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## Gene expression profile analysis of 4-phenylbutyrate treatment of IB3-1 bronchial epithelial cell line demonstrates a major influence on heat-shock proteins

Jerry M. Wright,<sup>1</sup> Pamela L. Zeitlin,<sup>2</sup> Liudmila Cebotaru,<sup>1</sup> Sandra E. Guggino,<sup>3</sup> and William B. Guggino<sup>1</sup>

Departments of <sup>1</sup>Physiology, <sup>2</sup>Pediatrics, and <sup>3</sup>Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287

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**Wright, Jerry M., Pamela L. Zeitlin, Liudmila Cebotaru, Sandra E. Guggino, and William B. Guggino.** Gene expression profile analysis of 4-phenylbutyrate treatment of IB3-1 bronchial epithelial cell line demonstrates a major influence on heat-shock proteins. *Physiol Genomics* 16: 204–211, 2004. First published October 28, 2003; 10.1152/physiolgenomics.00160.2003.—Most individuals with cystic fibrosis (CF) carry one or two mutations that result in a maturation defect of the full-length CFTR protein. The  $\Delta F508$  mutation results in a mutant protein that is degraded by the proteasome instead of progressing to the apical membrane where it functions as a cAMP-regulated chloride channel. 4-Phenylbutyrate (PBA) modulates heat-shock protein expression and promotes trafficking of  $\Delta F508$ , thus permitting maturation and membrane insertion. The goal of this study was to gain insight into the genetic mechanism of PBA action through a large-scale analysis of gene expression. The Affymetrix genome-spanning U133 microarray set was used to compare mRNA expression levels in untreated IB3-1 cell line cultures with cultures treated with 1 mM PBA for 12 and 24 h. The most notable changes in mRNA levels were transient elevations in heat-shock proteins. The majority of genes downregulated throughout the application period were functionally associated with control of gene expression. Another set of genes increased in expression starting at 24 h, suggesting these are downstream effects of altered gene expression initiated by PBA. More than one-third of the genes in this late expressing set were identified as having potential significance in understanding the pathology of CF. Our results demonstrate the usefulness of gene expression profile analysis in understanding the consequences of PBA treatment and provide insights in how this drug exerts its effect on the trafficking of CFTR.

microarray; chaperones; cystic fibrosis; RNA

CYSTIC FIBROSIS (CF) is a genetic disorder resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a cAMP-dependent apical membrane chloride channel that regulates fluid composition in the respiratory and gastrointestinal tracts (1, 5). The most common mutation associated with CF in the Caucasian population results from the deletion of phenylalanine at position 508 ( $\Delta F508$ -CFTR) (36). The resulting protein is capable of conducting chloride (32) but is retained in the endoplasmic reticulum (ER) and then degraded in the ubiquitin/proteasome (ERAD) pathway instead of being trafficked to the apical membrane (14). ER retention of the unfolded protein has been proposed to activate the unfolded protein response (UPR),

which ultimately leads to an inflammatory cascade in the CF lung (23, 46). In the maturation and trafficking process, CFTR interacts with many chaperone proteins, ER resident calnexin (33), as well as HSC70 (49), Hdj-2 (30), and HSP90 (28) in the cytosol. Upregulation of HSP70, a stress-induced chaperone, and downregulation of HSC70 can restore  $\Delta F508$ -CFTR trafficking (19, 39, 40). The  $\Delta F508$ -CFTR protein is functional but with a shorter half-life in the membrane and reduced probability of opening (9, 29). Approximately 50% of CF patients are homozygous for  $\Delta F508$ -CFTR and another 20% carry one copy of  $\Delta F508$ -CFTR. Thus correction of a trafficking defect becomes an extremely attractive therapeutic target, not only because a functional protein is already produced, but also because it will impact a majority of severe cases.

4-Phenylbutyrate (PBA) is a histone deacetylase inhibitor that activates transcription of a variety of genes important in regulation of cell development and proliferation through multiple signaling pathways (44). PBA is also known to modulate heat-shock protein chaperone expression, which permits  $\Delta F508$ -CFTR maturation and trafficking (40). In vitro, PBA restored chloride conductance by promoting trafficking of  $\Delta F508$ -CFTR to the cell surface in IB3-1 cells and primary nasal epithelia cultures (37). In vivo, systemic administration of PBA induces chloride transport as measured by changes in nasal potential difference (39, 50). PBA decreased heat-shock protein expression by decreasing the stability of HSC70 mRNA, and thus some of its effects are not clearly related to inhibition of deacetylase enzyme activity (38).

The goal of this study was to gain insight into the genetic components affected by PBA through a large-scale analysis of gene expression. Because PBA is an activator of transcription and the full range of altered gene expression is unknown, only genome-spanning microarrays are likely to capture the full range of response. The resulting information serves to simultaneously examine the expression pattern of known components of the trafficking system, identify candidate genes with possible roles in regulation of CFTR trafficking, and identify genes affected by altered trafficking of CFTR. Additional information is also needed on the effect of PBA treatment on the UPR and ERAD pathways, which are coordinated yet separate responses that cope with unfolded proteins in the ER (42). Although each of these responses could be studied in isolation, it is the overall response of the tissue assessed by expression profiling that provides a more global understanding of the impact of PBA treatment.

### METHODS

**Cell culture.** IB3-1 ( $\Delta F508$ /W1282X) bronchial epithelial cells (25) were cultured in LHC-8 as described previously (40). Briefly,

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Address for reprint requests and other correspondence: J. M. Wright, Dept. of Physiology, Johns Hopkins Univ. School of Medicine, Baltimore, MD 21287 (E-mail: [jwright@jhmi.edu](mailto:jwright@jhmi.edu)).

cells were grown in LHC-8, gentamicin-free medium in T75 flasks. Cells were fed with 10 ml of media every 2–3 days. After reaching 80% confluence cells were treated with 1 mM PBA. A T75 flask of confluent IB3-1 cells was rinsed twice with ice-cold Hanks' buffer, then scraped into 3 ml of ice-cold TRIzol (GIBCO-BRL), then rinsed with 3 ml ice-cold TRIzol, and the mRNA was isolated according to the TRIzol protocol.

**RNA microarray and data analysis.** Total RNA isolated from each sample was processed and hybridized to the A and B GeneChips of the U133 GeneChip Array Set according to the protocols described in the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Briefly, total RNA was used to generate double-stranded cDNA by using a T7-linked oligo(dT) primer. In vitro transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics). Target cRNAs generated from each sample were then processed per manufacturer's recommendation. Spike controls were added to fragmented cRNA before overnight hybridization. Arrays were then washed and stained with streptavidin-phycoerythrin, before being scanned on an Affymetrix GeneChip scanner at the Johns Hopkins Bayview core facility.

After scanning, array images were assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches. The 3'/5' ratios for GAPDH and  $\beta$ -actin were confirmed to be within acceptable limits, and BioB spike controls were found to be present in 100% of GeneChips, with BioC, BioD, and CreX also present in increasing intensity. GeneChip scanning and initial expression analysis was performed using MicroArray Analysis Suite 5.0 software (Affymetrix, Santa Clara, CA). When scaled to an average probe set intensity of 150, scaling factors for all arrays were within acceptable limits, as were background, Q values, and mean intensities. A total of five control cultures, three cultures with 12-h PBA application, and three cultures with 24 h PBA application were processed.

GeneChip expression data were exported to GeneSpring where per chip normalization to the 50th percentile expression level and per gene normalization to the median expression intensity in all samples was performed. Only probe sets scored present or marginal in at least 8 of the 11 samples were included in the analysis. Data were transformed to log ratio for display and analysis. GeneSpring 5.0 (Silicon Genetics, Redwood City, CA) and S-Plus 2000 (MathSoft, Cambridge, MA) software programs were used in data analysis and visualization. Significantly changing gene expression was set at  $P \leq 0.05$  using the GeneSpring *t*-test statistic with cross-gene error model activated.

**Function and literature analysis.** The lists of all probe sets changing significantly were subjected to intensive search to identify biological function. Probe set sequences from the Affymetrix web site were "blasted" against National Center for Biotechnology Information (NCBI) or Celera (21) databases to verify identity and update annotation. Individual genes may have multiple probe set sequences specific to different regions of the gene; each probe set was analyzed separately. The lists of all probe sets changing significantly were submitted to DRAGON (6) for automated annotation of conserved domains either predicted or known to exist in the protein. Further functional annotation of significantly changed probe sets was done manually using NCBI conserved domain database analysis program and the Celera database. The classification by conserved functional domains addresses the problem of assigning function to genes where protein sequences have been predicted but not yet studied. Gene Ontology classifications (2), automated online gene annotation (6), conserved protein family domains (4), reference literature, and expert knowledge were used to construct functional groupings of genes from the significantly changed Affymetrix array probe sets. Original array data images and supplementary data files are available online, at the

*Physiological Genomics* web site<sup>1</sup> and at [http://www.hopkins-genomics.org/cf/cf\\_research.html](http://www.hopkins-genomics.org/cf/cf_research.html). Processed image data files are online at the NCBI Gene Expression Omnibus (GEO) database under series accession number GSE620.

**Terminology.** The terminology for heat-shock proteins can be confusing, thus making it difficult to relate prior works to each other or to current Human Genome Organization (HUGO) (47) naming conventions. There is the originally described 70-kDa heat-shock protein referred to as HSP70, a constitutively expressed form known as HSC70, and a nonfunctional variant named HSC54. Before the distinction between the HSP70 and HSC70 proteins, as well as other similar heat-shock proteins, was well defined, the designation HSP70 sometimes referred to other heat-shock proteins. In addition, there is a conserved functional domain named HSP70, Pfam (4) designation PF00012, common to heat-shock proteins in the 70-kDa and 105-kDa groups. The 40-kDa family of cofactors for 70-kDa heat-shock proteins also has a confusing terminology history, and HSP90 has two variants referred to as alpha and beta forms. The HUGO designations for these genes are as follows: HSPA1A and HSPA1B are alternative forms of HSP70; HSPA8 variant 1 is HSC70, and HSPA8 variant 2 is HSC54; HSPCA and HSPCB are the  $\alpha$ - and  $\beta$ -forms of HSP90, whereas DNAJA1 is the designation for HSP40, also previously known as Hdj-2.

**Validation of RNA by RT-PCR.** Changes in selected RNA levels as determined in the microarray analysis were validated with semiquantitative RT-PCR using a thermocycler followed by gel electrophoresis. To ensure that RT-PCR was measuring the same portion of the gene mRNA as the microarray, Affymetrix probe set sequences were used as a point in primer creation. RNA extracted for microarray use was used as the source material. Gel images were scanned on a Fuji LAS 1000 imaging system (Fujifilm Medical Systems USA, Stamford, CT). cDNAs were generated by reverse transcription, and PCR analysis was performed using the following primers: for HSPH1, forward 5'-CGCAGATCTTCACCACCTACTCC-3', and reverse 5'-TTGTC-GGCCTCGCTGATCTT-3'; for HSP70, forward 5'-CGCAGATCTTCACCACCTACTCC-3', and reverse 5'-CCAACAGTCCACCTCAAAGACAA-3'; for  $\beta$ -actin, forward 5'-TGGAACGGTGAAGGTGACAGC-3', and reverse 5'-GCACGAAGGCTCATCAT-TCAA-3'.

## RESULTS

**Analysis of PBA application by expression profiling.** IB3-1 cells ( $\Delta$ F508/W1282X) express only  $\Delta$ F508 protein because the W1282X mRNA is unstable (17, 18). IB3-1 cell cultures untreated for 12 and 24 h or treated with 1 mM PBA for 12 or 24 h at 37°C were analyzed by oligonucleotide expression profiling. There were no significant differences in number of genes expressed among treatments. There were  $16,316 \pm 1,080$  probe sets scored present or marginal in the control samples,  $16,492 \pm 1,511$  in cultures treated with PBA for 12 h, and  $15,894 \pm 1,522$  for cultures treated with PBA for 24 h. A total of 194 (~1.2%) probe sets were significantly changed by PBA treatment (see Supplemental Table S1); of this set, 119 probe sets decreased in expression, whereas another 75 were upregulated for at least one time point. We were able to assign function to 120 (62%) of the 194 significantly changed probe sets. Three expression pattern groupings were evident by visual inspection (Fig. 1A) and verified by K-means clustering: sets downregulated by PBA application within 12 h and at 24 h (Fig. 1B); probe sets upregulated at 24 h (Fig. 1C); and probe

<sup>1</sup>The Supplementary Material for this article (Tables S1 and S2) is available online at <http://physiolgenomics.physiology.org/cgi/content/full/00160.2003/DC1>.

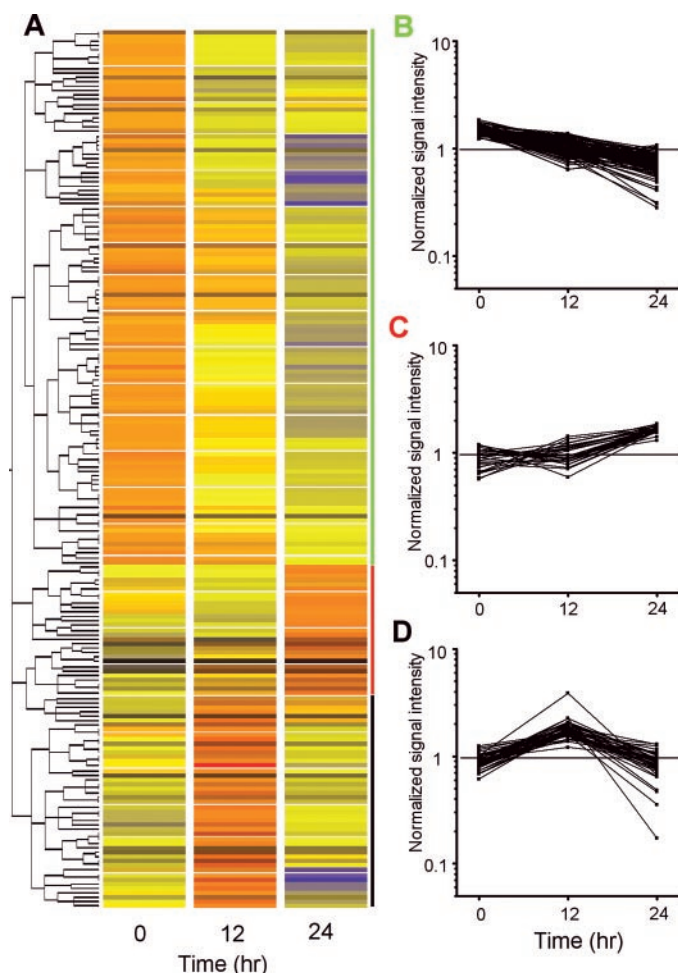


Fig. 1. A: expression profiles of 194 targets significantly altered in abundance by 1 mM 4-phenylbutyrate (PBA) after 12 and 24 h of application. B: probe sets with decreasing expression relative to control conditions (green vertical bar). Probe sets were upregulated at 24 h (red vertical bar) (C) and transiently increased at 12 h (black vertical bar) (D). There were 117 probe sets were present or marginal in all experiments. Another 77 probe set were present or marginal in at least 8 of the 11 samples and are indicated by darker horizontal shading. Intensity in the red and blue color range indicate upregulated and downregulated mRNAs, respectively; yellow indicates no change. Each row indicates a single gene; each column represents an experimental condition. Each box represents the average, normalized RNA intensity value for that condition.

sets transiently upregulated at 12 h (Fig. 1D). Attempts to extend the analysis by specifying additional clusters resulted in fragmentation of the downregulated set but provided no additional insight into functional relationships. The HSP70 probe set showed the largest increase, 3.8-fold (Fig. 1D). Some probe sets appeared to have declined in intensity by more than a factor of 4 (Fig. 1), but because the decrease resulted in absent calls, calculations could not be exact.

**Functional categorization of genes initially responding to PBA application.** Cluster 1 (Fig. 1B) contained genes significantly decreasing their expression with PBA application. From the listing of individual genes showing altered expression within the initial 12-h period, we identified functional groups of genes involved in common cellular processes (Table 1). Of the 119 probe sets in this cluster (Supplemental Table S1), we were able to assign function to 70 of them. There were some

differences in the amount of decline in expression intensity at 12 h, but all were clearly decreased in expression at 24 h (Fig. 1, A and B).

A majority (55) of the functionally identified genes in this cluster dealt with important gene expression processes at one of several levels: master switches that influence expression of numerous genes, regulation of transcription, and involvement in RNA processing (Table 2). CLDN1 is an epithelial membrane protein that appears to have numerous functions including regulation of cell polarity and permeability (41). Of six genes identified with possible signaling function, four (CIRH1A, TIP-1, HSPC163, and TAX1BP1) were involved in developmental signaling processes and categorized accordingly. Of the 10 downregulated transcription factors, 5 were negative regulators of transcription: KIAA1483, CNOT2, PAWR, POLE4, and R67076. R67076 resolved to a gene containing a KRAB domain in the Celera database but did not in the NCBI database. It is of interest that KIAA1483 contains the conserved domain BTB/POZ, which interacts with histone deacetylase in conjunction with effects on transcription.

Of the eight genes involved in protein processing and stress response in this first data set (Table 3), three have potentially significant impact on CFTR trafficking and ERAD. FLJ10808 and FLJ11011 are predicted proteins with conserved domains indicating involvement in targeting proteins for degradation by the proteasome. FLJ10808 is very similar to ubiquitin-activating enzyme E1, which catalyzes the first step in ubiquitin conjugation to mark cellular proteins for degradation. FLJ11011 is a ubiquitin conjugating enzyme that is another important step in the degradation marking process. Reduced expression of these two genes may have effects on trafficking of  $\Delta F508$ -CFTR by decreasing the amount targeted for degradation while it is associated with HSC70. Also reduced in expression is an E3 ubiquitin ligase, HRD1, which acts to protect cells against ERAD-induced apoptosis by marking unfolded proteins for ubiquitin-proteasome degradation processing (20). Blocking the ubiquitination process interrupts degradation of  $\Delta F508$ -CFTR, causing the immature form to accumulate in the cell but does not promote maturation (45). However, the net effect of accumulation of the immature form would be to give HSP70 greater opportunity to process the protein before it is degraded.

Cluster 2 (Fig. 1D) contained 39 probe sets significantly, but transiently, elevated within 12 h of exposure to PBA. There was a quite remarkable contrast to the downregulated lists. Ten of the probe sets were identified as genes associated with stress response (Table 4). Of these, there were five genes involved in the processes of folding and trafficking proteins in response to

Table 1. Functional summary of genes responding to PBA application

	Cluster 1	Cluster 2	Cluster 3
Regulation of gene expression	16	2	2
DNA binding/transcription factors	16	2	0
RNA processing	23	0	3
Protein processing	7	1	0
Stress response	1	7	0
Immune function	1	3	2
Enzymes	6	2	3

PBA, 4-phenylbutyrate.

Table 2. Downregulated gene expression

HUGO Name	GenBank ID	Description
<i>Regulation of gene expression</i>		
TAX1BP1	NM_006024	Anti-apoptotic
NDE1	NM_017668	Cell cycle
WDR4	NM_033661	Cell cycle
NOLC1	NM_004741	Cell cycle transcription regulation
CIRH1A	NM_032830	Cell differentiation
FLJ20232	NM_019008	Cell differentiation
HSPC163	NM_014184	Cell differentiation
JUB	BC034968	Cell differentiation
TIP-1	NM_014604	Cell differentiation/signaling
CLDN1	NM_021101	Cell proliferation
TMPO	NM_003276	Cell proliferation
MAT2A	NM_005911	Growth and differentiation
GAS5	BC038733	Growth arrest
FGF2	NM_002006	Growth factor
MGC13096	NM_032346	Programmed cell death
IPO11	NM_016338	Regulation of cell division
<i>Transcription/DNA binding</i>		
CITED2	NM_006079	Coactivator of transcription factor
D1S155E	NM_007158	DNA binding protein
CIP29	BC007099	DNA binding domain
C7orf3	XM_049384	Eukaryotic initiation factor
ID1	NM_002165	Inhibitor of transcription factors
HMGA1	AF176039	Non-histone regulation of transcription
TIM50L	XM_053074	Transcription
TAZ	NM_015472	Transcription coactivator
ZNRD1	NM_014596	Transcription factor
GAS6	NM_000820	Transcription factor
SOX4	NM_003107	Transcription factor
POLE4	NM_019896	Transcription repression
	R67076	Transcription repression
KIAA1483	NM_020861	Transcriptional repression
CNOT2	NM_014515	Transcriptional repression
PAWR	NM_002583	Transcriptional repression
<i>RNA processing</i>		
FLJ10534	NM_018128	Required for 40S ribosome biogenesis
MRPS22	NM_020191	Ribosomal protein
FLJ10534	NM_018128	Ribosomal protein
	XM_114317	Ribosomal protein
MRPS18C	NM_016067	Ribosomal protein
RPL7A	NM_000972	Ribosomal protein
MKI67IP	NM_032390	RNA binding domain
U5-116KD	NM_004247	RNA binding domain
HSU53209	NM_013293	RNA binding domain
RBM8A	NM_005105	RNA binding domain
CGI-110	NM_016047	RNA binding domain
HNRPC	NM_031314	RNA metabolism
KIAA1595	XM_045520	RNA metabolism domain
MGC4308	NM_032359	RNA metabolism domain
POLR2A	NM_000937	RNA polymerase
FUSIP1	AK001479	Splicing factor
SLU7	NM_006425	Splicing factor
HNRPA2B1	NM_002137	Splicing factor
HGRG8	NM_016258	Splicing factor
FRSB	NM_005687	tRNA synthetase
AARS	NM_001605	tRNA synthetase
CARS	NM_139273	tRNA synthetase
EEF1E1	NM_004280	tRNA transfer

HUGO, Human Genome Organization.

stress: HSP70 (2 probe sets) and HSPA2, members of the 70-kDa family of heat-shock proteins; APG-1 and HSPH1 (2 probe sets), members of the 105/110-kDa heat-shock proteins; and C14orf3, an activator of HSP90 ATPase. The transient rise in mRNAs was verified by semiquantitative RT-PCR for

Table 3. Downregulated protein processing and stress response genes

HUGO Name	GenBank ID	Description
<i>Protein processing</i>		
SNX5	NM_152227	Membrane trafficking, protein sorting
NLN	NM_020726	Metallopeptidase
PPIL1	NM_016059	Peptidylprolyl isomerase
CGI-85	NM_016028	Protein-protein interactions with phosphatases
XPO5	AF271159	Protein trafficking
FLJ11011	AK024050	Ubiquitin conjugating enzyme
FLJ10808	NM_018227	Ubiquitin-activating enzyme
<i>Stress response</i>		
HRD1	AA844682	Degrades unfolded proteins in ER

ER, endoplasmic reticulum.

HSP70, HSPH1 (Fig. 2), and APG-1 (data not shown). The other four probe sets in this functional group resolved to a set of closely related metallothioneins. Metallothioneins are protective proteins produced in response to a variety of stressful stimuli or as part of an inflammatory response with multiple functions including protection against heavy metal and oxidant damage, redox control, and metabolic regulation (26).

Other proteins of potential interest to CF research also appeared in this cluster. CGI-100 contains the conserved domain emp24/gp25L/p24, which is typically involved in ER trafficking and is found in *cis*-Golgi network (12). Also increased was TLR4, toll-like receptor 4, which is required for response to some bacterial proteins and is a target of stimulation by extracellular HSP70 (22, 43). The increase in expression of TLR4 was not necessarily related to the stress response but may be related given its similar pattern of expression and functional involvement with HSP70.

*Functional categorization of genes with delayed response to PBA application.* Cluster 3 contained 29 probe sets significantly increased above the median value at 24 h (Supplemental Table S1); genes of 19 probe sets could be assigned function. This set contained genes responding to a variety of factors including decreased transcription inhibition, corrected CFTR trafficking, and altered expression of other controlling proteins. Although the functional groups had only few members that had no apparent connections between groups (Table 5), this cluster had several genes potentially of significant interest to CF research.

Table 4. Transiently upregulated stress response and protein processing genes

HUGO Name	GenBank ID	Description
<i>Stress response</i>		
C14orf3	NM_012111	HSP90 ATPase
HSPA1A	NM_005345	HSP70 kDa family
APG-1	NM_014278	HSP105/110 kDa family
HSPH1 (2)	NM_006644	HSP105/110 kDa family
HSPA2	NM_021979	HSP70 kDa family
	AL031602	Metallothionein family
MT1F (2)	NM_005949	Metallothionein
MTIL	NM_002450	Metallothionein
<i>Protein processing</i>		
CGI-100	NM_016040	Protein trafficking

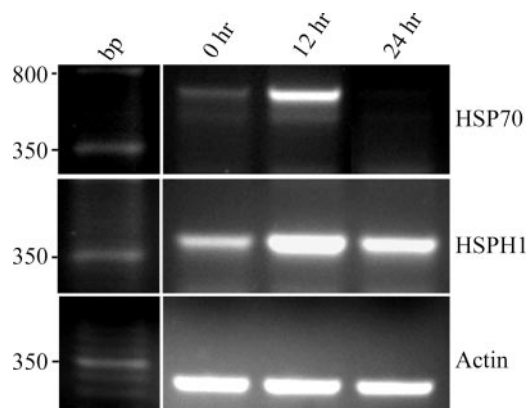


Fig. 2. RT-PCR. The increase in mRNA for HSP70 and the 110-kDa heat-shock protein HSPH1 induced by 1 mM PBA application was transient with maximal mRNA expression at 12-h exposure to PBA.

GSTA4, glutathione *S*-transferase A4, and GPX3, glutathione peroxidase 3, were elevated at 24 h. Glutathione is involved in a variety of functions as a thiol antioxidant, participating in antioxidant and defensive functions, modulation of cell proliferation, and modulation of signal transduction cascades (31). A lower activity of selenium-dependent GPX3 has been noted in CF patients (34), and altered or reduced processing of glutathione has been suggested as a contributing factor in the chronic lung inflammation and infections seen in CF patients (10, 35). GST mRNA is known to be significantly increased in human colon tumor cell lines within 24 h by treatment with 0.5–2 mM butyrate (13). Although it is unclear whether the elevation of these genes in our study is a result of PBA effects on transcription or altered  $\Delta F508$ -CFTR trafficking, increased expression of these genes is potentially of therapeutic benefit.

A central regulatory element in control of inflammatory responses, BCL6, was also elevated. It acts as a sequence-specific repressor of transcription and has been shown to modulate the transcription of START-dependent IL-4 responses of B cells. Absence of this gene in mice results in a chronic inflammatory state (11). Also of interest in this cluster were genes whose predicted proteins have potential involvement with regulation of ion movement in CF cells: CTL2, a transporter-like protein, and SLC22A1L, which appears to be an organic cation transporter.

Endocytosis of transmembrane proteins, including ENaC and CFTR, is an important process in regulation of channel activity with implications in understanding ion channel abnormalities in CF (16). Several genes from different functional groups that are involved in regulation of endocytosis were found to be upregulated. TSC2 functioning as a Rab5 GTPase activating protein (GAP) *in vivo* negatively regulates Rab5-GTP activity in endocytosis. ED3 localizes on endocytic tubular and vesicular structures and regulates their microtubule-dependent movement.

PBA treatment did not change status of the ER unfolded protein response. Because of the proposed link between elevated ER UPR response and an inadequate immunologic response resulting in chronic bacterial infection (46), we specifically looked for alteration in expression of genes involved in UPR and CFTR processing in the ER. A list of ER resident proteins induced by the UPR was derived from Lee (27) and

mapped to Affymetrix array probe sets. Additional ER resident genes, KDEL receptor, calnexin, RAP and HSP47, involved in ER protein trafficking and maturation were also inspected. Twenty-four of the 27 genes were detected (Supplemental Table S2); there was no significant change in intensity of any of the probe sets at 12 or 24 h (data not shown).

*Comparison of IB3-1 PBA results with gene array data from CF mouse model.* Recently, samples from transgenic mice with a null mutation in CFTR and the corresponding control animals were analyzed by Xu et al. (48). We looked for common patterns between the IB3-1 cell model and the mouse model using NCBI LocusLink to match mouse genes from Xu et al. (48) with the corresponding human gene. In the comparison lung samples from CFTR(–) and CFTR(+) mice, 45 genes consistently differentially regulated could be matched with the human ortholog; of these, 30 were expressed at levels too low to be detected in IB3-1 cells, whereas there was no significant change in expression of the remaining 15. In the CFTR(–) mice, human CFTR cDNA is expressed in the intestinal epithelium under control of the fatty acid-binding promoter that corrects intestinal pathology. In the list of genes significantly differentially expressed in gut-corrected vs. uncorrected mice (48), seven genes were below detection in IB3-1 cells and six others did not change significantly. HSP105 was elevated in the uncorrected gut and was elevated transiently at 12 h in the

Table 5. Genes upregulated at 24 h

HUGO Name	GenBank ID	Description
<i>Transporter</i>		
CTL2	NM_020428	Choline transporter-like
SLC22A1L	NM_002555	Organic cation transporter-like
<i>Glutathione</i>		
GPX3	NM_002084	Glutathione peroxidase 3
GSTA4	NM_001512	Glutathione <i>S</i> -alkyltransferase A4
<i>Immunity</i>		
HLA-E	X56841	NK cell recognition of tumor cells
BCL6	NM_001706	Regulator of immune system, modulates B cell development
<i>Enzyme</i>		
AKR1C1	NM_001353	Dihydrodiol dehydrogenase 1
<i>Signaling</i>		
TSC2	NM_000548	Rab5 GTPase activating protein
GRB10	NM_005311	Eukaryotic signaling protein
NR1D2	NM_005126	Nuclear (hormone) receptor subfamily 1, group D, member 2
FAAH	NM_024306	Fatty acid hydroxylase
CSPG5	NM_006574	Chondroitin sulfate proteoglycan 5, FGF domain
HS6ST	NM_004807	Heparan sulfate 6- <i>O</i> -sulfotransferase, EGF domain
<i>Structure</i>		
COL5A1	NM_000093	Type V collagen, cell adhesion domain
FBLN1	NM_006486	Fibulin 1, incorporated into a fibrillar extracellular matrix
EHD3	NM_014600	Regulates microtubule-dependent movement
<i>RNA processing</i>		
AARS	NM_001605	t-RNA synthetase
POLR2A	NM_000937	RNA polymerase II 220-kDa subunit
CARS	NM_139273	cysteinyI-tRNA synthetase

IB3-1 cell line after PBA treatment. This was the only match between the organ samples and the cell line culture and may be coincidental since the gene is produced in response to cellular stress. This appears to be the case in PBA treatment of IB3-1 cells and is likely to occur in the mouse model, because uncorrected CFTR(-) mice rarely survive weaning, due to digestive problems.

## DISCUSSION

Although it is recognized that PBA has significant and specific effects on  $\Delta F508$ -CFTR trafficking in conjunction with altering the expression of HSP70, there are also wide-ranging effects on gene expression, many of which may have importance in understanding CF pathology. We have identified genes from the three functional expression patterns with potential involvement in CFTR trafficking or are of interest in understanding CF pathology. Differentially regulated genes include those related to protein trafficking, degradation processing, cellular response to bacterial infection, control of inflammatory responses, and endocytosis of surface proteins. Although PBA is a transcription activator, there was not a clear trend toward generalized increases in expression in the data sets. There was a suggestion at 12 h of some specific upregulation of transcription by PBA, the effects of which may have implications in the drug dosing regime. At 24 h the difference disappeared. This suggests that the initial increase in gene expression is rapidly obscured by differential regulation of downstream targets. This is reassuring with respect to clinical use, since widespread “un-silencing” of fetal or cell proliferation genes might be of concern.

The early downregulation of inhibitory transcription factors suggests a delayed onset of altered expression in downstream targets which may play a role in later expression of genes important to regulation of CFTR trafficking and maturation. There was no clear functional relationship among genes upregulated at 24 h, yet 7 of 19 functionally identified genes (36%) were of immediate interest with potential importance in understanding CF pathology. Whether individual genes in this third set are induced by PBA or governed by correctly trafficked  $\Delta F508$ -CFTR remains to be investigated.

*IB3-1 as a model drug treatment system.* In addition to useful information on the effects of PBA on gene expression, the results of this genome-spanning microarray study can be helpful in evaluation of future therapeutic compounds using this model system. We have high confidence in the overall results reported by the gene array data, because alterations in gene expression patterns were consistent with the known capability of PBA to alter cell development. The results were in agreement with previous work from several laboratories working with selected genes (13) and smaller microarrays (38) using PBA or closely related compounds. We chose to verify the HSP70 mRNA expression pattern by RT-PCR, because we have previously demonstrated that PBA elevates HSP70 protein in IB3-1 (7). The low-amplitude changes in RNA and protein expression in response to application of mild histone deacetylase inhibitors has been documented in other laboratories (13, 15).

*Transient induction of stress response genes and CFTR trafficking.* The transient nature of heat-shock gene expression has implications in trafficking of  $\Delta F508$ -CFTR. The amount of

HSP70 protein would be predicted to initially rise, then decline at some later point depending upon the half-life of the protein. This in turn may affect the amount of mature and correctly trafficked  $\Delta F508$ -CFTR. However, the trafficking of  $\Delta F508$ -CFTR also appears to be affected by the ratio of HSP70 to HSC70 (7), and HSC70 protein declines in the continual presence of PBA (38). Thus, although there is an indication that steady-state delivery of PBA may not achieve the maximum desired response over time, there are significant confounding factors with an unknown degree of impact on trafficking that need further study. A transient clinical response has been observed in butyrate treatment of sickle cell disease and was addressed by using a pulsed application of butyrate for a 4-day period followed by a 10- to 24-day period with no drug exposure to achieve clinically effective levels of fetal hemoglobin in circulation (3). Hence, it appears worthwhile to compare pulse vs. sustained drug application and determine how the different protocols affect heat-shock protein production and trafficking of  $\Delta F508$ -CFTR over a period of weeks.

There are several lines of evidence that indicate the transient increase in heat-shock genes is not an artifact. Microarray data indicate simultaneous transient changes in gene expression by several families of genes involved in cellular stress response. The mouse homolog of APG-1 is known to transiently increase mRNA expression in response to changes in osmolality, another well-documented cellular stress (24). PBA effects on heat-shock proteins were not limited to the HSP70 gene but were part of a larger response by metallothioneins and members of both the 70-kDa and 110-kDa heat-shock families of chaperones (Table 4). Our results also agree with previously known data on GSX4, which is upregulated in response to butyrate application (13). There are several possible mechanisms to account for a transient effect, including acidification of intracellular pH with butyrate application (8).

Our findings on HSP70 and HSC70 mRNA expression are consistent with what is known about the process of correcting  $\Delta F508$ -CFTR trafficking. PBA has been demonstrated to restore trafficking of functional  $\Delta F508$ -CFTR to the cell surface in primary culture of nasal epithelial cells from CF patients and in the IB3-1 cell line (37). Research into the details of CFTR trafficking indicated that HSP70 family proteins and the HSP40 cofactors are important during the maturation process (30). It was further determined that PBA reduced the amount of HSC70-CFTR complex associated with degradation of CFTR (40). More recently, it was established that increased production of HSP70 by PBA application or other means is effective in promoting  $\Delta F508$ -CFTR maturation and trafficking  $\Delta F508$ -CFTR (7). From the evidence, it appears that it is the ratio of HSP70 to HSC70 which controls the functional expression of  $\Delta F508$ -CFTR, with HSP70 promoting maturation and HSC70 promoting degradation. Consistent with earlier work (38), we did not detect a significant change in HSC70 mRNA levels, but we observed an increase in HSP70 mRNA which is also consistent with previous work (7).

Monitoring changes in expression patterns across the genome expands greatly our knowledge base on the effects of PBA correction of  $\Delta F508$ -CFTR trafficking. The observation that the effects on heat-shock protein expression are consistent with previously known effects of butyrate and PBA on cultured cell lines provided additional confidence with the overall analysis. The analysis provides numerous new leads for investiga-

tion of CF pathology and indicates that altering the drug delivery protocol may improve clinical efficacy of the drug. Overall, our results demonstrate the usefulness of gene expression profile analysis in understanding the consequences of PBA treatment and provide insights in how this drug exerts its effect on the trafficking of CFTR.

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#### DISCLOSURES

A licensing agreement exists among the Johns Hopkins University, Ucyclus Parma, Inc. (Medicis), and P. L. Zeitlin. The terms of this arrangement are being managed by the University in accordance with its conflict of interest policies. The macroscopic sweat test for cystic fibrosis is covered by patent no. 5976499, issued Nov. 2, 1999. Also, there is a patent pending (no. PO1 H151811) for the pharmacological stimulation of CFTR expression.

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