

# The kuru infectious agent is a unique geographic isolate distinct from Creutzfeldt–Jakob disease and scrapie agents

Laura Manuelidis<sup>1</sup>, Trisha Chakrabarty, Kohtaro Miyazawa, Nana-Aba Nduom, and Kaitlin Emmerling

Section of Neuropathology, Yale University Medical School, 333 Cedar Street, New Haven, CT 06510

Communicated by Sheldon Penman, Massachusetts Institute of Technology, Brookline, MA, May 27, 2009 (received for review March 27, 2009)

**Human sporadic Creutzfeldt–Jakob disease (sCJD), endemic sheep scrapie, and epidemic bovine spongiform encephalopathy (BSE) are caused by a related group of infectious agents. The new U.K. BSE agent spread to many species, including humans, and clarifying the origin, specificity, virulence, and diversity of these agents is critical, particularly because infected humans do not develop disease for many years. As with viruses, transmissible spongiform encephalopathy (TSE) agents can adapt to new species and become more virulent yet maintain fundamentally unique and stable identities. To make agent differences manifest, one must keep the host genotype constant. Many TSE agents have revealed their independent identities in normal mice. We transmitted primate kuru, a TSE once epidemic in New Guinea, to mice expressing normal and ≈8-fold higher levels of murine prion protein (PrP). High levels of murine PrP did not prevent infection but instead shortened incubation time, as would be expected for a viral receptor. Sporadic CJD and BSE agents and representative scrapie agents were clearly different from kuru in incubation time, brain neuropathology, and lymphoreticular involvement. Many TSE agents can infect monotypic cultured GT1 cells, and unlike sporadic CJD isolates, kuru rapidly and stably infected these cells. The geographic independence of the kuru agent provides additional reasons to explore causal environmental pathogens in these infectious neurodegenerative diseases.**

sporadic CJD | bovine spongiform encephalopathy | viral adaptation | prion protein receptor | tissue culture infection

**K**uru, a progressive and fatal neurodegenerative disease in New Guinea, was the first human transmissible spongiform encephalopathy (TSE) shown to be caused by a virus-like agent (1). Kuru reached local epidemic levels in the 1960s and revealed remarkable similarities to scrapie, an endemic infection of sheep (2, 3). Infection with the kuru agent was linked to ritual cannibalism and probably spread into the bloodstream from superficial wounds smeared with infected brain and from direct consumption of infected tissues (1). Kuru died out with the cessation of these rituals and hence is not created spontaneously by the human host. As with endemic scrapie (4), the infectious disease clearly requires an environmental source or external pathogen for its reproduction.

Originally, human TSE agents such as kuru and sporadic Creutzfeldt–Jakob disease (sCJD) were thought to be distinguished from sheep agents by transmission to primates but not to rodents (1). Transmission of sCJD to several rodent species in the 1970s, including normal mice, undermined this difference and opened the way for direct comparison of various TSE agents in normal mice expressing WT murine prion protein (PrP) (5, 6). Serial propagation of different TSE isolates in a foreign species demonstrated that these agents, as classical viruses, could adapt and evolve, as shown by their increased virulence and shorter incubation times on serial passage (7). Despite this adaptation, each agent maintains its distinctive identity, and reinoculation of the initial host recreates the original disease (8–11). In normal mice with WT PrP, one can reliably distinguish many different

TSE agents by (i) the incubation time to clinical disease, (ii) the distribution of pathological brain lesions, (iii) the relative involvement of the lymphoreticular system, and (iv) tissue PrP pathology; some agents provoke major PrP amyloid deposits, whereas others do not. Major differences in agent-determined incubation times (80 vs. 350 days) and extremely restricted versus widespread brain lesions are obvious in comparisons of western sCJD and geographically limited Japanese CJD isolates (12, 13). Similarly, many different sheep scrapie agents also appear to possess an independent genome with only exceptional sudden mutation (14, 15). The propagation of a variety of TSE agents in monotypic murine cultures further extends the reality of individual agent-encoded identities not determined by host PrP (16).

Standard WT mouse models are very useful for studying human TSEs because they faithfully recapitulate key features of human disease in a relatively short time frame of ≤1 year. In humans, CJD agents can exist as persistent latent infections that are clinically silent for >30 years, probably hiding in the lymphoreticular system, and brought out by later stresses, including aging itself (17, 18). Whereas normal mice with WT murine PrP have been used to reproducibly discriminate many distinct TSE agents, transgenic (Tg) mice expressing PrP sequences from other species such as cow and human have been used to evaluate only a few agents. Foreign PrPs in Tg mice are typically expressed at higher than normal levels, sometimes inducing their own pathological consequences (19). Thus, Tg PrP models are not ideal for extensive agent comparisons. Furthermore, agents such as bovine encephalopathy (BSE) spread unpredictably with respect to PrP sequences and can maintain an unanticipated species preference. This U.K. BSE agent clearly causes a variant form of CJD (vCJD) in younger people; it induces the same unique pathology in WT mice when transmitted from cow brain and from human brain (11). Whereas this unfortunate cross-species transmission was considered a reasonable possibility from the virus-like biology and experimental transmissions of TSEs, it was dismissed by proponents of the prion hypothesis (20).

According to the prion hypothesis, normal host PrP misfolds to become an infectious agent by rare spontaneous events, by inherited germ-line mutations, or by inoculation or ingestion of misfolded PrP in a tissue homogenate (21, 22). The conversion of PrP into an infectious form requires interaction with a homologous PrP. The “revolutionary” aspect of this paradigm is that the infectious agent contains no nucleic acid. Hence, host PrP must encode individual agent properties through its misfolding (assayed as digested PrP-res bands on Western blots). PrP-res is absent in uninfected samples. However, (i) samples without PrP-res, such as blood (23, 24), microglia (25), and

Author contributions: L.M. designed research; L.M., T.C., K.M., N.-A.N., and K.E. performed research; L.M., T.C., K.M., N.-A.N., and K.E. analyzed data; and L.M. wrote the paper.

The authors declare no conflict of interest.

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<sup>1</sup>To whom correspondence should be addressed. E-mail: laura.manuelidis@yale.edu.

This article contains supporting information online at [www.pnas.org/cgi/content/full/0905825106/DCSupplemental](http://www.pnas.org/cgi/content/full/0905825106/DCSupplemental).

**Table 1. Comparison of different TSE agents in mice with WT PrP**

	Country–origin (no. of species before mouse)	Diagnosis	Agent name	Mouse incubations CD-1 (Tga20) $\geq$ passage 2	Mouse brain pathology	Mouse brain PrP-res
1	U.S.–human (++)	sCJD	SY-CJD	370 (307) days	Restricted	Type 1
2	Italy–human (+)	sCJD	LU-CJD	380 (324) days	Restricted	Type 1
3	U.K.–human	GSS	MA-CJD	380 (350) days	Restricted	Type 1
4	Japan–human (+)	GSS	FU-CJD	120 (70) days	Widespread + amyloid	Type 1
5	Japan–human	GSS	YAM-CJD	130 days	Widespread + amyloid	Type 1
6	U.K.–human	vCJD	vCJD	185 (126) days	BSE pattern + amyloid	Type 2
7	U.K.–cow	BSE	BSE	160 days	BSE pattern + amyloid	Type 2
8	kuru–human (+)	kuru	kCJD	440 (154) days	kCJD specific	Type 1
9	U.K.–sheep	Scrapie	22L-sc	140 (87) days	Scrapie variant	Type 1
10	U.K.–sheep	Scrapie	Ch (RML)-sc	120 days	Scrapie variant	Type 1
11	U.K.–sheep (++++)	Scrapie	263K-Sc	330 (310) days	263K specific	Type 1

Key features of transmissible spongiform encephalopathy (TSE) agents propagated in mice with country of origin, original species, and disease diagnosis. The agent name indicates the host from which it was isolated, e.g., CJD for human. Sporadic Creutzfeldt–Jakob disease (sCJD) causes a rapidly progressive dementia globally in older people. In Gerstmann–Sträussler–Sheinker disease (GSS), patients with a 102L prion protein (PrP) mutation have symptoms for  $\approx$ 5 years with cerebellar PrP amyloid plaques. A plus sign (+) indicates prior nonmurine species passages. SY-CJD was first passaged in guinea pigs and hamsters (++) (6). LU-CJD was passaged in hamsters (+), whereas MA-CJD brain homogenate was inoculated directly in mice. The Japanese GSS-102L PrP samples yield short mouse incubations and widespread brain lesions with the same PrP-res band pattern as sCJD (11, 12), and FU-CJD was first passaged in rats. The human variant CJD (vCJD) and cow bovine spongiform encephalopathy (BSE) isolates produce type 2 PrP-res (Fig. 2) and a unique BSE lesion profile (11). Mutant 263K-sc was cloned at limiting dilution in 5 different species and then again selected for low mouse pathogenicity [ $>$ 600 versus 65 days in hamsters (15)]. When retransmitted to mice, its biological properties were changed profoundly, but PrP-res remained type 1 (11). All mice were inoculated with 1% brain homogenates as detailed in refs. 5 and 6 and *SI Methods*.

digestive material (26) can transmit infection, (ii) very different agents show indistinguishable PrP-res band profiles, and (iii) PrP-res band patterns vary with tissue type while the agent remains invariant (10). Dramatically altering the PrP-res pattern by cell culture also does not alter an agent's distinctive identity because it reproduces the original disease in mice (9). Although host PrP is required for infection and probably acts as a receptor or scaffold for the infectious agent (27), it is unlikely to be the agent itself. The amount of PrP-res does not correlate with infectivity levels in many in vivo TSE models, and infectious particles can be isolated from most PrP and PrP-res without loss of titer (17, 28, 29). Most problematic for prion proponents is the lack of any significant or reproducible infectivity in purified, recombinant, or misfolded amyloid forms of PrP (10).

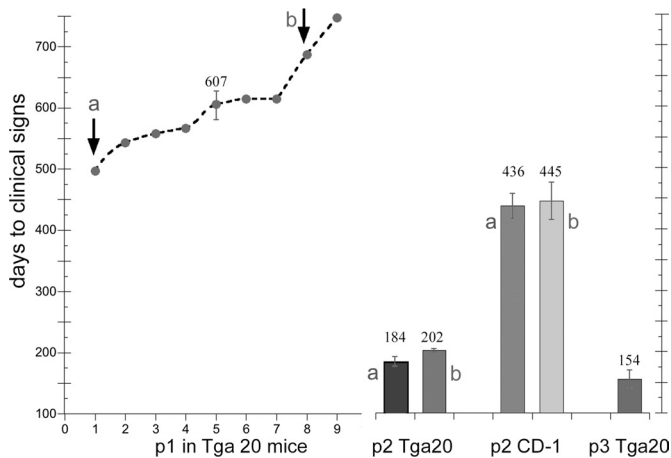
The key prion assumption that only a highly similar PrP sequence between the donor and the recipient will provide fertile ground for infection, or PrP conversion, has fueled many studies of Tg mice with PrP inserts that correspond to the donor. This concept has not been borne out experimentally [e.g., the vCJD agent that replicated in a human brain for  $>$ 5 years transmitted readily to normal mice but poorly to Tg mice expressing only human PrPs (11)]. Kuru has not been transmitted to any standard small rodents, including mice. However, transmission of kuru to several Tg mouse lines expressing human PrPs but not to normal mice has been reported (22). From incubation times, the authors concluded they had “established that kuru prions have prion strain properties equivalent to those of classical (sporadic and iatrogenic) CJD prions” and further stated that kuru probably originated from chance consumption of an individual with sCJD.

Our kuru transmission and neuropathology data are not in accord with this conclusion and are based on agent-induced incubation times, PrP-res profiles, brain and spleen pathology, and species susceptibility and culture transmissions. We compare kuru to a variety of other TSE agents in normal CD-1 and in Tga20 mice that express 8-fold higher than normal levels of murine PrP. These studies show the kuru agent is a unique geographic isolate unrelated to the sCJD agent. It is also different from the BSE vCJD agent, the geographic Japanese CJD agent, and representative scrapie agents derived from sheep and goats. Furthermore, kuru infected monotypic GT1

murine cells stably support infection by a variety of different TSE agents (11). These in vitro studies again showed that the kuru agent is different from 3 independent sCJD isolates, including one from a patient with a very rare 102L PrP mutation proposed to infect via the germ line (30). The fundamental realization that kuru represents yet another unique and geographically restricted TSE agent provides additional reasons to explore nonhost environmental pathogens as causal agents in these infectious neurodegenerative diseases.

## Results

Transmissible spongiform encephalopathy agents can differ dramatically in their virulence for species, by incubation time and by neuropathological sequelae. Because PrP sequence variants and other host factors can modulate susceptibility to infection and disease phenotype, we here compare kuru to other agents using mice and GT1 neuronal cultures expressing standard WT PrP. For simplicity, we designate all human agents according to the natural host species from which they were isolated (Table 1). This table summarizes some of the major features that distinguish various CJD and scrapie agents, including kuru (kCJD), in both CD-1 and Tga20 mice. First, there are obvious differences in incubation time that persist after serial passages. Notably, the same prolonged incubation time with limited brain lesions is generated by 3 different sCJD isolates with diverse species passage histories regardless of passage number (passages 2–8). In contrast, kCJD produced severe clinical signs and neuropathological lesions at short times (154 vs.  $>$ 300 days). Mice with sCJD also develop a unique stereotypic scratching syndrome, whereas kCJD and other agents elicited very different behaviors (*Movies S1–S4*). Kuru and sCJD agents also provoke markedly different patterns of neurodegeneration in CD-1 mice, as summarized in Table 1. These incubation and neuropathological differences were not apparent from the PrP-res band sizes (both type 1). In addition, human vCJD and BSE from cow brain showed no clear relation to any other CJD or scrapie agents by either incubation time or neuropathology. Unlike any of the other agents, BSE and vCJD isolates both provoke a diagnostic 19-kDa PrP-res band. This diagnostic band was remarkable



**Fig. 1.** Transmission of kuru Creutzfeldt-Jakob disease (kCJD) to mice. Graph shows days of incubation in the 9 intracerebrally inoculated Tga20 mice at passage 1 (p1) [607 ± 26 (SEM)]. Brain homogenates from passage 1 mice a and b with an ≈200-day difference in incubation time then were inoculated into both Tga20 and CD-1 mice. Brains a and b yield the same incubation time. Reductions in passage 2 (p2) incubations indicate agent adaptation in both mouse genotypes, with Tga20 mice showing a greater reduction. By passage 3 (p3), Tga20 mice provide a rapid kCJD model.

because it is present in many different infected species with nonhomologous PrPs (cow, human, other primates, and mouse). Thus, the vCJD agent induces a PrP response that is not host-encoded but probably results from agent-specific binding to the host PrP receptor as diagrammed in ref. 27.

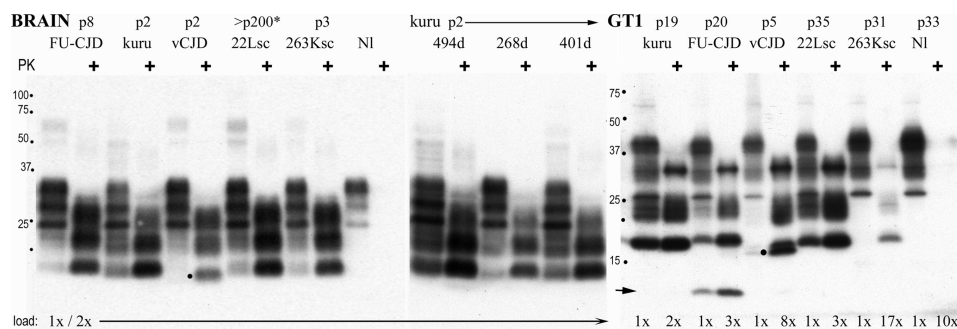
**Sequential Incubation Time Changes with kCJD.** Fig. 1 details serial passages of kCJD from a macaque brain homogenate (31) to Tga20 mice overexpressing WT murine PrP. All inoculated mice developed clinical symptoms and brain disease. As expected, the primary passage across species is very prolonged but completely effective. These data contrast with the lack of transmission to normal FVB mice with clinical signs scored for a shorter time (22). Two representative kCJD mouse brains at passage 1, including one with the shortest and another with a prolonged incubation (Fig. 1, a and b, respectively), were used

for passage 2 infection of Tga20 and CD-1 mice. Both samples yielded significantly reduced incubation times at passage 2 ( $t < 0.0001$ ). The Tga20 incubation times were cut by two-thirds.

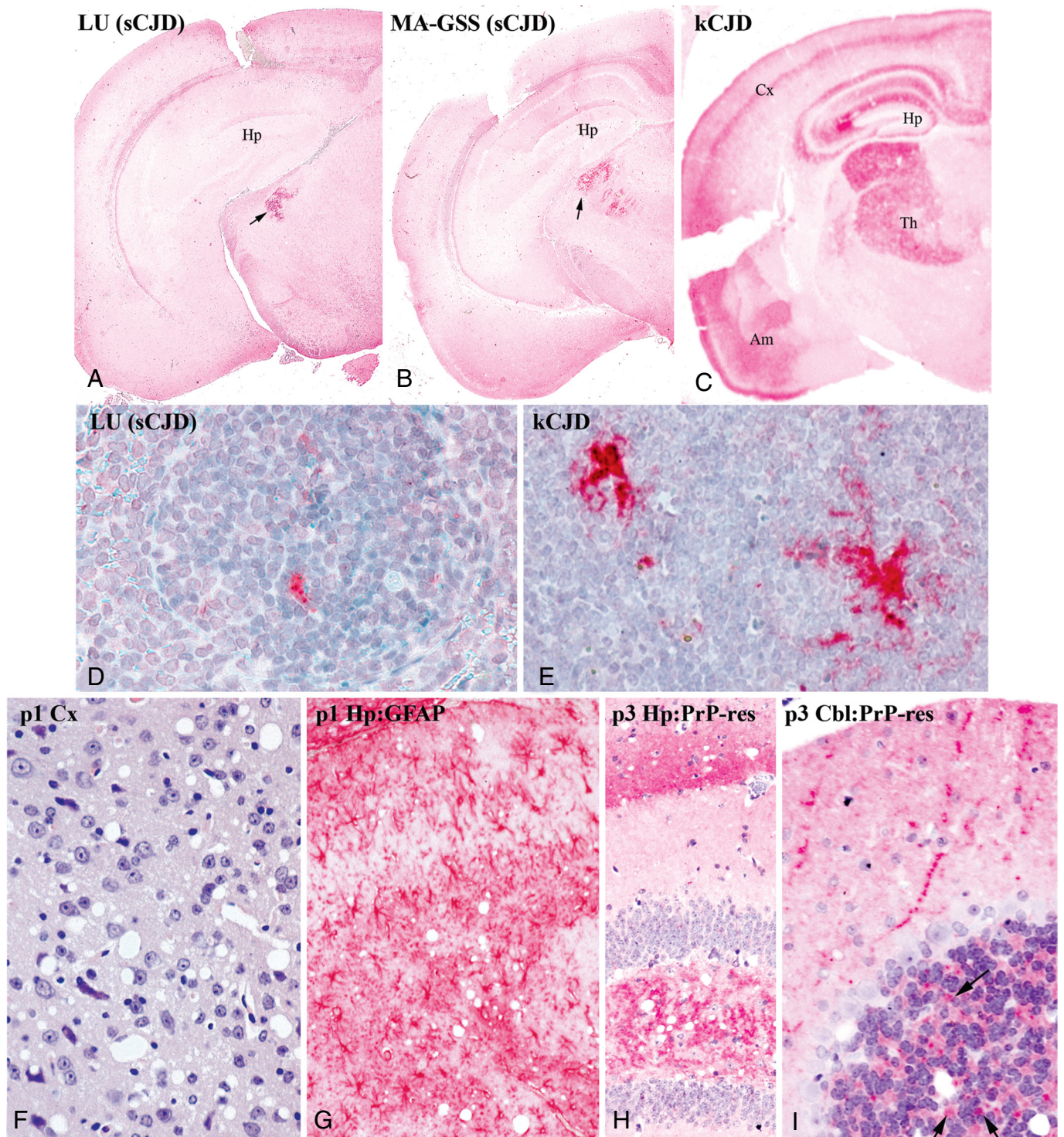
The much longer incubation times in CD-1 versus Tga20 mice at passage 2 are likely due to the relatively lower expression of murine PrP in CD-1 mice, as would be expected for a viral receptor. Nevertheless, both groups of recipient CD-1 mice at passage 2 (Fig. 1) showed a significantly shorter incubation time than passage 1 Tga20 mice (440 vs. 607 days), indicating adaptation or selection of the foreign agent. At passage 2, there was a >100-day difference between kCJD and sCJD in Tga20 mice (195 vs. 335 days, Table 1). Because Tga20 mice are highly inbred, the kCJD agent rather than the invariant murine genes must encode this dramatic and progressive reduction. A third passage of kCJD in Tga20 mice decreased the incubation time to 154 days, far below that seen even after 8 murine passages of the sporadic SY-CJD isolate (>300 days) and, notably, 50 days shorter than that of kuru in humanized PrP mice (22).

**PrP-res Profiles in Brain.** The PrP and PrP-res profiles in kCJD remained the same in serial passages despite the agent's adaptation to its new murine host. There was no evidence that host PrP-res folding encoded this progressively enhanced agent virulence. Fig. 2 shows that Japanese FU-CJD, 22L-sc, and the cloned mutant 263K-sc agents all provoke the same basic type 1 PrP-res profiles as kCJD in brain. The only obvious difference was a greater proteinase K sensitivity of the most glycosylated PrP band (at 27 kDa) in kCJD. This profile was unchanged by propagation in various normal inbred, outbred, and Tga20 mice. The SY-CJD agent also induces the same basic PrP-res brain profile (32). Only the vCJD agent reveals a clearly different diagnostic PrP-res band of 19 kDa (at dot). Fig. 2 also shows representative samples encompassing the entire span of kCJD incubation times and clinical durations in CD-1 mice. Only minor differences in PrP-res amounts, as would be expected for animals killed at varying stages of clinical disease, are seen. In contrast, pathological PrP-res amounts in brain can differ 10-fold by agent type, as shown for SY-CJD and FU-CJD (32).

**Infection of Monotypic GT1 Cultures.** Because we found that GT1 tissue cultures support stable infection by a wide variety of CJD and scrapie agents (11, 16), evaluating the relative transmission



**Fig. 2.** The prion protein (PrP) and PrP-res bands [proteinase K (PK)+ lanes] in CD-1 mouse brain homogenates and in GT1 cultures assayed on Western blots (SI Methods). Only the variant Creutzfeldt-Jakob disease (vCJD) agent shows a unique ≈19-kDa band (at dot). Kuru samples show one mouse with a long (55-day) clinical course (494-day lane) with more PrP-res than mice with typical 15–20 day signs; all kuru (kCJD) mice (268- and 401-day lanes), including Tga20 mice, show the same PrP-res profile. The 22L-sc CD-1 mouse (asterisk) was inoculated with 22L-sc-infected N2a cells displaying an N2a-specific but not agent-specific PrP band pattern (9–11), yet these cells reproduced their original 22L-sc incubation time and neuropathology (9–11) and the type I brain PrP pattern in mice (22L-sc lane). Protein loads and mouse passages are noted. The GT1 lanes show GT1-cell-specific PrP and PrP-res patterns (e.g., the heavily glycosylated top band in undigested brain homogenates is at 34 kDa, whereas it is at 39 kDa in GT1 homogenates). Kuru brain homogenates rapidly, reproducibly, and stably infected GT1 cells, as shown by the high levels of PrP-res (kCJD lane, >4 experiments), whereas sCJD gave only subliminal infection with the same homogenate exposure (SI Methods). The 13-kDa PrP-res marker in GT1 cells (arrow) is diagnostic for Japanese isolates in GT1 cells but not brain (FU-CJD lanes), but the 19-kDa doublet, found only in vCJD brain infections (at dot), is again seen in GT1 cultures. The band profile in kCJD is comparable with those of 22L-sc and 263K-sc in GT1 cells but required much higher amounts of protein to be loaded to each lane, as noted. Uninfected lanes (NI) show no PrP-res.



**Fig. 3.** Sporadic Creutzfeldt–Jakob disease (sCJD) and kuru (kCJD) tissue differences. The first row (A–C) shows low-magnification images of brain from passage 2 CD-1 mice with sCJD versus those with kCJD. All mice became sick at similar times (377, 384, and 403 days) yet still show that specific neuronal nuclei are affected. Both sCJD isolates (LU and MA) induce pathological prion protein (PrP) in the medial thalamus (dark red at arrow) like SY-CJD (12). Kuru infection (C) provokes extensive PrP deposits in a much larger thalamic region (Th) and in hippocampus (Hp), a band of cortical gray (Cx), and temporal lobe/Ammon's horn (Am). Profound differences in spleen PrP are seen with sCJD (D) versus kCJD (E) in passage 2 CD-1 mice. The third row (F–I) shows kCJD brains in Tga20 mice with spongiform lesions in temporal cortex at passage 1 (F), intense gliosis with red-stained astrocytes (G), and abnormal PrP deposits in hippocampus at passage 3 (H). Cerebellum (I) shows pathological PrP deposits consistent with degenerating synaptic boutons (arrows) that become more prominent with further digestion (SI Methods) (i.e., they contain PrP amyloid).

capability of kCJD in GT1 cells was advantageous. Sporadic SY-CJD barely elicits detectable PrP-res and displays this pathological marker only transiently (9). Sporadic LU-CJD and MA-

CJD also failed to elicit PrP-res. In marked contrast, kCJD readily infected GT1 cells when tested with 3 different kCJD brain homogenates, including CD-1 and Tga20 samples from



tious particle rather than the host's PrP sequence defines cross-species virulence. The sCJD agent is also relatively incapable of adaptive evolution in mice. Even after 8 passages, it has a very prolonged incubation time of >300 days, in contrast to kCJD and vCJD. Current and previous data do not support a conversion of homologous PrP to an infectious form, but are in accord with the proposal that host PrP is a required agent receptor that can modulate disease expression. Host receptor differences often modulate viral infection and disease progression. The kCJD and other TSE agents here maintain their fundamental identity, despite their adaptation to a new species and to monotypic cells with disparate cell-type-specific PrP bands.

The PrP-res band patterns often are presumed to encode agent-specific information, yet they are a relatively poor indicator of the biological diversity and stability of TSE agents. In the present studies, only the vCJD agent elicited a consistent PrP-res marker in different species and cell types that bred true. Eleven other independent isolates did not (11, 32). In contrast, incubation times are clearly distinctive for each agent group and nonoverlapping when both CD-1 and Tga20 values are considered. The extreme precision of incubation time with each type of agent is really quite remarkable and remains unexplained by prion theory. It also begs the question of what type of viral (nucleic acid) sequence could lead to this diversity, and this further underscores both complex and subversive interactions between these TSE agents and their hosts. The differential susceptibility of neuronal subtypes with the same PrP to the various TSE agents presents yet another conundrum for prion theory.

There are many aspects of TSE agent biology and structure that remain unresolved, but these agents clearly encode individual virus-like properties. None of these diverse biological properties can be explained parsimoniously by the prion hypothesis or by actual PrP observations. The many distinct geographic TSE agents, the local outbreak of epidemic kuru and BSE, the rare but sudden mutation or progressive selec-

tion of new virulent strains (14, 15), and the endemic perpetuation of infection only in exposed hosts (as in sheep scrapie) strongly implicate a viral structure in the environment (10). On a structural level, the presence of nucleic acid in all infectious preparations and the consistent observation of virus-like ultrastructural particles support this hypothesis. Although host PrP as well as other factors may modulate susceptibility to infection and disease phenotype, there is also no simple genetic inheritance pattern, nor evidence for a spontaneous conversion of PrP into an infectious form.

The clear distinction of kuru from western sCJD, its occurrence only in New Guinea, and the observation that Japanese CJD is limited to Asia make one suspect that additional geographic TSE isolates in the environment may be uncovered. The U.S. stopped its scrapie eradication program years ago, before comparative rodent and culture models were introduced. Since then, scrapie agents with new properties may have evolved, possibly facilitating their spread to deer in the U.S. Geographic French scrapie agents are different (31), and Europe appears to be free of U.S. cervid TSE. An understanding of the origin, relative virulence, individual properties, and molecular structure of TSE agents remains a fundamental problem and a public health issue. The distinctive biology of a wide variety of TSE agents revealed in mice and in monotypic cultures already has defined intrinsic agent properties, and simplified cultures may allow one to follow and develop ways to limit the spread of different environmental TSE agents.

## Methods

Standard methods for intracerebral inoculation, Western blot analysis and immunocytochemistry were as described in refs. 6, 7, and 9. See *SI Methods* for more details.

**ACKNOWLEDGMENTS.** We thank Brian Mullins for help with the mice. This work was supported by National Institute of Neurological Disorders and Stroke Grant R01 012674 and National Institutes of Health National Institute of Allergy and Infectious Diseases Grant R21 A1076645. K.M. was supported by a Japanese JSPS fellowship.

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