was set at ×8. Standard procedures were used to correct for fluorescein spectral overlap into the PI channel (*28*) and for compensation for cellular autofluorescence [S. Alberti, D. R. Parks, L. A. Herzenberg, *Cytometry* **8**, 114 (1987)]. We excluded small debris and large clusters of cells from analysis using forward scatter gates set from 100 to 920 (scale 0 to 1000); in experiments where PI but not CBAM was used to identify live cells, more restrictive gates (~200 to 920) were used to help exclude debris. Data was collected on 3×10^4 cells per sample.

28. D. R. Parks, L. L. Lanier, L. A. Herzenberg, in *The Handbook of Experimental Immunology*, D. M. Weir, L. A. Herzenberg, C. C. Blackwell, L. A. Herzenberg, Eds. (Blackwell, Edinburgh, United Kingdom, 1986), vol. 1, pp. 29.1–29.21; W. Moore and R. Kautz, *ibid.*, pp. 30.1–30.11.
29. M. A. Krasnow, E. E. Saffman, K. Kornfeld, D. S. Hogness, *Cell* **57**, 1031 (1989). We used pP_{ac} DNA as carrier in the transfections, and the culture medium was replaced 5 hours after DNA addition. In pP_{ac}lacZ, *lacZ* coding sequences are next to the *D. malanogaster act5C* promoter (M. Koelle and D. S. Hogness, personal communication).
30. FDG staining was as described (*8*) with the following modifications. Cells (10^6 to 10^7 per milliliter) were loaded with FDG by placement in hypotonic SM (SM diluted 1:1 with distilled H_2O) containing 1 mM FDG for 1.5 or 2 min at 23°C. We restored isotonicity by adding a tenfold excess of cold (4°C) SM containing PI (10 μg/ml). The β-galactosidase reactions continued at 4°C for 1 to 60 min. In most experiments, reactions were stopped by addition of PETG to 1 mM. CBAM, when used, was added just after the cold SM and the cells were further processed as described (*26*).
31. We centrifuged cells treated with FMG (5 min, 400*g*) and resuspended them in SM containing PI before FACS to reduce background fluorescence from free FMG.
32. C.-N. Chen and T. Kornberg, personal communication.
33. N. H. Patel *et al.*, *Cell* **58**, 955 (1989).
34. M/CM is a 1:1 mixture of M [SM supplemented with 18% (v/v) heat-inactivated fetal bovine serum and penicillin-streptomycin] and CM (conditioned medium, prepared by culturing cells from 4- to 6-hour-old embryos in M for 1 day and then removing cells by centrifugation and filtration through a 0.45-μm filter).
35. We thank D. Parks, S. Fiering, L. Herzenberg, and Y. Hiromi for valuable discussions; Y. Hiromi, M. Koelle, C.-N. Chen, and T. Kornberg for unpublished strains and plasmids; and E. Martin-Blanco, T. Kornberg, and A. Boulet for antibodies. M.A.K. is a Lucille P. Markey Scholar in Biomedical Science, S.C. was supported by National Research Service Awards training grant CA09151, and G.M. is a Howard Hughes Medical Institute Predoctoral Fellow. Supported by grants from the Lucille P. Markey Charitable Trust and the National Institutes of Health to M.A.K.

27 June 1990; accepted 15 October 1990

Inhibition of Morphine Tolerance and Dependence by the NMDA Receptor Antagonist MK-801

KEITH A. TRUJILLO* AND HUDA AKIL

The *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor is an important mediator of several forms of neural and behavioral plasticity. The present studies examined whether NMDA receptors might be involved in the development of opiate tolerance and dependence, two examples of behavioral plasticity. The noncompetitive NMDA receptor antagonist MK-801 attenuated the development of tolerance to the analgesic effect of morphine without affecting acute morphine analgesia. In addition, MK-801 attenuated the development of morphine dependence as assessed by naloxone-precipitated withdrawal. These results suggest that NMDA receptors may be important in the development of opiate tolerance and dependence.

OPIATE DRUGS SUCH AS MORPHINE are widely used in the clinical management of pain. Their clinical usefulness is limited, however, by tolerance and dependence. Tolerance is a decreased effect of a drug with repeated administration; a consequence of tolerance to opiates is the need to increase the dose of the drug to sustain the clinical analgesic effect. Dependence is also a state produced by repeated administration of a drug, but is only expressed after administration is terminated; an opiate-dependent subject undergoes an unpleasant withdrawal or abstinence syndrome in the absence of further administration of drug. Tolerance and dependence are thought to result from neuronal adaptations produced by repeated drug exposure (*1, 2*). The behavioral and neural plasticity seen after repeated exposure to opiates is experience-dependent and in many respects is similar to learning—in fact, learning has been suggested to be important in the development of tolerance and dependence (*3, 4*). Despite many years of study, the mechanisms underlying opiate tolerance and dependence are still relatively poorly understood.

Excitatory amino acid systems have a role in behavioral and neural plasticity. In particular, the *N*-methyl-D-aspartate (NMDA) receptor is involved in neuronal development, long-term potentiation, kindling, learning, and memory (*5*). In the present studies we examined the potential role of NMDA receptors in the development of tolerance and dependence by determining the effects of an NMDA receptor antagonist on the behavioral changes that occur with repeated administration of morphine to rats (*6*).

Animals receiving 10 mg of morphine per kilogram of body weight (10 mg/kg of morphine) displayed maximal analgesia on days 1 and 3 of treatment whether they were treated with saline or MK-801. In saline-treated animals the analgesic response to morphine displayed rapid development of tolerance, reaching baseline latencies by days 7 to 9 (Fig. 1A). In contrast, animals treated with 0.1 mg/kg of MK-801 showed considerably less tolerance, maintaining an analgesic response throughout morphine treatment. This effect was dose-dependent; animals treated with 0.03 mg/kg of MK-801 showed tolerance to the analgesic effect of morphine that was similar to that in saline-pretreated animals, while animals treated with 0.1 or 0.3 mg/kg of MK-801 were analgesic throughout the testing period (Fig. 2A). The increased analgesia seen in the MK-801–treated group was not due to analgesic actions of MK-801 alone or to an acute analgesic interaction between MK-801 and morphine. First, animals treated acutely with MK-801 (0.03, 0.1, 0.3, or 3.0 mg/kg) showed no change in tail-flick latency, demonstrating the lack of effect of this drug alone on pain responsiveness (Fig. 3). Additionally, animals treated acutely with MK-801 (0.03, 0.1, or 0.3 mg/kg) and then with a mildly analgesic dose of morphine (1.0 mg/kg) had tail-flick latencies no greater than saline-pretreated animals, demonstrating the lack of acute interaction between these drugs (Fig. 3). Despite the absence of analgesia observed with MK-801 alone or in combination with an acute injection of morphine, we considered the possibility that this drug may have unmasked "hidden" analgesia in the morphine-tolerant animal and may therefore have affected the expression rather than the development of morphine tolerance. However, the data from day 10 of treatment indicate that this was not the case; animals that had been treated with saline and morphine on days 1 through 9, then challenged with MK-801 and morphine on day 10 showed no greater analgesia than that seen the previous day, demonstrating that MK-801 does not affect the analgesia in an already morphine-tolerant animal (Fig. 1B). Moreover, animals that had been treated with MK-801 and morphine on days 1 through 9, then given morphine alone on day 10, displayed considerable analgesia on day 10 despite the lack of MK-801 pretreatment on this day (Figs. 1B and 2B), suggesting that MK-801 need not be present during testing to observe analgesia in the chronically treated

Mental Health Research Institute, The University of Michigan, 205 Washtenaw Place, Ann Arbor, MI 48109–0720.

*To whom correspondence should be addressed.

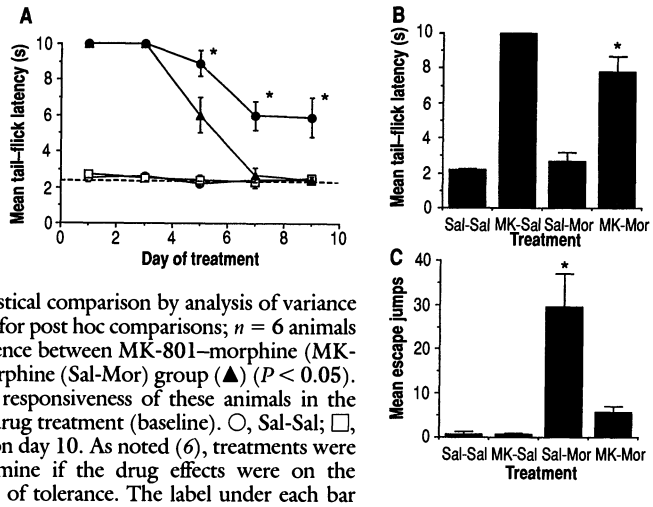

Fig. 1. Effects of MK-801 on opioid tolerance and dependence. (**A**) Effect of MK-801 (0.1 mg/kg) on the development of tolerance to the analgesic effect of morphine (10 mg/kg) over the 9 days of treatment. See (*6*) for methods. Scores are expressed as mean tail-flick latency ± SEM. Tail-flick latencies were converted to maximal percent effect for statistical comparison by analysis of variance (*13*). Dunnett *t* tests were used for post hoc comparisons; $n = 6$ animals per group; *, significant difference between MK-801–morphine (MK-Mor) group (●) and saline-morphine (Sal-Mor) group (▲) ($P < 0.05$). The dashed line indicates the responsiveness of these animals in the tail-flick test in the absence of drug treatment (baseline). ○, Sal-Sal; □, MK-Sal. (**B**) Analgesia scores on day 10. As noted (*6*), treatments were reversed on day 10 to determine if the drug effects were on the development or the expression of tolerance. The label under each bar indicates the treatment the animals received on days 1 through 9. *, Significant difference between MK-Mor group and Sal-Mor group ($P < 0.05$). The Sal-Mor group was not significantly different from Sal-Sal group. (**C**) Escape jumps during naloxone-precipitated withdrawal on day 10. The label under each bar indicates the treatment the animals received on days 1 through 9. Scores are expressed as mean number of escape jumps ± SEM. *, Sal-Mor group significantly different from all other groups ($P < 0.05$). The MK-Mor group was not significantly different from the Sal-Sal or the MK-Sal group.

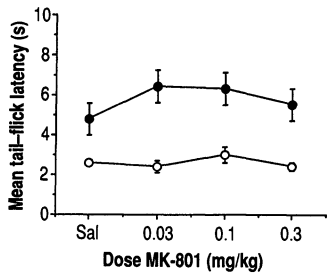

Fig. 3. Acute effect of MK-801 on morphine analgesia. Animals were administered a single injection of saline or MK-801 (0.03, 0.1, 0.3, or 3.0 mg/kg ip) 30 min prior to saline (○) ($n = 4$ to 6 animals per group) or morphine (●) (1 mg/kg sc; $n = 10$ animals per group). Tail-flick latencies were determined 60 min after the second injection. Scores are expressed as mean tail-flick (TF) latency ± SEM. Morphine produced moderate analgesia in animals pretreated with saline. MK-801 treatment had no effect on tail-flick latency when administered before saline and did not significantly influence morphine analgesia.

animal. These results suggest that MK-801 inhibits the development of tolerance to the analgesic effect of morphine.

In addition to interfering with tolerance, MK-801 pretreatment inhibited the naloxone-precipitated abstinence syndrome. Animals that had received repeated administration of saline and morphine displayed numerous escape jumps (*8*) in response to an injection of the opiate antagonist naloxone. In contrast, animals that were treated with MK-801 during repeated morphine administration showed very few jumps after naloxone administration (Figs. 1C and 2C). As with the above experiments on tolerance, control studies demonstrated that the effect of MK-801 was due to the repeated administration of the drug with morphine and not due to an acute interaction between the MK-801 and morphine on the day of withdrawal. First, animals that had received saline and morphine on days 1 through 9 displayed numerous escape jumps even when they received MK-801 before naloxone-precipitated withdrawal, suggesting that an acute dose of MK-801 does not interfere with escape jumps in a morphine-dependent animal (Fig. 1C). Second, animals that had received MK-801 and morphine on days 1 through 9 displayed few escape jumps despite the fact that they did not receive MK-801 on day 10 prior to precipitated withdrawal (Figs. 1C and 2C). Thus, MK-801, administered during repeated treatment with morphine, interferes with the development of dependence (*9*).

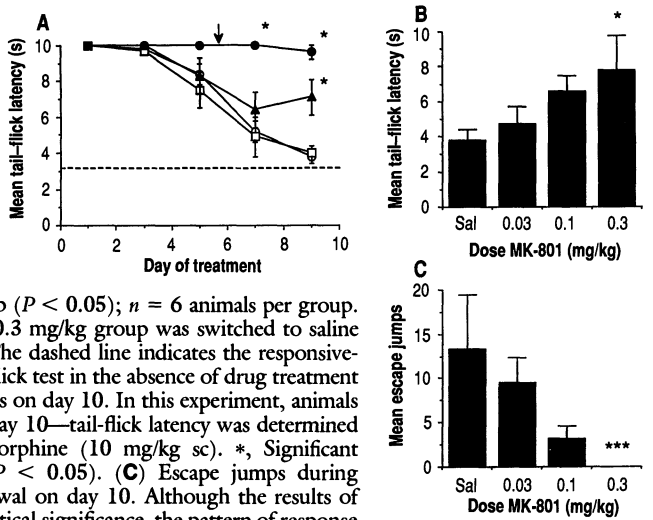

Fig. 2. Dose response of MK-801 on morphine tolerance and dependence. (**A**) Dose-response of MK-801 on the development of tolerance to the analgesic effect of morphine (10 mg/kg). See (*6*) and Fig. 1 legend for methodology and abbreviations. ○, Sal; □, 0.03 mg/kg MK; ▲, 0.1 mg/kg MK; ●, 0.3 mg/kg MK. *, Significant difference from Sal group ($P < 0.05$); $n = 6$ animals per group. The arrow indicates that the 0.3 mg/kg group was switched to saline pretreatment on day 5 (*14*). The dashed line indicates the responsiveness of the animals in the tail-flick test in the absence of drug treatment (baseline). (**B**) Analgesia scores on day 10. In this experiment, animals received no pretreatment on day 10—tail-flick latency was determined 60 min after injection of morphine (10 mg/kg sc). *, Significant difference from Sal group ($P < 0.05$). (**C**) Escape jumps during naloxone-precipitated withdrawal on day 10. Although the results of this study did not achieve statistical significance, the pattern of response suggests that MK-801 may inhibit escape jumps in a dose-dependent manner (the lack of significance appears primarily due to fewer escape jumps in the Sal-pretreated (control) group compared to this group in Experiment 1). ***, The three remaining animals pretreated on days 1 to 5 with 0.3 mg/kg MK-801, and days 6 to 9 with saline (*14*) showed inconsistent effects on escape jumping; one animal jumped only three times, one jumped 26 times, and the third jumped 53 times.

The inhibition of tolerance and dependence by MK-801 suggests that the NMDA receptor is involved in the behavioral changes and therefore presumably in the neural adaptations produced by repeated morphine administration. Although there are several possibilities for the actions of MK-801, both direct and indirect, we would like to consider two. First, it is possible that NMDA receptors are directly involved in the neuronal changes that occur in tolerance and dependence (*1, 2*). This model posits that NMDA receptors on opiate-responsive neurons may have an important role in the plasticity arising from repeated opiate administration. MK-801 would interfere with tolerance and dependence by preventing the primary cellular adaptations that occur in response to chronic opiates (*10*). Alternatively, learning processes have been found to be important in tolerance and dependence (*3, 4*). Since NMDA receptor antagonists disrupt learning and memory (*5*), MK-801 may impair tolerance and dependence by interfering with associative mechanisms involved in these behavioral changes (*11*). In contrast to the first model, which postulates an effect of MK-801 directly on the opiate-responsive neuron, in this latter model the drug may act at a site distal to the primary effect of the opiate; at a brain site involved in the development of drug-environment associations. These mechanisms are not mutually exclusive; MK-801 may act both at the cellular site of

action of morphine and on the associative processes involved in the development of tolerance and dependence.

Our results suggest a potentially important role for NMDA receptors in the development of opiate tolerance and dependence. MK-801 also interferes with the development of sensitization (or reverse tolerance) to stimulant drugs (*12*), suggesting that excitatory amino acid systems may be involved in experience-dependent changes produced by repeated exposure to a variety of drugs. Adjunctive treatment with drugs that interfere with tolerance and dependence may prove valuable for extending the usefulness of opiates clinically.

REFERENCES AND NOTES

1. D. H. Clouet and K. Iwatsubo, *Annu. Rev. Pharmacol.* **15**, 49 (1975); H. O. J. Collier, *Nature* **283**, 625 (1980); C. W. Sharp, Ed., *NIDA Res. Monogr. 54* (1984); S. M. Johnson and W. W. Fleming, *Pharmacol. Rev.* **41**, 435 (1989).
2. G. K. Aghajanian, *Nature* **276**, 186 (1978); R. Andrade *et al.*, *Eur. J. Pharmacol.* **91**, 161 (1983); R. A. North *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5487 (1987); M. J. Christie, J. T. Williams, R. A. North, *Mol. Pharmacol.* **32**, 633 (1987); M. J. Christie, J. T. Williams, R. A. North, *NIDA Res. Monogr.* **78**, 158 (1987).
3. A. Wikler, *Am. J. Psychiatry* **105**, 329 (1948); *Arch. Gen. Psychiatry* **28**, 611 (1973); *Pavlovian J. Biol. Sci.* **11**, 190 (1976); S. Siegel, *Science* **193**, 323 (1976).
4. S. Siegel, in *Research Advances in Alcohol and Drug Problems*, Y. Israel, F. B. Glaser, H. Kalant, R. E. Popham, Eds. (Plenum, New York, 1983), p. 207; T. B. Baker and S. T. Tiffany, *Psychol. Rev.* **92**, 78 (1985); A. J. Goudie and C. Demellweek, in *Behavioral Analysis of Drug Dependence*, S. R. Goldberg and I. P. Stolerman, Eds. (Academic Press, Orlando, FL, 1986), p. 225; B. A. Ray, Ed., *NIDA Res. Monogr. 84* (1988).
5. C. W. Cotman and L. L. Iversen, *Trends Neurosci.* 7, 263 (1987); G. L. Collingridge and R. A. J. Lester, *Pharmacol. Rev.* **40**, 143 (1989); J. C. Watkins and G. L. Collingridge, Eds., *The NMDA Receptor* (Oxford Univ. Press, New York, 1989).
6. Adult male Sprague-Dawley rats were used in all experiments. In acute studies, animals received an intraperitoneal (ip) injection of saline (1 ml/kg) or MK-801 followed 30 min later by a subcutaneous (sc) injection of saline or morphine sulfate. The analgesic response to morphine was assessed by the tail-flick test (7) 60 min after the second injection. For chronic studies, animals received injections twice daily (8:00 and 17:00) for 9 days. On odd-numbered days, analgesic response was assessed by the tail-flick test 60 min after the morning morphine injection. In experiment 1, treatment groups included (pretreatment:treatment) (i) saline:saline; (ii) saline:10 mg/kg morphine; (iii) 0.1 mg/kg MK-801:saline; and (iv) 0.1 mg/kg MK-801:10 mg/kg morphine. Control experiments were performed on day 10 in the same group of animals to determine whether MK-801 affected the development of tolerance or whether it simply altered the behavioral expression of the analgesic response. On this day treatments were reversed so that animals that had been treated with MK-801 followed by morphine on days 1 through 9 were challenged with saline followed by morphine, and animals that had been treated with saline followed by morphine on days 1 through 9 were challenged with MK-801 followed by morphine. In addition, animals that had received chronic treatment with MK-801 followed by saline were challenged with MK-801 followed by morphine. Immediately after the tail-flick test on day 10, animals were injected with naloxone (2 mg/kg sc) to precipitate withdrawal. The withdrawal syndrome was assessed by placing each rat in a 45-cm-high plexiglass box and recording the incidence of escape jumps (*8*) for 15 min. In experiment 2 the dose-dependent effects of MK-801 were examined. On days 1 through 9, animals were treated with saline or MK-801 (0.03, 0.1, or 0.3 mg/kg ip) followed 30 min later by morphine (10 mg/kg sc). Tail-flick latencies were determined on odd-numbered days, as described above. In this experiment, animals received no pretreatment on day 10, but received a morphine challenge, followed by the tail-flick test and naloxone-precipitated withdrawal 60 min after the morphine injection.
7. F. E. D'Amour and D. L. Smith, *J. Pharmacol. Exp. Ther.* **72**, 74 (1941). Two to three successive determinations were made for each rat, and the mean of these scores was used as the tail-flick latency for that animal. A 10-s cutoff was used to minimize tissue damage from the heat lamp.
8. D. L. Francis and C. Schneider, *Br. J. Pharmacol.* **41**, 424P (1971); E. Wei, *Psychopharmacology* **28**, 35 (1973); J. Bläsig *et al.*, *ibid.* **33**, 19 (1973).
9. We do not believe that our results were due to motor impairment of the animals. The tail-flick response is a specific indicator of opiate analgesia in rats and is very resistant to nonspecific impairment [J. W. Lewis, G. Baldrighi, H. Akil, *Brain Res.* **424**, 65 (1987)]. In addition, control experiments on day 10 suggest that motor impairment was not responsible for the observed effects. Our results cannot be explained by altered morphine metabolism produced by MK-801 since there was no increase in withdrawal signs in MK-801–treated animals as would be expected if morphine concentrations were increased during the course of treatment. Finally, it is unlikely that the effects were due to MK-801–dependent hypothermia [A. Buchan and W. A. Pulsinelli, *J. Neurosci.* **10**, 311 (1990)] since MK-801 does not, by itself, produce a decrease in body temperature [D. G. Nehls *et al.*, *Brain Res.* **511**, 271 (1990)].
10. Electrophysiological studies on single locus ceruleus neurons have suggested that excitatory amino acids may be involved in opiate withdrawal. [K. Rasmussen and G. K. Aghajanian, *Brain Res.* **505**, 346 (1989); C.-S. Tung, J. Greenhoff, T. H. Svensson, *Acta Physiol. Scand.* **138**, 581 (1990)].
11. There are two distinct types of drug tolerance: associative tolerance and nonassociative tolerance. Nonassociative tolerance is produced by high doses of opiates and is not affected by environmental or behavioral manipulation of the animal. Associative tolerance is very sensitive to behavioral and pharmacological manipulations that modulate learning and memory, and in fact may result primarily from learning processes such as classical conditioning, instrumental conditioning, or habituation [(*4*); S. T. Tiffany and P. M. Maude-Griffen, *Behav. Neurosci.* **102**, 534 (1988); R. Dafters and J. Odber, *ibid.* **103**, 1082 (1989)]. The present experiments did not distinguish between associative and nonassociative tolerance.
12. R. Karler *et al.*, *Life Sci.* **45**, 599 (1989); H. E. Criswell *et al.*, *Brain Res.* **512**, 284 (1990).
13. L. S. Harris and A. K. Pierson, *J. Pharmacol. Exp. Ther.* **143**, 141 (1964).
14. The highest dose of MK-801 (0.3 mg/kg) in combination with 10 mg/kg morphine proved to be toxic to the animals—three animals died on the first day of treatment and others appeared sick and lost weight over the days of treatment. Because of this toxicity, the 0.3 mg/kg dose of MK-801 was discontinued after the morning of day 5, and the three remaining animals in the group were pretreated with saline for the remainder of the experiment. Despite the discontinuation of MK-801 treatment, these animals showed nearly complete inhibition of morphine tolerance, remaining strongly analgesic to morphine throughout the study (after the discontinuation of morphine pretreatment these animals rapidly regained their health). Animals receiving 0.03 and 0.1 mg/kg of MK-801 in combination with 10 mg/kg of morphine appeared quite healthy throughout treatment, and had no untoward weight loss.

15. We thank S. Watson, D. Bronstein, J. Herman, and T. Robinson for helpful comments on the manuscript and L. Fox and J. Olds for encouragement and inspiration. Supported by National Institute on Drug Abuse (NIDA) National Research Service Award DA05336 to K.A.T., NIDA grant DA02265, National Institute of Mental Health grant MH422251, the T. Raphael Research Fund, and the Lucille B. Markey Charitable Fund to H.A.

23 July 1990; accepted 19 October 1990

Critical Structural Elements of the VP16 Transcriptional Activation Domain

W. DOUGLAS CRESS AND STEVEN J. TRIEZENBERG*

Virion protein 16 (VP16) of herpes simplex virus type 1 contains an acidic transcriptional activation domain. Missense mutations within this domain have provided insights into the structural elements critical for its function. Net negative charge contributed to, but was not sufficient for, transcriptional activation by VP16. A putative amphipathic alpha helix did not appear to be an important structural component of the activation domain. A phenylalanine residue at position 442 was exquisitely sensitive to mutation. Transcriptional activators of several classes contain hydrophobic amino acids arranged in patterns resembling that of VP16. Therefore, the mechanism of transcriptional activation by VP16 and other proteins may involve both ionic and specific hydrophobic interactions with target molecules.

VP16 (ALSO TERMED V_{mw65} OR α-TIF) is a protein component of the herpes simplex virus type 1 (HSV-1) virion that specifically and potently activates transcription of the viral immediate-early (IE) genes (*1*). Molecular genetic studies of VP16 have distinguished two functional domains that are relevant to transcriptional activation. The specificity for IE genes is conferred by the interaction of an NH_2-terminal portion of VP16 with host proteins that bind IE cis-regulatory elements (*2*). The

Department of Biochemistry, Michigan State University, East Lansing, MI 48824–1319.

*To whom correspondence should be addressed.