Genes Expressed During the Differentiation of Pancreatic AR42J Cells Into Insulin-Secreting Cells

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Pancreatic AR42J cells have the feature of pluripotency of the common precursor cells of the pancreas. Dexamethasone (Dx) converts them to exocrine cells, whereas activin A (Act) converts them into endocrine cells expressing pancreatic polypeptide. A combination of Act and betacellulin (BTC) converts them further into insulin-secreting cells. The present study identifies some of the genes involved in the process of differentiation that is induced by these factors, using the mRNA differential display and screening of the cDNA expression array. The expression levels of 7 genes were increased by Act alone, and a combination of Act and BTC increased the expression of 25 more genes. Of these, 16 represented known genes or homologues of genes characterized previously. Nine of the identified genes were unrelated to any other sequences in the database. An inhibitor of the mitogen-activated protein kinase pathway, PD098059, which blocks the differentiation into insulin-secreting cells, inhibited the expression of 18 of the 25 genes, suggesting that the proteins encoded by these genes are associated with the differentiation into insulin-producing cells. These include known genes encoding extracellular signaling molecules, such as parathyroid hormone-related peptide, cytoskeletal proteins, and intracellular signaling molecules. Identification and characterization of these differentially expressed genes should help to clarify the molecular mechanism of differentiation of pancreatic cells and the gene products that enable the β-cells to produce insulin. ***Diabetes*** **48:304–309, 1999**

Both the endocrine and exocrine cells of the pancreas arise from epithelial cells in the pancreatic duct (1,2). A number of recent studies using targeted disruption of the genes encoding the various transcription factors have shown them to play an important role in determining cell type during development. Mice lacking insulin promoter factor-1 failed to develop a pancreas (3). Islet-1, a LIM homeodomain-containing protein, is necessary for the development of the dorsal pancreas and is required for the generation of islet cells (4). Inactivation of BETA2/NeuroD or Pax4 causes a striking reduction in the number of insulin-producing cells and a failure to develop mature islets (5,6). These factors are thought to be involved in a complex regulatory network and cascade to exert their function. To clarify the molecular mechanism of differentiation in the pancreatic cells, we have attempted to identify the genes involved in this regulatory system.

Rat AR42J cells were derived from a chemically induced pancreatic tumor and have the feature of pluripotency of the common precursor cells of the pancreas (7,8). When exposed to dexamethasone (Dx), they become more like acinar cells (9). As we have shown recently, when treated with activin A (Act) and betacellulin (BTC), these cells differentiate into insulin-producing cells (10). In this way, AR42J cells resemble the common precursor cells in the developing pancreas and provide an excellent in vitro model system to study the differentiation of both exocrine and endocrine cells.

In this study, by the methods of mRNA differential display and screening the pancreatic islet cDNA expression array, we identify a group of genes whose expression is closely related to the differentiation of AR42J cells.

RESEARCH DESIGN AND METHODS

Materials. Recombinant human Act and activin-free fetal calf serum (FCS) were provided by Dr. Y. Eto of Central Research Laboratory, Ajinomoto Inc. (Kawasaki, Japan). Recombinant human BTC was prepared as described (11). PD098059 was obtained from Funakoshi (Kyoto, Japan). Human parathyroid hormone-related protein (PTHrP) cDNA was provided by Professor A. Broadus of Yale University (New Haven, CT), and the expression vector with metallothionein promoter pSVneoHMTII$_A$del-Ter was provided by Dr. K. Ikeda of the National Institute for Longevity Science (Aichi, Japan).

Cell culture. AR42J cells were originally provided by C. Logsdon of University of Michigan. AR42J-B20 cells, subclones of AR42J cells that convert into insulin-producing cells after treatment with a combination of Act and BTC, were obtained as described previously (9). They were cultured in Dulbecco's modified Eagle's medium containing 20 mmol/l HEPES/NaOH (pH 7.4), 5 mmol/l $NaHCO_3$, and 10% FCS at 37°C under a humidified condition of 95% air and 5% CO_2. Amylase activity in the cell extract was measured according to the method of Bernfeld (12). Cytoplasmic free calcium concentration was monitored by using fura-2 as described elsewhere (8). After the cells were treated with 10 nmol/l Dx (Dx-treated), 2 nmol/l Act (Act-treated), or 2 nmol/l Act and 2 nmol/l BTC (Act + BTC–treated) for 12, 24, and 48 h, total RNAs were extracted from the cells using the acid guanidinium thiocyanate-phenol-chloroform procedure.

mRNA differential display. mRNA differential display was performed as described previously (13). Briefly, 1 µg of total RNA was reverse-transcribed using 2.5 µmol/l of the anchored oligo-dT_{11}VN primer and SuperScipt Reverse Transcriptase (GIBCO/BRL, Grand Island, NY). Two microliters of the cDNA was amplified by polymerase chain reaction (PCR) using 2.5 µmol/l of the ^{32}P end-labeled anchored primer and 0.5 µmol/l of the arbitrary upstream primer in a Gene Amp PCR System 9600 (Perkin-Elmer, Norwalk, CT). The PCR conditions

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Act, activin A; BTC, betacellulin; Dx, dexamethasone; EST, expressed sequence tag; FCS, fetal calf serum; MAP, mitogen-activated protein; PCR, polymerase chain reaction; PTHrP, parathyroid hormone-related protein.

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used were 25 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 1 min, and extension at 72°C for 30 s, with a final extension for 10 min at 72°C. The PCR products were separated on a 5 or 8% denaturing polyacrylamide gel, and the gel was dried and exposed to X-ray film. Bands with increased or decreased intensity were excised from the gel, recovered, and re-amplified as described above. The re-amplified fragments were subcloned into pGEM-3Z (Promega, Madison, WI) and sequenced using an ABI PRISM Dye Terminator Cycle Sequencing FS Ready Reaction Kit and Applied Biosystems DNA sequencer model 373S (ABI, Foster City, CA).

Screening of pancreatic islet cDNA array. The 5′ sequence from the longer cDNA fragment is more likely to provide protein-coding information. To complement the method of mRNA differential display, rat pancreatic islet cDNA arrays on the membrane were screened by hybridization with ^{32}P-labeled cDNA populations prepared from mRNA of the differently treated cells. The cDNA proof for array screening was prepared by reverse transcription of 1 µg of poly(A)$^+$ RNA in the presence of [α-^{32}P]dATP (3,000 Ci/mmol, 10 mCi/ml). Each cDNA array included ~1,000 expressed sequence tags (ESTs) clones (14). To prepare the membrane of cDNA arrays, the insert of each EST clone (average insert size: ~1.5 kb) was amplified by PCR using T7 and SK vector primers that were denatured by the alkaline method and blotted in duplicate on a positively charged nylon membrane. The EST clones used were randomly selected in in vivo excised phagemid (pBluescript) forms from a unidirectional rat pancreatic islet cDNA library in λZAPII, which was constructed using poly(A)$^+$ RNA extracted from normal adult rat pancreatic islets and a Uni-ZAP XR vector system (Stratagene, La Jolla, CA). The cDNA library construction and in vivo excision were performed according to the manufacturer's instructions. The cDNA insert in the phagemids were partially sequenced from the 5′- and/or 3′-end to generate ESTs as described previously (15). The islet ESTs obtained have been deposited in the DNA Database of Japan (Acc. No. C06499~) to be freely available at dbEST (16).

Database analysis. The sequences obtained by mRNA differential display and screening of the cDNA array were compared with those in the nonredundant nucleotide and peptide databases at the National Center for Biotechnology Information using the BLAST network service (17,18).

Northern blotting. Of total RNA extracted from the cells, 20 µg was denatured and blotted onto a Hybond-N$^+$ nylon membrane (Amersham, Arlington, IL). The blot was hybridized with ^{32}P-labeled cDNA probe and washed at a final stringency of 0.1× standard saline citrate and 0.1% SDS at 60°C for 30 min before exposure to X-ray film.

Transfection. PTHrP cDNA was inserted into the expression vector pSV-neoHMTII$_A$del-Ter. For transfection, *N*-[1-(2, 3-dioleoyloxy)propyl]-*N*, *N*, *N*-trimethyl-ammonium methyl sulfate (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer's instruction. Forty-eight hours after the transfection, the cells were replated in Dulbecco's modified Eagle's medium containing 10% FCS and 400 µg/ml G418. Two weeks later, colonies were picked up, and the high-level expression was determined by Northern blotting.

RESULTS AND DISCUSSION

Identification of differentially expressed genes. We used 144 combinations of anchored oligo (dT) and arbitrary primers for differential display PCR. Reactions were performed in triplicate to minimize false positives. A total of 203 unique bands differentially responsive to stimuli of Dx, Act, and Act + BTC were selected and excised from the gel. After sequencing, 113 of the fragments that had both anchored oligo (dT) primer and arbitrary primer sequences at the ends were subjected to Northern blotting. Fragments with a short arbitrary primer sequence or unknown sequence at either end and without the anchored oligo (dT) primer sequence were not further characterized. No hybridizing signals were observed in 41 of 113 fragments, possibly because of the low levels of gene expression or nonapplicably small size (<150 bp) of the cDNA probe. Differential expression more than twofold was confirmed in 38 genes by Northern blotting (Fig. 1); 11 genes were identified in Dx-treated cells (referred to as D1 to D11), 7 genes in Act-treated cells (A1 to A7), and 20 genes in Act + BTC–treated cells (AB1 to AB20) (Table 1).

Through screening the pancreatic islet cDNA arrays (Fig. 2), we identified six upregulated genes (RBC 035, 124, 292, 522, 542, 684) in Act + BTC–treated cells (Table 1). Differential expression of these genes was confirmed by Northern blotting (Fig. 3). One of the genes—the gene for thymosin β10 (RBC 035)—was also identified in mRNA differential display (AB2).

Consistent with the pattern of differentiation of progenitor cells into either exocrine or endocrine cells, Northern blotting revealed that the upregulated genes identified in Dx- and Act + BTC–treated cells were all counterregulated when treated with Act + BTC and Dx, respectively (data not shown).

Database analysis. The sequence of each cDNA fragment was compared with those in the nonredundant nucleotide sequence database at National Center for Biotechnology Information using the BLAST network service. The results of the database search are shown in Table 1. The more 5′-upstream sequences of the seven PCR fragments (AB6, AB10, AB15, AB17, AB18, D6, and D7), which had no significant database match using BLASTN, were obtained by PCR screening of the cDNA library using vector primers and

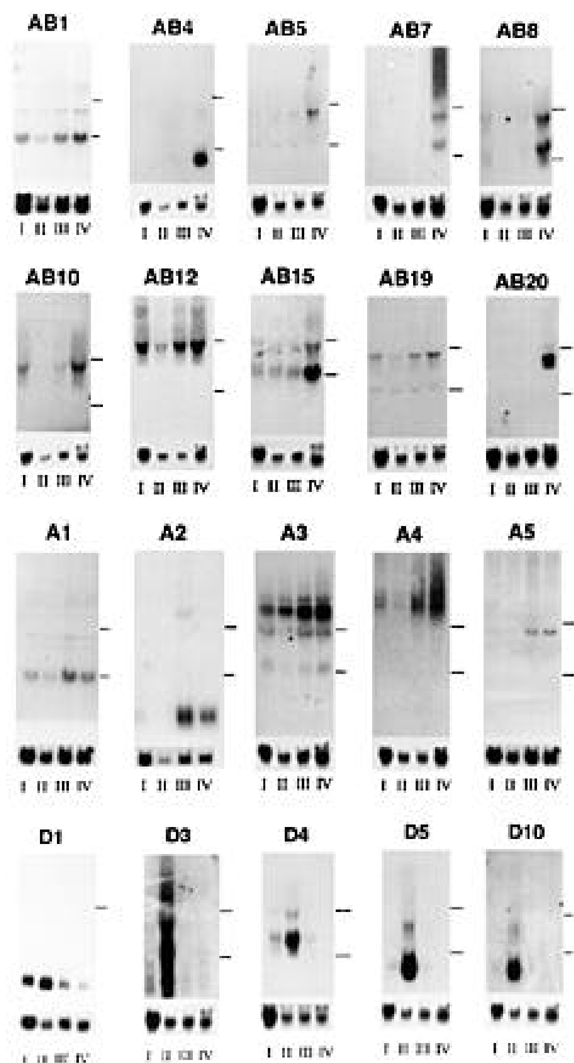

FIG. 1. Northern blot analysis of upregulated genes. Total RNA (20 µg) isolated from naive (*lane I*), Dx-treated (*lane II*), Act-treated (*lane III*), and Act + BTC–treated (*lane IV*) cells was blotted onto a nylon membrane and probed with ^{32}P-labeled cDNA from each gene identified by differential display. Results of Northern blot analyses of representative genes are shown. AB, A, and D were genes whose expression was increased in response to Act + BTC, Act, and Dx, respectively. These genes are listed in Table 1. The relative positions of 28S and 18S ribosomal RNA are indicated by the bars on the right lane of the gel. The blots were reprobed by ^{32}P-labeled G3PDH cDNA (lower panel).

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TABLE 1
Summary of AR42J cell difference products identified by mRNA differential display and screening cDNA expression array

Clone	Identification	Account no.	Length (bp)	%ID	Species	Effect of PDO98059
Upregulated genes in Act + BTC–treated AR42J cells						
Match with known genes						
AB1	β-Actin	J00691	359	99	Rat	
AB2/RBC035*	Thymosin β10	M17698	58	98	Rat	
AB3	PTHrP	M31603	297	97	Rat	
AB4	SPP-24 precursor	U19485	98	99	Rat	
RBC124*	Ca channel β subunit III	C07028	490	93	Rat	
RBC292*	Carboxypeptidase E	C06659	397	98	Rat	
Homologues of known genes and new members of gene families						
AB5	Tyrosine phosphatase	U42627	82	90	Rat	
AB6	Transcovalamine II	L02648	247 (958)	78	Human	
AB7	Mouse secreted protein	L33416	120	92	Mouse	
AB8	β-Tubulin	X60786	169	88	Hamster	
AB9	HNMP-1	U87948	167	91	Mouse	
AB10	Novel protein kinase PKN	D26180	218 (625)	62	Rat	
AB11	Integrin α6 subunit	X69902	284	95	Mouse	
AB12	Phospholipase A2	D78647	260	87	Mouse	
RBC522*	Amyloid precursor-like protein	C06825	348	90	Mouse	
RBC684*	Keratin D	C06952	438	91	Mouse	
Similar to other ESTs						
AB13		R68627	139	86	Human (placenta)	
AB14		D17889	430	83	Mouse (liver)	
AB15		AA161902		89	Mouse (embryo)	
RBC542*		C06842	522	97	Rat (heart)	
No match						
AB16			105			
AB17			143 (579)			
AB18			120 (1,149)			
AB19			105			
AB20			192			
Upregulated genes in Act-treated AR42J cells						
Match with known genes						
A1	γ-Actin	X52815	193	100	Rat	
A2	MIA/CD-RAP	U67884	385	98	Rat	
Homologues of known genes and new members of gene families						
A3	PAF acetylhydrolase	AF016049	228	86	Mouse	
Similar to other ESTs						
A4		W52883	335	92	Human (islet)	
No match						
A5			533			
A6			91			
A7			110			
Upregulated genes in Dx-treated AR42J cells						
Match with known genes						
D1	Acidic ribosomal phosphoprotein P1	X15097	363	98	Rat	
D2	Amylase	V01 225	114	97	Rat	
D3	Alanine aminotransferase	D10354	316	99	Rat	
D4	S-adenosyl L-homocystein hydrolase	U14937	193	100	Rat	
D5	Glycine methyltransferase	X06150	463	99	Rat	
Similar to other ESTs						
D6		D86666	131 (956)	97	Rat (ovary)	
No match						
D7			270 (808)			
D8			111			
D9			109			
D10			190			
D11			92			

%ID, the identity obtained by nucleotide database search. Species, the species to which sequence similarity was compared. For length, the numbers in parentheses indicate the length of elongated products generated by PCR cloning using a rat islet cDNA library. *Genes obtained by screening of the cDNA expression array.

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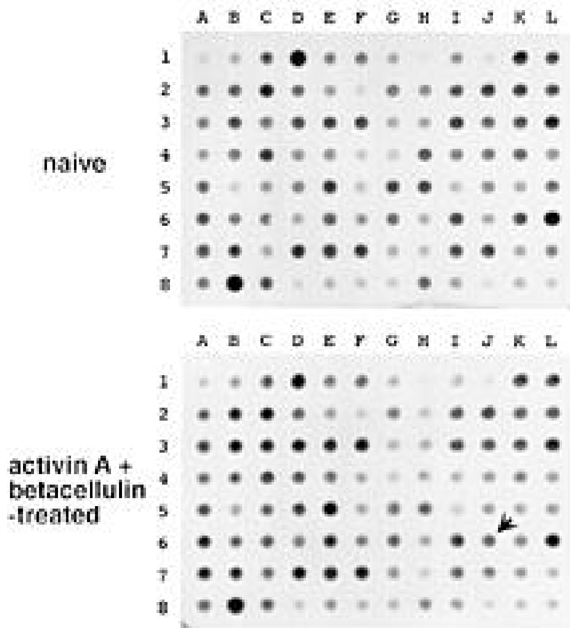

FIG. 2. Screening of pancreatic islet cDNA array. An example of the upregulated gene in Act + BTC–treated AR42J cells is indicated by the arrow. Hybridization was performed under standard conditions in the presence of COT-1 DNA (GIBCO/BRL). The final probe concentration in hybridization solution was >1 × 10^6 cpm/ml. The blot was washed with 0.1% SDS/0.1× SSC at room temperature and then at 58°C for 1 h, and it was exposed to X-ray film for autoradiography. Varied lengths of exposure time were tried: 6 h, overnight, and 3 days.

sequence-specific primers. The newly obtained sequences were also compared with those in the nucleotide and peptide databases using the BLASTN and BLASTX programs, respectively. The database search indicated that 24 of the 43 differentially expressed genes in the treated cells represented known genes or homologues of genes identified in other

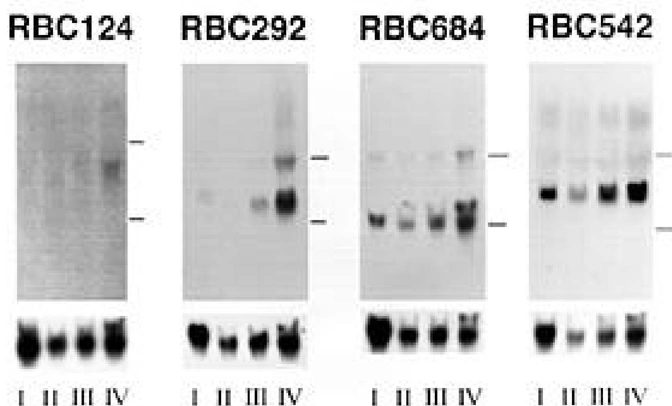

FIG. 3. Northern blot analysis of upregulated genes detected by screening of pancreatic islet cDNA array. Total RNA (20 µg) isolated from naive (*lane I*), Dx-treated (*lane II*), Act-treated (*lane III*), and Act + BTC–treated (*lane IV*) cells was blotted onto a nylon membrane and probed with ^{32}P-labeled cDNA from each gene obtained by screening of cDNA array. RBC124 to RBC542 were genes whose expression was increased in response to Act + BTC. For detail, see Table 1. The relative positions of 28S and 18S ribosomal RNA are indicated by the bars on the right lane of each gel. The blots were reprobed by ^{32}P-labeled G3PDH cDNA (lower panel).

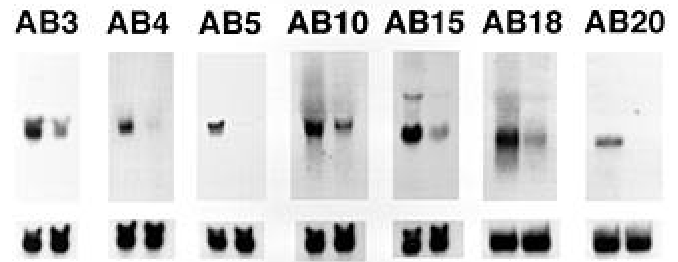

FIG. 4. Northern blot analysis of upregulated genes in Act + BTC–treated AR42J cells with or without PD098059. Total RNA (20 µg) isolated from Act + BTC–treated cells with (right) or without (left) 50 µmol/l PD098059 was blotted onto a nylon membrane and probed with ^{32}P-labeled cDNA from each gene. PD098059 was administered 30 min before the addition of Act + BTC. The blots were reprobed by ^{32}P-labeled G3PDH cDNA (lower panel).

species, and 11 were unknown. Upregulated genes in Dx-treated cells include five known genes and six unknown genes. The known genes, some of which are known to be related to exocrine function, include genes encoding acidic ribosomal phosphoprotein P1, pancreatic amylase, alanine aminotransferase, S-adenosyl L-homocystein hydrolase, and glycine methyltransferase. Upregulated genes in Act-treated cells include two known genes for γ-actin and MIA/CD-RAP, one homologue of the gene for platelet-activating factor acetylhydrolase, and four unknown genes. Upregulated genes in Act + BTC–treated cells include 6 known genes for β-actin, thymosin β10, PTHrP, SPP-24 precursor, Ca^{2+} channel β subunit III, and carboxypeptidase E; 8 homologues of the genes for tyrosine phosphatase (19), mouse secreted protein, β-tubulin, HNMP-1, integrin α6 subunit, phospholipase A_2, amyloid precursor-like protein, and keratin D; and 11 unknown genes. Of the eight homologues, the protein encoded by AB5 showed 90% nucleotide identity with the rat gene for tyrosine phosphatase, suggesting that this protein encodes a similar but distinct tyrosine phosphatase. The BLASTX search using the 5′-upstream sequences of the seven unknown products of AB6, AB10, AB15, AB17, AB18, D6, and D7 revealed that AB6 and AB10 encode a homologue of human transcovalamine II and a putative novel member of the protein kinase family, respectively.

The β-cell–specific genes—such as genes for insulin, glucokinase, and GLUT2, the expression of which was shown to be induced by the combination of Act + BTC in the previous study (10)—are not found in this study. This may be due to the low levels of expression of genes, which could be detected by the sensitive method of reverse transcription–PCR in the presence of the tracer of ^{32}P as described previously (10). The restrictions of the primers used may also be involved.

Characterization of candidate genes associated with differentiation into endocrine cells. When AR42J cells were treated with BTC, the mitogen-activated protein (MAP) kinase activity stayed increased for more than 2 h. On the contrary, PD098059, an inhibitor of the MAP kinase cascade (20), blocked differentiation of AR42J cells (20a). At 50 µmol/l, PD098059 completely blocked the differentiation, but at this concentration, PD098059 slightly decreased the number of living cells. We investigated to find if the signal transduction pathway via the MAP kinase cascade is responsible for con-

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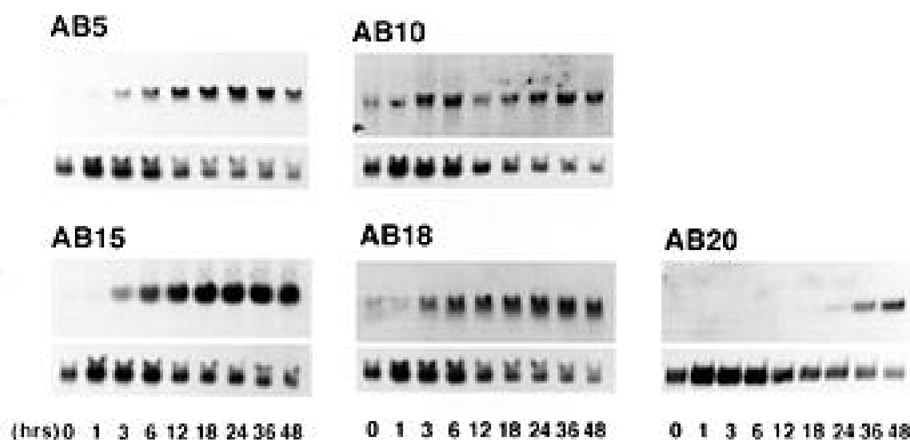

FIG. 5. Time course of the expression of AB5, AB10, AB15, AB18, and AB20. Total RNA (20 µg) was isolated from Act + BTC–treated cells at 0, 1, 3, 6, 12, 18, 24, 36, and 48 h, blotted onto a nylon membrane, and probed with ^{32}P-labeled cDNA from each gene. The blots were reprobed by ^{32}P-labeled G3PDH cDNA (lower panel).

version of AR42J cells into insulin-producing cells. The upregulation of 18 genes of the 25 upregulated genes identified in the Act + BTC–treated cells was significantly diminished by the addition of PD098059 (Fig. 4), which suggests that these genes are involved in the differentiation of AR42J cells into insulin-producing cells. The time course of expression of five genes—AB5 (tyrosine phosphatase-like protein), AB10 (protein kinase-like protein), AB15, AB18, and AB20, which showed a remarkable decrease of expression in response to PD098059—was monitored by Northern blotting (Fig. 5). The expression levels of AB5, AB10, AB15, and AB18 were rapidly increased in response to Act + BTC within 1 h, suggesting that the proteins encoded by these genes exert their function at an early phase in the differentiation of AR42J cells. On the other hand, AB20 was upregulated in the late phase after 24 h. Because proteins encoded by these genes are considered to be important for cell differentiation, the isolation of full-length cDNAs for these proteins is now underway.

The gene that encodes PTHrP (AB3) is included in the group of genes upregulated in Act + BTC–treated cells. Pancreatic islet cells have been shown to express both PTHrP (21) and its receptor (22). Because a study of transgenic mice showed that overexpression of PTHrP in pancreatic β-cells increases islet hyperplasia and hyperinsulinemia (23), we investigated the possible role of PTHrP in differentiation of AR42J cells. The cells were stably transfected with the expression plasmid containing a PTHrP cDNA and metallothionein promoter (AR42J/PTHrP cells). The addition of Zn^{2+} (100 µmol/l) in the culture medium to induce PTHrP stopped the cell growth and extension of neurite-like processes (data not shown) and decreased the amylase content in the cells (Fig. 6). Note that Zn^{2+} did not affect cell growth, morphology, protein content, or amylase content in mock-transfected cells (data not shown). Addition of tolbutamide to Zn^{2+}-treated AR42J/PTHrP cells resulted in the elevation of the cytoplasmic free calcium concentration (Fig. 7), which suggests that PTHrP induced the expression of ATP-sensitive potassium channels, the β-cell–specific potassium-conducting channels. However, because insulin gene transcripts were not observed in these cells by reverse transcription–PCR (data not shown), PTHrP could play a modulatory role in β-cell differentiation.

Carboxypeptidase E is a neuropeptide-processing enzyme that cleaves the precursor of insulin (24). In AtT-20 cells, a

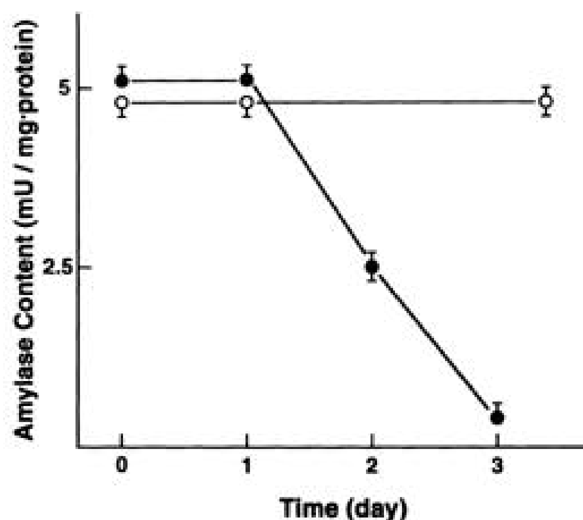

FIG. 6. Time course of amylase content in AR42J/PTHrP cells. AR42J/PTHrP cells were incubated with (●) or without (○) 100 µmol/l Zn^{2+} for the indicated time, and the amylase content was measured. Data are means ± SE for four experiments.

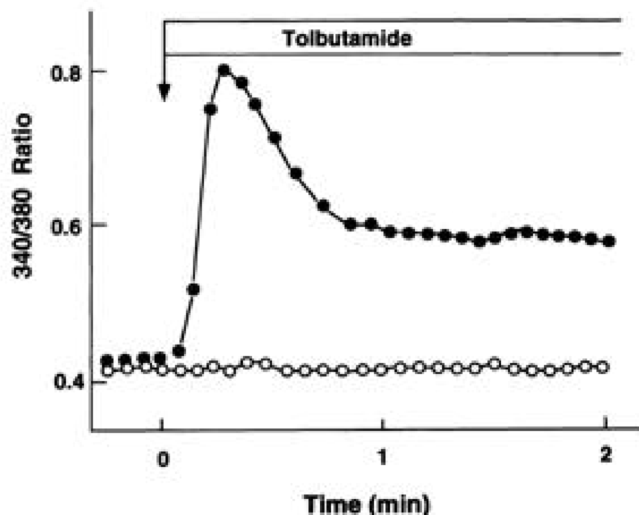

FIG. 7. Effect of tolbutamide on cytoplasmic free calcium concentration in Zn^{2+}-treated AR42J/PTHrP Cells. AR42J/PTHrP cells were treated with (●) or without (○) 100 µmol/l Zn^{2+} for 48 h. Changes in cytoplasmic free calcium concentration in response to 10 µmol/l tolbutamide were monitored using fura-2.

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mouse corticotropic cell line, carboxypeptidase E mRNA was found to be upregulated by corticotropin-releasing hormone and downregulated by dexamethasone (24). RBC124 encodes a neuroendocrine type of the voltage-dependent calcium channel subunit that plays an important role in Ca^{2+}-regulated insulin secretion by conducting an inward current of Ca^{2+}. AB9 encodes a homologue of HNMP-1, a member of the tetratrans-membrane-spanning protein encoded by PMP22, and is axon-associated (25). The amyloid precursor-like protein encoded by RBC522 has 42% amino acid identity and 64% similarity to the amyloid β protein precursor (26). Considered together, these increases in the mRNAs after the treatment suggest the differentiation of AR42J cells into neuroendocrine cells.

The AR42J cells show morphological changes during the course of differentiation in response to treatment with Act + BTC. The upregulated genes, AB1 (β-actin), AB2/RBC035 (thymosin β10), AB8 (homologue of β-tubulin), AB11 (homologue of integrin α6 subunit), and RBC648 (homologue of keratin D), are thought to be related to such changes of the cells. Because the expression levels of all except AB1 were decreased by treatment with PD098059, these cytoskeleton-related proteins appear to be important for the morphological changes of the cells that may be required in the conversion of cell type.

By the combination of mRNA differential display and screening of the cDNA expression array, a large subset of differentiation-related genes in AR42J cells are identified in this study. In the present study, we obtained RNA at 12, 24, and 48 h after treatment with differentiation factors. Preparation of RNA at earlier time points would provide a list of early genes induced by these factors. Although further characterization is required to understand their functional properties, molecular cataloging of the differentially expressed genes of AR42J cells should facilitate studies of molecular diversity, both before and after differentiation of the pancreatic cells, and provide clues to the roles of these genes in the development of the pancreatic β-cells and other islet cells.

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