Egr-1 Activates Basic Fibroblast Growth Factor Transcription
MECHANISTIC IMPLICATIONS FOR ASTROCYTE PROLIFERATION*

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The mechanisms controlling the proliferation of astrocytes are of great interest but are not well defined. We have previously shown that the endogenous neuropeptides, endothelin-3 (ET-3), and atrial natriuretic peptide (ANP), modulate the proliferation of astrocytes through positively and negatively regulating the transcription of the immediate-early gene egr-1 which transactivates basic fibroblast growth factor (bFGF) by unknown mechanisms. In these studies, we determined the involvement of MAP kinase (Erk) activation by ET-3 in the transcription of egr-1, and the molecular determinants by which Egr-1 transactivates bFGF. Transfection of astrocytes with a mitogen-activated protein (MAP) kinase (MAPK) expression vector increased the transcription of a cotransfected egr-chloramphenicol acetyltransferase (CAT) construct 3-fold. This induction was totally abolished by a dominant negative MAPK mutant. A 3-fold induction of egr-CAT expression by ET-3 was significantly reduced by treatment with ANP, or a cotransfected dominant negative MAPK plasmid. Using mobility shift assays, we showed that ET-3 induced the expression of Egr-1 protein which bound specifically to several early growth-related protein (Egr-1) binding sites on the bFGF promoter, and that this effect was significantly reversed by treatment with ANP. We also found that the Sp1 transcriptional factor was bound at these same sites, but was not stimulated by ET-3. Deletion experiments indicated that only the site at −160 bp of the bFGF promoter was significant for bFGF transactivation by Egr-1. We conclude that the astrocyte mitogen, ET-3, stimulates egr-1 transcription through a MAP kinase (Erk) related mechanism, and that Egr-1 transactivates bFGF through a specific noncanonical, Egr-1 site on the promoter. ANP inhibits each of these steps, providing a pathway for its anti-proliferative action.

Primary response or immediate-early genes are induced by mitogenic stimuli in the absence of de novo protein synthesis and often constitute the first step in the sequential expression of growth regulatory proteins. One such primary response gene, egr-1 (1), also known as NGF1-A (2), Krox24 (3), and TIS 8 (4), encodes a protein with three zinc finger motifs, suggesting that Egr-1 may mediate the growth response by regulating target gene expression. The evolutionary conservation of the egr-1 gene (1) as well as the broad spectrum of induction by phorbol ester and growth factors (1-5), neuronal stimuli (1, 2), ischemic injury (6), and by some differentiation signals (1) implicate Egr-1 as an important nuclear intermediary in signal transduction.

Astrocytes are known to play an important role in guiding the construction of the nervous system and controlling the chemical and ionic environment of nerve cells (7, 8). In this regard, astrocyte proliferation induced by exogenous or endogenous growth factors is important. One potent mitogen for astrocytes is bFGF, which is produced in a variety of neural cells including astrocytes (9, 10), where it may serve as an autologous growth factor. Recently, we have shown that Egr-1 is one of the key proteins in the regulation of astrocyte proliferation (11). It was determined that the endogenous mitogenic neuropeptide, ET-3, and the antigrowth ANP, can modulate the proliferation of astrocytes through positively and negatively regulating the production of Egr-1. We also showed that ET-3 and ANP modulate the transcription of bFGF by regulating the transcription of egr-1. Both these proteins were shown to be crucial for astrocyte proliferation.

To further understand the series of events underlying the regulation of astrocyte growth, we designed experiments to test how Egr-1 contributes to transcriptional activation/inhibition of the bFGF promoter, as modulated by ET-3 and ANP. We were interested in determining whether Egr-1 regulates activation of the bFGF promoter through direct interaction with DNA and what are the proximal signals which lead to the positive or negative modulation of egr-1 expression by these neuropeptides. These issues are potentially important beyond our model because Egr-1 is the first immediate early protein/transcriptional factor identified to activate bFGF transcription (11).

MATERIALS AND METHODS

Synthetic Oligonucleotides—Double-stranded oligonucleotides spanning the bFGF promoter between the nucleotides +34 to +44 (oligo A, 5’-CAGTAGGGGGCCGCGG-3’), −74 to −51 (oligo A1, 5’-GCGTTCGGCCTGCCCGCCGAGTCTAG-3’), and −177 to −151 (oligo A2, 5’-GGGCTGGGCCCCTTTGACCTTTT-3’) were obtained from Bio-Synthesis, Inc., Lewisville, TX (see Fig. 1). A double-stranded oligonucleotide (oligo egr) containing two Egr-1 consensus sequences (CCCGCCCGCGAGTCTAG) was synthesized by Santa Cruz Biotechnology, Inc.

1 The abbreviations used are: Egr, early growth related protein; ANP, atrial natriuretic peptide; bFGF, basic fibroblast growth factor; EMSA, electrophoretic mobility shift assays; ET, endothelin; MAP-K, mitogen-activated protein kinase; MEK, MAP-kinase kinase; SRE, serum responsive element; ZIPB, zinc finger protein binding; CMV, cytomegalovirus; bp, base pair(s).
Plasmids—The pBS.Apal plasmid contains the murine egr-1 DNA in pBluescript and was used for the in vitro transcription/translation of Egr-1 protein. This plasmid construct and the Egr-1 expression vector pCMV5-Egr-1 (12), and egr-CAT reporter plasmid containing the 1.2-kilobase pair segment of the 5'-upstream promoter region of the egr-1 gene (13) were kindly provided by Dr. V. P. Sukhatme. The plasmid containing the full-length human bFGF promoter-CAT construct (pF2.0CAT) as well as deletion mutants of bFGF promoter-CAT constructs (pFdcAT16/8, pFdcAT22/2, and pFdcAT7N) (14) were kindly provided by Dr. R. Florkiewicz. The MAPK expression vector and the dominant negative MAPK mutant (Y185F) were kindly provided by Dr. M. Cobb (15).

Cell Culture and Nuclear Extract Preparation—Primary astrocytes from 16-day gestational fetal rats were isolated, cultured, and characterized as previously reported (11). Astrocytes were incubated in the presence or absence of ET-3 and ANP for 2 h or 16–18 h. Nuclear protein extracts were prepared according to the method of Dignam et al. (16) with additional protease inhibitors, leupeptin and aprotinin (2 μg/ml; Sigma). Protein concentrations were measured using the Bio-Rad protein assay kit, and extracts were aliquoted and stored at –80°C.

EMSA and Supershift Assays—The oligonucleotides (oligo A, A1, and A2) containing the consensus sequence of the Egr-1 binding element was labeled with [γ-32P]ATP at the 5' end using T4 polynucleotide kinase. The [γ-32P]-labeled oligonucleotide probe was mixed with in vitro synthesized Egr-1 protein (Promega TNT reticulocyte lysate system) or nuclear extract protein in 20 ml of the binding solution (10 mM Tris/Cl, pH 7.5, 10% glycerol, 0.1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 2 μg poly(dA-dT), and incubated for 15 min at room temperature. For gel mobility shift assays with antibodies, the antibodies (2 μl; Santa Cruz Biotechnology Inc.) were added to the reaction mixture subsequent to addition of [γ-32P]-labeled oligonucleotide probe and incubated at 4°C for 1 h.

Cell Transient Transfection and Reporter Gene Assays—Transient transfection of astrocytes and COS-7 cells was performed using Lipofectamine (Life Technologies, Inc.) using the manufacturer's instructions. The Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum and 1% of antibiotic-antimycotic, was removed prior to transfection and cells were washed with plain medium without serum and antibiotics. Cultures were layered with liposome DNA complexes, incubated for 5 h, and after that the complex was removed and replaced with 10% fetal bovine serum medium, and the cultures were incubated for an additional 24 h. In some experiments the cells were maintained in 0.4% serum containing medium for 24 h and then exposed to either ET-3 (100 nM), ANP (100 nM), or ET-3 + ANP for 16 h. The egr-CAT reporter was included in each transfection. In some experiments double or triple transfections were performed. These experiments used a MAPK containing plasmid or a dominant negative MAPK (Dom Neg MAPK) plasmid. Transfection with this plasmid has been shown to lead to the overproduction of kinase-deficient protein (15). The MAPK, and Dom Neg MAPK plasmids are driven by the same CMV promoter; therefore as a control, pCMV5 vector was used. Comparable total plasmid DNA was present in all experiments. Transfected cultures were washed twice with isotonic buffer 24-48 h post-transfection and collected. Cell extracts were prepared by three cycles of freeze-thaw (dry icethanol) for 5 min each. Supernatants were collected after microcentrifuging for 5 min at 4°C and assayed for β-galactosidase or CAT activity. The conversion of [14C]chloramphenicol (DuPont NEN) into acetylated chloramphenicol was monitored as described previously (17).

To monitor transfection efficiency, a β-galactosidase expression plasmid pON249 (18) was simultaneously transfected into the cells, and β-galactosidase was detected using a chemiluminescent reporter assay system (Tropix, Inc.). The CAT values were divided by the β-galactosidase values to normalize for differences in the transfection efficiencies. All transfection data is presented as the mean ± S.D. from three culture dishes per experimental condition. The data shown in some figures is from a single representative experiment; similar results were obtained in at least three experiments for each preparation of cells and plasmids.

RESULTS

Modulation of Egr-1 Expression by MAPK and Neuroepithelides—To evaluate the need for MAPK in the signaling pathway activating egr-1 expression in astrocytes, we performed a series of transfection experiments which utilized a MAPK expression vector (MAPK) and a dominant negative plasmid which abolished MAPK activity (Dom Neg MAPK). We found that expression of wild type MAPK activated egr-1 expression 3-fold (Fig. 1A). This induction was totally abrogated when the cells were cotransfected with a dominant negative MAPK mutant. Moreover the latter plasmid caused a 40% reduction of the basic activity of egr-CAT.

We next addressed the role of MAPK-induced egr-1 expression in mediating ET-3 stimulation. We compared the level of egr-CAT expression in cells stimulated with ET-3 alone, and with cells treated with ET-3 + ANP or cells cotransfected with Dom Neg MAPK plasmid prior to ET-3 exposure. As shown in Fig. 1B, the 3-fold induction of egr-CAT expression by ET-3 was significantly reduced by treatment with ANP (50%) or when the Dom Neg MAPK plasmid was cotransfected (60%). Thus, we found that ET-3 requires MAPK activation to stimulate egr-1 expression in astrocytes.

Egr-1 Binding to the bFGF Promoter—Several genes that are regulated by Egr-1 protein share the similar consensus promoter sequences 5'-CGCCCGGCCGCGCCCGC-3' (19, 20). Examination of
formation (in the binding reaction mixture inhibited retardation complex because molarexcess of unlabeled homologous oligonucleotides

The proximal promoter region of the bFGF gene indicates that there are three imperfect but potential Egr-1 binding elements located at –160 bp (site A2) and –60 bp (site A1) upstream, and one at +40 bp (site A) downstream of the transcriptional start site, thus raising the possibility that bFGF gene expression may be modulated through the interaction of Egr-1 protein with the bFGF promoter (Fig. 2). Comparison of sequences of sites A2, A1, and A with the established consensus sequence for Egr-1 binding revealed that each site deviated by one or two nucleotides (underlined) from the Egr-1 consensus sequence: site A, CTCGCCCTGGC; site A2, GCCTGGGGTG; and site A, TAGGGGGCG. Additionally, it is necessary to stress that all three sites contain two extensively overlapping transcription factor motifs, one for Egr-1 and another for the zinc finger protein, Sp1 (GGCGGG) (Fig. 2). This common site we termed the ZiPB (zinc finger protein binding) motif. Previously, overexpression of Egr-1 using a GAL4-Egr-1 transgene increased binding of the ET-3-treated nuclear extract to the promoter, and that this expression is partially reversed by ANP treatment with ANP. Previously, we showed that ET-3 and ANP (100 nM) positively and negatively, respectively, modulate Egr-1 expression (11). Therefore, it is likely that the increased binding of the ET-3-treated nuclear extract to the bFGF promoter shown here reflects the stimulation of Egr-1 protein production, inhibited by ANP.

In order to determine if Egr-1 binds to sites A1 or A2, EMSAs were employed. As shown in Fig. 3, in vitro translated Egr-1 protein bound efficiently to oligonucleotide probes containing either site A1 or A2 and one DNA-protein binding complex was formed (Fig. 3, lane 1 and 7). This binding was saturable because molar excess of unlabeled homologous oligonucleotides in the binding reaction mixture inhibited retardation complex formation (lanes 2–4 and lanes 8–11). Moreover, the protein-DNA complex could be supershifted by anti-Egr-1 antibody (lanes 6 and 12). Similar results were obtained when oligonucleotide A was used as a probe (data not shown). These data strongly suggest that Egr-1 protein binds to sites within the human bFGF promoter.

ET-3 Stimulates and ANP Inhibits Egr-1 Binding to bFGF Promoter in Astrocytes—As we showed previously, ET-3 significantly stimulates both Egr-1 and bFGF mRNA and protein production (11). To elucidate the role of Egr-1, we examined the mechanism by which ET-3 and ANP modulate bFGF gene expression. We determined whether the bFGF ZIPBs can interact with astrocytic nuclear factors, and whether this interaction could be influenced by ET-3 and/or ANP action. We performed gel mobility shift assays using nuclear protein extracts from astrocytes not treated and treated with ET-3 and ET-3 + ANP (100 nM). Both probe A1 (Fig. 4A) and A2 (Fig. 4B) formed two DNA-protein binding complexes with nuclear extract prepared from nonstimulated astrocytes (lane 1), and three bands when cells were treated with ET-3 (lane 4) and ET-3 + ANP (lane 7). However, the intensity of the lower, ET-3-induced band was decreased when the probe was incubated with the nuclear extract prepared from the cells treated with ET-3 + ANP (compare lower bands lane 4 and 7). Anti-serum directed against Egr-1 specifically altered migration of only the lower complex formed by DNA and nuclear extract, prepared from astrocytes treated with ET-3 and ET-3 + ANP (Fig. 4, A and B, lanes 5 and 8). This indicates that Egr-1 gives rise to these inducible protein complexes. One of the slower DNA-protein complexes was supershifted with antibody directed against Sp1 protein (Fig. 4, lanes 3, 6, and 9). Sp1 protein binding to the ZiPB sites was constitutive, equal levels being detected in unstimulated and stimulated cells (Fig. 4, A and B, lanes 1, 4, and 7). The same experiments were conducted using oligonucleotide A as a probe and the same results were obtained (data not shown). These results indicate that ET-3 can stimulate the expression of nuclear protein which binds preferentially to the Egr-1 binding sites A2, A1, and A on the bFGF promoter, and that this expression is partially reversed by treatment with ANP. Previously, we showed that ET-3 and ANP positively and negatively, respectively, modulate Egr-1 protein production (11). Therefore, it is likely that the increased binding of the ET-3-treated nuclear extract to the bFGF promoter shown here reflects the stimulation of Egr-1 protein production, inhibited by ANP.

The DNA binding specificity of all complexes was further tested in competition experiments by using commercial oligonucleotides representing consensus sequences for Egr-1 (egr), Sp1, and mutated Egr-1 (megr) (see “Materials and Methods”). The EMSA was performed using nuclear extract prepared from astrocytes stimulated by ET-3 and probe A2 (Fig. 5) (we have...
obtained similar results using an oligonucleotide A1 or A as probes; data not shown). We found that the DNA binding of protein was saturable because the excess of unlabeled homologous oligonucleotides in the binding reaction mixture blocked the formation of all DNA-protein complexes (Fig. 5, lane 2). Competition of the lower complex, induced by ET-3 and identified with Egr-1 specific oligonucleotides (lane 3), are in agreement with the supershift observed with Egr-1 specific antibody (lane 6). Excess oligonucleotide containing a mutated Egr-1 consensus site did not abolish formation of this band (lane 4), showing specificity. These results confirm the observation that Egr-1 is a part of the lower DNA-protein complex formed on the bFGF promoter.

Addition of Sp1-binding oligonucleotides to the binding mixture effected formation of the two upper complexes (Fig. 5, lane 5), and as it has been shown already (Fig. 4, lanes 3, 6, and 9) antibody against Sp1 supershifted one of the upper complexes. It seems that the second upper band could represent binding by a second form of Sp1 protein, or a different member of the Sp family, which is able to bind to the same binding site but is not recognized by the Sp1 antibody used in these experiments.

Taken together, these results suggest that the ZiPB region of human bFGF promoter can bind at least two zinc finger proteins, Egr-1 and Sp1. Moreover, we found that ET-3 and ANP positively and negatively modulate the binding of Egr-1 protein to its target sequence on the bFGF promoter, but not that of Sp1.

Egr-1 Expression Stimulates bFGF Promoter Activity—To determine whether and how Egr-1 is capable of regulating bFGF reporter activity, in vivo cotransfection experiments were conducted with several CAT reporters containing 5'-flanking basic FGF promoter-CAT and its 5'-deleted constructs used for transfections. The AP1 and ZiPB regions are indicated. The ZiPB site is described in this report. B, relative CAT activity in astrocytes and COS-7 cells. Cells were cotransfected with the indicated plasmids in the presence or absence of an Egr-1 expression vector (pCMV5-Egr-1). All assays were normalized by β-galactosidase activity. Each bar represents the average of triplicate transfections per experiment, repeated three times.
GC boxes, and three Egr-1 binding sites, was activated by expression of Egr-1, nearly 6-fold in COS-7, and 7-fold in glial cells. The basic activity of an pFdCAT16/8 deletion construct (−480 bp to −179 bp) containing the regulatory elements described above, but lacking sequences from −1800 bp to −480 bp was much more responsive to Egr-1 than p2.0CAT, 12 and 14 times in COS-7 and glial cells, respectively. These results are consistent with a suggestion that the residues between −854 bp and −521 bp display negative regulatory function (14). The reporter pFdCAT22/2 (−160 bp to +179 bp) which does not contain A2 binding sites (see Fig. 6A) showed a significant decrease of responsiveness to Egr-1, 3.5-fold in COS-7 cells and 6-fold in astrocytes. However, pFdCAT6N, lacking A1 sites, was stimulated by Egr-1 to a similar magnitude, 5.6 times in COS-7 cells and 8 times in glia, while the promoterless pBLCAT4 plasmid was completely unresponsive. We suggest that deletion of −160 bp to −21 bp removed both the negative regulatory elements upstream of the A2 site along with the positive regulating A2 site, yielding a net effect that was neutral. Collectively, these data indicate that ZIPB of the human bFGF promoter, containing the A2 binding site element (at −160 bp), is necessary for maximal responsiveness of the bFGF gene to Egr-1 protein.

DISCUSSION

The regulation of egr-1 transcription is an important nuclear event in astrocyte proliferation, as modified by the neuropeptides, ET-3 and ANP (11). In fact, egr-1 is strongly activated by several glial mitogens (26). In the studies reported here we found that the ability of ET-3 to stimulate egr-1 transcription was dependent on MAP-kinase. Several laboratories, including our own, have reported that endothelin activates the MAP kinase cascade in astrocytes and other cells but the importance of this was not determined (27, 28). Our results are consistent with the recent report that vitamin D3 and phorbol ester activate egr-1 transcription through this serine/threonine kinase, in a setting where vitamin D3 is mitogenic (29). Since we found that ANP inhibits the ET-3 induced transcription of egr-1, this may have occurred through inhibiting MAP-kinase stimulation of egr-1. We recently showed that ANP inhibits ET-stimulated MAP kinase kinase (MEK) and MAPK making this mechanism likely (30). It is also known that ET can activate v-src (31) and that this cytoplasmic protein-tyrosine kinase can activate egr-1 in BALBc 3T3 cells (32), providing a possible upstream mechanism, connecting the activation of ET transmembrane receptors to this astrocytic nuclear action. We speculate that ET-induced v-src activation leads to MAPK activation, via MEK; additional signaling events upstream of MEK but distal to v-src are under investigation.

The egr-1 promoter contains five SREs (33), and it has been shown that transcriptional activation of this gene by extracellular signals is often mediated by SRE/Ets elements (32–35). Some of the studies suggested that tissue-specific and stimulus-dependent utilization of promoter elements may be important for egr-1 induction (36–39). SRE/Ets binding sites on the egr-1 promoter are occupied by multiprotein complexes that are similar to the ternary complex described for the c-fos SREs (40). Moreover, it has been shown that one of the Ets domain-containing proteins, Elk1 is a substrate for MAPK (41–44). We recently found that ET-3 activation of egr-1 through the MAPK pathway in astrocytes is mediated by SRE elements.2

Regarding the regulation of the bFGF gene, the promoter does not contain CAAT or TATA box motifs (14). It is known that this promoter contains AP1 binding sites and five GC boxes but the functional relevance of these sites is unknown. It has been described that an increased level of bFGF mRNA synthesis in response to phorbol ester occurs in human astrocytoma cell lines (45) and the activation of bFGF-reporter constructs by phorbol 12-myristate 13-acetate and forskolin occurs in adrenal medullary cells (46). Recently the bFGF promoter was reported to be regulated by the p53 protein at the transcriptional level (and its basal core promoter was found to be responsive to p53) (47). Within the human bFGF promoter we have identified three zinc finger protein binding sites (ZIPB) at −160, −60, and +40 bp, containing overlapping binding motifs for Egr-1 and Sp1 proteins. These motifs could be involved in the ability of Egr-1 to transactivate bFGF. We found that deletion of the Egr-1 binding site at −160 bp (designated A2) resulted in a significant loss of the ability of Egr-1 protein to stimulate bFGF transcription (reduction by 3-fold). Further deletion of Egr-1 binding sites (both A2 and A1 in the pFDcat6N construct) resulted in no further reduction in activity, suggesting that the Egr-1 binding site at −60 bp (A1) is unimportant for bFGF transactivation by Egr-1. A further analysis of the sequences of the A1 site led us to discover another putative binding site for Egr-1 (5′ CTCCCCCGC 3′) overlapped with the first one, and an Sp1 binding site (marked by dashed line in Fig. 2). However, results obtained from gel mobility shift assays indicated that this site is not able to bind Egr-1 protein (data not shown). It is known that the Wilms’ tumor suppressor gene product, WT-1, can bind to the same DNA sequences as Egr-1 (48) and displays opposite effects on the transcription of target genes containing Egr-1 binding sites (49, 50). The binding affinities of these two proteins to the same sequence varies (51, 52), but the classic WT-1 target motif has 20–30-fold higher affinity for WT-1. We observed that ZIPB sites located on A and A1 sites of the bFGF promoter display similarity of sequences to a DNA motif of higher affinity for the WT-1 protein (51). A potential role of the WT-1 protein in the interactive regulation with Egr-1 of human bFGF might be important. For instance, bFGF transcripts are abundantly expressed in >90% of human gliomas (53), and in glioma cells, ANP cannot inhibit and ET cannot stimulate the basal high expression of egr-1 (11). This is perhaps due to a loss of competitive regulation between WT-1 and Egr-1, as has been reported in Wilms’ tumor cells (49). Further work is needed to define this possible interaction.

We found that the results of the gel mobility shift assays supported the functional reporter studies but raised additional issues. The two zinc finger proteins Egr-1 and Sp1 were shown to bind to the oligonucleotide representing bFGF promoter sequences. In unstimulated and stimulated astrocytes, the ubiquitous transcription factor Sp1 (54) binds to these regions, while in activated cells, the induced Egr-1 protein binds these elements. ET-3 significantly increased binding of Egr-1 protein to all of the sites on the bFGF promoter, identified both by competition with an Egr-1 binding oligonucleotide, and by supershifting with antibody to this protein. ANP significantly inhibited the increased Egr-1 binding. This was likely to have resulted, at least in part, from the increase and decrease, respectively, of the production of the Egr-1 protein, as modulated by ET-3 and ANP, which we have previously shown (11). However, despite the changes in protein binding at the A, and A1 sites, there were no functional effects. We are unable at this time to discriminate functionally between the effect of Egr-1 and a possible action of Sp1 on bFGF expression. However, Egr-1 does not bind to Sp1 consensus sequences, and Sp1 does not bind to Egr-1 consensus sequence (19, 20, 22, 55). Methylation interference studies demonstrate that the two proteins exhibit different contact sites within DNA sequences containing overlapping Egr-1 and Sp1 sites (56), and similar overlap-
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binding site. The elucidation of how bFGF commits the astro-
neural cell proliferation is positively regulated, mainly
through the Egr-1 protein’s action at a noncanonical Egr-1
binding site. The elucidation of how bFGF commits the astro-
cyte to a cell division pathway, perhaps in conjunction with
egr-1 and MAPK, will provide a model to understand the pre-
cise events which govern positive and negative growth.

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