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PrP (prion) gene expression in sheep may be modulated by alternative polyadenylation of its messenger RNA

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Scrapie-associated fibrils and their major protein component, PrP or prion protein, accumulate in the brains and some other tissues of all species affected by transmissible spongiform encephalopathies or prion diseases. To investigate the role of PrP gene expression in the hosts of these diseases, we have analysed some characteristics of PrP gene RNA transcripts in sheep and cattle tissues and made comparisons with PrP RNA transcripts in human and mouse tissues. Two PrP messenger RNAs of 4·6 kb and 2·1 kb, the result of alternative polyadenylation, were found first in sheep peripheral tissues and also occurred at low levels in sheep brain and bovine tissues, but not in human and mouse tissues. Our results from transfection assays of murine neuroblastoma cells with constructs expressing different regions of ovine PrP messenger RNA revealed the presence of sequences in the 3′ untranslated region of the gene that modulate protein synthesis.

Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases combine the molecular genetics and epidemiology associated with non-infectious, genetic disorders (gene mutations, familial forms of disease) with the characteristics of virus diseases (transmissibility, strain variation). The natural hosts of these fatal, neurodegenerative diseases are ruminants (i.e. scrapie and bovine spongiform encephalopathy, BSE) and man (i.e. Creutzfeldt–Jakob disease), but TSEs have also been found in captive carnivores and in rodents after experimental exposure (Bruce *et al*., 1991; Prusiner *et al*., 1996). No TSE virus or agent genome has yet been identified; however, the host protein PrP has been consistently found to be associated with infectivity and the modulation of disease phenotypes (Hope, 1994; Weissmann *et al*., 1996).

The PrP gene is essential for the development of TSEs. Mice lacking a functional PrP gene do not develop disease when challenged with TSE isolates (Büeler *et al*., 1993). Disease incubation periods are inversely related to PrP gene dosage in PrP transgenic mice (Manson *et al*., 1994; Carlson *et al*., 1994) and are also linked to PrP coding region polymorphisms. The major PrP codons (amino acids) associated in a complex manner with TSEs in sheep are 136 (A or V), 154 (R or H) and 171 (Q or R). For example, sheep with a $AA_{136}RR_{171}$ genotype are relatively disease-resistant compared to $VV_{136}QQ_{171}$ homozygotes, which is the most scrapie-susceptible PrP genotype (Hunter, 1997). The genetic control of TSEs is therefore a function of PrP protein sequence and expression level.

Gene expression is often regulated through the post-transcriptional processing of mRNA. The length of the untranslated region (UTR) can, for example, modulate the mRNA stability and sequences within the 5′UTR and 3′UTR can modulate mRNA translation efficiency (Jackson & Standart, 1990). Alternative polyadenylation leading to size differences of several hundred bases amongst the alternative transcripts has been described for several genes. For example, alternative transcripts modulate translation by changing translational efficiency, as shown for amyloid protein precursor (DeSauvage *et al*., 1992), or by changing mRNA stability (translation initiation factor eIF-2α) (Miyamoto *et al*., 1996), which can additionally be tissue-specific as is the case for ADP-ribosylation factor (Mishima *et al*., 1992).

The PrP mRNA of sheep is encoded by three exons and,

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The sequence of the ovine PrP allele reported in this paper has been deposited in the GenEMBL database under accession no. AJ223072.

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isolated from brain, has an apparent length of 4·6 kb with a 150 nucleotide (nt) 5′UTR, a 768 nt coding region and a 3220 nt 3′UTR (Goldmann *et al*., 1990; Westaway *et al*., 1994). A similar structure has been described for bovine PrP mRNA (Inoue *et al*., 1997; Horiuchi *et al*., 1998), whereas rodent and human PrP transcripts have shorter 3′UTRs of about 1300 nt (Goldmann, 1993). Despite these considerable differences in length, regions of high sequence similarity in rodent, human and ruminant 3′UTRs could be identified. However, two regions of about 0·5 kb and 1·4 kb are found only in the 3′UTRs of ruminant PrP (Goldmann *et al*., 1990) and could be involved in ruminant-specific RNA processing and translation. Only preliminary data on developmental PrP expression in sheep have so far been available (Hunter *et al*., 1994), although an association between developmental variation in PrP expression and susceptibility to scrapie may be a major factor in the assessment of infection risks and pathogenesis. In this context, differences in PrP regulation between sheep breeds may also be important modulators of scrapie susceptibility. In this paper we report the differential expression of ovine PrP mRNAs, in comparison with bovine, mouse and human PrP mRNA expression patterns.

Methods

■ **Tissue samples.** Adult (A) animals were aged 2–5 years. Six developmental time-points were analysed from Neuropathogenesis Unit (NPU) Cheviots, foetal (F) at 98 and 134 days gestation and lamb (L) at 3, 11, 17, 24 days old), and three time-points from Suffolks (for flock reference see Foster & Dickinson, 1989), F at 105 and 125 days gestation and L at 5 days old. Samples were from individual sheep unless indicated otherwise (see legend to Fig. 1). Lymph node sample SLN1 and uterus sample SU1 were from natural scrapie cases. All other sheep tissues were derived from NPU Cheviots except for brain sample SB14 and spleen sample SS1 from an Oldenburg × Cheviot sheep with natural scrapie provided by the UK Veterinary Investigation Centres, Thirsk. The kidney sample SK7 was the only one from a scrapie-infected NPU Cheviot sheep. Cattle tissues were provided by Veterinary Laboratories Agency, Addlestone, UK. Brain (medulla), kidney and spleen were derived from a healthy calf, and ovary and uterus were from a BSE-infected cow, both animals of (6 : 6) PrP genotype (Goldmann *et al*., 1991). Each mouse sample was a pool of several adult VM/DK mice. The human multiple tissue Northern blot (Clontech) was derived from adult, healthy individuals.

■ **Nucleic acid preparation and analysis.** DNA preparation, restriction enzyme analysis and plasmid cloning were performed as described in Sambrook *et al*. (1989). DNA was sequenced with Sequenase (Amersham). Total RNA from frozen tissues was extracted with guanidinium thiocyanate followed by centrifugation in caesium chloride solutions. Cytoplasmic RNA from N2a cells was extracted at 4 °C (Sambrook *et al*., 1989). Poly(A)-enriched RNA fractions were isolated by oligo(dT) chromatography or by PolyATtract MagneSphere separation (Promega). Poly(A)–RNA was separated in formaldehyde/agarose gels, transferred to nylon membrane and hybridized with ^{32}P-labelled DNA probes as described by Goldmann *et al*. (1990). Membranes were washed in 1 × SSPE/0·1% SDS at 50–65 °C. The human multiple tissue Northern blot was hybridized for 1 h at 68 °C in ExpressHyb hybridization solution (Clontech) and washed in 0·1 × SSC/0·1% SDS at 50 °C. Membranes were exposed to Kodak X-AR film with intensifying screens at −70 °C. X-ray films were analysed by densitometry. The values obtained for the sheep samples were then normalized for tissue weight taking into account the efficiency of the mRNA preparation to achieve a relative PrP mRNA quantification for tissues at different developmental stages. All other comparisons of RNA levels were related to µg RNA loaded on the gel. PCR and codon analysis for PrP genotypes was performed as described by Goldmann *et al*. (1994). The 5′RACE (rapid amplification of cDNA ends) protocol was used as recommended (GibcoBRL) utilizing PrP-specific oligonucleotides 316 (GCTCCACCA-CTCGCTCCATTATCTTG) for cDNA synthesis and oligonucleotide pair A023 (CTGACAGCCGCAGAGCTGAGAG)/13741 (GAGGCC-TGAGGTGGATAGCGGTTGC) for PCR amplification. The cDNA synthesis for the 3′RACE analysis was performed with oligonucleotide F336 (GCGGCCGCTTTTTTTTTTTTTTT) or 25082 (TCGATGACA-AGCTTAGGTATCGATATTTTTTTTTTTTTTT). PCR amplification on this cDNA used oligonucleotide pairs W1420 (TATGGAAGAGG-TGCCCTTGG)/F336 or W1420/6885 (CATCGATGACAAGCTTAG-GTATCGATA) for the 2·1 kb mRNA and pairs WG3740 (AACACA-GAATTATGACGTTGCCT)/F336 or WG3740/6885 for the 4·6 kb mRNA. Semi-quantitative PCR on chloramphenicol acetyltransferase (CAT) mRNA was performed with oligonucleotide 25082 for cDNA and the pair 20811 (CACTGGATATACCACCGTTGA)/6885 for amplification as described (Knuchel *et al*., 1994), resulting in an 800 bp fragment for all CAT RNAs and 470 bp for the competitor RNA. The identity of RACE DNA fragments was confirmed by direct sequencing of amplification products. All nucleotide positions refer to GenEMBL sequence M31313.

■ **Plasmid constructs and hybridization probes.** The plasmid constructs described are summarized in Table 1. Plasmids pCATbasic and pCATpromoter (both Promega) contain the CAT open reading frame (ORF) and simian virus 40 (SV40) 3′UTR without and with the SV40 promoter, respectively. The SV40 promoter and a 1024 bp *Mae*I restriction fragment with the CAT ORF and t antigen intron from pCATpromoter were combined with a 3825 bp *Pvu*II restriction fragment from clone pSc71 (position 432–4256) with the entire sheep PrP 3′UTR and PrP ORF codons 121–256 (GenEmbl AJ223072; Goldmann *et al*., 1990) to generate plasmid pFR. Plasmid pEYR was derived from pFR by deletion of PrP sequence positions 432–622 (codons 121–184). Plasmids pD15 and pD17 were generated from pEYR by deletion of PrP sequence positions (1515–4256, UTR regions C, D–G) and deletion of PrP sequence positions (1761–4256, UTR regions D–G), respectively. Plasmid pFR was subjected to unidirectional digestion with exonuclease III, resulting in clones pD20, pD27, pD30, pD34, pD36 and pD39 with 3′UTRs as shown in Table 1. Plasmids pD17 and pEYR were mutated by digestion with *Pac*I (position 1521) and subsequent religation with T4 DNA ligase; the resulting sequences are shown in Fig. 3(B) (pD17m2 to pD17m5 and pEYRDA, respectively). The internal competitor for semi-quantitative PCR had a 339 bp deletion in the CAT ORF. All constructs were verified by restriction mapping and sequence analysis. The sheep ORF probe p42 (position 425–910) and the 3′UTR probes p62 (sense) and p65 (antisense) (both position 910–1245) used for sheep and bovine RNA hybridizations were derived from clone pScr23.4 (Goldmann *et al*., 1990). The mouse PrP probe pNBF was derived from clone pNZTB (Westaway *et al*., 1987). The human PrP probe, p4hu (codons 115–stop), was derived from plasmid p4/22/10 (Kretzschmar *et al*., 1986).

■ **Transient transfection of N2a cells and CAT assay.** Mouse N2a neuroblastoma cells were maintained in Dulbecco's MEM+

Table 1. Relationship between PrP untranslated regions and CAT activity

Plasmid	Poly(A) signal* 1a	1b	2	3	4	UTR (kb)†	PrP (kb)‡	PrP–UTR regions§	CAT activity (mean ± SEM)‖
pEYR	+	−	+	+	−	3·8	3·2	ABCDEFG	12·4 ± 5·4
						1·2	0·7	ABC	
pEYRDA	−	+	+	+	−	3·8	3·2	ABCDEFG	4·2 ± 1.3
pD15	−	−	−	−	−	0	0		0·9 ± 0·3¶
pD17	+	−	−	−	−	1·2	0·7	ABC	12·3 ± 3·9
pD17m2	−	+	−	−	−	1·2	0·7	ABC	4·0 ± 1·0¶
pD17m3	−	−	−	−	−	0	0		0·9 ± 0·3¶
pD17m5	−	−	−	−	−	0	0		0·9 ± 0·3¶
pD20	−	−	+	+	−	2·4	2·1	EFG	3·3 ± 0·9
pD27	−	−	−	+	−	1·7	1·4	FG	12·2 ± 3·8
pD30	−	−	−	+	−	1·4	1·1	fG	NA
pD34	−	−	−	+	−	1	0·7	fG	33·6 ± 9·8
pD36	−	−	−	+	−	0·8	0·5	fG	44·9 ± 12·5
pD39	−	−	−	+	−	0·5	0·2	G	NA
pCATpromoter	−	−	−	−	+	0·9	0	NA	100
pCATbasic	−	−	−	−	+	NA	NA	NA	0·5 ± 0·05

* Polyadenylation signals present on the plasmids: 1a, (PrP)AATAAA1523; 1b, (PrP)TATAAA1523; 2, (PrP)AATAAA2222, (PrP)AATAAA2285 and (PrP)AATAAA2667; 3, (PrP)ATTAAA4063; 4, (SV40)AATAAA.
† Length of chimeric 3′UTR of *in vitro* produced RNAs processed at poly(A) signals 1a, 1b, 3 or 4 (see Results).
‡ Length of PrP sequence in chimeric UTR.
§ As defined by Goldmann *et al*. (1990). Capital letter, full region; small letter, partial region (see also Fig. 4).
‖ Mean values of relative CAT enzyme activity and standard error of the mean in 10 transfections with pCATpromoter set as 100% and pCATbasic as negative control. Activities marked ¶ were not found in the same series of experiments and were re-standardized for comparison. Transfections with pD30 and pD39 were done independently and have not been re-standardized.
NA, Not applicable.

Glutamax (Life Techs) with 10% FCS (Globepharm) and penicillin/streptomycin (1000 U/ml). Cells of 60–70% confluence were DNA transfected with 1·5 µg of various constructs and 1·5 µg pSV-*β*-galactosidase (Promega) in the presence of Tfx-50 lipofectin (Promega). Transfections were conducted over a 2 h period, cells were harvested after 48 h and were assayed for *β*-galactosidase (as standard for transfection efficiency) and CAT activity as described by Sambrook *et al.* (1989) and Baybutt & Manson (1997). Acetylated chloramphenicol separated on silica TLC plates was recovered and ^{14}C decay was measured in a scintillation counter. CAT activity from construct pCATpromoter was set at 100%.

Results

PrP mRNAs in sheep

Processing of PrP transcripts. Two British Suffolk sheep PrP gene sequences show nine consensus polyadenylation signals downstream of the ORF at positions 1253, 1523, 2222, 2285, 2667, 4038, 4063, 4587, 4678 ($A_{136}R_{171}$ allele, GenEMBL M31313; $A_{136}Q_{171}$ allele, GenEMBL AJ223072; Goldmann *et al*., 1990 and unpublished data). Signal sequences in positions 1253, 1523, 4038 and 4063 have also been confirmed for various PrP alleles from the Cheviot sheep breed (W. Goldmann, unpublished observations). The theoretical length of ovine PrP mRNA could therefore range from 1·5–5 kb, although ovine PrP mRNA from brain tissue has been reported as 4·1–4·6 kb (further referred to as 4·6 kb) (Goldmann *et al*., 1990; Horiuchi *et al*., 1995). Analysis by 3′RACE of poly(A)-enriched RNA brain samples from Cheviots showed that the majority of molecules were polyadenylated about 20 bp downstream of signal $ATTAAA_{4063}$, which is in agreement with our previous results from S1 protection assays on Suffolk sheep RNA (Goldmann *et al*., 1990).

In addition, a PrP mRNA of 2·1 kb has been described in peripheral tissue of Cheviot (Hunter *et al*., 1994) and Suffolk sheep (Horiuchi *et al*., 1995) (Fig. 1). To investigate whether the 2·1 kb RNA is due to alternative polyadenylation, poly(A)-enriched RNA samples from kidney and spleen were analysed by various means. From S1 nuclease protection assays with 3′UTR-specific probes (data not shown), Northern blot hybridizations with single-stranded probes (Fig. 1 B, lanes 1–5) and sequencing of 3′RACE products it was concluded that the 2·1 kb mRNA is processed at position 1546, 23 bp downstream of polyadenylation signal $ATTAAA_{1523}$. Furthermore, long range RT–PCR and 5′RACE identified the 5′UTR of the 2·1 kb and 4·6 kb mRNAs from lymph node as identical to each other and to that published for the 4·6 kb mRNA (Westaway *et al*.,

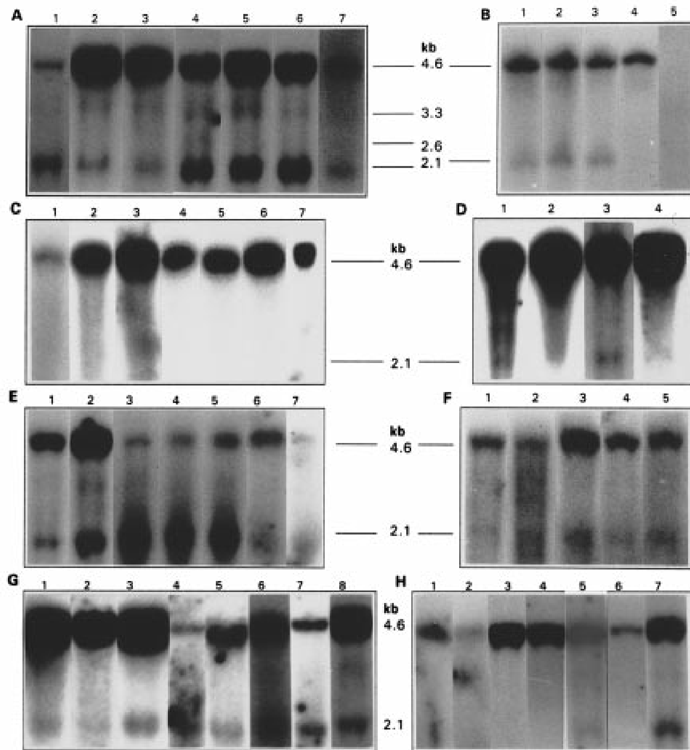

Fig. 1

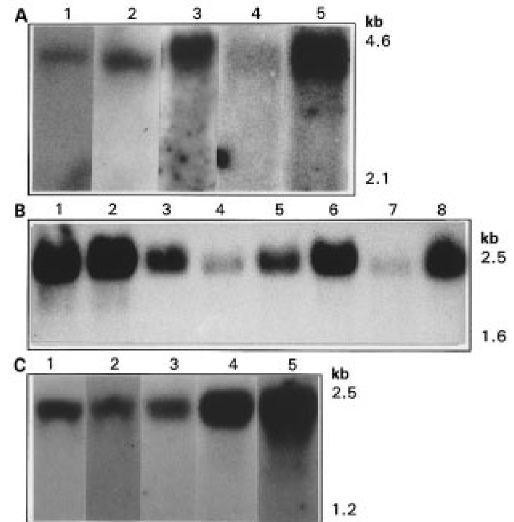

Fig. 2

Fig. 1. Northern blot hybridization of sheep PrP mRNA. (A) Kidney: (1) F98 (2) F134 (3) L3 (4) L11 (5) L17 (6) L24 (7) A, SK7. (B) Kidney: (1) L17 (2) L24 (3) A, SK7; brain: (4) L24 (5) L24. (C) Brain: (1) F98 (2) F134 (3) L3 (4) L11 (5) L17 (6) L24 (7) A. (D) Brain (long exposure): (1) L17 (2) L24 (3) A, SB14 (4) F105. (E) Spleen: (1) F134 (2) L3 (3) L11 (4) L17 (5) L24 (6) A, SS1 (7) A. (F) Thymus: (1) F134 (2) L3 (3) L11 (4) L17 (5) L24. (G) Heart: (1) F134 (2) L24; lung: (3) F134; lymph node: (4) pooled F98D, F134 (5) pooled L3, L11, L17, L24 (6) A, SLN1; placenta: (7) A; uterus: (8) A, SU1. (H) F105: (1) tonsil (2) spleen (3) brain; F125: (4) brain (5) kidney; L5: (6) brain (7) spleen. (A), (C)–(H): probe p42; (B) single-stranded probes: p62 (sense), lanes 1–4; p65 (antisense), lane 5.

Fig. 2. Northern blot hybridizations of bovine, human and mouse PrP. (A) Bovine PrP, calf: (1) brain (2) kidney (3) spleen; cow: (4) ovary (5) uterus. (B) Human PrP: (1) heart (2) brain (3) placenta (4) lung (5) liver (6) skeletal muscle (7) kidney (8) pancreas. (C) Mouse PrP: (1) brain (2) spleen (3) heart (4) lung (5) kidney.

1994). Both mRNAs were detected independent of the PrP genotype of the animal.

Tissue distribution and developmental expression of PrP mRNA. Samples from tissues with a proposed role in scrapie pathogenesis, such as brain and lympho-reticular tissues, from tissues that may play a part in maternal transmission, i.e. the reproductive system, and from internal organs with no apparent role in scrapie were screened for the presence of PrP mRNAs. These samples also comprised various developmental stages (foetuses, lambs and adult), different PrP genotypes (data not shown) and two sheep breeds, Cheviot and Suffolk. Northern hybridizations signals for the 4·6 kb and 2·1 kb mRNAs were detected in all analysed tissue types with the exception of the foetal tonsil sample, where only the 4·6 kb mRNA was detected (Fig. 1H, lane 1). However, the presence of the 2·1 kb mRNA in the foetal tonsil sample was confirmed by 3′RACE (not shown). When compared with the level of the 4·6 kb mRNA, the 2·1 kb mRNA was found in different tissue types at different levels and was highest in spleen, high in kidney, low in thymus, lymph node and heart, and lowest, if detectable at all, in brain. Most brain samples appeared negative for the 2·1 kb mRNA in Northern hybridizations.

In the Cheviot kidney samples, all time-points show significant levels of PrP mRNAs (Fig. 1A) and mRNA could easily be detected at 98 days gestation (F98) (Fig. 1A, lane 1). The PrP mRNA level (4·6 kb + 2·1 kb) was 100-fold higher at 134 days gestation (F134), doubled again in the L3 lamb and stayed relatively constant thereafter. The 4·6 kb/2·1 kb ratio was $\leqslant 1$ at F98, around 3 at F138 and L3, and around 2 at the later time-points. A Suffolk L5 kidney sample had a similar mRNA level to that seen in the Cheviot L3 kidney sample (data not shown). The level of an additional 3·3 kb RNA, most pronounced in these kidney samples (e.g. Fig. 1 A, lanes 4 and 5), was 1– 5% of the total PrP mRNA.

The 4·6 kb mRNA level also increased in brain, with a clear signal in all developmental stages (Fig. 1C, lanes 1–7 and H, lanes 3, 4, 6). The 2·1 kb mRNA was detected at low levels

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A.          1505                                    1546

(1)         ...UUAAGGUACCUUA_____AUUAAACUACCUUCUAGACACUG(aaa)n

(2)         ...TTAAGGTACCTTA_____ATTAAACTACCTTCTAGACACTGAGAGCA

(3)         ...TTAAGTTACCTTAATTTAATTAAACTACCTTCTAGACACTGAGAGCA

(4)         ...UUAAGUUACCUUAAUUUAAUUAAACUACCUUCUAGACACUG(aaa)n


B.           1505                                        1766

(pD17)      ...TTAAGGTACCTTAATTAAACTACCTTCTAG...ACTTTGGTCAC___

(pD17m2)    ...TTAAGGTACCTTA__TAAACTACCTTCTAG...ACTTTGGTCAC___

(pD17m3)    ...TTAAGGTACCTT___TAAACTACCTTCTAG...ACTTTGGTCAC___

(pD17m5)    ...TTAAGGTACCTTA_____ACTACCTTCTAG...ACTTTGGTCAC___

(pD15)      ...TTAAGGTACC_____________________________________

(pEYRDA)    ...TTAAGGTACCTTA__TAAACTACCTTCTAG...ACTTTGGTCAC...
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Fig. 3. Sheep and bovine PrP sequences. (A) (1) 3′UTR end of 2·1 kb sheep PrP mRNA (established for kidney, lymph node and tonsil RNA samples); (2) equivalent ovine PrP genomic sequence; (3) homologous bovine PrP genomic sequence; (4) 3′UTR end of 2·1 kb bovine PrP mRNA (established for ovary RNA sample). (B) 3′ End sequences of 3′UTR-deletion plasmids.

(0·5–2% of total PrP mRNA) in brain from the L17 Cheviot (Fig. 1D, lane 1), the F105 Suffolk and an adult, scrapie-affected Cheviot (Fig. 1D, lane 3). The presence of the 2·1 kb mRNA in brain sample SB14 (Fig. 1D, lane 3) was also confirmed by 3′RACE (not shown).

In the spleens from the same animals significant levels of PrP mRNA were again observed at all time-points (Fig. 1E). Whereas the 4·6 kb/2·1 kb ratio in spleens from F134, L3 and adults was $\geqslant 1$ (Fig. 1E, lanes 1, 2, 6 and 7) and therefore comparable with the kidney results, spleens from L11, L17 and L24 showed ratios of < 1 (Fig. 1E, lanes 3–5). Whether this result is characteristic for sheep spleen PrP expression or reflects differential degradation of the mRNA in these samples remains to be investigated.

In contrast to the spleen results, thymus showed a high 4·6 kb/2·1 kb ratio with an average of 4 for all five time-points. As opposed to the developmental PrP mRNA increase in kidney, calculations of thymus PrP mRNA levels based on tissue weight were eight- to tenfold lower in L11, L17 and L24 compared to the earlier time-points F134 and L3 (Fig. 1F).

Cheviot sheep expressed both 4·6 kb and 2·1 kb mRNAs in foetal heart, lung and lymph node (Fig. 1G, lanes 1, 3 and 4, respectively), in lamb heart, lymph node (Fig. 1G, lanes 2 and 5) and liver (not shown) and adult lymph node and placenta (Fig. 1G, lanes 6 and 7). Suffolk sheep expressed both mRNAs in spleen (Fig. 1H, lane 7), in lamb kidney (Fig. 1H, lane 5) and adult uterus (Fig. 1G, lane 8).

PrP mRNAs in other species

Bovine and goat tissues. The size of bovine and caprine PrP mRNA from brain tissue has been reported as 4·6 kb (Goldmann *et al.*, 1990; Inoue *et al.*, 1997). A 4·6 kb mRNA was also detected in calf brain, kidney, spleen (Fig. 2 A, lanes 1–3) and liver (not shown) as well as cow ovary and uterus (Fig. 2A, lanes 4 and 5). A PrP mRNA equivalent to the ovine 2·1 kb PrP mRNA was not detected in these bovine tissues using Northern hybridization on poly(A)–RNA samples (Fig. 2A). It was, however, possible to detect in total RNA from uterus and ovary a 3′RACE product that confirmed a poly(A) tail in the same position as described for the 2·1 kb sheep RNA (Fig. 3 A). The two goat spleens (adults) analysed showed both the 4·6 kb and 2·1 kb mRNAs with a similar ratio ($\geqslant 1$) as seen for adult sheep spleen samples. Goat brain samples appeared negative for the 2·1 kb mRNA, but highly positive for the 4·6 kb mRNA in Northern hybridizations (data not shown).

Human and mouse tissues. Human PrP mRNA has a size of 2·5 kb based on Northern hybridization of brain tissue, lymphocytes and cell lines (Kretzschmar *et al.*, 1986; Cashman *et al.*, 1990). Three additional consensus signals within the 3′UTR (positions 1765, 1978 and 2098) could result in shorter mRNAs. Poly(A)-enriched RNA fractions from eight different human tissues were hybridized with a human PrP probe and relative expression levels of the 2·5 kb PrP mRNA were analysed by measuring hybridization signals in relation to loaded RNA. The highest PrP mRNA level was found as expected in brain (Fig. 2B, lane 2), followed by heart and liver (20–30% of that in brain; Fig. 2B, lanes 1 and 5), lower levels were observed in placenta, lung, muscle, kidney and pancreas (10–15% of that in brain; Fig. 2B, lanes 3, 4, 6, 7, 8). None of these tissues show signals correlating with a smaller PrP mRNA; however, RNA was not available for 3′RACE analysis.

The most prominent PrP mRNA reported for mouse and hamster is 2·5 kb, but in peripheral tissues a 1·2 kb mRNA band has also been detected (Chesebro *et al.*, 1985; Robakis *et al.*, 1986), potentially polyadenylated close to position 1152 (Locht *et al.*, 1986). Mouse brain, heart, lung and kidney poly(A)-enriched RNA samples were analysed with a mouse PrP ORF probe and expressed similar high levels of 2·5 kb PrP mRNA (Fig. 2C, lanes 1, 3, 4, 5), whereas in spleen (Fig. 2C, lane 2) and liver (not shown) PrP mRNA was found at lower levels. In none of these tissues were signals of a 1·2 kb PrP mRNA observed.

Analysis of CAT activity under the control of ovine PrP sequences in transiently transfected N2a cells

We performed several independent transient transfections of murine N2a neuroblastoma cells with a total of 13 CAT–PrP/UTR chimeric constructs and with plasmids pCAT-promoter and pCATbasic as positive and negative control (Table 1), respectively, in order to establish whether a relationship between PrP 3′UTR sequences and regulation of gene expression exists.

Total RNA prepared 24 h after transfection with constructs pEYR, pD17 and pD36, respectively, were analysed by non-quantitative 3′RACE for the processing of the chimeric RNAs.

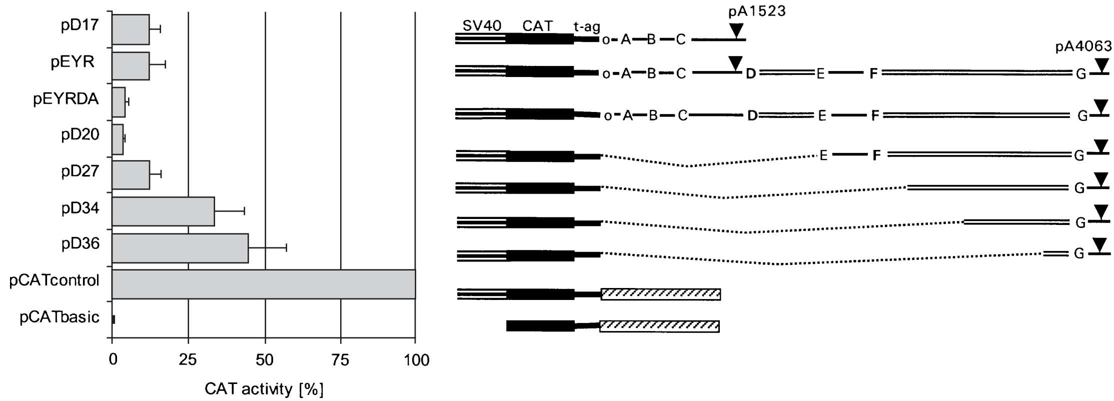

Fig. 4. CAT activity levels from cell transfections. Bar chart of relative CAT activities in N2a cells after transfection with constructs as indicated on the right (see also Table 1). SV40, SV40 promoter region; CAT, chloramphenicol acetyltransferase ORF; t-ag, SV40 UTR with t-antigen intron region; o, 200 bp PrP ORF; pA1523, pA 4063 polyadenylation signals AATAAA at positions 1523 and 4063. Thin single lines represent PrP conserved DNA regions A, B, C, E and G transcribed into mRNA UTR; double lines represent ruminant-specific regions D and F (see Goldmann *et al.*, 1990); dotted lines represent deletions; hatched box represents the SV40 UTR.

As expected, pD36 was transcribed into mRNA processed at poly(A) signal 4063 and pD17 was processed at signal 1523. In contrast, pEYR gave rise to two different chimeric mRNAs processed at poly(A) signals 1523 and 4063, respectively (data not shown). Total RNA prepared 36 h and 48 h after transfection with constructs pEYR, pD20, pD36 and pCAT-promoter, respectively, and analysed by semi-quantitative RT–PCR showed no significant difference in the RNA levels at those time-points amongst the four constructs.

The CAT activities measured for pEYR, pD17 and pD36 were 12·4%, 12·3% and 44·9%, respectively, compared to the 100% of pCATpromoter (Fig. 4). Deletion of all poly(A) signals downstream of position 1500 (constructs pD15, pD17m3, pD17m5) resulted in minimal CAT activity, the mutation of sequence $AATAAA_{1523}$ (construct pD17) to $TATAAA_{1523}$ (construct pD17m2), resulted in about 60% reduction of CAT activity. The same mutation ($TATAAA_{1523}$) introduced into pEYR (construct pEYRDA) resulted in the same decrease of CAT activity compared to pEYR transfections. The remaining activity from the pEYRDA transfection may indeed be generated from the 2·1 kb mRNA that is probably still produced, although presumably at a lower level than in the pEYR construct. These results suggested very low CAT expression from the full-length CAT–PrP/UTR chimeric mRNA (pEYRDA) and we therefore investigated the effect on CAT activity of deletions of 1·2 kb (pD20), 1·9 kb (pD27), 2·6 kb (pD34) and 2·8 kb (pD36) within the PrP/UTR regions A–F (Fig. 4). CAT activities were 3·3%, 12·2%, 33·6% and 44·9%, respectively (Fig. 4). Shortening the 3′UTR by 1·6 kb, from 2·4 kb to 0·8 kb, increased CAT activity 14-fold. However, although the pD20 mRNA is considerably shorter (by 1·2 kb) than the pEYRDA mRNA, both result in similar CAT levels, whereas an additional 0·7 kb deletion in pD20, resulting in pD27 mRNA, increased CAT activity fourfold. All four chimeric mRNAs that encoded only PrP/UTR regions G and F (pD30 to pD39, Table 1) showed very similar CAT activity, although the length of their UTRs varied by up to 1 kb.

Discussion

The results presented in this paper highlight three properties of PrP gene regulation that might influence TSE susceptibility and pathogenesis in sheep: (i) PrP gene transcripts were found in the foetus and throughout development to the adult sheep, in brain, spleen and kidney in a similar temporal pattern, but were also present in many other tissues. (ii) Two PrP gene transcripts (4·6 kb + 2·1 kb), encoding the same PrP ORF, are produced by alternative polyadenylation with tissue-specific levels, with the highest level of the 4·6 kb transcript in brain tissue and the highest level of the 2·1 kb transcript in spleen. The alternative 2·1 kb PrP transcripts were expressed significantly in ovine and caprine, but only marginally in bovine tissues. Alternative polyadenylation was not detected in human and murine tissues. (iii) *In vitro* experiments showed that the level of protein expressed from RNA transcripts with different ovine PrP gene 3′UTRs is related to sequences within the UTRs. The 3′UTR of the 2·1 kb mRNA did not preclude protein expression.

The 2·1 kb mRNA is produced by alternative polyadenylation rather than alternative splicing and it does not appear to differ from the 4·6 kb in its 5′UTR. Processing at the $AATAAA_{1523}$ signal resulting in the 2·1 kb transcript might be linked to the transposon element, which has a high C+G content and a high frequency of the dinucleotide GT known to function as additional RNA polymerase stop signals. The apparent lower efficiency of this process in bovine tissue may be related to sequence variation or, as may also be the case in sheep brain, the result of a variation in protein factors that are involved in RNA processing. There is some indication, especially in kidney, that minor quantities of other alternative

PrP transcripts (3·3 kb and 2·6 kb) exist in sheep, although their relevance remains to be investigated. The 3′UTR of the sheep 4·6 kb mRNA has previously been divided into regions A–G on the basis of sequence similarity to human (encoding regions A, B, C, E and G) and mouse PrP mRNA (encoding regions A, C, E and G) (Goldmann *et al.*, 1990). Although the 2·1 kb sheep mRNA is similar in length to human and mouse PrP mRNA, it may not be regarded as their homologue as it encodes only regions A to C.

The major mechanisms that link the kinetics of mRNA with protein synthesis involve the mRNA degradation rate, the rate of initiation and the rate of translation. Our cell transfections demonstrate conclusively that 3′UTR sequences of the sheep PrP gene can significantly affect production of protein. Whether this is due to mRNA stability or alteration of the initiation rate is of importance for the evaluation of the *in vivo* PrP expression from the two transcripts and, ultimately, for a therapeutic aim of interfering with the control of PrP synthesis in prion diseases.

The highest protein levels were expressed from constructs with the shortest 3′UTRs processed in region G, whereas the short 3′UTR processed in region C resulted in a distinctly lower level of protein. Taken together with the fact that the long 3′UTRs all resulted in low protein expression we speculate that the stability of these RNAs and a difference in the utilization of the two poly(A) signal sequences could explain these observations. Surprisingly, the RNA with the full-length PrP 3′UTR (pEYRDA) is poorly, if at all, translated in transfected N2a cells, which is in contrast to sheep brain *in vivo*, in which the major PrP transcript of 4·6 kb mRNA is associated with high levels of PrP protein. This suggests that the 4·6 kb RNA may be stabilized or translationally activated *in vivo* in sheep by factors that are missing in the murine neuroblastoma cells. We propose that sequence motifs encoded in regions D–F may interact with sheep-specific proteins *in vivo*. We have previously pointed out that the ruminant-specific regions (D and F) of PrP 3′UTR have homology to retroposons (Goldmann, 1993). A PrP gene analysis by Lee *et al.* (1998) confirmed that the 4·6 kb mRNA contains almost 2 kb of sequence originating from LINE, SINE or transposon elements, sequences that are not present on the 2·1 kb mRNA.

All sheep tissues analysed in this study produced PrP mRNA of 4·6 kb and, with the exception of some brain tissue samples, showed also PrP mRNA of 2·1 kb. There was no evidence for a tissue expressing only the 2·1 kb mRNA. The 2·1 kb mRNA was detected in some brain samples, as well as in heart and liver, contrary to previous reports (Hunter *et al.*, 1994; Horiuchi *et al.*, 1995). It remains to be seen whether the 2·1 kb mRNA-positive brain samples also shared other features that make them different from the 2·1 kb mRNA-negative samples. An intriguing possibility still to be examined is that the 2·1 kb transcript only occurs in specific brain areas.

The low level of the 2·1 kb mRNA in sheep brain appears comparable to the low level of expression of a 2·1 kb mRNA in bovine ovary, uterus (this paper) and brain (Horiuchi *et al.*, 1997). Whether the marked difference in the amount of the 2·1 kb transcript between, on the one side sheep and goats with high expression in peripheral tissues and low expression in brain, and on the other side cattle with low expression in all tissues is reflected in the PrP protein level is not known. The gross level of expression of the ovine 4·6 kb and 2·1 kb mRNA appears to be correlated with neither sheep breed nor PrP genotypes, of which $VV_{136}QQ_{171}$, $VA_{136}QR_{171}$ and $AA_{136}QQ_{171}$ were represented in the analysed samples (data not shown). Whether significant differences between TSE-infected and healthy animals exist remains to be seen when appropriate tissues from more animals have been collected. It should be noted that the 2·1 kb mRNA appears to be present independent of the health status of the animal.

It has previously been argued (Horiuchi *et al.*, 1995) that posttranscriptional regulation is the most likely explanation for the differences in PrP synthesis observed between ovine brain and kidney. A 20% PrP mRNA level in kidney compared with that of brain combined with 2·5% PrP protein level compared with brain was taken to demonstrate an eightfold less efficient translation of PrP mRNA in sheep kidney. However, our results may support an alternative model. The total PrP transcript level in kidney consists of 70–80% of the 4·6 kb mRNA and 20–30% of the 2·1 kb mRNA, which is 14–16% (4·6 kb mRNA) and 4–5% (2·1 kb mRNA) in brain equivalents. Significant translation of only the 2·1 kb mRNA would suggest PrP protein levels close to the observed figures. Whether this hypothesis, that the 2·1 kb mRNA level restricts PrP expression in kidney can be generalized for other peripheral sheep tissues, or whether the alternative hypothesis of a strong translational control of both mRNAs in peripheral tissues is correct will be a focus of future investigations.

The expression of PrP mRNA in a variety of peripheral human tissues such as placenta, kidney and heart suggests that there may be no major difference in the tissue distribution of PrP transcripts amongst prion disease-susceptible species. However, we have not found any indication for PrP mRNAs with alternative 3′UTRs in these human tissues. Whether there are different PrP transcripts generated from alternative splicing in the 5′UTR as has been shown for hamster (Li & Bolton, 1997) and bovine PrP (Horiuchi *et al.*, 1997; W. Goldmann, unpublished data) remains to be seen. Alternative 5′UTRs have not been observed in sheep, which may point to a subdivision of PrP regulation into species with 5′UTR-directed control (hamster, cattle) and 3′UTR-directed control (sheep, goat). Considering that it is currently not clear to which of these groups the human and mouse PrP gene belong, it may be advisable to approach the use of mice for modelling of molecular mechanisms involved in ruminant and human prion disease pathogenesis with caution.

Expression of PrP protein is crucial in the development of scrapie pathology and in the spread of scrapie infectivity. The detection of PrP mRNA in sheep brain at two-thirds of

gestation, incidentally very similar to murine PrP mRNA (Manson *et al*., 1992), and the presence of PrP mRNA in various tissues of foetuses and young lambs as well as in placenta implies that there is a risk of pre- or perinatal infection of the foetus/newborn lamb from an infected mother, unless there is tissue or developmentally specific downregulation of PrP protein synthesis. Our results have highlighted the potential for this kind of regulation of the sheep PrP gene.

Although disease linkage with PrP genotype based on protein amino acid polymorphisms is now well understood (Hunter, 1997) it is not always true that susceptible animals develop scrapie or that resistant animals never do so. Differences in PrP protein expression caused by differences in gene translation due to sequence variations between breeds may account for some of these observations. In this context, sequence data from different breeds indicate a relatively high mutation rate in the 3′UTR region of the PrP gene: the sequences of two British Suffolk alleles (GenEMBL sequences M31313 and AJ223072) differ in six positions, of which one is the previously published *Eco*RI polymorphism (Hunter *et al*., 1989). They also differ in about 30 positions to an American Suffolk sheep PrP sequence (Lee *et al*., 1998; GenEMBL U67922) and in several positions to Cheviot sheep sequences (W. Goldmann, unpublished observations).

Our studies suggest that by regulating PrP protein expression through translational regulation of the two major PrP transcripts, scrapie susceptibility of sheep may be altered. It may also become feasible to manipulate the translation of the 4·6 kb mRNA (i.e. PrP downregulation in brain) but leave active 2·1 kb mRNA in peripheral tissues. The next stage of this work is therefore a closer examination of the potentially complex correlation of PrP mRNA and protein expression patterns in ruminants.

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