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Proteomic Identification of AP2γ as a Rat Placental Lactogen II Trophoblast Cell-Specific Enhancer Binding Protein

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The factors that regulate the developmental expression of the rodent prolactin gene family in placenta remain poorly defined. We previously identified an enhancer element in the 5′ flanking region of one family member, rat placental lactogen II (rPLII), which could target reporter gene expression to the placenta in transgenic mice; this enhancer functioned in the Rcho rat trophoblast cell line but not in the rat pituitary GC cell line. In further experiments to identify the factors that bind this element, we have selectively enriched for DNA binding proteins in nuclear extract from Rcho cells using magnetic beads coupled to a 43-bp enhancer oligonucleotide. Tryptic peptides of bound proteins were analyzed by HPLC coupled off-line to matrix-assisted laser desorption ionization time of flight mass spectrometry. Several peptides of AP2γ, a key trophoblast cell-specific transcription factor, were identified. Gel mobility shift assays using AP2γ-specific antiserum and mutant enhancer oligonucleotides demonstrated binding specifically to the FP2 DNase I-protected region of the element, identifying an atypical binding site for this factor. In cotransfection assays in rat pituitary GC cells, AP2γ transactivated the enhancer via this region. Chromatin immunoprecipitation assays confirmed AP2γ occupancy of the enhancer region *in situ* in the nuclei of Rcho giant cells. These data support a role for AP2γ in the placental giant cell-specific expression of the rPLII gene and provide the first direct evidence for the involvement of a placental-specific transcription factor in the regulation of a member of this gene family. (*Endocrinology* 147: 4319–4329, 2006)

THE DEVELOPING PLACENTA of rats and mice expresses a family of pregnancy-specific proteins that are related by primary sequence and gene structure to the pituitary hormone, prolactin (PRL). The more than 20 orthologous genes, including the PRL gene itself, are organized into a single locus in each species; the gene order is identical and appears to reflect the degree of relatedness of coding sequence and intron/exon structure between adjacent genes, rather than groupings based on developmental expression patterns (reviewed in Refs. 1 and 2). Family members are expressed in trophoblast cell lineages, including primary and secondary giant cells, spongiotrophoblasts, and labyrinthine trophoblast giant cells; a few members are also expressed in the pregnant and pseudopregnant antimesometrial decidua. Each gene shows a characteristic temporal and cell-specific expression pattern, suggesting that transcription is tightly regulated during pregnancy. Given the variety of individual gene expression programs from implantation to parturition, the rodent PRL gene family has the potential to provide considerable insights into the general regulation of gene expression during the different stages of placental development. Despite this, the factors that regulate the expression of this family during pregnancy are still poorly understood.

Our studies have primarily focused on identifying the factors that regulate the unique developmental expression pattern of the rat placental lactogen II (rPLII) gene. In both rats and mice, PLII is initially expressed at midpregnancy in the primary and secondary trophoblast giant cells that surround the developing fetus and invade the uterine tissue. After chorioallantoic fusion, the PLII gene is also transcribed in trophoblast cells located in the labyrinth region (reviewed in Ref. 1). In previous studies, we identified a 3-Kb 5′ flanking fragment, immediately proximal to the rPLII transcription start site, which consistently targeted reporter gene expression to the placentas of transgenic mice, suggesting that it contained key placental-specific regulatory sites (3). We showed that an element within this region enhanced luciferase reporter gene expression from a minimal thymidine kinase promoter in the rat trophoblast Rcho cell line, a model of the junctional zone giant cell (4), but not in rat pituitary GC cells (5). DNase I protection assays defined two adjacent regions within this enhancer element, designated as FP1 and FP2. Changes to specific nucleotides within each of these regions reduced enhancing activity; mutations in both regions caused a complete loss of activity. Based on sequence similarities to known binding sites and DNA mutation analysis, we proposed that the FP1 region represented an Ets factor binding site and the FP2 was a putative activating protein-1 (AP-1) binding site (5). Our conclusions were supported by the fact that functional interactions between ad-

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Abbreviations: AP-1, Activating protein 1; ChIP, chromatin immunoprecipitation; ES, embryonic stem; hCG, human chorionic gonadotropin; MALDI TOF MS, matrix-assisted laser desorption ionization/time of flight MS; MS, mass spectrometry; MS/MS, tandem MS; PARP-1, poly (ADP-ribose) polymerase-1; PMSF, phenylmethylsulfonyl fluoride; poly dIdC, polydeoxyinosinic-polydeoxycytidylic acid; PRL, prolactin; QqTOF, quadrupole time of flight; rPLII, rat placental lactogen II; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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jacent Ets and AP-1 binding sites have been frequently observed (6).

Further experiments have now caused us to question the identity of AP-1 as the factor that binds the FP2 region. In this paper, we report a reassessment of the protein factors that interact with the rPLII enhancer using an unbiased proteomics approach to isolate and identify DNA binding factors directly. A 43-bp synthetic oligonucleotide, containing only the FP1 and FP2 DNase I protected nucleotides, was used to capture specific DNA-Rcho nuclear protein complexes on magnetic beads. Bound proteins were digested with trypsin, and after partial fractionation by HPLC peptides were analyzed by matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI TOF MS), mass matching to protein databases and sequencing by tandem mass spectrometry (MS/MS). Using this approach, we have identified the transcription factor AP2γ as a protein that binds the rPLII enhancer and further have shown by EMSA, DNA mutagenesis and transfection studies that AP2γ specifically binds within the FP2 region. Chromatin immunoprecipitation (ChIP) assays confirm that AP2γ occupies the rPLII enhancer region *in situ* in the chromatin of Rcho giant cells. AP2γ is a critical regulator of early placental development in the mouse (7, 8) and has been implicated in the regulation of a number of human and rodent genes that are expressed specifically in placenta (9–13).

Materials and Methods

Plasmids

The human AP2γ cDNA clone was a generous gift from Dr. Trevor Williams (University of Colorado, Denver, CO) and Dr. Helen Hurst (Imperial College, London, UK). For *in vitro* transcription/translation and cotransfection studies with AP2γ, a *Hin*dIII fragment from this clone was subcloned into the pCDNA3 expression vector (Stratagene, La Jolla, CA) and the orientation verified by sequencing (pCDNA3.AP2γ). The rPLII wild-type and mutant enhancer oligonucleotides (QIAGEN Operon, Mississauga, Ontario, Canada) were cloned into the firefly luciferase reporter vector, pTK81, which contains a minimal thymidine kinase promoter (14). Five micrograms each of the complementary strands for the 43-bp wild-type and mutant M3, M4, m6, and M4m6 oligonucleotides (Table 1) were annealed in 20 μl of 10 mM Tris-HCl (pH 8.5)/125 mM NaCl, phosphorylated with T_4 kinase, and purified with a GFX PCR DNA and gel purification kit (Amersham Pharmacia Biotech, Baie d'Urfé, Ouébec, Canada). Aliquots were ligated overnight at room temperature. The pTK81 vector was digested with *Sma*I, dephosphorylated with calf intestinal phosphatase, and ligated with varying amounts of the oligo concatamers. Clones containing inserts were identified by digestion with either *Sml*I or *Bst*NI (New England Biolabs, Pickering, Ontario, Canada). The number of cloned inserts was determined by PCR using a forward primer, WT84, from within the TK promoter region and a reverse primer, WT155, from a common region within the enhancer oligonucleotides (Table 1). Clones containing two copies of each oligo were sequenced (University of Calgary DNA Sequencing Laboratory, Calgary, Alberta, Canada), and those containing both copies in a 5′ to 3′ orientation were selected for transfection studies (pTKWT.luc, pTKM3.luc, pTKM4.luc, pTKm6.luc, pTKM4m6.luc). All plasmids were purified using a QIAGEN plasmid maxi kit. Transcription factor binding site analysis was carried out using MatInspector 2.2 and AliBaba 2 (http://www.gene-regulation.com/pub/programs.html#alibaba2).

Cell culture and transfections

The rat trophoblast Rcho cell line was cultured for 14–16 d as previously described (5). Transfections of wild-type and mutant enhancer luciferase constructs were carried out in 60-mm dishes using lipofectamine with Plus reagent according to the supplier's protocol (Invitrogen, Burlington, Ontario, Canada). Four micrograms of wild-type or mutant enhancer construct were transfected with 10 ng of pTK *Renilla* luciferase (Promega, Madison, WI). After 48 h, cell lysates were prepared as previously described (5), and luciferase activities were measured in a Berthold Lumat LB 9507 luminometer using a dual reporter assay system (Promega) according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity for each assay. The rat anterior pituitary cell line, GC, was cultured in DMEM with high glucose, supplemented with 10% fetal calf serum (Invitrogen). For AP2γ cotransfection studies, GC cultures were grown to approximately 50% confluency, replated at 2×10^5 cells per 35-mm dish, and transfected 24 h later using lipofectamine with Plus reagent. To optimize for AP2γ expression levels, preliminary experiments were carried out in which GC cultures were cotransfected with 3 μg of the wild-type enhancer luciferase constructs, 10 ng of pTK *Renilla*, and 0, 10, 25, 50, 100, or 500 ng of pCDNA3.AP2γ. DNA concentrations were kept constant by the addition of pCDNA3 vector. Luciferase activity was measured after 48 h and normalized to *Renilla* activity as for the Rcho assays. The optimum effect of AP2γ was seen at 50 ng, which was used for further transfection assays.

Nuclear extract preparation

Nuclear extracts from d 14 Rcho cells were prepared using a modified Dignam protocol (15). Lysis of the cells was extended to 25 min, and the concentration of protease inhibitors was altered to include 0.4 mM EDTA, 1 mM dithiothreitol, 2 mM PMSF, 10 μM pepstatin, 10 μM leupeptin, and 10 μM aprotinin. Rat placental nuclear extracts were prepared as previously described, using d 16 to d 18 frozen rat placenta (5). Protein concentrations were determined using Bradford's reagent (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

TABLE 1. Oligonucleotides used in this study

Oligo name	Sequence (5′–3′)
WT enhancer	CCAGGGTTATTTGCTCAAGGGTAAACAGGAAGTAGGGCTTGAA
M2	CCAGGGTgccgTGCTCAAGGGTAAACAGGAAGTAGGGCTTGAA
M3	CCAGGGTTATTctacCAAGGGTAAACAGGAAGTAGGGCTTGAA
M4	CCAGGGTTATTTGCTacgtGGTAAACAGGAAGTAGGGCTTGAA
M5	CCAGGGTTATTTGCTCAAGtcgcAACAGGAAGTAGGGCTTGAA
m6	CCAGGGTTATTTGCTCAAGGGTAAACActgAGTAGGGCTTGAA
M4m6	CCAGGGTTATTTGCTacgtGGTAAACActgAGTAGGGCTTGAA
AP2 consensus	GATCGAACTGACCGCCCGCGGCCCT
WT84	TCATGTCTGGATCCAAGC
WT155	GTACCCTTCAAGCCCTAC
Luciferase	CTTTATGTTTTTGGCGTCTTCCCA
ChIP 1F	AGATAGGTTAAGGGAGTCACTGGA
ChIP 1R	CGTCTTATGGGTTCTGTAAGGGTA

The application of each oligonucleotide is described in the text. *Lowercase* nucleotides in the M2, M3, M4, and m6 oligos represent changes from the WT enhancer sequence.

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In vitro coupled transcription-translation

The *in vitro* coupled transcription-translation of AP2γ was performed using the Promega TNT T7 coupled reticulocyte lysate system with pCDNA3.AP2γ according to the manufacturer's protocol.

EMSA

Complementary oligonucleotides containing the 43-bp wild-type enhancer sequence or a mutant version were annealed and end-labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase. Binding reactions were carried out at room temperature in 20 μl using 10 ng of labeled probe with 6 μg of Rcho or 10 μg of placental nuclear extract in 5 mM HEPES (pH 7.9), 25 mM NaCl, 1.25 mM $MgCl_2$, 50 μM EDTA, 125 μM dithiothreitol, 250 μM PMSF, 5% glycerol, and 0.1 μg/μl polydeoxyinosinic-polydeoxycytidylic acid (poly dIdC). EMSA reactions with *in vitro* transcribed and translated AP2γ were carried out in 10 μl with 2 μl of lysate. For competition studies, competitor oligonucleotides were incubated with the nuclear extract for 15 min on ice before addition of the labeled probe. For supershift assays, AP2γ antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) or nonimmune rabbit serum was incubated with nuclear extract or recombinant protein on ice for 30 min before the addition of labeled probe. All EMSA samples were separated on a 6% nondenaturing polyacrylamide gel, prerun at 200 V for 30 min before loading. Gels were dried and exposed for autoradiography with Kodak Biomax MS film.

DNA-protein binding reactions

Streptavidin-linked M-280 magnetic Dynabeads (Invitrogen) were washed according to the manufacturer's protocol, but with additional washes to reduce amounts of BSA present in the storage buffer. After each manipulation, beads were collected using a Dynal MPC-S magnetic separation apparatus (Invitrogen). Final washes were in coupling buffer [10 mM Tris-HCl (pH 7.5), 1 M NaCl, 1 mM EDTA). Complementary 43-bp wild-type oligonucleotides were annealed to form the double-stranded enhancer. The sense oligo included a 5′ biotin at the end of a triethylene glycol spacer (QIAGEN Operon). Oligonucleotide/Dynabead coupling reactions contained 50 pmol of the double-stranded biotinylated 43-bp enhancer oligo with 50 μg of magnetic beads in coupling buffer to a final volume of 50 μl. Tubes were mixed by gentle rotation at room temperature for 20 min. Beads were collected and washed twice in EMSA buffer without poly dIdC. Protein-DNA binding reactions were carried out in a final volume of 200 μl of EMSA buffer containing 0.1 μg/μl poly dIdC and 120 to 150 μg of Rcho nuclear protein. Samples were incubated at room temperature for 30 min, with gentle rotation. To remove nonspecifically bound proteins, beads were collected and washed once in two volumes of EMSA buffer containing 1.0 μg/μl poly dIdC with gentle mixing for 2 min and twice in EMSA buffer without poly dIdC. After the first set of washes, five binding reactions were pooled into one tube and washed three times in 0.1 M and twice in 10 mM ammonium bicarbonate for 2 min at 4 C with gentle rotation. The bound beads were resuspended in 70 μl of 10 mM ammonium bicarbonate, trypsin was added to a final concentration of 0.25 ng/μl, and the sample was incubated overnight at 37 C. Beads were removed after digestion using the magnet.

Mass spectrometry

Tryptic digests were divided into three aliquots and dried in a Savant Speed Vac concentrator. Each aliquot represented bound protein from approximately 250 μg of crude Rcho nuclear extract. Samples for MS analysis were treated similarly as outlined in Krokhin *et al.* (16). Briefly, pellets were dissolved in 6 μl of 0.2% trifluoroacetic acid (TFA), and chromatographic separation was carried out on a micro-Agilent 1100 series HPLC. Five-microliter samples were injected onto a 150 μm × 150 mm column (Vydac 218 TP C18, 5 μm) and eluted with a 1–80% acetonitrile gradient containing 0.1% TFA; column effluent (4 μl/min) was mixed on-line with 0.5 μl of MALDI matrix solution (160 mg/ml 2,5-dihydroxybenzoic acid in 3:1 water:acetonitrile containing 2% formic acid) and deposited by a computer-controlled robot onto a movable gold target at 40 1-min intervals. Each fraction was analyzed by single MS (*m/z* range, 560-5000) on a Manitoba/SCIEX quadrupole time of flight (QqTOF) mass spectrometer. The mass measurements using this instrument are very precise and sensitive enough for very small amounts of sample (17). Ions with sufficient intensity were selected for MS/MS on the same instrument.

Data analysis

Acquisition and analysis of spectra were carried out manually using TOFMA, an in-house program. Data analysis was carried out using the following programs (www.proteome.ca): *m/z* for automatic peak assignment [signal to noise ratio (S/N), 2.5]; ProFound for peptide mass fingerprinting on ions measurable over background (S/N, 2.5; error limit, 30 ppm); Sonar ms/ms program for MS/MS spectra analysis (S/N, 1.3; 2 Da error for the parent ion and 0.1 Da for fragments). Some MS/MS data files were also analyzed by Global Proteomics Machine software (http://www.thegpm.org) (18). Visual inspection of the spectrum was used to confirm identity. Both rodent and mammal databases were routinely searched (National Centre for Biotechnology Information and SwissProt) to ensure maximum probability of identification.

ChIP assays

ChIP assays were carried out following procedures outlined in Spencer *et al.* (19), with adaptations for Rcho cells. Briefly, two 150-mm plates of d 14 differentiated Rcho cell cultures (~2–3 × 10^7 cells) were cross-linked with 20 ml of 1% formaldehyde in RPMI 1640 medium per plate for 10 min at room temperature, followed by a 5-min wash at room temperature in PBS containing 125 mM glycine and two further rinses in ice-cold PBS. Cells were harvested with a rubber policeman into 5 ml per plate of ice-cold PBS, pelleted at 500 × *g* for 10 min at 4 C, and washed once in ice-cold PBS. At this stage, cell pellets were usually stored at −80 C. All PBS used for these procedures was Ca^{+2}/Mg^{+2}-free. For ChIP assays frozen pellets were thawed on ice and lysed in 1 ml of 50 mM Tris (pH 8.1), 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), and protease inhibitors [20 μM leupeptin, 18 μM pepstatin, 2 mM sodium vanadate, 20 mM sodium fluoride, 10 mM phenylmethylsulfonyl fluoride (PMSF)] for 30 min. Chromatin was sonicated to an average size of 500 bp in this buffer. The lysate was diluted 5-fold in ChIP dilution buffer [16.7 mM Tris (pH 8.1), 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA with 1 mM PMSF and 1 μg/ml leupeptin, aprotinin, and pepstatin]. An A_{260} measurement was taken, and 0.5 A_{260} units of diluted sample was set aside to represent DNA input sequences. Preliminary antibody pull-down assays and Western blots established that 1 μg of the same AP2γ antiserum used in EMSA studies (Santa Cruz Biotechnology) specifically and efficiently immunoprecipitated target protein at 2 and 4 A_{260} units of input DNA. For ChIP assays, 1 μg of antiserum was added to 2 A_{260} or 4 A_{260} units of DNA and incubated with rotation overnight at 4 C. Eight (2 A_{260}) or four (4 A_{260}) separate reactions were set up. Control samples of identical inputs were similarly incubated with 1 μg of nonimmune rabbit serum. A further control in which the cross-linked sample was treated with beads alone was also carried out. Twenty microliters of Protein A/G agarose beads (Santa Cruz Biotechnology) pretreated with 0.1 mg/ml sonicated salmon sperm DNA were added to each tube and incubated for 30 min at 4 C with gentle rotation. Beads were collected and washed in low salt, high salt, LiCl, and Tris/EDTA buffers as described (19). At this point, the individual 2 A_{260} or 4 A_{260} reactions were pooled and protein/DNA bonds were reverse cross-linked by incubation in 70 μl of Tris EDTA (pH 8.1), 0.5% SDS, 0.5 μg/μl proteinase K at 60 C overnight, followed by boiling for 5 min and digestion with 250 ng/μl of RNase A for 30 min at 37 C. The DNA fragments were purified on QiaQuick spin columns (QIAGEN) according to the manufacturer's protocol. The presence of the target rPLII enhancer fragment in the samples was assessed by PCR using a platinum Taq PCR kit (Invitrogen) and the ChIPF and ChIPR primers (Table 1), which amplify a 335-bp fragment containing the rPLII enhancer element. PCR was performed for 35 cycles.

Statistical analysis

Statistical analysis of the transfection and cotransfection data were performed using a two-tailed, unpaired *t* test and ANOVA with *post hoc* Bonferroni test (In-Stat, version 2.03; In-Stat, Newton, MA). A value of $P < 0.05$ is considered statistically significant.

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Results

EMSA studies with a synthetic oligonucleotide lead to a reassessment of the identity of the proteins that bind the rPLII enhancer

Our previous EMSA studies and functional analyses of the rPLII enhancer sequence used a 65-bp cloned genomic fragment containing nucleotides flanking those within the DNase I protected region as well as a small amount of polylinker sequence (5). Reporter gene expression studies with wild-type and mutated 65-bp fragments indicated that these extra nucleotides did not contribute to the enhancing activity. For further experiments, we synthesized a 43-bp double-stranded oligonucleotide containing only the FP1 and FP2 DNase I protected regions with immediately proximal nucleotides (Fig. 1A). To test that this shorter fragment functioned similarly to the 65-bp fragment, we cloned two copies of the synthetic oligonucleotide into the luciferase vector, pTK81 (14), for assessment by reporter gene transfection assays. Similar to the cloned fragment, the 43-bp synthetic oligonucleotide also acted as an enhancer, and enhancing activity was diminished or lost by changes to specific nucleotides within the FP1 and FP2 sites (Fig. 1, A and B) (5). We also tested the ability of the synthetic oligonucleotide to bind AP-1, as had been previously shown for the cloned fragment (5). The 43-bp oligonucleotide unexpectedly showed no complex formation with *in vitro* synthesized c-*fos*/c-*jun* dimers, and complex formation with Rcho nuclear extracts was unaffected by anti-c-*jun* antiserum (data not shown). Because the 43-bp oligonucleotide contained all the nucleotides within the DNase I protected regions, including the putative AP-1 binding site in FP2, we concluded that the previously observed *c-fos/c-jun* dimer binding did not occur within the enhancer sequence itself, but perhaps to a cryptic site within the additional sequence present in the cloned DNA. The loss of enhancing activity after mutation of specific nucleotides within the FP2 region could only be explained, however, if some or all of these nucleotides were involved in binding the authentic transcription factor.

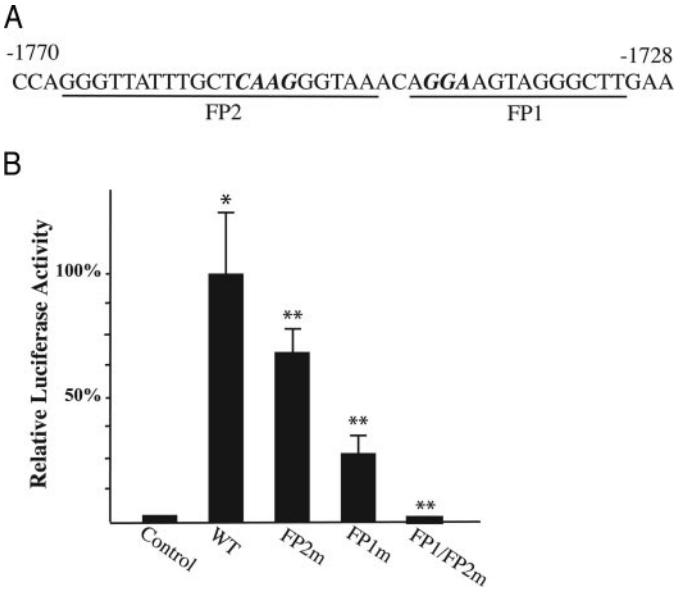

FIG. 1. A, 43-bp rPLII 5′ flanking fragment functions as an enhancer element in the rat trophoblast cell line, Rcho. A, A 43-bp double-stranded oligonucleotide representing the region from −1770 to −1728 of the rPLII gene was synthesized for studies to identify DNA binding proteins. Two previously identified DNase I protected sites within this region, FP1 and FP2, are *underlined*. Oligonucleotides with mutations in the FP1 and/or FP2 region, as indicated in *bold italics*, were also synthesized. B, Dimers of wild-type or mutated oligonucleotides were cloned into the luciferase reporter vector, pTK81, for transient transfection studies in Rcho cells. Luciferase activity was measured and values normalized to cotransfected *Renilla* luciferase activity. Data are plotted as the mean value and SD of the firefly/*Renilla* luciferase ratio (n = 14). The ratio of the wild-type construct was defined as 100%, and ratios of the other constructs as relative percentages. Activity of the wild-type enhancer construct is significantly greater than the vector control (*, $P < 0.001$). Individual mutations in the FP1 and FP2 regions resulted in lower activities compared with the wild-type construct (**, $P < 0.001$ and $P < 0.016$, respectively). The double mutation showed a complete loss in enhancing activity (**, $P < 0.0003$).

The core nucleotide binding sequence of the FP2 factor is localized to a 12-bp region

To define the essential core of the FP2 binding site more precisely, we synthesized a series of 43-bp oligonucleotides in which nonoverlapping sets of four nucleotides, extending across the FP2 region, were mutated. To confirm the importance of the GGA sequence within the FP1 site for protein binding and enhancer activity and to identify the DNA/protein complexes associated with this region, an oligonucleotide with changes to these nucleotides was also synthesized. The oligonucleotide changes are shown in Fig. 2A. All mutated oligonucleotides were tested by EMSA, both as competitors of the wild-type 43-bp enhancer element (Fig. 2B) and as probes (Fig. 2C), using nuclear extracts from differentiated Rcho cultures.

Six DNA/protein complexes were observed in the EMSA experiments using wild-type 43-bp probe (Fig. 2B, lane 1). Label was displaced from complexes 1 through 4 by excess unlabeled wild-type oligonucleotide (Fig. 2B, lane 2); complexes 5 and 6 were unaffected, suggesting that they were nonspecific. The M2 oligonucleotide (Fig. 2B, lane 4) also competed with complexes 1–4, suggesting that no critical DNA/protein interactions were disrupted by the M2 nucleotide changes. When oligonucleotides M3, M4, or M5, each with mutations in the FP2 site, were used, competition was seen with complex 3 and, to a lesser degree, with complex 4 (Fig. 2B, lanes 5–7). Conversely, the m6 oligonucleotide, mutated in the FP1 region, competed with complexes 1, 2, and 4, but not 3 (Fig. 2B, lane 3). Results of experiments in which wild-type and mutant oligonucleotides were used as labeled probes were consistent with the EMSA competition results, except that the m6 mutations appeared to affect formation of both complexes 3 and 4 (Fig. 2C). Oligonucleotides with the M3, M4, or M5 mutations in combination with the m6 mutation did not compete and formed no specific complexes when used as probes (data not shown). These results suggested that formation of complex 3 (and perhaps complex 4) required the GGA triplet in the FP1 site, whereas complexes 1 and 2 resulted from protein binding to nucleotides within a 12-bp sequence in the FP2 site, as defined by the M3, M4, and M5 mutations (Fig. 2A, sequence shown in *bold*). Al-

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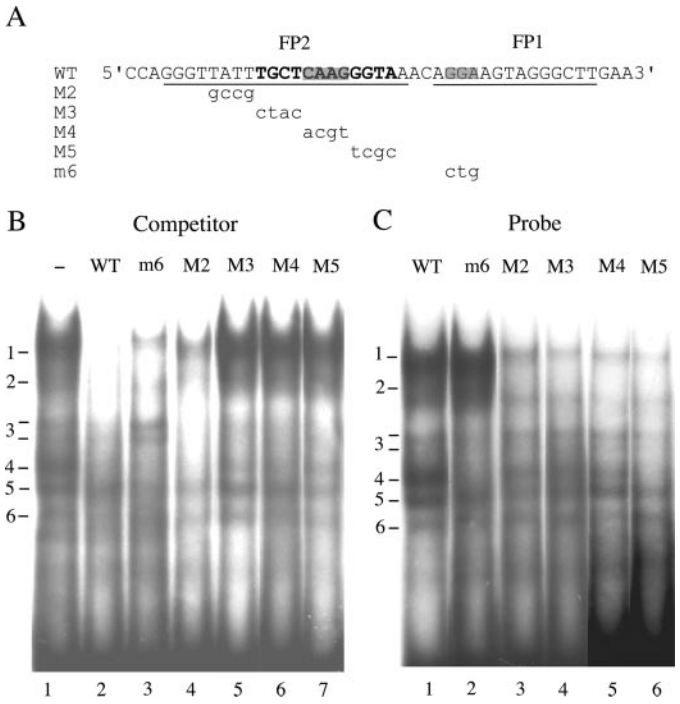

FIG. 2. EMSA studies identify specific protein-DNA complexes that are associated with core nucleotides within the FP1 and FP2 regions. A, A series of sequential 4-bp mutations were created in FP2 (M2-M5); the m6 mutation in FP1 altered a putative Ets factor core sequence, GGA. The *shaded* nucleotides indicate the FP1 and FP2 mutations tested in the transfection studies shown in Fig. 1. B, Competitive binding assays were carried out using a 200-fold molar excess of either wild-type or mutant oligonucleotides. Of the six DNA-protein complexes that were formed with the 43-bp wild-type probe (lane 1), complexes 1 to 4 were competed by both the wild-type (lane 2) and M2 mutated (lane 4) oligonucleotides. Only complex 3 was competed with the M3, M4, and M5 mutated oligonucleotides (lanes 5–7). Complexes 1, 2, and 4, but not 3, were competed by the m6 mutated oligonucleotide (lane 3). There was no competition of complexes 5 and 6 by any of the oligonucleotides, suggesting that they are nonspecific. C, Wild-type and mutated oligonucleotides were individually end-labeled as probes for EMSA studies with Rcho nuclear extracts. Compared with the wild-type probe in lane 1, complexes 3 (seen variously as one or two bands) and 4 were not formed with the m6 mutant probe (lane 2), and complexes 1 and 2 were reduced or lost with the M2, M3, M4, and M5 mutant probes (lanes 3–6). Complexes 5 and 6 were formed with all probes, supporting the conclusion that they are nonspecific. Taken together, these EMSA results suggest that complexes 1 and 2 result from protein binding to core nucleotides within a 12-bp region in FP2, as shown in *bold* in panel A. Complex 3 and perhaps complex 4 appear to result from binding to a site containing the GGA sequence of FP1.

though these experiments defined key nucleotides within the FP2 binding site and confirmed involvement of the GGA sequence in protein binding to the FP1 region, this additional information did not help in the identification of candidate rPLII enhancer binding proteins, based on transcription factor binding site database searches alone. To overcome this difficulty we undertook a direct proteomics approach using MS to identify the proteins that bind the rPLII enhancer.

MALDI TOF MS identifies AP2γ as an rPLII enhancer binding protein

To isolate the rPLII enhancer binding factors from Rcho giant cell nuclear extracts for MALDI TOF MS analysis, we used a DNA-affinity strategy in which the biotinylated, double-stranded wild-type enhancer oligonucleotide was immobilized on streptavidin-linked magnetic beads for protein binding and washing steps. To minimize sample loss, bound proteins were digested with trypsin while still on the beads; after removal of the beads, the peptide mixture was partially fractionated by HPLC, followed by automatic sample spotting off-line onto a MALDI target (20). The HPLC fractionation reduced sample complexity in any given spot and removed impurities, both of which are particularly important when working with the complex peptide mixtures generated from nuclear extracts. Mass measurements using the University of Manitoba/SCIEX MALDI QqTOF instrument are very precise and sensitive, making the analysis of small amounts of sample feasible. Peptide mass matching and MS/MS were carried out on selected ion peaks of sufficient signal intensity. Figure 3 outlines our procedure.

Because, as a result of our selection procedure, the samples were known to contain tryptic peptides derived from streptavidin, BSA (present in the magnetic bead storage buffer), and trypsin itself, those peptides were identified and removed from the data files manually. During the early phases of the work, we were somewhat hampered in our analyses by the fact that the rat protein and DNA databases were much less complete than those for the human and mouse. We found it useful to include the human and mouse proteomes in our database searches. A Sonar ms/ms program analysis of tandem mass spectra initially identified one ion at *m/z* 2087.159

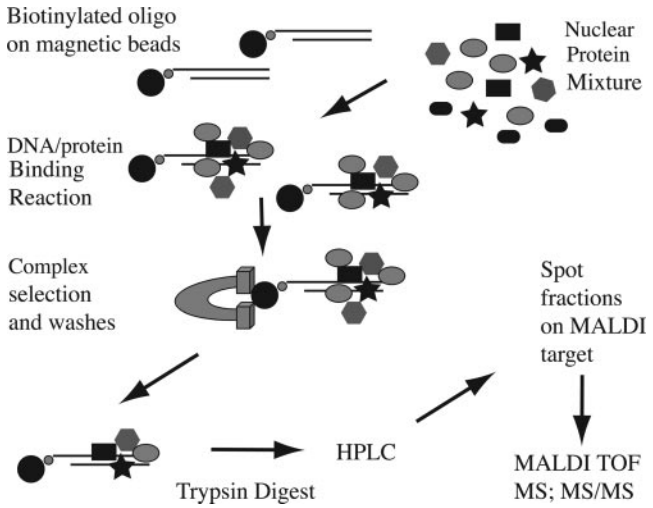

FIG. 3. An MS proteomics strategy was developed for the identification of rPLII enhancer binding proteins. Streptavidin-coated magnetic Dynabeads were coupled to the biotinylated, double-stranded 43 bp wild-type oligonucleotide and incubated with Rcho nuclear extracts in EMSA buffer. The protein-DNA complexes were enriched and washed on the beads, using a magnet to capture the beads after each manipulation. After the final wash, the beads with attached DNA/protein complexes were resuspended in 10 mM NH_4HCO_3 and digested overnight with trypsin at 37 C. The supernatant was collected and dried, and the pellets were dissolved in 0.2% TFA. Proteins were separated by HPLC. Fractions were collected, mixed with MALDI matrix solution, and deposited by computer-controlled robot onto a movable gold target at 1-min intervals. Spots from each fraction were analyzed by single MS (*m/z* range, 560-5000) on a Manitoba/SCIEX QqTOF mass spectrometer (17). Ions with sufficient intensity were selected for MS/MS on the same instrument.

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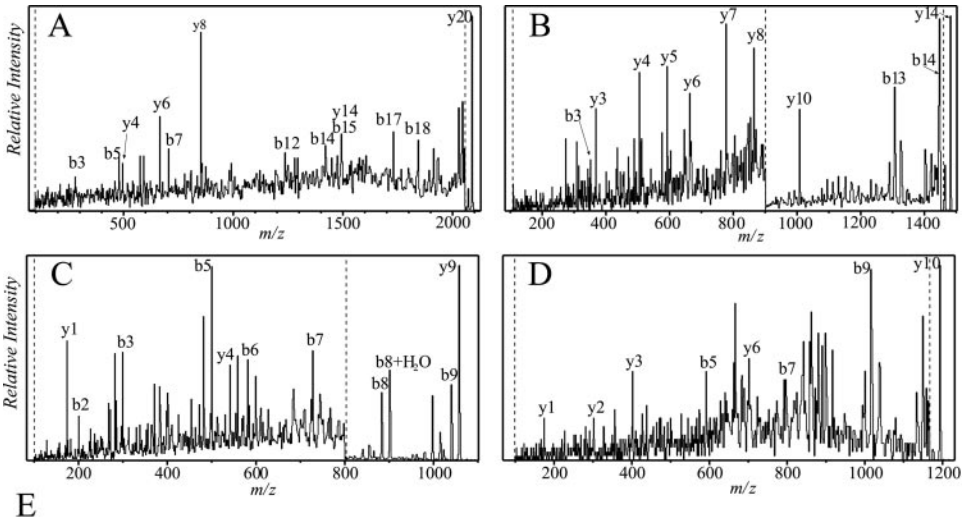
as a human and mouse AP2γ peptide. The mass fragmentation pattern of this peptide is shown in Fig. 4A. Subsequent annotation of the rat AP2γ cDNA and protein in databases (GenBank NM_201420 and NP_958823, respectively) showed that this peptide was also in the rat AP2γ protein sequence. Although five different AP2 family members have now been identified (22–26), peptide assignment could be made unequivocally to AP2γ, based on the presence of a histidine residue in the third position of this peptide [AAH-VTLLTSLVEGEAVHLAR]. The residue at this position is an asparagine in human and mouse AP2α, -β, -δ, and -ε (21–25).

Three further ions were also identified as being from rat AP2γ. The parent ion at *m/z* 1480.693 (Fig. 4B) is unique to rat AP2γ, whereas ions at *m/z* 1057.623 and 1192.668 (Fig. 4, C and D) represent overlapping peptides that are conserved in all known AP2 family members across several species. Mass matching analysis of our spectra to the most recent rat databases, using ProFound, has now identified tryptic peptides covering 26.8% of the rat AP2γ sequence (Fig. 4E). A complete list of the rat AP2γ peptides identified by mass matching, including the errors between predicted and observed tryptic peptide masses, is shown in Table 2.

In vitro and in vivo studies confirm functional AP2γ binding to the FP2 region of the rPLII enhancer

A comparison of the FP2 region of the rPLII enhancer with a consensus AP2 binding site (26) showed a sequence of marked similarity within the 12-bp region identified by our EMSA experiments (Fig. 2A). A major difference between the sequences was a C to T change at position 3 (Fig. 5A). Although a permitted change (11, 27), this is an unusual substitution and may suggest why database searches did not identify this sequence as a potential AP2 binding site. To confirm that AP2γ specifically recognized the FP2 region of the rPLII enhancer element, we performed further EMSA experiments with recombinant AP2γ protein and AP2γ-specific antiserum. *In vitro* synthesized human AP2γ formed a specific complex with the wild-type enhancer element (Fig. 5B, lanes 2 and 3), but not with an oligonucleotide containing either the M3 or M4 mutation in the FP2 region (data not shown). AP2γ antiserum specifically shifted complexes 1 and 2 in EMSA reactions with Rcho and placental nuclear extracts, whereas nonimmune rabbit serum had no effect (Fig. 5B, lanes 4–9); these same complexes were associated with binding to the FP2 region of the enhancer element (Fig. 2B). A consensus AP2 binding site oligonucleotide also competed only EMSA complexes 1 and 2 (Fig. 5C). These data support the MS identification of AP2γ as a transcription factor that binds to the rPLII enhancer in Rcho cells and in the rat placenta and further indicate that this binding is specific for nucleotides in the FP2 region.

To determine whether AP2γ could transactivate gene expression via the rPLII enhancer AP2 binding site, we carried out cotransfection studies in the rat pituitary GC cell line, which does not express AP2γ (Öztürk, A., unpublished observation). Our earlier studies had shown that the rPLII enhancer was not active in this cell line (5). It had previously been reported that high levels of AP2 have a negative effect on gene transcription, possibly due to self-inhibition or sequestering of coactivator proteins (28). To address this potential problem, varying amounts of the human AP2γ expression clone, pCDNA3.AP2γ, were first cotransfected with the wild-type enhancer/luciferase construct, pTKWT.luc, to titrate for optimum activity. A greater than 5-fold increase in luciferase expression was seen with pTKWT.luc at a concentration of 50 ng of AP2γ (Fig. 6A, lane 3). When a reporter



E

```
1   mlwkitdnvk yeedcedrhd assngnprip hlssagqhly spapplshtg vaeyqpppyf
61  pppyqqlays qsadhyshlg eayaaainpl hqpaptgsqq qawpgrqsqe gsslashhgr
121 sagliphisg legsavssrr eayrrsdlll phahaleagl aenlglhema hpieevqnvd
181 dphlllhdqt virkgpismt knplglpcqk dlvgvvmnps evfcsvpgrl sllsstskyk
241 vtvaevqrrl sppeclnasl lggvlrraks knggrslrek ldkiglnlpa grrkaahvtl
301 ltslvegeav hlardfayvc eaefpskava dyltrphlgg rnemaarksv llaaqqvcke
361 ftdllhqdrt pngnsrptpv letniqncls hfslithgfg sqaicaavsa vqnyikeali
421 tidksymnpg kqspadsnkt mekmekhrk
```

FIG. 4. MALDI TOF MS identifies AP2γ as an rPLII enhancer binding protein. Tandem mass spectra of four peptides that were identified by database searches as rat AP2γ are shown in panels A–D. A, Parent ion at *m/z* 2087.159 matched residues 295–314 of the mouse (NP_033361), 296–315 of the human (NP_00321), and 295–315 of the rat (NP_958823) AP2γ proteins. B, Parent ion *m/z* 1480.693 matched residues 107–120 of rat AP2γ. C, Parent ion *m/z* 1057.623 matched residues 242–250 of rat AP2γ. D, Parent ion *m/z* 1192.668 matched residues 240–249 of rat AP2γ. *Dotted lines* in the spectra indicate a change in intensity scale. E, The locations of all tryptic peptides identified by mass matching are shown in *bold* in the rat AP2γ protein sequence, representing 26.8% coverage. The peptides sequenced by MS/MS are shown in *shaded boxes* and represent 10% coverage. The *underlined* histidine at position 297 was key in identifying the protein as AP2γ. Other known members of the AP2 family contain an asparagine at a comparable location. A complete list of the mass matched peptides with expected and observed masses is shown in Table 2.

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TABLE 2. Rat AP2γ peptides identified by mass matching and MS/MS

Mass (Da)		Error (ppm)	Predicted sequence[c]	Rat AP2γ peptide[d]
Observed[a]	Expected[b]			
909.546	909.539	8	IGLNLPAGR	284–292
1056.604	1056.604	1	VTVAEVQRR[C]	241–249
1191.679	1191.661	15	YKVTVAEVQR[D]	239–248
1265.749	1265.745	3	LDKIGLNLPAGR	281–292
1272.630	1272.610	2	EFTDLLHQDR	360–369
1479.697	1479.68	11	QSQEGSSLASHHGR[B]	107–120
1682.914	1682.884	18	GPISMTKNPLGLPCQK	195–210
1836.947	1836.968	11	SAGLIPHISGLEGSAVSSR	121–139
1993.057	1993.070	7	SAGLIPHISGLEGSAVSSRR	121–140
2086.176	2086.153	11	AAHVTLLTSLVEGEAVHLAR[A]	295–314
2214.287	2214.248	18	KAAHVTLLTSLVEGEAVHLAR	294–314

The rat AP2γ protein sequence is from GenBank accession no. NP_958823.
[A, B, C, D] Peptides sequenced by MS/MS (Fig. 4) and identified using Sonar ms/ms and Global Proteomics Machine software programs.
[a] Mass of observed ion.
[b] Mass of ion as calculated from ProFound.
[c] Sequence of rat AP2γ tryptic peptides.
[d] Location of amino acid sequence in the rat AP2γ protein.

clone containing the FP2 M4 mutation in the enhancer element (pTKM4.luc) was similarly tested, there was no significant increase in luciferase activity (Fig. 6A, lane 5). A construct containing the M3 binding site mutation also showed no enhancement (data not shown). These data confirm that AP2γ has a positive effect on rPLII enhancer activity as a result of binding to the nucleotides within the FP2 region.

To address whether AP2γ binds the rPLII enhancer of an expressing placental giant cell *in situ*, we carried out ChIP assays using differentiated Rcho cells and the same AP2γ-specific antiserum that was used for the EMSA supershift studies. Figure 6B shows the results of a representative experiment. Chromatin samples immunoprecipitated with AP2γ antiserum at two different DNA input concentrations (Fig. 6B, lanes 3 and 4) gave a PCR product of the expected size of 335 bp. Control ChIP reactions with nonimmune rabbit serum (Fig. 6B, lane 5) and protein A/G agarose beads only (Fig. 6B, lane 6) showed either very low levels of the 335-bp band or no PCR product. A further primer set designed to amplify a 679-bp fragment between +2871 and +3550 of the rPLII gene produced no PCR product (data not shown). Together these data support the conclusion that the transcription factor that binds the FP2 region of the rPLII enhancer *in vivo* is AP2γ. Given the restricted expression of AP2γ to cells of the trophoblast lineage in rodents (7, 8) and the ability of the 5′ flanking fragment containing this rPLII enhancer sequence to target gene expression to the placenta in transgenic mice (3), AP2γ binding to this element is likely

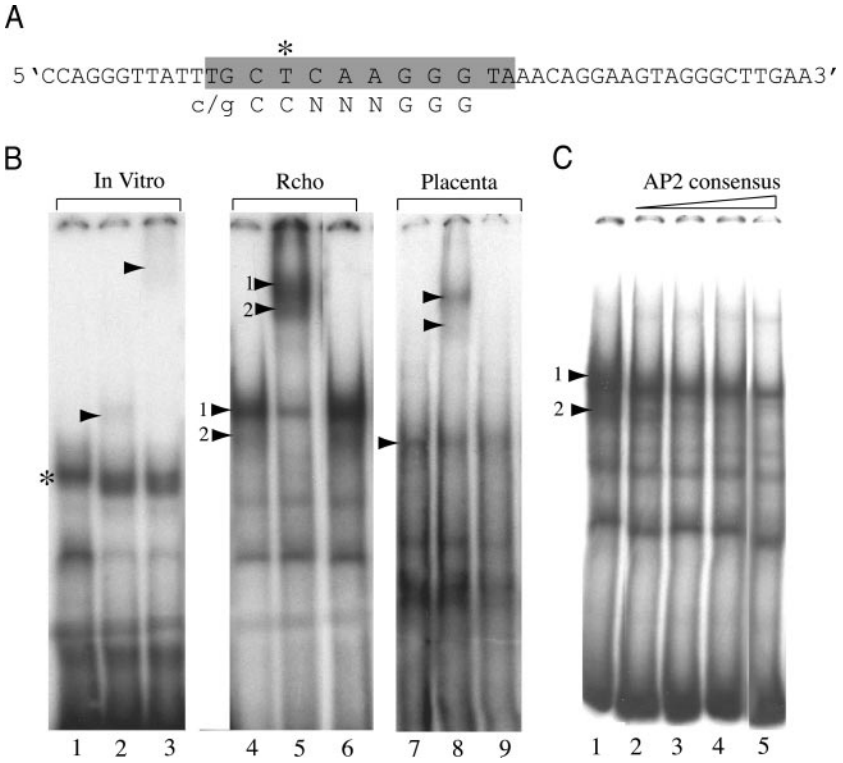

FIG. 5. AP2γ is the FP2 binding protein. A, A comparison of the 43-bp rPLII enhancer sequence with a consensus AP2 binding site shows a closely related region in FP2. The substitution of a highly conserved C by a T at position 3 in the rPLII element is marked by an *asterisk*. The *shaded box* indicates the 12 nucleotides that EMSA studies suggested could be part of an FP2 protein binding site. B, A series of EMSA supershift studies using AP2γ-specific antiserum were carried out with the wild-type 43-bp probe. A human AP2γ clone was transcribed and translated *in vitro* using a rabbit reticulocyte system. *Arrowheads* mark the EMSA complexes formed with *in vitro* translated AP2γ protein alone (lane 2) and with the addition of AP2γ-specific antiserum (lane 3). Lane 1 shows a control reaction without RNA. An *asterisk* marks a nonspecific band seen in all three lanes. With Rcho nuclear extracts, complexes 1 and 2 (lane 4) were shifted in the presence of the AP2γ-specific antiserum (lane 5), but were unaffected by the addition of nonimmune serum (lane 6). These are the complexes associated with binding to the FP2 region of the enhancer element, as shown in Fig. 2. Although EMSA complexes are less defined using nuclear extracts from d 18 rat placenta (lane 7), larger complexes are specifically formed in the presence of AP2γ antiserum (lane 8) but not nonimmune serum (lane 9). C, Rcho nuclear complexes 1 and 2 (lane 1) were specifically competed by an AP2 consensus oligonucleotide at 25-, 50-, 100-, and 500- fold molar excess (lanes 2–5, respectively).

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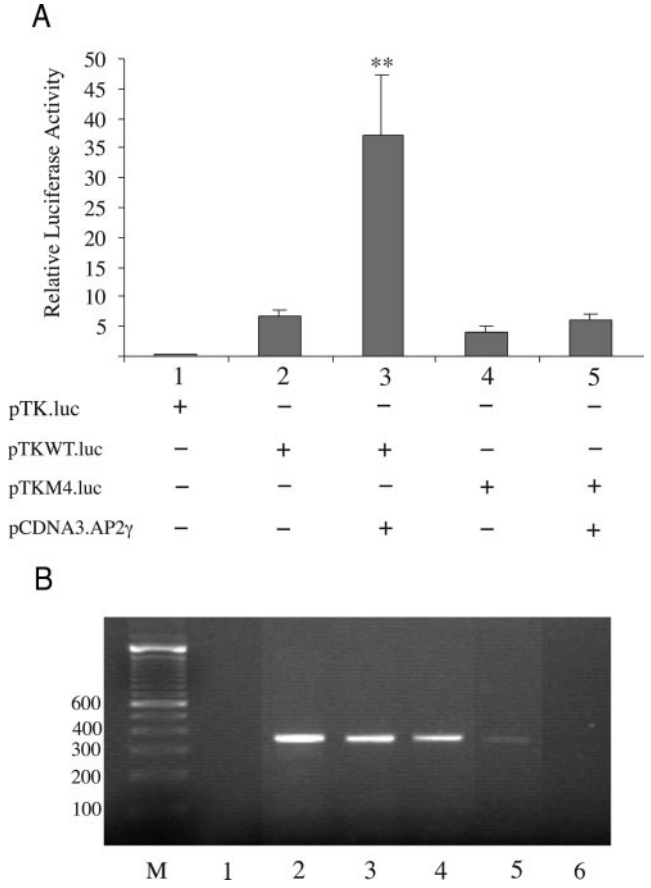

FIG. 6. AP2γ interacts functionally with the FP2 region of the rPLII enhancer *in vitro* and *in vivo*. A, Rat pituitary GC cells were transiently transfected with either the wild-type enhancer construct, pTKWT.luc, or the FP2 mutant construct, pTKM4.luc, with or without 50 ng of the human AP2γ expression clone, pCDNA.AP2γ. Firefly luciferase activity was normalized to cotransfected *Renilla* luciferase activity. The data are plotted as the mean value and SD of the firefly/Renilla luciferase ratios (n = 6). AP2γ increases the activity of the wild-type construct (lanes 2 and 3) (**, $P < 0.0001$), but has no effect on the activity of the M4 FP2-mutated construct (lanes 4 and 5), which is not significantly different from the wild-type construct without cotransfected AP2γ. B, ChIP assays were performed using cross-linked chromatin from differentiated Rcho cells and AP2γ-specific antiserum. A representative gel shows the predicted 335-bp PCR product for the input sample before immunoprecipitation (lane 2) and eight pooled 2 A_{260} (lane 3) and four pooled 4 A_{260} (lane 4) units of input sample immunoprecipitated with AP2γ antiserum. This band is barely visible or absent for the 16 A_{260} units of input sample immunoprecipitated with nonimmune rabbit serum (lane 5), or treated with Protein A and G Sepharose beads only (lane 6). Lane 1 is a PCR control containing no chromatin sample. Ten microliters of sample was amplified in each case, except for the nonimmunoprecipitated input sample, which was 4 μl. All PCRs were for 35 cycles.

to have an important role in the placental-specific expression of the rPLII gene during pregnancy.

Poly (ADP-ribose) polymerase-1 (PARP-1) and histone H1 subtypes are identified among the proteins selected by the rPLII enhancer oligonucleotide

In addition to AP2γ and background peptides derived from streptavidin, trypsin, and BSA, our mass spectra contained a number of ions that were identified by both mass matching and MS/MS data as peptides derived from PARP-1 and isoforms of histone H1. Twenty-seven rat PARP-1 peptides (25% of the protein) (GenBank NP_037195) were identified by mass matching; 13.8% of the sequence was verified by MS/MS. PARP-1 is an abundant nuclear protein, most widely recognized for its role in DNA repair processes (reviewed in Ref. 29). More recently, it has been shown to act as a component of enhancer/promoter complexes (29) and in particular has been identified as a coactivator of AP2α (30).

Peptides representing the N-terminal sequences of five different isoforms of rat histone H1 were also present in the sample. Three of these H1 isoforms were identified in the rat protein or genome databases as rat H1.4 (NP_579819), H1.0 (NP_036710), and an unknown H1 subtype (XM_225330). The other two sequences, although highly related to known H1 subtypes, were not in any databases. There is evidence that different histone H1 isoforms may play specific roles in the control of gene expression (31). Given the several subtypes identified in our experiments, it would seem more likely that histone H1 binds the short rPLII enhancer oligonucleotide because of a general affinity for DNA, and our MS data may reflect the different H1 subtypes present in the differentiated Rcho cells.

Discussion

In previous studies, we identified a 3-Kb 5′ flanking region of the rPLII gene that targeted reporter gene expression to the placenta in transgenic mice (3) and contained an enhancer element with trophoblast giant-cell specificity (5). The data we present in this paper identify AP2γ as the protein that binds the FP2 region of this rPLII enhancer element. This is the first direct evidence to support a role for a placental-specific transcription factor in the regulation of any gene in the large rodent PRL family.

Originally identified as the transcription factor that bound the trophoblast-specific element in the human placental chorionic gonadotropin α (hCGα) gene (32), AP2 has since been shown to have an important role in regulating the expression of a number of placenta-restricted genes across species (9–13). Five AP2 family members have been identified (21–25), of which both AP2α and AP2γ are expressed in human and rodent placenta (7, 33). Early experiments did not distinguish between AP2 family members, but a study by LiCalsi *et al.* (11) has since demonstrated the specific presence of AP2γ in protein complexes bound to the trophoblast-specific element of the hCGα and hCGβ genes. Detailed *in situ* hybridization studies in developing mouse embryos indicate that AP2γ mRNA is expressed in all cells of the preimplantation embryo and becomes restricted to trophoblast lineages after implantation (7, 10). In the mature rodent placenta, AP2γ is expressed in the primary and secondary giant cells at the fetal-maternal interface, spongiotrophoblast cells of the junctional zone and trophoblast cells of the labyrinth region (7); these are all cell types that express members of the PRL family (1). Targeted mutagenesis of the AP2γ gene in mice results in very early placental defects and fetal death by embryonic d 8.5 due to defective development of the extraembryonic lineages, in particular a reduction in the number of invasive giant cells at the fetal-maternal interface (7, 8).

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Although these embryos do not survive long enough to be tested, our results strongly support a role for AP2γ in the placental-specific expression of rPLII, and they reinforce a previously expressed idea that AP2γ may be a key regulator of placental gene expression throughout pregnancy (9, 10).

In situ hybridization data show that AP2γ is also expressed in the maternal antimesometrial decidua (7), the site of synthesis of the rat and mouse PRL-like protein B, decidua/trophoblast related protein, PRL-like protein J, and PRL itself (1). Such overlapping expression patterns suggest that AP2γ could potentially play a role in regulating the tissue-specific transcription of all PRL gene family members expressed during pregnancy. An argument against this possibility would seem to be the *in situ* hybridization data showing that the few giant cells formed in early AP2γ mutant mouse embryos still express placental lactogen I mRNA, the earliest PRL family member to be transcribed in the rodent placenta. These mutant cells also express adenosine deaminase, which previous *in vitro* and *in vivo* studies have shown to be regulated by AP2γ (10). To explain this latter inconsistency, Auman *et al.* (7) suggested either that low levels of other AP2 family members might compensate for loss of zygotic AP2γ or that maternal stores of protein may mask an earlier requirement for AP2γ. Conditional AP2γ mutant mice and the development of AP2γ-null mouse lines from embryonic stem (ES) cell-only derived embryos would be two potential means of directly addressing a general role for AP2γ in the expression of members of the PRL gene family throughout pregnancy.

AP2 family members bind a consensus motif that is usually represented by the sequence $^{C}/_{G}CCN_3GG^{G}/_{C}N$ (26). Recent PCR-based random selection studies of AP2α and AP2γ binding sites suggest a somewhat broader tolerance (34, 35) but in all instances showed an invariant C at position 3. The AP2γ site in the rPLII enhancer reported in this work, GCTCAAGGGT, has a T rather than a C in position 3. Similar substitutions have been reported only in the hCGβ FP4–2 (GCTTGAGGGT) (9) and c-erbB-2 (GCTGCAGGC) (27) AP2 binding sites. A randomly selected pool of AP2 binding sites did not recover molecules in which the 5′ GCC motif was replaced by GCT, and competitive binding studies indicate that a C to T change at position 3 results in weaker AP2 binding (35). Our ChIP data suggest, however, that even such a potentially weak site can act as a functional AP2γ binding element *in vivo*. In the rPLII enhancer, the factor that binds to the FP1 region may help stabilize AP2γ binding to this site.

The identity of the protein factor(s) binding to the FP1 site remains unknown. Database searches of our MS data showed at best only single mass matches with other known transcription factors. The GGAA sequence in FP1 marks this region as a potential binding site for a member of the family of Ets transcription factors, many of which are expressed in placenta. Using *in vitro* transcribed and translated proteins, we have shown by EMSA studies that several members of the Elf subfamily of Ets factors can specifically bind the enhancer FP1 region, whereas members of other subgroups, including Ets2 and Elk1, do not bind (data not shown). These results suggest an affinity for Ets proteins, in particular those of the Elf subgroup, but definitive evidence for a specific Ets factor binding *in vivo* is still lacking. Very recently, a null mutation of Elf-5/ESE-2a in mice has been shown to be embryonic lethal early in placental development (36); Elf-5 mRNA is present in Rcho cells (Duckworth, M. L., unpublished observation). Now that we have identified AP2γ as an rPLII enhancer binding protein, coimmunoprecipitation experiments, potentially coupled to MS analysis, may prove useful for isolating further DNA binding proteins and interacting factors in Rcho and rat placental cells.

The identification of PARP-1 as one of the proteins bound by the rPLII enhancer oligonucleotide is an intriguing one. PARP-1 is a highly abundant nuclear protein; until recently, its best known role was to catalyze the transfer of ADP-ribose chains onto glutamic acid, aspartic acid, and lysine residues of various nuclear acceptor proteins, including histone H1 (reviewed in Ref. 29). Our identification of both PARP-1 and five different isoforms of histone H1 may be the result of the large quantities of these interacting proteins present in nuclear extracts and binding to the rPLII enhancer oligonucleotide an artifact of our selection method. A recent proteomic analysis of nuclear hormone receptor coactivator complexes indicated that PARP-1 was present in many unrelated complexes, and in this study it was classified as a nonspecific association (37). Nonetheless, it is now well recognized that PARP-1 is able to modulate gene transcription not only by its effects on chromatin structure through interactions with histones, but also by directly altering the activity of enhancers and promoters either by binding to specific DNA sequences or through interactions with non-histone DNA binding factors (29). Of particular relevance for our studies, PARP-1 has been shown to bind specifically to AP2α, -β, and -γ (30) and, depending on the region of the PARP-1 protein that binds AP2, can either inhibit or activate transcription (38). There is also genetic evidence that PARP-1, like AP2γ, may play a direct role in the development of trophoblast cell lineages. PARP-1 null mice, although viable, display altered placental development, which includes reduced numbers of spongiotrophoblasts and increased numbers of giant cells (39). Mutant PARP-1 ES cells also show a markedly increased ability, compared with wild-type ES cells, to differentiate into trophoblast giant cells both in culture and within teratocarcinoma-like tumors formed in nude mice (39). When considered with the giant cell poor phenotype of AP2γ null placenta, these findings suggest that during normal placental development the levels and activities of AP2γ and PARP-1 will be carefully regulated to maintain appropriate developmental gene expression. Further studies will be needed to assess whether there is a specific functional interaction between AP2γ and PARP-1 that affects rPLII expression or whether its presence in our MS experiments is nonspecific.

Although many established methods are now available for identifying DNA regulatory sequences, it has proven far more challenging to identify the proteins that bind these sequences. MS has not yet been widely used to identify unknown proteins in DNA binding complexes, although it offers several advantages. Of particular importance for these types of studies are the sensitivity and specificity of MS technology (reviewed in Ref. 40). MALDI TOF MS has most often been used in combination with one- or two-dimensional gel separation of proteins to reduce sample complexity before mass analysis. This approach requires relatively large amounts of starting protein, not because of instrument sen-

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sitivity, but to have sufficient protein to visualize for gel isolation. The method we have used follows a newer trend of eliminating the use of gels for sample separation, thus taking full advantage of the sensitivity of MALDI TOF mass spectrometers, which are now capable of detecting proteins at amounts well below 100 fmol, and allowing the use of much smaller biological samples. The primary challenge in our studies came from peptide mass matching and MS/MS protein sequence analysis. Although rat AP2γ is now in protein databases, our initial identification came from a peptide that was identical with one found in human and mouse AP2γ. The specific difficulty imposed by incomplete rat databases is rapidly being resolved, but our experience illustrates the usefulness of broadening database searches to other related species.

Perhaps surprisingly, the identification of AP2γ as a factor that regulates the expression of rPLII is the first example of a placental-specific transcription factor having a direct role in the regulation of the rodent PRL gene family. The giant cell-specifying transcription factor, HAND-1, has been indirectly implicated in the expression of rat placental lactogen I, but no specific *cis*-acting element has been identified, and no promoter analysis has been carried out (41). Because AP2γ is expressed in all trophoblast cell types, it cannot be solely responsible for the complex developmental expression patterns seen during pregnancy for rPLII and potentially other members of the PRL gene family. It may, however, play a general role in the expression of these genes. The factors that lead to the individual expression patterns remain to be more thoroughly defined, but the simplicity of the HPLC-coupled MALDI TOF MS method that we have used in our studies should prove useful for these future studies, particularly when only small amounts of nuclear proteins are available for analysis. Rapid improvements in protein and DNA databases and the development of increasingly powerful MS data analysis programs will greatly facilitate this methodology.

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