and the third house with its surrounding water was examined for numbers of free-living and attached bacteria. In April 1984, one set of houses was examined periodically for changes in bacterial numbers and production, and chlorophyll *a* and ATP content. Oxygen concentration was measured with a Radiometer pO_2 electrode coupled to a PHM 71 amplifier fitted with a PHA934 oxygen module. In warmer water, an experiment of this kind would be negated by wall growth of bacteria, for example, bottle effect. The linear results in this experiment indicate that no significant bottle effect occurred. Chlorophyll *a* was measured by the method of C. S. Yentsch and D. W. Menzel [*Deep Sea Res.* **10**, 221 (1963)] as described by J. Strickland and T. R. Parsons [*Fish. Res. Board Can. Bull. 167* (1968)], using a Turner 110 Fluorometer. ATP of microorganisms was measured by the method of O. Holm-Hansen [*Limnol. Oceanogr.* **14**, 740 (1969)] as modified by D. M. Karl and O. Holm-Hansen [in *Handbook of Phycological Methods*, J. S. Cragie, Ed. (Cambridge Univ. Press, 1978), pp. 197–206], using a Science Applications, Inc., model 2000 ATP photometer. Bacteria were counted by the method of J. E. Hobbie, R. Daley, and J. P. Jasper [*Appl. Environ. Microbiol.* **33**, 1225 (1977)]. Bacterial production rates were measured by the method of J. A. Fuhrman and F. Azam (*3*). The [^{3}H-*methyl*]thymidine, 40 to 60 Ci/mmol, was obtained from New England Nuclear and counted in a Packard 300C scintillation counter.

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Functional Differences Between Two Classes of Sodium Channels in Developing Rat Skeletal Muscle

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Excitability is generated in developing skeletal muscle by the incorporation of sodium-selective ion channels into the surface membrane. Whole-cell and patch voltage-clamp recording from myotubes and their embryologic precursors, myoblasts, indicated that voltage-activated sodium current in myoblasts was more resistant to block by tetrodotoxin (TTX) than that in myotubes. Single-channel recording from both cell types showed two classes of sodium channels. One class had a lower single-channel conductance, activated at more hyperpolarized voltages, and was more resistant to TTX than the other. The proportion of TTX-resistant to TTX-sensitive sodium channels was higher in myoblasts than in myotubes. Thus, the difference in TTX sensitivity between myoblasts and myotubes can be explained by a difference in the proportion of the two classes of sodium channels. In addition, the lower conductance of TTX-resistant channels provides insight into the relationship between the TTX binding site and the external mouth of the sodium channel.

IN DEVELOPING SKELETAL MUSCLE, action potentials and the sodium currents that underlie them are initially quite insensitive to the specific sodium-channel blocker tetrodotoxin (TTX), but increase in their sensitivity to TTX as the cells mature (*1–6*). This change in TTX sensitivity with age could represent a gradual shift in the affinity of channels during development. Alternatively, if two populations of sodium channels with very different TTX affinities existed, an increase in the ratio of high-affinity to low-affinity channels could account for the results. Labeled-toxin binding and ion-flux studies support the explanation that there are two populations of sodium channels (*2, 4, 6*). However, two separate electrophysiological studies, based on direct measurement of functional sodium channels, support either the hypothesis of a gradual affinity shift (*3*) or the existence of two classes of channels (*5*). Here we show that functional forms of both TTX-sensitive and TTX-resistant channels coexist, both in early mononucleated myoblasts and in older multinucleated myotubes. The two classes of sodium channels retain their individual characteristics throughout development, while their relative proportions change. The amplitudes of the single-channel currents and the gating properties of the two classes are readily distinguishable. TTX-resistant channels carried less current at all voltages examined and could be activated at more negative membrane potentials.

The effects of TTX on macroscopic sodium currents were examined in myoblasts by whole-cell recording and in myotubes by averaging single-channel records from an outside-out patch (*7*). In both cell types the currents were activated by depolarizing steps. The addition of 312 n*M* TTX to the solution bathing a myoblast reduced the peak inward current by approximately 66 percent. (Fig. 1A). In a myotube patch, more than 95 percent of the inward current was abolished by 125 n*M* TTX (Fig. 1B), from which we calculate an equilibrium dissociation constant for toxin binding of less than 10 n*M* (assuming a single affinity binding site). In our experiments, the effect of TTX typically was greater on myotubes than on myoblasts, in agreement with previous reports (*2, 3, 6*). Other experiments showed that the shape of the dose-response relation of TTX inhibition of the sodium current in myoblasts was consistent with more than a single dissociation constant for block (*8*). At higher concentrations (>10 μ*M*) TTX reversibly abolished all inward current in both cell types; thus this current was passing through voltage-activated sodium channels.

Single-channel currents were obtained from both cell types with outside-out patches. Current records elicited by depolarizations to −40 mV revealed that single-channel currents tended to have either of two amplitudes (Fig. 2, upper panels). Large events were approximately 1.4 pA, and small events (arrowheads) were approximately 1.0 pA. Both classes of events were seen in seven out of eight patches from myotubes and in seven out of ten patches from myoblasts. In one myotube patch, only large events were seen and, in three patches from myoblasts, only small events were observed. The excised patches from myotubes typically had a higher density of functional channels than myoblasts. All single-channel events were completely blocked by 15 μ*M* TTX, which indicates that they were generated by sodium channels. Moderate concentrations of TTX (5 to 500 n*M*) reversibly reduced the

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Table 1. Comparison of sodium currents in myoblasts and myotubes. The number of experiments is in parentheses. Data are expressed as mean ± SEM. The data indicate that these parameters are indistinguishable between myoblasts and myotubes.

	Large events (μ_L) (pA)			Small events (μ_S) (pA)		
	−60 mV	−40 mV	−20 mV	−60 mV	−40 mV	−20 mV
Myoblast	1.60 ± .05 (2)	1.43 ± .03 (6)	1.17 ± .07 (2)	1.21 ± .03 (2)	1.02 ± .02 (6)	0.83 ± .01 (2)
Myotube	1.64 ± .03 (5)	1.40 ± .01 (7)	1.12 ± .03 (5)	1.20 ± .03 (5)	1.04 ± .01 (7)	0.78 ± .03 (5)

frequency of openings (Fig. 2, lower panels) and selectively eliminated the events of large amplitude; thus the larger conductance single channels are more sensitive to TTX.

Quantitative analysis of data obtained from an excised patch of myoblast membrane indicated a bimodal distribution of channel amplitudes (Fig. 3A). The data were fit by the weighted sum of two Gaussian densities that yielded mean amplitudes of the large (μ_L) and small (μ_S) events, their standard deviations (σ_L and σ_S, respectively), and a weighting factor for the fraction of large events (w_L). In the absence of TTX, w_L was 0.53. TTX (156 n*M*) markedly reduced w_L without significantly changing μ_L or μ_S (Fig. 3A, lower panel). The effects of TTX in this and other experiments were reversible. In the few experiments in which only one amplitude of channel current was detected, the distributions at −40 mV were well fit by a single Gaussian curve with a mean and standard deviation comparable to one of the two peaks in Fig. 3A. Furthermore, addition of TTX in these experiments reduced the frequency of openings without significantly changing the parameters of the distributions. TTX, at concentrations up to about 500 n*M*, had no effect on μ_L or μ_S. Therefore the toxin does not reduce the amplitude of single-channel currents but rather causes a complete block of channels of both current amplitudes. This confirms other evidence that TTX has slow blocking kinetics (*9*). The persistence of small amplitude events in the presence of moderate concentrations of TTX indicates that the TTX-resistant sodium channels in rat myoblasts and myotubes have a lower single-channel conductance than TTX-sensitive channels, and that both types of channel coexist throughout these stages of development. Although single sodium channels with two current levels have been reported previously from other cell types (*10*), their functional significance is unknown.

We measured the mean currents, μ_L and μ_S, over a range of membrane potentials (Fig. 3B). The current-voltage relationship for each type of event was fitted to a straight line by weighted linear regression. The slope conductance is 12.14 ± 1.25 pS (95 percent confidence interval) (*11*) for TTX-sensitive channels and 9.82 ± 0.62 pS for TTX-resistant channels. The single-channel amplitude of each class of sodium channel was not significantly different between myoblasts and myotubes at any voltage (Table 1). However, the absolute amplitudes and slope conductances of the two classes of sodium channels were significantly different within the voltage range we examined. In fact, there were no overlaps in individual estimates of μ_L and μ_S, even among different experiments (Fig. 3B).

We also found a difference in the gating properties between these two types of sodium channel. The proportion of large amplitude events, estimated by the parameter w_L, was significantly smaller at −60 mV than at −40 mV in each of six patches (three from myotubes and three from myoblasts) when the holding potential and prepulse voltages were held constant. If w_L were sampled at random from the same distribution at each voltage in each patch, the probability of w_L being less at −60 than at −40 mV would be 0.5 in each case. The probability of this occurring in all six patches is 0.5^6 or 0.016. Because very few events were observed at voltages more negative than −60 mV, we concluded that TTX-resistant channels begin to activate at more negative voltages than TTX-sensitive channels. The negatively shifted activation of TTX-resistant channels suggests that these channels may be important in the initiation of action potentials. Such a hypothesis has previously been proposed for a class of sodium channels in squid giant axons called "threshold channels" (*12*). Threshold channels activate at more negative potentials but, in contrast to TTX-resistant channels, they are highly sensitive to block by TTX.

It has been suggested that TTX-resistant channels appear first and are gradually supplanted by TTX-sensitive sodium channels during the development of muscle excitability in culture (*2, 4, 6*). We tested this hypothesis in 16 patches by two statistical analyses. The proportion of large amplitude events, w_L, was highly variable from patch to patch, ranging from 0 in patches with only small amplitude events, to 1.0 in a patch with only large events. w_L was estimated in the absence of TTX at −40 mV in each patch. The correlation of w_L with the age of the preparation (in hours after the

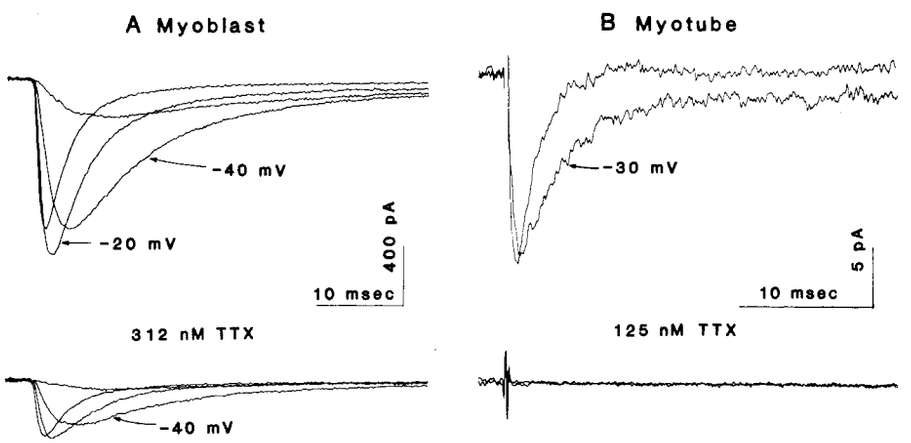

Fig. 1. Effect of TTX on macroscopic currents from a myoblast and averaged single-channel currents from a myotube. (A) Currents from a 2-day-old myoblast and (B) currents from a 6-day-old myotube in the absence (upper panels) and presence (lower panels) of TTX. The holding potential (V_H) and 100-msec prepulse (V_{PRE}) were the same in each experiment: V_H = −110 mV and V_{PRE} = −130 mV. The myoblast currents were elicited by pulses to test voltages of −60, −40, −20, and 0 mV. The myotube currents were elicited at −30 and −10 mV. In all experiments an interpulse interval of 1.5 seconds was used. Experimental procedures are described (*18*). All quantitative analysis was performed on a PDP 11/73 microcomputer (Digital Equipment Corp., Marlboro, MA). The currents were low-pass filtered at 8 and 2 kHz for myoblast and myotube, respectively. The myotube records are averages of 7 to 24 individual traces. The bath solution contained 160 m*M* NaCl, 10 m*M* Hepes, 5 m*M* glucose, 2 m*M* $CaCl_2$ (*p*H 7.3). The internal solution contained 140 m*M* CsF, 5 m*M* EGTA, 10 m*M* NaCl, 10 m*M* Hepes (*p*H 7.4). All experiments were performed at 9.5°C. Experiment numbers: (A) T47, (B) T10.

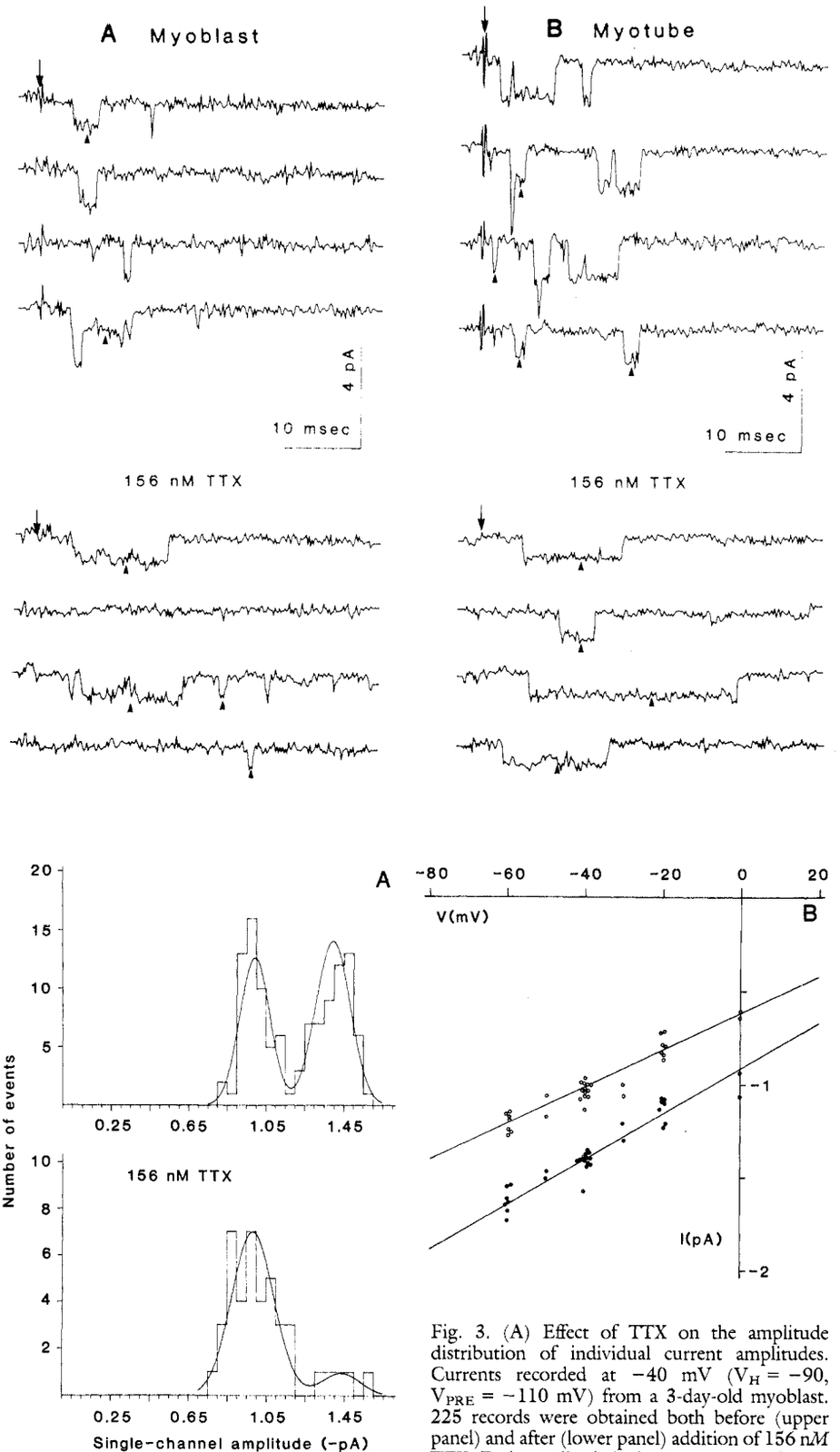

Fig. 2. Effect of TTX on single-channel currents. (A) Records from a 3-day-old myoblast and (B) records from a 6-day-old myotube in the absence (upper panels) and presence (lower panels) of 156 n*M* TTX. The test voltage was −40 mV in all records, which were low-pass filtered at 1.8 kHz. The test voltage began at the time indicated by a downward arrow. In the myoblast experiment $V_H = -110$ and $V_{PRE} = -130$ mV. For the myotube $V_H = -100$ and $V_{PRE} = -120$ mV. Both large- and small-sized currents (the latter indicated by upward arrowheads) were seen in both patches. The proportion of large events was estimated to be 0.62 and 0.86 for the myoblast and myotube, respectively, at this membrane potential. The addition of TTX reduced the frequency of all currents, and the few records with openings had predominantly small-sized currents. The estimate of the total number of channels per patch was (A) three and (B) nine. Experiment numbers: (A) T42, (B) T33.

initial plating) was determined to be significant ($P < 0.05$) by t test of Spearman's coefficient of rank correlation ($r_s = 0.482$) (*11*). We also examined the difference in w_L between myoblasts and myotubes. Seven patches were from myotubes (ages, 3 to 5 days; mean age, 3.6 days) and nine were from myoblasts (ages, 3 to 9 days; mean age, 5.3 days). The ranking of w_L from small to large was B,B,B,B,B,B,T,B,B,T,T,T,T,-B,T,T, where B and T represent the w_L values for myoblasts and myotubes, respectively. The ranking suggests that w_L tends to be smaller in myoblasts than in myotubes, and a rank-sum test shows that this trend is statistically significant ($P < 0.05$). Thus, myotubes usually have a greater proportion of large amplitude events than myoblasts and this result supports the hypothesis that TTX-resistant channels are supplanted by TTX-sensitive channels during development. Although the ratio of large to small amplitude events was typically low, we nevertheless observed large events in myoblasts as young as 3 days, the youngest cells used in our study. Therefore, functional sodium channels of both types can coexist in the plasma membrane, both prior to and after cell fusion. The coexistence of these two types of sodium channels has also been observed in denervated adult muscle (*13*), and suggests that TTX-resistant channels may play a role in certain pathologies, as well as in the development of muscle.

The smaller conductance of TTX-resistant sodium channels, which exist in developing muscle, provides insight into the relationship between the TTX receptor and the mouth of the sodium channel. It has been suggested that a negatively charged carboxyl oxygen is essential for the binding of TTX to sodium channels (*14*), in part because the *O*-methylation of such a group by trimethyloxonium (TMO) eliminates the TTX sensitiv-

Fig. 3. (A) Effect of TTX on the amplitude distribution of individual current amplitudes. Currents recorded at −40 mV ($V_H = -90$, $V_{PRE} = -110$ mV) from a 3-day-old myoblast. 225 records were obtained both before (upper panel) and after (lower panel) addition of 156 n*M* TTX. Each amplitude is the mean current from a single event (*18*). The five parameters for the double Gaussian curves were estimated by maximum likelihood from the individual amplitudes. In the absence of TTX, $\mu_S = .99 \pm .01$ (SEM) pA, $\sigma_S = .08 \pm .01$ pA, $\mu_L = 1.39 \pm .01$ pA, $\sigma_L = .09 \pm .01$ pA, and $w_L = .53 \pm .05$. In the presence of TTX, $\mu_S = .98 \pm .02$ pA, $\sigma_S = .11 \pm .01$ pA, $\mu_L = 1.43 \pm .05$ pA, $\sigma_L = .10 \pm .04$ pA, and $w_L = .12 \pm .05$. Experiment number: T35. (B) The current-voltage relationships of large- and small-sized currents. The data are combined from six myoblast and seven myotube patches. Filled and open circles represent respectively μ_L and μ_S, obtained by statistical fits. The standard error bars were usually smaller than the symbols and are not displayed. The weighted linear regression used these standard errors to produce the theoretical lines; the slopes are in the text.

ity of sodium current in both nerve and muscle (*15, 16*). TMO.treatment also causes a reduction of the single-channel conductance of sodium channels of adult nerve (*16, 17*). If the native TTX-resistant (or TMO-treated) sodium channel has a reduced affinity for sodium ions as well as for TTX, then other cations might have greater difficulty entering the channel. Consistent with this view is the observation that TMO-treated single channels are less sensitive to open-channel block by calcium ion (*17*). In fact, TTX-resistant channels are also relatively insensitive to calcium block (*8*). The similarity between TMO-treated and TTX-resistant channels suggests that, in both cases, the altered region for TTX binding is less effective at attracting sodium and calcium ions.

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Human Prion Protein cDNA: Molecular Cloning, Chromosomal Mapping, and Biological Implications

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A human complementary DNA whose protein product is considered to be the major component of scrapie-associated fibrils in Creutzfeldt-Jakob disease, kuru, and Gerstmann-Straussler syndrome has been identified and characterized. The extensive homology of this gene sequence to the hamster PrP 27- to 30-kilodalton prion protein complementary DNA clone, and its existence as a single copy in the human genome, leads to the conclusion that this is the human prion gene. This human prion gene has been mapped to human chromosome 20, negating a direct link between the prion protein and Down's syndrome or the amyloid of Alzheimer's disease.

SCRAPIE IN SHEEP AND GOATS, AND Creutzfeldt-Jakob disease (CJD), kuru, and Gerstmann-Straussler syndrome in humans, have been proposed to represent a group of degenerative encephalopathies caused by a small proteinaceous infectious particle called the prion that is resistant to inactivation by nucleic acid–modifying procedures (*1, 2*). A major protein component of the prion cosediments with hamster scrapie infectivity. This 27- to 30-kilodalton (kD) sialoglycoprotein, designated the prion protein (PrP 27–30), aggregates extracellularly into rodlike structures that resemble amyloid (*2–9*). It has been suggested that this PrP 27–30 component represents an accumulated or altered pathological marker protein that is not an integral part of the infectious agent (*10, 11*). Recently, a PrP 27–30–specific complementary DNA (cDNA) was cloned from scrapie-infected hamster brain (*12*). In addition, a scrapie prion protein–specific messenger RNA (mRNA) was identified in both scrapie-infected and uninfected brain (*13*). To investigate the human counterpart of hamster PrP 27–30 and its possible role in human disease, we isolated homologous cDNA clones, deduced the primary structure of the protein, and mapped the chromosomal location of the gene.

About 100 rat and human clones cross-reacted with two different synthetic oligonucleotide probes (legend to Fig. 1A); if no selection was introduced during cDNA cloning, this represents an mRNA abundance of 0.02% and 0.018% in human retina and rat brain tissues, respectively. Northern blot analysis showed that PrP mRNA is also found in placenta, kidney, vipoma, and neurofibroma. All ten randomly selected positive human clones revealed identical restriction patterns after single and combined digestions with Eco RI, Sac I, and Xba I endonucleases. The three overlapping λ clones with the largest inserts (1.8, 2.4, and 1.6 kb) were subcloned into M13 and the inserts sequenced (Fig. 1).

The cloned and sequenced human PrP 27–30 cDNA contains 2430 nucleotides, including eight poly(A) tail bases (Fig. 2). We translated each human and hamster cDNA in all three possible reading frames and compared all sets of resulting polypeptides. Only one translational reading frame (Fig. 2) of human and hamster cDNA did not contain premature termination and was consistent with the hamster polypeptide sequence. The human PrP cDNA, which has more 5′ sequences than the published hamster sequence, has two methionine codons in the additional 5′ region. We favor the second ATG codon at nucleotide position 84 as the translational start (Fig. 2) because it is

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