identified initially by comparing the retention times with those of known standards. Positive identification of FAME's was made by gas chromatography–mass spectroscopy with a 3 percent Dexsil column on a Finigan automated system.
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Induction of Hepatitis B Virus Core Gene in Human Cells by Cytosine Demethylation in the Promoter

Abstract. *A recombinant human cell line constructed by transfection of epithelial cells with a plasmid containing the hepatitis B virus core gene* (HBc) *was used to study the regulation of* HBc *gene expression. Methylation of a single Hpa II site 280 base pairs upstream from the structural gene was found to regulate the expression of the core gene. Expression increased in cells treated with 5′-azacytidine as a result of cytosine demethylation at this site, and there was a fivefold increase in the number of* HBc *gene transcripts in total cellular messenger RNA. The varied life cycle of hepatitis B virus in diseases such as viral hepatitis and liver cancer may therefore be attributable to the site-specific regulation of the gene involved in replication of the viral DNA and to the cytopathic effects elicited by this gene in human cells.*

Hepatitis B virus (HBV) infection is a serious worldwide health problem that is pathologically linked to viral hepatitis and liver cancer (*1*, *2*). There are an estimated 200 million individuals whose serum is positive for the surface antigen, HBsAg (*3*). The surface antigen and the core antigen (HBcAg) have different roles during HBV infection. Serologic data suggest that the constitutively regulated (*4*) gene for HBsAg (*HBs*) is needed for expansion of the focus of viral infection because antibodies to HBsAg are required for protection against the virus by immunization and are present during recovery from infection (*5*, *6*). In contrast, antibodies to the HBV core gene (*HBc*) product do not appear consistently during recovery and are frequently associated with virus replication and consequent infectivity of patients' sera (*7*).

The relation between *HBc* gene expression and the pathology of HBV infection is indicated by the cytopathologic effects of induced *HBc* gene expression in cells of the human epithelial cell line GTC2 (*8*). This line was constructed by transfection of carcinoma cells with a plasmid (pKYC200) that contains the *HBc* gene sequence without other HBV genes (*8*). This obviates the problems caused by the inability to infect mammalian cells with HBV in vitro and permits the study of *HBc* gene expression in human cells without interference from other HBV genes.

The importance of 5′-methyl cytosine to *HBc* gene regulation was first indicated by the cytotoxicity that follows induction of HBcAg expression in GTC2 cells or in an HBV-containing hepatocellular carcinoma cell line (*8*) [PLC/PRF/5 (*9*)] after treatment with 5′-azacytidine. The high degree of methylation of *HBc* gene sequences in PLC/PRF/5 (*10*) indicates that methylation of the *HBc* gene is biologically important. The demethylation of 5′-methyl cytosine at Hpa II–Msp I sites [CCGG (C, cytosine; G, guanine)] in total genomic HBV DNA has been studied in connection with generalized increases of gene expression in mammalian cells after they are treated with 5′-azacytidine (*11*). However, the low copy number of *HBc* gene inserts in GTC2 cells, the absence of other HBV sequences, and the ability to release the *HBc*-containing fragment from cellular DNA by digestion with Bam HI or Ava I (*8*) provide a model to determine the biologic importance of specific methylation sites within the 1.8-kilobase (kb) Bam HI fragment containing the *HBc* gene. Two Hpa II sites have been identified by sequence analysis of this Bam HI fragment of plasmid pKYC200 and have been mapped relative to the AUG start site (A, adenine; U, uracil) for *HBc* translation. One of these potential sites for regulation of methylation occurs 280 base pairs (bp) upstream from the *HBc* structural gene sequence ($HpaII^{-280}$), and the other site occurs 479 bp downstream from the sequence ($HpaII^{+479}$).

The methylation state at $HpaII^{-280}$ and $HpaII^{+479}$ was determined by digestion of DNA from nuclei of GTC2 cells with Bam HI or Ava I to release the transfected *HBc* gene. The DNA was subsequently digested with Hpa II before separation of the fragments by gel electrophoresis for Southern hybridization. The HBV-hybridizing fragments predicted at these two sites for each possible methylation state are listed in Table 1. Evaluation of site-specific methylation in the *HBc* gene of GTC2 cells after treatment with 5′-azacytidine is possible because the ratio of the HBV-hybridizing DNA fragments varies directly with the methylation state at the relevant Hpa II sites (Table 1). Internalization of the Hpa II sites being tested for methylation by digestion of cellular DNA with Bam HI or Ava I provides an internal control for measurement of methylation at these sites without interference from fragmented *HBc* gene inserts or from partially digested restriction fragments. This is because the fragments selected for analysis can only be produced by Hpa II digestion of complete *HBc* gene segments that do not contain inserted sequences (Table 1, Fig. 1). However, control digests of plasmid DNA indicated that the reaction conditions for Bam HI and Hpa II yielded

Table 1. Predicted sizes (in base pairs) of HBV-containing DNA fragments generated by enzymatic digestion of GTC2 cellular DNA. The methylation state at the Hpa II site is indicated by the presence (+) or absence (−) of 5′-methyl cytosine.

Restriction enzyme	Methylation state		Predicted size (bp)
	$HpaII^{-280}$	$HpaII^{+479}$	
Bam HI–Hpa II	+	+	1854
	−	−	171, 759, 924
	+	−	924, 930*
	−	+	171, 1683†
Ava I–Hpa II	+	+	975
	−	−	107, 109, 759
	+	−	109, 866
	−	+	107, 868

*Fragments at $HpaII^{+479}$. †Fragment at $HpaII^{-280}$.

complete digestion. The stoichiometric ratio of specific GTC2 DNA fragments was determined by densitometric analysis of autoradiographic films from Southern hybridization of GTC2 DNA fragments probed with ^{32}P-labeled HBV DNA (Fig. 1). By comparing the quantity of specific sizes of HBV-hybridizing DNA fragments with the sizes predicted from the transfected *HBc* gene sequence in various states of methylation at Hpa II sites (Table 1), the methylation state at HpaII^{-280} and HpaII^{+479} in GTC2 DNA was determined (Fig. 1).

Treatment of GTC2 cells with 5′-azacytidine enhances production of HBcAg (*8*). The cytotoxic effect of *HBc* induction in GTC2 cells causes an initial increase in HBcAg production at four to six divisions after cells are treated with 5′-azacytidine (primary culture, P_0). This increase reaches a maximum at approximately eight to ten divisions after treatment (P_1) (Table 2) (*8*). Treatment with 5′-azacytidine decreases the degree of cytosine methylation at the HpaII^{-280} site as indicated by the loss of the 1854-bp Bam HI–Hpa II and 975-bp Ava I–Hpa II fragments (Fig. 1, Table 2). The twofold increase in the number of 1683-bp Bam HI–Hpa II fragments that resulted from demethylation of the internal cytosine at HpaII^{-280} occurred when *HBc* gene expression was maximum (Table 2, Fig. 2). The twofold increase in the number of Ava I–Hpa II fragments (866 to 868 bp) was also due to demethylation at HpaII^{-280}. The greater importance of HpaII^{-280} with respect to HpaII^{+479} is shown further by the small increase in the 759-bp fragment predicted by demethylation of both sites (Fig. 1). Hypomethylation at the HpaII^{+479} site was occasionally detected by these assays. However, methylation at HpaII^{+479} was variable in individual digests and random with respect to *HBc* gene expression, which indicates that its methylation state was not related to *HBc* gene expression.

Demethylation at the HpaII^{-280} site in the *HBc* gene promoter region was required for maximum production of HBcAg when GTC2 cells were cultured in L4-5S growth medium. The site-specific methylation pattern of GTC2 DNA was the same for cells subcultured in RPMI 1640 medium with 10 percent fetal bovine serum (HUT medium) or LHC-4 medium (*12*) with 5 percent fetal bovine serum (L4-5S medium) (Table 2). Although hypomethylation of HpaII^{-280} increased *HBc* gene expression when cell cultures were grown in L4-5S medium, HUT medium did not induce expression of the *HBc* gene after GTC2 cells were treated with 5′-azacytidine (Fig. 2) (*8*). Steroid hormones, growth factors, trace metals, and bovine pituitary extract were added to L4-5S medium but not to HUT medium (*12*).

In efforts to determine the temporal relation of HpaII^{-280} demethylation to increased *HBc* gene transcription, whole cell RNA was extracted from GTC2 cells at various times after treatment with 5′-azacytidine and analyzed by hybridization to the 1854-bp Bam HI HBV fragment (Fig. 2). The amount of 5′-methyl cytosine in the genome of GTC2 DNA was measured (*13*) before cells were treated with 5′-azacytidine, at P_0, and at P_1 to determine overall changes in methylation during this experiment (Table 2). The amount of 5′-methyl cytosine was minimum at P_0 and returned to normal by P_1 (Table 2), when site-specific demethylation at HpaII^{-280} and *HBc* gene expression were maximum (Table 2, Fig. 2). The *HBc*-specific transcripts in extracts from GTC2 cells grown in HUT medium were nearly undetectable, which is consistent with the low amounts of HBcAg produced under this growth condition. GTC2 cells grown in L4-5S medium produced 30 times more *HBc*-specific transcripts. Treatment with 5′-azacytidine further increased the number of transcripts by a factor of about 5. Virtually no *HBc*-specific RNA was detected in postcytotoxic response cultures (P_2) or in cultures treated with 5′-azacytidine that were maintained in HUT medium (Fig. 2). Although the number of transcripts specifically derived from the guanine phosphoribosyltransferase (*gpt*) gene was about twice as high for cells grown in L4-5S medium as for those grown in HUT medium, 5′-azacytidine treatment did not appreciably increase (less than a factor of 2) the level of *gpt* gene transcription. This is consistent with other experiments indicating that the SV40 early promoter is not induced by hypomethylation (*14*). That cells must grow in L4-5S medium for gene expression to be induced by 5′-azacytidine indicates an additional regulatory requirement for *HBc* gene expression. Such effects of growth condition and hormones on induced expression of genes regulated by methylation are not without precedent (*13*, *15*).

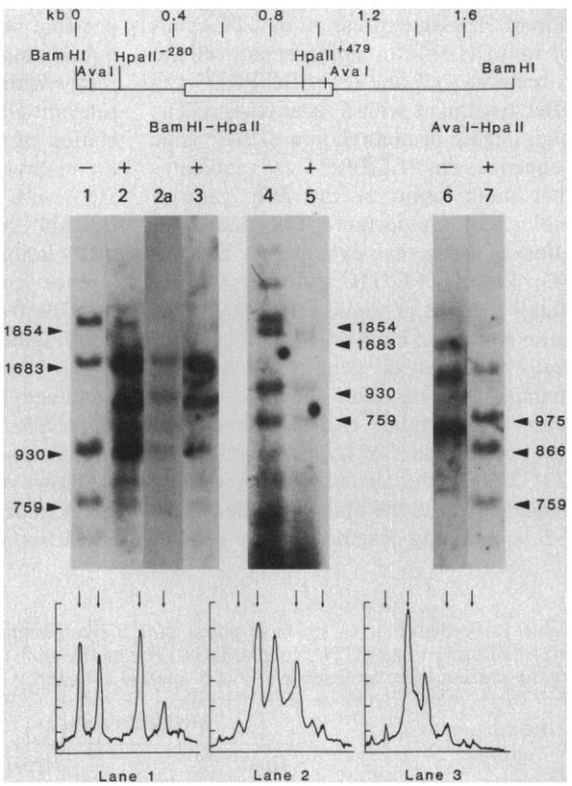

Fig. 1. Assay for site-specific demethylation of HBV DNA. GTC2 cultures were grown in HUT medium and plated (~10^4 cells per square centimeter) in HUT medium 24 hours before 5 μ*M* 5′-azacytidine was added. After 24 hours, the treatment medium was replaced with L4-5S or HUT. Cells were subcultured at 6- to 7-day intervals (four to five cell divisions). DNA (~10 to 20 μg per lane) was digested with a five- to tenfold excess of Hpa II and either Bam HI or Ava I (Boehringer Mannheim). The location of relevant sites for Hpa II mapping of the 1854-bp Bam HI fragment that contains the *HBc* gene from plasmid pKYC200 (*8*) is shown above the gel. The *HBc* gene–coding sequences are expanded to indicate the size relation of the gene to various products of digested GTC2 DNA. Southern hybridization analysis (*18*) of GTC2 DNA was conducted with ^{32}P-labeled pAM6 (*19*) probe DNA as described (*8*). The probe hybridized to GTC2 DNA digested with Bam HI–Hpa II (lanes 1 to 5) or with Ava I–Hpa II (lanes 6 and 7). Lanes 1, 4, and 6 contain DNA from GTC2 cells grown in L4-5S medium, and lanes 2, 3, 5, and 7 contain DNA from cells in parallel cultures treated with 5 μ*M* 5′-azacytidine and grown for eight to ten divisions in L4-5S medium. The arrows indicate bands described in Table 1. Lanes 2 and 2a show the range of film exposures used to compare various bands by densitometric measurements; the 1854-bp fragment can be measured at the exposure density for lane 2, but other bands are overexposed (lane 2a). Integrated areas under densitometric tracings (shown below the gel) similar to those in lanes 1 to 3 were used to determine the relative amount of DNA for the fragments listed in Table 2. Molecular sizes for the bands are in kilobases.

Table 2. Measurement by densitometry of relative amounts of GTC2 DNA restriction fragments. Southern hybridization autoradiographs were analyzed on a scanning-integrating densitometer (Beckman model Du-8). Relative areas (percent) are indicated for the different fragments generated by Bam HI–Hpa II or Ava I–Hpa II digestion of the 1854-bp HBV fragment in GTC2 DNA. Values are the average (± standard error of the mean) of at least two measurements from two separate experiments. Statistical analysis was not possible for passage 2 after cells were treated with 5′-azacytidine because only two samples were used. The ratios of positive to negative (P/N) values for HBcAg was determined in sonicated cell extracts by radioimmunoassay (*8*). A P/N ratio of 2.1 or greater is considered a positive response in the radioimmunoassay. Each passage represents approximately four to five cell divisions. Abbreviations: P_0, primary (treated) culture; P_1, passage 1 (maximum HBcAg production); P_2, passage 2 (postcytotoxic response); N.T., control cultures not treated with 5′-azacytidine; N.D., not determined. The total amount of 5′-methyl cytosine in the genome after treatment of GTC2 cells with 5′-azacytidine was determined as described (*13*).

Culture medium	Passage number*	P/N ratio	Relative amounts of Bam HI–Hpa II fragments (percent)				Relative amounts of Ava I–Hpa II fragments (percent)			Total genomic 5′-methyl cytosine (percent)
			1854 bp	1683 bp	924 and 930 bp	759 bp	975 bp	866 and 868 bp	759 bp	
HUT	N.T.	1.8 ± 0.2	43 ± 2	31 ± 1	22 ± 3	4 ± 1	61 ± 3	30 ± 2	9 ± 3	2.66 ± 0.17
L4-5S	N.T.	2.4 ± 0.2	41 ± 4	28 ± 3	22 ± 3	9 ± 1	57 ± 6	26 ± 5	17 ± 4	2.62 ± 0.13
L4-5S	P_0	2.7 ± 0.2	11 ± 3	53 ± 1	26 ± 3	10 ± 3	28 ± 4	49 ± 4	23 ± 1	0.45 ± 0.18
L4-5S	P_1	3.8 ± 0.5	4 ± 1	61 ± 6	19 ± 3	12 ± 4	8 ± 1	68 ± 2	24 ± 2	2.81 ± 0.20
L4-5S	P_2	2.0 ± 0.2	38	31	22	9	41	33	26	N.D.

*After treatment with 5 μ*M* 5′-azacytidine.

The presence or absence of 5′-methyl cytosine at the HpaII^{-280} site of the *HBc* gene thus effects the regulation of the induced expression of HBcAg. However, we cannot rule out the possibility that changes in methylation at additional sequences might also affect *HBc* gene expression. The sequences surrounding the HpaII^{-280} site contain the native promoter for the *HBc* gene (*8*). The observation that *gpt* transcription was not affected by treating cells with 5′-azacytidine, whereas *HBc* gene transcription increased by a factor of 5, is consistent with *HBc* gene expression from a native HBV promoter 5′ to the *HBc* structural gene. Sequence analysis of pKYC200 shows that no fusion product starting outside this fragment is possible because there are no promoters in the 5′-flanking SV40 DNA and no uninterrupted reading frames long enough to encode for HBcAg before base pair 451 of this fragment (*16*). Constitutive transcripts from the HBV surface antigen gene, *HBs*, end just upstream from, or a few base pairs inside, the *HBc* gene (*4*, *17*) when HBV is in its circular virion form.

A mechanism for 5′-azacytidine induction by HpaII^{-280} hypomethylation of the *HBc* gene must take into account the following: (i) induction of *HBc* gene expression by 5′-azacytidine requires approximately six to ten cell divisions before reaching a maximum; (ii) the cell death caused by treatment with 5′-azacytidine ends after the first passage of treated cultures; (iii) the degree of cytosine methylation in the genome is lowest at P_0, and induced *HBc* gene expression is highest at P_1 when the overall degree of genomic methylation has returned to normal; and (iv) more than 90 percent of GTC2 cells induced by 5′-azacytidine express HBcAg when induction of the *HBc* gene reaches a maximum, as shown by immunofluorescence (*8*). The induction of a specific gene in 90 percent of the cells must involve an indirect mechanism, because a lethal dose of 5′-azacytidine would be required to demethylate simultaneously a single site in so large a portion of the cell population. If treatment with 5′-azacytidine is followed by a reduced capacity of the cell to methylate the HpaII^{-280} site during the recovery of normal degrees of generalized methylation, the pattern of treatment, hypomethylation, and induction of the *HBc* gene expression would follow the sequence observed. This would occur if the initiation of activated transcription reduced the ability of the DNA cytosine methylase to maintain the methylation state at HpaII^{-280} by exclusion.

Regulation of the *HBc* gene may be critical to host cell viability because of the cytotoxicity of *HBc* gene expression. Although several hepatocellular carcinoma cell lines have been isolated that

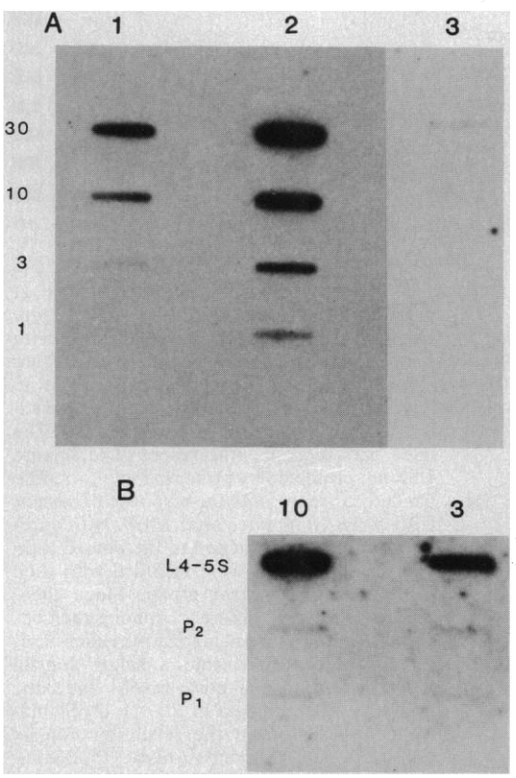

Fig. 2. Slot-blot analysis of *HBc* transcription in total GTC2 cellular RNA. RNA was isolated from GTC2 cells by guanidine thiocyanate extraction and cesium chloride centrifugation (*20*). RNA samples (1 to 30 μg) were denatured in 2.2*M* formaldehyde and 15× standard saline citrate for 15 minutes at 68°C, transferred to nitrocellulose filters by means of a slot-blot apparatus (Schleicher and Schuell), and hybridized to the ^{32}P-labeled 1854-bp Bam HI fragment of HBV DNA. (A) Exposure times were 8 hours for rows 1 and 2 and 24 hours for row 3. RNA from GTC2 cells grown in L4-5S medium (row 1), in L4-5S medium with 5′-azacytidine added (row 2), or in HUT medium (row 3) is shown. The amount of RNA in each band is given to the left (in micrograms). (B) Exposure time was 72 hours. RNA from GTC2 cells grown in L4-5S medium (L4-5S), in L4-5S medium with 5′azacytidine added (P_2), or in HUT medium with 5′-azacytidine added (P_1) is shown. The amount of RNA in each row is given at the top (in micrograms). The susceptibility of positively hybridizing RNA samples to mild alkaline hydrolysis (0.1*N* NaOH, 37°C, 1 hour) before neutralization and binding to nitrocellulose indicates that cellular DNA did not contaminate the fractions analyzed.

carry multiple HBV genomic inserts, only PLC/PRF/5 and GTC2 cells treated with 5′-azacytidine grown in LHC4 medium are known to express the *HBc* gene in vitro (*8*). Hepatocellular carcinoma cells containing HBV express the *HBs* gene constitutively, producing the HBV surface antigen, HBsAg, without cytopathologic effects (*4*). The sequences coding for HBcAg in PLC/PRF/5 cells are highly methylated, whereas HBV virion DNA and HBV DNA extracted from liver samples of infected patients is unmethylated (*10*). The contrasting methylation pattern of the *HBc* gene in cells carrying HBV integrated as a provirus in the human genome and in HBV DNA extracted from virions and tissues of acutely infected patients suggests that methylation of $HpaII^{-280}$ in the 5′-flanking region of the *HBc* gene may be important to the viability of host cells. Thus, host factors regulating *HBc* gene expression may have a pivotal role in establishing the intricacies of HBV host range and the variations in susceptibility that are related to nutrition, sex, age, and immunologic abnormalities.

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Endogenous Anticonvulsant Substance in Rat Cerebrospinal Fluid After a Generalized Seizure

Abstract. *Cerebrospinal fluid taken from rats subjected to electroshock-induced seizures and injected into the cerebral ventricles of rats that had not been shocked increased the seizure threshold of the recipients. The anticonvulsant activity of the donor cerebrospinal fluid was antagonized by opioid antagonists and enhanced by peptidase inhibitors. These results suggest the existence of an endogenous anticonvulsant substance in rat cerebrospinal fluid, possibly opioid in nature, which is activated as a consequence of a seizure and which may play a critical role in postseizure inhibition.*

Postseizure inhibition has been shown both experimentally (*1, 2*) and clinically (*3, 4*) to be a physiological regulator in the self-limitation of seizures. Although the clinical significance of the postseizure refractory state for preventing the continuous recurrence of seizures in the epileptic is clear, the mechanism underlying this state is not known. Because opioid peptides, endogenous ligands for opiate receptors, have marked anticonvulsant activity against a variety of experimentally induced seizures (*2, 5–7*) and are activated as a consequence of seizures (*5, 7, 8*), it was suggested that seizure-activated endogenous opioid systems function to decrease the probability or severity of subsequent seizures (*9, 10*). Working under this hypothesis, we demonstrated that endogenous opioid systems play a selective role in the postictal rise in seizure threshold (*3*) and that the opioid antagonist naloxone stereoselectively attenuates and morphine tolerance prevents the postseizure protection produced by repeated maximal electroshock (MES) seizures in rats (*11*). We suggested that the brain's opioid systems may act postictally as endogenous anticonvulsants and thus play an important role in maintaining a refractory state after a seizure. More recently, it was reported that the anticonvulsant effects of electroconvulsive shock or foot-shock stress against kindled seizures are mediated by the activation of endogenous opioid systems (*12*). We report here the presence of an endogenous "anticonvulsant substance" in the cerebrospinal fluid (CSF) of rats subjected to MES seizures, whose bioactivity is completely antagonized by opioid antagonists and enhanced by peptidase inhibitors. We suggest that a seizure activates in the central nervous system (CNS) an endogenous substance, possibly opioid in nature, that acts as an endogenous modulator of CNS excitability, particularly in the regulation of postseizure inhibition.

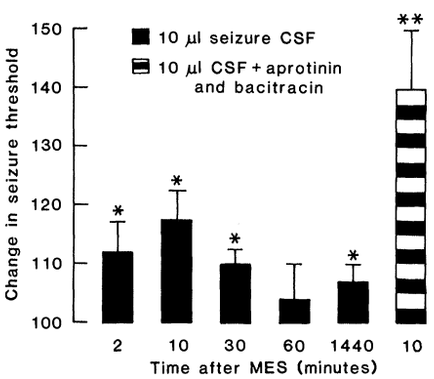

Fig. 1. Effects of seizure CSF collected at various times after MES on the seizure thresholds of recipient rats challenged with flurothyl. Data (means + standard errors) are expressed as the percentage of increase in seizure threshold beyond the baseline threshold for the control CSF–treated rats. The filled bars represent the response to seizure CSF not protected by protease inhibitors. The banded bar represents the response to seizure CSF taken 10 minutes after MES, with aprotinin and bacitracin added to the pooled sample. Control groups were included with each experimental treatment group. Since there was no significant difference among the control seizure measurements made in each trial, they were combined into a single control group and used for all comparisons (the same was done with the data in Fig. 2). (*) Significantly different from the result for control CSF ($P < 0.05$, Student's t-test). (**) Significantly different from the result for control CSF plus aprotinin and bacitracin ($P < 0.01$).

Male Sprague-Dawley rats (275 to 350 g; Zivic-Miller Laboratories) were randomly designated as donor or recipient animals. Donors were subjected to single transauricular MES (2 seconds at 60 Hz and 50 mA), causing a generalized seizure characterized by an initial tonic extension of the limbs followed by clonic jerking. At various times after an MES seizure, donor rats were anesthetized with ketamine HCl (100 mg/kg intraperitoneally), and CSF (referred to as seizure CSF) was collected with a 21-gauge needle inserted into the cisternal space. Seizure CSF from three to six donor animals was pooled and kept on ice throughout the experiment; control CSF was also collected from donor rats given sham MES.

In recipient animals (n = 12 to 15 per group) a 30-gauge cannula was stereotaxically implanted in the right lateral cere-