have been found in several nuclear proteins that bind to nucleic acids (*16*). This basic stretch is invariant among the HTLV-III isolates examined (*8*). No homology exists between the potential product of this region and other sequenced proteins, including the *tat* products of HTLV-I, HTLV-II, and BLV.

The conserved nature of this open reading frame among different AIDS virus isolates, the inclusion in spliced mRNA, and the dramatic reduction in *trans*-activating ability of proviruses with this reading frame deleted suggest that the potential protein product is involved in *trans*-activation. Since some of the deletion mutants containing this open reading frame exhibit slightly decreased *trans*-activating ability compared to the wild-type provirus, these data do not exclude the possibility that additional viral products play auxiliary roles in *trans*-activation. These results are consistent with studies of the HTLV-III *trans*-activating factor by means of complementary DNA (cDNA) expression vectors (*15*).

Characterization of the genomic regions of HTLV-I and HTLV-III encoding *trans*-activating factors, as well as their target sequences in the LTR, allows a comparison between *trans*-activation in these two viruses. In both viruses, *trans*-activation is accompanied by an increase in steady-state levels of LTR-directed RNA, with no replication of the template DNA (*14, 17*). Both the HTLV-I and HTLV-III target sequences in the LTR are located near the promoter element (TATA box) and include sequences 3′ to the mRNA start site (*14, 17*). *Trans*-activating factors do not stimulate the enhancers of either HTLV-I or HTLV-III (*14, 17, 18*). Both *trans*-activating factors can be made from multiply spliced mRNA's, which is unusual for retroviral messages (*4, 8, 15*). However, the location of the *trans*-activating regions on the genome and the sizes and characteristics of the potential protein products differ. Another difference is that sequences 5′ to the promoter of HTLV-I are necessary for the response to *trans*-activating factors (*17*). This is not the case for HTLV-III, in which the LTR sequences responsive to *trans*-activation are located between −17 and +80 (*14*). Thus, while the mechanism of *trans*-activation of the HTLV-I LTR appears to be an increase in the rate of transcription initiation, the mechanism whereby HTLV-III LTR-directed gene expression is increased in infected cells remains an open question.

The expression of host cellular genes might also be regulated by the *trans*-activating factors synthesized by the HTLV's. The expression of the HTLV-III *trans*-activating factor in specific T-lymphocyte subpopulations may result in the inappropriate expression of lethal or growth-suppressive genes. The availability of plasmids exclusively expressing the *trans*-activating factor should allow a direct test of this hypothesis.

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Immunocytochemical Detection of Acetylcholine in the Rat Central Nervous System

Abstract. *A specific antibody to acetylcholine was raised and used as a marker for cholinergic neurons in the rat central nervous system. The acetylcholine conjugate was obtained by a two-step immunogen synthesis procedure. An enzyme-linked immunosorbent assay was used to test the specificity and affinity of the antibody in vitro; the results indicated high affinity. A chemical perfusion mixture of allyl alcohol and glutaraldehyde was used to fix the acetylcholine in the nervous tissue. Peroxidase-antiperoxidase immunocytochemistry showed many acetylcholine-immunoreactive cells and fibers in sections from the medial septum region.*

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Although the importance of acetylcholine (ACh) among the putative neurotransmitters has long been recognized, little is known about its distribution in the central nervous system (CNS). What little we do know about the organization of CNS cholinergic neurons has been derived from the visualization of acetylcholinesterase, the degradative enzyme for ACh (*1*), and of choline acetyltransferase, the biosynthetic enzyme for ACh (*2*).

These approaches have been helpful, but the most desirable method would be direct visualization of ACh. Until now, however, a specific marker for the ACh molecule has been lacking. We report here the synthesis of an antibody to ACh and its use as a specific marker for cholinergic neurons in the CNS.

Two conditions must be met to obtain specific antibodies against small molecules: (i) the native structure of the molecule must be conserved in the immunogen and (ii) the antigenic determinant group should not exceed the sequence of three amino acid residues or their equivalent (*3*). Acetylcholine cannot be chemically conjugated (*4*) like other neurotransmitters (*5*). Therefore, to produce antibodies to ACh and fulfill the criteria for immunorecognition, we had to develop an immunological strategy compatible with the chemical structure of this molecule (Fig. 1).

Acetylcholine conjugates were synthesized with choline, glutaric anhydride, and polypeptides in order to conserve the structure of ACh (*6*). The conjugates were injected into four rabbits by a modi-

fication of the method of Vaitukaitis *et al.* (*7*). Every 3 weeks for 6 months the rabbits received choline–glutaryl–bovine serum albumin or choline–glutaryl–human serum albumin. Ten days after the last injection the specificity of the ACh antibody was tested in vitro and in tissues. The in vitro studies were done with an enzyme-linked immunosorbent assay (ELISA) (*8*) adapted for our purposes (Fig. 2) (*9*). The most immunoreactive compound, choline–glutaryl–poly-L-lysine, showed a displacement between 10^{-9} and $10^{-6}M$, indicating a high affinity. ACh and ACh analogs (choline and phosphatidylcholine) were poorly recognized. These results appear to be in good agreement with previous results for other antibodies against small molecules (*3*, *5*).

Immunocytochemical application of these antibodies to the CNS required a perfusion mixture that would (i) fix the ACh molecule in the tissue, (ii) mimic the hapten structure, and (iii) preserve the morphology of the tissues. Since the ACh molecule is an ester of choline, it cannot be directly fixed with standard fixatives such as glutaraldehyde or paraformaldehyde. Therefore, it was again necessary to find a chemical reaction that would not only activate the molecule but conserve its structure—that is, a reaction to induce desacetylization of ACh and simultaneously allow reacetylization by the intermediate of a radical

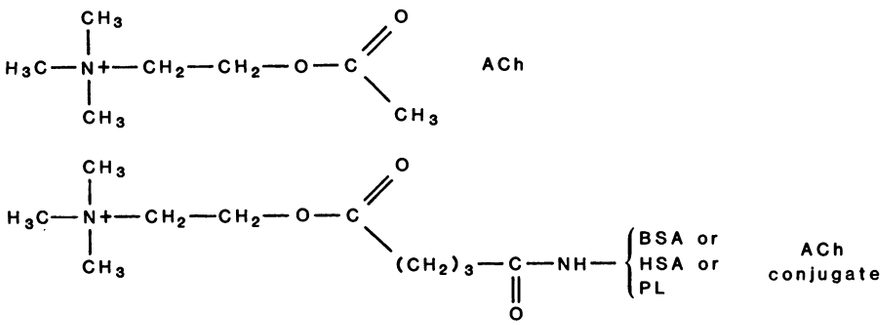

Fig. 1. Structures of ACh and choline-GA-protein (ACh conjugate).

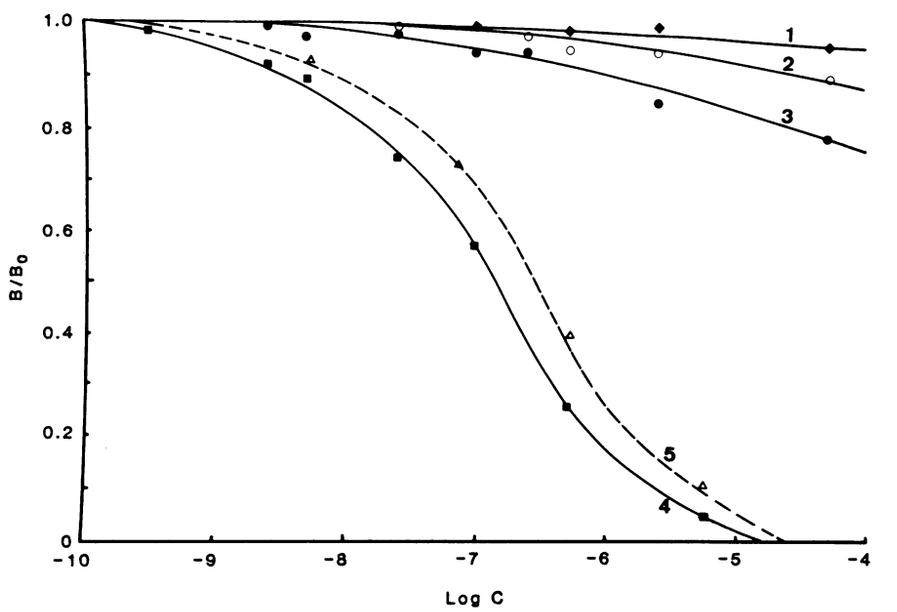

Fig. 2. Specificity of ACh antibodies, as determined with an ELISA (*9*). Before the tests the antiserum was purified on GA-BSA and GA-HSA to ensure that the coated GA-proteins did not contribute to the measured absorbance. Competition experiments were performed between choline-GA-BSA coated on the well plates and phosphatidylcholine (curve 1), choline (curve 2), ACh (curve 3), or choline–GA–poly-L-lysine (curve 4) previously incubated with antiserum. Curve 5 is a displacement curve obtained by methodology similar to that described above, the only difference being that the conjugate ACh–allyl alcohol–glutaraldehyde–protein was first incubated with the antiserum. Competition experiments were performed between choline-GA-BSA coated on the well plates and ACh–allyl alcohol–glutaraldehyde–protein previously incubated with ACh antibodies. Curve 5 is almost superimposable on curve 4. The other compounds synthesized with allyl alcohol, glutaraldehyde, or both—ACh–allyl alcohol–protein, ACh-glutaraldehyde-protein, choline–allyl alcohol–protein, choline-glutaraldehyde-protein, and choline–glutaraldehyde–allyl alcohol–protein—were not recognized by ACh antibodies. These compounds were synthesized in vitro. The coupling reactions were identical to those used during histological fixation. Addition of [^{3}H]ACh or [^{3}H]choline allowed calculation of the molar coupling ratio of ACh or choline to protein after dialysis against water. B/B_0 is the ratio of the absorbances with competition (B) to that without competition (B_0). Each data point represents the mean for three experiments. Standard deviations were too small to be included on the graph.

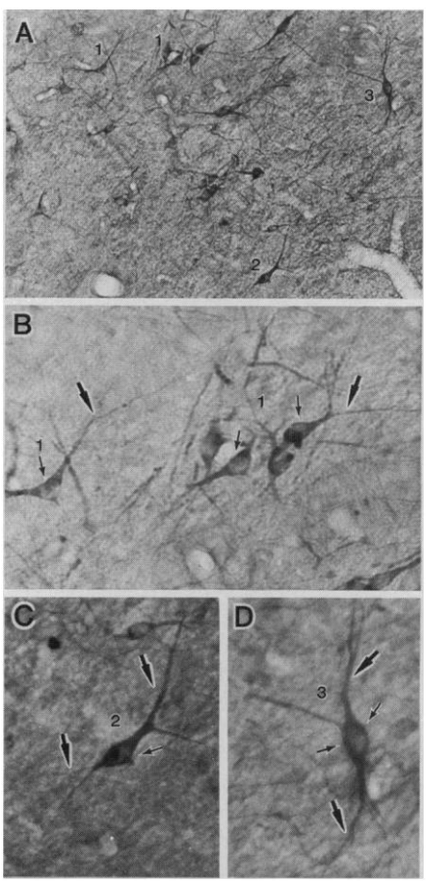

Fig. 3. (A to D) Photomicrographs of frontal sections (50-μm) of the medial septum region. The perfusion mixture consisted of 5 percent glutaraldehyde and 0.5*M* allyl alcohol in cacodylate buffer (*p*H 11). The sections were incubated overnight at 4°C with ACh antibody (1:1500) and processed for peroxidase-antiperoxidase immunocytochemistry. (A) Numerous ACh-immunoreactive cells and processes throughout the region (×300). The neurons indicated by 1, 2, and 3 are shown at higher magnification (×750) in (B), (C), and (D). (B) Cluster of ACh-immunoreactive cells (small arrows) and fibers (large arrows). (C) A representative cholinergic neuron. Notice the dense, uniform staining throughout the cytoplasm (small arrow) that extends into the fine processes (large arrow) emanating from the cell body. Branching of the dendrites can be observed (large arrow). In the upper right-hand corner a nonimmunoreactive cell appears to be in contact with this immunoreactive one. (D) An oval-shaped cell with numerous branching processes (large arrow) emanating from it.

long enough to attach ACh to tissue proteins. For these reasons we used a perfusion mixture of allyl alcohol and glutaraldehyde at *p*H 11, which allowed desacetylization of ACh and, simultaneously, transesterification. A bifunctional agent, glutaraldehyde allowed the newly formed hapten to be attached to tissue proteins.

Ten male rats (250 to 300 g) were perfused transcardially with glutaraldehyde (0.5*M*) and allyl alcohol (0.5*M*) in 0.1*M* cacodylate buffer (*p*H 11). The brains were removed, sectioned, and postfixed in the perfusion mixture without allyl alcohol. Sections (50 μm) were made with a Vibratome through the region of the medial septum. The sections were incubated at 4°C overnight with ACh antibody (1:1500), serum from unimmunized animals, or ACh antibody preadsorbed on choline–glutaryl–poly-L-lysine at the same dilution and were then processed for peroxidase-antiperoxidase immunocytochemistry. Figure 3 shows ACh-immunoreactive cells and fibers in the region of the medial septum. No positive staining was observed with the preadsorbed or pre-immune serum. Hence the perfusion mixture fulfilled our requirements in that (i) ACh was retained in the CNS tissue, (ii) the areas in which ACh was detected correspond to areas in which cholinergic neurons have been identified by other techniques (*1*, *2*), and (iii) as shown in Fig. 3 the ACh-immunoreactive cells maintained the morphological integrity of cholinergic neurons.

As a specific marker for cholinergic neurons in the CNS, the ACh antibody has several advantages over available immunocytochemical markers. Although antibodies to choline acetyltransferase are considered to be reliable markers for cholinergic neurons (*2*), preparation of the enzyme involves numerous purifications and specificity tests. Furthermore, application of the antiserum is restricted to species closely related to the one from which the enzyme was obtained. Such laborious procedures and restrictions are not encountered when raising ACh antibodies, since the antiserum is acquired after immunization with conjugates obtained through chemical reactions. We have successfully used the antibody to visualize cholinergic neurons in invertebrates (*10*).

The high specificity of the ACh antibody may enable the processes of cholinergic neurons to be visualized as well as the cell bodies. In a preliminary investigation fibers in the substantia nigra and striatum were stained with the antibody.

Recently we presented data (*11*) showing that the perfusion mixture can be used for the fixation of norepinephrine, dopamine, and serotonin in the rat CNS. This advantage of our procedure may enable interactions of the cholinergic system to be elucidated at both the light and electron microscopic levels with respect to other neurotransmitters. Unraveling these anatomic and functional relations may improve our understanding of neurological disorders such as Alzheimer's disease, parkinsonism, and Huntington's chorea, in which the role of ACh and other neurotransmitters is receiving more and more attention.

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9. Polystyrene well plates (Nunc) were coated with GA-BSA solution or choline-GA-BSA solution in 0.05*M* carbonate buffer (*p*H 9.6) at 4°C. After 16 hours they were filled with phosphate-buffered saline (PBS) containing 0.05 percent Tween 100 plus 1 percent bovine serum and incubated for 30 minutes at 37°C. The specific colored reaction measured from choline-GA-BSA enabled displacement curves to be established by competition. The antibody (1:5000) with or without the compounds was applied for 90 minutes at 37°C. Then the well plates were incubated for 45 minutes with goat antiserum to horseradish peroxidase–labeled rabbit antibody (1:2500) plus 1 percent bovine serum. Plates were washed with PBS and Tween 100 between each step. Peroxidase was assayed by incubation (10 minutes) with *o*-phenylenediamine dihydrochloride (10 mg/ml) in 0.1*M* citrate phosphate buffer (*p*H 5) containing 0.03 percent hydrogen peroxide. The reaction was stopped by adding 50 μl of 4*M* H_2SO_4 per well and the absorbance was read at 492 nm on a Multiskan Titertek. The blank values were subtracted from the experimental ones.
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Ribonuclease P Catalysis Differs from Ribosomal RNA Self-Splicing

Abstract. *Two RNA-catalyzed reactions have been described, the* Tetrahymena *self-splicing ribosomal RNA and ribonuclease P. The* Tetrahymena *self-splicing reaction proceeds through a transesterification cascade that is dependent upon nucleophilic attacks by ribose 3′-OH groups. Periodate oxidation of the catalytic (or substrate) RNA, which destroys the nucleophilicity of RNA 3′ termini, did not inhibit ribonuclease P activity. Thus, catalysis by ribonuclease P differs from the self-splicing reaction.*

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Novel biopolymer-catalyzed reactions involving the manipulation of nucleic acid phosphodiester bonds by catalytic RNA's have been reported. The first example, discovered by Cech and coworkers, is the "self-splicing" reaction of an intron-containing precursor of 26*S* ribosomal RNA (rRNA) from *Tetrahymena thermophila* (*1*). The 413-nucleotide intervening sequence is precisely excised from the mature rRNA through the action of the RNA itself, when guanosine is used as a cosubstrate. There is no requirement for a protein in this reaction.

The second example of an RNA-cata-