Protein kinase A enhances, whereas glycogen synthase kinase-3β inhibits, the activity of the exon 2-encoded transactivator domain of heterogeneous nuclear ribonucleoprotein D in a hierarchical fashion

Mate TOLNAY[1], Yuang-Taung JUANG and George C. TSOKOS

Walter Reed Army Institute of Research, Silver Spring, MD 20910, U.S.A., and Uniformed Services University, Bethesda, MD 20814, U.S.A.

Heterogeneous nuclear ribonucleoprotein D (hnRNP D) is implicated in transcriptional regulation. Alternative splicing of exons 2 and 7 generates four isoforms of the protein. We report here that only isoforms that contain the product of exon 2 (amino acids 79–97) were able to transactivate. Moreover, the exon 2-encoded protein domain alone was sufficient to drive transcription. TATA-binding protein and p300 interacted with a synthetic peptide corresponding to exon 2, and both proteins co-precipitated with hnRNP D. Stimulation of protein kinase A (PKA) and protein kinase C (PKC) synergistically induced the transactivating ability of hnRNP D, and the exon 2-encoded domain was sufficient for this inducibility. In kinase assays PKA phosphorylated Ser-87 of hnRNP D, whereas glycogen synthase kinase-3β (GSK-3β) phosphorylated Ser-83, but only if Ser-87 had been pre-phosphorylated by PKA. Phosphorylation of Ser-87 enhanced, whereas phosphorylation of Ser-83 repressed, transactivation. Overexpression of GSK-3β inhibited transactivation by hnRNP D, but stimulation of PKC negated the inhibitory effect of GSK-3β. We suggest that a hierarchical phosphorylation pathway regulates the transactivating ability of hnRNP D: PKA activates hnRNP D, but at the same time renders it sensitive to inhibition by GSK-3β; the latter inhibition can be suspended by inactivating GSK-3β with PKC.

Key words: hnRNP D, phosphorylation, protein kinase C, transcription.

INTRODUCTION

The heterogeneous nuclear ribonucleoproteins (hnRNPs) were first described as a group of chromatin-associated RNA-binding proteins [1,2]. hnRNP D is an abundant, ubiquitous protein that is present in both the nucleus and the cytoplasm [3]. The human and murine proteins are 97% identical, suggesting a conserved function for hnRNP D. hnRNP D exists in four isoforms, as a result of alternative splicing of exons 2 and 7 [4,5]. Exon 2 encodes 19 amino acids in the N-terminus, whereas exon 7 encodes 49 amino acids in the C-terminus. All isoforms contain a glycine-rich region in the C-terminus and two RNA-binding domains (RBDs), each consisting of about 90 amino acids, which are highly conserved among members of the 2 × RBD-Gly family. The different hnRNP D isoforms have distinct intracellular localizations, and at least certain isoforms shuttle continuously between the nucleus and cytoplasm [6]. The presence of the exon 2-encoded region seems to determine relative affinity for nucleic acids, and isoforms that contain it bind double- and single-stranded DNA in a specific manner [7], whereas isoforms that lack it have higher affinity for AU-rich RNA sequences [5,6].

Several groups have implicated hnRNP D in transcriptional regulation. We and others reported that hnRNP D recognizes a motif in the promoter region of the human complement receptor 2 gene and suggested its involvement in transcriptional regulation [6,8]. hnRNP D induces the Epstein–Barr virus C promoter in co-operation with a viral protein, EBNA2 [9]. Overexpression of E2BP, a protein that is highly homologous to hnRNP D, stimulates the hepatitis B virus enhancer II as well as the thymidine kinase promoter [10,11]. hnRNP D contains a transactivator domain in the N-terminus and a region in the C-terminus that is required for DNA binding, in addition to the RBDs [7]. Antibodies against a peptide that corresponds to the N-terminal half of exon 2 inhibit DNA binding, which implies that the exon 2-encoded region also influences interactions with DNA [7]. hnRNP D forms a complex with the TATA-binding protein (TBP) *in vivo*, suggesting that it affects transcription by interacting with the TFIID protein complex [12]. hnRNP D forms heterodimers with nucleolin in the B-cell-specific transcription factor LR1, which is implicated in transcriptional regulation [13].

In the cytoplasm, hnRNP D recognizes AU-rich motifs present in the 3′ untranslated region of many labile mRNA species, including those of cytokines and oncoproteins, and accelerates their degradation [6,14]. Those isoforms of hnRNP D that lack the product of exon 2 are more efficient in destabilizing mRNA with AU-rich motifs *in vivo* [6]. Interestingly, hnRNP D is also present in protein complexes that stabilize mRNA, suggesting that it may be a general mRNA turnover factor that is involved in both the stabilization and decay of mRNA [15,16]. In addition, hnRNP D interacts with telomerase and also with telomeric DNA, raising the possibility that it plays a functional role in telomere maintenance [17,18].

Phosphorylation of hnRNP A1, another member of the 2 × RBD-Gly family, influences its ability to bind to RNA

Abbreviations used: CAT, chloramphenicol acetyltransferase; CKII, casein kinase II; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; dcAMP, dibutyryl cAMP; DTT, dithiothreitol; GSK-3β, glycogen synthase kinase-3β; hnRNP, heterogeneous nuclear ribonucleoprotein; PKA, protein kinase A; PKC, protein kinase C; RBD, RNA-binding domain; TBP, TATA-binding protein.

[1] To whom correspondence should be addressed: Department of Cellular Injury, Walter Reed Army Institute of Research, Bldg. 503, Rm. 1A32, 503 Robert Grant Ave., Silver Spring, MD 20910-7500, U.S.A. (e-mail mtolnay@hotmail.com).

[19,20]. hnRNP D is phosphorylated *in vivo*, suggesting that protein kinases may regulate its activity [21]. Indeed, we showed that stimulation of protein kinase A (PKA) and protein kinase C (PKC) synergistically induces hnRNP D to transactivate a reporter plasmid driven by an hnRNP D motif [8]. Stimulation of the PKA pathway increases the DNA binding of hnRNP D [8,9], but the specific phosphorylation event(s) are unknown. Moreover, it is not known whether the transactivating ability of hnRNP D is regulated by phosphorylation, and how the PKC signalling pathway is involved.

In the present study we define the location of the transactivator domain of hnRNP D and the phosphorylation events that regulate its activity. We report that the exon 2-defined region of hnRNP D is responsible for transactivation and that the phosphorylation of serine residues can enhance or suppress the activity of the protein.

EXPERIMENTAL

Plasmids and mutagenesis

Fusion proteins hnRNP D-20–GAL4 and hnRNP D-27–GAL4 in the pSG424 plasmid, as well as the GAL4-CAT reporter plasmid (where CAT is chloramphenicol acetyltransferase), were described previously [7]. hnRNP D-00–GAL4 was generated by deleting the region corresponding to exon 2 by using Stratagene's Quick-Change protocol and the hnRNP D-20–GAL4 plasmid as template. The plasmid containing the exon 2–GAL4 fusion protein was made by inserting a synthetic oligonucleotide, corresponding to amino acids 79–98 of hnRNP D-20, into the *Bam*HI and *Kpn*I sites of the pSG424 vector. The fusion protein also contained a PQLPGILP linker peptide. hnRNP D point mutants, in mammalian and/or bacterial expression plasmids, were generated by using Stratagene's Quick-Change protocol. Sequences were confirmed by DNA sequencing. The glycogen synthase kinase-3β (GSK-3β) expression plasmids were a gift from Dr Hagit Eldar-Finkelman (Tel-Aviv University, Tel Aviv, Israel) [22]. All plasmids used for transfection were purified on columns from Qiagen.

Purification of recombinant proteins

We used Novagen's pET expression system. hnRNP D-20 with a His_6 tag, in the pET-21a(+) plasmid, was obtained from Dr F. Ishikawa (Yokohama National University, Yokohama, Japan). We induced the expression of the proteins in BL21(DE3) pLysS bacteria (Novagen) with 1 mM isopropyl β-D-thiogalactoside for 4 h. Recombinant wild-type or mutated hnRNP D-20 was purified on Ni^{2+} columns (Novagen), as suggested by the manufacturer. The concentrations of the purified recombinant proteins were measured using a protein microassay from Bio-Rad.

Cell cultures

IM-9 B lymphoblastoid cells (A.T.C.C.) were cultured in the presence of RPMI 1640 with 10% (v/v) fetal bovine serum, 2 μM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

Transient transfection assays

Plasmid DNA has been introduced into IM-9 B lymphoblastoid cells by electroporation, using a Bio-Rad Gene Pulser. We used 5.5 μg of expression plasmid and 4 μg of GAL4-CAT reporter plasmid for 5×10^6 cells in 0.25 ml of RPMI 1640 medium with 10% (v/v) fetal bovine serum, in a 0.4 cm electroporation cuvette at settings of 250 V and 960 μF (time constants obtained were 36–44). In experiments where GSK-3β was overexpressed, we used 5 μg each of GSK-3β and hnRNP D expression plasmids and 2 μg of reporter plasmid for 5×10^6 cells. Following transfection, cells were washed and cultured for 42 h. In some experiments cells were treated with 1 mM dibutyryl cAMP (dcAMP) and/or 100 ng/ml PMA 24 h after transfection, for 18 h. Freeze–thaw lysates containing equal amounts of protein were assayed for CAT activity, as described previously [23]. Data were analysed using a Molecular Imager (Bio-Rad) and Quantity One software (Bio-Rad).

In vitro kinase assays

Each sample contained 250 ng of recombinant hnRNP D or 1 nmol of peptide HSNSSPRHSEAATAQREEWK (synthesized by Research Genetics) along with 5 units of recombinant murine PKA catalytic subunit (Cell Signaling), 0.05 unit of rabbit GSK-3β (Upstate Biotechnology), 0.15 m-unit of human recombinant CKII (casein kinase II) catalytic subunit (Calbiochem) or 0.03 unit of rat brain PKC catalytic subunit (Calbiochem). With GSK-3β, PKA and PKC we used a buffer that contained 40 mM Tris, 10 mM $MgCl_2$, 1 mM EGTA, 10 mM NaF and 2 mM dithiothreitol (DTT), pH 7.2. With CKII we used a buffer that contained 20 mM Tris, 50 mM KCl, 10 mM $MgCl_2$ and 4 mM DTT, pH 7.5. To phosphorylate recombinant hnRNP D we incubated the samples for 30 min at 30 °C in the presence of 1 mM unlabelled ATP and 1.3 μl of [^{32}P]ATP (3000 Ci/mmol) in a total volume of 30 μl. One-half of each sample was subjected to SDS/PAGE, and dried gels were analysed on a Molecular Imager using Quantity One software. To phosphorylate the peptide we incubated the samples for 30 or 60 min at 30 °C in the presence of 5 nmol of unlabelled ATP and 3.3 pmol (1 μl) of [^{32}P]ATP in a total volume of 40 μl. The total reaction volume was spotted on to P81 filter paper (Whatman), washed four times with 0.75% phosphoric acid and then once with acetone, and the incorporated radioactivity was measured using a β-radiation counter.

Peptide affinity purification

IM-9 cells were grown in 1-litre culture flasks with continuous stirring. Nuclear proteins were extracted as described in [24]. The HSNSSPRHSEAATAQREEWK peptide with biotin attached covalently to the N-terminus through a C_{12} aminocaproic acid spacer was synthesized, then purified to 95% purity by Research Genetics. We incubated 5 mg (1.8 nmol) of peptide with 12 mg of streptavidin-coated magnetic porous glass beads (CPG Inc., Lincoln Park, NJ, U.S.A.) in 7.4 ml of binding buffer containing 10 mM Hepes, 0.15 M NaCl, 2 mM DTT and 0.005% Polysorbate-20, pH 7.4, for 1 h at 25 °C with continuous mixing. We also used beads without peptide, as a control. Beads were washed four times with 4 ml of binding buffer, using a magnet. The washed beads were combined with 60 mg of nuclear protein extract in a siliconized tube, in a total volume of 20 ml of binding buffer that also contained 1 mM PMSF and a protease inhibitor cocktail [Sigma; final concentrations: 0.15 μM aprotinin, 0.5 μM leupeptin, 0.5 mM EDTA, 0.7 μM E-64, 65 μM bestatin and 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride], and incubated for 1 h at 25 °C with continuous mixing. The beads were washed into a siliconized Eppendorf tube with binding buffer and then washed three more times with 1 ml of binding buffer. Before the last wash the beads were transferred into a new siliconized tube. Proteins were eluted for 8 min at 25 °C in 0.1 ml of buffer containing 10 mM glycine/HCl and 0.005% Polysorbate-20, pH 3, and subsequently in 0.1 ml of buffer containing 10 mM

glycine/HCl and 0.005% Polysorbate-20, pH 1.5. Aliquots of the eluted samples were subjected to SDS/PAGE and silver-stained.

Co-precipitation and Western blots

Aliquots of 1 μl of sample eluted from peptide or control beads with the pH 1.5 glycine/HCl buffer, as well as 9 μg of total nuclear protein extract, were subjected to Western blot analysis, as described in [12]. The membrane was blotted sequentially with an antibody against human TBP diluted 1:1000 (Santa Cruz Biotechnology) and with an antibody against human p300 diluted 1:200 (Santa Cruz Biotechnology). No TBP or p300 was detected in samples eluted with the pH 3 glycine/HCl buffer (results not shown).

IM-9 B cells were transfected by electroporation with plasmids expressing hnRNP D-20–GAL4 or hnRNP D-00–GAL4, and cultured for 44 h. Cells were lysed by incubation on ice for 30 min in a buffer containing 50 mM Tris, pH 7.4, 300 mM NaCl, 1% Triton X-100, 5 mM EDTA, 2 mM DTT, 2 mM PMSF, 2 μg/ml aprotinin and 2 μg/ml leupeptin. An aliquot of 800 μg of protein for each sample was resuspended in a buffer containing 10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P40, 1 mM PMSF, 2 μg/ml aprotinin and 2 μg/ml leupeptin, and pre-cleaned with normal rabbit IgG and Protein A/G PLUS–agarose beads (Santa Cruz Biotechnology) at 4 °C for 0.5 h. The supernatants were immunoprecipitated with 1 μg of GAL4-specific rabbit antibody (Santa Cruz Biotechnology; N-19) and Protein A/G PLUS–agarose beads for 1 h at 25 °C. Eluted proteins were subjected to Western-blot analysis, using an antibody against human TBP diluted 1:1000. To detect hnRNP D–GAL4 in cells that had been transfected with plasmids expressing different hnRNP D isoforms or carrying mutations, 40 μg of cellular protein was subjected to Western-blot analysis, using GAL4-specific rabbit antibody (Santa Cruz Biotechnology; SC-577) diluted 1:400.

Total cellular proteins were purified from IM-9 cells that had been treated with 1 mM dcAMP, or left untreated. Cells were lysed by incubation on ice for 30 min in a buffer containing 50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Nonidet P40, 2 mM PMSF and 0.5% sodium deoxycholate, pH 7.9. Aliquots of 150 μg of protein extract for each sample were resuspended in a buffer containing 20 mM Hepes, pH 7.9, 50 mM NaCl, 0.1% Nonidet P40, 10% glycerol, 10 mM EDTA, 2 mM EGTA, 1 mM DTT, 10 mM sodium orthovanadate, 1 mM PMSF and 20 μg/ml leupeptin, and pre-cleaned with Protein A/G PLUS–agarose beads at 4 °C for 1 h. The supernatants were immunoprecipitated with hnRNP D-specific rabbit serum diluted 1:100 (anti-P3a; specific to hnRNP D-20 and hnRNP D-00 [7]) and Protein A/G PLUS–agarose beads, at 4 °C overnight. Eluted proteins were subjected to Western-blot analysis, using an antibody against p300 diluted 1:1000 (Upstate Biotechnology). To detect hnRNP D, 10 μg of cellular protein was subjected to Western-blot analysis, using hnRNP D-specific rabbit serum diluted 1:1000 (anti-P3a).

Computer prediction of phosphorylation sites

We used the PhosphoBase program for predictions [25].

RESULTS

Exon 2 of hnRNP D encodes a transactivator domain that is necessary and sufficient to drive transcription

We have shown previously by truncating hnRNP D-20 (our nomenclature reflects the presence/absence of inserts encoded by alternatively spliced exons 2 and 7, i.e. hnRNP D-20 contains the product of exon 2 but lacks that of exon 7) that the transactivator domain of the protein is located within amino acids 70–98 [7]. This finding raised the possibility that the transactivator domain is encoded, partly or fully, by exon 2, which corresponds to amino acids 79–97. Fusion proteins containing the DNA-binding domain of GAL4 and different isoforms of hnRNP D were cloned into expression vectors and monitored for transactivation using a reporter plasmid driven by GAL4 sites, following co-transfection. We used this experimental system to avoid interference by endogenous hnRNP D that is present ubiquitously in all cells. We found that hnRNP D-00, which lacks the products of both alternatively spliced exons, was unable to transactivate (Figure 1A). However, the presence of the 19-amino-acid product of exon 2 in hnRNP D-20 allowed transactivation. The presence of the 49-amino-acid product of exon 7 in hnRNP D-27 did not increase the ability of the protein to transactivate over that of hnRNP D-20. Thus the product of exon 2 is indispensable for transactivation. We found that the different hnRNP D isoforms fused to GAL4 were expressed at similar levels following transfection (Figure 1B; differences of $> 10\%$ between samples), indicating that the observed differences in transactivation reflect changes in protein activity, not in

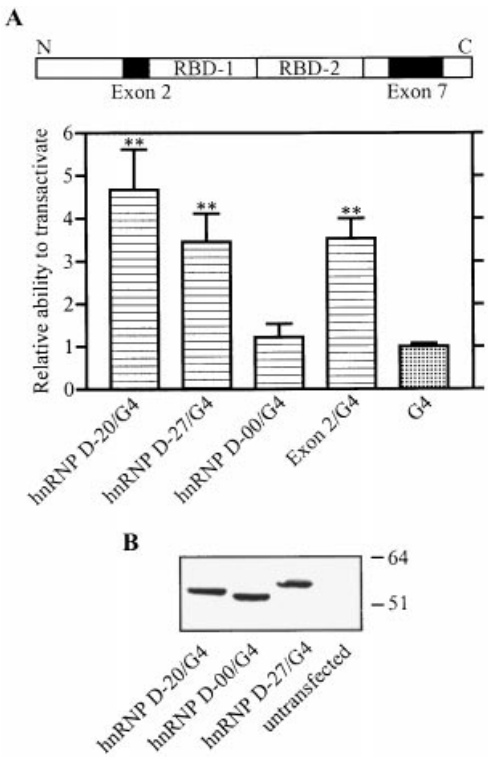

Figure 1 Exon 2 of hnRNP D encodes a transactivator domain

(**A**) We co-transfected IM-9 B cells with plasmids that express different isoforms of hnRNP D (our nomenclature reflects the presence/absence of the exon 2- and 7-encoded alternative inserts) or amino acids 79–98 of hnRNP D (exon 2) fused to the DNA-binding domain of GAL4 (G4), and a CAT reporter plasmid driven by GAL4 sites. CAT activities were measured 42 h after transfection. The transactivating abilities of fusion proteins were compared with that of a GAL4 control. Means $\pm$ S.E.M. are shown. $^{**}P < 0.005$ compared with GAL4 control by one-way ANOVA. A schematic depiction of hnRNP D (top) shows the positions of alternatively spliced exons 2 and 7, as well as the two conserved RBDs. (**B**) Cells were transfected with plasmids that express different hnRNP D–GAL4 fusion proteins, as indicated. We cultured the transfected cells for 25 h, then purified cell proteins and subjected them to Western-blot analysis using an antibody specific for GAL4.

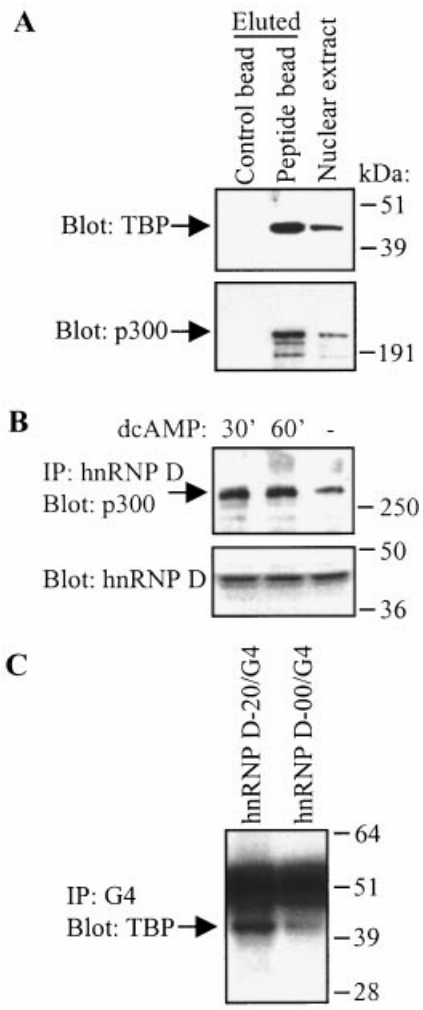

Figure 2 TBP and p300 interact with hnRNP D-20

(**A**) We attached a biotin-labelled 20-amino-acid synthetic peptide corresponding to amino acids 79–98 of hnRNP D to streptavidin-coated magnetic microbeads (see the Experimental section for details). Subsequently, peptide beads or control beads without peptide were incubated with nuclear protein extracts of IM-9 B cells for 1 h, and following extensive washes the interacting proteins were eluted. Aliquots of 1% of the eluted samples or 10 μg of total nuclear extract were run on SDS/polyacrylamide gels and subjected to Western-blot analysis using antibodies specific for TBP or p300. (**B**) We treated IM-9 B cells with 1 mM dcAMP for 30 or 60 min as indicated, or left them untreated, and then purified the cellular proteins. Samples were immunoprecipitated (IP) by an hnRNP D-20-specific antibody and subjected to Western-blot analysis using an antibody specific for p300. hnRNP D-20 levels are shown in the lower panel, as a control. (**C**) IM-9 B cells were transfected with plasmids expressing hnRNP D-20–GAL4 or hnRNP D-00–GAL4. We cultured the transfected cells for 44 h, then purified the cell proteins. Samples were immunoprecipitated with a GAL4 (G4)-specific antibody and subjected to Western-blot analysis using an antibody specific for TBP. The arrow indicates the band that corresponds to TBP. The upper band was due to rabbit antibody that leached off the agarose beads. All blots were analysed by a Molecular Imager using Quantity One software.

protein expression level. In order to test whether the exon 2-encoded alternative insert is sufficient to drive transcription we fused it to GAL4, separated by a flexible linker peptide. This short fusion protein also contained one additional amino acid of hnRNP D (Lys-98). Remarkably, the product of exon 2 was able to drive transcription at a level similar to that with full hnRNP D-20 (Figure 1A). The ability of the exon 2-encoded region to drive transcription out of the context of hnRNP D suggests that its conformation is intact, or that an intact conformation is not essential for activity.

The transactivator domain of hnRNP D interacts with TBP and p300

A transactivator domain typically interacts with the RNA polymerase II transcription apparatus directly or through mediator proteins. In order to identify proteins that interact specifically with the transactivator domain of hnRNP D, we used a synthetic peptide corresponding to that domain. The 20-amino-acid peptide was covalently linked to biotin and subsequently attached to streptavidin-coated magnetic microbeads. The peptide–beads were incubated with nuclear protein extracts and the interacting proteins were eluted. The samples obtained from peptide–beads contained several proteins that were not present in samples obtained from control beads, as revealed by protein staining of SDS/polyacrylamide gels (results not shown).

We first tested whether TBP, which we had shown previously to co-precipitate with hnRNP D [12], interacted with the peptide. Western-blot analysis demonstrated the presence of TBP in the sample that had been eluted from peptide–beads, but not that from the control beads, suggesting that TBP interacts with the transactivator domain of hnRNP D (Figure 2A). Using the same approach, we detected p300 in the sample that had been eluted from peptide–beads (Figure 2A). We estimated, based on the amounts of proteins that were run and the band intensities, that approx. 8% of total nuclear TBP and p300 adhered to the peptide–beads. We confirmed by immunoprecipitation, using specific antibodies, that hnRNP D and p300 formed complexes *in vivo* (Figure 2B). Furthermore, the interaction between hnRNP D and p300 was enhanced 2–3-fold following dcAMP treatment of cells, suggesting that their interaction is positively regulated by the PKA signalling pathway.

Next we tested the importance of the exon 2-encoded protein domain in the interaction with TBP and p300 *in vivo*. We transfected cells with GAL4-tagged hnRNP D-20 or hnRNP D-00, and used a GAL4-specific antibody to precipitate interacting proteins. We found TBP to associate specifically with hnRNP D-20, indicating that the product of exon 2 is required for protein interaction (Figure 2C). The weak band that we detected in the hnRNP D-00–GAL4 sample could be due to dimerization of the GAL4 fusion protein with endogenous hnRNP D isoforms that contain the product of exon 2. The dimerization domain of hnRNP D is located in the C-terminus, and is present in all isoforms [26]. We were unable to detect association of p300 with any GAL4-tagged hnRNP D isoform (results not shown), possibly due to competition of endogenous hnRNP D, or other p300-interacting proteins.

PKA and PKC regulate the ability of hnRNP D to transactivate

We have shown previously that the PKA and PKC signalling pathways regulate the overall activity of hnRNP D [8]. It is not known, however, whether the transactivating ability of hnRNP D is affected by these pathways. Experiments were performed to determine how these signalling pathways regulate the transactivating ability of hnRNP D-20 and that of the isolated transactivator domain *in vivo*. We co-transfected GAL4 fusion proteins and GAL4 reporters into B cells, then treated the cells with dcAMP and/or PMA to selectively induce PKA and PKC respectively. dcAMP alone significantly induced the transactivating ability of both full hnRNP D-20 and the isolated

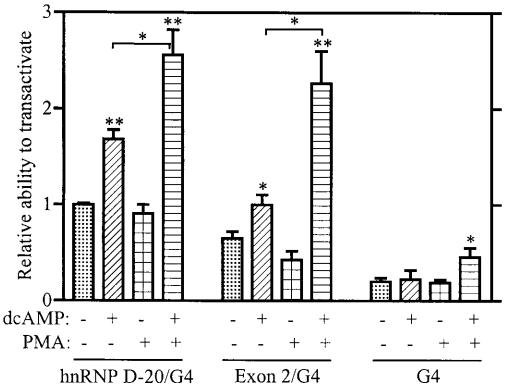

Figure 3 The transactivating ability of hnRNP D-20 is inducible

We co-transfected IM-9 B cells with plasmids expressing hnRNP D-20–GAL4, exon 2–GAL4 or GAL4, and a CAT reporter plasmid driven by GAL4 sites. Following transfection, cells were cultured for 24 h, then treated with 1 mM dcAMP and/or 100 ng/ml PMA for 18 h, or left untreated, and analysed for CAT activity. Means ± S.E.M. of relative transactivating abilities are shown. $^{*}P < 0.05$, $^{**}P < 0.005$ compared with unstimulated samples, by one-way ANOVA.

transactivator domain, while PMA alone did not have any effect (Figure 3). Treatment of cells with both dcAMP and PMA resulted in a response that was significantly enhanced over that following dcAMP treatment alone, using either full hnRNP D-20 or the exon 2-encoded domain. We conclude from these experiments that the ability of hnRNP D-20 to transactivate is regulated by the PKA and PKC signalling pathways, and that the transactivator domain contains all the elements required for regulation. It is also clear that PKA is a primary regulator, while PKC works in a PKA-dependent manner.

PKA and GSK-3β phosphorylate specific residues of the transactivator domain of hnRNP D

hnRNP D-20 contains a single amino acid, Ser-87, that is predicted to be a PKA phosphorylation site. Importantly, Ser-87 is located in the middle of the transactivation domain and thus, if it is a true PKA substrate, it could account for the functional regulation of hnRNP D-20 by PKA. Ser-83 is predicted to be a GSK-3β phosphorylation site, whereas Thr-91 is a potential CKI phosphorylation site, both in the transactivator domain. GSK-3β is known to require pre-phosphorylation of the C-terminal +4 serine, while CKI requires pre-phosphorylation of the N-terminal −4 serine for activity [25]. It is intriguing that both GSK-3β and CKI thus may depend on phosphorylation of Ser-87. The computer program also predicted seven additional PKC and CKI sites and six CKII sites outside the transactivator domain.

To start addressing the involvement of the predicted protein kinases, we performed *in vitro* kinase assays using recombinant hnRNP D-20. PKA phosphorylated wild-type hnRNP D-20, whereas GSK-3β alone did not (Figure 4). However, the simultaneous presence of PKA and GSK-3β resulted in enhanced phosphorylation of hnRNP D-20, suggesting that GSK-3β phosphorylated in a PKA-dependent manner (Figure 4A). PKC as well as CKII strongly phosphorylated hnRNP D-20. We focused on Ser-83 and Ser-87 because they are located in the transactivator domain. Our data are consistent with the assumption that Ser-87 is a PKA site, whereas Ser-83 is a PKA-dependent GSK-3β site. In order to support our interpretation with direct experimental evidence, we produced recombinant

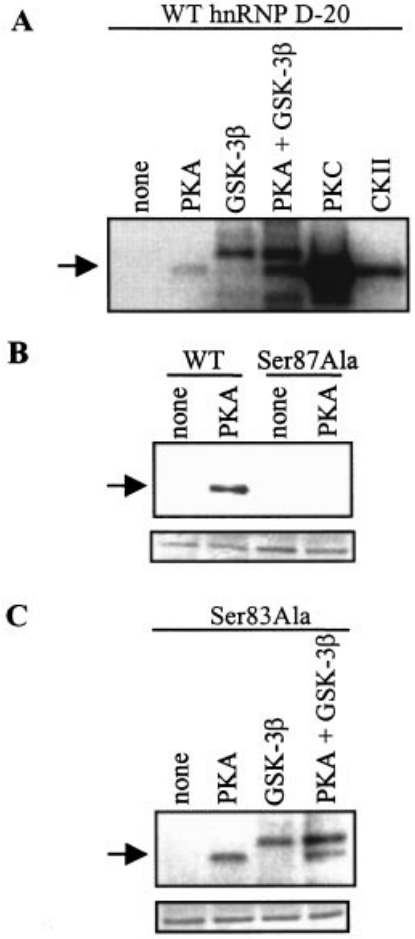

Figure 4 *In vitro* phosphorylation of wild-type and mutant hnRNP D-20

We produced recombinant wild type (WT) hnRNP D-20 or hnRNP D-20 with Ser-87 → Ala or Ser-83 → Ala substitutions. Equal amounts of hnRNP D were incubated with protein kinases, as indicated, in the presence of [^{32}P]ATP. Samples were subjected to SDS/PAGE and analysed by a Molecular Imager using Quantity One software. An arrow indicates the position of the band corresponding to hnRNP D-20. A slower migrating band was present in samples with GSK-3β, and was due to a contaminating protein present in the GSK-3β enzyme preparation, as this band appeared even if hnRNP D-20 was not included in the kinase reaction (results not shown). Note that the contrast in each panel was adjusted to reflect the relative differences between samples of that panel. Thus bands of different panels cannot be compared. The lower panels in (**B**) and (**C**) show hnRNP D protein levels by Coomassie Blue staining.

mutant hnRNP D-20 proteins in which Ser-83 or Ser-87 was replaced with alanine. The Ser-87 → Ala mutation abolished phosphorylation of hnRNP D-20 by PKA, strongly suggesting that Ser-87 is the only substrate of PKA (Figure 4B). The Ser-83 → Ala mutation did not affect phosphorylation by PKA, but prevented the increased phosphorylation that was observed with wild-type protein in the concurrent presence of GSK-3β (Figure 4C). These results indicate that Ser-83 is indeed the substrate of GSK-3β, and that the phosphorylation of Ser-83 requires pre-phosphorylation of Ser-87.

Next we performed phosphorylation experiments using the synthetic 20-amino-acid peptide, which corresponds to the transactivator domain, to verify and extend our experiments in which we used full hnRNP D. The presence of PKA resulted in the addition of approx. 0.25 phosphate group per peptide, which supports the presence of one acceptor site per peptide (Figure 5).

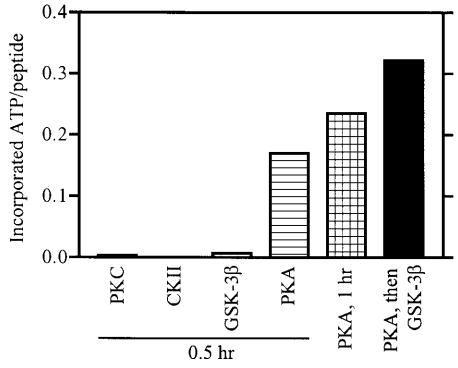

Figure 5 *In vitro* phosphorylation of a synthetic peptide that corresponds to exon 2 of hnRNP D

We incubated 1 nmol of synthetic peptide corresponding to amino acids 79–98 of hnRNP D with protein kinases and 5 nmol of unlabelled ATP for 0.5 or 1 h, as indicated. The incorporation of ATP into the peptide was traced by using 3.3 pmol of [^{32}P]ATP, which was added to the reaction at the beginning of the incubation, or after 0.5 h of preincubation (black bar). In the right-hand sample (black bar) the peptide was preincubated with PKA in the presence of unlabelled ATP for 0.5 h, then [^{32}P]ATP and GSK-3β were added and the sample was incubated for an additional 0.5 h. Thus for this sample the incorporation shown represents incorporation of ATP during the second 0.5 h incubation period, when [^{32}P]ATP was present.

PKC and CKII could not phosphorylate the peptide, arguing against the presence of corresponding phosphorylation sites in the transactivator domain. GSK-3β alone did not phosphorylate the peptide; however, following pre-phosphorylation of the peptide with PKA and unlabelled ATP, GSK-3β did phosphorylate the peptide (Figure 5). It should be noted that [^{32}P]ATP and GSK-3β were added in the sample after 0.5 h of pre-phosphorylation with PKA and unlabelled ATP. The difference in the incorporation between the samples phosphorylated by PKA for 0.5 and 1 h subtracted from the incorporation obtained in the PKA/GSK-3β sample reflects the incorporation that was due to GSK-3β. These calculations indicate that the amount of incorporation of ATP that was obtained in the presence of GSK-3β was about the same as that in the presence of PKA, suggesting a 1:1 stoichiometry.

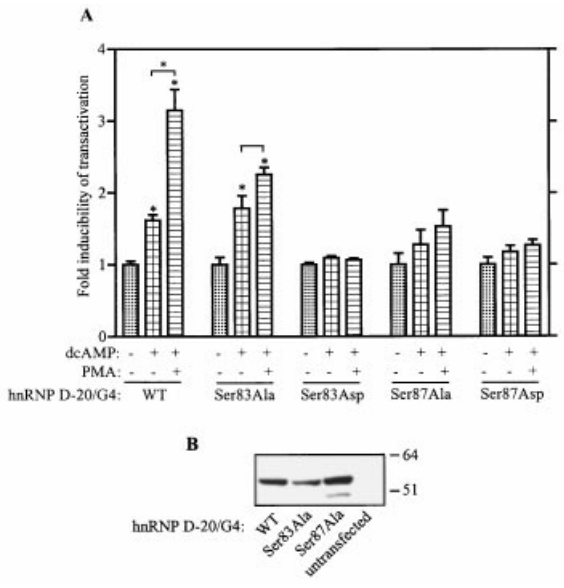

Figure 6 Mutations of Ser-83 and Ser-87 differentially affect the inducible transactivating ability of hnRNP D-20

(**A**) We co-transfected IM-9 B cells with plasmids expressing wild-type (WT) or mutated hnRNP D-20–GAL4 (G4) fusion proteins and a CAT reporter plasmid driven by GAL4 sites. Following transfection, cells were cultured for 24 h, then treated with 1 mM dcAMP and/or 100 ng/ml PMA for 18 h, or left untreated, and analysed for CAT activity. Means ± S.E.M. of the fold inducibility of transactivation are shown, where the transactivating ability of each mutant in uninduced cells was given a value of 1. $^*P < 0.05$ compared with unstimulated samples, using one-way ANOVA. (**B**) Cells were transfected with plasmids expressing different hnRNP D-20–GAL4 fusion proteins, as indicated. We cultured the transfected cells for 25 h, then purified the cell proteins and subjected them to Western-blot analysis using an antibody specific for GAL4.

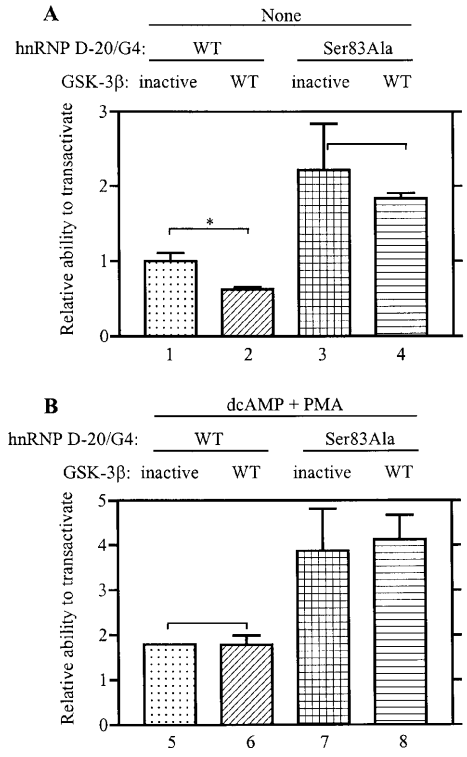

Figure 7 Overexpression of GSK-3β inhibits transactivation by hnRNP D-20

We co-transfected IM-9 B cells with plasmids expressing wild-type (WT) or Ser-83 → Ala mutant hnRNP D-20–GAL4 (G4), wild-type or inactive GSK-3β and a CAT reporter plasmid driven by GAL4 sites, as indicated. Following transfection, cells were cultured for (**A**) 42 h (no treatment) or (**B**) 24 h, then treated with 1 mM dcAMP and 100 ng/ml PMA for 18 h, and analysed for CAT activity. Note that the Figure is divided into panels (**A**) and (**B**) for clear presentation, but all samples are compared with sample 1 of (**A**). Means ± S.E.M. of relative transactivating abilities are shown. $^*P < 0.05$, using one-way ANOVA.

PKA enhances, whereas GSK-3β suppresses, the transactivating ability of hnRNP D

We analysed the *in vivo* functional effects of the detected phosphorylation events by fusing hnRNP D-20 to GAL4 after changing Ser-83 and Ser-87 of the transactivator domain to alanine or aspartic acid. We stimulated the cells with dcAMP and/or PMA to test how mutations of the PKA (Ser-87) and GSK-3β (Ser-83) phosphorylation sites affect the inducibility of transactivation. We found that changing Ser-87 to alanine or aspartate rendered hnRNP D-20 unresponsive to both dcAMP treatment and dcAMP/PMA co-treatment (Figure 6A). These findings suggest that phosphorylation of Ser-87 plays a critical role in inducing hnRNP D-20 by cAMP *in vivo*, and that the effect

of PMA is coupled to that of cAMP. The importance of Ser-87 phosphorylation in inducing the transactivating ability of hnRNP D-20 is supported by the 2-fold enhanced activity of the Ser-87 → Asp mutant, as compared with the wild-type protein, in uninduced cells (results not shown). The most likely reason for the enhanced transactivating ability of the Ser-87 → Asp mutant in uninduced cells is that the aspartic acid mimics a phosphate group. Mutation of Ser-83 to alanine allowed hnRNP D-20 to respond to dcAMP, but abolished the response to PMA. Thus Ser-83, which was phosphorylated *in vitro* by GSK-3β, is required for the PMA/PKC signalling pathway that results in the activation of hnRNP D-20. The Ser-83 → Asp mutant could not be induced by dcAMP, and this may be due to the inability of PKA to recognize Ser-87 as a substrate in the presence of a negative charge at position 83. We confirmed that substitution of Ser-83 or Ser-87 with alanine did not substantially affect protein expression levels (Figure 6B).

In order to address directly the *in vivo* role of GSK-3β in regulating the transactivating ability of hnRNP D-20, we overexpressed GSK-3β in B cells and monitored the activity of wild-type and Ser-83 → Ala mutant hnRNP D-20. An inactive GSK-3β, which contained two mutations in its ATP-binding domain and had no kinase activity [22], was used as a control. Overexpression of GSK-3β significantly ($P = 0.03$) inhibited transactivation by wild-type hnRNP D-20 (Figure 7A, compare samples 1 and 2). hnRNP D-20 with the Ser-83 → Ala mutation was resistant ($P = 0.48$) to the inhibitory effect of GSK-3β (Figure 7A, compare samples 3 and 4). It should be noted that the Ser-83 → Ala mutant had higher transactivating activity than the wild-type protein in several ($n = 5$) experiments; this could be due to unknown structural or regulatory factors. We conclude from these experiments that GSK-3β inhibits the ability of hnRNP D-20 to transactivate, and that this effect is mediated through phosphorylation of Ser-83. These results also provide the first direct evidence that GSK-3β phosphorylates Ser-83 of hnRNP D-20 *in vivo*.

PKC affects the ability of hnRNP D to transactivate by inactivating GSK-3β

We found that PMA/PKC enhance the transactivating ability of hnRNP D-20 in a PKA-dependent manner (see Figure 3). Based on the dependence of the action of both PKC and GSK-3β on PKA, PKC may exert its effect through GSK-3β. It is well documented that the kinase activity of GSK-3β is diminished if PKC phosphorylates Ser-9 of GSK-3β [22,27]. Thus active PKC may suspend the inhibitory effect of GSK-3β on the transactivating ability of hnRNP D-20. Indeed, stimulation of cells with PMA together with dcAMP blocked the inhibitory effect of GSK-3β on wild-type hnRNP D-20 (Figure 7B, compare samples 5 and 6 with samples 1 and 2 of Figure 7A), but did not affect hnRNP D-20 containing the Ser-83 → Ala substitution (Figure 7B, compare samples 7 and 8 with samples 3 and 4 of Figure 7A). These findings strongly suggest that PKC enhances the ability of hnRNP D to transactivate by inhibiting the activity of GSK-3β.

DISCUSSION

hnRNP D has recently been identified as a transcriptional regulator of several cellular and viral genes. In the present study we determined that the transactivator domain of hnRNP D is encoded by exon 2, which is spliced alternatively. Key general transcription factors, such as TBP and p300, interacted with this short transactivator domain. Moreover, the transactivating ability of hnRNP D was regulated in a hierarchical fashion by a complex interplay of three protein kinases. Our novel findings will help integrate hnRNP D into signalling pathways that involve specific protein kinases, and will increase our understanding of the *in vivo* functions of the different hnRNP D isoforms.

Although hnRNP D is one of the most abundant proteins in the nucleus, and its high degree of evolutionary conservation suggests functional importance, little is known about its physiological function and the mechanisms of its regulation. Several studies suggested that the four isoforms of hnRNP D, which are generated by alternative splicing, might play distinct roles in the cell. We have shown that hnRNP D-20, which contains the product of exon 2, has high affinity for DNA and is able to transactivate promoters *in vivo* [7,12]. In contrast, isoforms lacking the product of exon 2 have a higher affinity for RNA, and regulate mRNA turnover in the cytoplasm [5,6]. In the present study we defined that the transactivator domain of hnRNP D is encoded entirely by exon 2, because a single copy of the exon 2-encoded polypeptide could drive transcription when fused to the DNA-binding domain of GAL4. A homology search of GenBank against the 19-amino-acid transactivator domain found two human proteins, E2BP [10] and hnRNP UP2 [28], with a completely identical sequence, and no other protein with more than 45% identity, indicating that the transactivator domain of hnRNP is quite unique. Interestingly, E2BP is implicated in transcriptional regulation of the hepatitis B virus enhancer II [10]. The transactivator domain of hnRNP D and E2BP may belong to a novel class.

The transactivator domain of hnRNP D is one of the shortest known. The minimal transactivator domain of c-Myb consists of 25 amino acids [29], whereas that of VP16 contains 15–20 amino acids [30]. It is interesting that amino acids 70–98 of hnRNP D-20, which include the entire transactivator domain, were shown by NMR to be highly flexible and disordered in solution [31]. Such intrinsically unstructured protein domains can confer the ability to bind to several different targets without sacrificing specificity, and also provide a simple mechanism for inducibility by phosphorylation that can maximize complementarity between interacting proteins, to overcome the thermodynamic consequences of induced folding [29,32,33]. Several proteins implicated in transcription, including p300, CREB (cAMP response element-binding protein), CBP (CREB-binding protein), p53 and VP16, contain such unstructured domains (reviewed in [32]). Indeed, a likely reason why the short transactivator domain of hnRNP D was functional out of the context of the protein and was able to interact with protein targets as a synthetic peptide is that it is intrinsically unstructured.

TBP and p300 interacted with an exon 2-encoded synthetic peptide and co-precipitated with hnRNP D *in vivo*. Moreover, the *in vivo* association of TBP and hnRNP D depended on the presence of the exon 2-encoded domain. Although these experiments did not demonstrate a direct interaction of these proteins with hnRNP D, they did show that TBP and p300 form a complex with hnRNP D *in vivo* and that the interactions are dependent on the exon 2-encoded transactivator domain. Complex-formation between hnRNP D and p300 was enhanced if the PKA signalling pathway of the cell had been stimulated, suggesting that formation of the complex may play a functional role in the PKA-dependent induction of hnRNP D activity. Phosphorylation of p300 by PKC has recently been shown to repress the activity of p300 [34], suggesting that functional interaction of hnRNP D and p300 might be modulated by both PKA and PKC in an intricate manner. Considering the central role of TBP as the DNA-binding unit of the basal transcription apparatus, and the role of p300 in modulating chromatin structure through its

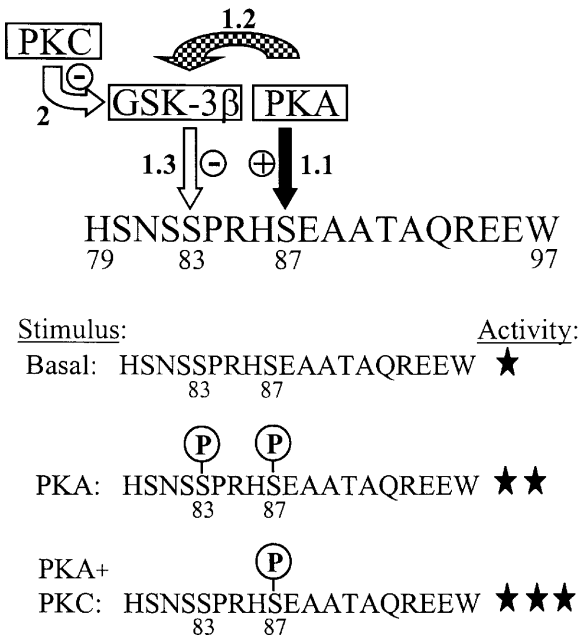

Figure 8 The transactivating ability of hnRNP D is regulated by hierarchical phosphorylation

The exon 2-encoded transactivator domain of hnRNP D is shown. In the upper section, phosphorylation of Ser-87 and Ser-83 of hnRNP D by PKA or GSK-3β, as well as phosphorylation of GSK-3β by PKC, are indicated by black arrows (activity enhanced) or white arrows (activity inhibited). We have indicated the sequence of events with numbering. Steps 1.1–1.3 are grouped to indicate that all are triggered by event 1.1, because GSK-3β is constitutively active in resting cells. Step 1.2 (hatched arrow) indicates that the activity of GSK-3β depends on phosphorylation of Ser-87 of hnRNP D. The resulting three phosphorylation states of the transactivator domain of hnRNP D are shown in the lower section, together with corresponding relative activity levels, which are depicted by an increasing number of stars.

histone acetyltransferase activity, the association of these proteins with hnRNP D is likely to be functionally critical. We are in the process of purifying and identifying additional proteins that interact with the transactivator domain of hnRNP D.

We studied the role of phosphorylation in regulating the transactivator potential of hnRNP D. We focused on the exon 2-encoded transactivator domain, because it was found to be fully responsive to activators of the PKA and PKC signalling pathways even if the rest of the protein was removed (Figure 3). PKA phosphorylated a single serine, Ser-87, of hnRNP D-20 *in vitro*, as mutation of Ser-87 to alanine abolished phosphorylation of the recombinant protein. Stimulation of cells with a cAMP analogue, which induces PKA directly, increased the ability of full hnRNP D-20, as well as the isolated exon 2-encoded domain, to transactivate *in vivo* (Figure 3). Furthermore, mutation of Ser-87 to alanine or aspartate prevented hnRNP D-20 from responding to cAMP *in vivo*. These findings clearly indicate the functional importance of the phosphorylation of Ser-87 by PKA, and show that this modification enhances the ability of the protein to transactivate. *In vitro* phosphorylation of hnRNP D by PKA increases the DNA binding of the protein [8], a finding that is supported by other studies [9]. The observation that the product of exon 2, where Ser-87 is located, was required for both transactivation (Figure 1) and DNA binding [7] points to regulation of both functions by PKA, and hints at a potential connection between induced DNA binding and transactivation. In addition, we identified Ser-83 as a substrate of GSK-3β. GSK-3β is known to require pre-phosphorylation of the C-terminal +4 serine for activity [35,36]; this is the PKA-phosphorylated Ser-87 in the case of hnRNP D. Indeed, GSK-3β phosphorylated Ser-83 in a PKA-dependent manner *in vitro*. Moreover, phosphorylation of Ser-83 negatively regulated the ability of hnRNP D-20 to transactivate *in vivo*, since overexpression of GSK-3β reduced the transactivating ability of the wild-type hnRNP D-20, and the Ser-83 → Ala mutant was resistant to GSK-3β. GSK-3β inhibits several transcription factors, including c-Myc, c-Jun, c-Myb, heat shock factor 1 and NFATc (nuclear factor of activated T-cells) [35,37,38]. The activity of CREB, on the other hand, is induced by GSK-3β [39]. In addition, PKC and CKII strongly phosphorylated hnRNP D-20 *in vitro*. It is not likely that direct phosphorylation of hnRNP D-20 by PKC and CKII influences transactivation, because predicted phosphorylation sites lie outside the transactivator domain, which alone was sufficient to transactivate in an inducible manner. Indeed, PKC and CKII were unable to phosphorylate a synthetic peptide that corresponded to the transactivator domain. Furthermore, stimulating PKC with PMA alone did not affect transactivation. PKA, PKC and CKII also phosphorylate hnRNP A1 *in vitro* [19].

We have uncovered a complex regulation of hnRNP D transactivator potential by protein kinases (Figure 8). It is clear that phosphorylation of the transactivator domain is not an absolute requirement for activity, because hnRNP D was able to transactivate in uninduced cells, and the transactivating ability of a Ser-87 → Ala mutant was comparable with that of wild-type protein in uninduced cells (results not shown). Nevertheless, phosphorylation of Ser-87 by PKA enhanced transactivation without any additional stimulus, suggesting that this is the primary event. This event triggered phosphorylation of Ser-83 by GSK-3β, which negatively regulated transactivation. The level of GSK-3β activity, at least in the B cell line we used in our experiments, was sufficiently high not to allow maximal hnRNP D-dependent transactivation when only the PKA pathway was stimulated. Overexpression of GSK-3β nevertheless inhibited hnRNP D activity. PMA, which stimulates PKC directly, enhanced the transactivating ability of hnRNP D in synergy with cAMP. The effect of PMA depended on the cAMP/PKA pathway, as PMA alone did not influence transactivation by hnRNP D. The dependence of the ability of PMA to influence transactivation on PKA activity is not likely to be the consequence of a PKA-dependent phosphorylation of hnRNP D by PKC, because there is no known example of PKC requiring pre-phosphorylation for activity. Moreover, PKC could not phosphorylate the exon 2-defined peptide, but the isolated exon 2 domain was responsive to PMA/PKC. We suggest an indirect mechanism for the action of PKC. The simplest model that integrates PMA into hnRNP D transactivation is one in which PKC phosphorylates and thus inactivates GSK-3β. Similarly, GSK-3β is unable to inhibit the activity of c-Jun following phosphorylation by PKC [40]. There is ample evidence that PKC phosphorylates GSK-3β *in vitro* and *in vivo*, resulting in inhibition of GSK-3β kinase activity [41–43]. We suggest that the hierarchical phosphorylation of hnRNP D by protein kinases allows three activity levels of a single protein (Figure 8). The transactivating abilities that correspond to these phosphorylation states are within a relatively narrow range, indicating fine regulation. A hierarchical model of phosphorylation has been described for several GSK-3β substrates [37,44]. Notably, sequential phosphorylation by PKA and GSK-3β regulates the transactivator domain of CREB, both phosphorylation events being stimulatory [39]. The transactivator domain of CREB is intrinsically unstructured, similar to that of hnRNP D, but assumes an α-helical structure upon binding to CBP in a

phosphorylation-dependent manner [45]. A similar phosphorylation-induced conformational change that enhances communication with general transcription factors may take place in hnRNP D.

In summary, we have identified, for the first time, that the exon 2-encoded 19 amino acids of hnRNP D function as an isoform-specific transactivator domain. The exon 2-encoded protein domain was necessary and sufficient for transactivation, and was phosphorylated sequentially by PKA and GSK-3β at specific residues. We describe a novel regulation of hnRNP D activity by protein kinases which enriches our understanding of the physiological role of hnRNP D in the regulation of gene transcription, as well as the role of phosphorylation in regulating the activity of hnRNP proteins in general.

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