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Makorin RING Finger Protein 1 (MKRN1) Has Negative and Positive Effects on RNA Polymerase II–Dependent Transcription

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Through its transcriptional activities, the proto-oncoprotein c-Jun can regulate cellular proliferation, survival, and differentiation. We have established a novel yeast assay that screens for repressors of c-Jun transcriptional activity. This screen led to the identification of a ubiquitously expressed novel RING zinc finger protein, termed Makorin RING zinc finger protein 1 (MKRN1), recently shown to act as an E3 ubiquitin ligase. Overexpression of MKRN1 in mammalian cells inhibited the transcriptional activities of not only c-Jun, but also the nuclear receptors, the androgen receptor, and the retinoic acid receptors. Truncation analysis indicates that both the amino and carboxy termini are required for this transrepression activity. Surprisingly, when fused to the heterologous DNA-binding domain of GAL4, MKRN1 activates, rather than inhibits, a GAL4-responsive reporter plasmid. In addition, truncation of either the amino- or carboxy-terminal half of MKRN1 disrupts its transactivation activity, the same observation that was made on its transrepression activity. These results demonstrate that MKRN1 has transcriptional activity and suggest that its transrepression and transactivation functions are mediated by the same mechanism. Interestingly, disruption of MKRN1's ubiquitin ligase activity does not affect its inhibitory transcriptional activity. Thus, MKRN1 may represent a nuclear protein with multiple nuclear functions, including regulating RNA polymerase II–catalyzed transcription.

Key Words: c-Jun; Makorin ring finger protein 1; androgen receptor; retinoic acid receptor; transactivation; transrepression.

Introduction

c-Jun can regulate transcription via multiple mechanisms: transactivation, transrepression, and coactivation (reviewed in refs. *1–3*). The most widely studied is transactivation, which is dependent on either c-Jun homodimerization or heterodimerization with c-Fos to form the transcription factor AP-1 (activator protein-1) (reviewed in ref. *3*). In this dimeric complex, c-Jun is able to regulate the expression of genes indispensable for cell proliferation and differentiation (reviewed in refs. *1* and *2*). This transactivation function of c-Jun is regulated by Jun N-terminal kinase (JNK), a MAP kinase that can phosphorylate and thereby activate c-Jun (reviewed in ref. *4*). In transrepression, c-Jun has been shown to inhibit the transcriptional activity of many nuclear receptors, including the receptors for the hormones progesterone (PR), estrogen (ER), glucocorticoid (GR), and thyroid hormone (TRs), and the vitamins retinoic acid (RARs and RXRs) and vitamin D (VDR) (reviewed in refs. *5* and *6*). This activity allows c-Jun to regulate cellular differentiation that is under the control of nuclear receptors. As a coactivator, c-Jun can mediate the transcriptional activities of the androgen receptor (AR) and the two ETS transcription factors ERM *(7)* and PU.1 *(8)*. Interestingly, at least with regard to AR and PU.1, c-Jun's function as a coactivator can be separated from that of a transactivator, because a transactivation-deficient mutant, c-Jun $Ala^{63/73}$, is still active in coactivation *(8,9)*.

Many proteins have been shown to regulate c-Jun activity. Several bZIP proteins can dimerize with c-Jun via the leucine zipper motif and thus increase the complexity of AP-1. This group includes the multiple members of the Jun and Fos families of proteins (reviewed in ref. *2*), ATF proteins *(10)*, MAF proteins *(11,12)*, and NF-E2 proteins *(11, 12)*. In addition, a diverse group of non-BZIP proteins are known to regulate c-Jun activity. The GR *(13)*, nuclear factor of activated T cells (NFAT) *(14)*, and Ets family proteins interact with c-Jun by sharing composite DNA binding sites with AP-1 *(15)*. Smad proteins, which are critical for TGFβ signaling, can associate with specific DNA elements in association with partners like c-Jun *(16)*. c-Jun can also functionally interact in a mutually antagonistic manner with nuclear

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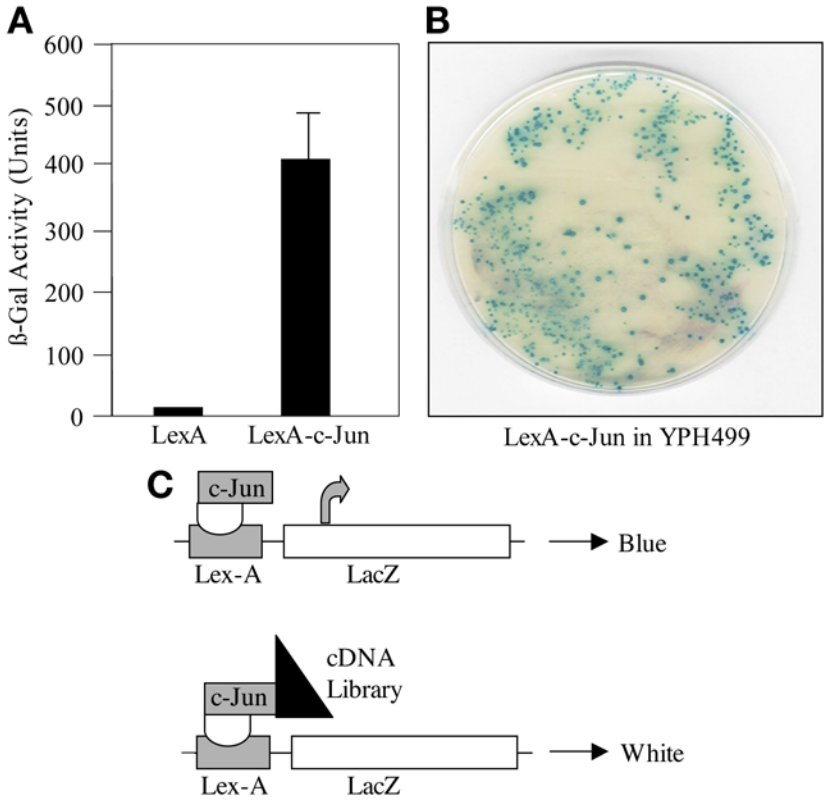

Fig. 1. c-Jun transcriptional activity in yeast and the yeast functional screen. (**A**) *YPH499* cells were transformed with 1 μg LexA or LexA-c-Jun and 1 μg reporter plasmid pSH18-34. c-Jun transactivation was measured by using a liquid β-gal assay. (**B**) *YPH499* cells were transformed with 1 μg each of LexA-c-Jun and pSH18-34 and grown on X-gal–containing medium. (**C**) A schematic diagram of the yeast functional screen for repressors of c-Jun transactivation. LexA-c-Jun activates the LacZ reporter and results in blue yeast colonies on X-gal medium. When a cDNA-expressed protein blocks c-Jun transactivation, there is no β-gal activity and yeast colonies remain white.

receptors (reviewed in refs. *5* and *6*), STAT proteins *(17)*, and MyoD proteins *(18)*. With respect to transcription complexes formed on promoters, c-Jun is known to interact with the general transcription factors TFIIE-34 *(19)* and TFIIF *(19)* and the coactivators JAB1 *(20)*, CBP/p300 *(21)*, and SRC-1 *(22)*. In contrast to these interacting proteins that stimulate c-Jun transactivation, several proteins have been demonstrated to inhibit c-Jun/AP-1 activity. JDP-2 was first defined as a c-Jun-interacting protein that functions as an AP-1 repressor *(23)*. The nuclear receptor corepressor NCoR acts as a transcriptional repressor of AP-1 in macrophage activation *(24)*. While c-Fos strongly stimulates c-Jun's transactivation properties, it is equally able to repress its coactivation of AR *(25–27)*.

Because of our lab's interest in c-Jun's multiple activities on transcription, we developed a novel screen for c-Jun repressors. This assay, which we call a yeast functional screen, led to the identification of a ubiquitously expressed RING zinc finger protein, termed Makorin RING zinc finger protein 1 (MKRN1) *(28)*. Based on its multiple functional domains, including four zinc fingers and one RING finger, MKRN1 was predicted to have roles in RNA processing and transcription. Recently, MKRN1 was demonstrated to act as a ubiquitin E3 ligase on hTERT and in this way modulate telomere length homeostasis *(29)*, the first and only published molecular activity for this protein. We provide evidence here that MKRN1 can also regulate RNA polymerase II–dependent transcription. It blocks the transcriptional activity of not only c-Jun/AP-1, but also several other RNA polymerase II–dependent transcriptional activators. When tethered to DNA via a heterologous DNA-binding domain (DBD), MKRN1 can activate transcription. Based on mutational analysis, the transrepression and transactivation functions of MKRN1 appear to be mediated by the same mechanism and the transrepression function does not depend on its ubiquitin ligase activity. Thus, MKRN1 may represent a protein with multiple nuclear functions, including stabilizing telomeres and regulating transcription.

Results

A Yeast Functional Screen for Inhibitors of c-Jun Transcriptional Activity

In order to screen for proteins inhibiting c-Jun transcriptional activity, we developed a modified yeast two-hybrid system that we call a yeast functional screen. In this approach, a LexA-c-Jun fusion protein was expressed in *Saccharomyces cerevisiae* and tested for transcriptional activity using an AP-1–responsive Lac Z reporter plasmid. As shown in Fig. 1A, LexA-c-Jun exhibited strong activity,

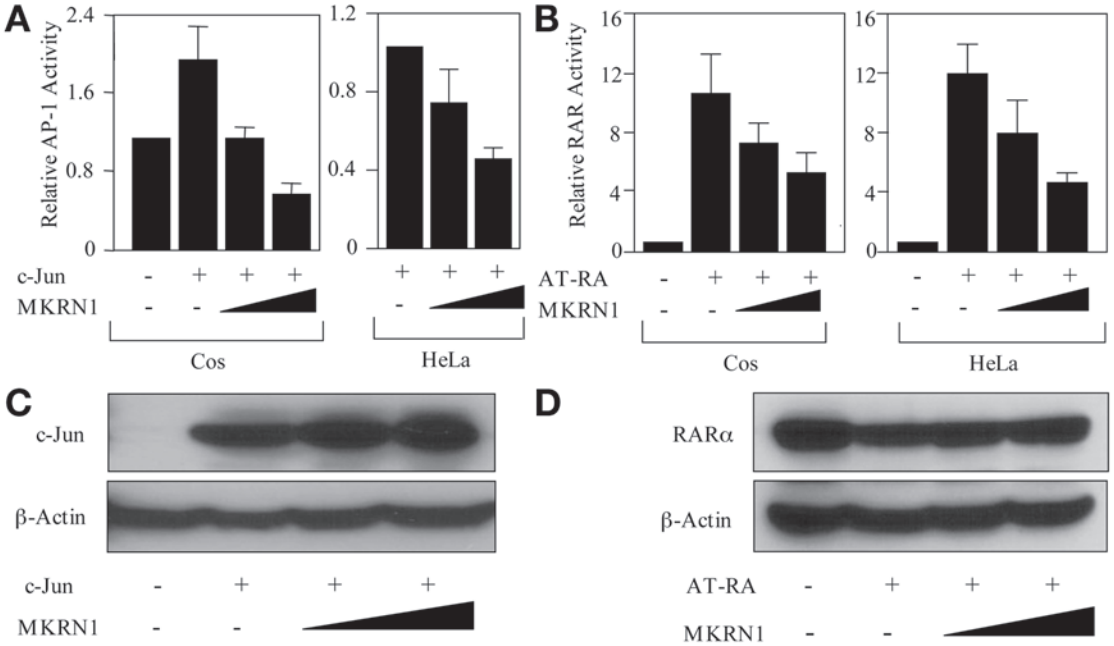

Fig. 2. MKRN1 represses c-Jun and hRARα transcriptional activities. (**A, B**) Cos and HeLa cells were transiently transfected with 1 or 5 μg MKRN1 and (**A**) 1 μg TRE-tk-CAT reporter plasmid and with or without 1 μg c-Jun or (**B**) 1 μg RARE-tk-CAT reporter plasmid and with or without 0.5 μg hRARα, as indicated. Cells transfected with hRARα were treated with 100 n*M* AT-RA as indicated. Note that the AP-1 and hRARα activities are represented relative to activity in the absence of transfected c-Jun or AT-RA, both of which were set to 1. Two-sample *t* test was performed to measure *p* values for MKRN1 inhibition of (i) c-Jun in Cos, $p = 0.040$, $p = 0.031$; (ii) c-Jun in HeLa, $p = 0.116$, $p = 0.004$; (iii) RAR in Cos, $p = 0.016$, $p = 0.014$; (iv) RAR in HeLa, $p = 0.030$, $p = 0.017$. (**C, D**) MKRN1 does not alter the expression of c-Jun or hRARα. HeLa cells were transfected with 1 or 5 μg MKRN1 and (**C**) 1 μg c-Jun or (**D**) 0.5 or 1 μg hRARα and subjected to Western blot analysis.

compared to the activity of LexA alone. When yeast cells transformed with LexA-c-Jun were grown on X-gal–containing medium, all the colonies were blue (Fig. 1B). To isolate inhibitors of c-Jun, a cDNA expression library from P19 mouse embryocarcinoma cells *(30)* was co-transformed into yeast together with LexA-c-Jun and these cells were grown on X-gal–containing medium (Fig. 1C). Among 500,000 transformed colonies, 84 remained white on the X-gal medium. From these, only five were able to cause a white phenotype upon plasmid isolation and retransformation. Of the five clones, four had mouse mitochondrial DNA sequences. Interestingly, the fifth clone encoded the carboxy terminus of mouse MKRN1 *(28)*. The 470-bp of our cDNA clone matches the 3' terminus of the MKRN1 cDNA, from bp 1038 to 1508 (data not shown). Full-length mouse MKRN1 protein consists of 481 amino acids and the isolated MKRN1 cDNA clone encodes amino acids 276–466.

MKRN1 Attenuates the Transcriptional Activities of AP-1 and RAR

Because our cDNA clone expressing a truncated MKRN1 exhibited a negative activity on c-Jun transactivation in yeast, we wanted to determine if full-length MKRN1 will affect AP-1 activity in mammalian cells. We used the human MKRN1 for these studies *(28)*. HeLa or Cos cells were co-transfected with or without c-Jun and different amounts of MKRN1 plasmid. The transcriptional activity of either endogenous or transfected c-Jun was measured using the TRE-tk-CAT reporter plasmid *(25)*. The results show that, in both HeLa and Cos cells, MKRN1 can inhibit the transcriptional activity of c-Jun in a dose-dependent manner (Fig. 2A). The results in Cos cells also demonstrate that MKRN1 can inhibit endogenous AP-1 activity (Fig. 2A).

Because mutual transcriptional interference has been observed between AP-1 and RAR (reviewed in refs. *5* and *6*), we were interested in studying a possible MKRN1 activity on RAR transactivation. Therefore, HeLa or Cos cells were transfected with increasing amounts of MKRN1 and RAR transcriptional activity was monitored using the RARE-tk-CAT reporter plasmid *(31)*. MKRN was able to block the transcriptional activity of hRARα in both cell lines (Fig. 2B). Interestingly, however, MKRN1 inhibited only about 50% of all AT-RA-induced activity, suggesting that MKRN1 is a partial repressor of RAR transactivation. These results show that the negative effect of MKRN1 is neither activator-specific nor cell-specific, and therefore, may be general.

Based on the predicted structure of MKRN1, this protein is suggested to be involved in RNA processing and/or stability *(28)*, making it possible that MKRN1 represses the activity of AP-1 and RAR by downregulating protein expression. To address this possibility, Western blot analysis was

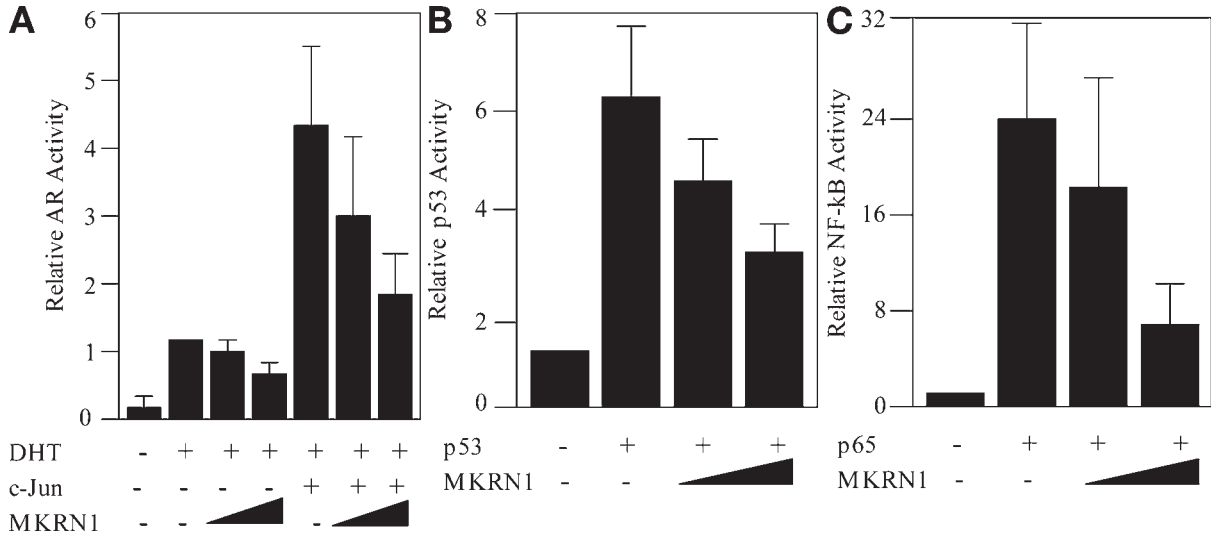

Fig. 3. MKRN1 has a general inhibitory effect on RNA polymerase II–dependent gene activators. Cos cells were transfected with 1 or 5 μg of MKRN1 and (**A**) 1 μg MMTV–CAT and 1 μg hAR with or without 1 μg c-Jun or (**B**) 1 μg p50-2-CAT with or without 1 μg of p53 or (**C**) 1 μg 3X-kB-CAT with or without 0.5 μg p65, as indicated; 100 n*M* DHT was used in A as indicated. Note that in all three experiments CAT activity is represented relative to activity of first condition (**B** and **C**) or the second condition (**A**), and this activity was set to 1. Two-sample *t*-test was performed to measure *p* values for MKRN1 inhibition of (i) AR with transfected c-Jun, $p = 0.104$, $p = 0.047$; (ii) AR with transfected c-Jun, $p = 0.092$, $p = 0.032$; (iii) p53, $p = 0.069$, $p = 0.017$; (iv) p65, $p = 0.109$, $p = 0.042$.

performed on cells transfected with either hRARα or c-Jun and increasing amounts of MKRN1, using the same conditions that block the transcriptional activity of these two proteins. MKRN1 had no detectable effect on the protein expression levels of either transfected c-Jun (Fig. 2C) or hRARα (Fig. 2D). These results argue against a MKRN1 effect on mRNA or protein stability.

MKRN1 Has a General Negative Effect on RNA Polymerase II–Dependent Transcription

The inhibition of AP-1 and RAR transcriptional activity suggests that MKRN1 may have a general negative effect on RNA polymerase II–dependent transcription. To further study this possibility, we analyzed the effect of transfected MKRN1 on several other RNA polymerase II–dependent transcriptional activators, including p53, NFκB (p65), and the human androgen receptor (hAR). Cos cells were transfected with expression plasmids for these transcription factors and the respective reporter plasmids (MMTV-CAT for hAR, p50-2-CAT for p53, and 3X-kB-CAT for p65). Increasing amounts of transfected MKRN1 resulted in a dose-dependent inhibition of all three transcription factors (Fig. 3). Interestingly, p65 exhibited the greatest sensitivity to the negative effects of MKRN1 (compare Figs. 3A,B, and C), showing that the magnitude of the MKRN1 negative effect can vary among different transcriptional activators. With respect to AR, we have previously shown that c-Jun acts as a coactivator for androgen-dependent transcription *(9,25,26)*. MKRN1 was able to inhibit AR in either the absence or presence of transfected c-Jun (Fig. 3A), showing that c-Jun–coactivated hAR is not resistant to MKRN1 inhibition. Together, these results further argue that MKRN1 may have a general negative effect on RNA polymerase II–dependent transcription.

Multiple Domains of MKRN1 Are Required for the Negative Activity

The MKRN1 protein harbors four C_3H zinc fingers motifs and a single C_3HC_4 RING zinc finger *(28)*. Three of the four zinc fingers are located on the amino-terminal half of the protein, while the RING zinc finger and the fourth zinc finger reside in the carboxy-terminal half of the protein (Fig. 5A). In order to determine the regions that are involved in mediating MKRN1's inhibitory functions, we generated two deletion mutants of MKRN1. These mutants include MKRN1(1–275), that encompasses the amino-terminal region with three zinc finger domains, and MKRN1(276–482), that contains the C-terminal region with one zinc finger and the RING zinc finger signature domain (Fig. 4A).

Using once again reporter gene assays, we monitored the activities of the two MKRN1 mutants on the transactivation functions of AP-1, RAR, and AR. As expected, transfection of full-length MKRN1 into Cos cells exerted a negative effect on the transactivation functions of both endogenous AP-1 and transfected c-Jun (Figs. 4B,C). In contrast, neither the C-terminal mutant MKRN1(276–482) nor the N-terminal mutant MKRN1(1–275) had a significant effect on c-Jun activity (Figs. 4B,C). Similar results were obtained with AR, whose activity was not significantly affected by either MKRN1 truncation mutant (Figs. 4B,C). With respect to RAR transactivation, MKRN1(1–275) did not have a significant effect (Fig. 4C). Interestingly, however, MKRN1(276–482) was almost as active in repression as was full-length MKRN1 (Fig. 4B). Interestingly, smaller truncations from

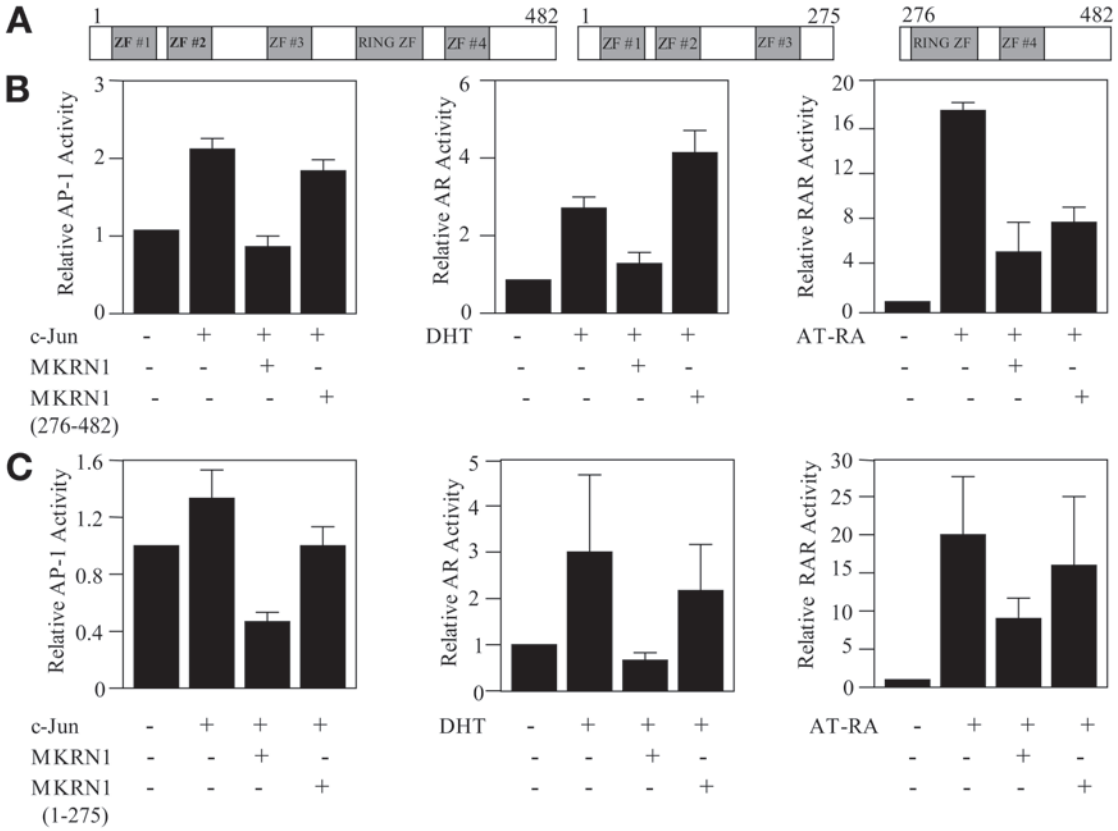

Fig. 4. Deletion of either the amino-terminal or carboxy-terminal region of MKRN1 significantly reduces its transrepression activity. (**A**) A schematic diagram of MKRN1 truncation mutants. Note that MKRN1(1–275) mutant is encoded by amino acids 1–275 and harbors three zinc fingers, while MKRN1(276–482) is encoded by amino acids 276–482 with the signature RING zinc finger and one zinc finger motif. (**B, C**) Cos cells were transfected with 1 μg TRE-tk-CAT, RARE-tk-CAT, or MMTV–CAT, 1 μg c-Jun, 0.5 μg hRARα, or 1 μg hAR, 5 μg full-length MKRN1, and 5 μg either (**C**) MKRN1(276–482) or (**D**) MKRN1(1–275). Note that, in each experiment, CAT activity is represented relative to activity of first condition, and this activity was set to 1. Two-sample *t* test was performed to measure *p* values for MKRN1 inhibition of (**B**) (i) c-Jun, $p = 0.029$, $p = 0.303$; (ii) AR, $p = 0.024$, $p = 0.080$; (iii) RAR, $p = 0.030$, $p = 0.043$; (**C**) (i) c-Jun, $p = 0.027$, $p = 0.300$; (ii) AR, $p = 0.047$, $p = 0.381$; (iii) RAR, $p = 0.050$, $p = 0.650$.

either the amino or carboxy terminus also disrupt the negative activity of MKRN1 (data not shown). Taken together, these results suggest that multiple domains of MKRN1 are necessary and must remain intact in order to retain the repressive functions of this protein on RNA polymerase II–dependent transcription. Additionally, the results suggest that the carboxy-terminal half may harbor a negative function that acts in an activator-specific manner.

The Ubiquitin Ligase Activity of MKRN1 Is Not Required for its Repression of RNA Polymerase II–Catalyzed Transcription

Recent work reported that MKRN1 has ubiquitin ligase activity, and this activity is mediated by its RING zinc finger *(29)*. To determine if this activity was required for the MKRN1 negative effect on RNA polymerase II–catalyzed transcription, the RING zinc finger motif was mutated to make MKRN1(H307E) (Fig. 5A). This mutation was previously shown to disrupt the ubiquitin ligase activity of MKRN1 *(29)*. Interestingly, MKRN1(H307E) was able to inhibit RARα-dependent transcription just as well as wild-type MKRN1 can (Fig. 5B), demonstrating that the transrepression activity of MKRN1 can be functionally separated from its ubiquitin ligase activity.

MKRN1 Bound to DNA Can Activate Transcription

To determine if MKRN1 contains intrinsic transcriptional repression domains, as do the co-repressors NCoR *(32)* and SMRT *(33)*, we used the same approach that was used with those two proteins. Hybrid proteins were generated that contained the GAL4 DNA-binding domain [GAL4(DBD)] fused to full-length MKRN1(1–482), MKRN(1–275), or MKRN1(276–482) (Fig. 6A). Cos cells were transiently transfected with a plasmid expressing one of the three MKRN1 fusion proteins and the GAL4-responsive reporter plasmid 17Mer-tk-CAT. Surprisingly, we found that full-length MKRN1 fused to the GAL4(DBD) activates in a dose-dependent manner transcription of the reporter gene (Fig. 6B). However, for the MKRN1 truncation mutants, both fusion proteins failed to yield any significant positive activity on the reporter gene (Fig. 5B). These results are in agreement with the results shown in Fig. 4, in which the amino- and carboxy-terminal truncation mutants showed no significant negative effect on several transcriptional activators. The

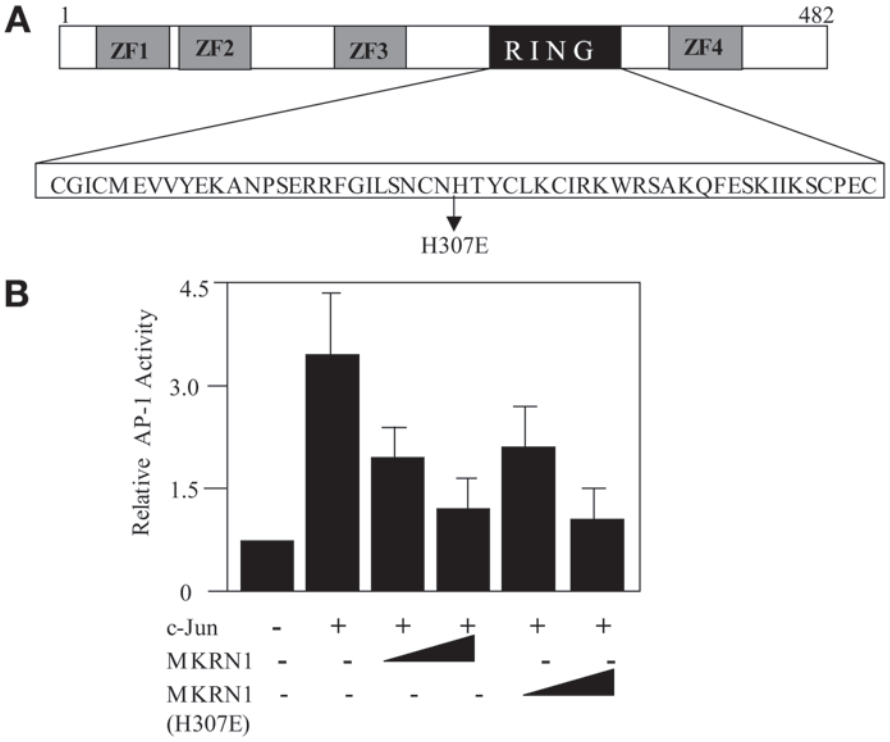

Fig. 5. Disruption of MKRN1's E3 ligase activity of MKRN1 does not alter its transrepression activity. (**A**) Schematic diagram of the MKRN1(H307E) point mutant. (**B**) To test the effect of MKRN1(H307E) on AP-1 stimulated transcription, Cos cells were cotransfected with 1 μg TRE-tk-CAT reporter, 1 μg c-Jun, and 1 or 5 μg wild-type or mutant MKRN1. CAT activity is represented relative to activity of endogenous AP-1, which was set to 1. Two-sample *t* test was performed to measure *p* values for MKRN1 inhibition of c-Jun by (i) wild-type MKRN1, $p = 0.076$, $p = 0.016$; (ii) MKRN1(H307E), $p = 0.039$, $p = 0.007$.

yeast activator protein, full-length GAL4, was used as a positive control. The strong transcriptional activity by full-length MKRN1 was not due to the fused GAL4(DBD), because this domain alone had no detectable activity (Fig. 6).

All the fusion proteins were expressed at equal levels as confirmed by the Western blot analysis (Fig. 7A), ruling out the possibility that expression differences are responsible for activity differences between full-length MKRN1 and truncation mutants. Moreover, immunohistochemistry analysis was used to show that all GAL4 fusion proteins are expressed and localized to the nucleus of transfected Cos cells (Fig. 7B), clearly demonstrating that the absence of transcriptional activity by the MKRN1 truncation mutants is not due to defective nuclear localization. All together, these results suggest that MKRN1 protein is likely a transcriptional activator involved in mediating RNA polymerase II–dependent gene activation.

MKRN1 Is Ubiquitously Expressed

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses were used to measure the expression of MKRN1 in mammalian cells. HeLa and Cos cells express significant levels of MKRN1 mRNA, which is markedly increased upon transfection of MKRN1 plasmid (Fig. 8A). MKRN1 protein was detected in a variety of mammalian cells, including Cos, NIH 3T3, P19, CHO, and LNCaP cells (Fig. 8B). Although the expression levels vary among the different cell lines, these results demonstrate that the MKRN1 protein is ubiquitously expressed, consistent with published work *(28)*.

Discussion

MKRN1 represents the ancestral gene of a family of intronless genes encoding proteins with a unique composition and order of zinc finger motifs *(28)*. This gene family consists of seven intronless loci distributed throughout the human genome and phylogenetic analysis has revealed the existence of MKRN1 orthologs in a wide variety of organisms from invertebrates to vertebrates *(28)*. ESTs have been found in plants and fungi that most likely represent MKRN1 orthologs, suggesting an ancient origin for this gene and vital cellular role(s) for its protein product. A possible important cellular function for MKRN1 is further supported by the high identity (92%) between the human and murine orthologs and the ubiquitous protein expression in human and mouse tissues *(28)*.

The multiple types and number of zinc finger motifs in MKRN1 possibly suggest multiple cellular functions for this protein. Zinc finger domains are found in many different proteins having a variety of cellular functions, including transcription, RNA processing, and DNA replication

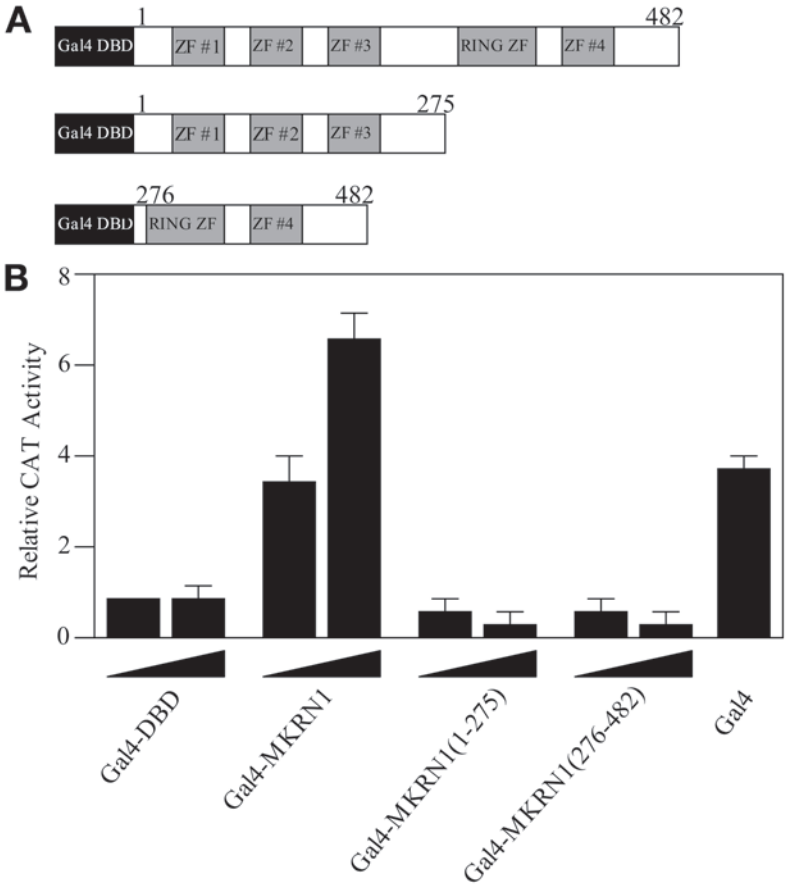

Fig. 6. MKRN1 activates transcription when bound to DNA. (**A**) A schematic diagram of MKRN1 fusions with the Gal4(DBD). (**B**) Cos cells were cotransfected with 1 μg 17-Mer-tk-CAT, 1 or 5 μg each Gal4(DBD), Gal4–MKRN1, Gal4–MKRN(1–275), and Gal4–MKRN1(276–482). 5 μg full-length Gal4 were used as positive control. CAT activity is represented relative to the activity of the first condition, and this was set to 1. Two-sample *t* test was performed to measure *p* values for transcriptional activities of Gal4–MKRN1 fusion proteins as compared to Gal4–DBD: (i) Gal4–MKRN1, $p = 0.045$, $p = 0.049$; (ii) Gal4–MKRN1(1–275), $p = 0.050$, $p = 0.005$; (iii) Gal4–MKRN1(276–482), $p = 0.042$, $p = 0.027$.

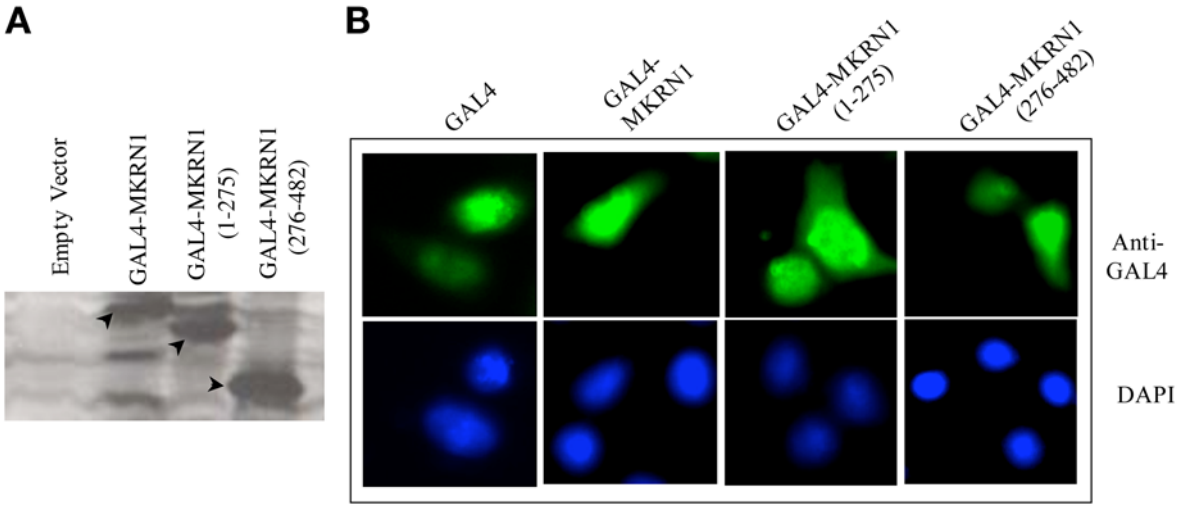

Fig. 7. Gal4–MKRN1 fusion proteins localize to the nucleus. Cos cells were transfected with 5 μg Gal4–MKRN1, Gal4–MKRN1(1–275), or Gal4–MKRN1(276–482) and subjected to (**A**) Western blot analysis or (**B**) immunofluorescence. For both experiments, the same anti-Gal4(DBD) antibody was used. Note that in B, there was an additional transfection with 5 μg full-length Gal4 and DAPI nuclear staining is shown in the lower panel.

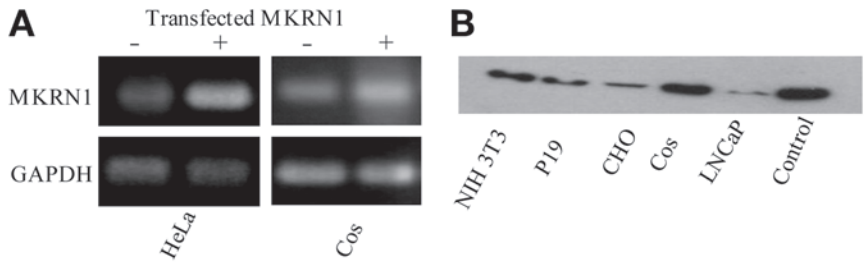

Fig. 8. MKRN1 mRNA and protein expression are ubiquitously expressed in cells. (**A**) HeLa and Cos cells were transiently transfected with or without 5 μg MKRN1 as indicated, and isolated total RNA was used in RT-PCR analysis to measure endogenous and exogenous MKRN1 gene expressions. GAPDH was used as control. (**B**) Endogenous expression of MKRN1 in different cell types was measured by Western blot analysis using an anti-MKRN1 antibody. Cos cells transfected with 5 μg MKRN1 were used as a positive control. Note that the specificity of the anti-MKRN1 antibody has been previously demonstrated *(28)*.

(reviewed in ref. *34*). Several different types of zinc finger domains have been described, including C_2H_2, C_2HC_4C, C_3H, and C_3HC_4 (reviewed in ref. *35*). MKRN1 contains four C_3H zinc fingers, one C_3HC_4 finger, and a novel Cys–His arrangement *(28)*. The C_3HC_4 type is more commonly referred to as the RING zinc finger domain, which appears to be mainly involved in protein–protein interactions (reviewed in ref. *36*). More current evidence suggests that the RING zinc finger is a signature domain for E3 ligases that mediate ubiquitination (reviewed in ref. *37*). Indeed, MKRN1 was recently shown to have E3 ligase activity on hTERT, the catalytic subunit and rate-limiting component of the telomerase holoenzyme *(29)*. This represents the first and only published work ascribing a biological activity to MKRN1.

In this report, we provide evidence for a second important nuclear function of MKRN1: transcriptional regulation. We identified MKRN1 in a yeast functional screen as a transcriptional repressor of c-Jun. Our transfection results in mammalian cells are consistent with these yeast data. Interestingly, the negative activity of MKRN1 is not targeted at only c-Jun, but affects various RNA polymerase II–dependent transcriptional activators, including the yeast activator GAL4 *(3)*. These results may suggest that MKRN1 has a general effect on RNA polymerase II–catalyzed transcription. This is reminiscent of the general repressor activity of p53 on RNA polymerase III–dependent transcription *(38)*. At this time, we do not know whether MKRN1 can affect either RNA polymerase III or RNA polymerase I activity. Certainly, our finding that the protein expression levels of exogenous c-Jun and RARα are not affected by overexpressed MKRN1 argue against a possible MKRN1 influence on translation, as it has been shown for p53 activity on RNA polymerase III *(38)*, or protein ubiquitination, as it has been shown for MKRN1 on hTERT *(29)*. Indeed, mutation of MKRN1 to abolish its E3 ligase activity on hTERT *(29)* has no effect on this protein transrepression function. Thus, it is likely that the negative activity of MKRN1 is mediated through the transcription process and is independent of protein ubiquitination.

What could be the mechanism of MKRN1 inhibition? MKRN1 may harbor inhibitory activity analogous to the nuclear receptor co-repressors NCoR and SMRT. By virtue of their association with unliganded nuclear receptors, NCoR and SMRT are recruited to target promoters, to which they bring a co-repressor complex that has histone deacetylase (HDAC) activity (reviewed in ref. *39*). Several lines of evidence argue that this is not the mechanism of inhibition utilized by MKRN1. First, based on immunoprecipitation experiments, no physical interaction could be detected between MKRN1 and its target transcriptional activators (e.g., c-Jun, RAR, AR) (data not shown). Second, MKRN1 does not appear to possess HDAC activity, because our results show that trichostatin A (TSA) has no effect on MKRN1-mediated transcriptional repression (data not shown). Finally and most importantly, MKRN1 has no inherent transcription repressor activity when tethered to DNA via a heterologous DBD, as do NCoR *(32)* and SMRT *(33)*. Surprisingly, this MKRN1 fusion protein has significant transcriptional stimulatory activity, suggesting that MKRN1 may be a transcriptional activator. If this is the case, MKRN1 may be inhibiting RNA polymerase II–dependent activators through a transcriptional interference or "squelching" process that has been proposed to be responsible for the mutually antagonistic interactions between AP-1 and some nuclear receptors (reviewed in refs. *5* and *6*). In this paradigm, a transcriptionally competent MKRN1 would interact with and sequester a common and limiting coactivator away from other transcriptional activators, thereby inhibiting these proteins. A prediction from this paradigm would be that there is a direct correlation between MKRN1's transactivation and transrepression activities. Our limited truncation analysis supports this, because deletions of MKRN1 that disrupt its transactivation function have the same effect on transrepression.

If MKRN1 is a transcriptional activator, it may have a DBD. While no direct evidence exists to support or refute this possibility, it is noteworthy that the MKRN1 protein has a collection of invariant Cys and His residues in the form of

$CX_2CX_5HX_9HX_3CX_3$. It has been suggested *(28)* that this motif may be structurally similar to the Zn_2C_6 binuclear cluster that is found in the DBDs of GAL4 *(40)* and PPR1 *(41)*. Future studies can determine whether this structural similarity makes MKRN1 a sequence-specific DNA-binding protein.

Regardless of whether MKRN1 binds to DNA or not, it is interesting that multiple regions of the protein appear to be required for both transactivation and transrepression. Deletion of either the amino or carboxy terminus interferes with both MKRN1 activities. These data may suggest that the functional MKRN1 molecule is a multimeric complex, perhaps a homodimer, that requires both ends of a monomer for multimerization, as it has been suggested for AR dimerization *(42,43)* or p53 tetramerization (reviewed in ref. *44*). MKRN1 may also act as a monomer that requires multiple domains for its transcriptional activities, perhaps some, if not all, of its multiple zinc finger domains. Another possible explanation is that large truncations of MKRN1 may disrupt the conformation of the truncated protein and thereby inhibit its activity. To address these different possibilities, we have begun to do smaller truncations of MKRN1. Preliminary results show that even these smaller deletions disrupt both MKRN1 activities (data not shown), suggesting that multiple regions of the protein are involved. Future work will utilize random point mutagenesis to map the regions of MKRN1 that are important in this protein's transcriptional activities. Interestingly, the MKRN1 regions necessary for transcriptional repression may be different in mammalian cells as compared to yeast cells. This is suggested by our findings that MKRN1 amino acids 276–466 are sufficient for inhibiting c-Jun transactivation in yeast while amino acids 276–482 cannot inhibit c-Jun in mammalian cells.

The presence of MKRN1 orthologs among a wide array of organisms and its ubiquitous tissue expression suggest that this protein may serve a vital cellular role. Previous data demonstrate that MKRN1 can regulate telomere stability by acting as an ubiquitin E3 ligase *(29)*. It has been suggested that MKRN, based on the presence of multiple C_3H zinc finger domains, may also bind RNA and be part of ribonucleoprotein particles *(28)*. We provide evidence here that MKRN1 can also regulate both positively and negatively the process of transcription. Telomere stability, RNA binding, and transcriptional regulation are basic processes in all eukaryotic cells. These molecular activities of MKRN1, which our results suggest are functionally separable from one another, may be responsible for the physiological functions of this protein. Interestingly, one such function was recently reported in which MKRN was found essential for proper kidney and bone development in the mouse *(45)*.

Materials and Methods

Plasmids

Mammalian expression plasmids for c-Jun *(26)*, hRARα *(30)*, p53 *(46)*, hAR *(26)*, and p65 (NFκB, nuclear factor kappa B) *(47)* have previously been described. To make MKRN1(1–482), GAL4-MKRN1(1–482)/pCMX (described below) was digested with *Eco*RI and *Xma*I the same two sites found in pTL3, a mammalian expression plasmid derived from pSG5 *(25)*. MKRN1 amino acids 1–275 were amplified by polymerase chain reaction (PCR) using the upstream oligonucleotide 5'-GATCGAATTCATGGCGGAGGCT GCA-3' and the downstream oligonucleotide 5'-GATCCC CGGGCTAGCGCTGCACGGCAAA-3' and inserted into pTL3, yielding MKRN1(1–275). MKRN1(276–482) was similarly constructed, using the upstream oligonucleotide 5'-GATCAAGCTTATGAGCAAGGACATGGTG-3' and downstream oligonucleotide 5'-GATCCCCGGG-CTATA GATCCAAGTC-3'.

To generate GAL4-MKRN1(1–482), MKRN1 amino acid 1–482 were PCR amplified using the upstream oligo 5'-GATCGAATTCATGGCGGAGGCTGCA-3' and downstream oligo 5'-GATCCCC GGGCTATAGATCCAAGT C-3' and inserted into pCMX-GAL4. The plasmid pCMX-GAL4 expresses the DBD (amino acids 1–147) of the yeast protein GAL4 *(26)*. To make GAL4-MKRN1(1–275), amino acids 1–275 were PCR amplified using upstream oligo 5'-GATCGAATTCATGGCGGAGGCTGCA-3' and downstream oligo 5'-GATCCCCGGGCTAGCGCTGCACGG CAAA-3' and ligated into pCMX-GAL4. GAL4-MKRN1 (276–482) was constructed by PCR amplification of MKRN1 amino acids 276–482 using the upstream oligonucleotide 5'-GATCGAATTCAGCAAGGACATGGTG-3' and downstream oligonucleotide 5'-GATCCCCGGGCTATAGATC CAAGTC-3' and inserted into pCMX-GAL4.

To generate the MKRN1(H307E) mutant protein, two complementary, MKRN1 sense 5'-ATCCTCTCCAACTG CAACGAAACCTACTGTCTCAAGTGC-3' and antisense 5'-GCACTTGAGACAGTAGGTTTCGTTGCAGTTGG AGAGGAT-3' oligonucleotide primers were used (from Integrated DNA Technology). The underlined nucleotides show mutation target site. The codon for histidine 307, CAC, was replaced with the glutamate-encoding codon, GAA. Histidine 307 is found on the RING zinc finger domain at the C-terminal region of MKRN1. For PCR reactions, the expression vector, pTL3, containing wild-type MKRN1 insert was used as template. For site-directed mutagenesis experiments, the Quikchange Site-Directed Mutagenesis kit (Stratagene) was used. All newly generated constructs were confirmed by DNA sequencing (done by Ohio State University Plant Microbe Genomics Facility).

All reporter plasmids used have the reporter gene, chloramphenicol acetyl transferase (CAT), driven by different promoters. The promoters include the RA-inducible RARE-tk (retinoic acid responsive–thymidine kinase) *(31)*, c-Jun-inducible TRE-tk (TPA responsive-tk) *(9,25)*, AR-inducible, MMTV (mouse mammary tumor virus) *(26)*, GAL4-inducible 17Mer-tk promoters *(26)*, p53-inducible, p50-2 *(46)*, and the NFκB-inducible 3X-kB-CAT *(47)*.

Cell Transfections and CAT Assays

HeLa and Cos cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Hyclone). Cells were plated in 60-mm dishes and grown to 50–60% confluency and transiently transfected using the calcium phosphate precipitation method *(4)*. Treatment with either DHT (dihydroxytestosterone) or AT-RA (all-*trans* retinoic acid), the respective ligands for hAR and RAR, were carried out 24 h posttransfection at a final concentration of 100 n*M*. Cells were washed with phosphate-buffered saline (PBS) prior to harvesting for CAT assays. β-Galactosidase (β-gal) (from 2 μg pCH110 plasmid) activity was determined and used for CAT assay standardization *(25)*. CAT assay results were quantified using the Molecular Imager FX (Bio-Rad) scanning of the autoradiograms of at least three repeats for each transfection. Thus, each CAT value represents the average of three repetitions plus the standard deviation. For all transfections, pTL1 was used to bring the final plasmid amount to 10 μg per dish.

Western Blot Analysis

For Western blot analysis, NIH3T3, P19, CHO, Cos, and LNCaP cells were grown in 100-mm dishes and subjected to calcium phosphate precipitation method of transient transfection *(25)*. Ligand treatments were performed 24 h posttransfection. Whole-cell extracts were prepared and subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis as described *(31)*. Normalization was done by using the same amount of protein for each extract and confirmed by Western blotting for β-actin. The blots were probed with an anti-hRARα antibody (sc551; Santa Cruz Biotechnology), anti-c-Jun antibody (sc45; Santa Cruz Biotechnology), anti-MKRN1 antibody (2-A7), or the anti-GAL4(DBD) (sc577; Santa Cruz Biotechnology) and secondary conjugate antibody, anti-rabbit IgG–Alexa Fluor 488 (Molecular Probes). The ECL chemiluminescence detection kit (Amersham Pharmacia Biotech) was used to develop the blots.

Semiquantitative RT-PCR

To prepare RNA for the RT reaction, total RNA from either HeLa or Cos cells was extracted using the Trizol reagent (Life Technologies). For the RT-PCR (reverse transcription-PCR) assays, cDNA was prepared from the isolated RNA using the M-MLV RT, according to the manufacturer's instructions (Fisher). The PCR reactions were carried out using MKRN1-specific primers, the upstream primer 5'-GAGCAGGTTCAGAGGACTGG-3' and the downstream primer 5'-CACTCTCCCACTGCAGCATA-3'. GAPDH-specific primers (upstream primer 5'-CGACCACTTTG-TCAAGCTCA-3' and the downstream primer 5'-AGGG GAGATT CAGTGTGGTG-3') were used in the RT-PCR reaction as a control. RT-PCR was carried out for 30 cycles using the following conditions: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The PCR products were electrophoretically separated on a 2% agarose gel and stained with ethidium bromide.

Immunohistochemistry

To confirm expression of the GAL4-MKRN1 fusion proteins and their nuclear localization, immunofluorescence assays were carried out. Cos cells were grown on culture well-chambered coverglasses (Molecular Probes) and transfected with plasmids expressing different GAL4-MKRN1 chimeras using the calcium phosphate precipitation method *(25)*. The cells were fixed with formaldehyde and immunofluorescence was carried out as described (protocol from Molecular Probes). To detect the GAL4–MKRN1 fusion proteins, we used the primary antibody anti-GAL4 (DBD) (sc577; Santa Cruz Biotechnology) and secondary conjugate antibody, anti-rabbit IgG–Alexa Fluor 488 (Molecular Probes); 300 n*M* 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) was used for nuclear staining and the antifade reagent, proLong Gold (Molecular Probes), to mount the specimen for fluorescence microscopy (Zeiss Fluorescence Microscope).

Yeast Functional Screen

The yeast transformation protocol was described previously *(48)*. A 20-mL culture of *YPH*499/pSH18-34 *(49)* was grown and transformed with c-Jun/pEG202in Glu/CM-Ura-His liquid dropout medium overnight at 30°C. The culture was diluted into a 300-mL Glu/CM-Ura-His liquid dropout medium with 2×10^6 cells/mL and incubated at 30°C until OD_{600} reading reached 0.5, after which it was centrifuged for 5 min at 1000–1500*g*. The liquid was discarded and the cells resuspended in 1.5 mL TE buffer/0.1 *M* lithiumm acetate; 1 μg of P19 library *(30)* and 50 μg of sheared salmon sperm carrier DNA were added to each of 30 sterile 1.5 mL microcentrifuge tubes. Fifty microliters of the resuspended yeast cells were added to each tube and they were incubated for 30 min at 30°C and DMSO was then added to 10%. The samples were heat-shocked for 10 min at 42°C. For 28 tubes, the complete contents of one tube was added per 24 × 24-cm Glu/CM-Ura-His-Leu/X-Gal dropout plate and incubated at 30°C. For the remaining two tubes, 360 μL of each tube was spread on 24 × 24-cm Glu/CM-Ura-His-Leu/X-Gal dropout plate. The remaining 40 μL from each tube was used to make a series of 1/10 dilution in sterile water. Dilutions were plated on 100 mm Glu/CM-Ura-His-Leu dropout plates. All plates were incubated at 30°C until colonies appeared (2–3 d). Colonies were monitored for color (blue or white). Among 500,000 transformed colonies, 84 white colonies appeared on the X-gal medium. Plasmid was isolated from each of the white colonies and used for retransformation. Only five of the isolated plasmids were able to cause a white phenotype upon retransformation.

Of these five clones, four had mouse mitochondrial DNA sequences. Interestingly, the fifth clone encoded the amino terminus of mouse MKRN1 *(28)*.

Yeast Liquid β-Gal Assay

A single yeast colony was inoculated in 3 mL YPD (or appropriate selective) medium and incubated overnight at 30°C, 20–50 μL of each overnight culture was inoculated in 4 mL YPD medium (or appropriate selective medium and/or inducing conditions) and grown to mid- or late-log phase. This was subjected to a liquid β-gal assay using ONPG (2-nitrophenyl β-D-galactopyranoside) as described *(50)*.

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