experiments should increase proportionate to t^2, where t is the time after gramicidin addition [S. B. Hladky, in *Drugs and Transport Processes*, B. A. Callingham, Ed. (Macmillan, London, 1974), pp. 193–210]. This effect is observed, however, only if the surface density of $\beta^{6.3}$-helical monomers increases as a linear function of t. The approximately linear increase in channel activity over time results because gramicidin channels form by several parallel sequential processes. See also D. C. Buster, J. F. Hinton, F. S. Millett, D. C. Shungu, *Biophys. J.* **53**, 145 (1988).
18. M. C. Bano, L. Braco, C. Abad, *FEBS Lett.* **250**, 67 (1989).
19. The gramicidin dimerization constant in ethanol is ~30 μM (*4*), and ~0.1% of the total gramicidin in the (14 nM) stock solution is intertwined dimers. This amount is in principle enough to account for all of the channel activity seen here.
20. D. W. Urry, M. M. Long, M. Jacobs, R. D. Harris, *Ann. N.Y. Acad. Sci.* **263**, 203 (1975).
21. I. Amdur and G. G. Hammes, *Chemical Kinetics: Principles and Selected Topics* (McGraw-Hill, New York, 1965), pp. 16–19.
22. We distinguish between slow permeation of gramicidin (monomers) across the bilayer, as evidenced by the maintained asymmetry in monolayer composition, and transfer of gramicidin at the start of the experiment, which constitutes a separate and transient process.
23. D. B. Sawyer, R. E. Koeppe II, O. S. Andersen, *Biophys. J.* **57**, 515 (1990).
24. J. T. Durkin, O. S. Andersen, F. Heitz, Y. Trudelle, R. E. Koeppe II, *Biophys. J.* **51**, 451a (1987).
25. Intertwined dimers of gA are left-handed helices (*6*), while the channel-forming $\beta^{6.3}$-helical dimers are right-handed helices [A. S. Arseniev, I. L. Barsukov, V. F. Bystrov, A. L. Lomize, Y. A. Ovchinnikov, *FEBS Lett.* **186**, 168 (1985); L. Nicholson and T. A. Cross, *Biochemistry* **28**, 9379 (1989); O. S. Andersen *et al.*, *Biophys. J* **57**, 100a (1990)].
26. The relative position of Trp in hydrophobicity scales varies with the methods used to establish the scales [D. M. Engelman, T. A. Steitz, A. Goldman, *Annu. Rev. Biophys. Chem.* **15**, 321 (1986)]. Methods that rely on the partitioning of Trp between water and nonpolar solvents (such as ethanol or octanol) tend to categorize Trp as quite hydrophobic, but these solvents have hydrogen-bond accepting groups. Methods that rely on the statistical distribution of residues in proteins of known structure or vapor-phase distribution tend to categorize Trp as only modestly hydrophobic. We also note that Trp has a high affinity for the membrane-solution interface [R. E. Jacobs and S. H. White, *Biochemistry* **28**, 3421 (1989)].
27. J.-L. Mazet, O. S. Andersen, R. E. Koeppe II, *Biophys. J.* **45**, 263 (1984).
28. H. Michel and J. Deisenhofer, *Curr. Top. Membr. Transp.* **36**, 53 (1990).
29. V. R. Lingappa, *J. Clin. Invest.* **83**, 739 (1989).
30. J. Kyte and R. F. Doolittle, *J. Mol. Biol.* **157**, 105 (1979).
31. Supported in part by NIH grants GM21342 and GM34968. We thank M. B. Becker, P. Daumas, J. T. Durkin, L. L. Providence, and D. B. Sawyer for helpful discussions and comments on the manuscript. A preliminary report has appeared [A. K. Maddock, R. E. Koeppe II, O. S. Andersen, *ICSU Short Rep.* **9**, 24 (1989)].

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Binding of the Wilms' Tumor Locus Zinc Finger Protein to the EGR-1 Consensus Sequence

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The Wilms' tumor locus (WTL) at 11p13 contains a gene that encodes a zinc finger–containing protein that has characteristics of a DNA-binding protein. However, binding of this protein to DNA in a sequence-specific manner has not been demonstrated. A synthetic gene was constructed that contained the zinc finger region, and the protein was expressed in *Escherichia coli*. The recombinant protein was used to identify a specific DNA binding site from a pool of degenerate oligonucleotides. The binding sites obtained were similar to the sequence recognized by the early growth response–1 (EGR-1) gene product, a zinc finger–containing protein that is induced by mitogenic stimuli. A mutation in the zinc finger region of the protein originally identified in a Wilms' tumor patient abolished its DNA-binding activity. These results suggest that the WTL protein may act at the DNA binding site of a growth factor–inducible gene and that loss of DNA-binding activity contributes to the tumorigenic process.

WILMS' TUMOR (WT) IS A MALIGNANCY of the kidney that occurs in children and is associated with aniridia, mental retardation, and urogenital malfunctions (the WAGR syndrome) (*1–3*). As with retinoblastoma, both hereditary and sporadic forms of WT have been described (*4*). Several cytogenetic studies have linked a specific chromosomal abnormality at the 11p13 locus to the heritable form of the tumor (*2, 3*). Furthermore, loss of heterozygosity at polymorphic loci that surround 11p13 has been observed in sporadic WT (*5*). Introduction of a normal human chromosome 11 into a WT cell line suppresses some aspects of the malignant phenotype (*6*), further suggesting that a tumor suppressor gene on chromosome 11 is involved in the genesis of WT.

Candidate complementary DNAs (cDNAs) for the WT gene have been isolated (*7, 8*). One was obtained with a long-range restriction map of the WAGR region, which allowed localization of the WT gene to a 345-kilobase region of chromosome 11 (*9*); a cDNA clone was subsequently isolated from this region (*7*). In another approach, the presence of CpG islands adjacent to the WT locus (WTL) was exploited in conjunction with chromosome jumping to isolate a similar cDNA (*8*). The gene is expressed in high amounts in embryonic kidney and adult spleen (*7, 8*).

The predicted protein product of the WTL contains a region rich in glutamine and proline residues, and four contiguous zinc fingers of the Cys_2-His_2 class (*7, 8*). The presence of these two structural motifs implies that the WTL protein is a sequence-specific nucleic acid–binding protein that may function as a transcriptional regulator (*10*). In this report, we have initiated a biochemical characterization of the WTL protein by identifying a DNA target sequence that is recognized by the WTL protein.

Our strategy was to express the zinc finger domain of the WTL protein in *E. coli* and to use this protein as an affinity matrix for isolation of a DNA target sequence. The zinc finger region (termed WT-ZF) was reconstructed from synthetic oligonucleotides with the use of an overlapping PCR strategy for gene synthesis (Fig. 1A) (*11*). Six histidine residues were added to the NH_2-terminus to facilitate purification of the protein with a nickel-chelate affinity resin (*11, 12*). The purified WT-ZF protein migrated with an apparent molecular size of 21,500 kilodaltons (kD) on SDS-polyacrylamide gels (Fig. 1B, lane 2).

A modification of the method developed by Struhl and colleagues (*13*) was used to isolate DNA sequences that were recognized specifically by WT-ZF (Fig. 2A). A degenerate oligonucleotide that contained random nucleotides at 25 consecutive positions flanked by Xho I and Eco RI sites was synthesized. By self-annealing this oligonucleotide and extending with DNA polymerase in the presence of deoxynucleotide triphosphates (dNTP's), a completely degenerate library of double-stranded oligonucleotides was obtained. A probe was prepared with ^{32}P-labeled dNTP's that allowed verification of the presence of potential binding sites (Fig. 2A). The probe was then selected

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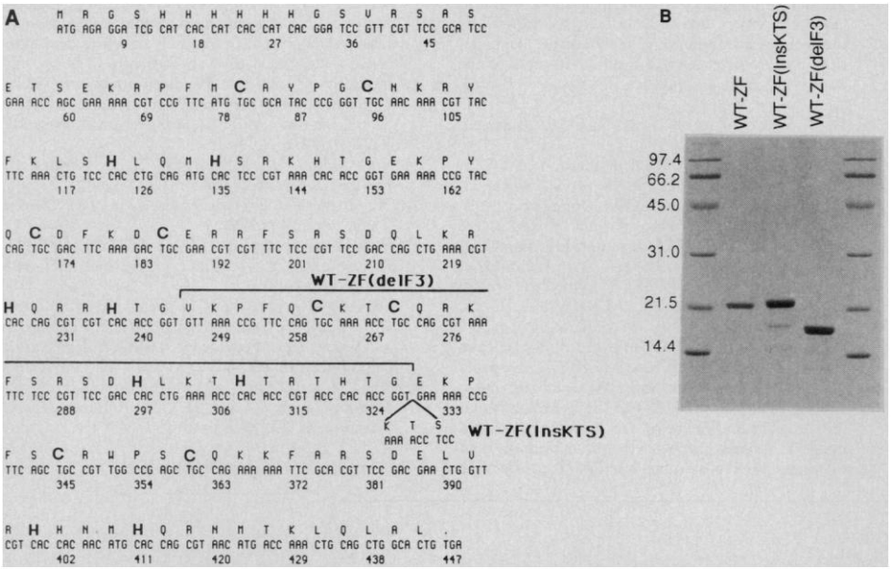
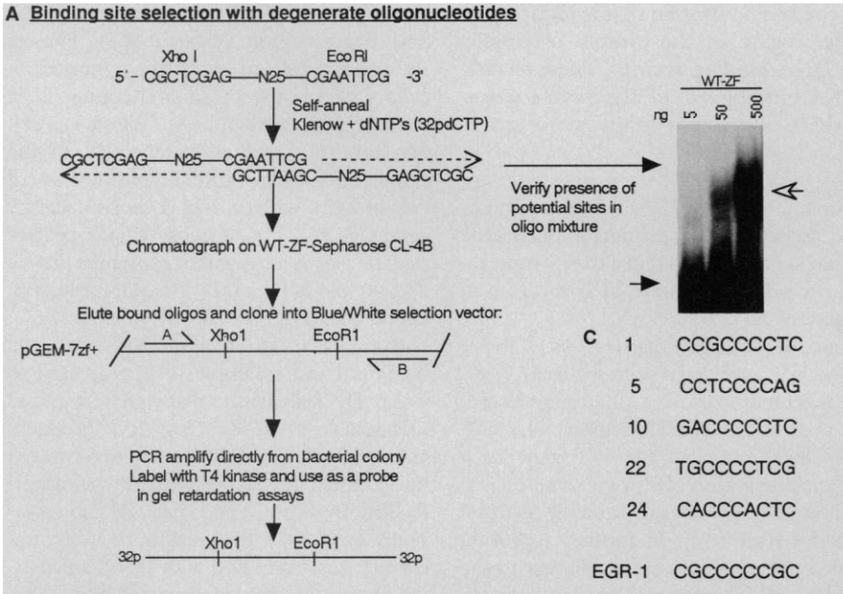


Fig. 1. WT-ZF: A synthetic gene that encodes the zinc finger region of the protein present at the Wilms' tumor locus. (**A**) The WT-ZF gene was constructed with synthetic oligonucleotides and an overlap-extension PCR method (*11*). The amino acid sequence was taken from the WT33 gene (*7*). The valine at position 13 corresponds to amino acid number 210 of WT33. The six histidine residues and the initiator methionine were added to facilitate expression and purification from *E. coli* with the use of nickel-chelate affinity chromatography (*12*). The cysteine and histidine residues which form the zinc fingers are shown in bold letters. The bracketed region indicates the amino acids that are deleted in WT-ZF(delF3) (*21*). WT-ZF(InsKTS) indicates the three–amino acid insertion that occurs as a normal splicing variant (*21*). (**B**) Expression and purification of WT-ZF proteins. The WT-ZF gene and the insertion [WT-ZF(InsKTS)] and deletion [WT-ZF(delF3)] mutations diagrammed in (A) [introduced into WT-ZF by PCR-mediated mutagenesis (*22*)] were cloned into the pDS56 *E. coli* expression vector (*12*). After DNA sequence analysis, recombinant proteins were produced and purified. Samples (2.5 μg) of each protein preparation were resolved on 12% SDS-polyacrylamide gels and the protein bands were stained with Coomassie blue. Protein molecular size standards (first and last lanes) were: rabbit muscle phosphorylase, 97 kD; bovine serum albumin, 66 kD; hen egg white ovalbumin, 45 kD; bovine carbonic anhydrase, 31 kD; soybean trypsin inhibitor, 21 kD; and hen egg white lysozyme, 14 kD.

by affinity chromatography on a WT-ZF affinity column (*14*). Oligonucleotides that bound to the column were eluted and cloned. Appropriate oligonucleotide primers and the polymerase chain reaction (PCR) were utilized to amplify a 70–base pair (bp) fragment for use in gel retardation assays (Fig. 2B) (*15*). Probes amplified from the vector alone did not bind to the WT-ZF protein. However, probes from clones 1, 5, 10, 22, and 24 were bound by recombinant WT-ZF in a gel retardation assay. The DNA sequence was derived from the PCR products and subjected to a comparative homology search. The probes that exhibited significant DNA-binding activity in the gel shift assay were relatively GC-rich and contained stretches of 4 to 5 C residues (Fig. 2C). These DNA sequences are similar to the consensus binding site of EGR-1 (also known as Krox 24, zif268, TIS-8, and NGF1-A) (*16*). EGR-1 is a serum-inducible, nuclear phosphoprotein that contains three zinc fingers (*16*). The zinc finger domain of WT-ZF exhibits 51% amino acid similarity to the EGR-1 zinc finger domain (*7*, *8*).

To determine if the WT-ZF protein recognized the EGR-1 DNA binding site, a synthetic oligonucleotide that contained a high-affinity, EGR-1 binding site (*17*) was used in gel retardation assays (Fig. 3A). Specific binding of the WT-ZF protein to this synthetic oligonucleotide was detected with as little as 0.2 ng of protein. The



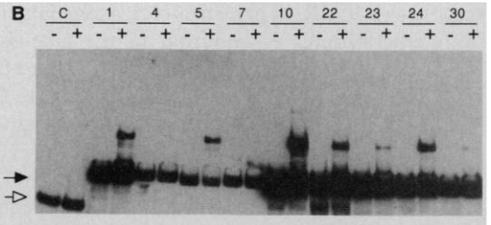


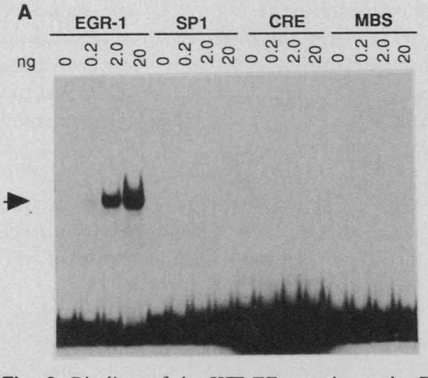
Fig. 2. Selection of consensus binding sites for the WT-ZF protein. (**A**) The strategy used for isolation of potential binding sites is a modification of (*13*). A synthetic 41-nucleotide (nt) probe was designed with a 25-nt internal segment that was degenerate for each of the four bases (A, C, G, or T). The degenerate oligonucleotide was converted to a double-stranded product by self-annealing and extension with DNA polymerase and cold dNTP's. A radioactive probe mix was used to verify the presence of potential binding sites in a gel retardation assay (*15*) with recombinant WT-ZF (inset). The solid arrow indicates the free probe; the open arrow indicates probe bound to the WT-ZF protein. The probe mixture was then chromatographed on a WT-ZF Sepharose CL-4B column (*14*). After three cycles of enrichment for oligonucleotides that bound to the column, the potential binding sites were cloned into a plasmid vector. Bacterial colonies that contained plasmids with inserts were used to directly amplify a 70-bp segment with PCR primers that flanked the cloning sites in the plasmid polylinker (*15*). (**B**) The PCR products that contained potential WT-ZF binding sites were used in gel retardation assays with recombinant WT-ZF protein. Binding reactions were performed with ^{32}P-labeled probe in the presence (+) or absence (−) of WT-ZF protein (200 ng). Open arrow, probe amplified from the vector alone; solid arrow, probes amplified from bacterial lysates that contained potential binding sites. (**C**) Potential sequences recognized by the WT-ZF protein. The probes that showed positive binding activity in gel retardation assays with WT-ZF were sequenced. The sequence of the region that contained the 25-nt degeneracy for each probe was subjected to homology alignment with the Compare program of MacMolly (Soft Gene Berlin). The region of each probe showing maximum similarity is indicated. The core consensus binding site for the EGR-1 protein (*16*) is shown at the bottom of the figure.

WT-ZF protein did not bind to the SP1 consensus sequence (another GC-rich binding site recognized by a zinc finger protein), the cyclic adenosine monophosphate (*c*AMP)– responsive element (CRE), or the myb binding site (MBS) (Fig. 3A) (*17, 18*). A number of controls were performed to confirm that the WT-ZF protein (and the EGR-1 protein) interacted specifically with the EGR-1 site: (i) an unlabeled EGR-1 oligonucleotide competed for binding to the EGR-1 site; (ii) the EGR-1 probe competed efficiently for binding with the original target sequences isolated from the degenerate oligonucleotide library (Fig. 2, B and C); (iii) the binding activity of the WT-ZF protein was abolished by the zinc chelator ortho-phenanthroline; (iv) binding activity was inhibited by an affinity-purified rabbit polyclonal antibody to WT-ZF (*19*); and, (v) the EGR-1 protein and the WT-ZF protein produced in rabbit reticulocyte lysate translation systems bound to the EGR-1 binding sequence (*20*). Thus, EGR-1 and WT-ZF proteins both recognized the EGR-1 consensus binding site. Furthermore, because WT-ZF binding activity was observed with the very small amounts of protein produced in the in vitro translation system, DNA binding did not require high concentrations of protein.

In order to determine whether WT-ZF recognized exactly the same core consensus sequence as EGR-1, mutations were introduced at various places in the EGR-1 binding site (Fig. 3B). Oligonucleotides M2 and M3 showed no binding activity with either protein, whereas M4 bound to both WT-ZF and EGR-1 (Fig. 3C). Mutant M1 showed diminished binding to WT-ZF, whereas binding to EGR-1 was unaffected. These results suggest that the DNA binding specificities of WT-ZF and EGR-1 proteins overlap, but may not be identical.

Recently a mutation was identified at the WTL of a WT patient (*21*). This mutation is a deletion of 25 bp that includes an exon-intron splice junction and results in the deletion of the third zinc finger. This mutation was introduced (*22*) into the synthetic WT-ZF gene [WT-ZF(delF3)], and the protein was purified (Fig. 1B, lane 3). The WT-ZF(delF3) protein exhibited only low DNA-binding activity with the EGR-1 oligonucleotide (Fig. 4). Thus, a naturally occurring mutation in the WTL severely reduced DNA-binding activity of the mutant WTL protein. A normal splicing variant has also been described in which three amino acids (Arg-Thr-Lys) are inserted between the third and fourth zinc fingers (Fig. 1A) (*21*). This alteration was introduced into WT-ZF to determine its effect on DNA-binding activity. The protein encoded by this splice variant (Fig. 1B, lane 4) exhibited impaired binding to the EGR-1 site (Fig. 4). However, we cannot rule out that the DNA binding specificity of the protein changed as a result of the three–amino acid insertion.

These studies have identified a DNA binding site recognized by the zinc finger protein encoded by the WTL. In addition, a mutation originally identified in a WT patient severely reduced DNA-binding activity of the protein. Thus, a mutation in a gene that has been identified genetically as a tumor suppressor gene (*7–9*) destroys a specific biochemical property of the encoded protein, that is, sequence-specific recognition of DNA.



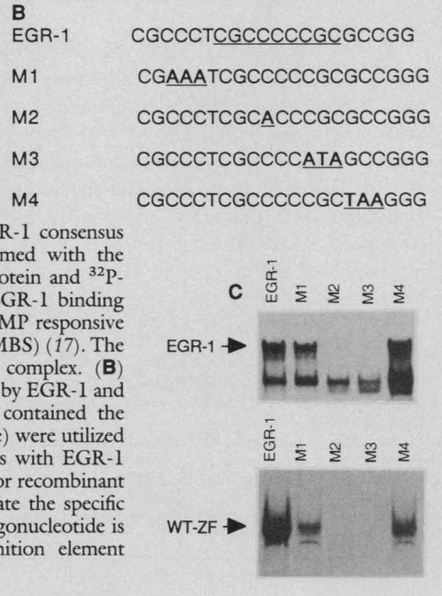


Fig. 3. Binding of the WT-ZF protein to the EGR-1 consensus sequence. (**A**) Gel retardation assays were performed with the indicated amounts (ng) of recombinant WT-ZF protein and ^{32}P-labeled oligonucleotide probes that contained an EGR-1 binding site (EGR-1), an SP1 binding site (SP1), a cyclic AMP responsive element binding site (CRE), or a myb binding site (MBS) (*17*). The arrow indicates the WT-ZF–probe nucleoprotein complex. (**B**) Similar DNA sequence requirements for recognition by EGR-1 and WT-ZF proteins. Synthetic oligonucleotides that contained the indicated mutations (shown underlined, in bold type) were utilized in gel retardation assays. (**C**) Gel retardation assays with EGR-1 protein expressed in reticulocyte lysates (top panel) or recombinant WT-ZF protein (bottom panel). The arrows indicate the specific nucleoprotein complexes. The wild-type EGR-1 oligonucleotide is shown at the top of (B) with the core recognition element underlined.

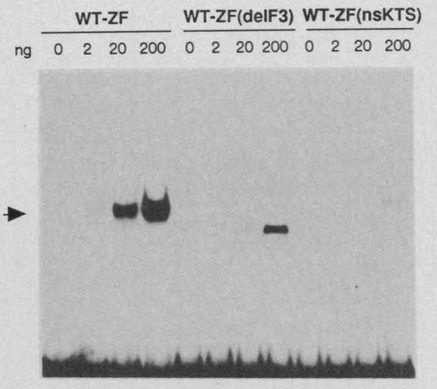


Fig. 4. Mutations in the WT-ZF protein block DNA-binding activity. The insertion [WT-ZF (InsKTS)] and deletion [WT-ZF(delF3)] mutations (Fig. 1A) were introduced into WT-ZF by PCR-mediated mutagenesis (*22*). The purified, recombinant proteins (Fig. 1B) were used in gel retardation assays with a ^{32}P-labeled EGR-1 probe. The arrow indicates the WT-ZF protein-DNA complex.

Because WT-ZF and EGR-1 can recognize the same DNA sequence, there may be a link between a tumor suppressor gene and the family of cellular immediate-early genes induced by growth factors (*23*). The EGR-1 family of genes, like the *fos* and *jun* families, are rapidly induced by mitogenic or differentiative stimuli and encode proteins that regulate gene transcription (*23*). The protein products of cellular immediate-early genes initiate changes in expression of specific target genes whose products are required for entry into the cell cycle or establishment of a differentiated phenotype.

The product of the WTL might act in an antagonistic manner to EGR-1 by binding to the same DNA recognition sequence. Alternatively, the WTL protein might be a tissue-specific regulator of transcription that initiates or maintains a particular differentiated cellular phenotype. A critical balance in the nucleus among members of the EGR-1 family of proteins and the products of the WTL might be required to maintain normal growth control. An alteration in this balance, which occurs when the WT gene is deleted or when the DNA binding properties of its encoded protein are inactivated, could initiate the neoplastic process.

REFERENCES AND NOTES

1. E. Matsunaga, *Hum. Genet.* **57**, 231 (1981).
2. R. W. Miller, J. F. Fraumeni, M. D. Manning, *N. Engl. J. Med.* **270**, 922 (1964).
3. V. M. Ricciardi, E. Sujansky, A. C. Smith, U. Francke, *Pediatrics* **61**, 604 (1978); U. Francke, L. B. Holmes, L. Atkins, V. M. Ricciardi, *Cytogenet. Cell Genet.* **24**, 185 (1979).
4. A. G. Knudson, *Proc. Natl. Acad. Sci. U.S.A.* **68**,

820 (1971); A. G. Knudson and L. C. Strong, *J. Natl. Cancer Inst.* **48**, 313 (1972).
5. A. Koufos *et al.*, *Nature* **309**, 170 (1984); S. H. Orkin, D. S. Goldman, S. E. Sallan, *ibid.*, p. 172; A. E. Reeve *et al.*, *ibid.*, p. 174; E. R. Fearon, B. Vogelstein, A. P. Feinberg, *ibid.*, p. 176.
6. B. E. Weissman *et al.*, *Science* **236**, 175 (1987).
7. K. M. Call *et al.*, *Cell* **60**, 509 (1990).
8. M. Gessler *et al.*, *Nature* **343**, 744 (1990).
9. E. A. Rose *et al.*, *Cell* **60**, 495 (1990).
10. P. J. Mitchell and R. Tjian, *Science* **245**, 371 (1989).
11. The methods for total gene synthesis with the use of overlap-extension PCR are described in the following articles: P. J. Dillon, P. Nelblock, A. Perkins, C. A. Rosen, *J. Virol.* **64**, 4428 (1990); P. J. Dillon and C. A. Rosen, *Biotechniques* **9**, 298 (1990); C. Abate, D. Luk, T. Curran, *Cell Growth Differ.* **1**, 455 (1990). Briefly, four overlapping oligonucleotides (120 bp in length) were synthesized, which spanned the zinc finger region of WT33 (*7*). Codon usage was optimized for *E. coli* [see P. M. Sharp *et al.*, *Nucleic Acids Res.* **16**, 8207 (1988)]. A standard PCR reaction that contained the four oligonucleotides was performed for ten cycles. A second PCR reaction run in the presence of short flanking primers gave rise to a 430-bp fragment, which was subcloned into the pDS56 *E. coli* expression vector (*12*). The vector provided the initiator methionine and six histidine residues to permit nickel-chelate affinity chromatography (*12*). The synthetic gene was sequenced on both strands with oligonucleotide primers that were complementary to flanking vector sequence. Log phase *E. coli* that harbored the WT-ZF expression vector were induced with isopropyl-β-D-thiogalactopyranoside (1 mM) and harvested 2 hours later by lysis in 6 M guanidine-HCl, pH 8.0. After nickel-chelate affinity chromatography (*12*), the purified protein was exhaustively dialyzed against binding buffer: 25 mM Hepes-KOH (pH 7.5), 100 mM KCl, 10 μM $ZnSO_4$, 0.1% NP-40, 1 mM DTT, and 5% glycerol. Protein yield averaged 3 to 5 mg per liter of bacteria.
12. E. Hochuli, W. Bannwarth, H. Dobeli, R. Gentz, D. Stuber, *Bio/Technology* **6**, 1321 (1988); C. Abate, D. Luk, R. Gentz, F. J. Rauscher III, T. Curran, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1032 (1990).
13. A. R. Oliphant, C. J. Brandl, K. Struhl, *Mol. Cell. Biol.* **9**, 2944 (1989).
14. The WT-ZF–Sepharose CL-4B affinity column was constructed as follows: pure recombinant WT-ZF (2 mg) was dialyzed against a coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl). Cyanogen bromide–activated Sepharose CL-4B (2 ml) was hydrated and prepared as described by the manufacturer, and incubated with the WT-ZF solution for 24 hours at 4°C. The resin was extensively washed with high and low salt buffers. Coupling efficiency approached 90%. Binding buffer (100 μl) (*11*) that contained double-stranded, degenerate probe (5 μg), poly(dIdC) (20 μg) and bovine serum albumin (0.2 mg/ml) was applied to the column. After consecutive washes with binding buffer that contained 200 and 400 mM KCl, bound oligonucleotides were released by incubating the resin in 0.5 M tris-HCl, 20 mM EDTA, 10 mM NaCl, 0.2% SDS (100 μl) at 50°C for 20 min. Recovered oligonucleotides were purified by phenol extraction-ethanol precipitation and either subjected to additional rounds of chromatography or digested with Xho I and Eco RI and ligated into pGEM-7Zf$^+$ (Promega). Ligations were transformed into competent DH5α bacteria and plated under blue-white selection conditions.
15. Bacterial colonies were inoculated into growth medium (1 ml) and grown at 37°C for 2 hours. Cultures were boiled for 5 min, centrifuged at 20,000*g* for 10 min, and the supernatant (5 μl) utilized for PCR. A standard PCR reaction was performed for 20 cycles except that the extension time at 72°C was reduced to 30 s. The PCR products were purified on preparative polyacrylamide gels (5%) and labelled with T4 kinase and [^{32}P]ATP under standard conditions. The primers used for PCR were complementary to the pGEM-7Zf$^+$ polylinker and were 5′-GCCCGACGTCGCATG-3′ (T7 side) and 5′-ATCGATTTCGAACCC-3′ (SP6 side). DNA binding reactions contained the indicated amounts of WT-ZF protein, poly(dIdC) (2 μg), and 50 to 70,000 cpm of ^{32}P-labeled probe in binding buffer (10 μl) (*11*). Nondenaturing gel electrophoresis was performed as described (*18*).
16. V. Sukhatme *et al.*, *Cell* **53**, 37 (1988); B. Christy and D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8737 (1989); X. Cao *et al.*, *Mol. Cell. Biol.* **10**, 1931 (1990); J. Milbrandt, *Science* **238**, 797 (1987); P. Lemaire *et al.*, *Mol. Cell Biol.* **10**, 3456 (1990); R. W. Lim, B. C. Varnum, H. R. Herschman, *Oncogene* **1**, 263 (1987).
17. Oligonucleotides for use in DNA binding assays were synthesized, gel purified, annealed, and labelled as described (*18*). The binding sites used were as follows: EGR-1, 5′-CGCCCTCGCCCCCGCGCCGGG-3′; SP1, 5′-TCCCGCCCCTAACTCCGCCCAT-3′; CRE, 5′-GCGCTCCTTGGCTGACGTCAGAGAGAGAG-3′; MBS 5′-GCTAAAAAACCGTTATAATGTG-3′.
18. F. J. Rauscher III, P. J. Voulalas, B. R. Franza, Jr., T. Curran, *Genes Dev.* **2**, 1687 (1988).
19. J. F. Morris and F. J. Rauscher III, in preparation.
20. F. J. Rauscher III, unpublished data.
21. D. A. Harber *et al.*, *Cell* **61**, 1257 (1990).
22. S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* 77, 51 (1989).
23. T. Curran and B. R. Franza, Jr., *Cell* **55**, 395 (1989); J. I. Morgan and T. Curran, *Trends Neurosci.* **12**, 459 (1989).
24. We thank H. Koprowski for his continuous support; C. Abate for much help and advice on nickel-chelate chromatography and PCR-mediated mutagenesis; R. Gentz for NTA resin and pDS vectors; P. Dillon and D. Luk for advice on PCR-mediated gene synthesis; V. Sukhatme for pBS.Apa I and anti-EGR-1 sera; R. Weinmann for the SP1 binding site; P. Reddy for the myb binding site and many helpful discussions; and G. Rovera, P. Reddy, and B. Knowles for critical reading of the manuscript. Supported by grant CA23413, core grant CA10817, and grant CA0917-15 from the NIH (J.F.M.).

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Mapping of Herpes Simplex Virus–1 Neurovirulence to $\gamma_1$34.5, a Gene Nonessential for Growth in Culture

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The gene designated $\gamma_1$34.5 maps in the inverted repeats flanking the long unique sequence of herpes simplex virus–1 (HSV-1) DNA, and therefore it is present in two copies per genome. This gene is not essential for viral growth in cell culture. Four recombinant viruses were genetically engineered to test the function of this gene. These were (i) a virus from which both copies of the gene were deleted, (ii) a virus containing a stop codon in both copies of the gene, (iii) a virus containing after the first codon an insert encoding a 16–amino acid epitope known to react with a specific monoclonal antibody, and (iv) a virus in which the deleted sequences were restored. The viruses from which the gene was deleted or which carried stop codons were avirulent on intracerebral inoculation of mice. The virus with the gene tagged by the sequence encoding the epitope was moderately virulent, whereas the restored virus reacquired the phenotype of the parent virus. Significant amounts of virus were recovered only from brains of animals inoculated with virulent viruses. Inasmuch as the product of the $\gamma_1$34.5 gene extended the host range of the virus by enabling it to replicate and destroy brain cells, it is a viral neurovirulence factor.

APPROXIMATELY ONE IN 250,000 adults per year acquires herpes simplex virus (HSV) encephalitis: an acute, frequently lethal disease with lasting sequelae even in individuals treated promptly with antiviral drugs (*1*). The morbidity associated with the HSV encephalitis has prompted considerable interest in determining which of the more than 70 viral genes impart to the virus the capacity to cause central nervous system (CNS) disease. We report that the protein encoded by the HSV-1 gene designated $\gamma_1$34.5 enables HSV-1 to cause lethal infection of the mouse brain.

The HSV-1 genome, schematically represented in Fig. 1, consists of two sets of unique sequences each flanked by inverted repeats (*2*). The long unique sequence is flanked by 9-kbp inverted repeats designated as *ab* and *b′a′*, respectively (*3*). The observation that the terminal 500-bp *a* sequence acts as a late (γ_1) HSV promoter led to the discovery of the $\gamma_1$34.5 gene. The coding sequences for the $\gamma_1$34.5 gene are located in the adjacent *b* sequences and specify a protein of 263 amino acids with an apparent molecular weight of 44,000 (*4–6*). The remarkable properties of this gene are that it lacks a canonical TATAA box and that its 5′ untranscribed domain, the *a* sequence, is

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