Detection of caprine arthritis-encephalitis- and maedi-visna viruses using the polymerase chain reaction

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Summary. The polymerase chain reaction (PCR) was used to demonstrate proviral DNA of lentiviruses of small ruminants in cultured cells. Primers for the Taq polymerase were selected in the GAG gene of Icelandic maedi-visna virus and POL gene of caprine arthritis-encephalitis (CAE) virus. Using PCR, proviral DNA of CAE virus was detected at 1 day post infection, 4 days before viral protein could be demonstrated using a sensitive immunoblotting protocol and 6 days before the appearance of syncytia. Primers derived from the published sequence of CAE virus successfully primed for the synthesis of homologous virus and Icelandic maedi-visna virus but not for maedi-visna virus isolated in The Netherlands. In contrast, primers derived from the GAG region of Icelandic maedi-visna virus allowed the amplification of DNA of homologous virus, maedi-visna virus isolated in The Netherlands as well as CAE virus.
Key words. Lentivirus; caprine arthritis-encephalitis virus; maedi-visna virus; polymerase chain reaction; DNA; protein; virus evolution; immunoblotting.

Caprine arthritis-encephalitis (CAE) in goats and maedi-visna in sheep are caused by closely related lentiviruses [1]. The main manifestations of CAE are arthritis, mastitis and, in young kids, encephalitis. Maedi-visna is characterized by interstitial pneumonia, mastitis and encephalitis. Similar in both animal species, the incubation time may extend over months or even years and disease symptoms develop slowly. The laboratory diagnosis of both infections is based primarily on the demonstration of antiviral antibody [2–5], but it may take several months before detectable antibodies are formed in infected animals [6, 7]. A rapid method for the isolation and identification of virus in cell cultures is not available. The polymerase chain reaction (PCR)[8] has previously been used successfully for the demonstration of human lentiviruses [9–12]. Using this technique, we show here that proviral DNA of CAE- and maedi-visna viruses can be identified in infected cells within 24 h post infection. In addition, we demonstrate extensive sequence differences in the amplified DNA of two strains of maedi-visna viruses.

Materials and methods

Cells and virus: Sheep plexus chorioideus cells (SPC) and maedi-visna virus (strain ZZV 1050) were originally obtained from Dr D. Houwers, Lelystad, The Netherlands. Secondary lamb carpus synovial cells were used for the propagation of CAE virus (strain 75-G 63, ATCC, Rockville, Maryland). The viruses were grown as described [13]. The cell culture medium was aspirated from 80% confluent monolayers in 75-cm^2 cell culture flasks containing approximately 1×10^6 cells and 5 ml undiluted, virus-containing cell culture supernatant was added to initiate infection. After an adsorption time of 90 min the previously removed medium was added. At various times post infection, the medium was discarded and the cells were harvested with a rubber policeman, centrifuged for 5 min at $1000 \times g$ and boiled in 1 ml phosphate buffered saline for 5 min.

Polymerase chain reaction: PCR was conducted on 25 µl of the boiled cell suspensions in an end volume of 100 µl using buffers and Taq polymerase supplied by Stehelin Company, Basel, Switzerland. Primers and deoxyribonucleotides were present at 1 µM and 1.5 mM, respectively. The samples were amplified for 30 cycles, each consisting of denaturation at 94 °C for 30 s followed by annealing at 56 °C for 60 s and extension at 74 °C for 60 s. 10 µl of the reaction mixture were then run on 4% polyacrylamide gels and DNA was stained with ethidium bromide. In some experiments, the sequence of amplified DNA was determined using Sequenase^R (United States Biochemical Corp., Cleveland, Ohio, USA).

Immunoblotting: Immunoblotting was performed as described [5]. Briefly, proteins of the cell suspensions (see above) were separated on 12% polyacrylamide minigels (Bio-Rad Laboratories, South Richmond, Calif., USA) under denaturing conditions [14], transferred to nitrocellulose and stained with a polyclonal CAE-positive goat serum.

Results and discussion

The strategy for selecting primers for amplification of caprine and ovine lentiviral DNA was based on several considerations. The highest degree of similarity between CAE- and maedi-visna viruses is in the GAG and POL genes coding for structural internal viral proteins and reverse transcriptase, respectively [15]. Moreover, different virus isolates differ less in these genes than in the ENV gene which codes for the surface glycoprotein [16]. To allow the detection of a wide spectrum of ovine and caprine lentivirus variants, we selected two pairs of primers (20 bases long, see table) complementary to regions in the GAG gene of maedi-visna virus [17] and the POL gene of CAE virus [18]. The former pair should allow the demonstration of maedi-visna- and the latter that of CAE viruses, with possible overlaps in both directions. To minimize nonspecific reactions, we selected virus-specific primer

Sequences of the primers used in this study

	Sequence	Position
Maedi-visna virus GAG region	5'-ATACAAATGCTAGCACAGAC-3' (+)	1459[a]
	5'-ATGTCCTGGTTTTCCACAAT-3' (−)	1673
CAE virus POL region	5'-TATTAGACATAGGAGATGCA-3'	260[b]
	5'-GATATCATCCATATATATTC-3'	498

[a]Sonigo et al.[17]; [b]Chiu et al.[18]; (+), sense; (−), antisense.

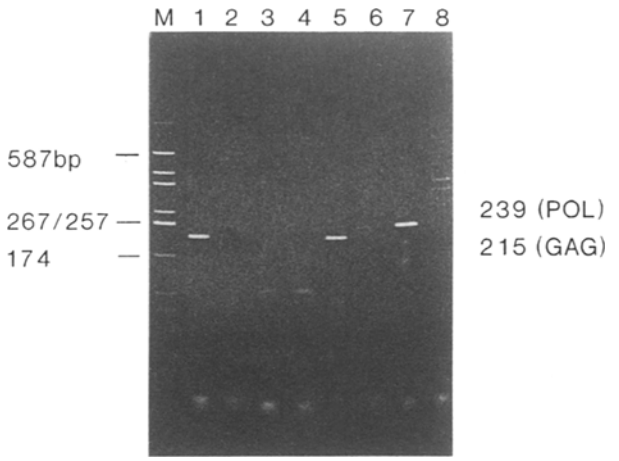

Figure 1. Polyacrylamide gel electrophoresis of enzymatically amplified DNA of maedi-visna- and CAE viruses. Sheep plexus chorioideus cells and lamb carpus synovial cells infected with maedi-visna- and CAE viruses, respectively, were harvested at 20 and 15 days post infection. The size of the PCR fragments was calculated from pUc 19 fragments obtained after digestion with HaeIII (M). Lane 1, GAG-specific primer in maedi-visna virus-infected cells; lane 5, same primer in CAE virus-infected cells; lane 3, POL-specific primer in maedi-visna virus-infected cells; lane 7, same primer in CAE virus-infected cells, lanes 2, 4, 6 and 8, effect of respective primers in uninfected cells.

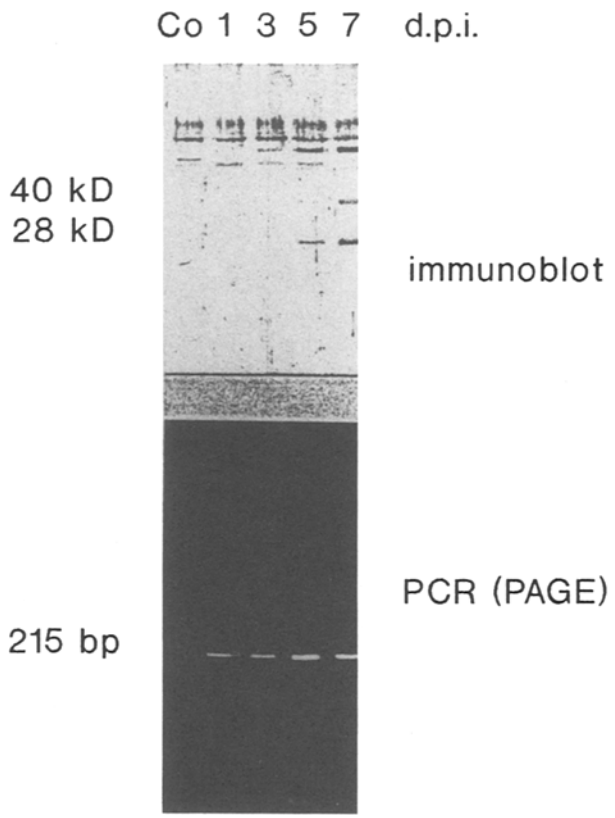

Figure 2. Time course experiment of appearance of proviral DNA and viral proteins in cells infected with CAE virus. Lamb carpus synovial cells were infected with CAE virus and cells were harvested from individual culture flasks at two-daily intervals. Viral DNA amplified by PCR using POL-specific primers was separated on polyacrylamide gels as described in the methods. Viral protein was run on SDS-polyacrylamide gels and detected by immunoblotting as described[5]. d.p.i.: days post-infection.

sequences with no similarity to known cellular genes and genes of other viruses, including retroviruses, as assessed by comparing the sequences of the primers with the Genbank[R] library. To increase the efficiency of PCR, we made the primers to frame relatively short parts of the viral genomes[19], i.e. 215 bp in the GAG and 239 bp for the POL genes (table). PCR using the primers located in the GAG region resulted in the amplification of DNA of both maedi-visna- and CAE viruses (fig. 1, lanes 1 and 5) whilst only CAE-specific DNA was amplified when the POL primers were used (fig. 1, lane 7). The amplified fragments were of the expected length.

To determine the sensitivity of the PCR procedure used for the detection of viral DNA, we conducted a time course experiment. Lamb carpus synovial cells were infected with CAE virus and cells harvested in 48-h intervals were assayed for viral protein using a sensitive immunoblotting protocol[5]. Proviral DNA was detected using the primers specific for the POL gene region. Virus-specific DNA was demonstrated already at 24 h post infection whilst immunoblotting indicated the presence of the viral p28 core protein starting at 5 days post infection (see fig. 2). Syncytia formation was observed microscopically only starting at day 7 post infection (not shown). These results indicated that PCR was clearly more sensitive than other methods used to detect the presence of CAE virus in infected cell cultures.

In the previous experiments, we had been using strain ZZV 1050 of maedi-visna virus which has not been sequenced and which was isolated in The Netherlands several years after the Icelandic strain 1514[20]. Each pair of the primers derived from the POL sequence of CAE virus is mismatched with the published sequence of strain 1514 in 1 base only, suggesting that the failure to prime for the synthesis of ZZV 1050 may be due to a more extensive sequence difference in that strain. Indeed, additional experiments showed that proviral DNA of maedi-visna vi-

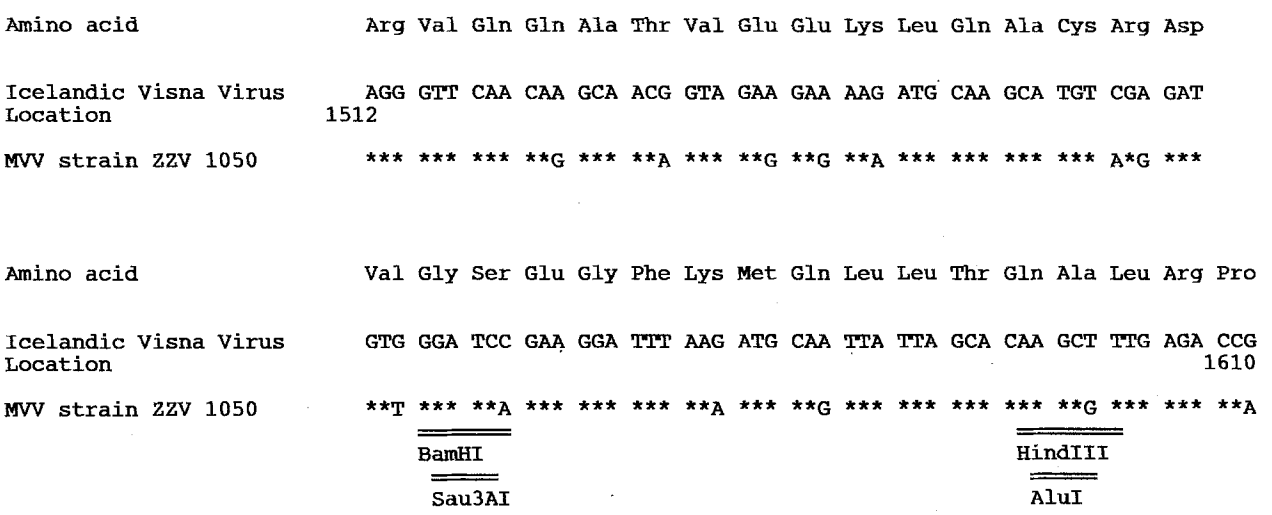

Figure 3. Nucleotide and amino acid comparison of two maedi-visna strains and loss of restriction sites in GAG region due to single base changes. The sequence of Icelandic maedi-visna virus contains restriction sites for BamHI, Sau3AI, HindIII and AluI [17]. In the DNA of the maedi-visna virus strain ZZV 1050 an exchange of C → A has occurred in the restriction site for BAMHI and T → G in the restriction site for HINDIII and AluI, resulting in the loss of all but the Sau3AI restriction site. *: identity of the two sequences.

rus strain 1514 could be amplified using the POL primers (results not shown). Additional evidence for a marked difference in the DNA sequences of these two strains of maedi-visna virus was obtained when we analyzed the amplified GAG DNA of maedi-visna virus strain ZZV 1050. Digestion with restriction enzymes revealed the loss of the sites for BamHI, HindIII and AluI in this strain. In contrast, Sau3AI cut the DNA into the fragments expected from the published sequence of strain 1514. This observation could be explained after sequencing of the part of the amplified DNA in which these restriction sites are located (see fig. 3). Thus, a change from C → A in position 1568 had no effect on the Sau3AI site but destroyed the BamHI site while the change from T → G in position 1601 resulted in the loss of the site for HindIII and AluI. It can also be seen that the sequence of strain ZZV 1050 differs in 13 of 99 bases shown in figure 3. Interestingly, these changes had no effect on the deduced protein sequence. The marked difference in the short segment of DNA shown in figure 3 is in line with the well-documented genetic heterogeneity of lentiviruses [21, 22]. Furthermore, the observation that DNA but not the deduced protein sequence was different suggests the existence of evolutionary pressures which may have prevented alterations in the protein. Consequently, PCR-based analyses including restriction enzyme digestion and sequencing of defined segments of the viral genome may represent a useful complement to epitope mapping in studies on viral evolution. Moreover, as suggested by the strain-specific failure of CAE virus-derived POL oligonucleotides to prime the synthesis of DNA of Dutch maedi-visna virus, PCR alone could represent a useful tool for virus identification. The most promising approach would consist in a strategy combining primers from more conserved regions with primers for more variable regions of the genome. This approach could be referred to as 'differential PCR', where the primers from conserved regions of the genome may be used for the initial grouping of virus isolates followed by fine typing with the help of primers from more variable parts of the genome.

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Calf thymus ribonuclease H IIa activity lacks ribonuclease H specificity

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Summary. Less purified fractions of ribonuclease H IIa activity of calf thymus display divalent cation-dependent ribonuclease H activity and divalent cation-independent ribonuclease activity. Because the ratio of the two enzyme activities does not change during successive chromatographic procedures, we suggest that ribonuclease H IIa activity is indeed able to degrade both ssRNA and the RNA moiety of RNA·DNA-hybrids. Ribonuclease H IIa activity can therefore be differentiated from calf thymus ribonuclease H I and H IIb by its lack of ribonuclease H specificity. The native molecular mass of ribonuclease H IIa activity is between 23 and 28 kDa. Under denaturing conditions a 23 kDa-protein band copurifies with the enzyme activity suggesting that this enzyme is monomeric.
Key words. Ribonuclease H activity; ribonuclease activity; calf thymus.

Ribonucleases H are enzymes which specifically degrade the RNA moiety of RNA·DNA-hybrids [1, 2]. Calf thymus contains two enzymes with ribonuclease H specificity, named ribonuclease H I and H IIb, and another ribonuclease H activity, named ribonuclease H IIa activity [3, 4]. Ribonucleases H I and IIb have been purified to near homogeneity and polyclonal antibodies have been raised against these proteins [5–7]. All three enzymes could be differentiated by their physical properties as well as by serological analyses [3–8]. It has been previously shown that the ribonuclease H IIa fraction is specific in degrading the RNA moiety of RNA · DNA-hybrids, but not able to degrade the DNA part of such hybrids and single- or double-stranded DNA. On the other hand, this enzyme fraction degrades poly(rA) quite efficiently [3, 4]. Therefore the question remained whether ribonuclease H- and ribonuclease-activities are intrinsic properties of the enzyme IIa fraction or whether they can be separated from each other.

Materials and methods

Assay for ribonuclease H activity. Ribonuclease H activity determinations were carried out exactly as described elsewhere [1, 3, 7, 8]. The assay mixture contained in a final volume of 500 µl 50 mM Tris-HCl pH 7.8, 50 mM $(NH_4)_2SO_4$, 0.4 mM $MnCl_2$, 0.02% 2-mercaptoethanol, and 20 µl (^{3}H)RNA·DNA-hybrid (2000 cpm, corresponding to 60 pmol of ribonucleotides). One unit of ribonuclease H is that amount of enzyme that renders 100 pmol of ribonucleotides acid soluble under optimal conditions in 10 min at 37 °C.

Protein determination. Protein determinations were carried out according to the method of Bradford [9].

Purification of ribonuclease H IIa activity. Preparation of calf thymus crude extract (fraction 0) and separation of the different ribonuclease H activities were performed exactly as described elsewhere [3, 4, 7]. After separation from the other ribonuclease H activities (H I and H IIb) the ribonuclease H IIa activity fraction (fraction 2a) contained less than 0.1% of the protein of the calf thymus crude extract. The specific activity of fraction 2a amounted to 355 units/mg of protein. Fraction 2a was separated on an S-sepharose fast flow column using a linear salt gradient (0–1000 mM KCl in buffer B (30 mM Tris-HCl pH 7.8, 30% glycerol (mass/vol.), 2 mM EDTA, and 0.1% 2-mercaptoethanol)). Ribonuclease H IIa activity elutes from this column at 220 mM KCl (fraction 3a). The proteins were concentrated by chromatography on a CM-sepharose fast flow column and step elution with buffer B + 500 mM KCl. Fractions containing ribonuclease H IIa activity (fraction 4a) were separated on a Sephadex G 75 gel filtration column. Fractions containing enzyme activity (fraction 5a) were dialyzed against buffer B and applied to a blue sepharose CL-6B column. Proteins were eluted with a linear salt gradient (0–1000 mM KCl in buffer B). Ribonuclease H IIa activity