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cDNA representational difference analysis of ileal Peyer's patches in lambs after oral inoculation with scrapie

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Abstract

cDNA representational difference analysis (RDA) was used to study gene expression profiles in the ileal Peyer's patch of a lamb 1 week after oral inoculation with the scrapie agent. Twenty-five differentially expressed cDNA fragments were identified and cloned. Sequence analysis indicated seven novel gene sequences. Other clones shared sequence homology with genes encoding ribosomal and mitochondrial proteins, the translation initiation factor EIF4GII and the bovine pancreatic thread protein. Reverse Northern was used to confirm the differential expression in another four lambs inoculated with scrapie and the tissue distribution of the novel genes was examined using Northern blot analysis.

Keywords: Scrapie; RDA; Peyer's patches; Differential expression; Reverse Northern

Scrapie is a transmissible spongiform encephalopathy occurring naturally in sheep and goats. The neuropathology of this disease is linked to the appearance of an insoluble and protease-resistant form of a host-encoded protein, the prion protein (PrP), which is normally expressed both in neuronal and in non-neuronal cells [1–5]. The disease-associated form of PrP, termed PrP^{Sc}, is considered to be responsible for the transmission and propagation of disease [6–9]. The conversion of PrP to PrP^{Sc} results in heavy accumulation of PrP^{Sc} in the brain of the infected animals and is most extensive at the terminal stage of the disease [10,11]. However, prior to the involvement of the central nervous system (CNS), PrP^{Sc} replicates within lymphoid organs as demonstrated by the presence of high levels of infectivity [12–14] and the accumulation of PrP^{Sc}, at least in most variants of the disease [15–17]. The accumulation of PrP^{Sc} in peripheral lymphoid tissues seems to facilitate neuroinvasion by the scrapie agent [18]. In scrapie of sheep, the alimentary tract is considered the major route of entry [19] and involvement of the gut-associated lymphoid tissues in uptake of the scrapie agent and early pathogenesis of the infection has recently been addressed [20,21]. The tissue of principal interest has been the organized lymphoid tissue of the alimentary tract including the tonsils, Peyer's patches (PP), and follicular aggregates of the large intestine [22,23]. In an experimental sheep model using oral exposure to scrapie, increased levels of PrP in the ileal PP were immunohistochemically detected as early as 1 week after the challenge [20]. The lymphatic follicles of the ileal PP consist predominantly of B-lymphocytes supported by a network of other cells including follicular dendritic cells, an important cell type in the pathogenesis of scrapie [24,25]. The ileal PP functions as a primary lymphoid tissue, being responsible for the proliferation and selection of B cells mediated through high mitotic and apoptotic activity going on in parallel [26,27]. On the luminal side of the intestine, the follicles are covered by the follicle-associated epithelium (FAE) with cells specialized in transepithelial transport of macromolecules. Uptake of pathological agents across FAE has been implicated in a number of diseases including salmonellosis and paratuberculosis [28,29].

Little information is available regarding the molecular mechanisms involved in the uptake and replication of the

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scrapie agent. An approach to define the underlying molecular mechanisms of a particular biological system is to identify differentially expressed mRNAs. In this report we have used cDNA representational difference analysis (RDA) [30] to detect transcripts and to identify genes that are differentially expressed in the ileal PP of lambs with susceptible genotypes 1 week after an experimental oral infection with scrapie [20]. To our knowledge, the present study is the first step towards an understanding of the molecular mechanisms involved in the uptake of the scrapie agent from the intestine in sheep.

Materials and methods

Animals. The sheep investigated were of the Norwegian white breed Rygja. The animal material has been described previously [20]. In short, lambs with PrP genotypes associated with enhanced (VV_{136}, RR_{154}, QQ_{171} and AV_{136}, RR_{154}, QQ_{171}) and reduced (AV_{136}, RR_{154}, QR_{171}, AA_{136}, RR_{154}, QR_{171} and AA_{136}, RR_{154}, QQ_{171}) susceptibility to scrapie were orally infected with the scrapie agent at an age of 6–8 weeks. Each lamb was given a single dose of 15 ml of a 30% (w/v) homogenate of 5 g brain tissue by stomach tube. The donor homogenate for each PrP genotype contained pooled frozen sheep brain tissue from clinical histopathologically confirmed cases of scrapie [20]. The tissues were pooled and mixed with saline. Matched control lambs received an oral dose of physiological saline on the same day and in the same manner as the lambs exposed to the scrapie agent. One week after the oral exposure to the scrapie agent, inoculated lambs and matched controls were necropsied and tissue samples were collected. Tissue samples were also collected from randomly selected healthy animals, older than 5 months of age, during slaughtering in an abattoir (Fellesslakteriet, Oslo).

Safety provisions and ethical aspects. The experimental inoculations of scrapie-infected material were conducted in the confined and controlled isolation facilities of the Norwegian School of Veterinary Science in Sandnes. Legal and ethical national requirements and code of practice were implemented in the animal experiments.

RNA extraction. Total RNA was extracted using a standard guanidinium thiocyanate/acidic phenol extraction [31] or the RNeasy Midi Kit from Qiagen (Qiagen GmbH, Germany). Poly(A)$^+$ mRNA was purified from total RNA using oligo-(deoxythymidine) paramagnetic beads (Dynal AS, Oslo, Norway).

Representational difference analysis. cDNA was synthesized from ileal PP mRNA from one control lamb and one inoculated lamb (1 week after challenge) with a PrP genotype (VV_{136}, RR_{154}, and QQ_{171}) associated with enhanced susceptibility to scrapie, using a cDNA synthesis kit according to the manufacturer's protocol (Invitrogen BV, Groningen, The Netherlands). The ileal PP of infected lambs had earlier been found to have increased PrP levels by immunhistochemistry [20]. RDA was performed essentially as described by Odeberg et al. [32]. Briefly, double stranded cDNA was digested with *Dpn*II and ligated to adapters (a 24-mer annealed to a 12-mer). Three successive rounds of subtraction and PCR amplification using hybridization tester-driver ratios of 1:100, 1:800, and 1:400,000 were performed to generate a third difference product (DP3). Adaptors were changed between sequential cross-hybridizations and digested adaptor-ends and uncleaved fragments were removed from the subtraction product by solid-phase purification (Dynabeads, Dynal AS). The DP3 fractions were run on an agarose gel and stained with ethidium bromide for visualization.

Sequence analysis. The DP3 fractions were cloned directly into the pGEM-T Easy vector (Promega, Madison, WI, USA). Colonies were picked and grown in microtiter plates, transferred to membranes, and screened with ^{32}P-labelled probes made from the original cDNAs. Only clones that hybridized with one of the probes were analyzed further, between 40 and 50 clones from each DP3. The inserts were PCR amplified with vector-specific primers (T7 and M13 reverse primers). Sequence analysis of the fragments was performed using dye-labelled nucleotides (Big-Dye, Perkin–Elmer, Foster City, CA, USA) on an ABI 377 DNA sequencer (Perkin–Elmer). The sequences were analyzed for homologies with known sequences using BLAST sequence similarity searching network service at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast).

Northern blot analysis. Reverse Northern analysis was performed as described by Tollet-Egnell et al. [33], with a few modifications. The PCR fragments (100 ng in 1 μl) corresponding to 25 specific RDA clones were spotted in triplicates onto Hybond N+ membranes (Amersham–Pharmacia Biotech, Buckinghamshire, UK). The membranes were denatured for 2 min, dried in a vacuum oven for 2 h, and then neutralized for 2 min. cDNAs corresponding to the housekeeping gene GAPDH and the ribosomal 18S gene were used as internal controls. To generate probes, ^{32}P-labelled cDNA was synthesized from 100 ng of ileal Peyer's patch mRNA from control and from scrapie-infected lambs (a pool from four animals in each group) with PrP genotypes associated with increased susceptibility for scrapie and from control and scrapie-infected lambs (a pool of mRNA from three animals in each group) with PrP genotypes associated with less susceptibility for scrapie as described [33]. The probes were denatured at 90 °C for 5 min before they were added to the hybridization solution. The membranes were prehybridized in Church buffer (0.5 M sodium-phosphate, 7% SDS, and 1 mM EDTA) at 55 °C for 3 h. After prehybridization, the probes were added and the membranes were hybridized in the same Church buffer at 55 °C overnight. The membranes were washed three times at 25 °C with 2× SSC/0.1% SDS for 10 min each and then three times at 25 °C in 0.1× SSC/0.1% SDS for 5 min each followed by an additional wash at 55 °C in 0.1× SSC/0.5% SDS for 15 min. The membranes were then exposed to X-ray film at −70 °C. Quantifications of autoradiograms were performed using ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA). The results were calculated in relation to the GAPDH and 18S rRNA cDNA levels on the respective filter. The experiment was repeated three times with similar results. For Northern blot analysis, total RNA was subjected to electrophoresis on a 1% agarose–formaldehyde gel and transferred to Hybond N$^+$ membranes (Amersham–Pharmacia Biotech) and RDA fragments were used as probes. The probes were radiolabelled using random priming (Rediprime II DNA Labelling System, Amersham–Pharmacia Biotech). The blots were prehybridized in Church buffer for 1 h at 65 °C and hybridized in the same Church buffer at 65 °C overnight. The membranes were washed three times in 40 mM sodium-phosphate/1% SDS at 65 °C for 20 min followed by exposure to X-ray film at −70 °C.

Results

cDNA RDA using ileal PP mRNA from lamb inoculated with scrapie agent

cDNA RDA was used to study whether uptake and/or replication of the scrapie agent affects gene expression in ileal PP. Both up- and down-regulation of gene expression were analyzed using ileal PP from the challenged lamb as driver (up) or tester (down) with ileal PP mRNA from the control lamb as tester or driver, respectively. Fig. 1 shows an agarose gel with the representation of cDNA from control and infected ileal PP mRNA together with the obtained difference products

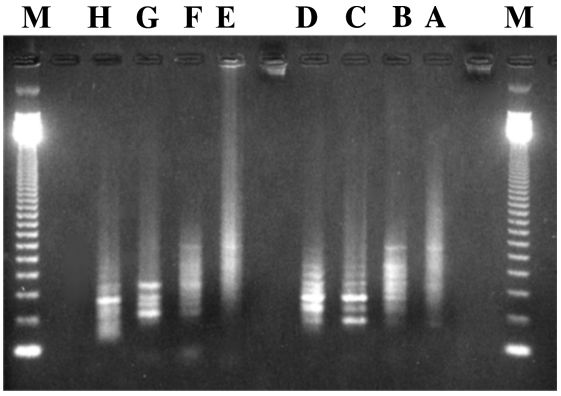

Fig. 1. Agarose gel electrophoresis of representations and difference products identified by cDNA RDA. Ethidium bromide-visible bands in a 1.4% agarose gel correspond to size markers (M), representations of control (A) and scrapie (E), difference products 1 (DP1) after one round of subtraction/amplification (B,F), difference products 2 (DP2) after two rounds of subtraction/amplification (C,G), and difference products 3 (DP3) after three rounds of subtraction/amplification (D,H). cDNA from control lamb was used as tester to generate the down-regulated difference products in lanes F–H. Similarly, cDNA from scrapie-infected lamb was used as tester to generate upregulated difference products in lanes B–D.

(DP1-3). This gel was analyzed by Southern blotting to evaluate the method using GAPDH cDNA as probe. GAPDH mRNA was present both in driver and tester but in very low amounts in the difference products (data not shown).

Approximately 100 colonies were identified representing unique clones from the DP3 fractions. Analysis of their sequences and comparison with those available in public databases indicated that 25 different sequences were represented (Table 1). Of these 25 clones, 17 showed homology to known genes including genes encoding several ribosomal and mitochondrial proteins, the translational initiation factor 4GII (EIF4GII), and the bovine pancreatic thread protein (PTP). The remaining clones were not represented in the database or represented only as expressed sequence tag (EST) sequences or human BAC clones.

Expression analysis of individual clones

Reverse Northern analysis was performed to confirm that the obtained cDNA RDA difference products indeed represented differentially expressed transcripts. The difference DP3 products were PCR amplified with vector-based primers and arrayed onto nylon filters together with GAPDH and 18S rRNA as internal controls. ^{32}P-labelled cDNA was synthesized from pools of ileal PP mRNA from four control lambs and four infected lambs, respectively, with PrP genotypes associated with enhanced susceptibility to scrapie and used

Table 1
RDA clones and their similarity to known genes

Clone	Homology	Accession	*E*-value	% ID	Region cloned
pGA1	h.BAC	AC026351	1e – 131	99	50,055–50,304
pGA2	h.clone	AC355478	2e – 8	100	137,812–137,844
pGA3	h.EST	BE644244	6e – 27	96	302–380
pGA4	Unknown				
pGA5	b.PTP	M59794	3e – 73	83	145–554
pGA6	o.EST	CD289354	4e – 58	98	121–240
pGA7	b.EST	CB532725	2e – 25	97	90–159
pGA8	h.RPS3a	NM_001006	8e – 66	91	602–784
pGA9	o.satellite	ZI7215	3e – 47	99	529–630
pGA10	h.RPL7	NM_000971	1e – 74	93	107–302
pGA11	h.β-actin	U39357	1e – 160	96	698–1033
pGA12	h.actin-γ2	XM010801	1e – 66	90	446–655
pGA13	o.cyto b	AF034730	1e – 145	98	329–614
pGA14	o.ATP synthase $F_0$6	NC001941	8e – 73	94	8174–8349
pGA15	o.RPS5	NM001009	3e – 83	88	332–648
pGA16	o.α-ATP synthase	M22465	1e – 87	90	944–1187
pGA17	b.OSCP	M18753	3e – 88	92	393–601
pGA18	h.RPS8	AK023362	2e – 50	89	1656–1684
pGA19	b.RPL12	AB098784	7e – 43	97	46–147
pGA20	b.RPLP2	NM_174788	2e – 59	91	121–280
pGA21	h.RPL26	NM_000987	1e – 80	92	140–360
pGA22	h.RPL27a	XM_017831	2e – 56	89	147–338
pGA23	h.EIF4G2	NM_001418	1e – 101	93	2115–2365
pGA24	r.RPL4	NM_022510	4e – 80	85	700–1010
pGA25	h.BAC	AC083886	5e – 05	80	28,689–28,814

Sequences have been compared using the BlastN program (http://www.ncbi.nlm.nih.gov/blast). The table shows the highest identity of the longest fragment that matched the database sequence. The *E*-value indicates the statistical significance for the database match. The % ID value shows the percentage of identical bases between the RDA product and the database sequence. h., human, b., bovine, r., rat, and o., ovine.

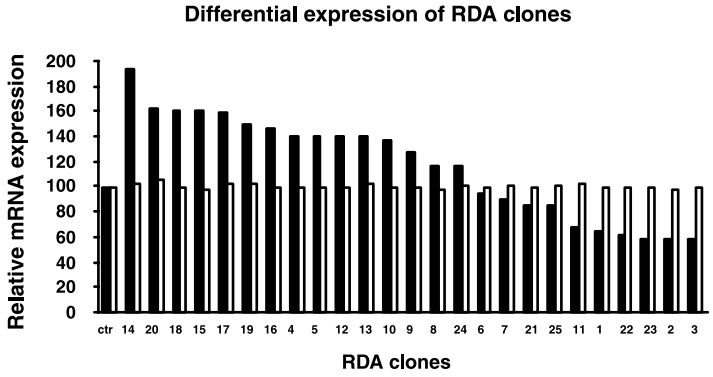

Fig. 2. Reverse Northern analysis. One hundred nanograms of PCR fragments corresponding to 25 RDA clones were spotted in triplicates onto nylon membranes together with GAPDH and r18S cDNAs as internal controls. The membranes were hybridized to ^{32}P-labelled cDNA probes generated from a pool of ileal PP mRNA (see Materials and methods) from control and challenged animals. The relative expression levels are normalized against the internal controls and the relative expression levels of the various RDA mRNAs in the control animals are set to 100. A representative experiment is shown. The experiment was repeated three times. Filled bars represent susceptible PrP genotypes, open bars represent less susceptible genotypes.

to probe the filters (Fig. 2). Relative levels of expression of the individual RDA transcripts were normalized to corresponding levels of mRNA for GAPDH and 18S rRNA on the same filters. Two clones (pGA6 and 7) showed no differential regulation. Similar experiments were performed with probes made from pools of mRNA from three challenged lambs and three control lambs, respectively, with PrP genotypes associated with reduced susceptibility to scrapie. No differential expression was detected in these animals (Fig. 2).

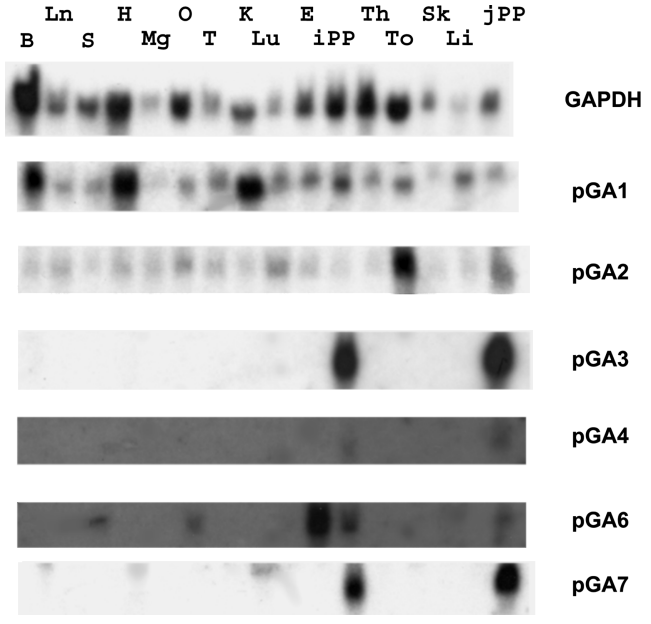

Fig. 3. Northern blot analysis. Fifteen micrograms of total RNA was isolated from various tissues according to Materials and methods, separated on 1% formaldehyde agarose gels, and blotted onto nylon membranes. Membranes were hybridized using pGA1, pGA2, pGA3, pGA4, pGA6, pGA7, and GAPDH as probes. Tissues are as follows: brain (B), retropharyngeal lymph node (Ln), spleen (S), heart (H), mammary gland (Mg), ovary (O), testis (T), kidney (K), lung (Lu), epididymis (E), ileal PP (iPP), thymus (Th), tonsil (To), skin (Sk), liver (Li), and jejunal PP (jPP).

In order to analyze the tissue expression for PTP and most of the unknown genes, multiple tissue blots were hybridized with the respective RDA fragments as probes. Fig. 3 shows that both pGA1 and 2 were ubiquitously expressed (transcripts approx. 0.95 and 0.98 kb, respectively) with the highest expression level in the tonsil while the expression of pGA6 was more restricted with the highest expression level in the epididymis (transcript approx. 1.4 kb). The expression of pGA3, 4, and 7 was restricted to the ileal and jejunal PP (transcripts approx. 0.9, 0.95, and 1 kb, respectively). This was also the expression pattern for PTP (pGA5, transcript approx. 0.9 kb, data not shown).

Discussion

The molecular mechanisms involved in the development of scrapie and other TSEs are poorly understood, especially in sheep. Differential expression has previously been studied in neuroblastoma cell-lines as well as in the CNS of hamster and mice infected with scrapie [34–39] and some of the genes found to be differentially expressed were related to apoptosis [36,39]. Recently, it was found that the mRNA level for the erythroid-differentiation-related factor (EDRF) was reduced in spleen of scrapie-infected mice, bone marrow of cattle with BSE, and whole blood from sheep with scrapie, compared with control animals [40]. This is to our knowledge, the only report so far on differential expression in non-CNS tissues.

Our interest lies in the early molecular events that take place in the gut-associated lymphoid tissues of lambs during scrapie infection and in the present work we aimed to identify genes differentially expressed in the

ileal PP 1 week after an oral inoculation with the scrapie agent. This particular tissue was chosen because immunohistochemical studies had demonstrated that the PrP level in the lymphoid follicles of the ileal PP was increased 1 week after oral exposure to the scrapie agent [20]. This finding suggested that pathological processes related to scrapie infection were taking place in this tissue at that time point [20]. Hence, cDNA RDA was used to identify genes whose expression was differentially regulated in the ileal PP exposed to scrapie agent. Since RNA from only one challenged and one control lamb was used in the cDNA RDA procedure, Reverse Northern was performed with probes made from a pool of mRNA from four animals to correct for individual variation in PrP mRNA levels. These animals had also been reported to have an increase in the PrP level in the lymphoid follicles of the ileal PP 1 week after challenge [20].

In this study we show differential expression of 23 genes, including five novel genes (pGA1, 2, 3, 4, and 25). None of the genes have previously been associated with scrapie or other TSEs. A number of clones represented homologies with genes encoding ribosomal proteins. Although ribosomal proteins are involved in the translational machinery, there is increasing evidence that they also have extra-ribosomal functions [41]. It has been suggested that certain ribosomal proteins might be involved in the regulation and/or induction of the apoptotic cascade [42–48]. We found up-regulation of two ribosomal genes, RPS3a (pGA8) and RPL7 (pGA10), whose expression levels have been associated with apoptotic events [42–45]. We also found elevated mRNA levels for RPS8, RPL12, and RPL2 and reduced mRNA level for RPL27a (pGA18, 19, 20, and 22, respectively). However, very little is known about the regulation and/or possible extraribosomal functions of these genes.

Other differentially expressed genes found in this study shared homologies with genes encoding proteins involved in the mitochondrial ATP production. The ATP synthase subunits 6 and α, and the oligomycin-sensitivity conferring protein (pGA14, 16, and 17, respectively), all parts of the F_0F_1 complex [52,53], showed increased expression indicating elevated ATP synthesis. Mitochondria play a major role in most forms of apoptosis and the changes in expression observed for these genes could be linked to apoptotic cell death in the ileal PP, especially since ATP levels and apoptosis seem interrelated [49–51]. Another mitochondrial protein, cytochrome *b* (pGA13), has previously been reported to be differentially expressed during apoptosis [54].

pGA23 showed homology with the C-terminus of both the translation initiation factor EIF4GII [55] and the translation repressor DAP-5 (also termed p97/NAT1), proteins with identical C-terminal parts [56,57]. Reduced transcription of DAP-5 has been shown to inhibit apoptosis [57] but the possible role of pGA23 in apoptosis has to await a more complete study of the gene.

The link between prion disease and apoptosis has been investigated in neuronal tissues and cells. It has been suggested that PrP^C has a protective role against apoptosis in neuronal cells, either as a suppressor and/or a regulator of apoptotic suppressors [58]. When PrP^C is transformed to the disease related form PrP^{Sc}, it is believed that the PrP^C level decreases [59]. The increased PrP found in the ileal PP follicles 1 week after scrapie inoculation was protease K sensitive [20,60]. However, this early accumulation of PrP could reflect the first changes in PrP metabolism and the possibility exists that these PrP molecules are transitional forms between PrP^C and PrP^{Sc} [61] without the normal ability to protect against apoptosis. At present it is not known whether alterations in apoptosis are induced in ileal PP in scrapie. However, the differential expression of the clones described above might indicate changes of the apoptosis in at least some of the cell populations in the ileal PP at this particular time point (1 week post-inoculation).

pGA 5 turned out to be the ovine homologue to the bovine PTP. Although the human homologue, PAP I, originally was identified due to elevated expression during experimental and clinical pancreatitis [62,63], the main site of normal expression is the small intestine where it is expressed in the Paneth cells in the crypts of the villi [64,65]. The function of PTP/PAP I has not been fully established. Several reports have suggested that increased expression of the protein is associated with antiapoptotic mechanisms [66,67] and with the regeneration process [68]. The increased expression of pGA5 suggests a decline in the apoptosis. The significance of this elevated expression will be studied further.

Additionally, transcripts for seven genes encoding as yet unknown proteins were identified and except for pGA6 and 7, their differential expression in inoculated animals with susceptible PrP genotypes, was confirmed by Reverse Northern. Of the novel genes identified, pGA3, 4, and 7 had expression restricted to the ileal and jejunal PP of the tissues tested. Isolating full-length clones for these genes will help us to delineate their function and role in scrapie infection.

As shown in Fig. 2, the differences in the expression levels of the differentially regulated genes found in the present study are relatively low. The increased levels of PrP detected in the follicles of ileal PP 1 week after oral challenge with scrapie only take place in a small number of follicles [20]. If the observed changes in the expression levels are due to accumulation of PrP, it is likely that this regulation occurs in those follicles with increased PrP levels. The isolation of individual follicles from tissue sections combined with quantitative real time PCR is in progress and will provide insight into these questions.

Since the control lambs did not receive normal brain material, one could argue that the observed differences

are caused by the exposure to brain material itself. However, in a group of lambs with PrP genotypes associated with reduced susceptibility to scrapie, no differences in expression of the genes found in the present study were observed between challenged and control animals. This indicates that the changes in gene expression observed in lambs with increased susceptibilities indeed are associated with scrapie infectivity and that different susceptibility to scrapie in animals with different PrP genotypes could be related to molecular events early in the pathogenesis.

To summarize, we have used the RDA approach to identify cDNA clones differentially expressed in the early phase of uptake and/or replication of scrapie agent in the ileal PP of sheep. The fact that transcription levels are regulated does not necessarily imply a significant physiological role in the uptake process of scrapie agent. Nevertheless, the approach permits the identification of potential mediators and modulators of the uptake process. Some of the results point towards alterations of apoptosis in the iPP after exposure to the scrapie agent. However, further studies are required to prove whether apoptosis is involved at this stage of the disease. Although our results certainly not include all changes in gene expression during this process, the genes we have isolated may provide information for understanding the molecular mechanisms involved in the early onset of scrapie.

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