Differences in Intestinal Gene Expression Profiles in Broiler Lines Varying in Susceptibility to Malabsorption Syndrome

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ABSTRACT Examination of the host gene expression response upon encounters with pathogens may provide insights into the cellular events following an infection. In addition, it may shed light on the basic mechanisms underlying differences in the susceptibility of the host. In this study gene expression in the chicken jejunum was investigated in 2 different broiler lines under control and malabsorption syndrome (MAS) affected conditions. The 2 broiler lines differ in their susceptibility for MAS. The gene expression was investigated at 6 different times postinoculation using a custom-made intestine specific cDNA microarray. More than 70 up- or downregulated genes were identified after a MAS inoculation in both broiler lines. However, the number of the up- and downregulated genes varied between the 2 lines, with more differences in expression in the most susceptible line. Marked differences were observed in expression profiles between the 2 broiler lines, in control as well as in the MAS affected birds. The microarray data were validated and confirmed by quantitative real time PCR. The differentially expressed genes included immune related genes, genes associated with food absorption, and genes that need to be characterized further before their role in MAS and MAS susceptibility can be understood.

(*Key words*: malabsorption syndrome, microarray, susceptibility, chicken, intestine)

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INTRODUCTION

Broiler strains can differ in susceptibility to infectious diseases, probably due to their genetic differences (Lamont, 1998; Zekarias et al., 2002b). During selection of strains, disease susceptibility was given minor attention, whereas major attention was given to specific economical traits as feed conversion and growth rate. Breeding chickens with an improved resistance to different infectious diseases would be beneficial, because the use of antibiotics is under pressure and will be forbidden in the near future. Therefore, it would be useful to consider genes involved in disease susceptibility as a trait in new breeding programs (Georges, 2001).

Genes associated with disease susceptibility may be discovered by comparing, on a genome-wide scale, susceptible and less susceptible lines under control and challenge conditions (Yonash et al., 1999; Liu et al., 2001). Identifying potential important genes for disease susceptibility in chickens may be done with a number of different techniques like QTL, differential display, SNP detection, microarrays or a combination of these techniques (Yonash et al., 1999; Liu et al., 2001; Moody, 2001; Morgan et al., 2001; Neiman et al., 2001; Smith et al., 2002). cDNA microarrays are a recommended technique to study mRNA expression profiles of many different genes simultaneously (Meltzer, 2001). Expressed sequence tags (EST) from chickens are available in the public database, and microarrays have been generated including a chicken jejunum cDNA microarray (van Hemert et al., 2003).

Malabsorption syndrome (MAS) is a worldwide disease that affects broilers during the first few weeks posthatch. It is a multifactorial disease and the exact agents causing the disease are not yet known, but different viruses and bacteria have been characterized (Songserm et al., 2002b). Experimental induction of MAS can be done by oral inoculation of 1-d-old broilers with homogenates obtained from digestive tissues of MAS-affected chickens. The disease is characterized by weight gain depression with nonuniform growth, defective feathering, and diarrhea with undigested food and watery content. Most lesions are present in the digestive organs, in particular, the small intestine. Because MAS mainly affects the intestine, it can be used as a model to study intestinal health and intestinal disturbances in young broilers.

Broiler lines differ in their susceptibility for MAS as measured by degree of growth retardation and the amount of lesions in the intestine after experimentally induced MAS.

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Abbreviation Key: EST = expressed sequence tag; MAS = malabsorption syndrome; PI = postinoculation; RT = reverse transcription.

Also, differences in the ratio of $CD4^+$:$CD8^+$ cells in the jejunum between lines in infected and control conditions were found (Songserm et al., 2002a; Zekarias et al., 2002a). The basic mechanisms underlying the cause(s) of the differences in susceptibility for MAS are unknown. The identification of differentially expressed genes in susceptible and less susceptible chickens under control and MAS-affected conditions may lead to a better understanding of the cellular processes that determine susceptibility to MAS and maybe to other diseases.

The transcriptional response in the intestine of broilers after MAS induction is reported here. Differences in gene expression in relation to MAS susceptibility are also described. Gene expression differences in the intestine were investigated using a cDNA microarray containing more than 3,000 EST derived from normalized and subtracted intestinal cDNA library (van Hemert et al., 2003). The findings were confirmed using quantitative reverse transcription (RT)-PCR.

MATERIALS AND METHODS

Chickens

Two broiler lines, S (susceptible) and R (resistant), were used in the present study.[2] The lines were described earlier as B and D, respectively (Zekarias et al., 2002a). Sixty 1-d-old chicks of each line (S and R) were randomly divided into 2 groups of 30 chicks. One group was orally inoculated with 0.5 mL of the MAS-homogenate [homogenate C in tryptose buffered broth (Songserm et al., 2000)] and the other (control) group was orally inoculated with 0.5 mL of Dulbecco's PBS. Five chicks of each group were randomly chosen and killed at 8 h, and d 1, 3, 5, 7, and 11 postinoculation (PI) and tissue samples were collected. Pieces of the jejunum were snap frozen in liquid nitrogen and stored at –70°C until further use. Adjacent parts of the jejunum were fixed in 4% formaldehyde and used for histopathology and immunohistochemistry. The study was approved by the Institutional Animal Experiment Commission in accordance with the Dutch regulations on animal experimentation.

The same protocol, lines, and groups, were used for a second experiment, although in that experiment, 3 chicks of each group were killed on d 1, 3, and 13 PI. The same tissues were sampled.

RNA Isolation

Pieces of the jejunum were crushed under liquid nitrogen. Fifty to 100 mg of tissue from the different chicks were used to isolate total RNA using Trizol reagent,[3] according to instructions of the manufacturer with an additional step. The homogenized tissue samples were resuspended in 1 mL of Trizol reagent using a syringe and 21-gauge needle and the lysate was aspirated through the syringe 10 times. After homogenization, insoluble material was removed from the homogenate by centrifugation at 12,000 × g for 10 min at 4°C. For the array hybridization, pools of RNA were made in which equal amounts of RNA from 5 different chickens of the same line, condition, and sampling time were present.

Hybridization of the Microarray

The microarrays were constructed as described earlier and contained 3,072 cDNA spotted in duplicate (van Hemert et al., 2003). Before hybridization, the microarray was prehybridized in 5% saline sodium citrate, 0.1% SDS, and 1% BSA at 42°C for 30 min. To label the RNA, the Micromax TSA labeling and detection kit[4] was used. The TSA probe labeling and array hybridization were performed as described in the instruction manual with minor modifications. Biotin- and fluorescein-labeled cDNA were generated from 5 μg of total RNA from the chicken jejunum pools per reaction. The cDNA synthesis time was increased to 3 h at 42°C, as suggested (Karsten et al., 2002). Posthybridization washes were performed according to the manufacturer's recommendations. Hybridizations were performed in duplicate with the fluorophores reversed. After signal amplification, the microarrays were dried and scanned in a GeneTAC2000.[5] The image was processed (geneTAC software[5]) and spots were located and integrated with the spotting file of the robot. Reports were created of total spot information and spot intensity ratio for subsequent data analyses.

Analysis of the Microarray Data

After background correction, data were presented in an M/A plot where $M = \log_2 R/G$, $A = \log_2(R \times G)$, R = fluorescence intensity of red (Cy5) labeled mRNA sample, and G = fluorescence, intensity of green (Cy3) labeled mRNA sample (Dudoit et al., 2002). An intensity-dependent normalization was performed using the lowess fit function in the statistical software package R (Yang et al., 2002). The normalization was done with a fraction of 0.2 on all data points.

A total of 48 microarrays were used in the first experiment. For each of the 6 sampling times, the following 4 comparisons were made: broiler line S control vs. broiler line R control, broiler line S MAS vs. broiler line R MAS, broiler line S control vs. broiler line S MAS, and broiler line R control vs. broiler line R MAS.

Two microarrays were used for each of the comparisons. Therefore, for each cDNA, 4 values were obtained, 2 for one slide and 2 for the dye-swap. Genes with 2 or more missing values were removed from further analysis. Missing values may have been due to a bad signal to noise ratio. A gene was considered to be differentially expressed when the mean value of the ratio was greater than 2 or less than –2 and the cDNA was identified with significance analysis of microarrays [based on significance analysis of

[2]Nutreco, Boxmeer, The Netherlands.
[3]Invitrogen, Breda, The Netherlands.
[4]PerkinElmer, Wellesley, MA.
[5]Genomic Solutions, Ann Arbor, MI.

TABLE 1. Sequences of primers used for LightCycler reverse transcription PCR

Gene name/ homology	Forward primer	Reverse primer
Avian nephritis virus	ATTGCACAGTCAACTAATTTG	AAAGTTAGCCAATTCAAAATTA
Calbindin	CATGGATGGGAAGGAGC	GCTGCTGGCACCTAAAG
Gastrotropin	TAGTCACCGAGGTGGTG	GCTTTCCTCCAGAAATCTC
HES1	TCTTCCCAGGCTGTGAG	GGTCACCAGCTTGTTCTTC
Interferon-induced 6-16 protein	CGATCATGTCTGGTGAGGC	AGCACCTTCCTCCTTTG
Lysozyme G	CGGCTTCAGAGAAGATTG	GTACCGTTTGTCAACCTGC
Meprin	TTGCAGAATTCCATGATCTG	AGAAGGCTTGTCCTGATG
Pyrin	CCTGCACTGACCCTTG	GTGGCTCAGGGTCTTTC

microarrays (SAM) (Tusher et al., 2001)] with a false discovery rate <2%. Because a ratio is expressed in a $\log_2$ scale, a ratio of greater than 2 or less than −2 corresponds to a more than 4-fold up- or downregulation, respectively.

Sequencing and Sequence Analysis

Bacterial clones containing an insert representing a differentially expressed gene were sequenced. First a PCR was performed. One reaction of 50 μL contained: 5 μL of 10× ExTaq buffer,[6] 1 μL of dNTP mixture[6] (2.5 m*M* each), 0.1 μL of nested primer 1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) and nested primer 2 (5′-AGCGTGGTCGCGGCCGAGGT-3′, 100 pmol/μL), 0.125 μL of ExTaq[6] (5 units/μL), 43.6 μL of sterilized distilled water and a bacterial clone from the library. The PCR was performed using a thermocycler programmed to conduct the following cycles: 2 min at 95°C, 40× (45 s at 95°C, 45 s at 69°C, 120 s at 72°C), 5 min at 72°C. The PCR amplification products were purified using Sephadex G50 fine column filtration.

One microliter of the purified PCR product was sequenced using 10 pmol of nested primer 1 and 4 μL of ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction[4] mix in a total volume of 10 μL. The sequence reaction consisted of 2 min at 96°C, 40× (10 s at 96°C, 4 min at 60°C). Sequencing was performed on an ABI 3700 DNA sequencer.[4] Sequence results were analyzed using SeqMan 5.00. Sequences were compared with the NCBI nonredundant and the EST *Gallus gallus* databases using BLASTN and BLASTX options (Altschul et al., 1997). A hit was found with the BLAST search when the E-value was lower than 1E-5. For unknown chicken genes, the accession number of the highest hit with the *Gallus gallus* EST database is given and a description of the highest BLASTX hit. For known chicken genes the accession number is given.

Quantitative LightCycler RT-PCR

For a reverse transcription, 200 ng of RNA was incubated at 70°C for 10 min with random hexamers[7] (0.5 μg). After 5 min on ice, the following was added: 5 μL of 5× first strand buffer,[8] 2 μL of 0.1 *M* dithiothreitol,[8] 1 μL of Superscript RNase H$^-$ reverse transcription[8] (200 units/μL), 1 μL of RNAsin[7] (40 units/μL), 1 μL of 2 m*M* dNTP mixture,[6] and water to a final volume of 20 μL. The reaction was incubated for 50 min at 42°C. The reaction was inactivated by heating at 70°C for 10 min. Generated cDNA was stored at −20°C until use.

PCR amplification and analysis were achieved using a LightCycler instrument.[9] For each primer combination, the PCR reaction was optimized (Stagliano et al., 2003). The primers are shown in Table 1. The reaction mixture consisted of 1 μL of cDNA (diluted 1:10), 1 μL of each primer (10 μ*M* solution), 2 μL of LightCycler FastStart DNA Master SYBR Green mix, and $MgCl_2$ (4m*M*) to a total volume of 20 μL. All templates were amplified using the following LightCycler protocol: Preincubation for 10 min at 95°C; amplification for 40 cycles: (5 s at 95°C, 10 s at annealing temperature, 15 s at 72°C). Fluorescent data were acquired during each extension phase. After 40 cycles, a melting curve was generated by heating the sample to 95°C followed by cooling down to 65°C for 30 s and slowly heating the samples at 0.2°C/s to 96°C while the fluorescence was measured continuously.

In each run, 4 standards of the gene of interest were included with appropriate dilutions of the cDNA, to determine the cDNA concentration in the samples. All RT-PCR amplified a single product as determined by melting curve analysis.

RESULTS

Differences Between Control and MAS-Induced Chickens

All chickens inoculated with the MAS homogenate exhibited growth retardation, which is the main clinical fea-

TABLE 2. Number of differentially expressed genes[1] in malabsorption syndrome (MAS) affected chickens at different sampling times postinoculation (PI) in different broiler lines

	8 h PI	d 1 PI	d 3 PI	d 5 PI	d 7 PI	d 11 PI
Genes upregulated after MAS infection						
Susceptible line, n	7	31	14	17	3	6
Resistant line, n	0	38	11	0	2	0
Genes downregulated after MAS infection						
Susceptible line, n	0	9	0	16	16	2
Resistant line, n	0	7	3	0	2	0

[1]A gene was declared differentially expressed when the mean value of the ratio was >2 or <2 and the gene was identified with significance analysis of microarrays with a false discovery rate <2%.

[6]TaKaRa, Kyoto, Japan.
[7]Promega, Leiden, The Netherlands.
[8]Life Technologies, Rockville, MD.
[9]Roche, Basel, Switzerland.

TABLE 3. Chicken genes upregulated (U) or downregulated (D)[1] 4-fold after a malabsorption syndrome (MAS) induction

Chicken gene	Description	Susceptible line	Resistant line
U73654.1	Alcohol dehydrogenase	D1	D1
AF008592.1	Inhibitor of apoptosis protein1	D1	
U00147	Filamin	D1	
X52392.1	Mitochondrial genome	D1 U5,11	
M31143.1	Calbindin	D1,5,7,11	U1, D7
AJ236903.1	SGLT-1	D5	
AJ250337.1	Cytochrome P450	D5,7	D1
M18421.1	Apolipoprotein B	D5,7	
M18746.1	Apolipoprotein AI	D5,7	
AF173612.1	18S rRNA	U8h	U3,7
AF469049.1	Caspase 6	U1	U1
U50339.1	Galectin-3	U1	U1
AJ289779.1	Angiopoietin 2C	U1,3,5	D1
L34554.1	Stem cell antigen 2	U1,5	U1,3
D26311.1	Unknown protein	U11	
AJ009799.1	ABC transporter protein	U3	D1
M10946.1	Aldolase B	U3	U1,3
AF059262.1	Cytidine deaminase	U5	U1
AJ307060.2	Ovocalyxin-32	U5	
M27260.1	78 kDa glucose regulated protein	U5	
AY138247.1	p15INK4b tumor suppressor		D7
AJ006405.1	Glutathion-dependent prostaglandin D synthase		U1

[1]U, D = genes up- or downregulated, respectively, at the sampling times indicated: 8 h, and 1, 3, 5, 7, and 11 d postinoculation.

ture of MAS. A significant reduction in BW gain relative to the controls was found in the susceptible chickens compared with that in the resistant chickens after MAS induction (data not shown). A comparison of the gene expression in the chicken intestine was made in control and MAS-induced chickens for the times 8 h, and 1, 3, 5, 7, and 11 d PI of both broiler lines. The hybridization experiments showed different numbers of up- and downregulated genes after the MAS induction (Table 2). In general, more genes were found differentially expressed in the MAS susceptible broiler line compared with the resistant line. At d 1 PI, most differentially expressed genes were found in both lines. The identity of the different up- and downregulated genes is shown in Tables 3 and 4. To investigate if these genes are generally induced or repressed after a MAS induction, hybridizations were repeated with samples from experiment 2, where the same chicken lines were used. Samples were available from d 1, 3, and 13 PI. Comparing the 2 experiments, the $\log_2$ (expression level of MAS-induced/expression level of control) differed by 0.4 on average. Of the ratios, 69% differed by less than 0.5, 18% between 0.5 and 1.0, 8% between 1.0 and 1.5, and only 5% differed by more than 1.5. When a gene was upregulated more than 4-fold in one experiment, it was also upregulated in the duplicate experiment, but not always more than 4-fold.

Differences Between MAS-Susceptible and Resistant Broiler Lines

The results of the comparison of infected vs. control chickens indicated that there are clear gene expression differences between the 2 chicken lines used. Therefore, samples from the 2 chicken lines were compared in control or in MAS-induced situations. In the control situation, no significant differences between the 2 broiler lines were found except at d 11. Here, 17 genes were identified which were expressed at least 4-fold higher in the susceptible line at d 11 with a false discovery rate lower than 2% (Table 5). In the MAS-induced situation at d 11, these genes differed nonsignificantly between the 2 lines; most $\log_2$ ratios of these expression differences were between –1.0 and 1.0, with only 2 exceptions.

For the MAS-affected situation, the only significant differences between the 2 broiler lines were found at d 7 PI with a false discovery rate lower than 2% and at least a 4-fold expression difference. However, at d 1 and 11 PI in the MAS-affected situation, genes were identified with a false discovery rate of 2.1 and 2.2%, respectively; these genes were considered to be significantly different in their expression levels. An overview of the genes differing between the 2 lines in the MAS-induced situation is given in Table 6. All these genes lacked significant expression differences in the control situation with $\log_2$ ratios between –1.0 and 1.0.

Confirmation of Gene Expression Differences

Array results can be influenced by each step of the complex assay, from array manufacturing to sample preparation and image analysis. Validation of expression differences is necessary with an alternate method. LightCycler RT-PCR was chosen for this validation, because it is quantitative, rapid, and requires only small amounts of RNA.

Eight differentially expressed genes were chosen for validation. They were differentially expressed in MAS-induced than in control chickens or were differentially expressed

TABLE 4. Chicken expressed sequence tags (EST) upregulated (U) or downregulated (D)[1] 4-fold after a malabsorption syndrome (MAS) induction

Chicken EST	Homology	Susceptible line	Resistant line
BU123833	Annexin A13	D1	
CD727681	Pyrin	D1	
BU420110		D1,7	
BU124420	Liver-expressed antibacterial peptide 2	D5	
BU217169	Sucrase-isomaltase	D5	D1,3
BU292533	Tubulointerstitial nephritis antigen-related protein	D5	
CD726841	Zonadhesin	D5	
BU123839		D5	D1,3
BU124534	Meprin	D5,7	
BU262937	Angiotensin I converting enzyme	D5,7	
BU288276	Mucin-2	D5,7	
BU480611		D5,7	U1
BU124511	Na+/glucose cotransporter	D7	
BU268030		D7	
BU464138		D7	
BU122834	Pyrophosphatase/phosphodiesterase	U8h	D1
BU122899	Fatty acyl CoA hydrolase	U8h,1	U1
BU467833	Interferon-induced 6-16 protein	U8h,1,3,5 D7	U1,3
—[2]	Avian nephritis virus	U8h,1,3,5,7 D11	U1,3 D7
BU138064	Retinoic acid and interferon inducible 58 kDa protein	U8h,1,5	U1,3
BX258371	Gastrotropin	U8h,5 D1	D1
AI982261	Ubiquitin-specific proteinase ISG43	U1	U1
BG712944	Aminopeptidase	U1	
BU125579	Cathepsin S	U1	
BU233187	Zinc-binding protein	U1	U1
BU240951		U1	U1
BU255435	β V spectrin	U1	U1
BU397837		U1	
BU492784	Putative cell surface protein	U1	U1
BX273124	Phosphofructokinase P	U1	
BU249257	Unnamed protein product	U1	U1
—		U1	U1
BU296697	IFABP	U1, D5,7	U1
BU302098	Cl channel Ca-activated	U1, D7	U1
BU410582	HES1	U1,11	U1,7
BU124153	Ca-activated Cl channel 2	U1,11 D5,7	U1
AJ452523	Mucin-like	U1,3	U1
BU118300	Hensin	U1,3	U1
—	Lymphocyte antigen	U1,3	U1
CD727020	Interferon induced membrane protein	U1,3,5	U1,3
BU401950	Lysozyme G	U1,3,5,7	U1,3
BU452240	14-kDa transmembrane protein	U1,3,5,7	U1,3
BU244292	Transmembrane protein	U1,5	U1
BX271857	Onzin	U1,5	U1,3
—		U11	
—	Immunoresponsive gene 1	U11	
BU305240		U3	
BU130996	Anterior gradient 2	U3,5	
BU378220		U5	U1

[1]U, D = EST up- or downregulated, respectively, at the sampling times indicated: 8 h, 1, 3, 5, 7, and 11 d postinoculation.

[2]— = no EST in the database (August 2003).

between the 2 chicken lines. Pools of RNA were tested for all time points. For the time with the largest differences in gene expression, 5 individual birds were tested in the LightCycler. In contrast to the microarray, relative concentrations of mRNA are measured in the LightCycler RT-PCR; the microarray detects expression differences. Therefore, the average was taken of the LightCycler results of the individual birds and then converted to $\log_2$ (infected/control). For all 8 genes tested, the results with the pools of RNA were similar for the LightCycler and the microarray (Table 7). For 7 of the 8 genes tested, the differences between the 2 groups were significant for individual birds ($P < 0.05$). Only for gastrotropin at d 1 PI, was the distribution of the results within the groups rather spread.

DISCUSSION

After induction of MAS, a number of up- and downregulated genes can be expected. This is the first report of gene regulation in the chicken intestine as a response to MAS induction. We studied 2 separate experiments in which chickens were induced with MAS to study the reproducibility of our findings and because no earlier reports about gene expression due to MAS were available. The results of the LightCycler RT-PCR were similar to the microarray data, as expected, because both methods measure RNA levels. In the LightCycler, concentrations are measured, whereas in a microarray experiment, relative expression differences are detected. The LightCycler was used here

TABLE 5. Genes expressed more highly in the susceptible line compared with the resistant line in control chickens at d 11

Expressed sequence tag	Chicken gene/homology	Log_2 ratio[1] in control situation	Log_2 ratio[1] in MAS-induced situation
BU123839	No homology	3.7	0.3
BU118300	Hensin	3.7	1.7
BX271857	Onzin	3.5	0.2
—[2]	Avian nephritis virus	3.3	–0.5
	Mitochondrial genome[3]	2.8	0.2
	Cytochrome C oxidase subunit 1[3]	2.5	0.1
BU123664.	No homology	2.3	–0.0
BU401950	Lysozyme G	2.3	1.1
BU467833	Interferon-induced 6-16 protein	2.3	0.2
	Plasma membrane calcium pump[3]	2.2	–0.1
BU124318	Immune associated nucleotide protein	2.2	–0.1
	Stem cell antigen 2[3]	2.2	0.0
—	Lymphocyte antigen	2.2	0.1
	Cytochrome C oxidase subunit III[3]	2.1	0.1
BX257981	No homology	2.1	0.3
—	No homology	2.0	0.6
—	No homology	2.0	0.9

[1]Log_2 ratio is log_2 (expression level susceptible/expression level resistant).

[2]— = no expressed sequence tag in the database (August 2003).

[3]Chicken gene.

and in other studies to validate microarray experiments (Rajeevan et al., 2001). In another study, the LightCycler confirmed the change in expression in only 71% of the genes (Rajeevan et al., 2001), whereas we confirmed the change in 100% of the genes. We used glass slides with a 2-color hybridization, whereas the other study used high-density filters for the microarray. Also, the threshold to detect differences was higher in our experiment, 4-fold compared with 2-fold differences. Thus, the genes described in this study are differentially expressed.

Gene Expression Differences After MAS Induction

Most upregulated genes were found at d 1 PI compared with the other sampling times investigated in both lines after a MAS induction. This could be because heterophil infiltration in the mucosa has started by d 1 PI. Another possible explanation for induced genes at d 1 is that MAS induction triggers epithelial apoptosis in the first days PI. Most downregulated genes were found at d 5 and 7 PI. During this time, cystic crypts and villus atrophy were found. Therefore, the amount of epithelial cells decreased. This may not represent a downregulation per se, but rather a decrease in the percentage of epithelial-specific genes in the total intestinal pool of RNA. It may be that those genes are really downregulated in the cells, but this needs to be investigated further.

Most identified induced or repressed genes are so far unknown in chicken or their function in the chicken intestine has not been determined. However, based on homology with known genes in other organisms a function can

TABLE 6. Genes and expressed sequence tags (EST) differentially expressed in one of the broiler lines after malabsorption syndrome (MAS) induction

EST	Chicken gene/homology	Day	Line[1]	Ratio MAS[2]	Ratio control[3]
	SGLT-1[4]	1	S	2.2	–0.0
BU233187	Zinc-binding protein	1	R	2.2	0.1
AJ295030	Aldo-ketoreductase	1	R	2.3	0.8
BU307467	Retinol-binding protein	1	R	2.4	–0.5
BX258371	Gastrotropin	1	R	2.6	0.7
CD727681	Pyrin	1	R	3.2	–0.3
—[5]	Avian nephritis virus	7	S	3.2	–0.2
BU401950	Lysozyme G	7	S	2.7	0.7
BU296697	IFABP	7	R	2.2	0.3
BU268030	No homology	7	R	2.2	0.1
	Cytochrome P450[4]	7	R	2.5	–0.2
	Glutathion-dependent prostaglandin D synthase[4]	7	R	2.5	0.9
BU124534	Meprin	7	R	2.7	–0.6
	Calbindin[4]	7/11	R	2.8/ 2.1	–0.4/–0.4
	Cytidine deaminase[4]	11	S	2.0	0.3

[1]Broiler line with higher expression after MAS induction. S = susceptible; R = resistant.

[2]Log_2 ratio in MAS-induced situation.

[3]Log_2 ratio in control situation.

[4]Chicken gene.

[5]— = no EST in the database (August 2003).

TABLE 7. Results of LightCycler real time-PCR for 8 genes compared with the microarray results

Gene name	Day	Array susceptible infected/control	LightCycler susceptible infected/control	Array resistant infected/control	LightCycler resistant infected/control
Anv	1	2.8	NA[1]	1.9	NA[1]
Calbindin	7	–3.5	–2.7	–1.2	–3.2
Gastrotropin	1	–2.7	–2.3	–2.3	–2.6
HES1	1	1.9	2.9	1.7	2.4
Interferon-induced 6-16 protein	1	2.4	3.0	4.1	3.1
Lysozyme G	1	3.4	11.2	3.8	13.4
Meprin	7	–3.3	–3.4	–0.6	–1.3
Pyrin	1	–4.2	–2.4	0.4	0.4

[1]All the control birds remained negative in the LightCycler experiment, therefore no ratio could be calculated.

be predicted. The identified induced and repressed genes are related to different cellular functions. Examples of apoptosis-related genes found after MAS induction are caspase 6, galectin-3, inhibitor of apoptosis protein 1, and angiopoietin 2C (You et al., 1997; Liu et al., 2002; Ruchaud et al., 2002). Genes related to food absorption are apolipoprotein A1 and B, sodium-glucose cotransporter, calbindin, and aldolase B. Immune-related genes are stem cell antigen 2 and interferon-induced proteins. Two different chloride channels were identified, as well as transmembrane proteins. Identified genes may have other functions than mentioned, because the prediction is based on homology. Furthermore, some genes have multiple functions, making it difficult to group such genes.

Interestingly, some upregulated genes were also found in a study of Marek's disease virus, such as interferon inducible proteins and stem cell antigen 2 (Morgan et al., 2001). In that study, gene expression following infection of chicken embryo fibroblasts with oncogenic Marek's disease virus was studied. Inteferon inducible proteins and stem cell antigen 2 have an immunological function and are involved in both and Marek's disease. It may be that a part of the same immunological pathway is activated. Gene expression in the chicken intestine after *Eimeria* infection has been studied (Min et al., 2003). The identity of the induced or repressed genes was different after MAS induction compared with an *Eimeria* infection. However, the infection models studied are quite different. *Eimeria* is a parasitic infection, whereas the etiology of MAS is still unknown, but both viruses and bacteria are involved. Furthermore, the source of the EST clones used for both studies differed, namely, a concanavalin-A-activated splenic lymphocyte and a lipopolysaccharide-activated macrophage cDNA library for the *Eimeria* study and a MAS-induced intestinal library in our study. It is not known if the same sequences are present on both microarrays.

Gene Expression Differences Between Broiler Lines

In the susceptible line, more up- and downregulated genes were found after MAS induction. The susceptible line had a more severe weight gain reduction when compared with the resistant line after MAS induction. This weight gain reduction is related to the severity of the lesions in the jejunum. After histopathological examination of the jejunum of the MAS-induced chickens, it was found that the susceptible line developed more severe cystic crypts and villus atrophy compared with the resistant line. The severity of the lesions in the jejunum due to MAS induction may be reflected in the number of up- and downregulated genes. After a MAS induction, some significant differences in gene expression were detectable between the 2 lines. Such differences were expected, because the lines differ in susceptibility for MAS in growth retardation and severity of intestinal lesions (Zekarias et al., 2002a). The lower expression of aldo-ketoreductase, gastrotropin, and intestinal fatty acid binding protein in the susceptible broiler line might cause poorer food absorption, resulting in the growth retardation. The lower expression of calbindin might cause a deficiency in calcium absorption resulting in the weak bones observed in MAS-affected broilers (van der Heide et al., 1981).

Prostaglandins are important in healing mucosal injuries and can downregulate the mucosal immune system (Wallace, 2001), so it is interesting to observe the higher expression of a prostaglandin D synthase in the resistant broiler line. A gene of the avian nephritis virus is more highly expressed in the susceptible line. This virus is probably present in the MAS homogenate, but is cleared faster from the resistant than from the susceptible broiler line. The susceptible broiler line has a longer upregulation of the antibacterial gene, lysozyme G. The higher expression in the susceptible line of cytidine deaminase at d 11 might be an indication of recovery of severe lesions.

Differences in gene expression in control conditions between the broiler lines were detected on d 11. These differences were not found in MAS-induced chickens. In the control chickens, there may be differences between the 2 broiler lines in the development of the intestine. In the MAS-induced situation, the disease is dominant, causing less regulation of genes involved in development. No differences between the broiler lines in gene expression in control conditions were detected at earlier sampling times. This means that the gene expression levels at earlier times are comparable in these 2 broiler lines in the control situation. Therefore, all differences found in the MAS-induced situation at earlier sampling times are due to MAS and not to other differences. The identified gene expression differences at d 11 have a role in energy metabolism, and immune system, or they are not yet characterized. Gene expression differences at d 11 might be important for the

rate of recovery of the intestinal lesions, which may also influence MAS susceptibility.

In summary, up- and downregulated genes after MAS induction were identified using a cDNA microarray. Gene expression differences were detectable between 2 chicken lines differing in susceptibility for MAS. Most of the induced and repressed genes are currently unknown in chickens and they need to be characterized further before conclusions can be made about their function during MAS induction and susceptibility.

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