2. M. A. Fedak and H. J. Seeherman, *Nature*, **282**, 713 (1979).
3. H. B. John-Alder, T. Garland, A. F. Bennett, *Physiol. Zool.* **59** (no. 5), 523 (1986).
4. R. J. Full, *Am. J. Physiol.* **251**, R775 (1986); ———, B. D. Anderson, C. M. Finnerty, M. E. Feder, *J. Exp. Biol.* **138**, 471 (1988).
5. C. F. Herreid, R. J. Full, D. Prawel, *Science* **212**, 331 (1981); G. A. Bartholomew, J. R. B. Lighton, G. N. Louw, *J. Comp. Physiol.* **155**, 155 (1985).
6. R. J. Full and C. F. Herried, *Am. J. Physiol.* **244**, R530 (1983); R. J. Full, *J. Exp. Biol.* **130**, 137 (1987).
7. R. J. Full, in *Energy Transformations in Cells and Animals*, W. Wieser and E. Gnaiger, Eds. (Thieme, Stuttgart, 1989).
8. J. R. B. Lighton, *Physiol. Zool.* **58** (no. 4), 390 (1985).
9. F. Depocas and J. S. Hart, *J. Appl. Physiol.* **10**, 388 (1957).
10. Frame speed of the videorecorder was calibrated by videotaping a close-up sequence of a moving stopwatch. The sequence was played back frame by frame and the frame count was compared to the video time image. In later experiments, a Panasonic time generator was used to put a time base (to the nearest 0.01 s) directly on tape.
11. T. M. Casey and R. Knapp, *Comp. Biochem. Physiol.* **86A**, 679 (1987).
12. T. M. Casey, unpublished observations.
13. N. C. Heglund and C. R. Taylor, *J. Exp. Biol.* **138**, 301 (1988); R. J. Full, D. A. Zuccarello, A. Tullis, *ibid.* **150**, 233 (1990).
14. A. F. Bennett, *Am. J. Physiol.* **247**, R217 (1984).
15. G. A. Bartholomew and T. M. Casey, *J. Therm. Biol.* **2**, 173 (1977).
16. R. McN. Alexander, *Am. Zool.* **24**, 85 (1984).
17. A. F. Bennett and J. A. Ruben, *Science* **206**, 649 (1979).
18. B. Heinrich, *Oecologia* **42**, 325 (1979).
19. Supported in part by NSF grant DCB88 02443 and by the New Jersey Agricultural Experiment Station, MacIntire-Stennis project 08337. I thank C. Benyak, P. Young, and M. Yurlina for assistance in data collection and B. Joos and M. L. May for critical comments on the manuscript.

16 October 1990; accepted 6 February 1991

An Erythromycin Derivative Produced by Targeted Gene Disruption in *Saccharopolyspora erythraea*

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Derivatives of erythromycin with modifications at their C-6 position are generally sought for their increased stability at acid pH, which in turn may confer improved pharmacological properties. A recombinant mutant of the erythromycin-producing bacterium, *Saccharopolyspora erythraea*, produced an erythromycin derivative, 6-deoxyerythromycin A, that could not be obtained readily by chemical synthesis. This product resulted from targeted disruption of the gene, designated *eryF* (systematic nomenclature, *CYP107*), that apparently codes for the cytochrome P450, 6-deoxyerythronolide B (DEB) hydroxylase, which converts DEB to erythronolide B (EB). Enzymes normally acting on EB can process the alternative substrate DEB to form the biologically active erythromycin derivative lacking the C-6 hydroxyl group.

PREPARATION OF MODIFIED ANTIBIOTICS in cases where the parent molecule is complex and polyfunctional can be achieved by alteration of the antibiotic's biosynthetic pathway. Mutasynthesis (*1*) has been practiced for many years. More recently, genetic engineering has been useful in the formation of novel hybrid antibiotics (*2, 3*). We have used recombinant DNA technology to produce an erythromycin (Er) derivative that was not readily obtainable through chemical means but that was desired for pharmacological analyses.

Integrative plasmids have been used in *Saccharopolyspora erythraea* for targeted gene disruption in the elucidation of the organization of genes in the Er cluster (*4–6*). These plasmids, which carry small pieces of chromosomal DNA, are directed into the homologous site on the chromosome by single, reciprocal (Campbell) recombination. Frequently this process results in the generation of stable mutants that no longer produce Er. In this study, integrants were analyzed in a relatively uncharacterized region of the gene cluster between the *eryH* and *eryG* loci (*6*). Two strains so generated were distinguishable from the others by the production of a new metabolite, which was identified initially as a single spot by thin-layer chromatography (TLC) (Fig. 1).

One mutant was chosen for further analysis, and the new metabolite was isolated from its growth medium and purified (7). Fast atom bombardment mass spectrometry revealed that the metabolite had a molecular mass of 718 daltons, and ^{1}H and ^{13}C nuclear magnetic resonance (*8*) indicated that the structure 6-deoxyerythromycin (deoxy Er) A (Fig. 2A). Other components of the culture broth were separated and purified, and their structures were determined. They comprised the analogous biosynthetic congeners to Ers B, C, and D. A family of minor components, the 6-deoxy-15-norerythromycins (nor Ers) A, B, C, and D (Fig. 2A) was also isolated, separated, and characterized spectrally. These minor components presumably arose from the incorporation of acetyl coenzyme A (CoA), rather than of propionyl-CoA, as the starter unit to the polyketide synthase (*9*). The aglycone resulting from a similar aberration of polyketide biosynthesis, 8, 8α-deoxyoleandolide, has been isolated (*10*).

The mutagenic plasmids used to generate these strains were found through restriction analysis to contain fragments of 500 to 1000 bp from the *eryH-eryG* region. It was shown by DNA blot analysis that the plasmids had inserted into the homologous region of the chromosome (Fig. 3, A and B).

The locus disrupted by the mutagenic

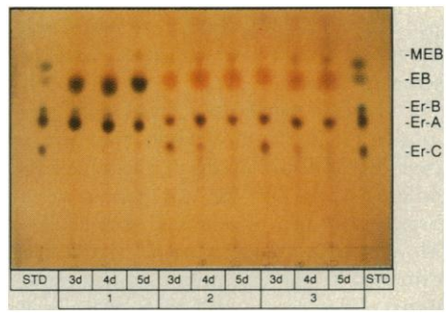

Fig. 1. Production of Ers by *S. erythraea* UW110 and by the insertion mutant, UW110::pMW56-H23, at 3, 4, and 5 days of growth, as analyzed by TLC. Lanes 1 (3d, 4d, and 5d), Er A–producing strain, UW110 (*met-4 leu-18 rif-63*), grown in T broth (7) at 32°C for 3, 4, and 5 days, respectively. The samples and chromatograph were prepared as described (*6*). Two major metabolites were visible, Er A and erythronolide B (EB). Lanes 2 and 3 (3d, 4d, and 5d): the mutant strain UW110::pMW56-H23, grown under the same conditions as the control strain, with thiostrepton selection (lanes 2), or without selection for the plasmid-borne *tsr* gene [thiostrepton (THIO) resistance] (lanes 3). Two major metabolites could be seen at 3 days that comigrated with the Er A and Er C standards, but that had a different (magenta) color than the Er A and Er C standards (STD). At 4 days and 5 days, the metabolite comigrating with Er C decreased in intensity, leaving only the spot comigrating with Er A. The results were not affected by thiostrepton selection. A light orange–colored spot was observed comigrating with EB, but did not appear in subsequent experiments and was not identified.

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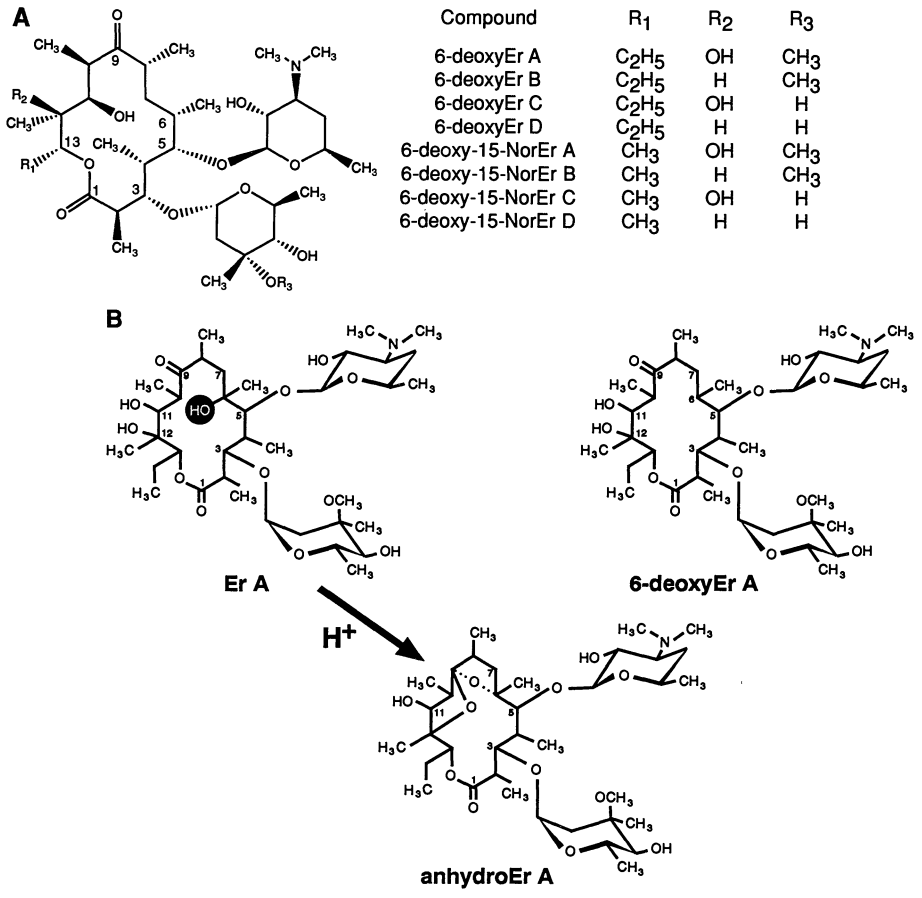

Fig. 2. (**A**) Structure (indicating stereochemistry) of 6-deoxyEr A and its biosynthetic precursors, 6-deoxyEr B, C, and D. Also shown are 6-deoxy-15-norEry A and its biosynthetic precursors. (**B**) Acid decomposition pathway of Er A to form anhydroEr A (*17, 18*), which involves the C-6 hydroxyl group (highlighted). 6-deoxy Er A is more resistant to acid inactivation because the C-6 hydroxyl group is absent (*21*).

plasmid (designated *eryF*) was found by nucleotide sequence analysis to contain an open reading frame of 1212 bp coding for a putative polypeptide consisting of 404 amino acids, with a calculated molecular weight of 45.1 daltons (*11*). Sequence analysis of the plasmid insert revealed that the plasmid had inserted internally to the *eryF* open reading frame and close to the NH_2-terminus, an event that would be predicted to disrupt the function of the gene (Fig. 3C). Computer-assisted homology searches of the GenEMBL databases with the deduced amino acid sequence of *eryF* revealed a significant similarity to cytochrome P450–type hydroxylases from bacterial and mammalian sources. The conservation of amino acid sequence was most striking in a 21–amino acid Cys fragment (Fig. 4) that is known to be associated with the heme iron-binding pocket (*12*). The large number of P450 genes known and sequenced thus far constitute a gene superfamily (*12*). On the basis of the criterion of Nebert and coworkers (*13*), in which members of the same P450 family must have ≥36% amino acid identity, *eryF* falls into a new P450 gene family and is given the systematic designation *CYP107*.

The partial gene structure surrounding *eryF* is also shown (Fig. 3C) and is suggestive of an operon containing *eryF* and at least two other open reading frames. Immediately upstream and possibly translationally coupled (*14*) to *eryF* is an open reading frame that we propose corresponds to the

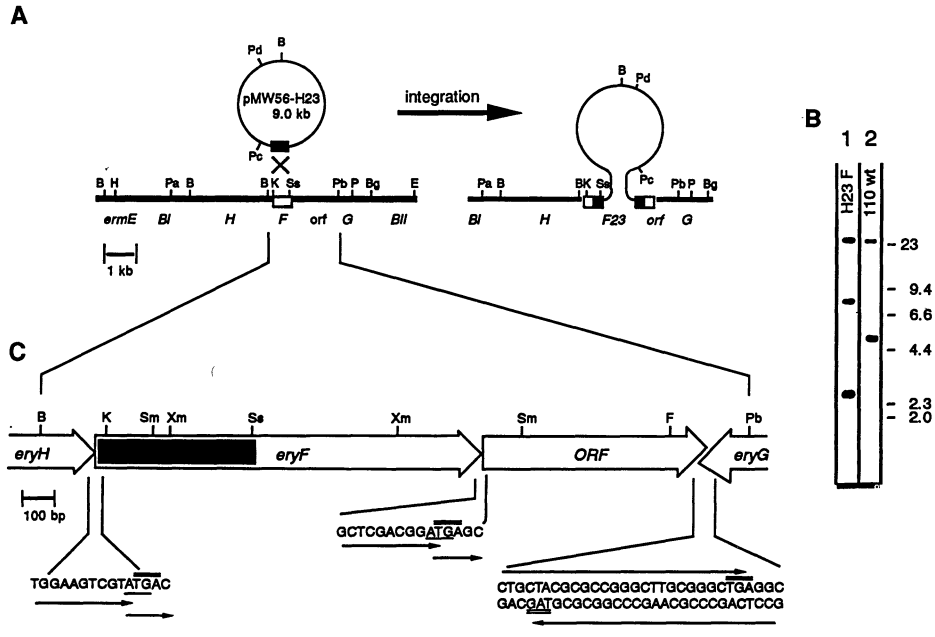

Fig. 3. (**A**) Physical map of *S. erythraea* DNA before (left) and after (right) integration of plasmid pMW56-H23 (circle) into the *eryF* locus (*F*) of the chromosome (horizontal line). Plasmid pMW56-H23 was isolated from a plasmid library constructed in *Escherichia coli* DH5α (*26*) and is shown carrying a small (503-bp) Sau 3AI fragment (black box) above the corresponding homologous region of the *S. erythraea* chromosome (open box). *BI*, *H*, *orf*, *G*, and *BII*, indicate approximate locations of corresponding *ery* genes (*6*). B, Bam HI; Bg, Bgl II; E, Eco RI; H, Hind III; K, Kpn I [the Kpn I site shown here is indicated as Kpn I site +12 in (*6*)]; P, Pa, Pb, Pc, and Pd, Pst I; Ss, Sst I. (**B**) Analysis of DNA from parent strain and transformant strain by DNA blot analysis. Total DNA from both strains was digested to completion with Pst I, separated by electrophoresis on a 0.7% agarose gel, transferred to a nylon filter, and probed with a 4.5-kb, radioactively labeled, nick-translated DNA fragment from the Bam HI at the 3′-terminus of *eryH* to the Eco RI of *eryBII*. Lane 1, transformant DNA [compare to (A), right]. The bottom band corresponds to the 2.5-kb right junction fragment (site Pc to site Pb) and the middle band corresponds to the 9.0-kb left junction fragment (site Pa to site Pd). Lane 2, parental DNA [compare to (A), left]. The bottom band corresponds to 5.5-kb target fragment, Pa to Pb. The large fragment (>23 kb) in both lanes corresponds to the fragment from site P to the next chromosomal Pst I site to the right (not shown). (**C**) Illustration of the gene structure of the region sequenced (GenBank M54983). Black box within *eryF* indicates the size and origin of the Sau 3AI clone contained in pMW56-H23 (nucleotide 173 to 676, GenBank M54983). The overlapping sequences between genes are shown at the bottom. Single lines indicate translational start sites; double lines indicate translational stop points; arrows indicate presumed direction of transcription. F, Fok I; Sm, Sma I; and Xm, Xmn I.

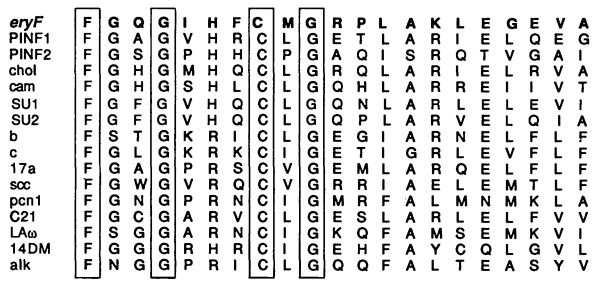

Fig. 4. Amino-acid sequence homology shared by *eryF* and 15 other cytochrome P450 hydroxylases (six prokaryotic and nine eukaryotic sequences) in their heme-binding domains [nucleotides 1187–1249, (*11*)]. PINF1, PINF2, *Agrobacterium tumefaciens* (*27*); chol, *Streptomyces spp.* (*28*); SU1, SU2, *Streptomyces griseolus* (*29*); cam, *Pseudomonas putida*; b, c, scc, pcn1, LAw, rat liver; 17a, C21, bovine adrenal cortex; 14DM, *Saccharomyces cerevisiae*; and alk, *Candida tropicalis* (*30*). Boxed residues indicate identity between proteins. Homology of deduced amino acid sequence of *eryF* to other sequences was determined with the assistance of the TFASTA program of the University of Wisconsin package (*31*). 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.

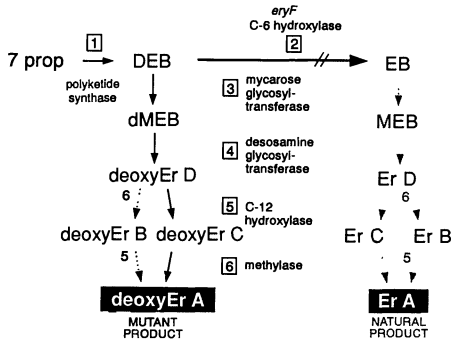

Fig. 5. The 6-deoxyEr A biosynthetic pathway (left) and the Er A biosynthetic pathway [right, (*9*)]. A mutation in step 2, *eryF* (indicated by //), allows 6-deoxyerythronolide B (DEB) to bypass the C-6 hydroxylation reaction and proceed directly to step 3, ultimately resulting in the formation of 6-deoxyEr A instead of Er A. Abbreviations: 7 prop, seven three-carbon–unit starter molecules, derived from proprionate; dMEB, 6-deoxy-3-α-mycarosylerythronolide B. The numbers in boxes indicate the step number in the pathway. Double arrows at steps 5 and 6 indicate that these reactions can occur in either order, although the preferred route is step 5, then step 6.

eryH locus identified previously (*6*). That *eryH* lies upstream of *eryF* is consistent with the apparent polarity of mutations in *eryH* on *eryF*, and with *eryH* involvement in synthesis or attachment of mycarose, a function shared with *eryBI* and *eryBII* genes (*6*). Immediately downstream and also translationally coupled to *eryF* is an open reading frame *ORF*, which bears significant homology in its deduced amino acid sequence to previously reported *S*-acyl fatty acid synthase thioesterases (*15*). Convergent to *ORF* is a fourth open reading frame, designated *eryG* in accordance with results (*4*) showing that the *eryG* transcript and an *eryG* insertion mutation overlap the Pst I site Pb (Fig. 3C). Finally, an unusual 24-bp overlap in the 3′ coding sequences of *ORF* and *eryG* was identified.

The finding that *eryF* encodes a cytochrome P450 hydroxylase is consistent with the production of 6-deoxyEr A by the *eryF* mutant. Inactivation of the C-6 hydroxylase by plasmid-mediated gene disruption of *eryF* apparently results in a bypass at step 2 and the production of 6-deoxyEr A as a "shunt" metabolite (Fig. 5). This indicates that the enzymes for the latter steps of the pathway do not discriminate against substrates with variations at C-6. Our results are also consistent with previous biochemical analyses, which indicate that a cytochrome P450–type hydroxylase is involved at step 2 of the biosynthetic pathway (*9, 16*).

The C-6 hydroxyl group is involved in the acid-catalyzed decomposition pathway of erythromycin (Fig. 2B), in which the biologically inactive derivative, anhydroerythromycin (anhydroEr) is rapidly formed at the pH of the stomach (*17*). Semisynthetic derivatives that are modified at C-6 or the participating C-9 ketone are unable to undergo this undesirable reaction (*18*). One example, 6-*O*-methylerythromycin A (clarithromycin), has shown improved in vitro and in vivo activity (*19, 20*), and is being used clinically. The preliminary results from this study of 6-deoxyEr A show that it is acid-stable like clarithromycin (*21*), but, unlike clarithromycin, it is two- to fourfold less active than Er in vitro (*22*). Upon oral administration in mice, however, where acid stability is an important factor, 6-deoxyEr A was as efficacious as Er A against three pathogenic strains (*23*).

The mutant that we created and characterized could have been isolated from classical mutational procedures but it was not, possibly because of the small size of the *eryF* target and the large numbers of survivors of mutagenesis that would have to be screened by TLC for the mutant to be found (*24*). This points to an advantage of the targeted mutagenic approach (*6*): much less screening is involved, because of the clustering of antibiotic biosynthetic genes, and, apparently, a greater diversity of mutant phenotypes can be uncovered. Clusters of genes for antibiotic biosynthesis have been uncovered in a variety of actinomycetes (*25*); therefore, the approach described here for the Er-producer could be extended to those species for development of new antibiotics.

REFERENCES AND NOTES

1. W. T. Shier, K. L. Rinehart, Jr., D. Gottlieb, *Biochemistry* **63**, 198 (1969); K. L. Rinehart, Jr *Pure Appl. Chem.* **49**, 1361 (1977).
2. D. A. Hopwood *et al.*, *Nature* **314**, 642 (1985); K. Epp, M. L. B. Huber, J. R. Turner, T. Goodson, B. E. Schoner, *Gene* **85**, 293 (1989).
3. J. B. McAlpine *et al.*, *J. Antibiot.* **40**, 1115 (1987).
4. J. M. Weber, B. Schoner, R. Losick, *Gene* **75**, 235 (1989).
5. N. Dhillon, R. S. Hale, J. Cortes, P. F. Leadlay, *Mol. Microbiol.* **3**, 1405 (1989); J. Tuan, J. M. Weber, M. A. Staver, J. O. Leung, L. Katz, *Gene* **90**, 21 (1990).
6. J. M. Weber *et al.*, *J. Bacteriol.* **172**, 2372 (1990).
7. The mutant was cultivated for 5 days at 32°C in 50-ml volumes of T broth (bactotryptone, 12 g; yeast extract, 24 g; glycerol, 4 ml; distilled water, 1 liter) in 500-ml Erlenmeyer flasks and aerated by shaking at 240 rpm, 5-cm displacement. On day 5, the cultures were combined and the cells were removed by centrifugation. To extract the antibiotic, we adjusted the broth to pH 10 with 4 M sodium hydroxide and then mixed with an equal volume of ethyl acetate. The concentrated extract was partitioned between 150 ml each of heptane and methanol, and the lower (methanol) layer was concentrated to a residue. The residue was purified by chromatography on a Sephadex LH-20 column (3.2 cm by 75 cm), which was preswollen, loaded, and eluted with a mixture of chloroform, heptane, and ethanol (10:10:1, v/v/v). Fractions (10 ml) were collected and analyzed by TLC (*6*). Fractions giving magenta spots with about the same R_f as erythromycins were collected and pooled according to R_f. These fraction pools were each concentrated to solid residues, which were individually subjected to high-speed countercurrent chromatography with an Ito Coil Planet Centrifuge [P.C., Inc.; for separation of complex mixtures of macrolides, as described (*3*)] in a mixture of carbon tetrachloride, methanol, and 0.01 M aqueous potassium phosphate buffer (pH 7.0) (1:1:1). More hydrophilic congeners were separated by further chromatography (upper-phase mobile, forward spin, tail as inlet, flow rate approximately 3 ml/min at 800 rpm, about 60% stationary phase retention). For more lipophilic congeners, the lower phase was used as the mobile phase by reversal of the spin. This led to 85% retention of the stationary phase. Fractions (10 ml) containing a single component were combined, concentrated, and partitioned between methylene chloride and dilute ammonium hydroxide (pH 9). The methylene chloride layers were concentrated to solid residues, and these were used for spectral analyses.
8. J. B. McAlpine *et al.*, in preparation.
9. J. W. Corcoran, in *Biosynthesis*, vol. 4 of *Antibiotics*, J. W. Corcoran, Ed. (Springer-Verlag, New York, 1981), pp. 132–174.
10. J. R. Martin *et al.*, *J. Antibiot.* **27**, 570 (1974).
11. Sequence deposited at GenBank as M54983. Sequence begins at the Bam HI site of *eryH*, closest to its 3′-terminus (Fig. 3C), continues through *eryF* and *ORF*, and ends at the Pst I site of *eryG*, closest to its 3′-terminus. Region cloned in pMW56-H23: nucleotides 173 to 676.
12. D. W. Nebert and F. J. Gonzalez, *Annu. Rev. Biochem.* **56**, 945 (1987).
13. D. W. Nebert *et al.*, *DNA* 6, 1 (1987).
14. H. Zalkin and D. J. Ebbole, *J. Biol. Chem.* **263**, 1595 (1988).
15. We used the TFASTA program from the University of Wisconsin GCG package to determine that the deduced amino acid sequence of ORF (GenBank M54983) shares a 28.6% identity over a 220–amino

acid overlap with the *S*-acyl fatty acid synthase thioesterase from the uropygial gland of mallard duck [A. J. Poulose, L. Rogers, T. M. Cheesebrough, P. E. Kolattukudy, *J. Biol. Chem.* **260**, 15953 (1985)] and a 38.1% identity over a 217–amino acid overlap with the medium-chain *S*-acyl fatty acid synthetase thioester hydrolase from rat mammary gland [J. Naggert, B. Williams, D. P. Cashman, S. Smith, *Biochem. J.* **243**, 597 (1987); R. Safford *et al.*, *Biochemistry* **26**, 1358 (1987)].
16. A. Shafiee and C. R. Hutchinson, *Biochemistry* **26**, 6204 (1987).
17. H. Sakakibara and S. Omura, in *Macrolide Antibiotics, Chemistry, Biology, and Practice*, S. Omura, Ed. (Academic Press, Orlando, FL, 1984), pp. 85–95.
18. H. A. Kirst and G. D. Sides, *Antimicrob. Agents Chemother.* **33**, 1413 (1989).
19. P. B. Fernandes, D. J. Hardy, D. McDaniel, C. W. Hanson, R. N. Swanson, *ibid.*, p. 1531; S. Naik and R. Ruck, *ibid.*, p. 1614.
20. P. B. Fernandes *et al.*, *ibid.* **30**, 865 (1986); D. J. Hardy *et al.*, *ibid.* **32**, 1710 (1988).
21. Aqueous solutions of Er A and 6-deoxyEr A, 0.5 mg/ml, were prepared in citrate buffer (pH 2.2), incubated at 37°C, and sampled at regular intervals. Samples and standards were analyzed and compared by TLC. After 2 min, the Er A solution had been converted (>95%) to biologically inactive anhydroEr. By comparison, 6-deoxyEr A displayed a half-life of approximately 6 hours under the same conditions.
22. D. J. Hardy *et al.*, unpublished data.
23. The strains were *Staphylococcus aureus, Streptococcus pyogenes*, and *Streptococcus pneumoniae* (R. N. Swanson *et al.*, unpublished data).
24. T. J. Paulus *et al.*, *J. Bacteriol.* **172**, 2541 (1990).
25. D. A. Hopwood, in *Genetics and Molecular Biology of Industrial Microorganisms*, C. L. Hershberger, S. W. Queener, G. Hegeman, Eds. (American Society for Microbiology, Washington, DC, 1989), pp. 12–20.
26. The procedure for construction and integration of plasmids is described in (*6*), under "scanning" gene disruption.
27. R. H. Kanemoto *et al.*, *J. Bacteriol.* **171**, 2506 (1989).
28. T. Ishizaki, N. Hirayama, H. Shinkawa, O. Nimi, Y. Murooka, *ibid.*, p. 596.
29. C. A. Omer *et al.*, *ibid.* **172**, 3335 (1990).
30. D. Sanglard and J. C. Loper, *Gene* **76**, 121 (1989).
31. J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984).
32. We thank J. DeWitt, T. Paulus, G. Maine, J. DiGangi, V. Leubke, and L. Hess for technical assistance; A. Buko and D. Whittern for mass and nuclear magnetic resonance spectrometry; D. J. Hardy and R. N. Swanson for release of data before publication; J. Tuan and M. Staver for cosmid clones of *S. erythraea* DNA; A. Walsh for helpful discussions and bibliographic searches; and L. Katz and S. Donadio for computer-assisted DNA sequence analyses and critical reading of the manuscript.

1 October 1990; accepted 4 January 1991

A Genetic Tool Used to Identify Thioredoxin as a Mediator of a Growth Inhibitory Signal

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Loss of sensitivity to growth inhibitory polypeptides is likely to be one of the events that participates in the formation of some tumors and might be caused by inactivation or loss of the genetic elements that transduce these extracellular signals. The isolation of such a gene was achieved by randomly inactivating genes by an anti-sense complementary DNA expression library followed by direct selection for growth in the presence of an inhibitory polypeptide. Thus, a gene whose inactivation conveyed growth resistance to interferon-γ (IFN-γ) was isolated. Sequence analysis showed complete identity with human thioredoxin, a dithiol reducing agent, implicated here in the IFN-γ–mediated growth arrest of HeLa cells.

TUMOR FORMATION IS OFTEN A MULTISTEP process in which many genetic alterations can be detected, including activation of proto-oncogenes (dominant alterations) as well as inactivation of tumor suppressor genes (recessive alterations) (*1*). Only a few tumor suppressor genes have been characterized, including *Rb*, *p53*, *DCC*, *NM23*, and *ras* suppressors (*2*), because there is no rapid selection method. To address this problem, we have developed a genetic tool for the rapid isolation of genes that inhibit proliferation in a specified restrictive environment. These genes would be logical candidates to test as possible tumor suppressor genes. We refer to this technique as the Technical Knock Out (TKO) selection; it is based on the random inactivation of genes via a cDNA library cloned into an anti-sense expression vector. The assumption is that the specific inactivation of a growth inhibitory gene would convey growth advantage in a specific restrictive environment. This growth advantage is the powerful forward selection that is used for isolation of the desired inactivation event from all the other random inactivation events.

Molecules capable of inhibiting cellular proliferation in cell culture and in the context of the entire organism include several structurally unrelated families of polypeptides, such as the interferon family [type I IFN (α and β) and type II IFN (IFN-γ)], tumor necrosis factors (TNFs) (α and β), the transforming growth factor–β (TGF-β) family, amphiregulin and oncostatin M (*3*). Some of these growth inhibitors, such as the IFNs and TGF-β, operate in a paracrine or an autocrine manner, especially in limiting the proliferation of differentiating tissues (*4*). In some hematopoietic tumors, the autocrine loop for type I IFN is inactivated by the deletion of the entire type I IFN gene cluster (*5*). The antiproliferative effects of IFNs, TGF-β, and TNFs were attributed in some cells to their ability to suppress c-*myc* (*6–9*). Furthermore, the failure of IFN to reduce c-*myc* expression in resistant tumor cells was complemented by fusion with sensitive cells, suggesting that it could result from a recessive genetic lesion that leads to neoplasia (*9*). We have used the growth inhibition of HeLa cells by IFN-γ as a model system for testing the TKO selection and for the isolation of a putative growth suppressor gene. IFN-γ also has a role in vivo as a major immune regulator, including anti-tumor regulation (*10, 11*). We used human recombinant IFN-γ, purified to homogeneity, in these studies (*8*).

The TKO selection requires the transfection of a large number of cells with a transcription unit that promotes high amounts of expression of an anti-sense cDNA library in the continuous presence of IFN-γ. We chose the Epstein-Barr virus (EBV) episomal vector (*12*) to transduce the cDNA library because it efficiently transfects human cells. We were able to reproducibly obtain stable transfection rate for HeLa cells of 2 to 10% by introducing this vector through the standard calcium phosphate transfection technique (*13*). The episomal vector can be mechanically separated from the bulk of the human chromosomal DNA by Hirt extraction (*14*) and used to transform bacteria. This property allows the rapid isolation and identification of the cloned anti-sense cDNA contained in any selected cell. In addition, the episomal vector contains a hygromycin B–resistance marker.

Three different EBV-based expression vectors that could drive the transcription of the chloramphenicol acetyl transferase gene (*CAT*) were transfected into HeLa cells and selected for resistance to hygromycin B. The pTKO1-CAT and pTKO2-CAT contain the simian virus 40 (SV40) early promoter (Fig. 1). The pTKO1-CAT also includes an IFN response element (IRS) (*15*), cloned downstream of the *CAT* gene, to increase expression in IFN-treated cells. The pTKO3-CAT contains the murine sarcoma virus long-terminal repeat (MSV LTR) promoter. Pools of hygromycin B–resistant cells consisting of more than 10^4 independent clones were generated and treated with IFN-γ.

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