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An alternatively spliced Pit-1 isoform altered in its ability to trans-activate

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ABSTRACT

Although alternative splicing has been shown to give rise to isoforms of a number of transcription factors, such isoforms have not previously been detected for the POU homeodomain protein Pit-1. Screening of a rat pituitary GH_3 cell cDNA expression library yielded a clone, termed pCMVPit-1a, encoding a 35.8 kD protein (Pit-1a) containing a 26 amino acid insert in the Pit-1 trans-activation domain. The position of the insert, plus Southern blot analysis, implied that Pit-1a mRNA arises by alternative splicing of the Pit-1 gene transcript. Pit-1a mRNA was detected in GH_3 rat pituitary tumor cells at levels about 1/7 that of Pit-1 mRNA. Pit-1a mRNA-specific sequences were also detected in rat and mouse pituitary, and in mouse thyrotropic tumor TtT cells. DNA mobility shift assays showed that Pit-1a binds specifically to Pit-1 binding sites in the proximal prolactin promoter, but produces DNA-protein complexes of markedly different mobilities than Pit-1. In stably transfected CHO cells which accumulated approximately equal levels of either of the two proteins, Pit-1 trans-activated a prolactin promoter-driven CAT construct, while Pit-1a yielded no detectable trans-activation, implying a trans-activation ratio for Pit-1a/Pit-1 of <0.05. Thus, the insertion of 26 amino acids of similar composition into the activation domain of Pit-1 has at once affected both the mode of binding of this protein and its ability to function as a trans-activator.

INTRODUCTION

The pituitary-specific protein Pit-1 (also known as GHF-1) is a member of the POU homeodomain protein family of transcription factors (1). Studies by a number of groups (2, 3, 4, 5, 6) have shown that Pit-1 can bind to and regulate the pituitary cell-specific expression of both the prolactin (PRL) and growth hormone (GH) genes. It has also been shown that Pit-1 is required for the normal development (7) and proliferation (6) of specific anterior pituitary cell types. It is becoming clear that, in addition to its developmental role in the pituitary gland, Pit-1 also plays an important role in hormonal regulation of pituitary gene expression. Evidence has been presented recently for a role of Pit-1 in regulation by thyrotropin-releasing hormone of the thyrotropin beta subunit gene (8), and by thyrotropin-releasing hormone and calcium of the prolactin gene (5, 9).

The 273 amino acids of Pit-1 can be divided into three functional regions. The first 73 to 80 amino acids ('trans-activation domain') are involved in trans-activation (10, 11, 12). The remainder of the protein is divided into two domains of conserved sequence homology joined by a non-conserved linker region. Amino acids 128–198, encompassing the POU-specific domain, confer high-affinity sequence-specific DNA binding and protein-protein interactions, while amino acids 213–273, encompassing the POU homeodomain, are necessary but not sufficient for high affinity DNA binding (11).

It has been shown recently that alternative splicing leads to multiple isoforms of a number of transcription factors, including another member of the POU homeodomain family, Oct-2 (13). However, no such isoforms of Pit-1 have yet been described. We report here the cloning and detection in rat and mouse pituitary cells of an alternatively spliced Pit-1 gene transcript encoding a protein with a 26 amino acid insert in the Pit-1 trans-activation domain, and the DNA-binding and trans-activational properties of the encoded protein, termed Pit-1a.

MATERIALS AND METHODS

Isolation of a cDNA clone for Pit-1a

Poly(A)$^+$ RNA was isolated (14, 15) from the GH_3 rat pituitary tumor cell line (16) and used to construct a cDNA library using primer adapter technology as described (14). Fifteen μg of poly(A)$^+$ RNA was used for first strand synthesis using an XbaI-T primer (5′ AATTCTCTAGATTTTTTTTTTTTTT 3′) with methyl deoxycytidine triphosphate in the reaction mixture. The first strand was then tailed with 12–15 deoxyguanosine residues. Second strand synthesis was carried out (17) using an XbaI-C primer (5′ AATTCTCTAGACCCCCCCCCC CCCC 3′). After second strand synthesis the double stranded cDNA was filled in using T4 polymerase, digested with XbaI, separated from primers on an Sp6 (Pharmacia) spin column and cloned into the XbaI site of the expression vector pCDM8 (Invitrogen). Approximately 45,000 recombinant clones were screened by colony hybridization (15) for Pit-1 homologous sequences. The probe was synthesized by polymerase chain reaction (PCR) amplification of the Pit-1 encoding nucleotides (nt) −21 to 985 (unless otherwise noted, numbering is designated

with the first methionine codon of the mRNA as +1) in the plasmid pSV2Pit-1 (18). The amplified DNA was gel purified and labeled by random priming (19, 20). After two rounds of colony hybridization, a recombinant plasmid was isolated; restriction mapping indicated that the insert was in the 3′ to 5′ orientation with respect to the CMV promoter. The insert was removed with XbaI and re-cloned in the reverse orientation to construct pCMVPit-1a.

DNA analysis

Double-stranded plasmid DNA was sequenced with Sequenase as recommended by the supplier (United States Biochemical). Southern blotting was performed as previously described (21). The various probes used were generated by PCR amplification of pCMVPit-1a sequences. All amplified DNA fragments were gel purified and labeled by random primer synthesis. To detect the 78 bp Pit-1a specific sequence, two primers in the Pit-1a insert were used in PCR experiments: one including nt 143−157 (5′ TCCCGTCTATTTTGT 3′) and the other including nt 220−206 (5′ CTGTATTTCCCATCG 3′). To locate sequences encoding Pit-1 and Pit-1a, two exon 1-specific primers were used, one including nt 1−20 of mouse Pit-1 cDNA (5′ ATGAGTTGCCAATCTTTCAC 3′, ref.7), the other including rat cDNA nt 75−43 (5′ CAGAGGCAGGGCAGCAGAAGCTCAGAATTCAG 3′).

RNA analysis

RNase protection experiments were performed as previously described (22). To detect Pit-1 sequences, ^{32}P-labeled anti-sense RNA was synthesized from a plasmid, pRPit-1, a pSp72 (Promega)-based vector containing an EcoRI fragment of the Pit-1 encoding insert of pSV2Pit-1 (18) extending from nt 45 to 495. The anti-sense probe was transcribed using T7 RNA polymerase. To detect Pit-1a transcripts, pRPit-1a was similarly constructed using an EcoRI fragment extending from nt 45 to 573 in the Pit-1a sequence of pCMVPit-1a. In this case SP6 polymerase was used to synthesize the anti-sense probe.

For RNA analysis using PCR, RNA was isolated as described previously (14) from rat pituitaries (Sprague Dawley, a gift of S. DeRiemer), mouse pituitaries (Balb/c, a gift of T. Moran) and GH_3 cells. RNA from AtT20 cells was a generous gift of M. Tsutsumi; RNA from TtT cells was generously provided by M. Gershengorn. Total RNA from each source (5−10 μg) was used in a 40 μl cDNA synthesis reaction that included 10 ng of random hexamers (Promega), reverse transcriptase buffer (Bethesda Research Laboratories, BRL), 0.25 mM dNTPs, 12 units of RNasin (Promega), 200 units of Moloney murine leukemia virus reverse transcriptase (BRL), and 5mM DTT. After incubation at 37°C for 1−2hrs and heating to 65°C for 5min, 10 μl of the reactions were used directly for PCR. PCR mixes (50 μl) were prepared with reaction buffer supplied by Promega, 3′ and 5′ primers at concentrations of 0.8 ng/μl, 80 μM dNTP, 4 μCi of ^{32}P-labeled dATP and 1.25 units of Taq polymerase (Promega). The reactions were run for 30 cycles at 94°C for 1 min, 54°C for 45 sec and 72°C for 1.5 min.

To detect Pit-1 and Pit-1a sequences in rat mRNA samples, primers including nt −21 to nt −2 (described above) and nt 294 to nt 311 (5′ GCTGTTGGGTCCTCCGCC 3′) of the Pit-1 sequence were used. To detect Pit-1a sequences in mouse pituitaries and pituitary cell lines, a primer homologous to mouse Pit-1 cDNA (7) including nt 1−20, (5′ ATGAGTTGCCAATCTTTCAC 3′), and a primer homologous to the Pit-1a insert, nt 223−199 (5′ TCGCTGTATTTCCCATCGTTGTCAT 3′) were used in PCR reactions.

Nuclear extract preparation and Western analysis

Nuclear extract preparation and immunoblotting were carried out as described previously (18; see legend to Fig.4).

In vitro transcription and translation and electrophoretic mobility shift assays (EMSA)

Preparation of wheat germ lysate was carried out essentially as described previously (23). Rabbit reticulocyte lysate was from Promega. Preparation of capped mRNAs was performed as follows. For Pit-1, pSp6pit-1 (5) was linearized with StyI (New England Biolabs, NEB) and transcribed with SP6 RNA polymerase (NEB) to produce an mRNA of approximately 1500 nucleotides. Pit-1a RNA was obtained by linearizing pCMVPit-1a with BamHI (NEB) and transcribing with T7 RNA polymerase (NEB) to produce an mRNA of approximately 1800 nucleotides. Transcription reactions were performed as recommended by the suppliers with the addition of 0.5mM $m^7G(5')pp(5')GTP$. Reactions were incubated at 37°C (T7 RNA polymerase) or 40°C (SP6 RNA Polymerase) for two hours.

In vitro translation reactions were performed as described previously (23). Translation was measured by incorporation of ^{35}S-methionine into total TCA-precipitable material. Translation products were separated in 18% SDS-polyacrylamide gels. Labeled material was analyzed by autoradiography; unlabeled translation products were used immediately for gel-shift analysis.

DNA electrophoretic mobility shift analysis was carried out essentially as described in (24). Probes used for gel-shift analysis

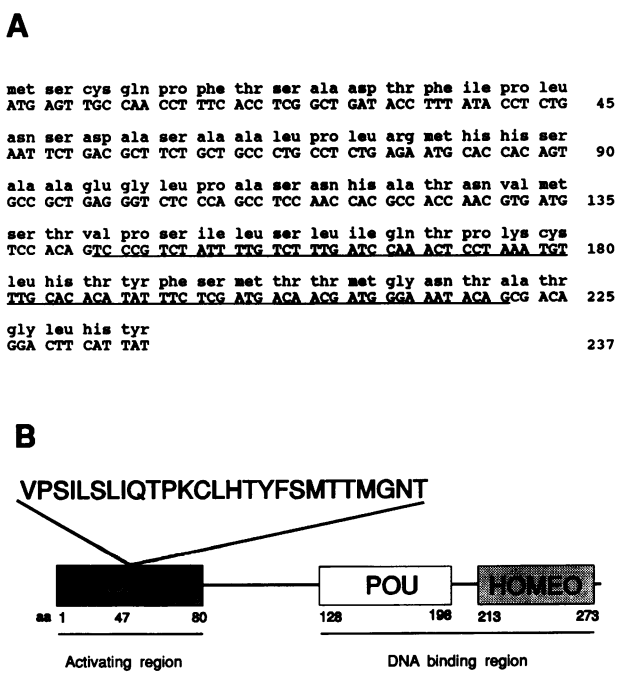

Fig. 1. Sequence analysis of Pit-1a cDNA. A) DNA sequence of the first two exons of Pit-1a cDNA. The new exon of Pit-1a is underlined. B) Location of the Pit-1a-specific amino acid sequence within the activation domain of the Pit-1 protein. The numbering of the amino acid sequence of Pit-1 starts with the first methionine codon (35).

were synthetic oligonucleotides corresponding to sequences from −39 to −63 (1P), −114 to −133 (2P), −144 to −167 (3P), and −185 to −204 (4P), relative to the transcription start site of the rat prolactin promoter. Single stranded oligonucleotides were annealed and labeled by end-filling with Sequenase (United States Biochemical). To each gel-shift reaction was added either 120 fmols (1P, 2P and 3P) or 62 fmols (4P) of labeled probe. To compensate for the difference in the translation efficiencies of the two mRNA products, 4.0 μl of the minus RNA control, 4.0 μl of the Pit-1a or 0.8μl of the Pit-1 *in vitro* translation reactions were used for the gel-shift reactions. In the last case, the volume was made up to 4 μl with the minus RNA reaction mixture. In the case of CHO cell nuclear extracts, 4μg of protein were incubated with the 1P probe. As a test for specificity, a mutant form of the 1P probe, 1P* (ref. 5), carrying four base substitutions in one of the Pit-1 consensus binding sequences was used as a competitor. Samples were loaded onto a low ionic strength native 10% polyacrylamide gel and electrophoresed at 150 volts. Gels were wrapped in plastic and the wet gels exposed to X-Ray film at −80°C with an intensifying screen.

Tissue culture and transfections

CHO cells were grown as described previously (25). Hela cells were grown in DMEM (Gibco) with 10% fetal calf serum. Transfections were performed as described previously (26). For transient chloramphenicol acetyl transferase (CAT) assays, 5 or 15 μg of the reporter plasmid p(−1957)PRL-CAT (27) were used for each 100 mm dish. Transfection with 5 μg of pSV2CAT was used as a positive control. All reactions were brought to 15 μg of DNA with calf thymus DNA. Lysates were prepared after 48 hours and CAT activity assayed by the production of ^{14}C-diacetylated chloramphenicol (28, 29). Radioactive spots on TLC sheets were quantified using a Molecular Dynamics Phosphoimager.

To construct cells that express Pit-1a, the dihydrofolate reductase (DHFR) deficient cell line DG44 (30) was co-transfected with 0.5 μg of the DHFR expression plasmid pDCH1P (22), 10 μg of the Pit-1a expression vector pCMVPit-1a and 9.5 μg of carrier DNA (calf thymus). After a 24 hour incubation period, the transfected cells were placed in medium lacking purines to select for DHFR$^+$ cells. To co-amplify *dhfr* and pCMVPit-1a sequences in the resultant DHFR$^+$ cells, colonies were pooled and plated in F12 medium supplemented with 7% fetal calf serum but lacking glycine, hypoxanthine and thymidine and containing 0.1 μM methotrexate. After 10 days colonies were pooled and selected in progressively higher concentrations of methotrexate to a final selection at 3 μM.

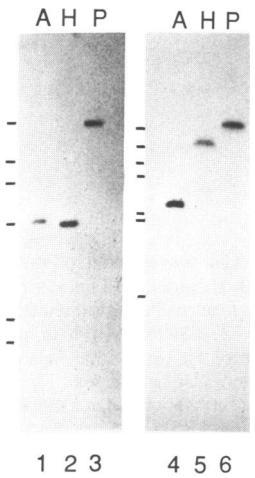

Fig. 2. Southern blotting analysis of Pit-1 and Pit-1a sequences in GH_3 cell DNA. A) Hybridization with a probe complementary to Pit-1 or Pit-1a exon 1. B) Hybridization with a probe consisting of the 78 bp Pit-1a exon sequence. A, AvaI; H, HindIII; P, PstI. Marks on the left indicate the position of HindIII digested lambda markers, from the top: 23.1, 9.4, 6.5, 4.4, 2.3, 2.0, 0.6 kb fragments.

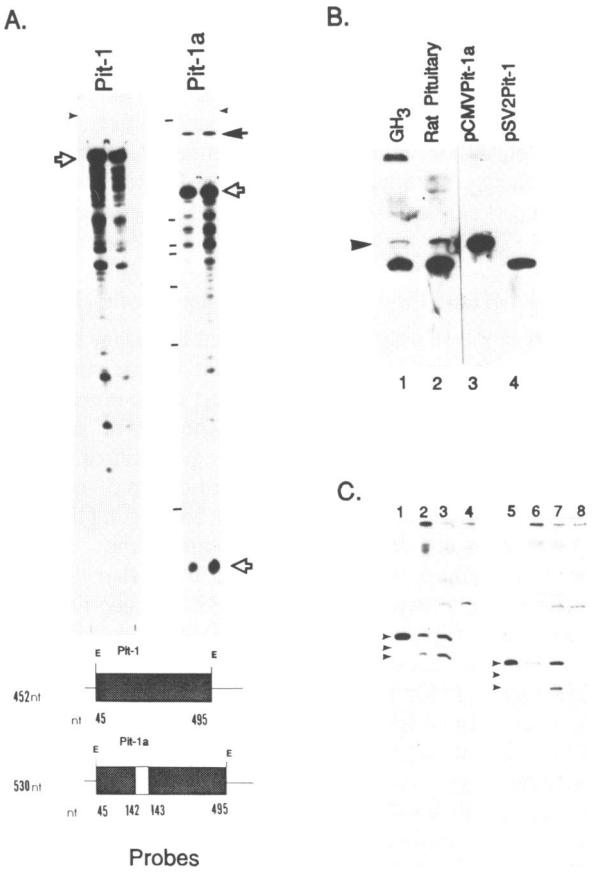

Fig. 3. Expression of Pit-1 and Pit-1a RNA in rat and mouse cell lines and tissues. A) RNase protection analysis of RNA isolated from rat GH_3 cells. The riboprobes used are indicated at the top and are depicted at the bottom. Pit-1 numbering applies to both probes, and is given relative to the first ATG codon of Pit-1. The shaded box indicates the 78 nt Pit-1a exon. The numbers on the left indicate the expected bands protected by Pit-1 mRNA for the Pit-1 probe or Pit-1a mRNA for the Pit-1a probe. Open arrows indicate bands protected by Pit-1 mRNA and the solid arrow indicates the band protected by the Pit-1a mRNA. The small arrowheads show the position of the undigested probe. Molecular size markers, indicated by short dashes, were end-labeled HaeIII fragments of φX174 DNA, from the top: 602, 310, 281, 271, 234, 194, and 118 bp. B) Reverse transcription and PCR amplification of Pit-1 and Pit-1a mRNA in GH_3 cells and rat pituitaries. The primers used in the PCR reaction amplify sequences extending from nt −21 to nt 311 of the Pit-1 cDNA sequence and nt −21 to nt 389 of Pit-1a. Plasmid DNA containing either Pit-1 (pSV2Pit-1) or Pit-1a (pCMVPit-1a) sequences were amplified as migration standards. C) Reverse transcription and PCR amplification of RNA from rat GH_3 cells (lanes 1 and 5), mouse pituitary (lanes 2 and 6), mouse TtT cells (lanes 3 and 7), mouse AtT-20/D16v cells (lanes 4 and 8). The 3′ primer used was specific for the Pit-1a sequence and the amplified fragments extend from nt 1 to 223 of the rat Pit-1a cDNA. Arrowheads indicate the location of Pit-1a specific fragments. The samples in lanes 5−8 were digested with EcoRI after amplification.

RESULTS

Cloning and sequence analysis of a splicing variant of Pit-1

A mammalian expression library was constructed using poly $(A)^+$ mRNA from the rat somatomammotrophic tumor cell line GH_3. When the library was screened for Pit-1 homologous sequences using a Pit-1 cDNA probe, positive colonies appeared at a frequency of 10^{-4}. Polymerase chain reaction (PCR) analysis showed that one of the resultant plasmids contained a Pit-1 coding region sequence larger than Pit-1. Sequencing of this plasmid revealed that the cDNA in this clone, termed pCMVPit-1a, was identical to the coding region for Pit-1 with the exception of the presence of a 78 nucleotide in-frame insert at position 142 of Pit-1 (Fig. 1A). The predicted amino acid sequence shows a 26 amino acid insert in the trans-activation domain of Pit-1 (Fig. 1B). A computer search yielded no significant homology between the insert and any known proteins. A search for known peptide signals or motifs indicated a possible kinase C phosphorylation site at threonine 57 of the predicted Pit-1a sequence. The high proportion of hydroxy amino acids in the insert is similar to that of the entire trans-activator domain (31).

The Pit-1 variant Pit-1a is generated by alternative splicing

The insert in Pit-1a occurs at the reported boundary between the first and second exons of the mouse Pit-1 gene sequence (7), suggesting that the presence of this insert is the consequence of an alternative splicing mechanism. The Pit-1a sequence is identical to that of Pit-1 at every other position of the coding region as well as at least 25 nucleotides of the 5′ untranslated region of the transcript (data not shown), supporting the idea that both transcripts are derived from the same gene.

We used Southern blot analysis to probe further the question of whether these two transcripts could be specified by two different genes. GH_3 cell DNA was digested with AvaI, HindIII or PstI, enzymes that do not cut in the cDNA sequence of either Pit-1 or Pit-1a. If Pit-1a were specified by a second gene, then one would expect each gene to yield a distinct restriction fragment upon treatment with an enzyme that cuts in the flank and introns surrounding exon 1. As can be seen in Fig. 2A an exon 1-specific probe detects only a single restriction fragment in each case. This is the result expected if a single gene codes for Pit-1 and Pit-1a. A Pit-1a-specific probe also hybridized to single restriction fragments produced by these enzymes. In the case of AvaI and HindIII these fragments were of different sizes than the fragments that hybridized to the exon 1 probe, whereas in the case of PstI, a large (20 kb) fragment of similar size was produced (Fig. 2B). It appears that AvaI and HindIII cut within the intron that must separate exon 1 and the Pit-1a-specific exon in the genomic sequence of the Pit-1 gene.

Taken together, these three findings (insert position, sequence identity, and Southern analysis) argue strongly for alternative splicing as the origin of the Pit-1a transcript.

Pit-1a is expressed in various rodent pituitary cells and tissues

To determine whether Pit-1a transcripts are expressed in GH_3 cells, RNase protection analysis was performed using riboprobes for Pit-1 or Pit-1a. With the Pit-1 probe (Fig. 3A, left) one major protected fragment (open arrow) was detected with the expected size of 452 nt for the Pit-1 transcript. With the Pit-1a probe (Fig.3A, right), a protected 530 nt fragment, the expected size for the Pit-1a transcript, was detected (solid arrow), as well as two fragments of sizes 352 bp and 97 bp expected for the Pit-1 transcript using this probe (open arrows). Densitometric analysis of the data shown in Fig. 3A showed that, in the GH_3 cells, Pit-1 mRNA is approximately 7-fold more abundant than Pit-1a mRNA.

To ask whether the Pit-1a transcript is also expressed in the normal rat pituitary, total mRNA was isolated from adult rat pituitaries as well as GH_3 cells and used for reverse transcriptase-PCR analysis. In Fig. 3B it can be seen that Pit-1a transcripts are present in both GH_3 cells (lane 1, arrow) and the rat pituitary (lane 2, arrow).

To perform similar investigations of the Pit-1a transcript in mouse pituitary, mRNA was isolated from mouse pituitary, thyrotropic mouse pituitary tumor TtT cells (32), and mouse corticotrophic AtT-20/D16v cells (33). Using another set of primers, one homologous to the first 20 bp of mouse Pit-1 cDNA and the other homologous to the last 22 bp of the rat Pit-1a exon, sequences homologous to Pit-1a were detected in mouse pituitary mRNA and the thyrotropic TtT cells but not in the corticotropic AtT-20/D16v cells (Fig. 3C); the last also do not contain detectable Pit-1 mRNA (data not shown). Two major and one minor transcripts were amplified in both mouse pituitary (lane 2) and TtT cell mRNA (lane 3), indicating the presence of Pit-1a encoding transcripts in both samples. It is seen that the higher molecular weight mouse bands co-migrated with the rat GH_3 cell Pit-1a amplified sequence (lane 1). To determine the specificity of the lower molecular weight bands, the amplified DNA was digested with EcoR1, which cuts at nt 45 of exon 1 of mouse Pit-1. As can be seen in Fig. 3C (lanes 5−8), all the amplified bands were cut with EcoRI, suggesting that the mouse pituitary contains at least three forms of Pit-1a mRNA.

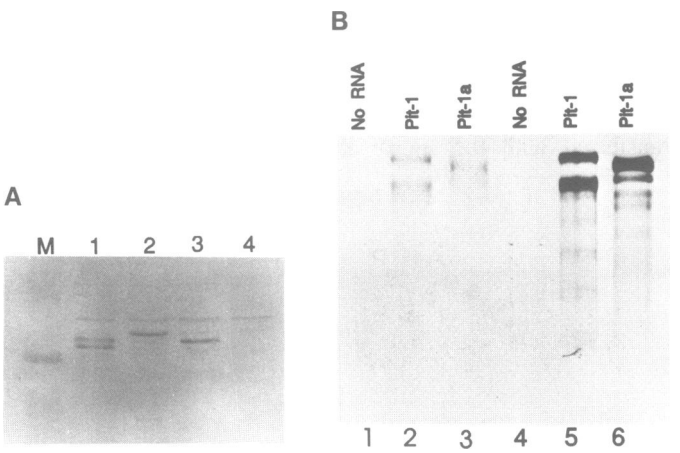

Fig. 4. Isoforms of in vivo and *in vitro* synthesized Pit-1a. A) Western immunoblot of nuclear proteins (50 μg) from various cell lines, separated by polyacrylamide gel electrophoresis, blotted onto nitrocellulose and reacted with anti-Pit-1 antiserum. Lane 1: GH_3 cells; lane 2: CHO/pCMVPit-1a pooled, amplified, stable transfectants; lane 3: CHO/pSV2Pit-1 stable transfectant clone; lane 4: CHO cells (a negative control). M , molecular size marker. The 30 kD species is the only one visible here. B) *In vitro* transcription and translation of Pit-1 and Pit-1a RNA. Each *in vitro* transcribed capped RNA (0.5 μg) was translated in a wheat germ or rabbit reticulocyte system in the presence of ^{35}S-methionine. Equal amounts of TCA-precipitable counts were loaded in each set of lanes and the products separated by SDS-PAGE. Lanes 1 and 4: minus RNA controls; lanes 2 and 5: products from Pit-1 transcripts; lanes 3 and 6: products from Pit-1a transcripts. Translation products were from rabbit reticulocyte lysates (lanes 1−3) and wheat germ extracts (lanes 4−6). The gel was visualized by fluorography. Sizes of the polypeptides were estimated by comparision with co-electrophoresed Rainbow high molecular weight markers (Amersham).

Analysis of the protein products of Pit-1a mRNA

To begin to characterize the protein encoded by the Pit-1a transcript, DG44 dhfr$^-$ CHO cells were stably transfected with the expression construct pCMVPit-1a plus *dhfr*, and selected as described in Materials and Methods. Initial experiments with these cells showed that, although the mRNA for Pit-1a could be detected by PCR, Western blot analysis yielded no detectable Pit-1a protein (data not shown). By contrast, we have previously found that Western blot analysis of CHO cells transfected with 1/10th as much pCMVPit-1 yielded readily detectable levels of Pit-1 (18). These results, plus our ability to detect at most only a trace of Pit-1a in nuclear extracts of GH_3 cells (data not shown) suggested that Pit-1a may be a less stable protein than Pit-1. To achieve a high level of expression of Pit-1a in the present experiments, the *dhfr* and pCMVPit-1a sequences in the transformed cells were co-amplified by selection with methotrexate (see Materials and Methods). The resultant colonies were then pooled and employed for further analysis of Pit-1a.

The results of Western blot analysis of nuclear extracts, employing antiserum against amino acids 136–150 of the POU specific domain of the Pit-1 protein (18), are shown in Fig. 4A. Extracts of GH_3 cells yielded the expected 33 and 31 kD Pit-1 doublet (lane 1), which has been previously suggested to arise from inititation of translation at the first and second methionine codons, respectively (34). In agreement with our previous observations (18), extracts of CHO cells stably transfected with pSV2Pit-1 yielded only the larger (33 kD) form of Pit-1 (Fig. 4A, lane 3). Extracts of the CHO cells containing amplified levels of pCMVPit-1a yielded comparable levels of a single protein approximately 36 kD in size (Fig. 4, lane 2), consistent with translation initiation at the first methionine of the Pit-1a gene transcript in CHO cells.

To further characterize the translation product(s) of Pit-1a mRNA, Pit-1 and Pit-1a transcripts were translated *in vitro* and analyzed by SDS polyacrylamide gel electrophoresis (Fig. 4B). In either reticulocyte lysates (lane 2) or wheat germ extracts (lane 5), the Pit-1 transcript directed the synthesis of two proteins approximately 33 and 30 kD in size, again consistent with the translation initiation at, respectively, the first and second methionine codons. By contrast, in either reticulocyte lysates (lane 3) or wheat germ extracts (lane 6), the Pit-1a transcript programmed synthesis of two major proteins approximately 32.5 and 31 kD in size, consistent with translation initiation at, respectively, the second and third methionine codons. An alternative explanation for the latter results, premature translation termination, seems unlikely since the same results were obtained in two translation systems. It thus appears that, for both Pit-1 and Pit-1a, various isoforms can arise from the use in different systems of alternative translational start sites.

Pit-1a binds to Pit-1 binding sites in the prolactin promoter

Pit-1 has been shown to bind to four sites, designated 1P, 2P, 3P, and 4P, in the proximal rat prolactin promoter (5, 35, 36). DNA mobility shift was employed to examine the ability of either Pit-1 or Pit-1a to bind to oligonucleotides corresponding to Pit-1 binding sites. Results using nuclear extracts of permanently transfected CHO cells and an oligomer corresponding to the strongest Pit-1 binding site, 1P, are shown in Fig. 5A. Pit-1 yielded a major shifted band (or doublet) plus minor bands of greater mobility (lane 4), each of which was competed by excess

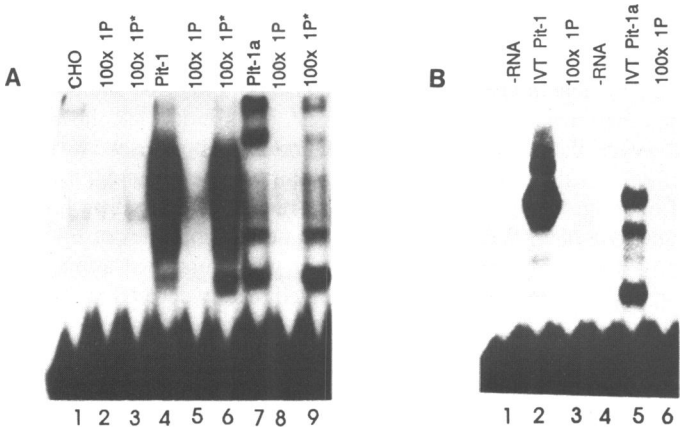

Fig. 5. Gel-shift analysis of Pit-1 and Pit-1a. Nuclear extracts from transfected CHO cell populations or unlabeled *in vitro* translated proteins were assayed for their ability to bind to an end-labeled double-stranded oligonucleotide corresponding to the 1P DNase-1 footprint site of the rat prolactin proximal promoter. Equal amount of nuclear extracts or Pit-1 or Pit-1a *in vitro* translated proteins were allowed to bind and then analyzed by electrophoresis in native 10% polyacrylamide low ionic strength gels. The wet gel was subjected to autoradiography. Panel A, nuclear extracts, (4 μg protein): lanes 1–3, untransfected CHO DG44 cells; lanes 4–6 CHO/pSV2Pit-1 cells, a stable transfectant clone producing Pit-1; lanes 7–9, CHO/pCMVPit-1a pooled, amplified, population of stable transfectants producing the same amount of Pit-1a (these are the same nuclear extracts used for the Western immunoblots shown Fig.4A). Lanes 1, 4, and 7: no competitor DNA; lanes 2, 5, and 8 include a 100-fold molar excess of unlabeled 1P probe; lanes 3, 6, and 9 include a 100-fold molar excess of unlabeled mutated probe, 1P* (5). Panel B: *in vitro* synthesized Pit-1 and Pit-1a: lanes 1 and 4, unprogrammed wheat germ lysate; lanes 2 and 3, Pit-1; lanes 5 and 6, Pit-1a. Reactions in lanes 3 and 6 contained a 100-fold molar excess of unlabeled probe 1P as competitor.

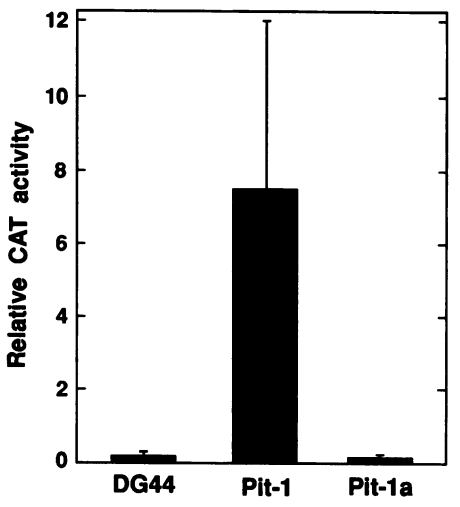

Fig. 6. Transient expression of the prolactin promoter in CHO cell lines expressing either Pit-1 or Pit-1a. The indicated cell lines were transfected with 15 μg of p(−1957)PRL-CAT or 5 μg of pSV2CAT DNA. Lysates were prepared after 48 hours and assayed for CAT activity. The results are expressed as the percent of the activity obtained with pSV2CAT in a parallel transfection, and are the averages of duplicate or triplicate transfections. The actual average percent conversion of chloampheinicol to the diacetylated derivative obtained with pSV2CAT were 28% (DG44), 49% (Pit-1) and 56% (Pit-1a). The recipient cells were: DG44, control DHFR-deficient CHO cells; Pit-1, a clonal DG44 derivative, SV5R1 (18), that expresses Pit-1; and Pit-1a, a pooled population of stably transfected DG44 cells expressing Pit-1a from amplified copies of pCMVPit-1a (see Materials and Methods). Standard errors of the mean are shown.

cold 1P (lane 5), but not by a mutant form of 1P (lane 6). Pit-1a yielded four bands of wide-ranging mobilities (lane 7); none of the Pit-1a bands were close in mobility to the major Pit-1 band. Each of the Pit-1a bands was less intense than the major Pit-1 band, and each represented specific binding as evidenced by competititon experiments (lanes 8 and 9). The origin of the most rapidly migrating band seen with Pit-1a, which has been observed consistently, is not presently clear; however, since it is competed by excess cold 1P, it presumably represents specific Pit-1a binding. We conclude that Pit-1a can bind specifically to site 1P, but in a markedly different manner than Pit-1. To rule out the possibility that the Pit-1a banding pattern was being caused by CHO proteins that had been induced by Pit-1a, we repeated the DNA mobility shift assay using *in vitro* synthesized Pit-1 and Pit-1a. As in the case of the *in vivo* synthesized proteins, *in vitro*-synthesized Pit-1 produced a major band and *in vitro*-synthesized Pit-1a yielded several bands of wide-ranging mobilities. The patterns produced by Pit-1a in the two systems were similar despite the fact that because of alternative translation initiation, *in vitro*-synthesized Pit-1a was a shorter polypeptide than its *in vivo* counterpart. Similar results were obtained using the *in vitro* translated proteins with binding sites 2P, 3P, and 4P (data not shown).

Pit-1a yields no detectable trans-activation of a co-transfected prolactin promoter

Previous studies have shown that Pit-1 can trans-activate transfected prolactin promoter constructs in various non-pituitary cell lines, in HeLa (2, 3), Rat 2 (3), and CHO (18) cells. We therefore compared the ability of Pit-1 and Pit-1a to trans-activate the prolactin-CAT construct p(−1957)PRL-CAT in CHO cells. In initial experiments, either a Pit-1 or a Pit-1a expression vector was co-transfected with p(−1957)PRL-CAT into either HeLa or CHO cells. No CAT activity above background was detected in the cells receiving the Pit-1a expression vector. However, since Pit-1a protein could not be detected in these transient expression experiments, it was not possible to conclude that Pit-1a cannot trans-activate the PRL promoter.

More definitive results were obtained by using as recipients for p(−1957)PRL-CAT, CHO cells that had been *stably* transfected with either a Pit-1 or a Pit-1a expression vector. For the former, a Pit-1-producing CHO cell line described previously (18) was employed, while for the experiments with Pit-1a, we used a population of CHO cells containing a stably transfected Pit-1a expression vector that had been amplified with methotrexate. Western blot analysis (Fig.4A) indicated that these two cell lines produce comparable levels of Pit-1 and Pit-1a. As can be seen in Fig. 6, the transfected PRL-CAT construct was active in the Pit-1 expressing CHO cells, but not in the CHO cells expressing Pit-1a. From this data we estimate that Pit-1a has less than 5% of the trans-activating capacity of Pit-1.

DISCUSSION

In the present studies, we report the detection and analysis of the mRNA for a Pit-1 isoform, termed Pit-1a. Our results imply that Pit-1a mRNA is generated from the Pit-1 gene by alternative splicing, yielding a transcript coding for a 35.8 kD protein containing a 26 amino acid insert in the Pit-1 trans-activation domain. This insert contains a high proportion of hydroxy amino acids, and is thus similar in composition to the Pit-1 trans-activator region (31).

The Pit-1a cDNA that we have cloned from GH_3 cell mRNA is not simply a cloning artifact, since reverse transcriptase-PCR analysis showed that Pit-1a mRNA is present in both the rat pituitary somatomammotropic tumor GH_3 cells and normal rat pituitaries. Furthermore, transcripts containing the exon encoding the Pit-1a insert are not limited either to the rat or to cells of the somatomammotrophic lineage, since both mouse pituitaries and mouse thyrotropic pituitary tumor TtT cells express transcripts containing the Pit-1a-specific exon.

As described in Results, we estimate that in the GH_3 cells Pit-1a mRNA is only about 1/7 as abundant as Pit-1 mRNA. Since Pit-1 is such an abundant protein in GH_3 cell nuclei, one might expect Pit-1a to be readily detectable by Western blot analysis of nuclear extracts of these cells. However, with this technique we have detected in GH_3 cells at most a trace of the predicted 36 kD Pit-1a protein (data not shown). Other investigators (3) employing the same antiserum, have also reported only occasional detection by Western blotting of an approximately 35 kD protein in GH_4C_1 cell nuclear extracts. Furthermore, as reported in Results, detection of Pit-1a in CHO cells stably transfected with a Pit-1a expression vector required amplification of the vector, while Pit-1 was readily detected, without vector amplification, in CHO cells stably transfected with only 1/10 the amount of the corresponding expression vector (18). These differences from the predicted accumulation of the two proteins in the CHO cells (and by extension also in the GH_3 cells) could result from an effect of the Pit-1a insert on either translational efficiency or protein stability.

Since the insert in Pit-1a is in the trans-activation domain, Pit-1a might be expected to bind to Pit-1 DNA binding sites, but possess altered trans-activational properties. In agreement with the former prediction, DNA shift analysis showed that Pit-1a synthesized *in vivo* or *in vitro* can bind specifically to the PRL proximal promoter Pit-1 binding site 1P in the proximal prolactin promoter (Fig. 5). The *in vitro* product could also bind to three additional Pit-1 binding sites in this promoter (2P, 3P, 4P, data not shown). However, the mobilities of the DNA-protein complexes were very different with the two proteins, suggesting a very different mode of binding or a differential ability to recruit additional proteins to the complex. When the trans-activational properties of Pit-1a and Pit-1 were compared (Fig. 6), it was found that although expression of Pit-1 in CHO cells yielded readily detectable trans-activation of p(−1957)PRL-CAT expression, expression in these cells of comparable levels of Pit-1a yielded no detectable trans-activation of this PRL-CAT construct. We estimate from these results that the ability of Pit-1a to trans-activate the prolactin promoter in the CHO cells is at most 5% that of Pit-1. The quantitative differences in overall binding that are apparent in Fig. 5 are not great enough to explain this inability of Pit-1a to trans-activate. We conclude that despite the fact that the 26 amino acid addition in Pit-1a has a composition similar to that of the rest of the activator region, this insertion destroys the ability of the protein to trans-activate the PRL promoter. The marked change in DNA shift pattern exhibited by Pit-1a suggests that the insertion of the 26 amino acid sequence causes this isoform to interact with different protein molecules than Pit-1.

It has been suggested previously that the two Pit-1 bands approximately 31 and 33 kD in size detected by gel analysis of nuclear extracts of GH_3 cells represent isoforms arising from alternative initiation site usage in Pit-1 mRNA (34). In the present studies, we have shown that another Pit-1 isoform, Pit-1a, arises from alternative splicing of the Pit-1 gene transcript.

Furthermore, depending upon the translation system employed, initiation of Pit-1a synthesis may apparently occur either only at the first methionine (in CHO cells, Fig. 4A) or at either the second or third methionines (during translation *in vitro*, Fig. 4B). The Pit-1 gene thus apparently yields a family of protein isoforms, arising from a combination of alternative transcript splicing and alternative translation initiation site usage within each mRNA. The physiological significance of the resultant diversity of Pit-1 isoforms, and in particular of the Pit-1a isoform described here, is not presently known. However, it has very recently been found that, in addition to the known role of Pit-1 in directing developmental pituitary-specific expression of both the prolactin and growth hormone genes, this transcription factor is also a gene-proximal protein in the pathway(s) of action of thyrotropin-releasing hormone and Ca^{2+} on expression of genes for pituitary protein hormones (5, 8, 9). It is thus possible that the multiple isoforms of Pit-1 are required for performance of the multiple cellular functions of this protein.

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