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Conserved Mechanism of *PLAG1* Activation in Salivary Gland Tumors with and without Chromosome 8q12 Abnormalities: Identification of *SII* as a New Fusion Partner Gene[1]

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ABSTRACT

We have previously shown (K. Kas *et al.*, Nat. Genet., *15:* 170–174, 1997) that the developmentally regulated zinc finger gene pleomorphic adenoma gene 1 (*PLAG1*) is the target gene in 8q12 in pleomorphic adenomas of the salivary glands with t(3;8)(p21;q12) translocations. The t(3;8) results in promoter swapping between *PLAG1* and the constitutively expressed gene for β-catenin (*CTNNB1*), leading to activation of *PLAG1* expression and reduced expression of *CTNNB1*. Here we have studied the expression of *PLAG1* by Northern blot analysis in 47 primary benign and malignant human tumors with or without cytogenetic abnormalities of 8q12. Overexpression of *PLAG1* was found in 23 tumors (49%). Thirteen of 17 pleomorphic adenomas with a normal karyotype and 5 of 10 with 12q13–15 abnormalities overexpressed *PLAG1*, which demonstrates that *PLAG1* activation is a frequent event in adenomas irrespective of karyotype. In contrast, *PLAG1* was overexpressed in only 2 of 11 malignant salivary gland tumors analyzed, which suggests that, at least in salivary gland tumors, *PLAG1* activation preferentially occurs in benign tumors. *PLAG1* overexpression was also found in three of nine mesenchymal tumors, *i.e.*, in two uterine leiomyomas and one leiomyosarcoma. RNase protection, rapid amplification of 5′-cDNA ends (5′-RACE), and reverse transcription-PCR analyses of five adenomas with a normal karyotype revealed fusion transcripts in three tumors. Nucleotide sequence analysis of these showed that they contained fusions between *PLAG1* and *CTNNB1* (one case) or *PLAG1* and a novel fusion partner gene, *i.e.*, the gene encoding the transcription elongation factor SII (two cases). The fusions occurred in the 5′ noncoding region of *PLAG1*, leading to exchange of regulatory control elements and, as a consequence, activation of *PLAG1* gene expression. Because all of the cases had grossly normal karyotypes, the rearrangements must result from cryptic rearrangements. The results suggest that in addition to chromosomal translocations and cryptic rearrangements, *PLAG1* may also be activated by mutations or indirect mechanisms. Our findings establish a conserved mechanism of *PLAG1* activation in salivary gland tumors with and without 8q12 aberrations, which indicates that such activation is a frequent event in these tumors.

INTRODUCTION

We have previously identified (1, 2) a new, developmentally regulated zinc finger gene, designated *PLAG1*,[4] as the target gene in 8q12 in pleomorphic adenomas of the salivary glands with t(3;8)(p21;q12) translocations. The translocation results in promoter swapping between *PLAG1* and the constitutively expressed gene for β-catenin (*CTNNB1*) in 3p21, leading to activation of *PLAG1* expression and reduced expression of *CTNNB1*. The breakpoints invariably occur in the 5′ noncoding regions of both genes. The deduced PLAG1 protein contains seven NH_2-terminal C_2H_2 zinc finger domains and a serine-rich COOH terminus, which acts as a transcriptional activator (3). *PLAG1* is developmentally regulated with expression mainly restricted to certain fetal tissues. β-catenin is a protein functioning as an interface in adherens junctions and in the WG/WNT signaling pathway (4, 5). β-catenin has also been implicated in tumorigenesis (4).

Recently (6), we identified a second translocation partner gene of *PLAG1* in pleomorphic adenomas with a recurrent t(5;8)(p13;q12) translocation, namely *LIFR*. *LIFR* encodes the ubiquitously expressed receptor for the leukemia inhibitory factor (7). The translocation results in up-regulation of *PLAG1* gene expression under control of the *LIFR* promoter, *i.e.*, a mechanism similar to that seen in adenomas with 3;8-translocations.

In addition to the above-mentioned subgroup of pleomorphic adenomas with abnormalities involving 8q12 (39% of the cases), there are at least three other cytogenetic subgroups that are characterized by (*i*) rearrangements of 12q13–15 (8% of the cases); (*ii*) sporadic clonal changes not involving 3p21, 8q12 or 12q13–15 (23% of the cases); and (*iii*) an apparently normal karyotype (30% of the cases; Refs. 8–10). The gene consistently rearranged in adenomas with 12q13–15 abnormalities is the high mobility group protein gene, *HMGIC* (11–13). This gene is also rearranged in a variety of benign mesenchymal tumors with 12q13–15 abnormalities (11, 14–16).

Our previous studies of *PLAG1* were restricted to pleomorphic adenomas with 8q12 abnormalities (1, 6). Here we present results showing that *PLAG1* activation is not confined to adenomas with 8q12 abnormalities but is also found in tumors with normal karyotype and 12q13–15 abnormalities as well as in individual cases of malignant salivary gland tumors and mesenchymal tumors. In addition, we show that *PLAG1* may also be activated by cryptic rearrangements in cases with normal karyotypes, leading to fusions between *PLAG1* and *CTNNB1* or a novel fusion partner gene.

MATERIALS AND METHODS

Tumor Material and Cytogenetic Analysis. Fresh tumor tissue was obtained from patients at the time of surgery. Chromosome preparations were made from short-term primary cultures as described previously (17). Forty-

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[4] The abbreviations used are: *PLAG1*, pleomorphic adenoma gene 1; UTR, untranslated region; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase gene; RT-PCR, reverse transcription-PCR; 5′-RACE, rapid amplification of 5′-cDNA ends.

Table 1 *Northern blot analysis of PLAG1 gene expression in salivary gland tumors and mesenchymal tumors*

Tumor type	No. of cases	Cytogenetic findings	Overexpression of *PLAG1*
Salivary gland tumors			
Benign			
Pleomorphic adenoma	17	normal diploid karyotype	13
Pleomorphic adenoma	10	12q13–15 aberrations	5
Malignant			
Adenoid cystic carcinoma	1	−9	1
Adenoid cystic carcinoma	1	del(16)(q13–21)	0
Adenoid cystic carcinoma	1	del(6)(q24) + other abnormalities	0
Adenoid cystic carcinoma	1	+9	0
Adenoid cystic carcinoma	1	t(17;18)(p12;q11.2) + other abnormalities	0
Adenocarcinoma	1	del(6)(q22q24)	0
Adenocarcinoma	1		0
Epidermoid carcinoma	1	del(6)(q25)	0
Epidermoid carcinoma	1		0
Myoepithelial carcinoma	1		0
Carcinoma ex pleomorphic adenoma	1	del(6)(q22) + other abnormalities	1
Mesenchymal tumors			
Benign			
Uterine leiomyoma	3		2
Soft tissue chondroma	1	t(X;12;8;13)(q24;q15;q11;q14), der(8)del(8)(p22)	0
Aggressive fibromatosis	1	del(8)q13;q22) + other abnormalities	0
Infantile fibromatosis	1	46,XY	0
Fibrous dysplasia	1	t(3;8)(p21;q13) + other abnormalities	0
Malignant			
Malignant fibrous histiocytoma grade III		der(1)t(1;?;8)(q42;?;q13), der(8)t(8;13)(q13;q12) + other abnormalities	0
Leiomyosarcoma	1		1

seven primary tumors were selected for molecular analysis, including 17 pleomorphic adenomas with a normal diploid karyotype, 10 pleomorphic adenomas with chromosome 12q13–15 aberrations, 11 malignant salivary gland tumors, 3 uterine leiomyomas, and 1 case each of soft-tissue chondroma, aggressive fibromatosis, infantile fibromatosis, fibrous dysplasia, malignant fibrous histiocytoma, and leiomyosarcoma. Karyotypic information was available from 40 of the 47 tumors. The diagnoses and relevant karyotypic data are presented in Table 1.

Preparation of RNA and Northern Blot Analysis. Total RNA was extracted from frozen tumor samples using the TRIZOL (Life Technologies) method. Northern blot analysis was performed according to standard procedures (18). Probes for filter hybridizations were radio-labeled with [α-^{32}P]dCTP using the Megaprime DNA labeling system (Amersham). The *PLAG1* probe used was a 3.7 kb *Eco*RI cDNA fragment consisting of 438 bp of the 5′-UTR, the complete coding region, and approximately 1800 bp of the 3′-UTR. This probe detects a 7.5-kb *PLAG1* specific transcript. The actin probe used as an internal control was a 2.0-kb human β-actin cDNA probe (Clontech).

RNase Protection Assay. The antisense riboprobes used for the RNase protection assay are schematically illustrated in Fig. 2. Preparation of probes and hybridization conditions were as described previously (6). The protected riboprobes were purified by ethanol precipitation, resuspended in 80% (v/v) formamide, and analyzed by electrophoresis using a 5% polyacrylamide gel containing 7 M urea. End-labeled *Msp*I fragments of pcDNA3 (Invitrogen) were used as single-strand molecular weight markers.

5′-RACE and Nucleotide Sequence Analysis. 5′-RACE was performed according to the protocol of the Marathon cDNA amplification kit (Clontech) with minor modifications. For first-strand cDNA synthesis, 5 μg of total RNA was used together with the *PLAG1*-specific cDNA synthesis primer MV2-low located in exon 5 of *PLAG1* (Table 2). The double-stranded cDNA was then ligated to the cDNA adaptor and amplified using the adaptor primer AP1 and the *PLAG1*-specific MV5 primer also located in exon 5 of *PLAG1* (Table 2). A second round of PCR was performed using the nested adaptor primer AP2 and the MV6 primer in exon 4 of *PLAG1* (Table 2). The resulting PCR products were purified from agarose gels and cloned into the pCR2.1 vector (Invitrogen). Nucleotide sequence analysis was performed with an A.L.F. DNA sequencer (Pharmacia/LKB) using the T7 polymerase sequencing kit (Pharmacia/LKB). The resulting sequences were analyzed using Lasergene (DNASTAR) and basic local alignment search tool searches (National Center for Biotechnology Information).

RT-PCR Analysis. For cDNA synthesis, 5 μg of total RNA was reverse-transcribed using the SuperScript Preamplification System according to the manufacturer's manual (Life Technologies). An aliquot of 0.25 μg of the resulting first-strand cDNA was amplified using the appropriate primer sets. Primer sequences and annealing temperatures for all of the PCR primers used are shown in Table 2. All of the PCR amplifications were performed using the GeneAmp PCR system 9600 (Perkin-Elmer). For detection of the *CTNNB1/PLAG1* fusion transcript, the first round of PCR was carried out with the *CTNNB1* primer CAT-UP and the *PLAG1* primer MV5. The second round of PCR was performed on a 20-fold diluted sample with the *CTNNB1* primer NECAT-UP and the *PLAG1* primer MV6. For detection of the reciprocal fusion transcript *PLAG1/CTNNB1*, the following nested primer sets were used: START-UP (*PLAG1*) and CAT3 (*CTNNB1*), and START-RACE (*PLAG1*) and CAT3NEST (*CTNNB1*). The *SII/PLAG1* fusion transcript was detected after two rounds of PCR using the primer sets SII-UP (*SII*) and MV5 (*PLAG1*), and S2–764S (*SII*) and MV6 (*PLAG1*), respectively. For the reciprocal *PLAG1/SII* fusion transcript, the first round of PCR was performed using the *SII* primer S2–1105AS and the *PLAG1* primer START-UP, and the second round was performed using the *SII* primer S2–972AS and the *PLAG1* primer START-RACE. For detection of the *LIFR/PLAG1* fusion transcript, the primer sets LIFR-NEUP (*LIFR*) and MV5 (*PLAG1*), and LIFR-CAU (*LIFR*) and MV6 (*PLAG1*) respectively, were used. For detection of the normal *PLAG1* transcript, the first round of PCR was carried out with the *PLAG1* primers START-UP and MV5 and the second round was carried out using the primers START-RACE and MV6. The normal *SII* transcript was detected using the primers SII-UP and S2–1105AS in the first round of PCR and S2–764S and S2–972AS in the second round. The normal *CTNNB1* transcript was amplified

Table 2 *Primer sequences used for 5′RACE and RT-PCR analyses*

Primer	Gene	Nucleotide sequence 5′-3′	T_a (°C)
START-UP	*PLAG1* (exon 1)	CAATGGCTGCTGGAAAGAGG	60
START-RACE	*PLAG1* (exon 1)	GGCCGGAGGGAGGATGTTAA	60
MV6	*PLAG1* (exon 4)	TGCACTTGTAGGGCCTCTCTCCTG	68
MV5	*PLAG1* (exon 5)	CAGGAGAATGAGTAGCCATGTGC	68
MV2-low	*PLAG1* (exon 5)	CTGCACTTGACCCACCCCTTGGAT	
CAT-UP	*CTNNB1* (exon 1)	TGTGGCAGCAGCGTTGGCCCG	68
NECAT-UP	*CTNNB1* (exon 1)	ACGGAGGAAGGTCTGAGGAGCAG	68
CAT-3	*CTNNB1* (exon 3)	AAGGAGCTGTGGTAGTGGCAC	63
CAT3NEST	*CTNNB1* (exon 3)	GCCGCTTTTCTGTCTGGTTCCA	63
SII-UP	*SII* (nt 733-753)	CATGCGGTGGTGGGGGTTGCT	68
S2-764S	*SII* (nt 762-782)	GGGGTCGCTCCTGCTGTGTCT	68
S2-1105AS	*SII* (nt 1104-1082)	AGGTCTTTCTCAGTTGATGGCCC	60
S2-972AS	*SII* (nt 972-949)	TGCAGTAATTCCAGGGTCATAGGA	60
SII-low	*SII* (nt 910-888)	CGCGTTCTTCTTCTGCACCATCT	68
LIFR-NEUP	*LIFR* (exon 1)	AGAACGTGTCTCTGCTGCAAGGC	68
LIFR-CAU	*LIFR* (exon 1)	CTCCTAATCCCAGCTCAGAAAGG	68
GAPDH2	*GAPDH* (nt 4-22)	GTGAAGGTCGGAGTCAACG	50
GAPDH3	*GAPDH* (nt 286-303)	GGTGAAGACGCCAGTGGACTC	50

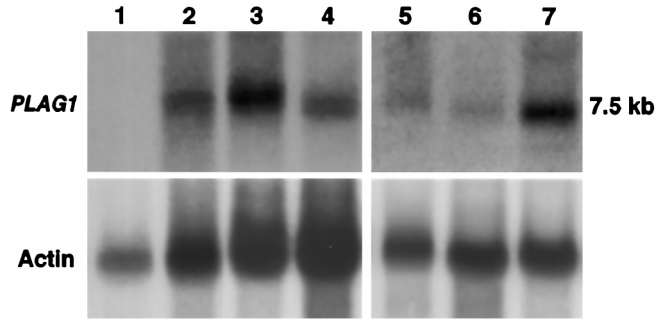

Fig. 1. Northern blot analysis of *PLAG1* expression in pleomorphic adenomas. Twenty μg of total RNA were size-fractionated electrophoretically, transferred to Hybond N filters, and hybridized to a 3.7-kb *PLAG1* cDNA probe. RNAs tested included samples from normal salivary gland tissue (*Lane 1*); adenoma C895, which has a t(5;8)(p13;q12) (*Lane 2*); and five adenomas with a normal karyotype: C954 (*Lane 3*), C1067 (*Lane 4*), C1102 (*Lane 5*), CG557 (*Lane 6*), and CG568 (*Lane 7*). To evaluate both the quantity and quality of the RNA samples tested, the same filter was rehybridized with a 2.0-kb human β-actin cDNA probe.

using the primer sets: CAT-UP and CAT3, and NECAT-UP and CAT3NEST. The identities of the PCR products were confirmed by nucleotide sequence analysis. As control for intact RNA and cDNA, an RT-PCR reaction for expression of the housekeeping gene *GAPDH* was performed on all of the cDNAs used (19).

RESULTS

Northern Blot Analysis of *PLAG1* Gene Expression in Tumors. The expression pattern of the *PLAG1* gene in 47 benign and malignant salivary gland tumors and mesenchymal tumors was studied using Northern blot analysis (Table 1). In normal salivary gland tissue, *PLAG1* expression was detectable only by RT-PCR and RNase protection analyses and not by conventional Northern blot analysis (Figs. 1 and 2). In tumors, however, *PLAG1* overexpression was detected by Northern blot analysis in 49% (23 of 47) of the cases. In pleomorphic adenomas, *PLAG1* overexpression was observed in 13 of 17 tumors with a normal karyotype (Fig. 1) and in 5 of 10 tumors with rearrangements of 12q13–15. In contrast, only 2 of 11 malignant salivary gland tumors overexpressed *PLAG1*, *i.e.*, 1 adenoid cystic carcinoma and 1 carcinoma ex pleomorphic adenoma. Among the mesenchymal tumors, two of three uterine leiomyomas and one leiomyosarcoma overexpressed *PLAG1*. The karyotypes of these tumors are not known. None of the four mesenchymal tumors with known cytogenetic aberrations affecting 8q11–13 overexpressed *PLAG1*.

RNase Protection Analysis of the *PLAG1* Transcript in Five Tumors with a Normal Karyotype. Five of the pleomorphic adenomas with a normal karyotype that overexpressed the 7.5-kb *PLAG1* transcript (C954, C974, C1067, C1102, and CG568) were selected for further analysis using the RNase protection assay (Fig. 2). To detect the normal 7.5-kb *PLAG1* transcript, we used a riboprobe corresponding to the noncoding exons 1 and 3 of *PLAG1* (riboprobe b) because exon 2 is often missing as a result of alternative splicing. To detect the *CTNNB1/PLAG1* fusion transcript, we used riboprobe c which contains exon 1 of *CTNNB1* and exon 3 of *PLAG1*. In normal salivary gland tissue, a protected fragment of 207 nucleotides was obtained with riboprobe b (*Lane 5*), indicating the presence of a normal *PLAG1* transcript containing exons 1 and 3. A protected fragment of 207 nucleotides was also detected in adenomas C974, C1102, and CG568 (*Lanes 11*, *17*, and *20*), which suggests that these tumors contain a normal *PLAG1* transcript. In contrast, similar analysis of RNA from adenomas C954 and C1067 failed to detect this fragment (*Lanes 8* and *14*). Instead, protected fragments of 67 bp were found in both tumors suggestive of the presence of chimeric mRNAs containing ectopic sequences fused to exon 3 of *PLAG1*. In C1067 this chimeric mRNA corresponds to a *CTNNB1/PLAG1* fusion transcript because riboprobe c gave a protected fragment of 219 bp (*Lane 15*) indicative of such a transcript. In adenoma C954, a *CTNNB1/PLAG1* fusion transcript could not be detected (*Lane 9*), indicating that in this case, novel ectopic sequences are fused to exon 3 of *PLAG1*.

Identification of *SII* as a New Fusion Partner Gene of *PLAG1*. Adenomas C954, C1067, and CG568 were selected for further characterization of the putative chimeric *PLAG1* transcripts using 5′-RACE analysis. Nucleotide sequence analysis of PCR products from adenoma C954 confirmed the presence of ectopic sequences fused to exon 3 of *PLAG1*. Basic local alignment search tool search of these sequences showed that they were identical to those of the gene encoding the transcription elongation factor SII (GenBank accession number X73534). The ectopic sequences were also assigned to chromosome 3 by PCR-analysis of National Institute of General Medical Sciences monochromosome hybrid Mapping Panel 2 (data not shown). This assignment is in agreement with the published localization of *SII* to 3p21.3–p22 (20). The fusion point in *SII* was at nucleotide position 910 resulting in a fusion of 5′ noncoding se-

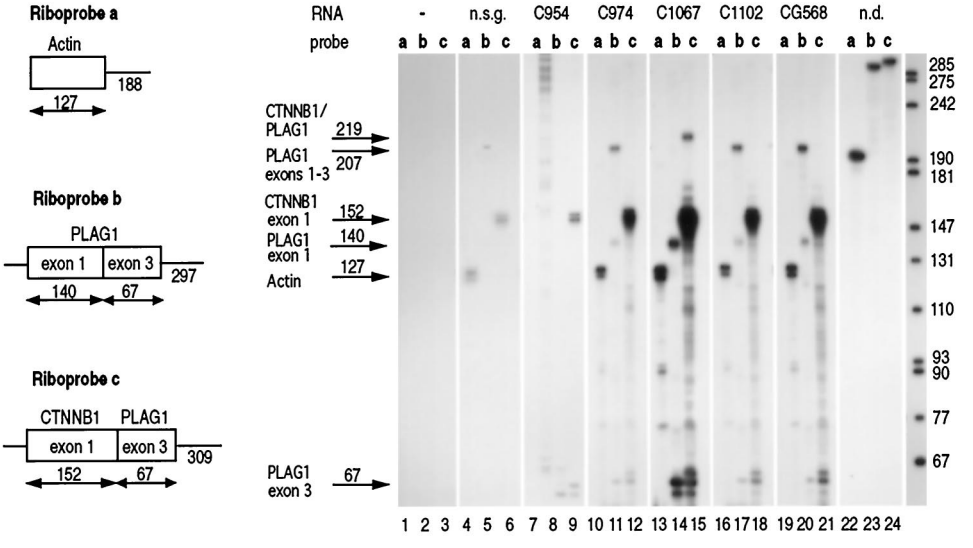

Fig. 2. RNase protection analysis of *PLAG1* fusion transcripts in five pleomorphic adenomas with a normal karyotype. On the *left*, the plasmid inserts used to generate the three riboprobes a, b, and c are schematically illustrated. The sizes of the various fragments are indicated. The three riboprobes were used in hybridization experiments using 5 μg of total RNA from yeast tRNA (*Lanes 1–3*), normal salivary gland tissue (*n.s.g.*; *Lanes 4–6*), adenoma C954 (*Lanes 7–9*), adenoma C974 (*Lanes 10–12*), adenoma C1067 (*Lanes 13–15*), adenoma C1102 (*Lanes 16–18*), and adenoma CG568 (*Lanes 19–21*). An actin probe was used as an internal control to show that similar amounts of RNA have been used in the various experiments (*Lanes 4*, *7*, *10*, *13*, *16*, and *19*). Undigested riboprobes (*n.d.*) are shown in *Lanes 22–24*. *Arrows* point to the protected fragments. As molecular weight markers, pcDNA3 digested with *Msp*I was used. On the *right*, the sizes (in bp) of the different fragments are indicated.

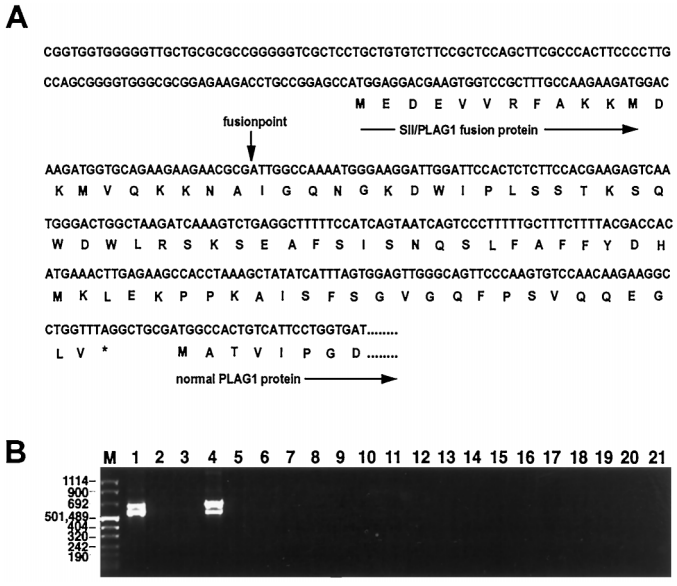

Fig. 3. Detection of *SII/PLAG1* fusion transcripts by 5′-RACE and RT-PCR in pleomorphic adenomas. *A*, nucleotide and deduced amino acid sequence data of the *SII/PLAG1* fusion in adenoma C954. *Vertical arrow* indicates the fusion point. The fusion transcript is composed of 5′ noncoding sequences as well as 63 nucleotides of the coding region of *SII* fused to the acceptor splice site of exon 3 of *PLAG1*. The fusion transcript encodes a truncated SII/PLAG1 protein of 90 amino acids as well as an apparently normal PLAG1 protein. The *asterisk* indicates the stop codon. *B*, analysis of *SII/PLAG1* fusion transcripts by RT-PCR in 20 salivary gland tumors overexpressing *PLAG1* as determined by Northern blot analysis. cDNAs tested include samples from 13 pleomorphic adenomas with a normal karyotype (*Lanes 1–13*), 5 pleomorphic adenomas with 12q13–15 abnormalities (*Lanes 14–18*), and 2 malignant salivary gland tumors (*Lanes 19–20*) as well as a control sample without cDNA template (*Lane 21*). Primer sets specific for the *SII/PLAG1* fusion transcripts give rise to a product of 557 bp consisting of 5′ *SII* sequences fused to exons 3 and 4 of *PLAG1*, as well as a 662-bp product consisting of 5′ *SII* sequences fused to exons 2, 3, and 4 of *PLAG1*. Fusion transcripts were detected in two adenomas with a normal karyotype (C954 and C974; *Lanes 1* and *4*). As molecular weight markers (*M*), the DNA Molecular Weight Marker VIII (Boehringer Mannheim) was used.

quences as well as 63 nucleotides of the coding region to the acceptor splice site of *PLAG1* exon 3 (Fig. 3*A*).

5′-RACE and nucleotide sequence analyses of PCR products from adenoma C1067 confirmed the findings of the RNase protection assay and showed that this tumor indeed contained a hybrid transcript consisting of exon 1 of *CTNNB1* fused to exon 3 of *PLAG1*.

5′-RACE and nucleotide sequence analyses of amplified fragments from adenoma CG568 revealed that this tumor only expressed an apparently normal *PLAG1* transcript and no fusion transcript.

RT-PCR Analysis of *PLAG1* Gene Fusions. Using RT-PCR, we also screened the 20 benign and malignant salivary gland tumors that expressed *PLAG1* (Table 1) to search for additional cases with hidden *CTNNB1/PLAG1*, *SII/PLAG1*, and *LIFR/PLAG1* gene fusions. Amplification with primers specific for exon 1 of *CTNNB1* and exon 4 of *PLAG1* revealed *CTNNB1*/*PLAG1* fusion transcripts only in adenoma C1067 and in control RNA from adenoma CG588, which has the classical t(3;8)(p21;q12) (1). In both tumors, two products of 509 bp and 614 bp were observed, consistent with hybrid transcripts containing exon 1 of *CTNNB1* fused to either exons 3 and 4 of *PLAG1* or to exons 2 to 4 of *PLAG1* (data not shown). The reciprocal *PLAG1*/*CTNNB1* fusion transcript could only be detected in adenoma CG588. Amplification with primers specific for *SII* and *PLAG1* revealed fusion transcripts of 557 bp and 662 bp, respectively, in two adenomas with normal karyotypes, C954 and C974 (Fig. 3*B*). Sequence analysis of these products showed that they consisted of hybrid transcripts containing 149 nucleotides (including 5′ noncoding sequences and 63 nucleotides of the coding region) of *SII* fused to either exons 3 and 4 of *PLAG1* or to exons 2 to 4 of *PLAG1*. The reciprocal fusion transcript *PLAG1*/*SII* could only be detected in C954. Amplification with primers specific for the *LIFR/PLAG1* fusion transcript resulted in two PCR products of 474 bp and 579 bp, respectively, only in control RNA from adenoma C895, which has a t(5;8)(p13;q12) with a known *LIFR/PLAG1* fusion (6), demonstrating that none of the tumors analyzed expressed this fusion transcript. The normal transcripts for *PLAG1*, *CTNNB1*, and *SII* were detected in C954, C974, and C1067 as well as in normal salivary gland tissue. A positive *GAPDH* RT-PCR, resulting in a 299 bp fragment, was obtained in all of the cases analyzed by RT-PCR.

DISCUSSION

PLAG1 was originally identified as the gene consistently rearranged in pleomorphic adenomas with chromosome translocations involving 8q12 (1, 2, 6). In this paper we show that *PLAG1* is activated not only in adenomas with 8q12 abnormalities but also in tumors with a normal karyotype and 12q13–15 abnormalities as well as in individual cases of malignant salivary gland tumors and smooth muscle tumors. In addition, we show that *PLAG1* may also be activated by cryptic rearrangements in cases with normal karyotypes, leading to fusions between *PLAG1* and *CTNNB1* or a novel fusion partner gene *SII*.

Overexpression of *PLAG1* as determined by Northern blot analysis was found in 76% of pleomorphic adenomas with a normal karyotype. In none of these cases was there any cytogenetic evidence of rearrangements of band 8q12, which indicates that *PLAG1* in these cases is activated by mechanisms other than gross chromosomal changes. Further analysis of these tumors using RNase protection, 5′-RACE, and RT-PCR analyses revealed chimeric transcripts with ectopic sequences fused to exon 3 of *PLAG1* in three tumors. One of the tumors contained a chimeric transcript resulting from a fusion of exon 1 of *CTNNB1* to exon 3 of *PLAG1*, *i.e.*, the same fusion as in tumors with the classical t(3;8)(p21;q12) (1). In two other adenomas we could demonstrate that the *SII* gene is a novel and recurrent fusion partner of *PLAG1*. The fusion points in *SII* were in both cases at nucleotide position 910, resulting in a fusion of 5′ noncoding sequences and 63 nucleotides of the coding region to exon 3 of *PLAG1*. Because all three of the tumors had a normal karyotype, these fusions must result from cryptic rearrangements. In the tumor with the *CTNNB1/PLAG1* fusion, this rearrangement was, however, too small to be detected by fluorescence *in situ* hybridization using yeast artificial chromosomes and cosmids containing these genes (data not shown). In all of the three tumors, the rearrangements led to up-regulation of *PLAG1* gene expression under control of the *CTNNB1* and *SII* promoters, respectively. Together with *CTNNB1* (1) and *LIFR* (6), *SII* is the third fusion partner gene known for *PLAG1*.

The *SII* gene (also known as *TCEA1*) encodes the transcription elongation factor SII (21, 22). The *SII* locus has previously been assigned to human chromosome segment 3p21.3–22 (20), *i.e.*, to the same region as *CTNNB1*. The gene encodes a ubiquitously expressed protein with a predicted molecular mass of 38 kDa (22, 23). SII belongs to the group of RNA polymerase II general elongation factors that are proteins involved in the regulation of the transcription of most, if not all, eukaryotic protein-coding genes (reviewed in Ref. 22). SII facilitates elongation of transcripts by preventing RNA polymerase II from terminating transcription prematurely at transcriptional blockages. The *SII* promoter contains two putative GC-box type and two CCAAT-box consensus sequences as well as an *Alu* sequence (21). There are also several potential binding sites for transcription factors such as Sp1, MEP-1, TCF-2, and E2A.

The fusion points in *SII* were at nucleotide position 910 in both tumors. In the corresponding gene in *Xenopus*, which contains 10

exons and 9 introns, this position coincides with the location of the first intron (24). Similarly, the related human gene *TCEA2* has also an intron at this position (25). The fact that the fusions occurred at the same nucleotide position in both tumors suggests that the breakpoints might have occurred in an intron. This is at variance with the findings of Park *et al.* (21), who reported that the human *SII* gene lacks introns. Further analysis of the genomic organization of *SII* will be necessary to resolve this discrepancy. To the best of our knowledge, this is the first time *SII* has been implicated in human neoplasia.

The *SII/PLAG1* fusions are the first examples where the breakpoints in a *PLAG1* fusion partner gene interrupt the coding sequence (the breakpoints in *CTNNB1* and *LIFR* invariably occur in the 5′ noncoding regions). The resulting fusion transcripts encode a truncated SII/PLAG1 protein of 90 amino acids as well as an intact PLAG1 protein (Fig. 3*A*). Because the coding region of *PLAG1* is not interrupted, the consequences of the fusion is the same as for those involving *CTNNB1* and *LIFR*, *i.e.*, exchange of the *PLAG1* promoter by an ubiquitously expressed promoter, leading to ectopic expression of *PLAG1*. Recently, a similar observation was made in B-cell non-Hodgkin's lymphomas with t(3;6)(q27;p21), in which, as a result of the translocation, the entire *H4* gene or part of the coding region including 5′ regulatory sequences replaced the 5′ noncoding region of the zinc finger gene *BCL6*, resulting in transcriptional deregulation of *BCL6* (26).

Our finding of a reciprocal fusion transcript between *SII* and *PLAG1* in one tumor demonstrates that the activation of *PLAG1* by promoter swapping occurs not only in tumors with the classical t(3;8)(p21;q12) (1) but also as a result of cryptic rearrangements in tumors with a normal karyotype. In the two other tumors with *CTNNB1/PLAG1* and *SII/PLAG1* fusions, no reciprocal transcripts could be detected, not even after multiple rounds of PCR, whereas the normal *PLAG1*, *CTNNB1*, and *SII* transcripts were detected in both tumors. The mechanisms in these two cases may, therefore, be classified as promoter substitution, resulting from a nonreciprocal rearrangement such as, for example, an insertion. Indeed, this was recently observed in a pleomorphic adenoma with normal karyotype, which was shown to have a hidden insertion of the 12q15 segment into 9p23, resulting in a fusion of the last coding exon of the *NF1B* gene to exons 1 to 4 of the *HMGIC* gene (13).

In adenoma CG568, 5′-RACE analysis revealed an apparently normal *PLAG1* transcript, suggesting that *PLAG1* in this case may be activated by a mechanism other than gene fusion. In C974, the RNase protection analysis revealed protected fragments of 207 bp (corresponding to a normal *PLAG1* transcript) and 67 bp (corresponding to exon 3 in the *SII/PLAG1* fusion transcript), respectively, whereas in C954 and C1067, only the 67 bp fragments (corresponding to the *SII/PLAG1* and *CTNNB1/PLAG1* fusion transcripts) were observed. These observations raise the question of whether normal *PLAG1* alleles independently or concomitantly with gene fusions may be activated by *e.g.*, mutations as previously described for *BCL6* (27–29).

Overexpression of *PLAG1* was also observed in 50% of the pleomorphic adenomas with 12q13–15 abnormalities. Previous studies have shown that the gene consistently rearranged in adenomas with 12q13–15 involvement is *HMGIC* (11–13). A crucial question was, therefore, to find out whether *PLAG1* and *HMGIC* may be affected in the same tumors. In two of the five tumors with *PLAG1* activation, the status of *HMGIC* was known. Both tumors contained *HMGIC/NF1B* fusion transcripts (13), demonstrating that, indeed, both genes may be affected in the same tumor. The frequency and role of such coexpression remains, however, to be determined. The fact that *PLAG1* is affected not only in adenomas with 8q12 abnormalities but also in adenomas with 12q13–15 abnormalities and normal karyotype further emphasize the importance of *PLAG1* in salivary gland tumorigenesis.

In contrast to the high frequency of overexpression of *PLAG1* in benign salivary gland tumors, only 2 of 11 malignant salivary gland tumors analyzed overexpressed *PLAG1*, *i.e.*, 1 carcinoma ex pleomorphic adenoma and 1 adenoid cystic carcinoma. In the former case, it is likely that *PLAG1* was already activated in the benign pleomorphic adenoma before malignant transformation. In the latter case, we cannot rule out that this may also represent a carcinoma ex pleomorphic adenoma because it is known that adenoid cystic carcinomas may develop from the epithelial components of preexisting pleomorphic adenomas (30). It should be pointed out that none of the two tumors with *PLAG1* activation had any cytogenetic evidence of rearrangements of 8q12. These observations suggest that the activation of *PLAG1* in salivary gland tumors is largely confined to benign pleomorphic adenomas and rarely occur in malignant salivary gland tumors, with the exception of carcinoma ex pleomorphic adenoma.

To find out whether *PLAG1* activation is restricted to tumors of epithelial origin, we also analyzed a series of mesenchymal tumors. Three of 9 tumors analyzed overexpressed *PLAG1*. All of the three cases were smooth muscle tumors. Whether *PLAG1* overexpression in mesenchymal tumors is limited to smooth muscle tumors remains, however, to be determined. There is an interesting similarity between pleomorphic adenomas and uterine leiomyomas in that both tumor types contain subgroups with rearrangements of *HMGIC* (11, 14, 15). In the present cases, it was, however, not possible to determine whether *HMGIC* was affected, but it is plausible that in uterine leiomyomas also, both *PLAG1* and *HMGIC* may be affected in the same tumors. Because most pleomorphic adenomas with 8q12 abnormalities overexpress *PLAG1*, it was of interest to see whether mesenchymal tumors with similar abnormalities also have activation of *PLAG1*. However, in none of the four cases analyzed did we find any evidence of *PLAG1* overexpression, which indicates that *PLAG1* is not the target gene in proximal 8q in these cases.

The results of this investigation clearly demonstrate that activation of *PLAG1* is a frequent genetic event occurring in all of the major cytogenetic subgroups of pleomorphic adenomas as well as in certain mesenchymal tumors. In addition to activation by chromosomal translocations or cryptic rearrangements in cases with normal karyotypes, our findings indicate that *PLAG1* may also be activated by other mechanisms such as mutations or indirect mechanisms. These mechanisms may operate independently in different tumors or concomitantly in the same tumor. In recent functional studies of the *PLAG1* gene (3), it was established that PLAG1 possess transcriptional activation capacity, raising the possibility that benign salivary gland tumors may originate because of the activation of particular target genes by ectopically overexpressed *PLAG1*.

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