The human histone gene expression regulator HBP/SLBP is required for histone and DNA synthesis, cell cycle progression and cell proliferation in mitotic cells

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Summary
Histone proteins are essential for chromatin formation, and histone gene expression is coupled to DNA synthesis. In metazoans, the histone RNA binding protein HBP/SLBP is involved in post-transcriptional control of histone gene expression. In vitro assays have demonstrated that human HBP/SLBP is involved in histone mRNA 3′ end formation and translation. We have inhibited human HBP/SLBP expression by RNA interference to determine its function during the mitotic cell cycle. Inhibition of HBP/SLBP expression resulted in the inhibition of histone gene expression and DNA synthesis, the inhibition of cell cycle progression in S phase and the inhibition of cell proliferation. These findings indicate that human HBP/SLBP is essential for the coordinate synthesis of DNA and histone proteins and is required for progression through the cell division cycle.

Key words: Cell division cycle, Histone gene expression, DNA synthesis

Introduction
Progression through the mitotic cell cycle requires the duplication of DNA and histone protein content during S phase. Histone proteins are required for the packaging of DNA into chromatin. This is essential for chromosome maintenance and replication and the regulation of gene expression. In metazoans, histone proteins are provided by the expression of the multicopy replication-dependent histone genes. The expression of these genes is coordinated with DNA synthesis and is restricted to S phase by the modulation of mRNA levels, resulting in approximately 35- to 50-fold higher levels of histone mRNA during S phase (Schümperli, 1988; Marzluff and Duronio, 2002). The histone hairpin binding protein HBP/SLBP (hereafter referred to as HBP) is involved in the control of histone gene expression (Wang et al., 1996; Martin et al., 1997). HBP is an S-phase protein whose expression is controlled by translational activation of HBP mRNA in late G1 phase and HBP destruction at exit from S phase (Whitfield et al., 2000; Zheng et al., 2003). HBP binds to the highly conserved hairpin structure found in the 3′ untranslated region (3′UTR) of replication-dependent histone mRNA. The hairpin structure is required for histone-specific RNA 3′ end formation, translation and for the regulation of mRNA stability (Müller and Schümperli, 1997).

Biochemical studies have demonstrated that HBP is required for the cell cycle-regulated histone mRNA 3′ end formation. Histone mRNA ends are formed by the cleavage of pre-mRNA 3′ of the hairpin structure by an unknown nuclease. The nuclease is positioned by a complex composed of HBP (Wang et al., 1996; Martin et al., 1997), the U7 snRNP and a Zn-finger protein (reviewed by Müller and Schümperli, 1997; Dominski and Marzluff, 1999; Marzluff and Duronio, 2002). The complex interacts at two sequence elements with the pre-mRNA: (i) the RNA binding protein HBP binds specifically to the conserved histone RNA hairpin structure (Michel et al., 2000; Battle and Doudna, 2001; Zanier et al., 2002) and (ii) U7 snRNP interacts with a sequence element downstream of the hairpin by base-pairing (Schaufele et al., 1986). After mRNA cleavage, HBP remains associated with histone mRNA and stimulates translation (Ling et al., 2002; Sanchez and Marzluff, 2002). It has been proposed that the limitation of HBP expression to S phase is instrumental for the restriction of histone gene expression to S phase (Whitfield et al., 2000; Zheng et al., 2003).

HBP is a single-copy gene in humans, Drosophila melanogaster and Caenorhabditis elegans and is essential for development in Drosophila and C. elegans (Sullivan et al., 2001; Kodama et al., 2002; Pettit et al., 2002). Histone mRNA 3′ end formation is disturbed in Drosophila Hbp mutants, resulting in the inhibition of histone synthesis. Similarly, histone synthesis is inhibited in C. elegans lacking CDL-1, the C. elegans HBP orthologue. Lack of maternal HBP leads to developmental arrest at an early stage in both organisms after several cell cycles. Our knowledge of the function of human HBP is based on studies with cell free systems or is inferred from observations in other model organisms. The biological role of this protein in mitotic cells has not been directly addressed. We present the results of an investigation of human
HBP function during the mitotic cell cycle using RNA interference (RNAi) to inhibit HBP expression. The inhibition of HBP expression resulted in the inhibition of histone gene expression and DNA synthesis, inhibited progression through the cell division cycle and prevented cell proliferation. Our observations demonstrate that HBP is an essential protein required for histone and DNA synthesis and is indispensable for cell cycle progression and cell proliferation.

Materials and Methods

Constructs for RNA interference

The human U6 promoter region from –265 to +1 was PCR amplified from human DNA and inserted into pGEM-T Easy (Promega), resulting in plasmid pGEM-U6P. Constructs expressing shRNAs for RNA interference were derived from pGEM-U6P by PCR using the SP6 primer and a downstream primer encompassing the 3’ end of the U6 promoter, an inverted repeat encoding the shRNA, a TTTTT terminator signal and a MluI restriction site (Paddison et al., 2002). The PCR fragments were inserted into pGEM-T Easy and then either used directly, excised as cassette with MluI, or inserted as MluI fragment into the unique MluI site of pEGFP-C3 (Clontech). The shRNAs HP1, HP2, HP3 and cHP were expressed from the cassettes U6pHP1, U6pHP2, U6pHP3 and U6cHP, respectively. shRNAs HP1, HP2, HP3 target different regions of the HBP open reading frame and cHP was a control derived from HP1 (Fig. 1A). U6pT was used as a further control and contained the U6 promoter followed by the TTTTT terminator sequence.

Cell culture conditions

HeLa cells (a gift from N. Proudfoot, University of Oxford) were grown under standard conditions (5% CO2, 37°C) in DMEM supplemented with 10% fetal calf serum and 100 µg/ml streptomycin (Gibco-BRL).

RNA interference procedure

HeLa cells were seeded 60% confluent and grown until >90% confluent. Transfections were then carried out using lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Transfection efficiency was monitored in parallel transfections with pEGFP-C3 (Clontech). EGFP expression was detected by fluorescence microscopy and was routinely between 50% and 70%. When RNA interference was done with pEGFP-derived plasmids, transfection efficiency was determined directly using EGFP expression as an indicator. Subsequently, cells were diluted 1:3 6-12 hours after transfection, and were then analysed at the indicated times post transfection. For treatment with hydroxyurea, HeLa cells were diluted 12 hours after transfection and then treated with 2 mM hydroxyurea (HU; Sigma) after a further 12 hours. After 16 hours HU treatment cells were washed with fresh medium and released into S phase. For mitotic arrest, cells were treated with 40 ng/ml nocodazole for 12 hours. Mitotic cells were harvested by shake-off, collected by centrifugation, washed once with PBS and released into fresh medium. Cells were transfected 1 hour after release, and washed with PBS 8 hours after release to remove unattached cells.

Antibodies and western blotting

Polyclonal rabbit anti-HBP antibodies were raised against peptide CLTEPLRDFSAMS coupled to KLH. Other antibodies used were mouse anti-tubulin monoclonal antibody (Sigma), rabbit polyclonal anti-cyclin A antibody (606-138; Upstate Biotechnology), Secondary anti-rabbit and anti-mouse antibodies coupled to horseradish peroxidase were purchased from Sigma and Amersham Pharmacia Biotech. For protein analysis, HeLa cells were collected, washed once in phosphate-buffered saline (PBS), and lysed in 1% sodium dodecyl sulphate by boiling for 5 minutes. Lysates were fractionated by SDS-PAGE and transferred onto Hybond-P membrane (Amersham Pharmacia Biotech) by electroblotting. Probing with antibodies was done using standard protocols and detection was by chemiluminescence (ECL, Amersham Pharmacia Biotech). Between probing, blots were stripped using standard protocols. Recombinant HBP (rHBP) was expressed in insect cells using a baculovirus expression system as described previously (Martin et al., 2000).

Northern blotting

Total RNA was prepared using Trizol reagents (Gibco-BRL) according to the manufacturer’s instructions. Normally 10-15 µg total RNA was fractionated by electrophoresis through glyoxal agarose gels and transferred onto Hybond-N membrane (Amersham Pharmacia Biotech) by capillary transfer and UV cross-linked to the membrane. mRNAs were detected by hybridisation with 32P-labelled DNA probes prepared by the random prime labelling method (Roche). Hybridisation was according to standard procedures (Church and Gilbert, 1984). Probes were the human HBP cDNA Neil fragment (Martin et al., 1997), a histone H2BJ-fragment excised with BamHI and HindIII from plasmid H2B-CYFP (a gift from Dr I. Ellenberg, EMBL) and human histone H2AC, H3A, H4A and H1C DNA fragments spanning nucleotides 15-392, 1-380, 57-312, 1-619 of the open reading frame, respectively. The H2AC, H3A, H4A and H1C fragments were amplified by PCR, inserted into pGEM-T Easy and released by restriction with EcoRI. 32P-end-labelled oligonucleotide ACGGATCTGATCGTCTTCGAACC was used to detect 18S rRNA. Between probing, blots were stripped using standard protocols. RNAs were visualised by autoradiography or using a Fujifilm FLA3000 Phosphoimager and analysed using Aida 2.0 software (Raytest GmbH).

Flow cytometry

To analyse cell cycle progression, cells were stained with propidium iodide (Spector et al., 1998). Control samples of asynchronous HeLa cells were used to define ranges for peaks with G1/G0 and G2/M-phase cells, and S-phase cells with intermediate DNA content were between these peaks. Sub-G1 cells were from 0 to the lower boundary of the G0/G1 range. The settings were transferred to the test samples and the percentage of cells in each phase was calculated using CellQuest software and is given as the percentage of the gated population of cells. To characterise DNA synthesis during S phase, DNA synthesis and DNA content were determined by pulse-labelling with BrdU for 30 minutes and by staining with 7-aminoactinomycin D, respectively, using the BrdU Flow Kit according to the instructions of the manufacturer (BD Biosciences). Data analysis was done using CellQuest software. FACS analysis was done with a FACs Calibur Cytometer at the Aberdeen University Flow Cytometry Facility.

Colonoy forming assay

Confluent HeLa cells were transfected with 5 µg DNA in 6-well plates. 12 hours after transfection, cells were detached and resuspended in 8 ml of medium. 0.5 ml of the cell suspension (containing 1.2×10^5 cells) was then seeded in wells of 24-well plates. After further 12 hours, G418 was added to a final concentration of 1.5 mg/ml. At the indicated times, cells were washed with PBS, fixed with ethanol for 2 minutes, stained with 0.2% crystal violet for 2 minutes and washed twice with PBS. 24-well plates were visualised using a Zeiss STEMI-2000-C dissection microscope and images were recorded using Optronics Magnafire-SP equipment (Optronics).
**Results**

The functional characterisation of HBP in vitro has shown that HBP is a central regulator of histone gene expression in metazoans. To investigate the role of HBP during the mitotic cell cycle we prepared constructs for the inhibition of HBP expression by RNAi. We expressed short hairpin RNAs (shRNAs) (Paddison et al., 2002) HP1, HP2 and HP3 that target human HBP mRNA for degradation by RNAi from cassettes composed of the U6 promoter, the sequence coding for the shRNA and an RNA polymerase III terminator sequence. The cassettes were produced by PCR amplification from pGEM-U6P, a pGEM vector containing the U6 RNA promoter and the amplification products were re-inserted into pGEM-T Easy. For the PCR amplification we used the SP6 primer as upstream primer, in combination with downstream primers that overlapped the end of the U6 promoter region and encompassed the shRNA coding sequence followed by a termination signal for RNA polymerase III and a MluI restriction site. For transfection into HeLa cells we used either the pGEM-T Easy derivatives, the cassettes excised with MluI or the MluI fragments inserted into pEGFP-C3. The structure of these cassettes is shown in Fig. 1A. We used several shRNAs to exclude the possibility that our results were due to off-target effects of a particular construct (Jackson et al., 2003; Scacheri et al., 2004) and the regions of the HBP/SLBP mRNA targeted by the shRNAs as well as the shRNA sequences are shown in Fig. 1A. As additional controls we used either the U6 promoter cassette (Up6), a U6 promoter-terminator cassette (U6pT) that lacks any shRNA sequence, or a construct expression cHP, a variant of HP1 that is changed by point mutations at two positions and is therefore not targeting the HBP mRNA any more.

To detect HBP expression we used a polyclonal rabbit anti-HBP serum raised against a C-terminal epitope of human HBP. Fig. 1B shows that the anti-HBP serum recognised endogenous HBP migrating with the mobility of a ~42 kDa protein in HeLa cell extract. Recognition of HBP was specific as this protein was not detected when the incubation with the primary antibody was omitted or when the primary antibody was preincubated with the antigen peptide. Other bands were non-specific as they appeared with all combinations of primary and secondary antibodies. To test for the inhibition of endogenous HBP expression, 90% confluent HeLa cells were subjected to HBP RNAi by transfection with U6pHP1, U6pHP3 or with the controls U6p and U6pT in pGEM, or subjected to a mock transfection procedure without DNA. The cells were then diluted to allow for growth and after 24 hours protein extracts were analysed by probing western blots with anti-HBP serum. Fig. 1C,D shows that HBP levels were clearly reduced in U6pHP1- and U6pHP3-treated cells, compared to mock-transfected and control-transfected cells. As RNAi inhibits gene expression by targeting mRNA for degradation, we

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**Fig. 1.** Inhibition of HBP expression by RNA interference. (A) Top: Schematic drawing showing the location of the shRNA target sequences in the 810 nucleotide HBP open reading frame. Boundaries of sequences coding for domains involved in histone RNA binding (RBD) and RNA processing (RP) and for the putative translation stimulation domain (T) are indicated (Wang et al., 1996; Dominski et al., 1999; Sanchez and Marzluff, 2002). The position of the sequences coding for residues responsible for the control of HBP stability (St) are also shown (Zheng et al., 2003). Note that the drawing is not to scale. Middle: Schematic diagram showing the structure of the RNA interference cassette inserted into the pGEM-T Easy cloning site. Shown are pGEM-T Easy flanking regions, Sp6 RNA polymerase promoter (Sp6), U6 RNA promoter (U6P) and termination signal (TTTTT). U6 promoter and terminator flank the inverted repeat coding for the shRNAs. MluI: restriction sites used for the excision of the cassettes and the subcloning into pEGFP-C3. Bottom: Sequences of the shRNAs used to inhibit HBP expression by RNAi. The control hairpin cHP deviates at two positions (indicated in lower case letters) from HP1. (B) HeLa cell extract was analysed by western blotting using anti-HBP serum. Strips were probed with both primary and secondary antibody, secondary antibody only, or with primary antibody preincubated with the antigenic peptide, and the secondary antibody. The HBP position is marked by the arrow; * marks a major non-specific band with the mobility corresponding to a ~38 kDa protein. The positions of molecular mass standards are indicated. (C) HeLa cells were transfected with either pGEM-U6pHP1 or pGEM-U6p or mock-transfected (no DNA). Protein samples prepared 24 hours after transfection were analysed by western blotting using anti-HBP and subsequently anti-tubulin antibodies as loading control. Blots were stripped between subsequent probings. * as in B. rHBP is recombinant human HBP. (D) HeLa cells were transfected with pGEM-U6pT, pGEM-U6pHP1 or pGEM-U6pHP3 and treated as described for B.
investigated the effect of our treatment on HBP mRNA levels by transfecting HeLa cells with the U6pHP1, U6pHP2 or U6pHP3 cassettes or the control U6pT and U6pcHP cassettes. RNA samples were prepared 24 hours after transfection and analysed by northern blotting. Fig. 2A shows a near twofold reduction of HBP expression in U6pHP1-, U6pHP2- and U6pHP3-treated cells compared to cells treated with the two control cassettes, indicating that the cassettes are sufficient to specifically inhibit HBP expression. As HBP is implicated in S-phase-specific histone gene expression, we also investigated the effect on the expression of histone H3 and found that histone H3 mRNA levels were reduced to a similar extent as HBP expression (Fig. 2A). To investigate this link between HBP expression and histone gene expression in more detail we compared HBP and core histone and linker histone mRNA levels in U6pHP1 and control treated cells. HBP mRNA levels in cells subjected to RNA interference were reduced by ~50% 24 hours and 48 hours after transfection when compared to the mock-transfected or U6pT-transfected controls (Fig. 2B,C). The treatment with U6pHP1 led to a corresponding reduction of histone H2A, H2B, H3, H4 and H1 mRNA levels and was similar to the transfection efficiency in these experiments (60%). We conclude that the expression of HBP is a prerequisite for histone gene expression.

Histone gene expression is required for cell division. To test whether HBP expression is also required for cell proliferation we performed colony-forming assays. The U6pT, U6pcHP, levels in U6pHP1 and control treated cells. HBP mRNA levels in cells subjected to RNA interference were reduced by ~50% 24 hours and 48 hours after transfection when compared to the mock-transfected or U6pT-transfected controls (Fig. 2B,C). The treatment with U6pHP1 led to a corresponding reduction of histone H2A, H2B, H3, H4 and H1 mRNA levels and was similar to the transfection efficiency in these experiments (60%). We conclude that the expression of HBP is a prerequisite for histone gene expression.

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U6pHP1, U6pHP2 and U6pHP3 cassettes were inserted as MluI fragments (Fig. 1A) into the vector pEGFP-C3 at the unique MluI restriction site that lies outside the regions required for neomycin phosphotransferase gene and EGFP expression and DNA replication (Fig. 3A). The resulting plasmids carry the neomycin resistance gene, which allows for the selection of transfected cells using G418. Fig. 3B shows that G418 selection effectively killed mock-transfected cells within 3 days. HeLa cells transfected with pEGFP-Up6T and -U6pcHP, on the other hand, were able to grow and formed clearly visible colonies within 3 days that grew further in the next 4 days, indicating that the introduction of the cassette and the expression of the control hairpin did not prevent cell division. In contrast, treatment with pEGFP-Up6HP1, -U6pHP2 or -U6pHP3 resulted in a pronounced inhibition of proliferation and no colony growth was observed in these cultures. But some expressed in U6pHP1 or Up6T cassettes were similarly arrested in mitosis by treatment with nocodazole. 1 hour after release from nocodazole, cells were transfected with either pGEM-U6pHP1 or pGEM-U6pT or mock transfected. The cell cycle distribution was then analysed 8, 10, 12, 14, 16 and 26 hours after release. Cell cycle progression of transfected cells was identical up to 16 hours and therefore only selected time points are shown in Fig. 4. A substantial proportion of mock-treated cells had entered S phase 16 hours after release. Most cells had gone through mitosis 26 hours after release and were no longer synchronised. Up to the 16-hour time point, cells treated with both U6pHP1 or Up6T cassettes were similarly delayed compared to the mock-treated cells, presumably because of the transfection with DNA. At the final 26-hour time point, the proportion of S-phase and G2/M-phase cells in U6pHP1-treated cells was reduced compared to the U6pT-treated cells, indicating that progression into S phase was inhibited. This was accompanied by an accumulation of sub-G1 cells. Sub-G1 cells were also observed in control-transfected cells, but as the proportion of sub-G1 cells was significantly higher in cells transfected with U6pHP1, we conclude that inhibition of HBP expression led to cell death.

This led us to investigate the effect of inhibition of HBP expression on S-phase progression. Cells were transfected with pGEM-U6pHP1 or pGEM-U6pHP3 or the control plasmids pGEM-U6pT and pGEM-U6cHP and then arrested by addition of hydroxyurea (HU). HU treatment arrests cells at G1/S-phase transition or during S phase and inhibits histone gene expression, but has no adverse effect on HBP

| Time (hours) | sub-G1 | G1/G0 | S   | G2/M |
|--------------|--------|-------|-----|------|
| 8 no DNA     | 4      | 69.7  | 7.4 | 17.0 |
| U6pT         | 4.1    | 61.9  | 9.0 | 22.6 |
| U6pHP1       | 3.6    | 62.1  | 8.4 | 22.3 |
| 16 no DNA    | 7.3    | 55.9  | 25.1| 10.1 |
| U6pT         | 9.0    | 60.5  | 15.8| 13.5 |
| U6pHP1       | 10.3   | 59.5  | 15.9| 12.9 |
| 26 no DNA    | 5.7    | 60.6  | 16.7| 16.8 |
| U6pT         | 9.3    | 35.5  | 33.6| 21.2 |
| U6pHP1       | 29.3   | 37.3  | 26.7| 6.5  |

Fig. 4. Inhibition of HBP expression inhibits cell cycle progression. HeLa cells were arrested by treatment with nocodazole and subsequently released. One hour after release, cells were transfected with either pGEM-U6pT or pGEM-U6pHP1 or mock transfected without DNA. Samples were prepared for FACS analysis by staining with propidium iodide at the indicated times after release. (A) FACS profiles 8, 16 and 26 hours after release from nocodazole block. The boundaries that define cells with sub-G1, G1/G0, S- and G2/M-phase DNA content were determined using asynchronous HeLa cells and are shown in the 8 hours/noDNA samples. Identical settings were used for the analysis of the other samples. (B) Cell cycle distribution. Shown is the proportion of cells with sub-G1, G1/G0, S- and G2/M-phase DNA content. Results are representative of two independent experiments.
expression (Whitfield et al., 2000). Subsequent to the arrest, cells were released and cell cycle progression was monitored. As shown in Fig. 5, RNAi with U6pHP1 and U6pHP3 delayed progression through S phase compared to the two control treatments, and ~1.5 times more cells had still G0/G1-phase DNA content 7 hours after release. To follow cell cycle progression in more detail, we compared the progression of cells transfected with either U6pHP1 or U6pT plasmids at shorter intervals after release from block with HU. Fig. 6 shows that the majority of U6pT-transfected cells were in mid-S phase 3 hours after release and had progressed to late S phase in the following 5 hours. 16 hours after release, the majority of cells had advanced to late S phase, G2/M phase or G0/G1 phase. In comparison, cell cycle progression of most U6pHP1-transfected cells was delayed for up to 5 hours. At 8 hours the majority of cells were approaching mid-S phase, and after 16 hours a significant fraction of cells remained that had S-phase DNA content. During this time we did not observe significant cell death caused by HBP RNAi (data not shown), indicating that the changes in cell cycle progression were not due to HBP-RNAi-mediated cell death. To determine whether the cell cycle progression of U6pHP1-treated cells was associated with a change in HBP expression we tested HBP levels at selected time points. As shown in Fig. 6C, HBP expression was inhibited at all time points tested up to 16 hours, indicating that the cells entered S phase despite a lack of HBP expression. This suggests that inhibition of HBP expression delays, but does not prevent cell cycle progression into S phase in HeLa cells. In addition we investigated the expression of cyclin A as a marker for transition into S phase. Fig. 6C shows that cyclin A expression was equal in the 0-hour samples, but then increased in the control-treated samples, compatible with the progression through S phase observed. After 8 hours, cyclin A levels were similar in U6pHP1- and U6pT-treated cells, and the increase in cyclin A in U6pHP1-treated cells was in agreement with the cell cycle progression observed. Cyclin A is also involved in mitosis. At the 16-hour time point, cyclin A levels in U6pT-treated cells were high, presumably reflecting the high proportion of G2/M phase cells in this sample. Although the cell cycle analysis of HBP RNAi-treated sample revealed that ~50% of cells were in S phase at this time point, cyclin A levels were markedly lower in these cells. The molecular mechanism resulting in the low cyclin A levels in these cells is not clear but we believe that this is linked to an inefficient progression through S phase in HBP RNAi-treated cells. Cyclin A expression and histone gene expression are required for DNA synthesis and are inhibited by HBP RNAi. We therefore decided to test whether HBP expression is required for DNA synthesis. We observed that HBP RNAi resulted in an approximate twofold reduction of DNA synthesis in

![Fig. 5. Inhibition of HBP expression delays S-phase progression. HeLa cells were transfected with either pGEM-U6pHP1, pGEM-U6pHP3 or the control constructs pGEM-U6pcHP and pGEM-U6pT and subsequently arrested by HU treatment as described in Materials and Methods. Samples were prepared for FACS analysis by staining with propidium iodide immediately prior to the release from HU block (0 hours) or 7 hours after release. (A) FACS profiles. The boundaries that define cells with G0/G1-, S- and G2/M-phase DNA content were determined as described in Fig. 4A. (B) Cell cycle distribution. Shown is the percentage of cells with G0/G1-, S- and G2/M-phase DNA content. Results are representative of two independent experiments.](image-url)
HBP/SLBP is required for cell cycle progression

Discussion

We used RNA interference to investigate the role of human HBP during the mitotic cell cycle. We demonstrate that the expression of shRNAs that target HBP mRNA for degradation inhibited HBP expression, and this inhibition resulted in (i) the reduction of histone gene expression, (ii) the inhibition of DNA synthesis; (iii) the inhibition of cell cycle progression during S phase; and (iv) the inhibition of cell proliferation. This indicates that the expression of HBP is required for cell division and is a prerequisite for histone and DNA synthesis, two coordinated processes essential for normal cell cycle progression.

We observed these effects independently with two or three shRNAs targeting different regions of the HBP open reading frame, indicating that the effects are due to the inhibition of HBP expression and not caused by off-target activity of a particular shRNA. As we used transient transfection, our experimental protocol was limited by the transfection efficiency of the cells used. However, we were routinely able

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**Table 1. Inhibition of HBP expression inhibits DNA synthesis during S phase**

| Time (hours) | % Cells synthesising DNA (A) | % Cells with S phase DNA content (B) | A/B * |
|-------------|-----------------------------|-----------------------------------|------|
| 0.5         | U6pHP1 12.2                 | 11.8                             | 1.03 |
|             | U6pT 16.5                    | 17.0                             | 0.97 |
| 2           | U6pHP1 11.1                 | 21.3                             | 0.47 |
|             | U6pT 36.9                    | 37.2                             | 0.99 |
| 4           | U6pHP1 10.0                 | 28.8                             | 0.39 |
|             | U6pT 45.5                    | 46.0                             | 0.99 |
| 6           | U6pHP1 13.1                 | 35.9                             | 0.36 |
|             | U6pT 58.8                    | 59.0                             | 1.00 |
| 8           | U6pHP1 8.1                  | 44.0                             | 0.18 |
|             | U6pT 57.7                    | 58.0                             | 0.99 |
| 12          | U6pHP1 13.6                 | 45.7                             | 0.35 |
|             | U6pT 36.0                    | 38.6                             | 0.93 |
| 16          | U6pHP1 9.0                  | 37.8                             | 0.24 |
|             | U6pT 28.2                    | 28.7                             | 0.98 |

Cells were transfected with either pGEM-U6pT or pGEM-U6pHP1, treated with HU and subsequently released and analysed as described in Materials and Methods. *The A/B ratio is an indicator of DNA synthesis activity in S-phase cells. Results are representative of two independent experiments.
to transfect between 50% and 70% of cells and therefore conclude that the corresponding reductions in histone and HBP mRNA levels, the clear effects on cell cycle progression and the severe inhibition of DNA synthesis observed were caused by the inhibition of HBP expression.

The analysis of cell cycle progression by FACS revealed in some but not all experiments a rapid accumulation of sub-G1 cells, indicating that the inhibition of HBP RNAi can result in cell death (Fig. 4). We also observed sub-G1 cells when incubation time was extended further in experiments using HU to arrest cells. Our data are compatible with cell death being a consequence of the inhibition of cell cycle progression in cells lacking HBP. However, we are currently not sure about the nature of the cell death observed.

Inhibition of HBP expression resulted in the repression of histone gene expression. The mechanism leading to this repression is not known. It can be accounted for by a lack of transcription or a defect in RNA 3′ end formation resulting in mRNA decay in HBP RNAi-treated cells. In yeast, defects in mRNA 3′ end formation destine mRNAs for decay by the nuclear exosome (Hilleren et al., 2001) and there is evidence for the existence of a similar process in mammalian cells (Moore, 2002). However, such a rapid degradation of misprocessed histone mRNA, resulting from the inhibition of HBP expression, would be in contrast to the situation in Drosophila embryos where a lack of HBP has been found to result in longer polyadenylated mRNA that was formed by using a polyadenylation signal downstream of the hairpin for end formation (Lanzotti et al., 2002). Alternatively, it is possible that transcription was inhibited by a feedback mechanism linking histone and DNA synthesis. Such a feedback mechanism was first proposed to account for reduction of histone mRNA when cells cease DNA synthesis (Butler and Mueller, 1973; Sariban et al., 1985; Peltz and Ross, 1987). Finally, our data do not exclude the formal possibility that HBP is required for cell cycle progression in late G1 phase, independent of histone gene expression.

Our experiments demonstrate a stringent requirement of cell cycle progression for HBP expression. HBP RNAi inhibited cell cycle progression into and through S phase (Figs 4–6) and resulted in a severe reduction of DNA synthesis (Table 1). This is in contrast to the more relaxed requirement for HBP during the early development of Drosophila and C. elegans where several cell cycles occurred in the absence of HBP (Sullivan et al., 2001; Kodama et al., 2002; Pettitt et al., 2002). However, the cell divisions in these animal models were not normal and nuclei were enlarged, compatible with a decondensation of chromatin caused by the lack of histones. It is probable that these cell cycles occurred because of a relaxed cell cycle control during early development of the model animals. In contrast, in mitotic cells the requirement for HBP, presumably in its role as regulator of histone gene expression, is such that cell cycle progression is immediately affected, reflecting the more stringent control of cell cycle progression in mitotic cells.

Coupling of DNA and histone synthesis has been previously reported, but the molecular links between these two processes are not well understood. It is well documented that the inhibition of DNA synthesis results in a rapid inhibition of histone gene expression (Heintz et al., 1983; Graves and Marzluff, 1984; Baumbach et al., 1984). This led to the proposal of an auto-regulatory feedback mechanism that controls histone mRNA abundance in response to excess histones that accumulate when cells stop DNA synthesis (Butler and Mueller, 1973; Heintz et al., 1983; Sariban et al., 1985; Peltz and Ross, 1987). More recently, it was demonstrated that the inhibition of DNA synthesis due to ionising irradiation resulted in the inhibition of histone gene expression in a p53/p21-dependent manner (Su et al., 2004). However, the requirement of DNA synthesis for histone gene expression has been more difficult to explore. Recent work has demonstrated that the transcription activator of histone genes NPAT/p220 is required for the transition from G1 into S phase (Wei et al., 2003; Ye et al., 2003). Furthermore, the inhibition of histone gene transcription by overexpression of the repressor HIRA resulted in the inhibition of DNA synthesis (Nelson et al., 2002). This inhibition was overcome when histone mRNA levels were increased by the overexpression of HBP (Nelson et al., 2002). Our work demonstrates that HBP expression is a prerequisite for both histone and DNA synthesis. It identifies a new link between the machinery controlling histone gene expression and DNA synthesis and future experiments will address the nature of the molecular mechanisms linking these two processes. In conclusion, we have demonstrated that human HBP is an essential protein required for histone gene expression, DNA synthesis, cell cycle progression and cell proliferation.

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References

Battle, D. J. and Doudna, J. A. (2001). The stem-loop binding protein forms a highly stable and specific complex with the 3′ stem-loop of histone mRNAs. RNA 7, 123-132.

Baumbach, L. L., Marashi, F., Plumb, M., Stein, G. and Stein, J. (1984). Inhibition of DNA replication coordinately reduces cellular levels of core and H1 histone mRNAs: requirement for protein synthesis. Biochemistry 23, 1618-1625.

Butler, W. B. and Mueller, G. C. (1973). Control of histone synthesis in HeLa cells. Biochin. Biophys. Acta 294, 481-496.

Church, G. M. and Gilbert, W. (1984). Genomic sequencing. Proc. Natl Acad. Sci. USA 81, 1991-1995.

Dominski, Z. and Marzluff, W. F. (1999). Formation of the 3′ end of histone mRNA. Gene 239, 1-14.

Dominski, Z., Zheng, L. X., Sanchez, R. and Marzluff, W. F. (1999). Stem-loop binding protein facilitates 3′-end formation by stabilizing U7 snRNP binding to histone pre-mRNAs. Mol. Cell. Biol. 19, 3561-3570.

Graves, R. A. and Marzluff, W. F. (1984). Rapid reversible changes in the rate of histone gene transcription and histone mRNA levels in mouse myeloma cells. Mol. Cell. Biol. 4, 351-357.

Heintz, N., Szce, H. L. and Roeder, R. G. (1983). Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. Mol. Cell. Biol. 3, 539-550.

Hilleren, P., McCarthy, T., Roshalm, M., Parker, R. and Jensen, T. H. (2001). Quality control of mRNA 3′-end processing is linked to the nuclear exosome. Nature 413, 538-542.

Jackson, A. L., Bartz, S. R., Schelt, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P. S. (2003). Expression profiling reveals off-target gene regulation by RNAi. Nat. Biotechnol. 21, 615-637.

Kodama, Y., Rothman, H. J., Sugimoto, A. and Yamamoto, M. (2002). The stem-loop binding protein CDL-1 is required for chromosome condensation, progression of cell death and morphogenesis in Caenorhabditis elegans. Development 129, 187-196.
Lanzotti, D. J., Kaygun, H., Yang, X., Duronio, R. J. and Marzluff, W. F. (2002). Developmental control of histone mRNA and dSLBP synthesis during Drosophila embryogenesis and the role of dSLBP in histone mRNA 3′ end processing in vivo. *Mol. Cell. Biol.* 22, 2267-2282.

Ling, J., Morley, S. J., Pain, V. M., Marzluff, W. F. and Gallie, D. R. (2002). The histone 3′-terminal stem-loop-binding protein enhances translation through a functional and physical interaction with eukaryotic initiation factor 4G (eIF4G) and eIF3. *Mol. Cell. Biol.* 22, 7853-7867.

Martin, F., Schaller, A., Eglite, S., Schümperli, D. and Müller, B. (1997). The gene for histone RNA hairpin-binding protein is located on human chromosome 4 and encodes a novel type of RNA binding protein. *EMBO J.* 16, 769-778.

Martin, F., Michel, F., Zenklusen, D., Müller, B. and Schümperli, D. (2000). Positive and negative mutant selection in the human histone hairpin-binding protein using the yeast three-hybrid system. *Nucleic Acids Res.* 28, 1594-1603.

Marzluff, W. F. and Duronio, R. J. (2002). Histone mRNA expression: multiple levels of cell cycle regulation and important developmental consequences. *Curr. Opin. Cell Biol.* 14, 692-699.

Michel, F., Schümperli, D. and Müller, B. (2000). Specificities of Caenorhabditis elegans and human hairpin binding proteins for the first nucleotide in the histone mRNA hairloop. *RNA* 6, 1539-1550.

Moore, M. J. (2002). Nuclear RNA turnover. *Cell* 108, 431-434.

Müller, B. and Schümperli, D. (1997). The U7 snRNP and the hairpin-binding protein: key players in histone mRNA metabolism. *Semin. Cell Dev. Biol.* 8, 567-576.

Nelson, D. M., Ye, X., Hall, C., Santos, H., Ma, T., Kao, G. D., Yen, T. J., Harper, J. W. and Adams, P. D. (2002). Coupling of DNA synthesis and histone synthesis in S phase independent of cyclin/cdk2 activity. *Mol. Cell. Biol.* 22, 7459-7472.

Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J. and Conklin, D. S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16, 948-958.

Peltz, S. W. and Ross, J. (1987). Autogenous regulation of histone mRNA decay by histone proteins in a cell-free system. *Mol. Cell. Biol.* 7, 4345-4356.

Pettitt, J., Crombie, C., Schümperli, D. and Müller, B. (2002). The Caenorhabditis elegans histone hairpin-binding protein is required for core histone gene expression and is essential for embryonic and postembryonic cell division. *J. Cell Sci.* 115, 857-866.

Sanchez, R. and Marzluff, W. F. (2002). The stem-loop binding protein is required for efficient translation of histone mRNA in vivo and in vitro. *Mol. Cell. Biol.* 22, 7093-7104.

Sariban, E., Wu, R. S., Erickson, L. C. and Bonner, W. M. (1985). Interrelationships of protein and DNA syntheses during replication of mammalian cells. *Mol. Cell. Biol.* 5, 1279-1286.

Scacheri, P. C., Rosenblatt-Rosen, O., Caplen, N. J., Wolfsberg, T. G., Umayam, L., Lee, J. C., Hughes, C. M., Shannugam, K. S., Bhattacharjee, A., Meyerson, M. et al. (2004). Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc. Natl. Acad. Sci. USA* 101, 1892-1897.

Schaufele, F., Gilmartin, G. M., Bannwarth, W. and Birnstiel, M. L. (1986). Compensatory mutations suggest that base-pairing with a small nuclear RNA is required to form the 3′ end of H3 messenger RNA. *Nature* 323, 777-781.

Schümperli, D. (1988). Multilevel regulation of replication-dependent histone genes. *Trends Genet.* 4, 187-191.

Spector, D. L., Goldman, R. D. and Leinwand, L. A. (1998). *Cells – A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Su, C., Gao, G., Schneider, S., Helt, C., Weiss, C., O’Reilly, M. A., Bohmann, D. and Zhao, J. (2004). DNA damage induces downregulation of histone gene expression through the G(1) checkpoint pathway. *EMBO J.* 23, 1133-1143.

Sullivan, E., Santiago, C., Parker, E. D., Dominiski, Z., Yang, X., Lanzotti, D. J., Ingledue, T. C., Marzluff, W. F. and Duronio, R. J. (2001). Drosophila stem loop binding protein coordinates accumulation of mature histone mRNA with cell cycle progression. *Genes Dev.* 15, 173-187.

Wang, Z. F., Whitfield, M. L., Ingledue, T. C., III, Dominiski, Z. and Marzluff, W. F. (1996). The protein that binds the 3′ end of histone mRNA: a novel RNA-binding protein required for histone pre-mRNA processing. *Genes Dev.* 10, 3028-3040.

Wei, Y., Jin, J. and Harper, J. W. (2003). The cyclin E/Cdk2 substrate and Cajal body component p220(NPAT) activates histone transcription through a novel LisH-like domain. *Mol. Cell. Biol.* 23, 3669-3680.

Whitfield, M. L., Zheng, L., Baldwin, A., Ohta, T., Hurt, M. R. and Marzluff, W. F. (2000). Stem-loop binding protein, the protein that binds the 3′ end of histone mRNA, is cell cycle regulated by both translational and posttranslational mechanisms. *Mol. Cell. Biol.* 20, 4188-4198.

Ye, X., Wei, Y., Nalepa, G. and Harper, J. W. (2003). The cyclin E/Cdk2 substrate p220(NPAT) is required for S-phase entry, histone gene expression, and Cajal body maintenance in human somatic cells. *Mol. Cell. Biol.* 23, 8586-8600.

Zanier, K., Luyten, I., Crombie, C., Müller, B., Schümperli, D., Linge, J. P., Nilges, M. and Sattler, M. (2002). Structure of the histone mRNA hairpin required for cell cycle regulation of histone gene expression. *RNA* 8, 29-46.

Zheng, L., Dominiski, Z., Yang, X. C., Elms, P., Raska, C. S., Borchers, C. H. and Marzluff, W. F. (2003). Phosphorylation of stem-loop binding protein (SLBP) on two threonines triggers degradation of SLBP, the sole cell cycle-regulated factor required for regulation of histone mRNA processing, at the end of S phase. *Mol. Cell. Biol.* 23, 1590-1601.