Analyses of p53 Target Genes in the Human Genome by Bioinformatic and Microarray Approaches*

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The completion of the human genome sequence (International Human Genome Sequence Consortium (2001) Nature 409, 860–921; Venter, J. C., et al. (2001) Science 291, 1304–1351) allows for new ways to analyze global cellular regulatory mechanisms. Here we present a strategy to identify genes regulated by specific transcription factors in the human genome, and apply it to p53. We first collected promoters or introns of all genes available using two methods: GenBank† annotation and a computationally derived transcript map. 4,852 genes analyzed in this way contained at least one p53 consensus binding sequence. Of 13 genes randomly selected for mRNA analysis, 11 were shown to respond to p53 expression. Five promoters were analyzed by chromatin immunoprecipitation, which revealed that all were bound by p53 in vivo. We then analyzed 33,615 unique human genes on cDNA microarrays, identifying 1,501 genes that respond to p53 expression. A parameter was derived that demonstrates that in silico prediction greatly enriches for genes that are activated and repressed by p53 and assists us to suggest other signaling pathways that may be connected to p53. The methods shown here illustrate a novel approach to analysis of global gene regulatory network through the integration of human genomic sequence information and genome-wide gene expression analysis.

Mutational inactivation of the p53 gene product is one of the most common genetic changes seen in human cancers. Overexpression of wild-type p53 protein in p53-deficient cells can arrest cell proliferation, reverse a tumorigenic phenotype, and sometimes induce apoptosis or differentiation (1, 2). p53-dependent apoptotic pathways are critical to the development of tumor (3). The biochemical activity of p53 most closely associated with tumor suppression is its function as a transcription factor. A number of investigators have identified a consensus DNA binding sequence for wild-type p53. El-Deiry et al. (4) used p53 to select DNA fragments from genomic DNA and found that selected fragments have two copies of the decamer motif 5′-RRRCWWGYYY-3′ separated by 0 to 13 base pairs of random sequence. In the motif, R = G or A, W = T or A, Y = C or T. Several human genes whose expression is positively regulated by wild-type p53 have been identified. These include mmd2 (5), box (6), gadd45 (7), proliferating cell nuclear antigen (8), p21/WAF1 (9), insulin-like growth factor-binding protein 3 (IGFBP3) (10), the muscle creatine kinase gene, thymospondin-1 (11), vascular smooth muscle a-actin gene (ACTA) (12), epidermal growth factor receptor (13), and type IV collagen gene (MMP-2) (14). Although the number of identified p53 target genes keeps growing, it is conceivable that large fraction of the p53 target genes have not yet been identified. The ultimate challenge to p53 biology is to define the complete gene regulatory network. In facing this challenge, the complete human genome “draft” sequence is an invaluable resource (15, 16). Knowledge of genes containing p53 consensus binding sequence in the regulatory region will greatly assist the identification of p53 target genes, which could be potential targets for cancer chemotherapeutic drugs. Bioinformatics methods become immediately more useful if they can be integrated with high-throughput gene expression analysis on the same scale. In this report we described analysis of global p53 gene regulatory network through computational methods and genome-wide gene expression analysis.

MATERIALS AND METHODS

Computational Analysis—All noncommercial software used in these studies was written in PERL 5.0. The results of subsequent analyses were organized in a relational data base (Sybase, SQL Server Release 11.0, Emeryville, CA, Sybase Inc.).

Cell Culture—The human lung cancer cell line A549, the human ovarian cancer cell lines PA1 and 2774, and the human osteogenic sarcoma cell line Saos2 were obtained from American Type Cell Collection (ATCC). The cell line, 2774qw1, was a single clone derived from human ovarian cancer cell line 2774. Culture conditions of all cell lines followed the instruction from ATCC.

Treatment of Cells with Recombinant Adenovirus Expressing p53 and RNA Isolation—To introduce exogenous p53, 2774qw1 cells were infected with recombinant adenovirus expressing p53 or adenovirus containing empty vector for 1 h. The recombinant adenovirus was then removed and cells were washed 1–2 times with phosphate-buffered saline (Life Technologies, Inc.). Cells were then refed with fresh culture medium and cultured at 37 °C and 5% CO2 for 2–24 h as indicated. Cells were harvested and total RNA was isolated using a Qiagen Oligotex kit for microarray experiments.

Quantitative RT-PCR—The quantitative RT-PCR of 13 genes was performed as described previously (18). The primers and probe specific to 13 genes were designed (see Supplementary Material, Table S1). Human β-glucuronidase gene, whose expression was not influenced by p53 across the test samples, was used as an internal positive control to ensure that equivalent amounts of RNA were included in each assay.

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† The on-line version of this article (available at http://www.jbc.org) contains Tables S1–S3.

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1 The abbreviations used are: IGFBP, insulin-like growth factor binding protein; EF, enrichment factor; TGF, transforming growth factor; RT-PCR, reverse transcription polymerase chain reaction; ChIP, chromatin immunoprecipitation; rAd-p53, recombinant adenovirus expressing p53; rAd-control, adenovirus containing empty vector.
Chromatin Immunoprecipitation (ChIP) Assays—Approximately \(1 \times 10^7\) A549, PA-1 and Sasso2 cells were treated with 500 nM adriamycin or MeSO (only for PA-1) for 24 h, respectively. ChIP assays were performed using a modification of a protocol provided by Bruno Amati's laboratory in DNAX (19). Precleared chromatin was immunoprecipitated with a monoclonal antibody against p53 (Ab-6, Oncogene Research Products), or no antibody added as a negative control. The precipitated chromatin was immunoprecipitated in DNAX (19). Precleared chromatin was immunoprecipitated using a modification of a protocol provided by Bruno Amati’s laboratory in DNAX (19)

**RESULTS AND DISCUSSION**

**Genome-wide Identification of Human Genes Containing a p53 Consensus Sequence(s) in Their Regulatory Regions**—To identify potential p53 target genes in human genomic sequences, the “FindPatterns” program (Wisconsin Sequence Analysis Package Version 10) was used to search the primate division of GenBank™ Release 120 (October, 2000) for the p53 consensus sequence, 5’-RRCWWGYYY (n = 0 – 13) RRCWWGYHY 3’ (4). In this motif, R = G or A, W = T or A, Y = C or T, n = any base. Some p53 target genes (human mdm2, IGFBP3, and gadd45) have functional p53 response elements in their intron regions (5, 7, 20). The data base search therefore includes intrinsic sequences. We took advantage of annotated features present in GenBank™ entries to restrict matches to

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**Functional p53-binding site documented in literature**

| Gene                        | GenBank No. | Position (promoter) | Sequence | Ref. |
|-----------------------------|-------------|---------------------|----------|------|
| Human bax                   | U17193      | 488                 | TCAACAGTTA | 6    |
| Human proliferating cell nuclear antigen | J05614 | 1035                | AGACAAGGCTT | 2    |
| Human MMP-2                 | U96098      | 11                  | ACATATGGCC | 8    |
| Human ACTA                  | J05193      | 584                 | GAACATGTCT | 2    |
| Human epidermal growth factor receptor | J03206 | 40                  | CAATATGGCC | 12   |
| Human mdm2                  | U28935      | 138 (intron 1)      | AGGCAAGGCC | 13   |
| Human IGFBP3                | M35878      | 5064 (intron 1)     | AGACACGGTC | 5    |
| Human p21/WAF1              | U24170      | 2303                | AGTAAAGGTC | 2    |

Note: These are rapidly expanding areas of the investigation and are likely to be incomplete. The regulatory region is defined as 5’-flanking region (2 kilobase cutoff) and the first 3 introns. Sequences of primers and probes are available in Supplementary Material, Tables S2 and S3.
the regulatory region of the genes, which consisted of 6,541 annotated human 5′-flanking sequences and 24,659 annotated human intron sequences. On the basis of the statistics of known p53 target genes (20), no more than 3 mismatches to the canonical consensus sequence were permitted in the selection. We collected 13 nucleotide sequences known to be functional p53-responsive elements in 10 genes; 23 out of 26 decamers bear a cytosine at position 4, and 25 out of 26 decamers have a guanine at position 7 (Table I). Hence, the sequence matches were further sorted with no mismatch allowed for C at position 4 or G at position 7. Using this approach, we identified 1,121 genes with at least one p53 binding sequence in the region 2,000 base pair upstream of the transcription start site, and 558 genes with at least one p53 binding site within the first 3 introns.

The efficiency of the computational analysis was evaluated with known p53 target genes (Table I). The p53 response elements of box, MMP-2, mdm2, IGFBP3, TGF-α, and gadd45 were recognized by this analysis. Additional p53 consensus sequences were found in the promoter or intron regions of these genes. Although they have a nucleotide in the positions 4 or 7 other than C or G in their p53 response elements, proliferating cell nuclear antigen, ACTA, and epidermal growth factor receptor genes were also selected because additional binding sequence matches were found in their regulatory sequences.

This annotation-based approach is limited by the availability of annotated human gene sequences in GenBank™. To circumvent this shortcoming, a transcript mapping approach was used to locate the promoter sequence on a genomic template. A collection of human mRNA was first extracted from the primate division of GenBank™ flat file (October, Release 120) primate division. The draft human genome sequence was used to identify or to extend the 5′-flanking region of many existing mRNA sequences for which the annotated gene structures are not available or are very short in their respective individual GenBank™ records. To ensure that the 5′ end of an available mRNA is close to the transcription start site, only mRNAs that encode the N-terminal of the protein were used for transcript mapping. Both finished and draft human genomic sequences were used as genomic templates. The analysis was done when ~94% of the genome was deposited (63% as working draft sequence) into GenBank™. Within the 22,034 promoters mapped in this way, 4,428 genes were identified that have at least one potential p53-binding site. For a sample of 150 promoters annotated in GenBank™, 132 (88%) were perfectly predicted by the transcript mapping, suggesting that the transcript mapping procedure could properly predict most promoters. Nevertheless, a small portion of predicted promoters (12%) still differ from GenBank™ annotated promoters. This resulted from either a duplicated copy, a different transcript start site of the annotated gene due to gene duplication, or a 5′ end splice variant. To remove redundancy, we collapsed all candidate genes identified by these two approaches, resulting in 4,852 candidate genes in the p53 Target Database.

Small Scale Verification of the in Silico Prediction by Tagman Analysis and Chromatin Immunoprecipitation Technique—To assess the validity of the in silico prediction, 13 genes were randomly selected from the p53 Target Database and tested for their p53 responsiveness using real-time quantitative RT-PCR. Total RNA samples were purified from human ovarian cancer cell line, 2774pw1, at various times post-infection with rAd-p53 or rAd-control. As shown in Fig. 1, the expression of myoglobin, cardiotrophin-1, catechol o-methyltransferase, IGFBP4, and α1-acid glycoprotein 2 genes was induced very early (2 h post-infection of rAd-p53). In contrast, the activation of carboxyl ester lipase and α-histidine decarboxylase genes were observed relatively late (8 h post-infection). α-Fetoprotein and interleukin 8 receptor α genes were repressed by p53 (Fig. 1A). It is interesting to note that although it has not previously been demonstrated for human α-fetoprotein, expression of the mouse homologue of the α-fetoprotein gene is repressed by p53 through sequence specific DNA binding in the promoter (21). ATP synthase-β and mitochondrial ATP synthase C genes slightly changed at the mRNA level following the expression of p53 (data not shown). It is also interesting to note that α1-acid glycoprotein 2 and complement component C1-inhibitor genes, which only have p53 DNA consensus sequences in their introns, were also activated by p53 (Fig. 1), confirming that sequences of introns should be included in searching for p53 DNA binding consensus sequences.

The 11 genes shown to respond to p53 at the mRNA level in Fig. 1 contain p53-binding sites in their regulatory regions, suggesting that they might be directly bound by p53. To test this, we took advantage of the chromatin immunoprecipitation technique that allows us to study p53 protein-DNA interaction in vivo. Five genes that respond to p53, α1-acid glycoprotein 2, catechol o-methyltransferase, myoglobin, and cardiotrophin-1, were evaluated in this way. One or two putative p53-binding sites were selected to design...
FIG. 2. p53 interacts with predicted p53 DNA-binding sites in vivo detected by chromatin immunoprecipitation technique. A, human ovarian cancer cell line, PA-1, was treated with 500 nM adriamycin (Adr) or MeSO (DMSO) for 24 h, respectively. ChIP was performed using antibody recognizing p53 (open bars) or no antibody as a negative control (closed bars). Immunoprecipitated DNA was analyzed by quantitative PCR using p53-binding site specific primers as described under “Materials and Methods.” The ChIP assays were repeated in two independent experiments, and similar results were observed. Representative data are shown. Genes are marked as follows: cardiotrophin, CAR; myoglobin, MYO; catechol-O-methyltransferase, COMT; i-histidine decarboxylase, HDC; α1-acid glycoprotein 2 (intron 1), AGP2(I1); α1-acid glycoprotein 2 (intron 3), AGP2(I3).

For comparison, percent of total input DNA of samples treated with adriamycin was normalized with that of sample immunoprecipitated with no antibody from sample treated with MeSO (DMSO).

B, ChIP assays were performed using antibody recognizing p53 (open bars) or no antibody as a negative control (closed bars) in human lung cancer cell line, A549, that expresses wild-type p53 and in human osteosarcoma cell line, Saos2, which has null p53 as a negative control. Both cell lines were treated with 500 nM adriamycin for 24 h.
site-specific primers for each gene. A human PA-1 ovarian carcinoma cell line was used because it expresses endogenous functional wild-type p53 (22), which is induced by the treatment with the DNA damaging agent adriamycin. Using antibodies against p53, chromatin was immunoprecipitated from PA-1 cells treated with 500 nm adriamycin or Me2SO for 24 h. The ChIP assays showed that genomic fragments containing p53-binding sites for all sites tested were enriched after immunoprecipitation using the p53-specific antibody, and the enrichment after treatment of adriamycin are greater than control samples (Fig. 2A). Negligible quantities of chromatin were recovered when no antibody was used. Similar results were observed with the lung carcinoma cell line, A549, which expresses wild type p53 (Fig. 2B). Furthermore, only background levels of the promoter fragments were precipitated from a human osteogenic sarcoma cell line, Saos2 (null for p53), in the presence of p53 antibody (Fig. 2B). Two known p53 target genes, p21/WAF1 and proliferating cell nuclear antigen, were checked for the interactions of p53 with their promoters and were both positive (data not shown). The results demonstrated that endogenous p53 protein interacts directly with predicted p53 binding sequences in these 5 genes, and that in silico prediction can greatly assist us in focusing on regions likely to confer p53-mediated expression. The biological properties of these genes are consistent with the functions that have been attributed to p53, such as, cell cycle checkpoints, apoptosis, inhibition of angiogenesis, and genetic stability (23–25). The data suggest that these genes could be mediators of p53 expression through transcriptional regulation.

Integration of the p53 Target Database and Genome-Wide Expression Analysis—High-density DNA microarrays appear to be the sole approach to study gene expression at the scale comparable to that of the p53 Target Data base. In an attempt to identify p53 target genes on a genomic scale, Incyte GeneAlbum microarrays (∼60,000 cDNAs) were hybridized with the same batch of mRNA used in Taqman analysis described above. 1,501 genes (4.4%) were found to be responsive to p53 using a 2.5-fold change up or down as a cutoff. Approximately 80% of these were repressed by p53.2 Here we focus on the overlap of p53 target genes predicted in silico with those identified by hybridization to microarrays. However, there was no single established method to estimate the significance of an observed degree of in silico prediction. Accordingly, we developed the EF to test the validity of our in silico predictions. $T_a$ is the ratio of genes directly regulated by p53 in human genome to those that are not regulated by p53. $T_i$ is the similar ratio for genes in our p53 Target Database. The EF is defined as the comparison of these ratios (EF = $T_a/T_i$), such that an EF > 1 indicates enrichment for genes regulated by p53 in the p53 Target Database. Since the actual number of genes in the human genome are not known, we used the genes on the microarrays as a large representative set for this analysis. As described under “Materials and Methods,” the low boundary for the EF can be estimated from the following equation,

$$EF > \frac{[R_{target} / T_{target} - R_{target}]}{[R_{random} / T_{random} - R_{random}]}$$

(7)

where $R_{target}$ is the number of p53-responsive genes observed in the microarray experiments that are in the p53 Target Data base, $T_{target}$ is the total number of genes on the microarrays that are in the p53 Target Data base, $R_{random}$ is the number of p53-responsive genes observed in microarray experiments, and $T_{random}$ is the total number of genes on the microarrays.

The six Incyte GeneAlbum microarrays used in hybridization represent 33,615 individual genes ($T_{random}$) based on “assembling” of the sequences into gene-based groups. Of 4,852 unique genes in the p53 Target Database, 3,387 of them ($T_{target}$) are represented on the GeneAlbum microarrays. In the microarray experiments using 2774qw1 ovarian cancer cells infected with rAd-p53, 217 genes ($R_{random}$) showed activation following p53 expression. Of those, 68 genes ($R_{target}$) were found to have at least one p53 DNA binding sequence in their regulatory region, and the resulting EF is greater than 3.2. It suggests that the p53 Target Database enriched p53 direct target genes at least 3.2-fold compared with a randomly selected gene pool. We observed that 1,205 genes ($R_{random}$) are repressed following p53 expression, suggesting that transcriptional repression is also an important mechanism through which p53 exerts its function. Of those, 296 genes ($R_{target}$) that are represented in the p53 Target Database, and the EF for the repressed genes was estimated to be 2.6.

It is well known that p53 mediates transcriptional activation through sequence specific DNA binding (5, 7, 9, 10, 12, 14). In contrast to transcriptional activation, the molecular basis of transcriptional repression by p53 is poorly understood. This is because most genes that have been reported were repressed by p53 have no classical p53-binding sites in their promoter. In general, genes repressed by p53 can be classified into two types. For the first type of genes, the mechanism is ascribed to sequestration of components of the basal transcription machinery by p53 through protein-protein interactions in the absence of direct DNA binding (26–28). However, p53 mediated repression of target gene expression by direct DNA binding has been demonstrated in several instances (21, 29, 30). In these cases, p53 binds to the DNA-binding site, which overlaps the binding site of another transactivator protein. The repression by p53 results from displacement of the activator binding. Our data gave higher EFs than background for both activated and repressed genes, indicating that p53-activated genes as well as p53-repressed genes, at least some of them, are directly regulated through p53 specific DNA binding. The EF for p53-repressed genes is lower than that for p53-activated genes, supporting the notion that repression by p53 has multiple mechanisms with or without requirement of sequence specific DNA binding by p53. This could result in “dilution” of overall EF values for repressed genes. Obviously, the complexity of regulation of p53 repressed genes is significant and requires further study.

To verify these observations, another data set of microarray experiment, derived from human leiomyosarcoma cell line,

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2. S. Liu, manuscript in preparation.
SKUT-1, infected with rAd-p53 or rAd-control, was subjected to the EF analysis. Similar trends were observed (data not shown), suggesting that the EF is a valid measure for the selectivity of in silico prediction and is sensitive enough to reflect the specificity of p53-binding sites.

In principle, genes directly regulated by p53 should respond to p53 expression more quickly than secondary responsive genes. However, it is difficult to determine the best sampling time to enrich for p53 direct target genes, since an early response might only become detectable at later time points with the accumulation of transcripts. With the help of the p53 Target Database, we estimated the relative EFs for genes differentially expressed at different time points following p53 expression in 2774QW1 (Fig. 3). For the p53-activated genes, we observed that EF remains above 2.5 over the time course. This may result in silico prediction and is sensitive enough to reflect the specificity of p53-binding sites.

The p53 Target Database could not only be used to delineate p53 target genes, but also could assist in identify other signaling pathways that are potentially connected to the p53 pathway. Assuming p53 mediates certain growth factor-induced signals, the differential expression data derived from growth factor stimulation experiments would give increased EF values since p53 target genes would be part of the affected genes. Perou et al. (34) used human normal mammary epithelial cells subjected to a set of experimental perturbations, including addition of TGF-β1 for 24 h, withdrawal of EGF for 2 days, addition of interferon alpha (IFN-α) for 24 h, and addition of IFN-γ for 24 h. To evaluate whether p53 mediates the signaling induced by these growth factors, the microarray data (using 2.5-fold change as a cutoff) was linked with the p53 Target Database, and subjected to EF analyses. Interestingly, the
TGF-β1 treatment (24 h) data showed a relative high EF (2.3) whereas the data from other treatments gave EFs close to 1. When the list of genes effected by TGF-β1 was closely examined, it was interesting to note that 16 out of 22 genes have p53 binding sequences in their regulatory region (two of them have no genomic sequence available at this moment, Table II). The fact that the majority of TGF-β1-induced genes contain p53-binding sites suggests that p53 might be a mediator of the growth suppressive effects of TGF-β1. This is supported by the evidence that p53 expression was induced during TGF-β1 initiation growth inhibition and apoptosis (35).

The methods developed here are not limited to identifying the p53 gene regulatory network, but can also be applied to any other transcription factors of interest. We have extended the computational analysis of human genomic sequences to identify DNA-binding sites for forkhead transcription factor, which plays a critical role in cell survival signaling. TGF-β can be phosphorylated by Akt/PBC, a serine-threonine kinase that activates expression analysis for all transcription factors in future will greatly assist us to define global gene regulatory networks.

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