JAC, a direct target of oncogenic transcription factor Jun, is involved in cell transformation and tumorigenesis

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Using subtractive hybridization techniques, we have isolated a gene termed *JAC* that is strongly and specifically activated in avian fibroblasts transformed by the v-*jun* oncogene of avian sarcoma virus 17 (ASV17), but not in cells transformed by other oncogenic agents. Furthermore, *JAC* is highly expressed in cell lines derived from *jun*-induced avian fibrosarcomas. Kinetic analysis using a doxycycline-controlled conditional cell transformation system showed that expression of the 0.8-kb *JAC* mRNA is induced rapidly upon activation of the oncogenic v-*jun* allele. Nucleotide sequence analysis and transcriptional mapping revealed that the *JAC* gene contains two exons, with the longest ORF confined to exon 2. The deduced 68-amino acid chicken JAC protein is rich in cysteine residues and displays 37% sequence identity to mammalian high-sulfur keratin-associated proteins. The promoter region of *JAC* contains a consensus (5′-TGACTCA-3′) and a nonconsensus (5′-TGAGTAA-3′) AP-1 binding site in tandem, which are both specifically bound by the Gag-Jun hybrid protein encoded by ASV17. Mutational analysis revealed that the two AP-1 sites confer strong transcriptional activation by Gag-Jun in a synergistic manner. Ectopic expression of *JAC* in avian fibroblasts leads to anchorage-independent growth, strongly suggesting that deregulation of *JAC* is an essential event in *jun*-induced cell transformation and tumorigenesis.

jun oncogene | signal transduction | gene expression | protein–DNA interaction | transcriptional control

The v-*jun* oncogene was originally identified as the transforming principle of avian sarcoma virus 17 (ASV17) (1). Expression of v-*jun* leads to transformation of avian fibroblasts (2) and to induction of fibrosarcomas in chicken (2, 3). The cellular progenitor of the retroviral v-*jun* oncogene, the c-*jun* protooncogene (4), encodes a major component of the transcription factor complex AP-1, which represents a collection of dimers consisting of Jun, Fos, or ATF protein family members (2, 5–7). Gene regulation by AP-1 is important for cell proliferation and differentiation, particularly during epidermal development (8), but constitutive AP-1 activation can lead to tumor formation (2, 6–8). Distinct AP-1-regulated keratin genes are consistently overexpressed in various carcinomas (9), and the transactivation function of AP-1 is required for tumor promotion in epidermal cells (10). Dermal fibrosarcomas have been observed in v-*jun* transgenic mice upon wounding (11), and the c-*fos* protooncogene is required for malignant progression of skin tumors (12).

Jun proteins can bind to DNA as functional homodimers, or as heterodimers with Fos or ATF proteins. Jun-Jun and Jun-Fos dimers bind preferentially with high affinity to enhancer elements with the consensus sequence 5′-TGAC/GTCA-3′ located in various AP-1-responsive genes (6, 7), but nonconsensus binding motifs differing in single nucleotides have also been identified (13, 14). There is increasing evidence that cellular transformation induced by the v-Jun protein involves the aberrant expression of specific genes that are normally regulated by endogenous c-Jun as a component of AP-1. Approaches aimed at the identification of genes specifically deregulated in *jun*- or *fos*-transformed fibroblasts have led to the identification of several *jun* target genes (2, 7, 15), including the direct transcriptional targets *BKJ*, encoding a β-keratin-related protein (16, 17), glutaredoxin (18), the gene encoding heparin-binding epidermal growth factor-like growth factor (HB-EGF) (19), and the *TOJ3* gene encoding a protein highly related to microspherule protein 1 (MCRS1) (20). Remarkably, the *HB-EGF* and *TOJ3* genes were shown to induce partial cell transformation when expressed in a retroviral context (19, 20).

Here, we describe the isolation of a *jun* target gene, termed *JAC*, that is highly expressed in v-*jun*-transformed fibroblasts and in v-*jun*-induced fibrosarcomas, and that is directly and specifically regulated by oncogenic Jun proteins at the transcriptional level. Ectopic expression of *JAC* in avian fibroblasts leads to anchorage-independent growth, strongly suggesting that deregulation of *JAC* is an essential event in *jun*-induced cell transformation and tumorigenesis.

Materials and Methods

Cells and Retroviruses. Cell culture and transformation of chicken or quail embryo fibroblasts (CEF, QEF) was performed as described (16, 17, 21, 22). Avian retroviruses used were ASV17 (1), NK24 (23), MC29 (22, 24), MH2A10 (25), and Rous sarcoma virus (RSV) (26). For generation of the tumor cell cultures T-ASV17 and T-RCAS-vJun, viral stocks of ASV17 or virus released from Rcas-vJun-transfected CEF, respectively, were injected intravenously into 10-day-old chicken embryos (3). Two months later, fibrosarcomas were recovered and expanded into cell cultures. The quail cell line Q/d3 (21), conditionally transformed by v-*jun*, and QEF or CEF transformed by the retroviral constructs RCAS-VJ1 and RCAS-VJ0 (27) have been described (16, 17, 21). For construction of DNA templates encoding amino-terminally FLAG-tagged (28) proteins, a double-stranded oligodeoxynucleotide encoding the nine-amino acid peptide tag DYKDDDDKD was inserted between codons 1 and 2 of *JAC* or *HBEGF*, respectively. For construction of RCAS-JAC, RCAS-FLAG-JAC, and RCAS-FLAG-HBEGF, the coding regions of chicken *JAC* and sequences encoding the FLAG-JAC or FLAG-HBEGF fusion proteins were inserted into the RCAS-BP vector (subgroup A) (29), respectively. Colony assays performed in 0.3% (wt/vol) Sea Plaque agarose (BioWhittaker)

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Abbreviations: CEF, chicken embryo fibroblasts; QEF, quail embryo fibroblasts; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF172321, AF172322, and AF239161).

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were done as described (21, 22), using cells 3 weeks after DNA transfection with RCAS constructs.

DNA Cloning and Nucleic Acid Analysis. Subtracted probe preparation, screening, Northern and Southern analysis, and nucleotide sequencing were performed as described (16, 17). For subtracted probe generation, cDNA synthesized on poly(A)$^+$ RNA from clonal cell cultures derived from ASV17-transformed CEF (17) was hybridized with poly(A)$^+$ RNA from normal CEF. This probe was used to screen a cDNA library derived from ASV17-transformed CEF (17) as described (16) yielding 40 positive phage clones, from which 29 represented the *JAC* gene. Hybridization probes included the full-length chicken *JAC* cDNA, the cDNA insert fragments of quail *JAC* (21), quail *BKJ* (16), and quail glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (30). For the isolation of *JAC* genomic clones, a λEMBL3 library of partially *Mbo*I-digested chicken genomic DNA was screened as described (17) with ^{32}P-labeled chicken *JAC* cDNA as a probe.

DNA Binding and Protein Analysis. Electrophoretic mobility-shift assays were carried out as described (17). The prokaryotic expression vector pET-CV is a derivative of pET-CJ (17) and encodes a chimeric c-Jun/v-Jun protein in which the 168 carboxyl-terminal amino acids of c-Jun (4) are replaced by the homologous 168-amino acid segment of v-Jun (1). This protein was expressed at higher levels in *Escherichia coli* than the original v-Jun protein (data not shown). Preparation of nuclear extracts from subconfluently grown cells was done as described (31). A ^{32}P-labeled 40-bp double-stranded oligodeoxynucleotide encompassing nucleotide positions 3461–3500 from the *JAC* promoter region including the AP-1 consensus site 5′-TGACTCA-3′, but excluding the nonconsensus 5′-TGAGTAA-3′ motif, and a mutant oligodeoxynucleotide derivative in which the consensus site is substituted by the sequence 5′-AGACCCA-3′ were prepared as described (17). Binding reactions with nuclear extracts contained 0.25 μg/μl poly(dI-dC)·poly(dI-dC). To test binding specificity, polyclonal antisera directed against avian Jun and Fos proteins were added 15 min after the binding reaction was initiated, and incubation was continued for another 15 min. Antisera directed against the amino-terminal 77 amino acids of chicken JunD (32) or against the entire chicken c-Fos protein (33) were prepared analogously as described for the generation of c-Jun/v-Jun or Fra-2-specific antisera (34). Detection of Gag-related proteins has been described previously (17). *In vitro* transcription/translation reactions using derivatives of pBluescript II SK(+) (Stratagene) as templates was done as described (17). Immunoprecipitation of L-[^{35}S]cysteine-labeled proteins from cell lysates or from *in vitro* transcription/translation reactions using each 8-μg aliquots of monoclonal Anti-FLAG M2 antibody (Sigma) and SDS/PAGE was done as described (17) with the following modifications. Cells were lysed in a buffer containing 20 mM Tris·HCl (pH 8.0), 200 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A, and the lysate was clarified by centrifugation at 20,000 × *g*. After binding of antigen–antibody complexes to protein A Sepharose CL-4B (Amersham Pharmacia), the solid phase was washed three times in lysis buffer and two times in a buffer containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40.

Transactivation Analysis. For construction of the chloramphenicol acetyltransferase (CAT) reporter plasmid pCAT-*JAC*, a 725-bp *Ear*I fragment derived from the chicken *JAC* promoter (*p-JAC*) was inserted into the polylinker region of pCAT-Basic vector (Promega). For construction of pCAT-*JAC*Δ, a 172-bp *Ssp*I–*Nsi*I fragment encompassing both AP-1 binding sites was deleted from pCAT-*JAC*. Site-directed mutagenesis of AP-1 binding sequences was performed as described (35) with pCAT-*JAC* or pCAT-*JAC*-mutD (see below) used as a template, and mutator oligodeoxynucleotides leading to conversion of the distal (D) AP-1 binding site 5′-TGACTCA-3′ into a 5′-AGACCCA-3′ motif (pCAT-*JAC*-mutD), to deletion of the proximal (P) site 5′-TGAGTAA-3′ (pCAT-*JAC*ΔP, pCAT-*JAC*-mutDΔP), or to deletion of the distal site (pCAT-*JAC*ΔD). The reporter construct pCAT-*BKJ* [pCAT-*BKJ*(DP)] has been described (17). pRc/RSV (Invitrogen) derived eukaryotic expression vectors used in this study contain the coding regions of the v-*jun* (1) or v-*fos* (23) oncogenes fused to partial gag sequences (*gag-jun*, *gag-fos*) and derivatives thereof (v-*jun*, v-*fos*) lacking the amino-terminal *gag* portions, of the protooncogenes c-*fos* (33), *fra-2* (36), c-*jun* (4), *jun*D (32), and of the transforming *jun*D mutant *jdv* (32). CAT analysis was performed as described (17). Quantification of radioactive signals was done by using a PhosphorImager (Molecular Dynamics).

Results

Specific Expression of *JAC* in *jun*-Transformed Avian Fibroblasts. By using subtractive hybridization cloning of cDNAs, a gene was identified that is specifically activated in CEF transformed by ASV17 (1) carrying the v-*jun* oncogene. Because of its characteristic expression profile (see below), this gene was termed *JAC* (for *jun*-activated gene in CEF). To test for a correlation with *jun*-induced cell transformation, the expression of *JAC* was tested in a variety of transformed and nontransformed CEF. Northern analysis using RNAs from CEF transformed by various oncogenes and *JAC* cDNA as a probe revealed the highly specific expression of a 0.8-kb mRNA in v-*jun*-transformed cells but not in normal cells or in cells transformed by the v-*fos*, v-*myc*, v-*myc*/v-*mil*, or v-*src* oncogenes (Fig. 1*A*). The activation of *JAC* was observed at a strikingly high level in all clonal cultures derived from individual ASV17-induced foci of transformed CEF (Fig. 1*A*). Intriguingly, *JAC* was also found to be expressed at high levels in cell cultures derived from chicken fibrosarcomas, indicating that *JAC* is also activated during *jun*-induced tumorigenesis (Fig. 1*B*). A conditional quail cell transformation system in which transcription of the ASV17 v-*jun* allele is controlled by a doxycycline-dependent transactivator (21) was used to determine the kinetics of *JAC* activation upon conditional induction of oncogenic *jun*. Fig. 1*C* shows that both *JAC* and the direct *jun* target gene *BKJ* (16, 17) were fully activated within 3 days following induction of v-*jun* expression, although the kinetics of the early onsets of induction varied.

Structural Organization of the Chicken *JAC* Gene. Nucleotide sequence analysis of various overlapping cDNA clones and of PCR products derived from primer-extended cDNA resulted in the compilation of a full-length 622-bp sequence (GenBank accession no. AF172321), with the longest ORF of 68 codons encoding a putative protein of M_r 7,044 with 18 cysteine residues (26.5%) and an estimated isoelectric point of 7.3 (Fig. 2*A*). A 564-bp *JAC* cDNA was also isolated from quail (GenBank accession no. AF172322) encoding a 66-amino acid protein product with 77% sequence identity to the presumed chicken orthologue. Computerized sequence comparison of the chicken *JAC* amino acid sequence with database entries revealed identities of up to 37% with a large gene family encoding the cysteine-rich human and rodent high-sulfur keratin-associated proteins ranging in their molecular masses from 9.5 to 53.9 kDa and forming part of a rigid matrix in which microfibrils of keratins are embedded (37). To determine the structural organization of the *JAC* gene, the nucleotide sequence of a 6,758-bp chicken genomic DNA segment (GenBank accession no. AF239161) hybridizing with a *JAC* cDNA probe was determined (Fig. 2*A*). The *JAC* gene contains two exons at positions 3835–3881 and 4315–4889, with the coding region confined to exon 2. Similar to mammalian high-sulfur keratin-associated protein-encoding genes (37), the *JAC*

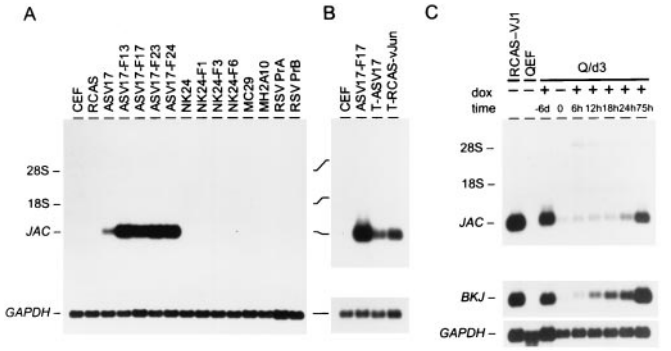

Fig. 1. Activation of *JAC* expression in *jun*-transformed fibroblasts. (*A* and *B*) Northern analysis of *JAC* expression in CEF, in CEF transfected with an empty retroviral vector (RCAS), or in CEF transformed by the avian retroviruses indicated, and in tumor cell lines derived from v-*jun*-induced chicken fibrosarcomas. ASV17 (v-*jun*) (1), NK24 (v-*fos*) (23), MC29 (v-*myc*) (22, 24), MH2A10 (v-*myc*/v-*mil*) (25), or the Prague A and B strains of RSV (v-*src*) (26) carry the oncogenes listed in parentheses. Samples of 2.5 μg of poly(A)$^+$ RNAs were derived from mass and clonal (F) cultures of ASV17- or NK24-transformed fibroblasts and of the v-*jun*-transformed tumor cell lines T-ASV17 and T-RCAS-vJun. (*C*) Kinetic analysis of *JAC* activation by using a conditional cell transformation system (Q/d3) containing a v-*jun* allele controlled by a doxycycline-dependent transactivator (21). Samples of 5.0 μg of poly(A)$^+$ RNAs were analyzed. At day −6, 6×10^6 transformed Q/d3 cells per 100-mm dish were depleted of doxycycline, and after 6 days, the drug was readded to the reverted cells (day 0). Normal QEF and QEF transformed by the retroviral construct RCAS-VJ1 (27) carrying the v-*jun* oncogene were used as controls. Filters were successively hybridized with ^{32}P-labeled cDNA probes derived from chicken *JAC* (*A*, *Upper*: 1.1×10^6 cpm/ml, 4-h exposure; *B*, *Upper*: 1.0×10^6 cpm/ml, 23-h exposure), from quail *JAC* (*C*, *Top*: 1.0×10^6 cpm/ml, 70-h exposure), from quail *BKJ* (*C*, *Middle*: 1.0×10^6 cpm/ml, 5-h exposure), and from quail *GAPDH* (*A*, *Lower*: 1.4×10^6 cpm/ml, 7-h exposure; *B*, *Lower*: 0.9×10^6 cpm/ml, 16-h exposure; *C*, *Bottom*: 1.0×10^6 cpm/ml, 16-h exposure). Autoradiography was done with an intensifying screen. The sizes of the mRNAs are as follows: *JAC*, 0.8 kb; *BKJ*, 0.8 kb; and *GAPDH*, 1.4 kb. The positions of residual ribosomal RNAs are indicated in the margins.

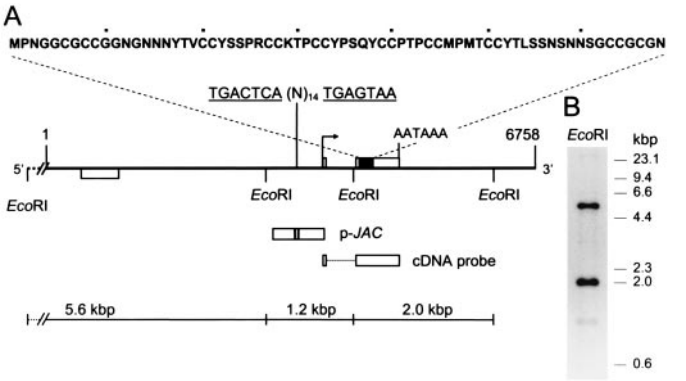

Fig. 2. Structure of the chicken *JAC* gene. (*A*) In the schematic diagram of the *JAC* gene structure, the two exons are depicted as boxes extending above the bar with the coding region shown in black. The 68-amino acid sequence of the predicted JAC protein is depicted above. The positions of the AP-1 binding sites in the 5′ upstream region, of the polyadenylation signal, and of the transcriptional initiation site (arrow) are indicated. The box extending below the bar near the 5′ end of the genomic segment indicates the location of a *JAC*-related region (see text). The complexities of the promoter fragment (*p-JAC*) containing both AP-1 binding sites and used for functional analysis (compare with Fig. 4), and of the full-length chicken *JAC* cDNA used as a hybridization probe, are also indicated. (*B*) Southern analysis of a cloned 16-kbp genomic chicken DNA fragment encompassing the *JAC* gene locus, using *Eco*RI for DNA digestion and ^{32}P-labeled *JAC* cDNA (1.4×10^6 cpm/ml) as a probe. The filter was finally washed at 60°C in a solution containing 0.1× SSC, 0.1% (wt/vol) SDS, and 1 mM EDTA and then autoradiographed for 9 h with an intensifying screen. The positions of DNA size markers are indicated in the margin.

coding region lacks introns. A TATA box is present 30 bp upstream of the experimentally determined transcriptional start site (data not shown) located at position 3835. Intriguingly, the 5′ upstream region contains one authentic 5′-TGACTCA-3′ heptamer motif for high-affinity AP-1 binding (2, 6, 7) at position 3478–3484 and, only 14 bp further downstream, a nonconsensus AP-1 binding site 5′-TGAGTAA-3′, representing a motif that was identified as a functional element in some AP-1-regulated genes (13, 14). When the 622-bp *JAC* cDNA probe was used, three *Eco*RI fragments hybridizing under stringent conditions were detected by Southern analysis of chicken genomic DNA (Fig. 2*B*). In addition to the expected exon-containing fragments, a 5.6-kbp fragment was detected that contains a 525-bp region with sequences similar to *JAC*, suggesting that gene duplication may have occurred during evolution of the *JAC* locus.

Specific Binding of v-Jun to the AP-1 Binding Sites. To directly demonstrate a specific interaction of v-Jun with the consensus AP-1 binding site present in the *JAC* promoter, a 40-bp double-stranded oligodeoxynucleotide encompassing this 5′-TGACTCA-3′ motif was used as a DNA probe for electrophoretic mobility-shift analysis. A chimeric recombinant c-Jun/v-Jun protein containing the carboxyl-terminal DNA binding domain from v-Jun bound efficiently to the DNA probe (Fig. 3*A*). The specificity of the protein–DNA interaction was emphasized by competitive inhibition using the unlabeled DNA probe in excess and by the lack of binding to a corresponding DNA fragment carrying two nucleotide substitutions in the heptamer motif (Fig. 3*A*). AP-1 binding activity was also detected in nuclear extracts prepared from normal CEF and from the v-*jun*-transformed tumor cell culture T-ASV17, resulting in the formation of specific protein–DNA complexes (Fig. 3*B*). To define the nature of these bound proteins, antibodies directed against c-Jun, JunD, c-Fos, Fra-2, or the viral Gag-Jun protein were added to the binding reactions, leading to interference of specific protein–DNA interactions (Fig. 3*C*) typical for Jun or Fos antibodies (38). In CEF extracts, all known members of the avian cellular Jun and Fos protein families were identified in the protein–DNA complex, with JunD and Fra-2 as the major participants. In T-ASV17 extracts, DNA binding was strongly inhibited by antisera directed against v-Jun/c-Jun or Gag proteins, whereas weak or no interference was observed employing antisera specific for Fra-2, c-Fos, or JunD, respectively (Fig. 3*C*). Because c-Jun protein is absent in v-*jun*-transformed cells (17), Gag-Jun, Fra-2, and c-Fos remain as major binding proteins. The additional band migrating below the complex formed by nuclear T-ASV17 proteins could be caused by truncated AP-1 components or result from yet unknown proteins. As representative size markers for protein–DNA complexes formed by nuclear extract proteins, DNA probes complexed with JunD/Fra-2 or Gag-Jun/Fra-2 dimers derived from *in vitro* translated proteins were applied, which differed in their mobility according to their M_r values (Fig. 3*D*). Electrophoretic mobility-shift assay analyses using an oligodeoxynucleotide containing the nonconsensus binding site yielded similar results to those described above for binding to the consensus site (data not shown).

Transcriptional Activation of the *JAC* Promoter by v-Jun. To determine whether Jun directly activates the chicken *JAC* promoter, the 5′ upstream region of the *JAC* gene (*p-JAC*, compare with Fig. 2*A*) was tested by CAT analysis. Transactivation of the promoter construct pCAT-*JAC* (see Fig. 4*E*) and of the analogous *BKJ* promoter construct pCAT-*BKJ* (17) was tested in

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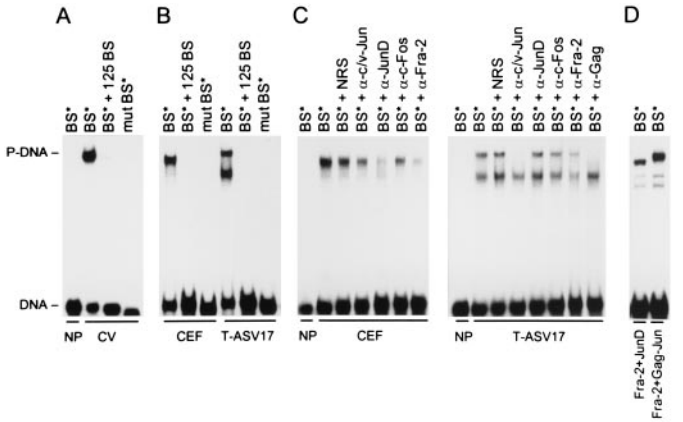

Fig. 3. Specific binding of Jun to the *JAC* promoter. A ^{32}P-labeled double-stranded oligodeoxynucleotide (4 nM) derived from the *JAC* promoter containing the consensus AP-1 binding site 5′-TGACTCA-3′ (BS*) or a mutant derivative thereof (mut BS*) were used as probes (1.0×10^5 cpm) in electrophoretic mobility-shift assays. (*A*) The probes were incubated with 140 ng of renatured recombinant chimeric Jun protein (CV) containing the DNA binding domain from v-Jun, or (*B* and *C*) with 5 μg of nuclear protein extracts derived from normal CEF or from the v-*jun*-transformed tumor cell line T-ASV17. In *A* and *B*, incubation was done in the absence or presence of the same oligodeoxynucleotide in unlabeled form (BS) as a specific competitor (125-fold excess); in *C*, incubation was carried out in the absence or presence of 0.33 μl of specific antisera (α) directed against the avian c-Jun/v-Jun, JunD, c-Fos, Fra-2 proteins, or the retroviral Gag polypeptide (NP, no protein; NRS, normal rabbit serum). (*D*) Binding of JunD/Fra-2 and Gag-Jun/Fra-2 heterodimers derived from *in vitro* transcription/translation reactions of DNA constructs carrying the coding regions from JunD, Fra-2, or Gag-Jun to the AP-1 consensus site. Autoradiography was performed for 4 h (*A* and *B*), for 13 h (*C*), or for 8 h (*D*) with an intensifying screen. The positions of free DNA and of protein–DNA (P-DNA) complexes are indicated in the margin.

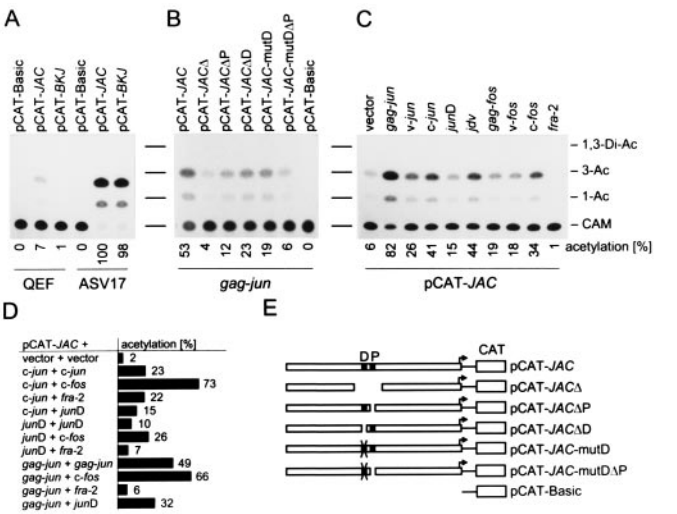

Fig. 4. Transcriptional activation of the *JAC* promoter by Jun. (*A*) Samples of 2.5 μg of DNA from CAT gene constructs containing either wild-type *JAC* (*p-JAC*, compare with Fig. 3*A*) or *BKJ* (17) promoters (pCAT-*JAC*, pCAT-*BKJ*) or no insert (pCAT-Basic) were transiently transfected into normal QEF and into QEF transformed by the retrovirus ASV17 encoding the Gag-Jun hybrid protein. Extracts containing equal amounts of proteins (30 μg) were used for acetylation of [^{14}C]chloramphenicol. (*B*) Samples of 0.5 μg of CAT gene constructs depicted in *E* containing either the wild-type *JAC* promoter or derivatives thereof lacking the proximal (P) or distal (D) AP-1 binding site (black boxes), or containing a mutated distal binding site (X), were used for transfection into QEF, together with 2.0 μg of the expression construct pRc-VJ0 encoding a Gag-Jun hybrid protein. (*C*) Samples of 0.5 μg of pCAT-*JAC* containing the wild-type *JAC* promoter were used for transfection into QEF together with 2.0 μg of pRc/RSV-derived expression constructs carrying various coding regions derived from members of the *jun* or *fos* gene families as indicated. (*D*) CAT analysis performed as in *C*, using 1.0-μg aliquots of two pRc/RSV-derived expression constructs. Extracts containing equal amounts of 20 μg or 40 μg total protein were tested in *B* and *D*, or in *C*, respectively. Reaction products were resolved by ascending TLC followed by autoradiography (3-h exposure). Positions of chloramphenicol (CAM), of acetylated products (1-Ac, 1-acetyl[^{14}C]chloramphenicol; 3-Ac, 3-acetyl[^{14}C]chloramphenicol; 1,3-Di-Ac, 1,3-diacetyl[^{14}C]chloramphenicol), and percentages of total acetylation are indicated in the margin and below the lanes, respectively.

normal QEF and in QEF infected by ASV17 encoding the Gag-Jun oncoprotein (Fig. 4*A*). The analysis revealed that Gag-Jun very efficiently transactivated both promoters, whereas in normal QEF only residual CAT activities were observed, apparently because of endogenous AP-1 complexes. To determine the functionality of the AP-1 binding sites present in the *JAC* promoter, derivatives of pCAT-*JAC* lacking one or both binding sites, or containing a mutated consensus binding site unable to bind Jun (compare with Fig. 3 *A* and *B*), were transiently cotransfected into QEF together with the pRc/RSV-derived expression plasmid encoding Gag-Jun (Fig. 4*B*). Strong transcriptional activation of the *JAC* wild-type promoter was detected with Gag-Jun, whereas deletion of the proximal site in addition to mutational inactivation of the distal AP-1 binding site (pCAT-*JAC*-mutDΔP) led to almost complete suppression of CAT activity, demonstrating the specificity of these Jun-binding motifs. When tested separately, each of the AP-1 binding sites was found to confer transcriptional activation (pCAT-*JAC*ΔP, pCAT-*JAC*ΔD), albeit at lower levels than the combined sites, indicating a synergistic effect of the tandem organization of the two AP-1 binding sites. The comparison of transcriptional activation potentials of the Gag-Jun (1), Gag-Fos (23), and JDV (32) oncoproteins and of the avian Jun and Fos proteins, c-Jun (4), JunD (32), c-Fos (33), and Fra-2 (36), showed that overexpressed Gag-Jun was the most efficient transactivator of the *JAC* promoter (Fig. 4*C*). Interestingly, Fra-2 was the only AP-1 component that did not activate but rather suppressed the *JAC* promoter when compared with the endogenous AP-1 activity level (Fig. 4*C*). Analysis of distinct AP-1 dimer combinations composed of overexpressed Jun and Fos proteins showed that only Gag-Jun homodimers, or heterodimers consisting of Gag-Jun/c-Fos or c-Jun/c-Fos, conferred strong transcriptional activation of the entire *JAC* promoter (Fig. 4*D*). Because Gag-Jun/Fra-2 heterodimers do not contribute to transcriptional activation of the *JAC* promoter (Fig. 4*D*), these results suggest that transactivation in v-*jun*-transformed cells is mediated either by Gag-Jun/c-Fos heterodimers or by Gag-Jun homodimers.

Overexpressed *JAC* Induces Cell Transformation. The strong and specific activation of *JAC* in v-*jun*-transformed cells prompted us to test whether overexpression of *JAC* by itself would adopt some parameters of the transformed phenotype. Hence, the chicken *JAC* coding sequence was inserted into the replication-competent retroviral RCAS vector (29), and the construct RCAS-JAC was transfected into CEF and QEF. The construct RCAS-HBEGF carrying the transforming *jun*-target gene *HB-EGF* (19) was transfected as a control. Transfections of the RCAS-VJ0 or RCAS-VJ1 constructs (27) encoding Gag-Jun or v-Jun proteins, respectively, resulted in full transformation manifested by focus formation after 1 week, whereas no prominent foci were induced by RCAS-JAC. However, when suspended into semisolid medium, cells overexpressing the *JAC* gene were capable of anchorage-independent growth similar to cells transfected with RCAS-HBEGF (Fig. 5 *A* and *C*). Although *JAC*-induced colonies were smaller in size than colonies derived from cells transformed by the *jun* oncogene, these results clearly indicate that cells transfected by RCAS-JAC display some of the

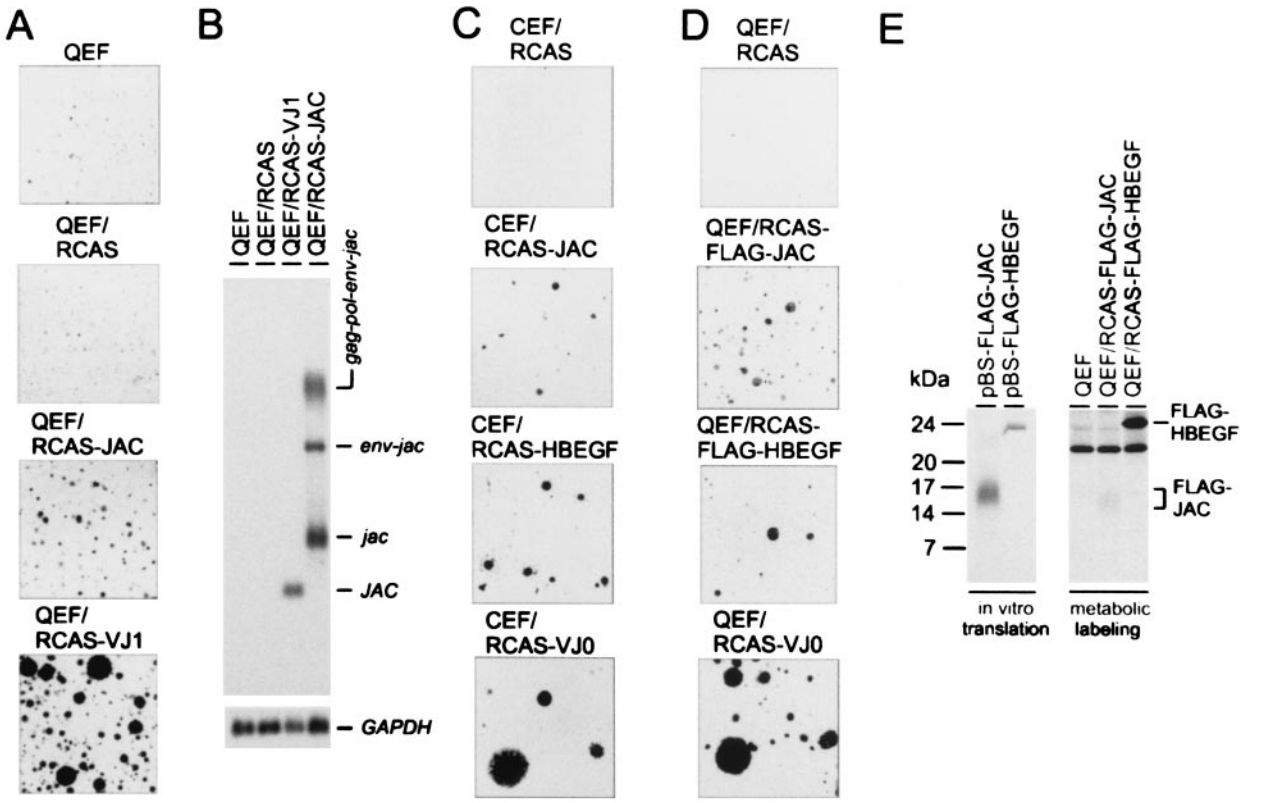

Fig. 5. Cell transformation by ectopic expression of *JAC*. (*A* and *C*) Agar colony formation by RCAS-JAC, RCAS-HBEGF, RCAS-VJ0, and RCAS-VJ1 transfected QEF (*A*) or CEF (*C*) carrying the coding regions of chicken *JAC*, *HB-EGF*, *gag-jun* (VJ0), or v-*jun* (VJ1). Equal numbers of cells, 1×10^5 (*A*) or 1×10^4 (*C*), including QEF or RCAS-transfected cells as controls, were seeded in soft agar on 35-mm dishes. Bright-field micrographs (×7.1) were taken after 14 days. (*B*) Northern analysis of 2.5-μg samples of poly(A)$^+$ RNAs isolated from the cell cultures shown in *A*. RNAs were hybridized with ^{32}P-labeled cDNA probes derived from quail *JAC* (1.4×10^6 cpm/ml, 69-h exposure) and from *GAPDH* (1.6×10^6 cpm/ml, 21-h exposure). The sizes of the mRNAs are as follows: endogenous *JAC*, 0.8 kb; retroviral genomic *gag-pol-env-jac*, 7.7 kb; retroviral subgenomic *env-jac*, 3.5 kb; retroviral subgenomic *jac*, 1.2 kb; and *GAPDH*, 1.4 kb. (*D*) Agar colony formation by RCAS-FLAG-JAC, RCAS-FLAG-HBEGF, and RCAS-VJ1 transfected QEF. Equal numbers of cells (5×10^4), including RCAS-transfected QEF as controls, were seeded in soft agar on 35-mm dishes. Bright-field micrographs (×7.1) were taken after 14 days. (*E*) SDS/PAGE (13%, wt/vol) of L-[^{35}S]cysteine-labeled and immunoprecipitated FLAG-JAC and FLAG-HBEGF proteins by using a monoclonal antibody directed against the amino-terminal positioned FLAG epitope. (*Right*) Equal aliquots of extracts (1.0×10^7 cpm) derived from metabolically labeled QEF transfected with RCAS-FLAG-JAC or RCAS-FLAG-HBEGF were analyzed. (*Left*) Proteins from *in vitro* transcription/translation reactions using the plasmid constructs pBS-FLAG-JAC or pBS-FLAG-HBEGF were analyzed. Fluorographs were exposed for 21 days (*Right*) or 5 h (*Left*). Positions of protein size markers are indicated in the margin. All exposures were done with intensifying screens.

typical parameters of the transformed state. Expression of *JAC* from the retroviral construct and ectopic activation of *JAC* in QEF transformed by RCAS-VJ1 was verified by Northern analysis (Fig. 5*B*). To monitor protein expression, RCAS-FLAG-JAC and RCAS-FLAG-HBEGF constructs encoding amino-terminal-tagged JAC or HB-EGF proteins were transfected into QEF, leading to colony formation of transformed cells in soft agar (Fig. 5*D*). The tagged proteins were immunoprecipitated from metabolically labeled lysates by using an antibody directed against the FLAG epitope (Fig. 5*E Right*). Whereas the overexpressed FLAG-HBEGF with a calculated M_r of 23,651 displayed an apparent M_r of 24,000 in SDS/PAGE, the FLAG-JAC protein with a calculated M_r of 8,154 migrated with an apparent size of ≈15,000. A similar anomalous electrophoretic mobility was also observed for the *in vitro* translated and immunoprecipitated FLAG-JAC protein (Fig. 5*E Left*).

Discussion

Oncogenic transcription factors are positioned at the end of known signal transduction cascades converting upstream incoming signals into changes of gene expression (2, 6). To understand the underlying molecular mechanisms leading to neoplastic transformation, the identification and functional characterization of genes aberrantly regulated by oncogenic gene regulators has become an indispensable prerequisite (2, 39, 40). However, the functional dissection of transformation pathways is severely complicated by the increasing evidence that cell transformation apparently involves changes in the expression patterns of multiple genes and even may depend on multiple interactions of transcription factors and coactivators (6, 40–42). Hence, the identification of transformation-relevant target genes acting as effectors downstream of oncogenic transcription factors represents an important step in deciphering this complex network. Several AP-1 target genes have been identified, but the possible role of most of those genes in *jun*-induced cell transformation has not been assessed yet. However, the recent identification of genes, like *BKJ* (16, 17), glutaredoxin (18), *HB-EGF* (19), or *JAC*, which are directly regulated by Jun and whose expression profiles correlate precisely with the *jun*-transformed cellular phenotype, strongly supports the hypothesis that cell transformation induced by oncogenic transcription factors is a consequence of aberrant transcriptional regulation of distinct target genes. Based on these criteria, and on its capacity to induce partial cell transformation, the *JAC* gene described here presumably belongs to the class of genes that are directly involved in *jun*-induced cell transformation and tumorigenesis.

The structural analysis of the chicken *JAC* locus revealed the presence of two AP-1 binding sites in the 5′ upstream region. The

noncanonical 5′-TGAGTAA-3′ motif has previously been identified as a key regulatory element in the promoter of the AP-1-controlled tissue inhibitor of metalloproteinases-1 gene (*Timp-1*) (13). Synergistic transcriptional activation by the presence of two AP-1 binding sites in the *JAC* promoter (compare with Fig. 5) may be explained by their close proximity and may suggest that two bound AP-1 complexes functionally interact with the basal transcription apparatus.

The elucidation of the biochemical function of the JAC protein will be necessary to assess the role of *JAC* in oncogenesis in molecular terms. Striking features of the predicted JAC protein are the high content of clustered cysteine residues, the aberrant electrophoretic mobility of the ectopically expressed protein, and its relationship to mammalian high-sulfur keratin-associated proteins (37). In analogy to those proteins, the physiological role of JAC could be that of a cytoskeletal matrix protein involved in avian epidermal development.

Because several transformation-relevant *jun*-target genes have now been identified, it is likely that only the combined action of distinct target gene products will constitute the fully transformed cellular phenotype caused by v-*jun*. Strong support for this hypothesis comes from the observations that the targets *HB-EGF* (19) or *TOJ3* (20), and the *JAC* gene described here, have the potential to induce some of the transformation parameters typical for the *jun*-transformed cellular phenotype. The identification and functional characterization of all relevant *jun*-target genes, either activated or suppressed, will reveal the molecular mechanisms of the *jun*-specific transformation program and will also identify possible overlaps with transformation pathways induced by other oncogenic transcription factors.

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