"Promoter Array" Studies Identify Cohorts of Genes Directly Regulated by Methylation, Copy Number Change, or Transcription Factor Binding in Human Cancer Cells

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ABSTRACT: DNA microarrays of promoter sequences have been developed in order to identify the profile of genes bound and activated by DNA regulatory proteins such as the transcription factors c-Jun and ATF2 as well as DNA-modifying methylases. The arrays contain 3083 unique human promoter sequences from +500 to -1000 nts from the transcription start site. Cisplatin-induced DNA damage rapidly leads to specific activation of the Jun kinase pathway leading to increased phosphorylation of c-Jun and ATF2-DNA complexes at hundreds of sites within 3 hours. Using three statistical criteria, approximately 269 most commonly phosphorylated c-Jun/ATF2-DNA complexes were identified and representative cases were verified by qPCR measurement of ChIP-captured DNA. Expression was correlated at the mRNA and protein levels. The largest functional cohort was 24 genes of known DNA repair function, most of which exhibited increased protein expression indicated coordinate gene regulation. In addition, cell lines of prostate cancer exhibit stable methylation or copy number changes that reflect the alterations of the corresponding primary tumors. 504 (18.5%) promoters showed differential hybridization between immortalized control prostate epithelial and cancer cell lines. Among candidate hypermethylated genes in cancer-derived lines, eight had previously been observed in prostate cancer, and 13 were previously determined methylation targets in other cancers. The vast majority of genes that appear to be both differentially methylated and differentially regulated between prostate epithelial and cancer cell lines are novel methylation targets, including PAK6, RAD50, TLX3, PIR51, MAP2K5, INSR, FBN1, GG2-1, representing a rich new source of candidate genes to study the role of DNA methylation in prostate tu-

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mors. Earlier studies using prototype promoter arrays examine approximately 7% of the proximal regulatory sequences while the current gene regulatory events surveyed here occur on a large scale and may rapidly effect the coordinated expression of a large number of genes.

KEYWORDS: promoter microarray; prostate cancer; breast cancer; DNA repair; methylation; gene regulation profile; location analysis

INTRODUCTION

Knowledge of the genome sequence and the regions of functional promoter sequences have made it possible to identify cohorts of genes that are coordinately regulated during normal cell responses or as part of a disease process. The first step is the fabrication of arrays of known promoter sequences. These arrays can be used to identify the genes corresponding to regulatory sequences that have been isolated from biologically relevant experiments by, for example, chromatin immunoprecipitation (ChIP) or by using a differential digestion strategies.[1,2] In the case of yeast, most of the regulatory sequences occur in the intergeneic regions and arrays containing these sequences provide the identity of any yeast regulatory sequence.[3] In the cases of mammalian cells where the vast majority of noncoding DNA may not be related to regulatory functions, the construction of mammalian promoter arrays had to be restricted to regions where annotation of regulatory function is available. Advances in technology will likely make pangenomic surveying of nearly all regulatory regions of the mammalian genome possible in the near future. Here we review recent experience using a prototype array containing 3083 unique sequences of 1 to 1.5 kB from regions of proximal promoter sequences. The sequences chosen are particularly suitable for the analysis of AP-1 regulated genes and regulation dependent on GC-rich sequences such as those utilized by the Sp1 and Egr1 transcription factor family or regulation by methylation.[1,2,4] The array has been applied to study gene regulation of human breast and prostate cancer cells. We have used a "chIP-on-chip" strategy to examine the regulation of genes of breast cancer cells following genotoxic stress. In addition the arrays have been used to identify genes regulated by methylation or change of copy number in human prostate cancer cells. Although sampling a very limited portion of the genome, the results indicate a versatile method and further indicate a surprisingly large scale nature of gene regulation by diverse mechanisms.

"Chromatin immunoprecipitation" or ChIP refers to methods for the isolation of chromatin specifically bound by a protein of interest by immunoprecipitation using an antibody to that protein. The method utilizes a simple cross-linking step to covalently stabilize DNA-protein complexes which are usually carried out as the first step by briefly treating cells with formaldehyde. The potentially unwieldy mass of crosslinked chromatin created by such a procedure is obviated by treatment of the crosslinked chromatin with restriction enzymes or by sonication, which rapidly produces a relatively uniform population of fragments of protein-DNA complexes that are readily precipitated by conventional immunoprecipitation. For such a relatively simple method, the potential yield of exciting new information about gene regulation is impressive. First, the crosslinking step may be applied directly to living cells and so physically "captures" the proteins interacting with DNA at the site and in the context of the functioning living cell. The information is more direct than by prior ap-

proaches such gel shift assays or even DNAase I mapping methods which require cell disruption. Second, potentially all DNA sites bound by a protein of interest are isolated providing the opportunity for pangenomic analysis. Third, the isolated crosslinked chromatin may be treated to remove either protein or DNA, thereby providing material for analysis of DNA such as identification of the genes of the bound regulatory sequences or analysis of protein such as the determination of activating modifications or determination of co-precipitated proteins. The purified DNA may be used for library preparation and cloning. Perhaps the most informative use is hybridization to arrays of known promoter sequences, "promoter arrays" in order to identify the gene origin of ChIP-captured sequences. The known identity of sites of hybridization on such arrays determines the identity of the gene from which the ChIP-captured DNA was derived. In the case of precipitation of a regulatory protein, this is the identification of all genes whose regulatory sequences were interacting with the factor under the conditions of the living cells at the time of crosslinking.

Formaldehyde-mediated DNA-protein crosslinking was first used by Solomon and Varshavsky[5] as a probe for *in vivo* chromatin structure. The method was adapted for the isolation and determination of numerous individual sequences of the SATB1

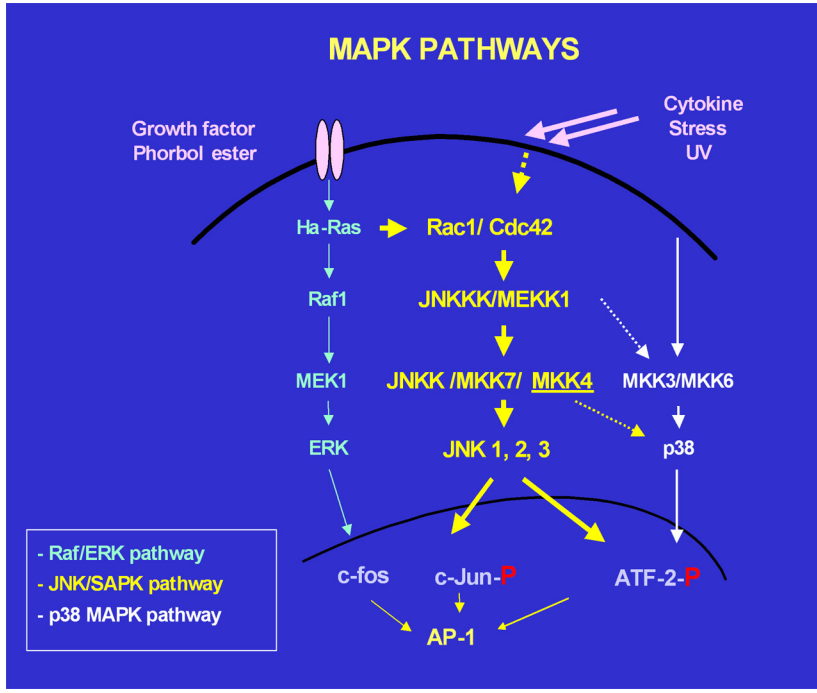

FIGURE 1. Diagrammatic representation of activation of c-Jun and AFT2 by N-terminal phosphorylation of JNK. JNK in turn is activated by phosphorylation as the end result of a kinase cascade of enzymes homology to the MAP/Erk andp38 map kinase pathways.

binding protein[6] and subsequently extended to a means of preparing a library of all DNA fragments bound by a protein of interest.[7] Similar procedures were developed by others[8,9] (for a review see ref. 10).

Mammalian promoter arrays that sample small portions of the human genome have been developed by several workers (e.g., refs. 11–14) and well as in our lab.[1,2,4] Moreover, proximal promoter regions are commonly rich in GC-islands, common sites of gene silencing by methylation, and so promoter arrays may be used to identify sites of methylation.[2,13]

Oligonucleotide array technology is rapidly approaching the point where it will be possible to sample the genome at densities approximately every 10^3 bases (NimbleGen Systems, Inc.) thereby providing the possibility of identifying the vast majority of ChIP-captured sequences. Until that time, array construction must focus on systems of interest. We have developed arrays that contain proximal promoter sequences of most known or suspected AP-1 binding sites or that have GC-rich elements commonly utilized by the Sp1- and Egr1-family of transcription factors as well as many proximal promoter sequences of genes implicated in prostate cancer. Moreover, these sequences are common sites of methylation. These arrays have been used to examine the role of the Jun kinase(JNK)/stress-activated protein kinase pathway and methylation in prostate cancer. The studies indicate that physiologic events are accompanied by rapid and very large scale gene-binding and regulation events involving many hundreds of genes that are under the regulation of a specific signal transduction pathway.

GENE REGULATION BY THE JNK PATHWAY: ANALYSIS OF GENOTOXIC STRESS

JNK in DNA Repair

JNK phosphorylates and activates the transcriptional activities of c-Jun, ATF2, and other transcription factors in response to variety of stresses including DNA damage.[15–18] Genotoxic stress leads to the activation of JNK and this activation has been shown to participate in various responses in different cell systems such as such as apoptosis, cell cycle regulation, enhanced cell survival, and enhanced DNA repair.[19–21] The pathway of activation of JNK following genotoxic stress is not known precisely, but likely involves recognition of DNA damage by a large complexes containing the ATM and ATR kinases which are activated and required for activation of JNK.[22–24] JNK in turn acts on a group of transcription factor substrates such as c-Jun, ATF2, Elk-1 and others by phosphorylation of N-terminal serine and threonine residues (FIG. 1). C-Jun and homologs of the c-Jun family of transcription factors, JunB and JunD, interact with DNA as heterodimers with members of the c-Fos family of transcriptions. c-Jun also interacts with DNA as a homodimer or as a heterodimer with ATF2. Phosphorylation of c-Jun at serine residues 63 and 73 or phosphorylation of ATF2 and threonine residues 69 and 71 greatly enhances the transactivation potential of these factors,[25–27] thereby leading to altered gene expression. We have shown that JNK leads to increased DNA repair of cisplatin-damaged DNA in several human tumor cell lines and that specific inhibition of JNK sensitizes cells to cell killing by cisplatin.[19]

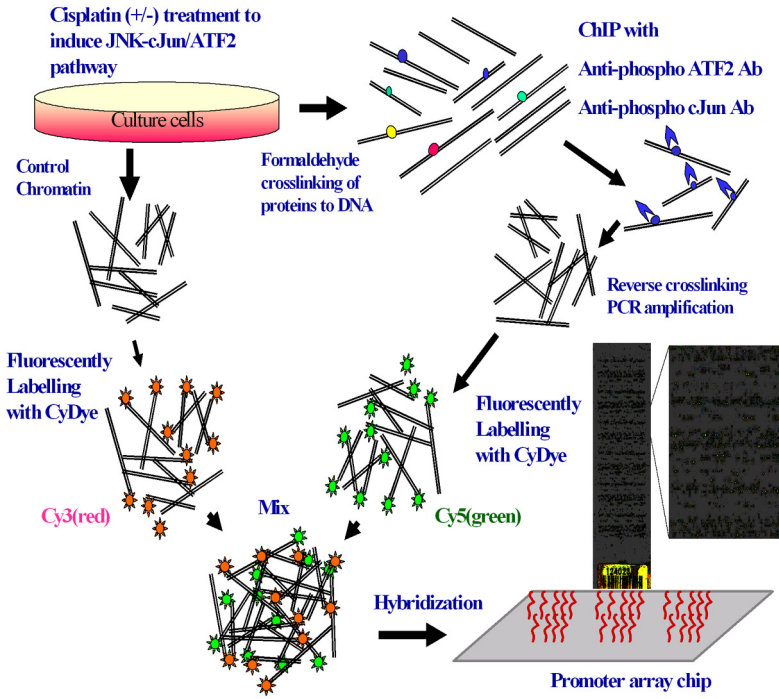

FIGURE 2. Schematic representation of ChIP-on-chip protocol.

Arrays for Identification of JNK-Regulated Genes

Our hypothesis is that this response utilizes the induction of a specific and coordinately expressed cohort of genes which includes genes of DNA damage recognition and repair.[1,28] To test this hypothesis, we have explored the use of ChIP-on-chip as applied to the phosphorylated substrates of JNK. We have used antibodies specific for c-Jun phosphorylated at two of the three N-terminal activation positions, serine residues 63 and 73 and ATF2 phosphorylated at threonine residues 69 and 71. Promoter arrays were used to identify the profile of genes whose promoters formed phospho-c-Jun or phospho-ATF2 complexes. The protocol is summarized in FIGURE 2. Cisplatin is an attractive test agent since the crystal structure of the principal DNA-cisplatin adduct is well defined through crystallographic studies.[29,30] Cisplatin forms intrastrand covalent links between N7 atoms of adjacent purine residues. The isomer, transplatin, is incapable of forming these crosslinks and serves as an excellent control. The cell system first examined by ChIP-on-chip was human breast cancer cells BT474.[1] These cells contain amplified erbB2 gene and greatly overexpress HER2/Neu receptors and, therefore, are a model of aggressive breast cancer. Resistance to DNA-damaging agents is an important mechanism limiting therapy of this form of breast cancer.[31] Indeed, treatment with cisplatin leads to rapid activation of JNK, increased phosphorylation of c-Jun and ATF2, and increased transactivation of reporter constructs within 3–6 hours. We applied ChIP-captured

FIGURE 3. *See following page for legend.*

DNA using both antibodies for phospho-c-Jun or phospho-ATF2 to prototype promoter arrays consisting of nearly 5000 features of which 3083 are unique human proximal promoter sequences. The typical sequence used extends from −1000 to +500 about the transcription start site. We identified approximately 370 genes found in the literature from SAGE, expression analysis, and individual gene studies to be regulated by or suspected as regulated by AP-1 components (see refs. 32–37 and references therein). To determine significant array hybridization intensities, several precautions are necessary. The arrays are printed in triplicate and all experiments are carried out in duplicate and repeated with the order of dyes reversed providing 12 estimates of all intensities. This allows for accurate use of T-tests. Hybridization of the ChIP-captured DNA to the array is carried out in competition with DNA from untreated cells. The hybridization results for "negative control" sequences on the array consisting of plant, viral, and bacterial sources are subtracted from all values. Since array hybridization intensity is assumed to be directly proportional to the amount of a particular sequence of ChIP-captured DNA, to select increases of interest, we use the criteria that all intensities should be at least 1.5× the intensity of binding of DNA from control cells (fold-change > 1.5) and that the T-test yield $P < 0.05$. Moreover, for array hybridization data of many sources it is commonly found that the standard errors are artificially low for small intensities, which enhances their apparent significance. The "B" values of Smyth[38] attempts to correct for this effect and we employ the criterion of $B > 2.5$.

Using this set of three criteria applied to the data for cisplatin-treated cells just 3 h after treatment; we find that there are 269 "significant" gene-binding events (FIG. 3, cisplatin). Very few comparable intensities are observed in transplatin-treated cells and even fewer comparable intensities are observed in mock-treated cells or cells treated with cisplatin but using a control nonimmune serum. Thus the implication of these results is that DNA damage by cisplatin leads to a rapid and large-scale formation of DNA complexes with phosphorylated-c-Jun and ATF2 transcription factors. Approximately 50 of the identified genes are known or suspected AP-1 regulated genes (FIG. 3, red gene names [color appears online only]) whereas the majority are "new" AP-1-regulated gene candidates. Since the array "samples" only 3083 sites, the projection for the entire genome is that cisplatin treatment may lead to a rapid and massive phosphorylation of c-Jun and ATF2 promoter complexes, on the order of 3300 ($269 \times 40{,}000/3083$). It appears important, therefore, to determine that these are specific and valid estimates. The profile may be nearly entirely eliminated by prior addition of the small molecular JNK inhibitor SP600125 (FIG. 3). Moreover, if the cells are treated with a mixture of siRNAs that have been shown to specifically eliminate the synthesis of the major isoforms of

FIGURE 3. Example of identification of genes whose promoters exhibit significantly increased phosphorylation of c-Jun- and ATF2-DNA complexes. Red [color appears online only], gene previously known or suspected to be regulated by API-1. Black, novel candidate genes reported by the promoter arrays as significantly increased in phosphorylation of c-Jun and ATF2-DNA complexes upon stimulation of cells with cisplatin but not transplatin. Note that several genes listed here exhibit one or more manifestation of nonspecific binding as indicated by high promoter array signals in the presence of transplatin, or JNK inhibitor, or mock stimulation. The JNK inhibitor is SP600125. (The conditions are as described in Hayakawa *et al.* 2004.[1])

FIGURE 4. *See following page for legend.*

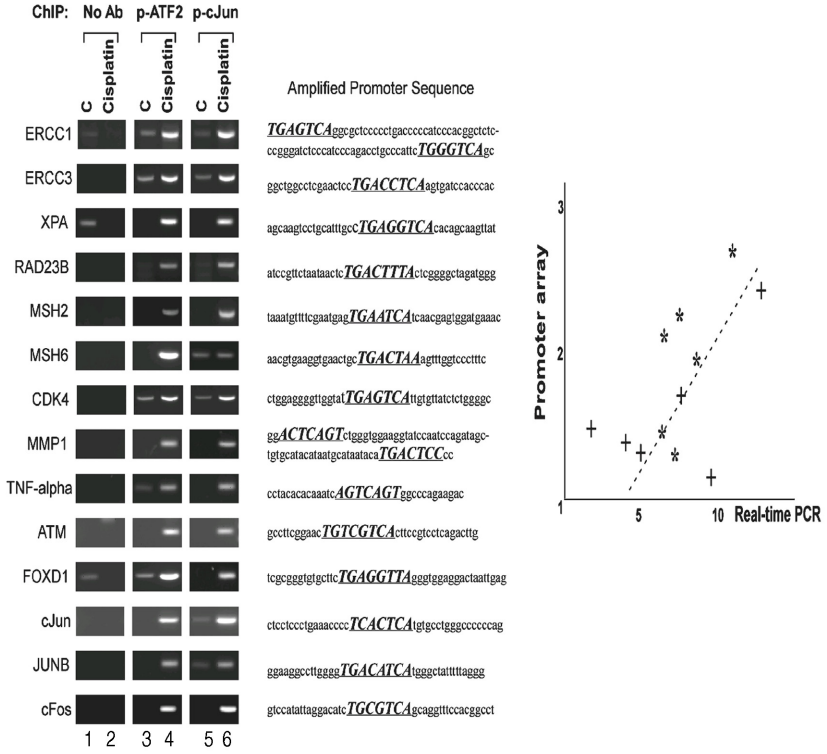

FIGURE 5. Validation of hybridization intensity. *Left*, agarose gel results for semi-quantitative PCR of ChIP-captured DNA for representative reported by the promoter array to be significantly increased in ChIP-captured DNA from cisplatin-treated cells. All the representative genes contain one or more putative c-Jun/ATF2 binding sites (capital letters) and surrounding sequences shown here were amplified. *Right*, Most of the same genes were examined in the ChIP-captured DNA by qPCR and the results are plotted (x-axis) against promoter array intensity (y-axis).

FIGURE 4. Replicate experiment of that shown in FIGURE 3 carried out in parallel with inhibition of JNK-regulated phosphorylation by siRNA $_1$. Red [color appears online only], gene previously known or suspected to be regulated by AP-1. Black, novel candidate genes reported by the promoter arrays as significantly increased in phosphorylation of c-Jun and ATF2-DNA complexes upon stimulation of cells with cisplatin but not transplatin. Note that several genes listed here exhibit one or more manifestation of nonspecific binding as indicated by high promoter array signals in the presence of transplatin, or JNK inhibitor, or mock stimulation. The JNK siRNA inhibition was achieved by prior treatment of the cells for 24 h with a mixture of siRNA designed to eliminate the two major isoforms of JNK (JNK1 and JNK2).[1]

JNK, the cisplatin-stimulated profile is again eliminated (FIG. 4). These experiments argue that the DNA damage profile is specific. To ensure that the intensities reported by the array reflect what is in the ChIP-captured DNA pool, we examined representative genes by semi-quantitative (sqPCR) and quantitative (qPCR) using primers expected to correspond to promoter sequences of the ChIP-captured DNA which supports the validity of the arrays (FIG. 5). This pattern of results supports the conclusion that JNK specifically mediates the rapid and large-scale formation of phosphorylated promoter regulatory complexes.

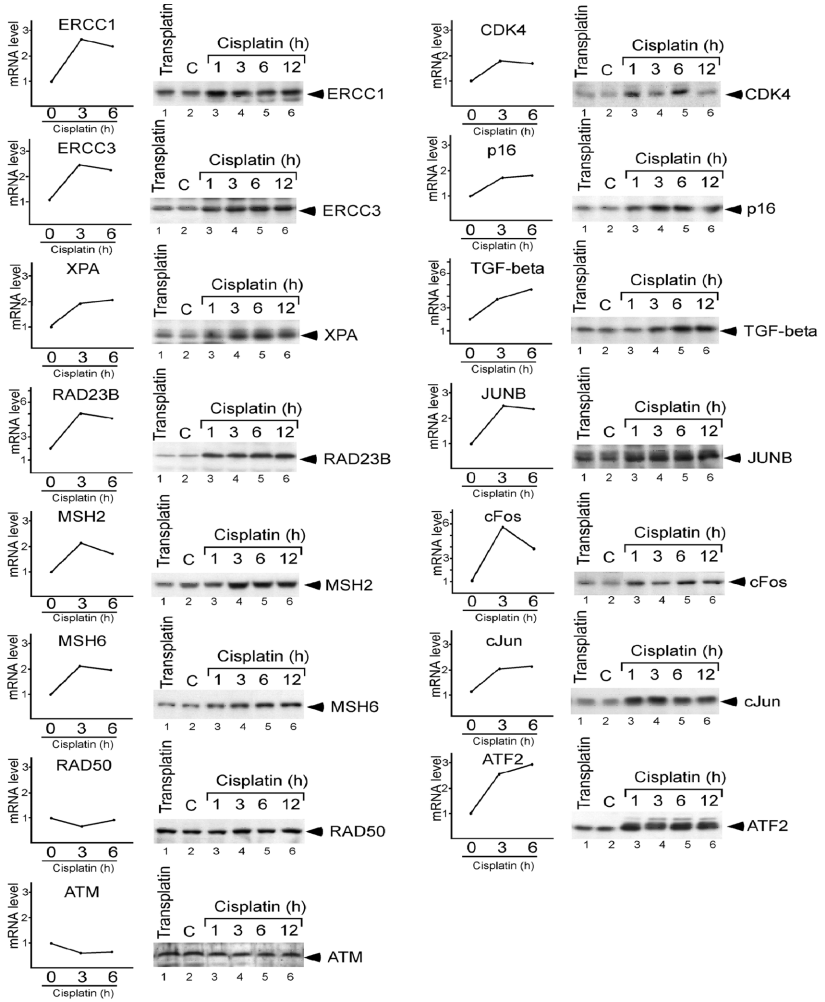

FIGURE 6. Comparison of qPCR results of ChIP-captured DNA with protein expression for representative genes over the 6 h period following initiation of genotoxic stress by treatment with cisplatin.

TABLE 1. Distribution of phospho-ATF2 and phospho-c-Jun DNA complexes among genes known or suspected to be AP-1 regulated and among all other genes of the s080 prototype promoter array

Promoter array (total 3083)	AP-1 group (249)	Remains (2834)
ChIP:phospho-ATF2 181	67 (26.9%)	113 (4.0%)
ChIP:phospho-cJun 210	68 (27.3%)	141 (5.0%)
"Common" of pATF2 and p-cJun 122	50 (20.1%)	71 (2.5%)

Formation of Activated ATF2/c-Jun-DNA Complexes Promotes a Net Increase in Transcription and Translation

Three observations indicate that, even though promoters contain dozens of regulatory elements, the single event of formation of phosphorylated c-Jun and ATF2 containing DNA complexes is associated with changes in transcription that are almost always in the positive direction (TABLE 1, FIG. 6). Second, nearly all of the genes on the promoter array are also represented on the Affymetrix U133a arrays. When total RNA isolated from cisplatin treated cells is applied to an Affymetrix array, the number of significant changes in transcript level over all common genes of the two arrays is 4.6% (FIG. 6). However, for the subset of genes reported to have increased formation of either phospho-c-Jun-DNA complexes or phospho-ATF2-DNA complexes the percent with significantly altered transcript levels by the Affymetrix criteria is a net positive 27% and 35%, respectively. Third, qPCR measurements confirmed that mRNA levels for the representative genes were elevated and maximum at or near the known time of maximum transcriptional activation following stimulation with cisplatin for all but two of the genes, RAD50 and ATM (FIG. 6, insert graphs).[1] Finally, protein expression for the same set of representative genes revealed increased protein levels following cisplatin stimulation compared to unstimulated cells which was maximal at 3 h and remained elevated for an extended period for most cases. The exceptions are again RAD50 and ATM50. Thus, among the representative genes examined there is a strict correspondence between mRNA and protein expression. The absence of increased protein for RAD50 and ATM50 may be related to large basal levels apparent for unstimulated cells which may not be elevated readily above the basal levels. The results suggest that phosphorylation of c-Jun and ATF2-DNA complexes have significant regulatory impact.

Functional Properties of Differentially Bound and Activated Genes following Cisplatin Treatment Are Dominated by DNA Repair and Related Roles

There are 121 genes that are significantly differentially bound by both phospho-ATF2 and phospho-c-Jun. An additional 60 genes significantly differentially bound by ATF2 (181 total), and a further 90 genes significantly differentially bound by c-Jun makes a total of 211. We examined their functional properties by use of *David*, a web-accessible program that integrates functional genomic annotations of multiple sources.[39] In order to broadly survey for functional generalities, the annotated features of each gene are used to classify each gene among *all* appropriate biologic processes of a set of 30 processes. The results for the biologic processes with the most

TABLE 2. Functional classification of genes significantly bound by phospho-ATF2 and phospho-c-Jun[a]

	Category	181 ATF2–bound genes	211 c-Jun–bound genes	121 Genes common to ATF2/c-Jun binding
1	**DNA Repair and Related**	**319**	**239**	**132**
2	physiologic process	109	124	77
3	cellular process	90	100	60
4	Metabolism	78	87	58
5	cellular physiologic process	72	78	47
6	cell growth and/or maintenance	62	67	39
7	**cell proliferation**	**42**	**47**	**30**
8	Transcription	34	37	23
9	cell communication	33	45	24
10	cell cycle	33	38	24
11	**regulation of transcription**	**33**	**36**	**22**
12	**transcription, DNA-dependent**	**33**	**36**	**22**
13	regulation of transcription, DNA-dependent	32	35	22
14	Response to stimulus	31	31	22
15	**signal transduction**	**29**	**38**	**21**
16	Development	27	35	21
17	**response to stress**	**25**	**24**	**17**
18	protein metabolism	24	30	20
19	**regulation of cell cycle**	**23**	**29**	**19**
20	**apoptosis, cell death, programmed**	**60**	**57**	**45**
	Classifications subtotal	1228	1260	772
	Unclassified	39	47	27
	Classifications total	1668	2232	1113
	Unique	181	211	121
	classifications/gene	9.2	10.6	9.2

[a]The numbers entered here are the number of significant gene assigned to the given c classification category. Classification categories are according to 30 Biological Processes of Dennis *et al*. 2003 with stringency set to 1, which utilizes general terms of gene annotation leading maximum number of classifications. The results for the most frequently assigned classifications are shown here. Classification category 1 (DNA repair and related) is a pool of classifications: DNA damage, DNA recombination, mismatch repair, double strand break repair, nucleotide excision repair, and related classifications. Classification category 20 also is a pool of the three classifications apoptosis, cell death, and programmed cell death.

TABLE 3. The 24 DNA Repair and Related Gene Promoter bound by QATF2 (no shading), c-Jun (light shading), or both (dark shading)

	Gene Symbol	Gene Description
1	CHES1	checkpoint suppressor 1
2	ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1
3	ERCC3	and 3 (xeroderma pigmentosum group B complementing)
4	FOXD1	forkhead box D1
5	GADD45G	growth arrest and DNA-damage-inducible, gamma
6	HIST1H2AC	histone 1, H2ac
7	PMS2	PMS2 postmeiotic segregation increased 2 (*S. cerevisiae*)
8	RAD23B	RAD23 homolog B (*S. cerevisiae*)
9	RAD50	RAD50 homolog (*S.cerevisiae*)
10	TOPORS	topoisomerase I binding, arginine/serine-rich
11	CKN1	Cockayne syndrome 1 (classical)
12	DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)
13	G22P1	thyroid autoantigen 70kDa (Ku antigen)
14	LIG1	ligase I, DNA, ATP-dependent
15	MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (*E. coli*)
16	MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*)
17	MSH6	mutS homolog 6 (*E. coli*)
18	UNG2	uracil-DNA glycosylase 2
19	XPA	xeroderma pigmentosum, complementation group A
20	ADPRT	ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase)
21	ATM	ataxia telangiectasia mutated (includes complementation groups A, C and D)
22	DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked
23	GTF2H1	general transcription factor IIH, polypeptide 1, 62kDa
24	NP	nucleoside phosphorylase
25	TREX1	three prime repair exonuclease 1

assignments are summarized in TABLE 2. Several well-known JNK functions are well represented (TABLE 2, bold) such as the roles of JNK in cell proliferation, stress responses, transcription, and apoptosis indicating the consistency of the identified genes and method of functional assignment by *David*. Other important biologic processes include physiologic and cellular processes. However, the most common function is indicated by a group of closely related processes including DNA repair, Mismatch repair, DNA damage response, DNA recombination, Double Strand Break Repair, and DNA metabolism collectively termed DNA repair and related events (TABLE 2). This collective group accounts for up to 19% (319) of all classification of the genes bound by phospho-ATF2-containing DNA complexes and 11% (239) for

phospho-c-Jun-containing complexes. This suggests the importance of DNA repair related functions especially among genes bound by phospho-ATF2.[1,40]

The unique set of genes corresponding to multiple DNA repair-related biologic processes corresponds to a set of 24 DNA genes summarized in TABLE 3. Ten DNA repair or related genes are common to binding by phospho-ATF2 and phospho-c-Jun (TABLE 3, dark shading) suggesting gene regulation occurs by ATF2-c-Jun heterodimers. Nine genes are bound by phospho-ATF2 but not phospho-c-Jun, a result that possibly indicates that these genes are regulated by ATF2 homodimers or ATF2 and unidentified partners.

The largest single group consists of 24 DNA repair-related genes. Several of these genes appear to be specifically related to the JNK as a genotoxic stress response pathway. ERCC1, ERCC2, XPA, RAD23B, MSH2, and MSH5 are among the representative sequences that were confirmed to form increased phosphorylated c-Jun and ATF2 DNA complexes upon cisplatin stimulation and also were confirmed to express increased mRNA and protein (FIG. 6). One of these, MSH2, as well as three others, MSH6, MLH1, and PMS2, are recognized members of the NMR DNA damage recognition complex.[41,42] Moreover, the roles of four other members, XPA, RAD23B, ERCC1, and ERCC3, have been shown to be participants in the repair of cisplatin-DNA adducts.[43,44] Similarly, the gene products of DMC1, ATM, and UNG2 have been implicated as facilitators of cisplatin-DNA adduct repair. Thus 12 of the 24 genes have an experimental basis supporting the conclusion that they are part of a cisplatin-stimulated response pathway and/or are targets of regulation by a JNK-mediated genotoxic stress pathway. The remaining genes reported by the promoter arrays as specific targets are novel candidates as participants as response genes of genotoxic stress.

Conclusions

The array results summarized here are derived from a very limited sampling of the regulatory sequences of the genome. Indeed, only 89 known DNA repair gene promoter sequences are represented. Moreover, only c-Jun and ATF2-DNA complexes down stream of the JNK pathway have been considered. It appears likely, therefore, that many dozens of additional DNA repair-related genes may be involved in the cisplatin-induced response. The recognition of the large-scale nature of specific activation of signal transduction maybe the major lesson learned from the exploratory studies summarized here.

METHYLATION AND COPY NUMBER CHANGE DETECTION BY PROMOTER ARRAYS: ANALYSIS OF PROSTATE CANCER CELL LINES

Proximal promoter regions are common locations of so-called “CpG” islands, sites of modification of cytosine residues by methylases, an effect commonly associated with “silencing” of transcription of the associated coding sequence. Promoter arrays can be used to identify these methylated sites in cells and tissues of interest.[2] Aberrant DNA methylation of CpG sites is among the earliest and most frequent alterations in cancer including prostate cancer.[45–47] Prostate cancer cell-specific gene

silencing is likely a major mechanism in the progression of the disease.[48] Several methods are used to determine the methylation status of a CpG island.[49,50] We have developed a simplified method based on the use of promoter arrays (FIG. 7). A methylation-sensitive restriction enzyme, *Hpa*II, was used to distinguish methylated from unmethylated DNA at all cleavage sites. For *Hpa*II, potential cleavage sites are common and therefore closely spaced in the CpG island as well as in the promoter region. If consecutive sites are both unmethylated, they can be cleaved and primers can be ligated. When the distance between the ligated primers is short enough, the fragment can be amplified efficiently by PCR. If, on the other hand, the DNA is methylated at one of the cleavage sites, the site will not be cut and longer and poorly amplified fragments will be produced. Thus, when *HpaII* digested and amplified DNA is hybridized to a promoter array, the intensities reported are proportional to the number of cells with unmethylated DNA for each sequence for which significant

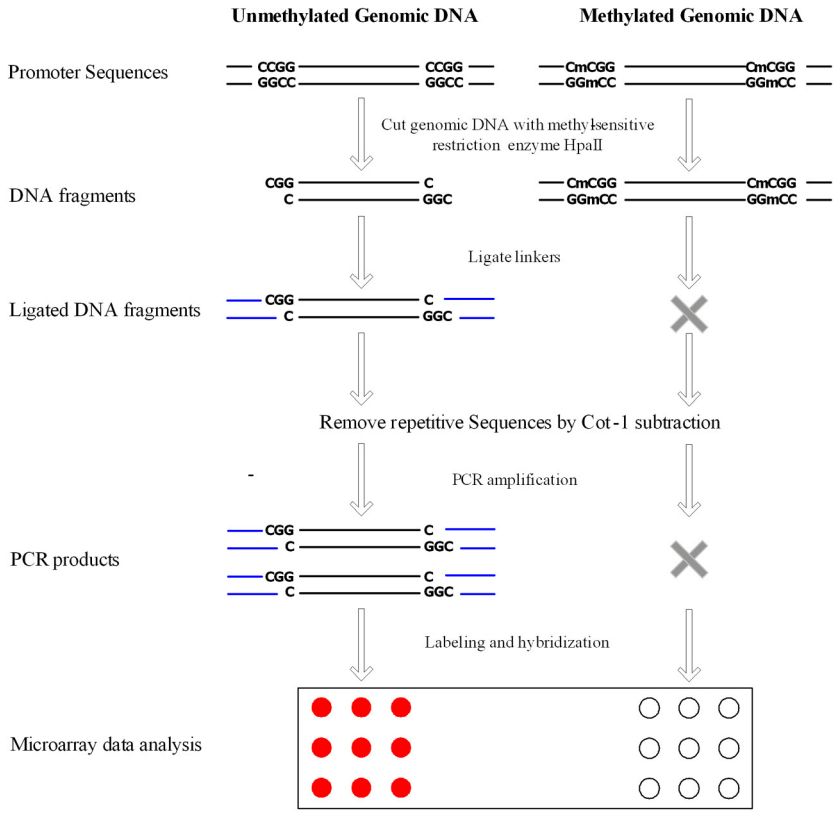

FIGURE 7. Schematic of the protocol for detecting differences in *Hpa*II fragment amplification between samples.

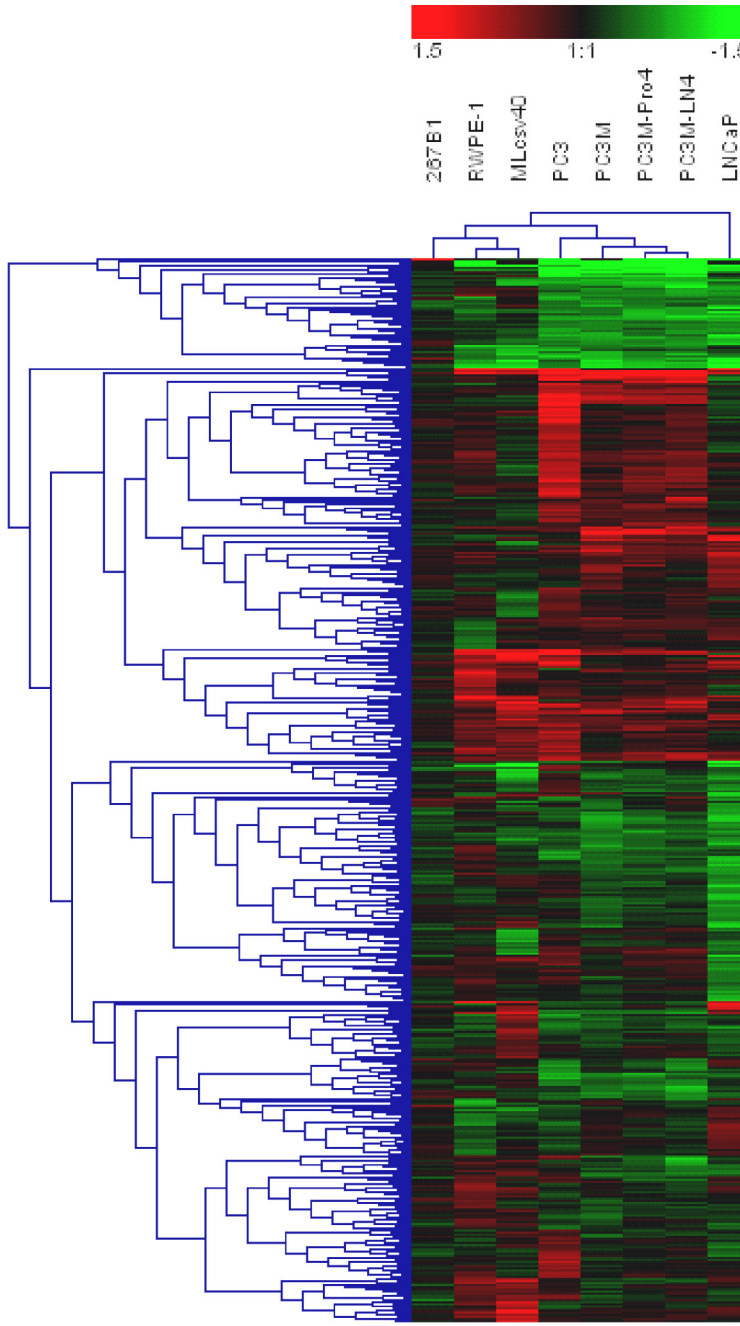

FIGURE 8. Cluster of hybridized amplified *Hpa*II fragments for eight cell lines.

hybridization occurs (FIG. 7). In addition to methylation status, the method of FIGURE 1 is also sensitive to any differences in the copy number of genes that may exist between the sample, such as a tumor cell or tissue, and normal control cells or tissues. Copy number changes may be distinguished from methylation changes by a variety of experimental methods, such as methylation specific-PCR or by treatment with 5-aza deoxycytidine as well as *in silico* analysis.

Differential Hybridization of Hpa*II Fragments*

We examined a series of prostate cancer cell lines including androgen-sensitive LNCaP cells and a series of cells increasing in metastatic potential based on the PC3 tumor lines: PC3, PC3M, and PC3M-Pro4, and PC3M-LN4 as well as three immortalized cell lines derived from prostate epithelium (FIG. 8). We observed 504 promoters that show statistically significant changes in hybridization between cancer and normal prostate cell lines. Hierarchical clustering of the hybridization patterns of these 504 promoters is displayed in FIGURE 8 (a complete list of the genes is found in Wang *et al*. 2005[2]). The clustering results show that PC3M-Pro4 and PC3M-LN4 are the most similar. Only one promoter, HAS3, appeared to be more differentially hybridized between PC3M-Pro4 and PC3M-LN4, possibly being hypermethylated in PC3M-Pro4. PC3M-Pro4 and PC3M-LN4 were clustered with PC3M, then with PC3. These four cell lines are less similar to LNCaP and normal cell lines. This is consistent with the origins of these cell lines.

A difference in *Hpa*II fragment hybridization intensity for a promoter between samples can occur due to methylation differences, differences in copy number, or due to restriction site polymorphisms. In cancer cell lines, relative to normal cell lines, there are fewer genes that showed an increased *Hpa*II fragment hybridization, characteristic of copy number increases or hypomethylation (251 promoters), and conversely there are more genes with lower *Hpa*II fragment hybridization, characteristic of copy number decreases or hypermethylation (286 promoters) (FIG. 8). An example of increased *Hpa*II fragment hybridization (hypomethylation or copy number increase) in cancer lines is the promoter of CTAG1, which is over-expressed in some lung and thyroid cancers,[51,52] although this overexpression has not been attributed to hypomethylation or copy number changes.

Methylation

We used 5-aza deoxycytidine (d5-AzaC) as a means of achieving global demethylation in order to determine if differences in hybridization could be partially reversed, indicating methylation as the underlying cause. For example when LNCAP cells are treated with d5-AzaC and the *Hpa*II fragment profile compared to that for untreated cells, reductions in hybridization intensities are detected for hundreds of genes. As a group, the shift of these genes to a more demethylated status is highly significant ($P < 0.001$). Methylation status was also examined by methylation-specific PCR for a group of 14 randomly selected genes. Eight out of 14 were hypermethylated in PC3M relative to 267B1 and one gene was hypermethylated in 267B1 all of which supported the array data. These events likely regulate transcription (FIG. 9, see also below: ***Correlation between RNA Expression and HpaII Fragment Hybridization***).

Detection of Copy Number Change

Of the remaining five genes that showed no changes or changes in the wrong direction, all were located on chromosome 5. When the *Hpa*II-ligation-PCR data for three cell lines are plotted in the order of their occurrence in the genome, the best candidate chromosome regions for widespread methylation or aneuploidy are apparent (FIG. 9A–C). This analysis suggests that the five genes that appeared to be differentially methylated, are, in fact, altered in copy number. Among the aneuploidy changes that are observed by hybridization to the promoter arrays and that have been reported previously are changes in chromosome 6 in LNCaP, chromosomes 8, 10, 14 in PC3[53] and many sporadic changes previously observed in prostate cancer.[54]

The results reported by the array as indicating copy number changes are supported by the comparison of PC3M cells to immortal prostate epithelial 267B1 cells using *Msp*I-ligation-PCR (FIG. 10D). *Msp*I is an enzyme which cuts at the same CCGG site as *Hpa*II but which is insensitive to methylation at most sites. The normalized ratio (PC3M/267B1) is plotted against the chromosomal position of each promoter (FIG.10D). This is a simple variation on the comparative genomic hybridization (CGH) method.[55] The correspondence of the major features for chromosomes 5, 10, 12, 14 and 15 is consistent with the results based on the *Hpa*II protocol.

Among the 504 promoters with significant differences between prostate cancer and the normal cell line, eight genes are known as methylation-regulated genes in prostate cancer; CD44, CDKN1A, ESR1, PLAU, RARB, SFN, TNFRSF6, TSPY, and 13 more are known in other cancers; ARHI, BCL-2, BRCA1, CDKN2C, GADD45A, MTAP, PGR, SLC26A4, SPARC, SYK, TJP2, UCHL1, WIT-1 (for references see Wang *et al*. 2005[2]). Similarly, methylation of SFN and PLAU in LNCaP but not PC3 has been reported before[56,57] which is consistent with the observations based on the promoter array analysis.

Other than dramatic differences in their growth properties and metastatic abilities[58,59] one of the most striking differences between PC3M and LNCaP, is that the latter is almost unique among prostate cancer cell lines in still being androgen dependent. In this experiment, 29 genes showed loss of hybridization in HpaII fragment in LNCaP and 19 genes showed loss of hybridization in PC3M, indicating hypermethylation or copy number loss. We looked for differences in hybridization between PC3M and LNCaP among 261 known and suspected androgen-regulated genes present on the array.[60] Among known or suspected androgen receptor–regulated genes that may be methylated or reduced in copy number in LNCaP relative to PC3M were GG2-1 (TNF-induced protein), GABARAPL2 (GABA(A) receptor-associated protein-like 2). In PC3M the list included FLJ13782 hypothetical protein, TSPY (testis-specific protein, Y-linked) and RPS4Y (ribosomal protein S4, Y-linked isoform).

Expression from the Y chromosome has been of interest in prostate cancer[61,62] and changes in methylation of EIF1AY, MGC26641, PRKY, RPS4Y, SHOX, TSPY, TSPYQ1 and VCY are observed in our experiments, whereas the few other Y chromosome genes on the array act as internal controls for this observation because they are seemingly not differentially methylated.

That an experiment involving three relatively normal prostate cell lines and five prostate cancer cell lines pointed to a large number of genes that were previously known to be differentially methylated in cancer, particularly prostate cancer, sup-

ports the observation that cell lines and primary tumors generally have similar overall distribution and frequencies of gene methylation[63] and that prostate cancer cell lines may have the same "hypermethylation fingerprint" as primary and metastatic prostate cancers.[64]

Correlation between RNA Expression and Hpa*II Fragment Hybridization*

The RNA expression levels of two cell lines, PC3M and 267B1, were obtained using Affymetrix U133A GeneChips. 51.6~53.5% genes were called as present for these samples. When methylation differences are plotted against gene expression differences between PC3M and 267B1 for all the genes that showed HpaII fragment hybridization differences and gene expression differences, there is a significant correlation (40 genes, $r = 0.68$, $P < 0.001$), FIGURE 9. The majority of genes that are differentially hybridized by amplified *Hpa*II fragments in the study are not considered in this comparison because these genes happen not to be sufficiently expressed as judged by the Affymetrix criteria. Twenty-seven genes, including three genes with no apparent CpG island in the promoter region, are less hybridized by *Hpa*II fragments (consistent with hypermethylation or copy number loss) in PC3M relative to 267B1. For these genes expression was also decreased in PC3M, as would be expected if methylation or copy number loss is associated with downregulation of expres-

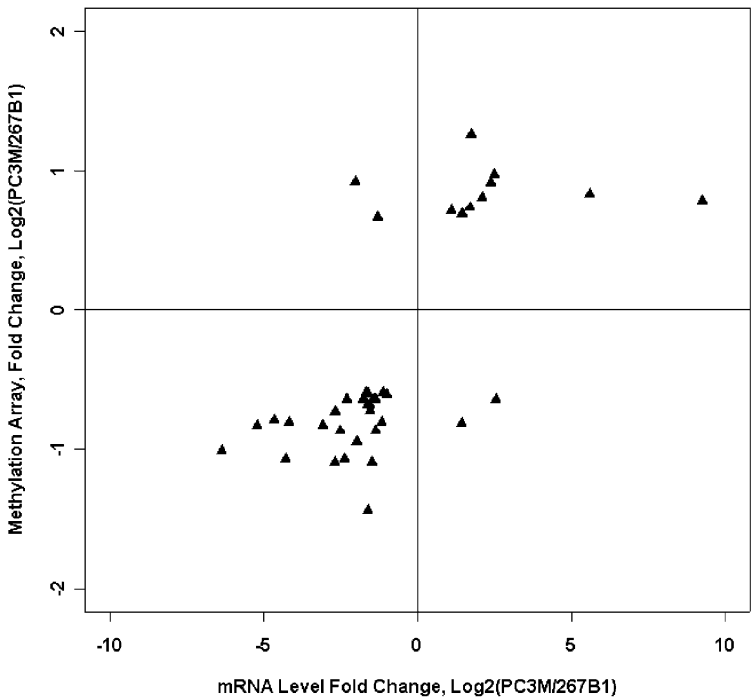

FIGURE 9. Comparison of amplified *Hpa*II fragment data to Affymetrix RNA expression data.

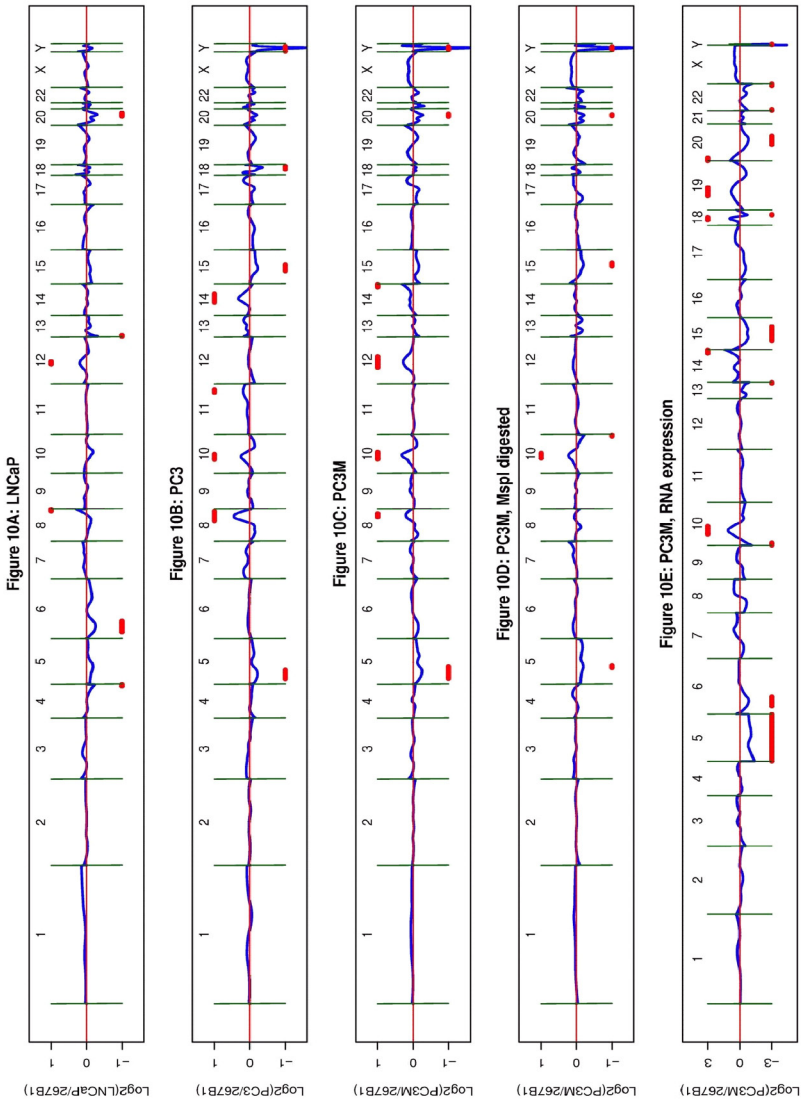

FIGURE 10. *See following page for legend.*

sion. There are nine genes, including two genes with no CpG island in promoter region, with increased hybridization of *Hpa*II fragments (consistent with hypomethylation or copy number increase) in PC3M relative to 267B1 and gene expression of these genes is higher in PC3M, also as expected. There were only four genes where the prediction of methylation or copy number loss was associated with an increase in gene expression level. It will be of interest to explore these exceptions further. Finally, the ratios of PC3M expression data relative to 267B1 was plotted against chromosome position in FIGURE 10E. Perhaps surprisingly, there are readily detectable global effects of aneuploidy on averaged RNA expression along the chromosomes.

Summary

The comparisons of the immortalized prostate epithelial and cancer cells lines revealed a high degree of differential hybridization, 18.5% of all promoter sequences represented on the array. Most differences could be eliminated by first treating dividing cells with the methylation inhibitor d5-azaC. Moreover, promoter methylation generally correlated with reduced RNA expression. As for the studies of activated transcription factor DNA complexes, the number of promoters detected is considerably larger than anticipated based on the number of individual genes reported to be regulated by methylation in prostate cancer. Similar to the results observed for active transcription factor-DNA complexes, while many known or suspected methylation targets were observed, the vast majority of genes that appear to be both differentially methylated and differentially regulated between prostate epithelial and cancer cell lines are novel methylation targets, including PAK6, RAD50, TLX3, PIR51, MAP2K5, INSR, FBN1, GG2-1, representing a rich new source of candidate genes to study the role of DNA methylation in prostate tumors. The use of promoter arrays appears to be a promising new avenue for the investigation of coordinated gene regulation. The promoter array described here has been expanded to over 10 K unique promoter sequences using the primer set developed by the Whitehead Institute.

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FIGURE 10. DNA copy number changes measured by CGH on promoter array. A-E, indicated cell lines. A-C, DNA digested with HpaI. D, DNA digested with MspI. E, Affymetrix expression analysis data given as relative mRNA level for genes by their chromosomal locations.

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