unrelated in primary sequence and overall architecture, the HSV-encoded gC (*10*), and the leukocyte integrin Mac-1 (*11*), have evolved a similar ligand recognition repertoire, both including complement proteins of the C3b cascade and factor X (*3, 12*), presumably through convergent evolution. Third, these studies directly link the ligand recognition of a broadly distributed leukocyte integrin such as Mac-1 (*3*) to established mechanisms of vascular injury. These are well exemplified by the recognized atherosclerotic risk associated with HSV infection of endothelial cells (*13*).

The synthetic peptidyl analogs of the ligand loops described here have the capacity to interrupt generation of thrombin on monocytes and HSV-infected endothelium, thereby preventing the pleiotropic consequences that include chemotaxis and mitogenesis (*14*), platelet and leukocyte adhesion to endothelium (*15*), and monocyte deposition of insoluble fibrin (*4*). Moreover, only one of these peptides minimally interferes with the mechanism of factor X activation mediated through the classic extrinsic pathways (*8*). Such specificity suggests that antagonists based on peptidyl analogs or more advanced derivatives may beneficially intervene in related forms of vascular injury without interfering with physiologic hemostatic mechanisms or leukocyte adhesion reactions.

REFERENCES AND NOTES

1. J. Niemetz and K. Fani, *Nature New Biol.* **232**, 247 (1971).
2. D. M. Stern, P. P. Nawroth, W. Kisiel, G. Vehar, C. T. Esmon, *J. Biol. Chem.* **260**, 6717 (1985); G. J. Broze, Jr., *J. Clin. Invest.* **70**, 526 (1982); P. B. Tracy, J. M. Peterson, M. E. Nesheim, F. C. McDuffie, K. G. Mann, *J. Biol. Chem.* **254**, 10354 (1979); I. Maruyama, H. H. Salem, P. W. Majerus, *J. Clin. Invest.* **74**, 224 (1984).
3. E. Ruoslahti, *J. Clin. Invest.* **87**, 1 (1991); M. A. Arnaout, *Blood* **75**, 1037 (1990); T. S. Springer, *Nature* **346**, 425 (1990).
4. D. C. Altieri and T. S. Edgington, *J. Biol. Chem.* **263**, 7007 (1988); D. C. Altieri, J. H. Morrissey, T. S. Edgington, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7462 (1988).
5. O. R. Etingin, R. L. Silverstein, H. M. Friedman, D. P. Hajjar, *Cell* **61**, 657 (1990).
6. The single-letter 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; and Y, Tyr.
7. Sequence-specific antibodies were raised in rabbits against selected synthetic peptides coupled in equal molar ratio to keyhole limpet hemocyanin. Sera reacting with immobilized factor X in solid phase radioimmunoassay were further purified by affinity chromatography over a 25-ml column of factor X coupled to agarose (2 mg/ml). The immunoglobulin G fraction from each antiserum was obtained by gel filtration on a Protein A–agarose column. In inhibition experiments, increasing concentrations of the various sequence-specific antibodies were preincubated with 15 nM ^{125}I–factor X for 30 min at 22°C before addition to suspensions of N-fMLP–stimulated monocyte THP-1 cells. Fifty per cent inhibition of ^{125}I–factor X binding was achieved with 10 μg/ml of each antibody to the three inhibitory peptides. Under the same experimental conditions, a sequence-specific antibody to the activation peptide of factor X (*21* to *41*) was ineffective.
8. A. Chattopadhyay and D. S. Fair, *J. Biol. Chem.* **264**, 11035 (1989).
9. S. C. T. Lam *et al.*, *ibid.* **262**, 947 (1987); S. A. Santoro and W. Lawing, *Cell* **48**, 867 (1987).
10. R. J. Frink, R. Eisenberg, G. Cohen, E. K. Wagner, *J. Virol.* **45**, 634 (1983).
11. T. K. Kishimoto, K. O'Connor, A. Lee, T. M. Roberts, T. A. Springer, *Cell* **48**, 681 (1987); A. L. Corbi, T. K. Kishimoto, L. Miller, T. A. Springer, *J. Biol. Chem.* **263**, 12403 (1988); M. A. Arnaout, E. Remold-O'Donnel, M. W. Pierce, P. Harris, D. G. Tenen, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2776 (1988).
12. H. M. Friedman, G. H. Cohen, R. J. Eisenberg, C. A. Seidel, D. B. Cines, *Nature* **309**, 633 (1984).
13. E. P. Benditt, T. Barrett, J. K. McDougall, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6386 (1983); M. R. Visser *et al.*, *ibid.* **85**, 8227 (1988); D. P. Hajjar, K. B. Pomerantz, D. J. Falcone, B. B. Weksler, A. J. Grant, *J. Clin. Invest.* **80**, 1317 (1987).
14. K. C. Glen and D. D. Cunningham, *Nature* **278**, 711 (1979); R. Bar-Shavit, A. Kahn, G. D. Wilner, *Science* **220**, 728 (1983).
15. S. R. Hanson and L. A. Harker, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3184 (1988); G. A. Zimmerman, T. M. McIntyre, S. M. Prescott, *J. Clin. Invest.* **76**, 2235 (1985).
16. For peptide competition experiments, 0.2-ml aliquots of THP-1 cells resuspended at 1.5×10^7 per milliliter in serum-free RPMI 1640 were stimulated with 10 μM N-fMLP (Sigma) in the presence of 2.5 mM $CaCl_2$, and simultaneously mixed with 15 nM ^{125}I–factor X and 0.5 mM doses of the various factor X synthetic peptides for 20 min at 22°C. The reaction was terminated by centrifugation through mixture of silicone oil (Dow Corning, New Bedford, MA) and nonspecific binding calculated in the presence of a 100-fold molar excess of unlabeled factor X was subtracted from the total to calculate net specific binding. ^{125}I–factor X specifically bound to N-fMLP–stimulated THP-1 cells in the absence of competing peptides was 57,000 ± 8,000 molecules per cell ($n = 10$).
17. M. R. Fung, C. W. Hay, R. T. A. MacGillivray, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3591 (1985).
18. J. Greer, *Proteins Struct. Funct. Genet.* **7**, 317 (1990).
19. Trypsinogen structure (2TGP) from Marquart *et al.* (*22*) as contained in the Brookhaven National Laboratory, F. C. Bernstein *et al.* [*J. Mol. Biol.* **112**, 535 (1977)], and E. E. Abola *et al.* [*Protein Data Bank*, in *Crystallographic Databases–Information Content, Software Systems, Scientific Applications*, F. H. Allen, G. Bergenhoff, R. Sievers, Eds. (Data Commission of the International Union of Crystallography, Bonn, Cambridge, and Chester, 1987), p. 107].
20. Trypsin (2PTC) from (*22*). Chymotrypsin (5CHA) from R. A. Blevins and A. Tulinsky [*J. Biol. Chem.* **260**, 4264 (1985)]. Elastase (1EST) from L. Sawyer *et al.* [*J. Mol. Biol.* **118**, 137 (1978)]. Kallikrein (2PKA) from W. Bode *et al.* [*ibid.* **164**, 237 (1983)].
21. T. K. Brunck, personal communication.
22. M. Marquart *et al.*, *Acta Crystallogr.* B39, 480 (1983).

23. Supported by National Institutes of Health grants R01 HL 43773, P01 HL 16411, HL 46408, HL 18828, and HL 45343. This is manuscript 6818-IMM from the Department of Immunology. D. S. Fair died 12 May 1990.

25 June 1991; accepted 11 September 1991

Epidermolysis Bullosa Simplex: Evidence in Two Families for Keratin Gene Abnormalities

J. M. BONIFAS, A. L. ROTHMAN, E. H. EPSTEIN, JR.*

Epidermolysis bullosa simplex (EBS) is characterized by skin blistering due to basal keratinocyte fragility. In one family studied, inheritance of EBS is linked to the gene encoding keratin 14, and a thymine to cytosine mutation in exon 6 of keratin 14 has introduced a proline in the middle of an alpha-helical region. In a second family, inheritance of EBS is linked to loci that map near the keratin 5 gene. These data indicate that abnormalities of either of the components of the keratin intermediate filament heterodipolymer can impair the mechanical stability of these epithelial cells.

EPIDERMOLYSIS BULLOSA SIMPLEX IS an unusual hereditary disorder in which patients develop blisters after relatively mild mechanical trauma. Cleavage is through the basal cells, unlike the more superficial cleavage that produces friction blisters in normal people. Those with the commonest forms of EBS have blisters predominantly acrally (EBS–Weber-Cockayne or EBS-WC) or in a more generalized distribution (EBS-Koebner or EBS-K). Skin fragility is temperature sensitive—it is worse in the summer, and preventive measures are confined to cooling the skin and avoiding trauma. Although the blisters heal relatively quickly and without scarring, blistering may be so painful as to be medically disabling (*1*).

Several years ago we were struck by similarities between EBS and heritable erythrocyte disorders such as pyropoikilocytosis, elliptocytosis, and spherocytosis. These similarities include autosomal dominant inheritance and temperature-sensitive cellular fragility. These red blood cell disorders result from molecular abnormalities of the cytoskeleton, which is limited in the mature erythrocyte to a submembranous position. Although keratinocytes do contain homologs of proteins of the erythrocyte membrane skeleton (*2*), their predominant cytoskeletal components are the tonofilaments—intermediate filaments composed of keratin

Department of Dermatology, San Francisco General Hospital, University of California, San Francisco, CA 94110.

*To whom correspondence should be addressed.

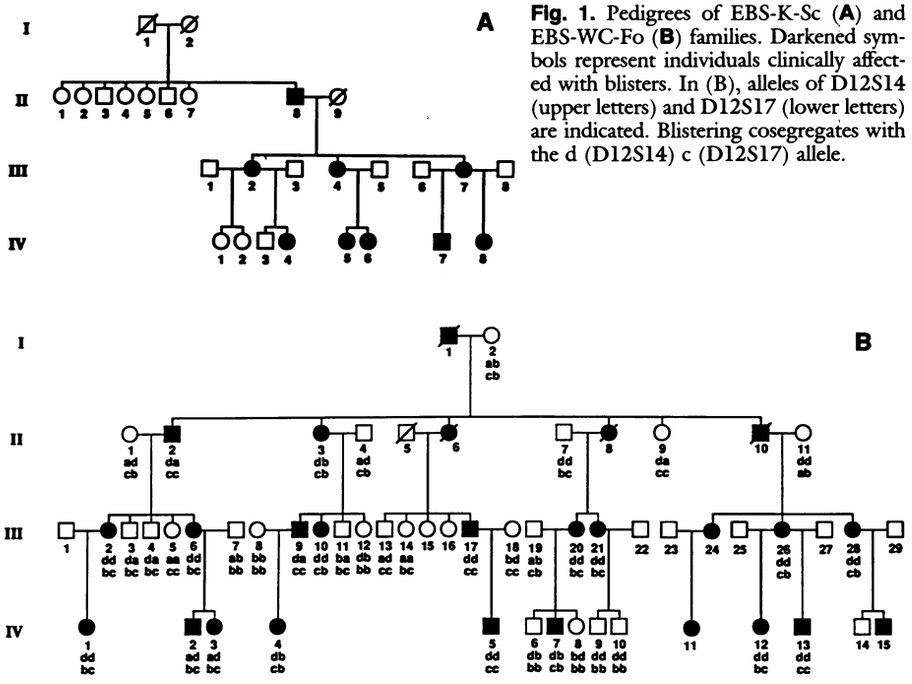

Fig. 1. Pedigrees of EBS-K-Sc (**A**) and EBS-WC-Fo (**B**) families. Darkened symbols represent individuals clinically affected with blisters. In (B), alleles of D12S14 (upper letters) and D12S17 (lower letters) are indicated. Blistering cosegregates with the d (D12S14) c (D12S17) allele.

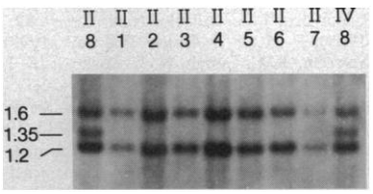

Fig. 2. Southern (DNA) blot analysis of genomic DNA from members of EBS-K-Sc family hybridized with 3′-untranslated K14 probe. Numbering above each lane as in Fig. 1A. The largest fragment (1.6 kb) is derived from the normal allele and is of a size compatible with known restriction sites (7), and the middle-sized fragment (1.35 kb) is unique to the affected persons. The smallest fragment (1.2 kb) is present in all genomic samples, does not hybridize with probes 5′ of bp 3857, and is probably derived from one of several K14-related genes (*5*).

molecules. On the basis of (i) the resemblance of the fragility and (ii) the morphological abnormalities of intermediate filaments in EBS (*3*), we reasoned that keratin gene disorders might underlie this disease, and so we have compared the inheritance of EBS with the inheritance of probes that identify DNA polymorphisms in regions to which keratin genes have been mapped.

Keratins can be divided into families of relatively acidic (type I) and relatively basic (type II) molecules, and they have been numbered according to size within each family (for example, K1 is larger than K2; K10 is larger than K11). One member of each family is required for polymerization. Although isolated acidic and basic keratins can pair promiscuously, epithelial cells in vivo express specific pairs that are characteristic of the epithelium and of the stage of differentiation of the cell. Thus, the predominant keratins of epidermal basal cells are K5 and K14 and those of suprabasal cells are K1 and K10. Each keratin molecule is the product of a single gene, and genes for acidic and basic keratins are clustered on chromosomes 17 and 12, respectively.

Polymorphisms of the genes for the predominant basal cell keratins K5 and K14 have not been described, so we tested for linkage to probes recognizing polymorphic loci that have been mapped to the regions where the type I keratin K14 (chromosome 17q12–q21) and several type II keratins (chromosome 12q11–q13) have been mapped (*4, 5*).

We studied two American families of Northern European ancestry (*6,* 7). Members of one family (EBS-K-Sc) (Fig. 1A) have generalized fragility, but most blisters occur on the hands and feet; members of the other (EBS-WC-Fo) (Fig. 1B) have blisters only acrally. Parents noticed blisters at an age as early as 1 month. Skin biopsies of one affected member of each kindred were studied, and electron microscopic examination confirmed the expected splitting through the basal cell layer that is characteristic of EBS.

In the Sc family, EBS is weakly linked to D17S74 and is excluded from the region of the chromosome 12q probes tested (Table 1). To survey the K14 gene in the Sc family directly, we hybridized a 3′-untranslated K14 probe (*8*) to patient DNA digested with nine restriction endonucleases. Eight gave a normal band pattern. However, Msp I digestion produced a 1.35-kb fragment in DNA from all affected members of the family but produced no fragment of this size in DNA from unaffected members. This K14 polymorphism is tightly linked to inheritance of EBS in this family, with a logarithm of likelihood ratio for linkage (lod score) of 3.0 (Table 1).

We wished to know whether the Msp I polymorphism is unique to this family and therefore tested DNA from 57 normal, unrelated Caucasians (114 chromosomes). None had the 1.35-kb Msp I fragment. The Sc family progenitor of this three-generation kindred (II-8) is the first affected in his family—his disease apparently is the result of a new mutation. His parents have died, but all seven of his siblings are alive. None of them has EBS, and DNA from all seven lack the 1.35-kb Msp I K14 fragment (Fig. 2), indicating with 128:1 odds that the 1.35-kb fragment and the disease arose in the same individual. Probes recognizing highly polymorphic loci (*9*) gave no evidence for nonpaternity in the eight members of generation II, and the progenitor's two D17S74 alleles each were present in several of his siblings.

A K14 exon-6 probe hybridizes with the normal 1.6-kb Msp I fragment as well as with the 1.35-kb fragment unique to the patient. Msp I digestion of patient DNA amplified with primers flanking exons 4 through 7 (*8*) produced the expected 575- and 520-bp fragments from the normal allele as well as three fragments (520, 333, and 242 bp) from the mutant allele. This maps the mutation creating the new Msp I site to within exon 6. After sequencing this region (*10*), we found a T to C substitution at bp 3542 (Fig. 3). This substitution produces the new recognition site for Msp I and is expected to change the amino acid at position 384 from leucine to proline. In vivo transcription of both normal and mutant alleles was confirmed by Msp I digestion of polymerase chain reaction (PCR)–amplified cDNA that had been prepared from skin samples taken from unblistered buttock skin (*11*).

In the Fo kindred, EBS is linked to chromosome 12q probes—with data combined from D12S14 and D12S17 in a haplotype analysis (Fig. 1), the lod score is greater than 7 (Table 1). We isolated a K5-specific human cosmid and found using two-color fluorescence microscopy that the locations of this cosmid and of D12S14 are nearly indistinguishable on in situ hybridization (*12*).

These data provide strong evidence for linkage of the inheritance of EBS to the inheritance of genes that encode the keratins of epidermal basal cells, the site of the phenotypic abnormality. Intermediate filament proteins share a similar molecular ar-

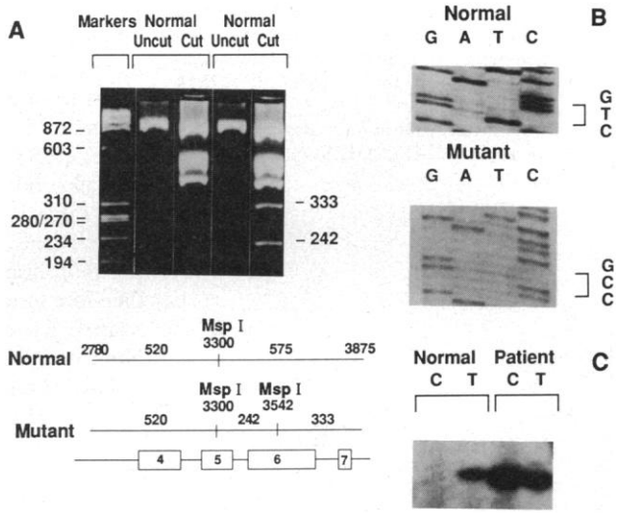

Fig. 3. (**A**) Mapping of polymorphic EBS-K-Sc K14 Msp I site. A 1095-bp fragment (bp 2780 to 3875) was amplified from genomic DNA (*8*), an aliquot was digested with Msp I, and digested and undigested DNA were separated by electrophoresis on agarose (3% NuSieve, 1% LE; FMC Corporation) (top). The Msp I map of this region is shown below; numbered boxes represent exons; thin lines represent introns. (**B**) Sequencing of region around bp 3542, indicating thymine to cytosine substitution. (Top) Sequence codes for Leu^{384} (CTG); (bottom) sequence codes for Pro^{384} (CCG). (**C**) Confirmation of sequencing by single nucleotide primer extension. With DNA from normal individuals, only the thymine is incorporated; with DNA from the heterozygous affected patients, both thymine and cytosine are incorporated (*20*).

Table 1. Pairwise lod scores between EBS and chromosome 12q and 17q loci. Chromosome 12q loci are ordered in the table according to their position on the genetic map from top (centromeric) to bottom (telomeric) (*21*). Paternal and maternal segregation are combined, and autosomal dominant inheritance with full penetrance and no sporadic cases (except the Sc progenitor) have been assumed.

Locus	Lod score at θ of				Peak lod (score/θ)
	0.001	0.01	0.05	0.10	
		Sc family			
D12S14	−9.59	−5.61	−2.88	−1.77	−0.07 /0.40
D17S74	−0.30	0.66	1.18	1.25	1.25 /0.09
K14	3.00	2.96	2.76	2.51	3.00 /0.00
		Fo family			
D12S2	−0.66	1.26	2.30	2.45	2.45 /0.09
COL2A1	1.75	3.66	4.64	4.67	4.71 /0.08
D12S15	2.14	3.06	3.42	3.27	3.42 /0.05
D12S14	4.17	4.13	3.91	3.61	4.17 /0.00
D12S17	3.52	3.52	3.42	3.17	3.52 /0.00
D12S14/D12S17 haplotype	7.43	7.35	6.97	6.42	7.43 /0.00
D17S74	−34.47	−19.56	−9.45	−5.42	0.07/40.00

chitecture, with four long regions of α helices separated by short nonhelical regions and flanked by longer nonhelical tails (*13*). Evidence for the function of the helical regions comes from conservation of sequences among keratins and from transfection studies, in which genes encoding shortened intermediate filament proteins do not disrupt the endogenous network when the proteins lack the nonhelical tails but do cause collapse of the network when even a few of the helical region amino acids are deleted (*14*). Thus, the effect of a proline for leucine substitution, which would be expected to disrupt the COOH-terminal α helix, is highly compatible with previous findings emphasizing the importance of this region to intermediate filament function.

Basal keratinocyte fragility causing neonatal death occurs in mice carrying a transgene encoding a shortened K14 (*15*). The phenotype of the human disease apparently caused by the proline for leucine substitution is much less severe in the Sc family than that caused by the deletion of 135 amino acids from the K14 COOH-terminus in these mice.

These data indicate that intermediate filaments in epidermal basal keratinocytes serve a structural role. They also indicate that EBS, like osteogenesis imperfecta (*16*), can be due to abnormalities of either of the components of a multimeric structural protein complex—of keratin filaments and of collagen fibrils, respectively. Thus, both disorders are a result of "dominant negative" mutations (*17*). Furthermore, the Sc family mutation resembles the common mutations causing osteogenesis imperfecta in that both appear to disrupt the normal helical structure of the polymer. Others have reported linkage in one kindred with the rare variant EBS-Ogna to the glutamic-pyruvic transaminase (GPT) gene, which is located on chromosome 8q (*18*), and linkage in one kindred with EBS-K to chromosome 1q (*19*). Although these patients may also have cytoskeletal protein abnormalities, genes encoding epidermal cytoskeletal components have not yet been mapped to these regions.

Note added in proof: Subsequent to submission, we have assessed DNA from 20 unrelated persons with EBS. None has the Msp I polymorphism described in the Sc family.

REFERENCES AND NOTES

1. J.-D. Fine, *Arch. Dermatol.* **124**, 523 (1988).
2. S. Mutha, A. Langston, J. Bonifas, E. H. Epstein, Jr., *J. Invest. Dermatol.* **97**, 383 (1991).
3. Y. S. Kitajima, S. Inoue, H. Yaoita, *Arch. Dermatol. Res.* **281**, 5 (1989); M. Ito, C. Okuda, N. Shimizu, T. Tazawa, Y. Sato, *Arch. Dermatol.* **127**, 367 (1991).
4. B. L. Bader, L. Jahn, W. W. Franke, *Eur. J. Cell Biol.* **47**, 300 (1988); S. R. Lessin, K. Huebner, M. Isobe, C. M. Croce, P. M. Steinert, *J. Invest. Dermatol.* **91**, 572 (1988); V. Romano *et al.*, *Cytogenet. Cell Genet.* **48**, 148 (1988); N. C. Popescu, P. E. Bowden, J. A. DiPaolo, *Hum. Genet.* **82**, 109 (1989); P. Heath *et al.*, *ibid.* **85**, 669 (1990); A. Wasseem, A. C. Gough, N. R. Spurr, E. B. Lane, *Genomics* **7**, 188 (1990).
5. M. Rosenberg, A. Ray Chaudhury, T.-B. Shows, M. M. LeBeau, E. Fuchs, *Mol. Cell. Biol.* **8**, 722 (1988).
6. Blood samples were collected, anticoagulated, and stored frozen. After thawing, DNA was extracted with phenol chloroform [J. Sambrook, E. G. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989)], digested with restriction endonucleases, separated by electrophoresis on agarose gels, and blotted onto membranes of nylon (Hybond-N, Amersham Corporation) or polyvinyldifluoride (Immobilon-N, Millipore Corporation) [W. Mann, V. S. Venkatraj, A. D. Auerbach, *Nucleic Acids Res.* **17**, 5410 (1989)]. After hybridization with ^{32}P-labeled probes, filters were analyzed by autoradiography. The DNA probe and enzyme combinations used were as follows: on chromosome 12, p12–16 and Eco RI (D12S2) [I. Basazs, M. Purrello, K.-H. Grezeschik, P. Szabo, *Hum. Genet.* **68**, 57 (1984)], WEAV214 and Hinf I (COL2A1) [E. J. Weaver and R. G. Knowlton, *Cytogenet. Cell Genet.* **54**, 1103 (1989)], pCMM1.2 and Taq I (D12S15) [C. Martin *et al.*, *Nucleic Acids Res.* **16**, 3596 (1988)], pEFD33.2 and Msp I (D12S14) (Y. Nakamura *et al.*, *ibid.*, p. 778), and pYNH15 and Msp I (D12S17) (Y. Nakamura *et al.*, *ibid.*, p. 779); on chromosome 17, pCMM86 and Hinf I (D17S74) (Y. Nakamura *et al.*, *ibid.*, p. 5223). Two-point linkage analysis was calculated with LIPED [J. Ott, *Analysis of Human Genetic Linkage* (Johns Hopkins Univ. Press, Baltimore, 1985)].
7. A portion of this linkage analysis has been published in abstract form. [J. M. Bonifas, A. L. Rothman, E. H. Epstein, Jr., *J. Invest. Dermatol.* **97**, 550 (1991)].
8. K14 probes were synthesized by PCR amplification of genomic DNA. The following primers were used: 3′-untranslated, 5′-TTGAATTCCCGCTCAGGCCTAGGAG-3′ (bp 4481 to 4497) and 5′-TTGGATCCGGTGAGGGTGAAGCAGGG-3′ (bp 4581 to 4564); exons 4 to 7, 5′-TTGAATTCGCAACAAGGTTAGACCTG-3′ (bp 2780 to 2798) and 5′-TTGGATCCAATGCCTAGACCTGCTTGG-3′ (bp 3875 to 3857); numbering as in D. Marchuk, S. McCrohan, and E. Fuchs [*Proc. Natl. Acad. Sci. U.S.A.* **82**, 1609 (1985)] (plus 5′ restriction endonuclease sites). The amplification buffer contained 2.5 mM (3′-untranslated) or 1.5 mM (exons 4 to 7) $MgCl_2$. Amplification was performed for 30 cycles with an

annealing temperature of 57°C (3′-untranslated) or for 6 cycles at an annealing temperature of 60°C followed by 24 cycles with an annealing temperature of 57°C (exons 4 to 7).
9. The DNA probe and enzyme combinations used in addition to pCMM86 and Hinf I (D17S74) were as follows: pMMZ10 and Msp I (D9S11), and pMCT96.1 and Hinf I (D9S14) [M. Lathrop *et al.*, *Genomics* **3**, 361 (1988)].
10. PCR products were precipitated with ethanol, cleaved with Eco RI and Bam HI, and cloned into pBluescript (Stratagene Cloning Systems). Clones containing wild-type and mutant alleles were distinguished by Msp I digestion of amplified insert. Double-stranded DNA was isolated from two clones each of normal and mutant alleles and sequenced (Sequenase, U.S. Biochemical Corporation). Sequencing primers used were as follows: 5′-TTGAATTCCGGCCACACTCACTAATCG-3′ (bp 3401 to 3419), 5′-TTGAATTCAGACCAAAGGTCGCTACTG-3′ (bp 3473 to 3491), and 5′-TTGGATCCGGATCATTAGATACATGGTGG-3′ (bp 3738 to 3718).
11. A shave biopsy specimen was frozen in liquid nitrogen, and RNA was isolated with a Micro-Fast Track mRNA isolation kit (Invitrogen Corporation, San Diego). The cDNA was prepared with random hexamers and was amplified with oligonucleotides corresponding to the 5′ end of exon 5 [5′-TTGAATTCAGAGGAGCTGAACCGCG-3′ (bp 3225 to 3242)] and to the 3′-untranslated region (bp 4581 to 4564) (7).
12. J. M. Bonifas *et al.*, data not shown.
13. P. Steinert and D. Roop, *Annu. Rev. Biochem.* **57**, 593 (1988).
14. K. Albers and E. Fuchs, *J. Cell Biol.* **108**, 1477 (1989); S. S. M. Chin, P. Macioce, R. K. H. Liem, *ibid.* **111**, 176a (1990).
15. R. Vassar, P. A. Coulombe, L. Degenstein, K. Albers, E. Fuchs, *Cell* **64**, 365 (1991).
16. B. Sykes, D. Ogilvie, P. Wordsworth, J. Anderson, N. Jones, *Lancet* **ii**, 69 (1986).
17. I. Herskowitz, *Nature* **329**, 219 (1987).
18. B. Olaisen and T. Gedde-Dahl, *Hum. Hered.* **23**, 189 (1973); L.-C. Tsui, M. Farrall, H. Donnis-Keller, *Cytogenet. Cell Genet.* **51**, 166 (1989).
19. M. M. Humphries *et al.*, *Genomics* **7**, 377 (1990).
20. Genomic DNA was amplified with primers flanking exons 4 to 7 (*6*) and purified by gel electrophoresis. A 19-bp oligonucleotide primer [5′-GGCAGCGTGGAGGAGCAGC-3′ (bp 3523 to 3541)] was added with either [^{32}P]dCTP deoxycytidine triphosphate or [^{32}P]TTP thymidine triphosphate, extended with Taq polymerase for one cycle, separated by electrophoresis on a denaturing 20% polyacrylamide gel, and analyzed by autoradiography [M. Kuppuswamy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1143 (1991)].
21. P. O'Connell *et al.*, *Genomics* **1**, 93 (1987).
22. WEAV214 was a generous gift of R. Knowlton; all other probes were obtained from the American Type Culture Collection. We thank M. Lee for assistance in calculation of the lod scores; J. W. Bare, C. J. Epstein, and M.-C. King for discussions; M. Bogan and M. Williams for culturing keratinocytes; D. Cox, J. Gitschier, and Y. W. Kan for suggestions on the manuscript; C. McMonigle for preparation of the manuscript; and the Fo and Sc families for their generous donations of samples of blood and skin. Supported in part by USPHS grants R01-AR28069 and R01-AR39953.

29 May 1991; accepted 9 September 1991

Functional Importance of Sequence in the Stem-Loop of a Transcription Terminator

SHEAU-WEI C. CHENG, EILEEN C. LYNCH, KENNETH R. LEASON, DONALD L. COURT, BRUCE A. SHAPIRO, DAVID I. FRIEDMAN*

Intrinsic transcription terminators of prokaryotes are distinguished by a common RNA motif: a stem-loop structure high in guanine and cytosine content, followed by multiple uridine residues. Models explaining intrinsic terminators postulate that the stem-loop sequence is necessary only to form structure. In the tR2 terminator of coliphage λ, single-nucleotide changes reducing potential RNA stem stability eliminated tR2 activity, and a compensatory change that restored the stem structure restored terminator activity. However, multiple changes in the stem sequence that should have either maintained or increased stability reduced terminator activity. These results suggest that the ability of the stem-loop structure to signal transcription termination depends on sequence specificity and secondary structure.

EARLY WORK ON GENE REGULATION focused interest on promoters, the sites of transcription initiation; further work has expanded interest to the study of termination and the generation of the 3′ ends of transcripts (*1*). Two classes of terminators have been defined for *Escherichia coli* (*2, 3*), those that require ρ protein (ρ-dependent) (*4*) and those that do not (intrinsic or ρ-independent) (*5*). The hallmark features of the DNA sequence of an intrinsic terminator are a region of hyphenated dyad symmetry, followed by multiple T residues, yielding an RNA with a stem-loop structure followed by a run of U residues (*6–8*). Lytic growth of coliphage λ requires read-through of multiple transcription terminators, including the tR2 intrinsic terminator (Fig. 1A). To assess the role of the hyphenated dyad symmetry in signaling termination, we systematically changed nucleotides in the λ tR2 terminator (*9*) and measured the in vivo effectiveness of these mutated terminators by using plasmid vector pKL600, designed to quantitate terminator activity (*10*) (Fig. 1B), and λ constructs with mutated tR2 regions, designed to test in situ terminator activity.

We cloned the tR2 terminator into pKL600 on a 434-bp Sau 3AI fragment, generating pKL600tR2$^+$, which places tR2 between the *lac* promoter (P_{lac}) and the *galK* reporter gene. This allows us to use *galK* expression as a measure of terminator activity. Mutations were introduced only in the sequences encoding the stem-loop structure; therefore, differences in *galK* expression yielded a direct measure of the effects on termination of alterations in this region of hyphenated dyad symmetry. A derivative of pKL600tR2$^+$ with an 18-bp deletion that removes most of the stem-loop region (pKL600tR2$^{\Delta 18}$) (Fig. 2) served as the terminator-deficient control; the expression of *galK* from this plasmid was set as the 100% read-through transcription or as the 0% termination base line. On the basis of this comparison, tR2$^+$ allowed 15% read-through transcription.

We next assessed the importance of the RNA stem structure using derivatives of pKL600tR2$^+$ with mutations in tR2 (Fig. 2). Mutants tR2^1 and tR2^7, with single base changes that weakened the stem structure, eliminated terminator activity. A double mutation (tR2^2), which re-created a G:C base pair eliminated in tR2^7, restored terminator activity. Mutations that reduce the number of distal U residues or the strength of the stem-loop structure of an intrinsic terminator reduce termination (*2, 7, 11*). This offers strong evidence that RNA structure plays a role in intrinsic terminator action. Two other mutants, tR2^5 and tR2^6, had compensatory changes in the nucleotide sequence of the stem structure that made the sequences of their stems equivalent to the wild type in number and quality of paired residues. Unexpectedly, tR2^5 and tR2^6 exhibited little terminator activity. This suggests that the secondary structure is not the only characteristic of the tR2 stem sequence that is important for transcription termination. We introduced mutations in the loop of tR2 (tR2^4) to determine if the sequence of the loop influenced termination. Although the number of nucleotides that composed the loop remained the same, the se-

S.-W. C. Cheng, E. C. Lynch, K. R. Leason, D. I. Friedman, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109.
D. L. Court, Molecular Control and Genetics Section, Laboratory of Chromosome Biology, ABL–Basic Research Program, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD 21702.
B. A. Shapiro, Imaging Processing Section, Laboratory of Mathematical Biology, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD 21702.

*To whom correspondence should be addressed.