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Endogenous viral complexes with long RNA cosediment with the agent of Creutzfeldt-Jakob Disease

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ABSTRACT

A class of viruslike agents that induces Creutzfeldt-Jakob Disease (CJD) and scrapie remains undefined at the molecular level. Several investigators believe this infectious agent is constituted by a single host protein or 'prion', and have emphasized data that would seem to exclude the presence of any viral nucleic acids. However, more rigorous evaluations in scrapie have shown reasonably abundant nucleic acids. Additionally, in highly purified 120S CJD preparations that have been treated with nucleases, RNAs as long as 6,000 bases have been detected. Few nucleic acids have been characterized in either scrapie or CJD, but previous cloning experiments delineated relatively short LTR regions of the endogenous IAP retrovirus in 120S CJD preparations. We therefore used specific primers encompassing the entire IAP genome to test for the presence of long viral RNAs, and here show ~5,000 contiguous bases of the IAP RNA genome can be recovered from reasonable amounts of starting brain. The 3′ *env* region of IAP is comparably truncated in CJD and normal preparations, and we find no evidence for IAP transduction of CJD-specific sequences. Because IAP cores can coencapsidate unrelated sequences, and are unusually resistant to physical and chemical treatments, it was relevant to find if cosedimenting cognate proteins of the IAP core, such as *gag*, could be detected. The predicted ~65kd acidic *gag* protein, showing appropriate antigenic and nucleic acid binding features, was apparent in both one and 2-D Western blots. This data strongly indicates specific viral complexes cofractionate with the CJD agent. Interestingly, these nuclease resistant IAPs do not appear to be in morphologically recognizable 'R' particles. This cosedimenting viral assembly therefore provides a paradigm for non-particulate CJD complexes in infectious preparations. In developing strategies to identify a CJD specific sequence, cosedimenting IAPs can be used to assess the quality, length and recovery of RNAs extracted from highly resistant viral complexes.

INTRODUCTION

Creutzfeldt-Jakob Disease (CJD), a late-onset dementia, is caused by an infectious and transmissible agent that can be latent for many years. We have established rodent models of CJD that are comparable to, but distinct from those established for scrapie of sheep[1,2]. Such models provide an opportunity to study pathogenetic mechanisms of neurodegeneration, and to explore the molecular attributes of this class of agents. The molecular nature of the infectious agent(s) in CJD and scrapie continues to be a subject of scientific controversy.

Some investigators strongly favor the proposal that a host-encoded membrane protein converts itself into a replicating infectious entity (prion hypothesis)[3], and have discarded the possibility of a more conventional viral (nucleic acid–protein) structure. Most recently this view has been supported by negative data from one laboratory claiming nucleic acids of >50 bases are not present in scrapie preparations stained with silver[4]. This data, if correct, would exclude a viral hypothesis. However, other investigators have found nanogram levels of mitochondrial DNAs that are ≥500bp[5], as well as other nucleic acids[6] in these *identical* scrapie preparations with ~10^8 infectious units (IU). Such large amounts of nucleic acid greatly exceed that which is necessary for a viral titer of this magnitude. Moreover, in highly purified CJD preparations exhaustively digested with nucleases, we detected a heterogeneous smear of nucleic acids up to 6,000 bases in length using ligation-PCR procedures combined with sensitive ^{32}P labeling techniques[7,8]. Because our strategies excluded host DNA, these long species derived from RNAs.

Several initial lines of evidence in CJD indicated these long RNAs originated in core-like (nucleic acid–protein) complexes that were impervious to nuclease digestion. In sucrose equilibrium gradients of nuclease treated material, a single infectious peak is reproducibly recovered separately from contaminating free nucleic acids and small proteins, and resolves with a density comparable to retroviral cores[7]. Furthermore, these infectious complexes show a viruslike size that can readily accommodate nucleic acids of conventional viral length as well as protective binding proteins. Empirical determinations in CJD reproducibly show a viral size of 120S in sucrose[8], with a mass of ~10^7 daltons by HPLC, and a physical diameter of ~30nm by

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sedimentation field flow analysis[9]. Non-specific aggregation of proteins with nucleic acids could possibly explain these data. However, a defined group of proteins that strongly bind to nucleic acids has also been resolved by both one and two-dimensional (2-D) gel electrophoresis in these CJD preparations[10]. During purification, these binding proteins can probably protect several different nucleic acid species from nuclease digestion, including those specific for the CJD agent. To us, the existing physical and molecular data indicates CJD is a viral complex, rather than an infectious protein or 'prion'.

The biological properties of scrapie and CJD, including agent strain variation, exponential replication, tissue specificity, and latency, additionally underscore a viral nature for the infectious agent (reviewed in 2). These biological properties, coupled with the known resistance of these agents to several physical and chemical treatments, and the non-inflammatory spongiform changes they provoke, led us to develop a retroviral working model for the CJD agent[11,12]. Reconstituted strong-stop experiments, designed to test for the presence of long terminal repeats (LTRs) that are characteristic of all retroviruses, yielded a group of selected LTR-size bands in infectious preparations[13]. To further examine such protected LTRs, we cloned and sequenced several cDNAs from highly purified infectious fractions, and identified ~900 terminal bases of the endogenous retroviral intracisternal A particle (IAP) genome[14]. IAPs are endogenous retroviruses associated with 'R' particles observed in some cells. As the endoplasmic reticulum to which they bind is removed, the remaining cores show an increased density, much like the CJD virus after membranes are removed. Because this recovered LTR, as other retroviral LTRs, contains all the necessary control and regulatory information to drive downstream sequences, including those that are transduced, it was germane to examine longer stretches of IAP RNA for recombinant CJD-specific inserts.

We here show ~5,000 contiguous bases of IAP RNA cosediment with infectivity despite exhaustive digestion with nucleases. Although there is no evidence for transduced CJD-specific sequences in PCR walks of this IAP element, and the same protected IAP RNAs are also present in parallel uninfectious brain fractions, these viral RNAs provide an important internal control for nucleic acid studies in this field. Using the described primers, other investigators can more rigorously assess their extraction and recovery techniques in commonly used experimental rodent models of CJD and scrapie. Notably, rodent IAPs are highly resistant to denaturing treatments, such as SDS and chaotropic salts, and thus provide a useful internal control in studies of highly resistant CJD and scrapie specific complexes.

The unusual resistance of IAPs is based, at least in part, on the extensive intramolecular disulfide bonding of the IAP *gag* protein within the core[15]. We therefore additionally sought evidence for the IAP *gag* protein in more purified CJD preparations. If present, such proteins would indicate isolation of true viral cores with CJD, rather than IAP nucleic acids protected in less specific transcriptional or translational complexes. We here show a protein with the predicted size, isoelectric point, antigenic, and nucleic acid binding features of the IAP *gag* protein in highly purified CJD preparations. Thus the long IAP RNA we here identify almost certainly derives from resistant viral complexes. We presume there are similar CJD-specific viral complexes waiting to be found.

MATERIALS AND METHODS

Source of materials

All components used in RNA/PCR walks were supplied in kit form from Perkin Elmer Cetus. Glycogen, EcoRI, BamHI and a random primed radioactive probe synthesis kit were purchased from Boehringer Mannheim. Recombinant clones representing the 5′ and 3′ halves of the Syrian hamster IAP genome were the generous gift of E.L.Kuff, as were three different rabbit polyclonal antibodies that bind the IAP *gag* protein[16]. All primers synthesized by the Yale Biotechnology Resource Laboratory were based on a published full-length IAP sequence[17].

Purification of CJD infectivity

Typically 12 CJD infected brains (~10 gms, standard CJD strain extensively passaged in hamsters[18]), were collected at terminal stages of disease. Preparations with high specific infectivity (~100,000 fold purification of IU with respect to nucleic acids) were obtained after nuclease digestion and separation of an ~120S gradient peak as described, with reproducible titers and molecular characteristics[7,8]. Briefly, brains homogenized in sarkosyl were centrifuged at 25,000×g, and the supernatant pelleted at 215,000×g for 2hr. This aggregated p215 pellet was washed, exhaustively digested with micrococcal nuclease, rewashed, and then disaggregated at pH 8.9 until they were devoid of PrP-res, i.e., the 'infectious form of the prion protein'[19]. For simplicity, we sometimes used sucrose step gradients that were equivalent to previous linear sucrose gradients[8] to resolve the ~120S infectious peak. Pooled 120S peak fractions were concentrated after ~4-fold dilution by resedimentation at 215,000g×3hr over a 20ml 80% sucrose cushion with essentially no loss of titer[8,9].

Extraction of RNA

Concentrated infectious preparations (3 gram equivalents of brain in 200ml) were digested overnight at 37°C with 500mg/ml proteinase K in 50mM Tris−HCl pH 8.0, 10mM EDTA and 0.5% sarkosyl. For sequence independent amplification, RNA was purified by brief DNAse I digestion under conditions that do not degrade RNA[8], and then extracted with phenol-chloroform. Alternatively, for IAP-specific amplification, RNA was separated from DNA using standard guanidinium−CsCl methods[20] with minor modifications, i.e., centrifugation in smaller tubes, and addition of sterile glycogen (0.5mg) carrier to the wet RNA pellet. The RNA was then carefully washed three times with 1 ml of 70% ethanol and 30% 0.3M sodium acetate pH5.2, lyophilized, resuspended in DEPC-treated water and stored at −70°C.

RNA/PCR amplification of IAP sequences

For sequence independent amplification, the described 'PRI-1' primer[21] was ligated at both ends to all CJD cDNAs synthesized by either random or oligo-dT priming[8]. Because the PCR products were of variable size in different experiments, we designed IAP specific primers for subsequent cDNA syntheses. The primer numbers below reference the sequence designation of the published Ono clone[17]. In this case, total RNA was primed with reverse complement (RC) IAP primers for first strand cDNA synthesis, and slightly different brain equivalents

of RNA were found to be optimal for each primer as follows: 0.5 gm for IAP 604RC and IAP 3886RC, 1.0 gm for IAP 3769RC, and 2.0gm for IAP 2360RC and IAP 7533RC. For cDNA synthesis, 0.75mM of reverse complement primer was added to a 20ml reaction mixture specified by the manufacturer. The mixture was covered with 75ml of mineral oil and sequentially incubated at 42°C or 1h, 99°C for 7 min and 4°C for 5 min. The PCR amplification was then carried out in the same reaction tube with the addition of the forward (F) primers at 0.15mM, and 1.5mM $MgCl_2$. Taq polymerase was introduced under the oil overlay ('hot start') during the first 72°C elongation step of a 40 cycle amplification reaction. Each cycle included the following steps: 95°C for 1 min, 55°C for 1 min and 72°C for 4 min.

The sequence of RNA/PCR IAP primers were as follows where RC denotes reverse complement and F denotes a forward orientation: **IAP 604RC:** 5'-TCGGCACCAGCCACGATGAAGGAG; **IAP 2360RC**: 5'-AGCCCTATGATAACCTTTC/ACCACA; **IAP 3769RC**: 5'-GCCTTGCGGCAGTACCTTCC; **IAP 3886RC**: 5'-GTGACAAATAAGAATATGTCA; **IAP 7533RC**: 5'-AACTTTGGAGTGGAGATAGAGGTC; **IAP 170F**: 5'-GTCATTGGGGTGAGTGCAAACC; **IAP 581F**: 5' CTCCTCATCGTGGCTGGTGCCG; **IAP 2337F**: 5'-TGTGGTAAAGGTTATCATAGGGCT; **IAP 3749F**: 5'-TGGAAGGTACTGCCGCAAGG; **IAP 3860F**: 5' TATATGGACGATATTCTTATTTG.

Probe hybridization to Southern blots

DNA probes, generated from a recombinant clone of Syrian Hamster IAP, and RNA/PCR walks between 3749F and 7533RC, were labeled with ^{32}P by random priming to $>10^8$ CPM/mg DNA. Identical hybridization conditions were used for all blots and probes. Prehybridization was done at 60°C for 4hr in the presence of 0.5M Na_2HPO_4 pH7.2, 1mM EDTA and 7% SDS. Hybridization was performed in the same solution with 10^6 CPM/ml of probe at 60°C overnight, followed by extensive washes in 0.1×SSC and 0.1% SDS at 55°C. Autoradiography was done with Kodak XAR film and DuPont intensifying screens at −70°C.

Western blots

For one and two-dimensional gel electrophoresis, concentrated 120S preparations were treated with detergents as previously described, and separated proteins were blotted on PDVF membranes for antibody binding studies[10,22]. Polyclonal rabbit anti-mouse IAP *gag* antibodies, were used at a dilution of 1:1000, and proteins were subsequently detected with alkaline phosphatase labeled secondary antibodies as done previously[22]. Alkaline phosphatase colorimetric development in 2-gels was generally done overnight to bring out all potential binding spots.

RESULTS

Purified 120S CJD preparations used in the present tudy reproducibly contain ~15% of the starting titer of whole brain, and concomitantly show an approximately million fold decrement in host nucleic acids. Thus the specific purification of CJD infectious units (IU) relative to starting nucleic acids is ~100,000 fold[8], and compares favorably with previously reported purifications of 263K scrapie that start with >25 fold higher levels of brain infectivity[7]. Because final RNA levels in 120S CJD preparations are 3−5ng per gram of starting brain, and ~80% of these RNAs are less than 150 bases, conventional cloning of larger RNAs is virtually impossible without incredible expense. For example, 12 infected hamster brains (~10 gm of brain) would yield only ~4ng of larger RNAs under ideal conditions. Moreover, cDNA synthesis, a prerequisite for cloning, has an efficiency of 20−30%, and thus larger cDNAs would be maximally 1ng. For establishing a single library, previous experiments indicate a minimum of 600 hamster brains (~50ng of larger RNAs) is required[21]. Furthermore, if one were to select a subset of RNAs, such as polyadenylated species, ~6,000 hamsters would have to be sacrificed after ~120 days of incubation. Obviously this is beyond the resources of most investigators. It was important, therefore, to develop strategies that could enrich the pool of nucleic acids for efficient recovery of larger viruslike RNAs that might include CJD-specific sequences. Recovery of long IAP RNAs was used to test this strategy on 120S preparations derived from 2−10 grams of brain.

We developed sequence-independent procedures to amplify all cDNAs synthesized from RNA[21]. Defined primers were ligated to double stranded cDNAs to provide adequate handles for subsequent PCR amplifications. Using RNA from nuclease-digested 120S preparations, a heterogeneous smear of amplified cDNAs, up to 6,000 bases in length, was detectable in Southern blots[8]. Further investigation of these amplified products revealed several long retroviral sequences after hybridization with specific recombinant probes. Fig. 1 demonstrates one of these products, a band of ~6,000 bp, which was detected with probes made from a representative cloned member of the Syrian hamster IAP family. The endogenous retroviral IAP sequence is mapped for reference in Fig. 2A and the IAP probe we used covered 7533 contiguous bases of the endogenous retrovirus, a length longer than the band detected in 120S preparations. This hybridization experiment provided the first evidence that specific viral sequences of substantial length were present in our nuclease treated 120S preparations. However, as can be seen from this blot, random primed cDNA syntheses yielded only smaller PCR products. To further facilitate the unambiguous identification of longer IAP RNAs, we exploited IAP specific primers for subsequent cDNA syntheses and PCR amplifications. Amplification of shorter stretches of the IAP genome, delimited

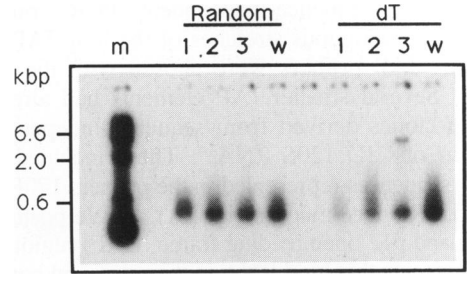

Figure 1. Sequence-independent amplification of cDNA primed with either oligo-dT or random primers, hybridized to a full length IAP insert labeled with ^{32}P. Sucrose fractions 1−3 (the peak of CJD infectivity at 120S) derived from 2 grams of starting brain, as well as a water blank are shown. One lane shows a discrete band of ~6kb. End-labeled λ HindIII restriction fragments (m) are indicated.

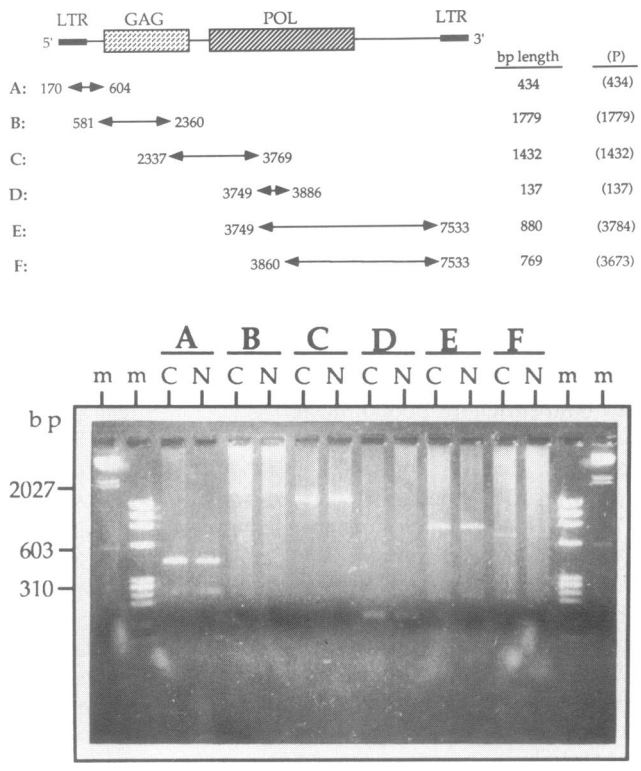

Figure 2. RNA/PCR walks of Syrian hamster IAP elements detected in CJD and normal 120S preparations (see Methods for gram equivalents of brain per reaction). **A.** diagrams the full length 7951 bp sequence[17]. LTRs and open reading frames for GAG and POL regions are indicated. Six different and overlapping PCR walks (A−F) in 120S preparations are mapped, and the length of each walk is shown for comparison to that predicted (p) for the full length genome. **B.** shows the PCR product of each walk (A−F) in CJD (C) and normal (N) preparations in a 1% agarose gel stained with ethidium bromide. Restriction fragment markers (λ HindIII and φX HaeIII) are loaded in flanking lanes (m) with relevant sizes indicated.

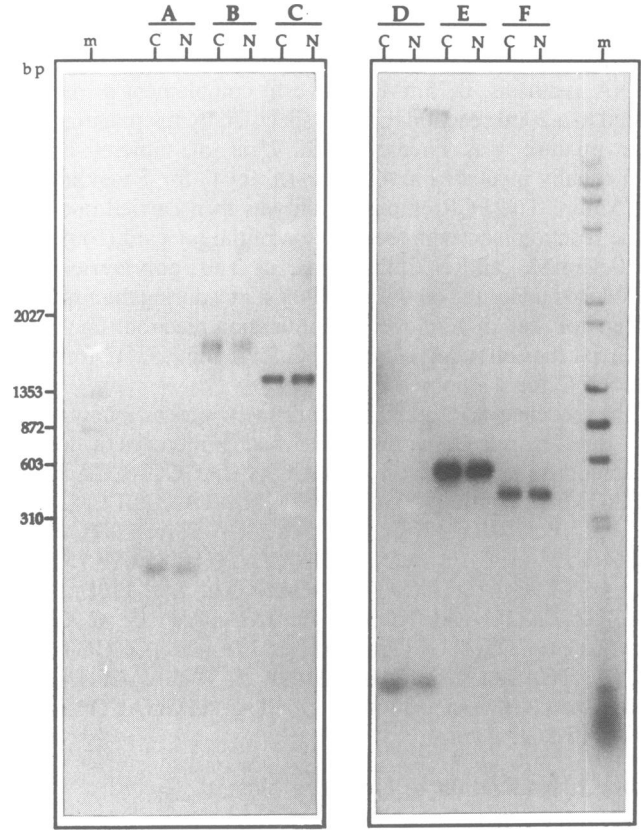

Figure 3. Southern blot hybridization showing IAP homology to RNA/PCR walks (A−F) from 120S preparations. The same CJD (C) and normal (N) products shown in Figure 2B were hybridized to the full-length IAP sequence labeled with ^{32}P. λ HindIII and φX HaeIII end labelled fragments are in lane m.

by IAP positional primers, should yield well-defined products. This approach is more rapid than conventional cloning. Additionally, these experiments could address the possibility that the 6kb band in Fig. 1 was due to some unappreciated artefact generated during sequence-independent PCR, rather than amplification of contiguous stretches of the long IAP genome.

Overlapping IAP primers were chosen with the following rationale: 1) Several smaller IAP elements had already been delineated in clones derived from sequence-independent PCR amplifications of CJD 120S RNA[14]. These included the LTR and adjacent sequences represented in the primers 170F, 640RC, 581F and 7533RC (see map in Fig. 2A). 2) We postulated that both the *gag* and p12 open reading frame (ORF) regions, known to code for proteins that bind RNA in the retroviral core, should be present in protected IAP viral complexes that cosediment with the CJD agent. Most of the *gag* region would be represented by the combination of primers 581F and 2360RC. Additionally, primer pairs 2337F and 3769RC would cover the p12 *cys−his* protein motif that binds nucleic acids, and is present in most retroviruses. 3) The reverse transcriptase domain was assessed using primers 3749F and 3886RC for a short region that is highly conserved in endogenous retroviruses[23]. The rest of reverse transcriptase, as well as potential ORFs that encode envelope (*env*) proteins was studied with overlapping primer sequences 3860F and 7533RC; the latter sequence was based on our previously cloned 3′ LTR inserts. This combination of primers moreover, could further verify that our cloned 7533RC sequence derived from a region just upstream to the 3′ LTR.

The chosen primers were initially tested on recombinant IAPs to establish conditions and starting amounts of DNA necessary for accurate PCR amplification. PCR products in CJD 120S cDNA were then compared with the predicted, and experimentally verified lengths of the 7533bp IAP example, as representatively shown in Fig. 2. A longer length would indicate transduction or insertion of extra bases that might be specific for CJD. This was not demonstrable in numerous repeated experiments. Thus transduction of CJD specific recombination with IAP RNA is unlikely. However, because PCR can often selectively amplify single homologous elements non-representatively, a recombinant CJD-IAP cannot be ruled out absolutely without exhaustive sequencing of individual elements. Although most of the products in CJD were of the same length as in the control IAP clone, those covering the more distal regions of reverse transcriptase through the 3′ LTR were clearly shorter in 120S preparations (walks E and F). Endogenous or integrated IAP family members, which number ~1,000 in both the Syrian hamster and mouse genomes[24], are often truncated in this

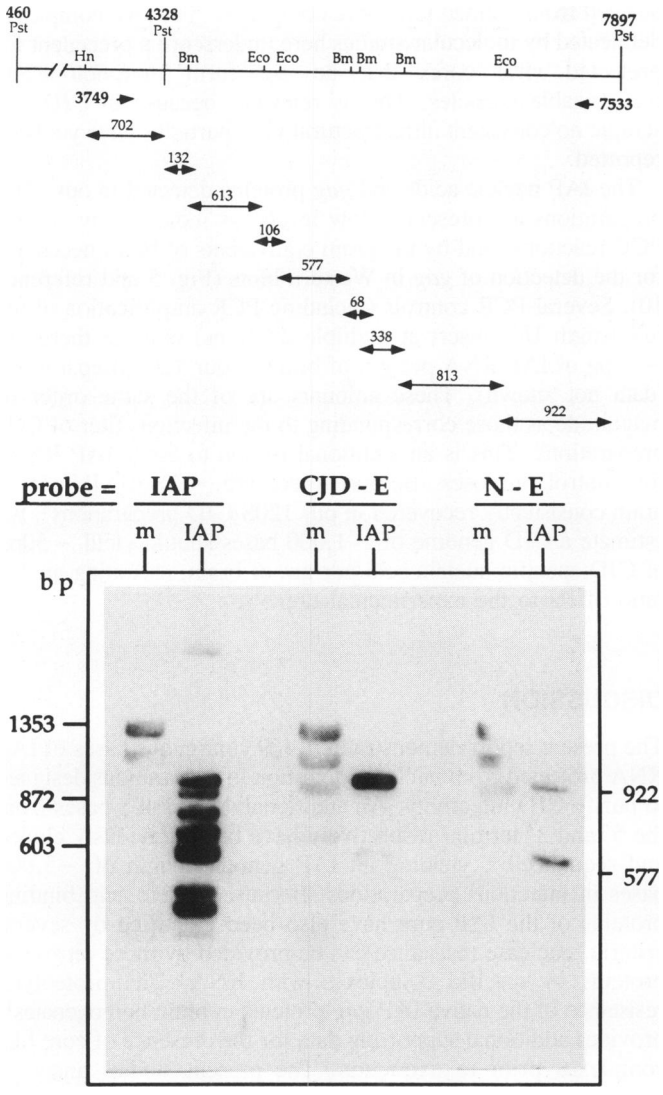

Figure 4. Analysis of the 3′ end of IAP sequences in 120S preparations. **A.** shows a restriction map of the 3′ half of the IAP reference genome containing a 3,569bp PstI fragment with internal BamHI (Bm) and EcoRI (Eco) sites localized. Predicted fragment sizes are indicated. **B.** shows the reference IAP genome, cleaved with EcoRI and BamHI, probed with itself (IAP), and with the 880bp product from walk E, derived either from CJD (C) or control (N) RNA. Only the 577bp (BamHI−BamHI) and the 922bp (EcoRI−PstI) regions are detected with probes from the 120S preparations. Fragments were resolved in 1.5% agarose, with end-labelled ϕX HaeIII markers (m) shown.

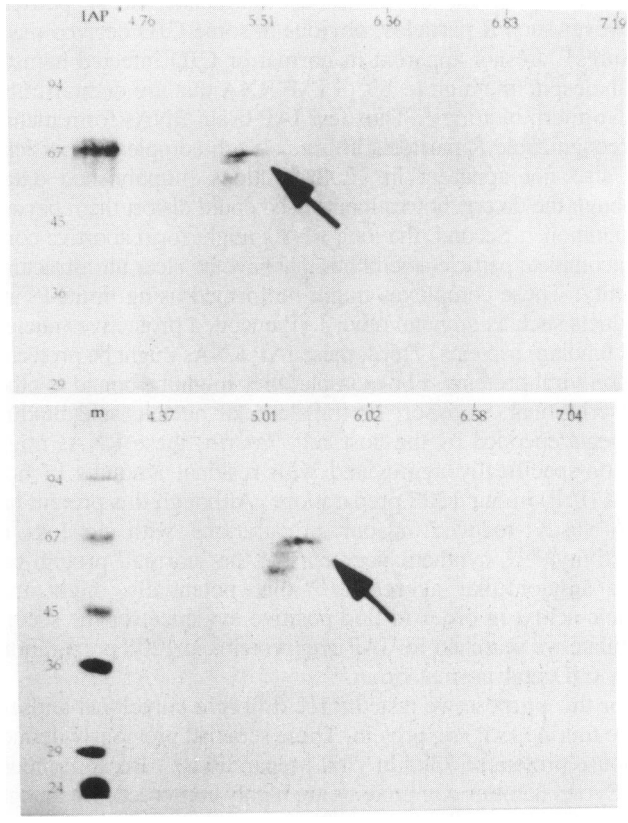

Figure 5. Western blot of 120S proteins resolved by 1 and 2-D electrophoresis using an IAP *gag* antibody. The top left (1-D) lane shows the ~65kd band, with only one other very minor small protein, detected with IAP antibody. Both 2-D blots (1.5gm equivalents per gel loaded) show a comparable major ~65kd spot at a pI of ~5.3 (arrows) in both normal (top) and CJD (bottom) preparations. The bottom blot, previously exposed to ^{32}P labeled nucleic acids, showed positive binding to the same 65kd spot[10]. More basic as well as lower molecular weight regions showed no signal and are omitted for photographic clarity. Second dimension size markers indicated are shown in lane m. First dimension pI determinations were done as previously described[10].

region[15]. We considered the possibility that selected IAP members might be transcribed preferentially and/or copurify with CJD infectivity. We therefore compared 3′ truncations in normal and CJD 120S preparations. The same length PCR products were seen in both (Fig. 2B). Thus these truncated IAP RNAs do not have any specificity for CJD infection.

The homology of IAP with the PCR products from CJD and normal 120S preparations was confirmed by Southern hybridization. Fig. 3 shows PCR walks A−F using the complete 7533bp IAP sequence as a probe. We further explored the more 3′ elements amplified from CJD and normal preparations. Restriction enzyme mapping allowed us to define the truncated IAP regions with greater precision. First the 3′ half of the reference IAP clone was purified, digested with appropriate restriction enzymes, and hybridized to itself to define maps of this element. The placement and length of these fragments are summarized in Fig. 4A which details a PstI fragment that contains the entire *env* region between base pairs 4328 and 7897. We then used PCR products of walk E from CJD and normal 120S preparations to probe the restriction digests of the IAP recombinant clone. These hybridization fragments were localized to the map as shown in Fig. 4. Only the 577 and 922bp fragments of the IAP recombinant were detectable with either the CJD or normal probe E. Thus in both CJD and normal 120S preparations the *env* region of IAP is almost completely deleted. Interestingly, the lack of an IAP envelope protein coding region confines endogenous IAP viruses to internal cellular compartments because they are defective for *env*-mediated budding and exit from the cell[15]. This attribute may explain why these nuclease resistant assemblies cosediment with CJD, which is also known to be cell associated.

Several types of complexes can be envisaged for the nuclease resistant, cosedimenting IAP RNAs shown above. First, the long IAP RNA may form a morphological 'R particle', the Syrian

hamster equivalent of the mouse intracisternal A particle. However such R particles, obvious in some CJD derived tissue cultures[25] are not apparent in normal or CJD infected hamster brain despite the high levels of IAP RNA that are demonstrable by Northern blotting[11]. Thus few IAP brain RNAs form mature or recognizable R particles in brain. Such complete R particles are also not apparent in 120S fractions (unpublished data), although the detergent treatments used could distort them beyond recognition[11]. Second, the long RNAs might form abortive core, or incomplete particle assemblies that have no clear ultrastructural identity. These complexes might be formed using limited viral products such as *gag* and other IAP encoded protective (nucleic acid binding) proteins. Third, these IAP RNAs might be protected by non-viral proteins. For example, they might be bound to other transcriptional, transport or translational nucleic acid binding elements encoded by the host cell. Fourth, these RNAs might be non-specifically aggregated with residual amounts of host Gp34 (PrP) in our 120S preparations. Although this protein has been vastly reduced in our preparations with no loss of infectivity[8,9,10], synthetic segments of the 'normal' protein can form amyloid-like aggregates[26] that potentially might trap nucleic acids. In order to find positive evidence for the second postulate we searched for IAP *gag* proteins in 120S preparations from CJD and normal brain.

For this purpose we tested three different polyclonal antisera to the murine IAP *gag* protein. These sera had previously defined a mouse protein of 73kd in viral preparations[27]. Because mouse and Syrian hamster *gag* proteins are highly conserved, we thought these antibodies might decorate the corresponding Syrian hamster *gag* protein, predicted to be ~65kd by sequence analysis. All three antibodies bound to the same ~65kd band in 1-D western blots. A representative profile for one of these is shown in Fig. 5 (left 1-D lane, top). Although two of the IAP antibodies reacted weakly with a few additional proteins (data not shown), the third antibody was able to define clearly a single 65kd band amidst the multiple proteins in whole hamster brain homogenates, i.e., it was remarkably specific[11]. Furthermore, this 65kd protein was shown to be more resistant to proteolytic digestion than prion protein in the same homogenate. This proteolytic resistance is most consistent with the presence of tight capsid–nucleic acid complexes, as only bound but not free capsid proteins of hepatitis B (one of the retroviridae) are similarly resistant to proteolysis[11,28].

We also analyzed the characteristics of this putative *gag* protein in 2-D protein blots of our 120S preparations. A protein of the same size showed an acidic charge (pI of ~5.3) as would be expected for the IAP *gag* protein. Fig. 5 shows this predominant protein in both normal and CJD 120S preparations. The 2-D spots detected by the IAP antibody were comparable in both preparations, although the second minor ~55kd spot (also at a pI of 5.3) was more prominent in CJD, possibly due to increased breakdown or slight discrepancies in protein loads. The major 65kd spot detected here has been previously identified by its positive ability to bind nucleic acids[10], another characteristic of *gag* proteins that gives further credence to the IAP *gag* identity of this spot. Finally, the amount of *gag* protein (estimated to be at ng levels per gm of brain) is in reasonable accord with the levels expected for the pg levels of IAP RNA detected here (vide infra). The cosedimentation of the IAP RNAs and their cognate core protein, as well the proteolytic studies indicating tight *gag*–nucleic acid complexes, lead us to conclude that the long IAP RNAs that we have extracted are largely if not entirely derived from defined viral core complexes. The IAP complexes delineated by molecular studies here underscore a precedent for protected viral cores that do not form morphologically recognizable particles. This is relevant, because in CJD and scrapie no consistent ultrastructural viral particles have yet been reported.

The IAP nucleic acids and *gag* proteins detected in our 120S preparations are present at low levels, as indicated by control PCR reactions, and by the gram equivalents of brain necessary for the detection of *gag* in Western blots (Fig. 5 and reference 10). Several PCR controls (including PCR amplification of the full length IAP insert at multiple dilutions) indicate there are ~50pg of IAP RNA per gm of brain in our 120S preparations (data not shown). These amounts are of the same order of magnitude as those corresponding to the infectious titer of CJD preparations. This is an additional reason to assay IAP RNAs for control purposes. Because there are $\sim 2 \times 10^7$ IU/gm of brain consistently recovered in our 120S CJD preparations[8], we estimate a CJD genome of ~1,000 bases should yield ~50pg of CJD-specific nucleic acid per gm of brain, assuming an 1:1 ratio of IU to the experimental titer[8,12].

DISCUSSION

The present report demonstrates 4,459 contiguous bases of IAP RNA protected from nuclease digestion in preparations designed to purify CJD infectivity. An additional 45 and 408 bases from the 5′ and 3′ termini respectively have been previously cloned and sequenced[14], yielding an IAP genome length of ~5,000 bases in infectious preparations. Because nucleic acid binding proteins of the IAP core have also been identified by several criteria, nuclease resistance can be provided by these retroviral proteins in specific complexes with RNA. The proteolytic resistance of the native IAP *gag* proteins in brain homogenates[11] provides additional supporting data for the presence of core like complexes prior to extraction. The present studies analyzed segmental, but overlapping portions of the IAP RNA genome, and sequences as long as 1,779 bases were retrieved. Additionally, almost full length IAP sequences of ~6kb appear to be present. Thus indirect assumptions about a lack of long viral nucleic acids in CJD (and less purified scrapie) preparations are unwarranted, and certainly cannot be used to validate the prion hypothesis.

The idea that scrapie is caused by a self-replicating protein[29], or a lipid–protein (membrane) complex devoid of nucleic acid[30], was proposed in 1967, with the implication of an independent protein world. Although this concept is intriguing, it may not be exemplified by the CJD and scrapie agents. Despite intensive attempts over the last 10 years to demonstrate an infectious protein, there is still no direct evidence for the prion hypothesis in scrapie. Recombinant prion proteins, however manipulated, have failed to show infectivity, and our high titer 120S preparations are devoid of PrP-res[19], the presumed 'infectious form' of this host protein. In accord with our original interpretation that PrP is involved in disease expression, more recent transgenic and other studies demonstrate pathology can be evoked by PrP without a transmissible agent[11,31,32]. Additionally, infectivity has been recovered from knockout mice challenged with unheated brain homogenates[33], and thus our suggestion that this host protein is a viral receptor[2] cannnot be dismissed.

On the other hand, no agent-specific nucleic acid has yet been demonstrable. However, there have been few sophisticated or systematic nucleic acid studies in this field. Even in very recent scrapie studies now acknowledging higher levels of nucleic acid, failed to use sensitive ^{32}P detection techniques or newer molecular amplification and cloning methods[4,34], and anlayses were performed in a setting where RNA would be degraded. We believe the primers and methods described here should help other investigators to pursue their own analyses of nucleic acids in a controlled and reproducible setting. They can readily verify the recovery of tightly sequestered IAP nucleic acids, assess the sensitivity of their detection techniques, and have confidence that the cDNAs they synthesize derive from a reasonable proportion of full-length RNA in an infectious preparation. Indeed, we now routinely use the primers described to assess rapidly the quality of our RNA, and the fidelity of our extractions and recoveries.

In the present study we found no evidence for transduction of a CJD specific sequence within the IAP RNA, although exhaustive experiments are required to rule out this possibility in absolute terms. If IAPs are involved in the life cycle and/or protection of CJD nucleic acids, it is more likely that a CJD-specific sequence is either co-packaged within IAP cores, or utilizes IAP products such as *gag* and reverse transcriptase for protection and replication. The demonstration of *gag* proteins as well as the reverse transcriptase encoding domain makes this a viable scenario. Co-packaging is also possible because IAPs have been shown to coencapsidate unrelated sequences[35]. Nevertheless, the current data can also reasonably suggest a completely independent CJD viral complex with at least some physical and molecular attributes that are comparable to those of IAP. Several additional *gag*-like proteins with *no* detectable affinity for the IAP antibody, but showing similar isoelectric points and nucleic acid binding characteristics common to *gag* proteins, have been identified in our 120S preparations[10]. Because some of these binding proteins are not apparent in normal 120S fractions, viral complexes unrelated to IAP, including those that may be CJD-specific, are probably present in our 120S purified preparations.

Our retroviral working hypothesis, initially based on several biological and physical properties shared by both CJD and retroviridae[11,12], led us to examine a broad group of retroviral elements. Notably the IAP LTR is only one of several LTR-like elements in purified fractions[13]. Therefore the assumption of an independent CJD complex has some foundation, and other retroviral sequences are currently being examined in more detail. Although we are not wedded to a retroviral hypothesis, we have emphasized RNA studies using a methodological approach that removes contaminating genomic DNA. Viral RNAs appear to be most relevant, given the irradiation data for both retroviruses and scrapie, and the known repair capacities of several viral RNA polymerases including reverse transcriptase (reviewed in 11). Viral RNA synthetic mechanisms, such as template switching during reverse transcription[36], can create full length cDNA from a fragmented viral genome. This established mechanism moderates conclusions predicated on the assumption that one will visualize a single discrete or full length CJD or scrapie specific viral band[34]. Aside from RNA breakdown produced by less fastidious manipulations, some viral RNAs may in fact be fragmented, or of heterogeneous size. Further improvements in capturing completely representative cDNAs that are sufficient for cloning will be most advantageous for identification of a CJD-specific sequence.

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REFERENCES

1. Manuelidis, E.E. and Manuelidis, L. (1979) Prog. Neuropath., 4, 1–26.
2. Manuelidis, L. and Manuelidis, E.E. (1989) Microb. Path., 7, 157–164.
3. Prusiner, S.B. (1991) Science, 252, 1515–1522.
4. Meyer, N., Rosenbaum, V., Schmidt, B., Gilles, K., Mirenda, C., Groth, D., Prusiner, S.B. and Riesner, D. (1991) J. Gen. Virol., 72, 1–113.
5. Aiken, J.M., Williamson, J.L., Borchardt, M. and Marsh, R.F. (1990) J. Virol., 64, 3265–3268.
6. Oesch, B., Groth, D.F., Prusiner, S.B. and Weissman, C. (1988) Ciba Found. Symp., 135, 209–233.
7. Sklaviadis, T., Akowitz, A., Manuelidis, E.E., and Manuelidis, L. (1990) Arch. Virol., 112, 215–229.
8. Akowitz, A., Sklaviadis, T., Manuelidis, E.E. and Manuelidis, L. (1990) Microb. Path., 9, 33–45.
9. Sklaviadis, T., Dreyer, R., and Manuelidis, L. (1992) Virus Res., 26, 241–254.
10. Sklaviadis, T., Akowitz, A., Manuelidis, L., and Manuelidis, E.E. (1993) Proc. Natl. Acad. Sci. USA, 90, 5713–5717.
11. Manuelidis, L. (1993) Ann. N.Y. Acad. Sci., *in press*.
12. Manuelidis, L., Murdoch, G. and Manuelidis, E.E. (1988) Ciba Found. Symp. 135, 117–134.
13. Murdoch, G., Sklaviadis, T., Manuelidis, E.E. and Manuelidis, L. (1990) J. Virol. 64, 1477–1486.
14. Akowitz, A., Manuelidis, E.E., and Manuelidis, L. (1993) Arch. Virol. 130, 301–316.
15. Kuff, E.L. and Leuders, K.K. (1988) Adv. Cancer Res., 51, 183–276.
16. Leuders, K.K. and Kuff, E. L. (1983) Nuc. Ac. Res., 11, 4391–4408.
17. Ono, M., Toh, H., Miyata, T. and Awaya, T. (1985) J. Virol., 55, 397–394.
18. Manuelidis, E.E., Gorgacz, E.J. and Manuelidis, L. (1978) Proc. Nat. Acad. Sci. USA, 75, 3432–3436.
19. Sklaviadis T., Manuelidis L., and Manuelidis, E.E. (1989) J. Virol., 63,1212–1222.
20. Manuelidis, L., Tesin, D.M., Sklaviadis, T. and Manuelidis, E.E. (1987) Proc. Natl. Acad. Sci. USA, 84, 5937–5941.
21. Akowitz, A. and Manuelidis, L. (1989) Gene, 81, 295–306.
22. Manuelidis, L., Valley, S. and Manuelidis, E.E. (1985) Proc. Nat. Acad. Sci. USA, 82, 4263–4267.
23. Shih, A., Misra, R. and Rush, M.G. (1989) J. Virol., 63, 64–75.
24. Taruscio, D. and Manuelidis, L. (1991) Chromosoma, 101, 141–156.
25. Manuelidis, E.E., Fritch, W.W., Kim, J.H. and Manuelidis, L. (1987) Proc. Natl. Acad. Sci. USA 84, 871–875.
26. Forloni, G., Angeretti, N., Chiesa, R., Monzani, E., Salmona, M., Bugiani, O. and Tagliavini, F. (1993) Nature, 362, 543–546.
27. Kuff, E.L., Callahan, R.and Howk, R.S. (1980) J. Virol., 33, 1211–1214.
28. Zhou, S., Yang, S.Q. and Standring, D.N. (1992) J. Virol., 66, 3086–3092.
29. Griffith, J.S. (1967) Nature, 215, 1043–1044.
30. Gibbons, R.A. and Hunter, G.D. (1967) Nature, 215, 1041–1043.
31. Xi, Y.G., Ingrosso, L., Ladogana, A., Masullo, C. and Pocchiari, M. (1992) Nature, 356, 598–601.
32. Diringer, H., Czub, M., Braig, H.R., Imrecke, W. and Blode, H. (1988) In Bauer, H. Klenk, H.D. and Scholtissek, C. (ed.), Modern Trends in Virology, Springer Verlag, Heidelberg, 43–48.
33. Bueler, H., Aguzzi, A., Sailer, R.A., Greiner, P., Autenried, M., Aguet, M. and Weissman, C. (1993) Cell, 73, 1339–1348.
34. Kellings, K., Meyer, N., Mirenda, C., Prusiner, S.B. and Riesner, D. (1992) J. Gen. Virol., 73,1025–1029.
35. Heidmann, O. and Heidmann, T. (1991) Cell, 64, 159–170.
36. Luo, G. and Taylor, J. (1990) J. Virol. 64, 4321–4328.