boundary spherules (Table 1) cannot be a coincidence. Although these impact events differ greatly in magnitude, both have produced remarkably similar deposits with high concentrations of Ir (*3*) and trace amounts of spherules. As in the late Pliocene deposits, K-T boundary spherules typically constitute <1% of the total sediment (*8*) and not all spherules contain spinels (*14*). The Ni-rich magnesioferrite compositions of these spinels have not been reported in other deposits and imply that they formed under similar conditions. The K-T boundary spinels must be relict mineral grains formed during an impact event (*15*). Furthermore, they probably crystallized from spherules with mafic compositions and thus indicate that either the K-T projectile or the impact target had a mafic component (for example, oceanic lithosphere).

REFERENCES AND NOTES

1. F. T. Kyte, Z. Zhou, J. T. Wasson, *Nature* **292**, 417 (1981).
2. F. T. Kyte and D. E. Brownlee, *Geochim. Cosmochim. Acta* **49**, 1095 (1985).
3. F. T. Kyte, L. Zhou, J. T. Wasson, *Science* **241**, 63 (1988).
4. Spherules were recovered from previously analyzed samples of E13-3 (magnetic separates) and E13-4 (fine fractions). We have not attempted to recover spherules from E10-2, the only other core with known impact debris (*3*). A single bulk sample of ~100 spherules with a mass of 0.6 mg was analyzed by NAA, primarily to determine the Ir content of the spherules. Analytical uncertainties for the NAA data are ±10 to 15%. Another ~100 spherules have been individually examined in a scanning electron microscope to characterize surface textures. Twenty-four spherules representing the spectrum of exterior morphologies were cut into polished sections to characterize interior textures and mineralogy by quantitative major element analyses with an electron microprobe.
5. S. V. Margolis, V. Barnes, P. Cloud, R. V. Fisher, *Proc. Lunar Sci. Conf.* **2** (part 1), 909 (1971); B. P. Glass, *Geol. Soc. Am. Bull.* **85**, 1305 (1974).
6. Because the size of most spinel crystals is below the limit of resolution of the electron microprobe, quantitative analyses were difficult to obtain. We used the abundance of Si as a measure of contamination of the analysis because it was clear that measurable Si indicated excitation of surrounding glass. We report only analyses in which SiO_2 concentrations were <1.5%, but these are representative of all spinels. More than 100 individual spinel crystals were analyzed in 24 separate spherule polished sections. All spinels showed similar enrichment in Ni, Cr, and Mg and low Ti.
7. S. E. Haggerty, *Short Course Notes* (Mineralogical Society of America, Washington, DC, 1976), vol. 3, p. Hg 101.
8. J. Smit and F. T. Kyte, *Nature* **310**, 403 (1984).
9. F. T. Kyte and J. Smit, *Geology* **14**, 485 (1986).
10. B. F. Bohor, E. E. Foord, R. Ganapathy, *Earth Planet. Sci. Lett.* **81**, 51 (1986).
11. E. Doehne and S. V. Margolis, *Geol. Soc. Am. Spec. Pap.*, in press.
12. T. N. Irvine, *Can. J. Earth Sci.* **2**, 648 (1968).
13. B. P. Glass and C. A. Burns, *Proc. Lunar Planet. Sci. Conf.* **18**, 455 (1988).
14. A. Montanari *et al.*, *Geology* **11**, 668 (1983).
15. Spherules are a nearly ubiquitous component in K-T boundary sediments and have a variety of mineral compositions (*13*). Several researchers have argued that they have a nonimpact origin; for example, H. R. Naslund, C. B. Officer, G. D. Johnson, *Geology* **14**, 923 (1986); G. A. Izett, *Geol. Soc. Am. Bull.* **99**, 78 (1987). Our results specifically apply only to spinel-bearing spherules in the K-T boundary, but the impact association is clear.

16. Curation of Eltanin cores is supported by the National Science Foundation (NSF). We are indebted to A. Kaharoeddin and D. S. Cassidy for assistance in obtaining sediment samples. This research was supported by NSF grant EAR-8904833 and a grant from the Institute of Geophysics and Planetary Physics–Lawrence Livermore National Laboratory.

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Characterization of a Human TAR RNA–Binding Protein That Activates the HIV-1 LTR

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Human immunodeficiency virus type 1 (HIV-1) gene expression is activated by Tat, a virally encoded protein. Tat trans-activation requires viral (trans-activation–responsive; TAR) RNA sequences located in the R region of the long terminal repeat (LTR). Existing evidence suggests that Tat probably cooperates with cellular factors that bind to TAR RNA in the overall trans-activation process. A HeLa complementary DNA was isolated and characterized that encodes a TAR RNA–binding protein (TRBP). TRBP activated the HIV-1 LTR and was synergistic with Tat function.

REGULATORY MECHANISMS INITIALLY described for viral systems often provide the first clue for the existence of counterpart mechanisms in cells (*1*). For example, recent molecular studies on HIV-1 have resulted in the description of novel modes of gene expression. There is compelling evidence that HIV-1 Tat and Rev proteins regulate viral transcriptional and posttranscriptional events through targeted RNA sequences (*2–6*). In particular, the association of Tat with TAR RNA (*7–9*) allows Tat to position itself optimally so as to activate DNA promoter sequences (*10, 11*). This process, which involves a bipartite DNA-RNA target, has yet to be demonstrated for a cellular transcription factor.

The HIV-1 TAR sequence is located between nucleotides +19 and +42 (+1 is defined as the transciptional start point) in the R region of the LTR (7). TAR RNA can fold into a stable stem-bulge-loop structure (*4*). Mutations that disrupt the stem, affect the loop or bulge, or destroy the overall secondary structure of the RNA interfere with Tat trans-activation (*5, 6, 12, 13*). Thus, a correct Tat-TAR interaction is essential for optimal expression of the LTR. Cell type–specific experiments, however, suggest that this interplay between Tat and TAR is not sufficient to explain the complete trans-activation process. The observation that Tat activates the HIV-1 LTR poorly in mouse (*14*) and hamster (*15*) cells as compared to human cells has led to the proposal that cellular proteins are important accessories in Tat trans-activation. Several human proteins that bind to TAR RNA have been identified (*16*); however, it is difficult to study the function and similarity of these proteins until the genes encoding them are isolated. We report here the characterization of a human cDNA sequence that encodes for a TAR RNA–binding protein that trans-activates the HIV-1 LTR.

To obtain cDNAs that code for TAR RNA–binding proteins we assayed a HeLa cell library with an RNA recognition site probe. This approach incorporated a modification of the procedure used to identify sequence-specific DNA-binding proteins (*17*). We substituted a uniformly ^{32}P-labeled TAR RNA in place of a DNA probe. This TAR RNA probe was used to screen a λ ZAP cDNA expression library (*18, 19*). In principle, all phage plaques that contain TAR RNA–binding proteins should bind the probe and become radioactively labeled. In the first round of "plaque hybridization" many plaques became radioactively labeled. We purified one plaque to homogeneity through two successive rounds of dilution and rescreening.

We characterized this cDNA clone in two ways. First, the insert, TRBP, was excised from the phage vector and completely sequenced. TRBP contains an open reading-frame, sufficient for 345 amino acids, that is positioned directly in frame to the β-galactosidase gene in λ ZAP (Fig. 1) (*18*). Thus TRBP (with a predicted size of 36,949 daltons) is expressed in this vector as a 44-kD fusion protein consisting of 402 amino acids. We then transferred the TRBP cDNA as a plasmid into *Escherichia coli* XL1 blue (*18*). In this setting, we could induce the fusion protein using isopropyl-1-thio-β-

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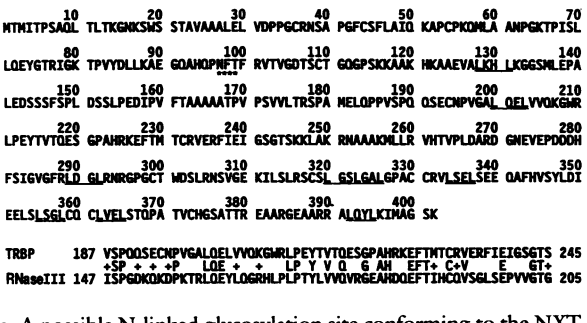

Fig. 1. Amino acid sequence of TRBP (**top**) and the amino acid similarity (**bottom**) between TRBP and *E. coli* ribonuclease III (RNase III). Amino acid sequence deduced from nucleotide sequencing of TRBP cDNA. Nucleotide sequence of the cDNA was determined by sequencing the TRBP insert cloned into pBluescript SK (Stratagene). The amino acid sequence shown reflects a fused translation product in which the first 39 amino acids are contributed from the plasmid *lacZ* gene. A possible N-linked glycosylation site conforming to the NXT sequence (*29*) is indicated with stars. Nine LXXL motifs are underlined. The cDNA sequence has been deposited with GenBank (*30*). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, unspecified; and Y, Tyr.

D-galactoside (IPTG). A 44-kD protein was induced in *E. coli* containing the TRBP plasmid (Fig. 2B, lane 2) but not in *E. coli* containing a control plasmid (Fig. 2B, lane 1). This abundant 44-kD protein was difficult to purify because most of it was poorly soluble (Fig. 2B, lane 6).

Because of purification difficulties, we used in situ filter-binding to define the binding of TRBP to different RNAs (Fig. 2, A and C). We found, not surprisingly, that TRBP bound strongly to a native TAR RNA probe (Fig. 2C, TAR RNA). TAR RNAs that were changed in the loop (Fig. 2C, TAR L135 RNA) or in the bulge (Fig. 2C, TAR B123 RNA) sequences or were disrupted in the base pairing at the bottom of the stem (Fig. 2C, TAR SΔ3-11 and TAR SΔ3-17 RNAs) remained good targets for TRBP. TRBP, however, associated poorly to a TAR mutant in which the double stranded helix between the bulge and the loop was perturbed (Fig. 2C, TAR BL234 RNA). It bound marginally to a non-TAR RNA [Fig. 2C, RxRE RNA (*20*)] and did not bind to DNA (Fig. 2C, TAR DNA and lambda DNA) probes. These findings suggest that TRBP binds to the double-stranded portion of the TAR hairpin between the bulge and the loop.

We examined the significance of TRBP binding to TAR RNA from a functional perspective. We positioned the TRBP reading frame downstream of the strong cytomegalovirus (CMV) IE promoter (*21*) to construct a eukaryotic expression plasmid (pCMVTRBP) (Fig. 3A). When pCMVTRBP was introduced with pLTRCAT [which contains an HIV-1 LTR driving a chloramphenicol acetyltransferase (CAT) reporter gene] into human epithelial cells, expression of the HIV-1 LTR was 20 to 60 times higher than control cotransfections with plasmid pBR322 or with a CMV promoter plasmid (Fig. 3, A and B). This result suggests that TRBP is a TAR RNA–binding protein that can trans-activate the expression of the HIV-1 LTR promoter.

To further evaluate the role of RNA

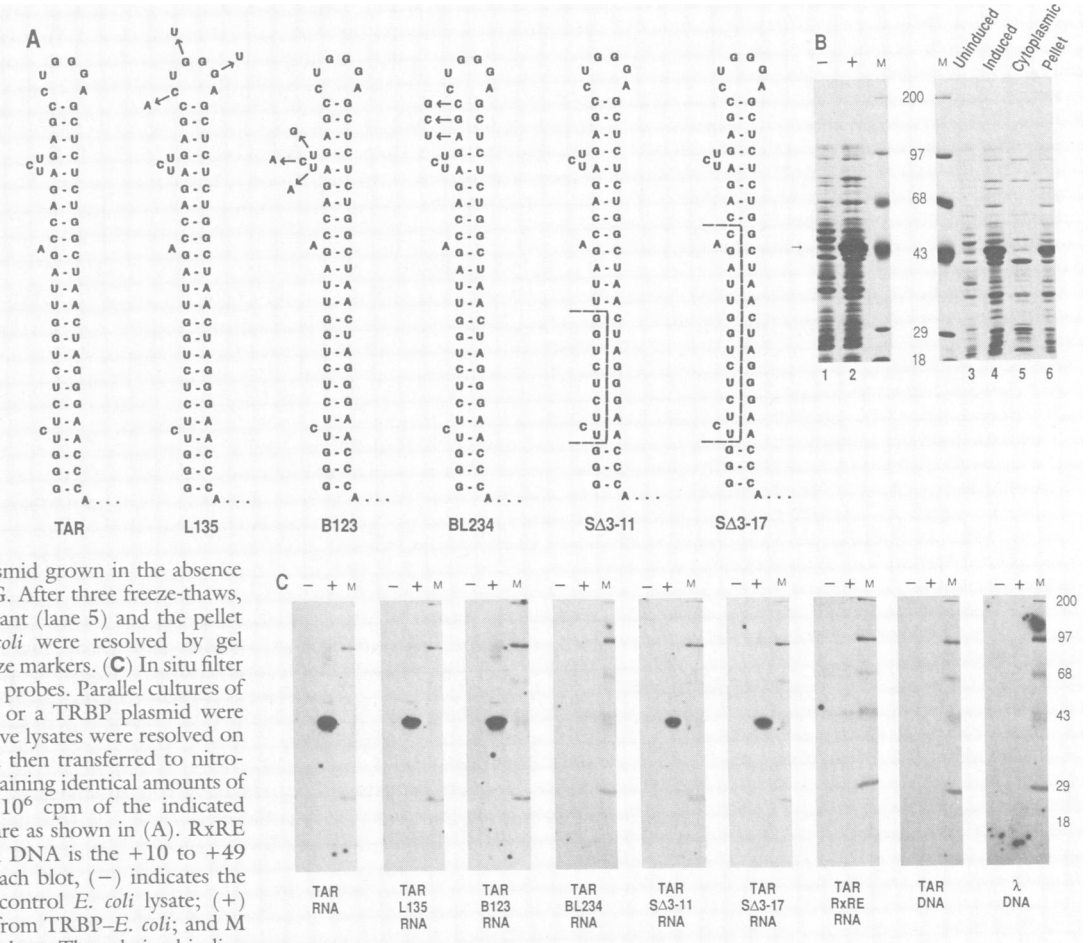

Fig. 2. TRBP binds to TAR RNAs but not to DNAs. (**A**) Structures of the different TAR RNAs used in the binding assay. Arrows indicate base changes and dotted lines denote deleted nucleotides. For the binding experiments, each of the RNAs was transcribed in vitro to comparable specific activities with T7 RNA polymerase in the presence of ^{32}P-labeled uridine triphosphate. TAR denotes a native TAR RNA. (**B**) Coomassie blue–stained profiles of proteins produced from *E. coli* that contain TRBP cDNA. *Escherichia coli* with a control plasmid (lane 1) and with a plasmid that contains TRBP cDNA (lane 2) were grown in the presence of IPTG. Total bacterial protein was solubilized and resolved by SDS–polyacrylamide gel electrophoresis. Lanes 3 and 4 compare the protein profile of *E. coli* with the TRBP plasmid grown in the absence (lane 3) or presence (lane 4) of IPTG. After three freeze-thaws, proteins in the cytoplasmic supernatant (lane 5) and the pellet (lane 6) of TRBP-producing *E. coli* were resolved by gel electrophoresis. M denotes protein size markers. (**C**) In situ filter binding of TRBP to RNA and DNA probes. Parallel cultures of *E. coli* harboring a control plasmid or a TRBP plasmid were induced with IPTG, and the respective lysates were resolved on a 10% SDS–polyacrylamide gel and then transferred to nitrocellulose filters. Nine filter strips containing identical amounts of protein were incubated with 1×10^6 cpm of the indicated probe. TAR RNAs used as probes are as shown in (A). RxRE RNA is as described (*20*) and TAR DNA is the +10 to +49 fragment from the HIV-1 R. For each blot, (−) indicates the lane containing proteins from the control *E. coli* lysate; (+) indicates the lane containing lysate from TRBP–*E. coli*; and M denotes ^{14}C-labeled protein size markers. The relative binding of each probe is as follows: TAR RNA (+++), TAR L135 RNA (+++), TAR B123 RNA (+++), TAR BL234 RNA (±), TAR SΔ3-11 RNA (++), TAR SΔ3-17 RNA (++), TAR RxRE RNA (±), TAR DNA (−), and λ DNA (−).

binding in TRBP function, we constructed pLTRΔTARCAT, which contains a deletion of 12 nucleotides that span the TAR loop sequence. This mutant RNA has a distinctly altered TAR RNA structure. In a titration series with increasing amounts of TRBP (Fig. 3C), we found that optimal trans-activation of the HIV-1 LTR did require an intact TAR RNA structure. Reduced activation of the HIV-1 promoter was observed in the absence of a normal TAR RNA (Fig. 3, A and C). This observation would, in part, be consistent with TRBP having separate binding and activation domains and suggests that RNA binding is not an absolute prerequisite for TRBP function. In this instance, TRBP binding to an RNA target could increase the local concentration of "activation domains" in proximity to the promoter. Promoter activation in the absence of binding could still be expected to occur, albeit less efficiently. Indeed, several eukaryotic promoters that do not contain TAR RNA sequences showed reduced [Fig. 3A, SV40 (*22*), HTLV-1 (*23*), Visna (*24*)] or no [Fig. 3A, CMV (*21*), TRE-TK (*25*)] responsiveness to TRBP.

The role of TRBP (and other cellular proteins) in the biology of HIV-1 infections is not entirely clear. TRBP mRNA is expressed as a single species in both mouse (3T3) and human (HeLa) cells (*26*). Currently we have no information on the amount of TRBP or its functional state in different cell types. The constitutive expression of TRBP in cells would be compatible with observations that Tat cooperates with preexisting cellular proteins in the trans-activation of the HIV-1 LTR (*3*). Furthermore, we observed evidence for a synergistic interaction between Tat and TRBP. Under conditions in which Tat activation of the HIV-1 LTR reached a saturated level, addition of TRBP "superinduced" the expression of the HIV-1 promoter (Table 1). This "super-induction" was not due to a TRBP effect that increased Tat expression from the pSVTat plasmid because the same result was obtained when Tat was produced from a TRBP nonresponsive promoter (Fig. 3, A and B), CMV.

TRBP is probably one of several cellular factors that interact with HIV-1 RNA on the viral RNA's entry into cells. Biochemical and functional studies on TAR RNA have implicated the bulge (*9, 13*) and loop (*12, 13*) sequences as important contact points for proteins. Our analysis suggests that TRBP binds to the double-stranded RNA helix that is positioned between the bulge and the loop structures. An isolated stretch of 59 amino acids (between positions 187 and 245) in TRBP shows a 52% similarity and 37% identity with the ribonuclease III protein of *E. coli* (Fig. 1). Ribonuclease III is known to bind to the double-stranded stem of *E. coli* RNA hairpins (*27*).

Table 1. Activation of pLTRCAT by TRBP, Tat, or both. Fold induction represents the average from three independent measurements, each normalized to the result without trans-activator. Transfections were performed in HeLa cells with pCMVTRBP, an HIV-1 Tat construction driven by the SV40 promoter, and an HIV-1 Tat construction driven by the CMV IE promoter.

Trans-activator	Fold induction ± SE
TRBP (5 μg)	20 ± 12
SVTat (1 μg)	250 ± 75
SVTat (2 μg)	220 ± 90
SVTat (5 μg)	204 ± 50
TRBP + SVTat (1 μg)	470 ± 196
CMVTat (1 μg)	260 ± 37
TRBP + CMVTat (1 μg)	880 ± 250

Computer comparisons of the TRBP cDNA with known databases revealed no other significant similarities. TRBP may thus represent one member of a novel family of cellular proteins that is capable of influencing gene expression through binding to RNA. Mapping experiments with somatic cell hybrids (*28*) have revealed the presence of three or four TRBP genes or pseudogenes in the mammalian genome. None of these sequences map to human chromosome 12. It is therefore unlikely that TRBP is related to the hypothetical Tat-cooperating factor in human cells that is absent from rodent cells (*14, 15*).

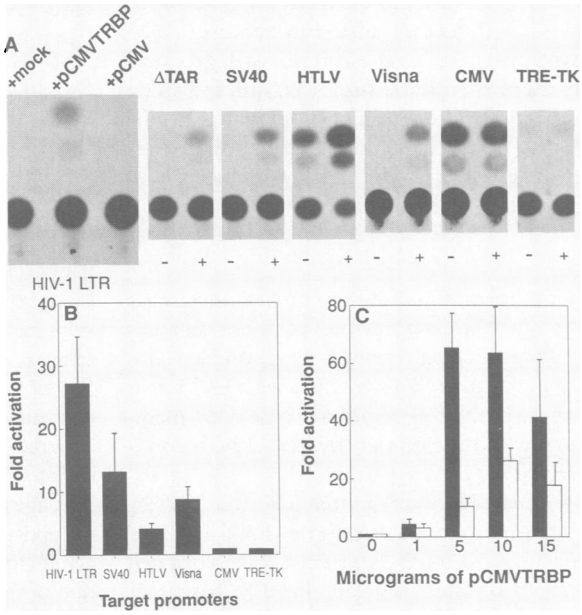

Fig. 3. TRBP activates expression from the HIV-1 LTR and other eukaryotic promoters. The plasmid pCMVTRBP is a eukaryotic expression vector containing TRBP cDNA driven by the CMV IE promoter (*21*). The pCMV contains only the CMV promoter. (**A**) Cotransfection of pLTRCAT into HeLa cells with pBR322 (mock), pCMVTRBP, or pCMV. CAT activities were measured by the acetylation of ^{14}C-chloramphenicol. Conditions for the assays were standardized with a cotransfected CMV–β-gal plasmid (because the CMV promoter activity was unaffected by TRBP). Right panels show the result of cotransfections of different promoter-CAT gene fusions with (+) or without (−) 5 μg of pCMVTRBP. The promoters tested were HIVΔTARLTR (*10*), SV40 (*22*), HTLV-1 (*23*), Visna (*24*), CMV (*21*), and TRE-TK (*25*). (**B**) Quantitation of the relative level of activation of different promoters by TRBP. In each assay 450 μg of total protein was used. The results show the activation level of the different promoters and are the average ± SE from eight experiments (HIV-1 LTR) or two (all others). (**C**) Comparison of the activation of HIV-1 LTR and HIVΔTARLTR. Increasing amounts of pCMVTRBP (0 to 15 μg) and 1 μg of HIV-1 LTR (filled bars) or HIVΔTARLTR (open bars) were transfected into HeLa cells. In this assay series 50 μg of protein per reaction was used (after heat treatment of 65°C). The results compare the relative activation of the HIVLTR and HIVΔTARLTR to a normalized activation value of 1.0 for 0 μg of pCMV TRBP. The results are the average ± SE from three experiments.

REFERENCES AND NOTES

1. M. R. Green and M. L. Zapp, *Nature* **338**, 200 (1989).
2. B. R. Cullen, *Cell* **46**, 973 (1986); B. M. Peterlin, P. A. Luciw, P. J. Barr, M. D. Walker, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9734 (1986); S. Y. Kao, A. F. Calman, P. A. Luciw, B. M. Peterlin, *Nature* **330**, 489 (1987); A. P. Rice and M. B. Mathews, *ibid.* **332**, 551 (1988); M. F. Laspia, A. P. Rice, M. B. Mathews, *Cell* **59**, 283 (1989); B. R. Cullen and W. C. Greene, *ibid.* **58**, 423 (1989); M. Braddock *et al.*, *ibid.* p. 269; E. T. Dayton, D. M. Powell, A. I. Dayton, *Science* **246**, 1625 (1989); M. H. Malim *et al.*, *Cell* **60**, 675 (1990); S. Heaphy *et al.*, *ibid.* p. 685; D. N. SenGupta, B. Berkhout, A. Gatignol, A. Zhou, R. H. Silverman, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7492 (1990).
3. K.-T. Jeang, P. R. Shank, A. Kumar, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8291 (1988).
4. M. A. Muesing, D. H. Smith, D. J. Capon, *Cell* **48**, 691 (1987); A. Jakobovits, D. H. Smith, E. B. Jakobovits, D. J. Capon, *Mol. Cell. Biol.* **8**, 2555 (1988).
5. M. J. Selby, E. S. Bain, P. A. Luciw, B. M. Peterlin, *Genes Dev.* **3**, 547 (1989).
6. B. Berkhout, R. H. Silverman, K.-T. Jeang, *Cell* **59**, 273 (1989).
7. C. A. Rosen, J. G. Sodroski, W. A. Haseltine, *ibid.* **41**, 813 (1985); J. Hauber and B. R. Cullen, *J. Virol.* **62**, 673 (1988).
8. C. Dingwall *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6925 (1989).
9. S. Roy, U. Delling, C.-H. Chen, C. A. Rosen, N. Sonenberg, *Genes Dev.* **4**, 1365 (1990).
10. C. Southgate, M. L. Zapp, M. R. Green, *Nature* **345**, 640 (1990); M. J. Selby and B. M. Peterlin, *Cell* **62**, 769 (1990).
11. B. Berkhout, A. Gatignol, A. B. Rabson, K.-T. Jeang, *Cell* **62**, 757 (1990).

12. S. Feng and E. C. Holland, *Nature* **334**, 165 (1988); J. A. Garcia *et al.*, *EMBO J.* **8**, 765 (1989).
13. B. Berkhout and K.-T. Jeang, *J. Virol.* **63**, 5501 (1989); S. Roy, N. T. Parkin, C. Rosen, J. Iticitch, N. Sonenberg, *ibid.* **64**, 1402 (1990).
14. L. J. Seigel *et al.*, *Virology* **148**, 226 (1986); M. Newstein, E. J. Stanbridge, G. Casey, P. R. Shank, *J. Virol.* **64**, 4565 (1990).
15. C. E. Hart *et al.*, *Science* **246**, 488 (1989).
16. A. Gatignol, A. Kumar, A. Rabson, K.-T. Jeang, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7828 (1989); R. Gaynor, E. Soultanakis, M. Kuwabara, J. Garcia, D. S. Sigman, *ibid.*, p. 4858; R. A. Marciniak, M. A. Garcia-Blanco, P. A. Sharp, *ibid.* **87**, 3624 (1990).
17. H. Singh, J. H. LeBowitz, A. S. Baldwin, P. A. Sharp, *Cell* **52**, 415 (1988); C. R. Vinson, K. L. LaMarco, P. F. Johnson, W. H. Landschulz, S. L. McKnight, *Genes Dev.* **2**, 801 (1988); H. Singh, R. G. Clerc, J. H. LeBowitz, *BioTechniques* **7**, 252 (1989).
18. J. M. Short, J. M. Fernandez, J. A. Sorge, W. D. Huse, *Nucleic Acids Res.* **16**, 7583 (1988).
19. *Escherichia coli* XL1 blue was infected with 1×10^6 plaque-forming units of λ ZAP HeLa cDNA library (Stratagene) and plated. After 3 hours at 42°C, the plates were overlaid with nitrocellulose filters previously soaked in 10 mM IPTG and then incubated for 6 to 8 hours at 37°C. Filters were then immersed sequentially for 5 min in 6 M, 3 M, 1.5 M, and 0.75 M guanidine-HCl in binding buffer [50 mM tris-HCl, (pH 7.5); 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol]. Finally, the filters were equilibrated with binding buffer alone. For the binding assays, we incubated the filters for 60 min in 2.5% nonfat milk in binding buffer and then probed with ^{32}P-labeled TAR RNA (50 fmol/ml) in the presence of poly(dI-dC) (10 μg/ml) and yeast RNA (10 μg/ml). We used 1×10^6 cpm per filter with an incubation time of 1 hour at room temperature and then washed the filters extensively in binding buffer.
20. S. M. Hanley *et al.*, *Genes Dev.* **3**, 1534 (1989).
21. K.-T. Jeang *et al.*, *J. Virol.* **61**, 1559 (1987).
22. L. Laimins, P. Gruss, R. Pozzatti, G. Khoury, *ibid.* **49**, 183 (1984).
23. J. Brady, K.-T. Jeang, J. Duvall, G. Khoury, *ibid.* **61**, 2175 (1987).
24. D. H. Gubzda, J. Hess, J. Small, J. E. Clements, *Mol. Cell. Biol.* **9**, 2728 (1989).
25. R. Chiu, M. Imagawa, R. J. Imbra, J. R. Bockoven, M. Karin, *Nature* **329**, 648 (1987).
26. A. Gatignol and K.-T. Jeang, unpublished data.
27. H. Nashimoto and H. Uchida, *Mol. Gen. Genet.* **201**, 25 (1985); J. C. A. Bardwell *et al.*, *EMBO J.* **8**, 3401 (1989).
28. C. A. Kozak, unpublished observations.
29. R. D. Marshall, *Annu. Rev. Biochem.* **41**, 673 (1972); E. Bause, *Biochem. J.* **209**, 331 (1983).
30. The sequence has been deposited with GenBank (accession number M60801).
31. We thank M. Martin and W. Leonard for critical readings of the manuscript, and C. Buckler for computer analysis. Supported in part by the intramural AIDS antiviral targeted program from the office of the director of the NIH.

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In Vitro and in Vivo Consequences of VLA-2 Expression on Rhabdomyosarcoma Cells

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Cloned integrin α_2 subunit complementary DNA was expressed on human rhabdomyosarcoma (RD) cells to give a functional VLA-2 ($\alpha_2\beta_1$) adhesion receptor. The VLA-2–positive RDA2 cells not only showed increased adhesion to collagen and laminin in vitro, but also formed substantially more metastatic tumor colonies in nude mice after either intravenous or subcutaneous injection. These results show that a specific adhesion receptor (VLA-2) can markedly enhance both experimental and spontaneous metastasis. In contrast to the metastasis results, there was no difference in either the in vitro growth rate or apparent in vivo tumorigenicity of RD and RDA2 cells.

MEMBERS OF THE INTEGRIN FAMILY of adhesion receptors, comprised of at least 15 distinct αβ-subunit heterodimers (*1, 2*), mediate cell binding to major components of the extracellular matrix (ECM). For example, among the β_1 subfamily of integrins (VLA proteins), VLA-1, -2, and -3 mediate cell binding to collagen, VLA-3, -4, and -5 bind fibronectin, and VLA-1, -2, and -6 bind laminin (*2*). Integrins in the β_1 subfamily may be involved in tumor cell metastasis because the dissemination of tumor cells and their subsequent growth in secondary sites require extensive interaction with ECM proteins, both in the vascular basement membrane and interstitial stroma at the secondary site (*3*).

As evidence of a potential role for β_1 integrins in metastasis, monoclonal antibodies (MAbs) to VLA proteins can block cell migration and invasion through basement membranes in vitro (*4*), and VLA protein expression has been variably correlated with invasiveness in vitro (*5, 6*). Also, small synthetic peptides derived from cell adhesion molecules can block both cellular invasiveness in vitro and experimental metastasis in vivo, presumably by acting as ligand analogs competing for adhesion receptor binding sites (7). However, in vivo studies have not yet identified the specific adhesion receptor or receptors important for metastasis. Without addressing the issue of metastasis, other investigators have found that cell transformation (*8, 9*) and increased tumorigenicity (*10*) correlate with alterations in β_1 integrin expression.

Because cells usually express multiple integrins with overlapping ligand specificities, MAb blocking studies and correlational changes in integrin profiles are difficult to interpret. Also, the adhesion receptors that facilitate cell growth at a primary tumor site (that is, show tumorigenicity) are not necessarily the same as those involved in dissemination to tissue sites distant from the primary tumor (that is, metastasis).

This study focuses on the in vitro and in vivo roles of VLA-2, an adhesion receptor that usually binds both collagen and laminin, but on some cell types only binds collagen (*11*). To fully evaluate the in vivo effects of VLA-2, we examined not only tumorigenicity, but also both "spontaneous" and "experimental" metastasis. In the former, a tumor cell migrates into and through surrounding tissue, traverses a nearby vascular wall (or lymphatic channel), travels through the circulation, extravasates by again migrating through a vascular wall, and finally begins to grow in a new tissue location. In "experimental metastasis," tumor cells are injected intravenously and then escape from the circulation and colonize a tissue site, in a model system that mimics the latter steps of spontaneous metastasis.

To study the functions of VLA-2, we introduced the full-length cDNA clone for the α_2 subunit (*12, 13*) into the rhabdomyosarcoma tumor cell line RD by means of the mammalian cell expression vector pFneo (*14*). The expression of VLA-2 or transfected RD (RDA2) was demonstrated by immunoprecipitation (Fig. 1B) and by immunofluorescence staining (Fig. 1A). Although β_1 and other α subunits are present in both RD and RDA2 cells, α_2 expression was observed only in the RDA2 cells. Because the α_2 gene product was coprecipitated with the β_1 subunit (Fig. 1B) and the amount of β_1 expressed at the cell surface increased (Fig. 1A) over that of RD cells, the α_2 subunit must have associated with the endogenous β_1 subunit of RD cells. Flow cytometry experiments showed that transfection of the α_2 gene caused no alteration in the surface levels of VLA-1, -4, -5 and -6, normally found on RD cells. Together these results support the previous suggestion (*15*) that a pool of excess β_1 subunit is available for association if the amount of α should increase. Also, our results agree with findings from other integrin transfection studies which showed that α or β subunits from the β_2 and β_3 integrin

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