

Visualization of viral candidate cDNAs in infectious brain fractions from Creutzfeldt-Jakob disease by representational difference analysis

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Creutzfeldt-Jakob Disease (CJD), a neurodegenerative and dementing disease of later life, is caused by a viruslike entity that is incompletely characterized. As in scrapie, all more purified infectious brain preparations contain nucleic acids. However, it has not been possible to visualize unique bands that may derive from a viral genome. We here used a subtractive strategy known as representational difference analysis (RDA) to uncover such sequences. To reduce the complexity of starting target nucleic acids, sucrose gradients were first used to select nuclease resistant particles with a defined 120S size. In CJD this single 120S gradient peak is highly enriched for infectivity, and contains reduced amounts of PrP (*Proc. Natl. Acad. Sci.* 92, 5124–8, 1995). Parallel 120S fractions from uninfected brain were made to generate subtractor sequences. 120S particles were lysed in GdnSCN, and ng amounts of released RNA were purified for random-primed cDNA synthesis. To capture representative fragments of 100–500 bp, cDNAs were cleaved with Mbo I for adaptor ligation and amplification. In the first experiment with moderate RDA selection, it was possible to visualize clones from CJD cDNA that did not hybridize to control cDNA. In the second experiment, more exhaustive subtractions yielded a discrete set of CJD derived gel bands. Competitive hybridization showed a subset of these bands were not present in either the control 120S cDNA or in the hamster genome. This represents the first demonstration of apparently CJD-specific nucleic acid bands in more purified infectious preparations. Although exhaustive cloning, sequencing and correlative titration studies need to be done, it is encouraging that most of the viral candidates selected thus far have no significant homology with any previously described sequence in the database.

Keywords: scrapie; bovine spongiform encephalopathy; slow virus; RNA

Introduction

Creutzfeldt-Jakob Disease (CJD), classified for many years with other late-onset neurodegenerative diseases of unknown etiology, was parenthetically thought to be infectious by Jacob (cf. Manuelidis and Manuelidis, 1989). Spongiform changes are typically present at terminal stages of disease, but there is an absence of the lymphocytic infiltrates evoked by most acute viral infections of the brain. In 1959, Hadlow rejuvenated an infectious concept (Hadlow, 1959), noting the pathological similarities between the CJD-like disease kuru, and scrapie, a far more prevalent infectious disease of sheep

recognized for at least 500 years. The transmission of human kuru and CJD to primates by Gibbs and Gajdusek demonstrated unequivocally the infectious origin of these human diseases (cf. Gajdusek, 1977). The serial propagation of CJD to rodents in our laboratory yielded more accessible models of disease that have been used to answer medically important questions (Manuelidis and Manuelidis, 1979) and to solidify distinctions between different agent strains (Manuelidis *et al.*, 1988). The recent epidemic infection of thousands of normal cows in England via the oral route, with demonstrable infection of Peyer's patches in the ileum, has been presented (Bradley, 1996). The subsequent infection of young people with a new agent strain that has no other source than cows is a reminder that some strains are transmitted in a manner not predicted by transgenic PrP polymorphism experiments (Hope,

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1995; Collinge *et al*, 1995). Interestingly, these outbreaks have not been linked to any PrP gene mutations that some consider central for infection (Prusiner *et al*, 1995).

PrP is a 34 kd host encoded protein that continues to be proposed as the major if not exclusive component of the infectious agent (Baldwin *et al*, 1995). In this scenario, infectious PrP (the prion) arises from a PrP mutation that is either familial or somatic, or from an as yet to be discovered unique protein conformation (Prusiner *et al*, 1995). The molecular nature of the infectious agent however remains speculative, with no definitive data for either an infectious PrP or a viral genome (reviewed in Manuelidis, 1994). PrP itself is insufficient to transmit infectious disease (cf. Discussion) and no infectivity has ever been demonstrable in preparations that are devoid of nucleic acid (Akowitz *et al*, 1994). Indeed, independent extractions of PrP-rich scrapie fractions yielded far higher levels of longer nucleic acids than reported (e.g. Aiken *et al*, 1990). Moreover, originally ultrafiltration experiments in scrapie have indicated a viruslike size of >25 nm whereas undocumented claims that the agent has a non viral size of ≤ 100 kd are questionable, especially because one author acknowledges artifacts due to leaky filters (cf. Sklaviadis *et al*, 1992). In CJD systematic physical characterizations of infectivity by gradient centrifugation combined with molecular analyses have reproducibly indicated a viral structure of reasonable size. Protective capsid proteins and longer nucleic acids have been detected in more purified infectious preparations. Relevant data include (i) a homogeneous viruslike size of $\sim 10^7$ daltons or ~ 27 nm after nuclease digestion (Sklaviadis *et al*, 1992), (ii) a viruslike density of 1.27–1.28 in sucrose (Sklaviadis *et al*, 1990) and (iii) the observation that treatments that disrupt or solubilize nucleic acid-protein complexes destroy infectivity whereas removal of PrP does not (Manuelidis *et al*, 1995). A current report in scrapie similarly shows sedimenting infectious particles separate from the majority of PrP during sucrose gradient centrifugations (Riesner *et al*, 1996). Removal of PrP also facilitated the detection of small viruslike structures of ~ 12 nm with a density of >1.2 (Ozel *et al*, 1994).

Biological properties such as exponential replication (Manuelidis and Fritch, 1996), strain variation and mutation (Bruce and Dickinson, 1987; Bruce *et al*, 1991), long or evasive persistence in the host and cell specificity are most easily embraced by known viral mechanisms. Additionally, classical viral interference experiments in scrapie show that injection of one agent strain can protect an animal from infection by a second agent strain (reviewed in Manuelidis, 1994). Because capsid-like proteins are detectable in more purified CJD preparations (Sklaviadis *et al*, 1993), and a non-specific but

5000 nt long endogenous retroviral RNA can be recovered from infectious preparations after exhaustive nuclease digestion (Akowitz *et al*, 1994), it seemed reasonable to pursue a strategy that could uncover a viral genome of reasonable size. Although radiation data has been used to exclude a viral genome of >50 nt (reviewed in Rohwer, 1991), it is notable that several RNA viruses, including ~ 10 kb retroviruses, are completely resistant to radiation (cf. Manuelidis, 1994). We therefore sought to further characterize RNAs in infectious brain fractions. Visualization of discrete nucleic acid bands in infectious but not control preparations is an important first step in this process.

It is advantageous to purify the infectious agent away from most cellular nucleic acids and aggregating PrP for more effective RNA extractions. Additionally, because the titer in CJD is $\sim 2 \times 10^8$ per gram of brain, or $<10^5$ per μg of brain RNA, enriching infectivity will increase the likelihood of capturing agent-specific sequences. We have shown that a 120S sucrose gradient peak reproducibly contains $\sim 15\%$ of the starting brain titer, with an $\sim 100\,000$ -fold purification with respect to starting nucleic acids. Nonetheless, the presence of contaminating cellular sequences in this fraction makes a subtractive approach desirable. As with other viruses that are not released extracellularly to any large extent, it has not been possible to purify the CJD agent to homogeneity.

The low levels of RNA obtained from more purified 120S infectious brain preparations (~ 5 ng/gm), with comparably low levels of nucleic acid in parallel normal preparations (Akowitz *et al*, 1990), made direct visualization as well as standard subtraction methods impractical. Amplification of cDNA by PCR after adaptor addition is also of limited usefulness due to the heterogeneity and unequal representation of large and small fragments (Akowitz *et al*, 1990). Representational difference analysis (RDA) was recently developed for identifying unique sequences in genomic DNA (Lisitsyn *et al*, 1993). This procedure seemed most suitable for our purpose because it can produce large amounts of subtractor by ligation of adaptors and PCR amplification. Furthermore, the sequential use of different adaptors on the target (in this case CJD amplicons) also ensures exclusive amplification of remaining target sequences. These amplified target bands become visible with increasing rounds of RDA. We therefore adapted this method for representation of small amounts of RNA by reverse transcription and used the synthesized cDNA for RDA. We show here that after moderate selection by RDA, individual clones containing sequences that are not present in the normal 120S preparation or in the hamster genome can be isolated. In a second more stringent RDA selection, a discrete set of well-resolved bands was seen. Competitive hybridization indicated this pool contained several se-

quences not present in the normal 120S subtractor or in the hamster genome. We here cautiously call such sequences viral candidates and suggest that these or other similarly generated fragments may be useful for identifying a complete viral genome in these infections.

Results

Homogenization of CJD infected brain releases only ~20% of the starting titer into the 25 000 g supernatant, but is useful because it excludes most nuclear DNA. The titer in this supernatant is quantitatively recovered in a 215 000 g pellet (Sklaviadis *et al*, 1992). To further reduce the complexity of input RNA and ensure effective RNA extraction, micrococcal nuclease resistant particles were further separated by sucrose gradient centrifugation. In infected brain, ~85% of the loaded infectivity is resolved at a peak centered at 120S. In contrast, most PrP, other small proteins, and nucleic acid fragments remain in the minimally infectious top portions of the gradient (Sklaviadis *et al*, 1992; Akowitz *et al*, 1990). For comparison and subtraction, a parallel 120S preparation from uninfected brains was generated. Previous controls have shown that as little as 2.5 M GdnHCl solubilizes most nucleic acid complexes in 120S preparations and concomitantly destroys infectivity (Manuelidis *et al*, 1995). We therefore used more chaotropic GdnSCN solutions as well as exhaustive proteinase K digestion to release undegraded nucleic acids. The proteolytic step was included to reduce the length of proteins that might be covalently attached to a CJD viral genome. CsCl isolation of RNA was chosen to avoid potential losses of material at the interface in organic extractions, to separate larger RNAs and to exclude small amounts contaminating DNA that can derive from nuclease-resistant chromatin fragments. These methods yield 5000 nt-long RNAs from a copurifying nuclease resistant endogenous retrovirus in both control and infectious 120S preparations (Akowitz *et al*, 1994), a reasonable test for effective extraction and lack of RNA degradation in these samples with low RNA. We chose random hexamers to initiate cDNA synthesis because the CJD viral genome might not be polyadenylated. Additionally, oligo dT priming might generate only short terminal 3' sequences if extensive RNA base pairing obstructed complete reverse transcription. Reduced amounts of random hexamers may be critical for synthesizing cDNA fragments of >300 bp (see Methods). However, because starting RNA was low, we did not rigorously optimize this ratio.

RDA was designed to isolate unique genomic sequences from mixtures with enormous sequence complexity and therefore used 6 bp recognition enzymes to reduce this complexity. However, our

aim was to maintain complexity since the CJD preparation was already highly enriched for infectivity (Akowitz *et al*, 1990) and loss of any fragment could result in negative results. Furthermore 6 bp cutters might cleave only one end of a viral genome, especially if cDNA synthesis was not full length. This would preclude amplification by PCR that requires two ends cleaved by the same restriction enzyme for adapter addition. To generate multiple small fragments for ligation we chose the more frequent cutter Dpn II because it contains the same internal 4 bp recognition sequence as Bgl II and thus could be ligated to the described Bgl II adaptors (Lisitsyn *et al*, 1993). Additionally, large fragments of DNA can be underrepresented during repeated cycles of PCR, whereas smaller fragments of cDNA (200–500 bp) will be more equally represented with each round of amplification.

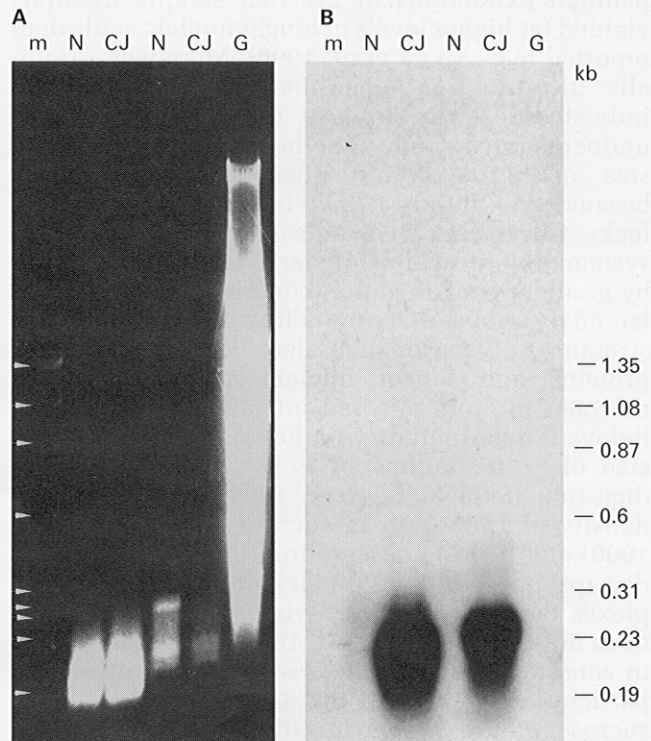


Figure 1 Ethidium bromide stained gel (A) and its Southern blot (B) of starting RBgl amplicons from normal driver (N) and unabstracted CJD (CJ) 120S cDNA and from Syrian hamster genomic DNA (G). Lane m contains Hae III ϕ x markers of standard size (arrowheads at 1353, 1078, 872, 603, 310, ~275, 234, 194, 118 bp). Samples of N and CJ amplicons either cut with Dpn II (~2 μ g, left pair), or without Dpn II cleavage of adaptors (≤ 0.5 μ g, right pair) were analyzed with 10 μ g of Mbo I cut genomic DNA. The corresponding blot shows a representative hybridization to the first cloned viral candidate of ~200 bp (see at markers). There is no significant hybridization to normal amplicons (N) or genomic DNA (G) at the predicted size before or after Dpn II cleavage (~250 and 200 bp respectively) while both starting CJD samples show intense hybridization. The second clone from the combined RDA-differential colony screening showed similar unique hybridization to CJD amplicons (data not shown).

After the first round of amplification with RBgl adaptors, a smear of DNAs from 100–600 bp, but predominantly between 150–300 bp, was generated using both normal and CJD cDNA (Figure 1A, lanes N and CJ). After moderate selection (see Methods), remaining CJD amplicons still yielded a smear of DNA from 150–500 bp, with a few faint bands between 200–300 bp (data not shown). However, these bands could not be definitively distinguished from the starting normal amplicons by size and differences were not enhanced after cleavage of the adaptors.

We therefore cloned these partially purified CJD amplicons and screened ~200 inserts by differen-

tial hybridization to nl driver and CJD amplicons. Each lane in Figure 2 shows 1–5 plasmid inserts stained with Ethidium bromide (A) and the corresponding blot hybridized first to nl driver (B) and then to CJD amplicons (C). Approximately 15% of screened inserts hybridized to the pool of CJD amplicons and most of these also hybridized with nl driver amplicons. However, several inserts hybridized only to the CJD probe (Figure 2C, arrows). Three CJD positive clones were arbitrarily selected for further study. Two of the purified inserts showed negligible hybridization to either nl amplicons or to large loads of Mbo I cleaved hamster genomic DNA in repeated studies using extended autoradiographic overexposures. This indicated their specificity for the infectious fraction. Figure 1B shows a representative hybridization of one of these inserts to starting normal driver (lanes N), to starting unabstracted CJD amplicons (lanes CJ), and to Mbo I cut genomic DNA (lane G). The two CJD specific inserts so identified were ~220 and ~110 bp after Dpn II cleavage. Thus a combination of RDA and cloning made it possible to detect and isolate the first viral candidates (unique cDNA fragments cosedimenting with infectivity).

Because individual bands were not well resolved by moderate RDA selection, possibly because initial PCR cycles were increased, and nl subtractor had to be reamplified for adequate yields (see Methods), we increased the stringency of selection. Increased driver in the third and fourth rounds of RDA yielded individual bands from CJD amplicons. Figure 3 shows that 7–8 bands were well resolved both before (lane 1) and after removal of the Bgl adaptors (lane 2). The size of remaining cDNA fragments in CJD ranged from ~120–450 bp after Dpn II cleavage.

To find if any of these visualized bands were restricted to the CJD infectious fraction, we labeled the total pool of fourth round cDNA fragments with ^{32}P and probed gel blots containing starting nl driver, starting (unabstracted) CJD amplicons, and total hamster genomic DNA cleaved with Mbo I. We also used a competitive hybridization strategy because one of these bands, or the faint residual background smear of DNA, might derive from or have homology with normal sequences. Figure 4 shows hybridization in parallel blots without cold competitor (A), and in the presence of 1000 fold excess of starting normal amplicons (B). In the presence of cold competitor the hybridization to nl amplicons (lanes N) and to genomic DNA (lanes G) was markedly reduced while the signal in CJD amplicons (lanes CJ) was minimally affected. Furthermore, the CJD amplicons still spanned a size range of 120–450 bases (arrows) while the weak nl signal was confined to a more limited size range. This data indicates that the CJD probe pool contained several distinct sequences that are not significantly represented in the nl driver or in the hamster genome.

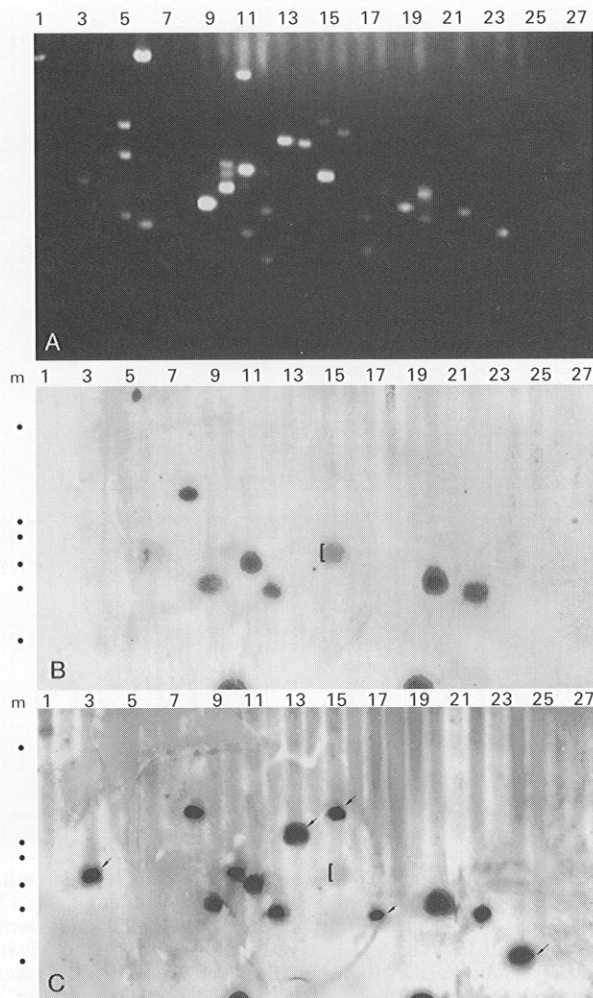


Figure 2 Representative differential screening of CJD amplicons. Four to five colonies were pooled for DNA extraction and cut to release inserts analyzed in each lane. (A) shows the Ethidium bromide stained gel used to make a blot. (B) shows hybridization to this blot probed with starting nl driver amplicons, and (C) shows hybridization with CJD amplicons (after stripping nl amplicons). In (C), the 5 arrows indicate inserts that were specific for CJD as determined by superimposition of the two autoradiographs. Lane 15 contains both a normal insert (bracketed) as well as a longer CJD specific insert. Indicated ϕ x markers (m) are 610, 275, 234, 194 and 118 bp.

To begin to investigate the nature of the subtracted CJD sequences, the gel was cut into 8 slices for elution and cloning and 12 clones were purified and partially characterized. Nine of these clones had independent non-homologous inserts from ~100–400 bp. A single gel band may contain more than one sequence, and exhaustive characterization of the subtracted cDNAs has not been done. Nonetheless, it is interesting that none of these inserts, or the two additional clones from the first experiment, hybridized to the cosedimenting endogenous retrovirus or to bacterial sequences, the most likely source of exogenous contaminants (Akowitz and Manuelidis, 1989, data not shown). This further documented the effectiveness of subtraction. Although we did not find the two shorter length sequences in the second more exhaustive RDA selection, we suspect their absence is probably due to the repeated more stringent size selection of larger fragments in the second RDA and/or to variations inherent in ligation-amplification reactions using small amounts of cDNA.

Preliminary sequencing thus far (LM and LA Manuelidis, unpublished data) has revealed only one clone with a clear genomic origin. This clone contains a 120 bp fragment with good homology

(91% in 57 bp) to the interspersed B1 Alu family of repeats in rodents. The presence of this sequence in the probe pool could certainly account for the background hybridization in the starting amplicons and genomic DNA. Although it is premature to speculate on possible sequences that may define a viral genome in CJD without more exhaustive isolations and titer correlations, it is intriguing that several clones had complete open reading frames as well as sequence motifs consistent with a capacity to bind nucleic acids. Most of the sequences determined thus far (representing a total of >1700 bp) also have no homology to genomic or viral sequences in the data base by FASTA and

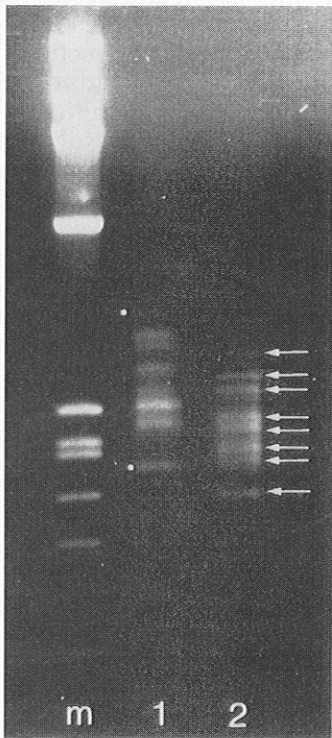


Figure 3 Ethidium bromide stained bands in CJD from ~120–450bp visualized after four cycles of RDA. CJD amplicons (5 of 200 μ l), uncut (lane 1) or cut with Dpn II (lane 2), run on a 3% agarose gel and stained with Ethidium bromide. Eight bands were visible after Dpn II cleavage (arrows) and the dots represent the corresponding upper and lower bands in the uncut sample. Markers (m) as in Figure 1.

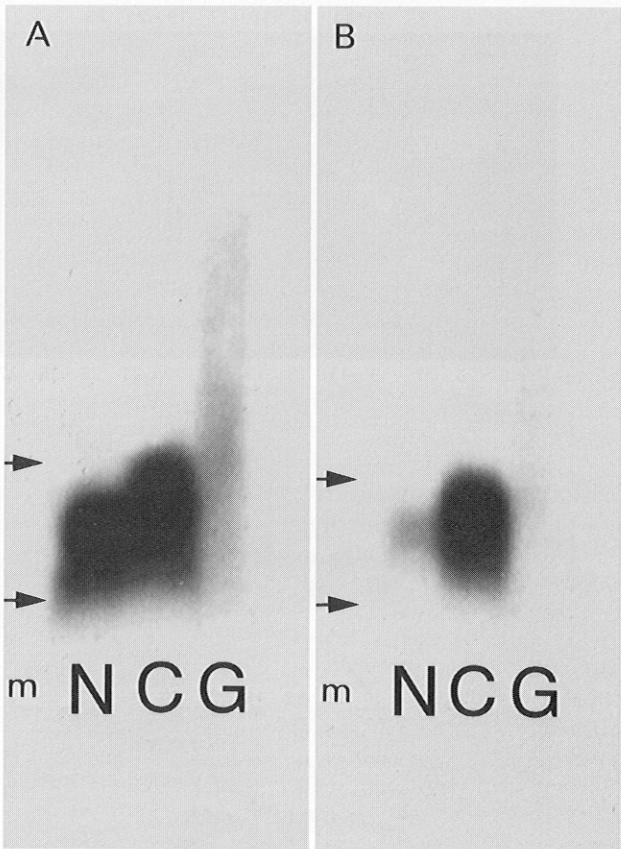


Figure 4 CJD-specific sequences demonstrated by competitive hybridization after four rounds of RDA. Blots contain 0.5 μ g 120S amplicons from starting normal driver (lanes N) and starting unselected CJD (lanes C), as well as 10 μ g of Mbo I cleaved hamster genomic DNA (lanes G). Replicate samples were blotted from a 2.5% agarose gel and hybridized with the pool of 4th cycle CJD amplicons (32 P labeled after cleavage with Dpn II) either without added competitor (A), or in the presence of 25 μ g of unlabeled normal amplicons to suppress non-specific hybridization (B). Both halves of the blot were exposed in parallel for the same time. The CJD signal is hardly suppressed by competition. In contrast, the normal amplicons show only a limited low intensity signal confined to a more discrete size range, and hybridization to genomic DNA is almost invisible with this overexposure. The overexposed autoradiograph is shown to bring out the faint nl signal, but lighter exposures confirmed the same size range for the CJ amplicons. Arrows are at ~120 and ~450 bp.

BLAST X searches. This lack of homology might be expected for this class of agents, but makes the selection of more meaningful candidates for rapid RT-PCR analysis difficult (*vide infra*).

Discussion

We have successfully adapted RDA for display of cDNA sequences using ng amounts of starting RNA. Nuclease protected sequences in preparations enriched for infectivity as well as in control 120S preparations were readily visualized even after digestion to smaller cDNA fragments. Not surprisingly, a smear of similar bands in both preparations precluded direct visualization of viral candidates. A subtractive approach was therefore followed. The necessary details presented here should help others pursue similar investigations of nucleic acid from infectious scrapie preparations. Keys to success included (i) careful extraction of RNA, (ii) modification of cDNA synthesis conditions and (iii) Dpn II cleavage to yield reasonable representation of fragments in RDA. Independently, Dpn II cleavage was also found to be advantageous for adequate differential representation of transcripts in total RNA from cells of different lineage (Hubank and Schatz, 1994), and was used to uncover two new flavivirus-like sequences from GB hepatitis serum (Simons *et al*, 1995). The latter study is especially pertinent because of the 60 clones isolated by RDA, only two were relevant. The identification of these clones was facilitated by their homology with other known viral sequences in the data base. In the case of CJD and scrapie, a related homologous viral sequence may not yet be entered in the data base, making the selection of relevant cloned sequences a more formidable task.

The major aim of this work was to find if one could first visualize candidate viral sequences in infectious preparations. The current results show several candidates can be displayed and isolated using random hexamer generated cDNA combined with RDA. Disaggregation of crude infectious preparations that allowed subsequent separation of most infectivity from the bulk of cosedimenting PrP, attention to physical and chemical properties of the infectious agent as judged by repeated bioassays (Akowitz *et al*, 1990; Manuelidis *et al*, 1995) and control studies assessing solubilization and recovery of long RNA from cosedimenting resistant viral structures (Akowitz *et al*, 1994) provided the necessary base of information for the current molecular studies. Because host PrP aggregates can form dense amyloid structures in CJD with proteinase K digestion *in vitro* (Manuelidis *et al*, 1986), we used conditions to minimize the formation of amyloid aggregates that might physically entrap viral particles. Although specific sequences might also be obtained using total RNA for RDA in

CJD and scrapie, most of these would probably represent differentially expressed genes associated with disease progression rather than viral candidates. The visualization of selected sequences in simplified infectious preparations, their lack of sequence homology with the major differentially expressed genes, eg, glial fibrillary acidic protein mRNA (Manuelidis and Fritch, 1996), and their survival after nuclease digestions that would destroy most cellular mRNAs, is much more suggestive of a viral origin.

This work extends previous nucleic acid analyses and clearly contradicts claims that there are only ≤ 50 nt long nucleic acids in nuclease treated infectious preparations (reviewed in Akowitz *et al*, 1994). Even after Mbo I or isoschizomer cleavages, cDNA sequences of several hundred base pairs were recovered from infectious preparations. We have shown several non-overlapping cDNAs that fulfill an important criterion for a viral genome, *viz.* they are present in more purified infectious brain fractions but not in parallel uninfected control fractions. They are also not detectable in the host genome by hybridization. This further suggests they are of exogenous origin and unrelated to pathological host responses. Although a few of the resolved bands could conceivably derive from an unknown PCR artifact, it is unlikely that all the subtracted sequences are artifactual. Thus this data provides a counterbalance to the assumption that unique nucleic acids will not associate with the agent (Prusiner *et al*, 1995).

In the prion hypothesis a host protein (PrP) is believed to become infectious and the possibility of a viral genome is not entertained (Prusiner, 1991). However, significant infectivity has not been demonstrable with the purified, recombinant or transgenic protein (Hsiao *et al*, 1994; Manuelidis, 1994). Additionally, synthetic PrP peptides yield proteinase-resistant amyloid fibrils like the 'infectious' or PrP-res form of the protein, but these resistant forms of PrP have not yet shown infectivity (Selvaggini *et al*, 1993). Similarly, reconstituted PrP-res in complex scrapie brain fractions (Bessen *et al*, 1995) have questionable infectivity because the reconstitution conditions reduce titer by more than 99.5% in both CJD and scrapie (Manuelidis *et al*, 1995; Riesner *et al*, 1996). Indeed, the scrapie investigators attributed the reformation of PrP-res to an artifact of incomplete denaturation. Therefore, while PrP is involved in disease pathogenesis (Manuelidis, 1994; Manuelidis and Fritch, 1996), the infectivity of PrP-res remains hypothetical. Moreover, the above results, as well as other data, have forced the conclusion that an additional unknown macromolecule ('protein X') is needed to convert PrP into a prion (Telling *et al*, 1995; Baldwin *et al*, 1995).

The visualization of RDA bands in CJD and the exclusion of common genomic and bacterial con-

taminants, is an encouraging first step for selecting viral sequences. However, without more exhaustive studies, RDA alone cannot provide a definitive identification of a viral genome. Unfortunately, some of the more intriguing sequences isolated thus far can not be assessed by RT-PCR because several GC rich sequences, such as those encoding nucleic acid binding motifs, lack adequate primer pairs for their rapid and sensitive identification in infected tissues. Additionally, RDA as currently formatted does not yield information about the sense orientation of cloned sequences, and this further complicates the evaluation of interesting sequences. We are currently addressing this problem using other molecular strategies to tag one end of the sense strand for RDA. Such methods should help orient multiple sequences for 'intervening' PCR of longer viral sequences and permit design of adequate primer pairs. Finally, further technical modifications to increase the robustness and reproducibility of random hexamer cDNA syntheses from ng amounts of RNA are being evaluated. Nevertheless, the present experiments show that refined nucleic acid strategies in CJD can be fruitful. Together with similar studies in scrapie and bovine spongiform encephalopathy it should be possible to identify sequences that are useful for the detection and prevention of these insidious infections in man and animals. The current approach can also be a powerful way to define other unsuspected or novel latent viruses that cause or contribute to late-onset dementias.

Materials and methods

Six brains from symptomatic (>20th serial CJD passage) and from normal uninfected hamsters of similar age (nl) were used to generate material for RDA. These preparations were treated in parallel with exhaustive micrococcal nuclease digestion and disaggregation as described (Akowitz *et al*, 1990) before sucrose gradient centrifugation to separate infectious particles with a size of 120S. An equivalent discontinuous step gradient was used. As in previous experiments, the majority of PrP and other small proteins (including micrococcal nuclease), were well separated from sedimenting infectious particles that were used here for nucleic acid extraction. Sedimenting particles from uninoculated brain were comparable, but contained no detectable PrP, as in previous experiments (Sklaviadis *et al*, 1993).

To extract RNA, the resuspended 120S particles were split into three aliquots. Three different extraction variants were used to ensure inclusion of representative RNAs. The first aliquot was lysed at 65°C in 6 M GdnSCN with 1.5% sarkosyl, 0.3 M β -mercaptoethanol, 15 mM Na Citrate pH 7.0. The second aliquot was lysed in 4 M GdnSCN for

15 min at 37°C, diluted to 1 M GdnSCN with 0.5% sarkosyl, 10 mM EDTA and EGTA, and further incubated for 2 h at 52°C with proteinase K (PK, 0.5 mg/ml). The third aliquot was digested with 0.5 mg/ml PK in 0.5% sarkosyl, 10 mM EDTA and EGTA for 1 h at 37°C. GdnSCN was then added to 4 M and this sample was incubated for 1 h at 52°C. The three aliquots were pooled and RNA was isolated by centrifugation over 5.7 M CsCl (Chirgwin *et al*, 1979). The RNA pellet was resuspended, extracted with butanol, precipitated with glycogen carrier and resuspended in RNase-free water. Minor variations of all of these methods have previously been shown to extract 5000 nt-long RNAs from cosedimenting viral particles (Akowitz *et al*, 1994), but we do not know their relative efficiencies for CJD RNA extraction. However, less chaotropic solutions of 2.5 M GdnHCl solubilize >75% of the nucleic acids and nucleic acid binding proteins (Manuelidis *et al*, 1995).

Two RDA experiments were done with these RNA preparations. RNA from 2.5 g of brain was used for each cDNA synthesis. After heating the RNA from 70–80°C over 10 min, the tubes were centrifuged and cDNA synthesis components including RNasin were added. Random hexamers were used to initiate cDNA synthesis from RNA using superscript (BRL kit). Hexamers were increased stepwise from 1 pg/ μ l to 250 pg/ μ l during reverse transcription (three increments to 10, 50 and 250 pg/ μ l over 2 h) in a volume of 10 μ l using the recommended buffers and incubation conditions. For second strand synthesis, *E coli* DNA polymerase and RNase H (Boehringer Mannheim) were diluted to 1/5 and 1/50 respectively over the recommended concentration using the supplied buffers for dilution. After 30 min, additional RNase H was added (1/5 dilution) for 30 min. Synthesis was completed with standard amounts of T4 DNA polymerase, and enzymes were inactivated at 70°C for 20 min.

For RDA, nl and CJD derived cDNAs were digested with Mbo 1 (or its isoschizomer Dpn II). Enzymes were inactivated at 70°C for 20 min and the RBgl24 and RBgl12 oligonucleotides ligated essentially as described (Lisitsyn *et al*, 1993). CJD and normal amplicons were generated by fill in (10 min at 72°C with Taq polymerase) and then amplified by PCR with RBgl24 (Lisitsyn *et al*, 1993) with minor modifications. Because there were low amounts of cDNA it was necessary to use 30 rather than 20 cycles of PCR (95°C for 1 min, 72°C for 3 min and 72°C for 5 min in the last cycle) with 200 rather than 400 μ l in each tube for the first amplification. Yields were \sim 10 μ g/200 μ l. Additional subtractor for the driver pool was made by dilution and additional PCR cycles from a diluted aliquot of the starting nl amplicons. After Dpn II cleavage, the RBgl adaptors were separated from the CJD target and nl driver amplicons. In the first RDA

experiment S-100 spin columns (Chroma) were used for this separation, while in the second RDA experiment S-400 columns were used to select longer CJD target cDNAs. Before each round of subtraction, CJD amplicons were ligated to NBgl or JBgl adaptors (alternated for each consecutive round of RDA) and precipitated with nl driver amplicons. DNA was denatured under oil for 5 min at 100°C in 4 µl 30 mM EPPS (Sigma) pH 8.0, 3 mM EDTA and then 1 µl 5 M NaCl was added at 67°C for a 20 h hybridization. For the first round of subtraction the ratio of CJD to nl amplicons was 1/80, i.e. 500 ng of CJD cDNA was hybridized with 40 µg of nl driver. Ten cycles of PCR, mung bean nuclease digestion and then 20 cycles of PCR were used to recover CJD enriched sequences essentially as described (Lisitsyn *et al.*, 1993). For each round of RDA this process of subtraction and regeneration was repeated with increasing ratios of nl driver. The first cDNA/RDA experiment used only moderate selection (three rounds of RDA with a final CJD to nl ratio of 1/10⁴). For the second cDNA preparation,

we increased the selective pressure by using four rounds of RDA, and increased the subtractor (ratios of 1/10⁵ and 1/10⁷ in the final two rounds).

For further isolation of viral candidates, Dpn II cleaved bands were cut from an agarose gel, eluted and cloned into Bluescript SK⁺ (Promega). Cloned inserts or Dpn II cleaved RDA products were labeled to ~10⁹ cpm/µg by random priming (Amersham kit) for probing Southern blots. Exhaustive washes at 68°C in 0.1 × SSC were used for high stringency hybridization, and autoradiographic overexposures (at -70°C with intensifying screens) were used to confirm negligible hybridization to normal sequences.

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