-activation. The mutants that lacked the RGG clusters (Δ 255-331) or part of the RGG cluster (Δ 255-280 and Δ 296-299) exhibited a significant reduction of the *trans*-activation function. Taken together, this line of analysis shows that the first and the second KH domains and the RGG clusters are essential for the *trans*-activation function of the hnRNP K protein. Because these sequences are also important for RNA binding, it is likely that the *trans*-activation function of hnRNP K involves RNA binding.

RNA Binding Is Not Sufficient for the *trans*-Activation of the Reporter Genes—Furthermore, the analysis of the mutants in transfection assays indicated that the RNA binding function may not be sufficient for *trans*-activation. Two C-terminal deletion mutants, C299 and C331, exhibited very similar RNA binding activities (Fig. 3). These two mutants were also detected in the nuclear extracts of the transfected cells (Fig. 6B). However, when analyzed in *trans*-activation assays, the mutant C299 consistently exhibited a much reduced activity compared with C331 (Fig. 6A). These results suggest the possibility that RNA binding alone may not be sufficient for the *trans*-activation function of the hnRNP K protein. Interestingly, the sequence between residues 299 and 331 is rich in proline. Proline-rich sequences have been implicated in protein-protein interactions. It is likely that this region of hnRNP K is involved in interactions with other proteins, which are important for the *trans*-activation function.

The *trans*-Activation by the hnRNP K Protein Involves an Increase in RNA Synthesis—The hnRNP K protein has been

shown to be localized in the nucleus (Matunis *et al.*, 1992). To investigate whether the stimulation of the CAT activity by a coexpression of hnRNP K protein is a nuclear event, the steady-state levels of CAT mRNA from two reporter constructs were monitored. CAT gene construct containing CRE or E2F site were transfected into NIH 3T3 cells along with the hnRNP K-expressing plasmid. The total cellular RNA from transfected cells was isolated and analyzed by RNase T2 protection assay as described under "Materials and Methods." The two panels in Fig. 7 represent two independent experiments. We could detect the specifically protected band (210 nucleotides) corresponding to the correctly initiated RNA (indicated by an arrow in Fig. 7). The band indicated by an asterisk in Fig. 7 most likely represents protected RNA from a secondary start site (at position +15 within the *FOS* gene sequences and upstream of the CAT coding region). The intensity of the correctly initiated band from the CRE-containing construct was enhanced by the coexpression of the hnRNP K protein in a dose-dependent manner. A densitometric scanning indicated a 12–15-fold increase in intensity of the correctly initiated band from the CRE reporter by the cotransfection of 5 μ g of hnRNP K expression plasmid. However, the intensity of the same mRNA band from the E2F-containing construct was not increased by the coexpression of the hnRNP K protein. Interestingly, the CAT enzyme activity from this construct was increased only 2–3-fold by the coexpression of the hnRNP K protein (data not shown), which probably reflects transcription from the secondary start site.

The hnRNP K protein did not alter the half-life of the re-

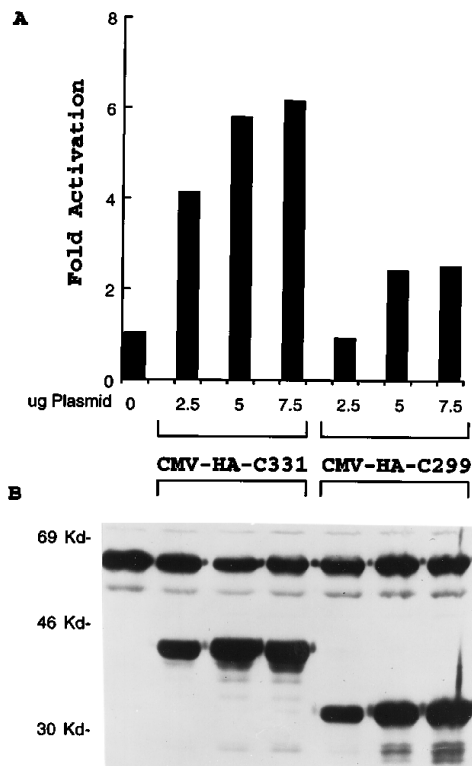


FIG. 6. RNA binding is not sufficient for trans-activation. *A*, increasing amounts of the HA tag-C331- or the HA tag-C299-expressing plasmid were transfected into NIH 3T3 cells along with pFC(-58)/CRE-CAT as a reporter construct. Each bar represents fold activation compared with the control plasmid (CMV-HA tag-poly(A)). *B*, increasing amounts of the HA tag-C331- or the HA tag-C299-expressing plasmid were transfected into NIH 3T3 cells. Nuclear extracts from the transfected cells were analyzed for the hnRNP K and the mutants. The Western blot was probed with a polyclonal antibody raised against a peptide of hnRNP K (see "Materials and Methods" for details) because 12CA5, monoclonal antibody against HA tag, gives a nonspecific band around 44 kDa that partly overlaps the band of HA tag-C331 (see Fig. 4). * indicates the endogenous hnRNP K, ** indicates the HA tag-C331, and *** indicates HA tag-C299.

porter RNA. We measured the decay rate of the CAT mRNA in the presence of the wild type or a mutant hnRNP K protein. Because the pFC(-58)/CRE construct had a very low basal level of expression, we sought to use a reporter construct that produces the same reporter mRNA at a high basal level. The plasmid pFC700 was used as reporter because it expressed the same CAT mRNA from a relatively stronger promoter. pFC700 construct exhibited only a marginal increase in the CAT activity by coexpression of the hnRNP K protein (not shown). Nevertheless, the high basal level expression allowed us to investigate the decay rate of the CAT mRNA. Before harvesting, the cells were incubated with 5 μ g/ml of actinomycin D for various time periods. The total cellular RNA was purified and digested with DNase I, and CAT mRNA was assayed by an RNase protection assay as described under "Materials and Methods." The upper panel in Fig. 8 shows the decay rate of CAT mRNA in cells cotransfected with wild type or a nonfunctional mutant hnRNP K-expressing plasmid. The band intensities were quantified by densitometric scanning. The lower panel of Fig. 8 shows a plot of log (percentage of mRNA remaining) versus time of treatment with actinomycin D. This experiment was reproduced several times, and we did not detect any significant difference in the decay rate of CAT mRNA in the presence of wild type or mutant hnRNP K protein. Taken together, these results suggest that hnRNP K increases the level of RNA synthesis from the reporter genes.

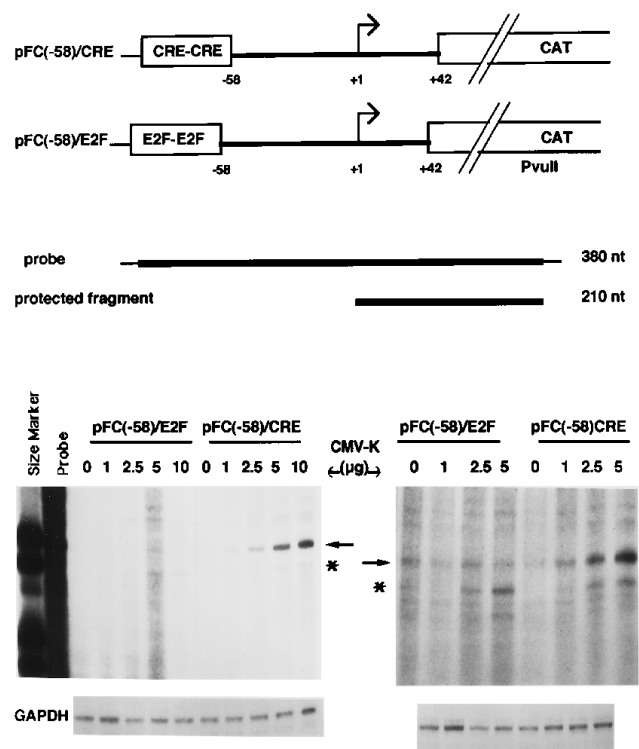


FIG. 7. The hnRNP K protein increases the steady-state level of CAT mRNA. The plasmid pFC(-58)/CRE-CAT was used as a responsive reporter, and the plasmid pFC(-58)/E2F-CAT was used as a nonresponsive reporter. The reporter plasmid (5 μ g) was transfected into NIH 3T3 cells along with the indicated amounts of the CMV-K plasmid. After transfection, total cellular RNA was isolated, and 25 μ g of the RNA, after a 10-min treatment with DNaseI, was analyzed for CAT specific transcript as described under "Materials and Methods." The arrow indicates correctly initiated CAT mRNA (210 nucleotides), and the band indicated by the asterisk most likely represents CAT mRNA from a secondary start site. The lower panel shows assays for the GAPDH RNA (see "Materials and Methods" for details). The two panels represent two independent experiments.

To investigate a role of the hnRNP K protein in altering the level of RNA synthesis, we carried out nuclear run-on assays using isolated nuclei from transfected NIH 3T3 cells. The transfection experiments were carried out using the E2F or the CRE sites containing constructs with and without hnRNP K-expressing plasmid. The nuclei from the transfected cells were isolated and labeled with [α - 32 P]UTP. The labeled RNAs from the four samples were isolated and were used to hybridize with specific probes bound to nitrocellulose membrane. Four nitrocellulose membranes, each containing the first 250 nucleotides of the CAT cDNA (0.5 μ g; Fig. 9, lower lanes) and a 800-nucleotide fragment corresponding to the cDNA of mouse 18 S rRNA (0.5 μ g; Fig. 9, upper lanes), were hybridized with the labeled RNA from the four samples. The probe for the rRNA served as an internal control because we did not detect any significant change in the rRNA level by the coexpression of hnRNP K protein. Clearly, the coexpression of the hnRNP K protein increased the level of RNA synthesis from the CRE-containing promoter.

DISCUSSION

The hnRNP K protein was shown to stimulate expression of the CAT gene from a *c-myc* promoter construct (Takimoto *et al.*, 1993). To investigate the cellular function of the hnRNP K protein, we carried out transient transfection experiments and analyzed the effects of an expression of hnRNP K on a variety of reporter genes. NIH 3T3 cells were used for these studies, because the endogenous level of hnRNP K in these cells is lower

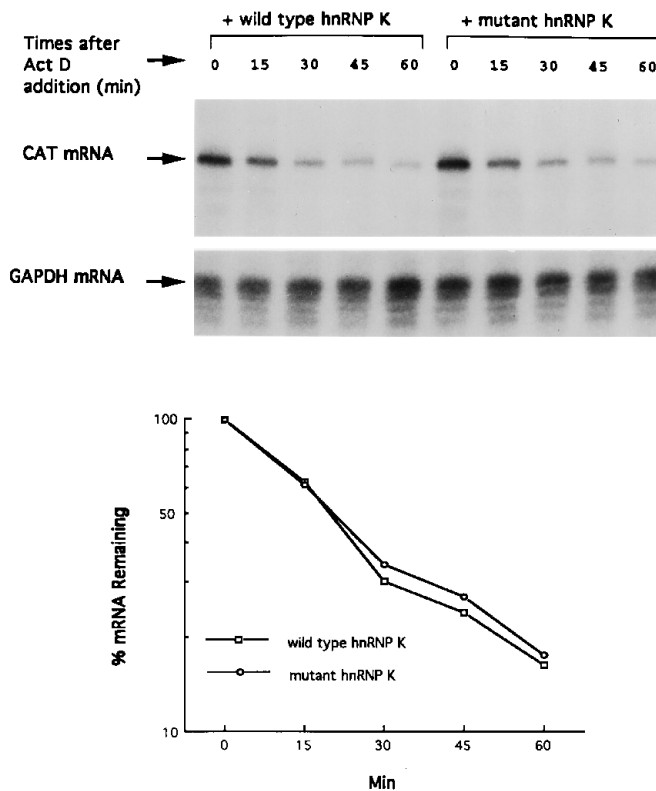


FIG. 8. **hnRNP K does not alter the decay rate of the CAT mRNA.** NIH 3T3 cells were cotransfected with the plasmid pFC700 (a CAT gene construct containing the human *c-FOS* promoter sequences from -700 to +40) and the wild type or a mutant ($\Delta 255-331$) hnRNP K expression plasmid. The transfections were carried out as described under "Materials and Methods." Before harvesting, the transfected cells were stimulated by adding 15% fetal bovine serum in the medium for 30 min followed by incubations with actinomycin D (5 μ g/ml) for the indicated period of time. The total cellular RNA was isolated and treated with DNase I. The CAT mRNA was assayed by using an anti-sense RNA probe (upper panel) as described under "Materials and Methods." The assays for the GAPDH RNA in the same samples are also shown. The lower panel shows a plot of log (% of mRNA remaining) versus the time of treatment with actinomycin D.

than that in several other cell lines (not shown). We observed that an expression of hnRNP K altered expression of reporter genes from a variety of promoters. Curiously, the adenovirus E2 gene promoter exhibited a reduction of activity. The majority of the promoter constructs, on the other hand, exhibited an increase in expression by the coexpression of the hnRNP K protein. Therefore, in this study we analyzed the *trans*-activation in greater detail.

To investigate a link between the RNA binding and the *trans*-activation functions, we analyzed the hnRNP K mutants in transient transfection assays. A CRE site-containing construct was used as a reporter gene. The results of these studies indicated that the mutants that are deficient in RNA binding are also impaired in the *trans*-activation function. However, RNA binding alone did not account for the *trans*-activation function. Because a C-terminal deletion mutant (C299) bound RNA efficiently but exhibited a much reduced *trans*-activation function. We do not think that this was due to a lack of expression or improper localization of C299. This mutant can be detected in the nuclear extracts of the transfected cells. Thus, we believe that in addition to RNA binding, there are other interactions that are involved in the *trans*-activation by the hnRNP K protein.

The increase in CAT enzyme activity from this construct correlated with an increase in the level of steady-state CAT

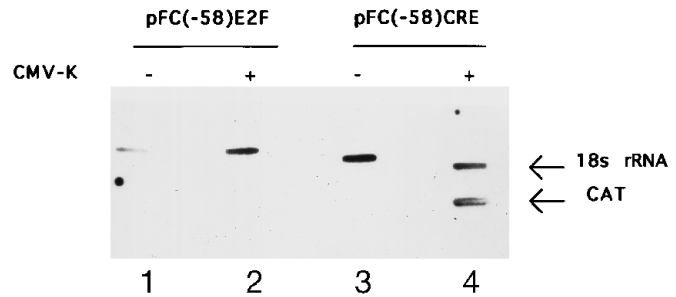


FIG. 9. **The hnRNP K protein increases the rate of transcription from a responsive target.** Four plates (10 cm) were used for each set of transfections. For blots 1 and 2, cells were transfected with pFC(-58/E2F)-CAT (5 μ g) in the presence (lane 1) or in the absence (lane 2) of the CMV-K plasmid (5 μ g). For blots 3 and 4, approximately equal number of cells were transfected with pFC(-58/CRE)-CAT in the presence (lane 4) or in the absence (lane 3) of the CMV-K plasmid (5 μ g). The cells from each set of transfections were pooled and nuclei were isolated. The nuclear run-on assays were performed as described under "Materials and Methods." The labeled RNA was hybridized with specific probes that were immobilized on nitrocellulose blots as described under "Materials and Methods." The specific probes contained a 250-nucleotide cDNA fragment (0.5 μ g) corresponding to the 5' end of the CAT gene (lower lanes) or a 800-nucleotide cDNA fragment (0.5 μ g) corresponding to the 18 S rRNA gene (upper lanes). The blots did not contain any plasmid sequences.

mRNA, indicating that the effect, at least partly, is at the level of RNA accumulation. We did not detect any significant alteration of the decay rate of CAT mRNA by a coexpression of the hnRNP K protein. These results suggested that coexpression of hnRNP K increases RNA synthesis from the reporter gene. To obtain further evidence for an increased rate of RNA synthesis, we performed nuclear run-on assays. Results of these assays confirmed the notion that the hnRNP K protein *trans*-activates reporter genes by increasing the level of transcription.

The molecular mechanism by which the hnRNP K protein increases the level of RNA synthesis is unclear. We can imagine three scenarios. First, it is possible that it activates transcription indirectly by increasing the availability of the transcription factors. Second, because hnRNP K binds single-stranded DNA, it might perform a function in transcription that is similar to what is carried out by single-stranded binding protein in DNA replication. Third, it is possible that hnRNP K enhances RNA synthesis by binding to the newly synthesized chain of mRNA.

An increase in the availability of transcription factors by hnRNP K can be accomplished in several ways. For example, it is possible that the hnRNP K protein alters the decay rate of the mRNAs of the transcription factors, resulting in an increase in the levels of the transcription factors. Because we consistently observed a large induction through the ATF/CRE site, we compared the levels of the transcription factors CREB, ATF1, ATF2, ATF3, and ATF4 in hnRNP K-transfected and untransfected cells by immunoblot assays. No alteration in the levels of these factors was observed (data not shown). Additionally, we did not detect any alteration of the decay rate of the CAT mRNA, implying that the hnRNP K protein does not alter the half-life of mRNA. It is also possible that the transcription factors remain sequestered in an RNA-bound form, and the overexpression of an RNA-binding protein releases these transcription factors, making them available to activate promoters. Such a possibility is unlikely because in that case any RNA-binding protein would stimulate RNA synthesis. We did not detect any *trans*-activation by coexpressing hnRNP A1 (not shown). Also, the mutant C299, which bound RNA, was impaired in its ability to *trans*-activate a reporter gene (Fig. 6).

It is noteworthy that in two different instances this RNA-binding protein was shown to associate with promoter-ele-

ments (Ostrowski *et al.*, 1994; Takimoto *et al.*, 1993). Although we have not detected a sequence-specific stable interaction with the promoters used in this study, it is possible that hnRNP K interacts with promoter complexes after a melting has occurred during the initiation complex formation. The single-stranded DNA binding function may have a role in stabilizing an open complex configuration during transcription. Such a possibility can not be ruled out; however, requirement for a single-stranded binding protein in transcription is yet to be shown.

An attractive model is that this pre-mRNA-binding protein binds to the newly synthesized RNA and enhances the rate of synthesis. There is precedence for RNA-binding protein involved in transcription. For example, the HIV encoded Tat protein is an RNA-binding protein that stimulates transcription from the HIV LTR (see Cullen (1991) for a review). We speculate that the hnRNP K protein, after binding near the 5' end of the synthesizing chain of pre-mRNA, interacts with the transcription machinery and enhances the rate of RNA synthesis. Nuclear matrix has been shown to play a role in RNA transcription. The actively transcribing genes have been shown to associate with the nuclear matrix (Hutchinson and Weintraub, 1985; Stief *et al.*, 1989). Because hnRNP binding to pre-mRNA is coupled to transcription, it is possible that the nuclear matrix play a role in loading the pre-mRNA-binding proteins onto the nascent chain of mRNA. We envision that the hnRNP K binds mRNA as soon as hnRNP K-recognition motif is synthesized and that this interaction then allows other interactions with the transcription machinery leading to an increased level of transcription. A clear definition of the RNA element recognized by the hnRNP K protein, as well as determination of the role of the RNA element, will provide an insight into the mechanism by which hnRNP K protein increases RNA synthesis.

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