served in DHFR from bacterial to mammalian enzymes (*16, 17*); these key amino acids (* in Fig. 2) are well conserved in the HVS DHFR sequence.

The central role of DHFR in DNA synthesis is demonstrated by its increase in activity in rapidly dividing cells and the sensitivity of such cells to folate analogs. Among the folate-requiring enzymes, TS is unique in its consumption of 1 mole of reduced folate for every mole of dTMP generated. This stoichiometry requires coordinated regulation of the two enzymes. This has been accomplished in T4 bacteriophage by overlapping the TS and DHFR genes (*14*) and in the protozoan parasite *Leishmania* by production of a bifunctional TS-DHFR polypeptide (*20*). The DHFR and TS genes of HVS are located near the opposite ends of the unique sequence L-DNA. Both of these regions are consistently retained in HVS-transformed T-cell lines in which large segments of the L-DNA central region are lost (*33*). The DHFR gene is apparently not required for immortalization or replication in vitro by HVS since replication-competent virus strains have been constructed that delete all or part of the DHFR coding sequences yet retain their ability to immortalize T cells and to replicate in permissive monolayer cell lines (*34, 35*). The presence of DHFR and TS genes in HVS and HVA, however, could facilitate virus reactivation or replication in resting T cells. The maintenance of TS and DHFR genes may be particularly important in providing adequate dTMP pools for viruses such as HVS and HVA, which have unusually high A+T content. A possible role for HVS DHFR in persistent infection, latency, and reactivation from latency can now be investigated by using susceptible New World primates as host.

REFERENCES AND NOTES

1. F. Deinhardt and H. Wolf, *IARC Sci. Publ.* **24**, 169 (1978); B. Roizman in *The Herpesviruses,* B. Roizman, Ed. (Plenum, New York, 1982), vol. 1, pp. 1–23.
2. B. Fleckenstein *et al.*, *J. Virol.* **25**, 361 (1978).
3. K. R. Cameron *et al.*, *ibid.* **61**, 2063 (1987); U. A. Gompels, M. A. Craxton, R. W. Honess, *ibid.*, in press.
4. D. R. Dubbs and S. Kit, *Virology* **22**, 493 (1964).
5. M. Ponce de Leon, R. J. Eisenberg, G. H. Cohen, *J. Gen. Virol.* **36**, 163 (1977); B. M. Dutia, *ibid.* **64**, 513 (1983).
6. D. J. M. Purifoy, R. B. Lewis, K. L. Powell, *Nature (London)* **269**, 621 (1977).
7. V. G. Preston and F. B. Fisher, *Virology* **138**, 58 (1984).
8. C. M. Preston and M. G. Cordingley, *J. Virol.* **43**, 386 (1982).
9. S. Caradonna, D. Worrad, R. Lirette, *ibid.* **61**, 3040 (1987).
10. R. W. Honess *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3604 (1986).
11. W. Bodemer, H. H. Niller, N. Nitsche, B. Scholz, B. Fleckenstein, *J. Virol.* **60**, 114 (1986).
12. H. H. Niller *et al.*, in *Viruses and Human Cancer,* R. C. Gallo, W. Haseltine, G. Klein, H. zur Hausen, Eds. (Liss, New York, 1987), pp. 443–450.
13. L. F. Johnson in *Recombinant DNA and Cell Proliferation,* G. S. Stein and J. L. Stein, Eds. (Academic Press, New York, 1984), pp. 25–47; J. R. Bertino and D. Johns in *Cancer Chemotherapy,* I. Brodsky, Ed. (Grune & Stratton, New York, 1972), pp. 9–22.
14. S. Purohit, R. K. Bestwick, G. W. Lasser, C. M. Rogers, C. K. Mathews, *J. Biol. Chem.* **256**, 9121 (1981); S. Purohit and C. K. Mathews, *ibid.* **259**, 6261 (1984).
15. G. Bornkamm, H. Delius, B. Fleckenstein, F. J. Werner, C. Mulder, *J. Virol.* **19**, 154 (1976); A. T. Bankier *et al.*, *ibid.* **55**, 133 (1985); R. C. Desrosiers and B. Fleckenstein, *Adv. Viral Oncol.* **3**, 307 (1983).
16. J. N. Masters and G. Attardi, *Gene* **21**, 59 (1983).
17. D. R. Setzer, M. McGrogan, R. T. Schimke, *J. Biol. Chem.* **257**, 5143 (1982); G. F. Crouse, C. C. Simonsen, R. N. McEwan, R. T. Schimke, *ibid.* **257**, 7887 (1982).
18. P. H. Lai, H. C. Pan, J. M. Gleinser, D. L. Peterson, R. L. Blakely, in *Chemistry and Biology of Pteridines,* R. L. Kisliuk and G. M. Brown, Eds. (Elsevier/North-Holland, Amsterdam, 1979), pp. 437–440.
19. A. A. Kumar, D. T. Blankenship, B. T. Kaufman, J. H. Freisheim, *Biochemistry* **19**, 667 (1980).
20. S. M. Beverley, T. E. Ellenberger, J. S. Cordingley, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2584 (1986); R. Grumont, W. L. Washtien, D. Caput, D. V. Santi, *ibid.*, p. 5387.
21. M.-J. Chen *et al.*, *J. Biol. Chem.* **259**, 3933 (1984).
22. J. H. Nunberg, R. J. Kaufman, A. C. Y. Chang, S. N. Cohen, R. T. Schimke, *Cell* **19**, 355 (1980); A. M. Carothers, G. Urlaub, N. Ellis, L. A. Chasin, *Nucleic Acids Res.* **11**, 1997 (1983).
23. M.-J. Chen, T. Shimada, A. D. Moulton, M. Harrison, A. W. Nienhuis, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7435 (1982).
24. J. K. Yang, J. N. Masters, G. Attardi, *J. Mol. Biol.* **176**, 169 (1984); P. J. Mitchell *et al.*, *Mol. Cell. Biol.* **6**, 425 (1986).
25. R. Baer *et al.*, *Nature (London)* **310**, 207 (1984).
26. A. J. Davidson and J. E. Scott, *J. Gen. Virol.* **67**, 1759 (1986).
27. D. McGeoch, personal communication.
28. B. Barrell, personal communication.
29. Southern blots of Hind III– and Eco RI–digested viral DNAs (0.5 μg per lane) were hybridized with ^{32}P-labeled nick-translated Hinf I DNA fragment (nucleotides 3975 to 4883 of L-DNA) representing the HVS DHFR coding sequences. Filters were hybridized at 67°C in 4× SSC containing 0.1% SDS and washed first for 2 hours at 65°C in 2× SSC containing 0.1% SDS, 25 m*M* sodium phosphate, *p*H 6.8, and 1.5 m*M* sodium pyrophosphate, and then for 10 minutes at 65°C in 1× SSC.
30. P. Medveczky, E. Szomolanyi, R. C. Desrosiers, C. Mulder, *J. Virol.* **52**, 938 (1984).
31. R. C. Desrosiers and L. A. Falk, Jr., *J. Gen. Virol.* **56**, 119 (1981).
32. J. Kamine *et al.*, *J. Virol.* **52**, 532 (1984).
33. S. Schirm *et al.*, *ibid.* **49**, 938 (1984); R. C. Desrosiers, *ibid.* **39**, 497 (1981).
34. R. C. Desrosiers, R. L. Burghoff, A. Bakker, J. Kamine, *ibid.* **49**, 343 (1984).
35. R. C. Desrosiers, *et al.*, *Science* **228**, 184 (1985); R. C. Desrosiers, D. P. Silva, L. M. Waldron, N. L. Letvin, *J. Virol.* **57**, 701 (1986); R. C. Desrosiers, unpublished data.
36. S. Henikoff, *Gene* **28**, 351 (1984).

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Synaptic Reorganization in the Hippocampus Induced by Abnormal Functional Activity

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Abnormal functional activity induces long-lasting physiological alterations in neural pathways that may play a role in the development of epilepsy. The cellular mechanisms of these alterations are not well understood. One hypothesis is that abnormal activity causes structural reorganization of neural pathways and promotes epileptogenesis. This report provides morphological evidence that synchronous perforant path activation and kindling of limbic pathways induce axonal growth and synaptic reorganization in the hippocampus, in the absence of overt morphological damage. The results show a previously unrecognized anatomic plasticity associated with synchronous activity and development of epileptic seizures in neural pathways.

THE HIPPOCAMPUS RECEIVES CONverging neocortical, subcortical, limbic, and brainstem inputs via the perforant pathway to dentate granule cells that in turn project to CA3 hippocampal neurons via mossy fiber axons (*1*). These pathways undergo synaptic reorganization in response to lesions (*2*) and also demonstrate long-lasting increases in synaptic efficacy in response to certain patterns of afferent activity (*3, 4*). Repeated activation of neural pathways can induce seizures and a permanent epileptic state (kindling) (*5*). Using the Timm method to identify hippocampal mossy fibers (*6*), we observed that synchronous perforant path activation and kindling of limbic pathways induce mossy fiber sprouting and synaptic reorganization in the dentate gyrus, in the absence of overt CA3/CA4 lesions.

The Timm method is a histochemical technique that stains neural elements con-

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taining heavy metals (*6*). As a consequence of the unusually high Zn^{2+} content of mossy fibers relative to surrounding neuropil (*7*), the trajectory of mossy fiber axons and the location of their synaptic terminals can be readily identified at the light and ultrastructural level with the Timm method. The mossy fiber pathway projects mainly to hippocampal pyramidal neurons in CA3/CA4 (*1, 8*), but sparse Timm staining may also be observed in the inner molecular layer of the dentate gyrus, near the tips of the supra- and infrapyramidal blades, and the crest (*9, 10*).

We examined the pattern of Timm staining in the hippocampus and dentate gyrus of rats after synchronous activation and kindling of the perforant path. The normal pattern of Timm staining was observed in unstimulated rats implanted with perforant path electrodes for 3 months (five of five rats) (Fig. 1A) and in normal rats (six of six rats). Kindling dramatically altered the pattern of Timm staining in the dentate gyrus. Eighteen hours after three kindled generalized seizures, aberrantly located Timm granules were observed in the supragranular molecular layer (12 of 13 rats) (Fig. 1B). Timm granules were distributed throughout the supragranular layer of the dentate gyrus, where they are not normally present, including the intervening regions between tips of the supra- and infrapyramidal blades, and the crest (Fig. 1C). Supragranular Timm granules were not observed in unstimulated implanted controls and normal rats, and therefore the development of aberrantly located granules was caused by kindling stimulation and was not related to electrode placement or staining methods. Kindling induced aberrantly located supragranular Timm granules in the dentate gyrus bilaterally, but the granules were more prominent ipsilateral to the stimulation site. The supragranular Timm granules were observed as long as 5 months after the last kindled seizure, an indication that the effect is long-lasting. In agreement with numerous previous studies (*5, 11*), there was no evidence of a CA3/CA4 lesion after three kindled, generalized seizures (Fig. 2, A and B).

Supragranular Timm granules were also observed bilaterally after three generalized seizures in rats kindled in the olfactory bulb (six of six rats) and in rats kindled in the amygdala (six of six rats) (*12*). Thus, epileptiform activity propagated in the perforant path is sufficient to induce aberrant supragranular Timm granules; direct perforant path stimulation is not required. Electron microscopic analysis (*13*) in four of four kindled rats demonstrated that the aberrantly located Timm granules were located in synaptic terminals (Fig. 2C). This finding suggests that development of supragranular Timm staining in association with limbic kindling results from sprouting, axonal growth, and synaptic reorganization.

The Fink-Heimer method (*14*) was used to detect any subtle neuronal or terminal degeneration that was not apparent in the cresyl-stained sections and that could potentially cause synaptic reorganization. Degenerating neural elements were not observed in the hippocampus and dentate gyrus after three generalized seizures in amygdala-kindled rats (five of five). At the ultrastructural level, terminal degeneration was not observed in the dentate gyrus in eight of eight rats kindled in the perforant path. If the sprouting induced by repetitive kindled seizures is due to subtle neuronal loss, it might be expected that neuronal loss would be more prominent after additional kindled seizures. However, in a rat that experienced 220 generalized seizures evoked by kindling of the olfactory bulb, a dense band of supragranular Timm granules was observed, but the neuronal population in CA3/CA4 still appeared normal.

Morphological evidence of synaptic reorganization was apparent early in the course of kindling after 5 or 6 days of twice daily stimulation, before the development of generalized convulsions. Sprouted supragranular mossy fiber terminals were observed after just five to ten afterdischarges evoked by perforant path stimulation, when the animals were experiencing only focal seizures (four of four rats).

Some animals received low-frequency perforant path stimulation that failed to evoke afterdischarges or seizures. Fifteen days of twice-daily stimulation with 62 1.0-mA biphasic pulses of 1.0-msec duration delivered at 0.2 Hz also induced supragranular Timm granules in the dentate gyrus (six of six rats) (Fig. 2D). However, the supragranular Timm granules induced by low-frequency stimulation were fewer and less prominent than those observed after kindling. Thus, chronic synchronous activation of the perforant path was sufficient to induce sprouting, even in the absence of evoked epileptiform activity.

The results demonstrate that synchronous

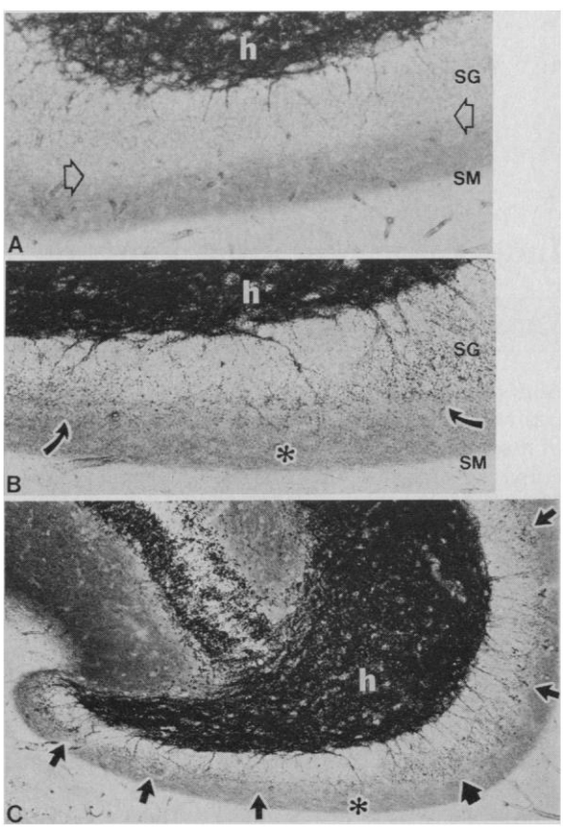

Fig. 1. Supragranular Timm staining and synaptic reorganization in the dentate gyrus induced by kindling of the perforant path. Anesthetized male Sprague-Dawley rats (250 to 350 g) were implanted with permanent bipolar electrodes in the perforant path near the angular bundle. Two weeks later, the unrestrained awake animals received a 1.0-second train of 62-Hz biphasic constant-current 1.0-msec pulses at intensities in the range of 500 to 1100 μA; afterdischarge and seizures were monitored by standard techniques (*3*). Stimulation was delivered twice daily (5 days per week) until three generalized seizures were evoked. Rats kindled via the perforant path experienced a generalized seizure (class 5) after 15.8 ± 5.0 afterdischarges. The rats were then anesthetized and perfused at least 18 hours after the last seizure. Adjacent 40-μm horizontal brain sections were obtained from septal to temporal levels of the hippocampus and were alternately stained with the Timm method (*10*) and cresyl violet. (**A**) The normal pattern of Timm staining in the dentate gyrus of an unstimulated rat implanted with a perforant path electrode for 3 months. Timm granules are absent in the supragranular dentate gyrus (open arrows). Similar findings were observed in four other unstimulated implanted controls and in six of six rats without permanent electrodes. (**B** and **C**) After the kindling procedures, Timm granules were observed in the supragranular layer of the dentate gyrus in the region between the tips and crest through the full septal-temporal axis of the hippocampus. The location of the high-power view in (B) is indicated by the asterisk in (C). Arrows indicate supragranular Timm granules. The larger arrow to the right of the asterisk identifies the crest area. Abbreviations: h, hilus of the dentate gyrus; SG, stratum granulosum; SM, stratum moleculare. Magnification: ×290 in (A) and (B); ×145 in (C).

functional activity can induce reorganization of hippocampal pathways in the absence of overt neuronal loss or degeneration. In the case of lesion-induced synaptic reorganization, neuronal loss in CA3/CA4 results in degeneration of the commissural-associational pathway from pyramidal and hilar neurons of CA4 to the inner molecular layer of the dentate gyrus (*8*) and is followed by sprouting of mossy fiber axons of dentate granule cells that form morphologically intact, physiologically functional excitatory synaptic terminals in the denervated zone (*10, 15*). Intense perforant path stimulation that evokes afterdischarges for 24 hours causes selective loss of Timm stain in the mossy fiber pathway and loss of CA3/CA4 neurons that receive afferent input from granule cells (*16*). Similarly, intense high-frequency perforant path stimulation (10-second trains of single stimuli at 20 Hz every minute for 24 hours) causes degeneration of hilar and pyramidal neurons, including somatostatin-staining hilar neurons (*16*). These more vigorous stimulation methods clearly induce neuronal loss; lesion-induced sprouting is likely in these circumstances.

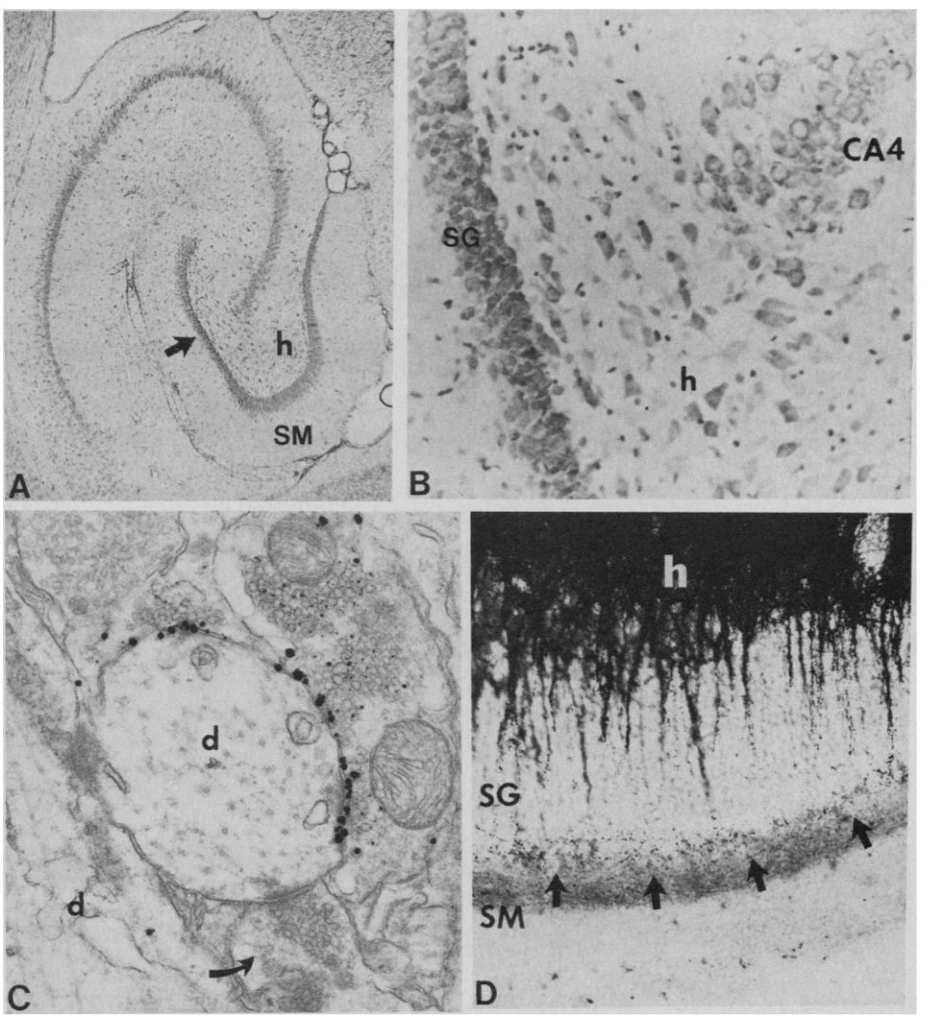

Fig. 2. Perforant path kindling induced synaptic reorganization in the absence of overt morphological damage. (**A**) Low-power view of the hippocampus and dentate gyrus from a rat killed 18 hours after the last of three generalized seizures and stained with cresyl violet. The dentate granule cell layer and the distribution of pyramidal and hilar neurons appear normal. The arrow indicates the dentate granule cell layer and the adjacent hilar area. (**B**) Higher magnification of the hilar region [indicated by the arrow in (A)] revealed normal appearing hilar neurons. (**C**) Electron micrograph (*13*) demonstrates reaction product (black) located at synaptic clefts and intermixed with synaptic vesicles in axon terminals. The labeled terminals form synapses with a dendrite (d) cut in cross section. Another dendrite cut in longitudinal section is also indicated by (d). Note the unstained bouton adjacent to labeled terminals (arrow), indicating that the reaction is specific for a subset of terminals. (**D**) Low-frequency stimulation consisting of 62 pulses of 1.0-msec duration and 1.0-mA intensity delivered at 0.2 Hz twice daily for 15 days also induced synaptic reorganization. A horizontal section of the dentate gyrus from septal hippocampus demonstrates supragranular Timm granules (arrows). Abbreviations: h, hilus of the dentate gyrus; SG, stratum granulosum; SM, stratum moleculare. Magnification: (A) ×50; (B) ×320; (C) ×14,000; and (D) ×290.

In contrast, the kindling methods employed in this study did not evoke sustained intense seizure activity and produced no overt morphological degeneration. We cannot exclude the possibility that synchronous perforant path activation and kindling may have produced subtle neuronal alterations in CA3/CA4 that were not apparent with the light and the electron microscopic techniques used in this study. However, the prominence of sprouting in the absence of obvious CA3/CA4 neuronal loss, the lack of evidence of terminal degeneration with ultrastructural analysis and with the Fink-Heimer technique, and the development of synaptic reorganization after low-frequency stimulation that failed to evoke epileptiform activity strongly suggest that abnormal patterns of functional activity alone are sufficient to induce axonal sprouting and synaptic reorganization in the hippocampus.

This observation was dependent on the Timm technique (*6*), a histochemical method that stains mossy fiber synaptic terminals due to their Zn^{2+} content (*7*). In support of our findings, previous studies have demonstrated increases in hippocampal and cortical zinc after amygdala kindling (*17*). Although evidence that zinc is released from mossy fiber synaptic terminals during excitatory activation (*18*) raises the possibility that the abnormal supragranular Timm staining is a short-term phenomenon caused by zinc reuptake in terminals where it is not normally found, this interpretation is unlikely. Since the supragranular Timm granules persist for at least 5 months after the last stimulation, the effect is long-lasting and most likely represents sprouting and synaptic reorganization.

Recent evidence indicates that the cellular oncogene c-*fos* participates in genetic events associated with functional activity in neurons (*19*). Kindling stimulation and brief seizure activity evoked by Metrazole induce dramatic increases of c-*fos* messenger RNA and c-*fos* protein in nuclei of dentate granule cells that give rise to mossy fiber axons (*20*). Furthermore, c-*fos* transcription has been associated with neurite outgrowth in PC12 pheochromocytoma cells (*21*). The c-*fos* transcription induced by kindling and Metrazole seizures could be involved in long-term responses to epileptic activity, such as the sprouting and axonal growth reported in this study.

Axonal sprouting and resulting synaptic reorganization in limbic pathways may contribute to development of kindled seizures. If the stimulus-induced alterations in connectivity increase excitability, hippocampal pathways could become progressively susceptible to epileptiform events. Indeed, the observation of altered circuitry early in the

course of kindling, before development of generalized seizures and, more generally, after synchronous activation of neural pathways, is consistent with the hypothesis that abnormal activity can induce structural reorganization that promotes epileptogenesis. Repeated seizure-induced alterations in hippocampal connectivity could also cause neuronal and behavioral dysfunction in chronic epileptic disorders.

Our observations have been confined to the hippocampal mossy fiber pathway, but similar phenomena may occur in other pathways of the brain.

REFERENCES AND NOTES

1. L. W. Swanson, *Neurosci. Res. Prog. Bull.* **20**, 624 (1982).
2. C. W. Cotman, M. Nieto-Sampiedro, E. W. Harris, *Physiol. Rev.* **61**, 684 (1981).
3. T. Sutula and O. Steward, *J. Neurophysiol.* **56**, 732 (1986).
4. E. Maru and G. V. Goddard, *Exp. Neurol.* **96**, 19 (1987); *ibid.*, p. 33; I. Mody and U. Heinemann, *Nature (London)* **326**, 701 (1987); R. M. Douglas and G. V. Goddard, *Brain Res.* **86**, 205 (1975).
5. G. V. Goddard, *Nature (London)* **214**, 1020 (1967); ———, D. C. McIntyre, C. K. Leech, *Exp. Neurol.* **25**, 295 (1969).
6. G. Danscher, *Histochemistry* **71**, 1 (1981).
7. C. J. Frederickson *et al.*, *Brain Res.* **273**, 335 (1983).
8. L. W. Swanson, J. M. Wyss, W. M. Cowan, *J. Comp. Neurol.* **181**, 681 (1978).
9. J. R. West and S. L. Dewey, *Neuroscience* **13**, 377 (1984).
10. J. V. Nadler, B. W. Perry, C. W. Cotman, *Brain Res.* **182**, 1 (1980); S. Laurberg and J. Zimmer, *J. Comp. Neurol.* **200**, 433 (1981).
11. J. O. McNamara, *Adv. Neurol.* **44**, 303 (1986).
12. Rats kindled via the olfactory bulb and amygdala were implanted with permanent electrodes and were stimulated by the methods used for perforant path kindling [see Fig. 1 for details] (*3*). Rats receiving olfactory bulb implants experienced a kindled generalized (class 5) seizure after 7.6 ± 3.6 afterdischarges. Rats receiving amygdala implants experienced class 5 seizures after 9.0 ± 2.8 afterdischarges.
13. Electron micrographs of the dentate gyrus were obtained from rats kindled by perforant path, olfactory bulb, and amygdala stimulation. Eighteen hours after the last kindled generalized seizure, the rats were perfused in accordance with the Timm method as modified for electron microscopy (*10*).
14. R. P. Fink and L. Heimer, *Brain Res.* **4**, 369 (1967).
15. D. L. Tauck and J. V. Nadler, *J. Neurosci.* **5**, 1016 (1985).
16. R. S. Sloviter, *Brain Res.* **330**, 150 (1985); *Brain Res. Bull.* **10**, 675 (1983); *Science* **235**, 73 (1987).
17. I. Mody and J. Miller, *Can. J. Physiol. Pharmacol.* **65**, 159 (1985); E. J. Karsarskis, T. M. Forrester, J. T. Slevin, *Epilepsy Res.* **1**, 227 (1987).
18. S. Y. Assaf and S.-H. Chung, *Nature (London)* **308**, 734 (1984); G. A. Howell, M. G. Welch, C. J. Fredrickson, *ibid.*, p. 736; L. Aniksztejn, G. Charton, Y. Ben-Ari, *Brain Res.* **404**, 58 (1987); S. Peters, J. Koh, D. W. Choi, *Science* **236**, 589 (1987).
19. P. Goelet, V. F. Castellucci, S. Schacher, E. R. Kandel, *Nature (London)* **322**, 419 (1986); M. Berridge, *ibid.* **323**, 294 (1986); S. P. Hunt, A. Pini, G. Evan, *ibid.* **328**, 632 (1987).
20. M. Dragunow and H. A. Robertson, *ibid.* **329**, 441 (1987); J. I. Morgan, D. R. Cohen, J. L. Hempstead, T. Curran, *Science* **236**, 192 (1987).
21. T. Curran and J. I. Morgan, *Science* **229**, 1265 (1985); D. Schubert, M. LaCorbiere, C. Whitlock, W. Stallcup, *Nature (London)* **273**, 718 (1978).

22. We appreciate the helpful comments of L. Haberly, R. Kalil, S. Kornguth, P. Lipton, P. Smith, H. Schutta, and O. Steward, as well as the technical assistance of C. Hurtenbach, P. Lewandowski, I. Parada, and S. Feig. This work was supported by NIH grants KO7-NS00808 and R29-NS25020, and by the Epilepsy Foundation of America.

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The Fos Complex and Fos-Related Antigens Recognize Sequence Elements That Contain AP-1 Binding Sites

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The Fos protein complex and several Fos-related antigens bind directly or indirectly to a common sequence element that is similar to the consensus binding site for HeLa cell activator protein 1 (AP-1). This element is present in a negative regulatory sequence in the differentiation-sensitive adipocyte gene, aP2; in a transcriptional enhancer for the Gibbon ape leukemia virus; and in a region of the human immunodeficiency virus (HIV) long terminal repeat partially characterized as a negative regulatory element. The protein level and binding activity of Fos and Fos-related antigens increase rapidly after calcium ionophore treatment of a CD4$^+$ human lymphoblast cell line, H9. These data suggest that several proteins may associate with the AP-1 binding site. Moreover, temporally regulated control of the level of each protein could represent a mechanism for modulation of these putative mediators of gene expression.

THE PROTO-ONCOGENE c-*fos* encodes a nuclear phosphoprotein (Fos) that is associated with chromatin and that displays DNA binding in vitro (*1*). Fos is expressed at relatively low levels in the majority of cell types; however, it can be rapidly and transiently induced by a great variety of extracellular stimuli (*2*). It has been proposed that Fos might function as a nuclear "third messenger" molecule in coupling short-term signals elicited by cell-surface stimulation to long-term alteration in cellular phenotypes by regulating expression of specific target genes (*3*). Indeed, v-*fos* has been shown to stimulate transcription of selected promoters in trans (*4*). Recently, evidence was presented that Fos participates in a nuclear protein complex with a sequence element in a control region of the adipocyte (3T3-F442A) differentiation-sensitive gene, aP2 (*5*). We noticed considerable similarity between the results of oligonucleotide gel retention assays done with the fat-specific element 2 (FSE2) by Distel *et al.* (*5*) to those of Quinn *et al.* (*6*) with an enhancer element from the Gibbon ape leukemia virus (GALV) long terminal repeat (LTR). These two oligonucleotides share a sequence motif within the region (−357 to −278) of the HIV-LTR that acts as a negative regulatory element (NRE) (*7*). This common sequence element is remarkably similar to the consensus binding site AP-1 (*8–10*) (see Fig. 1). To investigate a possible association of Fos with these nucleotide sequences we have applied an assay system that permits direct analysis of cellular proteins that interact with specific nucleic

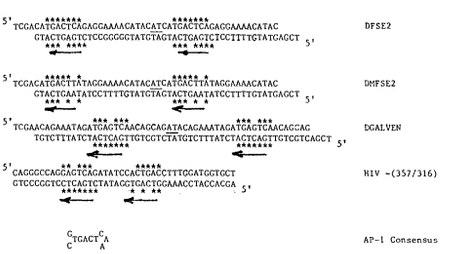

Fig. 1. Sequences of synthetic oligonucleotides used in DNAP assays. DFSE2 is a two-site iteration of the aP2 gene control element (bases −122 to −98 from transcription start site) as described by Distel *et al.* (*5*). DMFSE2 is a two-site iteration of a mutant of FSE2. The sites of mutation are −111 and −109. This mutant was designed to minimally disrupt the AP-1 site of FSE2. DGALVEN is a two-site iteration of the region identified in the GALV Seato strain LTR as sufficient for enhancer activity by Quinn *et al.* (*6*). HIV −(357/316) is a single iteration of these bases in the HIV-LTR as described previously (*7*). The * designates bases of significant sequence similarity to the reported AP-1 consensus (*8–10*). The underlined bases indicate the two-base insertion between the two sequence elements. Oligonucleotides were synthesized, purified, and biotinylated as described (*11*). Under the complementary strand arrows indicate the 5′ to 3′ orientation of the AP-1 related sequences.

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