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Sp Family Members and Nuclear Factor-Y Cooperatively Stimulate Transcription from the Rat Pyruvate Kinase M Gene Distal Promoter Region via Their Direct Interactions*

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The three distal transcriptional regulatory elements of the rat pyruvate kinase M gene, referred to as boxes A, B, and C, are located around −270 base pairs upstream from the transcriptional initiation site. Electrophoretic mobility shift assays with specific competitors and antibodies show that both box A and box B bind to Sp1 and Sp3 and that box C binds nuclear factor-Y (NF-Y). Luciferase reporter assays revealed that although box A and box B alone have no independent effect on luciferase activities, box C alone stimulates transcription. However, the inclusion of all three elements lead to maximal activity because of a synergistic effect, mainly between box B and box C, suggesting that functional synergism between Sp1/Sp3 and NF-Y is critical for the pyruvate kinase M (PKM) gene distal promoter activity. In fact, co-transfection of a dominant negative mutant of NF-YA (NF-YA29) resulted in a decrease in reporter activity in a box C-dependent manner. In addition, the overexpression of Sp1 or Sp3 and NF-Y in *Drosophila* SL2 cells synergistically stimulated PKM gene distal promoter activity. Using a mammalian two-hybrid system in HeLa cells, it was shown that both Sp1 and Sp3 interacted with NF-YA but not NF-YB and NF-YC. Moreover, glutathione *S*-transferase pull-down assays revealed that only *in vitro* translated ^{35}S-labeled NF-YA interacted with both Sp1 and Sp3 *in vitro*. A subunit interaction domain of NF-YA, which forms a heterotrimer with NF-YB and NF-YC, is not required for these interactions with Sp1 or Sp3. Thus, we conclude that Sp1, Sp3, and NF-Y stimulate the transcription of the PKM gene via their interactions.

Four isoenzymes of pyruvate kinase (PK, EC 2.7.1.40),[1] a rate-controlling glycolytic enzyme, are known to exist in mammals, and they are commonly referred to as M_1-, M_2-, L-, and R-types, respectively (1). These isoenzymes are produced from two genes, *PKM* (2–4) and *PKL* genes (5). In the case of the rat, the *PKM* gene encodes for both M_1- and M_2-type PK isoenzymes, whereas the *PKL* gene encodes for both the R- and L-types (2, 3, 5). The rat *PKM* gene is 20 kb in length and consists of 12 exons and 11 introns. Exons 9 and 10 contain nucleotide sequences that are specific to M_1-PK and M_2-PK, respectively. The other exons are common to both isoenzymes. Although M_1-PK is expressed in the skeletal muscle, heart, and brain, M_2-PK is expressed in broad variety of tissues. Thus, these isoenzymes are produced from a common primary transcript by mutually exclusive alternative splicing that selects exon 9 or exon 10 in a tissue-specific manner.

PKM gene expression is regulated under a variety of conditions (6). Although the M_2-PK isoenzyme is the only one that is detectable in early fetal tissues, it is gradually replaced by tissue-specific isoenzymes such as M_1-, L-, and R-types as development proceeds. In contrast, when the cells or tissues that express these tissue-specific isoenzymes are de-differentiated or develop a neoplasm, their expression is reduced or disappears, and M_2-PK expression is activated or enhanced.

Two DNase I-hypersensitive sites of the *PKM* gene have been detected in rat hepatoma cells (7). These sites, HS-1 and HS-2, are not observed in hepatocytes, which do not express the *PKM* gene. HS-1 is located in the first intron, and HS-2 is in the vicinity of the transcription initiation sites. Accordingly, an alteration in chromatin structure may be involved in the transcriptional activity of the *PKM* gene. It has been shown that transcriptional regulatory elements of the rat *PKM* gene are located within the −279 base pairs upstream of transcription initiation sites using transient transfection assays of the PKM promoter/chloramphenicol acetyltransferase (CAT) reporter plasmid (7). The HS-2, but not the HS-1 site, was found to be involved in promoter activity. Two regulatory regions in the PKM gene promoter exist, the distal and proximal regions. The distal region consists of three *cis*-acting elements, which are referred to as boxes A (−279 to −265), B (−256 to −242), and C (−235 to −216). The deletion of this region results in a dramatic decrease in transcriptional activity. Thus far, their binding proteins are unknown (7). In contrast, the proximal region consists of multiple GC boxes, GC box 1 (−48 to −39), GC box 2 (−86 to −77), and GC box 3 (−133 to −124) (8). Both GC boxes 1 and 3 are functionally active. GC box 2 is functionally silent in the case of FTO-2B rat hepatoma cells but not in C_2C_{12} rat myocytes (8, 9). All GC boxes are bound by Sp1 and Sp3 (8).

Sp1 is an ubiquitously expressed transcription factor that plays a major role in the transcription of a number of gene promoters (10). Recently, the closely related proteins Sp2, Sp3,

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[1] The abbreviations used are: PK, pyruvate kinase; CAT, chloramphenicol acetyltransferase; NF-Y, nuclear factor-Y; SL2, Schneider line 2; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; DBD, DNA-binding domain; AD, activation domain; SRE, sterol response element; Y box, inverted CCAAT box; CBP, cyclic AMP response element-binding protein-binding protein; P/CAF, p300/CBP-associated factor; kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; mut, mutated.

and Sp4 have been cloned (11, 12). These proteins are members of the Sp family (13). Sp2 does not recognize the same sequences as Sp1 (11), and Sp4 expression is restricted to brain tissue (14). Both Sp1 and Sp3 are ubiquitously expressed in mammalian cells and compete for common target sequences, including the GC box and GT box, respectively, with similar binding affinities (10–12, 14–16). Although Sp1 is a transcriptional activator (10), Sp3 is both a transcriptional activator and a transcriptional repressor (14, 15). The bifunctionality of Sp3 is dependent upon the promoter context as well as the cellular background (17).

Nuclear factor-Y (NF-Y), which is also referred to as the CCAAT-binding factor, binds to an inverted CCAAT box (5′-ATTGG-3′) in many gene promoters and transactivates their transcription (18, 19). NF-Y, which is highly conserved among species, is ubiquitously expressed and is composed of three subunits, NF-YA, NF-YB, and NF-YC, all of which are necessary for DNA binding (18, 20). NF-YB and NF-YC interact tightly with one another, and their association precedes association with NF-YA and binding to the cognate DNA sequences (21).

The present study reports on the identification and characterization of the box A-, B-, and C-binding proteins. Whereas the Sp family members, Sp1 and Sp3, bind to both box A and box B, respectively, NF-Y binds to box C. In addition, we report that these transcription factors contribute in a cooperative manner to stimulate the transcription from the PKM gene promoter via protein-protein interactions.

EXPERIMENTAL PROCEDURES

Materials—The dual luciferase reporter assay system, SP1 oligonucleotide, pGEM3, pGL3-Basic, pGL3-Control, pRL-SV, pRL-TK vectors, and the T7 TNT Quick-coupled transcription/translation system were purchased from Promega. The Qiagen plasmid kit was purchased from Qiagen. LipofectAMINE PLUS and Schneider's medium were purchased from Life Technologies, Inc. pGEX-4T-2 and pGEX-5X-1 vectors, glutathione-Sepharose 4B, rainbow colored protein molecular weight marker, and Thermo Sequenase II dye termination cycle sequencing kit were purchased from Amersham Pharmacia Biotech. [γ-^{32}P]ATP (111 TBq/mmol) and Tran^{35}S-Label (43.48 TBq/mmol) were obtained from NEN Life Science Products and ICN, respectively. Anti-Sp1 (SC-420X) or anti-Sp3 (SC-644X) antibodies were purchased from Santa Cruz. A Bio-Rad protein assay kit was obtained from Bio-Rad. An *Escherichia coli* strain, TOPP3, and pBluescriptII SK vector were obtained from Stratagene.

Cells and Cell Culture—dRLh-84 cells (22), a rat hepatoma cell line, CV-1 cells, an African green monkey kidney cell line, HepG2 cells, a human hepatoma cell line, and HeLa cells, a human cervical carcinoma cell line, were provided by the Japan Cancer Research Resources Bank. These cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator. Schneider Line 2 (SL2) cells, a *Drosophila* cell line, were purchased from the American Type Culture Collection. SL2 cells were grown in Schneider's medium supplemented with 10% fetal bovine serum at 25 °C.

Electrophoretic Mobility Shift Assays (EMSAs)—Preparation of nuclear extracts was carried out as described previously (23). The nucleotide sequences of oligonucleotides used in EMSA are listed in Table I. EMSAs were performed as described previously (23). For a competition analysis, an indicated amount of competitor DNAs was added to the binding mixture. After completion of the binding, the mixture was subjected to electrophoresis on a 6% polyacrylamide gel (19:1 = acrylamide:bis-acrylamide) in 44.5 mM Tris-HCl, pH 8.0, 44.5 mM boric acid, and 1 mM EDTA at 200 V for 1 h. For supershift assays, antibodies were first mixed with the nuclear extracts on ice for 1 h, and a ^{32}P-labeled probe was then added to the mixture, followed by a 30-min incubation. The mixture was then subjected to electrophoresis on a 4% gel polyacrylamide (30: 0.8 = acrylamide: bis-acrylamide), after which the gels were dried and exposed to Kodak X-AR film.

Anti-NF-YA monoclonal and anti-NF-YB polyclonal antibodies were generous gifts of Dr. Robert Mantovani (University of Milano, Italy) (24). The anti-hepatocyte nuclear factor 4 antiserum has been previously described (23).

TABLE I
Nucleotide sequences of oligonucleotides used in EMSAs
Mutated bases are underlined.

Oligonucleotides	Nucleotide sequences
Box A	5′-agctCTAGGGGTGGTGGCGGCGGT-3′ 3′-GATCCCCACCACCGCCGCCAtcga-5′
mut box A	5′-agctCTAGGGTTTGTGGCGGCGGT-3′ 3′-GATCCCAAACACCGCCGCCAtcga-5′
mut box A2	5′-agctCTAGGGGTGGTGATAACGGT-3′ 3′-GATCCCCACCACTATTGCCAtcga-5′
SRE-1	5′-gatcCTGATCACCCCACTGAGGAG-3′ 3′-GACTAGTGGGGTGACTCCTCctag-5′
Box B	5′-gatcCCGAGGGCGGGTCCTA-3′ 3′-GGCTCCCGCCCAGGATctag-5′
mut box B	5′-gatcCCGAGGATGGGTCCTA-3′ 3′-GGCTCCTACCCAGGATctag-5′
SP1	5′-ATTCGATCGGGGCGGGGCGAGC-3′ 3′-TAAGCTAGCCCCGCCCCGCTCG-5′
Box C	5′-ctagTCACCACTTCCCCATTGGCC-3′ 3′-AGTGGTGAAGGGGTAACCGGgatc-5′
mut box C	5′-ctagTCACCACTTCCCCCTGGTCC-3′ 3′-AGTGGTGAAGGGGGACCAGGgatc-5′
Y box	5′-gatcGTGCTGATTGGTTTTCCG-3′ 3′-CACGACTAACCAAAAGGCctag-5′

Plasmids—pMcat286 and pMcat197 plasmids were described previously (3, 7). 0.3- and 0.2-kb *Kpn*I/*Hin*dIII fragments from pMcat286 and pMcat197 were subcloned into the *Kpn*I/*Hin*dIII sites of the pGL3-Basic vector and designated as the pMPK287/Luc and pMPK194/Luc, respectively.

pGM-4 vector, a Rous sarcoma virus promoter-directed expression vector, was a gift from Drs. Paolo Monaci and Alfredo Nicosia (Instituto di Ricerche di Biologia Moleculare, Italy). pPac, pPac-Sp1, pPac-Sp3, pPac-USp3, and pGAL4-Sp1 vectors were generously provided by Dr. Guntram Suske (Philipps-Universität Marburg, Germany). A 2.3-kb *Bam*HI fragment of pPac-Sp3 was isolated and subcloned into the *Bam*HI site of the pGM-4 or the pGEX-4T-2 to produce a Sp3 expression vector directed by the Rous sarcoma virus promoter (pRSV-Sp3) or glutathione *S*-transferase (GST)-Sp3 fusion protein expression vector (pGST-Sp3), respectively. A 2-kb *Eco*RI/*Xba*I fragment of the pGAL4-Sp1 was isolated and subcloned into the *Eco*RI/*Xba*I sites of the pBluescriptII SK vector. A 2-kb *Eco*RI/*Not*I fragment of the resultant plasmid was ligated into the *Eco*RI/*Not*I sites of the pGEX-5X-1 to produce the GST-Sp1 fusion protein expression plasmid (pGST-Sp1). The pRSV-Sp1 plasmid, a Sp1 expression vector, was a generous gift from Dr. Yosiaki Fujii-Kuriyama (Tohoku University, Japan) (25). NF-YA29 plasmid, a dominant negative form of the NF-Y expression vector, was provided by Dr. Roberto Mantovani (26). pPacNF-YA, pPacNF-YB, and pPacNF-YC were gifts from Dr. Timothy F. Osborne (University of California, Irvine) (27). 5× Gal4 E1b/CAT and pHK3nVP16 vectors were generous gifts from Dr. Tony Kouzarides (University of Cambridge, UK) (28). The 5× Gal4 E1b/CAT was digested with *Xho*I, blunt-ended by the Klenow reaction, and digested with *Bam*HI. The insert DNA was subcloned into the *Sma*I/*Bgl*II sites of the pGL3-Basic vector to produce the 5× Gal4 E1b/Luc plasmid. pSG424, NF-YA·pET3a, NF-YB·pET3a, and NF-YC·pET3a vectors were kindly provided by Dr. Daryl K. Granner (Vanderbilt University) (29–31). To easily prepare overexpression constructs for GAL4 fusion protein, a synthesized double-stranded oligonucleotide consisting of customized multiple cloning sites with 5′ suppressed *Eco*RI and 3′ intact *Sac*I cohesive sites 5′-AATTGCATATGGAATTCCCGGGGATCCGTCGACGAGCT-3′ was inserted into the *Eco*RI/*Sac*I sites of the pSG424. This pSG424(N/R) vector contained the introduced multiple cloning sites consisting of *Nde*I/*Eco*RI/*Sma*I/*Bam*HI/*Sal*I/*Sac*I in that order. *Nde*I/*Eco*RI fragments from NF-YA·pET-3a, NF-YB·pET3a, or NF-YC·pET3a were ligated into the *Nde*I/*Eco*RI site of the pSG424(N/R), thus generating pSG-NF-YA, pSG-NF-YB, and pSG-NF-YC, respectively. The NF-YA·pET3a and pSG-NF-YA were digested with *Afl*II and blunt-ended to produce the NF-YA269·pET3a and the pSG-NF-YA269. These constructs express the amino acid sequence between 1 and 269 of NF-YA under an *in vitro* translation reaction or the truncated NF-YA fused to GAL4 DNA-binding domain (DBD) driven by SV40 promoter, respectively. The synthesized oligonucleotides, 5′-CCTCGAGCCCGGGGAATTCGAGCT-3′ and 5′-CTAGAGCTCGAATTCCCCGGGCTCGAGGGTAC-3′ were annealed, phosphorylated, and ligated into the *Kpn*I/*Xba*I sites of the pHK3nVP16 to produce the pVP16B1 vector. A 2.0-kb *Eco*RI/*Xba*I from pGAL4-Sp1 was inserted into the *Eco*RI/*Xba*I sites of the pVP16B1 to obtain pVP16-Sp1. A 2.3-kb

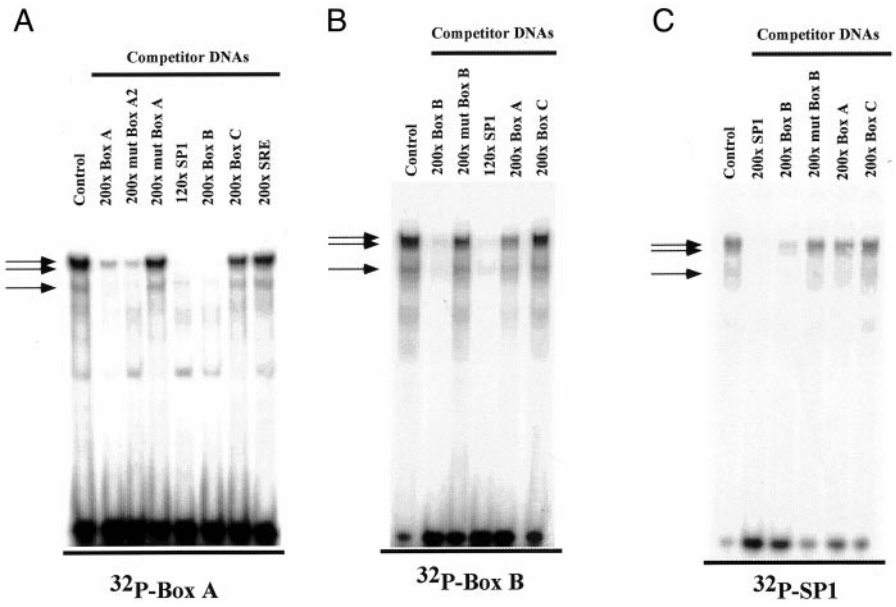

FIG. 1. **EMSA analyses of box A- and box B-binding proteins.** An end-labeled box A (*A*), box B (*B*), or SP1 oligonucleotide (*C*) was incubated with 5 μg of a nuclear extract of rat hepatoma dRLh-84 cells. Probe DNAs are shown at the *bottom*. The competitor DNAs shown at the *top* were used at the indicated fold molar excess. Protein-DNA complexes were separated by a 6% PAGE and subjected to autoradiography. The *arrows* on the *left* indicate the positions of the protein-DNA complexes.

*Bam*HI fragment from pPac-Sp3 was subcloned into the *Bam*HI site of the pHK3nVP16 to produce the pVP16-Sp3. The sequences of all plasmids were confirmed by dideoxy sequencing.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out according to the method of Künkel *et al.* (32). A *Kpn*I/*Hin*dIII fragment of pMcat286 was subcloned into the *Kpn*I/*Hin*dIII sites of the pUC118, and the resulting plasmid was used as a template for mutagenesis. Boxes A, B, and C were mutated alone or in combinations using the following primers, 5′-CCGCCGCCACAAAACCCTAGATCC-3′ for box A, 5′-GGCAGGACCCATCCTCGGGTCC-3′ for box B, and 5′-CATTCTGATGGACCAGGGGGAAGTGG-3′ for box C, respectively (mutated bases are underlined). These mutants were verified by DNA sequencing. *Kpn*I/*Hin*dIII fragments from mutated plasmids were subcloned into the *Kpn*I/*Hin*dIII sites of the pGL3-Basic to produce pMPK/Luc-287-mutA, pMPK/Luc287-mutB, pMPK/Luc287-mutC, pMPK/Luc287-mutA/B, pMPK/Luc287-mutA/C, pMPK/Luc287-mutB/C, and pMPK/Luc287-mutA/B/C. The preparation of pLcat62′ has been described previously (33). A *Kpn*I/*Hin*dIII fragment of the pLcat62′ was inserted into the *Kpn*I/*Hin*dIII sites of the pGL3-Basic vector to generate the DdeL reporter plasmid. Polymerase chain reactions were carried out using wild type or mutated pMPK/Luc plasmids as templates in a combination of 5′-LUC primer, 5′-CTAGCAAAATAGGCTGTCCC-3′, and 3′-MPK primer, 5′-CCGGGGTACCCGCATGGGTCATTCTGATG-3′. These products were digested with *Kpn*I and subcloned into the *Kpn*I site of the DdeL to obtain the WT/DdeL, mutA/DdeL, mutB/DdeL, mutC/DdeL, mutAB/DdeL, mutAC/DdeL, mutBC/DdeL, or mutABC/DdeL, respectively. The sequences of all plasmids were confirmed by dideoxy sequencing.

DNA Transfections—DNA transfections for HepG2 cells and HeLa cells were carried out by a calcium phosphate method (34). All plasmids used for transfection were prepared using a Qiagen plasmid kit, followed by CsCl gradient ultracentrifugation. 5×10^4 cells/well were inoculated in a 24-well plate on the day prior to transfection. CV-1 cells were transfected using LipofectAMINE PLUS. 2×10^5 cells/well were inoculated in a 24-well plate on the day before transfection. In both transfection methods, the indicated amount of reporter plasmid, pRL-SV or pRL-TK, and expression plasmid were used. The total amount of DNA was adjusted by adding pGEM3 plasmid, if required. 3 h (LipofectAMINE PLUS method) or 4 h (calcium phosphate method) after transfection, the medium was changed. The cells were harvested for determination of both firefly and sea pansy luciferase activities at 48 h after transfection.

DNA transfections for SL2 cells were carried out by a calcium phosphate method (35). Cells were plated at 1×10^6 cells/60-mm dish on day 0. On day 1, the cells were transfected with 2 μg of luciferase reporter plasmid and the indicated amount of pPac-derived expression vectors. The total amount of DNA (2.1 μg) was adjusted by an addition of the pPac plasmid. Cells were harvested 48 h after transfection, and assays were performed as described below.

Mammalian Two-hybrid Assays—The 5× Gal4 E1b/Luc was used as a reporter construct. pSG424, pSG-NF-YA, pSG-NF-YA269, pSG-NF-YB, and pSG-NF-YC plasmids were used as GAL4 DBD fusion protein expression plasmids. pHK3nVP16, pVP16-Sp1, and pVP16-Sp3 plasmids were used as VP16 activation domain (AD) expression plasmids. 0.3 μg of 5× Gal4 E1b/Luc plasmid, 0.1 μg of GAL4 DBD fusion protein expression plasmid, 0.1 μg of VP16 AD fusion protein expression plasmid, and 0.006 μg of pRL-SV were transfected into the HeLa cells with a calcium phosphate method, and luciferase activities were determined 48 h after transfection.

Luciferase Assays—Firefly and sea pansy luciferase assays were carried out according to the manufacturer's recommended protocol. Luciferase activities were determined by a Berthold Lumat model LB 9501. Firefly luciferase activities (relative light units) were normalized by sea pansy luciferase activities.

For SL2 cells, protein concentration was determined using the Bio-Rad protein assay reagents (36). Bovine γ globlin was used as a standard. Firefly luciferase activities were normalized by protein amount of cell extract.

GST Pull-down Assays—TOPP3 cells were transformed with the pGEX-5X-1, pGST-Sp1, or pGST-Sp3 fusion protein expression plasmid. Preparation of GST fusion protein, ^{35}S labeling of *in vitro* translated NF-YA, NF-YA269, NF-YB, or NF-YC, and pull-down analysis were previously described (30). Finally, the beads were resuspended in an equal volume of 2× SDS sample buffer, and each supernatant was loaded on a 10% SDS-PAGE gel, along with a prestained molecular weight marker. The gel was dried and exposed to Kodak X-AR film with an intensifying screen at −80 °C. The relative purity and amount of each fusion protein were determined by gel-staining with Coomassie Brilliant Blue R-250.

RESULTS

Sp Family Members Bind to Both Box A and Box B in Vitro, Respectively—EMSA was used to identify the proteins in nuclear extracts from rat hepatoma dRLh-84 cells that interact with boxes A, B, and C, respectively. Box A contains a GT box, a GC box, as well as sterol response element (SRE)-like sequences. Thus, the following oligonucleotides were used for competition experiments; mut box A oligonucleotide is mutated in a GT box, the mut box A2 is mutated in a GC box, along with overlapping SRE-like sequences, and SP1 and SRE-1 oligonucleotides contain a consensus GC box sequence and a consensus SRE sequence, respectively. Binding complexes of ^{32}P-labeled box A oligonucleotide nearly disappeared on the addition of a 200-fold molar excess of unlabeled box A and mut box A2 and a 120-fold molar excess of unlabeled SP1 oligonucleotides to the binding mixture (Fig. 1*A*). In contrast, the addition of a 200-fold molar excess of box C, mut box A, and SRE-1 oligonucleotides resulted in no competition. Interestingly, the addition of a 200-fold molar excess of unlabeled box B led to the disappear-

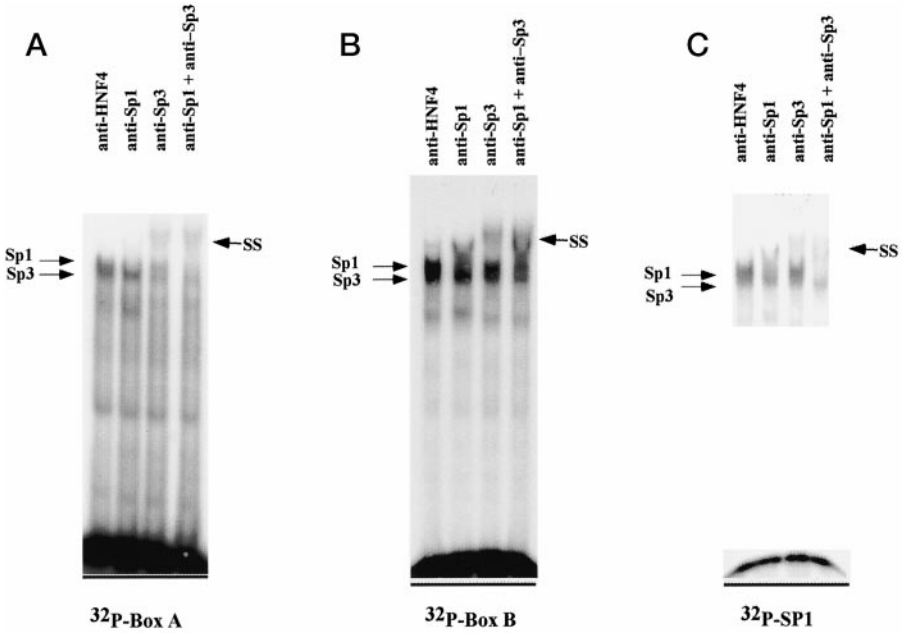

FIG. 2. **Both Sp1 and Sp3 bind to boxes A and B.** An end-labeled box A (*A*), box B (*B*), or SP1 oligonucleotide (*C*) was incubated with 5 μg of a nuclear extract of rat hepatoma dRLh-84 cells. The nuclear extract was preincubated with antiserum directed against hepatocyte nuclear factor 4 or Sp1 and/or Sp3 for 30 min prior to the addition of the probe. Protein-DNA complexes were separated by 4% PAGE and subjected to autoradiography. Probe DNAs are shown on the *bottom*. Antiserum are presented on the *top*. The *arrows* on the *left* and *right* indicate a Sp1-DNA complex or a Sp3-DNA complex and supershifted complex (*SS*) with antibodies, respectively.

ance of the binding complex. These results suggest that the box A-binding protein binds to a GT box sequence but not a GC box or an SRE in box A and that they are similar to the box B-binding protein.

DNA-protein complexes with labeled box B oligonucleotide mobilities similar to those of box A-protein complexes (Fig. 1*B*). These complexes disappeared on the addition of an excess of unlabeled box B or SP1 oligonucleotide but not on the addition of a box C oligonucleotide. The addition of a 200-fold molar excess of unlabeled box A resulted in only a slight reduction in the intensity of the bands. In contrast, mut box B oligonucleotide in which 5′-GGGCGG-3′ of box B is replaced by 5′-GGATGG-3′ failed to competitively bind to the probe. Lastly, when a labeled SP1 oligonucleotide was incubated with the same nuclear extracts, the mobilities of the resulting DNA-protein complexes were identical with those of box A- or box B-protein complexes (Fig. 1*C*). Whereas these complexes either disappeared or were decreased by the addition of a 200-fold molar excess of unlabeled SP1, box B, and box A oligonucleotides, the addition of either a 200-fold molar excess of unlabeled mut box B or box C had no effect. These results suggest that Sp1 or closely related proteins bind to a GT box in box A as well as GC box in box B and that the binding affinity of these proteins to each oligonucleotide is in the order SP1 > box B > box A oligonucleotides.

We then examined the issue of whether Sp family members are capable of binding to both boxes A and B using a specific antiserum against Sp1 and Sp3. As shown in Fig. 2*A*, the incubation of a hepatoma nuclear extract with the anti-Sp1 antiserum resulted in a faint supershifted band and a concomitant decrease in the abundance of the slowly migrating band. In addition, incubation with anti-Sp3 produced a supershifted band with parallel decreases in the abundance of the faster migrating bands. In contrast, no supershifted band was formed with anti-hepatocyte nuclear factor 4 antiserum. Identical results were obtained when the labeled box B and SP1 oligonucleotides were used as probes (Fig. 2, *B* and *C*). These results indicate that both Sp1 and Sp3 bind to box A and box B oligonucleotides, respectively.

NF-Y Binds to Box C in Vitro—Box C contains an inverted CCAAT box (Y box) sequence, 5′-CATTGGC-3′, which may be recognized by Y box-binding proteins including NF-Y or YB-1 family members. As shown in Fig. 3*A*, mixing the labeled box C oligonucleotide with a nuclear extract of dRLh-84 hepatoma cells resulted in the formation of a DNA-protein complex. This complex disappeared on the addition of a 200-fold molar excess of the unlabeled box C as well as Y box oligonucleotide, which contains the NF-Y-binding sequence of the mouse major histocompatibility complex class II gene. In contrast, the formation of this complex was not affected by the addition of mut box C, as well as unrelated box A or box B oligonucleotides. When the labeled Y box oligonucleotide was incubated with the same extract, the DNA-protein complex disappeared when a 200-fold molar excess of unlabeled Y box or box C was added but not mut box C, box A, or box B oligonucleotides (Fig. 3*B*). These results suggest that the box C-binding protein recognizes and binds to a Y box in box C.

We then examined whether box C was bound by NF-Y, a Y box-binding protein by supershift assays. When both box C and the Y box oligonucleotides were used as probes, a supershifted band and a parallel decrease in the intensity of the DNA-protein complex band were detected on incubation with anti-NF-YB antiserum but not with anti-Sp1 antiserum (Fig. 3*C*). In the case of anti-NF-YA monoclonal antibody, a faint supershifted band was detected in both experiments, and the intensity of the DNA-protein complex band was clearly reduced. These results indicate that NF-Y binds to box C.

Boxes A, B, and C Synergistically Activate the Transcription from the PKM Gene Distal Promoter—The role of three *cis*-acting elements of the PKM gene distal promoter was analyzed by luciferase reporter gene assays. Wild type or mutant of the PKM gene distal promoter region between −287 and −210 were linked to a heterologous minimal promoter from the rat L-type PK gene promoter and the firefly luciferase structural gene to exclude involvement of the PKM gene proximal promoter region in the promoter activity. The mutated sequences are shown in Fig. 4*A*. These mutations led to an impairment in the binding of transcription factors to each box as shown (Figs. 1 and 3). These constructs were transiently transfected into HepG2 human hepatoma cells, and their luciferase activities were determined (Fig. 4*B*). Relative luciferase activity of the minimal promoter construct is shown as 100%. The presence of wild type box A and box B alone and of both boxes A and B did not show any activity. In contrast, wild type box C alone increased luciferase activity by 3.4-fold. Although a combination of wild type boxes A and C did not exhibit further transcrip-

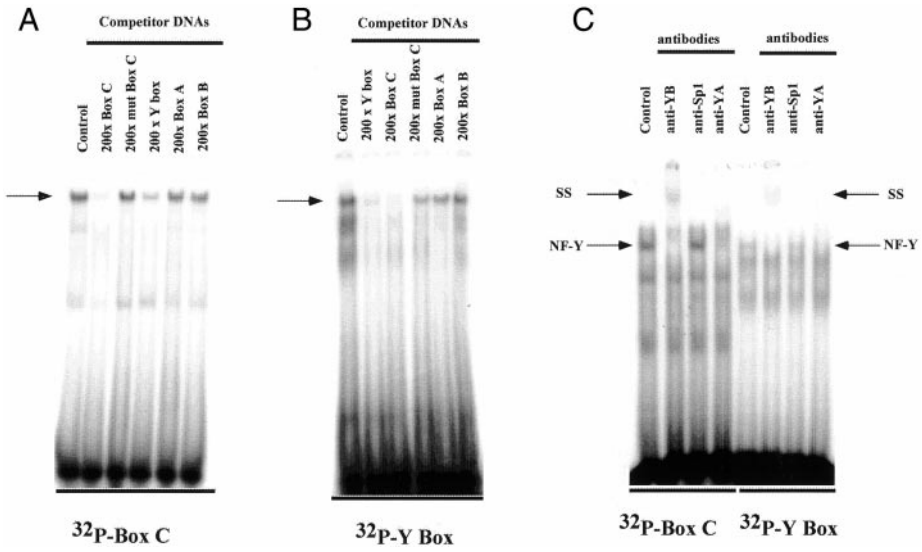

FIG. 3. **NF-Y binds to box C.** An end-labeled box C (*A* and *C*) and Y box (*B* and *C*) oligonucleotide was incubated with 5 μg of nuclear extract from rat hepatoma dRLh-84 cells. Probe DNAs are shown on the *bottom*. The competitor DNAs shown on the *top* were used at the indicated fold molar excess (*A* and *B*). Antiserum are presented on the *top* (*C*). The *arrows* on the *left* and *right* indicate the positions of the protein-DNA complex and super-shifted complexes (*SS*) with antibodies, respectively.

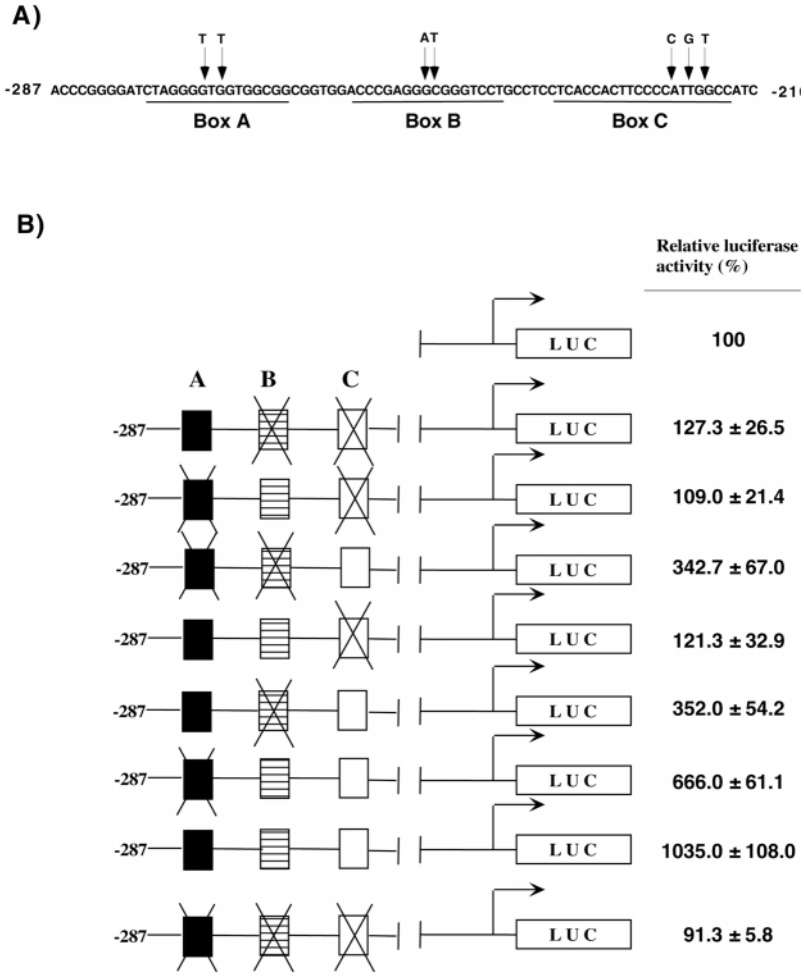

FIG. 4. **Effects of boxes A, B, and C on transcriptional activity of the rat PKM gene distal promoter.** HepG2 cells were co-transfected with 0.3 μg of reporter plasmid and 0.006 μg of pRL-SV. Mutated sequences are shown in *A*, and mutated boxes are depicted on the *left*. *B*, wild type, mutations of boxes A, B, or C of the PKM gene distal promoter, or combinations of all of the above were linked to the TATA box-containing minimal luciferase plasmid, DdeL (*rows 2–9*). A value of 100% indicates the luciferase activity of the DdeL (*row 1*). Each value represents the mean and standard error of at least three transfection experiments.

tional activity, a combination of wild type boxes B and C increased the activity by 6.6-fold. The inclusion of all three wild type boxes increased the activity by 10.4-fold. In contrast, a mutant of all three boxes had no activity. These results suggest that a functional synergism between three boxes, particularly box B and box C, is critical for the PKM gene distal promoter activity.

Dominant Negative NF-YA Decreases the Transcription from the PKM Gene Natural Promoter in CV-1 Cells—To examine the transcriptional effect of NF-Y on the PKM gene distal promoter, we carried out a co-transfection experiment of the dominant negative NF-YA with PKM/Luc reporter plasmids into CV-1 cells. To clarify the activity of distal elements between −287 and −195, the value for the luciferase activity of the pMPK287/Luc was subtracted from that of pMPK194/Luc and was shown to be 100%. As shown in Fig. 5, when pMPK287/Luc was co-transfected with NF-YA29, which expresses a dominant negative NF-YA under the control of the SV40 promoter, the distal promoter activity was decreased to the same level of that of the pMPK287/Luc-mutC, which has a

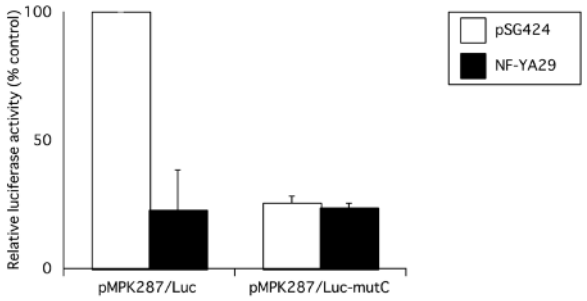

FIG. 5. **Dominant negative NF-YA29 inhibits the transcription of the PKM gene.** CV-1 cells were co-transfected with 0.2 μg of reporter plasmid, 0.006 μg of pRL-SV, and 0.2 μg of SV40 promoter-directed expression vector (shown on the *column*). pMPK287/Luc and pMPK287/Luc-mutC were used as reporters. pSG424 and NF-YA29 express GAL4 DBD and a dominant negative form of NF-YA, respectively. A value of 100% was assigned to the distal promoter activity of the pMPK287/Luc in the presence of pSG424. Each *column* and *bar* represent the mean and standard error of at least three transfection experiments.

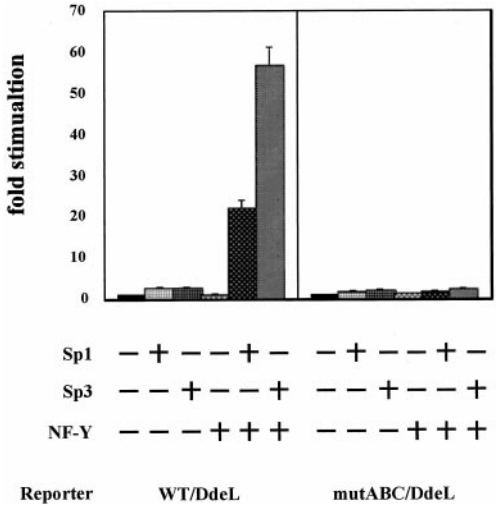

FIG. 6. **Synergistic activation by Sp1/Sp3 and NF-Y in *Drosophila* SL2 cells.** *Drosophila* SL2 cells were co-transfected with a reporter plasmid with or without expression vectors as indicated at the *bottom* of the figure. Wild type (WT/DdeL) and mutant (mutABC/DdeL) of the PKM gene distal promoter linked to the TATA box-containing minimal luciferase plasmid were used as reporters. 2 μg of reporter plasmid was co-transfected with 25 ng of pPac, 25 ng of pPac-Sp1, or 25 ng of pPac-USp3 with or without 25 ng of NF-YA, 25 ng of NF-YB, and 25 ng of NF-YC. Total DNA amount (2.1 μg) was adjusted by the addition of the pPac plasmid. Each *column* and *bar* represent the mean and standard error of at least three separate transfection experiments. The data are presented as fold stimulation where the value of luciferase activity normalized to total cell protein for the reporter alone is set at 1.0.

mutant box C. In contrast, the NF-YA29 did not affect the promoter activity of pMPK287/Luc-mutC. These results indicate that NF-Y regulates the transcription from the PKM gene promoter in a box C-dependent manner.

Both Sp Family Members and NF-Y Cooperatively Stimulate the Transcription from the PKM Gene Distal Promoter in Drosophila SL2 Cells—The effects of Sp family members and NF-Y on transcriptional activity of the PKM gene distal promoter were examined using *Drosophila* SL2 cells (Fig. 6). This cell line is devoid of endogenous Sp family members and NF-Y (27, 37). When wild type reporter construct (WT/DdeL) shown in Fig. 4*B*, was co-transfected with either Sp1 or Sp3, luciferase activities increased by 2.6- or 2.5-fold, respectively (Fig. 6). In contrast, the overexpression of NF-Y had only a marginal effect on the luciferase activity. However, inclusion of Sp1 or Sp3 and NF-Y dramatically enhanced the luciferase activities by 22- and 57-fold, respectively. These effects were not observed in the mutated reporter construct (mutABC/DdeL) shown in Fig. 4*B*. These results suggest that both Sp family members and NF-Y synergistically transactivate the transcription from the rat PKM gene distal promoter.

Both Sp1 and Sp3 Interact with NF-YA Specifically in Vivo Using a Mammalian Two-hybrid System—To examine the issue of whether a functional synergism between box B and box C in the PKM gene transcription is involved in protein-protein interaction between Sp1 or Sp3 and NF-Y, we employed a mammalian two-hybrid system. We prepared 5× Gal4 E1b/Luc, a reporter plasmid that contains five copies of the Gal4-binding sites and a TATA box from adenovirus E1b promoter upstream of the firefly luciferase coding sequences. Four GAL4 DBD expression vectors, pSG424, which expresses GAL4 DBD alone, and pSG-YA, pSG-YB, and pSG-YC, which express each of the NF-YA, NF-YB, and NF-YC subunits of NF-Y fused to C terminus of the GAL4 DBD, were employed. Three VP16 AD expression vectors, pHK3nVP16, which expresses VP16 AD alone, and pVP16-Sp1 and pVP16-Sp3, which express Sp1 and Sp3 fused to the C-terminal of VP16 AD, were also used. Various combinations of these plasmids were transfected into HeLa cells, and their luciferase activities were determined. The co-transfection the 5× Gal4 E1b/Luc with pSG-YA, pSG-YB, or pSG-YC resulted in increased luciferase activities in comparison with that of the 5× Gal4 E1b/Luc with pSG424 (data not shown). As shown in Fig. 7, only when pSG-YA was co-transfected with pVP16-Sp1 or pVP16-Sp3, the luciferase activities increased by 2.4- or 2.5-fold, respectively. These results indicate that both Sp1 and Sp3 interact with NF-YA but not with NF-YB or NF-YC *in vivo*.

NF-YA contains a subunit interaction domain to form the entire NF-Y protein through interaction with NF-YB and NF-YC. To determine whether an interaction of NF-YA with Sp1 or Sp3 occurs in the context of the whole NF-Y, we then prepared pSG-YA269, which expresses subunit interaction domain-truncated NF-YA. When pSG-YA269 was co-transfected with pVP16-Sp1 or pVP16-Sp3 but not pVP16, the luciferase activities increased by 3.5- and 2.0-fold, respectively (Fig. 7). These results indicate that both Sp1 and Sp3 interact with NF-YA subunit *per se*.

Both Sp1 and Sp3 Specifically Interact with NF-YA in Vitro as Evidenced by GST Pull-down Assays—^{35}S-Labeled NF-Y subunits, NF-YA, NF-YA269, NF-YB, and NF-YC were synthesized by *in vitro* transcription/translation system and incubated with GST, GST-Sp1, or GST-Sp3 immobilized onto glutathione Sepharose beads. The bound materials were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 8, the *in vitro* synthesized ^{35}S-labeled NF-YA and NF-YA269 were observed to bind to GST-Sp1 and GST-Sp3 but not GST alone. The amount of the material bound to GST-Sp3 was higher than that of GST-Sp1. In contrast, NF-YB and NF-YC were not bound to GST, GST-Sp1, and GST-Sp3. These results indicate that both Sp1 and Sp3 specifically interact with NF-YA subunit *in vitro* as well as *in vivo*.

DISCUSSION

Transcription factors not only bind to the cognate DNA sequences but also interact with one another to regulate gene transcription. In addition, these factors interact with non-DNA-binding proteins, such as co-activators and co-repressors. They, in turn, form a ternary complex, interact with basic transcription machinery, and confer transcriptional activity (stimulation or repression) to the gene promoter. In this study, we reported the identification of the *trans*-acting proteins of the PKM gene promoter and determined the synergistic transcriptional activation via their protein-protein interaction.

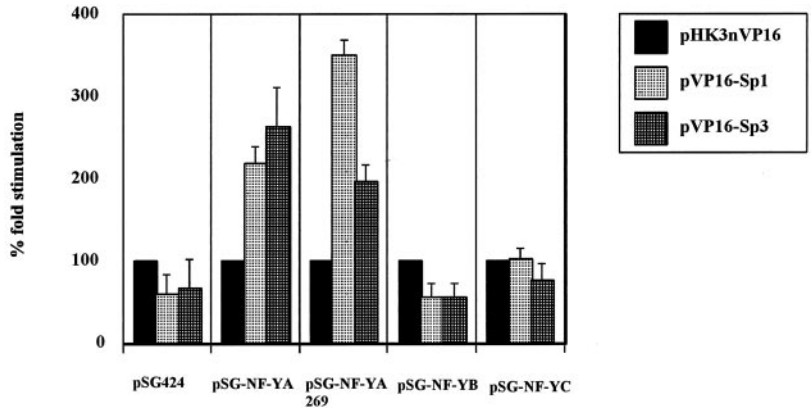

FIG. 7. **Interaction of NF-YA with Sp1 or Sp3 *in vivo* using a mammalian two-hybrid system.** HeLa cells were co-transfected with 5× Gal4 E1b/Luc plasmid, pRL-SV plasmid, GAL4 DBD fusion protein expression vector (shown on the *bottom*), and VP16 AD fusion protein expression vector (shown on the *right*). Although pSG424 express GAL4 DBD alone, pSG-NF-YA, pSG-NF-YA269, pSG-NF-YB, and pSG-NF-YC express NF-YA, NF-YA269, NF-YB, and NF-YC subunit of NF-Y fused to GAL4 DBD, respectively. Whereas pHK3nVP16 express VP16 AD, pVP16-Sp1 and pVP16-Sp3 express Sp1 and Sp3 fused to VP16 AD, respectively. The luciferase activity of each GAL4 DBD fusion protein in the presence of the VP16 AD is normalized to a value of 100. Each *column* and *bar* represent the mean and standard error of at least three transfection experiments.

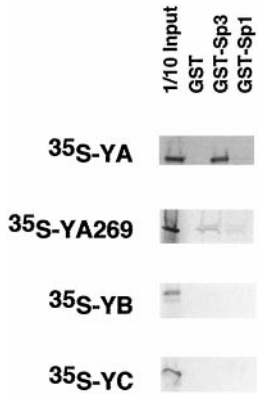

FIG. 8. **Interaction of NF-YA with Sp1 or Sp3 *in vitro* GST binding assays.** *In vitro* translated, ^{35}S-labeled, NF-YA, NF-YA269, NF-YB, or NF-YC (shown on the *left*) were incubated with agarose beads bound GST, GST-Sp1, or GST-Sp3 fusion protein (shown on the *top*). The beads were washed thoroughly, and the bound protein was analyzed by SDS-PAGE and autoradiography. The signal in the lane marked *1/10 Input* represents 10% of the protein added to the reactions shown in the other lanes.

Both Sp1 and Sp3 recognized and bound to a GT box sequence of box A and a GC box sequence of box B, respectively (Figs. 1 and 2). The binding affinity of the Sp family members to box B was higher than that to box A (Fig. 1). Boxes A and B alone had no independent effect on the transcription in transiently transfected HepG2 cells (Fig. 4). However, in the presence of box C, box B but not box A stimulated transcription. Only when all three boxes were included did box A participate in gene transcription. This difference in effects may be due to the binding affinity of Sp1 or Sp3 to each element and/or their relative position. In *Drosophila* SL2 cells, both Sp1 and Sp3 stimulated transcription from the PKM distal promoter (Fig. 6). Netzker *et al.* (8) reported that both Sp1 and Sp3 functioned as transcriptional activators at GC boxes 1 and 3 in the PKM proximal promoter in SL2 cells. Recently, these investigators also showed that both Sp1 and Sp3 stimulate PKM gene promoter activity at the GC box 4 (box B in our nomenclature) in these cells (38). We also observed that pMPK287/Luc, a construct of PKM promoter between −287 and +69 linked to the luciferase reporter plasmid, was stimulated by the overexpression of Sp1 or Sp3 in mammalian CV-1 cells (data not shown). Thus, both Sp1 and Sp3 cause stimulation of the PKM gene transcription in CV-1 cells as well as SL2 cells. It has been reported that Sp3 represses Sp1-mediated transcriptional stimulation in most genes (14) including dihydrofolate reductase (16), ornithine decarboxylase (39), and β-enolase genes (9). However, the repressor activity of Sp3 was not detected in some gene promoters, such as plasminogen activator inhibitor-1 and the c-*myc* gene (17, 40) as well as the PKM gene promoter.

Box C alone stimulated transcription from the reporter gene in HepG2 cells (Fig. 4*B*). Box C was bound by a CCAAT binding factor, NF-Y (Fig. 3) and the overexpression of the dominant negative of NF-YA decreased luciferase activity in a box C-dependent manner in the natural promoter context of the PKM gene (Fig. 5). Thus, NF-Y is a *bona fide* transcription factor for box C of the PKM gene promoter. A combination of box B and box C resulted in the synergistic activation of distal promoter activity as mentioned above (Fig. 4*B*), suggesting that a functional relationship between box B and box C exists. We also found the synergism in the natural promoter context of the PKM gene (data not shown). Functional synergism between the NF-Y and Sp1-binding sites is also exhibited in other genes such as fatty acid synthase, carnitine palmitoyltransferase Ia, *cdc25C*, thymidine kinase, and $p27^{Kip1}$ (41–45). However, an interaction between Sp1 or Sp3 and NF-Y has never been analyzed in detail. Here, we show the synergistic transcriptional activation from the PKM distal promoter by combinations of Sp1 or Sp3 and NF-Y in SL2 cells. The same synergism was observed when the pMPK287/Luc as a natural promoter construct was co-transfected with their expression vectors in SL2 cells (data not shown). We further showed that both Sp1 and Sp3 physically interact with NF-YA but not with NF-YB or NF-YC *in vivo* using a mammalian two-hybrid system. We also demonstrated that this specific interaction occurred *in vitro* using GST pull-down assays. Very recently, a physical interaction between NF-YA and Sp1 has been demonstrated (46). The results of this study showed that the amino acid sequence between 55 and 134 of NF-YA interacts with the amino acid sequence between 139 and 344 of Sp1. Our result, which showed that a subunit interaction domain of NF-YA is not required for an interaction with Sp1, is consistent with the results reported in Ref. 46. Although the interaction is not strong in both cases, this interaction could cause the synergistic transcriptional activation. However, we cannot rule out other possibility that a mediator or a bridging protein may be required for an enhancement of the interaction and the synergistic transcriptional activation. Further studies will be required for addressing this question. Thus, we conclude that

Sp1/Sp3 functionally binds to box B and physically interacts with NF-YA, when it is bound to box C. In addition, three binding sites of the Sp family members in the proximal region in the PKM promoter exist. We have not determined whether Sp1/Sp3, when bound to these sites, are involved in an interaction with NF-YA.

PKM gene expression is regulated under a variety of conditions (6). The level of M_2-PK mRNA in proliferating thymocytes that had been treated with concanavalin A and interleukin-2 was increased (47, 48). This induction coincides with the S phase of the cell cycle (47). Sp1 is regulated by phosphorylation and dephosphorylation. However, data on its function are conflicting (49–51). Phosphorylation of the C-terminal region of Sp1 is regulated in a growth/cell cycle-dependent manner that coincides with the temporal induction of dihydrofolate reductase gene transcription (49). Cyclic AMP-dependent protein kinase also catalyzes the phosphorylation of Sp1 and enhances its binding to the cognate sequence (50). Sp1 stimulates cAMP-dependent transcriptional stimulation from the *CYP11A* gene promoter in SL2 cells (52). However, it has been reported that the phosphorylated and the nonreduced form of Sp1 showed a decrease in binding to a DNA fragment that contained both GC boxes 1 and 2 of the PKM gene (51, 53). On the other hand, *cdc25C* gene transcription, which occurs late in the S/G_2 phase, is regulated by a cell cycle-dependent repressor element and an upstream activating sequences which contains three CCAAT motifs and two Sp1-binding sites. The cell cycle-dependent repressor element periodically represses *cdc25C* gene transcription by both NF-Y and Sp1. Therefore, the regulation of the PKM gene transcription in a cell cycle-dependent manner may be achieved via protein-protein interaction(s), in addition to the modification of each transcription factor. Hypoxia also causes stimulation of the PKM gene expression in cardiac myocytes and skeletal muscle cells (9, 54). It has been reported that hypoxia activates the PKM and β-enolase gene promoters by down-regulating Sp3 (9). In contrast to this report, however, Sp3 does not appear to be a transcriptional repressor but a transcriptional activator of the PKM gene. Thus, the mechanism for the hypoxia induction of the PKM gene expression must be re-examined.

The activities of Sp1 and NF-Y are altered by the treatment of cells with an inhibitor of histone deacetylase, trichostatin A (55, 56). Whereas some co-activators, including the p300/cAMP response element-binding protein-binding protein (CBP) and the p300/CBP-associated factor (P/CAF) *per se,* have histone acetyltransferase activity (57, 58), co-repressors that interact with nuclear corepressor and the silencing mediator of receptor transcription recruit histone deacetylase. These histone acetyltransferase and histone deacetylase activities alter histone acetylation status, thus resulting in the alteration of chromatin structure. Sp1 interacts with p300/CBP (59). NF-YB interacts with Tax1, a potent activator of human T-cell lymphotropic virus type 1 transcription or CBP (60). NF-YB and NF-YC both interact with the TATA-binding protein (61) and the complete NF-Y interacts with P/CAF and GCN5 (62). The overexpression of P/CAF stimulates the inverted CCAAT box-dependent transcription from the human multidrug resistance 1 gene promoter via a direct interaction between NF-Y and P/CAF (62). The interaction between NF-Y and GCN5 then results in the modulation of NF-Y transactivation potential by aiding the disruption of local chromatin structure, thereby facilitating the access of NF-Y to its binding sequence. Furthermore, it has been reported that acetylation/de-acetylation of histone or transcription factor affects gene transcription at the chromatin level. The issue of whether these factors are involved in the transcriptional activity or the formation of DNaseI-hypersensitive sites of the PKM gene by alterations of chromatin structure remains to be determined. Further studies will be required to address these questions.

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