DNA AND CELL BIOLOGY
Volume 15, Number 10, 1996
Mary Ann Liebert, Inc.
Pp. 827–844

Expression, Characterization, and Genomic Structure of Carp JAK1 Kinase Gene

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ABSTRACT

A 3.7-kb cDNA encodes the carp JAK1 kinase of 1,156 amino acid residues. The overall amino acid sequence identity between carp JAK1 and murine JAK1, JAK2, JAK3, and human TYK2 is 57%, 35.5%, 31.3%, and 42.4%, respectively. In addition, carp JAK1 shows higher sequence homology to mammalian JAK1 in both the kinase-like (JH2) and kinase (JH1) domains (approximately 70% identity). Therefore, carp JAK1 is a homolog of mammalian JAK1. To investigate the possible function of JH2 domain, full-length, and various truncated forms of carp JAK1 were produced in the baculovirus system. Our results demonstrate that c-JH1 and c-JH2 associate with each other and c-JH2 can be tyrosine-phosphorylated by c-JAK1 and by c-JH(1 + 2). The *JAK1* gene was also isolated from a carp genomic library and characterized. This gene is divided into 24 exons spanning at least 31 kb of genomic DNA. Exon 1 contains the 5′-untranslated region and exon 2 contains the putative translation initiation site. The 2.5-kb DNA region upstream of the transcription initiation site contains numerous potential binding sites for transcription factors including NF-IL6, HNF-5, AP1, GHF-5, and E2A. When this DNA fragment was placed upstream of the chloramphenicol acetyltransferase (CAT) reporter gene and transfected into a carp CF cell line, it could drive the synthesis of CAT enzyme 16 times more efficiently than the promoterless pCAT-Basic. Deletion analysis defined a positive regulatory region between −1,023 and −528. A smaller region (−181 to +59) without any typical TATA-box sequences, G + C-rich sequences, or other binding sequences for known transcription factors still had promoter activity. Constructs without this region did not have detectable promoter activity. This suggests that this region of DNA may play an important role in the expression of carp *JAK1* gene.

INTRODUCTION

TYROSINE PHOSPHORYLATION catalyzed by protein tyrosine kinases (PTKs) is a key step in transducing signals from external stimuli to the nucleus. Therefore, PTKs play very important roles in the regulation of cell proliferation and differentiation (Hunter and Cooper, 1985; Hanks *et al.*, 1988). On the basis of their structural similarities, PTKs can be divided into two major groups: receptor and nonreceptor PTKs. Receptor PTKs contain extracellular, transmembrane, and cytoplasmic domains. The extracellular domains are able to associate with ligands, where the cytoplasmic catalytic domains are responsible for transmission of the external signals to the cell. Upon binding of the ligand, the kinase activity of the receptor PTK is induced, resulting in phosphorylation of the receptor and cellular substrates on tyrosine residues (Ullrich and Schlessinger, 1990). By contrast, the nonreceptor PTKs, due to the lack of extracellular domains, cannot directly interact with extracellular ligands. Therefore, the physiological functions of these nonreceptor PTKs remained obscure for quite some time until the observation that $p56^{lck}$ was associated with the CD4 and CD8 co-receptors (Veillette *et al.*, 1988). This suggests that by binding nonreceptor PTKs, some transmembrane receptors lacking TK domains can act in a manner analogous to the receptor PTKs.

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The polymerase chain reaction (PCR) (Mullis and Faloona, 1987) has been employed to clone potential PTKs, using degenerate oligonucleotide primers corresponding to conserved peptide fragments in kinase domains. The JAK (Janus kinase) family was first identified by this strategy (Wilks, 1989; Wilks *et al.*, 1991). This family belongs to the nonreceptor PTKs and currently consists of JAK1 (Wilks *et al.*, 1991; Yang *et al.*, 1993), JAK2 (Harpur *et al.*, 1992), JAK3 (Witthuhn *et al.*, 1994), and TYK2 (Firmbach-Kraft *et al.*, 1990) in mammals. These kinases lack the Src homology domains SH2 and SH3 (Pawson and Schlessinger, 1993) but bear an unusual feature of having a kinase-like domain. The functional role of TYK2 was first demonstrated by its participation in interferon (IFN) signal transduction pathway and its association with the IFN-α receptor (Velazquez *et al.*, 1992). Recently, many cytokine receptors have been found to associate with one or two or more members of the JAK family, including the receptors for erythropoietin (EPO) (Witthuhn *et al.*, 1993), growth hormone (Argetsinger *et al.*, 1993), prolactin (Campbell *et al.*, 1994; Da Silva *et al.*, 1994; Rui *et al.*, 1994), interleukin 2 (IL-2) (Miyazaki *et al.*, 1994; Russell *et al.*, 1994; Witthuhn *et al.*, 1994), IL-3 (Silvennoinen *et al.*, 1993), IL-4 (Witthuhn *et al.*, 1994), IL-6 (Stahl *et al.*, 1994), IL-12 (Bacon *et al.*, 1995), oncostatin M, leukemia inhibitory factor (LIF) (Stahl *et al.*, 1994), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Quelle *et al.*, 1994), granulocyte colony-stimulating factor (G-CSF) (Nicholson *et al.*, 1994), and IFN-γ (Muller *et al.*, 1993; Watling *et al.*, 1993). Although lacking protein kinase domains, the above members of the cytokine receptor superfamily can couple ligand binding with the tyrosine phosphorylation of downstream effectors, which is mediated by association with the JAKs. The activated JAKs then phosphorylate cytoplasmic transcription factors STATs or the signal transducer and activators of transcription. After tyrosine phosphorylation, these transcription factors are homo- or heterodimerized, translocated into the nucleus, and induce transcriptional responses (Shuai *et al.*, 1993; Darnell *et al.*, 1994; Ihle *et al.*, 1994; Schindler and Darnell, 1995).

In fish, growth hormone and prolactin from many species have been isolated and cloned (Chang *et al.*, 1992a,b; Bernardi *et al.*, 1993), whereas only one IFN from flatfish has been purified and cloned (Tamai *et al.*, 1993). Based on a variety of studies from mammals, the JAK family members are shown to be involved in the signal transduction of growth hormone (Argetsinger *et al.*, 1993), prolactin (Campbell *et al.*, 1994; DaSilva *et al.*, 1994), and interferons (Velazquez *et al.*, 1992). Therefore, as an initial step to understand the signal transduction in fish, we started to isolate the carp JAK kinase family by PCR. As reported in this paper, a member of the JAK family was cloned and designated carp JAK1 kinase. The carp JAK1 kinase has a higher sequence homology in both JH1 and JH2 domains (70% identity) to human and murine JAK1 (Wilks *et al.*, 1991; Yang *et al.*, 1993). Because the function of the JH2 domain is unknown, we explored its activity in a baculovirus expression system, which has been used to express mammalian JAK2 kinase (Quelle *et al.*, 1994; Duhe and Farrar, 1995). We made constructs that encode full-length JAK1 (c-JAK1), the JH1 and JH2 domains alone (c-JH1 and c-JH2), and the JH1 and JH2 domains in combination [c-JH(1 + 2)] and expressed them in the baculovirus system. Coinfection and immunoprecipitation experiments showed that c-JH1 and c-JH2 could associate with each other, and that c-JH2 could be tyrosine-phosphorylated by c-JAK1 or c-JH(1 + 2). Our results may provide clues concerning the function of the JH2 domain.

Studies by others showed that expression of most members of the mammalian JAK kinase family such as JAK1, JAK2, and TYK2, is ubiquitous whereas only JAK3 is mainly expressed in hematopoietic cells (Ihle and Kerr, 1995). As a first step to explore the molecular basis underlying the regulation of carp *JAK1* gene expression, we also cloned and characterized the carp *JAK1* gene. We have mapped the carp *JAK1* transcription start site and characterized its promoter. With nested deletion mutants of the 5′-flanking region fused to the chloramphenicol acetyltransferase (CAT) reporter gene, the promoter activity was characterized by transfection into a fish cell line. To our knowledge, this is the first report to characterize the genomic structure and the promoter region of JAK kinases.

MATERIALS AND METHODS

Materials

Insect *Spodoptera frugiperda* (Sf9) cells were grown in Grace's medium containing 10% fetal calf serum (FCS; Life Technologies, Inc., Gaithersburg, MD). Monoclonal anti-phosphotyrosine IgG clone 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

Construction of a random-primed cDNA library

Total RNA was isolated from carp brain by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987), followed by the use of a QuickTrack mRNA isolation kit (Stratagene, La Jolla, CA). The isolated mRNA was used for the synthesis of random-primed cDNA.

Amplification of DNA fragments by PCR

Degenerate primers (Walks, 1989) were designed to fit the amino acid sequences that are conserved among PTKs (Hanks *et al.*, 1988; Hanks, 1991). The amino acid sequences of the two opposing primers are HRDLAAR (subdomain VI in Fig. 2, below) and DVWSFG (subdomain IX). The first-strand carp brain cDNA was used as template. The conditions for the reaction were 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min in 30 cycles.

cDNA library screening

An oligo-(dT)-primed carp liver cDNA library in the lamda ZAP II (Short *et al.*, 1988) obtained from Stratagene was screened by using a PCR-amplified DNA fragment as a probe (see Results). The probe was labeled using a DIG DNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany). Hybridization and washing were carried out according to the standard procedures (Sambrook *et al.*, 1989). Signals were detected using the DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim). Putative positive plaques were purified through several rounds of rescreening at lower densities and then excised as pBluescript plasmids according to the manufacturer's instructions. To isolate the full-length cDNA,

the 300-bp DNA fragment from the 5′ end of pJ21 (Fig. 1) was amplified by PCR, DIG-labeled, and then used as a probe to screen another random-primed carp brain cDNA library as described above.

Sequence analysis

cDNA clones were subcloned into plasmid pUC18 (Yanisch-Perron *et al.*, 1985) and sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using a Sequenase kit (United States Biochemical Corp. Cleveland, OH). Several programs from IntelliGenetics (Mountain View, CA) were used to analyze the nucleotide sequences.

DNA constructions

DNA fragments encoding the entire carp JAK1 were amplified with overlap extension PCR (Ho *et al.*, 1989) from J9 and J21 cDNA (Fig. 1) by using the following primers: primer F1, 5′-ATATCAGGATCCATGCCAGAACTAGCAGTCATG-3′ and primer R1, 5′-GTGGAAACTCAGCTGGCTGAC-3′; primer F2, 5′-GTCAGCCAGCTGAGTTTCCAC-3′ and primer R2, 5′-CAGTACAAGCTTCTCATCTTATTTCCATAGTTA-3′. The nucleotide sequences of primers R1 and F2 are complementary and they are located at the end of clone J9. Other DNA fragments encoding the kinase-like domain, kinase domain, and both domains were amplified with the PCR from J21 cDNA by using the following primers: c-JH2 (residues 572–875), 5′-ATATCAGGATCCCAGCTGAGTTTCCACCGCATC3′(F3) and 5′-CAGTACAAGCTTGAGGAACCTCTTCTCAAACAC-3′(R3); c-JH1 (residues 869–1,156), 5′-ATATCAGGATCCGTGTTTGAGAAGAGGTTCCTC-3′(F4) and 5′-CAGTACAAGCTTCTCATCTTATTTCCATAGTTA-3′(R2); c-JH(1 + 2) (residues 572–1,156), 5′-ATATCAGGATCCCAGCTGAGTTTCCACCGCATC-3′(F3) and 5′-CAGTACAAGCTTCTCATCTTATTTCCATAGTTA-3′(R2). Each primer encodes a unique restriction site (underline) (*Bam* HI, GGATCC; and *Hind* III, AAGCTT). The PCR products were then restricted with *Bam* HI and *Hind* III and ligated into pQE30 (QIAGEN Inc., Chatsworth, CA). DNA sequence analysis was performed to confirm the accuracy of the PCR products.

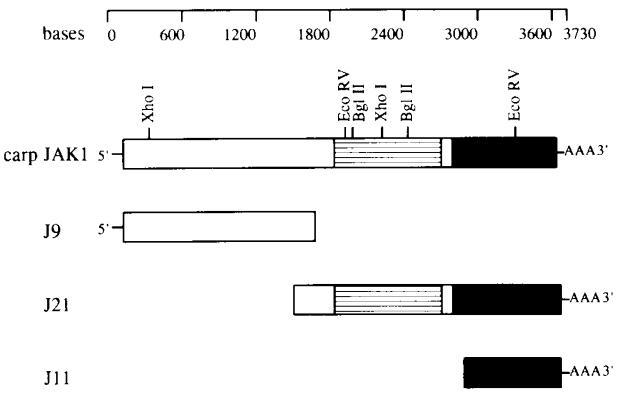

FIG. 1. Restriction maps of carp JAK1 cDNA clones. The kinase-like (JH2) and kinase (JH1) domains are represented by the horizontally striped and black boxes, respectively. Restriction enzyme sites are shown above carp JAK1 cDNA. The poly(A) tail is represented by AAA. The cDNA clones, J11 and J21, were isolated from a carp liver library whereas the clone J9 was isolated from a carp brain library.

Expression in Escherichia coli

All the resulting plasmids (pQEJAK1, pQEJH(1 + 2), pQEJH1, and pQEJH2) were transformed into *E. coli* strain JM109. The transformants were grown overnight at 37°C in LB broth and diluted 1:10 into fresh medium. After incubation at 37°C for 1 hr, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 m*M*, and incubation was continued for 3 hr. Cells were collected by centrifugation. Due to all the recombinant proteins contain a stretch of six histidines, therefore these proteins can be purified by using Ni^{2+}-nitrilotriacetate-agarose (Ni-agarose) (QIAGEN Inc.) according to the procedures as described (Huang *et al.*, 1995).

Antibodies

Purified recombinant protein c-JH1 or c-JH2 was dissolved in phosphate buffer saline (PBS) and mixed thoroughly with an equal volume of Freund's complete adjuvant for the first injection of Freund's incomplete adjuvant for the second and third injection. Approximately 100 μg of recombinant protein, for each injection, was subcutaneously injected into the back of guinea pig.

Expression in the baculovirus system

All of the pQE30 constructs described in the *E. coli* expression system were digested with *Eco* RI and *Xba* I and then ligated into the transfer vector PVL1393 (PharMingen, San Diego, CA). Plasmids (0.5 μg of each) were co-transfected into Sf9 cells with 0.1 μg of "Baculogold" virus DNA (PharMingen), which contained a lethal deletion. The parental baculovirus is unable to infect insect cells and only recombinant baculovirus containing the plasmid construct can replicate in Sf9 cells.

Sf9 cells were infected with various baculovirus at a multiplicity of infection (moi) of 10 plaque-forming units/cell. After 60 hr, cells were harvested, washed, and lysed in a buffer containing 150 m*M* NaCl, 1% Triton X-100, and 50 m*M* Tris pH 7.5. The Triton-insoluble cell pellets were solubilized in a buffer containing 1.5% *N*-lauroylsarcosine, 25 m*M* triethanolamine, and 1 m*M* EDTA pH 8.0 (Frankel *et al.*, 1991). Both Triton-soluble and -insoluble fractions were incubated with the Ni-agarose. After extensive washing with 0.5 *M* NaCl, proteins were eluted by 0.1 *M* EDTA pH 8.0. An equivalent amount of protein was subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis (PAGE), blotted to nitrocellulose paper, and probed with different antibodies.

Western blot

The method of Tricine $NaDodSO_4$-PAGE (Schagger and von Jagow, 1987) was used. The gel concentration was 7.5%. After $NaDodSO_4$-PAGE, the proteins were analyzed by immunoblot using anti-PY mAb or polyclonal antibodies specific for JH1 or JH2 domain according to the procedures as described (Huang *et al.*, 1995).

Immunoprecipitation

Sf9 cells were coinfected with vAcJH2 and vAcJH1. After 60 hr, cells were collected and solubilized by resuspension and incubation in the immunoprecipitation buffer (50 m*M* Tris pH 7.4, 150 m*M* NaCl, 1 m*M* Na_3VO_4 and 1% NP-40). Immunoprecipitation was performed by the addition of anti-JH1 or anti-JH2 antiserum at a dilution of 1:100 or with preimmune serum as a control reaction. The mixture (200 μl) was incubated at 4°C for 2 hr followed by an incubation with 50 μl of 50 mg/ml slurry of Protein A–Sepharose (Pharmacia, Uppsala, Sweden) for 1 hr. The Sepharose was collected and washed three times with the immunoprecipitation buffer and two times with 20 m*M* Tris pH 7.4, 1 m*M* EDTA/1 m*M* EDTA. Samples were then boiled in a 0.5% $NaDodSO_4$ sample buffer to disrupt the complexes. Products were subjected to $NaDodSO_4$-PAGE and analyzed by immunoblot using anti-PY mAb or polyclonal antibodies specific for the JH1 or JH2 domain.

Reverse transcription and PCR

mRNA (0.5 μg) isolated from carp brain and liver tissues was incubated at 65°C for 5 min in a buffer containing 50 m*M* Tris-HCl, 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 2 units of RNasin and 1.25 m*M* deoxynucleotide triphosphatates (dGTP, dATP, dTTP, and dCTP), and then kept on ice. Two hundred units of Superscript Moloney murine leukemia virus reverse transcriptase (RT; GIBCO BRL, Gaithersburg, MD) and oligo$(dT)_{12-18}$ primer (2 μg) and random hexamer primers (2 μg) were added and incubated at 37°C for 1 hr. The reaction was then stopped by incubation at 95°C for 5 min.

Five sets of specific primers, F1/R1, F1/R2, F3/R3, F4/R2, and F3/R2 were used and their sequences were described in the DNA Construction section. PCR was then carried out by addition of the heat-treated RT mixture, PCR buffer, and *Taq* polymerase (Promega, Madison, WI). Only the amplified DNA fragments by F3/R3 and F4/R2 from brain tissues and PCR product by F1/R1 from liver tissues were isolated from an agarose gel and subcloned into the pGEM-T vector (Promega). Other analytical methods were the same as described in the section of Sequence Analysis.

Isolation of carp JAK1 genomic clones

A commercially available carp liver lambda FIX II genomic library (Stratagene) was used to isolate 15- to 18-kb genomic DNA clones containing the gene that encodes carp JAK1. Full-length carp *JAK1* cDNA was labeled using a DIG DNA Labeling Kit (Boehringer Mannheim). Approximately 1×10^6 independent clones were plated at a density of 5×10^4 plaque forming units/150-mm Petri dish. Hybridization and washing were carried out as described above. Signals were detected using the DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim). Putative positive plaques were further purified through several rounds of rescreening at lower densities. Four distinct carp genomic DNA clones (designated J1, J2, J3, and J4) were isolated, and DNA was prepared from each clone. These carp genomic clones were digested with various restriction endonucleases. The DNA fragments were fractionated by electrophoresis in 1% agarose gels and transferred to nylon filters (Sartorius AG, Goettingen, Germany). The resulting DNA blots were probed under the above conditions of high stringency with the DIG-labeled whole carp *JAK1* cDNA.

Sequence analysis of genomic clones that encode carp JAK1

Phage DNA was digested with *Not* I, *Sac* I, *Sal* I, and *Xba* I and subcloned into either the pUC18 or pBluescript vector. Each subclone was sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Sequenase protocols from United States Biochemical. The nucleotide sequences of each DNA fragment were determined using T3 and T7 primers from both ends. Additional nucleotide sequences were determined using different sets of 20-nucleotide primers synthesized based on the known sequences from both ends of each DNA fragment. The sizes of the introns were determined either by restriction enzyme mapping of genomic clones or by PCR using sense and antisense specific primers that are from two different exons. The genomic DNA sequence was analyzed with the Genetics Computer Group software program. The transcription factor recognition site data bases (releases 7.3 and 6.5) were used to identify potential transcription factor motifs within the 5′-flanking region of the carp *JAK1* gene.

Primer extension analysis

An antisense oligonucleotide (5′-GTTTAATTTCGATCATTACAGACGTTTG-3′) complementary to the 5′ end of carp JAK1 cDNA clone J9 (Fig. 1) was labeled at the 5′ end with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a Stratagene NucTrap probe purification column. The labeled primer was annealed to 5 μg of poly(A)$^+$RNA prepared from adult carp liver total RNA and extendes with Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO BRL) as described previously (Sambrook *et al.*, 1989). The extended products were analyzed on a 5% polyacrylamide, 7 *M* urea sequencing gel. The size of the extended products was inferred from a sequencing ladder of the *JAK1* gene obtained from the same primer used for primer extension.

Plasmid construction for CAT functional analysis

A 3-kb *Sal* I fragment encompassing for 5′-flanking region was subcloned into pBluescript and various deletion mutants were generated from this clone by PCR using specific primer

FIG. 2. Amino acid sequence alignment of the carp, mouse (Yang *et al.*, 1993) and human (Wilks *et al.*, 1991) JAK1 proteins. Alignments were initially made by computer analysis and were subsequently aligned by inspection. Gaps are introduced to optimize alignment and shown as dots. The identical residues are represented by dashes. The putative kinase domains are delineated with arrows. Subdomains Ia–XIa of the kinase-line domain and subdomains I–XI of the kinase domain are denoted according to the previous reports (Hanks *et al.*, 1988; Hanks, 1991). The sequence of carp JAK1 has been deposited in the GenBank/EMBL Data Libraries, accession number L24895.

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cJAK1     MPELAVMELGRQLCGKMKKQRKAEMTVLTVMKGLEIHFYLPDTHQLEYFKDCHTAEDL    58
mJAK1     MQYLNIKEDCNAMAF-A--RSFK-T-VKQVVPEP-V-VT---L-REP-RLGSGEY---E-    60
hJAK1     MQYLNIKEDCNAMAF-A--RSSK-T-VNLEAPEP-V-VI---S-REP-RLGSGEY---E-    60

cJAK1     CVEGCQEDATSHLCADNLFALSEESQDLWYAPNHAFKITEETSIKLHYRMRFYFTNWHAP   118
mJAK1     -IRAA-ECSI-P--H.-----YD--TK------RIITVDDK--LR------------GT   119
hJAK1     -IRAA-ACRI-P--H.-----YD-NTK------RTITVDDKM-LR------------GT   119

cJAK1     VRTESPVWRHSLFKHKGVSVSPKGPEGTPLLDAASLEYLFAQGQYDFLRGLAPVRAPQNE   178
mJAK1     NDN-QS-----PK-Q-NGYEKKRV-EA------S-----------LIKC---I-D-KT-   179
hJAK1     NDN-QS-----PK-Q-NGYEKKKI-DA------S-----------LVKC---I-D-KT-   179

cJAK1     AEKHEIENECLGMAVLAITHHAKSNDLPLSGVGAETSYKRFIPDSLNRTIKQRNFSHVYV   238
mJAK1     QDG-D------------S---MMKKMQ-PELPKDI----Y--ET--KS-R---LLTRMR   239
hJAK1     QDG-D------------S---MMKKMQ-PELPKDI----Y--ET--KS-R---LLTRMR   239

cJAK1     YNNVFKNFLNEFNSKTIQDSNITLYDLKVKYLSTLETLTQGLGRETIEPKILKVSGESDG   298
mJAK1     I-----D--K---N---C--SVSTH-------A------KHY-A-IF-TSM-LI-S-NEL   299
hJAK1     I-----D--K---N---C--SVSTH-------A------KHY-A-IF-TSM-LI-S-NEM   299

cJAK1     SPALTLPSGDDG.LGYEVQVSGTTGISWRRKPVPNILIVKDKTKSKKNKADKQSKKEMTK   357
mJAK1     -RCHSND--NVL.YEVM-TGNLGIQWRQKPNV-PVEKEKNKLKRK-LEYNKHKKDD-RN-   358
hJAK1     NWFHSNDG-NVLYYEVM-TGNLGIQWRHKPNV-SVEKEKNKLKRK-LEYNKDKKDE-KN-   359

cJAK1     RKTVMTIFSDFFEITHIVIKESCATIYSQDNKTMELDLFYRDAALSFAALVDGYFRLTVD   417
mJAK1     LREEWNN--Y-P----------VVS-NK--N-N---K-SSHEE----VS---------A-   418
hJAK1     IREEWNN--Y-P----------VVS-NK--K-N---K-SSHEE----VS---------A-   419

cJAK1     AHHYLCTEVAPSSVVQNLENGCHGPICTEYAIHKLRQEGNEEGTYVLRWSCTDYNYIIMT   477
mJAK1     -------D---PLI-H-IQ-------------N------S---M---------FDN-L--   478
hJAK1     -------D---PLI-H-IQ-------------N------S---M---------FDN-L--   479

cJAK1     VVCIEMDLCESRPVPQYKNFQIETSPQGYRLYGTDTFRPTLKELLEHLQGQNLRTENLRF   537
mJAK1     -T-F-KSE.VLGGQK-F------VQKGR-S-H-SMDHF-S-RD-MN--KK-I---D-IS-   537
hJAK1     -T-F-KSEQVQGAQK-F------VQKGR-S-H-S-RSF-S-GD-MS--KK-I---D-IS-   539

                                                    ┌►*** Ia ****
cJAK1     QPVLVGLGQPRKISNLLVMTRDREPDSQRQPQVSQLSFHRILKEEIVQGEHLGRGTRTNI   597
mJAK1     VLKRCCQPK--E------A-KKAQEW.-PVYSM-----D----KDLI----------H-   596
hJAK1     MLKRCCQPK--E------A-KKAQEW.-PVYPM-----D----KDLI----------H-   598

                              **IIa**       *** IIIa ***   ***IVa***
cJAK1     YSGVLKLKSEDDDDMGGYSQEVKVILKVLGSGHRDISLAFFETASMMRQISHKHIALLYG   657
mJAK1     ---T-LDYKDEEGIAEEKK..I-------DPS----------A------V-----VY---   654
hJAK1     ---Y-MDYKD-EGTSEEKK..I-------DPS----------A------V-----VY---   656

                 ******Va******                              *****VIa******
cJAK1     VCVRHQENIMVEEFVQYGPLDLFMRRQSIPLSTAWKFQVAKQLAGALSYLEDKKLVHGNV   717
mJAK1     ----DV---------EG-------H-K-DA-T-P---K------S--------D------   714
hJAK1     ----DV---------EG-------H-K-DV-T-P---K------S--------D------   716

                       ****VIIa****          * *VIIIa**        ***
cJAK1     CSKNILVARDGLDGEGGPFIKLSDPGIPITVLSREECVDRLPWIAPECVQDTANLSIAAD   777
mJAK1     -T--L-L--E-I-SDI------------VS--T-Q--IE-I--------E-SK---V---   774
hJAK1     -T--L-L--E-I-S-C------------IT----Q--IE-I--------E-SK---V---   776

          *IXa****         ***** Xa ***                    *** XIa***
cJAK1     KWGFGTTLWEICYNGEIPLKDKKLTEKERFYAAQCQLASPDCEELAKLMTHCMTYDPRQR   837
mJAK1     --S------------------T-I------ESR-RPVT-S-K---D---R--N---N--   834
hJAK1     --S------------------T-I------ESR-RPVT-S-K---D---R--N---N--   836

          ┌◄                                          ┌►********** I ****
cJAK1     LFFRAIVRDIDMVEKQNPSIQP...VPMLEVDPTVFEKRFLKKIRDLGEGHFGKVELCRY   894
mJAK1     P-----M---NKL-E---D-VS.EKQPTT-----H-------R-----------------   893
hJAK1     P-----M---NKL-E---D-VSRKKNQPT-----H-------R-----------------   896

                ** ***II****           **III* *   **IV****         ***
cJAK1     DPRGDRTGELVAVKSLKPENREEQSSNLWREIHILRELYHENIVKYKGIWHEEGGRSIKL   954
mJAK1     --E--N---Q---------SGGNHIAD-KK--E---N------------CM-D--NG---   953
hJAK1     --E--N---Q---------SGGNHIAD-KK--E---N------------CM-D--NG---   956

          ***** V *******                        *** * ** VI *********
cJAK1     IMEFLPAGSLKEYLPRNKAHIDLKTLLNYAVQICQGMDLLASRNYIHRDLAARNVLVENE  1014
mJAK1     ------S--------K--NK-N--QQ-K--I---K---Y-G--Q-V------------S-  1013
hJAK1     ------S--------K--NK-N--QQ-K--V---K---Y-G--Q-V------------S-  1016

            ***VII*****                ***VIII*****        ***IX*****
cJAK1     NTVKIGDFGLTKSIKDNEGYYTVKDDLDSPVFWYAPECLIHCKFYRASDVWSFGVTMYEL  1074
mJAK1     HQ---------A-ETDKE-------R-------------Q----I----------LH--  1073
hJAK1     HQ---------A-ETDKE-------R------------MQS---I----------LH--  1076

                                          * *X****             **XI**
cJAK1     LTYCDISCSPMSVFLTMIGPTHGQMTVTRLVKVLEEGKRLPKPDGCSDRLYCLMRRCWEA  1134
mJAK1     -----SDF---AL--K---------------T-K------C-PN-P-EV-Q---K---F  1133
hJAK1     -----SDS---AL--K--------------NT-K------C-PN-P-EV-Q---K---F  1136

          ┌◄
cJAK1     TPEKRIDFKGLIANFQQMIDNQ        1156
mJAK1     Q-SN-TT-QN--EG-EALLK          1153
hJAK1     Q-SN-TS-QN--EG-EALLK          1156
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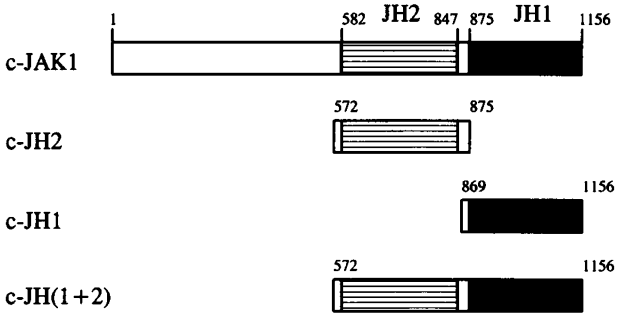

FIG. 3. Schematic diagrams of full-length and the various truncated forms of carp JAK1 constructs. The kinase-like and kinase domains are represented by horizontally striped and dark box, respectively. Truncated molecules were constructed as described in the text.

sets. PCR products were then cloned into polylinker regions of the reporter vector pCAT-Basic (Promega). Clone pJP1 contains the flanking region fragment JP1, nucleotides −2,541 to +59. JP1 was synthesized using PCR primers 5′-TTGAAGCTTTCCTCCTAGGATCAGG-CAGA-3′ (nucleotides −2,541 to −2,522) and 5′-GGGTCTAGAT-GCTTCAGTCGT-CATGATCAA-3′ (nucleotides +39 to +59) on the 3-kb *Sal* I fragment. The oligonucleotide primers have additional sequences of *Hin*d III and *Xba* I sites at each end, respectively. Fragment JP1 was purified from the gel, digested with *Hin*d III and *Xba* I, and then cloned into the same sites of pCAT-Basic. Clone spJP2, pJP3, pJP4, pJP5, and pJP6 were similarly constructed, except that the JP1 fragment was replaced with JP2 (nucleotides −2,541 to −181), JP3 (nucleotides −2,541 to −901), JP4 (nucleotides −1,023 to +59), JP5 (nucleotides −528 to +59), and JP6 (nucleotides −181 to +59), respectively. The pRSV-CAT (Gorman *et al.*, 1983) was used as a positive control.

Transfection, CAT, and β-galactosidase assay

Carp fin epitheloid cells, CF (Chen and Kou, 1986), were maintained in Leibovitz's L-15 medium supplemented with 10% fetal calf serum (FCS) at 27°C. Subconfluent cultures (approximately 60% confluent, 24 hr after plating) in 60-mm culture dishes were washed twice with Leibovitz's L-15 medium, and incubated with DNA–Lipofectamine complexes containing 20 μg of the different CAT constructs together with 5 μg of pSV-β-galactosidase (β-GAP) vector (Promega) in duplicate. Transfection was carried out for 5 hr, and cells were washed with fresh Leibovitz's L-15 medium and fed with the same medium supplemented with 10% FCS. After 40 hr, cells were harvested, washed in phosphate-buffered saline (PBS), and lysed with 25 m*M* Tris phosphate pH 7.8, containing 2 m*M* dithiothreitol, 2 m*M* EDTA, 10% glycerol, and 1% Triton X-100 at room temperature for 30 min. The total lysates were scraped from the dish and transferred to microcentrifuge tubes. Cell debris was removed by centrifugation and the extracts were frozen at −70°C until use. Protein concentration was measured by the Bio-Rad protein concentration quick-assay method (Bio-Rad, Richmond, CA). The pSV-β-Gal vector (Promega) carrying the SV40 promoter linked to the β-Gal gene was used to normalize the samples for differences in transfection efficiency. CAT and β-Gal activities in the extracts were measured according to the previous procedures (Herbomel *et al.*, 1984). The acetylated products of the CAT assay were separated by thin-layer chromatography developed with chloroform-methanol (95:5, vol/vol), visualized by autoradiography and quantitated by using a PhosphoImager (Bio-Imaging Analyzer BAS 2000, Fuji, Japan).

RESULTS

Isolation of carp JAK1 cDNA

We amplified carp brain cDNA by PCR using degenerate primers, an approach shown to be effective in isolating human and murine JAK1 gene (Wilks *et al.*, 1991; Yang *et al.*, 1993).

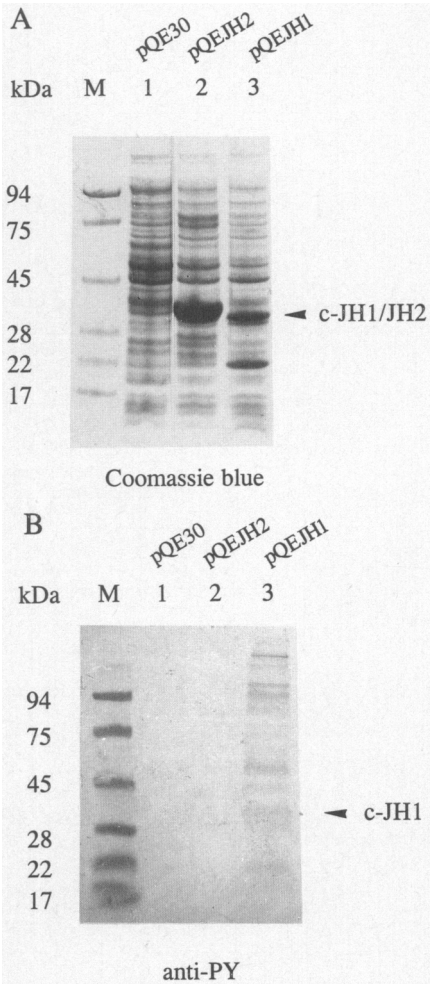

FIG. 4. Demonstration of tyrosine kinase activity of c-JH1 in *E. coli*. Strain JM109 cells were transformed with pQE30 (lane 1), pQEJH2 (lane 2), and pQEJH1 (lane 3), respectively. After induction with 1 m*M* IPTG for 3 hr, proteins were prepared and separated by $NaDodSO_4$-PAGE and stained with Coomassie blue (A) or immunoblotted with anti-PY mAb (B). Positions of c-JH1 and c-JH2 are shown on the right. Lane M, Prestained molecular mass markers.

Amplified fragments of about 220 bp were purified and ligated into pGEM-T (Promega). Among the 20 clones sequenced, 15 clones contained a single JAK-related sequence. Using this DNA fragment as a probe, we screened an oligo(dT)-primed carp liver cDNA library. Schematics of the two representative carp liver JAK1 clones obtained from this screen are shown in Fig. 1. On the basis of DNA sequence analysis, these two clones, pJ11 and pJ21, are identical except that pJ11 is 0.7 kb whereas pJ21 is 2.1 kb in length. It was found that pJ21 encodes the carp homolog of mammalian JAK1, containing residues from 465 to 1,156. To obtain the full-length cDNA clone, a 300-bp DNA fragment from the 5′ end of pJ21 was used as a probe to screen a carp brain cDNA library. This screening yielded pJ9 (1.9 kb), as shown in Fig. 1. Therefore, the full-length carp JAK1 cDNA sequences are derived from the two overlapping clones, pJ9 and pJ21, and contain a 5′-untranslated region of 186 nucleotides, a coding region of 3,468 nucleotides, and a 3′-untranslated region of 65 nucleotides. The total length of carp JAK1 cDNA is 3,719 nucleotides. (The sequence has been deposited in GenBank with an accession number L24895.)

Structure and the activity of carp JAK1 cDNA

The complete sequence of carp JAK1 cDNA encodes an open reading frame of 1,156 amino acid residues with a predicted molecular mass of 129 kD and the protein was termed carp JAK1. All members of the JAK family have seven homologous domains in the molecule that have been named as JHs or JAK homology domains. JH1 is a carboxy-terminal kinase-catalytic domain whereas JH2 is a kinase-like domain; the other five JHs are present in the far amino-terminal part. Amino acid sequence comparison of carp JAK1 with human and murine JAK1 (Wilks *et al.*, 1991; Yang *et al.*, 1993) is shown in Fig. 2. There is a higher sequence homology in both JH1 and JH2 (70% identity). The overall sequence identity between carp and human/murine JAK1 is about 57%. When the deduced amino acid sequences of carp JAK1 were compared with those of murine JAK2 (Harpur *et al.*, 1992), murine JAK3 (Witthuhn *et al.*, 1994), and human TYK2 (Firmbach-Kraft *et al.*, 1990), it was found that the sequence homology is lower in JH1 (50, 46, and 56% identity, respectively) and JH2 (45, 43, and 51% identity, respectively). The overall sequence identity between carp JAK1 and murine JAK2, murine JAK3, and human TYK2 is 35%, 31%, and 42%, respectively.

To test whether the JH1 or JH2 domains possess kinase-catalytic activity, we generated His-tag fusion proteins of the JH2 domain (c-JH2) and the JH1 domain (c-JH1) of carp JAK1 (Fig. 3), and expressed these fusion proteins in *E. coli* (Fig. 4A). The tyrosine kinase activity resulting from each fusion protein can be detected by anti-phosphotyrosine monoclonal antibodies (anti-PY mAb). Due to the lack of endogenous tyrosine kinases in *E. coli*, there is little or no cross-reactive background for the anti-PY Western blot (Fig. 4B, lane 1). Among the expressed fusion proteins (Fig. 4A), only c-JH1 displayed tyrosine kinase activity and several tyrosine-phosphorylated bands including a band corresponding to c-JH1 were detected (Fig. 4B, lane 3). By contrast, c-JH2 (lane 2) did not express any observable tyrosine kinase activity. These results are consistent with an earlier report (Wilks *et al.*, 1991) and suggest that only JH1 is a functionally active kinase domain. c-JH1 and c-JH2 were further purified by Ni-agarose affinity chromatography and used to generate polyclonal antibodies (see below).

Full-length and various truncated forms of carp JAK1 are produced in insect cells using the baculovirus system

Figure 5, A and B, show that c-JAK1, c-JH(1 + 2), c-JH1, and c-JH2 were present in both the Triton-soluble and Triton-

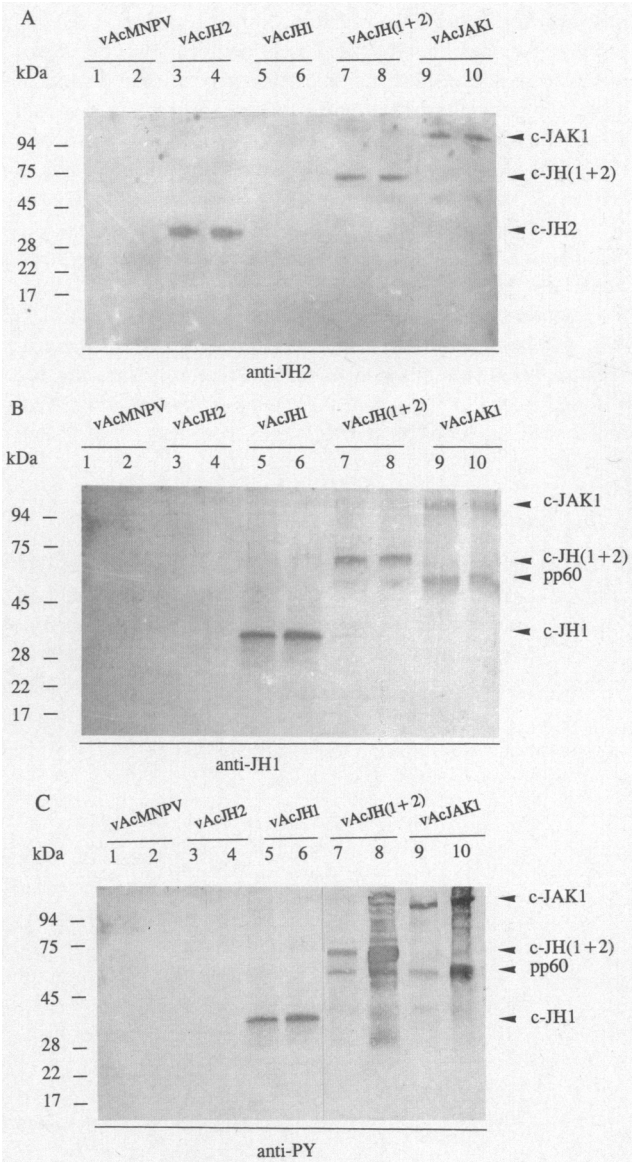

FIG. 5. Expression of full-length and various truncated forms of carp JAK1 in the baculovirus system. Sf9 cells were infected with wild-type baculovirus, AcMNPV (lanes 1 and 2) and a variety of recombinant baculovirus (lanes 3–10), respectively. After 60 hr, cells were harvested and separated into Triton-soluble (lanes 1, 3, 5, 7, and 9) and Triton-insoluble fractions (lanes 2, 4, 6, 8, and 10). Both fractions were further bound to Ni-agarose gels as described in the text and equivalent amounts of bound patients were separated by $NaDodSO_4$-PAGE, transferred to nitrocellulose, and immunoblotted with polyclonal antibodies specific for JH2 domain (anti-JH2; A), for JH1 domain (anti-JH1; B), and a mAb for phosphotyrosine (anti-PY; C). Positions of prestained molecular mass standards and their apparent sizes, in kilodaltons, are indicated on the left.

insoluble fractions as revealed by antibodies specific for JH2 (Fig. 5A) and for JH1 (Fig. 5B). Again, the anti-PY mAbs were used to detect the tyrosine kinase activity of each fusion protein. As a control, Sf9 cells were infected by wild-type baculovirus and there was little or no cross-reactive background for the anti-PY Western blot (Fig. 5C, lanes 1 and 2). As shown in Fig. 5C (lanes 3 and 4), c-JH2 did not display tyrosine kinase activity. Other fusion proteins, c-JAK1, c-JH(1 + 2), and c-JH1 all expressed tyrosine kinase activity (Fig. 5C, lanes 5–10) and autophosphorylation by themselves seemed to occur as we compared their electrophoretic mobilities in Fig. 5, A and B, and the corresponding mobilities in the anti-PY Western blot (Fig. 5C). Interestingly, in cells expressing c-JAK1 and c-JH(1 + 2), an additional protein of 60 kD was detected by anti-JH1 antibodies and anti-PY mAb (Fig. 5B, lanes 7–10, and Fig. 5C, lanes 7–10). Moreover, there were more tyrosine-phosphorylated proteins bound to Ni-agarose in the Triton-insoluble fraction of insect cells expressing c-JAK1 and c-JH(1 + 2) (Fig. 5C, lanes 8 and 10) than cells expressing c-JH1. The proteins analyzed were fractions bound to Ni-agarose, presumably *via* the His-tag of recombinant proteins. Therefore, they were either cleavage products of JAK1 related proteins or associated proteins.

c-JH1 and c-JH2 interact with each other

To demonstrate the association of c-JH1 and c-JH2, anti-JH1 antibody was used to precipitate c-JH1 from the extract of vAcJH1- and vAcJH2-co-infected cells, and the associated c-JH2 was detected by blotting with anti-JH2 antibody. As shown in Fig. 6A, c-JH2 is co-precipitated by c-JH1. Similarly, c-JH1 is also co-precipitated by c-JH2 (Fig. 6B). Because both proteins are highly expressed under these conditions, there is the possibility that this interaction is somehow nonspecific. Therefore, another recombinant baculovirus vAcCAT, which carries the chloramphenicol acetyltransferase (CAT) gene under the control of the same polyhderin promoter, was used to coinfect with vAcJH1 or vAcJH2. The immunoprecipitation data showed that neither anti-JH1 nor anti-JH2 antibody was able to precipitate CAT protein from the coinfected cell extract (data not shown). Moreover, neither anti-JH2 nor anti-JH1 antibody was able to precipitate c-JH1 or c-JH2 from the extracts from cells infected with vAcJH1 or vAcJH2 alone as shown in Fig. 6 (lane 1). Therefore, c-JH2 seems to interact specifically with c-JH1 and possibly by tyrosine-phosphorylated by c-JH1 (see texts below).

JH2 domain is tyrosine-phosphorylated by c-JAK1 and c-JH(1 + 2)

Because c-JH1 and c-JH2 were associated with each other (Fig. 6), we sought to investigate whether c-JH2 is a substrate for c-JH1 by co-infection experiments in which insect cells were co-infected with vAcJH2 and vAcJAK1 or vAcJH2 and vAcJH(1 + 2), instead of co-infection with vAcJH2 and vAcJH1. This avoided ambiguity in data interpretation due to the similar mobility of c-JH1 and c-JH2 on NaDodSO$_4$-PAGE. The experimental procedures were the same as those described in the vAcJH1 and vAcJH2 co-infection experiment. Proteins bound to Ni-agarose gels were analyzed by blotting with either anti-JH2 antibody or anti-PY mAb. The control experiment was carried out by infecting insect cells with either vAcJAK1 or

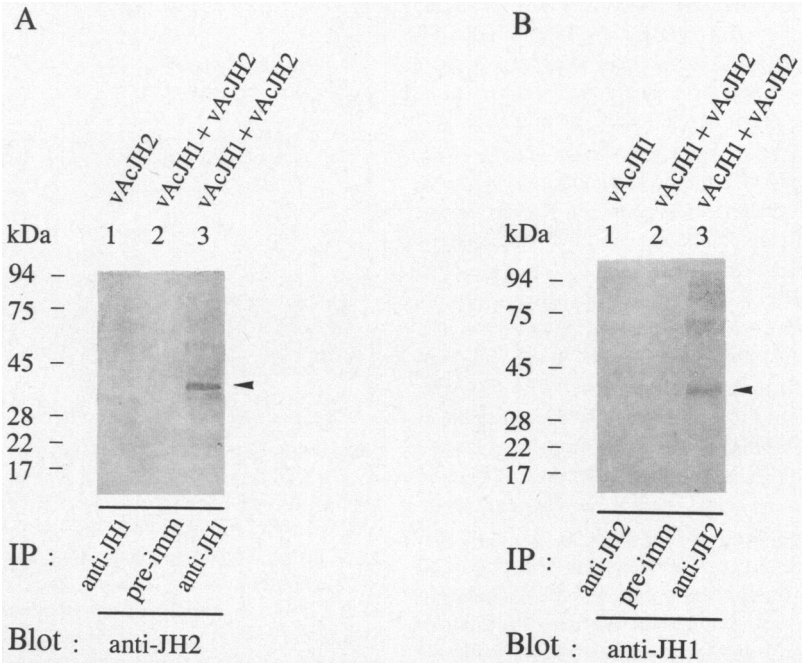

FIG. 6. Association of c-JH1 with c-JH2. Sf9 cells were infected with vAcJH2 alone (a, lane 1), vAcJH1 alone (B, lane 1), and co-infected with vAcJH1 and vAcJH2 (lanes 2 and 3), respectively. The Triton-soluble fractions were immunoprecipitated with normal guinea pig antiserum (pre-imm; lane2), anti-JH1 antibodies (A, lanes 1 and 3) and anti-JH2 antibodies (B, lanes 1 and 3). The associated proteins were analyzed by NaDodSO$_4$-PAGE, transferred to nitrocellulose, and immunoblotted with anti-JH2 (A) or anti-JH1 antibodies (B). Arrowhead indicates positions of c-JH1 and c-JH2, respectively.

vAcJH(1 + 2) alone. As shown in Fig. 7B, c-JH2 indeed was tyrosine-phosphorylated by c-JAK1 (lane 2) and by c-JH(1 + 2) (lane 4). This suggests that transphosphorylation of c-JH2 by c-JH1 may occur when they are expressed in high levels together.

RT-PCR of brain and liver mRNA

As described above, two overlapping clones, pJ9 and pJ21, were isolated from different tissues. To investigate whether tissue-specific alternative splicing occurs and to analyze the uncloned regions in the two tissues, five sets of specific primers were used to perform RT-PCR on mRNA derived from common carp brain and liver tissues. PCR products from both tissues were identical in length (Fig. 8). Only the uncloned regions in the two tissues, *i.e.*, PCR products from primers F3/R3 and F4/R2 (from brain), and the DNA fragment from F1/R1 (from liver) were sequenced. All of the nucleotide sequences were the same as those we deposited in GenBank with an accession number L24895. These sequences, as well as those from two overlapping clones, pJ9 and pJ21, suggest that at least two identical transcripts from carp brain and liver tissues encode the same full-length carp JAK1 kinase.

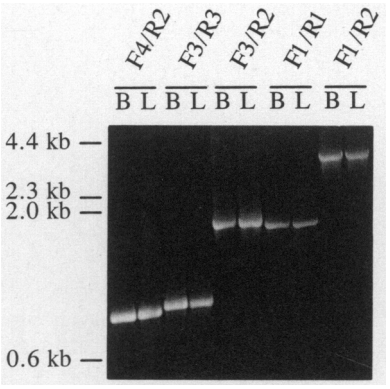

FIG. 8. RT-PCR analysis of the carp JAK1 kinase transcripts. mRNAs derived from brain (B) and liver (L) tissues were primed with oligo(dT) and random primers and subjected to reverse transcription. The resulting cDNA was amplified with five primer sets, *i.e.*, F3/R3, R4/R2, F3/R2, F1/R1, and F1/R2, and the PCR products were analyzed by electrophoresis on a 1% agarose gel. The sequence of each primer was described in the DNA Construction section.

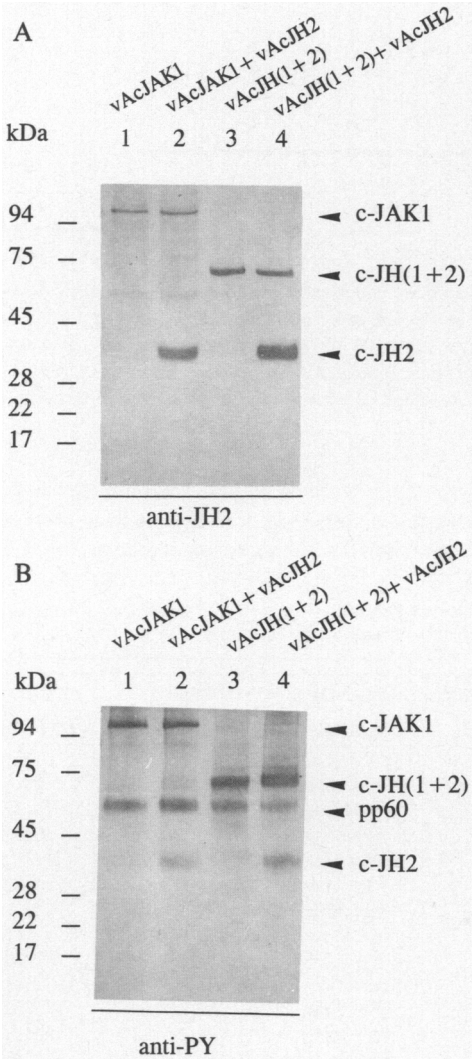

FIG. 7. Transphosphorylation of c-JH2 by c-JAK1 and c-JH(1 + 2). Sf9 cells were infected with vAcJAK1 alone (lane 1), both vAcJAK1 and vAcJH2 (lane 2), vAcJH(1 + 2) alone (lane 3), and both vAcJH(1 + 2) and vAcJH2 (lane 4), respectively. The Triton-insoluble fractions were prepared and then incubated with Ni-agarose gels and equivalent amounts of bound proteins were separated by $NaDodSO_4$-PAGE, transferred to nitrocellulose, and immunoblotted with anti-JH2 antibodies (A) or anti-PY mAb (B). Prestained molecular mass markers (in kilodaltons) are shown on the left.

Genomic organization of the carp JAK1 gene

As an initial step to investigate the regulation of carp *JAK1* gene expression, we also cloned and characterized the carp *JAK1* gene. Four positive phage clones, termed J1 to J4, were isolated from a Stratagene carp liver genomic library with a DIG-labeled full-length carp *JAK1* cDNA as a probe. We are unable to fill the gap between phage clone J1 and J2 by PCR amplification of genomic DNA with a set of oligonucleotides that correspond to one end of clone J1 and the other end of clone J2. To locate all exons, these phage clones were analyzed by Southern blotting, subcloning, and sequencing. As shown in Fig. 9, the restriction map of each genomic clone was constructed by digesting the phage DNA with a panel of restriction enzymes separately or in various combinations: *Sal* I, *Bgl* II, *Hind* III, *Xho* I, and *Eco* RI. On the basis of the nucleotide sequences of subcloned fragments, the carp *JAK1* gene is composed of 24 exons that spans at least 31 kb of DNA (Fig. 9). The sequences around the exon–intron boundaries were determined and are shown in Table 1. All exon–intron boundaries identified conformed to the GT/AG splice donor/acceptor rule (Breathnach *et al.*, 1978). Some exons were relatively small (88–108 bp), whereas the first and the sixth exons were large (237 bp and 349 bp). The size of introns varied considerably, ranging from >3 kb (intron 1) to 100 bp (intron 19). The first exon contained the 5′ untranslated region, and the second exon

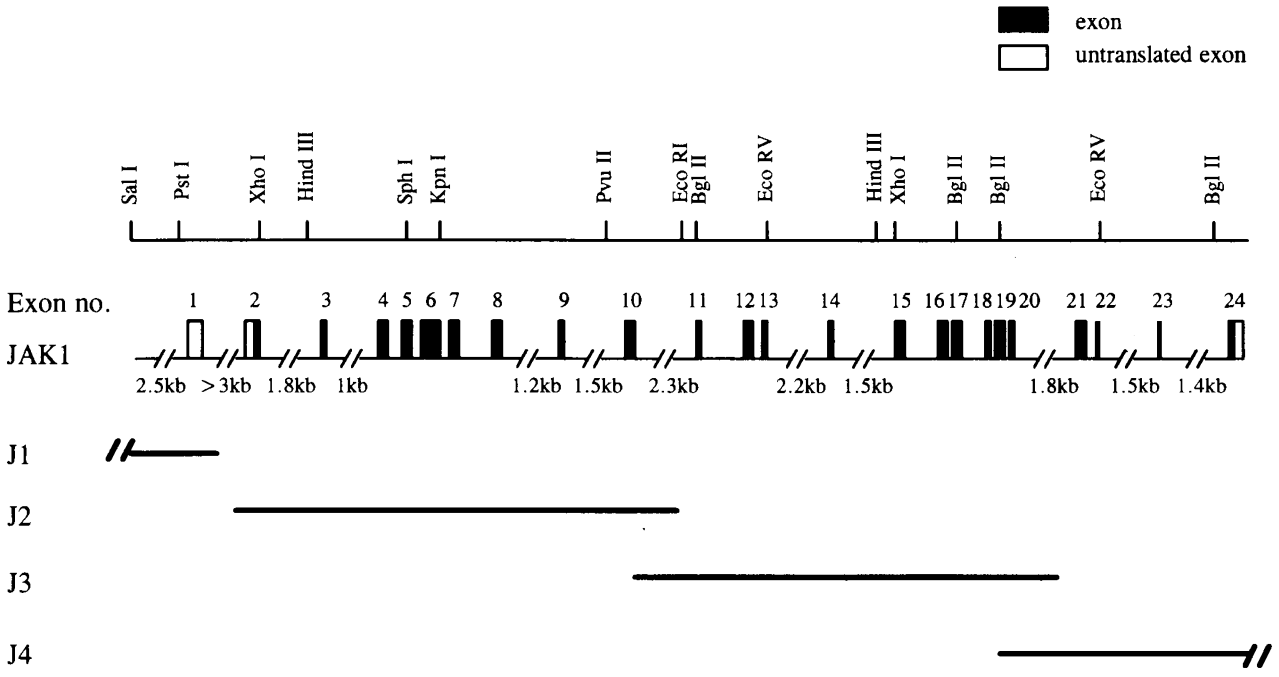

FIG. 9. Genomic organization of the carp JAK1 gene. Exons are indicated by boxes numbered from 1 to 24. Solid boxes indicate the carp JAK1 coding region whereas open boxes represent the 5′ and 3′ untranslated regions. Introns and the 5′- and 3′-flanking regions are indicated by the solid lines. The entire gene spans at least 31 kb in length and contains 24 exons. A restriction map was shown above the genomic structure. Three overlapping phage clones, J2–J4 and one nonoverlapping clone, J1, were isolated from a Lambda FIXII carp genomic library. A gap between phage clones J1 and J2 was not obtainable by PCR amplification of genomic DNA.

contained the putative translation initiation site. The largest intron (>3 kb) separates exons 1 and 2. The JH2 domain was located on exons 11–17 and the catalytic JH1 domain was located on exons 18–24. Exon 24 contained the last 33 amino acids as well as the 3′ untranslated region. The promoter and exon/intron sequences of carp *JAK1* kinase gene have been deposited in GenBank with 10 serial accession numbers, from U53685 to U53694.

TABLE 1. EXON-INTRON ORGANIZATION OF THE CARP JAK1 GENE

exon number	exon size (bp)	3'end of the exon	5'end of the intron	approximate size (bp)	3'end of the intron	5'end of the exon	amino acid interrupted
1	242	TGC CTG ACG AG	gtaaggacga	>3000	tatcctgcag	T GTC TGG ATG	
2	208	CAA GAA GAT GCC	gtaagcgaga	1800	ttttccccag	ACA TCT CAC	Ala67 (3)
3	125	TAC CGT ATG AG	gtgagtgggc	1000	gtttacacag	G TTT TAT TTT	Arg109 (2)
4	154	CTG TTT GCA CAG	gtgaggtaca	400	tatgttttag	GGC CAG TAT	Gln160 (3)
5	164	GCT GAG ACC AG	gtgaggtaca	148	atctttgtag	C TAC AAG CGA	Ser215 (2)
6	349	AAG CCT GTA CCG	gtgagtttag	250	cttttgatag	AAT ATT CTG	Pro331 (3)
7	180	AAT AAA ACT ATG	gtaatttcca	600	ttgcccacag	GAG TTG GAC	Met391 (3)
8	158	GGA CCT ATC TG	gtatgaccat	1200	ttctctacag	C ACA GAG TAT	Cys444 (2)
9	115	GTC TGC ATT GAG	gtacacacac	1500	tctctctcag	ATG GAC CTA	Glu482 (3)
10	199	CAA CCC AGA A	gtaccagcac	2300	tttccttcag	AA ATT TCC AAC	Lys549 (1)
11	110	GAG ATT GTA CAG	gtgatatttt	900	ttttattcag	GGT GAG CAT	Gln585 (3)
12	150	GAT ATC TCT CTG	gtaagatgca	187	ctcctctcag	GCT TTC TTT	Leu635 (3)
13	89	CAT CAG GAG AA	gtaagtacct	2200	ctgtctgtag	T ATC ATG GTG	Asn665 (2)
14	127	CTC AGC TAT CTG	gtaaagaaac	1500	aatcctgtag	GAG GAC AAG	Leu707 (3)
15	136	AGC AGA GAA G	gttgagagat	820	gtgtctacag	AG TGT GTG GAC	Glu753 (1)
16	152	AAG CTC ACA GAG	gtaacagcat	111	ctctgagcag	AAG GAG AGG	Glu803 (3)
17	152	GAG AAA CAG AA	gtatgaccta	457	ttctctccag	T CCT TCC ATT	Asn854 (2)
18	88	GAT CTT GGA GAG	gtattttctg	110	ctcttttcag	GGT CAC TTT	Glu883 (3)
19	193	CAC GAA GAA G	gtaaagccac	100	ttgtttcaag	GT GGG AGA TCC	Gly948 (1)
20	125	CAG ATC TGC CAG	gtaacatcat	1800	ttaactccag	GGC ATG GAC	Gln989 (3)
21	173	CCA GTG TTC TG	gtaagaatca	170	attttcccag	G TAT GCC CCA	Trp1047(2)
22	118	AGC CCT ATG TCG	gtaagtggcc	1500	aaccattcag	GTG TTC CTT	Ser1086(3)
23	111	TGC TCA GAC AGG	gtaatatata	1400	gttttgacag	TTG TAC TGT	Arg1123(3)
24	164	ATG ATT GAC AAT CAG TAACTATGGAAATAAGATGAGATGCAGACTCCACCTCTTTTGTAAGAGGAAGTTCCAGAGAGACCAAAAAAAAAAA					
						(the end of carp JAK1 gene)	

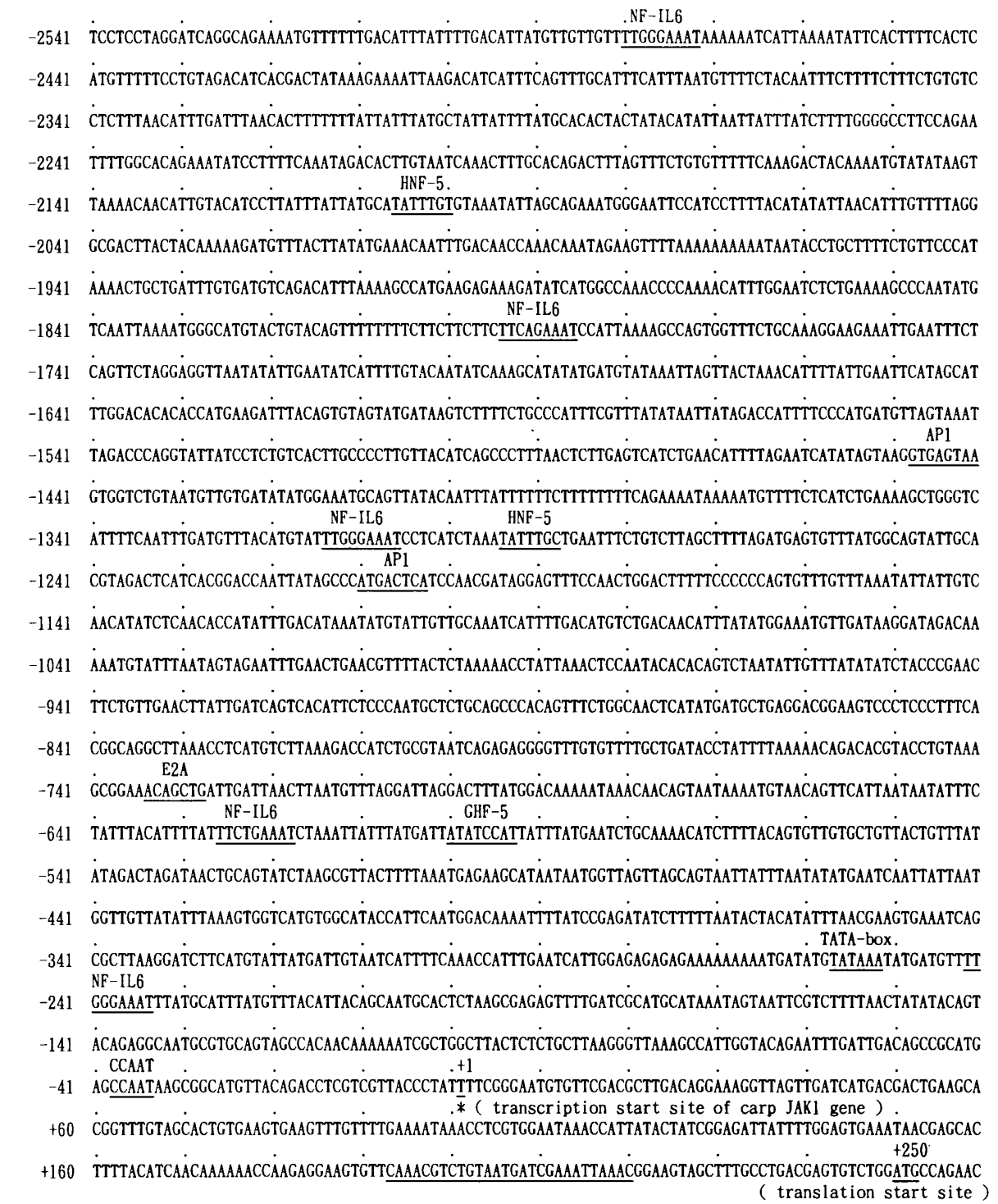

FIG. 10. Nucleotide sequence of the 5′-flanking region of the carp JAK1 gene. A 28-mer antisense oligonucleotide used for primer extension analysis is underlined. The candidate transcription start site by primer extension (see Fig. 11) is indicated with a nucleotide number (+1) and an asterisk, which is located at 249 bp upstream to the translation start site. Potential binding sites for a variety of transcription factors are also marked and underlined. The promoter and exon 1 sequences of carp *JAK1* kinase gene has been deposited in GenBank, accession number U53685.

Determination of the transcription initiation site

The transcription start site was determined by primer extension analysis using poly(A)$^+$RNA from carp liver. We used a 28-mer oligonucleotide labeled with ^{32}P at the 5′ end. The exact position of the extended product was determined by aligning the sequencing ladder obtained with the same primer. One major extended product was revealed and corresponded to the site at 249 bp upstream to the initiator methionine codon. For describing the 5′-flanking region of the carp *JAK1* gene, we

used a numbering scheme that the transcription start site of the carp JAK kinase gene is designated +1 (Fig. 10).

Characteristics of the 5′-flanking region of the carp JAK1 gene

To identify sequence elements possibly involved in the control of carp *JAK1* gene expression, the 2.8-kb DNA fragment of the 5′ upstream region of the *JAK1* gene relative to the translation start site was sequenced and shown in Fig. 11. Computer analysis of the sequence revealed numerous potential binding sites for transcription factors. One TATA box (TATAAA), which is generally located at a position around 30 bp upstream to the RNA start site (Breathnach and Chambon, 1981), is observed at 253 bp upstream to the RNA start site. Thus, the carp *JAK1* gene promoter belongs to the subclass of TATA-less RNA polymerase II promoters that are found in many protein tyrosine kinase genes (Patel *et al.*, 1990; Lichtenberg *et al.*, 1992; Uchiumi *et al.*, 1992; Kawagishi *et al.*, 1995; Perez-Castro *et al.*, 1995; Sideras *et al.*, 1994). This gene also has a CCAAT box at −35 to −40 relative to the RNA start site, however, not at the standard positions between −60 and −80 relative to the RNA start site (Chodosh *et al.*, 1988). Two putative binding sites for HNF-5 (Grange *et al.*, 1991) are present at −2,107 and −1,295. Other potential binding sites for a ubiquitously expressed transcription factor, NF-IL6 (Akira *et al.*, 1990), are observed at −2,481, −1,795, −1,315, −627, and −243. It has been shown that genes containing the NF-IL6 binding site are induced during inflammation (Akira *et al.*, 1994). Moreover, the proto-oncogene product E2A (Murre *et al.*, 1989) binding site is found at −735. A site for GHF-5 (Schaufele *et al.*, 1990), a ubiquitous gene activator that is induced in response to various extracellular signals, is found at position −601. The flanking region also contains DNA motifs for AP1 (Lee *et al.*, 1987) at −1,449 and −1,211.

Functional analysis of the 5′-flanking region

To verify whether the 5′-flanking region of the carp JAK1 kinase gene exhibits functional promoter activity, the genomic DNA fragment containing the 5′-upstream region (−2,541 to +59) was fused to the CAT reporter gene in pCAT-Basic to create pJP1-CAT (Fig. 12). Following transfection into carp CF cells (Chen and Kou, 1986), this chimeric gene produced about 16 times of the promoter activity of the pCAT-Basic, indicating that this fragment contains a functional eukaryotic promoter. A series of 5′ deletion mutants were then created from pJP1-CAT and analyzed. As shown in Fig. 12, the promoter activity of pJP4 (−1,023 to +59) was the same as that of pJP1 (−2,541 to +59). This indicates that deletion of the region between −2,541 and −1,023 did not have a detrimental effect on promoter activity. However, two further deletion mutants, pJP5 (−528 to +59) and pJP6 (−181 to +59), displayed significant loss of promoter activity that were about seven and six times of the promoter activity of the pCAT-Basic, respectively. This suggests that a positive enhancer element is located between −1,023 and −528. Interestingly, the minimal promoter element (−181 to +59) does not contain any typical TATA-box sequences or G + C-rich sequences associated with many promoters. In addition, other deletions without this region, pJP2 (−2,541 to −181) and pJP3 (−2,541 to −901), resulted in a severe loss in promoter activity and the activity was the same as that of the promoterless control.

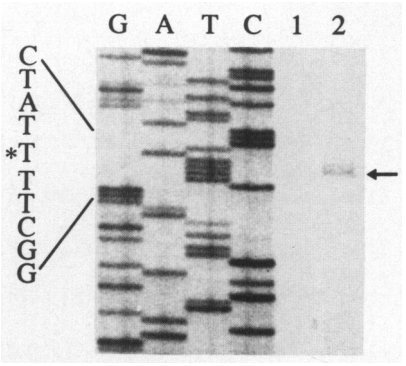

FIG. 11. Determination of the transcription initiation site of the carp JAK1 gene. ^{32}P-labeled primer (see Fig. 10) was annealed to 10 μg of yeast tRNA (lane 1, as a negative control) or 5 μg poly(A)$^+$RNA from carp liver (lane 2) and extended with reverse transcriptase. The sequencing ladder of the carp JAK1 gene labeled G, A, T, and C was obtained by using the same primer and electrophoresed on the same gel. The extended product and its position relative to the initiator ATG codon are indicated.

DISCUSSION

We have cloned a 3.7-kb cDNA that encodes carp JAK1 of 1,156 amino acids. The encoded protein has the characteristic kinase-like (JH2) and kinase (JH1) domains shared by all JAK family members. This clone has the highest sequence homology with mammalian JAK1, especially in the JH1 and JH2 domains (70% identity). The overall amino acid sequence identity between carp JAK1 and other JAK family members are: 57% (murine JAK1), 35% (murine JAK2), 31% (murine JAK3), and 42% (human Tyk2). On the basis of these homologies, we name this clone as carp JAK1.

The full-length clone of the carp JAK1 cDNA was not directly isolated by library screening, but was constructed from clones isolated from two different tissues. However, it represents the true carp JAK1 cDNA because we have employed RT-PCR to obtain the uncloned regions in the brain and liver tissues. The nucleotide sequences of the uncloned region in the brain were the same as those in clone pJ21 (isolated from a liver cDNA library). Similarly, the nucleotide sequences of the uncloned region in the liver were the same as those in clone pJ9 (isolated from a brain cDNA library). This also suggests that tissue-specific splicing does not occur in brain and liver tissues.

The baculovirus expression system has been used to express mammalian JAK2 kinase (Quelle *et al.*, 1994; Duhe and Farrar, 1995), and it appears that JAK2 kinase autophosphorylates and becomes activated when overexpressed in insect cells. In this report, all of the recombinant proteins, such as full-length carp JAK1 (c-JAK1), the JH1 (c-JH1), and combined JH2/JH1 [c-JH(1 + 2)], can display tyrosine kinase activity and self-autophosphorylation (Fig. 5C, lanes 5–10). It appears that once

kinase production exceeds a critical level, monomers of the kinase begin to form catalytically competent oligomers and then tyrosine autophosphorylation occurs spontaneously (Duhe and Farrar, 1995).

The JAK kinase members lack SH2 and SH3 domains, but contain a unique JH2 domain (Ziemiecki *et al.*, 1994). The functions of the JH2 domain are still unknown. In this study we have attempted to address this issue by using carp JAK1 as a model. Co-infection and immunoprecipitation data indicate that c-JH1 and c-JH2 associate with each other (Fig. 6). In addition, c-JH2 can be tyrosine-phosphorylated by c-JAK1 and by c-JH(1 + 2) (Fig. 7). This strongly suggests that the transphosphorylation does occur and c-JH2 is the potential substrate for c-JAK1, c-JH(1 + 2). The results also raise some questions. Is JH2 domain phosphorylated by the JAK kinases in cytokine-mediated signaling as we have observed in insect cells? What are the nature and relevance of c-JH2 tyrosine phosphorylation? Are these phosphorylation sites playing important roles in re-

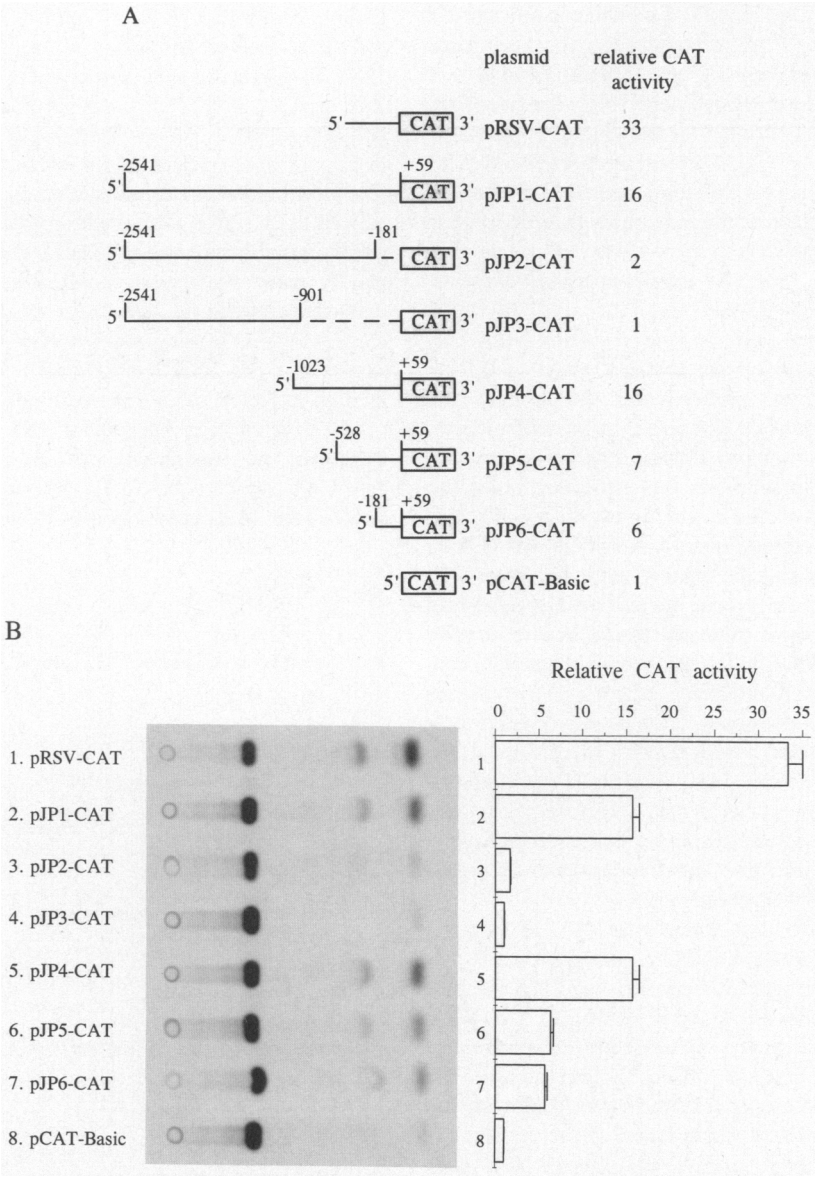

FIG. 12. Analysis of the promoter activity of the 5′-flanking region of the carp JAK1 gene fused to the CAT reporter gene. A. Schematic representation of various CAT transient expression constructs. DNA constructs are shown relative to the promoterless vector pCAT-Basic with the CAT gene depicted as an open box. Various lengths of the 5′-flanking region of the gene were fused with the CAT gene in pCAT-Basic (see Materials and Methods). B. Chimeric genes with different promoter activities. Each chimeric gene was cotransfected with pSV-β-Gal DNA into CF cells and assayed for CAT and β-Gal activities as described in Materials and Methods. CAT activity in an individual experiment was corrected for variation in transfection efficiency by normalizing the value to the β-Gal activity in the same extract. The normalized activity of each promoter construct was then expressed relative to that of pCAT-Basic, with pCAT-Basic assigned a relative activity of 1.0. The data represented the mean of triplicate transfection experiments for each plasmid.

cruiting additional signalling proteins to the cytokine receptor complex? Identification and characterization of tyrosine phosphorylation sites in c-JH2 are currently under investigation. The transphosphorylation between mammalian JAK kinases has been reported in a recent study on the interferon-γ signal transduction in which JAK2 can phosphorylate itself and JAK1 (Briscoe *et al.*, 1996). However, which domain of the JAK kinases is involved in this transphosphorylation is unknown. Therefore, our results may provide some information regarding this issue.

In cells expressing c-JAK1 and c-JH(1 + 2), a 60-kD protein (pp60) was detected by anti-JH1 antibodies and by anti-PY mAb (Fig. 5B, C, lanes 7–10). Although pp60 may be a degradation product of c-JAK1 or c-JH(1 + 2), pp60 could also be detected in the cell extract when insect cells were coinfected with vAcJH1 and vAcJH2 (data not shown). As reported recently, a relatively similar 62-kD protein has been found in the truncated rJAK2 immunoprecipitation complex (Duhe and Farrar, 1995). These two proteins are possibly the same protein that may be virally encoded or may be a Sf9 cellular product.

The association of an increased number of phosphotyrosine-containing proteins with c-JAK1 and cJH(1 + 2) (Fig. 5C) poses some intriguing questions. Are these proteins specifically associated with Ni-agarose or are proteins bound to the Ni-agarose phosphorylated to a greater extent. The proteins analyzed were fractions bound to Ni-agarose, presumably *via* the his-tag of recombinant proteins. If the former is correct, one might expect that those proteins are JH2-associated molecules. By contrast, an alternative explanation for the latter is that JH2 activates JH1, which indiscriminately phosphorylates cellular proteins resulting in nonspecific binding to the Ni-agarose. To test the possibility, Sf9 cells were infected with recombinant baculovirus, which contains truncated human insulin receptor gene (Villalba *et al.*, 1989). In the cell extract, there were constitutively active truncated insulin receptor and many tyrosine-phosphorylated proteins. However, these proteins could not be precipitated by Ni-agarose (data not shown). Therefore, the possibility that high numbers of c-JAK1- and c-JH(1 + 2)-associated proteins could be an artifact of the Ni-agarose column was ruled out. At present, we do not know how c-JAK1 and c-JH(1 + 2) associate with those tyrosine-phosphorylated proteins and this awaits further investigation.

In this study, we have also cloned at least 31 kb of a genomic region containing the carp *JAK1* gene. This gene consists of 24 exons that average 158 bp in size and 23 introns that range from 100 bp (intron 19) to >3 kb (intron 1).

The first intron interrupts the corresponding 5′ untranslated region of the carp *JAK1* mRNA sequence. Thus, not all of the exons contain translated sequences. An untranslated exon 1 is also found in many protein tyrosine kinase genes, such as the human *LCK* gene (Rouer *et al.*, 1989), mouse *HCK* gene (Ziegler *et al.*, 1991), mouse fibroblast growth factor receptor 3 gene (Perez-Castro *et al.*, 1995), human a-platelet-derived growth factor receptor gene (Kawagishi *et al.*, 1995), and human Bruton's agammaglobulinemia tyrosine kinase gene (Sideras *et al.*, 1994).

The members of the JAK kinase family lack SH2 and SH3 domains, but contain a unique JH2 domain (Ziemiecki *et al.*, 1994). Although the JH2 domain contains all the hallmarks of a protein kinase, its amino acid sequence differs significantly from that of the kinase-catalytic (JH1) domain (Fig. 13). In this study, we show that the locations of intron–exon boundaries in the JH2 domain are also different from that of the JH1 domain (Fig. 13). This suggests that the JH2 domain may not be derived from the JH1 domain by simple duplication.

On the basis of the sequence homology in the kinase domain, the nonreceptor PTKs are first divided into the Src, Fes/Fer, Abl/Arg, and JAK families (Hanks, 1991). Recently, other families such as Csk, Btk/Itk/Tec, Syk/Zap70, and FAK families are also included (Ziemiecki *et al.*, 1994). The complete genomic structures of some of them are known. Within the Src family, the genomic structures of *Src*, *Fgr*, *HCK*, and *LCK* are completely solved (Anderson *et al.*, 1985; Nishizawa *et al.*, 1986; Tanaka *et al.*, 1987; Rouer *et al.*, 1989; Patel *et al.*, 1990; Ziegler *et al.*, 1991). The intron–exon boundaries in the kinase domains are highly conserved among these members. As shown in Fig. 14, the locations of intron–exon boundaries in the kinase domain within several subfamilies of nonreceptor PTKs as well as carp JAK1 kinase are presented schematically. The intron–exon organization of carp JAK1 kinase is different from that of other nonreceptor PTKs. One exception is that the boundary between the carp JAK1 exons 18 and 19 within the consensus ATP-binding motif (Gly-X-Gly-X-X-Gly) is at the same site in Fes. This observation is consistent with the classification of JAKs into a separate family of nonreceptor PTKs.

The expression of mammalian JAK1, JAK2, and TYK2 is ubiquitous, whereas that of JAK3 is mainly in hematopoietic cells (Ihle and Kerr, 1995). To understand the possible regulation of carp *JAK1* gene expression, we have mapped its tran-

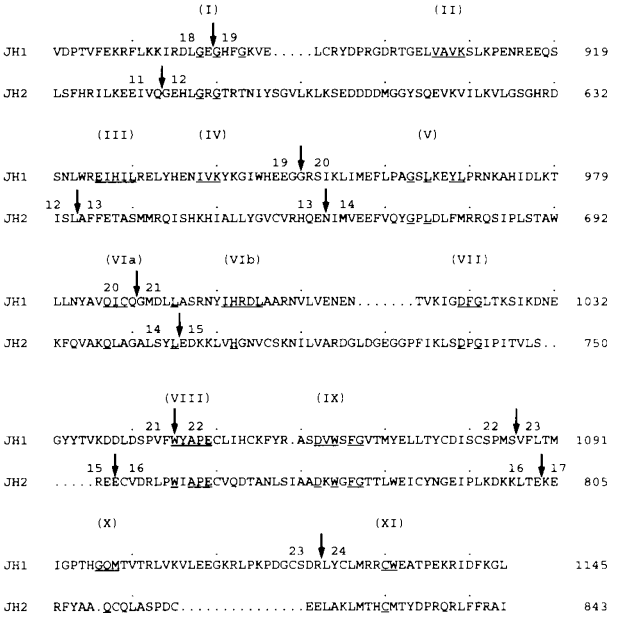

FIG. 13. Amino acid comparison and positions of the splice sites of the JH1 domain and JH2 domain of the carp JAK1 kinase. Numbers I–XI indicate 11 subdomains in the kinase domain as defined by Hanks (1991). Dots are introduced to optimize alignment. Splice positions are indicated by arrows with corresponding exon numbers. The PTK domain consensus sequences are underlined.

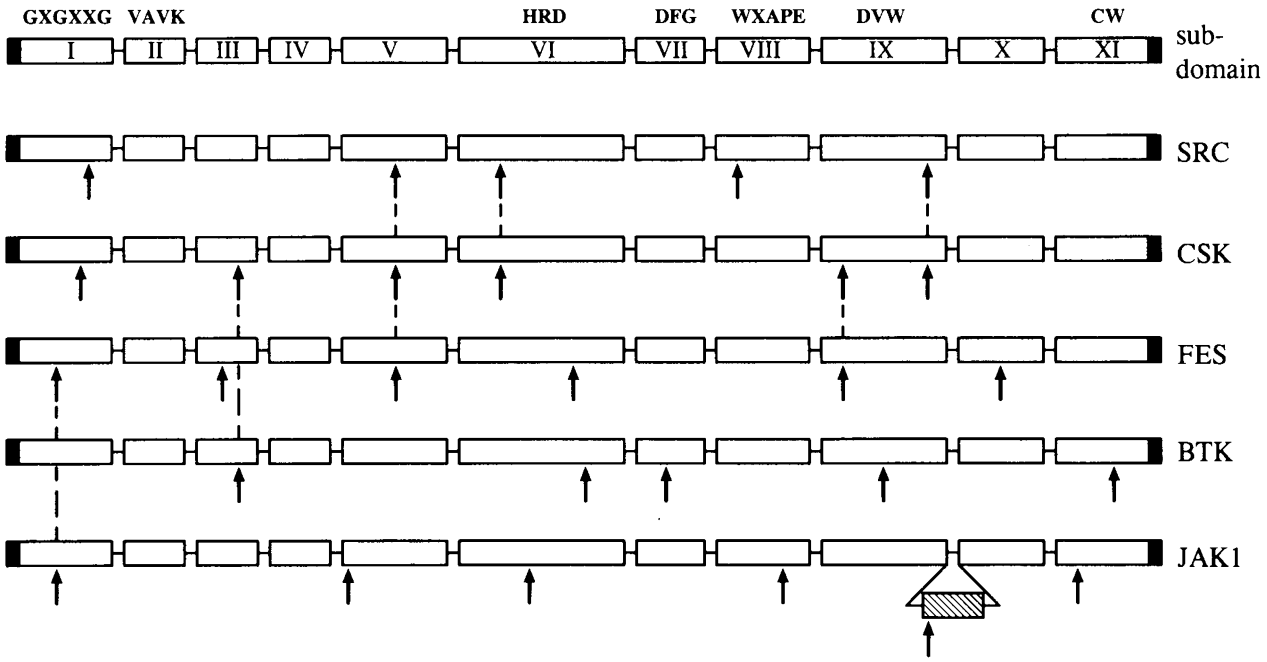

FIG. 14. Schematic comparison of the intron–exon boundaries in the kinase-catalytic domain of representative members of five subfamilies of nonreceptor PTKs. The kinase domain is divided into 11 subdomains as defined by Hanks (1991). The kinase domain of the carp JAK1 kinase has an insertion of 15 amino acid residues between subdomains IX and X. The amino acid sequences of the kinase domain of c-Src (Anderson *et al.*, 1985; Tanaka *et al.*, 1987), Csk (Brauninger *et al.*, 1993), Fes (Jucker *et al.*, 1992), Btk (Sideras *et al.*, 1994), and carp JAK1 were compared and the consensus sequence was shown on the top of the figure. Arrows indicate the location of exon boundaries. Vertical dotted lines indicate the boundaries that are found at the same corresponding position.

scription initiation site and characterized the promoter region. Primer extension reactions revealed that the putative transcription initiation site is 249 bp upstream of the translation initiation site. The putative carp *JAK1* promoter confers the transcription-promoting activity when placed in front of a CAT reporter gene. The deletion mutant study defined a positive regulatory region between −1,023 and −528 because its deletion decreased the CAT activity (Fig. 12). Some potential transcription factor binding sites are present in the carp *JAK1* promoter region, *i.e.*, AP1, E2A, GHF-5, HNF-5, and NF-IL6. However, their functional relevance awaits further characterization.

The promoter of carp *JAK1* gene is unique when compared with other PTKs, because of promoters of receptor PTKs such as platelet-derived growth factor receptor (Kawagishi *et al.*, 1995) and fibroblast growth factor receptor 3 (Perez-Castro *et al.*, 1995), or nonreceptor PTKs such as *HCK* (Lichtenberg *et al.*, 1992), c-*fgr* (Patel *et al.*, 1990), *lyn* (Uchiumi *et al.*, 1992), and *Btk* (Sideras *et al.*, 1994) do not contain TATA or CAAT boxes but have multiple SP1 sites and multiple transcriptional initiation sites. The smaller region (−181 to +59) of carp promoter does not contain typical TATA-box sequences, G + C-rich sequences, or other binding sequences for known transcription factors. However, this region still has promoter activity. Therefore, some important elements that regulate the expression of carp *JAK1* gene may be present in this region and are needed to be characterized.

ACKNOWLEDGMENTS

We are grateful to Dr. Hsing-Jien Kung for critically reading the manuscript and encouragement. We also thank Drs. Jeffrey Jong-Young Yen and Hsin-Fang Yang-Yen for helpful discussions and advice. We also acknowledge Mr. Shui-Tsung Chen for his excellent technical assistance in raising polyclonal antibodies. We are grateful to Dr. Chu-Fang Lo for providing recombinant baculovirus vAcCAT.

This work was supported by National Science Council grants NSC-82-0203-B-001-119, NSC-83-2321-B-001-072 and NSC-85-2311-B-001-046 (to C.J. Huang) and Department of Health grants DOH82-HR-C08 and DOH85-HR-507 (to C.K. Chou).

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Received for publication December 12, 1996; accepted April 17, 1996.