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ZFP100, a Component of the Active U7 snRNP Limiting for Histone Pre-mRNA Processing, Is Required for Entry into S Phase

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Metazoan replication-dependent histone mRNAs are the only eukaryotic mRNAs that are not polyadenylated. The cleavage of histone pre-mRNA to form the unique 3′ end requires the U7 snRNP and the stem-loop binding protein (SLBP) that binds the 3′ end of histone mRNA. U7 snRNP contains three novel proteins, Lsm10 and Lsm11, which are part of the core U7 Sm complex, and ZFP100, a Zn finger protein that helps stabilize binding of the U7 snRNP to the histone pre-mRNA by interacting with the SLBP/pre-mRNA complex. Using a reporter gene that encodes a green fluorescent protein mRNA ending in a histone 3′ end and mimics histone gene expression, we demonstrate that ZFP100 is the limiting factor for histone pre-mRNA processing in vivo. The overexpression of Lsm10 and Lsm11 increases the cellular levels of U7 snRNP but has no effect on histone pre-mRNA processing, while increasing the amount of ZFP100 increases histone pre-mRNA processing but has no effect on U7 snRNP levels. We also show that knocking down the known components of U7 snRNP by RNA interference results in a reduction in cell growth and an unsuspected cell cycle arrest in early G_1, suggesting that active U7 snRNP is necessary to allow progression through G_1 phase to S phase.

The synthesis of histone proteins is tightly coupled to DNA replication to ensure that the histone protein production will be sufficient to assemble newly replicated DNA into chromatin. In metazoans, the temporal control over histone protein production is achieved primarily through the modulation of the replication-dependent histone mRNA levels (26, 27). The regulation of histone mRNA occurs at multiple steps, including an increase in the rate of histone gene transcription at the beginning of S phase as well as a decrease in the half-life of histone mRNA at the end of S phase. However, the primary engine that drives the levels of histone mRNA to an S-phase level 35-fold higher than that in G_1 or G_2 phase is an increase in the efficiency of histone pre-mRNA processing during S phase (19, 46). Histone mRNAs end in a conserved stem-loop (SL) structure rather than a poly(A) tail. The 3′ end is a critical element for multiple steps in histone mRNA metabolism, including the translation (12, 32) and control of the degradation of the mRNA (29). The SL is not only required for each of these steps in mRNA metabolism but also sufficient to confer cell cycle regulation on a heterologous mRNA (19, 35).

The 3′ end of histone mRNA is formed by an endonucleolytic cleavage of the nascent histone pre-mRNA between the SL bound to SL binding protein (SLBP) and a second *cis* element called the histone downstream element (HDE) (Fig. 1) (13, 28). The HDE interacts with U7 snRNP by base pairing with the 5′ end of the U7 snRNA (4, 6, 33). The U7 snRNP is present at very low levels (500 to 1,000 molecules/HeLa cell compared to 5×10^5 to 5×10^6 molecules/cell for spliceosomal snRNPs), contains a noncanonical Sm binding site (18), and is known to function only in the processing of histone pre-mRNAs. The U7 snRNP contains five Sm proteins common to the spliceosomal snRNPs; however, in lieu of Sm D1 and D2, it contains two Sm-like proteins, Lsm10 and Lsm11 (30, 31). In addition, it contains the ZFP100 protein, which interacts with the SLBP/SL complex (7) and Lsm11 (30, 40). Cleavage requires a factor that contains CPSF-73 (9) and several other factors required for polyadenylation (22). SLBP levels oscillate throughout the cell cycle; SLBP is expressed just prior to entry into S phase, allowing the synthesis of the histone mRNA, and SLBP is degraded at the end of S phase (43, 46).

Here we report that ZFP100 is a limiting component for histone pre-mRNA processing in mammalian cells and that increased expression of ZFP100, but not of the U7 snRNP proteins Lsm10 and Lsm11 (30, 31), stimulates the expression of a novel green fluorescent protein (GFP)-based reporter containing the *cis*-acting sequences necessary for histone mRNA production. We also demonstrate that RNA interference (RNAi)-mediated knockdown of ZFP100 leads to a cell cycle arrest early in G_1 phase and that knockdown of the U7 snRNP proteins Lsm10 and Lsm11 also results in an accumulation of cells in early G_1 phase. This phenotype contrasts with the delayed progression through S phase that results from SLBP knockdown (38, 45) and suggests that the cell monitors the availability of active U7 snRNP prior to committing to enter S phase.

MATERIALS AND METHODS

Plasmid construction. The GFP-SL reporter clone was constructed by cloning the H2A-614 promoter in place of the cytomegalovirus (CMV) promoter of the pEGFPd4-N1 plasmid (Clontech, Mountain View, CA) and replacing the simian virus 40 poly(A) site with the H2A-614 SL and HDE. myc-tagged ZFP100 clones were made by the insertion of PCR-generated ZFP100 fragments cloned into the KpnI and ApaI sites of pcDNA4-TO-HisB (Invitrogen, Carlsbad, CA). All clones were sequenced, and the sequences of all primers are available upon request.

Cell culture and transfection. HEK 293T, HeLa, and U2OS cells were cultured using standard techniques with Dulbecco's modified Eagle's low-glucose medium supplemented with 10% fetal bovine serum. 293T cells were transfected

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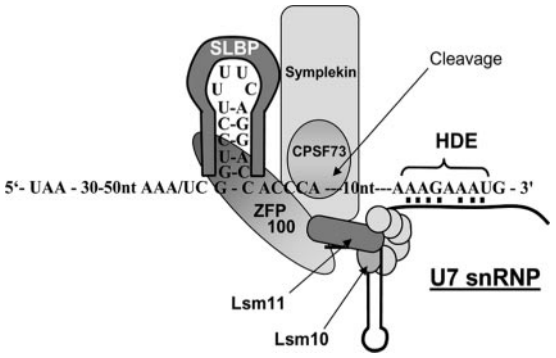

FIG. 1. Histone pre-mRNA processing. The two *cis* elements required for processing in the histone pre-mRNA are the SL and the HDE, which are bound by the SLBP and U7 snRNP, respectively. The three known unique components of the U7 snRNP, Lsm10, Lsm11, and ZFP100, are shown. A cleavage factor containing CPSF-73 and symplekin as well as other polypeptides is then recruited to catalyze the cleavage (9, 22). nt, nucleotide.

with plasmid using either Lipofectamine or Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Short interfering RNA (siRNA) transfections were performed using a modified version of a two-hit protocol previously published elsewhere (39).

Immunoblotting. 293T or HeLa cells transfected with myc-tagged ZFP100 were harvested by incubation with hot (approximately 80°C) sodium dodecyl sulfate (SDS) loading buffer, followed by five passes through a 22-gauge needle. Lysates were resolved by SDS-polyacrylamide gel electrophoresis and blotted with anti-myc monoclonal antibody (Invitrogen) at a dilution of 1:1,000. Lysates from U2OS cells were prepared by incubation with NP-40 lysis buffer (100 mM Tris [pH 8.3], 0.5% NP-40, 100 mM NaCl, 100 mM phenylmethylsulfonyl fluoride). Blots were probed with a 1:1,000 dilution of polyclonal antiserum raised against SLBP (41).

Northern blot analysis. Approximately 2 μg of total cell RNA was resolved on a 1.5% agarose gel containing both formaldehyde and MOPS (morpholinepropanesulfonic acid), followed by electrophoretic transfer to a HybondN nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was UV cross-linked and probed with a random-labeled PCR probe to H2A-614 open reading frame (ORF), 7SK, or GFP ORF overnight at 60°C, according to the instructions for the QuikHyb blotting agent (Stratagene, La Jolla, CA). U7 snRNA was detected as previously described (10).

RT-PCR. Total cellular RNA was isolated using TRIzol reagent. RNA was subject to reverse transcription (RT) using the manufacturer's protocols (New England Biolabs, Natick, MA) using random hexamer primers. Approximately 1/10 of the RT reaction was used as a template for PCR. The primers used were 5′-GAGACCAAGAACTCTCCTCTG-3′ and 5′-TCGCCTAGCAAACTATCT AAC-3′ for ZFP100, 5′-AGACCCGAGAGCTTTACCACT-3′ and 5′-AGGTG TCTTGGGACTTCTTTA-3′ for SLBP, 5′-GAATACGCATACTTTAGAAA T-3′ and 5′-CTCATTAGCTGTCCTGCATGC-3′ for h3′Exo, 5′-TGCGGGAT GAGAGCGTGGCCC-3′ and 5′-TGGATAATCTGCAGCTGCTGC-3′ for Lsm10, and 5′-GTGAACTCCATCGCTGTATC-3′ and 5′-ATGCTTTGCCTA GGACAGGT-3′ for Lsm11. Simultaneous PCRs for SLBP and 3′hExo were carried out for 27 cycles (see Fig. 5C, left panels), simultaneous PCRs for ZFP100 and 3′hExo were carried out for 35 cycles (see Fig. 5C, right panels), and the same RT was subject to PCR for either SLBP, h3′Exo, or Lsm10/11 (see Fig. 6B and D). PCRs were removed at 27 cycles for SLBP, 35 cycles for h3′Exo, and 36 cycles for Lsm11, and Lsm10 was removed at 42 cycles. In all cases, multiple cycle numbers were analyzed to ensure a linear range was analyzed, and in no cases were there products in the absence of reverse transcriptase.

Cell cycle analysis and fluorescence-activated cell sorter (FACS) analysis. Two days following the second siRNA transfection, U2OS cells were harvested using trypsin and fixed in 70% ethanol at −80°C. Cell cycle analysis was carried out as previously described (38). 293T cells analyzed for GFP fluorescence were harvested using trypsin and then added to 37% formaldehyde to achieve a final concentration of 0.5%. Cell fluorescence was quantified using a FACScan machine, and the data were analyzed using Summit.

RESULTS

The only processing reaction necessary to form mature histone mRNA is an endonucleolytic cleavage of the histone pre-mRNA to form the 3′ end of histone mRNA. There are three known factors required for processing diagrammed in Fig. 1: SLBP, which binds the stem loop, U7 snRNP, which base pairs with the HDE, and a cleavage factor that contains CPSF-73 (9) and several other factors required for polyadenylation (22). Here we demonstrate that ZFP100, a U7 snRNP protein that interacts with the SLBP/pre-mRNA complex (7), is a limiting factor for histone pre-mRNA processing and is required for entry into S phase.

A GFP histone stem-loop reporter gene. To readily visualize the effects on histone mRNA expression, we constructed a reporter gene encoding destabilized enhanced green fluorescent protein (EGFP) (4-h half-life) (23) to visualize and quantify the amount of expression of a gene encoding a histone-like mRNA within a mammalian cell. The GFP-SL gene contains a histone promoter, the destabilized GFP open reading frame, and a histone 3′ end located at the same position relative to the stop codon as in histone mRNAs (Fig. 2A). As a control, we used a GFP-pA gene that contains a CMV promoter, a GFP open reading frame, and the simian virus 40 early mRNA polyadenylation signal (Fig. 2A). Wild-type GFP is quite stable in cells having a half-life of about 26 h (5), whereas the destabilized GFP (half-life, 4 h) is advantageous as it provides a rapidly changing readout since the levels of histone mRNAs are rapidly regulated during the cell cycle (19).

To establish that the GFP-SL reporter mimicked endogenous histone mRNAs, we tested two criteria characteristic of histone mRNA: restriction of expression to S phase and degradation of histone mRNAs when DNA replication is inhibited. To determine whether the GFP-SL reporter was expressed primarily during S phase, we used two different techniques to mark S-phase cells: cotransfection of cells with a DsRed-SLBP fusion protein or labeling with bromodeoxyuridine (BrdU). SLBP is present in only S-phase cells and is rapidly degraded at the end of S phase (11, 43). We cotransfected either the GFP-SL reporter or the GFP-pA reporter, together with a gene expressing the DsRed-SLBP fusion protein into 293T cells, and assayed the expression using fluorescence microscopy (Fig. 2A and B, upper panels). The GFP-pA reporter was expressed efficiently, exhibiting high fluorescence in a large fraction of cells (Fig. 2A). The degree of expression was relatively constant from cell to cell. The GFP-pA reporter was highly expressed in many cells that were not expressing DsRed-SLBP (Fig. 2A, top row), consistent with this reporter not being cell cycle regulated. The GFP-SL reporter, in contrast, was expressed in far fewer cells. Importantly, all the cells expressing the DsRed-SLBP also expressed the GFP-SL reporter (Fig. 2B, top row). There was more-variable intensity of the GFP-SL reporter among different S-phase cells. This variability is likely due to the amount of time these cells have been in S phase. A fraction of the cells expressed the GFP-SL reporter but not DsRed-SLBP, and these cells are likely those that recently completed S phase and have entered G_2. The destabilized GFP used in these experiments has a 4-h half-life, and thus, the protein will persist into G_2 (with expression

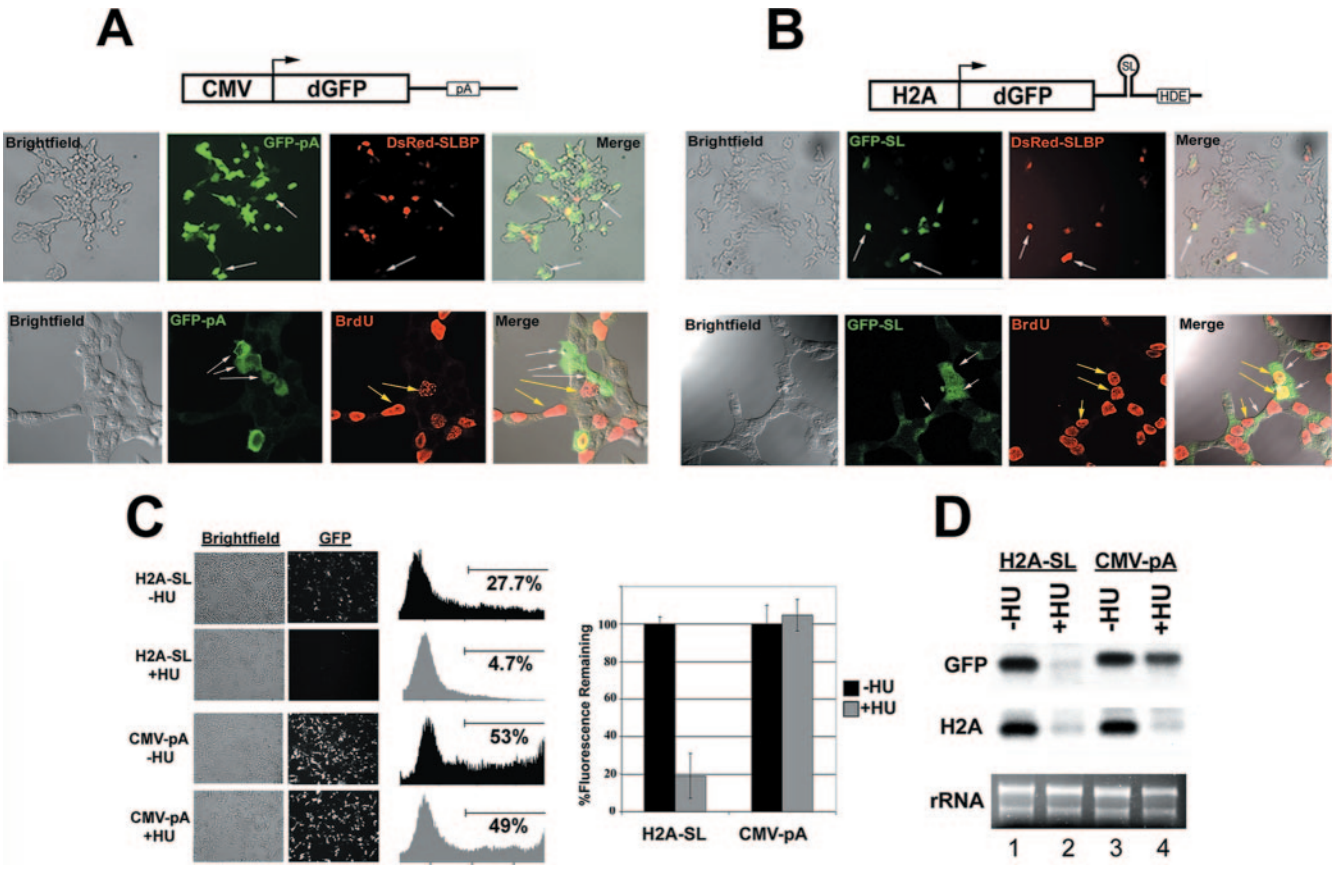

FIG. 2. The GFP-SL reporter gene mimics the expression of histone mRNA. (A) A schematic of the GFP-pA reporter (top). The top row of images is fluorescence microscopy of 293T cells cotransfected with the GFP-pA reporter, together with a plasmid expressing a DsRed-SLBP fusion protein. The white arrows indicate cells that express GFP and not DsRed-SLBP. The second row of images shows confocal microscopy of 293T cells transfected with the GFP-pA reporter and labeled with BrdU. The white arrows indicate cells that express GFP, and the yellow arrows denote BrdU-positive nuclei. (B) A schematic of the GFP-SL reporter (top). The top row of images is fluorescence microscopy of 293T cells cotransfected with the GFP-SL reporter, together with a plasmid expressing a DsRed-SLBP fusion protein. The white arrows indicate cells that express GFP and are also expressing DsRed-SLBP. The second row of images is confocal microscopy of 293T cells transfected with the GFP-SL reporter and labeled with BrdU. The white arrows indicate cells that express GFP, and the yellow arrows denote BrdU-positive nuclei. (C) 293T cells were transfected with the indicated reporter, and then 5 mM HU was added for 8 h. The left panels are the bright-field and fluorescence images. The FACS analysis of the cells detecting the amount of GFP fluorescence is shown in the middle panel, with the percentage of cells expressing a gated value of GFP denoted. The results were quantified by measuring total fluorescence. The *x* axis represents relative GFP units as assayed by the FACScan, and cell number is on the *y* axis. The percentage of cells expressing GFP and not treated with HU was set at 100%, and the others were quantified as a percentage of that value. The error bars on the graph on the right represent the standard deviations from three independent experiments. +, with; −, without. (D) The levels of the reporter mRNA and the endogenous histone H2a mRNA were determined by Northern blotting (top) of the cells analyzed in panel C. At the bottom is an analysis of total RNA resolved by agarose gel electrophoresis and detected by staining with ethidium bromide. +, with; −, without.

decreasing), whereas the DsRed-SLBP fusion protein is destroyed at the end of S phase.

To further confirm that the GFP-SL reporter was selectively expressed in S-phase cells, we also transfected either the GFP-pA or the GFP-SL reporter into 293T cells and then pulsed them with BrdU for 15 min. In the case of the GFP-pA reporter, many cells that were positive for the reporter were negative for BrdU incorporation (Fig. 2A, lower panel), whereas cells that were positive for the GFP-SL reporter were, in most cases, BrdU positive (Fig. 2B, lower panel). These two assays both indicate that the GFP-SL reporter expression is specifically enriched in S-phase cells.

Histone mRNAs are rapidly degraded when cells are treated with DNA synthesis inhibitors, such as hydroxyurea (HU) (17), and degradation is directed by the stem-loop (29). We treated cells transfected with each reporter with HU. Eight hours after HU treatment, there was a marked decrease in the fluorescence of the GFP-SL reporter as visualized by fluorescence microscopy (Fig. 2C, left panels), whereas there was no detectable change in the intensity of fluorescence from the GFP-pA reporter. To quantify the expression of the destabilized green fluorescent protein (dGFP), we used FACS analysis. This gives both a readout of the percentage of cells expressing dGFP and the average intensity of expression in individual cells. After HU treatment, there was a fivefold decrease in the average GFP intensity as well as a sixfold decrease in the percentage of cells residing within the gated value of GFP in GFP-SL reporter-transfected cells (Fig. 2C). There was no significant difference in the intensity of GFP fluorescence or in the percentage of cells residing within the gated value of GFP in the GFP-pA

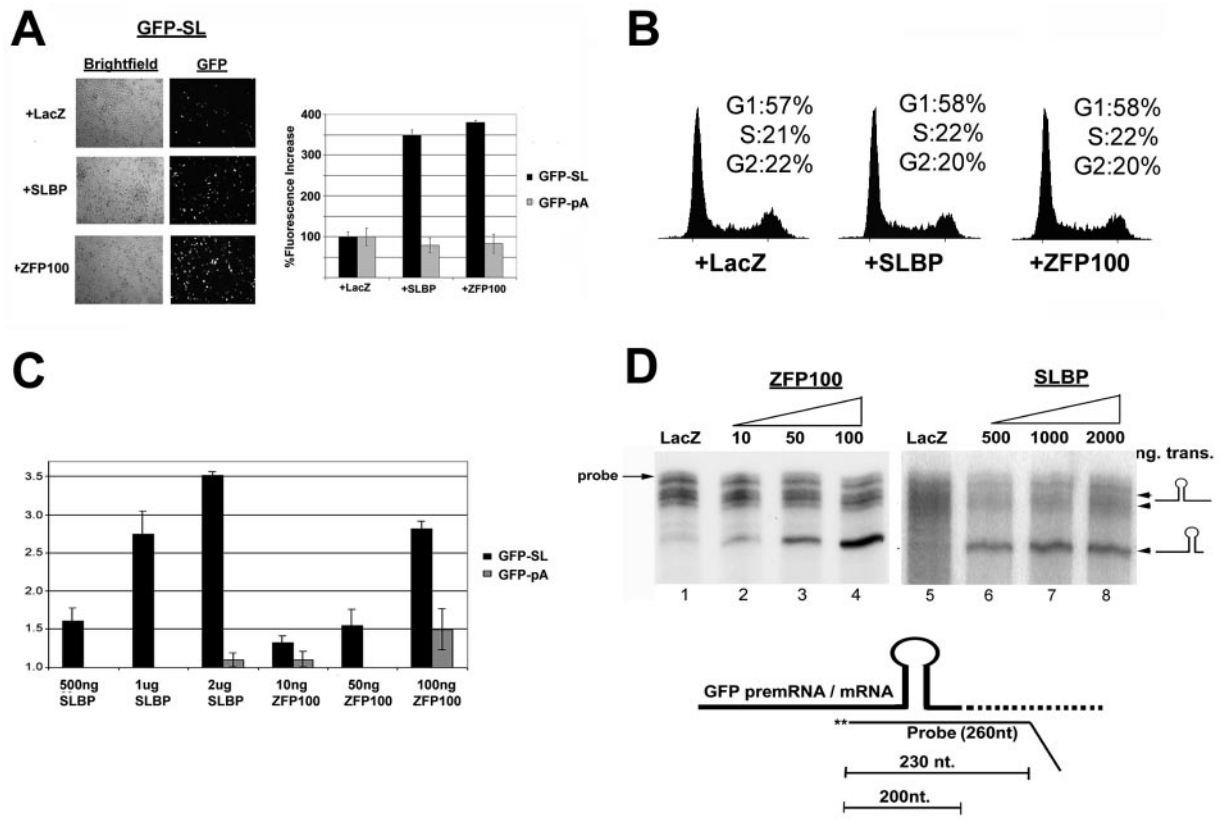

FIG. 3. Expression of ZFP100 or SLBP stimulates expression of the GFP-SL reporter. (A) The left panel represents brightfield and fluorescence images of the different cells transfected with H2A-dGFP-SL and the myc-tagged lacZ, SLBP, or ZFP100 for 24 h, followed by the transfection of the GFP-SL reporter. The graph on the right is the quantification of FACS analysis of the cells transfected with either GFP-pA or GFP-SL cells, together with (+) lacZ, SLBP, or ZFP100. Error bars represent standard deviations of a triplicate set of transfections. (B) Cells transfected with (+) myc-tagged lacZ, SLBP, or ZFP100 for 48 h were analyzed for cell cycle distribution by flow cytometry. (C) Cells were transfected with plasmids encoding either SLBP, ZFP100, or LacZ as a control at the doses labeled at the bottom of the graph. Twenty-four hours later, 2 μg of either GFP-SL or GFP-pA reporter plasmid was transfected into each well. The next day, cells were harvested and analyzed by FACS analysis to determine relative fluorescence level. The data are presented as severalfold increases in fluorescence over cells transfected with LacZ, and error bars are representative of a triplicate set of transfections. A transfection of more than 1 μg of ZFP100 plasmid was toxic to the cells (40). (D) Total cell RNA (5 μg) from a representative experiment similar to that in panel A with the cells transfected (trans.) with the GFP-SL gene was analyzed using an S1 protection assay that can detect both transcripts that end at the stem-loop and readthrough transcripts. Probe design is depicted in the schematic below, with the anticipated protected fragments labeled. nt, nucleotide. The position of the probe on the gel is indicated. Protected fragments longer than the stem-loop (arrowheads at right) represent readthrough transcripts. Lanes 1 and 5 are cells transfected with LacZ, lanes 2 to 4 are cells transfected with increasing amounts of ZFP100 plasmid, and lanes 6 to 8 are cells transfected with increasing amounts of SLBP plasmid. The amounts of plasmid are indicated above each lane.

reporter-transfected cells with and without HU treatment (Fig. 2C, right panel). Similarly, the RNA from the GFP-SL reporter was downregulated in response to hydroxyurea treatment to the same extent as was the endogenous histone H2A mRNA (Fig. 2D, lane 1 versus lane 2), whereas the GFP-pA-derived mRNA was unaffected (Fig. 2D, lane 3 versus lane 4). The combination of an S-phase enrichment of cells expressing the GFP-SL reporter coupled with the sensitivity to HU treatment allowed us to conclude that this reporter recapitulates endogenous histone mRNA expression in 293T cells.

Expression of SLBP or ZFP100 increases expression of the dGFP reporter. Increasing the concentration of any factors limiting for histone mRNA expression, which may include either or both 3′ end processing or translation, will likely increase the amount of GFP expressed from the GFP-SL gene. To test which factors might be limiting for expression, we cotransfected GFP-SL or GFP-pA, together with hemagglutinin-SLBP, myc-ZFP100, myc-Lsm10, and/or myc-Lsm11 or with myc-LacZ as a control (Fig. 3 and 4). The transfection of either SLBP or ZFP100 increased the intensity of fluorescence but gave only a slight increase in the number of fluorescing cells, while the transfection of myc-LacZ had no effect (Fig. 3A, left panels). This increase in fluorescence was not caused by an increase in the number of S-phase cells since cell cycle analysis revealed there was no significant change in cell cycle distribution (Fig. 3B). A quantification of this increase by FACS analysis demonstrated that the expression of SLBP increased the intensity of fluorescence threefold, whereas the expression of ZFP100 increased the fluorescence nearly fourfold (Fig. 3A, right panel). Neither SLBP nor ZFP100 had any effect on the expression of the GFP-pA reporter (Fig. 3A, right panel). The increase in fluorescence was dependent on the amount of SLBP or ZFP100 introduced (Fig. 3C). Note that

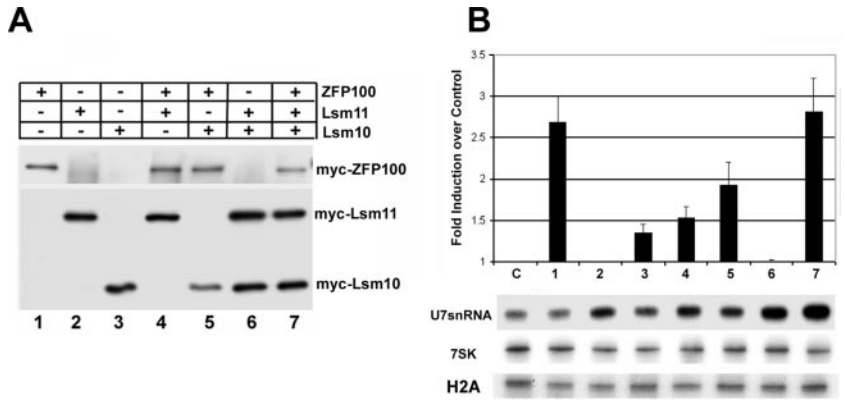

FIG. 4. Expression of Lsm10 and/or Lsm11 does not affect expression of the GFP-SL reporter. (A) Cells were transfected with myc-tagged ZFP100 (lane 1), Lsm11 (lane 2), Lsm10 (lane 3), or combinations of ZFP100 and Lsm11 (lane 4), ZFP100 and Lsm10 (lane 5), Lsm10 and Lsm11 (lane 6) or all three proteins (lane 7). Extracts from equal amounts of cells were resolved by gel electrophoresis and transferred to nitrocellulose, and the expressed proteins were detected with an anti-myc antibody. (B) The cells were also analyzed for GFP fluorescence (top). Cells cotransfected with myc-lacZ, which did not affect GFP expression, were used as a control. Total cell RNA was isolated from the cells and resolved by electrophoresis on polyacrylamide gels, and probed for U7 snRNA and 7SK RNA, or resolved by electrophoresis on agarose gels and probed for histone H2A mRNA (bottom). Error bars indicate standard deviations.

much lower levels of ZFP100 plasmid than SLBP plasmid were required to stimulate expression of the GFP-SL reporter, consistent with the low abundance of ZFP100 and the stoichiometric requirement for SLBP.

The increase in GFP-SL expression in response to ZFP100 transfection is reflected in an increase in the properly processed GFP-SL reporter mRNA ending at the 3′ end of the histone mRNA (Fig. 3D) (40). The expression of a different GFP-SL reporter driven by the CMV promoter was also stimulated by myc-ZFP100 to a similar extent (not shown), consistent with a stimulation of histone pre-mRNA processing. Thus, while increased GFP expression could result from increases in mRNA levels due to increased transcription or an increase in mRNA half-life or from an increased rate of translation, the stimulation observed by increased ZFP100 levels is most likely due to an increase in histone pre-mRNA processing. The interaction of ZFP100 with the U7 snRNP and its positive effect on processing in *Xenopus* oocytes (7) as well as its stable localization to Cajal bodies (40) strongly suggest a role in processing and not transcription or translation.

To measure the reporter mRNA ending at the histone 3′ end directly, we designed a probe to be used in an S1 protection assay that is complementary to a portion of the GFP ORF, the SL, and HDE as well as some of the downstream sequence present in the plasmid. RNA was analyzed from cells cotransfected with reporter and increasing amounts of myc-ZFP100 or hemagglutinin-SLBP [both plasmids contain the CMV promoter and the bovine growth hormone poly(A) signal] compared to the analysis of RNA isolated from cells cotransfected with LacZ. Increasing the expression of myc-ZFP100 led to a dose-dependent increase in the level of processed GFP mRNA (Fig. 3D, lanes 1 to 4). There are also longer mRNAs (Fig. 3D), which likely represent readthrough transcripts that might become polyadenylated and contribute to the background GFP expression in cells not transfected with ZFP100. This selective and dose-dependent increase in the level of processed GFP-SL message in response to increasing amounts of ZFP100 strongly supports the conclusion that the increase in GFP fluorescence as a result of the expression of ZFP100 reflects an increase in the efficiency of processing.

In contrast, the effect of SLBP expression could be due to a combination of stimulating processing, stabilizing the mRNA, and enhancing mRNA translation, all steps which SLBP is known to impact (26). The stimulation of GFP levels by SLBP was as large as or larger than the stimulation by ZFP100 (Fig. 3C), although the increase in mRNA was lower, and there was not a direct relationship between the amount of reporter mRNA and GFP expression (Fig. 3D, lanes 5 to 8). This suggests that there must also be an effect of SLBP on translation of the reporter mRNA, in addition to its effects on either processing or stability or both.

Expression of Lsm10 or Lsm11 does not activate the reporter. ZFP100 interacts with the U7 snRNP through the direct binding to the U7 snRNP protein Lsm11 (30) in a region of the amino-terminal portion of Lsm11 (40). Thus, the increase in activity of the reporter as a result of transfection of ZFP100 could result from an increase in the amount of active U7 snRNP. The assembly of U7 snRNP involves the incorporation of Lsm11 and Lsm10 into the U7 snRNP as a heterodimer (1). Normally, only a fraction of the U7 snRNA that is transcribed is incorporated into U7 snRNP (18), suggesting that incorporation of Lsm10 and Lsm11 is limiting for U7 snRNP formation. U7 snRNA that is not rapidly incorporated into U7 snRNP is rapidly degraded (18).

We determined the effects of increasing expression of Lsm10, Lsm11, and ZFP100 and combinations of these proteins on U7 snRNA levels as well as on the expression of the GFP-SL reporter. The expressions of the myc-tagged Lsm11, Lsm10, and ZFP100 proteins were comparable (Fig. 4A). However, the expression of Lsm11, Lsm10, or the two proteins together had little impact on the activity of the reporter (Fig. 4B, lanes 2, 3, and 6), suggesting that these proteins were not limiting for histone pre-mRNA processing. In support of this notion, when we cotransfected all three proteins, Lsm10, Lsm11, and ZFP100, this combination activated the expression

of the reporter to the same extent as did ZFP100 alone (Fig. 4B, lane 7). Cotransfection of ZFP100 with either Lsm10 or Lsm11 resulted in a stimulation of expression of the reporter which was lower than that observed with transfection of ZFP100 alone (Fig. 4B, lanes 4 and 5). We interpret the reduction in ZFP100 stimulation with either Lsm10 or Lsm11 alone to be the result of a toxic effect of overexpressing only one member of the Lsm11/Lsm10 heterodimer. Thus, the critical U7 snRNP protein required for stimulation of the expression of the GFP-SL reporter is ZFP100.

Expression of Lsm10 and Lsm11, but not ZFP100, increases the amount of U7 snRNP. We measured the amount of U7 snRNA, histone H2A mRNA, and as a control, 7SK RNA using Northern blot analysis of total RNA isolated from cells, following the transfection of the U7 snRNP proteins (Fig. 4C). None of the transfections resulted in any increase in the levels of endogenous H2A mRNA (Fig. 4C), consistent with the lack of effect on the cell cycle. Since the unique Sm sequence in the U7 snRNA determines the amount of U7 snRNP that accumulates in cells (18), it is likely that the levels of Lsm10 and Lsm11 play a role in the amount of accumulation of U7 snRNP. The expression of either Lsm10 or Lsm11 alone or together with ZFP100 increased U7 snRNA levels slightly above control levels. However, the overexpression of Lsm10 and Lsm11 together resulted in a threefold increase in U7 snRNA (analyzed by PhosphorImager analysis and normalized to the amount of 7SK RNA), although there was no effect on expression of the reporter gene (Fig. 4B, lane 6). In contrast, the overexpression of ZFP100 had no effect on U7 snRNA levels (Fig. 4B, lane 2), even though it stimulated the expression of the reporter gene nearly fourfold. These results support the notion that the Lsm10 and Lsm11 heterodimer is limiting for U7 snRNA accumulation but that additional factors, including ZFP100, are necessary for an active U7 snRNP particle. The expression of Lsm10, Lsm11, and ZFP100 together stimulated U7 snRNA accumulation and resulted in maximal expression of the GFP-SL reporter but was only slightly higher than the expression of ZFP100 alone. Taken together, these results support the conclusion that ZFP100, and not the total amount of U7 snRNP, is a limiting component for histone pre-mRNA processing and that the amount of ZFP100 is not limiting for the accumulation of U7 snRNA.

siRNA-mediated downregulation of ZFP100 arrests cells in early G_1. Histone mRNAs are present in high concentrations during only S phase and are necessary for the synthesis of the histone proteins required for rapid assembly of chromatin on newly replicated DNA. Knocking down any of the U7 snRNP proteins should interfere with histone pre-mRNA processing, resulting in a defect in histone protein synthesis. We expected that defects in histone mRNA production might cause either an arrest at the G_1/S border or slowed S-phase progression. In agreement with this expectation, we and others recently showed that RNAi-mediated depletion of SLBP results in slow progression through S phase (38, 45). In addition, interfering with chromatin assembly also results in slowing progression through S phase (44).

To test whether ZFP100 expression is required for cell cycle progression, we depleted the protein using two siRNAs that targeted unique regions of the ZFP100 open reading frame (Z1 and Z2). ZFP100 is not detectable by Western blotting in whole-cell lysates due to its low concentration. We therefore monitored ZFP100 accumulation with and without siRNA treatment by transfecting HeLa cells with a vector expressing a myc-tagged ZFP100 (Fig. 5A). Neither the control siRNA (C2) (Fig. 5A, lane 1) nor the S2 siRNA targeting SLBP (S2) (Fig. 5A, lane 5) had an effect on myc-ZFP100 expression. The two siRNAs targeting the ZFP100 mRNA (Z1 and Z2) both reduced the expression of myc-ZFP100 (Fig. 5A, lanes 2 and 3), as did the mixture of the two siRNAs (Fig. 5A, lane 4). None of the siRNAs had any effect on polypyrimidine tract binding protein expression used as a loading control. The Z1 siRNA reduced the levels of myc-tagged ZFP by about 90%, and the Z2 siRNA reduced the myc-tagged ZFP by more than 90%, as quantified by comparison with a dilution of the C2-treated lysate present on the same blot (Fig. 5A, lanes 6 to 9).

Knocking down ZFP100 in HeLa cells resulted in a reduced growth rate, followed by cell death. We tested the effect of knocking down ZFP100 in U2OS cells since these cells, unlike HeLa cells, are more amenable to cell cycle analysis due to their retention of intact p53 and pRb checkpoint systems. Untreated U2OS cells exhibited cell cycle profiles with about 50% of the cells in G_1 and 25% in S phase (Fig. 5B). Treatment of these cells with siRNAs against ZFP100 blocked proliferation, with a decrease in total cell number but not in cell viability (not shown). Treated cell populations displayed dramatic increases in the number of G_1 cells (Fig. 5B) and decreases in S-phase cells, while transfection with a control siRNA (C2) had no effect. The transfection of the Z1/Z2 siRNA mixture resulted in about 80% of the cells arresting in G_1 phase (Fig. 5B). U2OS cells transfected with the siRNA targeting SLBP also exhibited a reduction in cell proliferation. However, in contrast to the knockdown of U7 snRNP components, knockdown of SLBP resulted in an increased percentage of cells in S phase compared to the C2 siRNA (Fig. 5B, lower right panel), which is in agreement with our previously reported results (38).

Using an RT-PCR assay, we demonstrated that the different siRNAs specifically reduced their target endogenous mRNAs. As an internal control, we determined the levels of endogenous mRNA for 3′hExo (8) in the same assay with SLBP mRNA (Fig. 5C, lanes 1 to 3) or ZFP100 mRNA (Fig. 5C, lanes 4 to 6). 3′hExo is an exonuclease associated with histone mRNA (8) that serves as a nonspecific internal control and was not affected by any of the siRNA transfected. The S2 siRNA reduced the levels of the endogenous SLBP mRNA (Fig. 5C, lane 3), but it had no effect on the endogenous mRNA for ZFP100 (Fig. 5C, lane 6). The Z1/Z2 siRNA depleted the endogenous ZFP100 mRNA (Fig. 5C, lane 5) but had no effect on SLBP mRNA (Fig. 5C, lane 2).

In order to determine whether the ZFP100-depleted cells were arrested at the G_1/S border or in G_1 phase, we determined the levels of two cell cycle-regulated proteins, SLBP and E2F1. SLBP is cell cycle regulated, and the protein accumulates as cells approach entry into S phase (43). In particular, SLBP is present at high levels in cells arrested either by aphidicolin or by double-thymidine block at the G_1/S border (43). Although the amount of SLBP protein oscillates throughout the cell cycle, the level of SLBP mRNA varies much less (43). The cells with ZFP100 knocked down had a marked reduction in the levels of SLBP protein (Fig. 5D, lanes 2 to 4), with the amount of decrease with the Z1 and Z2 oligonucleotides cor-

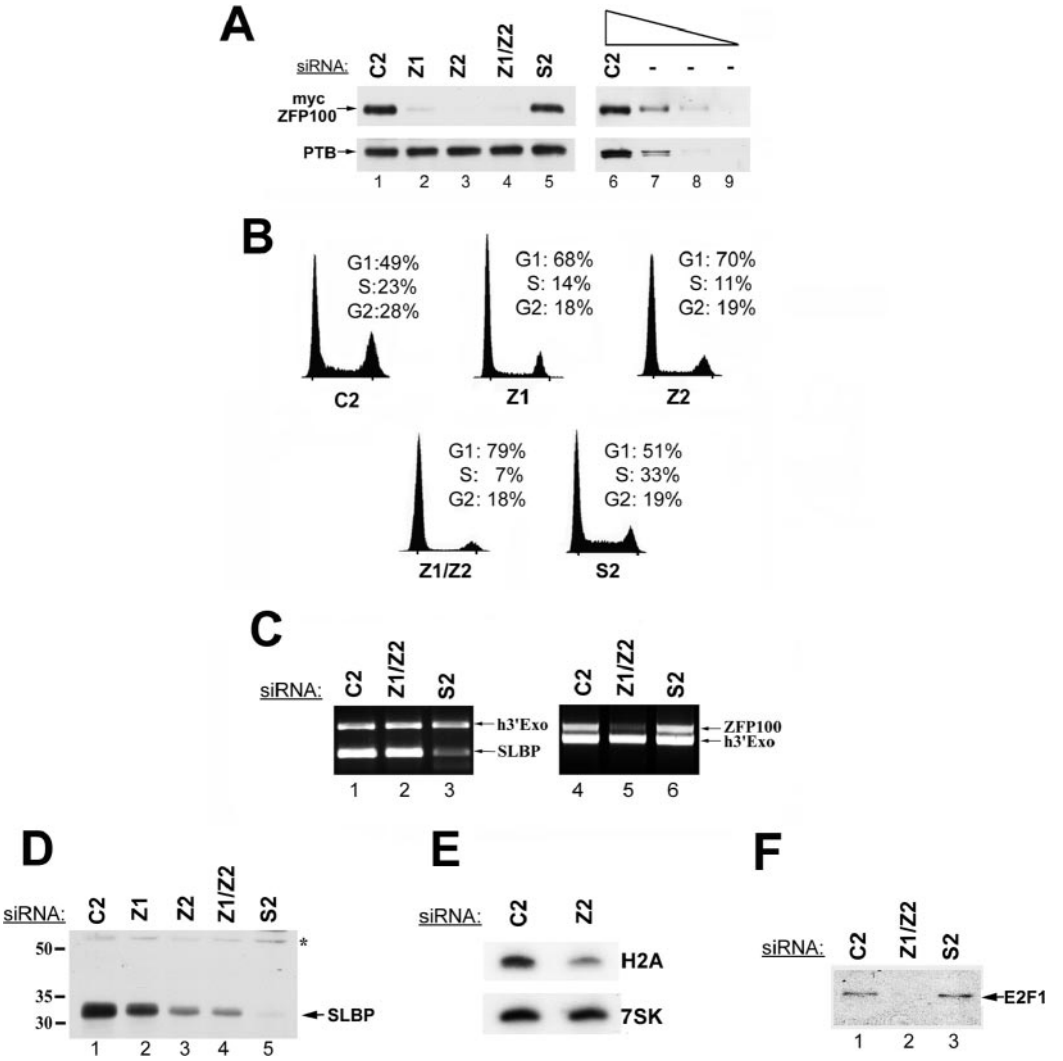

FIG. 5. Knockdown of ZFP100 arrests U2OS cells in G_1. (A) U2OS cells were treated with a control siRNA for 96 h (C2, lane 1), two different siRNAs (Z1 and Z2, lanes 2 and 3) against ZFP100, a combination of those two siRNAs (Z1/Z2, lane 4) or siRNA against SLBP (S2, lane 5). The cells were then transfected with a myc-tagged ZFP100. Lysates were prepared and resolved by SDS-gel electrophoresis, the myc-ZFP100 was detected by Western blotting with anti-myc (top), and polypyrimidine tract binding protein (PTB) was analyzed as a loading control (bottom). Lanes 6 to 9 show dilutions of the C2 lysate (100, 50, 20, and 10% of total) demonstrating that the myc-ZFP100 was knocked down at least 90%. (B) Cell cycle analysis of U2OS cells 96 h after transfection with the control siRNA C2, the Z1 and Z2 siRNAs against ZFP, a combination of Z1 and Z2, or the S2 siRNA against SLBP. The percentage of cells in each cell cycle phase is shown. (C) Multiplex RT-PCR analysis of RNA isolated from U2OS cells transfected with the respective siRNAs. The left panel contains amplified DNAs from primers to amplify SLBP mRNA and the 3′hExo mRNA (8) as a control. The right panel contains amplified DNAs from primers to amplify ZFP100. (D) Western blot analysis of lysates from U2OS cells transfected with either C2 siRNA (control), Z1, Z2, or a mixture of Z1 and Z2 or the S2 siRNA. Lysates were resolved by SDS-gel electrophoresis and probed with an anti-SLBP polyclonal antibody. The asterisk denotes a cross-reacting band. (E and F) Lysates from U2OS cells treated with the C2 (lane 1), Z1, and Z2 (lane 2) or S2 (lane 3) siRNAs were resolved by SDS-gel electrophoresis and probed with an anti-E2F1 antibody.

relating with the extent of G_1 arrest (Fig. 5D). Thus, the G_1-arrested cells are likely arrested early in G_1 phase, prior to the increase of SLBP protein that occurs in late G_1 (43).

Since SLBP and ZFP100 interact during histone pre-mRNA processing (7), it was also possible that the SLBP levels were directly coupled to ZFP100. E2F1 has an expression pattern similar to that of SLBP during the cell cycle and also accumulates in cells arrested at the G_1/S border (J. Dong and W. F. Marzluff, unpublished data). To further confirm that there was indeed an arrest early in G_1, we probed the same lysates for expression of the E2F1 protein (Fig. 5E). The E2F1 protein was not detected in ZFP100 siRNA-transfected cells; thus, reducing the activity of U7 snRNP resulted in a cell cycle arrest in G_1 prior to the synthesis of proteins necessary for entry into S phase.

siRNA-mediated downregulation of U7 snRNP components Lsm11 or Lsm10 also arrests cells in early G_1. The cell cycle arrest resulting from ZFP100 depletion may result from decreased U7 snRNP activity, but it could also reflect a function of ZFP100 distinct from its interaction with the U7 snRNP. To

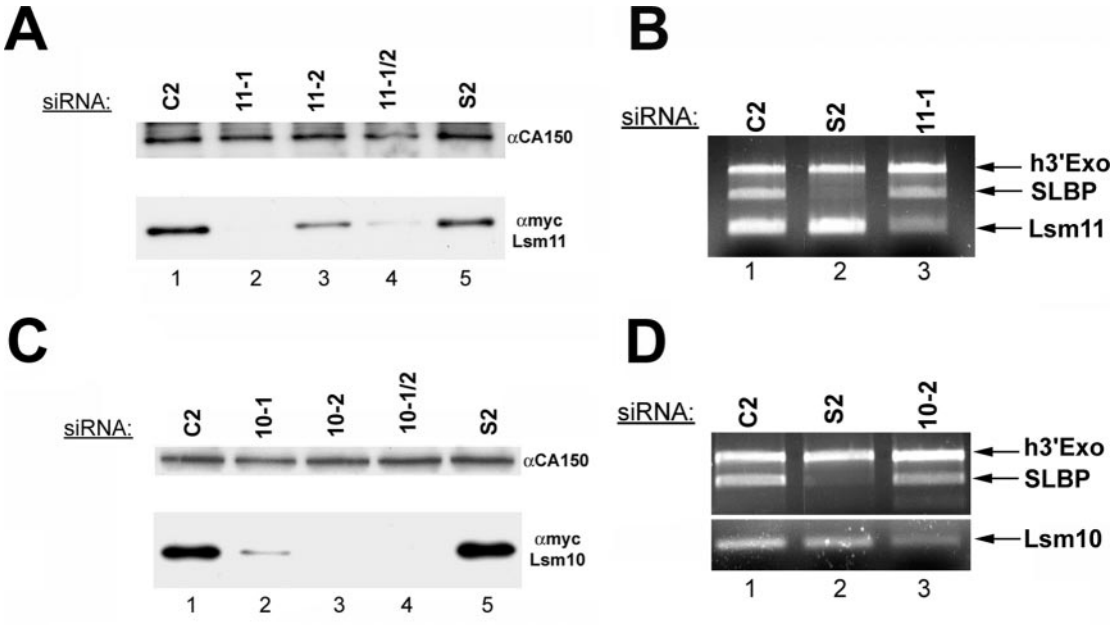

FIG. 6. RNAi-mediated knockdown of the U7 snRNP proteins, Lsm11 and Lsm10. (A) U2OS cells were transfected for 96 h with a control siRNA (C2, lane 1), two siRNAs against Lsm11 (11-1 and 11-2, lanes 2 and 3), a mixture of the two (11-1/2, lane 4), or an siRNA against SLBP (S2, lane 5). Cells were then transfected with a plasmid expressing myc-tagged Lsm11. Lysates were prepared and the levels of myc-Lsm11 measured by Western blotting using an anti (α)-myc antibody (bottom panel). As a control, the levels of CA150 (15) were analyzed (top) in the same lysates. αCA150; anti-CA150; αmyc; anti-myc. (B) RT-PCR analysis of RNA isolated from HeLa cells transfected with either control siRNA (C2), siRNA targeting SLBP (S2), or siRNA targeting Lsm11 (11-1). The same RT reaction was subjected to PCR with primers for the 3′hExo mRNA (8) as a control, SLBP, or Lsm11. (C) U2OS cells were transfected with a control siRNA (C2, lane 1), two siRNAs against Lsm10 (10-1 and 10-2, lanes 2 and 3), a mixture of the two (10-1/2, lane 4), or an siRNA against SLBP (S2, lane 5). Cells were then transfected with a plasmid expressing myc-tagged Lsm10. Lysates were prepared and the levels of myc-Lsm10 measured by Western blotting using an anti-myc antibody (bottom panel). As a control the levels of CA150 were analyzed in the same lysates (top). αCA150; anti-CA150; αmyc; anti-myc. (D) RT-PCR analysis of RNA isolated from HeLa cells transfected with either control siRNA (C2), siRNA targeting SLBP (S2), or siRNA targeting Lsm10 (10-2). The same RT reaction was subjected to PCR with primers for the 3′hExo mRNA (8) as a control, SLBP, or Lsm11.

rule out this possibility, we designed two sets of siRNAs targeting either Lsm10 or Lsm11. Similar to ZFP100, the low abundance of Lsm10 and Lsm11 precludes detection in whole-cell lysates by Western blotting; thus, both sets of siRNAs were cotransected with myc-tagged exogenous protein into HeLa cells (Fig. 6A and D). Both siRNAs targeting Lsm11 reduced the level of Lsm11 protein, with the L11-1 siRNA being more potent than L11-2 (Fig. 6A), while the L10-2 siRNA was more potent than the L10-1 siRNA (Fig. 6D). None of the siRNAs had any effect on the levels of the CA150 nuclear protein, used here as a loading control (15). We analyzed the effect of siRNA transfection on the endogenous Lsm11 mRNA (Fig. 6B) or endogenous Lsm10 mRNA (Fig. 6D) by using an RT-PCR assay of total cellular RNA. The L11-1 siRNA reduced the levels of the endogenous Lsm11 mRNA, while it did not have any effect on either the endogenous SLBP mRNA or h3′Exo mRNA (Fig. 6B). The L10-2 siRNA also specifically decreased the levels of endogenous Lsm10 mRNA, whereas it had no effect on the levels of SLBP or h3′Exo endogenous mRNA.

The transfection of any of these four siRNAs into U2OS cells led to reduced proliferation and a reduction in the level of SLBP that was comparable to, although not as dramatic as, knocking down ZFP100 (Fig. 7A and C). Cell cycle analysis demonstrated that cells transfected with the Z2 siRNA had 72 to 75% of cells in G_1 phase compared to 45% in the control. The knockdown of Lsm11 with either siRNA led to an intermediate phenotype with 55 or 57% of cells in the G_1 phase (Fig. 7B), and the knockdown of Lsm10 resulted in 52 to 60% of the cells in G_1 phase (Fig. 7D). In each case, the more potent siRNA (L11-1 and L10-2) resulted in the greatest accumulation of G_1 cells. The reduced amount of cells in G_1 arrest in the Lsm11 or Lsm10 knockdowns compared with that in the ZFP100 knockdowns may reflect either a less efficient knockdown or that ZFP100 is more critical to cell cycle progression. Evidence presented above demonstrates that ZFP is a limiting factor for histone pre-mRNA processing. For example, ZFP100 may be less stable than the core U7 snRNP components. In addition, since our data here and elsewhere (40) suggest that ZFP100 is critical for an active U7 snRNP, the amount of active U7 snRNP may not have been reduced as much by knocking down Lsm10 or Lsm11 as by knocking down ZFP100.

DISCUSSION

Histone protein synthesis is tightly coupled to DNA replication to ensure the efficient replication of chromatin. In metazoans, the histone mRNAs end in a unique 3′ end which plays a major role in coupling histone protein synthesis with DNA replication. One component of the coupling is the rapid degradation of histone mRNA when DNA replication is inhibited. The degradation is mediated by the 3′ end (29) and is inte-

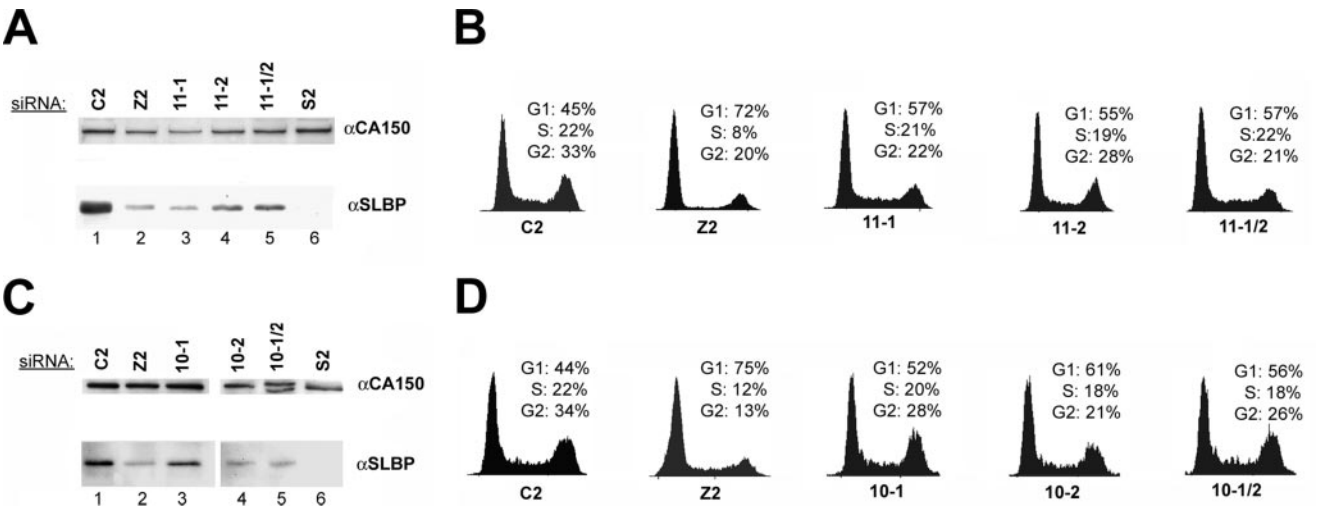

FIG. 7. Knockdown of Lsm10 or Lsm11 results in a G_1 arrest of U2OS cells. (A) U2OS cells were transfected with a control siRNA (C2, lane 1), an siRNA against ZFP100 (Z2, lane 2), two siRNAs against Lsm11 (11-1 and 11-2, lanes 3 and 4), a mixture of the two (11-1/2, lane 5), or an siRNA against SLBP (S2, lane 6). Lysates were prepared and analyzed by Western blotting for SLBP (bottom) and, as a loading control, CA150 (top). αCA150; anti-CA150; αSLBP; anti-SLBP. (B) Cell cycle analysis of U2OS cells transfected with siRNAs, C2, Z2, 11-1, 11-2, and a mixture of 11-1 and 11-2 (11-1/2). The percentage of cells in each cell cycle phase is indicated. (C) U2OS cells transfected with a control siRNA (C2, lane 1), an siRNA against ZFP100 (Z2, lane 2), two siRNAs against Lsm10 (10-1 and 10-2, lanes 3 and 4), a mixture of the two (10-1/2, lane 5), or an siRNA against SLBP (S2, lane 6). Lysates were prepared and analyzed by Western blotting for SLBP (bottom) and, as a loading control, CA150 (top). The two blots shown are two pieces of the same Western blot exposed at the same time. αCA150; anti-CA150; αSLBP; anti-SLBP. (D) Cell cycle analysis of U2OS cells transfected with siRNAs, C2, Z2, 10-1, 10-2, and a mixture.

grated with the S-phase checkpoint, requiring the activation of ATR (21). A second component that restricts histone mRNA expression to S phase is regulation of the levels of SLBP. SLBP accumulates to high levels during only S phase, even though SLBP mRNA is present throughout the cell cycle (43). The translation of SLBP mRNA is activated prior to entering S phase, and SLBP is rapidly degraded at the end of S phase (43), effectively restricting histone mRNA processing to S phase (46). SLBP is cell cycle regulated and, unlike histone mRNA, is not directly regulated by the inhibition of DNA replication (42). Since SLBP is also required for histone mRNA translation (32), the amount of SLBP also limits the amount of active histone mRNA that can accumulate in the cell.

While both histone gene transcription and the half-life of histone mRNA are regulated during the cell cycle, the major regulatory step is the processing of the 3′ end of histone mRNA (19, 24, 36). One aspect of this regulation is the cell cycle regulation of SLBP. However, there is evidence that a "heat-labile factor" required for histone pre-mRNA processing is also involved in cell cycle regulation (14). The "heat-labile factor" has recently been identified as symplekin, a component of the cleavage complex for both histone pre-mRNA processing (22) and polyadenylation (37). Since symplekin is involved in both polyadenylation and histone 3′ processing, it is not clear how it might specifically regulate histone 3′ processing. There is also evidence that there may also be regulation of the activity of U7 snRNP during the HeLa cell cycle (20), although these results have not been reproduced (3).

This study identifies ZFP100 as a limiting factor for histone pre-mRNA processing in vivo. We have also defined a critical role for an active U7 snRNP containing ZFP100 not only in histone mRNA metabolism but also, surprisingly, in the progression from G_1 into S phase. ZFP100 is one of three known U7 snRNP-specific proteins and was identified as a protein that interacts with the SLBP/histone pre-mRNA complex (7). The other U7-specific proteins, Lsm10 and Lsm11, are part of the Sm complex bound to U7 snRNA (30, 31) and are likely to be stable components of U7 snRNP in contrast to ZFP100, which is bound to the U7 snRNP through a specific sequence on the amino terminus of Lsm11 (31, 40). Thus, it is possible that ZFP100 is a component of the "active" U7 snRNP and need not be stably associated with the U7 snRNP. There are many proteins in splicosomal snRNPs that are associated with the active forms of the snRNPs but are not part of the purified stable core snRNP (2, 16, 25). Consistent with the possibility that ZFP100 is loosely associated with U7 snRNP is the observation that the purified U7 snRNP, which contains Lsm10 and Lsm11, does not contain ZFP100 (30, 31, 34).

The GFP-SL reporter mimics the expression of histone mRNA and provides a rapid way to measure the expression of a protein from an mRNA ending in the stem-loop. Expressing two proteins, ZFP100 and SLBP, increased the expression of GFP from the GFP-SL reporter three- to fourfold. Increasing the amount of ZFP100 increases the level of the reporter mRNA, which is consistent with its role in histone pre-mRNA processing. In contrast, increasing the amount of SLBP has a lesser effect on histone mRNA levels and likely stimulates translation as well as processing and/or stability of the reporter mRNA. Note that there was no increase in S-phase cells or in the number of cells expressing the reporter (Fig. 3B), so the increased ZFP100 concentration likely increases the efficiency of processing the reporter mRNA in S-phase cells.

In contrast, the expression of other U7-specific snRNP proteins, Lsm10 and/or Lsm11, had no effect on expression of the reporter. In particular, the expression of Lsm10 and Lsm11

together increased the overall amount of U7 snRNA two- to threefold (Fig. 4B) without increasing the expression of the GFP-SL reporter. The increase in U7 snRNA is likely due to more-efficient assembly of the U7 snRNP, which normally is only 20% efficient (18), likely as a result of limiting amounts of the Lsm10/Lsm11 heterodimer required for U7 snRNP assembly (1). In contrast, the expression of ZFP100 had no effect on U7 snRNA levels but increased activity of the reporter fourfold. Thus, the amount of ZFP100 (and likely the amount of active U7 snRNP) is limiting for histone mRNA expression.

In addition to ZFP100 being a critical factor for histone mRNA expression, surprisingly, an active U7 snRNP is critical for cell cycle progression. Knocking down U7 snRNP components, particularly ZFP100, results in cells arresting in G_1. The arrest is not caused by a failure to express sufficient histone mRNA at the G_1/S transition. This arrest is also not due to simply a defect in histone mRNA metabolism, since the depletion of SLBP results in slow progression through S phase rather than an arrest in G_1 phase (Fig. 5B) (38, 45) and S-phase arrest is also observed when one interferes with chromatin assembly (44). The G_1 arrest occurs early in G_1 prior to the point where the cell activates synthesis of SLBP and E2F1. Part of the decision to initiate progression into S phase therefore requires that there be sufficient amounts of active U7 snRNP. We envision that the pool of active U7 snRNP during the cell cycle is likely limited by the amount of active ZFP100 since the amount of U7 snRNA doesn't vary during the cell cycle (43). The arrest in G_1 due to insufficient active U7 snRNP may result from the cell "checking" its ability to synthesize sufficient histone mRNA to successfully progress through S phase. The molecular details that lead to this arrest are not known but likely integrate into other signals that lead to the decision of a cell to progress through G_1 to enter S phase.

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