Mouse Brain CD4 Transcripts Encode Only the COOH-Terminal Half of the Protein

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The T-cell surface glycoprotein CD4 is thought to function as a receptor for class II major histocompatibility complex molecules. Human CD4 is also the lymphoid cell receptor for human immunodeficiency virus, the causative agent of acquired immune deficiency syndrome. The observed infection of the central nervous system in acquired immune deficiency syndrome patients raises the possibility that CD4 is also present in nerve tissue and that a cell surface receptor for class II major histocompatibility complex antigens could play a role in central nervous system function. This possibility is reinforced by the detection of unique CD4-related transcripts in mouse and human brain tissue. In this study, the structure of the mouse brain CD4 transcript was determined. It is identical to the last two-thirds of the CD4 message and is capable of encoding a 217-residue protein that would consist of a truncated, 154-residue, cell surface region, together with the complete CD4 transmembrane and cytoplasmic regions. It would not include an amino-terminal hydrophobic leader peptide.

Mature T cells recognize antigen on the surface of cells bearing major histocompatibility complex (MHC) gene products. Recognition is mediated by the multicomponent T-cell receptor-T3 complex together with one of the two cell surface glycoproteins, CD4 or CD8. CD4 is expressed predominantly on helper T cells and is thought to stabilize the binding of the T-cell receptor with the antigen-MHC complex by interacting with invariant regions of class II MHC molecules. CD4 is a member of the immunoglobulin gene superfamily; the mature mouse protein consists of 431 amino acid residues and contains a 368-residue cell surface region that includes an amino-terminal immunoglobulinlike domain followed by a region with weak homology to the COOH-terminal half of a second immunoglobulinlike domain (16, 26). Two other members of the immunoglobulin gene superfamily, Thy-1 and OX-2, are expressed on both lymphoid and nerve cells (4, 9). Furthermore, class II MHC molecules, which are thought to interact with CD4, are expressed on the surface of oligodendrocytes in the brain (25). We therefore found it of interest that a transcript related to CD4 is expressed in mouse brain tissue, particularly since human CD4 is the receptor for human immunodeficiency virus, the lymphotropic, and possibly neurotropic, virus associated with acquired immune deficiency syndrome (AIDS; 17). Three groups of investigators recently reported the existence of CD4-related transcripts in mouse and human brain tissue (7, 17, 26) and speculated on the possible role of a brain-specific CD4 antigen in the apparently neurotropic behavior of human immunodeficiency virus. We report here the structure of the mouse brain transcript and discuss the implications of our findings for the existence of a distinct central nervous system receptor for the AIDS virus.

We isolated total and poly(A)⁺ RNA from brain, kidney, liver, spleen, and thymus of 4-week-old C57BL/6J mice to analyze the tissue-specific pattern of expression of CD4 (2). We separated the RNA on a 1.1% formaldehyde-agarose

gel, blotted it onto GeneScreen (New England Nuclear Corp., Boston, Mass.) nylon membrane, and hybridized it to nick-translated mouse CD4 cDNA clone p3C by the method of Church and Gilbert (3). We detected a 3.2-kilobase (kb) polyadenylated transcript in spleen and thymus tissue and a 2.3-kb polyadenylated transcript in brain tissue [Fig. 1; both transcripts were enriched by selection on oligo(dT) cellulose columns]. The 2.3-kb transcript appears to be brain specific since we did not see it in kidney, liver, spleen, or thymus. However, because it was expressed in brain at approximately 0.1% of the level of the 3.2-kb transcript in thymus and 1% of the level in spleen, it is possible that the small RNA also exists at these same low levels in spleen or thymus and simply cannot be detected above the background of degraded 3.2-kb transcript.

To determine the structure of the 2.3-kb transcript, we constructed a library of brain cDNA sequences cloned into the bacteriophage vector λ gt10. We probed the library with the mouse CD4 cDNA clone p3C (16) and established the structure of nine positive clones by nucleotide sequencing and fine-structure restriction mapping (Fig. 2). The clones overlap to span a region of 2.25-kb that is identical to the 3' two-thirds of the thymus CD4 cDNA clone pCD.L3T4.25 [16; nucleotide 871 to poly(A) tail]. The thymus cDNA clone pCD.L3T4.25 contains two polyadenylation signals separated by 7 nucleotides; we found examples of brain cDNA clones that used each of these signals.

The Northern blot (RNA blot) and cDNA cloning data alone do not rule out the possibility that the brain transcript contains a small 5' exon not included in the thymus transcript. We therefore performed primer extension and RNase protection experiments to determine the structure of the 5' end of the brain transcript. Because of the relatively low levels of this transcript in brain tissue, we were unable to detect unique primer extension products by using standard techniques. We therefore prepared poly(A)⁺ RNA specifically enriched for this species by hybrid selection (10) with the brain cDNA clone pCD5 (Fig. 2, lower panel). This clone

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FIG. 1. Northern blot of RNA isolated from brain (b), kidney (k), liver (l), spleen (s), and thymus (t) and probed with a mouse CD4 cDNA clone. Lanes: total, 12 μ g of total RNA; pA⁺, 6 μ g of poly(A)⁺ RNA; 1/10 t, 0.6 μ g of poly(A)⁺ RNA. The left panel shows a 6-day exposure of the probed blot to X-ray film, and the right panel shows a 1-day exposure of the same blot. The positions of the 28S and 18S rRNA species are indicated on the left.

consists of the last 1.4-kb of 3' untranslated sequence cloned into pBluescribe (Stratagene, San Diego, Calif.); it was chosen because it contains no sequences that could interfere with subsequent primer extension reactions. We used two end-labeled nonoverlapping synthetic oligonucleotides (the 27-mer, 5'-GTT TCC TTC AGC TGG AGC TTG AGG TCT-3' and the 30-mer, 5'-CTT CTC TGC CTT CCA CAT CAG CTC TCC CCA-3'; complementary to nucleotides 979 to 1,005 and 878 to 907, respectively, of the thymus cDNA clone pCD.L3T4.25) to prime reverse transcriptase reactions, with poly(A)⁺ and hybrid-selected C57BL/6J mouse brain RNA as templates. We performed the primer extension reactions as described by Hu et al. (13). A single major primer extension product appeared in each of the reactions in which we used hybrid-selected RNA as the template (Fig. 3). The products generated with the two oligonucleotides differ in length by the same number of nucleotides, 98, as the distance between the 5' ends of the oligonucleotides on the CD4 sequence. Thus, the two reactions serve as reciprocal controls and establish the position of the 5' end of the 2.3-kb

FIG. 2. Diagram of mouse CD4 cDNA clones. The structure of nine mouse brain cDNA clones is shown relative to the structure of the mouse thymus CD4 mRNA. The structure of the brain CD4-related mRNA, as deduced from the cDNA clones and from primer extension and RNase protection experiments, is shown beneath that of the thymus mRNA. The translated open reading frame of the thymus message and the potentially translated open reading frame of the brain message are indicated by boxes. Proposed structural features of the proteins: S, signal peptide; Ig, immunoglobulinlike domain; Ig', region with weak sequence homology to the COOH-terminal half of an immunoglobulinlike domain (26); M, transmembrane region; C, cytoplasmic domain.

FIG. 3. Primer extension analysis of the 5' end of the mouse brain CD4-related transcript. Lanes: 3' and 5', primer extension products primed with two different oligonucleotides (described in the text) staggered relative to each other in the CD4 sequence by 98 nucleotides; pA⁺, template for the primer extensions was poly(A)⁺ C57BL/6J mouse brain RNA; hyb. sel., template RNA was further enriched for CD4-related sequences by hybrid selection. Primer extension products were separated on a 6% polyacrylamide-urea gel. The sizes of the pBR322-MspI (pBR-Msp I) marker fragment bands shown are as follows (in descending order): 622, 527, 404, 309, 242 to 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, and 26 nucleotides (nt).

brain transcript at 26 nucleotides upstream from the most 5' nucleotide represented in the cDNA clones. We then performed an RNase protection experiment (31) to determine the identity of these 26 nucleotides. Hybridization of an antisense RNA fragment corresponding to nucleotides 702 to 994 of the T-cell-specific CD4 cDNA to brain RNA resulted in three sets of bands resistant to RNase digestion. The largest of these fragments, 150 nucleotides in length, corresponds to nucleotides 845 to 994. Therefore, RNase protection defines the same transcription start site as primer extension does, confirming that the 26 nucleotides upstream from the cDNA clone must be identical in brain and thymus. The combined results of the primer extension and RNase protection experiments establish that the 2.3-kb brain transcript initiates within the CD4 gene and that it simply represents a 5' truncation of the thymus CD4 mRNA (Fig. 4). The RNase protection experiment also confirms the observation of Maddon et al. (17) that the 2.3-kb transcript is enriched in the forebrain and shows that the smaller CD4 transcript is absent in the olfactory bulb and the cerebellum.

The shorter fragments, 125 and 100 nucleotides in length, seen in the RNase protection experiment may be due to alternative sites of initiation; however, because analogous products were absent in the primer extension, they are probably due to an artifact of the protection assay. The full-length 3.2-kb message was detected by RNase protection at low levels in RNA from all tissues. This is probably due to contamination of the tissues with blood, because the relative intensity of the band corresponding to the large transcript varied among experiments and because it was observed in tissues such as liver and brain that did not contain enough of the 3.2-kb transcript to be detected by Northern blotting.

FIG. 4. RNase protection analysis of the 5' end of the mouse brain CD4-related transcript. A uniformly labeled antisense SP6 transcript was hybridized to 4 μ g (thymus only) or 40 μ g of total RNA from tissues of 6-week-old C57BL/6J mice and digested with RNase A. Protected fragments were separated on a 5% acrylamide-urea gel. The first three lanes (Uncut, Spleen, and Thymus) represent 1-day exposures of the gel to X-ray film, and the last five lanes (Liver, Olfactory bulb, Forebrain, Midbrain/stem, and Cerebellum) are 7-day exposures.

The 2.3-kb mouse brain transcript appears to initiate at the T residue corresponding to nucleotide 845 of the CD4 cDNA clone pCD.L3T4.25 (Fig. 5). This is approximately in the middle of the CD4 coding region and occurs 57 nucleotides downstream from the nearest intron-exon junction in the CD4 gene (S. N. Gettner and D. R. Littman, unpublished observation). A possible promoter element, TATAAG, occurs 24 nucleotides upstream from the transcription start site. The longest open reading frame in the brain transcript

potentially encodes a 217-residue protein initiating with an in-frame (relative to CD4) AUG located 45 nucleotides downstream from the 5' end.

The predominant, 2.3-kb, CD4-related message in mouse brain is 900 nucleotides shorter than the T-cell message and would direct synthesis of a very different protein. This protein, 214 residues shorter than the mature thymus protein, would not contain an amino-terminal hydrophobic leader peptide; however, it would include a hydrophobic transmembrane region. The presence of a hydrophobic transmembrane region would presumably prevent the protein from folding properly if it cannot be inserted into the membrane (27). Therefore, it is unlikely that the encoded protein would function intracellularly. It is possible, however, that the 2.3-kb brain transcript is translated into a functional cell surface protein despite the fact that it does not encode an amino-terminal hydrophobic leader peptide; examples of secreted proteins without signal sequences have been reported (19, 20). In addition, it has recently been demonstrated that the sequence requirements for a signal peptide are not stringent (14), and it is possible that the leader peptide is simply not recognizable as such. One difficulty with this model is that a sequence capable of acting as a signal peptide in the truncated protein might act as a stop transfer signal in the full-length protein. Alternatively, the transmembrane region itself could function as an internal signal sequence. However, in this case, the brain CD4 protein would probably be inserted into the membrane in the opposite orientation, resulting in a 38-residue COOH-terminal extracellular region (24). The protein encoded by the mouse brain transcript would also be missing the 90-residue immunoglobulinlike domain found at the amino terminus of the mature CD4 protein. Because immunoglobulinlike domains are a common feature of cell surface proteins involved in cell recognition (11, 21), it is likely that this portion of the CD4 molecule plays a role in the recognition of class II MHC

FIG. 5. Sequence of the transcription start site of the brain CD4-related mRNA. A composite of mouse CD4 gene and cDNA sequence is shown with the point of initiation of the 2.3-kb transcript found in brain tissue. Lowercase letters indicate intron sequence (2). Translated amino acids and cDNA nucleotides are numbered according to Littman and Gettner (16). The heavy underlines indicate regions complementary to the two oligonucleotides used in the primer extension experiments. The light arrow indicates the region complementary to the primer extension products generated by using hybrid-selected mouse brain mRNA as a template. The most 5' cleavage site detected by RNase protection analysis is indicated above the sequence. The wavy arrow indicates the start site of transcription in brain tissue. A possible TATA box promoter element is boxed.

antigens. Therefore, the brain peptide, which does not have an amino-terminal immunoglobulinlike domain, would presumably function quite differently from CD4 even if it is expressed at the cell surface. Alternatively, the 2.3-kb transcript might not encode a functional protein. Upstream sequences may coincidentally resemble a brain-specific promoter, leading to serendipitous expression of the small RNA.

If the 2.3-kb brain transcript does encode a functional protein, then a single segment of the mouse CD4 gene has two overlapping functions—protein coding and transcription initiation—making it a unique example of a compact gene in a higher eucaryotic organism. This is a structural motif found in viruses (1), in which there is evolutionary pressure in favor of a compact genome. In organisms with large genomes, the expression of a single coding region from different promoters, or within different transcripts, usually results from differential RNA splicing (22, 30); presumably, the evolution of this pattern of gene expression is more rapid than the evolution of more compact genes with overlapping functions.

If the CD4 gene is an example of a compact gene in a higher eucaryote, then it might be expected to be under strong selective pressure to express both of its encoded protein products; thus, the overlapping gene structure would be expected to be conserved in closely related species. However, the analogous smaller transcript, if it exists in humans or rats, could not encode the same protein; the human and rat CD4 genes contain tryptophan and arginine codons, respectively, in place of the AUG found at the beginning of the long open reading frame in the mouse gene (5, 18). Furthermore, the nearest in-frame methionine codon is located 105 nucleotides downstream in the human gene and 120 nucleotides upstream in the rat gene. Maddon et al. (17) and Funke et al. (7) observed two different transcripts on Northern blots of human brain RNA probed with a human CD4 cDNA clone. One of these transcripts is 3.0-kb in length and appears to be identical to full-length T-cell CD4 message; the second transcript is smaller and comigrates with 18S rRNA in agarose gels. We have also observed this smaller transcript on Northern blots of human brain RNA. However, we do not believe that this message is analogous to the mouse brain transcript for the following reasons. (i) It was present in RNA samples from a number of different tissues. (ii) The intensity of the signal was reduced when the blots were hybridized and washed under more stringent conditions. (iii) Only the full-length transcript was detected by RNase protection experiments (data not shown). Thus, as suggested by Hoxie et al. (12), this band may represent cross-hybridization with residual 18S RNA. The presence of authentic CD4 message in human brain is likely to be due to macrophages, which are present in brain tissue and express CD4 (8). This view is consistent with the finding that macrophages are the predominant cell type infected with human immunodeficiency virus in brain tissue from AIDS patients (8, 15, 29). In contrast, mouse macrophages do not express CD4 (6), explaining why full-length CD4 message is not found in mouse brain tissue.

Definitive proof that the mouse brain CD4 transcript is nonfunctional will require genetic evidence; however, three features of the mouse brain CD4 transcript suggest that it is unlikely to be functional. First, the encoded protein lacks a conventional NH₂-terminal signal sequence. Second, the brain transcript initiates within a coding exon, requiring part of the gene to serve overlapping protein-coding and transcriptional promotion functions; this is an unusual structural

motif for a nonviral gene. Finally, the gene is not conserved across species; the human and rat CD4 genes cannot encode the same protein because they lack an initiator methionine at the correct position.

Tissue-specific transcription appears to be directed by combinations of factors that are unique to a particular cell type, while the individual factors themselves are expressed in a number of different cell types (23, 28). It may turn out that this system is fairly leaky and that the serendipitous recognition of random DNA sequences by *trans*-acting factors frequently causes the aberrant expression of genes in cell types in which they have no function. The expression of CD4 on human macrophages could be another example of an aberrant transcript. CD4 might be a tissue-specific pseudogene; although functional in one cell type, it is effectively a transcribed pseudogene in another. Sequences promoting aberrant transcription are analogous to junk DNA; i.e., as long as a particular pattern of expression is not detrimental, it will not be selected against even if it is nonfunctional. If this phenomenon turns out to be widespread, it will be important to rule out nonfunctional transcripts before drawing conclusions from certain experiments. In particular, such transcripts could complicate the interpretation of *in situ* nucleic acid hybridizations.

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ADDENDUM

S. D. Gorman, B. Tourville, and J. R. Parnes (Proc. Natl. Acad. Sci. USA **84**:7644–7648, 1987) have used an S1 nuclease assay to map the start of the mouse brain CD4 transcript to a site 2 nucleotides downstream from the start site we have identified.

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