

evidence that EPH-related receptors transiently concentrate in areas of cell-to-cell contact (17) and that they participate in refining intersegmental boundaries during hindbrain development (27). Although the mechanism by which membrane attachment participates in receptor activation has not been established, the activity of clustered soluble forms suggests that membrane attachment somehow facilitates dimerization or aggregation of ligands.

There is overlap in the binding specificities of these ligands for different EPH-related receptors. Because these cross-specificities preclude assigning a particular ligand solely to an individual EPH-related receptor, we suggest that this family of ligands be termed the EFLs (for EPH-family ligands) and that each factor be numbered sequentially; thus, B61 would be designated as EFL-1, EHK1-L as EFL-2, and ELK-L as EFL-3. For the currently known EFLs, membrane linkage seems to provide a specialized mechanism for coupling receptor activation to direct cell-to-cell contact. In addition to potential roles in hindbrain development, the particular distributions of EPH-related receptors in the developing and adult nervous system make them likely candidates for mediators of various neuronal processes that depend on cell-to-cell interactions (28, 29).

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18. The EHK1-Fc and ELK-Fc proteins resulted from fusion of the ectodomains of the rat EHK1 [residues 1 to 573 of the long form of this receptor (16)] and rat ELK [residues 1 to 540 (7)] receptors to the hinge, CH2, and CH3 regions of human IgG1 (starting with the residues Glu-Pro-Lys), with a spacer having the sequence Gly-Pro-Gly inserted between the ectodomains and the Fc region.
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32. To assay for binding to receptor-Fc proteins, we incubated cells plated confluent on 24-well dishes for 30 min with the indicated receptor-Fc proteins (used at either an approximate concentration of 1 μ g/ml or at the indicated concentrations; the receptor-Fc proteins were produced as supernatants from COS cells transiently transfected with appropriate expression constructs, and concentrations estimated by a two-site enzyme-linked immunosorbent assay, with a polyclonal antibody to human (anti-human) IgG as a capturing antibody and an alkaline phosphatase-conjugated anti-human IgG as a secondary antibody), washed twice with phosphate-buffered saline (PBS), and then incubated for an additional 30 min with radio-iodinated goat anti-human IgG (NEN/DuPont; 1 μ Ci/ml in PBS containing 10% calf serum). After two additional washes, cells were solubilized and bound radioactivity was determined. Total cpm bound (Fig. 1A), a ratio of cpm bound in the presence versus absence of receptor-Fc protein (Binding Ratio; Fig. 1B), or cpm bound after nonspecific binding was subtracted (Fig. 1C; nonspecific binding was assessed as binding in the absence of receptor-Fc protein) are presented. Expression cloning was largely performed as previously described (30).

with the following modifications: cDNA libraries were constructed in the JFE14 vector, and single-cell transfectants expressing ligands were detected by incubation with receptor-Fc fusion proteins, followed by methanol fixation, incubation with an alkaline phosphatase-conjugated second antibody and finally with alkaline phosphatase substrate. Positive cells were scraped from the tissue culture dish, and plasmid DNA was purified from these cells and rescued by electroporation. Plasmid DNA was then used for subsequent rounds of enrichment until single clones were isolated.

33. To assay membrane-bound ligand, we transfected COS cells with either vector alone (COS-Mock) or ELK-L expression vector (COS-ELK-L), detached from dishes with PBS + 1 mM EDTA, pelleted, resuspended in PBS, and layered on top of reporter cells. Soluble ligands were designed by replacement of the COOH-terminal GPI-recognition sequences of EHK1-L (residues 211 to 234) or B61 (residues 182 to 205), or the transmembrane and cytoplasmic domains of ELK-L (residues 235 to 353), with a myc-epitope tag (37). These soluble ligands were produced in COS cell supernatants and used as unclustered ligands, or clustered by incubation with both a mouse monoclonal antibody recognizing the myc epitope as well as anti-mouse immunoglobulin polyclonal antiserum.
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35. We thank L. S. Schleifer for enthusiastic support and members of the Discovery Group at Regeneron, particularly D. J. Glass and N. Stahl, for insightful discussions; D. M. Valenzuela, J. Griffiths, M. Gisser, and C. New for DNA sequencing and analysis; D. Datta and L. Pan for technical assistance; and R. Rossman, J. Cruz, and L. Defeo for cell culture expertise.

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The Function of KGF in Morphogenesis of Epithelium and Reepithelialization of Wounds

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The function of keratinocyte growth factor (KGF) in normal and wounded skin was assessed by expression of a dominant-negative KGF receptor transgene in basal keratinocytes. The skin of transgenic mice was characterized by epidermal atrophy, abnormalities in the hair follicles, and dermal hyperthickening. Upon skin injury, inhibition of KGF receptor signaling reduced the proliferation rate of epidermal keratinocytes at the wound edge, resulting in substantially delayed reepithelialization of the wound.

Cutaneous wound repair is a complex process that involves formation of granulation tissue, reepithelialization, and tissue remodeling (1). These processes are mediated by a large number of growth factors and cytokines that have been only partially identified (1). Recently we demonstrated a large induction of KGF expression in fibroblasts below the wound and at the wound edge (2). Because KGF is a highly specific and potent mitogen for keratinocytes (3), these findings suggest that dermally derived KGF stimulates wound reepithelialization in a paracrine manner. To address this possibility, we selectively blocked KGF receptor signaling by targeted expression of a dominant-negative KGF receptor mutant in the undifferentiated basal keratinocytes of transgenic mice.

We have previously demonstrated that mutated fibroblast growth factor receptors (FGFRs) that lack kinase activity block

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FGF receptor signaling by forming nonfunctional heterodimers with wild-type receptors upon ligand binding (4). These dominant-negative FGF receptors did not block signaling by other receptor tyrosine kinases (4). To block KGF function in the epidermis we chose a truncated FGFR2-IIIb variant, because this form of receptor is highly expressed in the keratinocytes of the epidermis and the hair follicles (2, 5). Furthermore, FGFR2-IIIb is the only known high-affinity receptor for KGF (6) and should therefore mediate the action of KGF in normal and wounded skin. We constructed a transgene by fusing the complementary DNA (cDNA) of a truncated form of FGFR2-IIIb downstream of the human keratin 14 promoter (7) (Fig. 1A) which directs high-level expression of transgenes to the undifferentiated keratinocytes in the basal layer of the epidermis and the outer root sheath of the hair follicles (7). We obtained three transgenic founder mice that expressed high levels of the transgene as assessed by ribonuclease protection assay of mouse tail skin RNA. Expression of the

transgene was at least 10-fold higher compared to that of endogenous FGFRs, a prerequisite for the dominant-negative mechanism of action (4). Transgene expression was skin-specific (Fig. 1B) and was restricted to basal cells of the epidermis (Fig. 2H) and to keratinocytes of the outer root sheath of the hair follicles. All transgenic mice appeared macroscopically normal. However, histological examination of more than 40 transgenic mice revealed severe phenotypic abnormalities in corpus skin of all mice tested. The phenotype was most pronounced in homozygous mice that expressed highest levels of the transgene, demonstrating the strong correlation between transgene expression and phenotype. The most marked feature of transgenic mouse skin was the severe atrophy of the epidermis (Fig. 2, B, C, and E), which is primarily based on the small size of the basal cells that express the transgene. In contrast to control mice (Fig. 2D), basal cells of transgenic mice were flattened and had pyknotic nuclei (Fig. 2E). The morphologic appearance of the transgenic mouse epidermis suggested a reduced steady-state proliferation rate, which was confirmed by labeling studies with bromodeoxyuridine (see

below). This indicates that the balance between proliferation and differentiation is disturbed, resulting in a premature onset of cornification starting in the lower suprabasal layers. In spite of the abnormal phenotypic appearance of the epidermis of transgenic mice, no gross abnormalities in the expression pattern of the differentiation-specific keratins K14, K10, and K6 were observed, as assessed by immunofluorescence staining of the skin with monospecific antibodies to these cytokeratins. The expression of endogenous keratin 14 in the cells that express the transgene shows that the phenotype is not caused by loss of endogenous K14 expression resulting from competition of the transgene for transcription factors. Furthermore, we can exclude the possibility that expression of any gene under this promoter nonspecifically creates the same phenotype, because expression of a series of other genes under the control of the same promoter created very different phenotypic abnormalities (8).

In addition to epidermal atrophy, transgenic mice exhibited abnormalities in the morphology of hair follicles (Fig. 2, B and C), and the number of hair follicles in transgenic mice was 60 to 80% lower than

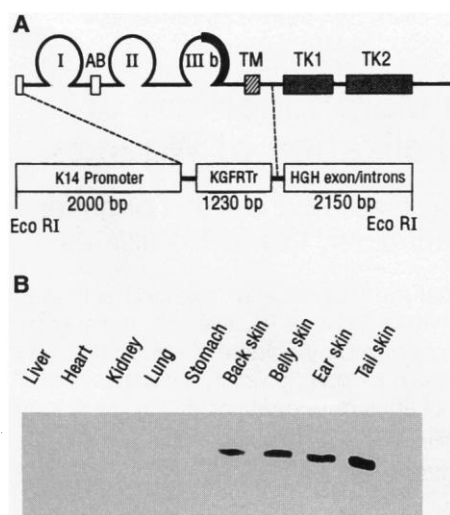


Fig. 1. (A) The FGFR2-IIIb transgene cDNA (top line) and the complete transgene (bottom line). A truncated human FGFR2-IIIb cDNA (KGFRTr) from which the tyrosine kinase domains (TK1 and TK2) were deleted was fused downstream of the human keratin 14 (K14) promoter and upstream of the regulatory elements of the human growth hormone (HGH) gene. Functional elements of the truncated receptor include immunoglobulin-like domains I, II, and IIIb, the acid box (AB, white box), and the transmembrane domain (TM, striped box). The IIIb exon which is specific for the KGF receptor variant of FGFR2 is indicated with a thick black line. **(B)** Expression pattern of the transgene in mouse tissues. Total RNA (50 μ g) from different tissues and organs of transgenic mice were analyzed by ribonuclease protection assay (2) for the presence of mRNA encoding the truncated human KGF receptor. Because of species-specific differences, mRNA encoding the endogenous KGF receptor was not detected.

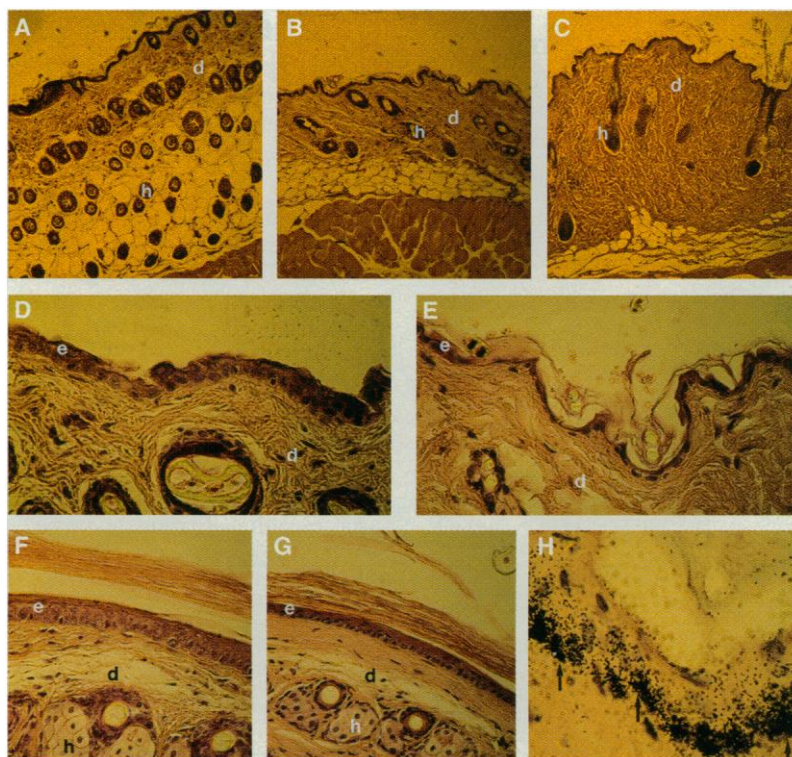


Fig. 2. Histological features and transgene expression in the skin of transgenic mice. Back skin of **(A)** a control mouse, **(B)** a heterozygous transgenic mouse, and **(C)** a homozygous transgenic mouse. Magnification, $\times 60$. Back skin of **(D)** a control mouse and **(E)** a homozygous transgenic mouse. Magnification, $\times 240$. Tail skin of **(F)** a control mouse and **(G)** a homozygous transgenic mouse. Magnification, $\times 180$. **(H)** In situ hybridization of the tail skin of a heterozygous transgenic mouse. Hybridization signals are seen as black dots and appear only in the basal cells. d, dermis; e, epidermis; and h, hair follicle. Skin from transgenic mice (3 months old) and control littermates was fixed in 4% paraformaldehyde and paraffin embedded. Sections (6 μ m) were stained with hematoxylin-eosin. Magnification, $\times 240$.

that of control littermates (Fig. 2A). These findings suggest that blocking of KGF receptor signaling had affected hair follicle

morphogenesis. Consistent with the morphological abnormalities in the hair follicles, we determined that hair regrowth was

substantially delayed by tearing out the hair on the backs of the animals after the first hair cycle.

Phenotypic abnormalities were also detected in the dermis of transgenic mice, which was characterized by severe thickening with a gradual replacement of adipose tissue by connective tissue (Fig. 2, B and C). Because the transgene is not expressed in interfollicular dermis, indirect mechanisms that are a consequence of the KGF receptor blockade must be responsible for this phenomenon. Thus, a blockade of KGF receptor function produced not only direct effects on keratinocytes but also indirect long-term effects that might result from abnormal regulation of other factors on inhibition of KGF receptor signaling.

The phenotypic abnormalities in transgenic mouse skin were age-dependent. No obvious histological changes were seen in newborn skin, and the first hair grew normally. Signs of epidermal atrophy were first observed 3 weeks after birth, and the full spectrum of phenotypic abnormalities described above were seen 6 weeks after birth. These findings suggest that the defect in KGF receptor signaling might be compensated for in young mice by other growth factors. In animals that were more than 18 months old, the differences in the epidermis between normal and transgenic mice were less pronounced, owing to the normal age-dependent reduction in epidermal thickness.

We also found epidermal atrophy in the skin of the tail and the ear (Fig. 2, F and G), but hair follicles and interfollicular dermis appeared normal. The less severe phenotype in tail and ear skin is most likely caused by a higher expression of endogenous KGF and KGF receptors in these areas (9), which might partially overcome the dominant-negative effect.

We have previously demonstrated a large induction of KGF expression in the dermis during wound healing (2), indicating that this factor plays an important role in the control of dermal-epidermal interaction during tissue repair. Furthermore, KGF has been shown to stimulate migration and proliferation of keratinocytes in vitro (3, 10), and application of purified KGF to a wound increased the rate of wound reepithelialization (11).

To examine the effects of a KGF receptor blockade on wound reepithelialization, we generated a single full-thickness wound on the backs of 10 normal mice, 10 heterozygous transgenic mice, and 6 homozygous transgenic mice (12). A substantially hyperthickened epithelium (Fig. 3A) formed at the wound edge of all control animals at day 5 after injury, indicating a high proliferation rate of the keratinocytes, and the wounds in these animals were al-

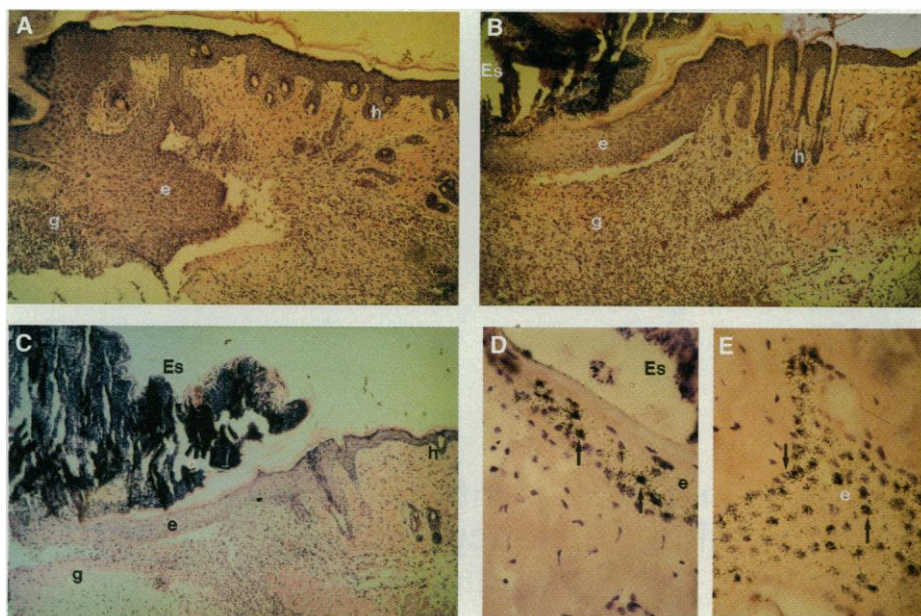


Fig. 3. Histologic features and transgene expression in full-thickness wounds of transgenic mice and control mice. A single full-thickness wound was made on the back of transgenic mice and control littermates (3 months old). Mice were killed at day 5 after injury. Wounds were isolated, bisected, fixed in 4% paraformaldehyde, and paraffin-embedded. Sections (6 μ m) from the middle of the wound were stained with hematoxylin-eosin. Wound edge of (A) a control mouse, and (B) a heterozygous and (C) a homozygous transgenic mouse. Magnification, $\times 60$. Transgene expression in the keratinocytes of (D) the epithelial tongue and (E) hyperthickened epithelium at the wound edge. Sections were hybridized with a transgene-specific 32 P-labeled riboprobe and counterstained with hematoxylin-eosin; e, epithelium at the wound edge; h, hair follicle; g, granulation tissue; and Es, eschar. Magnification, $\times 240$.

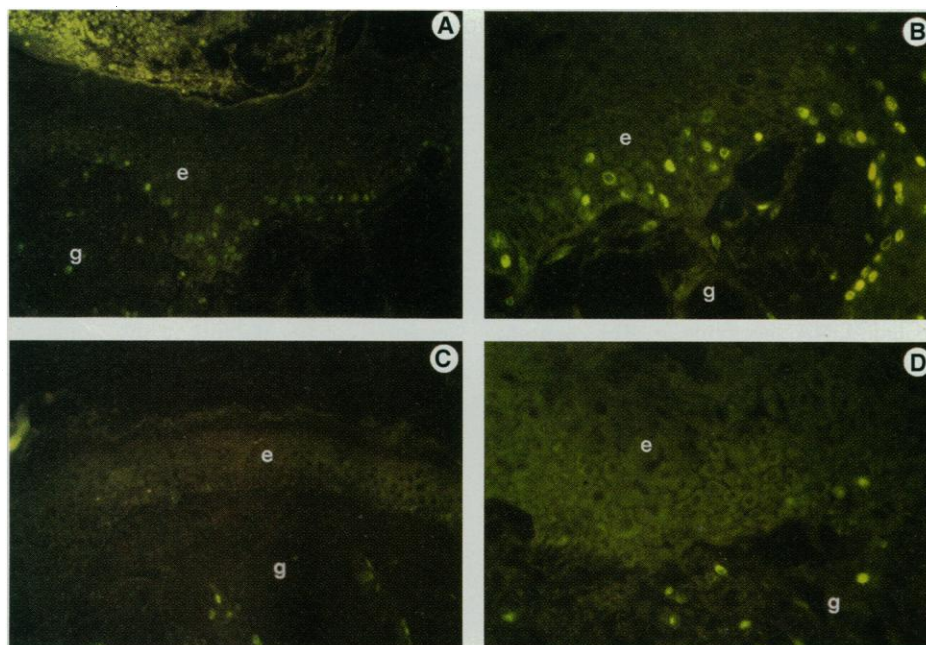


Fig. 4. Detection of proliferating cells in the wound tissue by BrdU labeling. A single full-thickness wound was made on the back of normal and transgenic mice. At day 5 after injury, mice were injected with BrdU and killed 2 hours later. Wounds were isolated, bisected, fixed in 70% ethanol, paraffin embedded, and sectioned. Sections from the middle of the wound were stained with a fluorescein isothiocyanate-conjugated antibody to BrdU. (A and B) Wound edge of a control mouse. (C and D) Wound edge of a heterozygous transgenic mouse; e, epidermis; g, granulation tissue. Magnification: (A) and (C), $\times 60$; (B) and (D), $\times 240$.

most completely reepithelialized. Epithelial hyperthickening was less pronounced in heterozygous transgenic animals (Fig. 3B), and reepithelialization was much less complete. This finding suggested that migration and proliferation of the keratinocytes in these mice were impaired. The reduced reepithelialization of the wound was most obvious in homozygous transgenic mice, where almost no hyperthickening of the epidermis at the wound edge was observed (Fig. 3C). The phenotypic abnormalities seen in the wound epithelium were consistent with high expression of the transgene in the keratinocytes of the migrating epithelium (Fig. 3D) and the hypertrophic epithelium at the wound edge (Fig. 3E).

To quantify the difference in the proliferation rate of the keratinocytes, we labeled the proliferating cells with 5-bromodeoxyuridine (BrdU) in vivo and subsequently stained them with an antibody to BrdU (13). At day 5 after injury, a large number of cells in the epidermis at the wound edge of control mice had incorporated BrdU (Fig. 4, A and B), whereas the number of proliferating keratinocytes at the wound edge of transgenic mice was 95 to 99% lower compared to that of control mice (Fig. 4, C and D). This reduced proliferation rate was seen in all transgenic mice tested. As an indication of specificity, the number of proliferating cells in the underlying granulation tissue was similar in transgenic and control mice at this stage of wound healing, demonstrating that transgene expression had only blocked proliferation of keratinocytes. The reduced proliferation rate of the keratinocytes that express the dominant-negative KGF receptor demonstrates that KGF receptor signaling is essential for wound reepithelialization.

The data presented here reveal a direct role of KGF receptor signaling in the morphogenesis of the epidermis and the hair follicles and an indirect role of KGF receptor function in the control of connective tissue formation in the dermis, indicating a complex epidermal-dermal interaction during morphogenesis of the skin. Our results demonstrate that KGF is essential for wound reepithelialization and that a blockade of KGF function is associated with wound-healing abnormalities.

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12. Homozygous and heterozygous transgenic mice as well as control littermates (3 months old) were used for wound-healing experiments. Mice were anesthetized with a single dose of Avertin. A single full-thickness wound of 5-mm diameter was made in the middle of the back by excising skin and panniculus

carneous. Animals were killed with ether at day 5 after injury and the complete wound was isolated. All animal experiments were carried out with permission from the local government of Bavaria (permission number 211-2531-16/93).

13. For BrdU-labeling studies, wound-healing experiments were carried out as described in (12). At day 5 after injury, mice were injected intraperitoneally with BrdU (Sigma; 250 mg per kilogram of body weight) and killed 2 hours after injection. Wound tissue was fixed in 70% ethanol and paraffin embedded.
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Determination of Intrinsic Transcription Termination Efficiency by RNA Polymerase Elongation Rate

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Transcription terminators recognized by several RNA polymerases include a DNA segment encoding uridine-rich RNA and, for bacterial RNA polymerase, a hairpin loop located immediately upstream. Here, mutationally altered *Escherichia coli* RNA polymerase enzymes that have different termination efficiencies were used to show that the extent of transcription through the uridine-rich encoding segment is controlled by the substrate concentration of nucleoside triphosphate. This result implies that the rate of elongation determines the probability of transcript release. Moreover, the position of release sites suggests an important spatial relation between the RNA hairpin and the boundary of the terminator.

Termination of RNA synthesis by *E. coli* RNA polymerase (RNAP) at intrinsic (or ρ -independent) terminators is a property of the core enzyme and probably of the highly conserved large subunits β and β' (1, 2). Essential elements of intrinsic terminators include DNA specifying an RNA hairpin, followed by a DNA segment encoding RNA rich in uridines at the end of the terminated RNA (1, 2). How these elements act is unknown, although a terminal U-encoding segment is also essential in eukaryotic RNAP III terminators (3), and

U-encoding sequences cause stalling by eukaryotic RNAP II (4). Terminator function may reflect the available energy of RNA and DNA strand associations: It has been suggested that RNA hairpin formation destroys part of an RNA-DNA hybrid that is essential for the stability of the elongating complex, after which the instability of the remaining U-rich RNA-DNA hybrid allows the complex to dissociate (1, 5). Alternatively, or in addition, the hairpin may interact with RNAP and promote termination either by disrupting RNA-enzyme associations required for complex stability or by inducing conformational changes that lead to termination (6, 7).

The efficiency of intrinsic termination may reflect competition between the rate of elongation and the rate of RNA (and eventually enzyme) release. As is true at ρ -dependent terminators (8), RNAP at or near the release site of an intrinsic terminator may be in a "pause," meaning a condition in which elongation is slowed or

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