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Neuronal Expression of Human Immunodeficiency Virus Type 1 Env Proteins in Transgenic Mice: Distribution in the Central Nervous System and Pathological Alterations

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It is now well documented that human immunodeficiency virus type 1 (HIV-1) induces encephalopathy in patients with AIDS. In vitro studies have implicated the envelope protein (gp120) as a factor which causes neuronal death. To better evaluate the role and elucidate the mechanisms of gp120 neurotoxicity, we have developed transgenic mice carrying a segment of the HIV-1 genome that expresses the viral gp160 protein under the control of the human neurofilament light gene promoter. In two separate lines of transgenic mice, the Env protein was found to be expressed in several nuclei of the brain stem and in the anterior horns of the spinal cord. The two lines showed identical patterns of Env expression. Neuropathological evaluation revealed numerous abnormal dendritic swellings in the immunostained motor neuron structures. Large and numerous neuritic swellings were also prominent in the nucleus gracilis and in the gracilis and cuneate fascicles. In addition, reactive astrocytosis was observed in several immunoreactive areas of the central nervous system. These transgenic mice offer a unique model to further investigate the role of HIV-1 Env protein in neuronal toxicity and to help elucidate the mechanisms that are involved.

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS. As a member of the *Lentivirinae* subfamily of retroviruses, it is recognized for its ability to target the immune system and the nervous system tissue (26, 27). In a large number (30 to 40%) of patients with AIDS, the virus entry in the central nervous system (CNS) initiates a slowly progressive dementing syndrome, termed HIV-1-associated motor/cognitive complex (13), which impairs cognitive and motor functions and induces behavioral disorders. At autopsy, up to 96% of these patients show neuropathological changes (5, 29, 40, 41) that typically define HIV-1 encephalopathy or leukoencephalopathy (39–41). The morphological characteristics of these two entities are well defined. For both, morphometric studies have clearly demonstrated a significant neuronal loss, reactive astrocytosis, and multinucleated cells (9, 17, 23, 32, 40–42). In addition to these CNS changes, the spinal cords of HIV-1-infected patients may also present alterations, such as myelitis or a peculiar vacuolar myelopathy for which no clear etiology has yet been determined. Expression and replication of HIV-1 have been reported to occur in macrophages and microglial cells of the human CNS but not in neurons (8, 32, 36). These findings appear rather paradoxical, considering the cognitive and motor dysfunctions, the dementing illness, and the neuronal loss observed in many HIV-1-infected patients. To account for all these disorders, several hypotheses, all based on in vitro studies, have been proposed. It was suggested that the neurotoxicity might be mediated by cytokines and arachidonic acid metabolites which are produced when HIV-infected CNS macrophages interact with the astrocytes (12). Other studies have shown that both native and recombinant gp120, added at very low concentrations to neuronal cultures, produce a striking increase in free calcium within the cells and cause cell death within 24 h, an effect which could be abolished by adding a dihydropyridine calcium channel antagonist (7), vasoactive intestinal peptide (3), anti-gp120 antibodies (7), or NMDA antagonists (3, 20). The neurotoxicity of gp120 may thus be conferred either through the NMDA receptor, via a second messenger, or directly by calcium channels (21, 36). Also, gp120 has sequence homology with vasoactive intestinal peptide, and therefore it might compete for the same binding sites and block this neurotransmission (19, 30). Despite the possible mechanisms suggested by all of these studies, many aspects related to AIDS neurophysiopathology could not be investigated in in vitro conditions. Recently, two groups have developed transgenic mice carrying the HIV genome or the gp120 gene under the control of, respectively, neuron- and astrocyte-specific promoters (37, 38). In both animal models, the best candidate among neurotoxic factors, gp120, was not detected in CNS tissue, and consequently, its role and mechanism of action could not be fully elucidated. In order to better define the role of gp120 in AIDS dementia, we chose to develop transgenic mice carrying the HIV-1 *env* gene under the control of the neuron-specific promoter of the human neurofilament light (NFL) gene with the hope to obtain expression of the protein in the targeted tissue. In the present report we describe these transgenic mice, the expression pattern of the Env protein in their CNS, and the preliminary findings of pathological evaluations.

MATERIALS AND METHODS

Recombinant plasmids. The plasmid harboring the transgene NFLgp160 (Fig. 1A) was derived as follows. A 4.8-kb *Sal*I-*Xba*I fragment comprising the segment encoding the Env protein of HXBc2 provirus was deleted from the Env expresser plasmid psvIIIexE7 (kindly provided by E. Cohen, Université de Montréal) and inserted into the 3′ end of a 2.2-kb fragment that contains the promoter of the human NFL gene (a gift of J. P. Julien). The HXBc2 fragment harbors the coding sequences of Env and of Rev, which is required for the transport of *env* mRNA from the nucleus to the cytoplasm (2). It also contains nonfunctional *vpu* and *nef* genes (because of a premature stop codon in their respective sequences), the entire 3′ long terminal repeat that provides the polyadenylation signal, and 1.3 kb of cellular sequences. The transgene was completed by fusing a 3.5-kb *Xba*I

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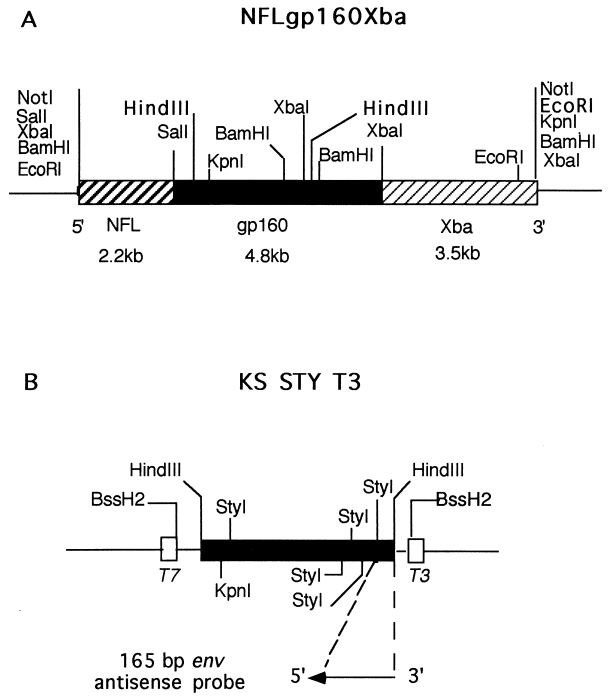

FIG. 1. Structure and restriction map of NFLgp160Xba and KS-StyT3 plasmids. (A) The pUC18 vector, harboring the 2.2-kb NFL promoter, was modified by introducing *Not*I sites at the unique *Kpn*I and *Eco*RI sites. The 4.8-kb *Sal*I-*Xba*I gp160 fragment of HIV-1 strain HXBc2 and the 3.5-kb *Xba*I fragment of the NFL gene were introduced into pUC18 at the *Sal*I and *Xba*I sites as described in Materials and Methods. The 2.2-kb *Sal*I segment containing the human NFL promoter was inserted in the *Sal*I site at the 5′ end of the HIV-1 4.8-kb fragment. The NFLgp160Xba transgene was deleted from the vector pUC18 by *Not*I digestion before microinjection. (B) The KS-StyT3 plasmid was constructed by insertion of the 2.2-kb *Hin*dIII subfragment of the HIV-1 *env* gene in the *Hin*dIII site of the Bluescript KS(+) vector.

fragment of the human NFL gene to the 3′ end of the HXBc2 segment. This fragment was added because it may contain positive regulatory elements which enhance the promoter activity (14). The transgene (10.5 kb) was excised from the pUC18 vector by *Not*I digestion. The KS-Sty construct (Fig. 1B) was derived to provide the probes for Southern, Northern (RNA), and RNase protection analyses. This construct harbors the 2.2-kb *Hin*dIII fragment of the *env* gene in the Bluescript KS(+) vector (Stratagene).

Cell cultures and transfections. The cell lines HeLa-CD4$^+$ and HeLa-CD8$^+$ used for transfection experiments were maintained in Dulbecco modified Eagle medium (Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL) and 0.1% gentamicin. The transfections were performed by the standard calcium phosphate technique as described by Shen et al. (33). Fifteen micrograms of plasmid DNA, coprecipitated with calcium phosphate, was applied for 12 to 16 h to rapidly growing cells plated onto a 10-cm-diameter plate containing 10 ml of Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 0.1% gentamicin. The cells were then rinsed with Dulbecco modified Eagle medium and incubated in fresh medium for 36 to 48 h.

HIV-1-infected and noninfected U937 cells, used, respectively, as positive and negative controls for *env* RNA expression, were maintained in RPMI medium (Gibco/BRL) supplemented with 10% fetal bovine serum and 0.1% gentamicin.

Immunoreactions. (i) Immunoperoxidase staining. For the immunoperoxidase reaction, Cos-1, HeLa-CD4$^+$, and HeLa-CD8$^+$ cells were seeded on glass coverslips and transfected as described above. Forty-eight hours later, the transfected cells and the appropriate controls were rinsed with phosphate-buffered saline (PBS), air dried, fixed in cold acetone for 10 min, and reacted first with a normal serum for 20 min and then with mouse monoclonal antibodies (Dupont/NEN) directed against gp41 or gp120. The immunoreactivity was revealed by use of the avidin-biotin-peroxidase (ABC) method with a biotinylated horse anti-mouse immunoglobulin G and an ABC complex (Vectastain; Vector) with diaminobenzidine as the chromogen. The cells were then rinsed with PBS, stained with hematoxylin for 10 s, washed in water, dehydrated in ethanol, and mounted on coverslips with DPX.

(ii) Immunoblot analysis. Brain stem lysates from normal and transgenic mice were prepared and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (18) and then blotted onto nitrocellulose with a Bio-Rad Mini-Protean apparatus. The blots were blocked with 20% skim milk in PBS and incubated with anti-gp120 and/or gp41 monoclonal antibodies and then with the appropriate secondary antibody conjugated to horseradish peroxidase. The immunoreactive bands were revealed by use of the ABC technique.

Microinjection of fertilized mouse eggs. The methods used to develop transgenic mice were those of Brinster et al. (4) and Julien et al. (16). The 10.5-kb NFLgp160Xba transgene was deleted from the plasmid by using *Not*I enzyme (Fig. 1A), purified with several phenol-chloroform extractions and ethanol precipitation, and finally microinjected at 2 μg/ml into the male pronuclei of fertilized eggs. Microinjected eggs were transferred to the oviducts of pseudopregnant females. All of the transgenic mice were developed and maintained in a pathogen-free facility.

DNA analysis of transgenic mice. The integration of the transgene into the mouse genome was assessed by Southern blot hybridization of genomic DNA extracted from tail samples of 3-week-old mice by using as a probe the 2.2-kb *Hin*dIII subfragment of the *env* gene, which was labeled with [^{32}P]dCTP by nick translation as described previously (31).

RNA expression in transgenic mice. The RNase protection assay and Northern blotting were used to analyze RNA expression in the transgenic and control mice.

RNase protection experiments were carried out to detect *env* mRNA expression and compare its levels in different organs. Transgenic and control mice were sacrificed, and tissue samples from the forebrain, cerebellum-brain stem, lung, liver, heart, and kidney were removed and immediately frozen in liquid nitrogen. The RNAs were prepared by homogenizing the different organs in 10 ml of 3 M LiCl–6 M urea per g as described by Auffray and Rougeon (1). The homogenates were kept on ice for 1 h, sonicated for 1 min, and incubated at 0°C overnight. The RNAs were harvested by centrifugation, rinsed with the LiCl-urea solution, pelleted again, and resuspended in 10 mM Tris-HCl (pH 7.6)–1 mM EDTA–0.5% SDS in volumes equivalent to 5 ml/g of original tissue. The RNAs were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1) followed by chloroform-isoamyl alcohol (24:1) and finally were precipitated in 2 volumes of ethanol–1/10 volume of 3 M Na acetate at −20°C. The purified RNAs were again pelleted, redissolved in sterile water, and kept at −80°C until used. As a positive control, RNAs from HIV-1-infected U937 monocytic cells were prepared under the same conditions. For RNase protection reactions, RNA samples (25 μg) were redissolved in 30 μl of hybridization buffer containing 2.5×10^5 cpm of an antisense *env* RNA probe labeled with [α-^{32}P]UTP (3,000 Ci/mmol) as described by Sambrook et al. (31). This probe was transcribed from *Sty*I-linearized KS-StyT3 plasmid by using T3 polymerase; it spanned 92 nucleotides specific to gp160 transcripts and 73 nucleotides belonging to the KS(+) plasmid. As a standard for these hybridization reactions, we used a 278-nucleotide antisense RNA probe, specific to the ubiquitus L32 riboprotein mRNA (11), labeled with ^{35}S-UTP (3,000 Ci/mmol). The hybridization products were digested with 0.1 and 5 μg of RNases T1 and A (Bethesda Research Laboratories), respectively, as described by Sambrook et al. (31) and were fractionated on 8% polyacrylamide–urea gels. The gels were dried and exposed to X-Omat AR films with intensifying screens for 2 to 8 days.

The Northern blot technique was used as described previously (31) to probe the RNAs with the following [α-^{32}P]dCTP-labeled probes: the 2.2-kb segment bearing the NFL gene promoter, the 3.5-kb fragment of the NFL gene, 1.3 kb of human sequences and, as a positive control for the reaction, the cDNA of the mouse NFL gene (15).

In vivo expression of HIV-1 Env proteins. The expression of the Env proteins in the nervous system tissues of transgenic mice was investigated by immunohistochemistry and immunoblotting, using monoclonal antibodies against gp41 and gp120 or serum from an HIV-1-infected patient. For immunohistochemistry, transgenic and control mice were anesthetized with an overdose of pentobarbital (Somnotol; 70 mg/kg intraperitoneally) and perfused transcardially with 0.01 M PBS (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain and spinal cord were removed, further fixed by immersion in the same fixative, and then cut into 40-μm-thick sections with a vibratome. The sections were rinsed extensively, preincubated with 10% normal goat serum for 2 h, and incubated overnight at room temperature with the primary antibodies. This was followed by washings with PBS and immunoreactivity detection by the ABC method (Vectastain; Vector). The sections were mounted on slides, air dried at 37°C overnight, dehydrated with ethanol, and mounted on coverslips with DPX. The stereotaxic atlas of Paxinos and Watson (28) for the rat and the atlas of Sidman et al. (34) for the mouse brain and spinal cord were used as anatomical references; the nomenclature used by Paxinos and Watson (28) was adopted for the present description. Furthermore, additional immunohistochemical experiments with the same antibodies were performed on 5-μm-thick sections from paraffin-embedded brain tissue.

For immunoblotting, brain stem lysates from normal and transgenic mice were prepared and separated by SDS-PAGE as described previously (18) and then blotted onto nitrocellulose with a Bio-Rad Mini-Protean apparatus. The blots were blocked with 20% skim milk in PBS and incubated with anti-gp120 and/or gp41 monoclonal antibodies and then with the appropriate secondary antibody conjugated to horseradish peroxidase. The immunoreactive bands were revealed by use of the ABC technique.

Neuropathological evaluation. CNS tissues of 3-month-old transgenic and control mice were also fixed in 10% formaldehyde and embedded in paraffin

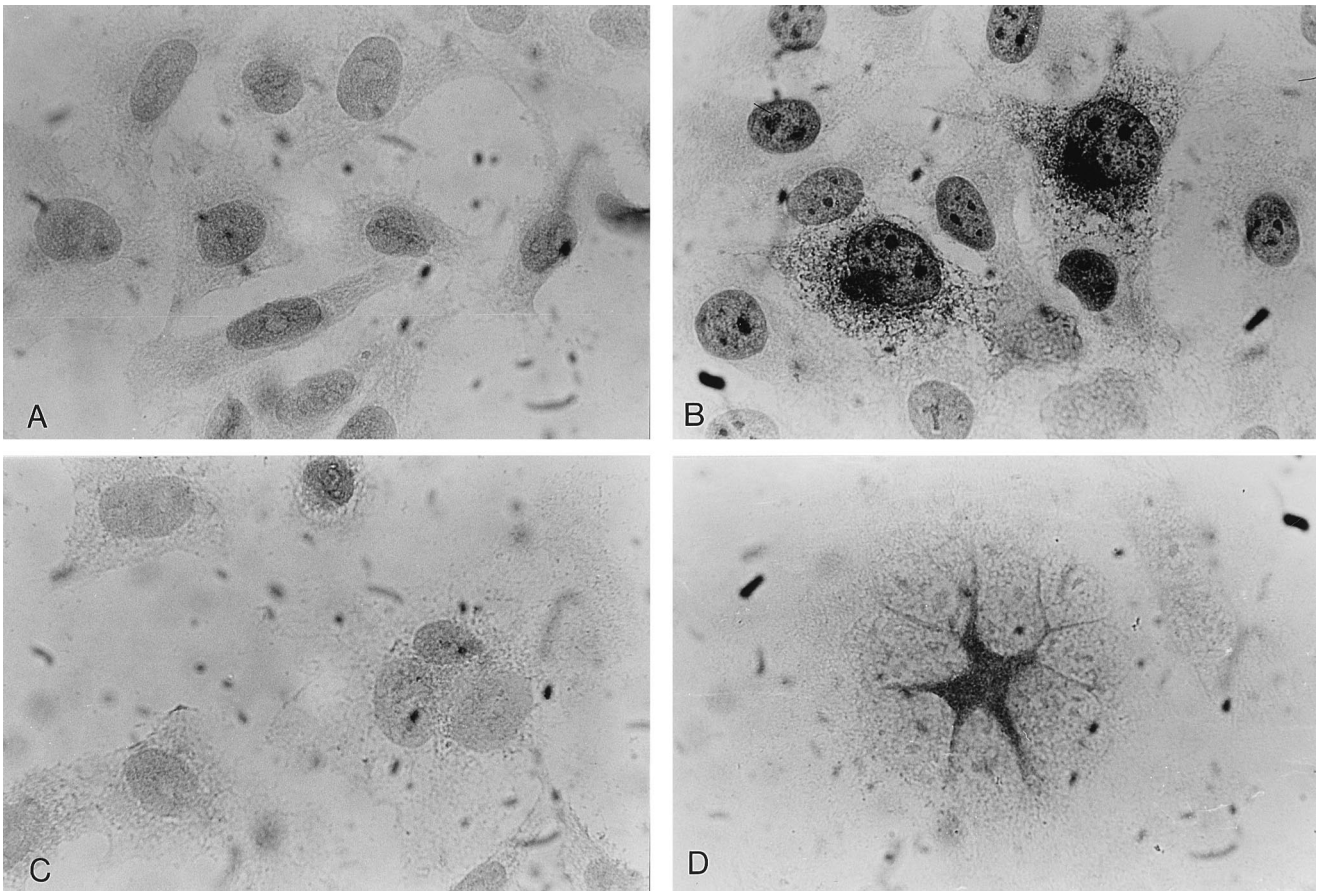

FIG. 2. Functional properties of the transgenic Env proteins. $CD4^+$ and $CD8^+$ HeLa cells transfected with the transgenic construct were immunoreacted with either normal or monoclonal antibodies against gp120 and gp41 by the ABC technique and examined for syncitium formation. (A) HeLa-$CD8^+$ cells immunoreacted with normal serum; (B) HeLa-$CD8^+$ cells treated with anti-gp120 or anti-gp41; (C) HeLa-$CD4^+$ cells immunoreacted with normal serum; (D) HeLa-$CD4^+$ cells treated with anti-gp120 or anti-gp41. Syncitia are observed only in HeLa-$CD4^+$ cells expressing the Env proteins (C and D). Magnification, ×2,000.

blocks for serial sectioning (5-μm-thick sections). The sections were stained with hematoxylin-phloxin-safran (HPS). Selected sections were further stained with modified Bielchowsky, Luxol-cresyl violet, and Holzer stains. Preliminary immunohistochemical exploration for glial fibrillary acidic protein (GFAP) and the phosphorylated neurofilament triplet was done by the ABC method. Finally, the horizontal suspension test was used as described previously (37) for preliminary evaluation of the motor functions of 3-month-old transgenic mice and controls. Five 3- to 4-month-old, heterozygous transgenic mice from each line and five age-matched controls were included in this evaluation.

RESULTS

In vitro expression of the transgene. Before the NFLgp160-Xba transgene was used to develop the transgenic mice, it was tested in transient-transfection experiments using Cos-1 cells and immunoprecipitation with HIV-1-infected patient serum. These assays clearly showed the expression of the precursor gp160 and its subproducts, gp120 and gp41 (data not shown). To further characterize the transgenic protein, we carried out transfection experiments with HeLa-$CD4^+$ and HeLa-$CD8^+$ cells to determine if the Env proteins encoded by the transgene have retained the ability to induce syncitium formation in $CD4^+$ cells. Immunostaining of NFLgp160Xba-transfected HeLa-$CD4^+$ and HeLa-$CD8^+$ cells with either anti-gp120 or anti-gp41 monoclonal antibodies showed that both types of cells expressed the Env proteins (Fig. 2B and D). Although present on the cell surface and all over the cytoplasm, the immunostained proteins were mostly concentrated in areas corresponding to the rough endoplasmic reticulum and Golgi compartments, as already reported (35a). No immunostaining was found in cells reacted with normal serum (Fig. 2A and C). As anticipated, several multinucleated cells, resulting from the fusion of Env-expressing cells with $CD4^+$ cells, were observed only among NFLgp160Xba-transfected HeLa-$CD4^+$ cells (Fig. 2C and D). These findings confirmed that the Env products encoded by the transgene are similar to the native moieties with respect to their processing and biological function.

Development of the transgenic mice. The integration of the transgene in the mouse genome was determined by Southern blot hybridization of genomic DNA extracted from mice tails. Of 25 animals tested, 4 were found to carry 1 to 30 full-length copies of the transgene per haploid genome (data not shown). All of the animals were maintained in a pathogen-free facility and, for 15 months now, have remained healthy. They all reproduced normally and transmitted the transgene in a Mendelian fashion, except for one founder which was found to be less fertile. The data described in this report were obtained with the heterozygous (844) and homozygous (854) transgenic mouse lines.

Expression pattern of the *env* mRNA in the transgenic animals. Tissue-specific expression of the NFL-driven env gene in transgenic animals was analyzed by RNase protection assay with RNAs extracted from the forebrain, cerebellum-brain stem, heart, liver, lung, and kidney of 3-month-old 844 and 854

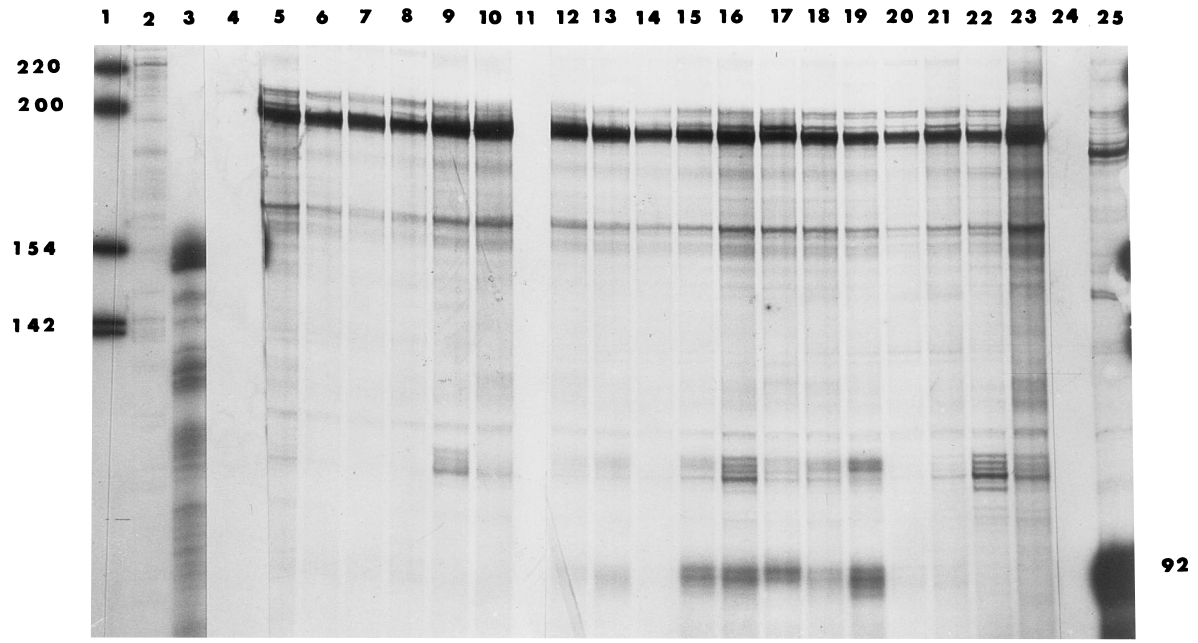

FIG. 3. RNase protection analysis of *env* mRNA expression in transgenic tissues. RNAs isolated from different tissues of normal mice and transgenic 844 and 854 mice and from HIV-1 infected U937 cells were hybridized to gp160 and L32 ribosomal gene-specific probes. The Env-specific ^{32}P-labeled antisense RNA probe was transcribed from *Sty*I-digested KS-Sty plasmid and protected 92 nucleotides of gp160 mRNA. The ^{35}S-labeled probe for internal control L32 ribosomal gene was transcribed from *Xba*I-linearized rpL3227.3.7 plasmid and protected 278 nucleotides of L32 mRNA. RNA samples from the forebrain, cerebellum-brain stem, heart, liver, lung, and kidney of normal mice are in lanes 5 to 11, respectively; in the same order, RNA samples from the same tissues of transgenic 844 mice are in lanes 12 to 17, and those from 854 mice are in lanes 18 to 23. Nucleotide contents of the molecular weight markers and of the protected segment of gp160 mRNA are indicated on the left and right, respectively. Lanes 2 and 3, samples of the synthesized probes, specific for the L32 ribosomal and gp160 genes, respectively.

mice and nontransgenic littermates. The reaction specificity was controlled with RNAs purified from HIV-1-infected U937 cells. Equal amounts of sample RNAs were simultaneously hybridized to an [α-^{32}P]UTP-labeled antisense RNA probe specific to Env transcripts and a ^{35}S-UTP-labeled antisense probe specific to L32 ribosomal protein mRNA. As illustrated on the autoradiogram in Fig. 3 (lanes 5 to 23), a signal with similar intensity corresponding to the protected segment of the L32 probe was observed in all of the tested RNA samples, thus indicating that the hybridization reaction mixtures contained approximately equivalent amounts of RNA. The hybridization of test samples with the probe specific for the gp160 transcripts led to the following results. (i) As expected, the RNAs from infected cells exhibited a protected fragment of 92 nucleotides (Fig. 3, lane 25). (ii) The forebrain, cerebellum-brain stem, heart, liver, lung, and kidney of normal mice (Fig. 3, lanes 5 to 10, respectively) were all negative). (iii) With the RNAs of the 844 transgenic mice, the probe specific for gp160 transcripts protected the anticipated 92-nucleotide segment in the forebrain and cerebellum-brain stem (Fig. 3, lanes 12 and 13, respectively) and also in nontargeted organs such as the heart, liver, lung, and kidney (lanes 14 to 17, respectively). Curiously, the level of *env* mRNA expression in the CNS tissues appeared to be lower than that in the ectopic organs. (iv) In contrast, among all of the tested tissues from heterozygous or homozygous mice of line 854, only the forebrain and cerebellum-brain stem exhibited the protected fragment (Fig. 3, lanes 18 and 19, respectively). No signal was observed in the other tissues (Fig. 3, lanes 20 to 23). Overall, these results clearly indicated that the transgene was transcribed in the CNS of both 844 and 854 mice, with a higher level of *env* mRNA expression in the cerebellum-brain stem than in the forebrain. As the transgene harbors additional non-HIV-1 DNA sequences, we also carried out Northern blot experiments to ascertain that only the HIV-1 *env* gene is transcribed in the transgenic animals. RNA samples from the cerebellum-brain stem, which demonstrated a higher signal for *env* mRNA in both transgenic lines, were blotted and hybridized with the following probes: the 2.2-kb segment bearing the NFL gene promoter, the 3.5-kb fragment of the human NFL gene, 1.3 kb of human sequences and, as a positive control, the cDNA of the mouse NFL gene (15). Except for the mouse NFL cDNA, which detected the expected endogenous neurofilament mRNAs, no signal was obtained with the probes, even after longer exposure of the filters (data not shown). Together, these results demonstrated that only the HIV-1 *env* sequences were transcribed from the NFLgp160 Xba construct in the transgenic CNS tissue.

Expression and distribution of Env proteins in the CNS of transgenic mice. The expression of *env* RNA in the CNS tissue prompted us to look for the presence of the protein. This was first examined by the Western blot (immunoblot) technique to determine if the transgenic protein was produced and processed. Protein lysates were prepared from the cerebellum-brain stem of normal and transgenic mice and from HIV-1-infected U937 cells and immunoreacted with anti-gp120 and/or anti-gp41 monoclonal antibodies. The cerebellum-brain stem tissues of the normal mice were totally negative (Fig. 4, lane 2). However, both HIV-1-infected cells and 844 and 854 transgenic tissues demonstrated clear bands corresponding to gp160 and gp41 (Fig. 4, lanes 1, 3, and 4, respectively). A faint band corresponding to gp120 was detected in infected cells and samples from 844 mice; the signal for the same protein in 854 mice was barely visible. In both transgenic samples, we also detected a band with an apparent molecular mass of ca. 85 kDa; this could correspond to a nonglycosylated Env precursor. These results showed that, in contrast to previously de-

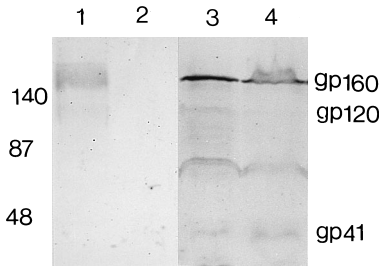

FIG. 4. Immunoblot analysis of protein lysates from the brain stems of transgenic and normal animals. The protein lysates were prepared as described in Materials and Methods and incubated with anti-gp120 and anti-gp41 monoclonal antibodies and then with the appropriate secondary antibody conjugated to horseradish peroxidase. Lanes: 1, HIV-1-infected U937 cells; 2, normal mice; 3 and 4, transgenic 844 and 854 mice, respectively.

scribed animal models (37, 38), our transgenic mice produce and correctly process the HIV-1 Env proteins in the targeted CNS tissue.

The distribution of the viral proteins in the CNS was then examined by immunostaining of serial sections of the brain and spinal cord from 3-month-old transgenic and control mice by using the same monoclonal antibodies as in the immunoblotting analysis and an HIV-1-infected patient serum. All three antibodies gave the same results, with the difference that the strongest reaction was obtained with the patient serum. The forebrain (telencephalon and diencephalon) of both lines of transgenic mice was essentially immunonegative, with the exception of the lateral hypothalamus in line 844, which displayed some immunostained cell bodies with the HIV-1 serum. The cerebellum was also completely immunonegative. In contrast, several nuclei of the brain stem and spinal cord exhibited immunoreactive perikarya (Fig. 5 and Table 1). Their distribution was consistent among transgenic mice of both lines and with all three antibodies. The motoneurons of cranial nerve nuclei (oculomotor nucleus, trochlear nucleus, trigeminal nucleus, facial nucleus, and hypoglossal nucleus) and the spinal cord (Fig. 5) were the most prominent in terms of the number of labeled neurons and staining intensity, with the immunoreactivity extending distally into the dendrites. Lower numbers of labeled neurons were observed in the mesencephalic, pontine, and medullar reticular formations (deep mesencephalic nucleus, caudal and oral parts of pontine reticular nucleus, ventral part of medullary reticular nucleus, gigantocellular reticular nucleus, and lateral reticular nucleus) red nucleus, superior colliculus, lateral and superior vestibular nuclei, and dorsal root ganglia. Thus, most brain regions which were shown in other studies to display perikaryal neurofilament immunoreactivity in normal adult mice (22, 25) were also immunopositive for Env proteins in NFLgp160Xba transgenic mice, with the notable exception of the cerebral and cerebellar cortices. However, a few brain areas (lateral hypothalamus, lateral reticular nucleus, and layers 4 to 8 of the spinal cord), which were immunopositive in one or both transgenic mouse lines, were found to be unlabeled for the neurofilament proteins in normal mice.

Neuropathological evaluation. The neuropathological evaluation of the CNS of 3-month-old 844 and 854 mice was done by using staining with HPS and modified Bielchowsky stain and immunostaining against HIV-1 Env proteins, GFAP, and the phosphorylated neurofilament triplet. The modified Bielschowsky and the phosphorylated neurofilament triplet reactions remained negative in the perikarya of gp160-positive nuclei of the brain stem and spinal cord anterior gray horn. These two stains, however, showed the expected positivity in axons of various tracts and nerve roots. The HPS staining did not demonstrate any sign of an inflammatory reaction, a migration disorder, or any developmental or acquired changes. (HPS staining was also used for evaluation of thoracic and abdominal organs; no inflammation or cytological changes were observed.) The anti-

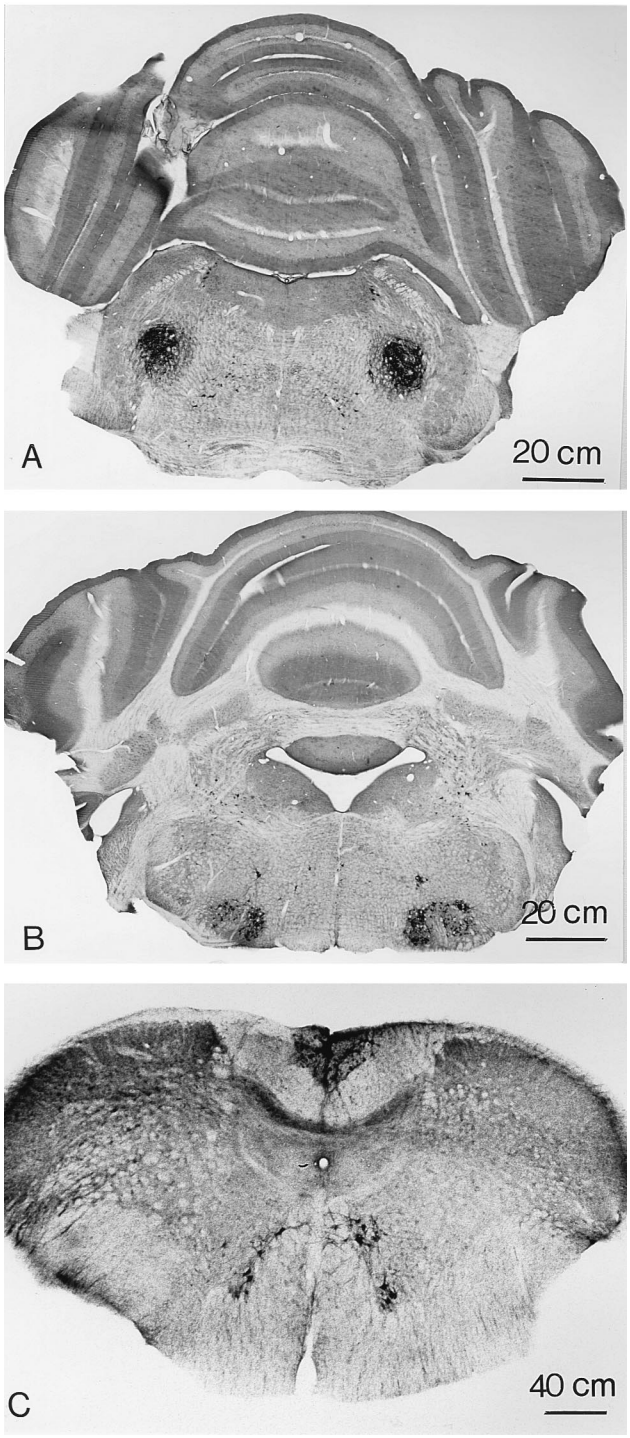

FIG. 5. Immunodetection of HIV-1 Env proteins in CNS sections from transgenic 844 and 854 mice with human anti-HIV-1 serum. (A) Immunolabeling of motor trigeminal neurons; (B) immunolabeling of the facial motor neurons; (C) immunostaining of neurons in the anterior gray horns of the spinal cord.

TABLE 1. CNS distribution of neuronal perikarya immunoreactive with HIV-1-infected patient serum or with monoclonal antibodies against gp41 or gp120[a]

Anatomical structure(s)	Reactivity of mouse line:	
	844	854
Hypothalamus (LH)	+	ND[b]
Mesencephalon (R, 3, SC, 4, DpMe, PCom)	+	+
Pons		
PnO/PnC, Mo5, Sp5, Acs5	+	+
Me5, 7, Acs7, LVe	+	+
VLL, PL	−	+
Medulla (12, Amb, MdV/Gi, LRt)	+	+
Spinal cord		
DRG, layers 4–8	+	+
Motoneurons	+	+

[a] The atlas of the mouse brain and spinal cord of (34) and the stereotaxic atlas for the rat (28) were used for anatomical references and nomenclature. Data were collected with transverse 50-μm-thick alternate serial sections through the whole brain and with sections of the cervical spinal cord and dorsal root ganglia. Neuroanatomical abbreviations: 3, oculomotor nucleus; 4, trochlear nucleus; 7, facial nucleus; 12, hypoglossal nucleus; Acs5, accessory trigeminal nucleus; Acs7, accessory facial nucleus; Amb, ambiguus nucleus; DpMe, deep mesencephalic nucleus; DRG, dorsal root ganglia; Gi, gigantocellular reticular nucleus; LH, lateral hypothalamic area; LRt, lateral reticular nucleus; LVe, lateral vestibular nucleus; MdV, medullary reticular nucleus, ventral part; Me5, mesencephalic trigeminal nucleus; Mo5, motor trigeminal nucleus; PCom, nucleus of the posterior commissure; PL, paralemniscal nucleus; PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part; Pr5, principal sensory trigeminal nucleus; R, red nucleus; Sp5, spinal trigeminal tract nucleus; SC, superior colliculus; VLL, ventral nucleus of the lateral lemniscus.

[b] ND, not determined.

HIV-1 immunostaining showed a similar topographic distribution of the Env proteins in sections from paraffin-embedded brain specimens, although these antibodies did not react as strongly as they did in nonembedded tissue. Most immunostained cells appeared normal in shape. The reaction was confined to the perikaryal area, with a variable extension into the dendrites (Fig. 6A). Initial segments of axons were also positive. Numerous small abnormal dendritic swellings were found in and around the most intensely stained motor nuclei, such as the motor trigeminal and the facial nuclei (Fig. 6B); they were also present in the anterior gray horn of the spinal cord. Interestingly, these dendritic swellings were not stained with antibodies against the phosphorylated neurofilament triplet or with the modified Bielschowsky method, which suggests that the neurofilaments were not involved in these changes. Additionally, HIV-1-immunoreactive axonal swellings were also observed in a region corresponding to the nucleus gracilis and to the gracilis and cuneate fascicles in the medulla and spinal cord (Fig. 6C). This positive immunoreaction indicated that the Env proteins were axonally transported. Similar swellings were occasionally found in the same regions of the CNS in control mice, but they were much more numerous and larger in transgenic animals. These abnormal swellings reacted positively for the phosphorylated neurofilament triplet and the modified Bielschowsky stain, but this reaction was variable in intensity. Finally some axonal swellings were found in other areas of the medulla and spinal cord (Fig. 6D). Besides these neuritic changes, the GFAP reaction suggested early reactive astrocytosis in several areas of the CNS. This was particularly evident around or close to the immunoreactive structures in the brain stem and spinal cord (Fig. 7c, d, g, and h). Interestingly, no reactive astrocytosis was observed in the cortex, where the expression of gp160 was undetectable, or in the normal control (Fig. 7a, b, e, and f). Thus, the astrocytosis we have observed appears to be associated with the expression of the Env proteins. Finally, the preliminary evaluation of the motor function of 3- to 4-month-old mice with the horizontal suspension test demonstrated no difference between the transgenic and the control animals.

DISCUSSION

The results described in this paper clearly showed that the NFLgp160Xba transgene was expressed in the two mice lines we have investigated. In both 844 and 854 mice, only HIV-1 *env* sequences were transcribed from the transgene. As anticipated with the NFL promoter, the *env* mRNA expression was restricted to the nervous system tissue in line 854; no transcriptional activity was detected in ectopic organs. In 844 mice, however, RNase protection experiments demonstrated the presence of *env* mRNAs not only in the CNS but also in the heart, liver, lung, and kidney. Such an ectopic expression could result from integration site effects, as already reported for various genes in different transgenic animals (35). The presence and localization of the Env proteins within the ectopic tissues have not yet been determined, but they do not appear to induce any effect as determined from the morphological aspects of these tissues after HPS staining. Notwithstanding these differences in mRNA expression patterns, the topographic distributions of the Env proteins in the CNS by immunostaining were identical for both 844 and 854 mice; they appeared mainly confined to the motor nuclei of the brain stem and anterior gray horns of the spinal cord (however, the presence of undetectable levels of the transgenic proteins in the immunonegative areas could not be ruled out). Additionally, in both mouse lines, immunoblotting as well as immunostaining demonstrated a lower signal for gp120 than for gp41, despite the fact that these proteins are produced at equimolar ratios. This could be due to the release of gp120 in the extracellular compartment or could result from its degradation, as previously suggested (38). In any case, the mere presence of gp41 is, in itself, evidence that gp120 is also produced.

The distribution pattern also shows that Env proteins were synthesized in neurons that express the highest levels of NFL protein. Additional structures, such as the lateral hypothalamus, the lateral reticular nucleus, and layers 4 to 8 of the spinal cord, where the NFL protein is normally not detected in perikarya, displayed Env-positive neuronal cell bodies. Surprisingly, no significant Env immunopositivity was observed in the cerebral cortex, although NFL protein is normally present in perikarya in layers II/III and V of the parietal cortex. This observation suggests either a lack of expression or the presence of an undetectable level of gp160 in the cortex. It could also be the effect of a neuronal loss due to neurotoxic properties of gp120. The loss of certain neuronal subpopulations in HIV-1-infected patients has been well documented; it was also reported to occur in GFAP-gp120 transgenic mice (38). If such a loss had occurred in NFLgp160Xba transgenic mice, one would predict that NFL immunoreactivity in the parietal cortex should also be reduced. To test this possibility, we performed immunostaining of alternate series of vibratome sections with anti-NFL and anti-HIV-1 antibodies. These experiments showed an apparently normal distribution of NFL-immunoreactive cell bodies in the cerebral cortex of the transgenic mice (data not shown), thus indicating that the absence of HIV-1 immunoreactivity in layers II/III and V in the parietal cortex does not seem to be related to neuronal loss in these structures. Studies are in progress to determine the Env protein expression pattern and effects in the cortex during embryonic development.

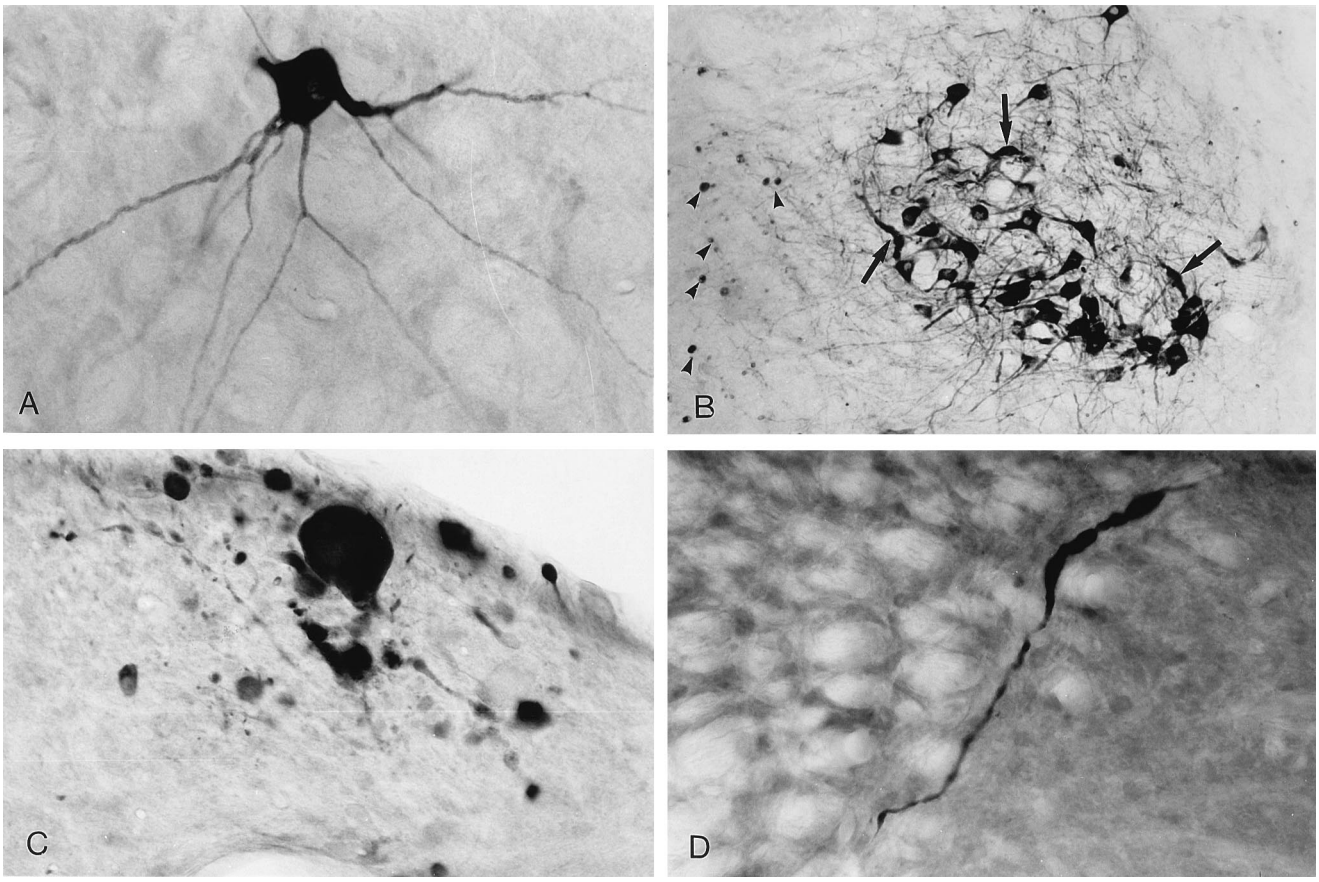

FIG. 6. Neuropathological changes associated with neuronal Env expression. Immunostaining was with human anti-HIV-1 serum. (A) Neuron of the reticular formation of the medulla. Intense immunostaining in the perikaryon with extension in the dendritic tree is seen. (B) Immunostaining of numerous neurons in the fifth motor nucleus. Elongated (arrows) or spherical (arrowheads) neuritic swellings are present in or around the nucleus. (C) Gracilis nucleus with numerous immunostained neuritic swellings of variable size. (D) Immunostained segment of one axon with row of swellings in the dorsal horn of the spinal cord.

Neuropathological evaluation of the two transgenic lines demonstrated cellular alterations manifested by two types of neuritic swellings. In brain regions with high levels of HIV-1 Env protein expression, i.e., the brain stem motor nuclei and the anterior gray horns of the spinal cord, such morphological anomalies were observed in the dendritic trees, sometimes quite distally. The negative reaction of the small neuritic swellings in the motor nuclei and anterior gray horns with the modified Bielschowski stain and with antibodies directed against the phosphorylated neurofilament triplet suggests that this anomaly did not involve neurofilaments. However, the involvement of the neurocytoskeleton should not be totally excluded until analyses of aging animals are completed. A second type of neuritic swelling was found in the gracilis nuclei and cuneate fascicles. These large, pleomorphic structures seem to represent an amplification of a normal physiological process, since similar, but fewer and smaller, spheroids were also present in normal mice. Their immunoreactivity with anti-HIV-1 antibodies shows that the Env protein was axonally transported. All of these changes appear to be moderate compared with those reported by Toggas et al. (38). They could represent a first step in the phenotypic manifestation(s) of neuronal dysfunction. Alternatively, these mild effects could be related to the low level of gp120 in neurons; indeed, the immunoreactions on the brain stem samples demonstrated a very weak signal for gp120 compared with that observed for gp41. Toggas et al. (38) have also observed that the severity of the neuropathological changes in GFAP-gp120 transgenic mice correlated with the level of gp120 mRNA expression. Finally, the pathological changes that we have found could be due to the relatively young age at which these animals have been analyzed. In addition to the neuritic changes, the GFAP staining demonstrated reactive astrocytosis in several CNS structures; recent data (unpublished) obtained from morphometric analyses of the spinal cord confirmed the presence of significant astrocytosis. These observations suggest that there may be a neuronal loss within and/or around the affected CNS regions of these young mice. This is being investigated by using a combination of immunological and histological stainings of semithin sections. Last, the absence of motor dysfunction as preliminarily observed with the horizontal suspension test appears consistent with the pathological findings in these young animals. Our interpretation is that either the neuronal changes are not substantial enough to result in phenotypic motor dysfunction or, as in the amyotrophic lateral sclerosis transgenic models (6), such a dysfunction could not be observed before the age of 8 to 12 months.

The neurocytological anomalies observed in the dendritic trees of motor neurons in our transgenic mice reproduced changes present in the cerebral cortex in patients with AIDS (23, 24). In patients, the possibility that these changes could have been caused by an inflammatory process rather than by the viral infection itself cannot be excluded (10). In NFLgp160Xba mice (so far), there was no inflammatory process, and hence the

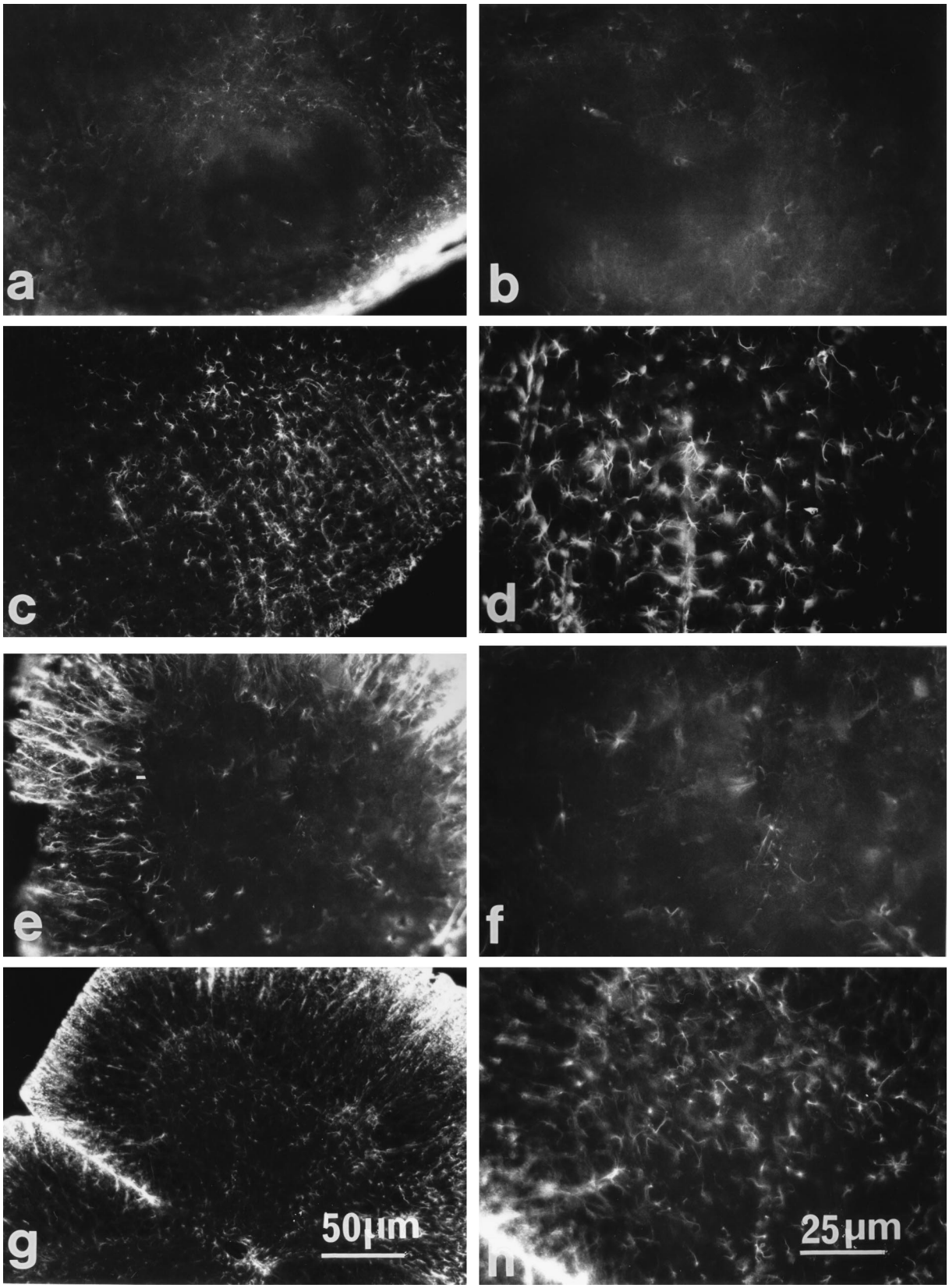

FIG. 7. GFAP reaction on CNS sections. The sections, prepared as for Fig. 3, were immunoreacted with fluorescein-conjugated anti-GFAP antibodies (Boehringer Mannheim) according to the instructions of the manufacturer. The astrocytosis revealed with these antibodies is closely associated with the neuronal structures expressing Env proteins. The seventh motor nuclei of normal (a and b) and transgenic (c and d) mice and the spinal cord anterior gray horns of normal (e and f) and transgenic (g and h) mice are seen.

observed changes could only be a direct effect of Env protein expression. In this context, these mice represent an interesting and valuable model for extensive analyses that could help define the role and the mechanisms of HIV-1 Env in neuronal toxicity. Furthermore, studies using offspring obtained from matings of different transgenic lines will help determine the effects of higher levels of gp120 on CNS morphology. Finally, the neuronal expression of Env proteins at levels detectable by immunocytochemistry in several CNS regions of these animals makes them a material of choice for studies of the neuropathological effects of gp120 during embryonic development and aging. These mice could also be a useful model for the development of efficient therapeutic agents.

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