Original Article

Modular Activation of Nuclear Factor-κB Transcriptional Programs in Human Diabetic Nephropathy

Holger Schmid,[1] Anissa Boucherot,[1] Yoshinari Yasuda,[1] Anna Henger,[1] Bodo Brunner,[2] Felix Eichinger,[1] Almut Nitsche,[2] Eva Kiss,[3] Markus Bleich,[4] Hermann-Josef Gröne,[3] Peter J. Nelson,[1] Detlef Schlöndorff,[1] Clemens D. Cohen,[1] Matthias Kretzler,[1] for the European Renal cDNA Bank (ERCB) Consortium*

Diabetic nephropathy (DN) is the leading cause of end-stage renal failure and a major risk factor for cardiovascular mortality in diabetic patients. To evaluate the multiple pathogenetic factors implicated in DN, unbiased mRNA expression screening of tubulointerstitial compartments of human renal biopsies was combined with hypothesis-driven pathway analysis. Expression fingerprints obtained from biopsies with histological diagnosis of DN (n = 13) and from control subjects (pretransplant kidney donors [n = 7] and minimal change disease [n = 4]) allowed us to segregate the biopsies by disease state and stage by the specific expression signatures. Functional categorization showed regulation of genes linked to inflammation in progressive DN. Pathway mapping of nuclear factor-κB (NF-κB), a master transcriptional switch in inflammation, segregated progressive from mild DN and control subjects by showing upregulation of 54 of 138 known NF-κB targets. The promoter regions of regulated NF-κB targets were analyzed using ModelInspector, and the NF-κB module NFKB_IRFF_01 was found to be specifically enriched in progressive disease. Using this module, the induction of eight NFKB_IRFF_01–dependant genes was correctly predicted in progressive DN (*B2M*, *CCL5/RANTES*, *CXCL10/IP10*, *EDN1*, *HLA-A*, *HLA-B*, *IFNB1*, and *VCAM1*). The identification of a specific NF-κB promoter module activated in the inflammatory stress response of progressive DN has helped to characterize upstream pathways as potential targets for the treatment of progressive renal diseases such as DN. *Diabetes* 55:2993–3003, 2006

Multiple factors have been implicated in the progression of DN. These include the action of profibrotic cytokines and reactive oxygen species and ongoing chronic inflammation. Most of the studies addressing these pathogenetic factors have focused on the analysis of a few relevant gene products. Gene expression profiling allows parallel study of thousands of genes in diseased tissue. These approaches can be seen individually as either too focused or too unsystematic for the identification of regulatory pathways involved in diseases such as DN. New bioinformatic methods now allow the analysis of data derived from unbiased whole-genome screening experiments for transcriptional elements linked to regulatory pathways. The identification of higher-order promoter structures, or promoter modules, linked to the regulation pattern of sets of genes is helping to identify specific transcriptional pathways that underlie the pathogenesis of disease. To test this "modular approach" in DN, we evaluated nuclear factor-κB (NF-κB)-based transcriptional pathways associated with the progression of disease.

Diabetic nephropathy is the most common single cause of renal insufficiency in the Western world. Glomerular changes, such as capillary basement membrane thickening, mesangial proliferation, and nodular glomerulosclerosis, are pathognomic for DN (1). Tubulointerstitial fibrosis is a predictor of progressive renal failure (2). Traditionally, DN has been considered a nonimmune, degenerative disease. However, in 1991, Bohle et al. (3) described the presence of monocytes, macrophages, T-cells, and fibroblasts associated with the tubulointerstitial changes seen in DN. More recent reports (revs. in 4,5) have suggested that inflammation may underlie disease progression in DN. The activation of NF-κB–linked regulatory pathways generally underlies inflammatory processes, and an increase in the nuclear translocation of NF-κB has been demonstrated in human DN (6,7).

It has become increasingly recognized that transcription factors such as NF-κB generally act synergistically with other transcription factors in order to impart selectivity and specificity to a transcriptional response. Two or more transcription factors in a specific orientation, separated by a given distance, working in concert within a promoter,

From [1]Medizinische Poliklinik, University of Munich, Munich, Germany; [2]Sanofi-Aventis Deutschland, Frankfurt, Germany; the [3]German Cancer Research Center, Heidelberg, Germany; and the [4]Physiology Institute, University of Kiel, Kiel, Germany.

Address correspondence and reprint requests to Clemens D. Cohen, MD, Division of Nephrology, Medizinische Poliklinik, University of Munich, Pettenkoferstr. 8a, 80336 Munich, Germany. E-mail: clemens.cohen@med.uni-muenchen.de. Or Matthias Kretzler, MD, Division of Nephrology, University of Michigan, 1570 MSRB II, 1150 W. Medical Ctr. Dr., Ann Arbor, MI 48109-0676. E-mail: kretzler@umich.edu.

Received for publication 11 April 2006 and accepted in revised form 3 August 2006.

H.S., A.B., and Y.Y. contributed equally to this work.

*A complete list of the members of the European Renal cDNA Bank Consortium can be found in the APPENDIX.

A.B. is currently affiliated with Sanofi-Aventis Deutschland, Frankfurt, Germany.

A.H., F.E., and M.K. are currently affiliated with the Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

CD, cadaveric donor; DN, diabetic nephropathy; FDR, false discovery rate; IRF, interferon regulatory factor; LD, living donor; MCD, minimal change disease; NF-κB, nuclear factor-κB; RMA, robust multichip analysis; SAM, Significance Analysis of Microarrays.

DOI: 10.2337/db06-0477

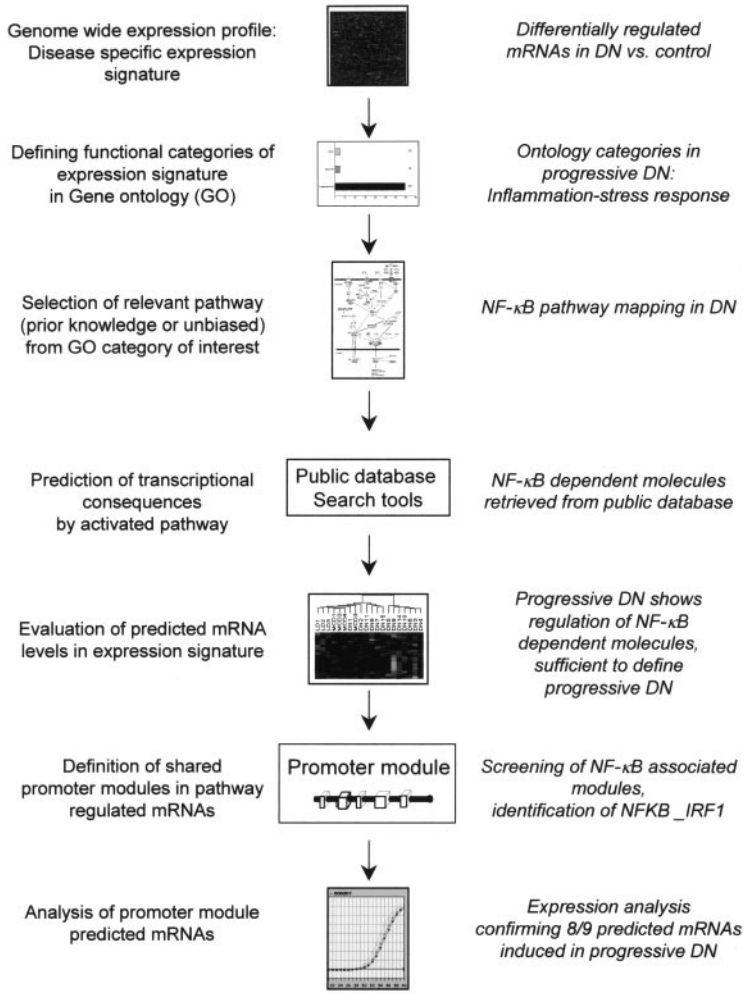

FIG. 1. Flow diagram of the experimental strategy to define the transcriptional regulation in progressive DN via an integrated systems biology approach.

are referred to as a promoter module and represent a core feature of an effective transcription initiation complex.

The aim of the present study was to identify potential regulatory pathways linked to NF-κB–based promoter modules that may underlie disease progression in DN. To this end, microdissected tubulointerstitial compartments from a set of biopsies with nodular glomerulosclerosis, the histopathological hallmark of DN, were subjected to genome-wide expression profiling. Unsupervised clustering of patients with DN by gene expression signatures allowed the definition of two principal subgroups of DN that correspond to patients showing mild or progressive DN, indicating specific molecular events in progressive versus early disease. The identification of specific promoter modules enriched in promoters of genes induced in progressive DN would help identify potential regulatory pathways linked to the progression of the disease and would, in theory, allow the prediction of additional genes potentially involved in the pathogenesis of DN. A schematic overview of the strategy used is provided in Fig. 1.

RESEARCH DESIGN AND METHODS

Kidney biopsies. Human renal biopsies were collected in a multicenter study, the European Renal cDNA Bank, after informed consent was obtained, according to the guidelines of the respective local ethics committees. Random aliquots taken from tubulointerstitial compartments of diagnostic renal biopsies were processed as described (8). All biopsies were stratified by the reference pathologist of the European Renal cDNA Bank according to histological diagnosis.

For oligonucleotide array–based gene expression profiling of DN, a total of 24 kidney biopsies from individual patients were included. Pretransplantation kidney biopsies from living donors (LDs) or cadaveric donors (CDs) were used as control renal tissue ($n = 7$). Renal tissue from patients with proteinuria, but normal glomerular filtration rate, and lacking any tubulointerstitial alterations served as an additional control group (minimal change disease [MCD], $n = 4$). The tubulointerstitium of 13 biopsies with nodular glomerulosclerosis, the histopathological hallmark of DN, was evaluated using the following semiquantitative grading system: 0, no; 1, minor; 2, moderate; 3, severe; and 4, most severe chronic tubulointerstitial changes. Here, 0 indicates normal histology with no pathologic lesions; 1 indicates small focal lesions, with minimal tubular atrophy and dilatation involving ≤15% of the renal parenchyma and interstitium; 2 indicates increased tubular dilatation and atrophy, mild inflammatory cell infiltrates with slight interstitial fibrosis, affecting 16–30% of the parenchyma and interstitium; 3 indicates increased tubular atrophy and moderate inflammatory cell infiltrate with interstitial fibrosis, involving 31–49% of parenchyma; and 4 indicates severe extensive tubulointerstitial changes and fibrosis involving ≥50% of the parenchyma and interstitium. Clinical and histological patient characteristics are given in Tables 1 and 2. The DN cohort was subdivided into "early" (representing histology scores 0–1) and "progressive" (scores 2–4) based on unsupervised clustering. The microarray data derived from the DN samples was found to reproducibly segregate these groups (see RESULTS).

For validation real-time RT-PCR analysis of biopsies from progressive DN patients ($n = 22$) and control subjects (LD, $n = 9$; CD, $n = 1$; and MCD, $n = 7$) were used (for clinical and histopathological characteristics, see Tables 1 and 2). Two (DN14 and DN15) of the 30 biopsies fulfilled the histological criteria for DN to be included in our study but revealed a discrepant clinical picture in the follow-up examination. DN14 showed a nodular glomerulosclе-

TABLE 1
Clinical and histological characteristics of reference biopsies analyzed by oligonucleotide array–based gene expression profiling and real-time RT-PCR

Sample name	Sex	Age (years)	Histology major diagnosis	Histology score	Creatinine (mg/dl)	Urine proteinuria (g/day)
Living donor						
Array						
LD1	F	66	LDx	NA	<1.1	<0.2
LD2	M	26	LDx w/o prev. damage	NA	0.9	<0.2
LD3	M	49	LDx w/o prev. damage	NA	<1.1	<0.2
Mean ± SEM		47 ± 9.6				<0.2
RT-PCR						
LD4	F	35	LDx	NA	<1.1	<0.2
LD5	M	39	LDx	NA	<1.1	<0.2
LD6	F	55	LDx	NA	<1.1	<0.2
LD7	M	41	LDx	NA	<1.1	<0.2
LD8	M	61	LDx	NA	<1.1	<0.2
LD9	F	58	LDx	NA	<1.1	<0.2
LD10	M	27	LDx	NA	<1.1	<0.2
LD11	F	54	LDx	NA	<1.1	<0.2
LD12	F	61	LDx	NA	<1.1	<0.2
Mean ± SEM		48 ± 14			<1.1	<0.2
Cadaveric donor						
Array						
CD1	M	50	CDx, minor int. fibrosis	NA	0.9	<0.2
CD2	M	54	CDx w/o prev. damage	NA	0.9	<0.2
CD3	M	61	CDx w/o prev. damage	NA	1.2	<0.2
CD4	F	51	CDx, minor int. fibrosis	NA	0.7	<0.2
Mean ± SEM		54 ± 2.1			0.9 ± 0.1	<0.2
RT-PCR						
CD5	NA	NA	CDx	NA	<1.1	<0.2
MCD/ no histological changes						
Array						
MCD1	M	32	Minimal-change GN	1	1.3	11.0
MCD2	F	32	Minimal-change GN	1	0.7	3.0
MCD3	M	16	Minimal-change GN	0	1.2	5.4
MCD4	M	20	Minimal-change GN in remission	0	0.9	0.2
Mean ± SEM		25 ± 3.6			1.0 ± 0.2	4.9 ± 2.3
RT-PCR						
MCD5	F	57	Minimal-change GN	0	1.1	10
MCD6	M	33	Minimal-change GN	1	1.4	9.1
MCD7	M	24	No histological changes	0	0.6	0.4
Mean ± SEM		38 ± 8			1.0 ± 0.2	6.5 ± 2.5

rosis associated with a light chain deposit disease, and DN15 showed an unclassified nodular glomerulosclerosis with mesangial matrix expansion. Both patients did not show overt diabetes in the available clinical datasets. Cluster analysis grouped DN14 in the progressive and DN15 in the early disease group. The samples were excluded from real-time RT-PCR analysis.

Microdissection and RNA isolation, microarray analysis, and real-time RT-PCR studies. Biopsy processing for gene expression was performed as described (8). For probe labeling, a modification of the Eberwein protocol was used (9). Affymetrix microarray analysis followed the protocol of the manufacturer. A detailed description of the protocols used is given in the online appendix (available at http://diabetes.diabetesjournals.org).

Microarray analysis. Image files were initially obtained through Affymetrix GeneChip software (MAS5). Subsequently, robust multichip analysis (RMA) was performed using RMAexpress. RMA is an R-based technique using the affymetrix microarray *.cel image file and is comprised of three steps: background adjustment, quartile normalization, and summarization. A background filter cutoff was defined using the highest signal value obtained from a nonhuman Affymetrix-control oligonucleotide multiplied by a factor of 1.2, corresponding in the current dataset to a log-based 2 value of 5.8.

Data analysis. Starting from the normalized RMA, the Significance Analysis of Microarrays (SAM; version 1.21, http://www-stat.stanford.edu/~tibs/SAM/) software was applied using a false discovery rate (FDR) of 1% to identify genes that were significantly differently regulated between the analyzed groups. For visualization of the results, a hierarchical cluster was performed using dChip-software (http://www.biostat.harvard.edu/complab/dchip/). The clustering algorithm applied the "1-Pearson correlation coefficient" for the distance metric and the "centroid-linkage" method using Unsupervised Hierarchical Cluster Analysis (10). The distribution of differentially regulated genes was attributed to functional categories using the GeneOntologyChart in DAVID (Database for Annotation, Visualization, and Integrated Discovery), version 2.0. The controlled hierarchical vocabulary of the GO Consortium provides a structured language that can be applied to the functions of genes and proteins in all organisms (http://www.geneontology.org/). Pathway analysis was performed using Ingenuity Pathway Application tools (www.ingenuity.com). The networks of regulated genes could be examined in the context of known metabolic and cell signaling cascades using "canonical pathways" that reflect previously characterized functional networks of genes.

NF-κB target genes and promoter module analysis. To evaluate the potential functional status of the NF-κB pathway, the expression of experimentally confirmed NF-κB downstream target genes were retrieved from the Rel/NF-κB transcription factor database (http://people.bu.edu/gilmore/nf-kb/target/index.html) and evaluated for expression on the DNA arrays.

A promoter module is defined as a functional unit consisting of two or more transcription factor binding sites conserved in order and distance. Experimentally verified promoter modules containing NF-κB binding sites were selected from the Module Library for Vertebrate Modules (Genomatix [www.genomatix.de], Munich, Germany). The proximal promoter regions of human NF-κB target genes were retrieved using the software ElDorado (Genomatix). The proximal promoter regions used were generally defined as 500 nucleotides upstream and 100 nucleotides downstream from the transcription start site. Transcription start sites were automatically assigned to genes based on 5′ cap site databases integrated into promoter identification pro-

TABLE 2
Clinical and histological characteristics of disease biopsies analyzed by oligonucleotide array–based gene expression profiling and real-time RT-PCR

Sample name	Sex	Age (years)	Histology major diagnosis	Histology score	Creatinine (mg/dl)	Proteinuria (g/day)	Diabetes type	Diabetes duration (years)	HbA_{1c} (%)	Retinopathy
DN										
Array										
DN1	M	45	DN	0	0.9	0.7	2	0.3	7.1	No
DN2	F	34	DN	1	1.4	0.3	1	31	NA	Yes
DN3	M	57	DN	3	1.6	9.7	1	20	8.7	Yes
DN4	M	61	DN	2	1.1	3.7	2	3	5.7	Yes
DN5	F	46	DN	2	2.4	0.4	2	25	7.5	Yes
DN6	F	67	DN	4	4.8	2.4	2	5	NA	Yes
DN7	M	73	DN	2	1.1	2.4	2	1	7.3	No
DN8	M	62	DN	2	1.2	2.5	2	5	5.6	No
DN9	M	67	DN	2	4.0	3.0	2	NA	NA	NA
DN10	M	62	DN	3	2.3	5.0	2	4	7.8	No
DN11	M	68	DN	3	3.5	2.4	2	4	7.2	No
DN14	**F**	**70**	**DN**	**4**	**4.0**	**5.0**	**—**	**—**	**—**	**No**
DN15	**M**	**46**	**DN**	**3**	**0.9**	**6.0**	**—**	**—**	**—**	**No**
Mean ± SEM		58.3 ± 3.4			2.2 ± 0.4	3.3 ± 0.7		10.9 ± 3.4	7.1 ± 0.3	
RT-PCR										
DN3	M	57	DN	3	1.6	9.7	1	20	8.7	Yes
DN5	F	46	DN	2	2.4	0.4	2	25	7.5	Yes
DN6	F	67	DN	4	4.8	2.4	2	5	NA	NA
DN10	M	62	DN	3	2.3	5.0	2	4	7.8	Yes
DN11	M	68	DN	3	3.5	2.4	2	4	7.2	No
DN16	M	58	DN	2	1.7	0.7	2	6.5	7.4	No
DN17	M	78	DN	4	3.6	3.1	2	32	NA	No
DN18	F	59	DN	4	3.2	21.4	2	12	NA	NA
DN19	M	58	DN	4	2.4	14.2	2	31	9.8	Yes
DN20	F	63	DN	4	3.3	7.0	2	18	7.9	No
DN21	M	63	DN	4	2.1	8.6	2	10	7.1	No
DN22	F	47	DN	4	7.0	NA	2	8	13.8	Yes
DN23	M	74	DN	3	2.5	0.3	2	8	4.6	No
DN24	F	63	DN	3	2.9	6.5	2	7	6.4	No
DN25	M	55	DN	4	2.8	7.5	2	6	7.8	Yes
DN26	M	57	DN	3	1.6	1.4	2	20	8.8	NA
DN27	M	63	DN	2	3.0	0.6	2	6	6.2	NA
DN28	M	66	DN	2	9.8	1.7	2	10	6.0	No
DN29	M	60	DN	4	2.2	2.0	2	8	6.9	No
DN30	M	75	DN	2	1.7	6.2	2	6	7.3	No
DN31	M	NA	DN	NA	1.2	10.0	2	<1	NA	NA
DN32	F	63	DN	4	2.0	5.5	2	23	6.4	NA
Mean ± SEM		62 ± 1.7			3.0 ± 0.4	5.5 ± 1.1		11.3 ± 1.7	7.6 ± 0.4	

Clinical data are presented for all patients involved in the study, either analyzed by microarrays, real-time RT-PCR, or both. Follow-up of the clinical data revealed in 2 (in boldface type) of the 30 patients with nodular glomerulosclerosis, no evidence for clinically overt diabetes but a light chain deposit disease in DN14 and unclassified nodular glomerulosclerosis with mesangial matrix expansion in DN15.

grams (Genomatix). The software ModelInspector (Genomatix) (11) was systematically engaged to identify modules in promoter regions. The Genomatix Promoter Database, containing 50.145 human promoter sequences, served as a control set. All analyses were performed with default parameters of the software tools.

Statistical analysis. Statistical analysis was performed using SPSS software (version 10.0; SPSS, Chicago, IL). Data were expressed as absolute values, means ± SEM. Multivariate ANOVA with a Bonferroni post hoc correction or χ^2 test were used where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Gene expression profiling identifies specific signatures of DN. Tubulointerstitial compartments of renal biopsies from patients with histological evidence of DN ($n = 13$) were analyzed by genome-wide expression profiling using Affymetrix oligonucleotide arrays HGU133A. Three pretransplant biopsies from related LD kidneys, four pretransplant biopsies from CDs, and four biopsies from patients with MCD without histological or clinical evidence of impaired renal function served as initial control samples. Of the 22,283 probe sets on the Affymetrix oligonucleotide arrays, 10,183 probe sets (45.7%) were expressed above background. Using the SAM algorithm, a multiple comparison analysis of LD, CD, MCD, and early and progressive DN with an FDR of 1% detected differential regulation of 1,349 from the 10,183 expressed probe sets (see online appendix Table S1 for gene expression data of respective probe sets).

Hierarchical clustering showed three principal branches, DN, CD, and MCD/LD, with only one outlier found in the MCD/LD cluster (DN1: DN patient with the best renal function) (Fig. 2). Inside the DN cohort, two discrete branches could be identified. One subgroup cor-

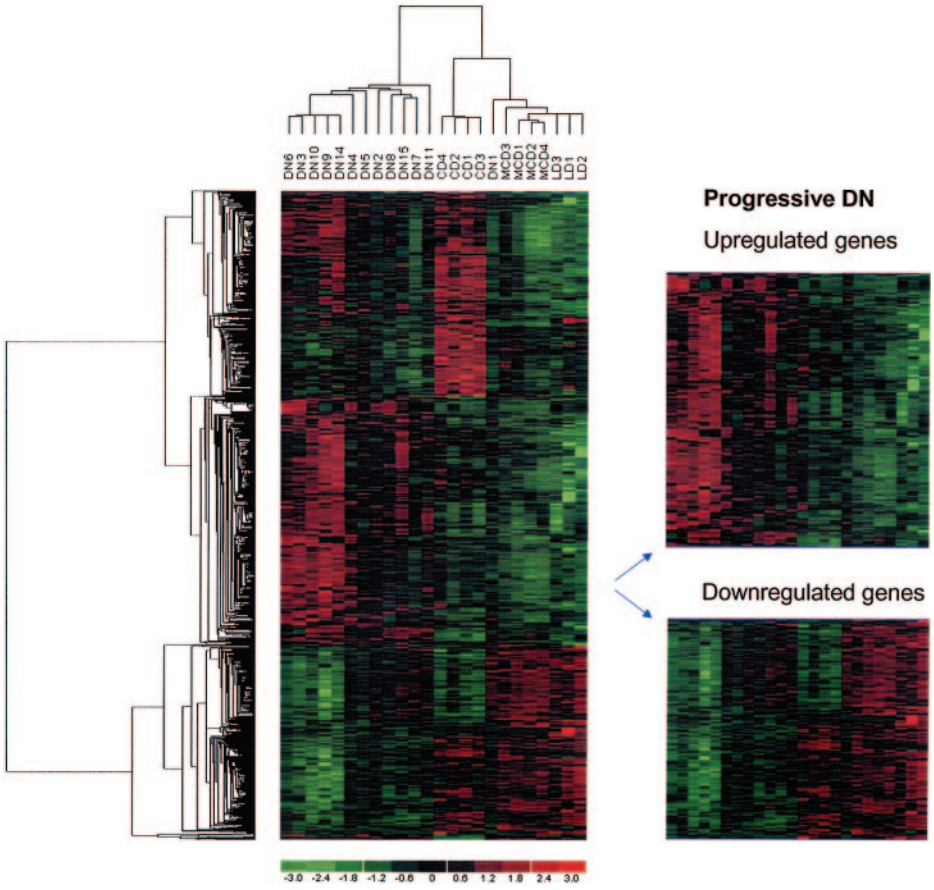

FIG. 2. Hierarchical cluster analysis of gene expression profiles from renal biopsies with early or progressive DN and different controls. Tubulointerstitial compartments of renal biopsies from patients with DN (n = 13) and MCD (n = 4), as well as pretransplant biopsies from related LD (n = 3) and CD (n = 4) kidneys, were analyzed. For clinical and histological patient characteristics, see Table 1. Gene expression profiles were analyzed using Affymetrix oligonucleotide arrays HGU133A. Of the 22,283 probe sets, 10,183 probe sets (45.7%) were expressed above background signal. Each row represents a gene; each column represents a biopsy. Transcript abundance is displayed on a red-green color scale, with red expression above and green below the median. Using the SAM algorithm, a multiple comparison analysis of LDs, CDs, MCD, and early and progressive DN with an FDR of 1% detected differential regulation of 1,349 from the 10,183 expressed probe sets. The cluster dendrogram sorts the patients with the most similar gene expression profiles together with the shortest branches. Hierarchical clustering showed three principal branches of DN, CD, and MCD/LD, with the earliest DN (DN1) as the only outlier in the MCD/LD cluster. Differentially regulated mRNAs are provided in the online appendix.

responded to patients with predominantly mild renal impairment (DN2, 7, 8, and 15; mean serum creatinine 1.4 ± 0.27 mg/dl, histology scores for tubulointerstitial damage 1–3 [see RESEARCH DESIGN AND METHODS]), and the second group showed more severe DN (DN3, 4, 5, 6, 9, 10, and 14; mean serum creatinine 2.8 ± 0.46 mg/dl, $P < 0.05$ compared with mild DN; histology scores 2–4). The gene expression signatures of LD and MCD biopsies were comparable, as both cohorts revealed stable renal function and no detectable tubulointerstitial damage. LD and MCD biopsies were then used as a combined control group to define genes associated with progressive tubulointerstitial damage. Interestingly, the gene expression profiles from CD biopsies showed considerable alterations when compared with those of LDs, most likely due to the events before organ harvest from the brain-dead donor. For this reason, the samples were not included as controls in the subsequent analysis.

The DN subgroups revealed 2,737 probe sets differentially regulated in progressive DN compared with the combined control group of LDs and MCD (after dChip analysis for redundant probe sets), whereas only 131 probe sets showed significant regulation in mild DN compared with controls.

Identification of a prominent inflammatory stress response in progressive DN. Analysis of the differentially regulated probe sets was performed to determine which inflammation-associated genes were regulated in DN. The GOCharts tool of DAVID was used to display the distribution of differentially expressed genes among functional categories. Of the 2,737 probe sets regulated in progressive DN, 282 were attributed to the functional category "response to stimulus," corresponding to genes involved in the inflammatory/stress response. By contrast, in early DN, 13 differentially regulated genes fell into the category of "response to stimulus."

Genes of the NF-κB pathway are upregulated in progressive DN. The differentially regulated stress response genes in progressive DN were then categorized into canonical pathways using the Ingenuity Pathways Knowledge Base, a structured database of biological networks. A special emphasis was placed on genes/pathways linked to the biology of NF-κB, a key transcriptional regulator in inflammatory/stress response. A group of genes directly linked to the NF-κB signaling pathway were evaluated for differential mRNA regulation in the DN dataset. Of the 85 known members of the NF-κB signaling pathway, 23 (27%) showed significant regulation in pro-

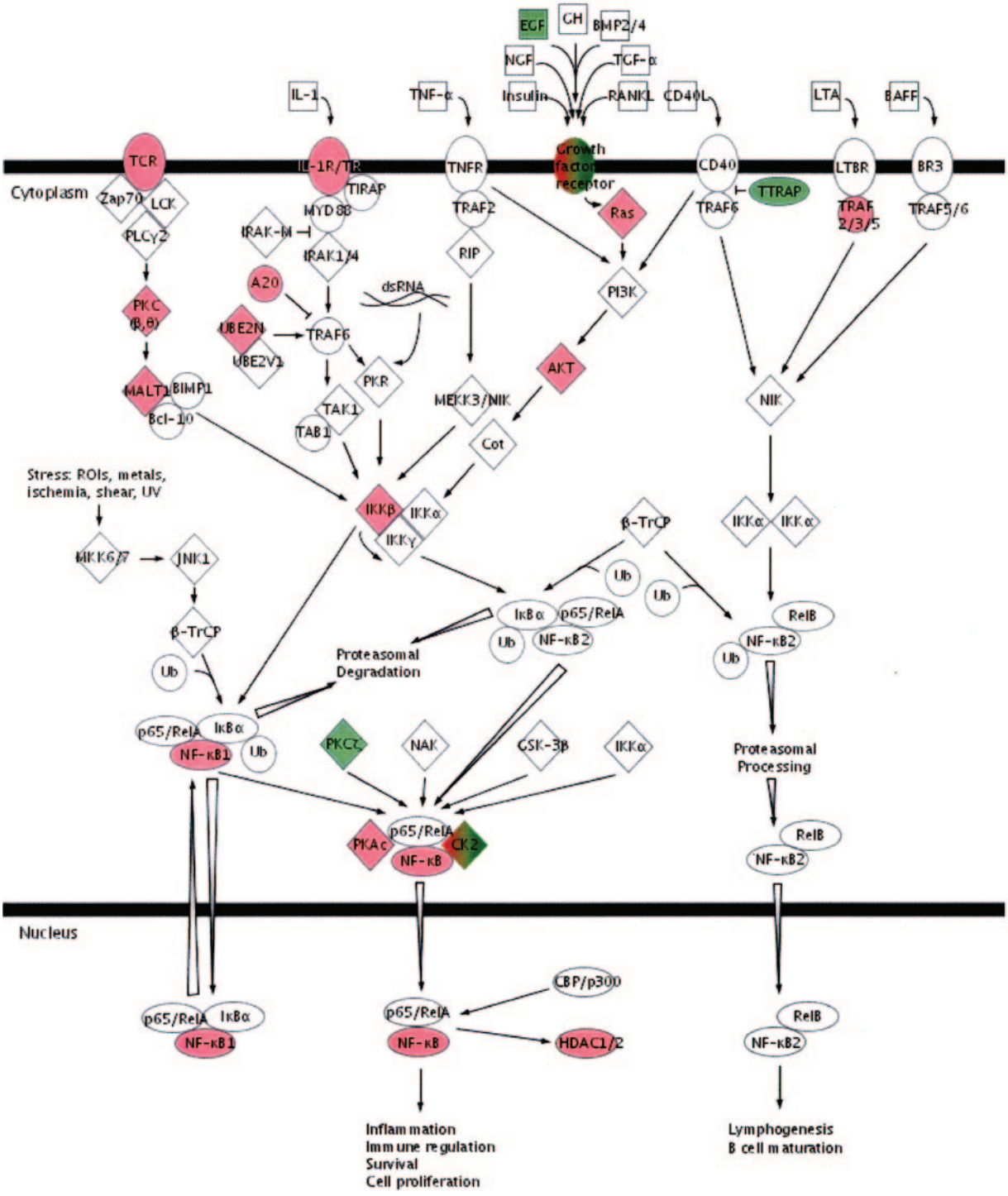

FIG. 3. The canonical NF-κB signaling pathway shows a prominent induction in progressive DN. To identify underlying control mechanisms of stress response genes in progressive DN, differentially regulated molecules were categorized into canonical pathways. Starting from 2,738 probe sets regulated between progressive DN and the combined controls (MCD/LD), 1,725 genes could be annotated to biological pathways using the Ingenuity Pathways Knowledge Base. Evaluating the master switch of stress responses, the canonical NF-κB signaling pathway, a differential regulation of 23 of 85 members (27%) in progressive DN compared with none in early DN could be detected. Upregulated genes are marked in red, downregulated genes in green, and green/red symbols correspond to differential regulation of gene isoforms.

gressive DN, whereas none were regulated in the early DN samples (Fig. 3). Thus, the NF-κB pathway is induced in progressive DN.

A subset of known NF-κB–regulated genes were upregulated in progressive DN. Activation of the NF-κB pathway leads downstream to the transcriptional activation of NF-κB–dependant genes. The gene expression data were then analyzed for the presence of known NF-κB target genes as defined by a comprehensive master set of experimentally confirmed NF-κB target genes (http://people.bu.edu/gilmore/nf-kb/target/index.html). Of the 232 compiled NF-κB target genes, 138 (59%) showed mRNA expression above background in the tubulointerstitial compartments of the kidney biopsies studied. To test the biological relevance of NF-κB induction, the DN biopsies were sorted using unsupervised cluster analysis, by ex-

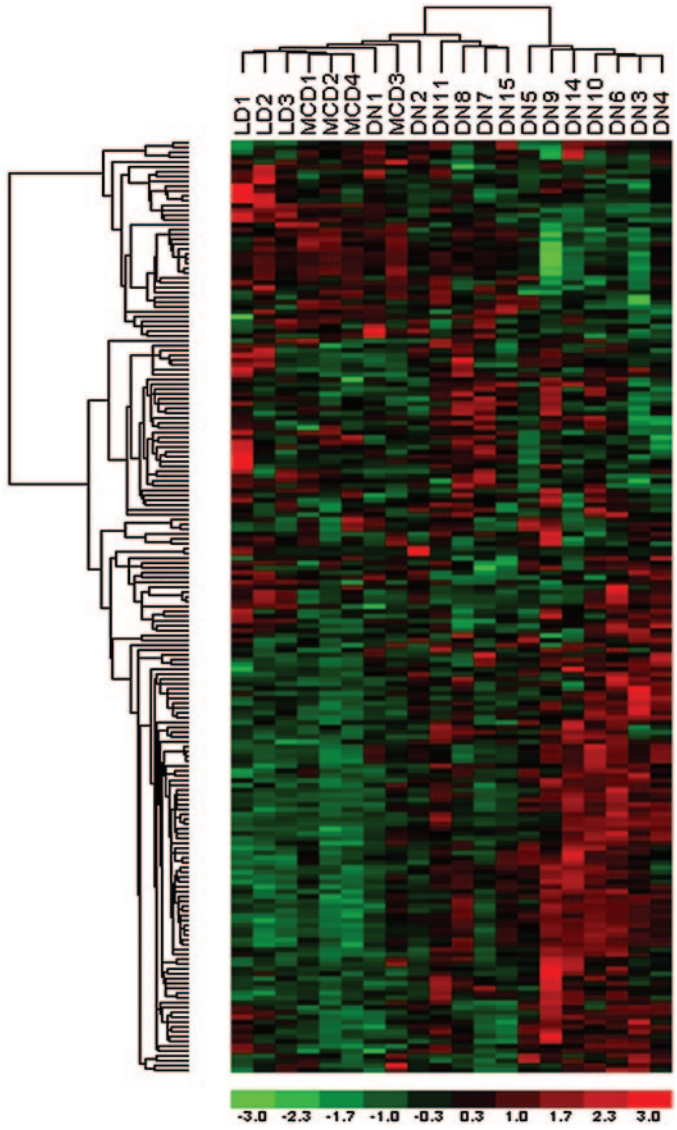

FIG. 4. NF-κB–dependant target molecules are significantly regulated in progressive DN. Of 232 NF-κB–dependant target genes, 138 (59.5%) showed mRNA expression above background in the kidney expression arrays. An unbiased cluster dendrogram using these 138 genes segregated progressive DN from the combined controls (MCD/LD) and early DN. Of the 138 expressed NF-κB–dependant mRNAs, 54 genes (39%) were significantly induced compared with the combined controls (LD/MCD). In contrast, only one of the 138 genes was upregulated in the early DN, underlining the role of NF-κB in the progression of DN.

pression of the 138 detectable NF-κB target genes. This resulted in the segregation of the progressive DN biopsies from the control and mild DN samples (Fig. 4). Of the 138 expressed NF-κB–dependant mRNAs, 54 genes (39%) were found to be significantly induced in progressive DN compared with controls. Only 1 of the 138 genes was found to be upregulated in mild DN. The results are consistent with the activation of NF-κB pathways in progressive DN.

Involvement of specific NF-κB transcription factor modules in progressive DN. As only a subgroup of NF-κB target genes were found to be differentially regulated on the array, the next question was to identify whether previously characterized NF-κB promoter modules linked to signal-specific gene expression were associated with the mRNA patterns seen in progressive DN. Promoter modules represent hierarchical promoter structures defined by a conserved combination, order, and spacing of specific transcription factor binding sites. These promoter structures allow the integration of signaling cascades that ultimately impart specific transcriptional regulation. The identification of a promoter module whose target genes are overrepresented in progressive DN would identify important pathways that converge at the nucleus (represented by a promoter module) that impart a selective regulation of a class of genes. A total of 51 verified NF-κB promoter modules have been identified and experimentally validated (12,13) (Module Library, version 3.8; Genomatix). The proximal promoter regions of the differentially regulated NF-κB genes were analyzed for the presence of these NF-κB modules using the tool ModelInspector (Genomatix).

Three subgroups of genes were characterized for the presence of NF-κB promoter modules. These included genes that were *1*) known NF-κB–regulated genes induced in progressive DN ($n = 54$), *2*) known NF-κB genes not found upregulated in DN ($n = 84$), and *3*) a database of 50,145 human promoter regions (Genomatix). Groups 1 and 3 were used as control sets to identify specific promoter modules that were enriched in the promoters of NF-κB target genes upregulated in progressive DN.

Of the 51 known NF-κB–associated promoter modules (Genomatix), only 6 could be specifically associated with the promoter regions of the NF-κB target genes upregulated in progressive DN compared with the noninduced NF-κB target genes. Seventeen additional modules were detected in the promoter regions of both upregulated and noninduced NF-κB target genes (Table 3). Eleven NF-κB modules were found only in the noninduced NF-κB target genes.

The NFKB_IRFF_01 module was the only example significantly overrepresented in the proximal promoter regions of upregulated NF-κB target genes compared with the noninduced NF-κB target genes ($P < 0.05$). The module was identified in the promoter regions of 5 of the 54 upregulated NF-κB target genes and in none of the noninduced genes. Additionally, this module was 41 times more frequent in the upregulated NF-κB target genes than in the human promoter database of 50,145 (Table 3).

The module NFKB_IRFF_01 represents a NF-κB binding site on the plus and interferon regulatory factor (IRF) binding site on the minus DNA strand separated by 14–24 bp. The module was originally identified by analysis of the HLA class I heavy-chain gene promoter (14). Accordingly, it was detected in the list of upregulated NF-κB target genes in the proximal promoter regions of *HLA-A*, in an additional computationally predicted alternative promoter of *HLA-A* and in the proximal promoter regions of *HLA-B*, *B2M*, and *CD74*.

Prediction of gene upregulation by the NFKB_IRFF_01 promoter module. Linkage of the NFKB_IRFF_01 module to progressive DN was then examined. A comprehensive literature search of the PubMed database was performed to identify other genes regulated by IRF and NF-κB. Nine genes were identified from a total of 21 publications studying IRF and NF-κB target gene regulation. These were *B2M*, *CCL5*, *CXCL10*, *EDN1*, *HLA-A*, *HLA-B*, *IFNB1*, *NOS2A*, and *VCAM1* encoding the gene products β2-microglobulin, C-C chemokine ligand 5 (RANTES), C-X-C chemokine ligand 10 (IP-10), endothelin 1, major histocompatibility complex class I-A and -B, interferon β-1, inducible nitric oxide synthase 2A, and vascular cell adhesion molecule-1, respectively.

The predicted regulation of these genes in DN was reanalyzed using the array data. Hybridization signals of

TABLE 3
ModelInspector analysis demonstrates a clear overrepresentation of the NF-κB module NFKB_IRFF_01 in 54 upregulated NF-κB–dependant target genes

	Induced NF-κ B genes		Non-ind. NF-κ B genes		All human promoters	
Modules	Number of matches	Probability (per 10,000 bp)	Number of matches	Probability (per 10,000 bp)	Number of matches	Probability (per 10,000 bp)
AP1F_NFKB_01	2	0.29	1	0.11	53	0.02
AP1F_NFKB_02	4	0.58	1	0.11	41	0.01
AP1F_NFKB_03	0	0.00	1	0.11	145	0.04
AP1F_NFKB_04	1	0.15	1	0.11	19	0.01
AP1F_NFKB_EBOX_01	0	0.00	1	0.11	2	0.00
CEBP_NFKB_01	0	0.00	1	0.11	34	0.01
CEBP_NFKB_02	2	0.29	1	0.11	121	0.03
CEBP_NFKB_04	1	0.15	1	0.11	111	0.03
CEBP_NFKB_05	1	0.15	0	0.00	139	0.04
CEBP_NFKB_06	5	0.73	3	0.34	>1,000	0.43
CEBP_NFKB_NFAT_02	0	0.00	1	0.11	204	0.06
CEBP_NFKB_STAT_01	0	0.00	1	0.11	1	0.00
CREB_NFKB_01	1	0.15	1	0.11	138	0.04
CREB_NFKB_03	1	0.15	0	0.00	127	0.04
GATA_GATA_NFKB_NFKB_01	1	0.15	0	0.00	1	0.00
IRFF_NFKB_01	1	0.15	0	0.00	106	0.03
IRFF_NFKB_03	0	0.00	2	0.23	70	0.02
NFKB_AP1F_01	4	0.58	6	0.68	>1,000	0.58
NFKB_AP1F_SP1F_01	0	0.00	2	0.23	14	0.00
NFKB_CEBP_01	1	0.15	4	0.46	596	0.17
NFKB_CREB_01	6	0.88	4	0.46	>1,000	0.58
NFKB_ETSF_01	0	0.00	1	0.11	54	0.02
NFKB_IRFF_01	**5**	**0.73**	**0**	**0.00**	**62**	**0.02**
NFKB_NFKB_02	0	0.00	1	0.11	5	0.00
NFKB_NFKB_03	3	0.44	1	0.11	40	0.01
NFKB_RBPF_01	3	0.44	6	0.68	344	0.10
NFKB_SORY_01	14	2.04	11	1.25	772	0.22
NFKB_SORY_02	10	1.46	5	0.57	348	0.10
NFKB_STAT_01	0	0.00	2	0.23	26	0.01
NFKB_STAT_02	7	1.02	9	1.02	>1,000	0.76
NFKB_STAT_NFKB_01	2	0.29	3	0.34	265	0.08
SP1F_NFKB_01	0	0.00	1	0.11	32	0.01
STAT_NFKB_02	1	0.15	0	0.00	27	0.01
YY1F_NFKB_01	1	0.15	1	0.11	50	0.01

Numbers of matches by ModelInspector (Genomatix) analyses of individual experimentally verified NF-κB containing promoter modules in proximal promoter sequences of 54 DN upregulated NF-κB target genes, 84 DN noninduced NF-κB target genes, and the whole human promoter library, including 50.145 proximal promoter sequences are depicted. Total length of analyzed proximal promoter sequences were 68.468 bp in upregulated NF-κB target genes, 87.856 bp in noninduced NF-κB target genes, and 35.125.826 bp in the whole human promoter library. Probabilities of matches with individual modules were shown as frequency of matches observed in 10.000 bp of analyzed proximal promoter sequences. Only the module NFKB_IRFF_01 (in boldface type) was significantly overrepresented in the proximal promoter sequences of induced NF-κB target genes compared with noninduced target genes ($P < 0.05$).

sufficient intensity were observed for five of the above mentioned genes: *B2M*, *HLA-A*, *HLA-B*, *CXCL10/IP10*, and *VCAM1*; the first three genes were already identified as indicator genes in the initial screen for relevant NF-κ B promoter models (*B2M*, *HLA-A*, and *HLA-B*). The remaining two genes revealed significant induction in progressive DN. For additional confirmation, mRNA expression of *CXCL10/IP10* was also verified in a larger cohort of DN patients using real-time RT-PCR. Comparable with the array data, *CXCL10/IP10* was found to be significantly induced in the tubulointerstitium of patients with progressive DN (Fig. 5*A*). For the remaining four genes (*CCL5/RANTES*, *EDN1*, *IFNB1*, and *NOS2A*), expression levels on the array were too low for quantification. Expression of the four genes was then evaluated using a more sensitive real-time RT-PCR analysis in the larger cohort of biopsies with DN. As predicted, *CCL5/RANTES*, *IFNB1*, and *EDN1* showed a significant induction of expression in progressive DN (Fig. 5*B*). The further testing of one of these genes, *CCL5/RANTES*, showed a significantly ($P = 0.002$) correlated mRNA expression level with proteinuria (measured in grams per day). NOS2A mRNA expression was too low for real-time mRNA quantification. The correct prediction of eight of nine genes (*B2M*, *CCL5/RANTES*, *CXCL10/IP10*, *EDN1*, *HLA-A*, *HLA-B*, *IFNB1*, and *VCAM1*) supports the functional role of the NF-KB_IRFF motif in progressive DN.

DISCUSSION

The final common pathway for progressive renal diseases such as DN is the development of tubular atrophy and chronic interstitial fibrosis, which is generally preceded by or associated with an inflammatory infiltrate. Here, increased steady-state mRNA levels of inflammatory genes are shown to be associated with interstitial fibrosis and

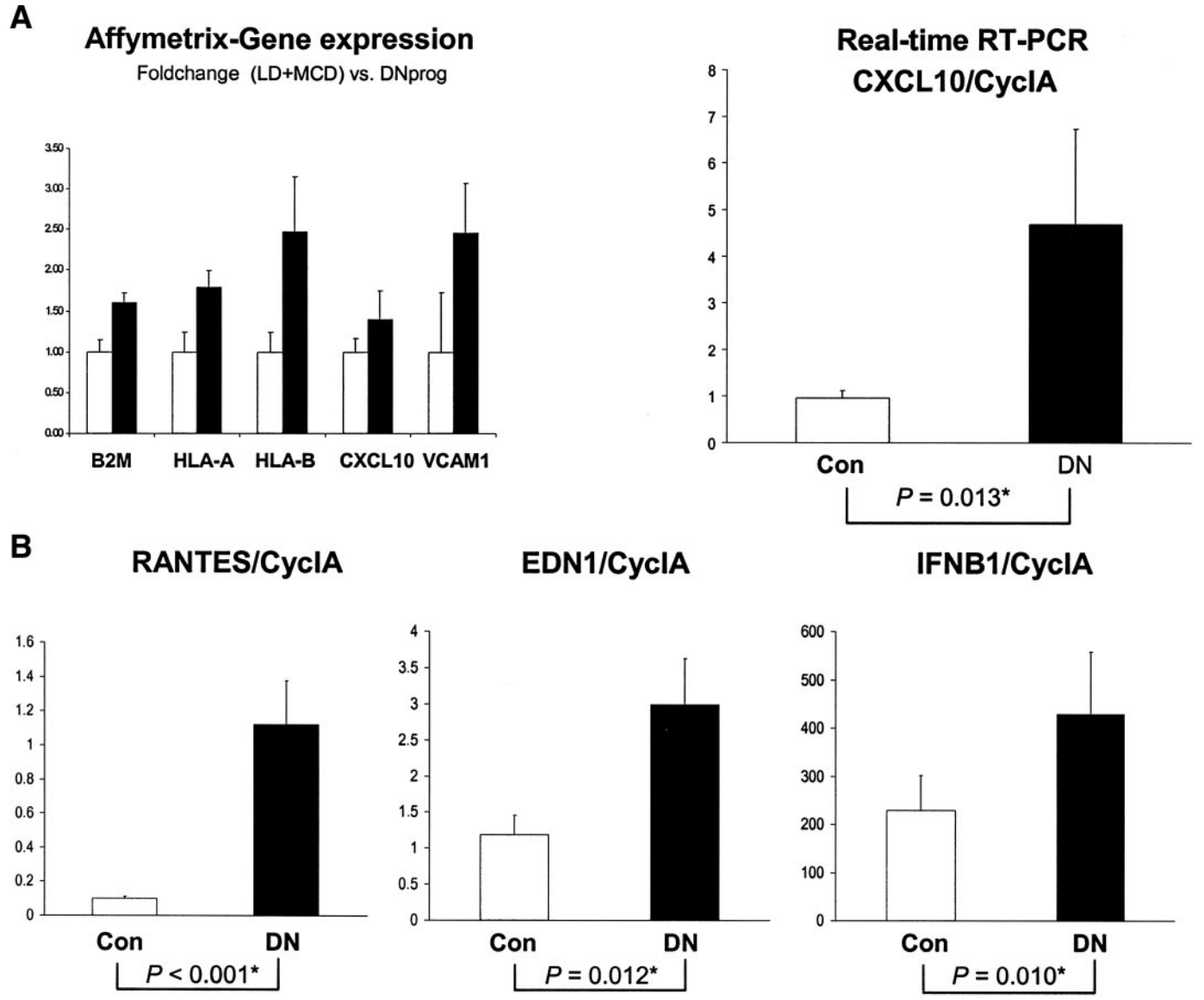

FIG. 5. Confirmation of predicted gene regulation by the NFKB_IRFF_01 promoter module. *A*: Microarray and real-time RT-PCR confirm the induction of *B2M*, *HLA-A*, *HLA-B*, *VCAM1*, and *CXCL10/IP10* in progressive DN. *B2M*, *HLA-A*, *HLA-B*, *VCAM1*, and *CXCL10/IP10* expression levels in progressive DN extracted from the array studies are given as ratios to the combined control group (LD/MCD) in the *left panel*. For confirmation and validation of the hybridization experiments, mRNA expression for *CXCL10/IP10* was studied in a cohort of progressive DN patients ($n = 22$) and different control subjects (LD, $n = 9$; CD, $n = 1$; and MCD, $n = 7$) using real-time RT-PCR (*right panel*). mRNA expression ratios were normalized to Cyclophilin A. Comparable with the array data (*left panel*) *CXCL10/IP10* mRNA was induced in the tubulointerstitium of patients with advanced DN (DN vs. control, $P = 0.013$*). Con, control. *B*: mRNA expression for *CCL5/RANTES*, *EDN1*, and *IFNB1* is significantly induced in patients with progressive DN. mRNA expression for *CCL5/RANTES*, *EDN1*, and *IFNB1* was studied in a cohort of progressive DN patients ($n = 22$) and different control subjects ($n = 17$) by real-time RT-PCR. mRNA expression ratios normalized to Cyclophilin A revealed a significant induction in the tubulointerstitium of patients with advanced DN (*CCL5/RANTES*: DN vs. control, *$P < 0.001$; *EDN1*: DN vs. control, *$P = 0.012$; *IFNB1*: DN vs. control, *$P = 0.010$*). Con, control.

progressive human DN. The transcription factor NF-κB helps to control the expression of numerous genes activated during inflammation. NF-κB is induced by various cell stress–associated stimuli including growth factors, vasoactive agents, cytokines, and oxidative stress (15–17). NF-κB in turn controls the regulation of genes encoding proteins involved in immune and inflammatory responses (i.e., cytokines, chemokines, growth factors, immune receptors, cellular ligands, and adhesion molecules). The activation and nuclear translocation of NF-κB in human DN has been recently demonstrated in intrinsic cells of the kidney in human DN (6,7).

To define the specific NF-κB–associated pathways at work in progressive DN, gene expression datasets were evaluated for NF-κB–dependant target genes. The subset of NF-κB–dependant genes found to be induced in DN were further evaluated for the overrepresentation of specific NF-κB–containing regulatory elements or modules within the proximal promoter sequence of these regulated genes. The identification of a specific module would help identify a regulatory process involved in the progression of DN (i.e., decrease in glomerular filtration and increase in serum creatinine among others) through the selective regulation of genes involved in the pathogenesis of the disease.

Computational analyses of promoter characteristics have become feasible as a consequence of access to genome sequences and experimentally verified transcrip-

tion factor binding patterns. The identification of specific frameworks of transcription factor binding sites linked to gene coregulation remains challenging, as the characteristics of such frameworks cannot be defined by sequence homology. It is now widely accepted that position weight matrices defining the specific order, spacing, distance, and orientation for transcription factors allow the best in silico characterization of promoter motifs (11,18). Systematic promoter module analysis of the proximal promoter regions of upregulated known NF-κB–dependant genes identified a NF-κB module (NFKB_IRFF_01) associated with progression of DN. This module was found overrepresented in progressive DN–associated upregulated NF-κB–dependant promoters at a 45-fold higher frequency than could be expected by chance.

The NFKB_IRF_01 module was originally characterized by Johnson and Pober (14) while searching for *cis*-acting elements mediating the effect of tumor necrosis factor-α and interferon-γ. Ohmori and Hamilton (19) demonstrated the cooperative interaction between transcription factors binding to an interferon-stimulated response element, a promoter sequence found in several interferon-inducible genes, with NF-κB binding at a proximal site that leads to synergistic induction of the *CXCL10/IP-10* gene. Later complementary data regarding synergistic effects of IRF and NF-κB were described in the induction of *B2M*, *CCL5/RANTES* (20), *EDN1* (21), *IFNB1* (22), *NOS2A* (23), and *VCAM1* (24).

The computational approach was verified by evaluating mRNA expression levels of nine genes predicted to be regulated by the NFKB_IRFF_01 module based on a comprehensive search of the literature. For eight of these nine genes (*CCL5/RANTES*, *CXCL10/IP10*, *EDN1*, *VCAM1*, *HLA-A*, *HLA-B*, *IFNB1*, and *B2M*) the predicted induction in progressive DN was confirmed by analysis of the specific expression values in the array experiment or by real-time RT-PCR analysis. Only *NOS2A* expression was too low for a reliable mRNA expression analysis. A functional role of these predicted genes in the development of DN is supported by published data. For example, upregulation of *CCL5/RANTES* and NF-κB activation has been reported in tubular cells in DN (6). Furthermore, polymorphisms associated with the NF-κB binding sites in the promoter for *CCL5/RANTES* are reported to be a risk factor for the development of DN in patients with type 2 diabetes (25). For *CXCL10/IP-10*, an increased interstitial staining signal has been reported in obese diabetic Zucker rats, a model of diabetes-related nephropathy (26). These results suggest a general role for these chemokines in the inflammatory tubulointerstitial infiltrate of progressive DN. Soluble vascular adhesion model-1 has been reported to be correlated with the degree of microvascular complications in diabetes (27) and interstitial inflammation in DN (28). Finally, upregulation of HLA-A and -B and of β2-microglobulin is consistent with an inflammatory state and increased cell turnover in kidneys with progressive DN. Consistent with the potential involvement of an IRF/NF-κB module in DN, we found that these genes were only induced in the progressive DN group out of a cohort of 54 patients (i.e., all patients analyzed by microarray and real-time RT-PCR without repetitive analysis of the same patient).

Inflammation underlies the progression of many chronic disease processes. The role of inflammation in the progression of DN can be implied from the studies described above. The importance of the inflammatory component has been further substantiated in intervention studies, demonstrating an amelioration of diabetic tissue damage by anti-inflammatory drugs such as mycophenolate mofetil or low-dose methotrexate (29,30). A reduction in NF-κB activation and subsequent reduction in macrophage and T-cell infiltration may help explain the observed benefit. As general immunosuppressive interventions may not be a long-term therapeutic option in DN, the identification of the underlying inflammatory pathways responsible for a diabetes-specific inflammatory response in DN may allow development of a more selective immunomodulation intervention in the future.

In conclusion, a modular systems biology approach to human DN enabled the identification of a prominent inflammatory signature in progressive DN, paralleled by a differential regulation of the NF-κB pathway. Computational promoter analysis allowed the characterization of a specific promoter module effectively predicting the mRNA regulation of NFKB_IRFF_01–dependant genes in DN. Integrating gene expression profiling with promoter modeling defined a central role of inflammation, including chemokines, as mediators and potential therapeutic targets in human DN. Systematic promoter module analysis on gene expression data revealed specific transcriptional regulatory mechanisms associated with tubulointerstitial inflammation in DN. These data argue for a pivotal and still underestimated role of inflammatory processes in the development and progression in renal disease as DN and for a central role of the NF-κB/IRF "master switch."

APPENDIX

Members of the European Renal cDNA Bank at the time of the study

C. Cohen, H. Schmid, M. Kretzler, and D. Schlöndorff, Munich, Germany; P. Ronco and J.D. Sraer, Paris, France; M.P. Rastaldi and G. D'Amico, Milan, Italy; F. Mampaso, Madrid, Spain; P. Doran and H.R. Brady, Dublin, Ireland; D. Mönks and C. Wanner, Würzburg, Germany; A.J. Rees and P. Brown, Aberdeen, Scotland; F. Strutz and G. Müller, Göttingen, Germany; P. Mertens and J. Floege, Aachen, Germany; N. Braun and T. Risler, Tübingen, Germany; L. Gesualdo and F.P. Schena, Bari, Italy; J. Gerth and G. Wolf, Jena, Germany; R. Oberbauer and D. Kerjaschki, Vienna, Austria; B. Banas and B.K. Krämer, Regensburg, Germany; W. Samtleben, Munich, Germany; H. Peters and H.H. Neumayer, Berlin, Germany; K. Ivens and B. Grabensee, Düsseldorf, Germany; and V. Tesar, Prague, Italy.

ACKNOWLEDGMENTS

This study was supported in part by the State of Bavaria "Bayerischer Habilitationsfoerderpreis" to C.D.C., EU FW V: QLG1-CT-2002-01215, the German Human Genome Project (DHGP), DFG Kr 1492/6-4 and the Else Kröner Fresenius Foundation to M.K. and C.D.C., DFG FG 406/project D to H.-J.G., and EU INNOCHEM and SFB 571 C2 to P.J.N.

The expert technical assistance of Ingrid Bayer, Sandra Irrgang, and Karin Frach is gratefully acknowledged. We thank Bruno Luckow for helpful discussion and Martin Seifert, Genomatix, Germany, for support.

REFERENCES

1. O'Connor AS, Schelling JR: Diabetes and the kidney. *Am J Kidney Dis* 46:766–773, 2005
2. Gilbert RE, Cooper ME: The tubulointerstitium in progressive diabetic

kidney disease: more than an aftermath of glomerular injury? *Kidney Int* 56:1627–1637, 1999
3. Bohle A, Wehrmann M, Bogenschutz O, Batz C, Muller CA, Muller GA: The pathogenesis of chronic renal failure in diabetic nephropathy: investigation of 488 cases of diabetic glomerulosclerosis. *Pathol Res Pract* 187:251–259, 1991
4. Galkina E, Ley K: Leukocyte recruitment and vascular injury in diabetic nephropathy. *J Am Soc Nephrol* 17:368–377, 2006
5. Navarro JF, Mora C: Role of inflammation in diabetic complications. *Nephrol Dial Transplant* 20:2601–2604, 2005
6. Mezzano S, Aros C, Droguett A, Burgos ME, Ardiles L, Flores C, Schneider H, Ruiz-Ortega M, Egido J: NF-kappaB activation and overexpression of regulated genes in human diabetic nephropathy. *Nephrol Dial Transplant* 19:2505–2512, 2004
7. Sakai N, Wada T, Furuichi K, Iwata Y, Yoshimoto K, Kitagawa K, Kokubo S, Kobayashi M, Hara A, Yamahana J, Okumura T, Takasawa K, Takeda S, Yoshimura M, Kida H, Yokoyama H: Involvement of extracellular signal-regulated kinase and p38 in human diabetic nephropathy. *Am J Kidney Dis* 45:54–65, 2005
8. Cohen CD, Frach K, Schlondorff D, Kretzler M: Quantitative gene expression analysis in renal biopsies: a novel protocol for a high-throughput multicenter application *Kidney Int* 61:133–140, 2002
9. Phillips J, Eberwine JH: Antisense RNA amplification: a linear amplification method for analyzing the mRNA population from single living cells. *Methods* 10:283–288, 1996
10. Eisen MB, Spellman PT, Brown PO, Bolstein D: Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 95:14863–14868, 1998
11. Frech K, Danescu-Mayer J, Werner T: A novel method to develop highly specific models for regulatory units detects a new LTR in GenBank which contains a functional promoter. *J Mol Biol* 270:674–687, 1997
12. Werner T: Cluster analysis and promoter modelling as bioinformatics tools for the identification of target genes from expression array data. *Pharmacogenomics* 2:25–36, 2001
13. Kim T, Yoon J, Cho H, Lee WB, Kim J, Song YH, Kim SN, Yoon JH, Kim-Ha J, Kim YJ: Downregulation of lipopolysaccharide response in Drosophila by negative crosstalk between the AP1 and NF-kappaB signaling modules. *Nat Immunol* 6:211–218, 2005
14. Johnson DR, Pober JS: HLA class I heavy-chain gene promoter elements mediating synergy between tumor necrosis factor and interferons. *Mol Cell Biol* 14:1322–1332, 1994
15. Karin M, Greten FR: NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 5:749–759, 2005
16. Li Q, Verma IM: NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2:725–734, 2002
17. Yamamoto Y, Gaynor RB: Role of the NF-kappaB pathway in the pathogenesis of human disease states. *Curr Mol Med* 1:287–296, 2001
18. Cohen CD, Klingenhoff A, Boucherot A, Nitsche A, Henger A, Brunner B, Schmid H, Merkle M, Saleem MA, Koller KP, Werner T, Gröne HJ, Nelson PJ, Kretzler M: Comparative promoter analysis allows *de novo* identification of specialized cell junction associated proteins. *Proc Natl Acad Sci U S A* 103:5682–5687, 2006
19. Ohmori Y, Hamilton TA: The interferon-stimulated response element and a kappa B site mediate synergistic induction of murine IP-10 gene transcription by IFN-gamma and TNF-alpha. *J Immunol* 154:5235–5244, 1995
20. Genin P, Algarte M, Roof P, Lin R, Hiscott J: Regulation of RANTES chemokine gene expression requires cooperativity between NF-kappa B and IFN-regulatory factor transcription factors. *J Immunol* 164:5352–5361, 2000
21. Woods M, Wood EG, Bardswell SC, Bishop-Bailey D, Barker S, Wort SJ, Mitchell JA, Warner TD: Role for nuclear factor-kappaB and signal transducer and activator of transcription 1/interferon regulatory factor-1 in cytokine-induced endothelin-1 release in human vascular smooth muscle cells. *Mol Pharmacol* 64:923–931, 2003
22. Kirchhoff S, Wilhelm D, Angel P, Hauser H: NFkappaB activation is required for interferon regulatory factor-1-mediated interferon beta induction. *Eur J Biochem* 261:546–554, 1999
23. Kinugawa K, Shimizu T, Yao A, Kohmoto O, Serizawa T, Takahashi T: Transcriptional regulation of inducible nitric oxide synthase in cultured neonatal rat cardiac myocytes. *Circ Res* 81:911–921, 1997
24. Neish AS, Read MA, Thanos D, Pine R, Maniatis T, Collins T: Endothelial interferon regulatory factor 1 cooperates with NF-kappa B as a transcriptional activator of vascular cell adhesion molecule 1. *Mol Cell Biol* 15:2558–2569, 1995
25. Nakajima K, Tanaka Y, Nomiyama T, Ogihara T, Ikeda F, Kanno R, Iwashita N, Sakai K, Watada H, Onuma T, Kawamori R: RANTES promoter genotype is associated with diabetic nephropathy in type 2 diabetic subjects. *Diabetes Care* 26:892–898, 2003
26. Blanco S, Vaquero M, Gomez-Guerrero C, Lopez D, Egido J, Romero R: Potential role of angiotensin-converting enzyme inhibitors and statins on early podocyte damage in a model of type 2 diabetes mellitus, obesity, and mild hypertension. *Am J Hypertens* 18:557–565, 2005
27. Koga M, Otsuki M, Kubo M, Hashimoto J, Kasayama S: Relationship between circulating vascular cell adhesion molecule-1 and microvascular complications in type 2 diabetes mellitus. *Diabet Med* 15:661–667, 1998
28. Ina K, Kitamura H, Okeda T, Nagai K, Liu ZY, Matsuda M, Fujikura Y: Vascular cell adhesion molecule-1 expression in the renal interstitium of diabetic KKAy mice. *Diabetes Res Clin Pract* 44:1–8, 1999
29. Utimura R, Fujihara CK, Mattar AL, Malheiros DM, Noronha IL, Zatz R: Mycophenolate mofetil prevents the development of glomerular injury in experimental diabetes. *Kidney Int* 63:209–216, 2003
30. Yozai K, Shikata K, Sasaki M, Tone A, Ohga S, Usui H, Okada S, Wada J, Nagase R, Ogawa D, Shikata Y, Makino H: Methotrexate prevents renal injury in experimental diabetic rats via anti-inflammatory actions. *J Am Soc Nephrol* 16:3326–3338, 2005