Neuroantibodies: Molecular cloning of a monoclonal antibody against substance P for expression in the central nervous system

(antibody expression vectors/tissue-specific promoters)

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ABSTRACT   We present a strategy to study functional and/or developmental processes occurring in the nervous system, as well as in other systems, of mice. This strategy is based on the local expression of specific monoclonal antibodies (mAbs) by cells of the nervous system. As an application of this strategy, we report the cloning of the anti-substance P rat mAb NC1/34HL. Functional substance P-binding antibodies were reconstituted from the cloned variable domains by using vectors for expression in myeloma cells. With these and other vectors a general system for the cloning and expression of mAbs under a series of promoters (of the rat VGF8a gene, the neurofilament light-chain gene, and the methallothionein gene) has been created. The activity of these plasmids was confirmed by expressing the recombinant NC1/34HL mAb in GH3 pituitary cells, PC12 pheochromocytoma cells, and COS cells. DNA from the described constructs can be used to target the expression of the NC1/34HL mAb to the central nervous system of transgenic mice. This procedure will allow us to perturb substance P activity in a controlled way in order to dissect its multiple roles.

Functional and developmental studies on the mammalian nervous system would greatly benefit from the ability to specifically interfere with selected neuronal subpopulations or pathways. Injection of antibodies has been used with success in this aim, as, for example, in the classic immunosympathectomy experiment (1). The availability of hybridoma cell lines (2), together with existing gene-transfer techniques, suggests that, in principle, engineering the expression of specific antibodies in the nervous system of an organism (neuroantibodies) to perturb or modulate the activity of selected neuronal pathways should be possible.

As a prelude to this use of antibodies, we recently showed that immunoglobulins can be efficiently expressed as secreted (3) or intracellular (4) proteins by mammalian nonlymphoid cells. In particular, the efficiency of antibody secretion by cells related to the nervous system was shown comparable to that of lymphoid cells (3). Thus, the local secretion (by nervous system cells) of specific monoclonal antibodies (mAbs) could be used in functional and developmental studies on the otherwise intact nervous system of transgenic mice. To apply such an experimental strategy, we undertook a study of the physiological role(s) of the neuropeptide substance P (SP), a peptide belonging to the tachykinin family, that has been associated with the transmission of sensory and nociceptive information in the spinal cord, as well as with neurogenic inflammation (for reviews, see refs. 5 and 6). The activity of SP-containing primary afferents is under presynaptic inhibitory control by enkephalinergic interneurons. Antidromic release of SP from sensory

afferents is responsible for vasodilatation and plasma extravasation in neurogenic inflammation. SP is also present in central nervous system (CNS) areas, such as the substantia nigra and hypothalamus, where its function is unknown, and in peripheral neuronal structures, where it helps regulate smooth muscle motility. Evidence suggesting roles for SP as a growth factor (7) or as a messenger between the nervous and the immune systems (8) has also been presented. We report here the construction of general vectors for the expression of any mAb of interest in nonlymphoid mammalian cells, particularly in cells of the nervous system. These vectors have been used to clone the variable regions of the NC1/34HL mAb[†] (9), which recognizes the COOH-terminal part of SP. The NC1/34HL mAb inhibits the interaction of SP with its receptors *in vitro* as well as *in vivo*. Several different stable cell lines expressing the cDNA sequence encoding the NC1/34HL mAb were derived, and the antigen specificity of the antibody secreted by these cells was confirmed.

A discussion of the neuroantibody approach as a general tool for neurobiological studies is presented.

MATERIALS AND METHODS

Plasmids. Plasmids M13-VHPCR1, M13-VKPCR1, pSV-gpt-Huγl, pSV-hyg-HuC$_κ$, and pSW1-VHPOLY-TAG1 were supplied by Greg Winter (Medical Research Council, Cambridge, U.K.); plasmids pNF, pMT, and p[$^-$806 + 693]VGF8a were provided by Nicholas Cowan (New York University), Arturo Leone (Universitá di Napoli, Naples), and Roberta Possenti (Consiglio Nazionale delle Ricerche, Rome), respectively.

Cloning of Immunoglobulin-Variable Regions by PCR. Total cytoplasmic RNA or mRNA was prepared from ≈5 × 10^8 hybridoma cells, as described (10). Genomic DNA was prepared by resuspending 5 × 10^6 hybridoma cells in 0.5 ml of water, boiling 5 min, and centrifuging.

First-strand cDNA synthesis was done as described (11). PCR on 50–200 ng of cDNA or genomic DNA with the primers described in ref. 12 was essentially accomplished as in ref. 13. The amplified DNA was force-cloned into the M13-VHPCR1 and M13-VKPCR1 vectors (12) (M13-IgV$_H$SP and M13-IgV$_κ$SP) and sequenced (14).

The κ light-chain variable (V$_κ$) region was also cloned by conventional cDNA cloning (11, 15) starting from NC1/34HL mRNA, priming the first-strand cDNA synthesis from the constant κ light-chain region (C$_κ$) with the oligonucleotide

5'-AAG ATA GAT AAG CTT GGT GCA GCA TCA GCC-3' (16).

Plasmid Description. Plasmids were constructed for the expression of recombinant NC1/34HL heavy and light chains under the control of the immunoglobulin promoter (pSVgpt-IgV$_H$SP and pSVhygro-IgV$_\kappa$SP, respectively), the VGF8a promoter region (pSVgpt-VGF8aV$_H$SP and pSVhygro-VGF8aV$_\kappa$SP), and the neurofilament 68-kDa light-chain (NF68L) promoter (pSVgpt-NFLV$_H$SP and pSVhygro-NFLV$_\kappa$SP). All these plasmids were based on expression vectors described in ref. 12.

Cells, Transfections, and Immunofluorescence. Cells were grown, transfected, and analyzed by immunofluorescence, as described (3, 4, 17). The methallothionein promoter was induced by incubating transfected and control cells with 10 μM CdSO$_4$ for 24–48 hr.

ELISA and Immunoblotting. SP conjugated to bovine serum albumin (SP–BSA) was provided by J. Jarvis (Medical Research Council, Cambridge, U.K.). Tachykinin peptides (SP and the COOH-terminal fragments SP4-11, SP6-11, SP7-11, and eledoisin) were from Sigma. ELISA and immunoblotting were done as described (18, 19). Binding of antibodies was shown using biotinylated anti-human and anti-rat constant γ heavy-chain region (C$_\gamma$) antibodies and peroxidase–streptavidin (Amity, Milan).

The secretion from *Escherichia coli* cells of isolated heavy-chain variable region (V$_H$) domains from the NC1/34HL mAb (plasmid pSW1-V$_H$SP-TAG1) was obtained as described (13). Secreted V$_H$ domains were shown by immunoblotting (19) with the 9E10 mAb (from H. Pelham, Medical Research Council, Cambridge, U.K.) (20) directed against the myc peptide tag (21).

RESULTS

Cloning and Expression of the Variable Regions of mAb NC1/34HL. The variable regions of the rat mAb NC1/34HL (9) were PCR-amplified (12) from cDNA and genomic DNA derived from the hybridoma and sequenced after cloning (Fig. 1). The sequences obtained differ from all other known antibody variable regions (22), and the heavy chain appears most similar to the mouse III C V$_H$ family. The amino acid sequences corresponding to the regions of PCR primers differ in the cloned antibody from those in the light chain of mAb NC1/34HL (as determined from the mAb NC1/34HL light chain cloned by standard cDNA cloning procedures): Met-4 and Leu-106–Asn-107. However, such differences do not affect antigen-binding specificity, as demonstrated below.

The expression plasmids pSVgpt-IgV$_H$SP and pSVhygro-IgV$_\kappa$SP were stably transfected into NSO myeloma cells, and the expressed heavy and light chains were assayed by indirect immunofluorescence (see Fig. 3) and ELISA (see below).

To obtain the expression of anti-SP antibodies in cell lines related to the nervous system, the available expression vectors (12) were modified to include 5' regulatory sequences from the VGF8a gene (23) (which encodes a protein highly expressed in the CNS) and the NF68L gene (24) (Fig. 2). These are general vectors that can be used to express any variable region of interest cloned by using this PCR system; this fact will facilitate the future exploitation of our experimental strategy. The vectors have been used to express the recombinant NC1/34HL antibodies in cell lines permissive for the appropriate promoters: GH3 pituitary cells and PC12 pheochromocytoma cells. Staining by indirect immunofluo-
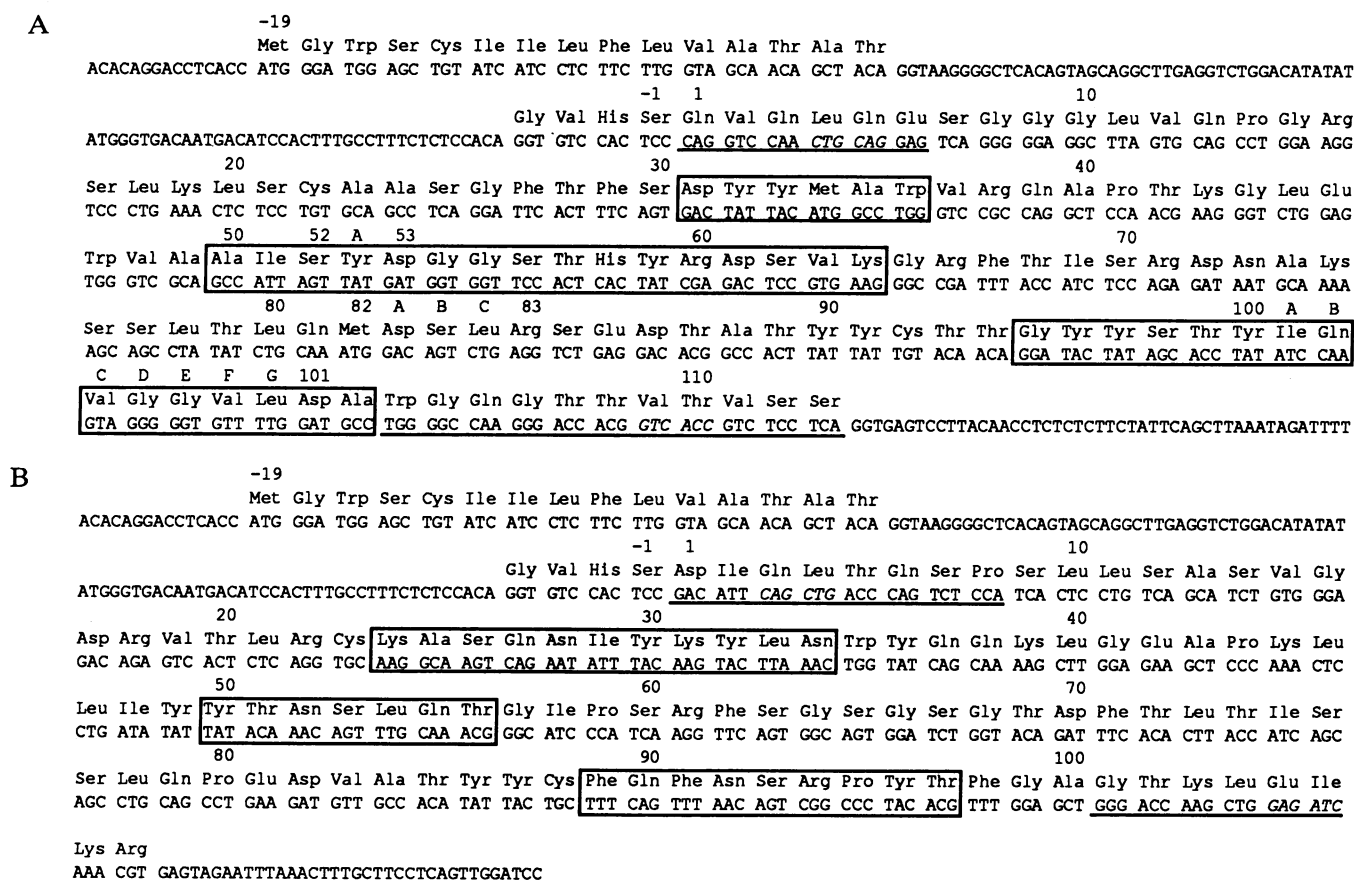

FIG. 1. Nucleotide and deduced amino acid sequence of NC1/34HL V$_H$ (*A*) and V$_\kappa$ (*B*). PCR primers are underlined; the enclosed restriction sites are in italics. Sequences up- and downstream of the amplification primers, including the leader sequence for secretion and intron sequences, are from M13-PCR vectors (12). Complementarity determining regions (CDRs) are boxed.
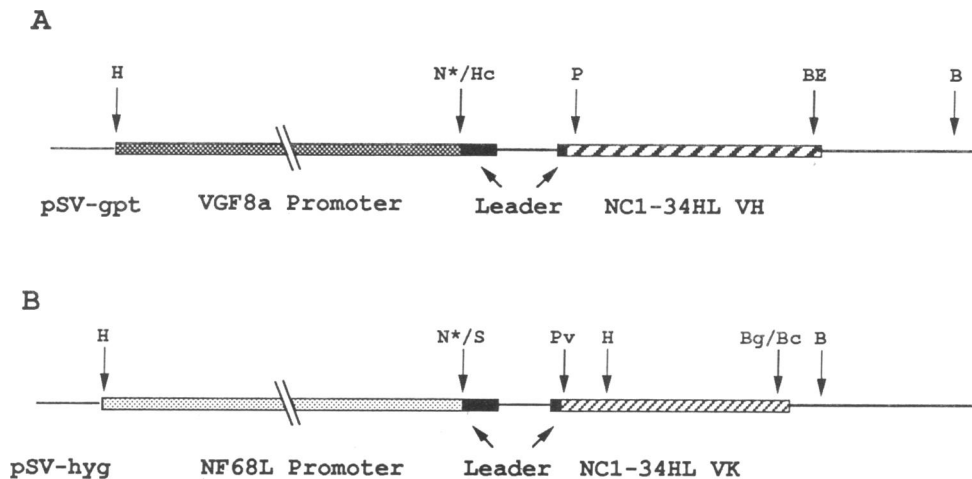
FIG. 2. Vectors for expression of NC1/34HL antibody. The region of the vectors encompassing the promoter region, the leader sequence for secretion, and the variable region are shown. The constant region (human $C\gamma$ or C_κ, as appropriate) is encoded by sequences 3' to the *Bam*HI site in the V–C intron. Only one of the two vectors prepared is shown for each promoter [promoter of the VGF8a gene (*A*) and of the neurofilament light chain (*B*)], but corresponding vectors for the other chain have been prepared. H, *Hin*dIII; N*, *Nco* I made blunt; Hc, *Hin*cII; P, *Pst* I; BE, *Bst*EII; B, *Bam*HI; S, *Sma* I; Pv, *Pvu* II; Bg, *Bgl* II; Bc, *Bcl* I.

rescence of stable GH3 transfectants (Fig. 3*B*) showed strong antibody expression driven by the VGF8a promoter. The activity of the NF68L constructs was assayed in PC12 cells, where nerve growth factor treatment has been reported to induce the expression of the endogenous neurofilament light-chain gene (25). Transient transfection of PC12 cells after incubation with nerve growth factor results in predicted expression of heavy- and light-chain polypeptides driven by the NF68L promoter (Fig. 4); expression levels, however, were consistently lower than those obtained with the corresponding VGF8a-driven constructs (data not shown).

In some cases inducibly controlling the expression of the antibodies may be useful. Vectors have been prepared for the expression of any variable region of interest under the control of the promoter of the metallothionein I gene (26), which is inducible by heavy metal ions. Analysis of transfectants shows that this promoter can, indeed, drive the regulated

expression of immunoglobulins in GH3 (Fig. 4*B*) and COS cells (data not shown).

Antigen-Binding Properties of the Recombinant NC1/34HL Antibodies. To verify the specificity of the cloned antibody, the supernatants of stable NSO (data not shown) and GH3 transfectants (Fig. 5*A*) were assayed by ELISA for SP binding. The results show that the recombinant antibody retains the same binding specificity for SP as the parental antibody (Fig. 5*B*). This result was confirmed in competition
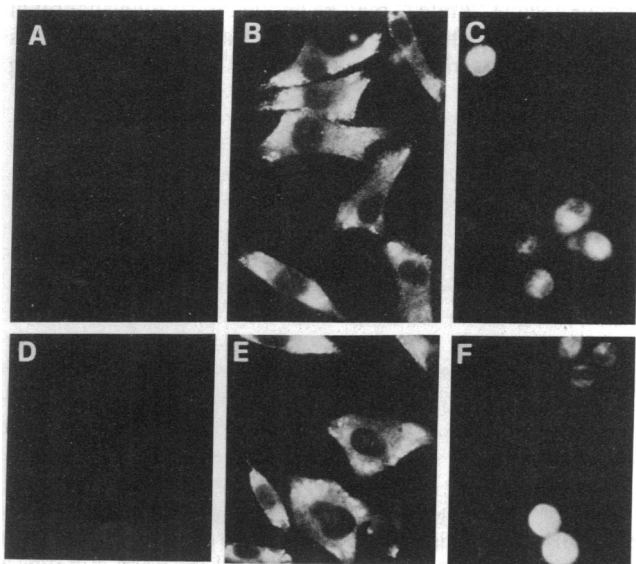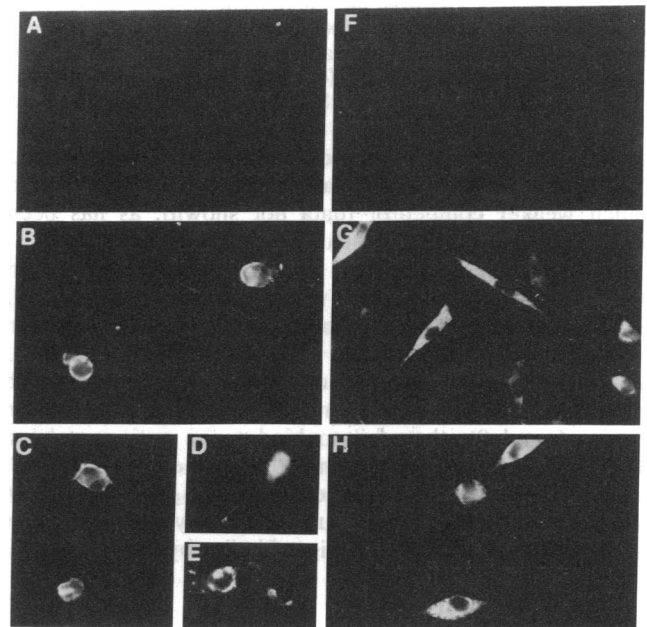

FIG. 4. Immunofluorescence of cells expressing immunoglobulin chains driven by the neurofilament and methallothionein promoters. Expression of recombinant antibody chains in PC12 cells driven by the NF68L promoter (*A–E*). PC12 cells, incubated with nerve growth factor for 1 (*B* and *C*) and 3 (*D* and *E*) days were assayed 48 hr after transfection for expression of the recombinant heavy (*B* and *D*) and light (*C* and *E*) chain. Untransfected PC12 cells are shown in *A*. Expression of recombinant antibody chains in GH3 cells are driven by the inducible methallothionein promoter (*F–H*). Transfected GH3 pituitary cells were incubated in the presence (*G* and *H*) or absence (*F*) of 10 μM cadmium and stained 48 hr later for expression of antibody heavy (*G*) and light (*H*) chain.

FIG. 3. Immunofluorescence of GH3[pSVgpt-VGF8aV$_H$SP/pSVhygro-VGF8aV$_\kappa$SP] and NSO[pSVgpt-IgV$_H$SP/pSVhygro-IgV$_\kappa$SP] transfectants. Untransfected GH3 (*A* and *D*), transfected GH3 (*B* and *E*), and NSO transfectants (*C* and *F*) were stained with antibodies against human heavy (*A–C*) or light (*D–F*) chain.
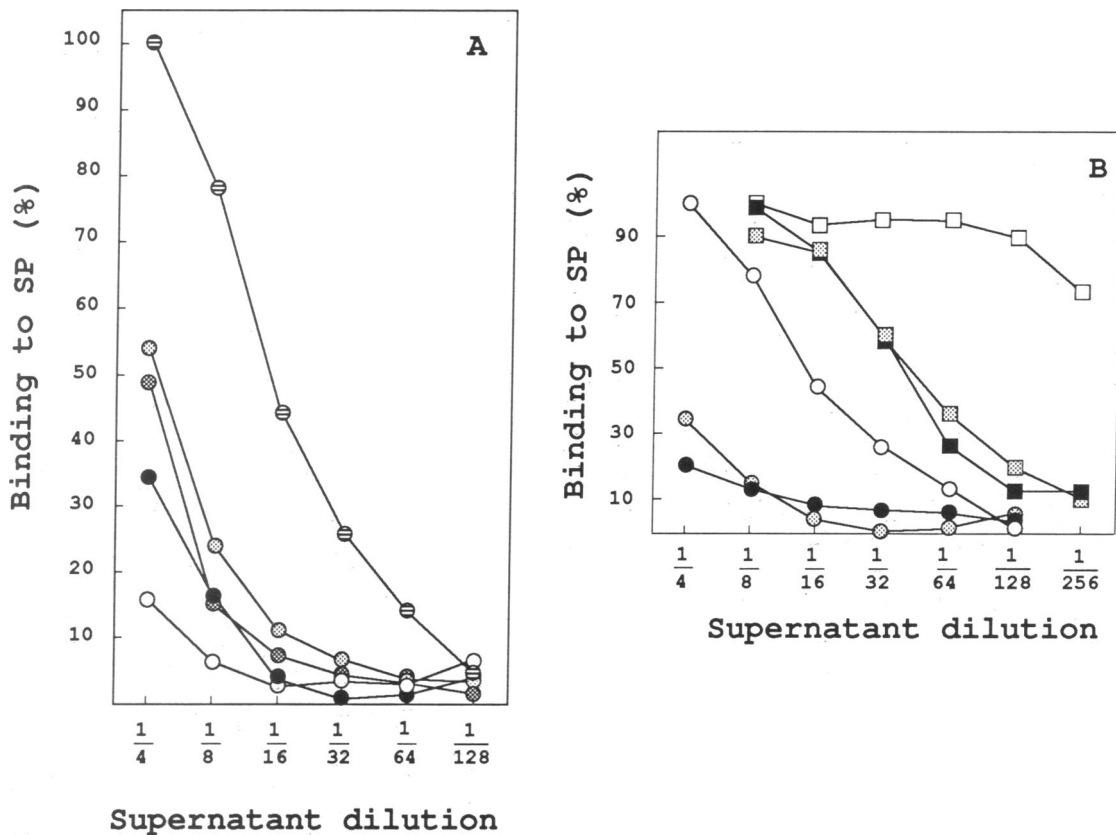
FIG. 5. Antigen-binding activity of recombinant (from a pool of GH3 stable transfectants) and parental NC1/34HL antibody to solid-phase-bound SP assayed by ELISA. (*A*) Competitive binding to SP was studied by preincubating the antibodies with different C-terminal peptide fragments (dissolved in 20 mM acetic acid and diluted in phosphate buffered saline with bovine serum albumin at 200 μg/ml). When appropriate, cell supernatants (100 μl) were incubated with the relevant peptides overnight at 4°C (4.5 pmol of peptide), before challenge with the solid-phase SP bovine serum albumin. Binding was evaluated as percentage of maximum. ⊖, GH3; ●, GH3 + SP; ◑, GH3 + SP-(7–11); ◓, GH3 + SP-(6–11); ○, GH3 + SP-(4–11). (*B*) Binding of recombinant (circles) and parental (squares) whole antibodies to solid-phase SP (empty symbols) after preincubation with SP (dotted symbols) or with SP plus molar excess of NC1/34HL single-domain antibody (dAb) (filled symbols).

experiments with the 5-, 6-, and 8-amino acid COOH-terminal SP fragments (Fig. 5). The related peptide eledoisin proved a much weaker competitor (data not shown), as has been described for NC1/34HL mAb (9).

V$_H$ alone has recently been shown in some cases to retain the binding specificity of the whole antibody molecule (13). It was therefore of interest to assess whether this situation was also true for the isolated V$_H$ domains from NC1/34HL mAb (antiSP-dAb). Fig. 6 shows that although antiSP-dAb molecules are quantitatively retained by an anti-tag column (lanes 6 and 8), they fail to bind to an antigen column (SP-Sepharose, lane 7). A competition experiment (Fig. 5*B*) also confirmed that the isolated anti-SP-V$_H$ domains do not recognize the SP peptide. Thus, both heavy and light chains are needed to reconstitute antigen binding by the NC1/34HL mAb.

DISCUSSION

A seminal series of experiments by Levi-Montalcini (reviewed in ref. 1) showed that antibody injections can represent an extremely valuable experimental strategy to perturb physiological processes in the development and function of the nervous system. The availability of hybridoma cell lines secreting mAbs of desired specificity (2), together with the demonstration of the efficient expression of immunoglobulin genes in nonlymphoid cells (3), allows, in principle, extension of this approach by using gene-transfer techniques (for review of these neurobiological applications, see ref. 27).

We now propose that targeting the expression of specific immunoglobulin genes to the CNS of transgenic mice, thereby using local secretion of selected antibodies as an immunological "knife," may be applied to studying neuronal development or physiology. As an initial application of this strategy, we describe here experiments aimed at expressing
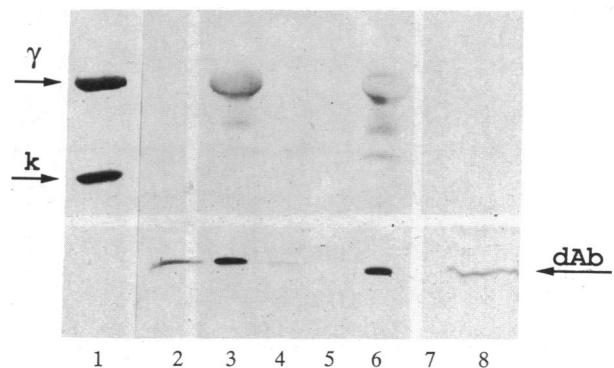

FIG. 6. Antigen-binding activity of isolated V$_H$ domain of the NC1/34HL mAb. Supernatants from *E. coli* cells secreting isolated V$_H$ domains from the NC1/34HL antibody (lanes 6 and 7) or from a nonrelevant antibody (lanes 3 and 4) were chromatographed on SP-Sepharose (lanes 6 and 3) columns. Bound material was analyzed by immunoblotting with mAb 9E10. Lanes: 2 and 8, unchromatographed supernatant from nonrelevant and NC1/34HL V$_H$ domains, respectively; 5, NC1/34HL V$_H$ domain chromatographed through a nonrelevant antigen column. γ, γ heavy chain; κ, κ light chain.

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a mAb directed against the neuropeptide SP in the nervous system of transgenic animals.

Among the different methods used to inhibit the function of selected genes in mammalian cells and the whole organism that are presently being pursued, those of gene disruption by homologous recombination (28), competition with mRNA function by antisense RNA (29) or ribozyme (30), or expression of dominant negative mutants of a gene (31) appear the most promising. Each of these methods, however, suffers from some drawbacks related to technical problems or to experimental design. The use of ectopic antibody expression, which we are proposing, is in some way similar to the expression of dominant negative mutants of a gene (31) but is more general in that it exploits the virtually unlimited repertoire of identical molecules, the antibodies. By the use of suitable promoters and intracellular tags (4), this approach will allow the expression of inhibitory antibody molecules in the appropriate cellular compartment or extracellular space (ref. 4 and see below) of any tissue of interest.

We have chosen the SP system as a model in which to test this strategy. The spectrum of activities described or suggested for SP is extremely widespread (5, 6). The expression of anti-SP antibodies in the CNS should provide a specific tool to interfere with SP function in neuronal targets (i.e., the CNS), where pharmacological perturbing agents such as capsaicin (32, 33) fail to act. The constructs described here should allow the appropriate targeting of antibody expression. In particular, the VGF8a gene (23) encodes a secreted protein that is highly expressed in many areas of the nervous system where SP is also found (34, 35). The NF68L constructs should yield a more widespread expression of the NC1/34HL antibodies throughout the CNS, but not elsewhere, as reported for a hybrid transgene (36). A complementary pattern of expression can be obtained by placing the NC1/34HL chimeric antibody transgene under the control of the immunoglobulin promoter, thus allowing us to study the role of SP in the immune system, as well as in other peripheral tissues. The failure of the isolated V_H of the NC1/34HL antibody to recognize SP could be exploited to make the expression of the NC1/34HL antibody both tissue-specific and inducible—by placing one antibody chain under the control of the promoter of the methallothionein gene (see Fig. 3) and the other chain under the control of a tissue-specific promoter. A more localized expression of the antibodies might be achieved by grafting embryonic cells infected with a recombinant retrovirus harboring the sequences that code for this antibody.

The neuroantibody approach described can be taken one step further by our recent demonstration of functional expression of antibodies (4, 17) or antibody domains (37) in different intracellular compartments of mammalian cells, such as cytosol or nucleus. This selectivity further expands the potential application range of the neuroantibody technique to many biological systems and to the CNS, in particular.

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