

# Pharmacogenomics of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and the Cystic Fibrosis Drug CPX Using Genome Microarray Analysis

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Accepted September 16, 1999.

## Abstract

**Background:** Cystic fibrosis (CF) is the most common lethal recessive disease affecting children in the U.S. and Europe. For this reason, a number of ongoing attempts are being made to treat the disease either by gene therapy or pharmacotherapy. Several phase 1 gene therapy trials have been completed, and a phase 2 clinical trial with the xanthine drug CPX is in progress. The protein coded by the principal CFTR mutation,  $\Delta F508$ -CFTR, fails to traffic efficiently from the endoplasmic reticulum to the plasma membrane, and is the pathogenic basis for the missing cAMP-activated plasma membrane chloride channel. CPX acts by binding to the mutant  $\Delta F508$ -CFTR and correcting the trafficking deficit. CPX also activates mutant CFTR channels. The comparative genomics of wild-type and mutant CFTR has not previously been studied. However, we have hypothesized that the gene expression patterns of human cells expressing mutant or wild-type CFTR might differ, and that a drug such as CPX might convert the mutant gene expression pattern into one more characteristic of wild-type CFTR. To the extent that this is true, a pharmacogenomic profile for such corrective drugs might be deduced that could simplify

the process of drug discovery for CF.

**Materials and Methods:** To test this hypothesis we used cDNA microarrays to study global gene expression in human cells permanently transfected with either wild-type or mutant CFTR. We also tested the effects of CPX on global gene expression when incubated with cells expressing either mutant or wild-type CFTR.

**Results:** Wild-type and mutant  $\Delta F508$ -CFTR induce distinct and differential changes in cDNA microarrays, significantly affecting up to 5% of the total genes in the array. CPX also induces substantial mutation-dependent and -independent changes in gene expression. Some of these changes involve movement of gene expression in mutant cells in a direction resembling expression in wild-type cells.

**Conclusions:** These data clearly demonstrate that cDNA array analysis of cystic fibrosis cells can yield useful pharmacogenomic information with significant relevance to both gene and pharmacological therapy. We suggest that this approach may provide a paradigm for genome-based surrogate endpoint testing of CF therapeutics prior to human administration.

## Introduction

Pharmacogenomics of genetic diseases is a relatively unexplored but promising field, in which the genetic consequences of effective gene or drug therapy can potentially be assessed at the cellular level in terms of specific patterns of global gene expression (1,2). It is hoped that

such patterns could be employed as biologically relevant surrogate endpoints when searching for more effective drugs or genes (3). Cystic fibrosis (CF), the most common lethal autosomal recessive disease affecting the U.S. and European populations (4), is an attractive target for pharmacogenomic studies. It is one of the first diseases for which gene therapy has been attempted (5,6) and various drug therapies are currently being developed (7,8). CF is most frequently associated with a homozygous  $\Delta F508$  mutation in the CFTR gene (9-11), and a single copy of the mutant

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gene is carried by approximately 5% of the population (12,13).

Among the several potential CF drugs being studied (14), the xanthine drug CPX is presently in phase II clinical trials in CF patients. For that reason, we have focussed our attention in the present paper specifically on CPX. The rationale for administration of CPX to CF patients has been severalfold. First, the drug activates wild-type and mutant  $\Delta F508$ -CFTR channels in cells (15–20) and planar lipid bilayers (21). In addition, CPX also promotes correction of the intrinsic trafficking defect of the  $\Delta F508$ -CFTR molecule in the endoplasmic reticulum (8). The mutant CFTR is misfolded (22), and it has been hypothesized that the interaction of the misfolded protein with CPX results in corrected folding and proper trafficking to the plasma membrane (8,23). However, CFTR is known to interact with numerous other cellular proteins (24–26), and CPX also interacts with classical adenosine receptors (27). It is thus possible that CPX may affect CFTR trafficking and function by additional actions that are not immediately apparent from the simpler *in vitro* and *in vivo* experiments. Furthermore, different CFTR mutations have profoundly different disease phenotypes (28), including a propensity to develop gastrointestinal and other cancers (29–36). We have therefore hypothesized that the gene expression patterns of human epithelial cells expressing mutant or wild-type CFTR might differ, and that a drug such as CPX might convert the mutant gene expression pattern into one more characteristic of wild-type CFTR.

To test this hypothesis, we have turned to genomic techniques permitting disease-dependent global quantitative analysis of gene expression (37–48). The data presented here clearly demonstrate that the expression of either wild-type or  $\Delta F508$ -CFTR in cells has both common as well as specific differential consequences for global gene expression. We have used these data to identify not only the specific consequences of expressing either wild-type or  $\Delta F508$ -CFTR in cells, but also the global genomic consequences of gene transfer, as would occur as a consequence of “gene therapy.” In addition, we have identified differential effects of CPX on global gene expression, depending on whether wild-type or mutant CFTR is being expressed. Most importantly, CPX does indeed induce a subset of genes in mutant CFTR cells to move in the direction of expression levels found in wild-type CFTR cells. Furthermore, we have learned that

CPX can induce massive overexpression of certain genes, independently of the mutational form of CFTR concurrently being expressed. The most profound example of this effect is the CPX-dependent overexpression of two protein kinase A (PKA) regulatory subunits, a tyrosine kinase, and several different heat shock proteins. Such responses can be used as diagnostic markers of CPX action, and we therefore believe that this approach to the pharmacogenomics of CPX may provide important, testable insights into CPX action in CF. This approach may also provide a paradigm for genome-based surrogate endpoint testing of CF drugs prior to human administration.

## Materials and Methods

### *Cells and Culture Conditions*

**PERMANENT TRANSFECTION OF HEK293 CELLS BY WILD-TYPE AND  $\Delta F508$ -CFTR.** Wild-type and  $\Delta F508$ -CFTR cDNA (6) were subcloned into the eukaryotic expression vector pCEP4 (Invitrogen, San Diego, CA) between the *NheI* and *XhoI* restriction sites according to the method of Tao and colleagues (49) to create the recombinant vector pCEP4(CFTR). Following generation of the permanently transfected cell lines, hygromycin was omitted from all subsequent culture conditions.

**CULTURE OF CELLS WITH CPX.** HEK293 cells, either parental or transfected with either wild-type or  $\Delta F508$  CFTR, were split 1:10 and grown to confluence in 100 cm<sup>2</sup> tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) and 1% glutamine. CPX was prepared as a 10-mM stock solution in DMSO, and after reaching cell confluence the medium was replaced with either fresh medium containing 0.1% dimethylsulfoxide (DMSO) (control) or medium with 0.1% DMSO and 20  $\mu$ M CPX. The cells were then incubated as indicated for either 24 hr or 48 hr with CPX. In a different experiment, cells were incubated for 24 hr with CPX and then moved back to control medium for an additional 24 hr to study the consequences of drug washout. At the end of the incubation period, the medium was completely aspirated and the flasks frozen at  $-20^{\circ}\text{C}$ , awaiting RNA extraction.

*cDNA Array Technology*

**PREPARATION AND LABELING OF RNA.** Total RNA was prepared by the method of Chirgwin et al. (50) from HEK293 cells, wild-type-transfected HEK293 cells, and  $\Delta F508$ -CFTR-transfected HEK293 cells, with and without CPX. The total RNA was then subjected to DNase I digestion to eliminate genomic DNA contamination. Subsequently, two rounds of purification of poly A+ RNA from total RNA was performed using the mRNA isolation kit from Invitrogen (Carlsbad, CA) as recommended by the manufacturer. The quality of the RNA was tested by running a formaldehyde-denatured agarose gel and quantitated by measuring the optical density at 260 nm.  $^{32}\text{P}$ -labeled cDNA probes were synthesized from 1  $\mu\text{g}$  of poly A+ RNA from each sample using MMLV reverse transcriptase, dNTP mix, and the cDNA synthesis (CDS) primer mix comprising the oligonucleotide sequences for the 588 genes spotted on the human Atlas<sup>®</sup> cDNA array (Clontech, Carlsbad, CA). The reaction was carried out in a thermocycler set at 50°C for 25 min and terminated by the addition of 0.1 M EDTA, pH 8.0, and 1 mg/ml glycogen. The  $^{32}\text{P}$ -labeled cDNA probes were purified from unincorporated  $^{32}\text{P}$ -labeled nucleotides by using a CHROMA SPIN-200 column (Clontech, Palo Alto, CA), as recommended by the manufacturer. The human Atlas cDNA expression array was prehybridized using Express Hyb (Clontech) at 68°C for 1 hr and hybridized overnight at 68°C with the denatured and neutralized  $^{32}\text{P}$ -labeled cDNA probes. The membrane was washed three times with 2  $\times$  sodium chloride and sodium citrate (SSC), 1% SDS at 68°C for 30 min each, and twice with 0.1% SSC, 0.5% SDS at 68°C for 30 min each. The Atlas array was then exposed overnight to X-ray film or for 6 hr to a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA) plate. All data shown here were derived solely from the first use of each microarray blot.

**IMAGING AND QUANTITATION OF THE cDNA ARRAY.** Atlas cDNA array blots were assayed on a Storm PhosphorImager using proprietary PhosphorImager screens. Each blot was exposed for 6 hr. The intensity of each grid point, at a resolution of 200  $\mu\text{m}$ , was quantified, including the ubiquitin standard and the blanks. The intensity data were then loaded into a Microsoft Excell spreadsheet. To determine the absolute density, each grid point was corrected by subtraction of the background and divided by the intensity of the ubiq-

uitin gene, a standard stable housekeeping gene present on each blot. Thus each data point, in duplicate, could be compared quantitatively to identical points on duplicate blots. Accuracy of identical samples on duplicate cDNA targets on the same blot was  $\sim 1.3\%$ . The average interblot accuracy for independently prepared duplicate samples on duplicate cDNA targets was estimated to be at least 12%. Statistically significant changes were in excess of these errors. To further validate the analysis, the data were also independently evaluated using the PSCAN program for point identification and the JMP program for graphical organization as described by Munson and colleagues (<http://abs.cit.nih.gov>). Each experiment was assayed at least in duplicate, as indicated.

**DATA MINING USING THE GENE RATIO ANALYSIS PARADIGM (GRASP).** The analysis of the effects of a specific CFTR mutation on global gene expression is performed as follows. Orthogonal graphs are prepared by plotting the ratios of specific gene expression in  $\Delta F508$ -CFTR-transfected cells to expression in parental cells on the vertical axis against the equivalent ratio for the same gene in wild-type CFTR-expressing cells on the horizontal axis (see Fig. 2a for an example). For each graph, we calculate the averages and the standard deviations of the distribution of the logarithms of the individually measured gene expression ratios on the horizontal and vertical axes. We also perform this calculation for the differences between the logs of each pair of ratios of all genes (see Figs. 2b–d for examples). The criterion of being more than 1 standard deviation (1 SD) away from the respective average is chosen as an index of those genes in parental cells whose expression levels had been changed by the transfection of either wild-type or  $\Delta F508$ -CFTR. We use a 2 SD criterion for determining that a change is significant. The SD values are calculated separately for expression ratios between wild-type or mutant cells relative to parental cells, or for differences in expression level ratios between wild-type and mutant cells. Using the SD as a discriminator in this way is one of several novel contributions of the GRASP approach to mining gene expression data. Note that the genes whose relative expression levels occur on the diagonal are those genes whose expression levels are mutation independent, or are simply related to transfection, per se.

The second step in the GRASP data mining approach is to represent the effects of drugs or

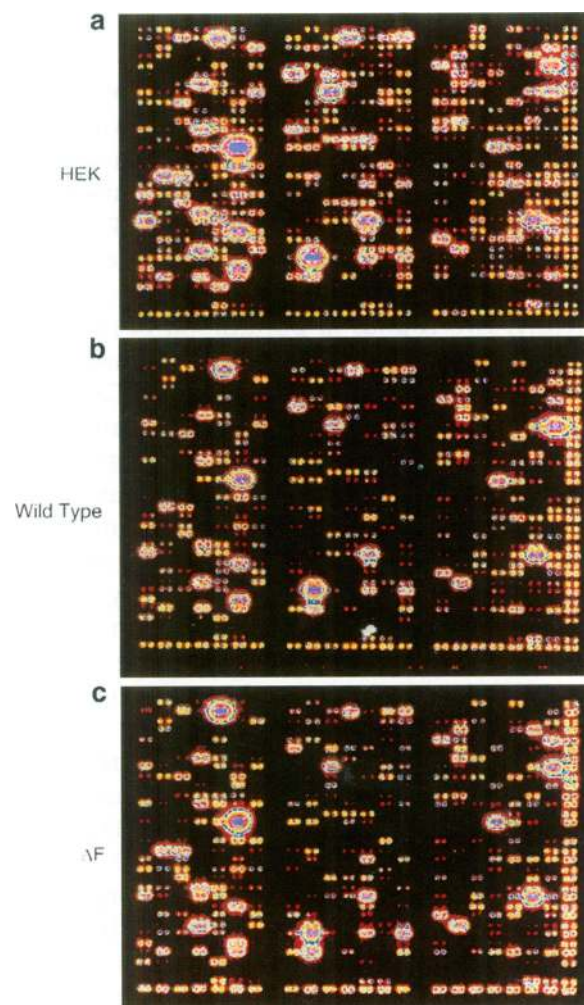
genes as a vector in genomic space. We define here a genomic vector as originating on the location specified by the gene's expression levels in the absence of drug, and terminating at the location specified by the gene's expression levels in the presence of the drug. The length of this genomic vector is a measure of the efficacy of the drug's effect on the expression of that particular gene. The angle is related to the differential effect of the drug depending on the mutational state of CFTR. For example, the vectors of those genes for which the effects of CPX on their expression level are not specifically dependent on the CFTR mutation state have angles close to the main diagonal (i.e.,  $\sim 45^\circ$  in the positive direction or  $\sim 225^\circ$  in the negative direction). Conversely, the vectors for the genes whose expression is significantly dependent on the mutational status of CFTR are sorted to the 2nd quadrant ( $90 \leq \theta \leq 180^\circ$ ) or to the 4th quadrant ( $270 \leq \theta \leq 0^\circ$ ). Vector lengths can be discriminated by their number of SDs from the average length (see Fig. 4 for an application). We emphasize here that in order to prepare these vectors, a common basis for comparison is needed for mutant and wild-type CFTR expressing cells, with or without CPX. Expression by the parental cell line is thus the only common basis available.

## Results

### *Differential Effects of Recombinant Wild-Type CFTR and $\Delta F508$ -CFTR on Global Gene Expression in HEK293 Cells*

The molecular consequences of wild-type or  $\Delta F508$ -CFTR genes for global gene expression in a human epithelial cell line are shown in Figure 1. This figure shows a comparison among cDNA arrays of parental cells (Fig. 1a), transfected with either wild-type CFTR (Fig. 1b) or  $\Delta F508$ -CFTR (Fig. 1c). Using a statistically weighted graphical inspection approach, we have been able to detect those genes whose expression levels change specifically and significantly upon transfection of either wild-type CFTR or  $\Delta F508$ -CFTR, and to sort them quantitatively from those genes whose expression levels remain essentially the same as in the parental cell controls.

The results of this search are shown in Figure 2a, in which ratios of expression levels of all 588 mRNAs are plotted using respective data from all three cell types. In the figure, the horizontal axis is the ratio of expression levels of

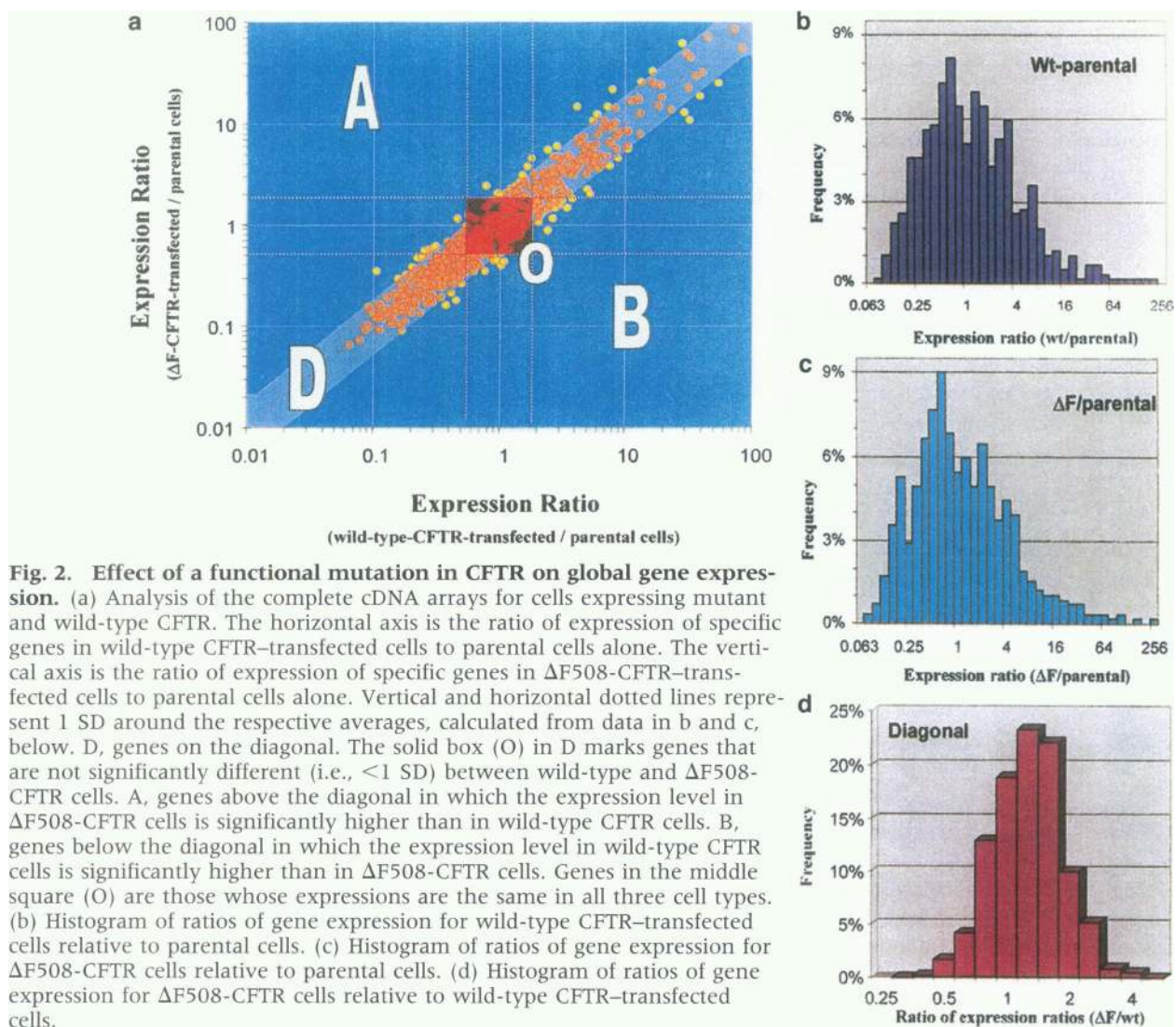


**Fig. 1.** cDNA array analysis of wild-type and  $\Delta F508$ -CFTR transfected HEK293 cells. (a) cDNA array of mRNA from parental HEK293 cells, used to derive wild-type and  $\Delta F508$ -CFTR-transfected cells in b and c. (b) cDNA array of mRNA from HEK293 cells transfected with wild-type CFTR. (c) cDNA array of mRNA from HEK293 cells transfected with  $\Delta F508$ -CFTR.

given genes in wild-type CFTR-transfected cells relative to parental cells alone. The vertical axis in this figure is the ratio of expression of the same genes in  $\Delta F508$ -CFTR-transfected cells relative to parental cells alone. As described in Materials and Methods, SDs have been calculated from the distributions of ratios of gene expression. Histograms of these ratios are explicitly shown for wild-type CFTR relative to parental cells (Fig. 2b),  $\Delta F508$ -CFTR relative to parental cells (Fig. 2c), and  $\Delta F508$ -CFTR relative to wild-type CFTR cells (Fig. 2d).

The first step in this data-sieving operation is to eliminate all genes whose expression ratios differ from 1.0 by  $< 1$  SD in both transfected cells.





The genes in this category are shown in the center box O of Figure 2a. Their removal results in the elimination of 340 (58%) of total genes in the array. The interpretation to be placed on these genes is that they are not significantly affected by either the transfection process or by the mutational state of CFTR. However, importantly, the remaining 248 genes (42% of the total) are affected by our experimental interventions, and it is therefore necessary to distinguish between the two root causes of expression variation.

To this end, we start by finding those genes whose expression is unaffected by the mutational state of CFTR. These genes have expression ratios for wild-type or mutant CFTR that differ by  $<1$  SD from each other, and reside along the diagonal, D, in Figure 2a. These genes sense the fact of transfection but are not affected

by the mutational and/or functional state of CFTR. This results in the elimination of an additional 153 genes (26%). The remaining 95 genes (16%) are those whose expression levels change by  $>1$  SD because of the mutational status of CFTR. These latter genes belong to two classes, distinguished by whether they are above (A) or below (B) the diagonal (D). We can discriminate even further within this group by selecting a subset of 26 genes (4.3%) whose expression is significantly changed by  $>2$  SDs from the norm (see Table 1).

The classes of differences among these remaining 26 genes can be distinguished on the basis of which domain of the graph the differences are found (viz., A or B), and how close a gene is to one axis relative to another. For example, of these 26 genes, 12 are in the vi-

**Table 1. Effect of a functional mutation in CFTR on global gene expression**

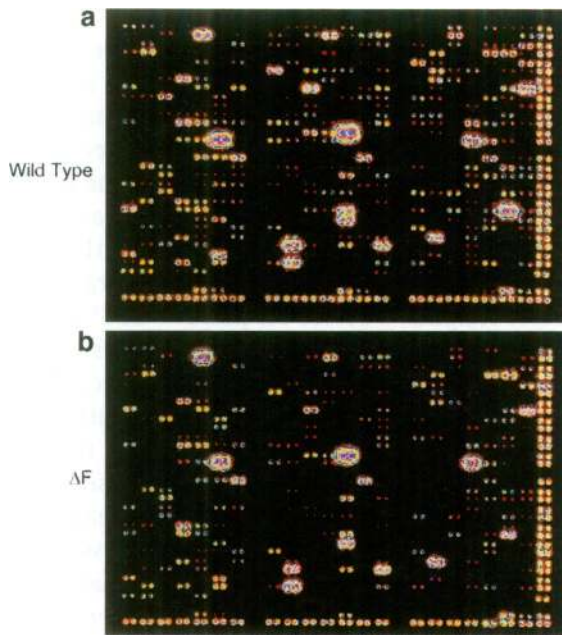
Location	Name of Gene	Expression Level <sup>a</sup>			Significance	S	Gene-Bank Accession No.
		HEK	wt	$\Delta F$			
Close to the y-axis ( $\Delta F$ changes while wt ~ parental)							
E2n	Interleukin-6 receptor	0.74	0.77	2.42	***	3.0	M20566
D3b	DNA binding protein (DB1)	10.79	7.96	21.58	**	2.6	D28118
E3m	Cytokine receptor (EB13)	0.60	0.46	1.16	**	2.5	L08187
A7l	Cell cycle control protein (C-1)	1.59	1.65	3.77	**	2.2	U41816
D4j	DNA-binding protein (NF-E1)	27.40	28.05	60.83	**	2.1	M76541
A4c	Breast cancer susceptibility (BRCA2)	0.43	0.57	1.22	**	2.0	U43746
E1j	PDGF-alpha receptor	1.98	2.43	0.88	**	2.7	M21574
E5f	Lymphocyte activation antigen CD30	3.96	5.32	2.03	**	2.6	M83554
A7e	Cell cycle control protein Tob	1.43	1.02	0.39	**	2.5	D38305
B7m	Heat shock protein (HSP 70)	0.47	0.37	0.16	**	2.3	M11717
E1k	PDGF-beta receptor	5.95	5.04	2.22	**	2.2	M21616
E7n	Semaphorin (CD100)	1.33	0.96	0.44	**	2.1	U60800
Close to the x-axis (wt changes while $\Delta F$ ~ parental)							
E3n	Interleukin 12 receptor component	0.26	0.11	0.34	***	3.1	U03187
D7a	GATA-3 transcription factor	3.92	1.81	4.59	**	2.5	X55122
E2j	NGFR-related B-lymphocyte activation molecule	14.21	32.25	11.00	**	2.9	X60592
F4e	Connective tissue growth factor	0.27	0.45	0.18	**	2.5	M92934
F5d	Neutrophil-activating peptide ENA-78	0.58	1.08	0.43	**	2.5	X78686
C6g	Replication factor C 40-kDa subunit (A1)	0.71	2.64	1.15	**	2.2	M87338
Distant from both x- and y-axes							
A5c	AXL tyrosine kinase receptor	1.52	4.16	14.67	***	3.3	M76125
A6j	Cyclin H	1.18	2.41	5.52	**	2.2	U11791
C6c	ATP-dependent DNA helicase II 70-kDa subunit	0.57	0.95	2.07	**	2.1	M32865
F4a	Growth/differentiation factor GDF-1	0.37	0.26	0.55	**	2.0	M62302
D6i	HOX A1 homeodomain protein (HOXA1)	2.50	3.43	1.45	**	2.3	U10421
D6l	DNA binding protein (ard-1)	37.06	54.18	25.42	**	2.0	U14575
B1f	Coagulating factor II receptor	18.55	29.24	13.01	**	2.1	M62424
F7n	Beta-preprotachykinin	0.59	0.76	0.34	**	2.1	X54469

HEK, HEK-293, human embryonic kidney cell line; wt, wild type; S, standard deviation.

<sup>a</sup>Relative to average expression level of all 588 genes.

cinity of the y- (vertical) axis. These genes in the vicinity of the y-axis are located in the space enclosed between the positive y-axis,  $\pm 1$  SD, and above the  $\pm 1$  SD diagonal; and in the space enclosed between the negative y-axis,  $\pm 1$  SD, and the  $\pm 1$  SD diagonal, but below the

1 SD line on the x-axis. These 12 genes are preferentially expressed in  $\Delta F508$ -CFTR cells. However, in wild-type CFTR cells, their expression levels are not significantly different from expression of these same genes in the parental cells. Thus these genes would appear

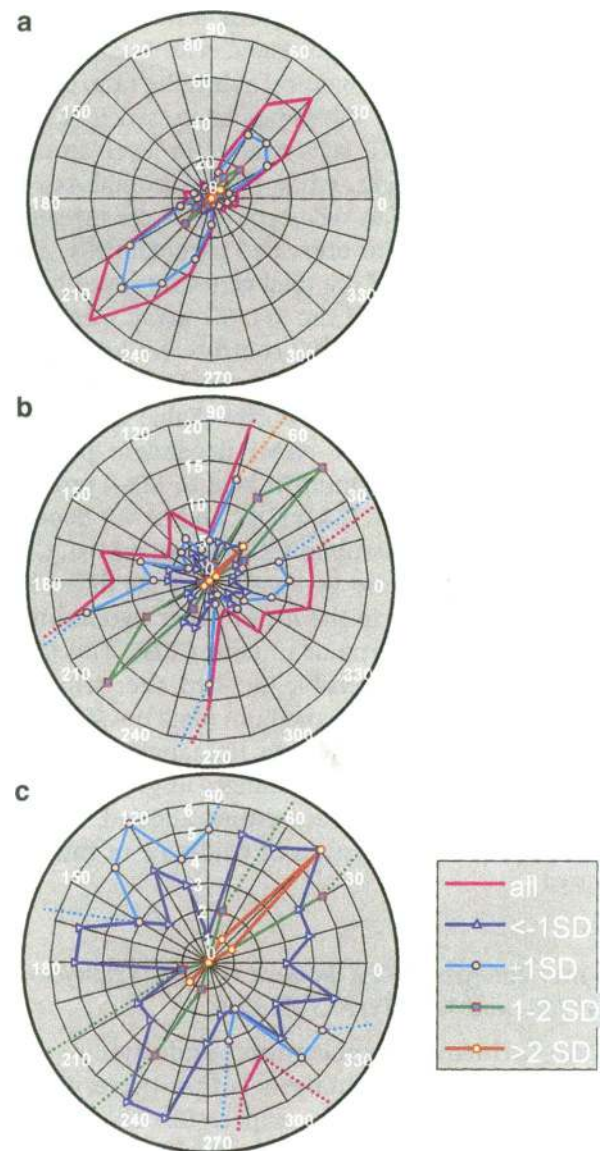


**Fig. 3. cDNA array analysis of wild-type and  $\Delta F508$ -CFTR cells treated with CPX.** (a) cDNA array of mRNA from wild-type CFTR cells treated with 20  $\mu\text{M}$  CPX. (b) cDNA array of mRNA from  $\Delta F508$ -CFTR cells treated with 20  $\mu\text{M}$  CPX.

to be reporting on the consequences for them of a dysfunctional  $\Delta F508$ -CFTR. Six of the 26 genes are in the vicinity of the x- (horizontal) axis. These genes are preferentially expressed in wild-type CFTR cells. However, in  $\Delta F508$ -CFTR cells, their expression levels are not significantly different from expression of these same genes in the parental cells. Thus these genes would appear to be reporting on consequences for them of having a functional wild-type CFTR in the cell. Finally, eight genes are away from both axes and outside the 2 standard deviation limit from the diagonal. Expression of these latter genes are differentially affected by both wild-type and mutant CFTR. The names and classification of these genes are given in Table 1. At the simplest level of interpretation, these data mean that “gene therapy” with wild-type CFTR makes a difference for many more genes than just CFTR, and that the mutational state of the transfected CFTR has its own specific consequences for global gene expression.

#### *Effects of CPX on Global Gene Expression by Cells Expressing Either Wild-Type or $\Delta F508$ -CFTR*

CPX was administered for 48 hr to both  $\Delta F508$ - and wild-type CFTR-transfected cells, and analytic



**Fig. 4. Radial histogram of the effect of CPX on global gene expression of wild-type and  $\Delta F508$ -CFTR-transfected cells.** (a) Low-resolution radial distribution plot of CPX induced changes in gene expression. Angles are directions in orthogonal genomic space in which ratios of gene expression were changed by exposure to CPX. The radius represents numbers of genes in that category. The colors represent all genes (red); genes whose vector length is changed by  $<1$  SD from the average (blue); genes whose vector length is changed by 1 SD from the average (yellow); genes whose vector length is changed by 1–2 SDs (green); and those changed by more than 2 SDs (brown). (b) Higher-resolution radial distribution plot of CPX-induced changes in gene expression. (c) Highest-resolution radial distribution plot of CPX-induced changes in gene expression.

cDNA microarrays were prepared (Fig. 3a,b). Even by eye, substantial, differential changes can be seen. A quantitative analysis of these data was then



performed in a manner identical to that developed in Figure 2 to analyze untreated cells. For the new plot (not shown), the vertical axis is the ratio of the expression level of the genes expressed in CPX-treated  $\Delta F508$ -CFTR cells relative to levels in untreated parental cells, while the horizontal axis is the ratio of levels in wild-type CFTR cells treated with CPX relative to untreated parental cells. Approximately 10% of the total genes surveyed have expression levels that are  $>1$  SD away from the  $\Delta F508$ - or wild-type CFTR controls, while only 28 genes (4.8%) are expressed at levels of 2 or more SDs from their respective norms (Table 2). Prominent among these are cAMP-dependent protein kinase regulatory subunit RI-beta (6 SDs), protein tyrosine kinase (JAK1, 4.9 SDs), and cAMP-dependent protein kinase subunit RII-beta (2.4 SDs). Curiously, many of the genes that are modified by CPX fail to appear in the list of genes significantly modified by wild-type or mutant CFTR. The CPX effect is virtually reversible in 24 hr after removal of CPX from the culture medium (data not shown). A principal conclusion from this analysis is that CPX can elicit substantial changes in gene expression from genes that are either affected or not affected by the expression of wild-type or mutant CFTR.

#### *Vector Analysis of CPX-Induced Genomic Changes in $\Delta F508$ - and Wild-Type CFTR Cells*

One principal expectation underlying this pharmacogenomic analysis is that there might exist a pattern of gene expression that could signal whether a given drug might be a therapeutic candidate. At the simplest level, one might ask what genes were caused by CPX to move from an "aberrant" expression level in  $\Delta F508$ -CFTR cells to one more characteristic of that gene's expression in wild-type CFTR cells. Logically, a set of such genomic markers might be useful in predicting whether a specific drug might be of therapeutic use; conversely, the possibility of unwanted side effects might also be discerned. To test this hypothesis, our strategy has been to view graphs such as that shown in Figure 2a as "genomic space," before and after CPX treatment, respectively. This allows the construction of a drug-related "difference vector" between the location of the data point in the absence of CPX and the location in the presence of CPX (see Materials and Methods for more details).

A low-resolution radial histogram of the angular distribution of these difference vectors is

shown in Figure 4a. The labels on the circumference of the circle represent the angles of the vectors from the horizontal axis. The radius of the circle represents the numbers of genes at that angle. The given angle is that direction in genomic space (see Fig. 2) into which the relative gene expression moved when CPX was added. The colors delineate genes whose lengths are at different SDs from the logarithmic average of all vector lengths. Vectors on the  $45^\circ$ - $225^\circ$  axis are those genes whose expression levels change equally, positively, or negatively, on account of the effect of CPX on cells expressing either mutant or wild-type CFTR. For example, the gene for the cAMP-dependent protein kinase regulatory subunit RII-beta (see Table 2) appears at the  $45^\circ$  angle because both mutant and wild-type CFTR cells responded equally and dramatically to CPX by increased expression of that gene. Gene vectors on the  $45^\circ$ - $225^\circ$  axis are thus sorted by definition into the 1st quadrant ( $0^\circ < \theta < 90^\circ$ ) or 3rd quadrant ( $180^\circ < \theta < 270^\circ$ ), respectively. Genes with the longest drug vectors (viz., 1-2 or  $>2$  SD) are almost entirely along the  $45^\circ$ - $225^\circ$  axis, while those with drug vector lengths  $<1$  SD are distributed randomly about the center (see Fig. 4c, blue line). The latter vectors represent the noise in the system.

By contrast, the higher-resolution histograms (see Fig. 4b,c) show smaller numbers of vectors whose angles fall into the 2nd quadrant ( $90^\circ \leq \theta \leq 180^\circ$ ) or the 4th quadrant ( $270^\circ \leq \theta \leq 360^\circ$ ). The importance of a gene vector being sorted into the 2nd or 4th quadrant is that these are the genes whose response to CPX is mutation dependent. The distribution of vectors of average length (i.e., those that are within 1 SD of the logarithmic mean) are colored cyan in the histogram, and show a significant population in the 2nd and 4th quadrants. This population consists of 69 genes (12%). As an alternative to simply listing all 69, we have selected a subset of these genes (see Table 3) that have been shown above to be changed significantly ( $\geq 2$  SDs) by either mutation or CPX.

As shown in Table 3, the advantage of data mining using drug vector analysis is that the vector angle allows us to distinguish between CPX effects that are either predominantly on mutant cells, predominantly on wild-type cells, or on both mutant and wild-type cells but in opposite directions. As an example of the latter situation in the 2nd quadrant, changes in the  $120^\circ$ - $150^\circ$  range delineate genes (e.g., BRCA2) in which CPX exposure leads to an expression increment in mutant CFTR cells but a



**Table 2. Effects of CPX on global gene expression in wild-type and  $\Delta F508$ -CFTR cells**

Location	Name of Gene	Expression Level <sup>a</sup>					Significance	S
		HEK	wt	$\Delta F$	wt + CPX	$\Delta F$ + CPX		
B5m	PKA regulatory subunit R1 $\beta$	5.78	2.44	2.56	203.3	234.8	***	6.0
B5l	Protein-tyrosine kinase (JAK1)	0.39	0.19	0.19	10.42	8.20	***	4.9
B5n	Extracellular signal-regulated kinase 2	2.21	1.24	0.77	18.89	27.82	***	4.7
B6m	MAPKAP kinase (3pK)	0.40	0.18	0.11	4.16	2.42	***	4.1
B6n	Receptor tyrosine kinase ligand LERK-3 (EPLG3)	0.30	0.12	0.12	2.40	1.65	***	3.4
D4j	DNA-binding protein (NF-E1)	27.40	28.05	60.83	3.75	4.64	***	3.4
B7k	Heat shock protein hsp86	0.31	0.17	0.19	1.76	2.41	***	3.3
E5h	Intercellular adhesion molecule-1 (ICAM-1)	4.70	3.51	3.92	34.72	42.56	***	3.1
B4d	Active transcription factor CREB	0.61	0.29	0.29	1.31	2.57	**	2.9
D3b	DB1	10.79	7.96	21.58	2.18	2.50	**	2.8
F6b	Interleukin-6 (IL-6) precursor	1.19	0.39	0.43	5.73	3.07	**	2.6
A1a	<i>c-myc</i> oncogene	0.56	0.26	0.30	1.91	2.03	**	2.5
F2n	Macrophage inflammatory protein GOS19-1	0.09	0.07	0.07	0.39	0.43	**	2.5
B5k	cAMP-dependent protein kinase subunit RII-beta	0.24	0.16	0.16	1.32	0.95	**	2.4
C5d	CD40 receptor associated factor 1 (CRAF1)	0.53	1.11	1.98	8.84	11.80	**	2.4
D2b	Basic transcription factor 44 kDa subunit	19.53	16.11	25.90	6.19	4.40	**	2.3
F3h	Nerve growth factor HBNF-1	1.57	2.92	4.64	0.66	0.81	**	2.3
D6h	Nuclear factor NF90	41.78	38.40	18.80	5.58	3.41	**	2.3
D2n	ZFM1 protein alternatively spliced product	0.70	0.83	0.67	2.67	3.59	**	2.2
D2a	CCAAT displacement protein cut homolog alt splice 1	4.42	1.41	2.21	0.83	0.43	**	2.2
D4l	Homeobox protein (HOX-11)	1.65	1.57	2.54	0.34	0.51	**	2.1
C3b	Glutathione S-transferase pi	1.51	0.57	0.53	8.34	2.55	**	2.1
F5l	Interleukin-1 alpha precursor (pre IL-1)	0.60	1.02	1.98	0.43	0.42	**	2.0
A1d	<i>c-kit</i> protooncogene	0.44	0.32	0.37	1.11	1.77	**	2.0
C3i	Fas ligand (FASL)	0.98	1.35	2.44	0.37	0.52	**	2.0
B4k	Mutant lymphocyte-specific protein tyrosine kinase (LCK)	0.34	0.14	0.13	0.77	0.59	**	2.0
C2k	Retinoid X receptor beta (RXR-beta)	7.51	5.51	5.88	1.96	1.27	**	2.0
F2l	Bone morphogenetic protein 2a	0.25	0.18	0.32	1.14	1.41	*	2.0

HEK, HEK-293, human embryonic kidney cell line; wt, wild type; S, standard deviation.

<sup>a</sup>Relative to average expression level of all 588 genes.

decrement in wild-type CFTR cells. The opposite is the case in the 4th quadrant for genes in the 300°–330° range. We are careful to emphasize that without further information we cannot, a priori, know which specific angles are of potential therapeutic relevance.

## Discussion

The data presented here clearly demonstrate that the cellular expression of either wild-type or  $\Delta F508$ -CFTR has global genomic consequences, the nature of which depends on the mutational

**Table 3. Candidate surrogate endpoint genes responding<sup>a</sup> to CPX as a function of either mutant or wild-type CFTR<sup>b</sup>**

Location on Blot	Name of Gene	Angle of CPX Vector <sup>c</sup>	Expression Level <sup>c</sup>				
			HEK	wt	$\Delta F$	wt + CPX	$\Delta F$ + CPX
<i>2nd quadrant genes</i>							
CPX predominantly affects $\Delta F$							
A3l	Retinoblastoma susceptibility	90	1.04	0.40	0.41	0.45	1.38
A5n	p55CDC	90	15.52	7.79	9.86	7.80	25.06
F7n	Beta-preprotachykinin	90	0.59	0.76	0.34	0.73	0.72
C6i	DNA polymerase alpha-subunit	105	0.75	2.18	1.31	1.50	3.56
CPX affects wt and $\Delta F$ oppositely							
D4h	Transcriptional enhancer factor (TEF1) DNA	120	0.57	0.53	0.82	0.40	1.48
A4b	APC	120	2.28	1.89	3.17	0.86	9.30
A4c	Breast cancer susceptibility (BRCA2)	135	0.43	0.57	1.22	0.41	1.73
A4a	fgr protooncogene encoded p55-c-fgr protein	135	0.97	1.23	1.53	0.78	2.25
CPX predominantly affects wt							
D4g	Transcription factor ETR101	180	2.78	6.07	12.45	3.33	12.44
A5b	DP2 dimerization partner of E2F	180	29.41	103.8	153.5	40.9	155.4
A1h	v-erbA related ear-2 protein	180	1.63	6.82	8.97	2.48	9.01
E1j	PDGF-alpha receptor	180	1.98	2.43	0.88	0.75	0.86
D6i	HOX A1 homeodomain protein (HOXA1)	180	2.50	3.43	1.45	0.86	1.24
<i>4th quadrant genes</i>							
CPX predominantly affects $\Delta F$							
F6h	Interleukin-14 (IL-14)	270	2.12	5.67	3.92	6.00	1.98
F6g	Interleukin-13 (IL-13)	285	40.68	73.34	84.72	84.11	35.14
CPX affects wt and $\Delta F$ oppositely							
A7l	C-1	330	1.59	1.65	3.77	2.34	3.18
CPX predominantly affects wt							
E6g	Contactin 1 precursor (CNTN1)	345	1.56	0.71	0.71	1.52	0.62
D2f	DNA-binding protein TAXREB67	360	1.48	1.43	1.52	4.04	1.65

<sup>a</sup>Relative to average expression level of all 588 genes.

<sup>b</sup>Genes having differences  $\geq 2$  SD from any of the respective means.

<sup>c</sup>All these CPX genomic vectors are within 1 SD from the average vector length.

wt, wild type; HEK, HEK-293, human embryonic kidney cell line.

status of the CFTR gene. This is a remarkable and somewhat unexpected finding in view of the implicit assumption that gene transfer, as might occur in gene therapy of the wild-type CFTR gene, ought only to have the consequence of increasing the level of functional CFTR protein in the target cell. Instead, we find here that many changes occur, including several which are mu-

tation dependent. However, the significance of individual changes in gene expression will only be discerned as we employ ever-larger genome arrays, and especially as we query additional CF cells and tissues.

A further exceptional finding is that administration of the CF drug CPX has mutation-dependent and mutation-independent conse-

quences for global gene expression. Using our novel approach of genomic drug vectors, we have been able to seive out a group of 69 genes (12% of the total), which, in the presence of CPX, are induced to move significantly and differentially depending on the mutational status of CFTR. In the absence of more biological information we cannot discern which directions are better or worse, since any of these may have positive or negative effects on the physiology of the CF cell. The list in Table 3 is a subgroup of those 69 genes selected on the basis of having a difference of >2 SDs from any one of the respective means. We note that this list overlaps only occasionally with those genes known on the basis of the present study to be associated with expression of mutant or wild-type CFTR alone (cf. Table 1). In the future, when more data on the physiological sequellae of overexpression or underexpression of each gene become available, it may become possible to use this approach to predict the consequences of different potential drug or gene therapies. It might even be possible to do this on an individual patient basis.

It is perhaps not so unexpected that CPX might also induce changes in expression of certain genes in a mutationally independent manner. However, the magnitude of some of these changes, ranging up to 6 SDs, is truly exceptional. In this category we find two PKA regulatory subunits and one tyrosine kinase. In principal, the induction of these genes by CPX might be important for CF. For example, PKA is necessary to activate CFTR channels. However, tyrosine kinases can also activate CFTR channels independently of PKA (results from many investigators summarized in ref. 51). On the other hand, it is also possible that some consequences of mutation-independent CPX action could be due to drug action on classical adenosine receptors. However, the parental HEK293 cells have only low levels of A<sub>1</sub> receptors (52), and CPX is widely reported to have antiproliferative effects on those epithelial and other cell types where the A<sub>1</sub> receptors do occur (53–55). Therefore, discerning whether these CPX-dependent effects are therapeutically wanted or unwanted in terms of repair of specific CFTR functions will require much further study.

Another interesting aspect of these results is that of those genes shown by our present work to be significantly changed by CFTR mutation or by CPX, surprisingly few have been historically associated with CF. The Atlas microarray is far from complete with regard to CF. However, it

does contain at least 25 genes that have been thought at one time or another to be associated with CF, and we thought it would be of interest to summarize the data for them in Table 4. Indeed, most of these genes are not affected by the mutation, except for HSP70, which is reduced, and muc18 (a mucin) and IL10, which are increased. By contrast, in the presence of CPX, many of these genes are increased. The most profound among these include PKA regulatory subunits; the tyrosine kinase JAK1; AXL tyrosine kinase; a panepithelial glycoprotein; HSP60, HSP70, and HSP86; MUC18; caspase9; IL6; and, marginally, the A<sub>1</sub>-adenosine receptor. Presumably, with further study of larger arrays and under different conditions, it will be possible to identify a more complete set of CF-relevant reporter genes, and to construct a CF chip or blot suitable for CF drug discovery. Since we are presently analyzing only 588 of the 100,000 potential genes in the human genome, we can extrapolate by proportion that there could be as many as 5000 CF-relevant genes.

One advantage of the GRASP approach to accurate data seiving is that the intrinsic high number of genes in each blot can be employed in concert to profoundly raise the statistical strength of the analysis. On the basis of our own analysis of the ATLAS system in this series of experiments (see Materials and Methods for statistics), data from individual cDNA targets could just as easily been used. However, the problem with this simple approach is that the number of genes that are observed to change expression in a mutation- or drug-dependent manner becomes very large. Then, to determine whether a specific change, no matter how large or small, is truly significant, a large number of experiments must be performed. Given the current cost of an individual blot, this ceases to be a viable option. However, by asking, instead, whether any specific change is greater than the SD calculated for the entire sample, we take into account the entire sample when deciding on the significance of the specific change. This is the basis of the GRASP genome seiving technique.

There is also an additional advantage to presenting the expression data as a ratio to the same gene expressed in the parental cell line. The parental cell line in the denominator is genetically very similar to the cells in the numerator, except for the transfected CFTR gene. The parental cell thereby provides a biologically relevant normalization factor. We might point out here that such a specific comparison is intrinsically difficult to

**Table 4. Effects of CFTR mutation and CPX on expression of available CF-relevant genes**

Location	Name of Gene	Expression Level <sup>a</sup>					CPX Vector	
		HEK	wt	$\Delta F$	wt + CPX	$\Delta F$ + CPX	Length (S) <sup>b</sup>	Angle
Kinases and phosphatases								
B5l	Protein-tyrosine kinase (JAK1)	0.39	0.19	0.19	10.42	8.20	2.7	45
B7f	Tyrosine kinase (Tnk1)	1.56	1.27	1.49	1.02	0.98	-0.3	240
B2i	Protein kinase C $\delta$ -type	1.18	0.70	0.49	0.68	0.51	-3.0	135
B5j	Protein kinase C (PKC) type $\beta$ I	0.32	0.16	0.17	0.56	0.44	1.1	30
B5k	PKA subunit RII- $\beta$	0.24	0.16	0.16	1.32	0.95	1.8	45
B5m	PKA regulatory subunit RI- $\beta$	5.78	2.44	2.56	203.33	234.82	2.8	45
B6b	PKA catalytic subunit type $\alpha$	3.39	5.94	6.25	2.78	2.95	0.7	225
B7g	Protein tyrosine phosphatase (CIP2)	0.26	0.24	0.23	0.35	0.27	-0.5	30
A5c	AXL tyrosine kinase receptor	1.52	4.16	14.67	1.54	7.18	0.8	210
B6n	Receptor tyrosine kinase ligand (LERK-3)	0.30	0.12	0.12	2.40	1.65	2.3	45
Heat shock proteins								
B7l	Heat shock protein HSP27	1.83	1.56	1.19	4.50	2.39	0.9	30
B7i	Heat-shock protein 40	1.55	1.56	1.53	1.45	1.15	-0.9	255
B7j	Chaperonin (HSP60)	0.64	0.47	0.47	1.06	1.11	0.8	45
B7m	Heat shock protein (HSP 70)	0.47	0.37	0.16	0.81	0.52	1.0	60
B7k	Heat shock protein hsp86	0.31	0.17	0.19	1.76	2.41	2.1	45
Adenosine receptors								
C1h	A2a adenosine receptor	0.53	0.68	0.53	0.55	0.43	-0.9	225
C1i	A1 adenosine receptor	3.01	3.22	2.27	2.17	1.06	0.4	240
C1j	A3 adenosine receptor	0.56	0.47	0.37	0.71	0.50	-0.3	30
Inflammatory								
F2j	RANTES pro-inflammatory cytokine	9.30	32.49	45.30	23.54	51.80	-0.7	150
F6b	Interleukin-6 (IL-6) precursor	1.19	0.39	0.43	5.73	3.07	2.0	30
F6f	Interleukin-10 (IL-10)	4.16	4.64	7.42	5.02	2.59	0.6	270
Transcription factors								
D2g	cAMP-responsive element-binding protein	1.17	1.28	1.10	0.70	0.67	0.3	225
B3a	NF $\kappa$ B transcription factor p65 subunit	0.56	0.39	0.33	0.79	0.62	0.5	45
Miscellaneous								
A5i	MUC18 glycoprotein	0.59	0.27	0.50	0.45	1.17	0.6	60
C4l	Caspase-9 precursor	1.50	1.07	1.27	8.36	4.76	1.7	30

HEK, HEK-293, human embryonic kidney cell line; wt, wild type; S, standard deviation.

<sup>a</sup>Relative to average expression level.

<sup>b</sup>Length of vector expressed as number of S above (positive) or below (negative) the average length.

realize with human tissue samples because there is usually no "parental" cell population available for analysis.

Finally, from a drug development vantage point, another important advantage to the

GRASP approach is that the genomic vector approach yields a radial distribution useful for discerning changes that are specific either to the drug or to the mutation. This approach emphasizes movements directed toward or away from



the wild-type expression phenotype. These types of vectors, when taken as a group, thus constitute a quantitative, dynamic form of cluster analysis (56) that has substantial advantages for mining genomic data. We therefore believe that this type of analysis may prove to be of fundamental importance for the development of surrogate endpoints for drug discovery, not only in CF but for other genetic diseases as well.

## Acknowledgments

The authors thank Dr. A. Namboodiri, Dr. S. Galdwicky, Dr. L. Fossom, Dr. X. Leighton, and Ms. M. Glasman for substantive help and discussions, and acknowledge financial support from the NIH (RO1-DK53051), the Cystic Fibrosis Foundation, and the Juvenile Diabetes Foundation, International.

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