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Human Supt5h protein, a putative modulator of chromatin structure, is reversibly phosphorylated in mitosis

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Abstract **The *Saccharomyces cerevisiae* proteins Spt4p, Spt5p and Spt6p are involved in transcriptional repression by modulating the structure of chromatin. From HeLa cells we have purified a human homologue of Spt5p, Supt5hp, and show here that the protein is reversibly phosphorylated in mitosis. The cloned cDNA predicts a protein of 1087 residues with 31% identity to yeast Spt5p. It includes an acidic N-terminus, a putative nuclear localization signal and a C-terminal region containing two different repeated motifs. One of them, with the consensus sequence P-T/S-P-S-P-Q/A-S/G-Y, is similar to the C-terminal domain in the largest subunit of RNA polymerase II.**

Key words: Transcriptional regulation; Chromatin structure; Cell cycle; C-terminal domain; Phosphorylation

1. Introduction

The structure of chromatin is modified by proteins that interact with histones and promote either a relaxed state of chromatin, suitable for transcription, or a more compact and transcriptionally inactive state [1,2]. Factors, like the large Snf/Swi complex, facilitate the initiation of transcription by interaction with nucleosomes and the RNA polymerase II complex [3].In *Saccharomyces cerevisiae* a deficiency in the activating Swi/Snf complex is suppressed by mutations in the *SPT4*, *SPT5* and *SPT6* genes [4–6], indicating that the wild-type proteins are involved in transcriptional repression. Mutations in these genes were also shown to alter transcription of a subset of histone genes in *S. cerevisiae* [7]. Since double mutant combinations of *spt4*, *spt5* and *spt6* are generally lethal in haploid strains, the corresponding wild-type proteins may form a heterotrimeric complex. The co-immunoprecipitation of Spt5p and Spt6p also indicates a physical interaction of these proteins [8]. The chromatin remodelling-mechanism of the postulated Spt protein complex may in part be explained the finding that Spt6p interacts directly with the globular domain of histone H3 and that it functions as a nucleosome assembly factor which assists histones in binding to DNA [9].

The *SPT4* gene encodes a small protein of 102 amino acid residues, whereas Spt5p (1063 residues) and Spt6p (1726 residues) are considerably larger proteins with highly acidic N-terminal regions. Null mutants of *SPT4* are viable [10], while the deletion of *SPT5* or *SPT6* has a lethal effect, indicating that a main function of the complex is carried out by the *SPT5* and *SPT6* gene products. Recently, *SUPT4H* and *SUPT6H*, the human homologues of the *SPT4* and *SPT6* genes, have been cloned [11–13]. We report here the purification of Supt5hp, a human homologue of yeast Spt5p, its mitotic phosphorylation, and the cloning and sequencing of the *SUPT5H* cDNA.

2. Materials and methods

2.1. Generation of antibodies and purification of Supt5hp

HeLa cells were cultivated as described before [14]. For cell cycle studies, cells were synchronized in S-phase by a double thymidine block [15] and the subsequent arrest in mitosis was achieved by a nocodazole block [16]. Monoclonal antibody 8001 was one of a set raised against proteins from mitotic HeLa cell lysates. It was characterized as IgMκ and used to monitor the purification of Supt5hp. This was then used to generate polyclonal antibody AS40 by immunizing chicken and preparing antibodies from the eggs.

Initially, monoclonal antibody 8001 was used to monitor Supt5hp isolation. In subsequent routine procedures the more sensitive antibody AS40 was used. Fractions containing Supt5hp were identified in immunoblots stained with either of the two antibodies, and the intensity of the stain was determined densitometrically. All purification steps were performed at 4°C. Frozen HeLa cells (4×10^{10}, 100 g wet weight) were thawed, resuspended in 200 ml of buffer A (20 mM bis-Tris-propane-HCl (Sigma), pH 6.9, containing 20% glycerol, 1 mM dithiothreitol and protease inhibitors, swollen on ice for 30 min and homogenized by 35 strokes in a S-type Dounce homogenizer (Braun, Melsungen). Insoluble material was removed by centrifugation (30 min at $70\,000\times g$). The pellet was resuspended in 100 ml of buffer A containing 50 mM NaCl, and again centrifuged for 30 min at $70\,000\times g$. The two supernatants were pooled, solid ammonium sulfate was added with continuous stirring up to 30% saturation. The suspension was further stirred for 1 h at 4°C. After spinning down precipitated protein (30 min at $70\,000\times g$), the pellet was dissolved in 25 ml of buffer A containing 100 mM NaCl and applied to an DMAE-Fractogel 650 S column (1.2×10 cm, Merck) equilibrated with buffer A containing 100 mM NaCl. Bound protein was eluted by applying a linear gradient of 250 mM–1 M NaCl at a flow rate of 1.2 ml/min. The fractions containing Supt5hp were diluted 10-fold in buffer B (50 mM Mes–NaOH, pH 6.9, containing 20% glycerol and 1 mM dithiothreitol) and applied to an SO_3-Fractogel 650S column (0.5×5 cm, Merck) equilibrated with buffer B containing 140 mM NaCl. Bound protein was eluted by applying a linear gradient of 140 mM–2 M NaCl at a flow rate of 0.4 ml/min. The fractions containing Supt5hp were diluted 10-fold in buffer A containing 200 mM NaCl, and applied to a MonoQ column (0.5×5 cm, Pharmacia) equilibrated with the same buffer. Bound protein was eluted by applying a linear gradient of 200–400 mM NaCl at a flow rate of 0.4 ml/min. After electrophoresis, SDS gels were stained with Coomassie blue R250 and scanned in a laser densitometer, with bovine serum albumin as standard.

2.2. Amino acid sequences of peptides and cloning of SUPT5H

Ten micrograms of the purified protein was cleaved in 0.1 M NH_4HCO_3 and 1 M urea with N-Asp protease for 2 h at 25°C. The reaction was stopped by addition of 15% (v/v) TFA. Another aliquot of 10 μg was cleaved in 75% formic acid with CNBr for 20

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h at 25°C. The resulting peptides were separated by reversed phase HPLC (C18) in 0.1% TFA, 5–72% acetonitrile and sequenced with a gas-phase sequencer (Model 470A, Applied Biosystems). The Tblastn program (part of the HUSAR program package, DKFZ) was used to search the databases for nucleotide sequences corresponding to the peptide sequences obtained.

Total RNA was obtained from logarithmically growing HeLa cells using the RNA-Clean kit (AGS, Heidelberg, Germany). Reverse transcriptase-mediated PCR was performed on total RNA using the SuperScript reverse transcriptase (Life Technologies). Clone 322 was amplified by RT-PCR using as forward and reverse primers: 5′-gac cga ccc ccc aag aaa ccc-3′ and 5′-ggg atc ctt gac tcc ctg gga-3′, respectively. PCR products were cloned directly into the pCRII vector (Invitrogen).

HeLa and testis libraries were plated on *E. coli* Y1090$^-$ with approximately 5×10^4 plaque-forming units per 150 mm plate, and were lifted on Hybond N$^+$ membranes (Amersham). Fifty nanograms of cDNA clones were labelled (DECAprimeII kit, Ambion) with 50 μCi [α-^{32}P]dCTP and hybridized to the membranes. Positive clones were selected and DNA was eluted (35 mM Tris/HCl, pH 7.5, 10 mM $MgSO_4$, 0.1 M NaCl, 0.01% gelatin). Phage DNA was replated and further rounds of screening were performed. Phage DNA of single plaques was purified and cut with *Eco*RI in the presence of 10 mM spermidine. The inserts obtained were purified as before, eluted and cloned into the *Eco*RI sites of pCRII. DNA sequencing was performed with an ALFexpress (Pharmacia) DNA sequencer and with an ABI sequencer Model 373A (Applied Biosystems).

5′-RACE was performed on testis cDNA (Marathon-Ready kit, Clontech) with the primer: 5′-ctc tag aat gtc ctc tgc tcc atc ctc cca ctg gtc ctc-3′. Twenty-six cycles were performed with 68°C for 1 min, 72°C for 2 min and 92°C for 1 min. Two microliters of the 50 μl reaction mixture were removed and used as a template for further reamplification under the same conditions. The PCR products were analyzed on agarose gels and cloned into pCRII vectors. EST clones from the IMAGE Consortium (LLNL) [17] were obtained from Research Genetics (Huntsville, AL).

3. Results

3.1. Purification of Supt5hp from HeLa cells

In a study to identify proteins involved in cell cycle regulation, we had generated a set of monoclonal antibodies raised against proteins from mitotic HeLa cells. One of them, antibody 8001, in immunofluorescence studies stained a nuclear epitope in interphase HeLa cells (data not shown) and detected a protein with an apparent mass of approximately 155 kDa in immunoblots (Fig. 1B, lane 10). Using this antibody to monitor enrichment, the protein we later identified as Supt5hp was purified from lysates of logarithmically growing HeLa cells by fractionation as described in Section 2 and enriched 32500-fold to apparent homogeneity (Fig. 1A and Table 1). In order to confirm the results obtained with monoclonal antibody 8001, the purified protein was used to raise polyclonal antibodies in a chicken. Antibody preparations collected from the eggs of this chicken were designated AS40. Both antibodies displayed an identical distribution pattern of a 155 kDa protein in the various protein fractions, both detected the purified

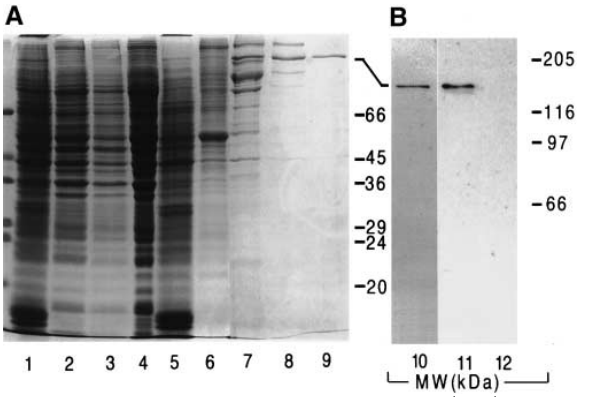

Fig. 1. Purification of Supt5hp from HeLa cells. A: 12.5% SDS-PAGE of protein fractions stained with Coomassie brilliant blue. B: 10% SDS-PAGE: Total soluble HeLa protein, immunostained with monoclonal antibody 8001 (lane 10), or immunostained using the polyclonal antibody AS40 (lane 11), or pre-immune immunoglobulin (lane 12).

155 kDa protein in immunoblots (Fig. 1A, lane 9), and both stained the nuclei of interphase HeLa cells. By comparative immunoblotting using AS40, we estimated the amount of the protein to be 20 000 copies/HeLa cell; representing ~0.0025% of the total cellular protein (data not shown).

3.2. Cloning of SUPT5H

We cleaved aliquots of the antigenic protein by N-Asp protease and CNBr. The resulting fragments were separated by HPLC and 15 of them were sequenced. A data base search using the Tblastn program yielded one cDNA clone (T18964) which, in its translated open reading frame, contained the sequence of one of the peptide fragments (residues 131–152 in Fig. 2). Based on this sequence, primers were designed and used to amplify a fragment of 342 bp (clone 322) from HeLa cDNA. A λgt10 HeLa library was screened with this fragment yielding a clone with a 792 bp insert, designated clone 13. Furthermore, another peptide fragment (residues 796–810 in Fig. 2) was found in the translated sequences of a number of expressed sequence tags in the GenBank database (R59954, R73139, R14898 and T80145). Clone R14898 represents a sequence of 1517 bp including a poly(A) tail and a polyadenylation signal. With pooled DNA from clones 13 and R14898 a λgt10 testis library was screened, yielding two overlapping clones of 972 bp and 1430 bp, respectively. The sequence was extended by 5′-RACE for a further 320 bp. A new database search with the complete sequence identified two additional EST clones (R18289 and W73325) of 2391 bp and 3276 bp, respectively, which were sequenced and confirmed the sequence already established. The sequence data are corroborated by the microsequencing data of the Supt5hp peptides.

Table 1
Purification of Supt5h from HeLa cells

Fraction	Total protein (mg)	Supt5hp (μg)	Purification factor	Recovery (%)
Crude lysate	7920	200		100
Soluble protein	3872	160	1.3	80
30% Ammonium sulfate precipitate	184	150	26.7	75
DMAE-Fractogel	4.2	120	943	60
SO_3-Fractogel	0.37	94	8300	47
MonoQ	0.06	60	32500	30

Crude lysate was obtained from 100 g of packed HeLa cells (4×10^{10} cells). Supt5h was quantitated by the staining of gels with Coomassie blue and of immunoblots with AS40 antibodies to Supt5h.

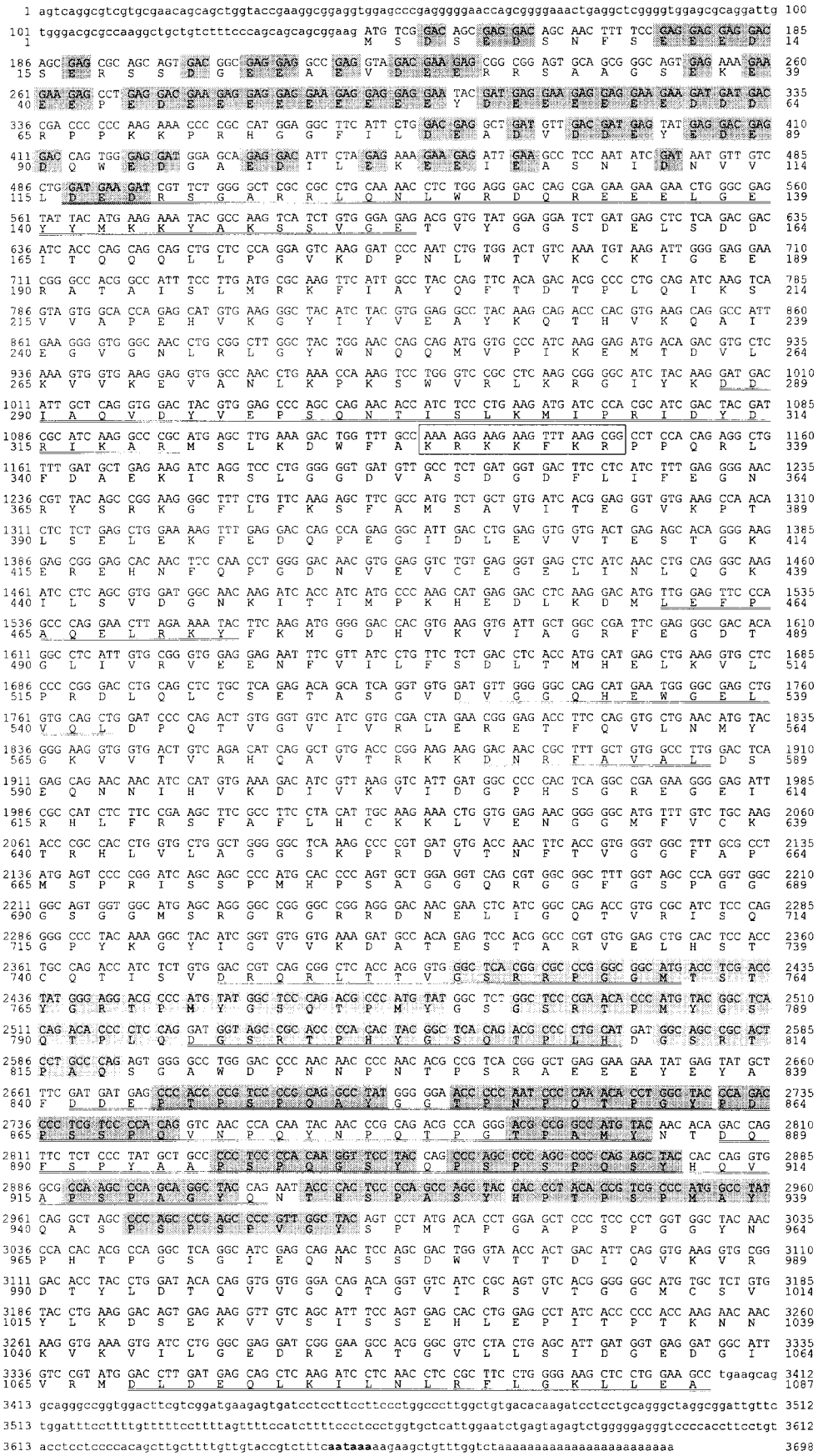

Fig. 2. cDNA and derived amino acid sequence of *SUPT5H*. The 5′- and 3′-UTRs are printed in lower case, the polyadenylation signal is highlighted in bold. Amino acid sequences confirmed by microsequencing of purified Supt5hp are underlined. Acidic residues of the N-terminal region are boxed, as is the putative NLS signal. The CTR1 and CTR2 repeats are boxed and marked differently.

3.3. SUPT5H cDNA and predicted structure of the encoded protein

The SUPT5H cDNA has a length of 3699 bp, with an open reading frame of 3261 bp, predicting a protein of 1087 amino acid residues (Fig. 2). The 3′-UTR of 270 bp contains a polyadenylation signal (AATAAA), preceding a poly(A) tail by 17 bp. A 5′-UTR region of 143 bp precedes a start ATG codon which is in agreement with other functional initiation sequences [18]. There is 31% overall amino acid identity between Supt5hp and its yeast homologue, with the domain 1–816 displaying a stronger identity of 34%, and the C-terminal 271 residues showing a weaker one of 25% (Fig. 3). Supt5hp, similar to its yeast counterpart, has a very acidic N-terminal domain of 118 amino acid residues 62 of which are either Asp or Glu (Fig. 4). Residues 77–81 contain an incomplete (L-D-E-A-D) DEAD-box motive (normally implicated in ATP binding) which, however, lacks the characteristic additional conserved domains normally flanking such a motif. A putative nuclear localization signal KRKKFKR (residues 328–334), may be responsible for the observed nuclear localization of Supt5hp. A C-terminal domain of 280 residues, rich in Ser, Pro and Tyr, contains two different C-terminal repeat motifs (Fig. 4), unrelated to the motif S-T/A-W-G-G-A/Q, present in yeast Spt5p. The first motif (CTR1) with the consensus sequence G-S-R/Q-T-P-X-Y is repeated 9 times and the second, less conserved motif (CTR2), with the consensus sequence P-T/S-P-S-P-Q/A-S/G-Y is repeated 10 times.

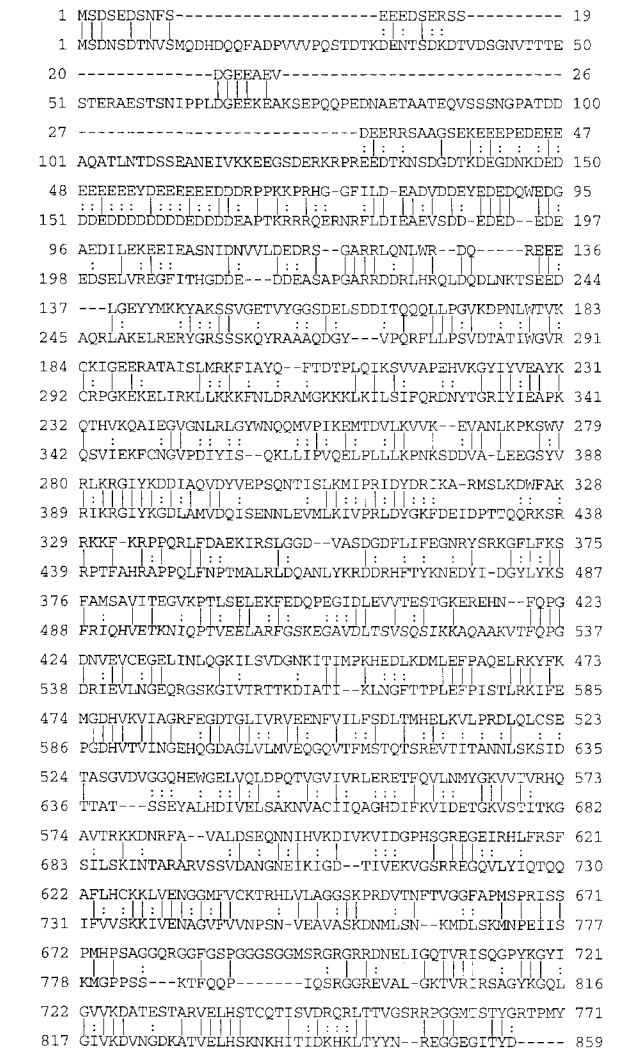

Fig. 3. Sequence homology of Supt5hp (this work, upper lines) and yeast Spt5p ([22], lower lines). The C-terminal regions of both sequences, with a less significant homology, are omitted. Identities are marked 'ı', related residues are labeled ':' and deletions introduced to maximize the homology are indicated by '-'.

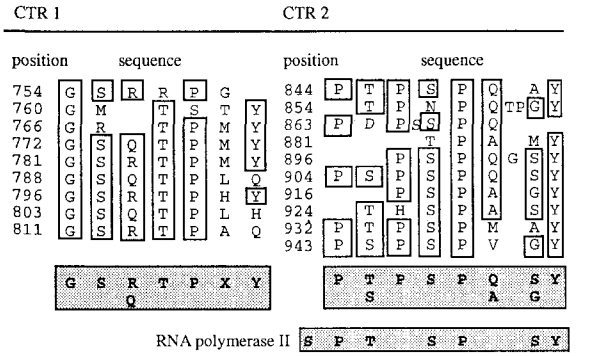

Fig. 4. Structure of the C-terminal repeats. The C-terminal repeat domains (CTR) 1 and 2 are listed and the consensus motifs are given. On the left side the number of the first residue of each repeat is displayed. Below these repeats the consensus sequence of the CTR of RNA-polymerase II is listed, which shows a similar pattern.

3.4. Cell cycle-dependent phosphorylation of the Supt5hp

To monitor the amount of Supt5hp throughout the cell cycle, we performed immunostaining with AS40 of lysates from synchronized HeLa cells. We found the amount to remain constant during the cell cycle, like that of actin, while the cyclin B level showed its characteristic fluctuation (Fig. 5A). During mitosis, Supt5hp appeared as a more slowly migrating form in SDS-polyacrylamide gels than in interphase. This effect is likely to result from protein phosphorylation, since it can be reverted upon treatment with calf intestinal phosphatase (Fig. 5B).

4. Discussion

Genetic evidence in the yeast *S. cerevisiae* suggests that Spt4p, Spt5p and Spt6p modify chromatin structure and thereby change transcriptional regulation of a large number of genes in vivo. We have purified Supt5hp, a human homologue of Spt5p and cloned its cDNA. The open reading frame of *SUPT5H* predicts a protein of 1087 amino acid residues, a calculated molecular mass of 121 kDa and a p*I* of 4.8, compared to the yeast Spt5p protein with 1063 residues, a calculated mass of 116 kDa and a p*I* of 5.0. Both proteins share 31% sequence identity. The apparent mass of the purified protein as determined by SDS-PAGE is somewhat higher than the calculated mass. This discrepancy is probably due to the abundance of acidic residues present in Supt5hp, causing a reduced electrophoretic mobility in SDS-PAGE. Similar differences between masses calculated from electrophoretic mobilities and from the derived sequence have been described for many other proteins [19–21].

A carboxyterminal domain of Supt5hp is rich in Ser, Pro and Tyr and contains two C-terminal repeat motifs (CTR). Nine variants of the consensus sequence G-S-R/Q-T-P-X-Y (CTR1) are found and 10 copies of the type P-T/S-P-S-P-Q/A-S/G-Y (CTR2). The yeast Spt5p also contains a C-terminal repeat, yet with a different consensus motif (S-T/A-W-G-G-A/Q). The importance of the repeat domain for the yeast protein

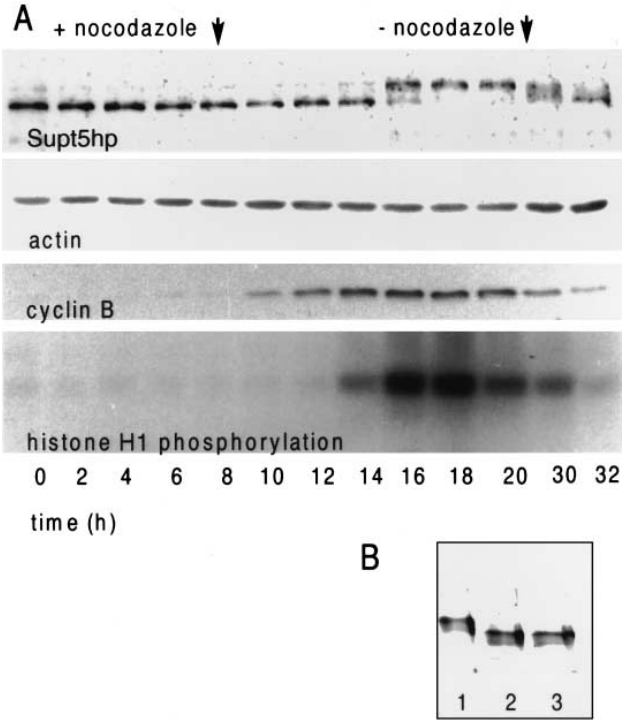

Fig. 5. Phosphorylation of Supt5hp in vivo. HeLa cells were synchronized using a double thymidine block in combination with a subsequent nocodazole block and samples were lysed at the times indicated after release from a second thymidine block. Eight hours after the release, nocodazole was added. At the indicated time just before removal of the 30 h sample, nocodazole was removed. A: Immunoblots. Supt5hp was detected using AS40. Note the change in electrophoretic mobility during mitosis. Equal loading was confirmed by Ponceau S staining and by the immunostaining of actin. As cell cycle markers, cyclin B levels were determined by immunostaining, and histone H1 kinase activity was determined in immunoprecipitates with a commercial antibody specific for the cdc2 kinase (autoradiograph). B: Dephosphorylation of mitotic Supt5hp. Cellular proteins from a sample taken at 8 h (lane 3) and 18 h (lanes 1 and 2) after release from the thymidine block were precipitated with chloroform/methanol and resuspended in calf intestine phosphatase buffer. Samples were incubated in the absence (lanes 1, 3) or presence (lane 2) of alkaline phosphatase and subsequently analyzed in an immunoblot with AS40.

is emphasized by the finding that deletion of the repeats impairs Spt5p function [22]. The second repeat region of Supt5hp is strikingly similar to the repeated motif (S-P-T-S-P-S-Y) found in the largest subunit of RNA polymerase II (accession number: X63564). Transcription is regulated by interaction of positive and negative effectors with this region [23]. Supt5hp in HeLa cells is phosphorylated in vivo during mitosis (Fig. 5A,B). It was shown that yeast Spt4p is involved in kinetochore assembly, which is needed for chromosome transmission in mitosis [24]. If this function is conserved from yeast to man, it is possible that also Supt5hp is involved in kinetochore assembly. Given this, its in vivo phosphorylation at the G2-M border might be functionally relevant. The CTR2 region is a candidate for such phosphorylation, as it is known that the C-terminal domain in RNA polymerase II can be phosphorylated by several protein kinases [25]. Another human amino acid sequence homologous to Spt5p has been published recently [26]. It lacks the second repeat motiv and diverges considerably from the one presented here, that is corroborated by peptide sequences derived from the purified protein.

Supt5hp binds ATP in photolabelling experiments (data not shown). This finding is particularly interesting, as proteins with a function implicated in the modulation of chromatin structure were shown to possess an ATPase activity [27,28]. We do not know yet whether the incomplete DEAD-box motif present in Supt5h protein is involved in ATP-binding by Supt5hp.

As yeast Spt4p and human Supt4hp are functional homologues [11], one may expect a similar functional conservation from yeast to human for Spt5p and Spt6p and their human homologues. Further functional studies will address this issue.

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