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Identification of a Competitive HGF Antagonist Encoded by an Alternative Transcript

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We identified a naturally occurring hepatocyte growth factor (HGF) variant, whose predicted sequence extends only through the second kringle domain of this plasminogen-related molecule. This smaller molecule, derived from an alternative HGF transcript, lacked mitogenic activity but specifically inhibited HGF-induced mitogenesis. Cross-linking studies demonstrated that the truncated molecule competes with HGF for binding to the HGF receptor, which has been identified as the c-*met* proto-oncogene product. Thus, the same gene encodes both a growth factor and its direct antagonist.

HGF WAS INITIALLY DETECTED AS A hormone-like activity capable of stimulating hepatocyte proliferation (*1*). This growth factor is expressed by stromal fibroblasts and is mitogenic for a variety of cell types, including melanocytes, endothelial cells, and cells of epithelial origin (*2*). HGF is also highly related or identical to scatter factor, an agent that stimulates the dispersion of epithelial and vascular endothelial cells (*3*). Studies have identified the HGF receptor as the c-*met* proto-oncogene product (*4*), a membrane-spanning tyrosine kinase (*5*). Structurally, HGF resembles plasminogen in that HGF has characteristic NH_2-terminal kringle domains (*6*) and a COOH-terminal serine protease–like domain (*7, 8*). HGF is synthesized as an 87-kD single chain polypeptide (p87). Like plasminogen, it can be cleaved into a heterodimeric form consisting of a heavy (~60 kD) and a light chain (~30 kD) held together by disulfide bonds (*1*).

We have reported that human fibroblasts in culture secrete HGF p87 as the predominant form of the growth factor (*2*). Survey of a number of HGF-producing cell lines confirmed that p87 was the major secreted product among the various HGF species. However, we also detected variable amounts of lower molecular mass HGF-immunoreactive species by using SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions. When similar experiments were performed under nonreducing conditions to prevent dissociation of any HGF heterodimers, we observed the high molecular mass HGF species, as well as lesser amounts of a 28-kD (p28) HGF-immunoreactive polypeptide. The concentration of p28 in medium from the SK-LMS-1 line (*9*) was striking (Fig. 1A). These results, as well as pulse-chase experiments (*10*), suggested that p28 was not derived from p87 but represented instead an independently synthesized HGF-immunoreactive protein.

To identify the transcript that encoded the p28 protein, we performed Northern (RNA) blot analysis with polyadenylated [poly(A)$^+$] RNA prepared from SK-LMS-1 cells. When the full-length HGF coding sequence was used as a probe, two major transcripts of 6.0 and 3.0 kb were detected (Fig. 1B) that encode the full-length growth factor (*2*). In addition, we detected an HGF RNA species of ~1.3 kb. The three transcripts were observed in normal placenta, as well as in fibroblasts derived from different tissues. However, the ratios of the transcripts showed considerable variation, with foreskin fibroblasts expressing the highest relative amount of the 1.3-kb message (Fig. 1B). Because only a portion of the 2.2-kb HGF coding sequence could be present in the small transcript, we reasoned that it might encode p28. To better define this transcript, we carried out Northern analysis with poly(A)$^+$ RNA from M426 human embryonic lung fibroblasts, separately hybridized with probes derived from either the HGF NH_2-terminal heavy chain (H) or the COOH-terminal light chain (L) regions. Whereas both probes detected the 6.0- and 3.0-kb transcripts, only the H probe was capable of recognizing the 1.3-kb message (Fig. 1B). These results suggested that this RNA species encoded a truncated version of the HGF molecule that contained sequences specific to its NH_2-terminal region.

To isolate cDNA corresponding to the 1.3-kb transcript, we differentially screened an M426 cDNA library (*11*) with both HGF H and L probes. Clones that specifically hybridized to the H probe were plaque purified. On the basis of sizes and physical maps of the inserts, we selected one 1.2-kb cDNA clone, pH45, for sequencing. The 1199-bp cDNA contained a short 5′ untranslated region of 75 bp, an open reading frame of 870 bp, and a 254-bp 3′ untranslated region containing a polyadenylation signal, AATAAA (Fig. 2A). The open reading frame predicted a 290–amino acid truncated version of HGF consisting of a signal peptide (S), an NH_2-terminal domain (N), and the first two kringle domains (K1 and K2) with a calculated size of ~30 kD, excluding the signal peptide. This sequence, designated HGF/NK2 (*11a*), was identical to that of HGF cDNA, including the 5′ untranslated region, until it diverged precisely at the end of the K2 domain. The HGF/NK2 open reading frame continued for two additional amino acids, followed by an in-frame stop codon (TAA). These findings suggested that HGF and HGF/NK2 cDNAs were derived from alternative transcripts of the HGF gene.

To define the splicing event that generated the truncated cDNA, we used the polymerase chain reaction (PCR) with primers P1 and P2 (Fig. 2A) to amplify specific HGF/NK2 sequences. Whereas the reaction yielded the expected 227-bp PCR product with HGF/NK2 cDNA, a ~600-bp fragment was detected when human genomic DNA was used as template. Sequencing of the latter PCR product revealed a ~400-bp intron flanked by consensus splice donor/acceptor sequences, CG/GT and AG/AG, at the intron-exon boundaries. These aligned precisely with the predicted splice junction in the HGF/NK2 cDNA clone (Fig. 2B).

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Thus, the 1.3-kb HGF/NK2 transcript is generated during precursor RNA processing by the joining of the K2 exon to an alternative exon K2T (Fig. 2A), containing a termination codon followed by a unique 3′ untranslated region.

To test whether the HGF/NK2 transcript encoded naturally occurring p28 protein, we transiently expressed HGF/NK2 cDNA in COS-1 cells. COS-1 cells transfected with the sense construct (pC45s) secreted a 28-kD HGF-immunoreactive recombinant protein that was not detected after transfection with the antisense construct (pC45as) (Fig. 3). The protein's size corresponded closely to that of p28 from M426 and SK-LMS-1 cells (Fig. 3). Under reducing conditions, the mobility of both the recombinant and naturally occurring proteins shifted to an apparent relative molecular mass of ~34 (*10*), providing further evidence that they were structurally indistinguishable. The two proteins also showed indistinguishable elution profiles on heparin-Sepharose chromatography with the peak for each at ~1.0 M NaCl (*10*). These findings established that p28 was the product of the HGF/NK2 transcript.

To investigate its biological activity, we isolated the HGF/NK2 protein from SK-LMS-1 culture fluids by a three-step procedure combining ultrafiltration, heparin-Sepharose chromatography, and TSK-sieving chromatography. The purified protein exhibited the characteristic mobility shift under nonreducing and reducing conditions and was immunoreactive with antiserum to HGF, confirming its identity as HGF/NK2 (*10*). Whereas HGF stimulated ^{3}H-labeled thymidine incorporation in B5/589 human mammary epithelial cells (*12*) with a half-maximal effect at ~0.25 nM, HGF/NK2 under identical conditions caused no enhancement of DNA synthesis at concentrations as high as 10 nM (Fig. 4A). In view of their structural similarity, we also tested the possibility that HGF/NK2 might act as a specific HGF inhibitor. When DNA synthesis induced by HGF was measured in the presence of increasing HGF/NK2 concentrations, a dose-dependent inhibition of [^{3}H]thymidine incorporation was observed (Fig. 4B). A 10- to 20-fold molar excess of HGF/NK2 over HGF was required for 50% inhibition. Similar results were obtained when human melanocytes were used as target cells (*10*). Moreover, the inhibition was HGF-specific because HGF/NK2 did not impair the mitogenic activity of epidermal growth factor (EGF) (Fig. 4B).

To elucidate the mechanism by which HGF/NK2 acted as an antagonist of HGF mitogenic activity, we performed cross-linking studies of [^{125}I]HGF/NK2 to B5/589 cells. We observed a single major cross-linked species of 170 kD (Fig. 5A). This cross-linked species was immunoprecipitated from cell lysates with antiserum to the COOH-terminus of the c-*met*/HGF receptor. Moreover, the immunoprecipitation reaction was effectively inhibited with the same COOH-terminal peptide, confirming that the 170-kD species contained the 145-kD β subunit of the processed c-*met* product cross-linked to HGF/NK2. When a similar cross-linking reaction was performed in the presence of increasing concentrations of either unlabeled HGF/NK2 or HGF, labeled HGF/NK2 cross-linking was effectively inhibited (Fig. 5B). On a molar basis, we estimated HGF to be three to five times more effective than HGF/NK2 as a competitor of [^{125}I]HGF/NK2 cross-linking. Under the same conditions, EGF failed to block HGF/NK2 cross-linking. All of these results demonstrate specific competitive binding of HGF/NK2 and HGF to the same cell surface receptor molecule.

Our studies demonstrate a mechanism for negative regulation of growth factor action. Secreted forms of certain growth factor receptors containing only their external domains may act to transport or protect the respective ligand (*13*). In contrast, HGF/NK2 competes directly with HGF for bind-

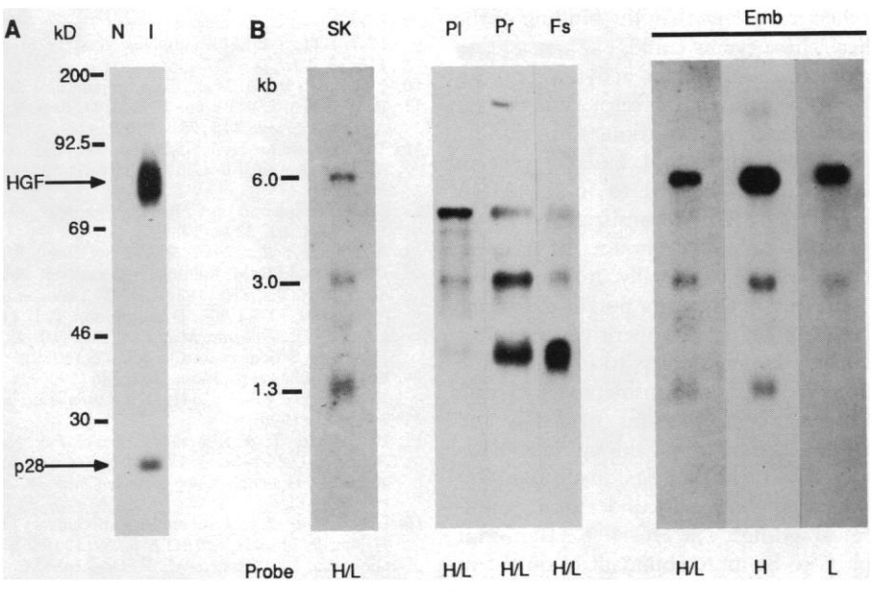

Fig. 1. Detection of p28 and its transcript. (**A**) ^{35}S-labeled methionine and cysteine conditioned medium from SK-LMS-1 cells was immunoprecipitated with nonimmune (N) and HGF-immune serum (I) (*23*). Proteins were subjected to 10% SDS-PAGE under nonreducing conditions. HGF and p28 are indicated by arrows. Molecular mass markers are shown in kilodaltons at left. (**B**) Northern analysis of RNA from human tissues and cell lines. We separated 2 μg of poly(A)$^{+}$ RNA from SK-LMS-1 (SK) cells, placenta (Pl) cells, and M426 (Emb) fibroblasts and 20 μg of total RNA from prostatic (Pr) and foreskin (Fs) fibroblasts by electrophoresis on 1% agarose gels, and then we hybridized the Northern blots with a probe to either the HGF coding region (H/L), the H region, or the L region (*26*). The sizes in kilobases of three major HGF-related transcripts are indicated at left.

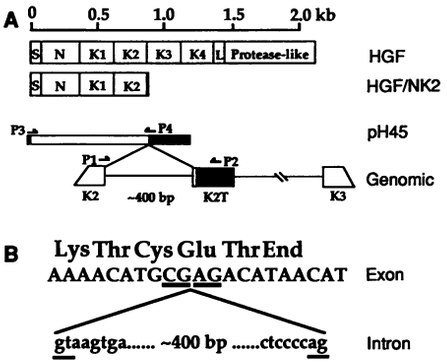

Fig. 2. Characterization of an HGF/NK2 cDNA. (**A**) Schematic representation of the domain structures of HGF and HGF/NK2 (open boxes). The 1.2-kb cDNA clone pH45 is composed of a coding region (open bar) and untranslated ones (filled bars). The splicing event for the generation of the HGF/NK2 sequence is depicted by the genomic region, with the alternative exon (K2T) located ~400 bp downstream of the K2 exon. Intronic region between K2T and the downstream K3 exon is of undefined length. Arrows represent the positions and directions of the PCR primers used (*29*). Abbreviations are as follows: K1 to K4, kringle 1 to 4; and L, linker region. Sizes in kilobases are shown at top. The double lines at end of HGF/NK2 indicate the two additional amino acids. (**B**) (Top) The cDNA and the predicted amino acid sequences of HGF/NK2 (Exon) at the point of divergence with HGF are shown with the splice site underlined. (Bottom) The corresponding genomic region (Intron) includes a ~400-bp intron with the consensus splicing signals at the exon-intron boundaries underlined.

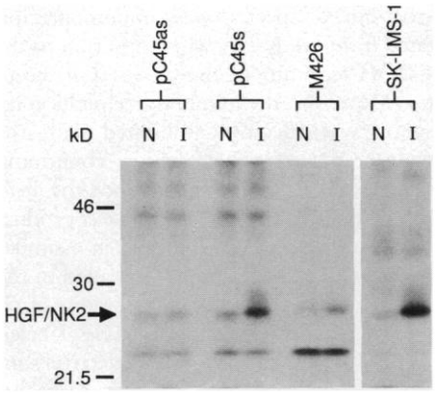

Fig. 3. Expression of HGF/NK2 cDNA in COS-1 cells. Conditioned media from COS-1 cells transfected with plasmid pC45as or pC45s, as well as M426 and SK-LMS-1 cells, were immunoprecipitated with nonimmune (N) or HGF antiserum (I) (*28*). Samples were analyzed under nonreducing conditions. HGF/NK2 is indicated by the arrow. Molecular size markers (in kilodaltons) are indicated at left.

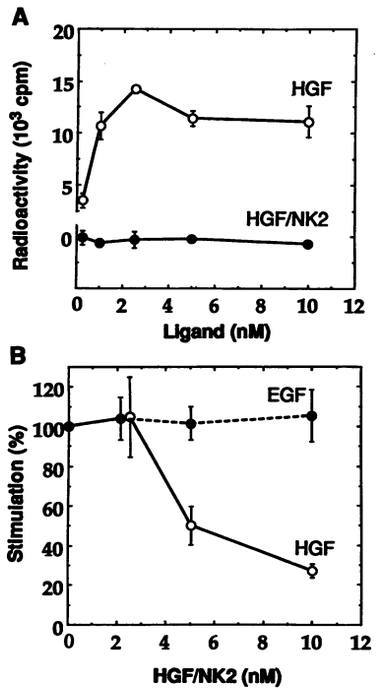

Fig. 4. Characterization of the purified HGF/NK2. HGF/NK2 was purified from conditioned medium of SK-LMS-1 cells as described (*29*). (**A**) Comparison of DNA synthesis stimulated by HGF and HGF/NK2. B5/589 cells were exposed to increasing concentrations of protein, and [^{3}H]thymidine incorporation was measured as described (*30*). (**B**) Effect of HGF/NK2 on HGF- and EGF-induced [^{3}H]thymidine incorporation in B5/589 cells. Results are expressed as the percentage of stimulation in the absence of HGF/NK2. HGF- and EGF-treated cells were tested at growth factor concentrations (0.2 nM and 0.3 nM, respectively) in the linear range of their dose-response curves. Typically, the stimulation was 10,000 to 20,000 cpm with a background of 2,000 cpm. Each data point is the mean ± SD of triplicate measurements; when no error bar is shown, the error was less than the symbol size.

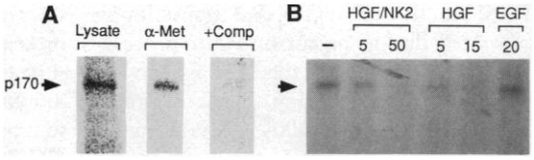

Fig. 5. Covalent affinity cross-linking of [^{125}I]HGF/NK2 to the HGF receptor. (**A**) B5/589 cells were incubated with [^{125}I]HGF/NK2 and treated with disuccinimidyl suberate (*31*). The sample in the left lane (lysate) was then prepared by solubilizing cells with boiling in SDS–sample buffer and resolved by 6.5% SDS-PAGE. Samples in the adjacent lanes were prepared by immunoprecipitation with a polyclonal antiserum to the COOH-terminal 28–amino acid residues of the human c-*met* product in the absence (α-met) or presence (+comp) of competing peptide. Immunoprecipitated proteins were adsorbed to immobilized protein G (Genex) and eluted with SDS–sample buffer before electrophoresis. We generated autoradiographic images by exposing the dried gel to a Phosphor Storage Screen (Kodak) and subsequently scanning the film by a Phosphor Imager (Molecular Dynamics). (**B**) [^{125}I]HGF/NK2 was incubated with B5/589 cells in the presence of HGF/NK2, HGF, or EGF at the nanomolar concentrations indicated at the top of the lanes. (The minus sign indicates the absence of growth factor.) Cultures were then incubated with the cross-linking agent, and total cell lysates were resolved by 6.5% SDS-PAGE under reducing conditions (*31*). The arrow in (A) and (B) indicates the 170-kD cross-linked complex of HGF/NK2 and the c-*met* receptor.

ing to a common cell surface receptor. The interleukin-1 (IL-1) antagonist IRAP is a nonmitogenic, competitive inhibitor of IL-1 (*14*). However, IRAP and IL-1 share only 26% (IL-1α) and 19% (IL-1β) amino acid sequence identity and are encoded by distinct genes. By contrast, HGF and HGF/NK2 protein sequences are >99% identical throughout the entire length of the smaller molecule and are encoded as alternative transcripts of the same gene.

Evidence that HGF/NK2 binds the c-*met*/HGF receptor establishes that no more than the NH_2-terminal and the first two kringle domains are required for receptor recognition. The different effects of HGF and HGF/NK2 on mitogenesis imply that the binding of one of these proteins to a common cell surface receptor triggers a different set of biochemical events than the binding of the other. These events could involve receptor oligomerization, kinase activity, or coupling to downstream effector systems that govern signal transduction. Our finding that a five- to tenfold molar excess of HGF/NK2 was needed to inhibit DNA synthesis by HGF is consistent with evidence that only low amounts of receptor occupancy are generally required for growth factors to exert a potent mitogenic effect (*15*). Thus, a competitive antagonist must be present in excess to inhibit ligand action. We observed considerable variation in relative concentrations of HGF and HGF/NK2 transcripts among fibroblasts analyzed, which suggests that an HGF/NK2 excess could exist under some physiological settings. The HGF/NK2 transcript appears to be more abundant in quiescent cells.(*10*). This would be consistent with a model in which HGF/NK2 modulates HGF growth-stimulating effects at late stages of wound healing and tissue repair as fibroblasts return to the resting state. Chronic *met*/HGF receptor activation has been observed in certain malignancies (*16*). Conceivably, HGF/NK2 could be used to inhibit the growth of tumors in which an HGF autocrine loop were implicated.

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23. Cells in 100-mm tissue culture plates were incubated with ^{35}S-labeled methionine and cysteine (0.1 mCi/ml; specific activity, 1150 Ci/ml; Du Pont Biotechnology Systems) in heparin (50 μg/ml) for 4 hours as described (*2*). Conditioned medium was concentrated 20-fold in a Centricon-10 microconcentrator (Amicon) and immunoprecipitated with nonimmune or HGF-neutralizing antiserum. Immunoprecipitates were absorbed onto Gamma-bind G agarose (Genex) and washed three times with 10 mM tris-HCl buffer containing 150 mM NaCl, 0.05% Tween 20, 0.1% SDS, 1% NP-40, 1 mM EDTA, and 10 mM KCl. Samples were analyzed under reducing (with 100 mM β-mercaptoethanol) and nonreducing conditions with 10% or 14% SDS-PAGE. Gels were fixed, treated with enlightening solution (Du Pont Biotechnology Systems), dried, and exposed to Kodak AR film at −70°C.
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26. Total and poly(A)$^+$ RNA were isolated as described (*17*). After separating samples by electrophoresis with 1% denaturing formaldehyde agarose gels, we transferred the samples to nitrocellulose filters (*17*). Blots were hybridized at 42°C for 12 hours to ^{32}P-labeled randomly primed DNA probes in 40% formamide, 6× saline sodium citrate (SSC), 5× Denhardt's solution, 50 mM sodium phosphate (pH 6.8), and sonicated salmon sperm DNA (250 μg/ml). After the hybridization reaction, we washed the filters twice in 1× SSC and 0.1% SDS at room temperature and in 0.1× SSC and 0.1% SDS at 55°C. Filters were dried and exposed to x-ray films for 5 to 8 days at −70°C. Hybridization probes were generated by PCR and purified on low melting temperature agarose gels. The nucleotide sequence of each probe was numbered according to the HGF sequence of Miyazawa *et al.* (7) as follows: H/L (heavy and light chains), −24 to +2187; H, +189 to +1143; and L, +1475 to +2122.
27. For routine PCR (*18*), 0.5 μg of human genomic DNA and 5 ng of plasmid DNA were subjected to 30 cycles of amplification with the following cycling conditions: 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C. For PCR cloning of genomic DNA, PCR was carried out with Bam HI linker primers, and amplified DNA fragments were digested with Bam HI. The resultant Bam HI fragments were purified on a low melting temperature agarose gel and subcloned into the M13mp18 vector for sequencing analysis (*19*). Primers used were as follows: P1, AGTACTGTGCAATTAAAACATGCG; P2, GTAGAAAAATGATTGTATGGACTGCTA; P3, AGGCACTGACTCCGAAC A GGATTCTTTCACCC AGGCATCTCCTCC; and P4, ATGGATCCTTATGTCTCGCATGTTTTAATGCACA.
28. The HGK/NK2 coding sequence was generated by PCR with the Bam HI linker primers P3 and P4 (Fig. 2A) and subcloned into the Bam HI site of the vector pCDV-1 (*20*) in both orientations. We sequenced the HGF/NK2 insert in a selected construct to ensure that the PCR product was correct. Plasmid DNA (10 μg each) was transfected by the calcium phosphate precipitation method (*21*) into COS-1 cells (*22*). At 48 hours, proteins in conditioned medium were processed for labeling, immunoprecipitation, and SDS-PAGE under reducing and nonreducing conditions as described (*23*).
29. Conditioned medium (6 liters) from SK-LMS-1 cells grown in 175-cm^2 T flasks were passed through a 0.5-μm filter (Millipore) and concentrated to 300 ml with a Pellicon cassette system with a 10-kD molecular mass cutoff (Millipore). Concentrated medium was loaded at a flow rate of ~150 ml/hour onto heparin-Sepharose resin (4-ml bed volume, Pharmacia) that had been equilibrated in 20 mM tris-HCl (pH 7.5) and 0.3 M NaCl. The sample was eluted with a modified linear gradient of increasing NaCl concentration. We subjected aliquots from each fraction to immunoblot analysis with antiserum to HGF (final dilution 1:500) to identify the presence of HGF/NK2. Pooled fractions were further resolved on a TSK G2000 sizing column (Pharmacia) in 20 mM tris-HCl (pH 6.8) and 1.0 M NaCl. The purity and identity of the HGF/NK2 protein were determined by silver-stain analysis (*24*) and immunoblotting under reducing and nonreducing conditions. Fractions containing >95% of HGF/NK2 were selected for biological analysis. Protein concentration was estimated by absorbance (A) measurements; we assumed that $A^{1\%}_{214} = 140$.
30. Microtiter plates (96-well) were coated with human fibronectin at 1 μg/cm^2 before we seeded the plates with B5/589 cells as described (*25*). The incorporation of [^{3}H]thymidine was determined during a 6-hour period beginning 16 hours after the addition of samples. Trichloroacetic acid–insoluble DNA was collected and counted. HGF was purified as reported (*2*), and human recombinant EGF was purchased from Upstate Biotechnology.
31. TSK-purified HGF/NK2 was iodinated by the chloramine-T method as described (*4*). This HGF/NK2 represented >99% of the labeled material in the preparation, as determined by SDS-PAGE analysis. Affinity cross-linking experiments were performed on six-well plates seeded with B5/589 cells at a density of 5 × 10^5 cells per well. HGF/NK2 (5 × 10^5 cpm; specific activity, ~200 μCi/μg) was added to each well with or without nonradioactive competitors in Hepes binding buffer [100 mM Hepes, 150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8.8 mM dextrose, heparin (2 μg/ml), and 0.1% bovine serum albumin; pH 7.4]. After we incubated the cells at room temperature for 45 min, we washed them twice in cold Hepes saline (pH 7.4). Disuccinimidyl suberate (Pierce) in dimethyl sulfoxide was added to a final concentration of 250 μM and incubated for 15 min. Samples were quenched with 100 μl of a solution containing 20 mM tris, 100 mM glycine, and 1 mM EDTA for 1 min and then rinsed in Hepes saline. Cells were extracted with Laemmli sample buffer and resolved by 6.5% SDS-PAGE under reducing conditions. Gel was dried and exposed to x-ray film at −70°C unless otherwise indicated.
32. We thank W. Taylor for advice concerning cell culture work, R. Halaban for assaying HGF/NK2 activity on human melanocyte cells, C. Florio and E. Fortney for technical assistance, J. Thompson for photography, and N. Lichtenberg for preparation of the manuscript.

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Regulation of a Segmentation Stripe by Overlapping Activators and Repressors in the *Drosophila* Embryo

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Gene expression stripes in *Drosophila melanogaster* embryos provide a model for how eukaryotic promoters are turned on and off in response to combinations of transcriptional regulators. Genetic studies suggested that *even-skipped* (*eve*) stripe 2 is controlled by three gap genes, *hunchback* (*hb*), *Kruppel* (*Kr*), and *giant* (*gt*), and by the maternal morphogen *bicoid* (*bcd*). A direct link is established between binding sites for these regulatory proteins in the stripe 2 promoter element and the expression of the stripe during early embryogenesis. The *bcd* and *hb* protein binding sites mediate activation, whereas neighboring *gt* and *Kr* protein sites repress expression and establish the stripe borders. The stripe 2 element has the properties of a genetic on-off switch.

THE PAIR-RULE GENE *EVE* ENCODES A homeobox protein important in the segmentation process. Mutations in *eve* produce severe segmentation defects, including the complete loss of segment borders in the middle body region (*1*). The *eve* protein is first detected 2 hours after fertilization, when it is uniformly distributed in all nuclei. This general expression gives way to a series of seven stripes along the length of the embryo before cellularization (*2*).

Individual stripes are regulated by separate cis elements contained within the *eve* promoter. For example, the first 1.7 kb of *eve* 5′ flanking sequence drives the expression of a *lacZ* reporter gene only within the limits of stripes 2 and 7; a 480-bp deletion between −1.6 and −1.1 kb abolishes expression of stripe 2 (*3, 4*). Stripe 3 depends on sequences located between −3.8 and −3 kb. We focus here on the regulation of stripe 2.

Genetic studies suggest that a total of four segmentation genes are responsible for stripe 2 expression (Fig. 1A). There are anterior and posterior expansions of the stripe borders in gt^- or Kr^- embryos, respectively, whereas the stripe is abolished or reduced in bcd^- or hb^- embryos (*5*). The four genetic regulators of stripe 2 expression may act directly on the *eve* promoter and modulate its transcription because proteins encoded by all four genes bind with high affinity to sequences in the *eve* promoter that are essential for stripe 2 expression (*5, 6*) (Fig. 1B).

To test whether the *bcd, hb, Kr*, and *gt* protein binding sites in the *eve* promoter directly mediate the interactions predicted by the genetic studies, we used site-directed mutagenesis (7) to disrupt some of these sites in a fusion gene that contains 5.2 kb of *eve* 5′ flanking sequence. This *eve-lacZ* fusion normally drives equally intense expression of stripes 2, 3, and 7 (*3, 4*). In this way, stripes 3 and 7 served as internal controls for perturbations of stripe 2 expression (*8*).

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