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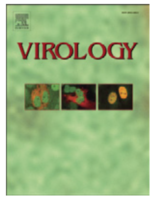

TOJ3, a v-*jun* target with intrinsic oncogenic potential, is directly regulated by Jun via a novel AP-1 binding motif

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The *TOJ3* gene was originally identified on the basis of its specific activation in avian fibroblasts transformed by the v-*jun* oncogene of avian sarcoma virus 17 (ASV17). Overexpression of *TOJ3* induces cellular transformation of embryonic avian fibroblasts, revealing an intrinsic oncogenic potential. Transforming activity has also been demonstrated for *MSP58*, the human homolog of *TOJ3*, and oncogenic cell transformation by *MSP58* is specifically inhibited by the tumor suppressor PTEN. To investigate the mechanism of aberrant *TOJ3* gene activation in *jun*-transformed fibroblasts, the entire quail *TOJ3* gene including 13 exons and the 5′ regulatory region was isolated. Functional analyses of the promoter by transcriptional transactivation assays revealed that the specific induction of *TOJ3* is mediated by a cluster of three noncanonical AP-1 binding motifs (5′-CAGCTCA-3′ or 5′-CACCTCA-3′) which share the 3′ half-site with the consensus motif (5′-TGA$^{C}/_{G}$TCA-3′). Electrophoretic mobility shift assays and chromatin immunoprecipitation analyses showed that Jun binds to these motifs with an affinity similar to that observed for binding to an AP-1 consensus site. Noncanonical binding sites are also present in the chicken and human *TOJ3*/*MSP58* promoter regions. These results confirm and extend the previous observation that *TOJ3* represents an immediate effector gene of Jun and may point to an essential role of *TOJ3*/*MSP58* in carcinogenesis involving aberrant AP-1 expression.

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Introduction

The dimeric AP-1 (activator protein-1) transcription factor complex containing c-Jun, c-Fos, or related proteins regulates the expression of genes relevant for cell proliferation and differentiation (Rauscher et al., 1988; Vogt, 2001, 2002; Eferl and Wagner, 2003). AP-1 binds with high affinity to the consensus sequence 5′-TGA$^{C}/_{G}$TCA-3′, or variants thereof, in the promoters of specific target genes (van Dam and Castellazzi, 2001; Eferl and Wagner, 2003). Numerous genes have been identified that are differentially expressed in cells transformed by the v-*jun* oncogene (Maki et al., 1987) of avian sarcoma virus 17 (ASV17), including genes which are directly regulated by AP-1 (van Dam and Castellazzi, 2001; Vogt, 2001; Hartl et al., 2003; Black et al., 2004; Iacovoni et al., 2004). However, only a few AP-1 targets have been identified that display intrinsic oncogenic potential, including *TOJ3*, originally discovered on the basis of its immediate and specific activation in avian fibroblasts transformed by the ASV17 v-*jun* oncogene (Bader et al., 2001). The 530-amino acid protein product (TOJ3) of the *TOJ3* gene is the avian homolog of microspherule protein 1 (MSP58/MCRS1), containing a bipartite nuclear localization motif and a phosphopeptide binding module (forkhead-associated domain, FHA) (Bader et al., 2001). Human MSP58 is localized in the nucleus and presumably acts in concert with distinct protein binding partners as a cell cycle-dependent transcriptional cofactor important for cell proliferation (Lin and Shih, 2002; Shimono et al., 2005; Du et al., 2006). Furthermore, MSP58 interacts with transformation associated proteins (Bruni and Roizman, 1998; Ren et al., 1998) or tumor suppressors (Okumura et al., 2005), including PTEN that frequently shows a loss of function in various human cancer types (Simpson and Parsons, 2001). Direct interaction with PTEN abrogates the oncogenic function of MSP58 (Okumura et al., 2005), suggesting that the *TOJ3*/*MSP58* genes may play an important role in human cancer.

Results and discussion

To explore the mechanism of aberrant transcriptional *TOJ3* activation, we have isolated the entire quail *TOJ3* gene. Using a 5′-fragment of *TOJ3* cDNA as a probe, a genomic quail library was screened leading to the isolation of two overlapping DNA fragments of 1141 and 3926 bp, respectively. The assembled 4538-bp nucleotide sequence encompasses the promoter region and 13 exons of the *TOJ3*

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gene (Fig. 1A). The promoter region contains 1089-bp of 5′-untranscribed region, the previously mapped transcription start site (Bader et al., 2001), and a potential TATA box at nucleotide positions −28 to −23. No consensus AP-1 binding site (5′-TGA$^{C}/_{G}$TCA-3′) was found, but 19 potential nonconsensus AP-1 binding motifs with the sequence 5′-CAGCTCA-3′ (18×) or 5′-CACCTCA-3′ (1×). In both motifs, the 3′-half (CTCA) of the consensus site is retained (Fig. 1B). Three of the 19 motifs are located in close proximity to the transcription start site (−100 to −94, −81 to −75, −62 to −56), referred to as the distal (D), central (C), or proximal (P) site. Comparison of the quail *TOJ3* promoter with the corresponding region of the chicken *TOJ3* gene (from contig NW_001471752 of the chicken genome sequence) showed a high degree of conservation from nucleotide positions −69 to +8 including the proximal 5′-CACCTCA-3′ motif (Fig. 1C). Furthermore, six 5′-CAGCTCA-3′ motifs are present in the chicken *TOJ3* promoter. *MSP58*, the human *TOJ3* homolog, is located on chromosome 12 and contained within contig NT_029419.11 of the human genome sequence. Notably, the overall *MSP58* gene topology is very similar to that of *TOJ3*, encompassing 15 exons and displaying a cluster of 15 variant AP-1 binding sites in the 5′ upstream region (not shown), some of which have been described as functional sites in other AP-1 targets.

For functional promoter analysis, DNA fragments encompassing the quail or chicken *TOJ3* promoter were inserted into chloramphenicol acetyltransferase (CAT) reporter plasmids yielding the constructs pCAT-qTOJ3 and pCAT-cTOJ3. Transient transfection of the pCAT-qTOJ3 or pCAT-cTOJ3 plasmids into ASV17-transformed quail embryo fibroblasts (QEF) revealed that both avian *TOJ3* promoters are strongly activated, even stronger than the promoter of the direct AP-1 target

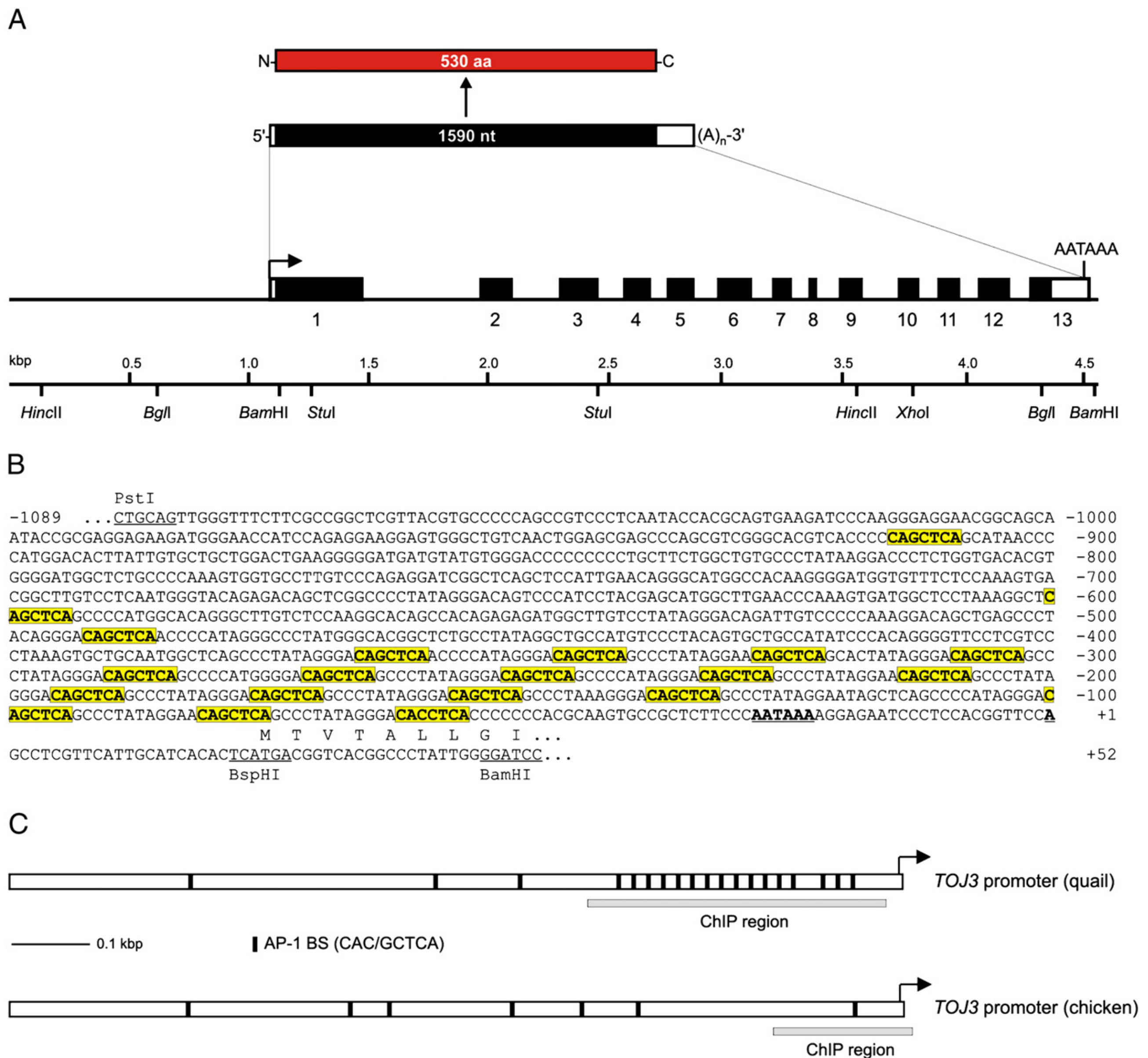

Fig. 1. Structure of the quail *TOJ3* gene. (A) The schematic diagram indicates the 13 exons with the coding region shown in black, the transcriptional initiation site (arrow), a polyadenylation signal, restriction enzyme cleavage sites, and the structures of mRNA and protein product. (B) Nucleotide sequence of a 1141-bp *TOJ3* promoter/exon1 fragment, containing 19 nonconsensus AP-1 binding motifs (5′-CA$^{C}/_{G}$CTCA-3′), a TATA box (5′-AATAAA-3′), the previously mapped transcriptional start site at position +1 (Bader et al., 2001), and the translational start site. (C) Diagram of the chicken and quail *TOJ3* promoters showing the positions of potential AP-1 binding sites (BS) and of the transcriptional start sites (arrows). BLAST analysis of quail *TOJ3* promoter sequences was used to identify the corresponding region in the chicken genome. The regions amplified for chromatin immunoprecipitation (ChIP) analyses are indicated. The nucleotide and deduced amino acid sequences reported here have been deposited in the GenBank database (accession no. EU116502).

BKJ (Hartl and Bister, 1995, 1998) containing two consensus AP-1 binding sites (Fig. 2A). No promoter activities were observed in v-*myc*-transformed cells or in normal QEF. To test directly if AP-1 components are necessary for transcriptional activation, the promoter construct pCAT-qTOJ3 was cotransfected with eucaryotic expression vectors specifying v-Jun, c-Jun, c-Fos, the Jun- and Fos-related proteins JunD and Fra-2, or v-Myc into normal QEF (Fig. 2B). The analysis revealed that high level expression of v-Jun led to strong activation of the *TOJ3*

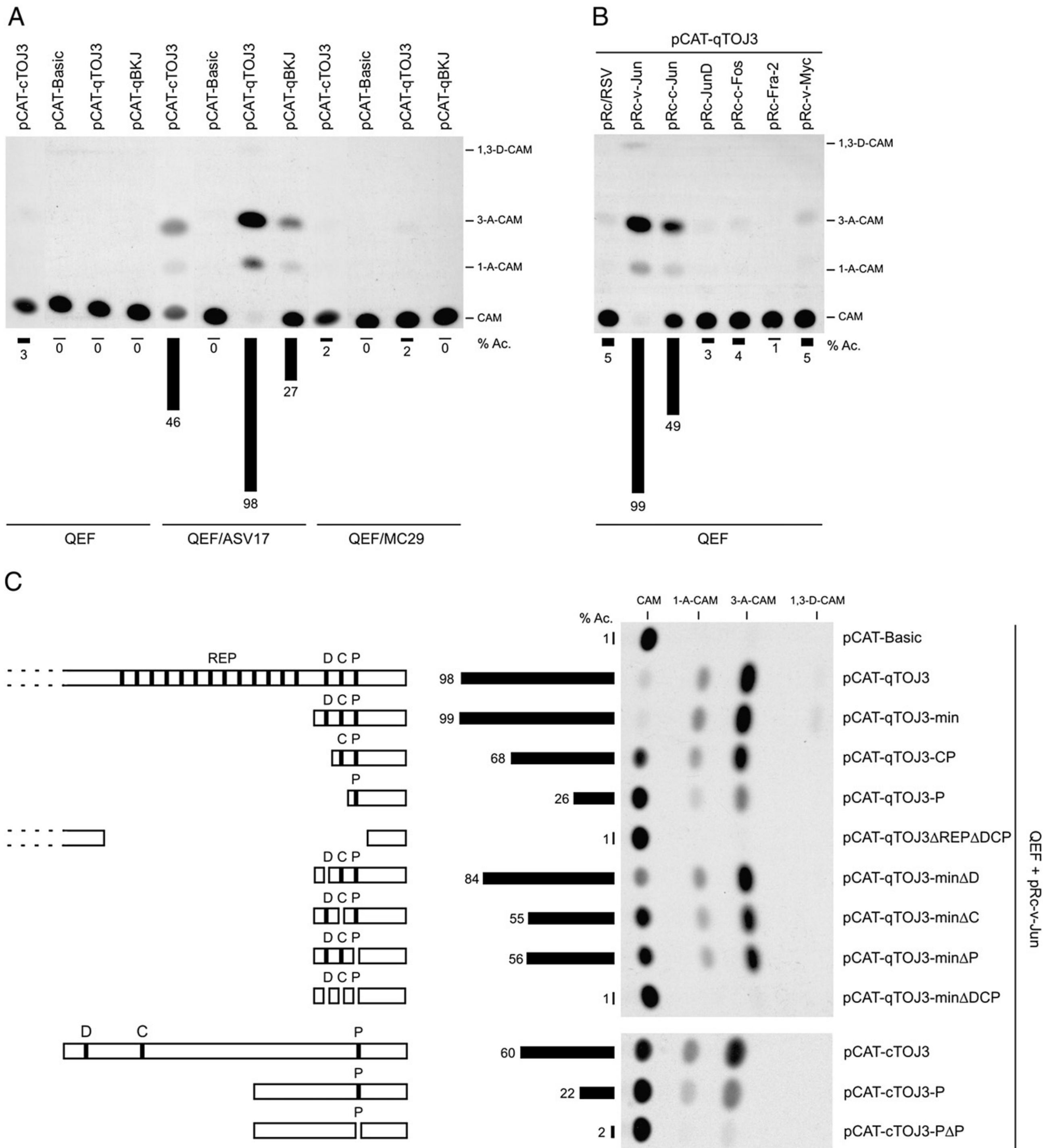

Fig. 2. Specific activation of the *TOJ3* promoter mediated by nonconsensus AP-1 binding sites. (A) 5.0 µg aliquots of DNA from the CAT gene constructs pCAT-qTOJ3 or pCAT-cTOJ3 containing the quail or chicken *TOJ3* promoter regions, respectively, were transfected into normal quail embryo fibroblasts (QEF) or into QEF transformed by the ASV17 or MC29 retroviruses encoding Gag-Jun (Maki et al., 1987) or Gag-Myc (Bister et al., 1977) hybrid proteins, respectively. Equal amounts of DNA from the empty pCAT-Basic vector (Promega) and from the pCAT-qBKJ promoter construct of the direct AP-1 target gene *BKJ* (Hartl and Bister, 1995, 1998) containing two consensus AP-1 binding sites (5′-TGACTCA-3′) were used as controls. Aliquots of total cell extracts containing equal amounts of protein (10 µg) were used for the acetylation reactions. Reaction products were resolved and visualized by autoradiography. Positions of [^{14}C]chloramphenicol (CAM), of acetylated products (1-acetyl-, 3-acetyl-, 1,3-diacetyl-[^{14}C]chloramphenicol), and extent (%) of total acetylation are indicated. (B) 2.5 µg aliquots of pCAT-qTOJ3 DNA were transfected into QEF together with equal amounts of DNA from the pRc/RSV empty vector, or from expression constructs encoding v-Jun, c-Jun, JunD, c-Fos, Fra-2, or v-Myc proteins. Cell extracts containing equal amounts of protein (10 µg) were tested. (C) Mutational analysis of quail and chicken *TOJ3* promoter activity. Equal amounts of the CAT gene constructs (2.5 µg) were transfected into QEF together with 2.5 µg aliquots of the pRc-v-Jun expression vector. CAT analysis was performed using cell extracts containing equal amounts of protein (2.5 µg).

promoter. Moderate activation was also observed upon ectopic expression of the cellular counterpart c-Jun, which is in agreement with a weak increase of *TOJ3* mRNA levels in c-*jun*-transformed cells (not shown). No activation was seen with overexpressed c-Fos, JunD, Fra-2, or v-Myc.

In order to localize the *TOJ3* promoter region essential for the specific activation by v-Jun, several pCAT-qTOJ3 and pCAT-cTOJ3 derivatives were generated with progressive deletions, including mutants of a minimal quail (pCAT-qTOJ3-min) or chicken *TOJ3* promoter (pCAT-cTOJ3-P) with deletions of the distal, central, or proximal AP-1 binding sites. These constructs were cotransfected into QEF together with an expression vector encoding the v-Jun protein (Fig. 2C). The analysis revealed that the 5′-deletion mutant pCAT-qTOJ3-min containing 131 bp with three potential AP-1 binding sites retained full promoter activity, whereas the construct pCAT-qTOJ3-ΔREPΔDCP lacking these sites has no activity. The integrity of the minimal promoter fragment is essential, since successive deletions yielding the constructs pCAT-qTOJ3-CP and pCAT-qTOJ3-P led to diminished CAT activity (Fig. 2C). To directly investigate if the three heptameric binding motifs are essential for transcriptional activation, mutants from pCAT-qTOJ3-min were generated in which the 7-bp sequences representing the distal (pCAT-qTOJ3-minΔD), the central (pCAT-qTOJ3-minΔC), or the proximal (pCAT-qTOJ3-minΔP) site were deleted individually. Inactivation of any site led to a reduction in transcriptional activity, whereas specific deletion of all three binding motifs (pCAT-qTOJ3-minΔDCP) led to complete abrogation of CAT activity, suggesting that these sites act synergistically. Consistently, a deletion mutant of the chicken *TOJ3* promoter construct containing only the proximal AP-1 binding site (pCAT-cTOJ3-P) gave rise to reduced CAT activity, whereas deletion of this site (pCAT-cTOJ3-PΔP) led to complete promoter inactivation (Fig. 2C).

To directly demonstrate specific interaction of Jun with the noncanonical AP-1 binding sites in the *TOJ3* promoter, double-stranded 23-mer oligodeoxynucleotides containing the 5′-CAGCTCA-3′ or 5′-CACCTCA-3′ motifs were used for electrophoretic mobility shift assays (EMSA) (Fig. 3A). A homodimer of recombinant Jun protein bound to this DNA probe with equal specificity compared to a control probe containing the consensus AP-1 site from the promoter of the direct Jun target *BKJ* (Hartl and Bister, 1995, 1998). The specificity of the protein-DNA interactions was demonstrated by adding the unlabeled DNA probe as a competitor (BS), or an oligodeoxynucleotide with a mutated AP-1 binding site (BS-mut) as a control (Fig. 3A). Competition with unlabeled DNA probe was also used to estimate the dissociation constant (K_D) of the protein-DNA complex as described previously (Papoulas et al., 1992; Hartl and Bister, 1998) (Fig. 3B). For binding of Jun to the consensus AP-1 site in the *BKJ* promoter (Hartl and Bister, 1998), a K_D of 0.8×10^{-7} M was determined, whereas for binding to the 5′-CAGCTCA-3′ or the 5′-CACCTCA-3′ element in the *TOJ3* promoter K_D values of 2.0×10^{-7} M or 1.1×10^{-7} M were measured, respectively.

To test if these sites were also recognized by AP-1 protein complexes formed *in vivo*, EMSA and supershift EMSA were performed using nuclear extracts prepared from ASV17-transformed QEF or from normal QEF (Fig. 4A). Specific binding activity was detected in nuclear extracts containing v-Jun. The specificity of binding was demonstrated by unlabeled probe competition and by complete (α-Jun) or partial (a-Fos) antibody inhibition. As reported previously, Jun or Fos antibodies inhibit DNA binding in EMSA analyses, rather than inducing supershifts (Hartl et al., 2001; 2006). Application of normal rabbit serum (NRS) did not interfere with DNA binding (Fig. 4A). The extracts both from normal or transformed QEF also contained proteins that formed uniform complexes with the probe. However, formation of these bands was not inhibited by Jun- or Fos-specific antisera, in contrast to the additional larger complexes observed only in extracts from v-*jun*-transformed cells (Fig. 4A). Further independent evidence for direct binding of Jun complexes to the *TOJ3* promoter was obtained by chromatin immunoprecipitation (ChIP) analysis (Fig. 4B), using cross-linked chromatin from QEF/ASV17 or CEF/ASV17 cells, Fos-, Jun-, or Gag-specific antibodies, and PCR primer pairs for amplification of a 381-bp or a 177-bp DNA segment encompassing the functional AP-1 binding sites of the quail or chicken *TOJ3* promoter, respectively (cf. Fig. 1C). PCR analysis specifically produced a 381-bp or a 177-bp DNA fragment when Jun- or Gag-specific antibodies were used for the ChIP analysis, corroborating the finding that the Gag-Jun hybrid protein of ASV17 binds specifically to the AP-1 motifs present in the quail and chicken *TOJ3* promoter. Low

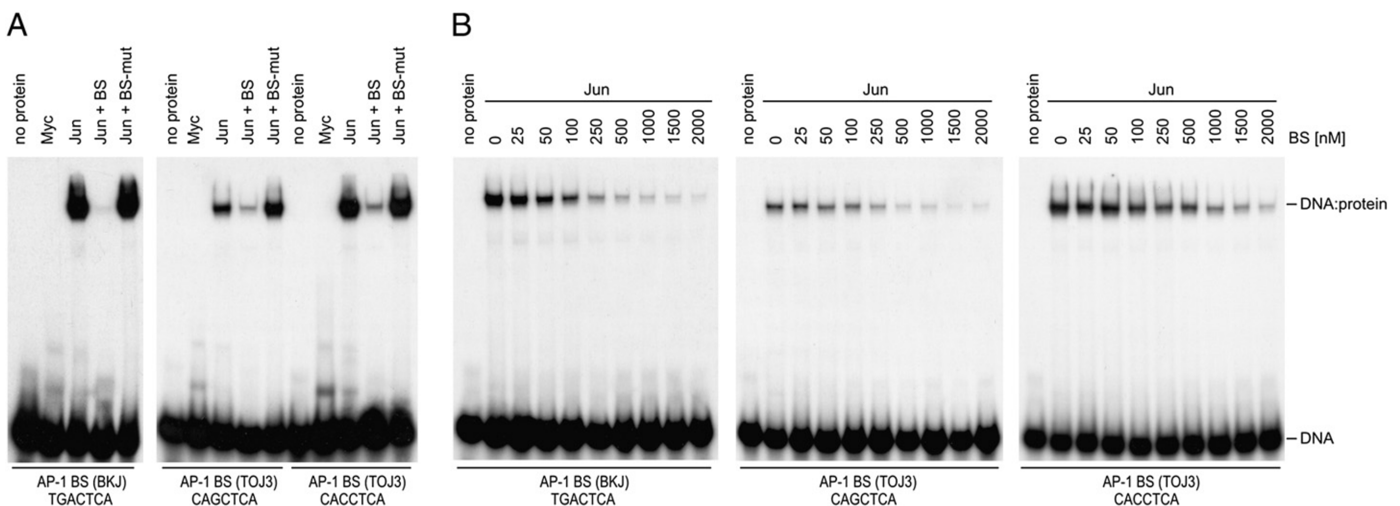

Fig. 3. Specific binding of recombinant Jun to the *TOJ3* promoter. (A) ^{32}P-labeled 23-bp double-stranded oligodeoxynucleotides (AP-1 BS; 10 nM) containing the consensus AP-1 binding site (5′-TCCAGGCCTGACTCAGCAGCCTC-3′) from the quail *BKJ* promoter (Hartl and Bister, 1998), or the central (5′-TATAGGAACAGCTCAGCCCTATA-3′) or the proximal (5′-TATAGGGACACCTCACCCCTATA-3′) nonconsensus AP-1 binding motif from the quail *TOJ3* promoter (cf. Fig. 1) were used as probes (1.5×10^5 cpm) for electrophoretic mobility shift assays (EMSA). The probes were incubated with no protein, or with solubilized and renatured proteins (0.8 μg) from washed inclusion bodies containing v-Myc recombinant protein Myc p16 (Kerkhoff et al., 1991) or chicken Jun recombinant protein CV (Hartl et al., 2001), in the absence or presence of a 125-fold excess (1.25 mM) of unlabeled wild type (BS) or mutated (BS-mut; 5′-TATAGGAAAGACCCAGCCCTATA-3′) AP-1 binding site oligodeoxynucleotide. Autoradiography was performed for 6 h using an intensifying screen. (B) Dissociation constants of Jun–DNA complexes were determined using the same DNA probes (5 nM) as in (A) and Jun recombinant protein CJ (0.6 μg). Binding to the labeled probes was competed by increasing amounts of unlabeled AP1-BS oligodeoxynucleotides as indicated, and the K_D values were calculated as described (Papoulas et al., 1992; Hartl and Bister, 1998).

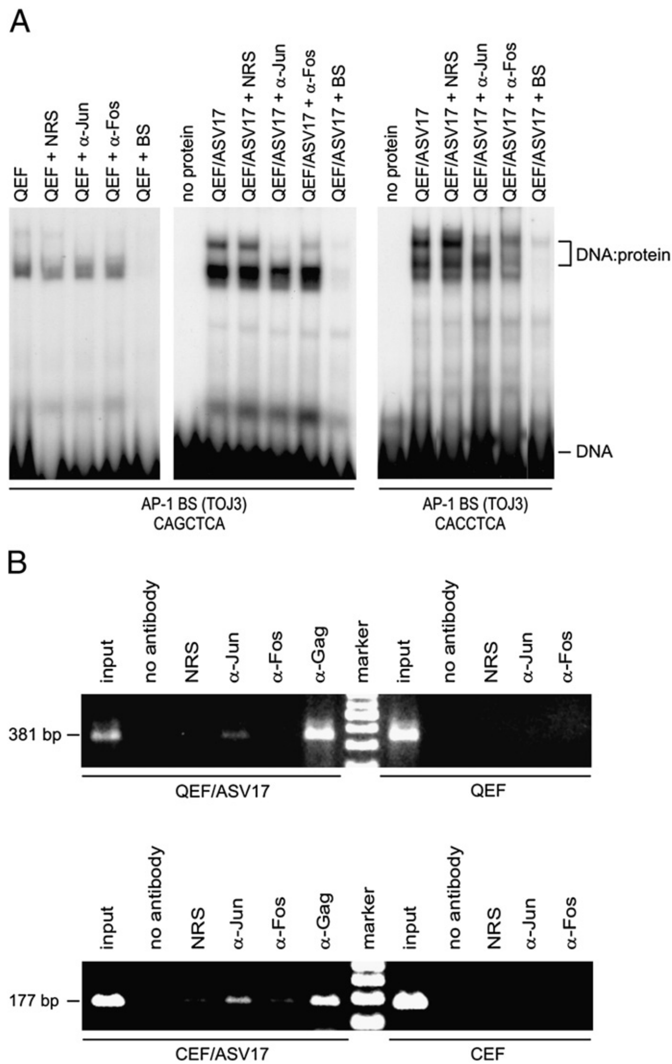

Fig. 4. Specific binding of Jun from ASV17-transformed cells to the *TOJ3* promoter. (A) EMSA analysis using the same ^{32}P-labeled *TOJ3*-specific DNA probes (5 nM; 4.0×10^4 cpm) as in Fig. 3 and equal amounts of nuclear proteins (14 μg) from normal QEF or from ASV17-transformed QEF. Incubations were also carried out in the presence of 1 μl aliquots of normal rabbit serum (NRS), or of specific antisera (α) directed against Jun or Fos proteins, or in the presence of unlabeled AP-1 BS competitor DNA (500 nM). Autoradiography was performed for 48 h using an intensifying screen. (B) Chromatin-immunoprecipitation (ChIP) analysis of sheared extracts derived from formaldehyde-treated ASV17-transformed QEF or CEF, or normal QEF or CEF. Immunoprecipitations were carried out using Jun-, Fos- or Gag-specific antibodies followed by PCR amplification of a 381-bp or a 177-bp fragment from the quail or chicken *TOJ3* promoter regions, respectively, containing the essential AP-1 binding sites (cf. Fig. 1B). Control reactions included samples treated with no antibody or with NRS. PCR products were analyzed by agarose (1.4%, wt/vol) gel electrophoresis, and visualized by ethidium bromide staining.

amounts of the PCR fragments were also produced when α-Fos serum was used, in agreement with the partial inhibition of complex formation in the EMSA analysis by Fos serum, indicating that the ASV17 Gag-Jun protein at least partially recruits endogenous Fos for dimerization. As a control, ChIP was performed using chromatin from normal QEF or CEF yielding no specific PCR products, in agreement with the lack of Jun- or Fos-related complex formation in the EMSA analysis using normal cell extracts (cf. Fig. 4A).

These results show that the *TOJ3* gene is a direct transcriptional target of Jun in *jun*-transformed cells. Notably, high-affinity interaction with the *TOJ3* promoter occurs via nonconsensus AP-1 binding motifs. In view of its intrinsic oncogenic potential (Bader et al., 2001), *TOJ3* belongs to the important group of AP-1 target genes which may contribute directly to malignant cell transformation. To reveal the specific role of the TOJ3 protein in carcinogenesis, elucidation of its biochemical function will be required.

Materials and methods

Cells and retroviruses

Preparation, culture, and transformation of chicken or quail embryo fibroblasts (CEF, QEF) derived from fertile Japanese quail (*Coturnix japonica*) or chicken (*Gallus gallus*) eggs have been described (Hartl and Bister, 1998; Bader et al., 2001; Hartl et al., 2001, 2006). Infection of QEF with the avian retroviruses ASV17 (Maki et al., 1987) or MC29 (Bister et al., 1977) encoding Gag-Jun or Gag-Myc hybrid proteins, respectively, and of CEF with ASV17 was performed as described previously (Hartl and Bister, 1998; Hartl et al., 2001).

DNA cloning and nucleic acid analyses

DNA labeling, plaque hybridization, and subcloning were performed as described (Hartl and Bister, 1998; Bader et al., 2001; Hartl et al., 2001, 2006). For the isolation and sequencing of overlapping DNA fragments encompassing the *TOJ3* gene, a quail genomic library was screened as described (Hartl and Bister, 1998) using a 165-bp NcoI fragment derived from the 5′-region of *TOJ3* cDNA as a probe (Bader et al., 2001). Enzymatic DNA sequencing from subcloned DNA fragments was performed as described using ^{35}S-α-dATP (Hartl and Bister, 1998) or fluorescence-labeld dideoxynucleotides (Applied Biosystems) in cycle sequencing reactions. Reaction products were resolved either by denaturing polyacrylamide gelelectrophoresis as described (Hartl and Bister, 1998) or by using a 16-capillary sequenator (Applied Biosystems), respectively. Bioinformatic analyses of nucleotide sequences and sequence interpretation were performed as described (Hartl and Bister, 1998; Bader et al., 2001; Hartl et al., 2001, 2006). BLAST analyses of quail *TOJ3* promoter and of cDNA sequences were used to identify the corresponding region in the chicken genome (accession no. NW_001471752). The nucleotide sequence of the quail *TOJ3* gene has been deposited in the GenBank database (accession no. EU116502).

Transactivation analysis

The construct pCAT-qBKJ containing the quail *BKJ* promoter has been described (Hartl and Bister, 1998; Hartl et al., 2001). For construction of the chloramphenicol acetyltransferase (CAT) reporter plasmid pCAT-qTOJ3, pCAT-Basic was cut with XbaI, filled in with Klenow polymerase, and then cut with PstI. A 1107-bp quail *TOJ3* promoter fragment generated by cleavage with BspHI at the 3′ end, treatment with mung bean nuclease, and cleavage with PstI at the 5′ end, was then inserted. To construct pCAT-cTOJ3, a chicken genomic DNA fragment corresponding to the quail *TOJ3* promoter (nt positions 1055–1511 of sequence contig NW_001471752) was amplified by PCR and inserted into the pCAT-Basic vector using the polylinker PstI/XbaI restriction enzyme sites. Sequencing of the promoter insert revealed four nucleotide substitutions and a single nucleotide deletion compared to the NW_001471752 database sequence (A/G, T/A, A/G, C/T, ΔC at positions 1116, 1180, 1365, 1393, 1401, respectively). pCAT-qTOJ3-min contains nucleotides −107 to +22 of the quail *TOJ3* promoter and was generated by PCR using pCAT-qTOJ3 as a template and primers containing HindIII and XbaI restriction enzyme sites for ligation into pCAT-Basic. pCAT-qTOJ3-CP and pCAT-qTOJ3-P encompass nucleotides −89 to +22 or −75 to +22 and were generated by PCR using pCAT-qTOJ3-min or pCAT-qTOJ3-CP as a template, and specific primers containing PstI and XbaI restriction enzyme sites. To construct pCAT-qTOJ3ΔREPΔDCP, PCR mutagenesis using overlapping primers and pCAT-qTOJ3 as a template was performed leading to deletion of nucleotides −383 to −49. In pCAT-qTOJ3-minΔD, pCAT-qTOJ3-minΔC,

pCAT-qTOJ3-minΔP, or pCAT-qTOJ3-minΔDCP, the distal (D), central (C), proximal (P), or all (DCP) heptamer motifs representing AP-1 binding sites (black boxes) were deleted using PCR mutagenesis. pCAT-cTOJ3-P was generated by deleting a PstI/PvuII fragment containing nucleotides 1055–1307 of sequence NW_001471752 from pCAT-cTOJ3. Deletion of the proximal (P) AP-1 binding site by PCR mutagenesis using pCAT-cTOJ3-P as a template yielded a cTOJ3-PΔP fragment which was inserted into a HindIII/XbaI-cut pCAT-Basic vector. The eucaryotic expression vectors pRc-c-Jun, pRc-JunD, pRc-v-Jun, pRc-c-Fos, pRc-Fra-2, pRc-v-Myc expressing the relevant proteins at approximately equal amounts have been described (Hartl et al., 2001, 2006). DNA transfer into cells by the calcium phosphate method, CAT analysis and quantification of radioactive signals using a phosphor imager was performed as described (Hartl and Bister, 1998; Hartl et al., 2001, 2006), and all results were confirmed in several independent experiments.

Protein–DNA interaction analyses

The preparation of solubilized and renatured proteins from washed inclusion bodies containing v-Myc recombinant protein Myc p16 (Kerkhoff et al., 1991) or chicken Jun recombinant proteins CV (Hartl et al., 2001) and CJ (Hartl and Bister, 1998), the preparation of nuclear extracts from subconfluently grown cells, the generation of ^{32}P-labeled double-stranded oligodeoxynucleotides, and electrophoretic mobility shift assays (EMSA) were carried out as described (Hartl and Bister, 1998; Hartl et al., 2001, 2006). Dissociation constants of Jun–DNA complexes were determined by competition of binding reactions with increasing amounts of unlabeled AP-1 BS oligodeoxynucleotides. For estimation of the dissociation constant (K_D), radioactive areas of the dried gel were quantified by phosphorimaging. Ratios of bound and free DNA concentrations were determined, and free protein concentrations were calculated from the equilibria equations of normal and competed binding reactions. K_D was then determined according to K_D=[DNA] [protein]/[DNA–protein] (Papoulas et al., 1992; Hartl and Bister, 1998).

Chromatin-immunoprecipitation (ChIP) analyses were carried out using sheared extracts derived from formaldehyde-treated ASV17-transformed QEF or CEF, or normal QEF or CEF as described (Hartl et al., 2006; Reiter et al., 2007). Immunoprecipitations were performed with Jun-, Fos- or Gag-specific antibodies followed by PCR amplification of a 381-bp or a 177-bp fragment from the quail or chicken *TOJ3* promoter regions, respectively, using the quail *TOJ3*-specific primer pair 5′-TCGTCCCTAAAGTGCTGC-3′ and 5′-TATTGGGAAGAGCGGCAC-3′ encompassing nucleotides −405 to −25, or with the chicken *TOJ3*-specific primer pair 5′-CCTACGGTGCTGCCGTAT-3′ and 5′-AAGAAAC-GAGGCTGGCAC-3′ encompassing nucleotide positions 1323 to 1500 of sequence contig NW_001471752.

Acknowledgments

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