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Retinoblastoma Binding Factor 1 Site in the Core Promoter Region of the Human *RB* Gene Is Activated by hGABP/E4TF1[1]

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Abstract

We previously reported two oncogenic point mutations present in the *RB* (retinoblastoma) gene promoter region, found at consensus Sp1 and ATF sites, respectively, and in two separate hereditary RB families. However, Sp1 protein was shown not to bind to the Sp1 site; this indicated that the Sp1 consensus site mutation was blocking the action of an alternative transcription factor, which we called RBF-1 (retinoblastoma binding factor 1). Subsequent purification of RBF-1 revealed it to be hGABP/E4TF1, a transactivator from the adenovirus early-region 4 promoter. In this study, we directly examined the effects of hGABP/E4TF1 on transactivation of the *RB* gene promoter through the RBF-1 site. As expected, hGABP/E4TF1 enhanced the core RB promoter activity, whereas it did not stimulate a mutant RBF-1 site. We therefore conclude that the most essential transcription factor in the human *RB* gene is likely to be hGABP/E4TF1.

Introduction

The *RB*[4] gene is a well known tumor suppressor, believed to suppress carcinogenesis in a variety of malignant tumors (1). When the function of the RB protein was found to be regulated by phosphorylation (2), it was speculated that transcriptional regulation of the *RB* gene might not be essential. However, transcription of RB was found to be drastically increased during cellular differentiation (3–5). In addition, we found two oncogenic germ-line mutations in the promoter region of the *RB* gene that repress transcription, suggesting that transcriptional inactivation may be important in predisposition to *RB* (6).

One of these mutation was found at a consensus Sp1 recognition sequence, and the other was found at the neighboring ATF recognition sequence. However, when we examined Sp1 protein binding, we found that it did not bind to the consensus Sp1 site of the normal RB promoter, implying the presence of an unknown transcription factor that we termed RBF-1 (6). The sequence of the RBF-1 site indicated that it may be an Ets binding sequence (7). Subsequently, we purified the protein that binds this site and found it to be the same as E4TF1-60 and E4TF1-53 (7). E4TF1, a member of the Ets transcription factor family (8), was originally identified as a transactivator of adenovirus E4 gene (9). Because E4TF1 is thought to be the human homologue of rat GABP (10), E4TF1 and its subunits have been renamed according to the hGABP nomenclature. hGABPα and hGABPβ1 correspond to E4TF1-60 and E4TF1-53, respectively.

In this study, we have examined the effects of hGABP on stimulation of the *RB* gene promoter. Using *Drosophila* SL2 cells, we cotransfected hGABP expression plasmids together with luciferase reporter plasmids containing normal or various mutant RB promoters. We chose these cells because, in contrast to mammalian cells, they are highly responsive to exogenous transcription factors (present study; Refs. 11 and 12). We found that hGABP stimulates the normal RB promoter. In addition, hGABP drastically enhanced promoter activity of a tetramer RBF-1 sequence. Furthermore, hGABP increased the core promoter activity containing only RBF-1, ATF, and E2F sites. However, hGABP did not increase activity from the same core promoter element when it harbored a point mutation at the RBF-1 site. These experiments showed that hGABP has a strong transactivating effect on the *RB* gene promoter, suggesting that hGABP is the main transactivator for the core promoter element of the *RB* gene.

Materials and Methods

Constructions of Reporter Plasmids. A series of plasmids was produced in which normal or mutated versions of the RB promoter region were inserted into the expression plasmid pXP2 (13) containing a luciferase reporter gene. pXRP1 contains 683 bp of a normal human RB promoter region between −686 and −4 bp, relative to the translation start site. pXRP2 contains a full-length RB promoter region with a point mutation at the ATF site (a G-to-T transversion, 189 bp upstream of the translation start site). pXRP3 contains a full-length RB promoter region with a point mutation at the RBF-1 site (a G-to-A transition, 198 bp upstream of the translation start site). These plasmids have been described previously (6).

An additional set of constructs was based on the shorter core region of the RBF-1 promoter that is composed of the RBF-1, ATF, and E2F sites and has the following sequence: 5′-GACGCCGCGGGC<u>G</u>GAAGTGACGTTTTC-CCGCGGTTGGACGC-3′. Plasmid pE1RP1 contains this normal core promoter region and was generated by subcloning the appropriate PCR fragment amplified from pXRP1 into pXP2 (13). pE1RP3 contains the same core promoter region, but the *underlined* G in the above sequence is replaced with an A (corresponding to the base mutated at the RBF-1 promoter site). RBF-1x4 contains four tandem repeats of RBF-1 site containing overlapping Sp1 sites (5′-CGCGGGCGGAAGTG-3′) and was generated by subcloning a PCR fragment of four tandem repeats of the RBF-1 site into pXP2. E4-luc contains the adenovirus E4 promoter region between 39 and −324 bp relative to the transcription start site linked to the luciferase coding region, which was generated by subcloning the appropriate PCR fragment amplified from E4 promoter into the luciferase expression plasmids PGL2-Basic (Promega). 4TF1-luc contains four tandem repeats of the TF1 site (CGGAAGTG) and TATA box derived from the E4 promoter linked to the luciferase coding region, which was generated by subcloning a DNA fragment consisting of four

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[4] The abbreviations used are: RB, retinoblastoma; RBF-1, retinoblastoma binding factor 1; ATF, activating transcription factor; E4, early region 4; GABP, GA binding protein; hGABP, human GABP; SL2, Schneider line 2; TF1, E4TF1 binding site.

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tandem repeats of the TF1 site and a TATA box derived from the E4 promoter into the luciferase expression plasmid PGL2-Basic.

Transfection Assay in SL2 *Drosophila* Cells. Using SL2 cells, 2 μg of reporter plasmid were cotransfected with various combinations of hGABP/E4TF1 expression plasmids for *Drosophila* cells (6 μg of A5cΔ hGABPα hGABPα expression plasmid, 6 μg of A5cΔhGABPβ1 hGABPβ1 expression plasmid alone, or a combination of them). The total amount of expression plasmids was adjusted to 12 μg by A5cΔp control plasmid. pXRP1, E4-luc, RBF-1x4, 4TF1-luc, pE1RP1, or pE1RP3 was used as reporter plasmid (Fig. 1). Three h before transfection, cells were replated onto 6-cm Petri dishes at a density of 3×10^6 cells/plate. Cells were transfected by the calcium phosphate precipitation method as described previously (14). Forty-eight h after transfection, luciferase activity was measured as described previously (15). Luciferase activity was normalized for the amount of protein in each cell lysate. Relative luciferase activity is shown as raw light units/1 μg of protein in cell lysates. The luciferase assays were performed in triplicate, and the data are shown as means ± SE ($n = 3$). Values were analyzed statistically relative to the control without E4TF1 expression plasmids using Student's *t* test.

Transfection Assay in Mammalian Cells. On day 0, cells were replated onto 6- or 10-cm Petri dishes at a density of $2–10 \times 10^5$ cells/plate. On day 1, Saos-2, MG-63, HTB-9, and C2 cells were transfected by the calcium phosphate precipitation method as described previously (16). A549, B104, and differentiated F9 cells were transfected by the DEAE-dextran method as described previously (17). In the cotransfection assay, 2 μg of pXRP1 (Fig. 1) were cotransfected with combinations of hGABP/E4TF1 expression plasmids for mammalian cells (2 μg of pSRα-hGABPα hGABPα expression plasmid and 2 μg of pSRα-GABPβ1 hGABPβ1 expression plasmid). As the control, 4 μg of pSRα-296 control plasmid were transfected. In the mutational analysis, 2 μg of pXRP1, pXRP2, or pXRP3 were used as the reporter plasmids (Fig. 1). Forty-eight h after the transfection, luciferase activity was measured as described previously (15). Luciferase activity was normalized by the amount of protein in each cell lysate. Relative luciferase activity represents the measured luciferase activity divided by the amount of protein in cell lysates and is normalized so that the cotransfection with control plasmid equals 100% or pXRP1 equals 100%.

Results

Formation of a heterodimer complex of hGABPα and hGABPβ1 is required for transcriptional activation of adenovirus E4 promoter *in vitro* (10, 18, 19). We therefore examined whether both factors are also necessary to exert transcriptional activity in the RBF-1 site of the *RB* gene. As shown in Table 1, the expression of hGABP was found

Table 1 *Comparison of the effects of hGABP on stimulation of the RB gene promoter activity in various mammalian cell lines*

Cell line	Cell type	Relative luciferase activity	
		Control	hGABP
Saos-2	Human osteosarcoma	100%	158%
MG-63	Human osteosarcoma	100%	67%
HTB-9	Human bladder carcinoma	100%	43%
C2	Mouse myoblast	100%	93%

to enhance the activity of the RB promoter in Saos-2 human osteosarcoma cells alone. However, the responsiveness of the RB promoter to hGABP was low and was not statistically significant.

To elucidate the effects of hGABP on stimulation of the RB gene promoter, we decided to use *Drosophila* SL2 cells as a host for the cotransfection assay. Because the responsiveness of the RB promoter to hGABP in mammalian cells was low, and because the above-mentioned insect cells lacked several transcription factors that might influence this process, a cotransfection assay using SL2 cells was expected to prove useful for the analysis of a variety of mammalian transcription factors (11, 12). We found that hGABP stimulated the normal RB promoter in these insect cells. In addition, hGABP drastically enhanced promoter activity of a tetramer RBF-1 sequence. As shown in Fig. 2*A*, neither hGABPα nor hGABPβ1 alone could stimulate RB promoter activity from pXRP1 or from the tetramer of RBF-1. In contrast, cotransfection of A5cΔhGABPα and A5cΔhGABPβ1 was found to enhance the activity of the RB promoter by about 10-fold and to enhance that of the tetramer of RBF-1 element by 13-fold (Fig. 2*A*). These results indicate that both hGABPα and hGABPβ1 are required to activate the RBF-1/E4TF1 site, similar to the stimulatory effect of hGABPα and hGABPβ1 on adenovirus E4 promoter.

Cotransfection with hGABP expression plasmids was found to stimulate the RB promoter activity by about 3-fold (Fig. 2*B*). As a positive control, we used an adenovirus E4 promoter-luciferase plasmid, E4-luc (Fig. 1), and confirmed that the hGABP expression plasmids could enhance the E4 promoter activity by about 3-fold (Fig. 2*B*). We generated a tetramer of the RBF-1 element-luciferase fusion plasmid (Fig. 1) to examine whether hGABP expression plasmids

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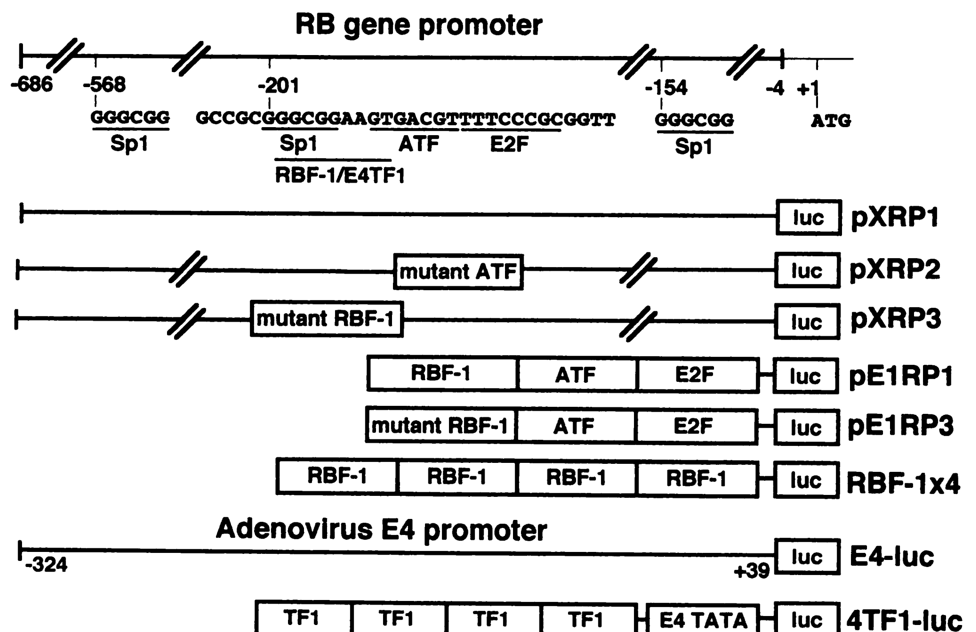

Fig. 1. Schematic representation of normal (*pXRP1*), mutant (*pXRP2* and *pXRP3*), and deletion (*pE1RP1*, *pE1RP3*, and *RBF-1×4*) RB promoter-luciferase plasmids and normal (*E4-luc*) and deletion (*4TF1-luc*) adenovirus E4 promoter-luciferase plasmids used in transfection assays. The *boxes* show the locations of the *cis*-acting elements (*RBF-1*, *ATF*, *E2F*, or *TF-1* site). The sequence is numbered relative to the start site of translation.

increase RB promoter activity through the RBF-1 site. The hGABP expression plasmids drastically increased the luciferase activity driven by the tetramer of the RBF-1 element to approximately 47-fold, whereas in the positive control, they only enhanced luciferase activity driven by a tetramer of hGABP/E4TF1-binding element of adenovirus E4 promoter (4TF1-luc; Fig. 1) by about 2-fold (Fig. 2*B*). These results suggest that hGABP stimulates the *RB* gene promoter activity through the RBF-1 site.

To confirm whether hGABP actually stimulates the RBF-1 site, we generated the following plasmids: pE1RP1, including only the core promoter region of RBF-1 and the neighboring ATF and E2F sites; and pE1RP3, a similar plasmid containing a point mutation at the RBF-1 site (Fig. 1). Whereas the hGABP expression plasmids A5cΔhGABPα and A5cΔhGABPβ1 stimulated the core promoter activity from pE1RP1 by about 3-fold, they did not enhance the same region containing a point mutation at the RBF-1 site from pE1RP3 (Fig. 2*B*). In contrast, when the hGABP expression plasmids enhanced full-length RB promoter activity from pXRP1 about 5-fold, they also increased the RB promoter activity from pXRP3 containing the full-length RB promoter region with a point mutation at the RBF-1 site (data not shown). This transactivation of the promoter activity from the larger pXRP3 promoter region by hGABP may be due to the presence of some other Ets family recognition motifs in the RB promoter region. We therefore conclude that the RBF-1 site is responsible for transactivation by hGABP but that there are likely to be other responsive sites of hGABP in the RB promoter region.

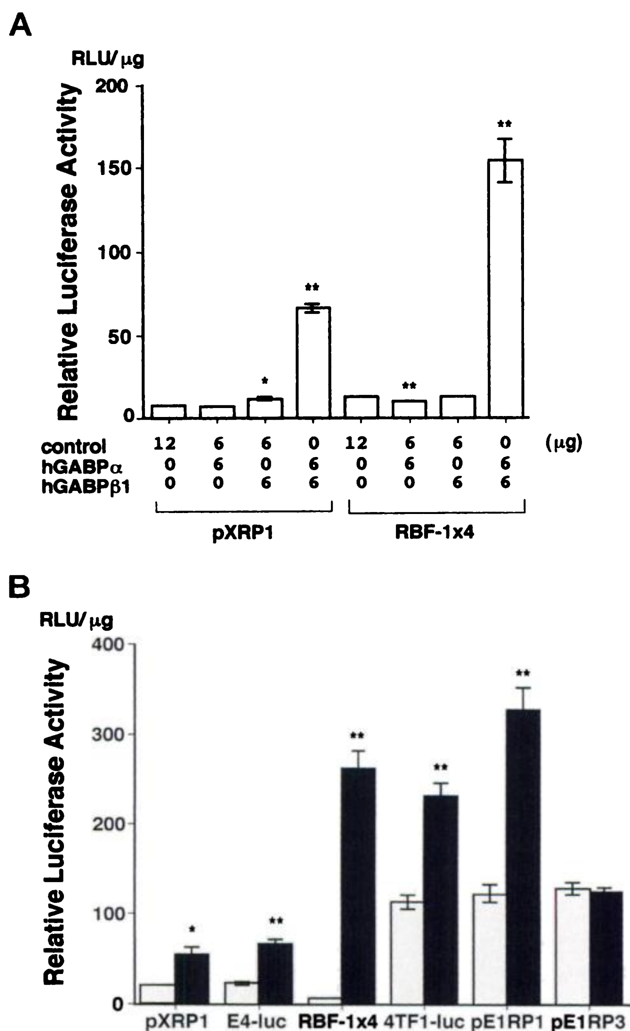

Fig. 2. *A*, Both hGABPα and hGABPβ1 are required for activation of the *RB* gene promoter in SL2 cells. pXRP1 or RBF-1×4 was cotransfected with various combinations of expression plasmids as indicated below. *, $P < 0.05$; **, $P < 0.01$; *control*, A5cΔp; *hGABPα*, A5cΔhGABPα;*hGABPβ1*, A5cΔhGABPβ1. *B*, hGABP stimulates the RB promoter activity in SL2 cells. Using SL2 cells, the various reporter plasmids were cotransfected with hGABP expression plasmids for SL2 cells. pXRP1, E4-luc, RBF-1×4, 4TF1-luc, pE1RP1, or pE1RP3 was used as a reporter plasmid (Fig. 1). *, $P < 0.05$; **, $P < 0.01$; □, control, A5cΔp′, ■, hGABP expression plasmids A5cΔ hGABPα and A5cΔ hGABPβ1.

Table 2 *Comparison of the effects of mutations at the ATF or RBF-1 site on the RB promoter activity*

Cell line	Cell type	Relative luciferase activity		
		pXRP1	pXRP2	pXRP3
A549	Human lung adenocarcinoma	100%	29%	14%
Saos-2	Human osteosarcoma	100%	19%	5%
B104	Rat neuroblastoma	100%	41%	21%
DF9	Rat teratocarcinoma (differentiated)	100%	17%	11%
C2	Mouse myoblast	100%	6%	5%

As reported previously, the core promoter region of the *RB* gene promoter includes a consensus Sp1 recognition sequence that overlaps the RBF-1 site (6). However, we originally pointed out that consensus Sp1 protein does not bind to the Sp1 site (6). Therefore, we investigated whether Sp1 stimulates the RBF-1 site and found that Sp1 does not stimulate the tetramer of the RBF-1 site containing the overlapping Sp1 site from RBF-1×4 plasmid (data not shown).

In a previous study, we showed that point mutations at both the RBF-1 and ATF sites decrease RB promoter activity (6). To determine which site of the RB core promoter is more important for promoter activity, we compared the promoter activities from pXRP1, pXRP2 (which has a point mutation at the ATF site), and pXRP3 (which has a point mutation at the RBF-1 site). In the cell lines examined, a point mutation at the RBF-1 site drastically decreased the RB promoter activity (Table 2). Similarly, a point mutation at the ATF site also decreased RB promoter activity (Table 2). However, reduction of the RB promoter activity by a mutation at the RBF-1 site was usually greater than that produced by mutation at the ATF site, although the reduction by each mutation was almost the same in C2 cells (Table 2). Therefore, we conclude that the RBF-1 site is the most essential site in the *RB* gene promoter region.

Discussion

In a previous report, we clarified that the RBF-1 site and the overlapping Sp1 and ATF sites are essential for core *RB* gene promoter activity, because naturally occurring point mutations at these sites markedly reduce this activity (6). In addition, we demonstrated that hGABP preferentially binds to the RBF-1 site (7). In the present study, we have shown that hGABP, which consists of hGABPα and hGABPβ1, can stimulate the *RB* gene promoter in SL2 cells. These results elucidate the etiology of hereditary RB that occurs as a consequence of a naturally occurring point mutation in this site.

In mammalian cells (Saos-2 human osteosarcoma, MG-63 human osteosarcoma, HTB-9 human bladder cancer, and C2 mouse myoblast), hGABP had little or no stimulatory activity on the RB promoter, but this was statistically insignificant (Table 1). These results are consistent with the fact that hGABP stimulates adenovirus E4 promoter in SL2 cells but not in mammalian cells (10). We speculate that this lack of activity might occur if hGABP, present in mammalian cells, saturates RBF-1 site. In studies of human Sp1, Courey and Tjian (11) described that the ideal host for a cotransfection assay would be a mammalian cell line that does not normally express the transcription factor under examination to provide a null background; however, Sp1 is likely to modulate the activity of essential genes required for cell viability. To overcome this problem, they used SL2 cells as a host for

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the cotransfection assay of Sp1. In our studies of hGABP, a similar problem was likely, because our results suggest that hGABP may modulate expression of the *RB* gene. Interestingly, it is through the RBF-1 site that bcl-3 stimulates this promoter (20). Taken together, these results reflect the importance of hGABP as a key functional transcription factor operating through the RBF-1 site.

In many cell lines, a point mutation at the RBF-1 site produces a marked decrease in RB promoter activity, which is usually greater than that at the ATF site (present study; Ref. 21). These results suggest that hGABP is the transcription factor that most strongly affects the expression of the *RB* gene. The *hGABPα* and *hGABPβ1* genes are located on chromosomal regions 21q21 and 7q11.21, respectively (22). Interestingly, structural abnormalities are frequently identified at chromosome region 7q11 in non-small cell lung cancer cell lines (23, 24) and at chromosome band 21q21–q22 in lipomas (25). We speculate that in some cases of human malignant tumors, inactivation of the *hGABP* gene might lead to transcriptional inactivation of the *RB* gene, thereby causing malignancies.

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