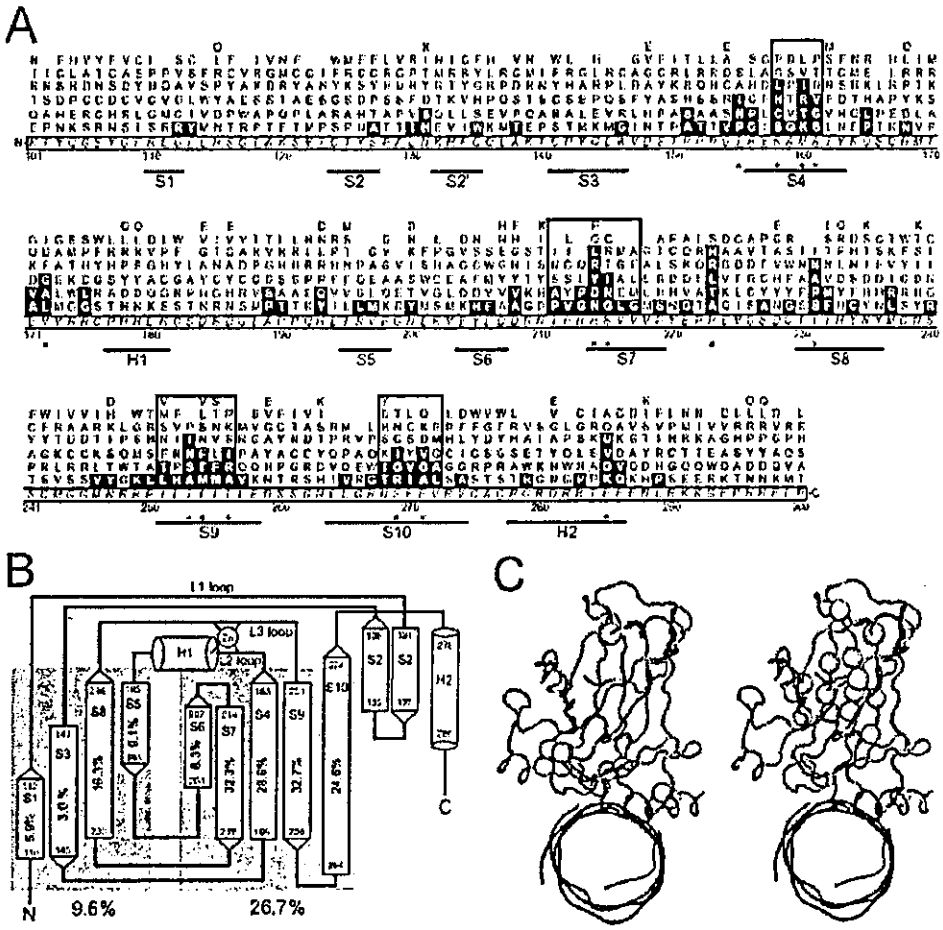

FIG. 4. **Location of the ts p53 mutants in the core DNA-binding domain.** *A*, precise map of the constructed p53 mutants and ts mutants (*white characters* in *black boxes*) in codons from 101 to 300. *Italicized characters*, original residues observed in wild-type p53; *open boxes*, four hot spots for ts mutants; *asterisk*, 15 hot spot residues for ts mutants; *bold lines*, secondary structures (11 β-strands and 2 α-helices). *B*, schematic representation of the core DNA-binding domain (14) and the fraction of the ts mutants in the two β-sheet structures. *Percentages*, fraction of the ts mutants within the constructed mutants in the indicated secondary structures; *small numbers*, codon numbers showing the NH_2-terminal and COOH-terminal ends of the indicated secondary structures. *C*, three-dimensional structure of p53 core DNA-binding domain with double-strand DNA oligonucleotides. Positions of the 10 tumor-derived hot spot residues (*left panel*) were compared with 15 representative ts hot spot residues (*right panel*). p53 and the interacting DNA structure were derived from Protein Data Bank file 1TUP (14), and views from the longitudinal axis of DNA are shown using CD3n 4.0 software (22).

p53 structure by their post-translational mechanisms. Second, as shown in Fig. 2, there is distinct strength in ts transactivation, and some reported ts mutants have been eliminated from our criteria because of a weak ts phenotype. In fact, several mutants clustered in Fig. 1*B* were not selected in our defined criteria. We also note that many previously identified ts mutants had a weak ts phenotype in our yeast screening (data not shown).

Promoter Specificity of the ts p53 Mutants—We have shown that several p53 mutants differ in transactivity spectrum in different p53 binding sites (18). Similarly, ts mutants differed in the ts transactivity spectra in different p53 binding sites (Fig. 3). We speculate that there are subtle differences in structural alterations caused by specific mutations and temperatures and that such alterations are responsible for the partial inactivation or reactivation of p53-binding to the distinct DNA sequences. In fact, there are similarities in the transactivity spectra among mutants in the same or contiguous residues (Fig. 3*A*), suggesting similar structural alterations. In particular, some showed ts in only one or two promoters, suggesting the possible application of such mutants in the conditional transactivation of specific promoters to study p53 downstream gene functions. Various ts transactivity spectra on different p53-responsive promoters were also observed in mammalian cells (Fig. 3*B*). The promoter selectivity of wild-type p53 by Ser^{46} phosphorylation has been shown as the mechanism of *p53AIP1* transactivation (25). Overall, from the results of this study and our previous observations (18), we propose that there may be other unknown potential mechanisms determining the promoter selectivity of wild-type p53 on p53 downstream promoters other than the *p53AIP1* gene. The ts transactivity against different promoters was similar in part but significantly different between human and yeast cells (data not shown). We speculate that there are several reasons for this discrepancy. First, the p53 binding elements, other than $p21^{WAF1}$ and *MDM2* used in the yeast study, were three copies of the specific p53-binding elements and differed from the genomic sequences used in the mammalian cell study. Second, the temperature for the identification of ts mutants in yeast was 30 °C, whereas it was 32 °C in mammalian cells. Third, post-translational modification and the interaction of other proteins may differ in yeast cells and mammalian cells. Finally,

TABLE II
Frequency of residue of ts mutant p53 in the DNA-binding domain before and after substitution

Residue	No. of constructed mutations	No. of ts mutants	Frequency of ts mutant
Before substitution (original residue at mutation site)			
Ile	42	9	21.43
Thr	76	16	21.05
Trp	5	1	20.00
Met	36	7	19.44
Glu	66	12	18.18
Ala	42	7	16.67
His	49	8	16.33
Arg	99	14	14.14
Pro	78	11	14.10
Phe	30	4	13.33
Val	76	9	11.84
Ser	86	9	10.47
Asn	63	6	9.52
Lev	75	6	8.00
Gln	36	2	5.56
Tyr	48	2	4.17
Gly	78	3	3.85
Asf	56	2	3.57
Cys	60	2	3.33
Lys	36	0	0
After substitution (substituted residue at mutation site)			
Gly	75	15	20
Ile	50	9	18
Ala	84	14	16.67
Leu	74	12	16.22
Pro	68	9	13.24
Asn	49	6	12.24
Met	33	4	12.12
Val	99	12	12.12
Thr	67	8	11.94
Tyr	47	5	10.64
Arg	86	9	10.47
Phe	43	4	9.30
His	55	5	9.09
Ser	98	7	7.14
Lys	42	3	7.14
Gln	38	2	5.26
Trp	21	1	4.76
Cys	43	2	4.65
Asp	56	2	3.57
Glu	35	0	0

the criteria to define ts mutants were strict and differed between yeast and mammalian cell systems. Therefore, there may be ts mutants defined by the yeast system but not by the mammalian cell system and *vice versa*, in addition to those showing ts phenotypes in both systems.

Ts Mutants and the Structure of the p53 Protein—We isolated 142 (6.1%) ts mutants from 2,314 p53 missense mutations. Most were mapped in the core DNA-binding domain (131, 91.5%), and a few were in the NH_2-terminal (4, 3.5%) or COOH-terminal (7, 4.9%) domains. The results indicated that the ts mutants isolated in this study may be mutants that directly affect sequence-specific DNA binding rather than mutants affecting the p53 function through post-translational modifications and protein-protein interactions. Within the DNA-biding domain, 50% (71 of 142) of the ts mutants were mapped on β-strands (S1–S10). As only 16% of the residues in the DNA-binding domain form β-strands, it is clear that the β-strands have more ts substructure than α-helices and loops in the DNA binding domain. Among the 11 β-strands, four areas in S4, S7, S9, and S10 are hot areas for ts mutants (see above), and, therefore, the ts mutants tended to be concentrated in one of the two β-sheets (Fig. 4*B*). In particular, residues Thr^{155}, Arg^{158}, Met^{160}, Ala^{161}, Val^{172}, His^{214}, Ser^{215}, Pro^{223}, Thr^{231},

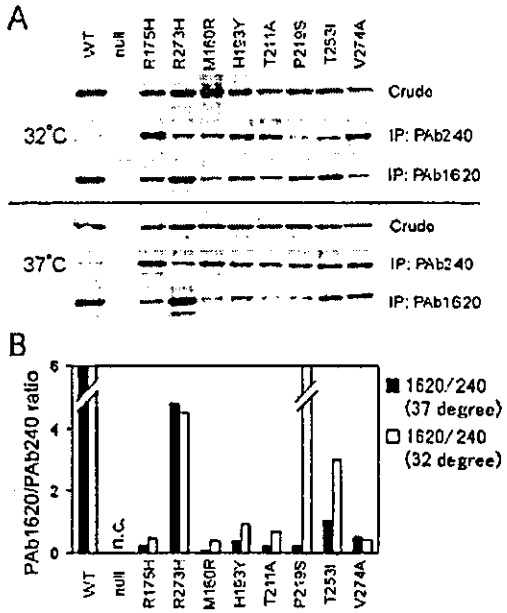

FIG. 5. **Expression of conformation-sensitive epitopes.** *A*, the indicated p53 proteins were expressed from the corresponding p53 expression vectors in the human osteosarcoma cell line, Saos-2, at 32 °C and 37 °C. The crude cell lysate was separated by SDS-polyacrylamide gel electrophoresis immediately or after immunoprecipitation (*IP*) using PAb240 or PAb1620. The p53 proteins were detected using an HRP-conjugated anti-p53 polyclonal antibody. *WT*, wild-type. *B*, the ratio of PAb1620 expression to PAb240 expression at 32and 37 °C. The expression of either PAb1620- or PAb240-reactive p53 proteins (shown in *panel A*) was quantitatively analyzed, and the ratio of the values are shown as graphs. *n.c.*, not calculated.

Thr^{253}, Ile^{254}, Thr^{256}, Ser^{269}, Glu^{271}, and Glu^{285} are ts hot spots (Fig. 4*A*). The ts hot spot mutants are spatially located relatively far from the p53-DNA interface, whereas the tumor-derived 10 hot spot mutations (except Thr^{220}) are formed or located close to the interface (Fig. 4*C*). Although the structural reason why the second β-sheet was more susceptible to ts is still unclear, this observation indicated that the β-sheet was a key structural element controlling the p53 function, suggesting the existence of a potential intramolecular mechanism in normal p53 regulation of the promoter selectivity after post-translational modification such as damage-sensitive phosphorylation.

The consideration of ts-specific amino acid residues before and after substitution is of great interest, because such information may provide a better understanding of the structure of ts mutants. In p53 protein, the majority of the sensitive residues before substitution comprised isoleucine, threonine, and tryptophane, all of which are hydrophobic residues preferentially used in the β-strands of many other proteins (26). On the other hand, the most frequent residues after substitution were glycine, isoleucine, alanine, leucine, and proline. Because these residues are smaller hydrophobic residues, it is possible that the structure of the β-strands is largely undisrupted. Less frequent residues were negatively charged, *i.e.* aspartic acid and glutamic acid. This result was reasonable, because these are known to be β-strand-disrupting residues (26). An unexpected result was frequent proline substitution, because proline is known to be a structurally stable residue and, therefore, should not be vulnerable to subtle structural change due to temperature shift.

The results of immunoprecipitation using conformation-sensitive antibodies were unexpected, because only a limited num-

ber of the mutations examined showed ts changes in the expression of epitopes. We speculate that most ts mutants partially recovered their structural alteration, but their structure and transactivation function were not completely restored. It will be interesting to examine whether such partial restoration of p53 function is sufficient to suppress tumor formation and/or progression when expressed under physiological conditions.

Frequency of ts Mutants in TP53 Mutation Databases—According to the latest International Agency for Research on Cancer (IARC) data base for tumor-derived somatic mutations (17), 1,135 distinct missense mutations, including 1,066 missense mutations with a single nucleotide substitution, are registered. These mutations have been reported 12,032 times in total. Among them, 10.3% (110 of 1,066) of mutants were thought to be ts mutants, and such ts mutations comprised 10.4% (1,254 of 12,032) of the total number of mutations. Therefore, we conclude that ts p53 mutation is not as rare as it was previously thought to be (19), and it may be a molecular target for the pharmacological rescue of p53 protein.

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Oral Session

Collaboration of Breast Cancer Clinic and Genetic Counseling Division for BRCA1 and BRCA2 Mutation Family in Japan

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Background: BRCA1 and BRCA2 mutations cause high breast cancer incidence rates as high as 80%. Although prophylactic therapy is still controversial, several prophylactic therapies have been proposed and tried for BRCA1 and BRCA2 mutation carriers. Prophylactic surgery, chemo-prevention and precise screening have been proposed as prophylactic therapy. All BRCA1 and BRCA2 mutation carriers need knowledge about their disease and the countermeasures that are used to protect against onset of disease. Counseling plays an important role in this regard for people with genetic diseases. Therefore, collaboration between breast cancer clinics and genetic counseling services is the most important issue in clinical practice. Our group consists of three national universities and a general hospital. In this article we describe our trial to construct a clinical system against hereditary breast cancer as an interim report for the Japanese Ministry of Health, Labour and Welfare.

Patients and Methods: Twenty familial breast cancer patients were registered in this study. The whole sequence of BRCA1 and BRCA2 were analyzed. If pathological mutations were detected, their first degree families were introduced to the counseling division at each institute when candidates visited counseling divisions.

Results and Discussion: Four cases of a deleterious mutation in BRCA1 or BRCA2 were detected among 20 cases. Their first degree relatives are now under consideration for visiting counseling divisions. The clinical system described in this study should play a role to protect BRCA1 or BRCA2 mutation carriers in Japan.

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Key words: Hereditary breast cancer, Familial breast cancer, BRCA1, BRCA2

Breast cancer is the most common malignancy observed in Japan and western countries. Treatment of groups at high-risk for breast cancer is also a challenging problem. Familial breast cancer is an important factor impacting the early and bilateral onset of breast cancer. Five to 10% of breast cancer patients meet the definition of familial breast cancer[1,2]. BRCA1 and BRCA2 mutations have been reported as major causes of familial breast cancer. Twenty to thirty percent of Japanese familial breast cancer patients show deleterious BRCA1 or BRCA2 mutation[3]. Further, it has been reported that 80-90 percent of BRCA1 and BRCA2 mutation carriers suffer from breast cancer in their lives[4]. This high risk of breast cancer incident suggests the importance of screening for BRCA1 and BRCA2 mutations and preventive measures for familial breast cancer. Counseling and prophylactic therapies are of importance in prevention of such genetic diseases. Familial breast cancer patients and their first degree relatives are anxious about their genetic status. There is no standard clinical system and method for prophylactic therapy for BRCA mutation carriers in Japan. Hospitals or medical systems that provide proper knowledge of familial breast cancer and preventive therapies are needed. A larger number of patients are required to establish interventions for Japanese BRCA1 or BRCA2 mutation carriers. In this study we aimed to construct a multi-center

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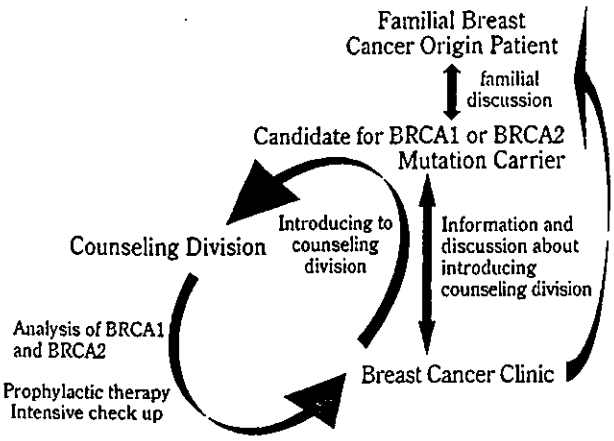

Fig 1. Counseling system for BRCA1 or BRCA2 mutation careers.

and inter-division cooperative system to smoothly introduce breast cancer clinic patients to the counseling division. This is an interim report for the grant of Japanese Health, Labour and Welfare.

Patients and Methods

Twenty familial breast cancer patients were registered between March 2002 and March 2003. Blood sampling and BRCA1/BRCA2 mutation analysis were done with informed consent according to the guidelines of the Japanese Ministry of Health, Labour and Welfare. Whole sequences of BRCA1 and BRCA2 were analyzed by the direct sequence method at Myriad Genetic Laboratories, Inc.

Clinical System

If obvious pathological mutations were detected, the patients were informed about the genetic counseling division at breast cancer clinics. Their first degree relatives were also informed about their genetic background and invited to visit genetic counseling services. When the first degree relatives visited genetic counseling divisions, they were informed about familial breast cancer and given the option to undergo genetic examination at breast cancer clinics.

Results

Deleterious Mutations of BRCA1 and BRCA2

Four cases of apparent deleterious BRCA1 and BRCA2 mutations were detected from among the total 20. These four carriers of deleterious mutations are now undergoing counseling to discuss along with their first degree relatives options for BRCA1 and BRCA2 examination and prophylactic treatments at the counseling divisions (Fig 1). An uncertain mutation thought to be a normal variant was also found in four cases.

Discussion

Treatment for BRCA1 and BRCA2 mutation carriers is a controversial issue. Prophylactic interventions for pathological BRCA1 and BRCA2 mutation carriers include prophylactic surgery, medication, irradiation and intensive follow-up. Prophylactic surgery consists of mastectomy and oophorectomy. Prophylactic mastectomy shows a prevention rate of 90%[5], while prophylactic oophorectomy shows a 50% prevention rate[6, 7]. Although prophylactic mastectomy shows a high rate of prevention as high as 90%, prophylactic surgical treatments are invasive interventions not only physically but also mentally for healthy people. Contant *et al.* proposed prophylactic mastectomy accompanied by immediate breast reconstruction (IBR). IBR can be one of solution for those hesitant to undergo prophylactic mastectomy[9]. Medication and intensive check-up as conservative treatments show relatively low prevention rate. Treatment with tamoxifen has an approximately 50% prevention rate[9]. Intensive check-ups may also allow BRCA mutation carriers to undergo breast-conserving surgery and have a better prognosis as they can be diagnosed at an earlier clinical stage.

A nonsense mutation of BRCA1 that was reported to be a Japanese founder mutation was detected as reported by Sekine *et al.*[10]. In addition, a variation in BRCA2 detected in four cases may be related to breast cancer onset.

There have been very few reports of prophylactic therapy in Japan. Proper prophylactic therapy has to be established for Japanese BRCA mutation carriers and prognosis and outcome of prophylactic therapy needs to be clarified. Our study group consists of two national university hospitals and a general hospital that cover each area. We have also been studying BRCA1 and BRCA2 mutations before this study. Together with the data from the former study, we are now beginning to analyze potential for BRCA1 and BRCA2 mutation carriers. Each institute in our study group has

a breast cancer clinic and a counseling division. If BRCA1 and BRCA2 mutation carriers decide to visit our breast cancer clinics and receive counseling about their genetic status, they can accept our introduction to clinicians in the counseling division (Fig 1). When the candidates decide to undergo genetic examination, we analyze their BRCA1 and BRCA2 sequences. The results of the examinations are reported to us. The results are given to the breast cancer clinics in each institute and the patient is informed. If they have a deleterious mutation, they visit the genetic counseling division again and receive information about familial breast cancer and prophylactic treatments.

Prophylactic mastectomy, oophorectomy, irradiation, medication and intensive check-ups have been proposed for the treatment of deleterious BRCA1 and BRCA2 mutation carriers. Prophylactic mastectomy may seem to be a radical therapy even if they show the highest prevention rate. Since the general prognosis of breast cancer occurring in BRCA mutation carriers is not different from that of sporadic breast cancer patients. Diagnosis before the onset of breast cancer should positively influence outcomes. In addition, Hoogerbrugge *et al.* reported that the breast tissue of BRCA mutation carriers has atypical hyperplastic change. Careful follow-up for BRCA1 and BRCA2 carriers is required unless they undergo prophylactic surgery[11].

Long term observation should be continued to clarify the actual onset rate and clinical outcome of breast cancer among Japanese BRCA1 and BRCA2 mutation carriers. Eventually this counseling system may be available for all Japanese families with deleterious BRCA1 and BRCA2 mutation carriers.

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SHORT COMMUNICATION

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Identification and evaluation of 55 genetic variations in the *BRCA1* and the *BRCA2* genes of patients from 50 Japanese breast cancer families

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Abstract We sequenced approximately 23 kb genomic regions containing all the coding exons and their franking introns of two breast cancer susceptibility genes, *BRCA1* and *BRCA2*, of 55 individuals from 50 unrelated Japanese breast cancer families. We identified 55 single-nucleotide polymorphisms (SNPs) (21 in *BRCA1* and 34 in *BRCA2*) containing nine pathogenic protein-truncating mutations (four in *BRCA1*and five in *BRCA2* from ten patients). Among the remaining 46 SNPs, allele frequencies of 40 were examined in both the breast cancer patients and 28 healthy volunteers with no breast cancer family history by PCR-RFLP or by direct DNA sequencing. Twenty-eight SNPs were common and were also found in the healthy volunteers and/or a SNP database. The remaining 18 were rare (allele frequency <0.05) and were not found in the healthy volunteers and/or the database. The pathogenic significance of these coding SNPs (cSNPs) remains to be clarified. The SNP information from this study will be useful in the future genetic testing of both *BRCA1* and *BRCA2* genes in the Japanese population.

Keywords Single-nucleotide polymorphism (SNP) · Breast cancer susceptibility gene · *BRCA1* · *BRCA2* · Japanese population · Direct DNA sequencing

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Introduction

Mutations in the *BRCA1* and the *BRCA2* genes have been linked with the susceptibility to breast and ovarian cancer (Miki et al. 1994; Wooster et al. 1995; Tavtigian et al. 1996). Mutation carriers of these genes are at high risk of breast and ovarian cancer (Narod et al. 1995; Ford et al. 1998; Thorlacius et al. 1998; Neuhausen 1999; Rebbeck 1999; Struewing et al. 1997; Anglian Breast Cancer Study Group 2000). The two genes have large coding sequences consisting of 48 exons in total, and a large number of mutations and SNPs are reported in the Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/) and the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html). The majority of mutations described are protein-truncating mutations containing frame-shift mutations and nonsense mutations. In the BIC database, 55.9% of genetic variants are reported as pathogenic mutations containing mainly protein-truncating mutations and a small fraction of pathogenic missense mutations, and 39.3% of variants are categorized as "unclassified variants." These variants contain coding SNPs (cSNPs) that result in amino-acid substitutions or SNPs located at exon–intron boundaries. We have also reported five protein-truncating mutations and 12 "unclassified variants" that have been found only once in 24 breast cancer families (Sakayori et al. 2003). To elucidate whether detected SNPs are pathogenic mutations or not, it is important to accumulate SNP information (both the type and allele frequency) in patients and the general population.

Table 1 Sequence variations detected in the *BRCA1* and the *BRCA2* genes

SNP ID[a]	Location	Variation[b]	Flanking sequence (5′ to 3′)	dbSNP ID[c]	BIC[d]	Volunteers[e]
*BRCA1*gene						
BRCA1-1	Intron 1	IVS1-115C > T	tggtttgtat C/T attctaaaac	rs3765640		+
BRCA1-2	Exon 3	K38 (silent) (114G > A)	tctccacaaa G/A tgtgaccaca	rs8176099	+	+
BRCA1-3	Intron 8	IVS8-58delT	tacatttttt T/– aaccctttta		+	+
BRCA1-4#	Exon 11	G275D (824G > A)	gagccatgtg G/A cacaaatact			
BRCA1-5	Exon 11	S694 (silent) (2082C > T)	gacatgacag C/T gatactttcc	rs1799949	+	+
BRCA1-6	Exon 11	L771 (silent) (2311T > C)	cagtatttca T/C tggtacctgg	rs16940	+	+
BRCA1-7#	Exon 11	2389–2390delGA*	ggcaaaaaca GA/– accaaataaa			
BRCA1-8	Exon 11	P871L (2612C > T)	tcatttgctc C/T gttttcaaat	rs799917	+	+
BRCA1-9	Exon 11	E1038G (3113A > G)	gtttttaaag A/G agccagctca	rs16941	+	+
BRCA1-10	Exon 11	K1183R (3548A > G)	agcgtccaga A/G aggagagctt	rs16942	+	+
BRCA1-11#	Exon 12	C1372X (4116T > A)*	catctgggtg T/A gagagtgaaa			
BRCA1-12	Exon 13	S1436 (silent) (4308T > C)	taagtgactc T/C tctgcccttg	rs1060915	+	+
BRCA1-13#	Intron 14	IVS14 + 14A > G	agaaacatca A/G tgtaaagatg		+	
BRCA1-14	Exon 16	S1613G (4837A > G)	atctgcccag A/G gtccagctgc	rs1799966	+	+
BRCA1-15#	Exon 16	M1628T (4883T > C)	tataatgcaa T/C ggaagaaagt	rs4986854	+	
BRCA1-16	Intron 18	IVS18 + 66G > A	tacacctaac G/A tttaacacct	rs3092994	+	+
BRCA1-17#	Intron 22	IVS22 + 33A > T	gagagggagg A/T cacaatattc			
BRCA1-18#	Intron 23	IVS23 + 8G > T	atggtaaggt G/T cctgcatgta		+	
BRCA1-19	Exon 5	L63X (188T > A)*	cagtgtcctt T/A atgtaagaat		+	
BRCA1-20	Exon 3	H41R (122A > G)	aagtgtgacc A/G catattttgcaaa			
BRCA1-21	Exon 8	470–471delCT*	tccaactct CT/– aaccttggaa		+	
*BRCA2*gene						
BRCA2-1	Exon 2	5′UTR-26G > A	tatttaccaa G/A cattggagga	rs1799943	+	+
BRCA2-2#	Intron 2	IVS2-16T > A	taaggtggga T/A tttttttta			
BRCA2-3#	Intron 2	IVS2-9T > G	ggatttttt T/G ttaaatagat			
BRCA2-4	Intron 4	IVS4 + 67A > C	tgttctataa A/C gatgaatctg			+
BRCA2-5	Intron 4	IVS4-89T > C	acaatttata T/C gaatgagaat		+	+
BRCA2-6	Intron 7	IVS7 + 183T > A	caaatacatt T/A agtggtagtc			+
BRCA2-7	Intron 8	IVS8 + 56C > T	tttggaatgc C/T ttgttaaatt		+	+
BRCA2-8#	Exon 10	F266 (silent) (798T > C)	gtcatggatt T/C ggaaaaacat			
BRCA2-9	Exon 10	N289H (865A > C)	gtcaatgcca A/C atgtcctaga	rs766173	+	+
BRCA2-10	Exon 10	H372 N (1114C > A)	aaatgtagca C/A atcagaagcc	rs144848	+	+
BRCA2-11	Exon 10	S455 (silent) (1365A > G)	taccaaaatc A/G gagaagccat	rs1801439	+	+
BRCA2-12#	Exon 10	T582P (1744A > C)	tttaatatcc A/C ctttgaaaaa		+	
BRCA2-13	Exon 11	H743 (silent) (2229T > C)	cagtacaaca T/C tcaaaagtgg		+	+
BRCA2-14	Exon 11	M784V (2350A > G)	aaacctagtc A/G tgatttctag		+	+
BRCA2-15	Exon 11	N991D (2971A > G)	tgattacatg A/G acaaatgggc	rs1799944	+	+
BRCA2-16	Exon 11	K1132 (silent) (3396A > G)	agtttagaaa A/G ccaagctaca	rs1801406	+	+
BRCA2-17#	Exon 11	S1140 (silent) (3420T > C)	tgcagaagag T/C acatttgaag			
BRCA2-18#	Exon 11	3830delA*	aagatagaaa A/– tcataatgat			
BRCA2-19#	Exon 11	L1522F (4566G > T)	ctactctgtt G/T ggttttcata			
BRCA2-20#	Exon 11	G2044V (6131G > T)	tcccaaaaag G/T cttttcatat		+	+
BRCA2-21	Exon 14	S2414 (silent) (7242A > G)	aaactaaatc A/G cattttcaca	Rs1799955	+	+
BRCA2-22#	Exon 11	6491–6495delAGTTG*	gacaaacaac AGTTG/– gtattaggaa			
BRCA2-23	Intron 16	IVS16 + 47A > G	gtattcccte A/G tccctctttc			
BRCA2-24	Intron 16	IVS16-14T > C	aatattctac T/C tttatttgtt		+	+
BRCA2-25	Exon 11	V2109I (6325G > A)	acttcctcgt G/A ttgataagag		+	
BRCA2-26#	Exon 20	S2835X (8504C > A)*	gagaagacat C/A atctggatta			
BRCA2-27	Intron22	IVS22-147A > G	cagataaagt A/G taaagttagt			
BRCA2-28	Exon 25	R3128X (9382C > T)*	AacctccagtggC/Tgaccagaatcc		+	
BRCA2-29	Exon 10	1278delA*	ttcagaaaa A/– gacctattag			
BRCA2-30	Exon 10	K322Q (964A > C)	aaatctacaa A/C aagtaagaactagc		+	
BRCA2-31	Exon 10	E425 (silent) (1275A > G)	aaatatttcaga A/G aaagacct			
BRCA2-32	Exon 11	V1269 (silent) (3807T > C)	catgattctgt T/C gtttcaatgt		+	
BRCA2-33	Exon 11	E1455 (silent) (4365A > G)	cagaaaccaga A/G gaattgcata			
BRCA2-34	Exon 18	K2729N (8187G > T)	tatgctgttaa G/T gcccagttagatcct		+	

[a]The SNP ID that have been found in this study. #Published previously (Sakayori et al. 2003)

[b]The nucleotide number in the coding region indicates the position downstream of the first nucleotide of ATG (initiation codon) in the *BRCA1* gene or the *BRCA2* gene. *: Nonsense or frame-shift mutation

[c]National Center for Biotechnology Information (NCBI) dbSNP database

[d]Breast Cancer Information Core (BIC) database, +: listed in BIC

[e]28 Japanese healthy volunteers, +: found at least one individual

Table 2 Allele frequencies of SNPs in the *BRCA1* and the *BRCA2* genes

SNP ID[a]	Allele frequency		
	Breast cancer patients[b]	Volunteers[c]	dbSNP[d]
*BRCA1*gene			
BRCA1-1	*C*=0.43, *T*=0.57	*C*=0.52, *T*=0.48	*C*=0.343, *T*=0.657
BRCA1-2	*G*=0.93, *A*=0.07	*G*=0.98, *A*=0.02	*G*=0.994, *A*=0.006
BRCA1-3	*T7*=0.65, *T6*=0.35*	*T7*=0.50, *T6*=0.50	
BRCA1-4	*G*=0.98, *A*=0.02	*G*=1.00, *A*=0.00	
BRCA1-5	*C*=0.62, *T*=0.38	*C*=0.56, *T*=0.44	*C*=0.657, *T*=0.343
BRCA1-6	*T*=0.62, *C*=0.38	*T*=0.54, *C*=0.46	*T*=0.678, *C*=0.322
BRCA1-8	*C*=0.62, *T*=0.38	*C*=0.54, *T*=0.46	*C*=0.619, *T*=0.381
BRCA1-9	*A*=0.62, *G*=0.38	*A*=0.54, *G*=0.46	*A*=0.725, *G*=0.275
BRCA1-10	*A*=0.62, *G*=0.38	*A*=0.54, *G*=0.46	*A*=0.703, *G*=0.297
BRCA1-12	*T*=0.62, *C*=0.38	*T*=0.54, *C*=0.46	*T*=0.747, *C*=0.253
BRCA1-13	*A*=0.98, *G*=0.02*	*A*=1.00, *G*=0.00	
BRCA1-14	*A*=0.62, *G*=0.38	*A*=0.54, *G*=0.46	*A*=0.696, *G*=0.304
BRCA1-15	*T*=0.98, *C*=0.02	*T*=1.00, *C*=0.00	*T*=0.995, *C*=0.005
BRCA1-16	*G*=0.63, *A*=0.37	*G*=0.54, *A*=0.46	*G*=0.693, *A*=0.307
BRCA1-17	*A*=0.98, *G*=0.02*	*A*=1.00, *G*=0.00	
BRCA1-18	*G*=0.99, *T*=0.01	*G*=1.00, *T*=0.00	
BRCA1-20	*A*=0.99, *G*=0.01	ND	
*BRCA2*gene			
BRCA2-1	*G*=0.55, *A*=0.45	*G*=0.49, *A*=0.51	*G*=0.762, *A*=0.238
BRCA2-2	*T*=0.97, *C*=0.03*	*T*=1.00, *C*=0.00	
BRCA2-3	*T*=0.98, *G*=0.02*	*T*=1.00, *C*=0.00	
BRCA2-4	*A*=0.84, *C*=0.16*	*A*=0.84, *C*=0.16	
BRCA2-5	*T*=0.84, *C*=0.16*	*T*=0.84, *C*=0.16	
BRCA2-6	*T*=0.32, *A*=0.68*	*T*=0.58, *A*=0.42	
BRCA2-7	*C*=0.96, *T*=0.04*	*C*=0.96, *T*=0.04	
BRCA2-8	*T*=0.98, *C*=0.02	*T*=1.00, *C*=0.00	
BRCA2-9	*A*=0.86, *C*=0.14	*A*=0.84, *C*=0.16	*A*=0.838, *C*=0.024, *G*=0.009, *T*=0.129
BRCA2-10	*A*=0.80, *C*=0.20	*A*=0.82, *C*=0.18	*A*=0.607, *C*=0.281, *G*=0.026, *T*=0.085
BRCA2-11	*A*=0.86, *G*=0.14	*A*=0.84, *G*=0.16	*A*=0.875, *G*=0.125
BRCA2-12	*A*=0.99, *C*=0.01	*A*=1.00, *C*=0.00	
BRCA2-13	*T*=0.86, *C*=0.14	*T*=0.84, *C*=0.16	
BRCA2-14	*A*=0.93, *G*=0.07	*A*=0.95, *G*=0.05	
BRCA2-15	*A*=0.85, *G*=0.15	*A*=0.84, *G*=0.16	*A*=0.970, *G*=0.030
BRCA2-16	*A*=0.47, *G*=0.53	*A*=0.50, *G*=0.50	*A*=0.705, *G*=0.295
BRCA2-17	*T*=0.99, *C*=0.01	*T*=1.00, *C*=0.00	
BRCA2-19	*G*=0.98, *T*=0.02	*G*=1.00, *T*=0.00	
BRCA2-20	*G*=0.97, *T*=0.03	*G*=0.98, *T*=0.02	
BRCA2-21	*A*=0.58, *G*=0.42	*A*=0.50, *G*=0.50	*A*=0.758, *G*=0.242
BRCA2-23	*A*=0.96, *G*=0.04*	*A*=1.00, *G*=0.00	
BRCA2-24	*T*=0.36, *C*=0.64	*T*=0.27, *C*=0.73	
BRCA2-25#	*G*=0.99, *A*=0.01	*G*=1.00, *A*=0.00	
BRCA2-27#	*A*=0.98, *G*=0.02*	*A*=1.00, *G*=0.00	
BRCA2-30	*A*=0.97, *C*=0.03	ND	
BRCA2-31	*A*=0.97, *C*=0.03	ND	
BRCA2-32	*T*=0.86, *C*=0.14	ND	
BRCA2-33	*A*=0.98, *G*=0.02*	ND	
BRCA2-34	*G*=0.98, *T*=0.02	ND	

[a]Identical to the SNP ID listed in Table 1, #: found in a relative but not in the proband
[b]110 alleles from 55 patients, *: data derived from 68 alleles (34 patients)
[c]56 alleles from 28 healthy volunteers, ND: not determined
[d]National Center for Biotechnology Information (NCBI) dbSNP database

In this study, we extended our study on the *BRCA1* and *BRCA2* sequencing project to 55 breast cancer patients from 50 Japanese breast cancer families. We evaluated the detected SNPs by comparing the allele frequency of the SNPs in healthy volunteers.

Subjects and methods

Fifty-five enrolled patients (including 50 probands and five relatives) with a history of breast cancer from 50 unrelated high-risk breast cancer families were selected according to the criteria defined by the Tohoku Familial Cancer Society (Sakayori et al. 2003). An additional 28 Japanese volunteers with no breast cancer family history were also enrolled to analyze the specific *BRCA1* and *BRCA2* variations detected in the breast cancer patients. We obtained informed consent from all patients and volunteers, and the independent studies were approved by the Familial Cancer Society and the Ethical Committee of Tohoku University Graduate School of Medicine. To identify the sequence variations in the *BRCA1* and *BRCA2* genes of the familial breast cancer patients, we sequenced approximately 23 kb genomic regions of the *BRCA1* (8.4 kb) and the *BRCA2* (14.6 kb) containing all coding exons and their franking introns using a method described previously (Sakayori et al. 2003). The identified SNPs found in the familial breast cancer

patients were also examined in the genomic DNA from the healthy volunteers by PCR-RFLP analysis using PCR primers, cycle conditions and restriction enzymes for PCR-RFLP analysis (http://www.idac.tohoku.ac.jp/dep/co/data/saka/brca02.htm) or by DNA sequence analysis. We used Genbank (U14680 and U61268 for *BRCA1*, U43746 and X95152-77 for *BRCA2*) and the BIC database as the reference sequences of *BRCA1* and *BRCA2* genes.

Results and discussion

By DNA sequencing analysis of the *BRCA1* and *BRCA2*genes for 55 patients from 50 unrelated breast cancer families; we detected 55 SNPs (21 SNPs in *BRCA1* and 34 SNPs in *BRCA2*). Among these SNPs, we found nine protein-truncating mutations (four in *BRCA1*and five in *BRCA2*) in ten patients containing six novel mutations that were not found in the BIC database (http://research.nhgri.nih.gov/bic/) (Table 1). The existence of mutations were also confirmed by the stop codon assay in yeast (Ishioka et al. 1997; Sakayori et al. 2003). The percentage of protein-truncating mutations in the examined families was 20% (ten of 50), comparable with several reports describing the frequency of protein-truncating mutations of the two genes in Japanese breast cancer families (Inoue et al. 1995; Takano et al. 1997; Inoue et al. 1997; Ikeda et al. 2001). Although the frequency was also similar to results from Western populations, the number of patients studied in our and other Japanese populations was smaller than that in Western countries. Therefore, it is necessary to study a larger number of patients to clarify the mutation frequency in Japanese familial breast cancer.

Among the remaining 46 SNPs (17 SNPs in *BRCA1* and 29 SNPs in *BRCA2*), 18 and 31 have been reported in the dbSNP database and in the BIC database, respectively, (Table 1). To evaluate whether the SNPs are also found in Japanese healthy volunteers, all but six SNPs were further examined by PCR-RFLP analysis or by DNA sequence analysis. Twenty-six SNPs were found in the volunteers at least once with different allele frequencies (Table 2). In these 40 SNPs, we predicted that 27 SNPs were common polymorphisms and probably played no direct role in the tumorigenesis of breast cancer. The remaining 13 SNPs were quite low in allele frequency (<0.05) and were not found in the healthy volunteers or the dbSNP database. In addition, there were six SNPs that failed to examine in the volunteer group. In these six SNPs, one was predicted to be common and five to be rare from the allele frequencies in the patients. Overall, we predicted the 18 rare SNPs are candidates of pathogenic mutations and that the remaining 28 were common polymorphisms and probably have no direct roll in tumorigenesis of breast cancer. In the 18 rare SNPs, five (BRCA1-13, BRCA1-17, BRCA1-18, BRCA2-2, BRCA2-3) were located at the exon–intron boundaries and two (BRCA2-23, BRCA2-27) were located in introns far from exon–intron boundaries. Although SNPs at the exon–intron boundaries may affect normal RNA splicing, our RT-PCR analysis showed negative data for splicing abnormalities. In the remaining 11 rare cSNPs, seven (BRCA1-4, BRCA1-20, BRCA2-12, BRCA2-19, BRCA2-25, BRCA2-30 and BRCA2-34) were nonsynonymous substitution resulting in the amino-acid substitutions, and four (BRCA2-8, BRCA2-17, BRCA2-31 and BRCA2-33) were synonymous (silent) changes. These SNPs may directly affect the functions of BRCA1 and BRCA2 proteins or affect normal splicing by acting as possible cryptic splicing sites. Unfortunately, we have failed to clarify this issue mainly because there are no reliable functional assays of either BRCA1 or BRCA2 protein for many nonsynonymous changes.

To confirm the pathogenic effect of rare SNPs, both the development of functional assays for these gene products and more intensive SNP analysis including an investigation into whether these SNPs cosegregate with breast cancer onset in families are necessary.

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Filamin A-Bound PEBP2β/CBFβ Is Retained in the Cytoplasm and Prevented from Functioning as a Partner of the Runx1 Transcription Factor

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The heterodimeric transcription factor PEBP2/CBF is composed of a DNA-binding subunit, called Runx1, and a non-DNA-binding subunit, called PEBP2β/CBFβ. The Runx1 protein is detected exclusively in the nuclei of most cells and tissues, whereas PEBP2β is located in the cytoplasm. We addressed the mechanism by which PEBP2β localizes to the cytoplasm and found that it is associated with filamin A, an actin-binding protein. Filamin A retains PEBP2β in the cytoplasm, thereby hindering its engagement as a Runx1 partner. The interaction with filamin A is mediated by a region within PEBP2β that includes amino acid residues 68 to 93. The deletion of this region or the repression of filamin A enables PEBP2β to translocate to the nucleus. Based on these observations, we propose that PEBP2β has two distinct domains, a newly defined regulatory domain that interacts with filamin A and the previously identified Runx1-binding domain.

The heterodimeric transcription factor PEBP2/CBF is composed of the DNA-binding protein Runx1/AML1 and the non-DNA-binding protein PEBP2β/CBFβ. The Runt domain, which is conserved among Runx family proteins, is responsible not only for the DNA-binding activity of Runx1, but also for its ability to dimerize with PEBP2β. Nuclear magnetic resonance and X-ray diffraction studies have allowed the determination of the three-dimensional structure of the Runt domain of Runx1 as well as the three-dimensional structures of PEBP2β and the heterodimer formed by both subunits (4, 9, 12, 21, 30, 36). These analyses showed that when it dimerizes with PEBP2β, the stabilized Runx1 protein can bind both the major and minor grooves of DNA (30). PEBP2β alone does not interact with DNA but enhances the DNA-binding activity of Runx1. Furthermore, *Runx1* and *PEBP2β* homozygous knockout mice exhibit identical phenotypes, with a failure of hematopoietic stem cell development during embryogenesis. This finding provides genetic evidence that dimer formation between Runx1 and PEBP2β is vitally important for transcription factor activity (22, 24, 25, 28, 34, 35).

In humans, both *Runx1* and *PEBP2β* are frequently targeted in leukemia-associated chromosomal abnormalities such as the t(8; 21) and inv translocations (16), which generate chimeric transcription factors that interfere with or abolish the transcriptional activity of endogenous PEBP2/CBF. For example, the inv (16)-derived PEBP2β-SMMHC protein consists of an amino-terminal fusion of the PEBP2β heterodimerization domain to the carboxy-terminal coiled-coil region of the smooth muscle myosin heavy chain.

In addition, while PEBP2/CBF was originally characterized as a transcriptional activator, recent studies have demonstrated that it can also function as a repressor, depending on the enhancer or promoter sequences it binds to and on the cofactors it interacts with. An interaction with p300/CBP or mSin3A converts Runx1 into an activator or a repressor, respectively (16, 19). Other factors such as YAP, Ear-2, ALY, Ets-1, MOZ, and Groucho/TLE also interact with Runx1 and modulate its activity (2, 5, 10, 13, 15, 17, 18, 37, 38). On the other hand, no such cofactors or modulators have been reported for PEBP2β.

Although the structure and functions of the PEBP2/CBF transcription factor have been extensively studied, little is known about how its activity is influenced by the subcellular localization of its constituent subunits. The Runx1 protein possesses nuclear localization signals and is found exclusively in the nucleus, whereas PEBP2β is located in the cytoplasm in most cells and tissues examined thus far (14, 32). The ability of Runx1 to bring PEBP2β into the nucleus has been demonstrated (1, 31). On the other hand, the mechanism that localizes PEBP2β to the cytoplasm is not known. We previously reported that cytoplasmic PEBP2β has a weak affinity for a cytoskeletal structure, namely, F-actin on stress fibers (32). We also observed that PEBP2β is located on or near the Z-line of muscle fibers, where many actin-associated proteins are abundant (7). Moreover, we found that the leukemogenic chimeric protein PEBP2β-SMMHC disorganizes cytoplasmic stress fibers and that the PEBP2β portion of this protein is necessary for interference (33). Based on these observations, we pro-

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posed that PEBP2β interacts with actin-associated proteins and that this interaction determines the cytoplasmic localization of PEBP2β (32, 33).

In the present study, we show that filamin A binds PEBP2β and retains it in the cytoplasm, thereby preventing it from acting as a partner for the Runx1 transcription factor. When filamin A is absent, PEBP2β moves into the nucleus and enhances Runx1-dependent transcription.

MATERIALS AND METHODS

Yeast two-hybrid screening. The Matchmaker Two-Hybrid System 3 (Clontech) was used according to the instructions in the manufacturer's manual. A bait plasmid was constructed by inserting the mouse *PEBP2β* cDNA next to the GAL4 DNA-binding domain of the vector pGBKT7. cDNA libraries prepared from 11- or 17-day-old mouse embryos were fused to the GAL4 DNA activation domain of the vector pGAD10 and used as prey plasmids. AH109 cells were used as host cells. Plasmid DNAs were recovered from positive colonies and sequenced by use of an ABI PRISM 310 genetic analyzer (Applied Biosystems).

Plasmid construction. A c-Myc or hemagglutinin (HA) tag was fused to the amino or carboxy terminus of filamin A-C, PEBP2β, or Runx1, as indicated in the text, by a PCR-based method. Carboxy-terminal deletion mutants of PEBP2β were constructed by PCRs using the common sense primer 5′-CGGAATTCAC CATGCCGCGCGTCGTCCCGG-3′ and the following antisense primers: 5′-G GAATTCCTACTGGAGAGACAGATTGGTTC-3′ for βΔC67, 5′-GGAATTC CTACTTGCCTGCTTCTCTCTC-3′ for βΔC94, and 5′-GGAATTCCTACTG GGCTCGCTCCTCATC-3′ for βΔC133.

For preparation of an internal deletion mutant, βΔ68–93, the following primers were used in appropriate combinations for two successive rounds of PCR: 5′-AAGGTATACTTGAAGGCTCCCATG-3′, 5′-CCAATCTGTCTCTCCAG AAGGTATACT-3′, and 5′-CAAGAAGACAGCAAGACCCTAGGAATTCC G-3′. All cDNAs were subcloned into the mammalian expression vector pCAGGS-neo.

Cell culture. HeLa cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% (vol/vol) fetal bovine serum. M2 and A7 cells were cultured in minimal essential medium (Life Technologies, Inc.) supplemented with 8% (vol/vol) newborn calf serum and 2% (vol/vol) fetal bovine serum (8). G418 (Sigma) was added to A7 cells at 0.3 mg/ml.

siRNA-mediated repression of filamin A. Five RNA oligonucleotides (iGENE) were synthesized by use of the following sense and antisense oligonucleotides: for filaminA-1499, 5′-CAGCUGACUUCAAGGUGUACACAAA-3′ and 5′-UUUGUGUACACCUUGAAGUCAGCUG-3′; for filaminA-4566, 5′-A GUACUGUAUGGAGAUGAAGAGGUA-3′ and 5′-UACCUCUUCAUCUC CAUACAGUACU-3′; for filaminA-5792, 5′-ACUACAGCAUUCUAGUCAAG UACAA-3′ and 5′-UUGUACUUGACUAGAAUGCUGUAGU-3′; for filaminA-6911, 5′-ACUACGAAGUCUCAGUCAAGUUCAA-3′ and 5′-UUGAACUUGA CUGAGACUUCGUAGU-3′; for filaminA-7140, 5′-CACAGAAAUUGACCAA GAUAAGUAU-3′ and 5′-AUACUUAUCUUGGUCAAUUUCUGUG-3′; and for the luciferase control, 5′-ACAUCACGUACGCGGAAUACUUCGA-3′ and 5′-UCGAAGUAUUCCGCGUACGUGAUGU-3′. Small interfering RNAs (siRNAs) were introduced into HeLa cells by the use of Lipofectamine 2000 (Life Technologies, Inc.). The cells were processed for immunological detection 72 h after transfection.

Immunoprecipitation and immunoblot analysis. Expression vectors harboring Myc-tagged filamin A-C and HA-tagged PEBP2β were cotransfected into HeLa cells by use of the Effectene reagent (Qiagen). Twenty-four hours after transfection, the cells were lysed with a buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (vol/vol) Nonidet P-40, and a mixture of protease inhibitors (Complete; Roche Molecular Biochemicals). If the lysate was to be incubated with an anti-human filamin A monoclonal antibody (Chemicon), the cells were lysed with a buffer consisting of 25 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 2 mM dithiothreitol, and a mixture of protease inhibitors. The cell lysates were incubated with the anti-c-Myc 9E10 antibody (Sigma), the anti-HA 3F10 antibody (Roche Molecular Biochemicals), or an anti-filamin A antibody, and the immunoprecipitates were adsorbed to protein G-Sepharose beads. The beads were washed five times with a buffer consisting of 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, 1% (vol/vol) Nonidet P-40, and a mixture of protease inhibitors or, in the case of anti-filamin A precipitates, with a buffer consisting of 25 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 2 mM dithiothreitol, and a mixture of protease inhibitors. Proteins were eluted by boiling the beads in sodium dodecyl sulfate (SDS) sample buffer, electrophoresed in an SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane. Immunodetection of membrane-bound proteins was performed with the anti-HA 3F10, anti-c-Myc 9E10, or anti-PEBP2β antibody (6), and products were visualized by use of the ECL Plus reagent (Amersham Pharmacia Biotech).

Cells subjected to siRNA analysis were lysed in a urea-Triton buffer, and immunoblot analyses were performed as previously described (7). The antibodies used were an anti-human filamin A antibody, a murine anti-β-actin monoclonal antibody (Sigma), and a peroxidase-conjugated goat antibody to mouse immunoglobulin G (IgG) (Cappel Products).

Immunofluorescence analysis. Cells grown on coverslips were transfected with expression plasmids. Twenty-four hours later, the cells were fixed with 2% (wt/vol) paraformaldehyde, permeabilized with 0.1% (vol/vol) Triton X-100, and blocked with 1% (wt/vol) bovine serum albumin in phosphate-buffered saline. Signals were detected by an indirect immunofluorescence technique using anti-HA 3F10 as a primary antibody and Cy3-conjugated goat anti-rat IgG (Chemicon) as a secondary antibody or using anti-c-Myc 9E10 or an anti-human filamin A antibody as a primary antibody and Alexa fluor 488–goat anti-mouse IgG (Molecular Probes) as a secondary antibody. Cells were viewed with a confocal laser scanning microscope (LSM410 or LSM 5 PASCAL; Zeiss).

Reporter gene assay. Runx1 and PEBP2β expression plasmids were cotransfected with the reporter plasmid pM-CSF-R-luc (39) by use of the Effectene reagent. pRSV-β-GAL was transfected together with the reporter plasmid as an internal control, and its activity was used to normalize transfection efficiencies. Twenty-four hours after transfection, the cells were lysed in a lysis buffer (Promega), and the luciferase activities in lysates were measured by use of a luciferase assay system (Promega) and a Luminescencer-JNR AB-2100 instrument (Bio-Instrument). β-Galactosidase activity was assayed by the use of chlorophenol red-β-D-galactopyranoside (Roche Molecular Biochemicals) as a substrate. Each assay was performed at least three times. In each case, a siRNA for filamin A or a *filaminA* cDNA was introduced into HeLa or M2 cells, respectively. The cells were incubated for 24 h and then transfected with the expression plasmids for Runx1 and PEBP2β together with the pM-CSF-R-luc reporter and the pRL-TK vector (Promega) as an internal control.

RESULTS

Identification of filamin A as a novel PEBP2β-interacting protein. To identify molecules that interact with PEBP2β, we employed a yeast two-hybrid screening system. cDNAs derived from murine embryos were used as preys, and *PEBP2β* was used as a bait. We screened nine million yeast transformants by an interaction trap strategy and obtained 110 colonies that were positive for growth on both histidine and adenine. Many of the cDNAs thus isolated corresponded to *Runx* genes. In addition, one clone that carried a portion of the cytoskeletal gene *filaminA* (also known as *ABP-280*) was identified. The region of *filaminA* that was recovered as a *PEBP2β*-interacting sequence consisted of the extreme carboxy terminus and the adjacent hinge 2 domain (Fig. 1A). This region is hereafter referred to as filamin A-C.

We next examined whether the PEBP2β protein interacts with filamin A in mammalian cells by transiently expressing Myc-tagged filamin A-C and HA-tagged PEBP2β in HeLa cells. As shown in Fig. 1B, PEBP2β was coimmunoprecipitated with filamin A-C from HeLa cell lysates when the two proteins were introduced by cotransfection. Similarly, filamin A-C was coimmunoprecipitated with PEBP2β (data not shown). Since HeLa cells express both the PEBP2β and filamin A proteins endogenously, their possible interaction was also examined. As shown in Fig. 1C, PEBP2β was coimmunoprecipitated with the intact filamin A molecule. These data indicate that PEBP2β forms a complex with filamin A in HeLa cells and that this is true not only for the exogenously introduced proteins, but also for those expressed endogenously.

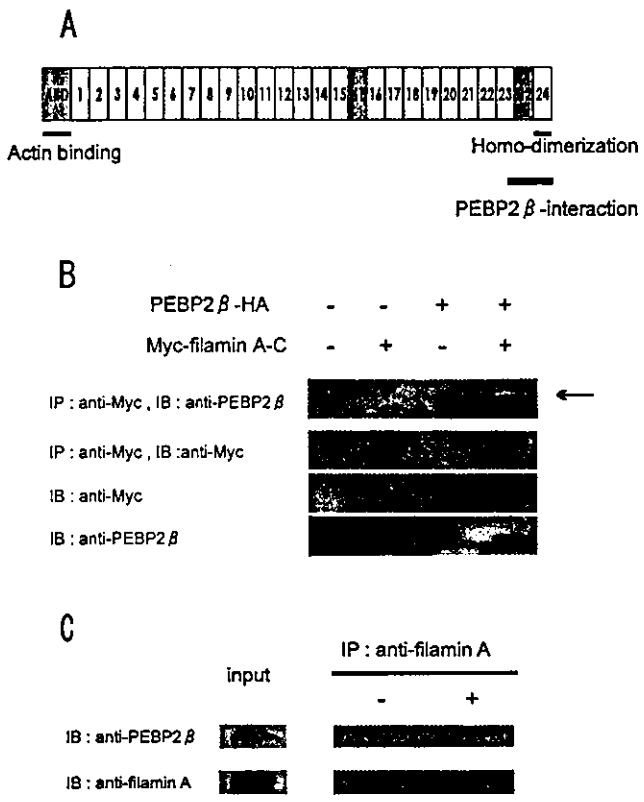

FIG. 1. Interaction of PEBP2β with filamin A. (A) Schematic diagram of structure of filamin A. The actin binding and homodimerization domains are located at the extreme amino- and carboxy-terminal ends, respectively. The repetitive domains are numbered. H represents a hinge region. A fragment of filamin A obtained in the yeast two-hybrid screening assay contained the PEBP2β-interacting region and is termed filamin A-C. (B) HeLa cells were transfected with the indicated combinations of expression plasmids, and cell lysates were immunoprecipitated (IP) with an anti-Myc antibody. Precipitates were resolved by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot (IB) analysis with the indicated antibodies. An aliquot of lysate was directly immunoblotted without immunoprecipitation to evaluate the level of protein expression. (C) Lysates of untransfected HeLa cells were immunoprecipitated (IP) with or without an anti-filamin A antibody. Precipitates were resolved by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot (IB) analysis with the indicated antibodies. An aliquot of lysate was directly immunoblotted without immunoprecipitation to evaluate the level of protein expression (input).

The central region of PEBP2β is necessary for interaction with filamin A-C and for cytoplasmic localization. To determine which region of PEBP2β is responsible for its interaction with filamin A, we generated a series of carboxy-terminal deletion mutants. Figure 2A illustrates the relationship between each deletion derivative and the known substructures of PEBP2β. The 141-amino-acid (aa) amino-terminal region of PEBP2β is composed of four α helices, seven β strands, and six loop structures. Since three isoforms of the PEBP2β protein share the 133 amino-terminal amino acids, we constructed a derivative lacking the carboxy-terminal region (βΔC133) by truncating the protein at residue 134. The βΔC94 derivative lacked the β5-L5 region, one of the Runt-interacting domains. The βΔC67 construct possessed only the 67 amino-terminal amino acids, and the βΔ68–93 construct retained all of the Runt-interacting domains (β1-L1, L2-β3, α1, β3, and β5-L5) but lacked the L3-β4-L4 region.

By using a yeast two-hybrid system, we found that the βΔC133 and βΔC94 derivatives, as well as full-length PEBP2β, interacted with filamin A-C (Fig. 2B). We could not, however, assess the binding ability of the βΔC67 construct, since cells containing this construct alone were able to grow on selection medium. Notably, the internal deletion construct, βΔ68–93, did not interact with filamin A-C. Thus, the region of aa 68 to 93 of PEBP2β is necessary for its interaction with filamin A.

We next examined the effect of carboxy-terminal deletions on the subcellular localization of PEBP2β. Each plasmid was transfected into HeLa cells, and the expression of transduced proteins as well as of endogenous filamin A was monitored by double immunofluorescence staining (Fig. 3). While full-length PEBP2β and the βΔC133 and βΔC94 proteins were all located in the cytoplasm, the βΔC67 and βΔ68–93 proteins were both found in the nucleus. Thus, the region of aa 68 to 93 is necessary for the cytoplasmic localization of PEBP2β, since constructs that lack this region move into the nucleus.

PEBP2β is located in the nuclei of filamin A-deficient cells. We reasoned that filamin A functions to retain PEBP2β in the cytoplasm. Therefore, we predicted that in its absence, full-length PEBP2β would not stay in the cytoplasm but would instead move into the nucleus. To test this idea, we performed experiments with a filamin A-deficient human melanoma cell line, M2, and a subline, A7, which was obtained by stably transfecting M2 cells with a human filamin A cDNA (8). A plasmid bearing full-length PEBP2β was transfected into these cells, and the expression of transduced protein as well as of endogenous filamin A was monitored by immunofluorescence (Fig. 4). PEBP2β was detected in the nuclei of filamin A-deficient M2 cells but in the cytoplasm of filamin A-expressing A7 cells. We also examined the effects of carboxy-terminal deletions on the subcellular localization of PEBP2β in M2 and A7 cells. In filamin A-expressing A7 cells, while the full-length PEBP2β, βΔC133, and βΔC94 proteins were all located in the cytoplasm, the βΔC67 and βΔ68–93 proteins were both found in the nucleus. On the other hand, in filamin A-deficient M2 cells, the full-length PEBP2β, as well as all carboxy-terminal deletion mutants, was located in the nucleus (data not shown).

To confirm these results, we next determined whether the repression of filamin A by the siRNA method influences the subcellular localization of PEBP2β in HeLa cells. Since the same results were obtained for five different siRNAs synthesized as described in Materials and Methods, we present here representative results obtained with the siRNA filaminA-7140. A luciferase siRNA was used as a negative control. We found by immunoblot analysis (Fig. 5A) that the level of filamin A in cells treated with the filaminA-7140 siRNA was 1/10 that in luciferase siRNA-treated cells. We determined the localization of PEBP2β in these cells by immunofluorescence staining (Fig. 5B) and found the protein in the nuclei of filamin A-repressed cells but in the cytoplasm of control filamin A-expressing cells.

These data indicate that filamin A sequesters the PEBP2β protein in the cytoplasm and that in its absence, PEBP2β moves into the nucleus.

Runx1 colocalizes with PEBP2β in the nuclei of filamin A-deficient cells. We next examined whether the reported ac-

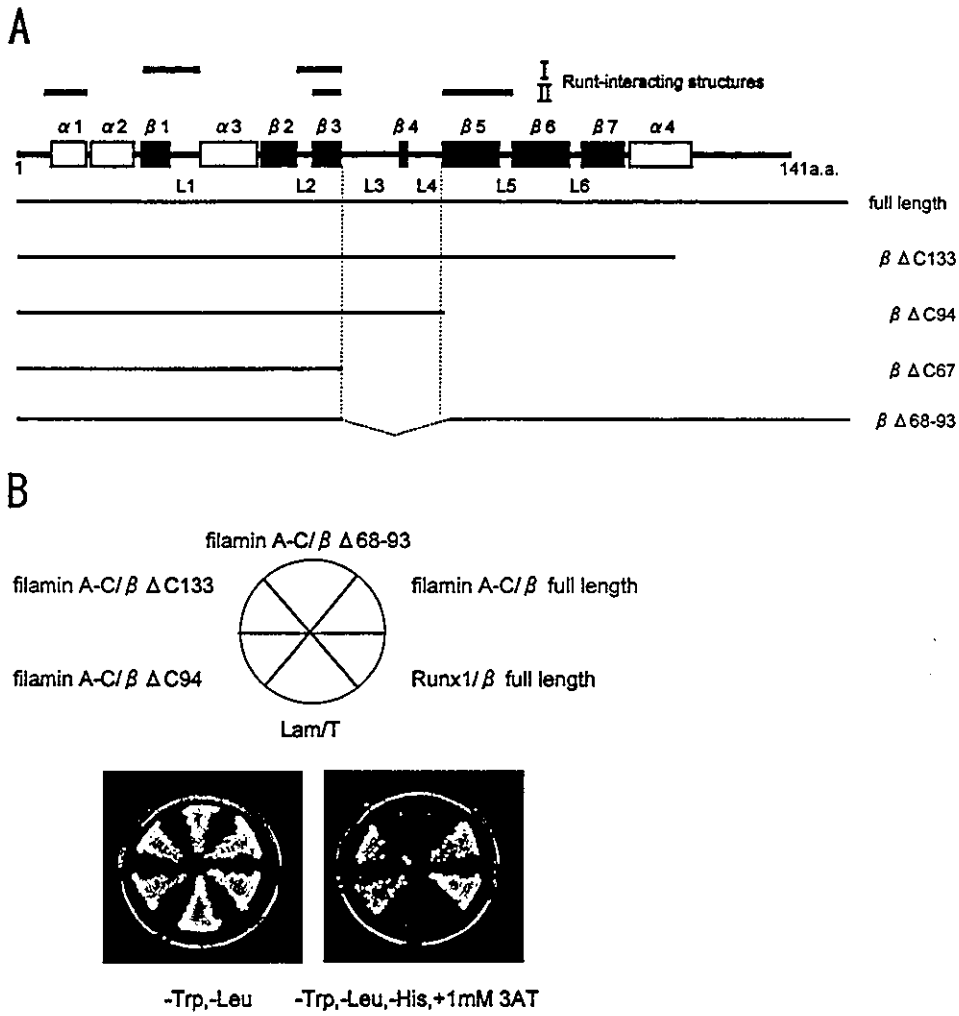

FIG. 2. Mapping of the filamin A-interacting domain of PEBP2β by yeast two-hybrid analysis. (A) Schematic diagram of domain structures found in the PEBP2β protein and description of carboxy-terminal deletion mutants of PEBP2β. α, β, and L represent α helixes, β strands, and loops, respectively. The thick lines at the top indicate areas I and II of the Runt-interacting region (30). (B) PEBP2β and its deletion mutants were tested for the ability to interact with filamin A-C. Yeast cells were transformed with the indicated combinations of plasmids and grown on nonselective (−Trp, −Leu) and selective (−Trp, −Leu, −His, 1 mM 3-amino-1,2,4-triazole) media. Lamin/large T antigen and Runx1/PEBP2β served as negative and positive controls, respectively.

tivity of Runx1 to bring PEBP2β into the nucleus is affected by the presence of filamin A. Plasmids expressing PEBP2β and Runx1 were cotransfected into the M2 and A7 cell lines, and the transduced proteins were detected by double immunofluorescence (Fig. 6A). Runx1 colocalized with PEBP2β in the nuclei of filamin A-deficient M2 cells. In addition, PEBP2β was coimmunoprecipitated with Runx1 from cotransfected M2 cell lysates (data not shown). In contrast, although Runx1 was found in the nuclei of filamin A-expressing A7 cells, PEBP2β was found in the cytoplasm. These data suggest that the presence of filamin A prevents Runx1 from translocating PEBP2β into the nucleus. On the other hand, filamin A did not have an effect on the subcellular localization of Runx1, which was localized to the nuclei of both M2 and A7 cells.

PEBP2β has been reported to be capable of stabilizing the Runx1 protein, which is rather unstable by itself, by forming a heterodimer (11). We examined the protein level of Runx1 in the presence or absence of PEBP2β by using A7 and M2 cells. As shown in Fig. 6B, the band intensities of Runx1 detected by immunoblotting were roughly equal in both PEBP2β-expressing and non-PEBP2β-expressing A7 cells (compare lanes 2 and 3). In contrast, the cotransfection of PEBP2β significantly increased the band intensity of Runx1 in M2 cells (compare lanes 5 and 6). Therefore, in the nuclei of filamin A-deficient M2 cells, where the two proteins are colocalized, PEBP2β appears to increase the stability of Runx1.

The transcriptional activity of PEBP2/CBF is enhanced in filamin A-deficient cells. Filamin A may affect the extent of PEBP2/CBF transcriptional activity by controlling the subcellular localization of PEBP2β. In order to assess this idea, we transfected M2 and A7 cells with an M-CSF-R-luc reporter, which allowed the contribution of PEBP2β to the transcriptional activity of the PEBP2/CBF heterodimer to be measured (39). As seen in Fig. 7A, the transfection of PEBP2β in addition to Runx1 caused a sixfold increase in luciferase activity in M2 cells compared to that in cells transfected solely with

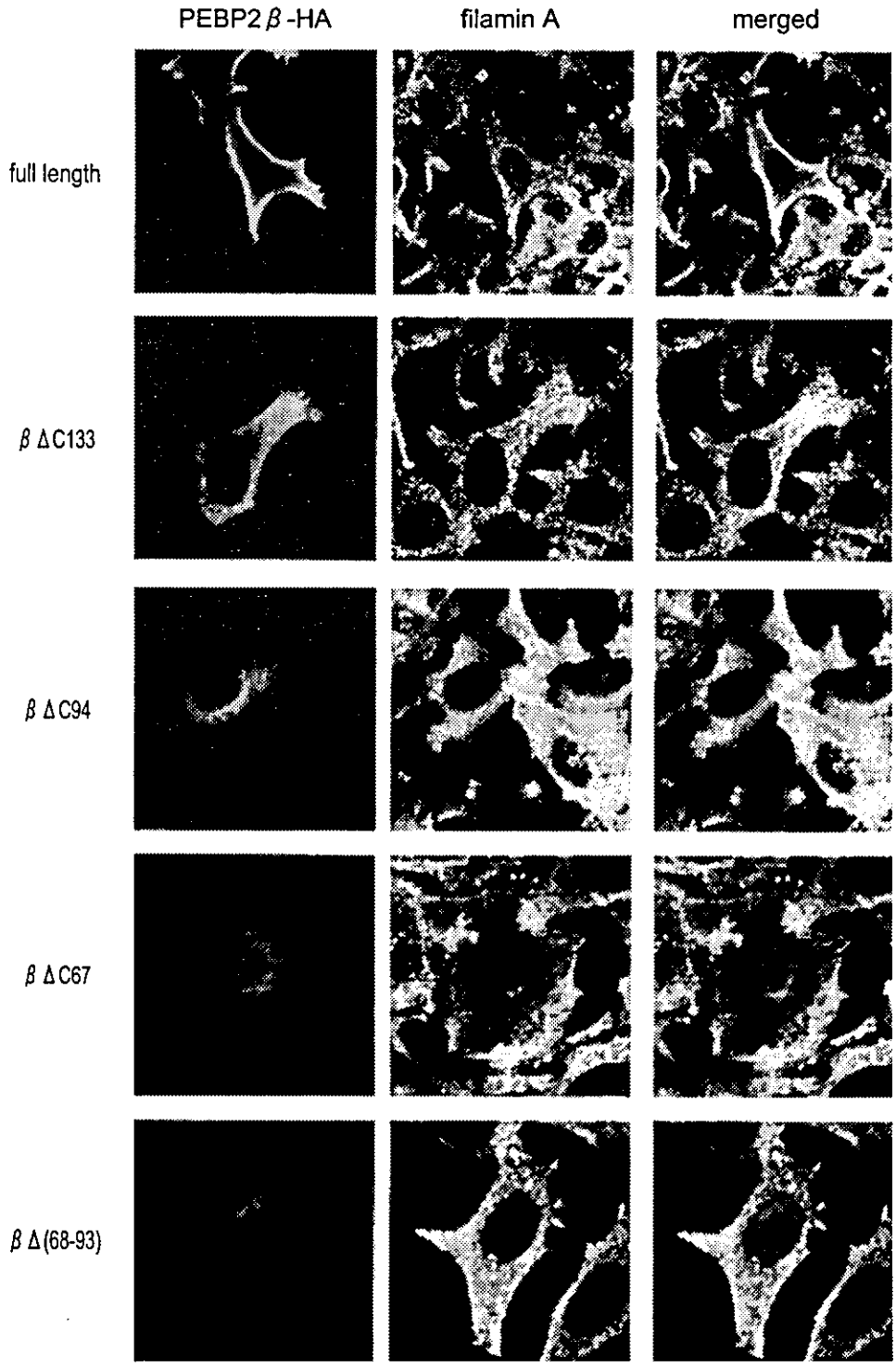

FIG. 3. Subcellular localization of PEBP2β protein derivatives. Each PEBP2β deletion mutant and full-length PEBP2β were transfected into HeLa cells, and cells were processed for double immunofluorescence staining. Red and green fluorescence represent PEBP2β and filamin A, respectively. Merged images are also presented.

Runx1. On the other hand, in A7 cells, the cotransfection of PEBP2β and Runx1 caused a 1.6-fold increase in luciferase activity compared to cells transfected only with Runx1.

We also performed several control experiments in parallel. For the data shown in Fig. 7B, HeLa cells were treated or not treated with the siRNA filaminA-7140 and then were transfected with reporter and expression plasmids. The siRNA treatment increased the luciferase activity from 1.6-fold (without siRNA) to 3.6-fold (with siRNA) (the significance of the difference was valid by a t test [$P < 0.002$]). For the data shown

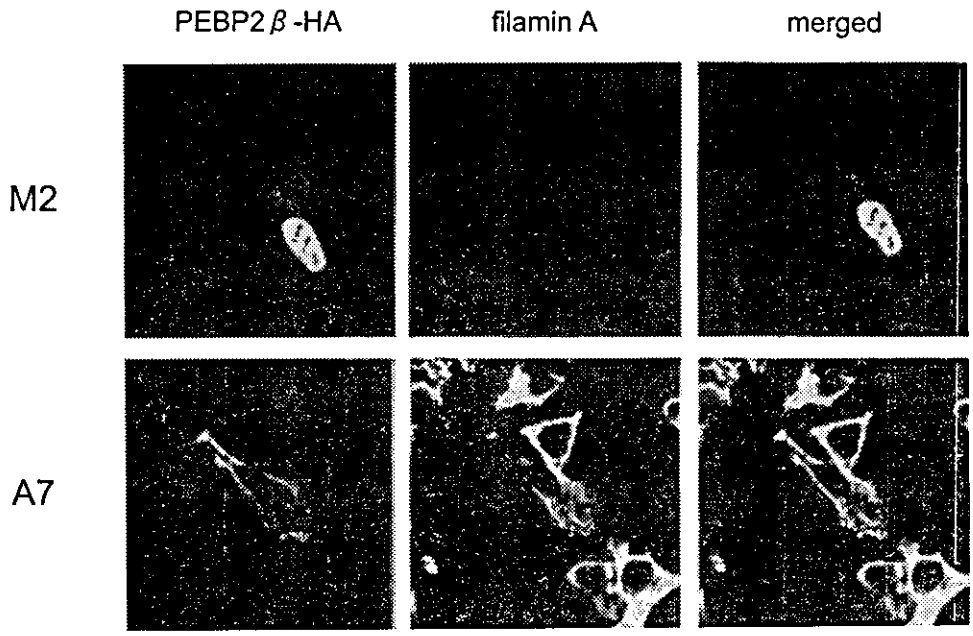

FIG. 4. Subcellular localization of PEBP2β protein in filamin A-deficient M2 cells. M2 and A7 cells were transfected with full-length PEBP2β, fixed, and processed for double immunofluorescence staining as described in the legend to Fig. 3.

in Fig. 7C, M2 cells were transfected with *filaminA* cDNA and then with reporter and expression plasmids. The coexpression of filamin A decreased the luciferase activity from 4.3-fold (without filamin A) to 3.4-fold (with filamin A) (the significance of the difference was valid by a t test [$P < 0.03$]).

Thus, the transcriptional activity of PEBP2/CBF is enhanced by an increase in the level of PEBP2β, but only in the absence of filamin A. Conversely, the presence of filamin A appears to decrease the transcriptional activity of PEBP2/CBF, probably by retaining PEBP2β in the cytoplasm. (Note that the βΔ68–93 protein did not induce transcriptional activation in a reporter assay [data not shown]. This was probably because its three-dimensional structure was unfavorably altered due to the deletion.)

DISCUSSION

Filamin A is a non-muscle-specific isoform of filamin that is ubiquitously expressed in many different cell types. As an actin binding protein, filamin A organizes a three-dimensional intracellular network of actin filaments and connects filamentous actin to plasma membrane glycoproteins. Furthermore, filamin A acts as a scaffold for intracellular proteins and is involved in various signal transduction pathways (29). In the present study, we demonstrated that filamin A binds to and retains PEBP2β in the cytoplasm, a finding which is in accord with our previous observations. For example, PEBP2β-specific staining has been observed along stress fibers and cell membrane processes in some cultured cells (32). This pattern of distribution of PEBP2β is similar to that of filamin A. Also, PEBP2β is located on or near the Z lines of muscle fibers (7). Filamin C, a muscle-specific isoform of the filamin family is present on Z lines (3) and therefore may specify the localization of PEBP2β to these structures. Indeed, we found that the cotransfected carboxy-terminal region of either filamin C or filamin B, another ubiquitously expressed isoform, was coimmunoprecipitated with PEBP2β. Moreover, the forced expression of full-length filamin B relocated PEBP2β from the nucleus to the cytoplasm of M2 cells (N. Yoshida and T. Watanabe, unpublished observations). It must be noted that since the endogenous expression of filamin B and filamin C in M2 cells was 1/10 that of filamin A or undetectable, respectively, their contribution, if any, to retaining PEBP2β in the cytoplasm may be minimal in this particular cell line.

By analyzing deletion mutants, we identified a region within the PEBP2β molecule that is important for its interaction with filamin A and for cytoplasmic localization. This region spans aa 68 through 93 and consists of loop-β strand-loop (L3-β4-L4) structures, but only 2 aa comprise the β4 strand. Therefore, this region as a whole does not appear to adopt a solid two-dimensional structure but is instead flexible. Furthermore, it contains a hydrophobic tryptophan residue (aa 73) embedded in a cluster of hydrophilic amino acid residues (30). These characteristics likely confer upon the region a tendency to interact with other molecules. In addition, an inspection of the three-dimensional structure of PEBP2β showed that the region responsible for interacting with filamin A and the Runx1-interacting region are situated on opposite sides of the molecule. The Runx1-interacting region is composed of an alpha helix and four β strands and probably adopts a rigid structure (30). Thus, based on the above observations, we propose a new model which holds that the PEBP2β protein consists of two structurally and functionally distinct domains. The first is a regulatory domain that has a loose structure and which perhaps interacts with various molecules. The binding of this domain by filamin A, for example, retains PEBP2β in the cytoplasm, thereby preventing it from being recruited as a component of a transcription factor complex. The second do-

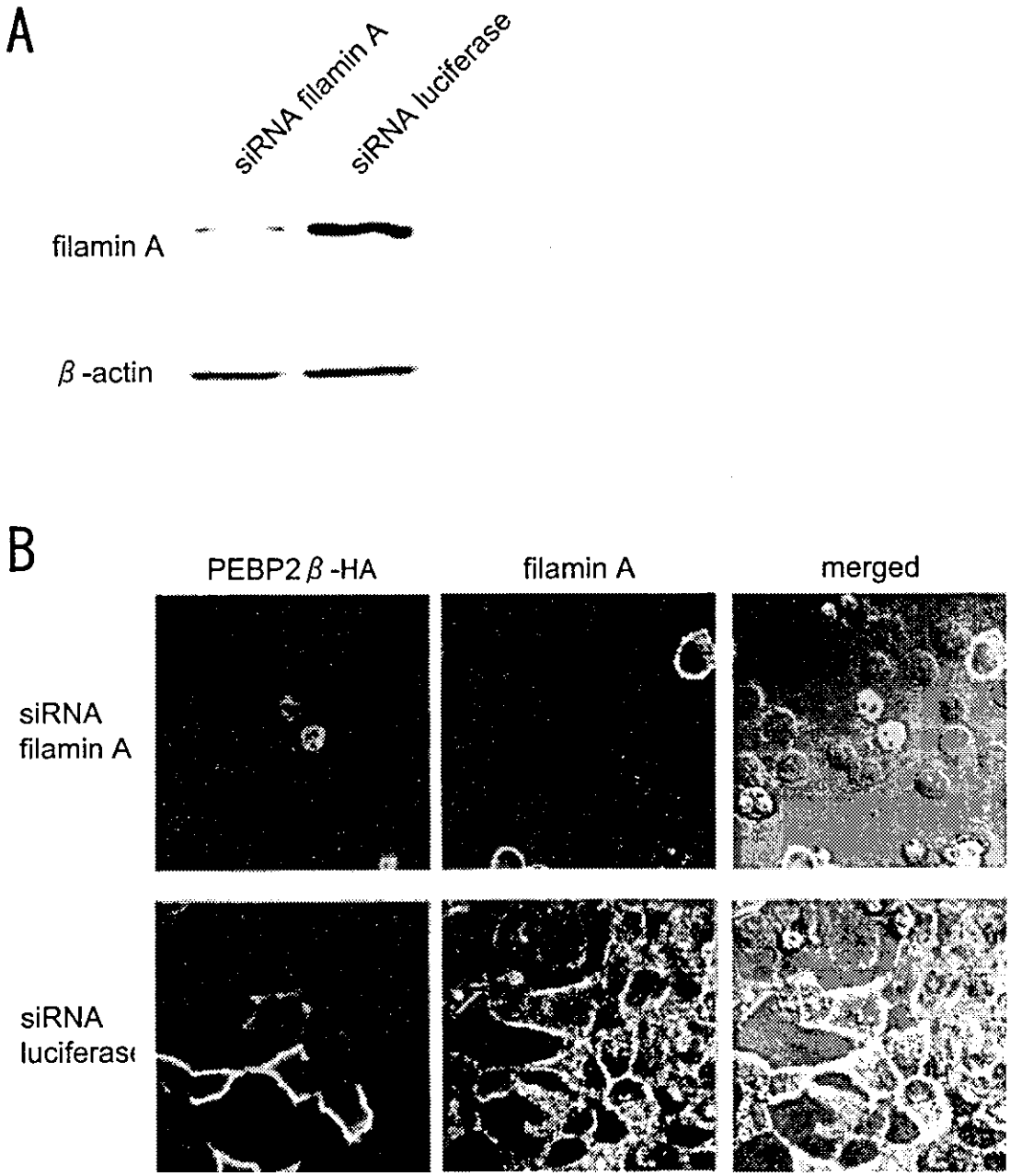

FIG. 5. Effect of filamin A repression on subcellular localization of PEBP2β protein. (A) Repression of filamin A expression by the siRNA method. HeLa cells were transfected with a siRNA targeted to filamin A or luciferase. The protein level in these cells was measured by immunoblot analysis. The top panel represents filamin A, and the bottom panel shows β-actin. (B) Cells were treated with a siRNA as indicated, transfected with *PEBP2*β cDNA, fixed, and processed for double immunofluorescence staining as described in the legend to Fig. 3. Merged images of two-color fluorescence were captured by differential interference contrast microscopy.

main is an executive domain that has a rigid structure and which perhaps interacts only with the Runx1 protein. When it is bound to Runx1, PEBP2β can function as a transcription factor in the nucleus.

It is not clear how PEBP2β moves into the nucleus in the absence of filamin A. Previously, the Runx1 protein was thought to bring PEBP2β into the nucleus (1, 31). However, Runx1 protein expression was not detected in M2 cells by immunoblot analysis (Yoshida and Watanabe, unpublished observation), and PEBP2β was detected in the nuclei of M2 cells that were not transfected with Runx1. An unidentified mechanism appears to be involved in the nuclear localization of PEBP2β.

Runx1 and *PEBP2*β are known to be indispensable for the development of hematopoietic stem cells, and a precise dose of each of the Runx1 and PEBP2β proteins appears to be necessary for the proper functioning of PEBP2/CBF during this process (6, 20, 23). For example, a haploinsufficiency of *Runx1* can impair the temporally and spatially regulated generation of hematopoietic stem cells in mouse embryos (6). Hematopoietic

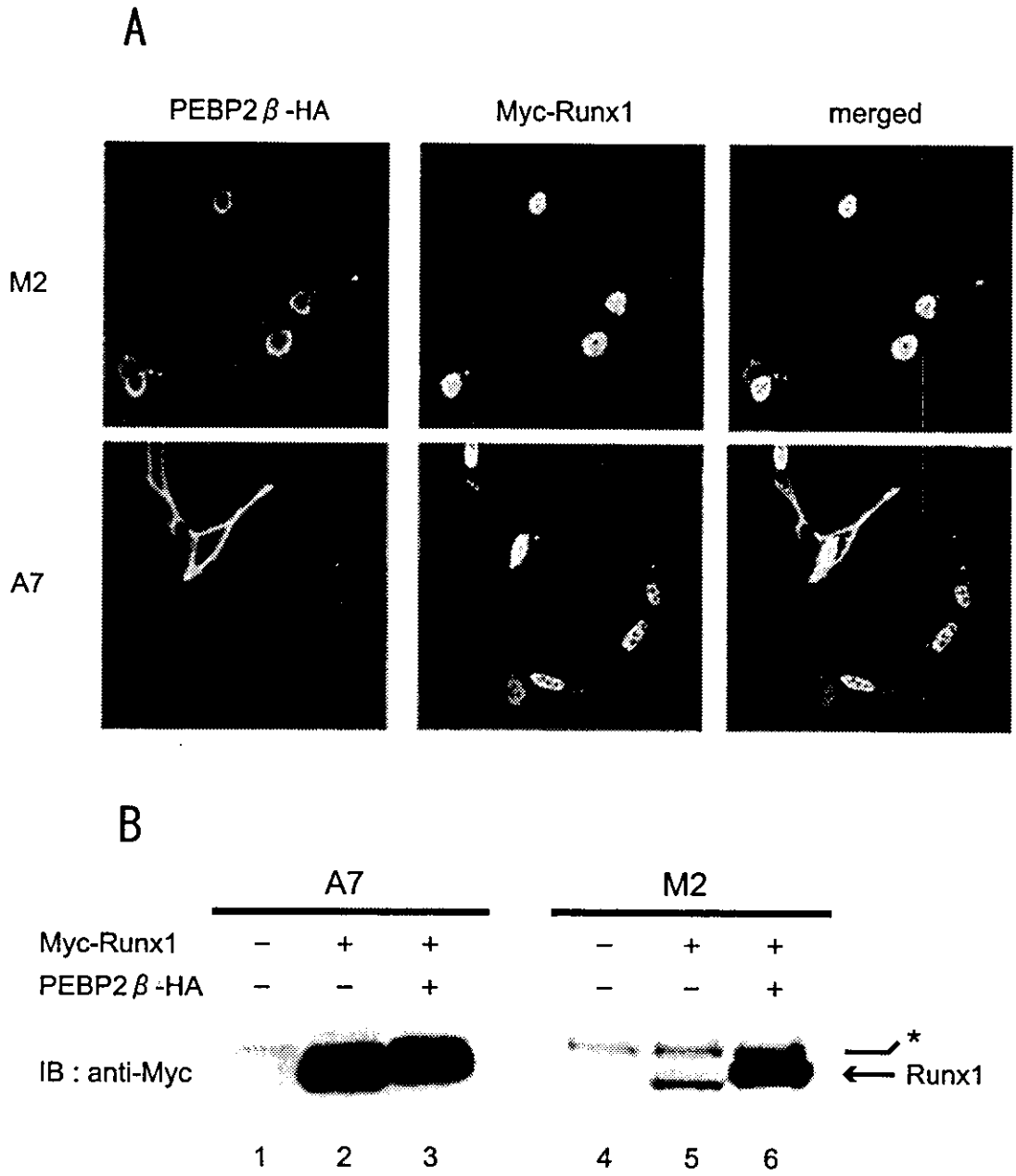

FIG. 6. Subcellular distribution of PEBP2β and Runx1 proteins in filamin A-deficient M2 cells and filamin A-expressing A7 cells. (A) M2 and A7 cells were cotransfected with *Runx1* and *PEBP2*β expression plasmids, fixed, and processed for double immunofluorescence. Red and green fluorescence represent PEBP2β and Runx1, respectively. Merged images of two-color fluorescence are also presented. (B) A7 and M2 cells were transfected with the indicated combinations of *Runx1* and *PEBP2*β expression plasmids. The protein level of Runx1 in these cells was measured by immunoblot (IB) analysis with an anti-Myc antibody. The bands indicated by the arrow represent Runx1, whereas those indicated by the asterisk represent nonspecific reactions.

stem cells develop from hemangioblasts, a specific subset of endothelial cells. Notably, hemangioblasts, which undergo transformation from flat endothelial cells to round hematopoietic cells, are considered to accompany alterations of cytoskeletal structures, including the actin and perhaps filamin A molecules. One can imagine that the mechanism described in the present study may tune the activity of PEBP2/CBF at the site of hematopoietic stem cell generation. Efforts toward understanding this mechanism are under way.

Filamin A regulates the subcellular localization of Smad2 and of the androgen receptor (26, 27), two transcription factors that are usually found in the cytoplasm. The treatment of cells with transforming growth factor beta leads to the phosphorylation of Smad2 and to the subsequent translocation of the phosphorylated form into the nucleus. Interestingly, Smad2 is neither phosphorylated nor translocated into the nucleus after transforming growth factor beta stimulation in cells lacking filamin A. Similarly, the androgen receptor, which moves into

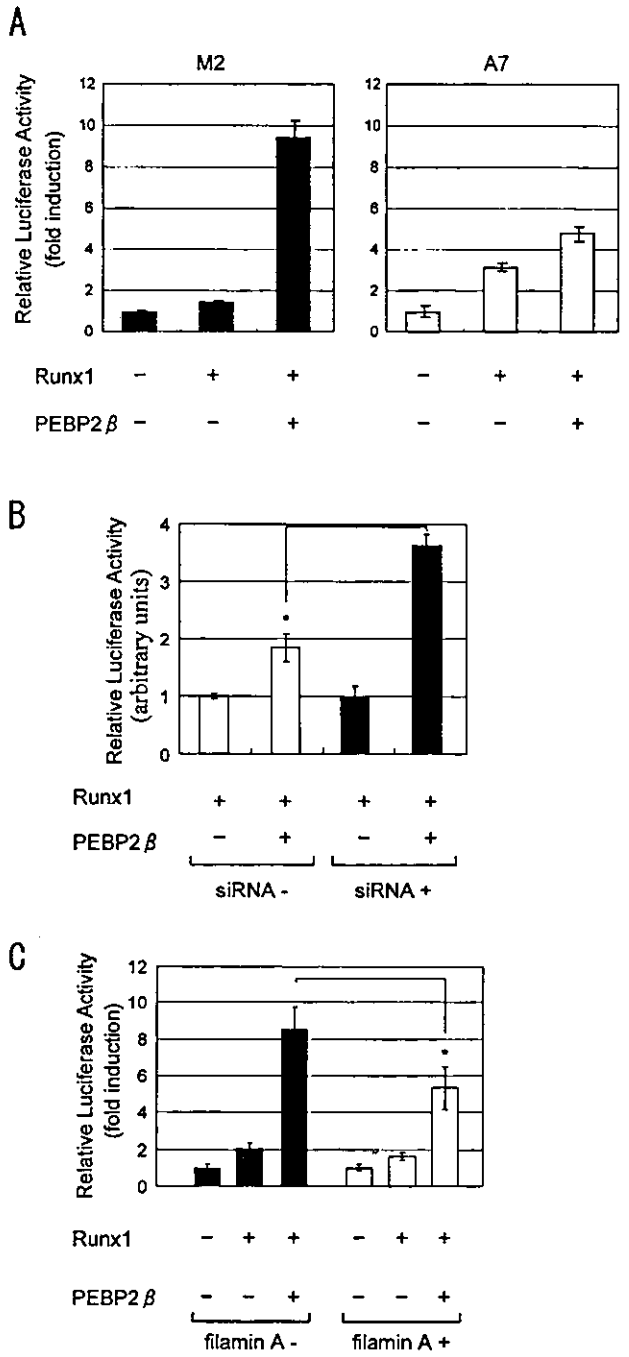

FIG. 7. PEBP2/CBF transcription activity in filamin A-deficient M2 cells and filamin A-expressing A7 cells. (A) M2 and A7 cells were cotransfected with an M-CSF-R-luc reporter construct and *Runx1* and/or *PEBP2*β expression plasmids as indicated. Luciferase activities in cell lysates are presented as averages ± standard deviations. (B) HeLa cells pretreated with a filamin A siRNA and untreated HeLa cells were cotransfected with an M-CSF-R-luc reporter construct and *Runx1* and/or *PEBP2*β expression plasmids as indicated. (C) M2 cells which were pretransfected with *filaminA* cDNA were cotransfected with an M-CSF-R-luc reporter construct and *Runx1* and/or *PEBP2*β expression plasmids as indicated.

the nucleus when bound to its ligand, remains in the cytoplasm of cells that do not express filamin A. Therefore, filamin A probably serves as a site at which a kinase and/or other ligand can also bind, and in the absence of filamin A, target molecules are not appropriately modified and thus are not translocated into the nucleus. On the other hand, PEBP2β is translocated into the nuclei of cells lacking filamin A, which enhances the transcriptional activity of PEBP2/CBF. A signal that can dissociate the interaction of PEBP2β and filamin A is not known at present. Thus, the mechanisms by which filamin A regulates the nuclear translocation and transcriptional activity of PEBP2β and of Smad2 and the androgen receptor appear to differ. Our present study has thus broadened the molecular scope of the interplay between cytoskeletal filamin A and transcription factors.

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