**PLAG1**, the Main Translocation Target in Pleomorphic Adenoma of the Salivary Glands, Is a Positive Regulator of **IGF-II**

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**ABSTRACT**

PLAG1, a novel developmentally regulated C\(_2\)H\(_2\) zinc finger gene, is consistently rearranged and overexpressed in pleomorphic adenomas of the salivary glands with 8q12 translocations. In this report, we show that PLAG1 is a nuclear protein that binds DNA in a specific manner. The consensus PLAG1 binding site is a bipartite element containing a core sequence, GRRGCC, and a G-cluster, RGGK, separated by seven random nucleotides. DNA binding is mediated mainly via three of the seven zinc fingers, with fingers 6 and 7 interacting with the core and finger 3 with the G-cluster. In transient transactivation assays, PLAG1 specifically activates transcription from its consensus DNA binding site, indicating that PLAG1 is a genuine transcription factor. Potential PLAG1 binding sites were found in the promoter 3 of the human insulin-like growth factor II (IGF-II) gene. We show that PLAG1 binds IGF-II promoter 3 and stimulates its activity. Moreover, IGF-II transcripts derived from the P3 promoter are highly expressed in salivary gland adenomas overexpressing PLAG1. In contrast, they are not detectable in adenomas without abnormal PLAG1 expression nor in normal salivary gland tissue. This indicates a perfect correlation between PLAG1 and IGF-II expression. All of these results strongly suggest that IGF-II is one of the PLAG1 target genes, providing us with the first clue for understanding the role of PLAG1 in salivary gland tumor development.

**INTRODUCTION**

Activation of the PLAG1 gene on chromosome 8q12 is the most frequent gain-of-function mutation found in pleomorphic adenomas of the salivary glands (1, 2). This mainly results from recurrent chromosomal translocations that lead to promoter substitution between PLAG1, a gene mainly expressed in fetal tissue, and more broadly expressed genes. The three translocation partners characterized thus far are the PLAG1, a gene mainly expressed in fetal tissue, and more broadly expressed in the salivary glands (1, 2). This mainly results from recurrent chromosomal translocation, the t(3;8)(p21;q12), the PLAG1/growth factor receptor gene partner, leads to ectopic expression of PLAG1 in the tumoral adult salivary glands, by a strong promoter derived from the translocation part, leading to ectopic expression of PLAG1 in the tumoral cells. This abnormal PLAG1 expression presumably results in a deregulation of PLAG1 target genes, causing salivary gland tumorigenesis.

The PLAG1 protein contains seven canonical C\(_2\)H\(_2\) zinc finger domains and a serine-rich COOH terminus that exhibits transactivation capacities when fused to the Gal4 DNA binding domain (4), suggesting that it may act as a transcriptional regulator.

To extend our knowledge on the function of the PLAG1 gene and the mechanisms by which it causes salivary gland adenomas, we decided to further investigate functional characteristics of PLAG1 and in particular its potential transcriptional role. We determined in which subcellular compartment PLAG1 exerts its function by immunofluorescence studies; determined whether PLAG1 could bind DNA in a sequence-specific manner and identified its consensus DNA binding site by performing CASTing experiments. The zinc fingers required for sequence-specific DNA binding were determined by deletion/mutation analysis; and we used the PLAG1 consensus was used to screen the eukaryotic promoter databank. Possible target genes were studied regarding the capacity of PLAG1 to bind and activate their promoter. Finally, we analyzed the expression of such target genes in normal salivary gland tissue and in pleomorphic adenomas with or without PLAG1 overexpression to determine whether these genes could be PLAG1 targets in salivary gland adenomas.

**MATERIALS AND METHODS**

Construction and Production of GST-PLAG1 Fusion Proteins. The PLAG1 NH\(_2\)-terminal region (N2-C244) as well as parts of it (N84-C244; N101-C244; N159-C244) were fused in-frame to GST by inserting in pGEX-5X-2 (Pharmacia) the DNA fragments obtained by the PCR with full-length PLAG1 cDNA as template. The NH\(_2\)-terminal oligonucleotides used to generate the various constructs were: G8N159, 5'-CGGGATTCTTGAGTCATCATCTGAAACGTCGACC-3'; G8N84, 5'-CCGGATCTTGAGTCATCATCTGAAACGTCGACC-3'; G8N101, 5'-CCGGATCTTGAGTCATCATCTGAAACGTCGACC-3'; G8N159, 5'-CGGGATTCTTGAGTCATCATCTGAAACGTCGACC-3' as COOH-terminal oligonucleotide G8C244, 5'-GGGGTCCGACCTGGTTCTTCATGGAGGAGT-3' and 2,5-diphosphate 2.5-32P]dCTP (DuPont NEN), and 10 units of Amplitaq (Perkin-Elmer-Cetus) and treated as follows: 5 min at 94°C, 20 min at 65°C, and 20 min at 72°C. Fifty μl of each deoxynucleoside triphosphate, 2.5 μl 32P-α-32P]dCTP (DuPont NEN), and 10 units of Amplitaq (Perkin-Elmer-Cetus) and treated as follows: 5 min at 94°C, 20 min at 65°C, and 20 min at 72°C. Fifty μl of each deoxynucleoside triphosphate, 2.5 μl 32P-α-32P]dCTP (DuPont NEN), and 10 units of Amplitaq (Perkin-Elmer-Cetus) and treated as follows: 5 min at 94°C, 20 min at 65°C, and 20 min at 72°C. Fifty μl of each deoxynucleoside triphosphate, 2.5 μl 32P-α-32P]dCTP (DuPont NEN), and 10 units of Amplitaq (Perkin-Elmer-Cetus) and treated as follows: 5 min at 94°C, 20 min at 65°C, and 20 min at 72°C.
precipitation. An aliquot was used for the subsequent amplification reaction in 100 μl of polymerase reaction buffer containing 200 pmol of each amplifier CAST-UP (5'-CTGCGGGAATTCCGGTACG-3') and CAST-Low, 200 μM deoxynucleotide triphosphates, and 2.5 units of AmpliTaq (Perkin-Elmer Cetus) with 1 μl of [α-32P]dCTP (DuPont NEN) with 25 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. The amplified products were subsequently used for a second round of selection performed as described above. After four rounds of selection, the subsequent three steps of selection were performed by EMSA with 100 ng of eluted GST-PLAG1 (N2-C244). After X-ray exposure of the dried gel, the shifted bands were cut out of the gel, and the double-stranded DNA was eluted 3 h at 50°C in 200 μl of polymerase reaction buffer. An aliquot of the eluate was used for amplification. After a total of seven amplification cycles, the oligonucleotides were cloned into the pGEM-T Easy vector according to the manufacturer’s protocol (Promega), and 23 independent clones were sequenced.

EMSA. The different probes were synthesized as complementary oligonucleotides with 4-bp sticky ends, annealed, subsequently end-labeled with [α-32P]dCTP and Klenow enzyme, and finally purified with the QiAquick Nucleotide removal kit (Qiagen). DNA-protein binding reactions were carried out for 10 min at room temperature in 30 μl of EMSA binding buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 50 μM ZnCl2, 10% glycerol, 1 mM MgCl2, 1 mM DTT, 1 μg polydeoxyadenylic acid-polythymidylic acid (Sigma), 1 μg of salmon sperm DNA, and 300 ng of BSA] with 10,000 dpm of probes (about 0.1 ng of oligonucleotides) and an equimolar amount of proteins. DNA-protein complexes were analyzed on nondenaturing polyacrylamide gels [6% acrylamide] with 1× TBE (pH 8.0) and 5% glycerol]. Electrophoresis was performed at 4 V/cm at 14°C.

Plasmid Constructions. The PLAG1 expression vector pCDNA3-PLAG1 was constructed by inserting into EcoRI/XhoI-digested pCDNA3 (Invitrogen) the complete open reading frame of PLAG1 preceded by its own Kozak consensus translation start site. This fragment was generated by PCR using Pfu polymerase (Stratagene) with the 5′ primer GGN3-5′-CCCAGATATCCCTGCTGAGGCTGT-3′ and the 3′ primer GSC500-5′-GGGCTCGAGACAATGTATTATGAACAC3′. The same blunt-ended fragment was also cloned in the blunt-ended EcoRI site of the PCAGGS vector (5) to get a second expression construct for PLAG1 (pCAGGS-PLAG1) with higher levels of expression in transfected cells. The three mutant PLAG1 proteins (PLAG1-F2mut, PLAG1-F3mut, and PLAG1-F7mut) were produced by replacing in pCDNA3-PLAG1 the first histidine in the C2H2 motif of the corresponding zinc finger (His81, His110, and His231, respectively) with an alanine. For this, we applied the QuickChange Site-directed Mutagenesis kit (Stratagene) according to the instructions of the supplier. All constructs were sequenced to confirm the fidelity of the PCR and the site-specific mutagenesis. The full-length PLAG1 protein as well as the three mutants were expressed by in vitro translation was monitored by SDS gel analysis of [35S]Met-labeled proteins. The full-length PLAG1 protein as well as the three mutants were expressed by the complete open reading frame of PLAG1 (17). The expression constructs were cotransfected in COS-1 kidney fibroblast cells (ATCC, CRL1650) were grown on glass chamber slides (Nunc) and transfected with 1 μg of pCAGGS-PLAG1 and 1 μg of PM3 plasmid, which encodes the Gal4 DNA binding domain (amino acids 1–147; Ref. 16). Twenty-four h after transfection, the cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, followed by two washes with PBS. Slides were then incubated in PBS-BT (PBS/0.5% blocking reagent (Boehringer Mannheim)/0.2% Triton X-100) for 30 min at room temperature. To simultaneously detect the Gal4 and Gal4 proteins, cotransfected cells were incubated at room temperature for 1 h in PBS-BT containing the rabbit polyclonal anti-PLAG1 together with the mouse monoclonal anti-Gal4 (SC-510, Santa-Cruz). The polyclonal anti-PLAG1 was obtained by immunizing rabbits with the peptide FSSTSYAISIPEKEQPL (amino acids 336–352 in PLAG1) and the specificity of the antibody was verified by Western blot analysis (data not shown). After three washes with PBS-T (PBS/0.2% Triton X-100), the slides were incubated in PBS-BT with FITC-labeled swine anti-rabbit (DAKO, F0205) and Texas red-labeled sheep antimouse (Amersham, N2031). This allowed simultaneous visualization of the PLAG1 (FITC, green) and Gal4 (Texas red) proteins. After three washes in PBS-T, slides were mounted in Citifluor containing 0.5 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) and analyzed with a Zeiss Axiopt microscope equipped with UV optics. Images were recorded with a CE2000 CCD camera (Photometrics), using Smart capture (Digital Scientific) and Iplab Spectrum (Signal Analytics) software.

RESULTS

PLAG1 Is Localized in the Nucleus. The presence of seven canonical C2H2 zinc fingers and the transactivation capacities of the COOH-terminal domain suggest that PLAG1 may act as a transcriptional regulator. A prerequisite for such a role is that the protein be localized, at least in some conditions, in the nucleus. To determine the subcellular localization of PLAG1, we have made an eukaryotic expression construct (pCAGGS-PLAG1) directing the synthesis of a full-length PLAG1 protein in transfected cells. Immunofluorescence staining of transfected COS-1 cells indicates that PLAG1 protein is confined to the nucleus as demonstrated by the green staining observed with the PLAG1-specific antibody (Fig. 1A), which perfectly coincides with the DAPI nuclear DNA staining (Fig. 1B). The PLAG1 protein also colocalizes with the well-characterized nuclear protein
The presence of a zinc finger domain in PLAG1 as well as its nuclear localization suggests that PLAG1 is a DNA binding protein. To test this hypothesis and to identify the putative PLAG1 binding consensus sequence, CASTing was performed as described in “Materials and Methods.” The protein used for this study was a chimeric protein containing the complete zinc finger domain (amino acids 2–244) fused in-frame to the GST. The fusion protein GST-PLAG1 (N2-C244) was immobilized on Glutathione-Sepharose beads and incubated with a pool of oligonucleotides containing a central region of 25 random nucleotides. The frequency of the number of oligonucleotides that carried a base at that position.

Fig. 1. Nuclear localization of the PLAG1 protein. Immunofluorescence of Cos-1 cells cotransfected with the PLAG1 expression vector construct, pCAGGS-PLAG1, and with the pM3 expression vector expressing the DNA binding domain of Gal4 (16). The cells were cotransfected with the PLAG1 antibody, 4’6-diamidino-2-phenylindole and the Gal4 antibody (see “Materials and Methods”). This allows the visualization in the same cells of PLAG1 (A), nuclear DNA (B), Gal4 (C), or all three (D).

Gal4 (Fig. 1, C and D). Nuclear localization of the endogenous PLAG1 protein was also established by immunofluorescence analysis of the fetal kidney 293 cell line and independently confirmed by Western blot analysis of 293 nuclear extracts (data not shown).

The PLAG1 Binding Site Is Composed of Two Essential Parts, a GRGGC Core and a G-Cluster. The presence of a zinc finger domain in PLAG1 as well as its nuclear localization suggests that PLAG1 is a DNA binding protein. To test this hypothesis and to identify the putative PLAG1 binding consensus sequence, CASTing was performed as described in “Materials and Methods.” The protein used for this study was a chimeric protein containing the complete zinc finger domain (amino acids 2–244) fused in-frame to the GST. The fusion protein GST-PLAG1 (N2-C244) was immobilized on Glutathione-Sepharose beads and incubated with a pool of oligonucleotides containing a central region of 25 random nucleotides. The pool of oligonucleotides selected by seven rounds of PLAG1 binding was cloned, and 23 independent clones were sequenced. The alignment of all the sequences with the program Macaw (National Center for Biotechnology Information) clearly revealed a consensus sequence composed of a core GRGGC followed 6–8 nucleotides further by a cluster of at least three guanidines (Fig. 2).

To assess the importance of the two motifs in the consensus, we performed EMSAs on a double-strand probe containing these two motifs and on four probes presenting mutations in the consensus (Fig. 3A). We found that the fusion protein GST-PLAG1 (N2-C244) binds strongly to the consensus (Fig. 3B, Lane 1), whereas mutations in the G-cluster (Fig. 3B, Lane 2) reduce drastically the binding (~8-fold) and mutations in the core nearly completely abolish it (~37-fold reduction; Fig. 3B, Lane 3) as well as mutations in both (Fig. 3B, Lane 4). The distance between the G-cluster and the core is also important because PLAG1 binds weakly to the probe containing the G-cluster separated by 2 bp instead of 7 bp from the core (WT2ml; Fig. 3B, Lane 5). These results indicate that both motifs in the consensus are important for the binding of PLAG1, with an importance more pronounced for the core compared with the G-cluster.

The relative importance of the two motifs was also tested in a series of competition EMSAs using WT2 as probe. As shown in Fig. 3C, WT2 competes much more efficiently than mCLU2 or mCO2, confirming the importance of the two motifs in the consensus. mCLU2 competes poorly but nevertheless better than mCO2, confirming by this way that the destruction of the G-cluster is not so deleterious for the binding as the destruction of the core. As expected, no competition could be observed, with mComClu2 presenting a mutation in both motifs.

Fingers 6 and 7 of PLAG1 Bind to the Core, Whereas Finger 3 Interacts with the G-Cluster. To determine which of the zinc fingers contributes to the binding to the consensus sequence, we examined the binding of bacterially expressed GST fusion proteins containing different combinations of zinc fingers (Fig. 3B, Lanes 6–25). A similar binding pattern is observed with the protein containing fingers 3 to 7 compared with the protein F1–F7, which contains the complete zinc finger region (compare Lanes 6–10 with Lanes 1–5), suggesting that fingers 1 and 2 are not directly involved in the binding. In contrast, the protein containing fingers 4–7 binds 3-fold less to the WT2 probe (Fig. 3B, Lane 11). More importantly, protein F4-F7 binds nearly as well as to the probe, presenting a mutation in the G-cluster (mCLU2, Fig. 3B, Lane 12) as to the WT2 probe, indicating that the G-cluster is not important for the binding of F4–F7. This is a good indication for the requirement of finger 3 for the interaction with this cluster. The F6–F7 protein binds nearly with the same affinity as F4–F7 to all of the different probes (Fig. 3B, compare Lanes 16–20 to Lanes 11–15), suggesting that fingers 4 and 5 are not directly involved in the binding. This F6–F7 protein still binds clearly to all of the probes containing an intact core motif (Fig. 3B, Lanes 16, 17, and 20), indicating that fingers 6 and 7 are sufficient for the interaction with the core. Finally, the protein F1–F5 does not show any clear binding (Fig. 3B, see Lanes 21–25), indicating the absolute requirement of the two last fingers F6 and F7. All of these results suggest that finger 3 of PLAG1 interacts with the G-cluster, whereas the core is recognized by fingers 6 and 7.

To confirm this model, we performed additional EMSAs using full-length PLAG1 protein translated in vitro in reticulocyte lysates instead of the GST fusion proteins expressed in bacteria. Three mutant PLAG1 proteins (F2mut, F3mut, and F7mut) were also selected by seven rounds of CASTing using GST-PLAG1 (N2-C244). The frequency of the number of oligonucleotides that carried a base at that position.

Fig. 1. Determination of the PLAG1 binding site. Alignment of the 23 oligonucleotides selected by seven cycles of CASTing using GST-PLAG1 (N2-C244). The frequency of each of the bases at each position is shown at the bottom of the figure, and N represents the number of oligonucleotides that carried a base at that position.

Consensus

<table>
<thead>
<tr>
<th>Core</th>
<th>Cluster</th>
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<tr>
<td>A</td>
<td>AGGGCCCGTGACCTGCTACC</td>
</tr>
<tr>
<td>B</td>
<td>ACTATAGGGGTTA</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
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Fig. 2. Determination of the PLAG1 binding site. Alignment of the 23 oligonucleotides selected by seven cycles of CASTing using GST-PLAG1 (N2-C244). The frequency of each of the bases at each position is shown at the bottom of the figure.
Fig. 3. The G-cluster is recognized by finger 3 and the core by fingers 6 and 7 of PLAG1. A, nucleotide sequences of the different oligonucleotides used in EMSA analysis. Mutations are underlined. B, EMSAs performed with equimolar amount (0.6 pmol) of bacterially expressed GST-PLAG1 proteins. GST-PLAG1 (N2-C244) (Lanes 1–5), (N84-C244) (Lanes 6–10), (N101-C244) (Lanes 11–15), (N159-C244) (Lanes 16–20) and (N2-C203) (Lanes 21–25) were incubated with the probes WT2 (Lanes 1, 6, 11, 16, and 21), mCLU2 (Lanes 2, 7, 12, 17, and 22), mCO2 (Lanes 3, 8, 13, 18, and 23), mCLUmCO2 (Lanes 4, 9, 14, 19, and 24), and WT2ml (Lanes 5, 10, 15, 20, and 25) as described in “Materials and Methods”. The percentage of binding of all these mutants on the different probes were compared with the binding of F1–F7 to the probe WT2 and is the mean of at least six experiments. C, competition experiments performed on the probe WT2 in presence of increasing amounts of unlabeled double-strand oligonucleotides (10, 30, 100, 300, and 1000 ng) using recombinant full-length PLAG1 expressed in vitro in reticulocytes lysates; D, EMSAs performed with recombinant PLAG1 proteins produced in vitro in reticulocytes lysates. Wild-type PLAG1 (Lanes 1–5), F2mut (Lanes 6–10), F3mut (Lanes 11–15), and F7mut (Lanes 16–20) were incubated with the probes WT2 (Lanes 1, 6, 11, and 16), mCLU2 (Lanes 2, 7, 12, and 17), mCO2 (Lanes 3, 8, 13, and 18), mCLUmCO2 (Lanes 4, 9, 14, and 19), and WT2ml (Lanes 5, 10, 15, and 20) as described in “Materials and Methods”. Equal efficiency of protein expression was obtained for the different constructs as demonstrated by SDS-PAGE of proteins labeled with [35S]methionine (data not shown). The percentages of binding of all of these mutants on the different probes were compared to the binding of the wild-type PLAG1 to the probe WT2 and is the mean of at least three experiments.
produced by replacing with an alanine the first histidine of the corresponding C\textsubscript{2}H\textsubscript{2} motif. This mutation hinders the coordination of the zinc and has been shown to prevent the formation of a functional zinc finger (17). The full-length PLAG1 protein expressed in reticulocyte lysate binds with the same specificity as the bacterial F1–F7 protein (compare Fig. 3D with 3B, Lanes 1–5). The destruction of the zinc finger 2 (PLAG1/F2mut) does not affect the binding specificity of this protein but decreases its affinity \textasciitilde3-fold (Lanes 6–10). In contrast, the finger 3 destruction decreases drastically the affinity of this protein as F3mut binds 17-fold less to the WT2 probe than the natural PLAG1 protein (compare Lane 11 with Lane 1). The specificity was also completely modified since F3mut binds equally well to WT2 (Lane 11), mCLU2 (Lane 12), and WT2ml (Lane 15). Thus, the presence or absence of a G-cluster does not affect the binding of F3mut, indicating that finger 3 is actually the finger required for the interaction with this motif. This conclusion is in agreement with the conclusions drawn from the EMSA experiments performed with the bacterial protein. Finally, destruction of finger 7 completely prevents any binding (Lanes 16–20), confirming the absolute requirement of this finger.

**PLAG1 Can Stimulate Transcription Through Its Consensus Binding Site.** To investigate whether PLAG1 binding sites could mediate a transcriptional activation by PLAG1, six copies of the minimal consensus (WT) were cloned upstream the herpes simplex virus thymidine kinase promoter, followed by the luciferase reporter gene. This reporter construct was then transfected into the fetal kidney 293 cell line in the presence or absence of the expression vector pCAGGS-PLAG1.

Table 1 shows that PLAG1 stimulates expression of this reporter construct \textasciitilde19-fold. This stimulation is completely abolished by mutations in the G-cluster or in the core since no stimulation was detectable with six copies of mCLU or mCO. This demonstrates that the activation is completely dependent of the presence of the two motifs.

**PLAG1 Binds IGF-II Promoter 3 and Up-Regulates Its Promoter Activity.** A computer search in the EPD (18) with the PLAG1 binding consensus GRGGC(N)\textsubscript{7} RGGK revealed potential PLAG1 binding sites in the promoter region of many genes (about 176 of the 1280 screened). Table 2 presents the list of all of the human promoters present in the EPD containing at least two PLAG1 DNA binding consensus in their promoter. The ability of each promoter to be induced by PLAG1 has been estimated by cotransfection of the fetal kidney 293 cell line with pCAGGS-PLAG1 or pCAGGS expression vectors, together with reporter constructs in which each promoter has been cloned in front of a luciferase gene (see "Materials and Methods"). PLAG1 induction levels are expressed as the ratio of luciferase activity obtained in the cell transfected with pCAGGS-PLAG1 expression vector versus the activity obtained in cells transfected with the empty vector pCAGGS. The data are means \pm SE of at least two independent transfection experiments, each performed in triplicate.
IGF-II Transcript Is Up-Regulated in Salivary Gland Tumors with PLAG1 Overexpression. The fact that PLAG1 is able to bind and activate the promoter $3$ of IGF-II suggested that IGF-II could be a target for PLAG1 in salivary gland tumors. To test this hypothesis, we analyzed the expression of these two genes in specimens of primary salivary gland tumors and of normal glands. As described previously (1), PLAG1 expression could not be detected by Northern blot analysis in normal salivary gland tissue (Fig. 5, Lanes 1, 5, and 8) and from adenomas c895 (Lane 2), c904 (Lane 3), cg650 (Lane 4), cg644 (Lane 6), and cg580 (Lane 7). B. recapitulation of the Northern blot analysis of normal salivary gland tissues and pleomorphic adenomas hybridized with probes specific for the genes encoding PLAG1, IGF-II, PDGF-B, GOS24, or c-Ha-Ras. The karyotype of the tumors has been described elsewhere (1, 2), and in tumors cg650 and cg601, the breakpoint occurs outside the PLAG1 region (20, 21).

Fig. 5. IGF-II P3 transcript is up-regulated in tumors with PLAG1 overexpression. A. Northern blot analysis of normal salivary gland (n.s.g.) tissues and pleomorphic adenomas hybridized sequentially with a 3.7-kb PLAG1 cDNA probe, an IGF-II exon 9 probe, and a 2-kb β-actin probe. RNAs tested included samples from three different normal salivary gland tissue specimens (Lanes 1, 5, and 8) and from adenomas c895 (Lane 2), c904 (Lane 3), cg650 (Lane 4), cg644 (Lane 6), and cg580 (Lane 7). B. recapitulation of the Northern blot analysis of normal salivary gland tissues and pleomorphic adenomas hybridized with probes specific for the genes encoding PLAG1, IGF-II, PDGF-B, GOS24, or c-Ha-Ras. The karyotype of the tumors has been described elsewhere (1, 2), and in tumors cg650 and cg601, the breakpoint occurs outside the PLAG1 region (20, 21).

Fig. 6. Prediction and schematic representation of PLAG1 binding consensus site. A. amino acids at position −1, 2, 3, and 6 (numbering with respect to the start of the α-helix) within the PLAG1 zinc fingers are shown in the first column. Bases predicted to be preferred for binding by these amino acids are shown in the second column (22, 23). The consensus found by CASTing is shown in the third column, and thick lines indicate that the predicted base matches with the selected one. B. comparison between the PLAG1 binding site and other reported consensus binding sites like the Zac1 consensus (27), one of the characterized WT-1 binding sites (33), the consensus sequence for Sp1 binding described as the decanucleotide 5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3′ (34) and the Egr-1/Zif268 consensus binding sequence (35).
DISCUSSION

In this report, we show that PLAG1, the major translocation target gene in pleomorphic adenoma of the salivary glands, codes for a nuclear protein that binds DNA in a sequence-specific manner. The minimal PLAG1 binding site is composed of two essential parts, a GRRG core separated by seven random nucleotides from a RGGK cluster. This bipartite binding site is quite unusual and can be explained by the particular way PLAG1 binds DNA. Indeed, we show that two noncontiguous regions in PLAG1 are essential for DNA recognition, finger 3 interacting with the G-cluster and fingers 6 and 7 recognizing the core. This model of interaction we found by deletion/mutation analysis (see Fig. 3) is corroborated by the stereochemical rules governing the DNA contacts of individual zinc fingers (22). These rules are based on different types of studies: (a) structural studies have shown that each finger module folds to form a compact ββα structure with the α-helix fitting into the major groove (reviewed in Ref. 23). Residues -1, 2, 3, and 6 (numbering with respect to the start of the α-helix) typically make key base contacts that are responsible for defining sequence specificity; (b) by phage-display selections and site-directed mutagenesis, correlations were established between the amino acids present in key positions -1, 2, 3, and 6 and the nucleotide sequences of their optimal binding sites. This led to a recognition code governing zinc-finger/DNA interactions. As depicted in Fig. 6A, the consensus we found by CASTing shows good agreement with the binding site predicted by such a recognition code. It is interesting to note that the fingers that are not involved in DNA recognition (e.g., fingers 1, 4, and 5) do not present in key position amino acids known to interact with DNA. The distance between the G-cluster and the core is also in good agreement with the presence of two noninteracting zinc fingers that are predicted to cover six nucleotides. However, alignment of all the sequences selected by seven rounds of CASTing indicates that the distance between the G-cluster and the core seems to vary from six to eight nucleotides (see Fig. 2). One explanation to this variability could be the presence of a longer linker region between fingers 5 and 6 (14 amino acids instead of the usual 7 amino acids). This longer linker may generate sufficient flexibility to allow an interaction with a cluster at different positions.

Recently, we and others identified two PLAG1-related proteins, PLAGL1 [also called Lot1 (24, 25) or Zac1 (26)] and PLAGL2 (24). PLAGL1 and PLAGL2 are highly homologous to PLAG1 in their NH2-terminal zinc finger domain (73 and 79% identity, respectively), whereas the COOH-terminal region is much more divergent. Strikingly, the best homology is found in fingers 6 and 7, suggesting that PLAGL1 and PLAGL2 should also recognize a core motif analogous to the PLAG1 core. In contrast, fingers 2–5 are much less conserved and still present conservation for the amino acids present in key positions (-1, +2, +3 and +6). This suggests that the three PLAG proteins would interact with similar consensus sequences. The consensus binding site for Zac1/PLAGL1 has been defined recently as GGGGGGCCCCC through a CASTing assay (27). Actually, the PLAG1 core is present in this large consensus sequence (Fig. 6B). However, no G-cluster was identified, suggesting that Zac1/PLAG1 does not interact in the same way as PLAG1. Both PLAG1 and Zac1/PLAGL1 are possibly involved in tumorigenesis, PLAG1 as a putative oncogene that contributes to pleomorphic adenomas whereas Zac1/PLAGL1 as a tumor suppressor candidate that regulates apoptosis and cell cycle arrest (26). It is thus interesting to further investigate the differences of specificities between these two related proteins to determine whether the opposite functions are the result of different target genes or different actions on the same set of genes.

The PLAG1 DNA binding consensus is highly GC-rich, which is a hallmark of most promoters of genes controlling cell growth. It is thus tempting to speculate that PLAG1 will exert its oncogenic effect via the activation of growth factors. The IGF-II is an excellent candidate because IGF-II is a peptide growth factor that plays an important role in embryonic development and also in carcinogenesis (28). The human IGF-II is a complex transcription unit that is regulated by activation of multiple promoters designated P1 to P4. Promoter 1 activity has been demonstrated only in adult liver, whereas promoters 2, 3, and 4 are coexpressed in a variety of fetal tissues, notably fetal liver, and at a lower level, in many adult tissues with the exception of adult liver. Promoters 3 and 4 are also highly active in numerous tumor tissues, suggesting that transcriptional up-regulation of P3 and P4 activities may be importantly involved in tumorigenesis. Our study is the first demonstration of IGF-II up-regulation in tumors of the salivary glands. This up-regulation is the result of a drastic up-regulation of promoter 3 activity, as demonstrated by the hybridization performed with a P3-specific probe. Several lines of evidence strongly suggest that IGF-II up-regulation in salivary gland tumors results from transcriptional activation by PLAG1: (a) five potential binding sites were found in promoter 3 of IGF-II; (b) PLAG1 binding was effectively demonstrated on the site P3-4; (c) IGF-II promoter 3 activity is up-regulated by PLAG1; (d) IGF-II is highly expressed in tumor cells that overexpress PLAG1 but could not be detected in tumors without PLAG1 up-regulation or in normal salivary gland tissues. Our study suggests that the oncogenic activity of PLAG1 results from its positive regulation of IGF-II expression, known to potently stimulate cell proliferation in human tumors through autocrine or paracrine mechanisms (28–30).

The PLAG1 consensus sequence is also reminiscent of G-rich sequences recognized by an important group of zinc finger proteins that include Sp1, Zif268/Egr1, and WT-1. As shown in Fig. 6B, the PLAG1 consensus binding motif overlaps with the consensus binding sequence for the transcription factor Sp1 and the tumor suppressor WT-1, suggesting that these proteins could at least partly regulate the same set of genes. In fact, the growth factor gene IGF-II has been shown to be also one of the targets of the tumor suppressor WT-1. High levels of IGF-II in Wilms’ tumor were attributed to the loss of function of WT-1, which normally represses IGF-II transcription (31). The WT-1 protein binds within the IGF-II P3 promoter to multiple sites with some overlapping with potential PLAG1 binding sites. This suggests that one control for IGF-II expression during development could be provided by a balance between activators (like PLAG1) and repressors (like WT-1) acting on overlapping sequences. Deregulation of one of these factors is probably an important step in tumor formation.

ACKNOWLEDGMENTS

We gratefully acknowledge G. Stenman for the specimens of primary salivary gland tumors and P. E. Holthuizen for her kind gift of the IGF-II exon 5 probe and for (IGF-II-P3)luc plasmid. We thank J. Remacle, S. Tejpar, and B. Peers for critical review of the manuscript.

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