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Identification of multiple genes in bovine retinal pericytes altered by exposure to elevated levels of glucose by using mRNA differential display

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ABSTRACT **Loss of capillary pericytes, a characteristic finding in diabetic retinopathy, is strongly associated with hyperglycemia. The pathologic aberrations associated with diabetic retinopathy are localized primarily in the retinal capillaries and are only poorly reversed by subsequent euglycemic control. Since hyperglycemia significantly inhibits pericyte growth in culture, we investigated the regulation of gene expression in retinal pericytes exposed to physiologic (5.5 mM) and pathologic (20 mM) glucose concentrations. By utilizing modifications of the mRNA differential display technique, over 14,000 mRNA species were screened, and 35 candidate clones were obtained. Partial DNA sequence demonstrated that 25 of these were distinct genes, including 7 known, 16 previously unreported, and 2 sequences with known homologues. Northern blot analysis demonstrated altered gene expression in 10 (40%), undetectable signals in 12 (48%), and nonregulation in 3 (12%). Genes with glucose-regulated expression included those encoding fibronectin (51% ± 15%, $P = 0.003$; mean percentage of control ± SD), caldesmon (68% ± 18%; $P = 0.026$), two ribosomal proteins (201% ± 72%, $P = 0.011$; 136% ± 16%, $P = 0.036$), Rieske FeS reductase (66% ± 17%; $P = 0.029$), three previously unreported sequences (57%, 167%, 271%), and molecules homologous to autoantigens (213%) and tyrosine kinases (down 16- to 33-fold). Caldesmon protein concentrations in pericytes and smooth muscle cells demonstrated decreases by Western blot analysis concordant with mRNA levels. These studies identify genes whose expression is significantly altered after 7 days of exposure to elevated glucose levels and provide new targets for understanding the adverse effects of hyperglycemia on vascular cells. In addition, this study provides strong support for the use of differential mRNA display as a method to rapidly isolate differentially expressed genes in metabolic systems.**

Clinically evident retinopathy eventually develops in the majority of patients with diabetes. One of the earliest and most specific histopathologic findings associated with hyperglycemia is the selective loss of retinal capillary pericytes (1). Indeed, pericyte loss occurs prior to clinically discernible retinopathy. Although the mechanisms underlying the development of diabetic retinopathy and pericyte loss are unknown, hyperglycemia has been suggested as one of the most important causal factors (2). Once established, retinal vascular abnormalities are poorly reversed by euglycemic control (3). Since retinal pericytes replicate slowly *in vivo* and their replication rates are further reduced by elevated glucose levels in culture (4), impaired reversibility might result from biochemical changes, possibly due to alterations in gene expression.

The identification of diabetes-induced alterations in gene expression has been undertaken primarily by the "candidate gene" approach (5–7). These methods only permit screening of known molecules on an individual basis. Standard techniques for concurrently isolating multiple differentially expressed genes, such as subtraction (8) and differential hybridization (9), require large quantities of mRNA and are both time consuming and labor intensive. Differential mRNA display (10, 11) promises to greatly reduce the time and materials needed for such procedures. However, this technique has only been reported for breast carcinoma-specific gene expression and has not yet demonstrated its applicability to other systems or its ability to distinguish genes whose expression is quantitatively modulated rather than absolutely induced or inhibited. In this study, we have successfully used modifications of differential mRNA display to rapidly isolate 10 glucose-regulated genes†† from retinal pericytes whose expression is modulated from 2- to 30-fold.

MATERIALS AND METHODS

Materials. Fetal bovine serum, plasma-derived horse serum, and calf serum were obtained from HyClone, Lampire Biological (Pipesville, PA), and GIBCO/BRL, respectively. Enzymes were purchased from GIBCO/BRL, [^{32}P]dATP and [^{35}S]dATP were obtained from Amersham, and Superscript reverse transcriptase was purchased from Invitrogen. PCR reactions were performed in Costar 96-well polycarbonate plates in an American Analytical OmniGene thermocycler using AmpliTaq DNA polymerase and PCR buffer from Perkins–Elmer/Cetus. PCR primers were purchased from Operon Technologies (Alameda, CA).

Cell Culture. Bovine retinal pericytes were obtained from fresh eyes as described (12) and cultured in Dulbecco's modified Eagle's medium (DMEM; 5.5 mM glucose) with 20% (vol/vol) fetal bovine serum. Prior to RNA isolation, the pericytes were grown to confluence and shifted into DMEM plus 1% (vol/vol) calf serum with glucose levels of either 5.5 mM, 22 mM, or 5.5 mM with 16.5 mM mannitol. Media were changed daily for 7 days, and the cells were stimulated with DMEM plus 10% fetal bovine serum for 8 hr (30 hr for Western blots). Pericyte growth is inhibited under similar conditions (13). Bovine aortic endothelial and smooth muscle cells were obtained from calf aorta as described (14) and

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††The sequence reported in this paper has been deposited in the GenBank data base (accession no. U09956).

cultured in DMEM with 10% (vol/vol) plasma-derived horse serum or 10% calf serum, respectively. All cells were grown on fibronectin-coated P-100 tissue culture dishes from Costar.

mRNA Differential Display. Differential display was performed as described (10, 11) with modifications. Total RNA was extracted using guanidium thiocyanate (15). DNase I was added to 25 μg of RNA in the presence of pancreatic RNase inhibitor in 1× PCR buffer and incubated at 37°C for 30 min. Four reverse transcription reactions were performed for each RNA sample using 0.2 μg of DNA-free total RNA in 1× reverse transcription buffer, 10 mM dithiothreitol, 20 μM each dGTP, dATP, dCTP, and dTTP, and 1 μM of either $T_{12}VG$, $T_{12}VA$, $T_{12}VT$, or $T_{12}VC$ oligonucleotide (where V is 3-fold degenerate for G, A, and C). The solution was heated to 65°C for 5 min and cooled to 37°C for 10 min, and then 200 units of reverse transcriptase was added. After incubation at 37°C for 1 hr, the mixture was heated to 95°C for 5 min prior to storage at 4°C. PCR was performed in 96-well polycarbonate plates. Reaction mixtures contained 0.1 volume of reverse transcription reaction, 1× PCR buffer, 4 μM each dGTP, dTTP, and dCTP, 2 μM dATP, 10 μCi (1 Ci = 37 GBq) of [^{35}S]dATP, 1 μM of the respective $T_{12}VN$ oligonucleotide (V = G, C, or A; N = G, A, T, or C), 0.2 μM specific arbitrary 10-mer, and 10 units of AmpliTaq DNA polymerase. Light mineral oil (Sigma) was overlaid, and the PCR reactions were performed: 1 cycle of 95°C for 1 min; 40 cycles of 94°C for 45 sec, 40°C for 90 sec, and 72°C for 30 sec (incremented 2 sec per cycle); and 1 cycle of 72°C for 15 min. DNA sequencing stop buffer (United States Biochemical) was added and heated to 80°C for 2 min prior to loading on 6% polyacrylamide sequencing gels. Gels were run at 65 W constant current, dried without fixation, and exposed directly to Kodak XAR-5 film overnight at room temperature. Any band clearly evident under one condition and absent in the other was subsequently confirmed by repeat reverse transcription and PCR using RNA isolated from new cultures.

Band Recovery. Bands demonstrating reproducible differences were excised under sterile conditions. The dried gel slice was placed in 10 mM Tris·HCl/1 mM EDTA and boiled for 30 min prior to rapid cooling to 4°C. Supernatant was removed and ethanol precipitated in the presence of glycogen. The pellet was resuspended in sterile water after a 95% (vol/vol) ethanol wash. Reamplification by PCR was performed using the appropriate primers and conditions noted above except for dNTP concentrations of 25 μM and no radioisotope. DNA was excised from 2% agarose gels, eluted using polyethylene spin columns (16), and used directly for probe generation or subcloning.

Northern Analysis. Radioactive probes were generated using Amersham Multiprime labeling kits and [^{32}P]dATP. Northern blot analysis was performed using ICN Biotrans nylon membranes (17) and ultraviolet crosslinking (Stratagene UV Stratalinker 2400). Analysis was first performed using probe generated directly from PCR reamplification. Those samples demonstrating differential expression were subcloned using the TA Cloning kit (Invitrogen) and Northern results were reconfirmed. Northern blots shown in the text are derived from these subclones. Analysis employed a Molecular Dynamics computing densitometer and PhosphorImager, and lane loading differences were normalized using 36B4 cDNA (10, 11, 18). Results are expressed as mean percentage of the control ± SD. Except where noted, experiments were performed using a minimum of three different pericyte isolations prepared from ≈20 pooled retinas each. Statistical analysis employed the paired *t* test.

Other Procedures. Western blot analysis was performed as described (19) using IgG purified polyclonal anti-caldesmon antibody (20) kindly provided by Michael P. Walsh (University of Calgary, Alberta, Canada) with equal loading of total protein (21, 22). DNA sequencing from the TA cloning vector used the United States Biochemical Sequenase kit version 2.0. Polyadenylylated RNA was obtained using oligo(dT) type III cellulose from Collaborative Biochemical Products (Bedford, MA) (23). Gene data base searches were performed at the National Center for Biotechnology Information using the BLAST network service.

RESULTS

A total of 24 arbitrary 10-mer primers were screened against all four $T_{12}VN$ primers. Each differential display lane yielded 150–250 discrete bands allowing evaluation of over 14,000 RNA species representing 70–90% (24) of the estimated 10,000–20,000 cellular mRNAs (25). Separate experiments utilizing the same primers produced >95% equivalent banding patterns while patterns were markedly different with differing primer pairs. In 35 instances, obvious differences between 5.5 mM and 22 mM glucose were reproducible upon two separate reverse transcription/PCR displays. All excised bands were successfully recovered by the boiling technique after a single 40-cycle PCR reamplification. Partial DNA sequence analysis revealed flanking sites complementary to the PCR primers in all cases except one where anomalous vector reinsertion occurred. All species were 111–404 bp in size, A+T rich, and contained putative polyadenylylation signals just 5′ to the poly(A) tail (25).

Comparison of the 35 candidate clones with gene data bases demonstrated 25 distinct genes including 7 known, 16 previously unreported, and 2 sequences homologous to known entities. Fragments corresponding closely to previously reported sequences included fibronectin, caldesmon, tuftelin, Rieske FeS reductase, and ribosomal 27S and 28S RNA. One clone was 67% homologous with human papillary thyroid carcinoma protooncogene tyrosine kinases (26), and another was 78% homologous to human 75-kDa autoantigen (27) at the nucleotide level.

These attributes are shown for two representative isolates with homologies to known molecules in Fig. 1. The cDNA sequences for fragments homologous to bovine fibronectin (Fig. 1*A*) and human aortic caldesmon (Fig. 1*B*) are shown. Each contains flanking primer sequences identical to those used in the differential display (boldfaced lettering) and putative polyadenylylation signals (underlined). Except for 10 bases adjacent to the 10-mer primer, clone 1 is 100% homologous to the terminal 3′ untranslated region of bovine fibronectin. The mispairings at the primer sites in both clones confirm the degenerate binding of these oligonucleotides previously noted (10, 11). Except for a 20-base region adjacent to the primers, clone 2 has 85% nucleotide homology and 76% exact (87% conservative) protein homology with human aortic caldesmon despite spanning an intron in the human sequence. However, at the extreme 3′ end the homology is lost and the poly(A)-directed primer is found at least 1.4 kb earlier in the bovine pericyte than predicted for the human caldesmon DNA sequence. Whether this results from PCR mispriming, species variation, tissue specificity, differential splicing, or another etiology awaits isolation and sequencing of the full-length bovine cDNA.

Northern blots were screened using ^{32}P-labeled DNA from both the PCR mixes and subcloned inserts with equivalent results. Using total RNA, differential expression was noted in 7 clones (28%), equal expression was observed in 2 (8%), and signals were not detectable with 16 clones (64%). With polyadenylylated RNA, 19% (3/16) of the previously undetectable clones now demonstrated glucose-regulated expression while 1 was expressed equally under both conditions. The remainder continued to be undetectable, presumable due to low mRNA levels. Thus, 40% (10/25) of unique and 29% (10/35) of all doubly confirmed candidate signals on differ-

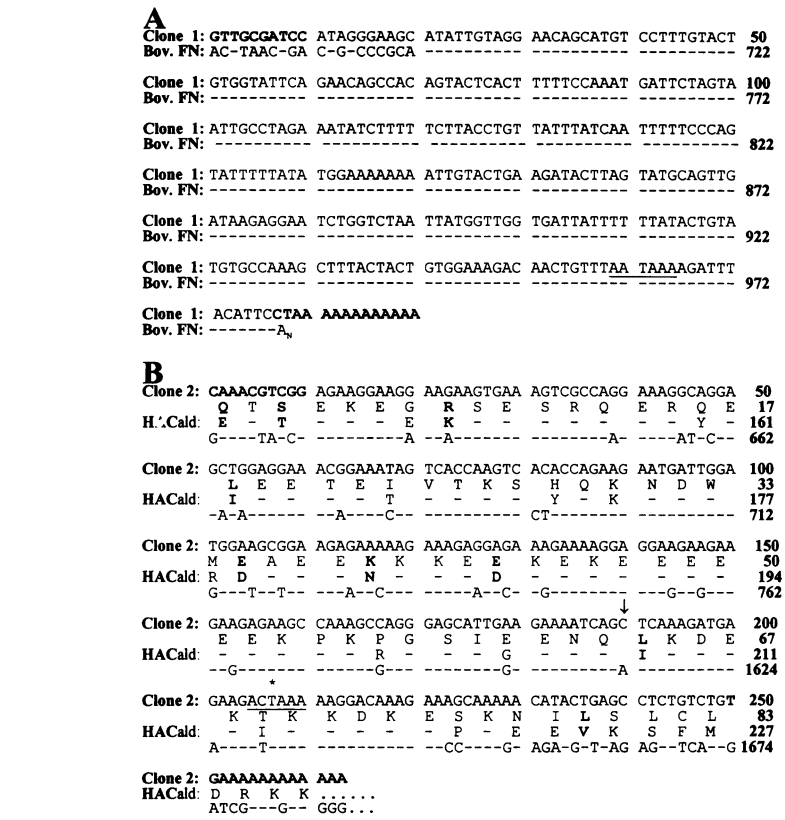

FIG. 1. Nucleotide sequence of two differentially expressed cDNA fragments. Homology of clones 1 and 2 with the 3′ untranslated region of bovine fibronectin (Bov. FN; *A*) (28) and human aortic caldesmon (HACald; *B*) (23) is shown. The deduced protein sequences of clone 2 and human aortic caldesmon are also given in *B*. Dashes represent identical residues, flanking primer sequences are in boldfaced letters, putative polyadenylylation signals are underlined, conservative amino acid changes are in boldfaced type, and numbering correlates with previous references (23, 28). Human aortic caldesmon has an intron at positions 851–1615 (vertical arrow), coding termination at position 2611, and five polyadenylylation sites between positions 3048 and 3610.

ential mRNA display resulted in clones exhibiting differential expression by Northern analysis. All changes observed by Northern blot were in the same direction predicted from the initial display, although the magnitude of this change was usually exaggerated by the differential display technique (Fig. 2). Despite duplicate confirmation, 52% of candidate clones yielded no detectable Northern signal while 12% exhibited equal expression.

The results for 6 of the 10 differentially expressed clones are shown in Fig. 2. RNA regulation and the relative exaggeration of these differences by differential display are evident. Three apparently differentially expressed signals in Fig. 2*A* (open arrows) were undetectable by Northern analysis. For the known genes, apparent mRNA size was consistent with that expected from prior reports (Table 1) (26, 27, 29–31).

The differentially expressed genes included those encoding fibronectin, caldesmon, Rieske FeS reductase, two ribosomal proteins, three previously unreported sequences, and molecules with sequence homology to human 75-kDa autoantigen and human papillary thyroid carcinoma protooncogene tyrosine kinases (Table 1). Similar alterations of gene expression were noted in aortic smooth muscle and endothelial cells. Tuftelin, another ribosomal 28S RNA and two unknown messages did not exhibit altered expression. Mannitol (16.5 mM), used as an osmotic control, did not effect expression of the genes studied.

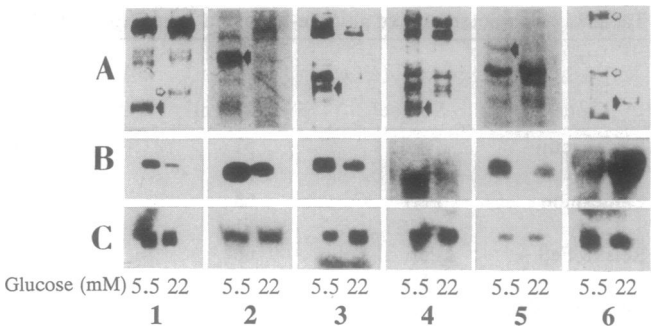

FIG. 2. Differential mRNA display (row A) and Northern blot analysis (rows B and C) of six differentially expressed cDNA fragments. Signals demonstrating altered expression by differential mRNA display are marked by arrows in row A. Row B shows Northern blot analysis using cDNA probes derived from reamplification of molecules marked by solid arrows in row A. Molecules isolated from open arrows were undetectable by Northern analysis. The same blots reprobed with 36B4 cDNA (18) as a loading control are shown in row C. Samples represent 20 μg of total cellular RNA per lane (except column 4: 15 μg of polyadenylylated RNA) from cells exposed to 5.5 mM or 22 mM glucose for 7 days as indicated. DNA sequence analysis characterized the fragments as fibronectin, caldesmon, unknown, unknown with tyrosine kinase homology, Rieske FeS reductase, and ribosomal 28S protein (columns 1–6, respectively).

To document that mRNA changes reflect protein levels, we evaluated caldesmon protein by Western blot analysis in both retinal pericytes and aortic smooth muscle cells (Fig. 3). Protein binding to polyclonal IgG anti-caldesmon antibody in cells exposed to 22 mM glucose was reduced (5.4–19%) compared with normoglycemic conditions in concordance with mRNA levels detected in the same cells. Quantitatively, changes in the protein level were less than the corresponding mRNA changes.

DISCUSSION

This study demonstrates that clinically relevant glucose concentrations can cause significant alterations of cultured retinal pericyte gene expression. Previous reports have established that retinal pericytes are lost in early stages of diabetic retinopathy (1), are not restored by subsequent euglycemic control (3), replicate slowly *in vivo*, and are growth inhibited by elevated glucose concentrations in culture (4). Thus, hyperglycemia may induce biochemical alterations in retinal pericytes, decreasing their physiologic function and/or viability. Impairment of these microvascular-supporting cells (32–36) may be an initial step in the pathogenesis of diabetic retinopathy. Subsequent endothelial compromise could increase vascular permeability, microaneurysm formation, capillary drop-out, or retinal neovascularization. Pericytes also inhibit endothelial cell growth (32, 34). Thus, loss of the pericyte might permit the increased cellularity seen with microaneurysm formation or neovascularization. Our studies demonstrate that significant hyperglycemia-induced alterations in gene expression do occur.

Four of the isolated glucose-regulated genes were known, although the expression of only one had been reported to be altered by glucose (5, 37). Fibronectin mRNA levels increase in diabetic rat heart and kidney cortex and remain elevated for weeks after restoration of normoglycemia. Similarly, high glucose levels increase human umbilical vein endothelial cell fibronectin mRNA ≈80%, with persistent elevation despite subsequent normoglycemia (5, 37). Although increased glycoproteins and basement membrane thickening are consistent histopathologic findings in many diabetic organs (38), quantitative increases in fibronectin protein are often not

Table 1. Characteristics of glucose-regulated gene expression

Clone*	Source†	Mean, % of control‡	Median	Range, % of control‡	SD	*P* versus control	Probe size, bp	Approximate mRNA size, kb
Fibronectin	Peri	51	50	36–66	15	0	320	7.5
	SMC	72	68	64–83	10	0	320	7.5
Caldesmon	Peri	68	66	49–90	18	0.03	264	4.3
	SMC	51	55	26–74	23	0	264	4.3
Reiske FeS	Peri	66	58	55–86	17	0.03	404	1.3
	SMC	76	77	69–83	9	0.02	404	1.3
Ribosomal 28S	Peri	201	200	150–252	72	0.01	206	4.7
	AEndo	191	170	124–280	80	0.01	206	4.7
Ribosomal 27S	Peri	136	137	124–147	16	0.04	244	4.7
TKH§¶	Peri	3	3	0.6–6.0	—	—	165	4.5
HAH¶‖	Peri	213	213	173–252	—	—	111	1.6
Unknown 1¶	Peri	271	271	182–360	—	—	116	1.8
Unknown 2¶	Peri	167	167	164–170	—	—	278	5.0
Unknown 3¶	Peri	57	57	54–61	—	—	247	6.0

*Identified by cDNA sequence homology.
†Peri, retinal pericytes; SMC, aortic smooth muscle cells; AEndo, aortic endothelium.
‡Control (mean) 99% ± 17% SD; mannitol (% of control) 105% ± 12%; $P = 0.572$.
§Tyrosine kinase homologous molecule.
¶Data from only two experiments.
‖Human autoantigen homologous molecule.

observed. Fibronectin in diabetic human glomerular basement membranes (39) and calf glomerular epithelial or endothelial cells (40) do not increase despite basement membrane thickening. Fibronectin mRNA in skin of diabetic patients is reduced (41) as are plasma protein levels relative to patients without disease (42, 43). We find a 49% decrease of fibronectin mRNA in retinal pericytes exposed to 22 mM glucose in contrast to previous studies with human umbilical vein endothelial cells. This difference could represent a distinct manner in which pericytes react to glucose as compared with other vascular cells (13) or be due to differing culture conditions.

Caldesmon binds F-actin, Ca^{2+}/calmodulin (44), tropomyosin (45), and meromyosin (46) and inhibits the actomyosin ATPase in muscle tissues. Phosphorylation of caldesmon by

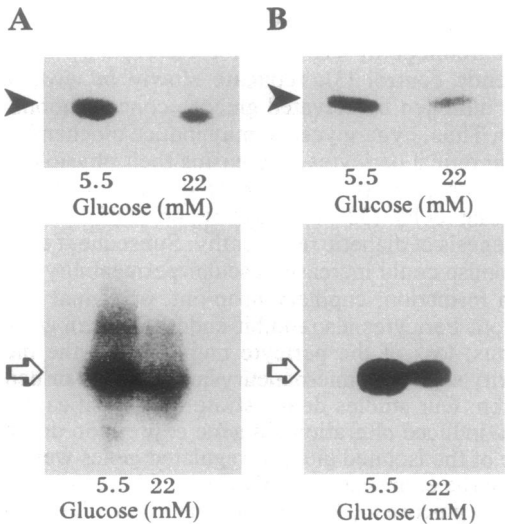

FIG. 3. Caldesmon protein and mRNA levels are reduced upon exposure to elevated glucose. (*Upper*) Western blot analysis (solid arrowheads) was performed on aortic smooth muscle cells (*A*) and retinal pericytes (*B*) after 7 days of exposure to 5.5 mM or 22 mM glucose using polyclonal IgG anti-caldesmon antibody. Lanes contain equal total protein levels. (*Lower*) Northern blot analysis (open arrows) of 20 μg of total RNA derived from the same cells probed with the isolated caldesmon cDNA is also shown.

cdc2 kinase (47) or protein kinase A or C (48) blocks caldesmon binding and restores actomyosin ATPase activity. We found that caldesmon mRNA and protein decreased upon exposure to elevated glucose in retinal pericytes. Since the half-life of caldesmon is several days, the magnitude of protein reduction might be even more pronounced after prolonged exposure to hyperglycemia as occurs in chronic disease.

Since retinal pericytes are contractile cells possibly involved in microvessel flow regulation (36, 49, 50), decreased caldesmon levels could reduce the effectiveness of actomyosin ATPase inhibition, permitting increased contraction and decreased vascular flow. Decreased retinal flow without changes in large vessel diameters has been demonstrated in rats after only 1 week of diabetes (51). Caldesmon also plays an essential role during cellular mitosis (52, 53) and receptor capping (54).

Although protein synthesis decreases with diabetes in most tissues (55–57), in retinal pericytes it increases (4) as do our clones encoding 27S and 28S ribosomal proteins. An autoimmune response against islet cells and insulin is important in the development of insulin-dependent diabetes mellitus (58, 59). We find that an mRNA species with 78% homology to human nuclear 75-kDa autoantigen is induced 2-fold upon glucose elevation. Whether this autoantigen is recognized by serum antibodies remains to be evaluated. An mRNA species with homology to tyrosine kinases and protooncogenes (26) is reduced 16-fold by a 1-week exposure to elevated glucose. Tyrosine kinases are intimately involved in mediating growth (60), and regulation of such a molecule may be important in pericyte physiology.

We isolated the gene encoding tuftelin from retinal pericytes. Tuftelin belongs to the enamelin family of molecules involved in mineralization and structural organization of extracellular enamel (61). The progenitor cells for osteoblasts and chondroblasts are uncertain, although a microvessel pericyte lineage has been suggested (28). Our finding of low tuftelin mRNA levels in the retinal pericyte offers strong support that pericytes are an osteogenic precursor.

Several modifications of the originally described mRNA differential display have increased its speed and ease of use. Doubling all dNTP concentrations except dATP yielded stronger signals without significant loss of polymerase fidelity. Autoradiograms were visualized after overnight expo-

sure rather than the 2–3 days required previously. Random priming using [^{32}P]dATP rather than [^{32}P]dCTP resulted in higher radiolabel incorporation due to the A+T rich nature of the 3′ terminal probes. As suggested by Liang *et al.* (24), use of $T_{12}VN$ primers decreased the number of primer combinations 3-fold. In addition, the use of 96-well plates dramatically increased screening speed. DNA isolated directly from agarose gels using spin columns reduced preparation time to 10 min. Use of boiling rather than electroelution to isolate DNA fragments from polyacrylamide gels was faster, more efficient, and did not require specialized equipment. Differential display signals not confirmed by Northern analysis were relatively common due to undetectable signals (48%) or lack of regulation (12%). Confirming the differential display twice improved positive yields 2-fold. Northern probes generated directly from the PCR mixture permitted rapid identification of isolates of interest without first subcloning the fragment. Northern analysis repeated after subcloning ensured correspondence with sequenced DNA.

The magnitude of differential gene expression was often exaggerated by differential display. This may reflect the extreme sensitivity of PCR. Most changes observed in our system were not the "all-or-none" response observed by Liang and coworkers in breast carcinomas (10, 11) or recently observed by us with insulinomas (L.P.A. and G.L.K., unpublished results). It appears that carcinomatous systems may be more readily evaluated by differential display, resulting in isolation of genes undergoing more extensive regulation than those observed when altering the cellular milieu.

In summary, these findings demonstrate that mRNA differential display can rapidly isolate genes whose expression is altered by metabolic changes in cultured cells. We have shown that a diverse array of genes are regulated in cultured retinal pericytes in response to elevated glucose levels. The identification of known genes has provided additional target molecules to probe the adverse effects of hyperglycemia on vascular cells. The regulation of these genes suggests that several processes may be impaired by hyperglycemia including contractility, protein synthesis, basement membrane composition, and redox potentials. Genes encoding new proteins, which may correlate with pericyte growth, have also been identified. If confirmed *in vivo*, these findings may enhance our understanding of the vascular cell response to hyperglycemia and the pathogenesis of diabetic ocular complications.

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