the FEBS Journal

Gradual phosphorylation regulates PC4 coactivator function

Hendrik R. A. Jonker[1], Rainer W. Wechselberger[1], Martijn Pinkse[2], Robert Kaptein[1] and Gert E. Folkers[1]

1 Department of NMR Spectroscopy, Bijvoet Center for Biomolecular Research, Utrecht University, the Netherlands
2 Department of Biomolecular Mass Spectrometry, Utrecht University, the Netherlands

Keywords
casein kinase II; PC4; phosphorylation; transcription; RNA polymerase II

Correspondence
G. E. Folkers, Bijvoet Center for Biomolecular Research, Section NMR spectroscopy, Utrecht University, Padualaan 8, 3584 CH, Utrecht, the Netherlands
Fax: +31 30 253 7623
Tel: +31 30 253 9930
E-mail: g.folkers@chem.uu.nl
H. R. A. Jonker, J.W. Goethe University - Center for Biomolecular NMR, Institute for Organic Chemistry and Chemical Biology, Marie Curie Strasse 11 (N160/312), D-60439 Frankfurt am Main, Germany
Fax: +49 69 798 29515
Tel: +49 69 798 29137
E-mail: henry@vinden.nl

(Received 14 December 2005, revised 30 January 2006, accepted 3 February 2006)

doi:10.1111/j.1742-4658.2006.05165.x

The unstructured N-terminal domain of the transcriptional cofactor PC4 contains multiple phosphorylation sites that regulate activity. The phosphorylation status differentially influences the various biochemical functions performed by the structured core of PC4. Binding to ssDNA is slightly enhanced by phosphorylation of one serine residue, which is not augmented by further phosphorylation. The presence of at least two phosphoserines decreases DNA-unwinding activity and abrogates binding to the transcriptional activator VP16. Phosphorylation gradually decreases the binding affinity for dsDNA. These phosphorylation-dependent changes in PC4 activities correlate with the sequential functions PC4 fulfils throughout the transcription cycle. MS and NMR revealed that up to eight serines are progressively phosphorylated towards the N-terminus, resulting in gradual environmental changes in the C-terminal direction of the following lysine-rich region. Also within the structured core, primarily around the interaction surfaces, environmental changes are observed. We propose a model for co-ordinated changes in PC4 cofactor functions, mediated by phosphorylation status-dependent gradual masking of the lysine-rich region causing shielding or exposure of interaction surfaces.

Eukaryotic class II gene expression is a complex biochemical process that involves the interaction of many proteins to control the activity of RNA polymerase II during initiation and elongation of transcription. Activation of transcription requires the formation of a pre-initiation complex (PIC) on the promoter region [1,2]. The assembly typically begins with binding of the TATA-binding protein (TBP) to the TATA-box promoter region, followed by recruitment of the general transcription factors (GTFs) in a stepwise fashion or by recruitment of pre-assembled holoenzyme (sub)complexes [3,4]. Many components of the human RNA polymerase II transcription apparatus are subjected to phosphorylation during the transcription cycle [5]. One major target of kinases is the C-terminal domain of the largest subunit of the RNA polymerase II enzyme itself [6]. It was postulated that phosphorylation switches RNA polymerase II from the initiation to

Abbreviations
CKII, casein kinase II (EC 2.7.1.37); GST, glutathione S-transferase; GTF, general transcription factor; HSQC, heteronuclear single-quantum coherence; PC4, human positive cofactor 4; PC4ntd/ctd, N-terminal or C-terminal domain of PC4; PC4p, completely CKII-phosphorylated PC4; PIC, pre-initiation complex; SEAC, serine and acidic-rich region of PC4ntd; TBP, TATA-box-binding protein; VP16, herpes simplex virion protein 16; VP16ad, activation domain of VP16.

elongation mode [7]. Furthermore, intermediately phosphorylated forms of RNA polymerase II have been observed that may have specific roles during the transcription cycle [5,8]. Thus (co-ordinated) phosphorylation turns out to be an important regulatory mechanism for many proteins throughout the transcription process [9–11].

The rate of transcription initiation can be enhanced by activators that directly or indirectly interact with the general transcription factors. Cofactors (or coactivators) positively (PCs) or negatively (NCs) mediate the interaction between the DNA-bound activators and the basal transcription machinery [12–14]. The general transcriptional cofactor PC4 stimulates RNA polymerase II transcription activation of a wide variety of transactivation domains that are classified as acidic (Gal4, AH, VP16, NF-κB), proline-rich (CTF-1/NF1), glutamine-rich (Sp1) or other classes of activators (E1a, IE) [15,16]. Furthermore, dsDNA binding has been reported to correlate with the ability of PC4 to stimulate activator-dependent transcription [17]. In contrast, PC4 exhibits inhibitory activity by interaction with melted dsDNA, hereafter referred to as bubble DNA [18]. This DNA template with an unpaired dsDNA region mimics the initiation open promoter site of transcription [19,20]. Repression of transcription can be relieved by increasing amounts of TAF_{II}s, TFIIH or a preassembled RNA polymerase II holoenzyme [18,21–24]. It has been reported that the helicase activity of ERCC3 (also referred to as XPB), present in TFIIH, is probably crucial for alleviating PC4-mediated repression [25].

PC4 is composed of two distinct functional domains each constituting approximately half of the protein. The homodimeric structure of the C-terminal half of PC4 (PC4ctd, residues 61–126) has been elucidated by crystallography [26], and the ssDNA-binding surface was identified by NMR [27]. The N-terminal half of PC4 (PC4ntd, residues 1–60) comprises a lysine-rich region in between repeats that are rich in serine residues [16]. The N-terminal serine-rich region (residues 3–20) also contains many acidic residues and is therefore termed the SEAC region. Phosphorylation of PC4 by casein kinase II (CKII) is restricted to seven (or eight) serine residues in this region, which negatively regulate the PC4 cofactor function [28]. The association with the TBP–TFIIA promoter complex was abrogated by phosphorylation [16] as well as its ability to interact with the activator VP16 [28]. Furthermore, phosphorylation of PC4 leads to a loss of dsDNA binding, but does not inhibit ssDNA binding [17]. Interestingly, it has been reported that PC4 can be phosphorylated by components of TFIID ($TAF_{II}250$) and TFIIH, showing that modification can take place in the context of the PIC, which suggests phosphorylation-dependent control of the cofactor activity during the transcription cycle [21,22]. As PC4 is predominantly present in the phosphorylated (inactive) form *in vivo* [28], it seems plausible that the phosphorylation status critically regulates the cofactor function. In agreement with this model, only unphosphorylated PC4 has the ability to inhibit cdk1, cdk2 and cdk7 mediated phosphorylation of RNA polymerase II [29]. As phosphorylation of the C-terminal domain of RNA polymerase II controls the transition from initiation to elongation, this suggests a PC4 phosphorylation status-dependent control of the transcription cycle. This is further emphasized by the TFIIH-dependent role of PC4 in promoter escape, revealing that the direct interaction between TFIIH and PC4 is influenced by PC4 phosphorylation, which is not performed by the TFIIH kinase [25,30]. Finally Calvo & Manley [31] have shown in chromatin immunoprecipitation experiments that Sub1, the yeast PC4 ortholog, is found in association with elongating RNA polymerase II. Furthermore, Sub1 influences transcription elongation by destabilizing Fcp1, the phosphatase that dephosphorylates RNA polymerase II C-terminal domain [31]. Altogether these observations strongly suggest that phosphorylation dynamically regulates the ability of PC4 to act as a cofactor.

To reveal how the PC4 cofactor function is affected by phosphorylation, we studied the unwinding characteristics and its interaction with DNA and VP16 for unphosphorylated as well as partially and fully phosphorylated PC4. Our results show that the phosphorylation status of PC4 affects the binding properties towards DNA, the VP16 activation domain (VP16ad), and the ability to unwind dsDNA. By NMR we show that phosphorylation of the SEAC region causes structural and/or environmental changes in the lysine-rich region, indicating gradual masking. These observations show that regulated phosphorylation of PC4 fulfils a crucial role in its ability to act as a cofactor throughout the transcription cycle.

Results

Quantitative analysis of DNA binding and unwinding of PC4 and PC4p

Previous experiments suggested that phosphorylation of PC4 can act as a molecular switch converting PC4 from a coactivator into a repressor [16]. To monitor the contribution of the phosphorylation state to the various activities of PC4, we first quantitatively

analyzed these for unphosphorylated PC4 and completely CKII-phosphorylated PC4 (PC4p) (Fig. 1A). We observed a small increase in ssDNA binding affinity on phosphorylation, with an apparent K_d of 6.4 ± 1.6 nM for PC4p compared with 10.1 ± 2.3 nM for PC4 (Fig. 1B). A similar increase in binding affinity was obtained with bubble DNA as probe (data not shown). Binding to dsDNA was significantly decreased by phosphorylation (Fig. 1C, $K_d = 344 \pm 146$ nM for PC4p and 75 ± 48 nM for PC4). The composition of the protein–DNA complexes was also different. A weak fast migration PC4p protein–DNA complex was detectable at lower protein concentration. At higher protein concentrations, a less stable complex was formed as the complex fell apart during electrophoresis. Binding of PC4 as well as PC4p to dsDNA is highly co-operative (Hill constant of 1.8 ± 0.4, indicative of a dimeric or higher-order PC4–DNA complex).

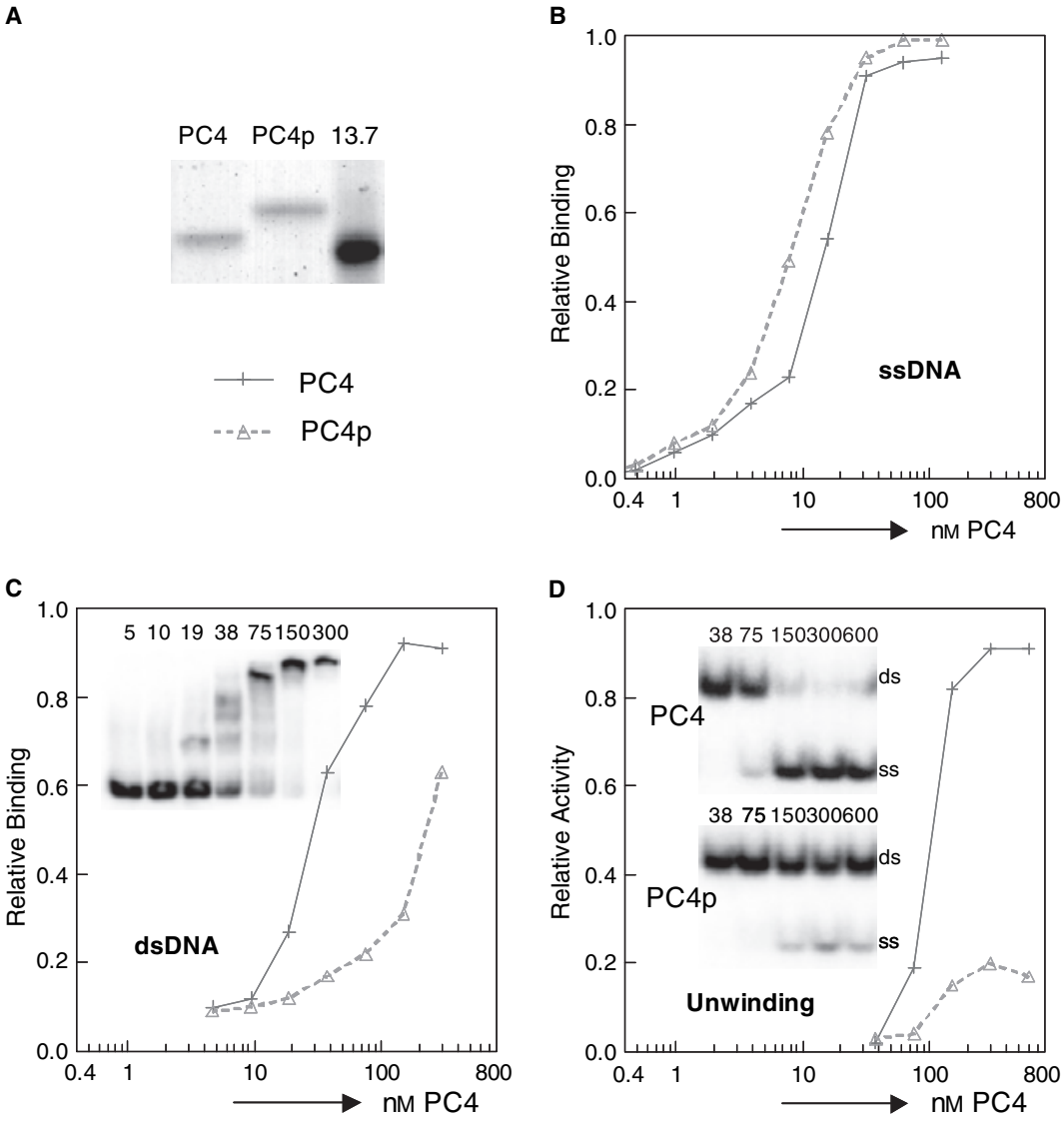

Fig. 1. Phosphorylation of PC4 affects its DNA binding affinity and unwinding activity. (A) Coomassie stained SDS/polyacrylamide gel of 100 ng protein samples after purification used for binding and unwinding studies. PC4p, PC4 protein after 24 h of CKII-mediated phosphorylation. As a reference, 1 μg RNase A (13.7 kDa) was loaded. (B,C) Representative binding experiment showing the calculated fractional occupancy of PC4 and PC4p at the indicated protein concentrations (nM) to ssDNA (B) or dsDNA (C). The inset of C shows the autoradiogram of this experiment for unphosphorylated PC4 binding to dsDNA. (D) Unwinding assay showing the unwound fraction of bubble DNA at the indicated PC4 protein concentrations (nM). The inset shows the autoradiogram of the unwinding assay using PC4 or PC4p at the indicated protein concentrations (nM).

More importantly, phosphorylation led to a striking decrease in DNA-unwinding activity (Fig. 1D). Under these experimental conditions, PC4p is unable to completely unwind all the substrate whereas PC4 can. At protein concentrations at which negligible unwinding activity was observed, more than half of the bubble DNA was bound by PC4 or PC4p, as was evident from unwinding experiments in which the unwinding reaction mixture was loaded in parallel on an electrophoretic mobility shift assay gel and an unwinding gel (data not shown). Interestingly the protein concentrations at which half-maximal DNA-unwinding activity was observed for both PC4 and PC4p (56 nM and 128 nM, respectively) are comparable to their apparent K_d values for dsDNA, suggesting that dsDNA binding is the rate-limiting step in the helix-destabilizing activity of PC4.

Preparation of partially phosphorylated PC4

To investigate the effects of phosphorylation in more detail, we studied the binding effects of partially phosphorylated PC4. Samples were obtained at different phosphorylation stages by ending the CKII-mediated phosphorylation reaction at different times. Electrospray ionization mass spectra of the purified protein samples clearly illustrate the time-dependent PC4 phosphorylation (Fig. 2). The enlarged part of the mass spectrum contains the $[M + 20H]^{20+}$ ion signal of PC4 at different time intervals during phosphorylation. At $t = 0$ only an ion signal at m/z 714.194 is observed, from which a mass of 14263.88 Da for the nonphosphorylated PC4 was determined (theoretical mass 14264.18 Da). This ion signal shows a time-dependent increase in multiples of 4.0 m/z units, indicating multiple mass increments of 80 amu (atomic mass units), which matches protein phosphorylation. After 1 h of phosphorylation, more than half of the sample contained at least one phosphorylated serine. After 4 h, hardly any unphosphorylated PC4 was present, and, on average, almost two serines were phosphorylated per molecule. Purification of the PC4 sample obtained after 8 h revealed two fractions. The smallest fraction eluted at the same salt concentration as fully phosphorylated PC4 and also co-migrated with PC4p on SDS/polyacrylamide gels. MS confirmed that this

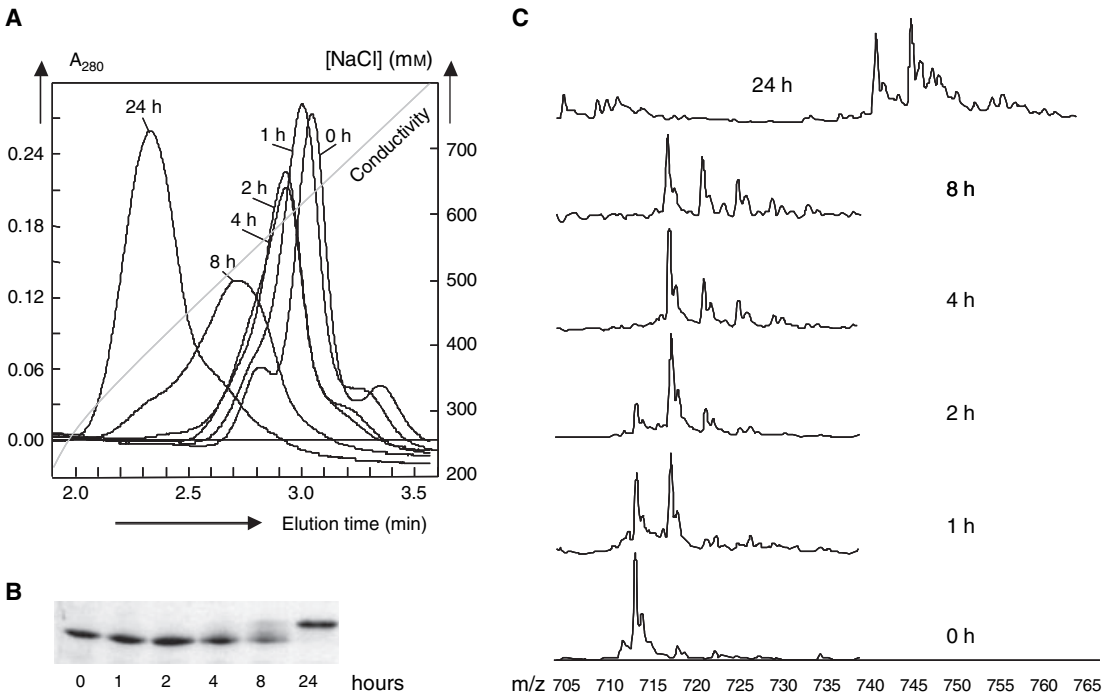

Fig. 2. Isolation of partially phosphorylated PC4 proteins. (A) Overlay of the heparin column elution profiles (A_{280} nm) for the purification of the PC4 samples that had been phosphorylated by CKII for the indicated time (hours). The right axis shows the measured conductivity (in mM NaCl). (B) Coomassie-stained SDS/polyacrylamide gel of 200 ng concentrated and purified PC4 that had been phosphorylated by CKII for the indicated time (in hours). (C) Enlarged areas of the electrospray ionisation mass spectra of PC4, showing the $[M + 20H]^{20+}$ ion signal at different phosphorylation stages. After prolonged incubation in the presence of CKII, substantial degradation is observed resulting in additional peaks at m/z 705–710 and 750–755.

fraction was indeed completely phosphorylated. The mass spectrum shown for the 8 h time point is the major fraction and is a mixture of PC4 with one to five phosphoserines. We failed to obtain more homogeneously phosphorylated PC4 samples using a different phosphorylation experiment or purification strategies. After 24 h, most of the PC4 sample was fully phosphorylated to an amount of seven or eight serines. The biochemical behavior of PC4 containing seven or eight phosphoserine residues appeared indistinguishable (data not shown), and therefore we refer to this sample as fully phosphorylated (PC4p). A small fraction of partially phosphorylated PC4 was detected (< 25%, Fig. 2A), which was not further analyzed.

Biochemical characterization of partially phosphorylated PC4

As we were unable to prepare homogeneously phosphorylated PC4 samples specifically phosphorylated at only one position, we used the partially phosphorylated protein samples to analyze the DNA binding and unwinding characteristics and the interaction with VP16ad (Fig. 3). When the majority of PC4 contained one or two phosphoserines, a small decrease in dsDNA binding affinity was observed (Fig. 3A, 1–4 h). A further small reduction was found after 8 h, when the sample contained a mixture of one to five phosphoserines per PC4 molecule. The strongest contribution to the decrease in dsDNA binding was observed when the phosphorylation was complete.

In contrast with the dsDNA binding results, the increased ssDNA binding affinity became evident when only one phosphoserine was present (Fig. 3B). Most of the phosphorylation-dependent increase in binding affinity was detected after 1 h of phosphorylation, and only a minor increase was observed on further phosphorylation. The unwinding activity was resistant to the presence of one phosphoserine residue as no decrease in unwinding was detectable after 1 h and only a small decrease was observed after 4 h of phosphorylation (Fig. 3C). The 8 h time point, which mainly contained multiple phosphorylated PC4, had an unwinding activity comparable to that of PC4p. On the basis of the difference in unwinding activity between the 4 and 8 h time points, it is most likely that the presence of only two or three phosphoserines in PC4 is sufficient to block the helix-destabilizing activity.

Finally we analyzed the interaction between PC4 and VP16ad using glutathione S-transferase (GST) pull-down assays. As shown in Fig. 3D, interaction between PC4 and VP16ad was not disturbed by phosphorylation of a single serine, as up until 4 h no decrease in binding affinity was detected. After 8 h of phosphorylation, PC4 contained a mixture of one to five phosphoserines, which resulted in multiple species on

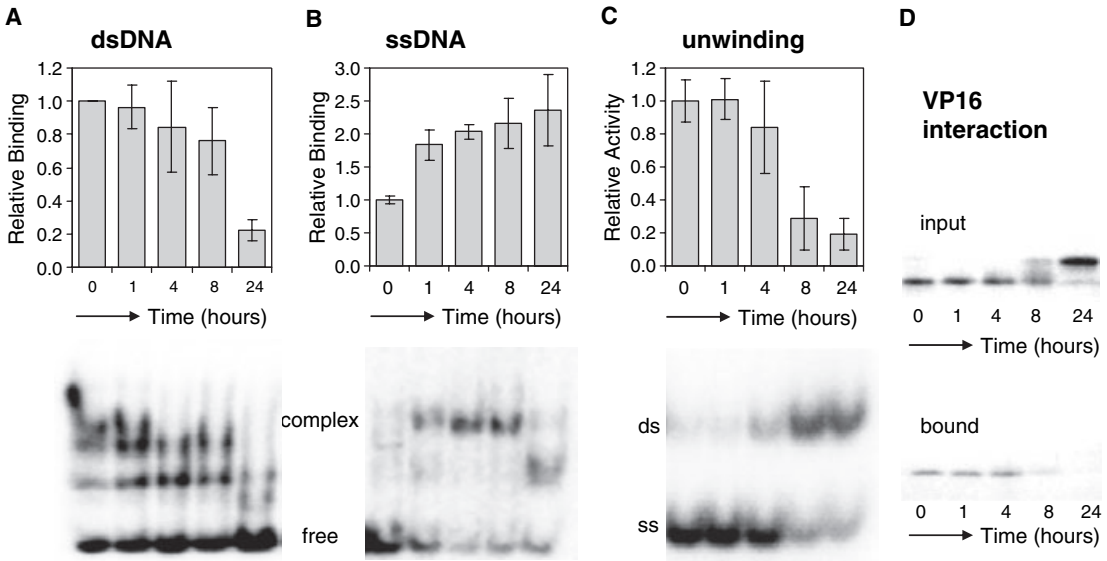

Fig. 3. Phosphorylation status-dependent DNA binding, unwinding and interaction with VP16. Comparison of the dsDNA (A) and ssDNA (B) binding affinity, the unwinding ability (C), and the VP16ad interaction measured by GST pull-down assays (D) of PC4 proteins that had been phosphorylated for 0, 1, 4, 8 and 24 h by CKII. The position of free DNA and the protein–DNA complex is indicated. For the unwinding assay (C), the position of bubble probe (ds) and unwound DNA (ss) is depicted. For quantification, the activity of unphosphorylated PC4 is set at 1. The activities of phosphorylated PC4 were calculated relative to the level of binding or unwinding of PC4. The upper panel of (D) shows 10% of the total amount of PC4 added in the GST pull-down assay; the lower panel shows the bound PC4.

SDS/polyacrylamide gel. Given the ratios of the phosphorylated species as determined by MS (Fig. 2C) and the intensities of PC4 proteins on the SDS/polyacrylamide gel (Fig. 3D), we conclude that the fastest migrating species contained only monophosphorylated PC4. This is further confirmed by co-migration of the fastest migrating band with PC4 that had been phosphorylated for 1 h and therefore lacked the multiple phosphorylated species. The smeary slower migrating bands represent a mixture of PC4 proteins with two to five phosphoserine residues as the 4-h time point, which contain less of these phosphorylated PC4 species, and also show less protein of lower mobility. On the basis of these observations, we conclude that monophosphorylated PC4 interacts with VP16ad, whereas the cofactor fails to interact significantly if two or more phosphoserine residues are present.

Characterization of PC4 phosphorylation by NMR

We next investigated the nature and structural consequences of PC4 phosphorylation in more detail by NMR. In the (^{1}H,^{15}N)-HSQC spectrum of full length PC4, the signals originating from PC4ntd appear as intense signals among the well-dispersed peaks assigned to PC4ctd [27]. The signals from the flexible PC4ntd were assigned at 400 mM KCl and 305 K for PC4 and PC4p. The ^{1}H, ^{15}N and ^{13}C chemical shift values of PC4ntd have been deposited in the BioMagResBank under accession number 6098 (http://www.bmrb.wisc.edu/). Figure 4 shows an overlay of the (^{1}H,^{15}N)-HSQC spectra of PC4 and PC4p. The amide signals of serines 8, 9, 10, 11, 12, 14, 16 and 18 have disappeared at their unphosphorylated positions in the (^{1}H,^{15}N)-HSQC spectrum and appear at the phosphorylated positions.

To determine which regions in PC4 are affected by phosphorylation, the chemical shift perturbations were mapped (Fig. 5). Although large changes in the chemical shift values were observed for the serine residues, there is no indication of the formation of well-defined structure elements, as the phosphoserine resonances appear at the random coil position [32]. In addition, the intense amide signals for PC4p are comparable to those of PC4. Not surprisingly, the most significant shifts, indicative of structural and/or environmental changes, are observed throughout the entire SEAC region. However, the first part of the lysine-rich region (residues 20–34) was strongly affected by phosphorylation. The direction of the chemical shift changes may indicate some enhancement of the α-helical conformation of this region. Interestingly in the PC4ctd core significant chemical shift changes were also observed that partially overlap with the region previously identified as the ssDNA-binding surface (Jonker *et al.* unpublished work; [27]).

NMR shows progressive phosphorylation of the SEAC region

During the phosphorylation reaction of PC4, a heterogeneous protein mixture containing zero to eight phosphoserines was detected (Fig. 2). As we were unable to determine the exact locations and order of the phosphorylated serines by MS, we performed the phosphorylation reaction in an NMR tube. The reaction was monitored by recording successive (^{1}H,^{15}N)-HSQC spectra in which the disappearance of serine amide signals and appearance of phosphoserine amide signals were visible. Using optimized NMR conditions (400 mM KCl, 50 mM potassium phosphate buffer, pH 5.6, 2 M D_5-glycine) in the presence of 10 mM $MgCl_2$, 10 mM ATP and CKII, no phosphorylation was observed. In lower salt concentrations, 175 mM KCl and 10% D_2O instead of 2 M D_5-glycine, phosphorylation was detected for Ser18 only (data not shown). At the optimized phosphorylation conditions at pH 7.5 [33], the serine amide signals could not be observed because of exchange with water, but the appearance of phosphoserines could be detected. A ^{15}N-labeled PC4 sample was phosphorylated at these optimal conditions and monitored by NMR. Samples were taken from a parallel phosphorylation reaction at distinct time points (0, 45 and 90 min and 3 h). When the (^{1}H,^{15}N)-HSQC spectra of the NMR sample did not show any changes (7 h), the reaction was terminated. All samples were exchanged to the optimized NMR conditions and (^{1}H,^{15}N)-HSQC spectra recorded (inset Fig. 4).

In the (^{1}H,^{15}N)-HSQC spectra of the partially phosphorylated PC4 samples, signals of phosphorylated serines are visible at intermediate positions (marked as 'Si'), which disappear again when more serines were phosphorylated. Whereas mapping of the upcoming phosphoserines is difficult because of these intermediate peak positions, the decrease in signal intensity of unphosphorylated serines can be readily determined (Fig. 6A). The SEAC region contains four primary consensus sites (serines 12, 14, 16 and 18) and three secondary and tertiary sites (serines 8, 9 and 11) for CKII phosphorylation [34]. Our results show that Ser10 can also be phosphorylated. The decrease in signal intensity clearly shows that the primary phosphorylation sites were indeed phosphorylated first. Ser18 is already ≈ 50% phosphorylated after 45 min, followed by serines 16, 14 and 12, within the first 2 h. One of

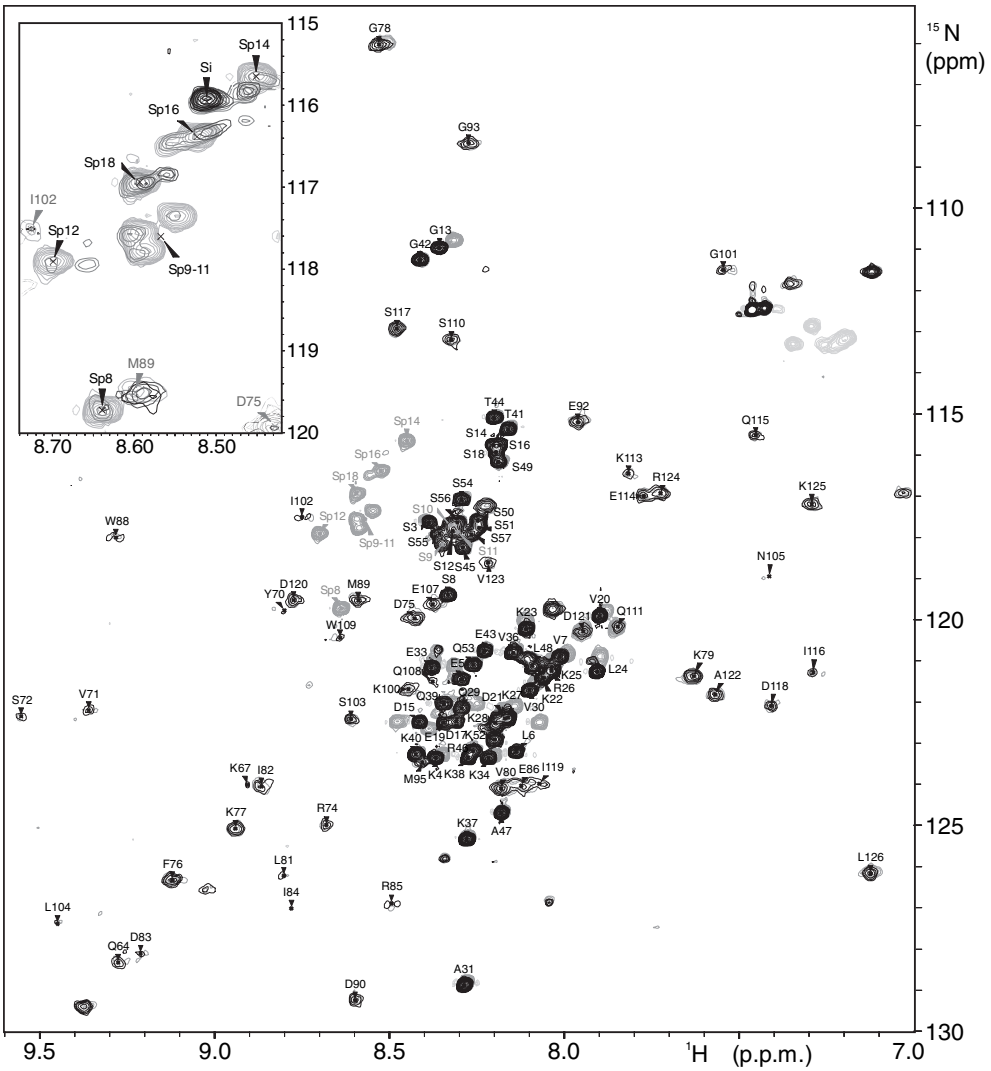

Fig. 4. Phosphorylation status monitored by NMR. Overlay of (^{1}H,^{15}N)-HSQC spectra (500 MHz) of PC4 (black) and PC4p (grey). The backbone assignments are indicated, where Sp represents the phosphorylated serines and Si a phosphorylation intermediate. The inset shows part of an overlay of (^{1}H,^{15}N)-HSQC spectra (500 MHz) recorded after 0.75, 1.5, 3 and 7 h of phosphorylation (in greyscale) to indicate the upcoming phosphoserine signals. All spectra were acquired at 305 K in the same buffer solution (400 mM KCl, 50 mM potassium phosphate buffer, pH 5.6, 2 M D_5-glycine).

serines 9, 10 or 11 (most probably the secondary phosphorylation site of Ser11) also appears to be 50%phosphorylated within this time period. The serines closest to the N-terminus of PC4 (serines 8, 9 and 10) are phosphorylated last. The NMR series thus show a progressive phosphorylation of the SEAC region from the C-terminus to the N-terminus.

Phosphorylation causes gradual chemical shift changes in the lysine-rich region

As phosphorylation creates additional negative charges in the SEAC region, close to the positively charged lysine-rich region, we decided to investigate the influence of the phosphorylation status on the lysine-rich region. Therefore, the amide ^{1}H and ^{15}N perturbations were combined and mapped for the partially phosphorylated samples to observe the changes during phosphorylation (Fig. 6B). Only residues in the 22–34 region of the lysine-rich region, which do not overlap in the (^{1}H,^{15}N)-HSQC spectra and show significant chemical shift changes, were analyzed. Residues E19, V20 and D21, located between the two affected regions, disappeared and arose at a new position in the (^{1}H,^{15}N)-HSQC spectrum. Residues in the N-terminal part of the lysine-rich region (residues K23 and L24) already

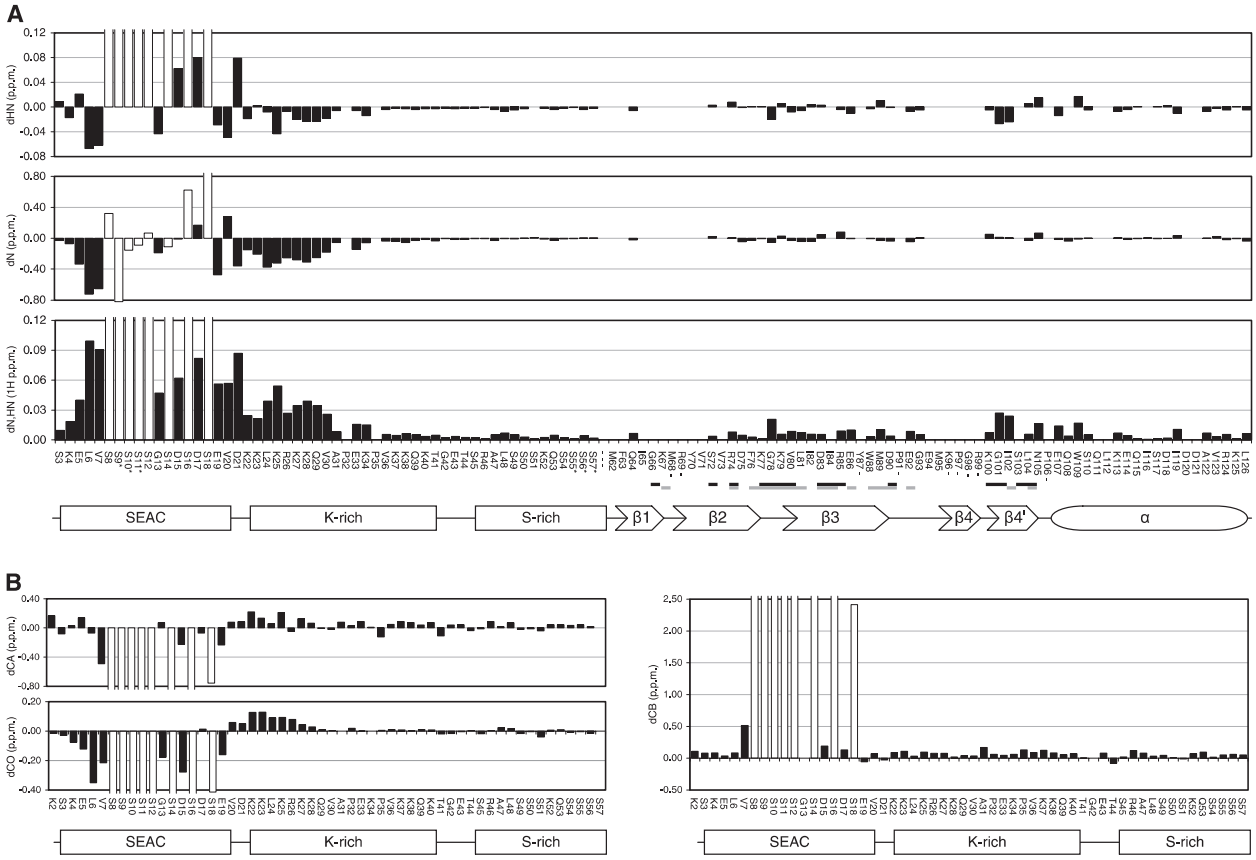

Fig. 5. Phosphorylation-induced chemical shift perturbations in PC4. A The amide ^{1}H and ^{15}N resonance shifts were mapped for PC4 and combined as Euclidian distances between peak maxima (in ^{1}H p.p.m.). Perturbations of the modified serines are indicated with white bars. The VP16ad (black) and ssDNA (gray) binding regions in PC4ctd are indicated on the *x*-axis by a line below the most affected residues (Jonker *et al.*, unpublished work; [27]). Mutations that influence dsDNA binding are R99G and K100G. (B) Chemical shift changes in ^{13}C resonances for Cα, Cβ and C′ were mapped for the N-terminal domain of PC4.

showed half-maximal perturbation (of the total amount) after 90 min when serines 18 and 16 were over 80% and 60% phosphorylated, respectively. When the phosphorylation of the SEAC region proceeded in the N-terminal direction, more residues of the lysine-rich region up to K34 were affected in the C-terminal direction, which indicates progressive structural and/or environmental changes throughout this region.

Shielding of the lysine-rich domain contributes to the phosphorylation-dependent changes in PC4 activities

The observed phosphorylation status-dependent environmental or conformational changes in the lysine-rich region (Fig. 6B), combined with the altered interactions as presented in Fig. 3, suggest gradual shielding of the functionally important lysine-rich region on phosphorylation. This implies that mutation of a few N-terminally located positively charged residues in the lysine-rich region would have a similar effect on PC4 activity as partially phosphorylated PC4, and mutation of a larger proportion of the lysine-rich region would result in a more pronounced change in activity. The mutants K22G/K23G and K25G/R26G were both impaired in dsDNA binding, as observed for partially phosphorylated PC4, and were slightly less efficient at interacting with VP16 (Fig. 7). More importantly, the dsDNA binding of the lysine-rich mutant (K22G/K23G/K25G/R26G/K27G/K28G) was compatible with fully phosphorylated PC4 (600 ± 310 nM versus 344 ± 146 nM for PC4p). Although phosphorylation had a more dramatic effect on interaction with VP16ad, clearly mutation of the positively charged residues decreased the interaction between PC4 and VP16ad.

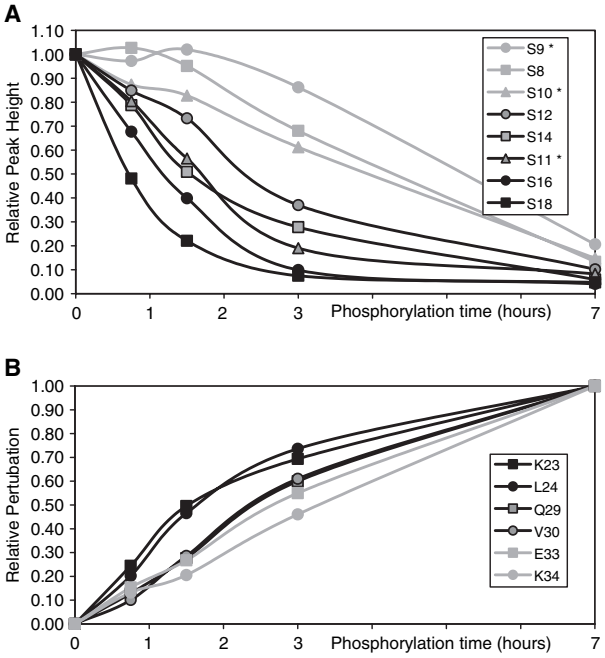

Fig. 6. Determination of the phosphorylation order and gradual effect on the lysine-rich region. (A) Quantification of the decrease in amide signal intensity of phosphorylated serine residue. The peak intensities were calculated relative to the peak intensity in the unphosphorylated form. The differences in protein concentration (due to buffer exchange) were corrected using the amide signal of Ser54 as internal standard. *Tentative assignment for the indicated phosphoserine. (B) Combined amide ^{1}H and ^{15}N chemical shift changes of residues in the lysine-rich region (K23, L24, Q29, V30, A31, E33, K34) due to phosphorylation. The perturbations were mapped relative to the maximum perturbation after 7 h, when the PC4 sample was completely phosphorylated.

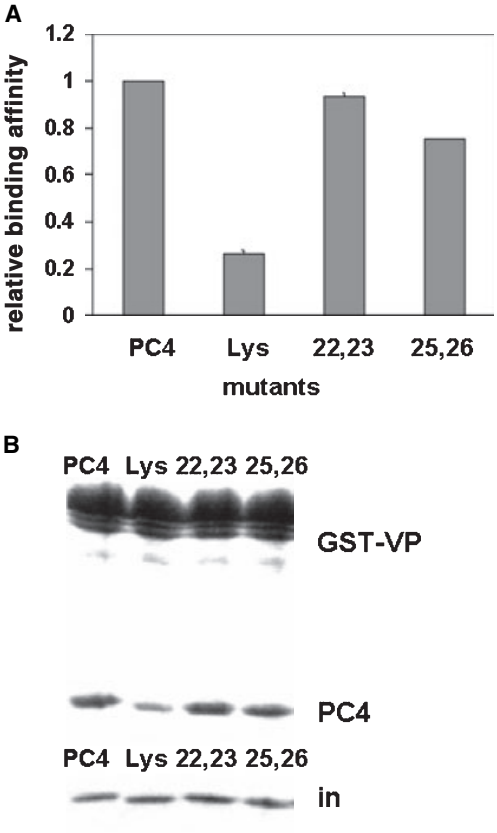

Fig. 7. Shielding of the lysine-rich region contributes to phosphorylation-dependent changes in PC4 activity. (A) Quantification of dsDNA binding of PC4, the lysine-rich mutant (K22G/K23G/K25G/R26G/K27G/K28G), and the double point mutants K22G/K23G and K25G/R26G. The relative binding affinities for the PC4 mutants were calculated relative to the interaction of PC4 with dsDNA. (B) A representative GST pull-down assay determining the interaction between PC4 mutants and VP16ad (upper panel); the positions of GST-VP16ad and PC4 are indicated. The lower panel shows 10% of the total amount of PC4 added in the GST pull-down assay.

As expected, the ssDNA binding by the K22G/K23G and K25G/R26 mutants was increased, but the lysine-rich mutant was impaired in ssDNA binding (data not shown). The imperfect similarity between phosphorylation of the serine-rich region and mutation of the lysine-rich region indicates that apart from shielding (dsDNA, VP16ad binding), additional mechanisms involving the phosphoserines contribute to the phosphorylation-dependent changes in ssDNA binding. We propose that this is the result of communication between PC4ntd and PC4ctd, which is influenced by phosphorylation (Fig. 5), but not by mutation of the lysine residues.

Discussion

The protein kinase CKII is a highly conserved multifunctional enzyme, which is known to activate and regulate RNA polymerase III transcription [35–37]. More than 300 CKII substrates have been documented, and many of these proteins have transcriptional functions [38]. Many components of the RNA polymerase II machinery can be phosphorylated by CKII, with dramatic effects on transcription [39,40]. The human chromatin protein DEK is phosphorylated by CKII *in vitro* and *in vivo*, which weakens its binding to DNA [41]. Furthermore, CKII is able to phosphorylate the histone H4 [42,43]. PC4 is subjected to *in vivo* phosphorylation events mediated by CKII [28] and possibly TFIID and TFIIH within the context of the PIC [21,22]. Recently it was found that CKII, together with PC4, establishes downstream core promoter element (DPE)-specific transcription of RNA polymerase II genes [44]. Although it is at present not

known whether *in vivo* partial phosphorylation of PC4 is functionally important, our *in vitro* data indicate that phosphorylation of the SEAC region by CKII influences the biochemical properties of PC4 in a phosphorylation status-dependent fashion. Phosphorylation of a single serine residue is enough to increase the ssDNA binding affinity. The unwinding activity and interaction with VP16ad was minimized when at least two or three phosphoserines were present in PC4, and, for maximal decrease in dsDNA binding, complete phosphorylation was required. Together these data indicate that the phosphorylation status differentially modulates PC4 activity. Consequently, phosphorylation may act as a molecular switch, resulting in a phosphorylation status-dependent change in the cofactor function of PC4 in transcription. We examined the mode of phosphorylation in detail by NMR. Up to eight serines (residues 8–12, 14, 16, 18) were modified in the SEAC region of PC4. Large chemical shift perturbations were observed in the first part of the lysine-rich region (residues 20–34). Furthermore, significant perturbations were detected in the β2–β3 and β4 sheet regions of PC4ctd, which are proposed to be caused by a changed environment due to temporary interactions between PC4ctd and PC4ntd. The affected PC4ctd regions are involved in the interaction with VP16, dsDNA and ssDNA (Jonker *et al.* unpublished work; [16,17,27]). As the binding interfaces in PC4ctd for the activator and DNA templates are not completely the same, phosphorylation may have different effects on the ability of PC4 to bind either of these substrates. We examined the nature of the phosphorylation event in detail by NMR, which allowed us to conclude that the SEAC region is gradually phosphorylated to the N-terminus, resulting in gradual chemical shift changes to the C-terminal side in the first part of the lysine-rich region. We provide evidence for the masking model as proposed by Kaiser *et al.* [17] and extend this model by suggesting that gradual masking of lysine residues by phosphoserine residues leads to specific changes in PC4 activity.

Functional consequences of PC4 phosphorylation

We have shown here that full length PC4 can bind to ssDNA, albeit with lower affinity than PC4p. The apparent K_d values reported here for binding to ssDNA (10 ± 2 nM) or bubble DNA (12 ± 4 nM, data not shown) are at least two orders of magnitude higher than reported for PC4ctd alone with identical probes under similar conditions [45]. Phosphorylation of PC4 results in a 2–3-fold increase in ssDNA binding affinity, indicating that phosphorylation may, at least partially, unmask the ssDNA-binding properties of PC4ctd. We found that phosphorylation of only one serine, probably Ser18 (Fig. 6), is enough to increase the ssDNA-binding properties to levels comparable to those of PC4p. We propose that the SEAC region temporary contacts PC4ctd and interferes with ssDNA binding caused by steric hindrance. These contacts are weakened by the interactions between the phosphoserine(s) and the lysine-rich region.

Binding to DNA is of functional relevance for the cofactor activity of PC4 [17]. A gradual decrease in dsDNA binding affinity is observed during phosphorylation, which correlates closely with changes observed in the lysine-rich region and points strongly to a regulatory mechanism. The strongest contribution to the dramatic decrease in dsDNA binding is observed when phosphorylation is complete. We propose that the overall positive charge of the lysine-rich region is progressively masked by phosphoserines, which causes the gradual decrease in binding affinity. This is in agreement with the effect of mutations in the lysine-rich region on dsDNA binding (Fig. 7A). Werten *et al.* reported that PC4 has the ability to unwind heteroduplex oligonucleotides in the absence of ATP [45]. We show here that PC4p has almost completely lost the ability to unwind bubble DNA and that at least two or three phosphoserines are required for inhibition. Both dsDNA binding and unwinding activity require similar PC4 concentrations and are decreased to a similar extent on phosphorylation. We propose that dsDNA binding is a prerequisite for unwinding, which does not exclude an important role for ssDNA binding.

The ability to bind dsDNA, and with a higher affinity ssDNA (Fig. 1), seems to be a hallmark of ATP-independent unwinding or helix-destabilizing proteins. The co-operative binding to dsDNA is reminiscent of the unwinding mechanism of the adenovirus DNA binding protein [46,47] and bacteriophage ϕ29 protein P5 [48]. The flexible C-terminal arm contacts a neighboring DNA binding protein molecule to form a stable and flexible protein chain that binds DNA. The co-operative protein–protein interactions have been proposed to be the driving force for the unwinding reaction by ssDNA binding proteins [47]. As PC4 dsDNA binding is co-operative in nature (Fig. 1), a similar mechanism may also account for PC4-dependent unwinding. Phosphorylation diminishes the unwinding activity and simultaneously decreases dsDNA binding. The decreased ability to form higher-order protein–DNA complexes on dsDNA may cause the lower activity. It has been shown that phosphorylation of replication protein A affects intersubunit inter-

actions which in turn modulate its dsDNA binding and melted dsDNA destabilization activities [49]. In this case a direct interaction between the phosphoserines and the DNA interaction surface was postulated. We have no direct evidence for contacts between the phosphoserine region of PC4ntd and PC4ctd.

The strongly electrostatically driven interaction between PC4 and VP16ad [50] is lost on phosphorylation of two or more serine residues, while monophosphorylated PC4 still interacts with VP16ad. Using NMR, we noticed that CKII-mediated phosphorylation of the first two serines (Ser16 and Ser18) leads to substantial chemical shift perturbations in the lysine-rich region up to approximately residue Leu24. Furthermore, the binding affinity decreased when lysine residues in this region were mutated to glycine (Fig. 7B). We propose that neutralization of the N-terminal positively charged lysine residues by the negative charges of the neighboring phosphoserines accounts for the decrease in binding affinity of PC4 for VP16ad.

Gradual masking of the lysine-rich region

It has been shown that the SEAC region and lysine-rich region both contribute to, respectively, the regulation and activity of PC4 function (Jonker *et al.*, unpublished work; [16,17]. While the SEAC region is gradually phosphorylated in the N-terminal direction, the adjacent lysine-rich region shows gradually increasing chemical shift perturbations towards the C-terminal side up to Lys34, which strongly suggests an interaction between the two regions. We propose that the SEAC region gradually covers the functional surface of the lysine-rich region by ongoing phosphorylation. This event triggers which parts of the lysine-rich region are available for interaction, thereby fine-tuning the interactions with DNA, activators and other targets in the basal transcription machinery. Binding experiments with PC4 mutants of lysine residues in the 22–28 region confirm this model (Fig. 7). In addition, phosphorylation has been shown to inhibit p300-mediated acetylation of PC4 [51]. Probably, the acetylation sites in the lysine-rich region are masked by phosphorylation and made inaccessible for p300-mediated acetylation. Furthermore, the cdk-inhibitory activity of PC4 correlated with the functional lysine-rich region and was abolished by phosphorylation [29]. The electrostatic interaction between the phosphorylated SEAC region and the positively charged lysine-rich region could not be unambiguously confirmed by NOE contacts. This is probably due to the highly flexible nature of the whole N-terminal domain (Jonker *et al.*, unpublished work), a characteristic that does not change much on phosphorylation. The equal peak intensities before and after phosphorylation exclude, furthermore, the possibility that the phosphorylation-dependent effects on PC4 activities are due to the formation of a stable complex between the phosphorylated SEAC region and the PC4ctd core. However, as we observed significant changes in the PC4ctd core after phosphorylation (Fig. 5A), we cannot exclude the possibility that modulation of weak temporary interactions with the flexible PC4ntd may also contribute to the observed effect on PC4 function.

Dynamic behavior of PC4 in transcription explained by the gradual masking model

The cofactor function of PC4 appears to be regulated by the phosphorylation mechanism in a distinct pathway that agrees with the functions PC4 is thought to fulfil from PIC assembly to elongation. In this model, activators recruit PC4 to the promoter [13], which allows PC4 to bind in concert with GTFs near the transcription start site and thereby stabilize the PIC [17]. Furthermore, when bound to dsDNA, PC4 can assist in promoter opening through its DNA-unwinding activity [27], although other GTFs, including TFIIH, are probably more important for this event. Upon promoter opening, PC4 can bind to bubble DNA [45], thereby forming a stalled PIC [21]. This repression can be relieved by the presence of the ERCC3 helicase activity of TFIIH [25], a factor that is essential for promoter opening and for the transition from transcription initiation to transcription elongation [52].

As phosphorylation of PC4 is thought to be performed by kinases present within the PIC [21,22], the subsequent phosphorylation events required for PC4 activities at the proper time within the transcription cycle can only occur when GTFs containing the kinase(s) required to phosphorylate PC4 are present. The PC4 concentrations required for these activities as well as the phosphorylation status-dependent changes in activities (Fig. 8) correlate with the timings of these events within the transcription cycle. First PC4 is recruited to the promoter region through interaction with activators (VP16 $K_d \approx 1$ μM [50]). Phosphorylation decreases the affinity for the activator, thereby shifting PC4 to bind to dsDNA ($K_d \approx 100$ nM). At this intermediate phosphorylation state (two or three phosphoserines), PC4 and/or other GTFs can unwind dsDNA. PC4 binds preferably to this DNA bubble ($K_d \approx 10$ nM). Because of the binding of PC4 to both dsDNA and ssDNA, transcription is stalled. Further phosphorylation results in the loss of dsDNA binding,

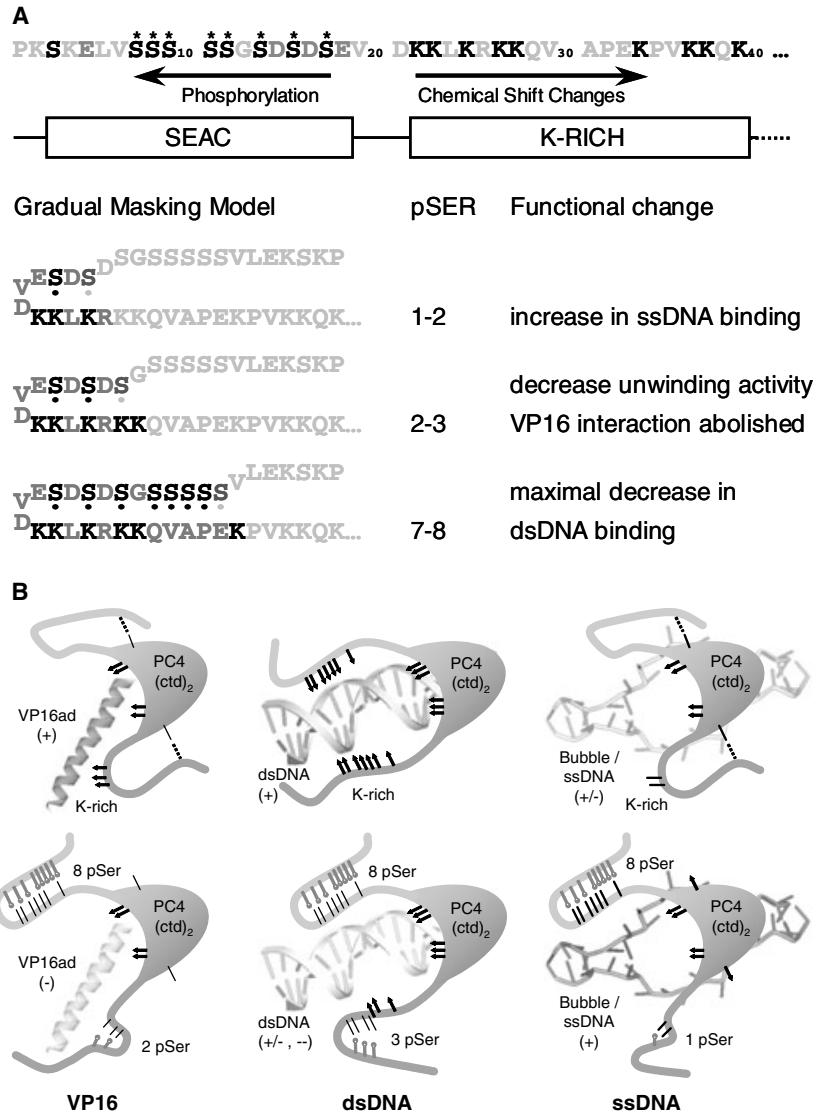

Fig. 8. Gradual masking model. (A) The biochemical properties (DNA and activator interactions, ATP independent unwinding activity) of PC4 are influenced in a phosphorylation status-dependent fashion through gradual masking of the functional lysine-rich region by the phosphoserines. (B) Schematic representation of PC4 binding to dsDNA, ssDNA and VP16ad influenced by phosphorylation. The PC4 dimer (dimeric PC4ctd core and two PC4ntd tails) contacts its target (black arrows represent the involved interacting residues of PC4). The interaction is repressed (solid lines represent PC4 residues that do not contact the target) by either different stages of serine phosphorylation (phosphoserines are represented as grey lines with a dot) or PC4ntd–PC4ctd contacts (dotted lines).

and the presence of TFIIH in the PIC enables transcription initiation through the helicase activity of ERCC3. Furthermore, it has been shown that PC4 can specifically inhibit the cdk1, cdk2 and cdk7 mediated RNA polymerase II phosphorylation [29], a phosphorylation event generally thought to be a critical step in the conversion from transcription initiation to elongation [7]. PC4p is no longer able to inhibit the phosphorylation of RNA polymerase II [29], arguing that regulated PC4 phosphorylation may also contribute to the conversion from initiation to elongation of transcription.

In summary, we propose that the phosphorylation status-dependent regulation of PC4 function is accomplished through gradual masking of the lysine-rich region by phosphoserines. This phosphorylation event is most likely performed by kinases present in the PIC, which allows PC4 to perform the adequate cofactor activity during the transcription cycle.

Experimental procedures

Protein, DNA and biochemical assays

The PC4 and VP16 protein constructs and mutants were prepared and quantified essentially as described previously (Jonker *et al.*, unpublished work; [27,45]. The oligonucleotides were purchased from Invitrogen (PO Box 3326, 4800 DH Breda, the Netherlands). The bubble consists of: 5′-GGGCGGCGGGTTTTTTTTGGCGGGGCGG and 5′-CCGCCCCGCCTTTTTTTTCCCGCCGCCC. For ssDNA binding, either of these strands was used; both show

essentially identical results. For dsDNA binding, a HIV/ML hybrid promoter fragment [17] was used. The electrophoretic mobility shift assays, unwinding assays and GST pull-down experiments were performed as described (Jonker *et al.*, unpublished work). The GST pull-down assays were carried out in the presence of 0.25 μM PC4. For the phosphorylation time course, the average binding affinity was determined in relation to unphosphorylated PC4, for which the percentage binding was set at 1.

Phosphorylation assay

For the phosphorylation assay, the PC4 sample buffer was exchanged to 1 mL phosphorylation buffer containing 200 mM KCl, 20 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM ATP and Complete (EDTA-free) protease inhibitor (Roche), using Centriprep 3K ultrafiltration filters (Millipore). The sample was prewarmed at 30 °C, and the reaction was started by the addition of 500 U CK II (Calbiochem). Samples were taken after 0, 8, 16, 30, 60, 120, 240, 480 and 1440 min, diluted in 1 mL 20 mM Tris/HCl (pH 7.5)/50 mM KCl/1 mM EDTA, and immediately purified using a linear KCl gradient on a heparin column (Poros). The unphosphorylated PC4 was eluted at ≈625 mM KCl, and completely phosphorylated PC4 eluted at 400 mM KCl.

MALDI and electrospray ionization MS

Electrospray ionization MS was carried out on a Micromass LC-T orthogonal time-of-flight instrument (Micromass UK Ltd, Manchester, UK) equipped with a 'Z-Spray' nanoflow electrospray source using in-house pulled and gold-coated borosilicate glass needles. Before analysis, protein samples were concentrated and desalted using Micro C18 ZipTips (Millipore, Bedford, MA, USA) following the manufacturer's protocol for peptide and protein concentration and clean up. Mass spectra were acquired in the positive ion mode, and a mass range of 200–2000 *m/z* was monitored.

NMR spectroscopy

The phosphorylation assay using ^{15}N-labeled PC4 was carried out at 303 K in 900 μL 20 mM Tris/HCl (pH 7.5) buffer containing 200 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, Complete (EDTA-free) protease inhibitor (Roche) and 10% D_2O. A 300-μL portion of this sample was used to set up the 500-MHz NMR spectrometer. A 150-μL sample was set apart (time point 0), and the phosphorylation was started by adding CKII in proportional amounts to both the prewarmed NMR sample and the remaining parallel batch. A series of 2D (^1H,^{15}N)-HSQC spectra were recorded for 7 h (14 × 30 min). Then 150-μL samples were taken from the parallel batch after 45, 90 and 180 min. The reaction was terminated by adding 5 mL cold (4 °C) 50 mM phosphate NMR buffer (pH 5.6) containing 400 mM KCl and Complete protease inhibitor (Roche). The final NMR samples were exchanged to this NMR buffer using Centriprep 3K filters (Millipore) and supplemented with 2 M D_5-glycine (for better long-term stability of the PC4 protein at higher temperatures [27]).

NMR experiments using 0.2–1 mM ^{15}N and $^{13}C/^{15}N$ labeled PC4 samples were essentially performed as described in Cavanagh *et al.* [53] and carried out at 305 K on Bruker Avance 500-MHz and 750-MHz spectrometers using triple-resonance probes. Most of the resonances for PC4ctd have been assigned before [27] and could be re-assigned for full length PC4 by overlaying (^1H,^{15}N)-HSQC spectra and by verification of NOE patterns from 3D NOESY-(^1H,^{15}N)-HSQC spectra. The ^1H, ^{15}N and ^{13}C resonances of residues 3–57 in PC4 and PC4p were assigned using 3D TOCSY-(^1H,^{15}N)-HSQC, 3D NOESY-(^1H,^{15}N)-HSQC and triple-resonance spectra (3D HNCACB, 3D HN(CA)CO and 3D HNCO) as described previously (Jonker *et al.*, unpublished work). The spectra were processed using the software package NMRPIPE [54] and analyzed using SPARKY 3 (T. D. Goddard and D. G. Kneller, University of California, San Francisco, CA, USA).

Acknowledgements

We thank Pauliina Uitto, Claudia Maier and Albert Heck from the Biomolecular Mass Spectrometry Department of Utrecht University for their MS support and useful discussions. Rob de Jong is acknowledged for critically reading the manuscript. We thank Ineke Braakman for the use of equipment. This work is financially supported by the Netherlands Organization for Scientific Research (NWO) and the Centre for Biomedical Genetics (CBG).

References

1 Orphanides G, Lagrange T & Reinberg D (1996) The general transcription factors of RNA polymerase II. *Genes Dev* **10**, 2657–2683.
2 Roeder RG (1996) The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem Sci* **21**, 327–335.
3 Koleske AJ & Young RA (1994) An RNA polymerase II holoenzyme responsive to activators. *Nature* **368**, 466–469.
4 Myer VE & Young RA (1998) RNA polymerase II holoenzymes and subcomplexes. *J Biol Chem* **273**, 27757–27760.
5 Kobor MS & Greenblatt J (2002) Regulation of transcription elongation by phosphorylation. *Biochim Biophys Acta* **1577**, 261–275.

6 Cadena DL & Dahmus ME (1987) Messenger RNA synthesis in mammalian cells is catalyzed by the phosphorylated form of RNA polymerase II. *J Biol Chem* **262**, 12468–12474.
7 Dahmus ME (1996) Reversible phosphorylation of the C-terminal domain of RNA polymerase II. *J Biol Chem* **271**, 19009–19012.
8 Bensaude O, Bonnet F, Casse C, Dubois MF, Nguyen VT & Palancade B (1999) Regulated phosphorylation of the RNA polymerase II C-terminal domain (CTD). *Biochem Cell Biol* **77**, 249–255.
9 Karin M & Hunter T (1995) Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr Biol* **5**, 747–757.
10 Hill CS & Treisman R (1995) Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* **80**, 199–211.
11 Brivanlou AH & Darnell JE Jr (2002) Signal transduction and the control of gene expression. *Science* **295**, 813–818.
12 Verrijzer CP & Tjian R (1996) TAFs mediate transcriptional activation and promoter selectivity. *Trends Biochem Sci* **21**, 338–342.
13 Kaiser K & Meisterernst M (1996) The human general co-factors. *Trends Biochem Sci* **21**, 342–345.
14 Burley SK & Roeder RG (1996) Biochemistry and structural biology of transcription factor IID (TFIID). *Annu Rev Biochem* **65**, 769–799.
15 Ge H & Roeder RG (1994) Purification, cloning, and characterization of a human coactivator, PC4, that mediates transcriptional activation of class II genes. *Cell* **78**, 513–523.
16 Kretzschmar M, Kaiser K, Lottspeich F & Meisterernst M (1994) A novel mediator of class II gene transcription with homology to viral immediate-early transcriptional regulators. *Cell* **78**, 525–534.
17 Kaiser K, Stelzer G & Meisterernst M (1995) The coactivator p15 (PC4) initiates transcriptional activation during TFIIA-TFIID-promoter complex formation. *Embo J* **14**, 3520–3527.
18 Werten S, Stelzer G, Goppelt A, Langen FM, Gros P, Timmers H, ThM, Van der Vliet PC & Meisterernst M (1998) Interaction of PC4 with melted DNA inhibits transcription. *EMBO J* **17**, 5103–5111.
19 Holstege FC, van der Vliet PC & Timmers HThM (1996) Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIH. *EMBO J* **15**, 1666–1677.
20 Holstege FC, Tantin D, Carey M, van der Vliet PC, Timmers H & ThM (1995) The requirement for the basal transcription factor IIE is determined by the helical stability of promoter DNA. *EMBO J* **14**, 810–819.
21 Malik S, Guermah M & Roeder RG (1998) A dynamic model for PC4 coactivator function in RNA polymerase II transcription. *Proc Natl Acad Sci USA* **95**, 2192–2197.
22 Kershnar E, Wu SY & Chiang CM (1998) Immunoaffinity purification and functional characterization of human transcription factor IIH and RNA polymerase II from clonal cell lines that conditionally express epitope-tagged subunits of the multiprotein complexes. *J Biol Chem* **273**, 34444–34453.
23 Wu SY, Kershnar E & Chiang CM (1998) TAFII-independent activation mediated by human TBP in the presence of the positive cofactor PC4. *EMBO J* **17**, 4478–4490.
24 Wu SY & Chiang CM (1998) Properties of PC4 and an RNA polymerase II complex in directing activated and basal transcription in vitro. *J Biol Chem* **273**, 12492–12498.
25 Fukuda A, Tokonabe S, Hamada M, Matsumoto M, Tsukui T, Nogi Y & Hisatake K (2003) Alleviation of PC4-mediated transcriptional repression by the ERCC3 helicase activity of general transcription factor TFIIH. *J Biol Chem* **278**, 14827–14831.
26 Brandsen J, Werten S, van der Vliet PC, Meisterernst M, Kroon J & Gros P (1997) C-terminal domain of transcription cofactor PC4 reveals dimeric ssDNA binding site. *Nat Struct Biol* **4**, 900–903.
27 Werten S, Wechselberger R, Boelens R, van der Vliet PC & Kaptein R (1999) Identification of the single-stranded DNA binding surface of the transcriptional coactivator PC4 by NMR. *J Biol Chem* **274**, 3693–3699.
28 Ge H, Zhao Y, Chait BT & Roeder RG (1994) Phosphorylation negatively regulates the function of coactivator PC4. *Proc Natl Acad Sci USA* **91**, 12691–12695.
29 Schang LM, Hwang GJ, Dynlacht BD, Speicher DW, Bantly A, Schaffer PA, Shilatifard A, Ge H & Shiekhattar R (2000) Human PC4 is a substrate-specific inhibitor of RNA polymerase II phosphorylation. *J Biol Chem* **275**, 6071–6074.
30 Fukuda A, Nakadai T, Shimada M, Tsukui T, Matsumoto M, Nogi Y, Meisterernst M & Hisatake K (2004) Transcriptional coactivator PC4 stimulates promoter escape and facilitates transcriptional synergy by GAL4-VP16. *Mol Cell Biol* **24**, 6525–6535.
31 Calvo O & Manley JL (2001) Evolutionarily conserved interaction between CstF-64 and PC4 links transcription, polyadenylation, and termination. *Mol Cell* **7**, 1013–1023.
32 Bienkiewicz EA & Lumb KJ (1999) Random-coil chemical shifts of phosphorylated amino acids. *J Biomol NMR* **15**, 203–206.
33 Hathaway GM & Traugh JA (1983) Casein kinase II. *Methods Enzymol* **99**, 317–331.
34 Pinna LA (1990) Casein kinase 2: an 'eminence grise' in cellular regulation? *Biochim Biophys Acta* **1054**, 267–284.

35 Hu P, Wu S & Hernandez N (2003) A minimal RNA polymerase III transcription system from human cells reveals positive and negative regulatory roles for CK2. *Mol Cell* **12**, 699–709.
36 Ghavidel A & Schultz MC (2001) TATA binding protein-associated CK2 transduces DNA damage signals to the RNA polymerase III transcriptional machinery. *Cell* **106**, 575–584.
37 Johnston IM, Allison SJ, Morton JP, Schramm L, Scott PH & White RJ (2002) CK2 forms a stable complex with TFIIIB and activates RNA polymerase III transcription in human cells. *Mol Cell Biol* **22**, 3757–3768.
38 Pinna LA (2002) Protein kinase CK2: a challenge to canons. *J Cell Sci* **115**, 3873–3878.
39 Cabrejos ME, Allende CC & Maldonado E (2004) Effects of phosphorylation by protein kinase CK2 on the human basal components of the RNA polymerase II transcription machinery. *J Cell Biochem* **93**, 2–10.
40 Abbott KL, Renfrow MB, Chalmers MJ, Nguyen BD, Marshall AG, Legault P & Omichinski JG (2005) Enhanced binding of RNAP II CTD phosphatase FCP1 to RAP74 following CK2 phosphorylation. *Biochemistry* **44**, 2732–2745.
41 Kappes F, Damoc C, Knippers R, Przybylski M, Pinna LA & Gruss C (2004) Phosphorylation by protein kinase CK2 changes the DNA binding properties of the human chromatin protein DEK. *Mol Cell Biol* **24**, 6011–6020.
42 Utley RT, Lacoste N, Jobin-Robitaille O, Allard S & Cote J (2005) Regulation of NuA4 histone acetyltransferase activity in transcription and DNA repair by phosphorylation of histone H4. *Mol Cell Biol* **25**, 8179–8190.
43 Cheung WL, Turner FB, Krishnamoorthy T, Wolner B, Ahn SH, Foley M, Dorsey JA, Peterson CL, Berger SL & Allis CD (2005) Phosphorylation of histone H4 serine 1 during DNA damage requires casein kinase II in *S. cerevisiae*. *Curr Biol* **15**, 656–660.
44 Lewis BA, Sims RJ III, Lane WS & Reinberg D (2005) Functional characterization of core promoter elements: DPE-specific transcription requires the protein kinase CK2 and the PC4 coactivator. *Mol Cell* **18**, 471–481.
45 Werten S, Langen FW, van Schaik R, Timmers H, ThM, Meisterernst M & van der Vliet PC (1998) High-affinity DNA binding by the C-terminal domain of the transcriptional coactivator PC4 requires simultaneous interaction with two opposing unpaired strands and results in helix destabilization. *J Mol Biol* **276**, 367–377.
46 van Breukelen B, Kanellopoulos PN, Tucker PA & van der Vliet PC (2000) The formation of a flexible DNA-binding protein chain is required for efficient DNA unwinding and adenovirus DNA chain elongation. *J Biol Chem* **275**, 40897–40903.
47 Dekker J, Kanellopoulos PN, Loonstra AK, van Oosterhout JA, Leonard K, Tucker PA & van der Vliet PC (1997) Multimerization of the adenovirus DNA-binding protein is the driving force for ATP-independent DNA unwinding during strand displacement synthesis. *EMBO J* **16**, 1455–1463.
48 Soengas MS, Gutierrez C & Salas M (1995) Helix-destabilizing activity of phi 29 single-stranded DNA binding protein: effect on the elongation rate during strand displacement DNA replication. *J Mol Biol* **253**, 517–529.
49 Binz SK, Lao Y, Lowry DF & Wold MS (2003) The phosphorylation domain of the 32-kDa subunit of replication protein A modulates RPA–DNA interactions: evidence for an intersubunit interaction. *J Biol Chem* **278**, 35584–35591.
50 Jonker HRA, Wechselberger RW, Boelens R, Folkers GE & Kaptein R (2005) Structural properties of the promiscuous VP16 activation domain. *Biochemistry* **44**, 827–839.
51 Kumar BR, Swaminathan V, Banerjee S & Kundu TK (2001) p300-mediated acetylation of human transcriptional coactivator PC4 is inhibited by phosphorylation. *J Biol Chem* **276**, 16804–16809.
52 Dvir A, Conaway JW & Conaway RC (2001) Mechanism of transcription initiation and promoter escape by RNA polymerase II. *Curr Opin Genet Dev* **11**, 209–214.
53 Cavanagh J, Fairbrother WJ, Palmer AG III & Skelton NJ (1996) *Protein NMR Spectroscopy*. Academic Press, San Diego, CA.
54 Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J & Bax A (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* **6**, 277–293.