Heparin-binding epidermal growth factor-like growth factor, a v-Jun target gene, induces oncogenic transformation

(differentially expressed genes/transcriptional deregulation/anchorage independence/focus formation)

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ABSTRACT Jun is a transcription factor belonging to the activator protein 1 family. A mutated version of Jun (v-Jun) transduced by the avian retrovirus ASV17 induces oncogenic transformation in avian cell cultures and sarcomas in young galliform birds. The oncogenicity of Jun probably results from transcriptional deregulation of v-Jun-responsive target genes. Here we describe the identification and characterization of a growth-related v-Jun target, a homolog of heparin-binding epidermal growth factor-like growth factor (HB-EGF). HB-EGF is strongly expressed in chicken embryo fibroblasts (CEF) transformed by v-Jun. HB-EGF expression is not detectable or is marginal in nontransformed CEF. Using a hormone-inducible Jun-estrogen receptor chimera, we found that HB-EGF expression is correlated with v-Jun activity. In this system, induction of v-Jun is followed within 1 hr by elevated levels of HB-EGF. In CEF infected with various Jun mutants, HB-EGF expression is correlated with the oncogenic potency of the mutant. Constitutive expression of HB-EGF conveys to CEF the ability to grow in soft agar and to form multilayered foci of transformed cells on a solid substrate. These observations suggest that HB-EGF is an effector of Jun-induced oncogenic transformation.

Viral jun is the oncogene of avian sarcoma virus ASV17; the v-Jun protein induces sarcomas in chickens and transforms chicken embryo fibroblasts (CEF) in culture (1, 2). The cellular Jun protein (c-Jun) is a member of the activator protein 1 (AP-1) transcription factor family and is highly responsive to extracellular signals that control proliferative and apoptotic programs (3, 4). AP-1 proteins function as homo- or heterodimers. They share common structural features; a leucine zipper serves as dimerization domain, and an adjacent basic region forms the DNA contact surface. These structures define the class of bZip proteins (5). Dimerization of Jun is required for DNA binding, and DNA binding is necessary for transcriptional activation. All three properties, dimerization, DNA binding, and transactivation, are essential for oncogenic transformation (6). However, the transforming activity of various Jun mutants is not correlated with their transactivation potential as measured in transient transfection assays with reporter genes that contain the consensus AP-1 DNA-binding sequence (7–9).

v-Jun differs from c-Jun by a 27-aa deletion, which defines the delta region, and by two amino acid substitutions (10). As a result of the delta deletion, v-Jun cannot be phosphorylated by the Jun kinase JNK and thus escapes a major regulatory mechanism that controls the activity of c-Jun (11). The amino acid substitutions in v-Jun affect DNA-binding ability and nuclear translocation (12–14). All mutations in v-Jun function in concert to activate and enhance the oncogenic potential of the protein in vivo and in vitro (15, 16). The oncogenicity of v-Jun probably results from the deregulation of specific target genes. There exist numerous Jun-regulated genes with AP-1-binding sites in their promoters. Examples are collagenase, stromelysin, proliferin, and IL-2 (17). Several laboratories have described genes that are up-regulated in v-Jun-transformed cells. These include quail btk, chicken jtab-1, and the genes encoding for glutaredoxin, neuromodulin, and phenobarbital-induced cytochrome P450 (18–20). Whether any of these Jun-regulated target genes play a role in determining the oncogenic phenotype of the cell is not known. The identification of targets that participate in the transformation process, therefore, remains an important challenge.

In this report we describe the isolation and characterization of a transformation-related v-Jun target, heparin-binding epidermal growth factor-like growth factor (HB-EGF). HB-EGF is up-regulated by v-Jun. In cells transfected with regulatable v-Jun, the expression of HB-EGF is tightly correlated with the hormone-dependent activity of Jun. Overexpression of HB-EGF induces transformation of CEF in culture. These data suggest that HB-EGF plays an important part in mediating Jun-induced oncogenic transformation.

MATERIALS AND METHODS

Cell Cultures. Primary CEF were prepared from White Leghorn embryos supplied by SPAFAS (Preston, CT) (21). Secondary cultures were seeded in growth medium (F-10 supplemented with 6% iron-enriched calf serum/2 mM L-glutamine/100 units/ml penicillin/100 μg/ml streptomycin) for transfection and infection. Transfected or infected CEF cells were maintained in F-10 supplemented with 10% donor calf serum/4% chicken serum/1× minimal essential medium vitamin solution/2 mM L-glutamine/100 units/ml penicillin/100 μg/ml streptomycin/8 μg/ml folic acid/0.4% DMSO. In the case of estrogen-treated cultures, estrogen dissolved in ethanol was added at the final concentration of 2 μM.

Plasmids and Viruses. The following plasmids have been described previously: the v-Jun expression plasmids RCAS(A)JVJ0 and RCAS(A)JVJ1 (2); the c-Jun plasmid RCAS(A)CJ3 (2); the estrogen receptor–Jun fusion RCAS(A)ΔJV-hER and its control plasmid RCAS(A)hER (22); the c-Jun deletion mutant RCAS(A)JUN41LZ, RCAS(A)CJ3–23, and RCAS(A)CJ3–34 (6, 7); the v-Jun deletion mutants RCAS(A)VJ3 (15); and the VP16-c-Jun chimera RCAS(A)VCJ3 (23). RCAS(A)VJR4A is a highly oncogenic v-Jun mutant in which the arginine located next to

Abbreviations: AP-1, activator protein 1; CEF, chicken embryo fibroblasts; HB-EGF, heparin-binding epidermal growth factor-like growth factor.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF131224).†

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the N terminus of the leucine zipper was converted to alanine (this plasmid was kindly provided by Makoto Nishizawa). Infectious retroviruses containing various oncogenes were either naturally occurring or recombinant viruses created by inserting the oncogene into the RCAS(A) retroviral vector (24–27). These viruses are ASV17 carrying v-jun, MC 29 with v-myc, NK24 expressing v-fos, PR-A with v-src, MH2 carrying v-myc and v-ml, RCAS(A)-v-P3K expressing an oncogenic version of the p110 subunit of PI3-kinase, RCAS(A)-MaFQ5H, which carries a strongly transforming mutant of the maF transcription factor gene, and RCAS(A)-Hras with the Harvey ras gene. RCAS(A)JunD is a nontransforming construct carrying chicken JunD.

**Transfection, Infection, Focus Assays, and Soft Agar Assays.** For transfection, 5 × 10⁴ secondary CEF were seeded on 35-mm plates and were incubated overnight in growth medium containing 2 µg/ml Polybrene. They were then transfected with various amounts of DNA by using the DMSO method (28). For infection, 10–50 µl of high-titer virus stocks was added to 10⁶ secondary CEF on 60-mm plates. In focus assays, cells were overlaid with nutrient agar 15–20 hr after transfection (0.6% Sea Plaque agar in F-10 supplemented with 3% FCS, 1% chicken serum, 2.5 mM l-glutamine, 100 units/ml FCS, 1% chicken serum, 2.5 mg/ml trypsin phosphate broth, 1 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1% DMSO) and fed every 2–3 days with the same agar medium until foci developed (7–14 days). Foci were stained with 2% (wt/vol) crystal violet (in 20% methanol). Soft agar assays were performed as described previously (29). In brief, transfected cells were grown under agar for 10–14 days; they then were trypsinized and resuspended in nutrient agar consisting of 0.3% Sea Plaque agar in F-10 supplemented with 10% donor calf serum, 4% chicken serum, 3 mg/ml trypsin phosphate broth, 1.4X minimal essential medium vitamin solution, 12 µg/ml tunicamycin, 80 units/ml penicillin, 80 µg/ml streptomycin, and 0.2% DMSO. Cells were fed with the same nutrient agar every 2–3 days. Agar colonies were counted after 3 weeks.

**Northern Blots.** Transfected or infected CEF were lysed with RNA STAT-60 reagent (Tel-Test, Friendswood, TX), and total RNA was isolated following the protocol provided by the company. Poly(A)⁺ RNA was further purified by using the Oligotex beads according to the protocol from the supplier (Qiagen). Two micrograms of poly(A)⁺ RNA was separated by gel electrophoresis and then transferred onto Hybond-N membranes (Amersham) as described previously (30). Hybridization was carried out at 42°C overnight in Hoofer buffer containing 50% formaldehyde/5× SSC/20 mM Na₂HPO₄/NaH₂PO₄, pH 6.7/7.7% SDS/1% polyethylene glycol (molecular weight, 20,000) and 0.5% bovine albumin. After hybridization, filters were washed three times for 15 min with 0.2× SSC/0.1% SDS at 55°C. Filters then were rinsed with 2× SSC and autoradiographed. Hybridization probes were prepared from purified DNA fragments that were labeled with [α-32P]dCTP by using a random priming kit (Boehringer Mannheim) followed by treatment with a nucleotide removal kit (Qiagen). Probes were added at the final activity of 1–3 × 10⁶ cpm/ml of hybridization buffer.

**Construction and Screening of cDNA Libraries.** Poly(A)⁺ RNA was isolated from pooled foci induced by v-Jun-transformed CEF. cDNA was synthesized from 5 µg of poly(A)⁺ RNA with oligo(dT) primers by using cDNA synthesis kit (Stratagene). Different sizes of cDNA were separated on a Sepharose CL-2B gel-filtration column. Fractions containing cDNA fragments larger than 500 bp were collected, cloned into the Uni-ZAP XR vector, and packaged with the Gigapack III packaging extract (Stratagene) to form the primary library of about 8 × 10⁶ independent clones. The average size of library inserts was 1.6 kb. Clones (1.6 × 10⁶) from the primary library were amplified to create a secondary library. The titer of the secondary library was about 10 × 10¹⁰ pfu/ml. For screening, phase plaques were transferred onto Hybond-C extra nitrocellulose filters (Amersham). Filters were denatured with 1.5 M NaCl/0.5 M NaOH, neutralized with 1.5 M NaCl/0.5 M Tris (pH 7.5), rinsed with 2× SSC, followed by baking at 80°C for 2 hr. Hybridizations of first, secondary, and tertiary screening were carried out in Hoofer buffer overnight at 42°C with a probe activity of 6 × 10⁶ cpm/ml. After hybridization, filters were washed three times for 20 min with 0.1× SSC/0.1% SDS at 60°C.

**RESULTS**

**Identification of VJT-6 as a v-Jun-Responsive Gene.** We have used the directional tag PCR subtraction method to search for genes that are up-regulated in v-Jun-transformed CEF but not in vector-infected CEF (20, 31). Several clones representing genes that are up-regulated in Jun-transformed CEF were isolated; among these is clone VJT-6 (viral Jun target 6). Northern blot analysis with VJT-6 as a probe revealed an mRNA of 1.3 kb that is strongly induced in

![Fig. 1](image-url). Expression of VJT-6 is regulated by v-Jun. (A) v-Jun-transformed CEF overexpress VJT-6. Two micrograms of poly(A)⁺ RNA from CEF infected with the RCAS(A) vector or RCAS(A)-v-Jun was analyzed by Northern blot and autoradiographed. (B) Induction of VJT-6 by the estrogen-regulated Jun-estrogen receptor chimera ΔVJ-hER. Twenty micrograms of total RNA from CEF expressing the hormone-binding domain of the human estrogen receptor (hER), v-Jun (VJ1), or ΔVJ-hER was used for Northern blots. –, Control treatment (10 µl of EtOH) for 48 hr; +, exposure to 2 µM estrogen in EtOH for 48 hr; and +/−, 48 hr of estrogen treatment, followed by 48 hr without estrogen. (C) Time course of VJT-6 induction by ΔVJ-hER. CEF infected with ΔVJ-hER were treated with estrogen for various time periods. Twenty micrograms of total RNA from each indicated time point was analyzed by Northern blot. For A–C, blots were probed with [32P]-labeled VJT-6 cDNA. Molecular markers are on the left (×1,000). Control blots for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) show equal loading of the lanes. B and C were generated with different exposure times; quantitative aspects of VJT-6 induction are not comparable between B and C.
v-Jun-transformed CEF (Fig. 1A and ref. 20). We then analyzed the expression of VJT-6 RNA by using a Jun-estrogen receptor chimera (ΔVJ-hER) whose transforming and transcriptional activity can be regulated strictly by estrogen (22). Exposure to estrogen for 48 hr stimulates VJT-6 RNA expression compared with untreated controls (Fig. 1B, lanes 7 and 8). Removal of estrogen for 48 hr reverses the induction of VJT-6 (Fig. 1B, lane 9). Estrogen did not have a significant effect on VJT-6 RNA expression in CEF expressing wild-type v-Jun or estrogen receptor alone (Fig. 1B, lanes 1–6). This result shows that induction of VJT-6 RNA correlates with v-Jun activation. In time-course experiments, activation of ΔVJ-hER with estrogen led to the elevation of VJT-6 mRNA starting at 1 hr. The increased level was retained up to 12 hr in the presence of estrogen (Fig. 1C). This rapid induction is not seen with all Jun targets (S.-L.F., unpublished results) and suggests that VJT-6 may be a direct target of v-Jun. The estrogen-induced stimulation of VJT-6 precedes detectable cellular transformation by ΔVJ-hER.

**Extensive Homology Between Full-Length VJT-6 and Mammalian HB-EGF.** The original VJT-6 clone isolated from the subtractive library has a size of about 360 bp and shows no homology to any known gene in the database. To isolate a full-length clone for further characterization, we used the original VJT-6 fragment as a probe to screen a λ phage cDNA library of v-Jun-transformed CEF cells. From a screen of 240,000 clones, 20 positives were obtained. Six of them were sequenced because their insert sizes were close to the estimated size of VJT-6 mRNA. Four independent clones, differing in the length of their 5′ untranslated regions, contained the same ORF, which encodes a 212-aa protein. A database search via BLASTP revealed that the predicted translation product shares extensive homology (66%) with the membrane-bound precursor form of human HB-EGF. These results suggest that VJT-6 may be the chicken homolog of HB-EGF. The alignment of Jun-induced HB-EGF with mammalian HB-EGF proteins and the predicted domains are illustrated in Fig. 2. The region representing the mature secreted HB-EGF is highly conserved among different species (about 80%), including the EGF-like domain (amino acids 77–152) and the heparin-binding sites (amino acids 97–117). The homology in the C-terminal transmembrane domain and cytoplasmic domain is also high. However, the N-terminal region (amino acids 1–77), proposed to represent the signal peptide and propeptide, is not conserved (about 30% homology). In vitro transcription/translation of the full-length HB-EGF cDNA gave rise to a single peptide consistent with the predicted size of 22.5 kDa (data not shown).

**Correlation of HB-EGF Expression with v-Jun Transformation.** The involvement of HB-EGF in v-Jun-induced transformation was examined with various Jun mutants. RCAS(A)VJC3, RCAS(A)CJ3–34, and RCAS(A)VJR4A are strongly transforming mutants similar to the v-Jun-expressing RCAS(A)VJ0 (7, 23). RCAS(A)CJ3 and RCAS(A)CJ3–23 are weakly transforming mutants that induce only 5–15% of transformed cell foci relative to that of

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**Fig. 2.** Alignment of putative chicken HB-EGF to mammalian HB-EGF proteins and the domains of HB-EGF. The amino acid sequences of mammalian HB-EGF proteins were downloaded from the Swiss-Prot database. The numbers indicate the amino acid numbers of the putative chicken HB-EGF. The dark gray areas indicate identical amino acids, and light gray areas indicate similar amino acids. The alignment was generated with the program CLUSTALW ALIGNMENT. The arrows mark the amino acid sequence of predicted, mature-secreted HB-EGF. The black dot designates the potential glycosylation site, threonine-89. The dashed lines mark the heparin-binding sites. The transmembrane domain is underlined. Swiss-Prot database accession numbers are Q99075 (human HB-EGF), Q061767 (rat HB-EGF), and Q06186 (mouse HB-EGF). The chicken HB-EGF has been deposited in GenBank (accession no. AF131224).
HB-EGF induced foci of transformed cells growing in multiple layers. The numbers of foci per μg DNA of RCAS-HB-EGF were equivalent to those seen with RCAS(A)-VJ0. However, the foci induced by HB-EGF were smaller than those of v-Jun. They appeared more diffuse, showed less multilayering, and lacked the pronounced parallel orientation of the transformed cells characteristic of v-Jun foci (Fig. 4). The results of agar colony tests are shown in Fig. 5. HB-EGF stimulated anchorage-independent growth with an efficiency of 19 ± 5% of that of v-Jun. HB-EGF-induced colonies were smaller than Jun-induced colonies but their growth in agar was clearly evident as compared with CEF transfected by vector alone. The morphology of HB-EGF-transformed CEF in liquid culture was also distinct (Fig. 6). In contrast to the flat, contact-inhibited normal CEF and to elongated v-Jun-transformed CEF growing in parallel alignment, HB-EGF-transformed cells grew in crisscrossed arrangements and were short and refractile. Expression of HB-EGF RNA by the RCAS(A) vector in the transformed cells was confirmed by Northern blot analysis (data not shown). Three major bands, 7.95, 3.75, and 1.45 kb, were detected. The band with the highest molecular weight represents the retroviral genome plus the length of the insert; two shorter transcripts result from alternative splicing. The data on transformation and anchorage-independent growth support the conclusion that HB-EGF is sufficient to induce a partial cellular transformation.

Differential Expression of HB-EGF in Various Oncogene-Transformed CEF. HB-EGF could be induced exclusively in Jun-transformed cells or it could be widely elevated in cells transformed by diverse oncogenes. The Northern blot shown in Fig. 7 documents a broad induction of HB-EGF. HB-EGF expression is especially elevated in cells transformed by v-Fos and v-Maf, which, like v-Jun, are bZip proteins. Both Maf and Fos can form heterodimers with Jun. Jun-Fos heterodimers bind to AP-1 consensus sites, and Jun-Maf heterodimers recognize a variant of the AP-1 consensus site (32, 33). The induction of HB-EGF by AP-1-related bZip proteins implicates these nuclear oncoproteins in transcriptional activation.

Fig. 5. Anchorage-independent growth of HB-EGF-expressing CEF. CEF expressing vector RCAS(A), positive control v-Jun, or HB-EGF were seeded in soft-agar assays as described in Materials and Methods. Experiments were repeated four times, and one typical experiment is shown.

Fig. 6. Morphology of HB-EGF-transformed cells. CEF expressing RCAS(A), RCAS(A)v-Jun, or RCAS(A)HB-EGF were grown into mass culture and photographed 3 weeks postinfection with phase-contrast optics at ×16 objective lens magnification.
or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as probes. CEF were infected with various onco-
genome. CEF were infected with various oncogenes. Twenty micro-
grams of total RNA was analyzed in Northern blots by using HB-EGF or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as probes. A recipient of Ras and Raf signals, it is possible that the a recipient of Ras and Raf signals, it is possible that the

3T3 cells transformed by v-Ras or v-Raf (39). Because c-Jun is a recipient of Ras and Raf signals, it is possible that the

v-Jun is not simply a gain-of-function mutant of c-Jun. In v-Jun-transformed CEF, c-Jun is down-regulated at the transcrip-

tion is correlated with serum- and TPA-stimulated Jun ex-

scriptional level; v-Jun acts as a dominant negative of c-Jun (9, 34). In transient transfection assays with CEF, v-Jun fails to transactivate reporter genes that carry the AP-1 consensus sequence TGACTCA; such reporters are strongly activated by c-Jun. However, both v-Jun and c-Jun act on reporters that contain a variant AP-1-binding site (9). The DNA-binding specificity of AP-1 proteins can be modulated by dimerization with the activating transcription factor (ATF) proteins (35, 36). In v-Jun-transformed cells Fos-related protein Fra-2 has been identified as Jun dimerization partners (37). There is evidence that dimerization with different partners has functional con-
sequences for transformation. Dimerization of Jun with mem-
bers of the Fos protein family (including Fos-related antigens) induces anchorage-independent growth but not growth factor independence whereas Jun linked to ATF-2 mediates growth factor independence without the ability to grow in agar suspension (38). Because of the altered target specificity of v-Jun and the down-regulation of c-Jun in v-Jun-transformed cells, such cells probably contain differentially regulated trans-
formation-specific genes. In recent years, several genes that are specifically up-regulated in v-Jun-transformed cells have been identified, but little is known about their role in the transformation process (18–20). None of them has been shown to induce cellular properties characteristic of the transformed phenotype. In contrast, expression of HB-EGF is not only v-Jun-responsive and up-regulated in v-Jun-transformed cells, it can induce, by itself, transformation-specific cellular prop-

erties.

Levels of HB-EGF RNA and protein are elevated in NIH 3T3 cells transformed by v-Ras or v-Raf (39). Because c-Jun is a recipient of Ras and Raf signals, it is possible that the

induction of HB-EGF in these transformants is mediated by c-Jun. The promoter of the murine HB-EGF gene contains a composite AP-1/Ets-binding site and an AP-1 consensus site (40, 41). Full induction of HB-EGF in v-Ras- or v-Raf-transformed cells depends on the presence of the composite AP-1/Ets-binding site (41). These data suggest that HB-EGF may also be regulated directly by Jun in avian cells. Although the promoter of the avian HB-EGF gene has not yet been isolated, indirect evidence supports this suggestion: (i) HB-EGF expression in avian cells is serum- and phorbol 12-myristate 13-acetate (PMA)-responsive, and HB-EGF induction is correlated with serum- and TPA-stimulated Jun expression (data not shown). (ii) Induction of HB-EGF by estrogen-regulated Jun occurs within an hour or less after addition of the hormone. (iii) Both chicken v-Jun and c-Jun can activate the promoter of mouse HB-EGF in luciferase assays. Mutation of the AP-1 consensus sequence within the com-
posite AP-1/Ets site of this promoter greatly diminishes this activation (data not shown).

HB-EGF is a mitogen for keratinocytes, hepatocytes, smooth muscle cells, and fibroblasts; its expression is elevated in human hepatocellular and gastric carcinomas, pancreatic cancers, gliomas, and glioblastomas (42, 43). Other members of the EGF family (e.g., EGF and transforming growth factor type α) can induce oncogenic transformation of fibroblasts by an autocrine mechanism (44–46). A similar mechanism may be operative in HB-EGF-induced transformation and may also play a role in the oncogenicity of Jun. CEF transformed by HB-EGF differ from Jun-transformed cells in morphology and growth patterns, and, unlike v-Jun, HB-EGF is not tumori-
genic in chickens (data not shown). The suggested role of HB-EGF in v-Jun-induced oncogenic transformation is a partial one; other Jun targets probably supplement the activity of HB-EGF to effect full transformation. The search for additional transformation-related targets of v-Jun is being continued.

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