Cell-Specific Expression of High Levels of Human S100 β in Transgenic Mouse Brain Is Dependent on Gene Dosage

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The β -subunit of S100 protein (S100 β) is highly conserved in the mammalian brain. The gene coding for human S100 β has been mapped to chromosome 21. In order to study the consequences of overexpression of the S100 β gene, transgenic mice were generated by microinjection of a 17.3 kilobase human genomic fragment containing the three exons and the transcription control elements of the human S100 β gene. Mice from four transgenic lines carried approximately 10–100 transgene copies. Northern blotting demonstrated a tissue-specific and gene dose-dependent expression of human S100 β mRNA in mouse brain. Increased expression of S100 β mRNA was correlated with an increased production of S100 β protein. Examination of brain sections by *in situ* hybridization and immunocytochemistry indicated that S100 β was localized globally to astrocytes, as well as to discrete neurons in the mesencephalic and motor trigeminal, facial, and lemniscus nuclei in both normal and transgenic mice. In peripheral tissues, human S100 β was expressed at 10–50-fold lower levels than in brain. The strict gene dosage dependence and cell specificity of transgene expression suggest the presence of a locus control region (LCR) in the human S100 β gene. The mice tolerated 10–100-fold higher than normal levels of S100 β gene expression in brain without any gross physical or behavioral abnormalities. The high-level expression and cell specificity of the S100 β promoter/LCR suggest that it may provide a valuable tool to direct the expression of other transgenic products to specific cell types in the CNS.

S100 protein is a 20 kDa calcium-binding protein widely distributed and conserved in the nervous system of vertebrates (Moore, 1965; Kessler et al., 1968; Calissano et al., 1969; Marks et al., 1983; Kuwano et al., 1984). It is a hetero- or homodimer of two distinct, but similar subunits, S100 α and S100 β , that have different tissue distributions (Isobe and Okuyama, 1978, 1981; Takahashi et al., 1984). The $\beta\beta$ -form of S100 protein predominates in the mammalian brain, where it is found principally in the cytoplasm of astrocytes (Haan et al., 1982; Kato and Kimura, 1985; Zimmer and Van Eldik, 1987). The presence of the protein in other locations in the brain is a subject of controversy. Early studies have reported the presence of S100 protein in the nucleus of specific brainstem neurons (Hyden and McEwen, 1966; Sviridov et al., 1972). Later studies have disputed a neuronal location for the protein (Matus and Mughal, 1975; Ghandour et al., 1981; Haan et al., 1982).

S100 protein accumulates during the maturation of the mammalian brain (Zuckerman et al., 1970; Cicero et al., 1972; Landry et al., 1989), and participates in several calcium-dependent interactions with other brain proteins (Patel and Marangos, 1972; Zimmer and Van Eldik, 1986; Baudier and Cole, 1988). Although its localization is primarily cytoplasmic, S100 protein has been also detected extracellularly in the CNS, suggesting that it may exert a paracrine or autocrine function following secretion from glial cells (Shashoua et al., 1984). S100 β has been shown to stimulate growth in glia (Selinfreund et al., 1991) and neurite extension in neurons (Kligman and Marshak, 1985; Kligman and Hsieh, 1987; Van Eldik et al., 1988; Winningham-Major et al., 1989), *in vitro*. The above results have led to the suggestion that S100 β may influence the development of the CNS by stimulating the proliferation of glia and differentiation of neurons (Marks and Allore, 1990; Selinfreund et al., 1991).

We have recently cloned and characterized the full-length human gene for S100 β (Allore et al., 1990). This gene is composed of three exons, the first of which specifies the 5' untranslated region, while the second and third each encode a single EF-hand calcium-binding domain. The promoter region contains several potential regulatory elements of transcription, including cAMP-responsive elements and multiple repeats similar in sequence and location to the β -globin direct repeat elements (Allore et al., 1990). The human S100 β gene has also been mapped to a subtelomeric location in the 21q22.3 region of human chro-

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Figure 1. Schematic diagram of the 17.3 kb ClaI-XbaI fragment spanning the human S100 β gene derived from genomic clones λ HS14.4 and λ HS100.1. A partial restriction enzyme map is shown. Cleavage sites for ClaI (C), EcoRI (E), HindIII (H), BamHI (B), and XbaI (X) are specified. Exons are numbered, with coding regions indicated by solid boxes and untranslated regions by open boxes. The location and size (nucleotides) of regions in mRNA corresponding to each of the three exons are shown. The regions corresponding to the probes KN3 and oligonucleotide 34b are indicated.

mosome 21 (Allore et al., 1988; Duncan et al., 1989; Burmeister et al., 1991). In order to develop a model to study the developmental consequences of an increased S100 β gene dosage, we have derived and characterized transgenic mice carrying multiple copies of the human S100 β gene. These mice express human S100 β mRNA in a gene dose-dependent and tissue-specific manner, and higher than normal levels of S100 β protein in brain. Furthermore, by comparing the transgenic mice with their normal (nontransgenic) littermates using *in situ* hybridization and immunocytochemistry, we were able to localize S100 β precisely to astrocytes and a subset of brainstem neurons.

Materials and Methods

Transgene assembly. The transgene was derived from two overlapping human genomic clones (λ HS 14.4 and λ HS 100.1) (Fig. 1) that jointly span 25 kilobases (kb) of DNA including the S100 β gene (Allore et al., 1990). Nonoverlapping fragments of the two clones were subcloned in multiple steps into pBS (Stratagene) to construct plasmid pWF9c containing 17.3 kb of human genomic DNA. In order to excise the transgene from pWF9c, the plasmid was cut at a ClaI site situated in the 5' polylinker (17 nucleotides upstream of the insert), and at a unique XbaI site situated at the 3' boundary of the insert. The resulting 17.3 kb ClaI-XbaI genomic DNA fragment consisted of 7 kb of DNA spanning the three exons of the human S100 β gene and 6.3 kb of the upstream and 4 kb of the downstream sequence. The fragment was purified by ultracentrifugation through a linear 10–40% sucrose gradient, precipitated with ethanol, and redissolved in H₂O (Ausubel et al., 1987).

Production of transgenic mice. The transgene was microinjected at a concentration of 1–2 μ g/ml into male pronuclei of fertilized CD1 mouse eggs. The injected embryos surviving micromanipulation were reimplanted in the oviducts of pseudopregnant foster females, and allowed to develop to term according to published procedures (Hogan et al., 1986).

Hybridization probes. For Southern blotting, a 243 base pair partial human cDNA fragment, KN3 (encompassing most of the S100 β coding region) (Allore et al., 1990), and an 0.8 kb partial rat cDNA fragment of the myelin-associated glycoprotein (MAG) (Arquint et al., 1987) were randomly labeled with α -³²P-dCTP. For Northern blotting, a ³²P-CTP-

labeled antisense riboprobe was derived from KN3 subcloned into an RNA synthesis vector, pBS (Stratagene), that had been linearized for *in vitro* transcription with BamHI. A ³²P-CTP-labeled rat β -actin antisense riboprobe was derived as previously described (Dunn et al., 1987).

For *in situ* hybridization, the following ³⁵S-labeled probes were used. Antisense riboprobes labeled with ³⁵S-UTP were derived from (1) the human S100 β cDNA clone KN3 described above, (2) a rat S100 β cDNA clone (Dunn et al., 1987) subcloned into pSP65 (Promega) and linearized with BamHI, and (3) exon 1 of the mouse neurofilament light chain gene (Lewis and Cowan, 1986) subcloned into pGEM-3Z (Promega) and linearized with BglII. Sense riboprobes were similarly derived after linearization of the vectors with appropriate restriction enzymes. A synthetic antisense 34 base oligonucleotide, spanning the (exon 1) sequence +21 to +54 of the human S100 β gene, was end labeled with ³⁵S-ATP. This probe is complementary to 34 nucleotides of the 5' untranslated region of the human S100 β mRNA.

Preparation and Southern blotting of DNA. DNA was prepared from mouse tail clippings as described in the literature (Hogan et al., 1986). Samples of DNA (5 μ g) were digested with restriction enzymes, fractionated on 1% agarose gels, and blotted onto nylon membranes (Zeta-probe, Bio-Rad). Southern blotting was performed essentially as described previously (Allore et al., 1988), using a mixture of ³²P-labeled human S100 β and rat MAG cDNA probes. The blots were exposed to preflashed x-ray film (Kodak X-Omat, AR) with an intensifying screen at –70°C. The number of human S100 β gene copies in mice from each of the transgenic lines was determined by densitometry and comparison with a human diploid placental DNA standard. Densitometry was performed on a Molecular Dynamics computing densitometer model 300A using the Molecular Dynamics IMAGE QUANT software release version 3.0.

Preparation and Northern blotting of RNA. Mice were anesthetized and killed by cervical dislocation. Brain and other organs were rapidly removed, rinsed in phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.1), frozen in liquid nitrogen, and powdered. RNA was prepared from powdered tissue by homogenization in 4 M guanidinium isothiocyanate and centrifugation through a cushion of cesium chloride (Sambrook et al., 1989). Northern blotting was performed essentially as described previously (Dunn et al., 1987), using a ³²P-labeled human S100 β antisense riboprobe (KN3). Blots were exposed to x-ray film and the relative amounts of S100 β mRNA were determined by densitometry as described above. In order

Figure 2. Tissue-specific expression of S100 β mRNA in transgenic mice. Total RNA (10 μ g per lane) was analyzed by Northern blotting, using a 32 P-labeled human S100 β antisense riboprobe KN3. RNA from the following sources was analyzed in the specified individual lanes: transgenic mouse (line H-2) brain (1), liver (2), heart (3), kidney (4); human brain (5); rat glioma (C6) cells (6); normal mouse brain (7). The positions of migration of human S100 β mRNA (1.0 kb) and mouse and rat S100 β mRNA (1.6 kb) are indicated.

to control for equivalent loading of RNA, the blots were rehybridized with a 32 P-labeled rat β -actin probe, reexposed to x-ray film, and analyzed by densitometry.

In situ hybridization. The analysis of 15 μ m cryostat sections of mouse brains for expression of mouse and human S100 β mRNA and of neurofilament light chain mRNA using *in situ* hybridization with appropriate 35 S-labeled antisense riboprobes was performed as previously described (Landry et al., 1989). Alternate sections were hybridized with an equal amount of control 35 S-labeled sense riboprobe. Nontransgenic littermates were used as controls for the transgenic mice.

The following modifications were instituted when the 34 base oligonucleotide probe complementary to part of the 5' untranslated human S100 β mRNA sequence was used. First, digestion of sections with proteinase K to facilitate probe penetration was not carried out. Second, hybridization temperature was lowered to 50°C from 55°C. Third, digestion with RNase A was omitted. Fourth, the final wash in 0.1 $\times$ saline-sodium citrate, 300 mM 2-mercaptoethanol was performed at 60°C instead of 70°C.

Following hybridization and washing, the sections were applied to Du Pont Cronex sensitive x-ray film or coated with Kodak NTB2 liquid emulsion, and exposed for varying periods of time. The slides were developed in Kodak D19 developer for 2.5 min and stained lightly with cresyl violet.

Western blotting of brain extracts for S100 β protein. Mouse brains were obtained as described above. Brain extracts were prepared from frozen brain powder as described previously (Marks et al., 1983), except that 2-mercaptoethanol was omitted from all buffers, and assayed for protein concentration (Lowry et al., 1951). Aliquots of the 100,000 $\times$ g supernatants were dissociated by boiling in the presence of 2% SDS, 5% 2-mercaptoethanol and subjected to electrophoresis in 12.5% polyacrylamide slab gels in the presence of 0.1% SDS and 2 mM EDTA. The gels were frozen at -70°C and soaked in transfer buffer (12.5 mM Tris HCl, 96 mM glycine) for 30 min to remove the SDS, and the proteins were transferred electrophoretically to polyvinyl membranes (Immobilon-P, Millipore). The blots were incubated at room temperature, first for 2 hr with PBS containing 10% skim milk to block nonspecific binding of antibody, and then for 1 hr with anti-S100 protein monoclonal antibody G12.B8 (Haan et al., 1982; a generous gift from Dr. B. Boss, Salk Institute) at a 1:2000 dilution in PBS containing 1% skim milk, and finally washed three times for 30 min each with PBS containing 0.02% Tween 20. Following washing, the blots were incubated for 1 hr

at room temperature with peroxidase-conjugated rabbit anti-mouse Ig antibody (Dako; 1:500 dilution) and washed as described above, and the peroxidase color reaction was developed by addition of substrate (1% diaminobenzidine in PBS containing 0.009% H_2O_2 for 1–2 min). After visualization of the S100 β band, the membranes were incubated with ^{125}I -protein A (2×10^5 cpm/ml in PBS containing 1% skim milk) for 2 hr at room temperature, washed as described above, and analyzed by autoradiography as described above for Southern blotting.

Immunohistochemistry. Immunoperoxidase staining of mouse brain, liver, heart, and kidney sections for localization of S100 β was performed as previously described (Kahn et al., 1983; Nakamura et al., 1983), except that an indirect immunoperoxidase technique was used. The organs were fixed in 10% formalin, embedded in paraffin, and cut at 6 μ m sections. The sections were deparaffinized with xylol, washed in three changes of absolute ethanol, and incubated with 0.3% H_2O_2 in methanol to inactivate endogenous peroxidase. Incubations with various sera and antisera were at room temperature in a humidified chamber. Between each incubation, the sections were washed with 5 mM Tris HCl, pH 7.4, containing 145 mM NaCl (TBS). Normal swine serum was applied at a 1:10 dilution in TBS for 10 min, followed by rabbit anti-S100 protein antibody (Labourette and Marks, 1975) at a 1:400 dilution or rabbit anti-neuron-specific enolase antibody (Dako) at a 1:250 dilution, for 60 min. The rabbit anti-S100 protein antibody reacts with S100 β but not S100 α (Kahn et al., 1991). Secondary antibody (peroxidase-conjugated swine anti-rabbit Ig antibody at a 1:20 dilution) was applied for 30 min. The peroxidase color reaction was developed by the addition of 0.05% diaminobenzidine and 0.02% H_2O_2 in TBS for 5 min. The sections were counterstained with hematoxylin, dehydrated, cleared, and mounted.

Results

Generation of transgenic mice

The transgene consisted of a 17.3 kb human genomic DNA ClaI - XbaI fragment containing the three exons of the S100 β gene, 6.3 kb of DNA 5' to the transcription initiation site, and 4 kb of DNA 3' to the polyadenylation signal (Fig. 1). Previous studies have demonstrated that this fragment contains the control elements required for accurate transcription of the human S100 β gene in transfected rat glioma cells (Allore et al., 1990).

Table 1. Relative expression of human S100 β mRNA in organs of transgenic mice

	Transgenic line			
	H-2	H-8	H-5	H-4
Brain	100	100	100	100
Liver	6	3.2	3.2	1.7
Heart	3	6	4.4	6.9
Kidney	10.5	9	9.8	3.2

Values for human S100 β mRNA were obtained from densitometry of Northern blots, and are expressed as relative amounts to the value obtained for brain, which was arbitrarily set at 100. Results shown are averages of two determinations performed on one mouse from each transgenic line.

The fragment was injected into male pronuclei of fertilized CD1 mouse eggs, and the surviving embryos were reimplanted in the oviducts of pseudopregnant foster females and allowed to develop to term. Offspring were screened for transgene incorporation by Southern blotting of tail DNA with a human S100 β specific probe. Eight founder transgenics, three males and five females, were identified. One founder male died 3 weeks after birth. Of the remaining founder mice, one of two males and two of five females failed to transmit the transgene to the F1 progeny, suggesting that these mice were mosaics that had not incorporated the transgene into the germline. The other male

and three females were successfully crossed with CD1 mice to generate four transgenic lines H-2, H-8, H-5, and H-4. Mice from these lines carried approximately 110, 70, 8, and 10 copies of the transgene, respectively (see below).

Tissue-specific expression of S100 β mRNA in transgenic mice

On Northern blots, the human S100 β antisense riboprobe KN3, which covers the evolutionarily conserved S100 β coding sequence, hybridized to the human S100 β mRNA of 1.0 kb and the mouse and rat S100 β mRNAs of 1.6 kb (Fig. 2) in agreement with the sizes of human and rat S100 β mRNA species that have been reported previously (Dunn et al., 1987; Marks et al., 1990). The size difference between the human and mouse S100 β mRNA species allowed identification of transcripts from the human S100 β transgene in the presence of the endogenous cross-hybridizing mouse transcript. Both the 1.0 kb and 1.6 kb mRNA species were present in transgenic mouse brain. The human S100 β mRNA (1.0 kb) was present in high amounts in brain and was detected in only very low amounts (10–50-fold less) in liver, heart, and kidney. The endogenous mouse mRNA (1.6 kb) was present in brain but was not detected in liver, heart, or kidney. The relative amounts of human S100 β mRNA present in the brain, liver, heart, and kidney in each of the transgenic cell lines are shown in Table 1. On immunoperoxidase staining, there was no S100 β immunoreactivity in the parenchymal cells of liver, heart, and kidney. A small number of S100 β -immu-

Figure 3. Analysis of the human S100 β gene and its products in transgenic mice.

A, Southern blotting was performed on EcoRI-digested samples of DNA (5 μ g per lane), using a mixture of 32 P-labeled rat MAG and human S100 β cDNA probes. The position of the genomic EcoRI fragments that hybridize to the rat MAG (4.4 kb) and human S100 β (3.2 kb) cDNA probes are indicated. **B**, Northern blotting of total brain RNA (5 μ g per lane) using a 32 P-labeled human S100 β antisense riboprobe KN3. The blots were exposed to x-ray film for 5 d at -70°C . The blots were rehybridized with a 32 P-labeled rat β -actin antisense probe, and reexposed to x-ray film for 20 hr. **B** is a composite of the two autoradiograms. The positions of migration of mouse β -actin mRNA (1.8 kb), mouse S100 β mRNA (1.6 kb), and human S100 β mRNA (1.0 kb) are indicated. **C**, Western blotting was performed on brain extracts (4 μ g/lane) following separation by SDS-PAGE under reducing conditions. The primary antibody used was mouse monoclonal anti-S100 protein antibody, followed by rabbit anti-mouse Ig antibody, and 125 I-protein A. The position of migration of S100 β protein (M_r 10K) is indicated. The origin of the DNA, RNA, and protein samples in the respective lanes is the following: transgenic lines H-2 (lanes 1 and 2), H-8 (lanes 3 and 4), H-5 (lanes 5 and 6), H-4 (lanes 7 and 8), control (nontransgenic) mouse (lane 9), human (lane 10). DNA, RNA, and protein samples were from the same individual mouse in each of the respective lanes 1–9.

(A) DNA

4.4 kb —

3.2 kb —

(B) RNA

1.8 kb —

1.6 kb —

1.0 kb —

(C) Protein

M_r 10k —

noreactive histiocytes were seen (data not shown). Other minor cell types that likely contribute to the low level of human S100 β mRNA expression in these organs include Schwann cells that surround the innervating nerve fibers (Kahn et al., 1991). The precise localization of S100 β in cell types outside the CNS is presently under investigation in the transgenic mouse lines.

Dosage-dependent expression of the human S100 β gene in transgenic mice

The results of Southern blot analysis of tail DNA, Northern blot analysis of brain RNA, and Western blot analysis of brain extracts of mice from each of four transgenic lines are shown in Figure 3. On Southern blotting (Fig. 3*A*), the rat MAG and human S100 β cDNA probes hybridized to 4.4 kb and 3.2 kb EcoRI fragments, respectively. At the stringency conditions used, the human S100 β cDNA probe hybridized to human placental DNA (Fig. 3*A*, lane 10) but not to control (nontransgenic) mouse DNA (lane 9). The signal obtained with the MAG probe was used to correct for loading differences in the DNA samples from the transgenic mice for estimation of gene copy number from densitometric analysis.

On Northern blotting (Fig. 3*B*), the rat β -actin antisense probe hybridized to a 1.8 kb mRNA species, and the human S100 β (KN3) antisense probe, to a 1.6 kb mouse S100 β mRNA and a 1.0 kb human S100 β mRNA species. Both S100 β mRNA species were present in transgenic mouse brain. The human S100 β mRNA signal was much stronger than the mouse S100 β mRNA signal. In addition, a minor hybridizing band was also seen below the mouse S100 β mRNA in the high transgene copy lines H-2 and H-8 (Fig. 3*B*, lanes 1–4). This band likely represents a minor transcript of the human S100 β transgene. The signal obtained with the actin probe was used to correct for the loading differences in the RNA from the transgenic mice for estimation of relative amounts of S100 β mRNA from densitometric analysis. The strength of the human S100 β mRNA signal on Northern blots increased in parallel with the human S100 β gene signal on Southern blots, suggesting a direct correlation between human S100 β gene copy number and the amount of human S100 β mRNA in the brain of mice from each of the four transgenic lines. In contrast, the mouse S100 β mRNA signal did not vary appreciably among the brains of these mice. On the basis of densitometric analysis, the mean amount of mouse S100 β mRNA in the brain of the eight transgenic mice shown in Figure 3*B* represented 0.14 ± 0.05 ($\pm$ SD) of the amount of human S100 β mRNA in the brain of transgenic mice with the lowest human S100 β gene copy number (line H-5). This correlates well with the average number of human transgene copies (eight) present in this cell line.

On Western blotting (Fig. 3*C*), S100 β protein from transgenic mouse, normal mouse, and human brain migrated with an M_r of 10,000 in SDS-PAGE under reducing conditions, in the same position as that of the subunits of purified bovine brain S100 protein (not shown). In general, there was good agreement between the strengths of the S100 β mRNA signal on Northern blots and the corresponding S100 β protein signal on Western blots for individual mice from the four transgenic lines, suggesting a direct correlation between levels of human S100 β mRNA and the corresponding levels of the translated protein.

The relationship between transgene copy number and levels of human S100 β mRNA in the four transgenic lines is shown in Figure 4. The levels of human S100 β mRNA increased directly with increased transgene copy number.

Figure 4. Gene dosage-dependent expression of human S100 β mRNA in transgenic mouse brain. Gene copy numbers of the human S100 β transgene were determined by densitometric analysis of Southern blots of tail DNA of transgenic mice (Fig. 3*A*) and comparison with a human diploid DNA standard as described in Materials and Methods. The amounts of the human S100 β mRNA were obtained by densitometric analysis of Northern blots of brain RNA (Fig. 3*B*) as described in Materials and Methods and are expressed as relative amounts to the lowest value obtained for a transgenic mouse (from line H-5) that was arbitrarily set at 1.0. The means of the relative amounts of S100 β mRNA are plotted against the means of the human S100 β transgene copy numbers for lines H-2 ($\diamond$), H-8 ($\circ$), H-4 ($\triangle$), and H-5 (∇). Vertical and horizontal error bars represent $\pm$ SEM for relative amount of S100 β mRNA and transgene copy number, respectively, and the number of individual mice analyzed is shown above each point. All mice analyzed were approximately 1.4 years of age. No variations were observed between male and female mice.

Regional distribution of S100 β mRNA in normal and transgenic mouse brain determined by in situ hybridization

Brain regions that express S100 β mRNA were identified by *in situ* hybridization (Fig. 5) using a rat S100 β antisense riboprobe in normal mice (Fig. 5*A,D,G*), and a 34 base human antisense oligonucleotide probe complementary to part of the 5' untranslated region of human S100 β mRNA in transgenic mice (Fig. 5*B,E,H*). The oligonucleotide probe was specific for human S100 β mRNA and did not hybridize with mouse mRNA in normal mouse brain (Fig. 5*C,F,I*). The distribution of mouse and human S100 β mRNA in normal and transgenic mouse brain, respectively, was remarkably similar. At low magnification (Fig. 5*A,B*), S100 β mRNA was found to be concentrated in specific brain regions such as cerebellum and defined brainstem nuclei including the mesencephalic and motor trigeminal, facial, and lemniscus. Focusing on the cerebellum at higher magnification (using dark-field microscopy) (Fig. 5*D,E*), S100 β mRNA was found in the molecular cell layer and deep white matter, but not in the granule cell layer. At a still higher magnification of the Purkinje cell layer (Fig. 5*G,H*), the signal corresponding to S100 β mRNA was localized over Bergmann glia situated between Purkinje neurons. This localization was particularly well defined in the transgenic mouse brain because of the high amount of the human S100 β mRNA (Fig. 5*H*).

Figure 5. Distribution of S100 β mRNA in normal and transgenic mouse brain determined by *in situ* hybridization. Sagittal sections of brain from normal and transgenic (line H-2) mice were hybridized *in situ* using 35 S-labeled antisense probes specific for mouse and human S100 β mRNA respectively. The origins of the DNA, RNA, and protein samples in the respective lanes are the following: A, D, and G, normal mouse hybridized with a rat S100 β antisense riboprobe; B, E, and H, transgenic mouse hybridized with a 34 base oligonucleotide probe complementary to the untranslated region of human S100 β mRNA; C, F, and I, normal mouse hybridized with the human oligonucleotide probe. A-C, Sections analyzed by autoradiography following exposure to x-ray film, showing specific hybridizing brain regions: cerebellum and brainstem nuclei including motor trigeminal (MT), mesencephalic trigeminal (MeT), facial (F), and lemniscus (L). D-F, Dark field of emulsion-coated slides, low magnification, showing specific hybridizing cell layers of cerebellum: molecular (M), Purkinje (P), granule (G), deep white matter (D). G-I, Bright field of emulsion-coated slides, high magnification of Purkinje cell layer showing Purkinje neurons (large arrowheads) and Bergmann glia (small arrowheads). Exposure times: transgenic mouse: B-3 d, E, H-10 d; normal mouse, A, D, G and C, F, I-30 d. Scale bar: A-C, 2 mm; D-F, 500 μ m; G-I, 10 μ m.

Expression of S100 β mRNA and protein in discrete neuronal populations

In order to examine the possibility that S100 β mRNA was expressed in discrete neuronal populations in brainstem nuclei, the pattern of hybridization obtained using a neuronal marker probe (mouse neurofilament light chain antisense riboprobe) was compared with that obtained using the rat S100 β antisense riboprobe (Fig. 6). In normal mice, the patterns were very similar over the trigeminal and facial nuclei (Fig. 6A,B). When consecutive sections of the mesencephalic trigeminal nucleus were probed with the S100 β (Fig. 6C) or neurofilament (Fig. 6D) probes, the signals appeared to be localized over identical cells, suggesting that discrete brainstem neurons express S100 β mRNA. Likewise, in transgenic mice, the pattern of labeling over the facial nucleus in a section hybridized with the human S100 β antisense riboprobe (KN3) (Fig. 6E), was very similar to that in an adjacent section hybridized with the neurofilament probe (Fig. 6F). At the stringency conditions used for *in situ* hybridization, KN3 did not hybridize to sections of normal mouse brain (Fig. 6G,H). There was no hybridization signal above background with any of the control sense riboprobes.

In order to correlate regional and cellular expression of S100 β mRNA with protein, sections of normal and transgenic mouse

brain were examined for the presence of S100 β protein by immunoperoxidase staining using a rabbit anti-S100 protein polyclonal antibody (Fig. 7). In all regions and cell types examined, staining was more intense in transgenic mice (Fig. 7B,D,F), than in normal mice (A,C,E). S100 β protein was present in individual astrocytes in hippocampus (Fig. 7A,B), in Bergmann glia between Purkinje neurons in cerebellum (Fig. 7C,D), and discrete neurons in brainstem (Fig. 7E,F). In normal mice, positive staining was confined to the nucleus of selected brainstem neurons. In the same regions of brainstem in transgenic mice, staining was of much greater intensity and involved both the nucleus and cytoplasm of neurons. Additional confirmation of the neuronal cell type was provided by Bielschowsky staining (Fig. 7G) and positive immunoperoxidase staining with antibody to neuron-specific enolase (Fig. 7H), but absence of staining with anti-glial fibrillary acidic protein antibody (not shown).

The agreement between the results of *in situ* hybridization and immunoperoxidase staining indicates a concordance between expression of S100 β mRNA and production of S100 β protein, suggesting that the protein is synthesized in the cells where it is found and not taken up after secretion from other sources. Moreover, the pattern of increased S100 β immunoreactivity in astrocytes and neurons was similar in brains of mice from each of the four transgenic lines, indicating that there

Figure 6. Localization of S100 β mRNA in neurons by *in situ* hybridization. Sagittal sections of brain from normal and transgenic (line H-2) mice were hybridized *in situ* using 35 S-labeled antisense riboprobes specific for rat and human S100 β mRNA, and mouse neurofilament light chain mRNA. *A–D*, Normal mouse hybridized with rat S100 β antisense riboprobe (*A*, *C*), and neurofilament antisense riboprobe (*B*, *D*). Brainstem nuclei, mesencephalic trigeminal (*MeT*), motor trigeminal (*MT*), and facial (*F*), are indicated. *C* and *D* (lower half), Enlargement of *in situ* hybridization to consecutive sections of the mesencephalic trigeminal nucleus; arrowheads indicate identical neurons showing positive hybridization signal with rat S100 β antisense riboprobe (*C*), and neurofilament antisense riboprobe (*D*). *E* and *F*, Consecutive sections of the facial nucleus in transgenic mouse brain, hybridized with a human S100 β antisense riboprobe (KN3) (*E*), and a neurofilament antisense riboprobe (*F*). *G* and *H*, Normal mouse hybridized with the human S100 β probe. *A*, *B*, *E*, and *H*, dark field; *C*, *D*, *F*, and *G*, bright field. Slides were coated with Kodak NTB2 emulsion, and exposed for the following times: *A* and *C*, 30 d; *B* and *D–H*, 21 d. Scale bar: *A*, *B*, *G*, and *H*, 400 μ m; *C* and *D*, top, 200 μ m; *C* and *D*, bottom, and *E* and *F*, 100 μ m.

was no extinction of specific expression of human S100 β in any of the gene-positive progeny.

Discussion

Presence of a locus control region in the human S100 β gene

In many transgenic mouse studies, expression from the inserted transgene has been found to vary among lines derived from different founder animals. In addition, expression from the transgene often did not correlate with the transgene copy number and was at a much lower level than expression from the endogenous homolog (Palmiter and Brinster, 1986). These quantitative differences are believed to reflect a position effect resulting from random integration of the transgene into the mouse genome (Palmiter and Brinster, 1986). Locus control regions (LCRs) (Stamatoyannopoulos, 1991) that circumvent these limitations have been identified in the flanking regions of a number

of human genes including β -globin and CD2 (Grosveld et al., 1987; Greaves et al., 1989). Similarly, introduction of the 17.3 kb S100 β gene fragment into transgenic mice resulted in a position-independent, copy number-dependent, expression of both S100 β mRNA and S100 β protein. Even though gene expression increased to levels 10–100-fold above normal, tissue and cell specificity were maintained. Expression of human S100 β mRNA in liver, heart, and kidney was detected at levels 10–50-fold lower than that observed in brain, and there was no S100 β immunoreactivity in the parenchymal cells in any of these (non-brain) organs. These results suggest that a sequence within the 17.3 kb fragment functions as an LCR in a manner analogous to the LCR described for the human β -globin gene (Grosveld et al., 1987). The β -globin LCR has been shown to activate both β -globin and heterologous genes in transgenic mice in a gene copy-dependent, erythroid-specific manner, independent of the

Figure 7. Immunohistochemical localization of S100 β protein in normal and transgenic mouse brain. Sagittal sections of brains from normal (*A*, *C*, *E*) and transgenic (*B*, *D*, *F*) mice were stained with rabbit anti-S100 protein antibody using the immunoperoxidase reaction. *A* and *B*, hippocampus; *C* and *D*, cerebellum; *E* and *F*, brainstem. *B*, *D*, and *F* show increased immunoreactivity (*B* and *D* within astrocytes, and *F* within neurons). In *E*, immunoreactivity is confined to neuronal nuclei, and in *F*, it is present in both neuronal nuclei and cytoplasm. *G* and *H* are brainstem regions of a normal mouse showing neurons stained with Bielschowsky stain (*G*) or with rabbit anti-neuron-specific enolase antibody (*H*). Scale bar: *A*–*D*, 200 μ m; *E*–*H*, 100 μ m.

site of transgene insertion (Grosveld et al., 1987; Blom van Assendelft et al., 1989). These unique gene-regulating properties appear to be due to two activities of the globin LCR, a chromatin domain organizing activity indicated by the appearance of DNase I-sensitive regions, and a transcription enhancer activity (Behringer et al., 1990; Collis et al., 1990). A similar activity, manifested in the CNS, particularly in astrocytes and selected brainstem neurons, appears to reside in the S100 β gene fragment studied here. Sites of DNase I sensitivity may provide useful markers for the location of the S100 β LCR, and an approach to the characterization of the specific LCR sequence.

Localization of S100 β in brain

Our *in situ* hybridization and immunocytochemistry experiments clarify a number of long-standing issues relating to the precise location of S100 protein in brain. The finding of S100 β in astrocytes (Bergmann glia) in cerebellum and hippocampus is in agreement with published data (Matus and Mughal, 1975;

Haan et al., 1982). On the other hand, the previously reported presence of S100 protein in the nucleus of discrete brainstem neurons has been the subject of controversy (Hyden and McEwen, 1966; Ghandour et al., 1981). Because of the nuclear location, a possible role for S100 protein in the control of gene expression was proposed (Hyden and McEwen, 1966). However, it was not known whether these neurons synthesized S100 protein or the protein was taken up by neurons after being produced and secreted by astrocytes (Hyden and McEwen, 1966). Our results indicate that since both S100 β mRNA and S100 β protein are present in selected neuronal populations, these cells actually synthesize S100 β .

The origin of the S100 β -expressing neurons in brainstem nuclei is not known. A presumptive common precursor for neuronal and glial cell lineage has been described in the prenatal mouse hypothalamus (De Vitry et al., 1980). This bipotential stem cell expresses S100 protein and could give rise to both neuronal and glial cell populations in which this protein is found.

Functional implications of increased S100 β levels

Our model compares favorably with the other transgenic mouse models derived to study the consequences of aberrant expression of individual genes located on chromosome 21 in humans. In the case of superoxide dismutase, there was no correlation between the number of integrated human gene copies and the observed level of gene expression, and the tissue distribution of the human enzyme differed from that of the product of the endogenous mouse gene (Epstein et al., 1987). Some transgenic mice in that study developed abnormalities in the neuromuscular junctions of the tongue resembling those seen in Down's syndrome subjects (Avraham et al., 1988). These abnormalities were age dependent, and nontransgenic littermates developed similar findings at a more advanced age (Avraham et al., 1988). In the case of the human β -amyloid protein gene, the transcript of the human transgene represented only a small fraction of the transcript of the endogenous mouse gene in one study (Wirak et al., 1991), and there was no apparent correlation between the integrated human gene copy number and the level of the expressed gene product in the other study (Quon et al., 1991). In the latter study, amyloid deposition was found in the brain of the transgenic mice, but no behavioral abnormalities were reported.

In contrast, in the present study, the human S100 β transgene was expressed at high levels in a copy number-dependent and cell-specific manner. The increased immunoreactivity of S100 β protein in individual astrocytes in the brain of transgenic mice mimics the increased S100 protein immunoreactivity observed in the brains of Down's syndrome and Alzheimer disease subjects (Griffin et al., 1989). Nonetheless, transgenic mice from each of the four lines did not manifest any obvious behavioral differences or gross pathological abnormalities at autopsy in comparison with their normal littermates. This implies that excessive expression of the S100 β gene ranging from approximately 10- to 100-fold over normal is generally well tolerated, but does not exclude the possibility that increased levels of S100 β promote subtle changes in development or behavior that will become apparent in more precise histological and functional assays.

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