1906 (1985).
21. K. C. McFarland *et al.*, *Science* **245**, 494 (1989).
22. W. H. Landschulz, P. F. Johnson, S. L. McKnight, *ibid.* **240**, 1759 (1988).
23. R. Turner and R. Tjian, *ibid.* **243**, 1689 (1989); W. H. Landschulz, P. F. Johnson, S. L. McKnight, *ibid.*, p. 1681; R. Gentz, F. J. Rauscher III, C. Abate, T. Curran, *ibid.*, p. 1695.
24. J. Field *et al.*, *Mol. Cell. Biol.* **8**, 2159 (1988).
25. J. Colicelli *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3599 (1989).
26. F. Sherman *et al.*, *Laboratory Course Manual for Methods in Yeast Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986).
27. pYCYR was constructed by first cloning the 4-kb Hind III fragment of pEF-CYR1 (*24*), encoding the COOH-terminal region of adenylyl cyclase, into the Hind III restriction site of YEp13 to yield the plasmid pYC. The 4.5-kb Bam HI–Xho I fragment of pEF-CYR1, containing the *ADH-1* promoter and region encoding the NH_2-terminal region of adenylyl cyclase, was then cloned into pYC, which had been digested with Bam HI and Xho I, to create pYCYR. pYCYR differs from pEF-CYR1 only in the vector used to carry adenylyl cyclase. pEF-CYR1 carries the *TRP1* auxotrophic marker, whereas pYCYR carries *LEU2*. Both plasmids use the yeast *ADH-1* promoter to express an adenylyl cyclase fusion protein. This protein has a small peptide epitope fused to the NH_2-terminus of adenylyl cyclase. The epitope is recognized by monoclonal antibody 12CA5, which can be used to monitor and purify the fusion protein (*24*). The codons deleted are indicated by means of the numbering system of Kataoka *et al.* (*14*). In some cases two regions were deleted. Precise deletions were made by reconstructing the parent plasmid with combinations of restriction endonuclease fragments and the desired DNA products of polymerase chain reactions [R. K. Saiki *et al.*, *Science* **239**, 487 (1988)]. Details of the deletions are described elsewhere (J. Colicelli, J. Field, R. Ballister, N. Chester, M. Wigler, in preparation). Each construct was first tested for function by complementation in adenylyl cyclase–deficient or RAS-deficient strains. Constructs were tested by immunoprecipitation of adenylyl cyclase activity with monoclonal antibody 12CA5. In some cases the enzyme was additionally tested by protein immunoblots or by affinity purification, in each case with the use of monoclonal antibody. After it was established that the deleted plasmids produced a catalytically functional adenylyl cyclase molecule, a frameshift mutation was introduced at the Nco I restriction site. This was performed by digestion with Nco I, filling in the ends with Klenow fragment, followed by ligation. This procedure was monitored by the creation of an Nsi I site formed by the ligation of the filled-in ends of the Nco I site.
28. We thank P. Bird for help in preparing the manuscript. Supported by NIH grant CA 39829, the Pfizer Biomedical Research Award, and the American Cancer Society. T.M. is supported by the Damon Runyon–Walter Winchell Cancer Fund Fellowship DRG-923. J.F. is supported by the Anna Fuller Fund and NIH. J.C. is supported by Life Sciences Research Foundation. M.W. is an American Cancer Society Professor.

24 August 1989; accepted 14 November 1989

Two Distinct Transcription Factors That Bind the Immunoglobulin Enhancer μE5/κE2 Motif

PAULA HENTHORN, MEGERDITCH KILEDJIAN, TOM KADESCH

Activity of the immunoglobulin heavy and κ light chain gene enhancers depends on a complex interplay of ubiquitous and developmentally regulated proteins. Two complementary DNAs were isolated that encode proteins, denoted ITF-1 and ITF-2, that are expressed in a variety of cell types and bind the μE5/κE2 motif found in both heavy and κ light chain enhancers. The complementary DNAs are the products of distinct genes, yet both ITF-1 and ITF-2 are structurally and functionally similar. The two proteins interact with one another through their putative helix-loop-helix motifs and each possesses a distinct domain that dictates transcription activation.

THE IMMUNOGLOBULIN HEAVY chain (IgH) enhancer activates transcription of rearranged heavy chain genes (*1*). In transfection experiments it stimulates transcription from a variety of promoters, but only in cells of the lymphoid lineage (primarily B cells). This activity is mediated through several protein binding sites. Four of these sites, μE1, μE2, μE3, and μE4, were defined initially by in vivo footprinting (*2*). Two others, octa (*3*) and μEBP-E (*4*), were first defined in vitro. Deletions or mutations of these sites generally reduce overall enhancer activity (*5–7*). However, mutation analyses also suggest that the sequence motifs identified by DNA binding assays do not account for all of the IgH enhancer activity (*5, 6*). In particular, a deletion that destroys both the μE1 and μE2 sites and removes the 24 bp between these two sites has a more deleterious effect in B cells than clustered point mutations that simultaneously destroy these two motifs (*6*). This deletion results in an increase in enhancer activity in mouse L cells, suggesting that the region between μE1 and μE2 may comprise a negative regulatory element as well (*6, 8*). An examination of the nucleotide sequence between μE1 and μE2 reveals an additional E-related motif, referred to as μE5 (*9*). Although this site closely resembles the κ light chain enhancer κE2 site, protein binding to the μE5 site has not been detected with crude nuclear extracts (*5, 10*).

To study the function of this region of the enhancer in more detail, we have isolated cDNAs that encode its cognate DNA binding proteins. We used an oligonucleotide bearing both the μE5 and μE2 sites to screen a B cell–derived λgt11 cDNA library (*11*). We analyzed in detail two phage isolates, denoted E2-2 and E2-5, that had binding activity for the oligonucleotide. A series of experiments employing mutant oligonucleotides confirmed that the DNA binding activity encoded by each phage was specific for the μE5 motif within the oligonucleotide, as well as for a κE2 motif carried on a different oligonucleotide (*12*).

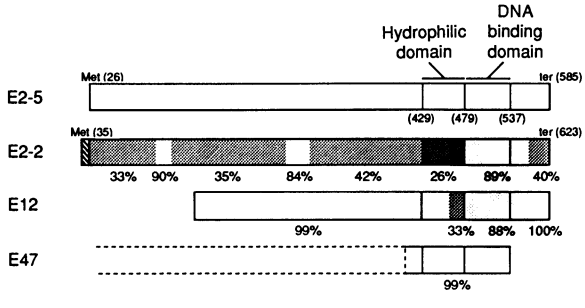


Fig. 1. Comparison of proteins that bind the μE5/κE2 site. A schematic comparison of the amino acid sequences deduced from the E2-5 cDNA, the E2-2 cDNA (GenBank accession numbers M30314 and M30313, respectively), and two related cDNAs (E12 and E47) isolated by Murre *et al.* (*9*) is shown. Amino acid positions are shown in parentheses, with the terminal Eco R1 sites of the E2-5 and E2-2 cDNAs representing amino acids one and two. Amino acid similarity of regions within each protein, relative to the protein encoded by E2-5, is indicated by percent identity and by relative shading, the lighter the shading, the more related (gaps were introduced to optimize the alignments). The dashed lines of clone E47 indicate the region of that cDNA not sequenced. A region of high charge density (hydrophilic domain) and the DNA binding domain as determined by Murre *et al.* (*9*) are shown. Phage clones E2-2 and E2-5 were isolated from a human B cell derived λgt11 cDNA library (Clontech, Palo Alto, California) as described (*11*), with the following modification. The probe used for screening, the μE5 + μE2 oligonucleotide (5′-AGAACACCTGCAGCAGCTGGCAGG-3′; μE5 and μE2 sites underlined), was end-labeled with [γ-^{32}P]-adenosine triphosphate and then ligated to form random concatamers. Nucleotide sequences (both strands) were determined using the dideoxy method and Sequenase T7 DNA polymerase (USB, Cleveland, Ohio) on single-stranded M13 subclones generated with various restriction enzyme fragments. Specific oligonucleotides were also synthesized to use as sequencing primers. Nucleotide sequences are available upon request.

Howard Hughes Medical Institute and Department of Human Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104–1257.

A schematic diagram of the proteins encoded by the E2-2 and E2-5 cDNAs is shown in Fig. 1, along with those encoded by E47 and E12 (*9*), two cDNAs shown previously to encode κE2 binding proteins. The E2-2 and E2-5 proteins share no regions of complete identity. There are, however, three blocks of nearly 90% identity (each corresponding to approximately 75% nucleotide sequence identity) on a background of a minimum of 26% identical amino acid residues. The E2-2 and E2-5 cDNAs encode related proteins that are encoded by distinct genes. On the other hand, E2-5 is nearly identical to E47 and, with the exception of the region identified as the DNA binding domain, to E12. The DNA binding domain of E2-2 is highly related to the similar region in E12. The DNA binding domains of E12 and E47 are comprised of putative helix-loop-helix (HLH) motifs that also specify protein dimerization (*9*). We therefore tested and confirmed that the E2-2 and E2-5 proteins are able to form heterodimers in vitro (*12*).

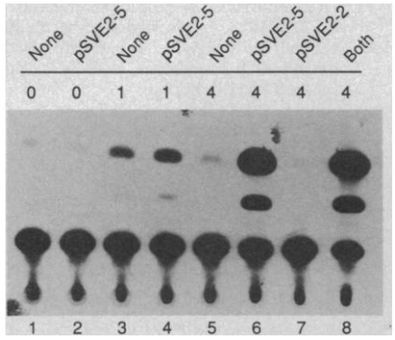
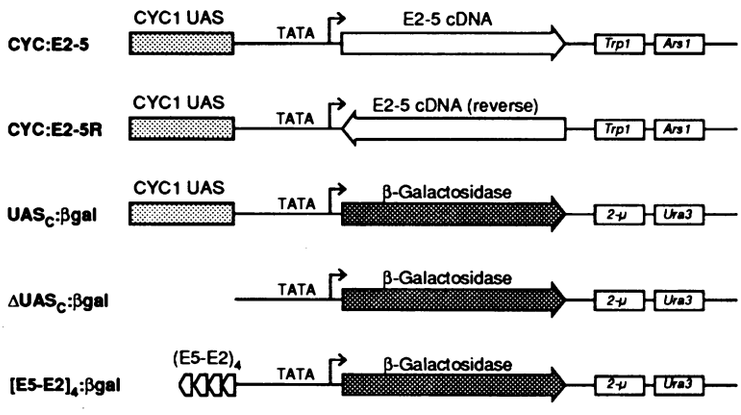


Fig. 2. The E2-5 protein product activates transcription through a μE5 + μE2 oligonucleotide in vivo. Expression plasmids pSVE2-2 and pSVE2-5 (*25*) were cotransfected into the B cell line P3-X63Ag8 (*6*) with reporter plasmids that contain the bacterial *cat* gene under the control of a promoter with varying numbers of μE5 + μE2 oligonucleotides (*25*). The expression plasmids used and the number of μE5 + μE2 oligonucleotides linked to the alkaline phosphatase TATA box (*13*) in each reporter plasmid are indicated above each line. Cells were transfected using 25 μg of expression plasmid, 25 μg of μE5 + μE2 oligonucleotide reporter plasmid, 10 μg of plasmid pSV2Apap (*26*) to normalize transfection efficiencies, and pUC19 DNA to bring the total DNA transfected to 100 μg. Cells were harvested after 48 hours and assayed for CATase (*14*).

To determine if the polypeptides encoded by E2-2 and E2-5 could activate transcription, we linked the entire open reading frames of the cDNAs to the 5′ untranslated region and ATG from the β-globin gene under control of the simian virus 40 (SV40) early promoter. These expression plasmids (pSVE2-2 and pSVE2-5) were then cotransfected into the mouse B cell line P3-X63Ag8 with reporter plasmids that contained various oligonucleotides placed upstream of an alkaline phosphatase gene TATA box (*13*) linked to the bacterial *cat* gene [expressing chloramphenicol acetyltransferase, CATase (*14*)] (Fig. 2). Whenever a reporter plasmid that had one or four copies of the μE5 + μE2 oligonucleotide was transiently transfected along with pSVE2-5, more CATase activity was detected (compare lanes 3 and 4 to lanes 5 and 6). CATase activity was reduced from a reporter plasmid that carried a mutated μE5 site (*12*). Hence, the E2-5 polypeptide is capable of activating transcription through the μE5 site in mammalian cells. We did not detect transcriptional activation in transfections with the pSVE2-2 expression plasmid (lane 7). Cotransfection of pSVE2-2 did not inhibit transcription activation by pSVE2-5 (lane 8). This lack of activation by the E2-2 encoded protein may be a result of weaker DNA binding affinity in vivo or because the E2-2 cDNA may not encode full-length protein. In fact, we have observed that a GAL4:E2-2 fusion protein (see below) is capable of weakly activating transcription through a μE5 site. Hence, it is possible that the GAL4 amino acids supply a function (perhaps nuclear localization) that is missing in the E2-2 encoded protein.

As an initial effort to establish a more well-defined system to study the E2-2 and E2-5 protein products, and ultimately to study their interactions with other IgH enhancer binding proteins, we examined their abilities to activate transcription in yeast. This was accomplished by constructing a set of E2-2 and E2-5 expression plasmids and a set of reporter plasmids, analogous to those described above, but compatible with yeast transformation and expression (Fig. 3). In the yeast expression plasmids, the entire coding sequences of E2-2 and E2-5 cDNAs were linked to the *CYC1* promoter [including the two upstream activating sequence (UAS) elements] and ATG (*15*). For negative controls, the E2-2 and E2-5 cDNAs were also placed in reverse orientation. The reporter plasmids contained the β-galactosidase gene under the control of the yeast *CYC1* promoter (*16*) with or without four tandem copies of the μE5 + μE2 oligonucleotide in place of the *CYC1* UAS ele-



Expression plasmid	Reporter plasmid	β-gal activity
---	UAS_c:βgal	410 ± 79
---	ΔUAS_c:βgal	26 ± 3
---	$[E5\text{-}E2]_4$:βgal	5 ± 1
CYC:E2-5	---	<1
CYC:E2-5R	---	<1
CYC:E2-5	$[E5\text{-}E2]_4$:βgal	330 ± 40
CYC:E2-5R	$[E5\text{-}E2]_4$:βgal	18 ± 6
CYC:E2-5	ΔUAS_c:βgal	7 ± 7

Fig. 3. The E2-5 protein product activates transcription in yeast. The structures of the various yeast expression and reporter plasmids used to transform yeast strain 29α (*27*) are shown on the left. Expression plasmids CYC:E2-5 and CYC:E2-5R contain the forward and reverse orientations, respectively, of the E2-5 cDNA insert under the control of the yeast *CYC1* promoter (*25*). The various reporter plasmids contain the β-galactosidase gene under the control of the *CYC1* TATA box and various elements upstream. UAS_c:βgal contains the normal *CYC1* UAS elements upstream of the *CYC1* TATA box. ΔUAS_c:βgal carries the *CYC1* TATA box only. $[E5\text{-}E2]_4$:βgal carries four copies of the μE5 + μE2 oligonucleotide upstream of the *CYC1* TATA box. Yeast strains were transformed, harvested, and assayed for β-galactosidase as described (*27*). β-galactosidase activity (arbitrary units) was expressed relative to the protein content of each extract. Protein concentrations were determined using the micro-assay of Bio-Rad (Richmond, California). Values represent the mean and standard deviations from at least five independent yeast transformants.

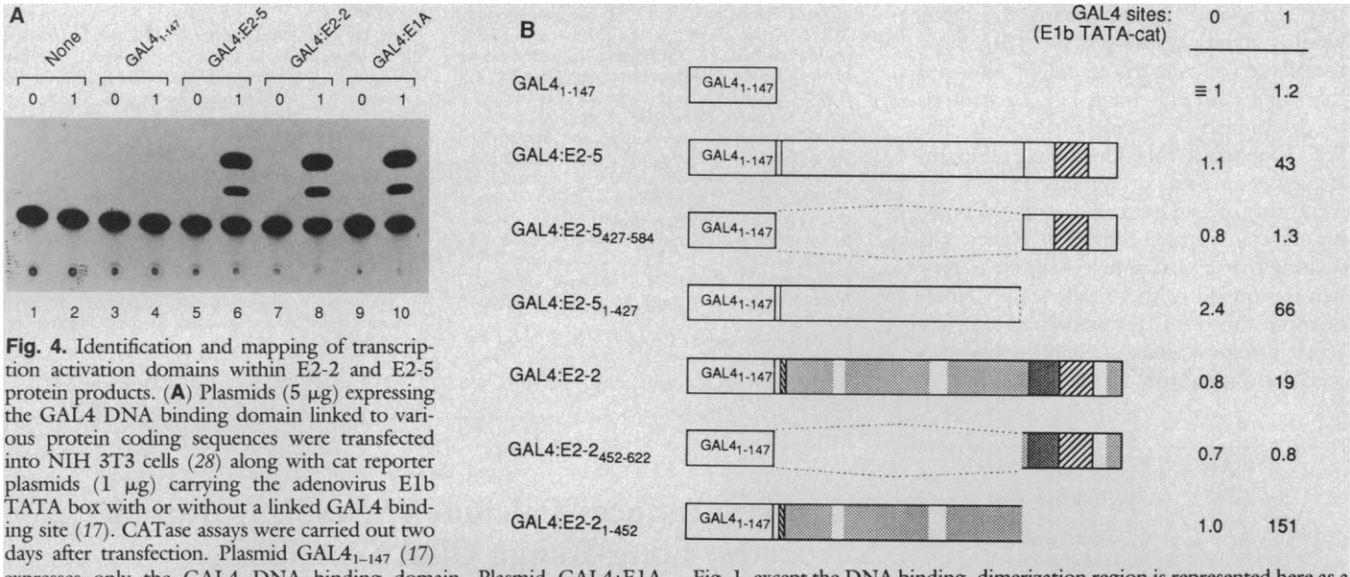


Fig. 4. Identification and mapping of transcription activation domains within E2-2 and E2-5 protein products. (**A**) Plasmids (5 μg) expressing the GAL4 DNA binding domain linked to various protein coding sequences were transfected into NIH 3T3 cells (*28*) along with cat reporter plasmids (1 μg) carrying the adenovirus E1b TATA box with or without a linked GAL4 binding site (*17*). CATase assays were carried out two days after transfection. Plasmid $GAL4_{1-147}$ (*17*) expresses only the GAL4 DNA binding domain. Plasmid GAL4:E1A expresses the GAL4 DNA binding domain linked to the transcription activation domain of the adenovirus E1a protein (*17*). Plasmids GAL4:E2-2 and GAL4:E2-5 express the GAL4 DNA binding domain linked to the entire coding sequences of the E2-2 and E2-5 cDNAs, respectively (*25*). The number of GAL4 binding sites in the reporter plasmids are indicated above each lane. (**B**) Plasmids were constructed to express fusion proteins of the GAL4 DNA binding domain linked to either the NH_2-terminal two-thirds or COOH-terminal one-third of the E2-2 or E2-5 coding regions (*25*). The fusion proteins are diagrammed schematically, using the bar diagrams as in Fig. 1, except the DNA binding–dimerization region is represented here as a cross-hatched box. Subscripted numbers indicate the E2-2 or E2-5 amino acid residues included in the various constructs. The plasmids were transfected into NIH 3T3 cells along with reporter plasmids as described above, except 5 μg of the β-galactosidase expression plasmid pCH110 (*29*) was also included to normalize transfection efficiencies. CATase values, adjusted relative to levels of β-galactosidase, represent the number average of two experiments and are expressed relative to the activity obtained with transfections of the $GAL4_{1-147}$ plasmid and the reporter plasmid carrying no GAL4 binding sites.

ments. Yeast were transformed with various combinations of expression plasmids and reporter plasmids and assayed for β-galactosidase (Fig. 3). Large amounts of β-galactosidase were obtained in yeast transformed with the E2-5 expression plasmid and the reporter plasmid carrying the μE5 + μE2 oligonucleotides. These levels were comparable to those obtained when β-galactosidase was expressed from an intact *CYC1* promoter with its cognate UAS elements. Less β-galactosidase was expressed if either of these plasmids was introduced alone or if the E2-5 cDNA was expressed in the wrong orientation. Similar to our results with mammalian cells, we also did not detect any transcription activation by expressing the E2-2 cDNA in a parallel set of experiments (*12*).

To further examine the transcription activation potential of the proteins encoded by E2-2 and E2-5, we constructed hybrid genes in which the sequences specifying the yeast GAL4 DNA binding domain were fused to the E2-2 and E2-5 cDNA coding regions. Plasmids that contained these fusion genes, which are under the transcription control of the SV40 early promoter, were introduced into mouse NIH 3T3 cells with reporter plasmids that contained the bacterial *cat* gene under the control of an adenovirus E1b TATA element, plus or minus an upstream GAL4 binding site (*17*). It was expected that the GAL4:E2-2 and GAL4:E2-5 fusion proteins would bind to the GAL4 binding site and activate transcription from the E1b TATA box if the E2-2 and E2-5 proteins contain transcription activation domains. When the coding regions of either E2-2 or E2-5 were linked to the GAL4 DNA binding domain, proteins were produced that stimulated transcription from an E1b TATA element linked to a GAL4 binding site (Fig. 4A, lanes 6 and 8). No activation was observed if the reporter lacked a GAL4 binding site (lanes 5 and 7) or if the expression plasmid carried only the GAL4 DNA binding domain (lanes 3 and 4). Activation was roughly equivalent to that obtained using GAL4 fused to the activation domain of the adenovirus E1a protein [lane 10 (*17*)].

The locations of the amino acids required for transcription activation were determined roughly by linking specific segments of the E2-2 and E2-5 coding regions to the GAL4 DNA binding domain. The segments were chosen to test specifically whether the DNA binding–protein dimerization motif was also required for transcription activation (Fig. 4B). The transcription activation domains of both E2-2 and E2-5 were localized to the NH_2-terminal portions of the proteins, distinct from the hydrophilic and DNA binding–protein dimerization domains.

Both proteins encoded by E2-2 and E2-5 cDNAs have the capacity to activate transcription in mammalian cells. Hence, they can be considered transcription factors. Since these proteins are distinct, yet structurally related, we will henceforth refer to them as "immunoglobulin transcription factors" ITF-1 (cDNA E2-5) and ITF-2 (cDNA E2-2).

ITF-1 and ITF-2 are members of a larger family of proteins. The E2-5 cDNA that encodes ITF-1 appears to be a longer version of two closely related cDNA clones, E12 and E47, isolated by Baltimore and coworkers (*9*). Together, these three cDNAs define a small group of proteins that are most likely encoded by alternatively spliced transcripts of a single gene. ITF-2 (encoded by E2-2) is closely related to ITF-1 in a region that shows homology to a number of other proteins, including Myc (*18*), myoD (*19*), myogenin (*20*), lyl-1 (*21*), TFE3 (*22*), and those encoded by the *Drosophila* genes *daughterless*, *twist*, and those of the *achaete-scute* complex (*9, 23*). A region common to all of these proteins has the potential to form an HLH structure. The HLH domain has been shown for some of these proteins to be responsible for DNA binding and protein dimerization (*9*), and for the formation of a variety of heteromeric complexes among proteins with related HLH domains (*24*). We have confirmed that ITF-1 and ITF-2 also interact with one another through their respective HLH domains. It has been proposed that interactions between different HLH-containing proteins may have regula-

tory significance by influencing the DNA binding affinity of the proteins (*24*). How these types of interactions might influence the activities of ITF-1 and ITF-2 remain to be determined. It is relevant to note that ITF-1 appears to function as a transcription factor on its own. It contains distinct elements that dictate both efficient DNA binding and transcription activation. Hence, it is unlikely that a second helix-loop-helix protein is required to specifically supply either of those functions. Furthermore, ITF-1 is active in yeast where it is doubtful that such specialized regulatory proteins exist.

REFERENCES AND NOTES

1. J. Banerji, L. Olson, W. Schaffner, *Cell* **33**, 729 (1983); S. D. Gillies, S. L. Morrison, V. T. Oi, S. Tonegawa, *ibid.*, p. 717; M. S. Neuberger, *EMBO J.* **2**, 1373 (1983); M. L. Atchinson, *Annu. Rev. Cell Biol.* **4**, 127 (1988).
2. A. Ephrussi, G. M. Church, S. Tonegawa, W. Gilbert, *Science* **227**, 134 (1985); G. M. Church, A. Ephrussi, W. Gilbert, S. Tonegawa, *Nature* **313**, 608 (1985).
3. H. Singh, R. Sen, D. Baltimore, P. A. Sharp, *Nature* **319**, 154 (1986); L. M. Staudt *et al.*, *ibid.* **323**, 640 (1986).
4. C. L. Peterson, S. Eaton, K. Calame, *Mol. Cell. Biol.* **8**, 4972 (1988).
5. M. Lenardo, J. W. Pierce, D. Baltimore, *Science* **236**, 1573 (1987).
6. M. Kiledjian, L.-K. Su, T. Kadesch, *Mol. Cell. Biol.* **8**, 145 (1988).
7. B. P. Tsao, X.-F. Wang, C. L. Peterson, K. Calame, *Nucleic Acids Res.* **16**, 3239 (1988); J. Perez-Mutul, M. Macchi, B. Wasylyk, *ibid.*, p. 6085.
8. C. Wasylyk and B. Wasylyk, *EMBO J.* **5**, 553 (1986); T. Kadesch, P. Zervos, D. Ruezinsky, *Nucleic Acids Res.* **14**, 8209 (1986); J. Weinberger, P. S. Jan, P. A. Sharp, *Mol. Cell. Biol.* **8**, 988 (1988).
9. C. Murre, P. Schonleber McCaw, D. Baltimore, *Cell* **56**, 777 (1989).
10. R. Sen and D. Baltimore, *ibid.* **46**, 705 (1986).
11. C. R. Vinson, K. L. LaMarco, P. R. Johnson, W. H. Landschulz, S. L. McKnight, *Genes Dev.* **2**, 801 (1988).
12. P. Henthorn, M. Kiledjian, T. Kadesch, unpublished observations.
13. M. J. Weiss *et al.*, *J. Biol. Chem.* **263**, 12002 (1988).
14. C. M. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982).
15. B. C. Rymond, R. S. Zitomer, D. Schumperli, M. Rosenberg, *Gene* **25**, 249 (1983).
16. L. Guarente and M. Ptashne, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2199 (1981); L. Guarente and T. Mason, *Cell* **32**, 1279 (1983).
17. J. W. Lillie and M. R. Green, *Nature* **338**, 39 (1989).
18. J. Battey *et al.*, *Cell* **34**, 779 (1983).
19. S. J. Tapscott *et al.*, *Science* **242**, 405 (1988).
20. W. E. Wright, D. A. Sasson, V. K. Lin, *Cell* **56**, 607 (1989).
21. J. D. Mellentin, S. D. Smith, M. L. Cleary, *ibid.* **58**, 77.
22. H. Beckmann, L.-K. Su, T. Kadesch, *Genes Dev.* **4**, in press.
23. M. Caudy *et al.*, *Cell* **55**, 1061 (1988); B. Thisse, C. Stoetzel, C. Gorostiza-Thisse, R. Perrin-Schmitt, *EMBO J.* **7**, 2175 (1988); R. Villares and C. V. Cabrera, *Cell* **50**, 415 (1987).
24. C. Murre *et al.*, *Cell* **58**, 537 (1989).
25. Plasmids were constructed using standard techniques (*30*). The βGE2-2 and βGE2-5 constructs were made by replacing the β-globin gene coding region of pT7βΔ6Sal (*31*) with the E2-2 and E2-5 protein-coding regions, respectively. The pSVE2-2 construct was made by replacing the dihydrofolate reductase cDNA of pSV2Adhfr (*32*) with the β-globin 5′ leader, ATG, and E2-2 coding region from βGE2-2. The pSVE2-5 construct was similarly made from βGE2-5. The bacterial *cat* gene reporter plasmids will be described elsewhere (*33*). CYC:E2-5 and CYC:E2-5R were constructed by replacing the GalK gene of YRpR1 (*15*) with the E2-5 cDNA in both orientations. UAS_c:βgal and ΔUAS_c:βgal are pLG669-Z and pLG670-Z (*16*), respectively. $[E5–E2]_4$:βgal was constructed by replacing the UAS sequences of pLG669–Z with four copies of the μE5 + μE2 oligonucleotide. All plasmids expressing GAL4 fusion proteins were derived from $pGAL_{1–147}$ (*17*).
26. P. Henthorn, P. Zervos, M. Raducha, H. Harris, T. Kadesch, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6342 (1988).
27. H. Beckmann and T. Kadesch, *Mol. Cell. Biol.* **9**, 4535 (1989).
28. F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973).
29. F. Lee *et al.*, *Nucleic Acids Res.* **12**, 4191 (1984).
30. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
31. C. Norman, M. Runswick, R. Pollock, R. Treisman, *Cell* **55**, 989 (1988).
32. S. Subramani, R. C. Mulligan, P. Berg, *Mol. Cell. Biol.* **1**, 854 (1981).
33. D. Ruezinsky, H. Beckmann, P. Henthorn, T. Kadesch, manuscript in preparation.

34. We would like to thank R. McCarrick-Walmsley for excellent technical assistance and D. Ruezinsky for the various reporter plasmids carrying the μE5 + μE2 oligonucleotide. Supported by funds from the Howard Hughes Medical Institute (to T.K.).

2 October 1989; accepted 14 December 1989

Glutamate Induces Calcium Waves in Cultured Astrocytes: Long-Range Glial Signaling

ANN H. CORNELL-BELL, STEVEN M. FINKBEINER, MARK S. COOPER, STEPHEN J SMITH*

The finding that astrocytes possess glutamate-sensitive ion channels hinted at a previously unrecognized signaling role for these cells. Now it is reported that cultured hippocampal astrocytes can respond to glutamate with a prompt and oscillatory elevation of cytoplasmic free calcium, visible through use of the fluorescent calcium indicator fluo-3. Two types of glutamate receptor—one preferring quisqualate and releasing calcium from intracellular stores and the other preferring kainate and promoting surface-membrane calcium influx—appear to be involved. Moreover, glutamate-induced increases in cytoplasmic free calcium frequently propagate as waves within the cytoplasm of individual astrocytes and between adjacent astrocytes in confluent cultures. These propagating waves of calcium suggest that networks of astrocytes may constitute a long-range signaling system within the brain.

ASTROCYTES GUIDE NEURONAL DEvelopment, metabolize ions and neurotransmitters, and regulate central nervous system vasculature (*1*), but until recently, there has been little indication that they play any role in rapid signal transmission (*2, 3*). This situation has begun to change with the finding that astrocytes possess ion channels opened on a millisecond time scale by glutamate, the common excitatory neurotransmitter (*3*). In this report we describe another prompt form of astrocytic response to glutamate and observations suggesting that networks of astrocytes may constitute an extraneuronal pathway for rapid long-distance signaling within the brain.

We first examined intracellular free calcium (Ca^{2+}_i) responses of cultured hippocampal astrocytes to prolonged applications of glutamate (*4, 5*) (Fig. 1). Virtually all cells responded to 100 μ*M* glutamate with an initial spike-like increase in Ca^{2+}_i, but later phases of the response to the continued presence of glutamate varied from cell to cell (Fig. 1, A to D). We have classified response types into three categories. (i) In the sustained oscillation response, cells exhibited a prolonged episode of Ca^{2+}_i oscillation, lasting from 300 to 1800 s (mean ± SD = 850 ± 300 s; measurements on 24 cells, five experiments). In some cells, the oscillation frequency remained relatively constant (Fig. 1E) (period of 13 ± 2 s; 192 measurements on seven cells, three experiments). In other cells, the oscillation frequency gradually decreased (Fig. 1F) (period of initial cycle of oscillation is 9 ± 2 s, increasing to 23± 5 s after 5 min, 31 measurements). (ii) In the damped-oscillation response, cells exhibited Ca^{2+}_i oscillations of decreasing frequency (Fig. 1G) (period of initial cycle of oscillation is 12 ± 4 s) that damped to a steady, elevated base line within 115 ± 48 s (230 measurements on 30 cells, three experiments). (iii) In the third response pattern, cells did not oscillate, but exhibited a step-response, where Ca^{2+}_i remained elevated

Section of Molecular Neurobiology, Howard Hughes Medical Institute and Yale University School of Medicine, New Haven, CT 06510.

* Present address: Department of Molecular and Cellular Physiology, Stanford Medical School, Stanford, CA 94305.