Location of the *Trans*-Activating Region on the Genome of Human T-Cell Lymphotropic Virus Type III

Abstract. *The retrovirus involved in acquired immune deficiency syndrome (HTLV-III/LAV) contains a region that is necessary for stimulation of gene expression directed by the viral long terminal repeat. This region is located between nucleotides 5365 and 5607, immediately 5′ to the envelope gene. A doubly-spliced message containing this region could encode an 86–amino acid protein with structural features similar to those of nucleic acid–binding proteins.*

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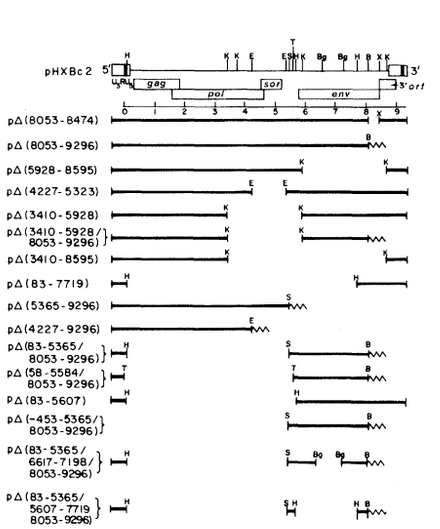
Human T-cell lymphotropic virus type III (HTLV-III/LAV) is the etiological agent of the acquired immune deficiency syndrome (AIDS) and associated diseases (*1*). Like HTLV types I and II and the related bovine leukemia virus (*2*), gene expression directed by the long terminal repeat (LTR) of HTLV-III is stimulated in *trans* in infected cells (*3*). The virus-associated *trans*-acting factors are specific for the infecting virus and have been postulated to play critical roles in increasing viral gene expression and possibly in pathogenesis (*2, 3*).

Trans-activation of the HTLV-I and HTLV-II LTR's is mediated by a novel gene product (*4*). In addition to the virion structural proteins and reverse transcriptase, the genomes of HTLV-I and -II encode 42- and 38-kilodalton (kD) proteins, respectively (*5*). These nuclear proteins (*6*) are encoded by a gene called *tat*, for *trans*-activating transcriptional regulation, located within the pX region between the envelope glycoprotein gene and the 3′ LTR (*7*).

Identification of the region in HTLV-III that is responsible for *trans*-activation was not possible by direct analogy with HTLV-I or HTLV-II since the organization of the HTLV-III genome differs from that of the other HTLV's. Five long open reading frames have been identified in HTLV-III (Fig. 1) (*8*). In addition to the *gag, pol,* and *env* genes, there are two open reading frames, one located immediately 3′ to the *pol* gene (*sor*) and one 3′ to the *env* gene (3′ *orf*) (*8*). Numerous other open reading frames that could encode polypeptides of 10 kD or smaller also exist (*8*).

Recently, a complete clone of HTLV-III proviral DNA was shown to produce infectious virions upon transfection into lymphocytes (*9*). Initially, we determined whether *trans*-activating factors specific for the HTLV-III LTR could be expressed by this complete proviral clone. Lymphoid and nonlymphoid cells were cotransfected with a plasmid that contained the complete HTLV-III provirus (pHXBc2) and an indicator plasmid that contained the HTLV-III LTR 5′ to the gene for chloramphenicol acetyltransferase (CAT) of *Escherichia coli* (pU3R-III CAT) (*3, 10*). Cotransfections were also done with the pSV2CAT plasmid, which contains the SV40 early promoter 5′ to the CAT gene (*10*). Cells of lymphoid and nonlymphoid lineage were used as recipients. At 48 hours after transfection, CAT enzyme activity, which correlates with CAT messenger RNA (mRNA) levels (*11*), was assayed, providing a measurement of promoter strength for the sequences 5′ to the CAT gene. All experiments to be described were performed at least twice with a range of values of not more than ±30 percent of the reported value. The effect of plasmid pHXBc2 on CAT activity directed by pSV2CAT was the same as that of a control plasmid that contained only the HTLV-III LTR sequences (pIII) (Table 1). By contrast, a marked stimulation of LTR-directed CAT activity was observed in pU3R-III CAT after cotransfection with pHXBc2 but not with the negative control DNA. We conclude that the entire HTLV-III provirus in plasmid pHXBc2 encodes *trans*-acting factors that stimulate HTLV-III LTR-directed gene expression.

The stimulation of LTR-directed CAT activity by the HTLV-III provirus in clone pHXBc2 was seen in all cell lines examined (Table 1). These included feline epithelial cells as well as human lymphoid and epithelial cell lines. Thus, neither efficient promoter activity nor *trans*-activation of the HTLV-III LTR is restricted to cells that normally serve as targets for viral infection. This observation demonstrates that, unlike the murine leukemia viruses (*12*), cell type–specific restriction of LTR-directed gene expression is not a major determinant of the tropism of these viruses.



	Conversion of chloramphenicol (%)	
	HeLa	H9
pHXBc2	55.3	24.0
pΔ(8053-8474)	53.5	ND
pΔ(8053-9296)	53.7	21.0
pΔ(5928-8595)	13.4	14.1
pΔ(4227-5323)	29.5	19.6
pΔ(3410-5928)	0.7	0.3
pΔ(3410-5928/8053-9296)	0.7	ND
pΔ(3410-8595)	0.7	0.3
pΔ(83-7719)	0.8	0.3
pΔ(5365-9296)	0.7	0.4
pΔ(4227-9296)	0.8	0.3
pΔ(83-5365/8053-9296)	64.4	35.2
pΔ(58-5584/8053-9296)	0.8	0.3
pΔ(83-5607)	0.7	0.3
pΔ(-453-5365/8053-9296)	2.6	ND
pΔ(83-5365/6617-7198/8053-9296)	66.8	ND
pΔ(83-5365/5607-7719/8053-9296)	67.2	ND

Fig. 1. Structure and *trans*-activation ability of the HTLV-III deletion mutants. The restriction map of the complete HTLV-III provirus in plasmid pHXBc2 is shown (H, Hind III; K, Kpn I; E, Eco RI; S, Sal I; T, Sst I; Bg, Bgl II; B, Bam HI; X, Xho I). The LTR's are indicated by boxes at either end of the provirus with the U3, R, and U5 regions depicted. Numbers correspond to the nucleotide numbers of the deletion endpoints and are based on the sequence of Ratner *et al.* (*8*), where the RNA cap site is designated +1. All deletion mutants were made with available restriction endonuclease cleavage sites (shown above the deletion endpoints), in some cases in the presence of ethidium bromide to promote partial digestion. All deletion mutants utilize HTLV-III LTR sequences responsive to *trans*-activating factors as promoters. The promoter for plasmid pΔ(83-5365/8053-9296) and derivatives consists of HTLV-III LTR sequences from −167 to +83. All 3′ *orf*-related sequences are deleted from this promoter, which retains the ability to respond to *trans*-activating factors (*14*). The zig-zag lines mark the position of polyadenylation and splice signals derived from the SV40 small t–antigen coding region. Transfections were carried out as described in the legend to Table 1. The percentage conversion of chloramphenicol to its acetylated forms was measured after transfection of HeLa (human epithelial) or H9 (human T lymphocyte) cells with the test plasmid and the pU3R-III CAT plasmid; a 1-hour time point and equivalent amounts of protein lysate were used for each reaction. ND, not determined.

The stimulation of HTLV-III LTR-directed CAT activity in the cotransfection experiments was less than that observed in the same cell lines infected with HTLV-III (*3*). This difference may be due to the lower levels of viral protein expressed transiently in transfected cells as compared with those produced in infected cells (*13*). To determine if the effect observed in the cotransfection experiments was qualitatively the same as that in infected cells, the response of CAT plasmids that carried alterations in the HTLV-III LTR sequences affecting in vivo *trans*-activation (*14*) was analyzed in cotransfection experiments with the pHXBc2 plasmid. The LTR mutants that could respond to *trans*-activating factors in virus-infected cells also responded to factors produced upon cotransfection with the pHXBc2 plasmid (Table 2). Those LTR mutants that had lost the ability to respond to the factors present in infected cells were not activated by cotransfection with the pHXBc2 plasmid. We conclude that the stimulation of HTLV-III LTR-directed CAT activity observed upon cotransfection with pHXBc2 DNA is qualitatively the same as the effect observed in cells infected with HTLV-III virions.

To understand what regions of the HTLV-III genome are necessary for determining *trans*-activation, deletions were introduced into the pHXBc2 plasmid. All deleted plasmids retained an LTR capable of *trans*-activation as a promoter element. Because the HTLV-I and HTLV-II *trans*-activating proteins are encoded by the 3′ end of the genome (*4*), our initial effort focused on testing deletions in this part of the HTLV-III genome (Figs. 1 and 2). Plasmids with deletions of major portions [pΔ(8053-8474)] or all [pΔ(8053-9296)] of the 3′ long open reading frame (3′ *orf*) had nearly the same *trans*-activating activity as the wild-type pHXBc2 plasmid. Plasmid pΔ(5928-8595), in which almost the entire *env* gene and the 3′ open reading frame are deleted, retained *trans*-activating ability, albeit at a reduced level compared to the pHXBc2 plasmid. In plasmid pΔ(83-5365/8053-9296), all of the 3′ long open reading frame sequences in the 5′ LTR were also deleted, yet the plasmid retained the ability to stimulate HTLV-III LTR-directed CAT expression (see below). These experiments exclude the 3′-*orf* and the complete *env* gene as sequences required for *trans*-activation.

Plasmid pΔ(4227-5323) has the entire potential coding region designated *sor* deleted. This plasmid retained most of the *trans*-activating ability of the wild-type plasmid. Thus, *sor* is not necessary for *trans*-activation.

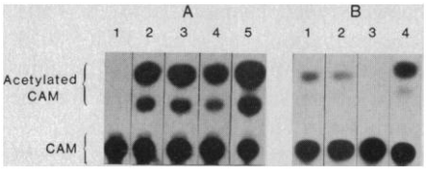


Fig. 2. Typical CAT assays after cotransfection into H9 (A) or HeLa (B) cells. Conversion of chloramphenicol (CAM) to its acetylated form after a 60-minute reaction is shown. CAT plasmids utilized for transfection were pU3R-III (lanes A1–A5), pSV2CAT (lanes B1 and B2), or pSV2/-17 (lanes B3 and B4). Cotransfected plasmids used were pIII (lanes A1, B1, and B3), pHXBc2 (lane A2), pΔ(8053-9296) (lane A3), pΔ(5928-8595) (lane A4), and pΔ(83-5365/8053-9296) (lanes A5, B2, and B4).

Deletion of the region from nucleotides 3410 to 5928 in the plasmids capable of *trans*-activating HTLV-III LTR gene expression consistently resulted in a loss of activity (Fig. 1). For example, although pΔ(4227-5323) was competent for *trans*-activation, plasmid pΔ(3410-5928) exhibited no *trans*-activating ability. The 3′ boundary of the necessary region was determined by placing SV40 polyadenylation signals at nucleotide positions 4227, 5365, and 8053. While pΔ(8053-9296) retained *trans*-activating ability, plasmids pΔ(4227-9296) and pΔ(5365-9296) were inactive. We conclude that the 3′ boundary of the region necessary for *trans*-activation lies between 5365 and 8053. The actual 3′ boundary is likely to be between 5365 and 5928 since plasmid pΔ(5928-8595) also showed *trans*-activating ability.

To define the 5′ border of the region necessary for *trans*-activation, the HTLV-III LTR was placed at nucleotides 5365, 5584, and 5607. Plasmid pΔ(83-5365/8053-9296) *trans*-activates the CAT plasmid containing the HTLV-III LTR, but plasmids pΔ(58-5584/8053-9296) and pΔ(83-5607) do not (Fig. 1). This indicates that the 5′ boundary of the region necessary for *trans*-activation lies between nucleotides 5365 and 5584. Furthermore, sequences from 5365 to 8053 are clearly sufficient for *trans*-activation,

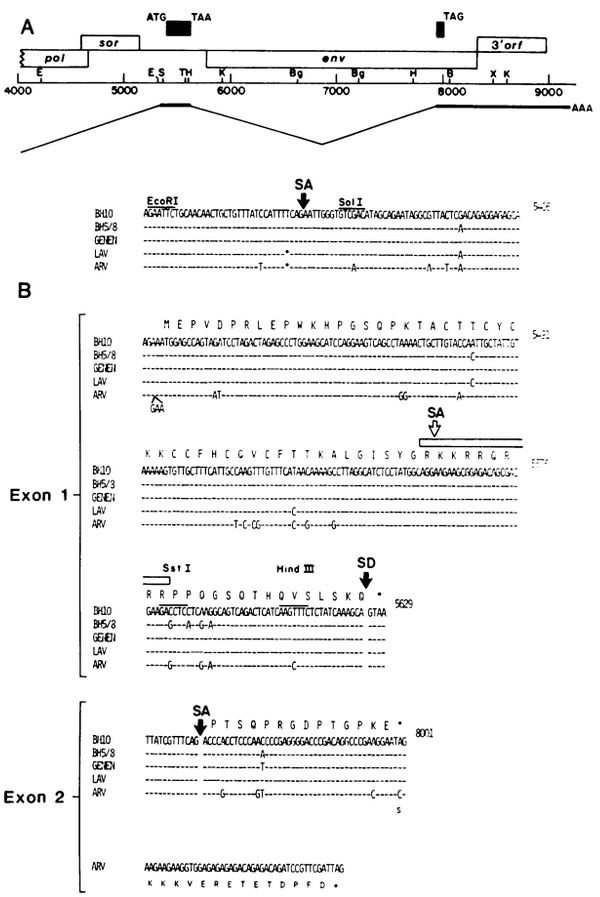


Fig. 3. The HTLV-III/LAV genome and potential protein product. The 3′ half of the HTLV-III/LAV genome is shown in the upper figure. The restriction endonuclease map is depicted, with enzyme abbreviations as in Fig. 1, and nucleotide positions corresponding to those of Ratner *et al.* (*8*). The two exons potentially encoding the *trans*-activating protein are shown as black boxes above the restriction map. The splicing pattern of the message for this potential coding region, based on the sequence of cDNA's cloned from HTLV-III–infected cells (*8*, *15*), is shown beneath the genome. Dark horizontal bars represent exons and thin diagonal lines introns (AAA, polyadenylation site). The nucleotide sequence of this region (*8*) is shown in the lower figure. Viral isolate designations are at the left. Restriction endonuclease cleavage sites and splice donors (SD) and acceptors (SA) are noted (*8*, *15*). The splice donors and acceptors depicted with dark arrows are those probably used for production of the *trans*-activating protein. The splice acceptor depicted with the open arrow is also used (*8*, *15*), probably for mRNA encoding the 3′ open reading frame. The translated amino acid sequence of the open reading frame in this region is shown above the nucleotide sequence. The open bar above the amino acid sequence delimits a highly conserved stretch of basic residues typical of nucleic acid–binding proteins located in the nucleus (*16*). The asterisks indicate stop codons.

since pΔ(83-5365/8053-9296) was as active as the wild-type pHXBc2 clone in the cotransfection assay. The pΔ(83-5365/8053-9296) plasmid *trans*-activated the same HTLV-III LTR mutants as did the wild-type plasmid (Table 2). Thus, *trans*-activation by pΔ(83-5365/8053-9296) is both quantitatively and qualitatively similar to that observed for the complete proviral clone.

Table 1. Cotransfection in different eukaryotic cell lines. Cotransfection of CAT plasmids and plasmids to be tested for *trans*-activating ability were carried out with 2 μg CAT plasmid DNA and approximately 2 μg test plasmid DNA, the latter adjusted so that molar equivalents of DNA were used. For adherent cells, 1×10^6 cells were transfected overnight by calcium phosphate-DNA coprecipitation (*2*). For cells grown in suspension, the DEAE-dextran technique (*19*) was employed, with 1×10^7 cells per transfection. Forty-eight hours after transfection, CAT lysates were prepared by freeze-thawing (*2*). CAT assays were done with equivalent amounts of protein from each lysate (approximately 200–300 μg protein per assay) as described (*10*), except that the final acetyl coenzyme A concentration in the reaction mix was 3 m*M*. At 10-, 30-, and 60-minute time points, reaction mixes were evaluated by thin-layer chromatography for percentage conversion of chloramphenicol to acetylated forms. The time points for the values shown in the table were chosen so that values fall within the linear range of the assay.

Cell line	Description	CAT plasmid	Cotransfected DNA	Conversion of chloramphenicol (%)
HeLa	Human cervical carcinoma epithelial cells	pSV2CAT	pIII	6.3
			pHXBc2	4.3
		pU3R-III	pIII	0.8
			pHXBc2	55.3
H9	Human T lymphocytes	pU3R-III	pIII	0.2
			pHXBc2	21.7
HUT78	Human T lymphocytes	pU3R-III	pIII	0.33
			pHXBc2	12.0
Raji	Human B lymphocytes	pU3R-III	pIII	0.66
			pHXBc2	76.0
CCCS+L−	Feline kidney epithelial cells	pU3R-III	pIII	0.5
			pHXBc2	52.0

Table 2. Effect of cotransfected DNA on CAT plasmids. Transfections and CAT assays were carried out as described in the legend to Table 1, with 1×10^6 HeLa cells as recipients. The construction of the CAT plasmids and an evaluation of their responsiveness to *trans*-activating factors present in cells infected with HTLV-III are as described (*14*). Relative CAT activity is the ratio of chloramphenicol conversion to acetylated forms obtained in H9 cells infected with HTLV-III to that obtained with an equivalent amount of transfected DNA, protein lysate, and reaction conditions in uninfected H9 cells (*14*).

CAT plasmid	Promoter	Relative CAT activity	Cotransfected DNA	Conversion of chloramphenicol (%)*
pSV2CAT	SV40 early region promoter	1.0	pIII	6.3
			pHXBc2	4.3
			pΔ(83-5365/8053-9296)	5.1
pU3R-III	HTLV-III LTR (−453 to +83)	400–800	pIII	0.8
			pHXBc2	55.3
			pΔ(83-5365/8053-9296)	65.2
p-167	HTLV-III LTR deleted to −167 from the cap site	400–800	pIII	2.9
			pHXBc2	95.7
			pΔ(83-5365/8053-9296)	98.2
p-167/+21	Same as above but R region deleted to +21	1.0	pIII	0.7
			pHXBc2	0.6
			pΔ(83-5365/8053-9296)	0.7
pRSV/−44	Rous sarcoma virus enhancers at −44 of HTLV-III LTR	170	pIII	0.7
			pHXBc2	55.8
			pΔ(83-5365/8053-9296)	61.2
pSV2/−17	SV40 enhancer and promoter at −17 of HTLV-III LTR	40	pIII	0.9
			pHXBc2	31.6
			pΔ(83-5365/8053-9296)	42.6
pHEP/−17	HTLV-I enhancer and promoter at −17 of HTLV-III	1.0	pIII	0.6
			pHXBc2	1.5
			pΔ(83-5365/8053-9296)	1.3

*Measured after a 1-hour reaction period.

These studies define a region from positions 5365 to 5928 that is necessary for the ability to *trans*-activate the HTLV-III LTR. Expression of this region is a prerequisite for efficient *trans*-activation, as a plasmid that contains this region without 5′ promoter sequences [pΔ(-453-5365/8053-9296)] was almost inactive in the cotransfection assay.

Knowledge of the HTLV-III splicing patterns provides insight into how this genomic region might be expressed. Multiple double-spliced messages with exons including this region have been identified in infected cells (*8, 15*). The nucleotide sequence contains a splice acceptor followed by a methionine codon. This codon initiates an open reading frame that could encode a protein 72 amino acids long (Fig. 3). Immediately 5′ to the stop codon is a splice donor (*8, 15*). The corresponding splice acceptor at position 7955 precedes an in-phase open reading frame capable of encoding an additional 14 amino acids. The potential protein product of the two exons is 86 amino acids long. Deletion of sequences contained within the intron did not affect the *trans*-activating ability of plasmids. For example, plasmids pΔ(83-5365/8053-9296) and pΔ(83-5365/6617-7198/8053-9296) exhibited comparable activities in stimulating HTLV-III LTR-directed CAT expression in the cotransfection assay (Fig. 1).

When the putative downstream exon is deleted, as in plasmid pΔ(5928-8595), a truncated protein of 72 amino acids encoded by the upstream exon can be made. Since plasmid pΔ(5928-8595) still showed *trans*-activating ability, the residues encoded by the second exon must not be essential to the activity of the putative protein product. In plasmid pΔ(83-5365/5607-7719/8053-9296), a stop codon has been placed at position 5612, precluding use of the second exon and prematurely terminating translation from the first exon. This plasmid still *trans*-activated, even though its potential protein product lacks 19 carboxyl terminal amino acids compared to the wild-type protein. The open reading frames are well conserved among the different HTLV-III/LAV isolates. However, the ARV-2 strain of HTLV-III is altered such that the second putative exon could encode 28 rather than 14 amino acids. These observations suggest that some heterogeneity in the carboxyl terminus of the putative *trans*-activating protein is permissible without compromising function.

Amino acid residues 49 through 57 in the potential product of this region comprise a strongly basic domain (Fig. 3). Such arginine- or lysine-rich regions

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.

References and Notes

1. M. Popovic *et al.*, *Science* **224**, 497 (1984); R. C. Gallo *et al.*, *ibid.*, p. 500; J. Schüpbach *et al.*, *ibid.*, p. 503; M. G. Sarngadharan *et al.*, *ibid.*, p. 506; F. Barré-Sinoussi *et al.*, *ibid.* **220**, 868 (1983); D. Klatzmann *et al.*, *Nature (London)* **313**, 767 (1984); J. A. Levy *et al.*, *Science* **225**, 840 (1984).
2. J. G. Sodroski, C. A. Rosen, W. A. Haseltine, *Science* **225**, 381 (1984); C. A. Rosen *et al.*, *ibid.* **227**, 320 (1985); J. Fujisawa *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2277 (1985).
3. J. G. Sodroski *et al.*, *Science* **227**, 171 (1985).
4. J. G. Sodroski *et al.*, *ibid.* **228**, 1430 (1985).
5. T. H. Lee *et al.*, *ibid.* **226**, 57 (1984); D. J. Slamon *et al.*, *ibid.*, p. 61.
6. W. C. Goh *et al.*, *ibid.* **227**, 1227 (1985).
7. M. Seiki *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2618 (1983); W. A. Haseltine *et al.*, *Science* **225**, 419 (1984); K. Shimotohno *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6657 (1984).
8. L. Ratner *et al.*, *Nature (London)* **313**, 227 (1985); R. Sanchez-Pescador *et al.*, *Science* **227**, 484 (1985); M. A. Muesing *et al.*, *Nature (London)* **313**, 450 (1985); S. Wain-Hobson *et al.*, *Cell* **40**, 9 (1985).
9. M. Fisher *et al.*, *Nature (London)*, in press.
10. C. M. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982).
11. C. M. Gorman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6777 (1982); M. D. Walker *et al.*, *Nature (London)* **306**, 557 (1983); J. M. Keller and J. C. Alwine, *Cell* **36**, 381 (1984); L. Herrera-Estrella *et al.*, *Nature (London)* **310**, 115 (1984).
12. D. Celander and W. A. Haseltine, *Nature (London)* **312**, 159 (1984).
13. J. Sodroski *et al.*, unpublished observations.
14. C. A. Rosen, J. G. Sodroski, W. A. Haseltine, *Cell*, in press.
15. S. K. Arya *et al.*, *Science* **229**, 69 (1985).
16. E. M. DeRobertis, R. F. Longthorne, J. B. Guron, *Nature (London)* **272**, 254 (1978); D. Kalderon *et al.*, *ibid.* **311**, 33 (1984); D. Kalderon, B. L. Roberts, W. D. Richardson, A. E. Smith, *Cell* **39**, 499 (1984).
17. C. A. Rosen, J. G. Sodroski, W. A. Haseltine, *Proc. Natl. Acad. Sci. U.S.A*, in press.
18. ———, in preparation.
19. C. Queen and D. Baltimore, *Cell* **33**, 741 (1983).

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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-