Nuclear Autoantigenic Sperm Protein (NASP), a Linker Histone Chaperone That Is Required for Cell Proliferation*

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A multichaperone nucleosome-remodeling complex that contains the H1 linker histone chaperone nuclear autoantigenic sperm protein (NASP) has recently been described. Linker histones (H1) are required for the proper completion of normal development, and NASP transports H1 histones into nuclei and exchanges H1 histones with DNA. Consequently, we investigated whether NASP is required for normal cell cycle progression and development. We now report that without sufficient NASP, HeLa cells and U2OS cells are unable to replicate their DNA and progress through the cell cycle and that the NASP−/− null mutation causes embryonic lethality. Although the null mutation NASP−/− caused embryonic lethality, null embryos survive until the blastocyst stage, which may be explained by the presence of stored NASP protein in the cytoplasm of oocytes. We conclude from this study that NASP and therefore the linker histones are key players in the assembly of chromatin after DNA replication.

Chromatin is a nucleoprotein complex containing repeating nucleosome units that allow the chromatin to be folded into higher-ordered structures and orchestrate numerous interactions through their histone tails. Nucleosomes typically are separated by short pieces of DNA that link the nucleosomes to one another, bind H1 linker histones, and maintain nucleosome spacing that is critical for normal development (1–3). DNA-binding proteins that regulate replication and repair of the genome must successfully interact with chromatin during each cell cycle. Consequently, the nucleosome-remodeling protein machinery and the DNA replication machinery coordinate their activities to ensure a faithful transition of chromatin from one cell cycle to the next (4, 5). One of the key players in this coordination of activity is CAF-1.3 The chromatin assembly factor that is necessary for progression through S phase (6, 7). CAF-1 exists in a multichaperone nucleosome-remodeling complex that contains, in addition to CAF-1 (p150, p60, p48), H3.1, H4, Asfa, Asfb, NASP, Chk2, HAT1, and importin 4 (8, 9). This complex is thought to coordinate the deposition of H3.1–H4 histones into new nucleosomes and signal the replication complex via phosphorylation events through various checkpoints that DNA replication (S phase) can proceed. The presence of nuclear autoantigenic sperm protein (NASP) in the CAF-1 multichaperone complex during nucleosome remodeling (9) implies that NASP may have a role in the completion of S phase in a normal cell cycle. H1 linker histones (H1) are required for the proper completion of normal development (2, 10) and NASP, an H1 chaperone, transports H1 into nuclei (11) and exchanges H1 with DNA (12). NASP has also been found to be a binding partner of Ku70/Ku80 and DNA-PK in HeLa cells (13), indicating that NASP may play an additional role in DNA repair events.

Previous studies demonstrated that NASP expression is regulated during the cell cycle (14) and that progression through the G1/S border is delayed by the overexpression of NASP (12). Linker histones not bound to DNA are bound to NASP (12), which is found in all dividing cells in either a somatic/embryo (sNASP) or testis/embryo (tNASP) form (14, 15). To address the role of NASP in cell proliferation and normal development, we used a small interfering RNA (siRNA) knockdown of NASP in HeLa and U2OS cells and inactivated the NASP gene by homologous recombination (NASP−/−) in mice. We now report that without sufficient NASP, HeLa cells and U2OS cells are unable to replicate their DNA and progress through the cell cycle and that the NASP−/− null mutation causes embryonic lethality. Interestingly, because NASP is expressed in the oocyte and NASP protein is present in the cytoplasm of the one-cell embryo, the embryos survive until the depletion of the maternally derived NASP protein. Our results imply that NASP orchestrates the supply of linker histones that are necessary for nucleosome remodeling during DNA replication or repair and support the conclusion that H1 linker histones are necessary for normal development (2, 3).

EXPERIMENTAL PROCEDURES

Materials—Miscellaneous chemicals were the highest possible molecular biology grade (Sigma). Restriction and modifying enzymes were purchased from New England Biolabs (Beverly, MA). DNA sequencing was performed by the University of North Carolina at Chapel Hill automated sequencing facility using an ABI PRISM model 377 DNA sequencer (Applied Bio-
systems, Foster City, CA). Sequence data were analyzed using DNAsis (Hitachi Corp., South San Francisco, CA) and Sequencher (Gene Codes Corp., Ann Arbor, MI) software. Oligonucleotides were synthesized at the University of North Carolina at Chapel Hill Nucleic Acids Core Facility. Purification of plasmid and PCR DNAs was performed using their respective kits from Qiagen (Valencia, CA). Anti-symplekin antibodies were obtained from BD Biosciences.

Targeting Vector—The mouse NASP arms of homology inserted into the pOsdupdel targeting vector were generated by PCR using mouse genomic DNA (strain 129 Sv/Ev) as template. PCRs were performed with Takara LA TaqDNA polymerase (Fisher) according to the manufacturer’s protocol. The cycle conditions were as follows: 1 min at 94 °C, 1 cycle; 20 s at 98 °C followed by 4.5 min at 68 °C, 30 cycles; 10 min at 72 °C, 1 cycle. The 7508-bp long arm was generated with NotI and PacI restriction sites (5’ and 3′, respectively), and the 1805-bp short arm had BbsI and PmlI restriction sites (5’ and 3′, respectively). Both the vector and inserts were sequentially digested with the appropriate restriction enzymes and purified, and the inserts were ligated into the vector overnight at 4 °C using T-4 DNA ligase. A clone of the correct size was picked, tested by various restriction digests, and sequenced to confirm its validity.

Both the vector and inserts were sequentially digested with the appropriate restriction enzymes and purified, and the inserts were ligated into the vector overnight at 4 °C using T-4 DNA ligase. A clone of the correct size was picked, tested by various restriction digests, and sequenced to confirm its validity. The DNA sequences of the PCR primers used to generate the NASP arms of homology are as follows: 1) long arm upstream primer, 5’-AGAGCCGGCGGCATATTCTCTAGTTGACACACTCAG-3’; 2) long arm downstream primer, 5’-GCGTTAAATATCTGGCAATCCAGAAGTGAC-3’; 3) short arm upstream primer, 5’-GCGCAGTGTAGTTATACCTGCTGAGAGGAGG-3’; and 4) long arm downstream primer, 5’-GCGGATCTAAGATTCCTCCAGCCTTTAGGCCAGC3’. Homologous recombination with this targeting construct deletes 9682 bp from the NASP gene, which includes exons 6–11 (see Fig. 4). This deletion includes exon 7, which is expressed only in the adult testis.

Targeting—The targeting plasmid was linearized with NotI, and mouse ES cells (2 × 10^7) embryonic stem cells, strain 129 Sv/Ev) were electroporated with 25 μg of vector. After positive-negative selection with ganciclovir and G418 (17), surviving colonies were picked and screened using PCR with one primer from the neo gene and the other from the region just 3’ to the 3’ arm of homology. Several clones were identified, and the correctness of the targeting was verified by Southern blots.

Genotypic Analysis—Genomic DNA for PCRs was extracted from 2-mm mouse tail clips by incubation in 100 μl of 25 mM NaOH/0.2 mM EDTA for 1 h at 95 °C followed by addition of 100 μl of 40 mM Tris-HCl. After a brief vortexing, the samples were centrifuged at 1000 × g for 10 min and the supernatant removed. Genomic DNA for Southern blots was isolated from 6-mm mouse tail clips by overnight incubation at 55 °C in 400 μl of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% SDS and 20 μl Proteinase K (10 mg/ml). After incubation, 200 μl of saturated NaCl was added and the mixture vortexed for 30 s followed by centrifugation at 14,000 × g for 20 min. Five hundred μl of supernatant was removed and precipitated with 100% ethanol and centrifuged at 14,000 × g for 10 min; the pellet was washed with 70% ethanol and centrifuged. The final air-dried DNA pellet was dissolved in 50 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)). Genotyping PCRs were performed using Takara LA Taq as described above except that 2 μl of genomic DNA (described above as DNA for PCR) was used as template, and the primers were as follows: for the wild-type allele sense and antisense, 5’-GAAGCAAGGGAGATGGTGAAGAGCAG-3’ and 5’-TTCACCTCTCAGAGGGTGAC3’; respectively, and for the targeted allele sense and antisense, 5’-GTTTCCACCATGTGAGCACCAGCAC3’ and 5’-TTGATGTGAGACACGAGCAC3’. Southern blots were performed on 10 μg of Sacl- and PstI-digested DNA (described above as DNA for Southern blots) as described previously (15) except that restriction digests contained 3 mM spermidine, and the 20 μl samples were incubated in 1 μl of 1 mg/ml RNase A for 1 h at 37 °C before loading onto the gel. The blots were probed with a 32P-labeled, random-primed PCR-generated cDNA that represents NASP exon 12 (see Fig. 3) and visualized using a Storm 860 PhosphorImager (Amer sham Biosciences).

Embryo Isolation, Blastocyst Culture, and Genotyping—NASP^+/− females were superovulated by injection with 5 units of pregnant mare serum gonadotropin (Sigma) followed 48 h later by 5 units of human chorionic gonadotropin (Sigma) and mated with NASP^+/− males. Successful matings were detected by the presence of a vaginal plug. Embryos at various stages in either the uterus or oviducts were collected for histological examination or flushed as blastocysts from the uterus. Flushed blastocysts were washed two times in medium M16 (Sigma) and then cultured in 96-well plates precoated with 1% gelatin in Glasgow’s minimal essential medium (Fisher) with 15% fetal bovine serum and leukemia inhibiting factor (0.2 units/ml) for up to 10 days. To isolate DNA for PCR template, individual blastocyst cultures were suspended by pipetting and incubating them in 20 μl of 25 mM NaOH/0.2 mM EDTA for 1 h at 95 °C followed by addition of 100 μl of 40 mM Tris-HCl. After a brief vortexing, the samples were centrifuged at 1000 × g for 10 min, and the supernatant was removed.

Immunohistochemistry—Embryos were fixed in situ in excised oviduct and uterine tissue by immersion in Bouin’s fixative for 1–4 days, paraffin-embedded, and sectioned (8 μm). Every fifth section was stained with hematoxylin and eosin to locate the embryos, and adjacent serial sections were stained with anti-NASP antibody (14) or with a NASP probe for in situ hybridization.

In situ Hybridization—A high affinity NASP antisense hybridization probe was designed using Oligo Analysis software, version 6.65 (Cascade, CO). The 5’-digoxigenen-labeled probe, 5’-CCCCGATGACAG-3’ (where boldface and underlined letters indicate locked nucleic acids), was prepared.
by Proligo Primers & Probes (Boulder, CO), and an identical unlabeled probe was prepared as a control to demonstrate the specificity of the probe when used in 100× excess.

In situ hybridization was performed in the University of North Carolina at Chapel Hill Laboratories for Reproductive Biology Immunohistochemistry Core Facility based on methods described by Schwarzacher and Heslop-Harrison (18).

Analysis of the NASP Transcript—Testes were collected from NASP−/− and NASP+/− mice, and after disruption in a Dounce homogenizer, total and poly(A)+ RNA were prepared using an RNeasy Mini Kit and an Oligotex mRNA Kit, respectively (Qiagen). Equal quantities of mRNA were electrophoresed and Northern blotted as described previously (14). After hybridization with a 32P-labeled, randomly primed NASP exon 12 probe, the blots were visualized as above with the PhosphorImager.

siRNA Transfection—HeLa cells were maintained as described (12). A series of siRNAs targeting the human NASP open reading frame were designed (Dharmacon, Lafayette, CO), and one (GCACAGUUCAGCAAAUCUAdTdT) was found to effectively deplete NASP from HeLa cells. Transfection with C2 siRNA, which had no cellular target, served as a negative control (19). HeLa cells (8.5–10 × 10^5 cells per well in a 24-well plate) were transfected with NASP and C2 siRNAs using a two-hit siRNA transfection method with Lipofectamine™ 2000 for 18 h as described (19). Twenty-four h after the first transfection, cells were trypsinized and split into 6-well plates. Forty-eight h after the first transfection, cells were retransfected. Ninety h after the initial transfection, cells were harvested for Western blot analysis. For rescue experiments, cells were transfected with tNASP as described previously (12) 72 h after siRNA transfection. Control cells were transfected with the transfection reagent (Effectene transfection reagent, Qiagen). siRNA transfection of U2OS cells was performed identically to that described above for HeLa cells. Anti-SLBP antibodies were used as described previously (20).

BrdUrd Cell Proliferation Assay—The BrdUrd Cell Proliferation Assay kit (Calbiochem) was used to measure BrdUrd incorporation. HeLa cells were trypsinized 90 h after the first transfection. Equal numbers of cells were placed in a propylene round-bottom tube and suspended in growth medium with 10 μM BrdUrd (37 °C, 5% CO2, 2 h) with regular shaking to prevent cells from anchoring. BrdUrd-containing medium was then removed by centrifugation, and the cells were washed twice with PBS, resuspended in growth medium, and plated in a 96-well plate for overnight attachment. Plates were processed according to the manufacturer’s recommendations. Absorbance was measured at 450 nm. An average reading was determined by measuring five independent wells. Significance was determined by the Student’s t test.

Fluorescence-activated Cell Sorting (FACS) Analysis—Control and transfected cells were collected 90 h after the first transfection. Equal numbers of cells were placed in a propylene round-bottom tube and suspended in growth medium with 10 μM BrdUrd (37 °C, 5% CO2, 2 h) with regular shaking to prevent cells from anchoring. BrdUrd-containing medium was then removed by centrifugation, and the cells were washed twice with PBS, resuspended in growth medium, and plated in a 96-well plate for overnight attachment. Plates were processed according to the manufacturer’s recommendations. Absorbance was measured at 450 nm. An average reading was determined by measuring five independent wells. Significance was determined by the Student’s t test.
transfection. Cells were trypsinized, washed with PBS, and fixed in 70% ethanol for ≥2 h on ice. Cells were washed in PBS and stained overnight (4 °C) with 50 μg/ml propidium iodide in PBS (containing 200 μg/ml RNase A and 0.1% Triton X-100). Samples were analyzed at the University of North Carolina at Chapel Hill Flow Cytometry Facility. For each sample, at least 10,000 cells were counted. After gating out doublets and debris, cell cycle distribution was analyzed using Summit version 3.1 software (Cytomation Inc., Fort Collins, CO).

Immunofluorescence Microscopy—For immunostaining, cells were trypsinized and split into a cell culture slide (Falcon, Bedford, MA) at 24 h posttransfection and retransfected at 48 h (as above). Following 6 h in growth media, cells were incubated for 18 h in 20 μM BrdUrd (staining 90 h after transfection). After exposure to BrdUrd (final concentration 20 μM) slides were briefly washed in PBS and fixed in 3% formaldehyde in PBS for 15 min, rinsed twice in PBS, and permeabilized with 0.5% Triton X-100 in PBS containing 1% fetal bovine serum for 5 min. Cells were washed and incubated with anti-NASP antibody for 1 h, washed in PBS, and incubated in secondary antibody (Alexa Fluor-568 goat anti-rabbit IgG conjugate; Molecular Probes, Eugene, OR). After a wash in PBS, cells were fixed a second time in 3% formaldehyde, incubated for 1 h with 4 M HCl (7), and incubated in Alexa Fluor-488-conjugated anti-BrdUrd (1:50) antibody (Molecular Probes).

RESULTS
NASP Is Required for Cell Proliferation—HeLa cells were transfected with NASP siRNA or a control (C2), nonspecific siRNA (20) at 0 and 48 h. Forty-two h later (90 h after initial siRNA transfection), HeLa cells were examined for NASP protein by Western blotting. As shown in Fig. 1A, tNASP and sNASP proteins were depleted from siRNA-transfected cells, whereas control (C2)-transfected cells contained both tNASP and sNASP. To determine the effect on DNA replication, cells were pulse-labeled for 2 h with BrdUrd 90 h after initial siRNA transfection. BrdUrd incorporation into DNA in NASP siRNA-transfected cells significantly decreased (**p < 0.017**), whereas incorporation into control (C2)-transfected cells was not significantly different (p > 0.8) from untreated cells (Fig. 1B). These results demonstrate that concomitant with the loss of NASP protein 90 h after initial siRNA transfection, there is a decrease in DNA replication.
With the loss of NASP protein, there was also a noticeable effect on cell proliferation, which was determined by directly counting cells. The total number of control (C2)-transfected cells increased significantly during the 90 h following initial transfection (~6-fold; Fig. 1C), whereas the total number of NASP siRNA-transfected cells increased during the first 66 h after transfection (4-fold) and did not significantly increase thereafter from 66 to 90 h (Fig. 1C). The number of NASP siRNA-transfected cells was significantly less than the number of control (C2)-transfected cells \( (p < 0.01) \) at 90 h (Fig. 1C). Examination of the live/dead cell ratio at 24 h posttransfection indicated that <1% of the cells were dead (data not shown). These results demonstrate that the NASP siRNA treatment did not eliminate cells from the culture dish but rather held them in a nonproliferation state.

The effect of a loss of NASP protein on the cell cycle was determined by FACS analysis of control (C2)- and NASP siRNA-transfected cells at 90 h following initial transfection. As shown in Fig. 1D, the number of cells in G1 phase of the cell cycle increased after NASP siRNA transfection concomitant with a decrease in the number of cells in S and G2 phases. These results indicate that a loss of NASP protein causes a delay in cell cycle progression; cells in G1 cannot progress past the G1/S border. Previously, when NASP was overexpressed in HeLa cells, a similar delay in cell cycle progression was observed (12). Consequently, attempts to rescue the siRNA treated cells by transfecting with tNASP cDNA resulted in a large increase in tNASP protein as shown by Western blots and a modest increase in BrdUrd incorporation, but the overall effect was a continued delay in cell cycle progression and increased numbers of cells in G1 as reported previously (Ref. 12 and data not shown).

Immunolocalization of BrdUrd and NASP in the nuclei of control cells after incubation with BrdUrd for 18 h, demonstrated that all cells contained both NASP and BrdUrd in their nuclei (Fig. 2, A and B); however, 90 h after NASP siRNA transfection, immunostaining for NASP protein had decreased in many cells (Fig. 2C, arrows), and BrdUrd staining was absent in these cells (Fig. 2D). We conclude from these experiments that the loss of NASP from the nucleus coincided with the loss of BrdUrd incorporation into DNA and the completion of S phase in HeLa cells. However, because of a reservoir of NASP protein, it takes at least 3–4 days before the lack of NASP protein affects cell proliferation.

Comparison of NASP and SLBP Knockdown in U2OS Cells—SLBP binds histone mRNA stem loops and is required for histone message synthesis and translation (21). Cells synthesize SLBP just prior to entering S phase (22) and require SLBP for proper DNA replication (20). When treated with SLBP, siRNA

![FIGURE 3. NASP and SLBP knockdown in U2OS cells. A, Western blot of NASP and SLBP in U2OS cells treated with C2 siRNA (control siRNA having no cellular target) (lane 1), NASP siRNA (lane 2), or U2OS siRNA (lane 3). Proteins were detected 90 h after siRNA transfection. Symplekin was the loading control protein. Western blot was probed with anti-NASP, anti-SLBP, or anti-symplekin antibody. B, cell cycle changes induced by transfection of U2OS cells with NASP, SLBP, or control (C2) siRNA. FACS analysis demonstrates an increase in NASP siRNA treated cells in G1 phase and a decrease of cells in S-G2; in contrast, SLBP siRNA-treated cells demonstrate an increase of cells in S phase and a decrease of cells in G1.]

21530 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 30 • JULY 28, 2006
cells are treated with aphidicolin or hydroxyurea; cells reach the G₁/S border, and SLBP reaches maximal levels (24).

**NASP Knock-out**

**NASP Is Required for Normal Development**—If NASP (GenBank™ accession number AF349432) is knocked out for normal completion of the cell cycle, then it should be required for normal development. To test this possibility, the NASP gene was inactivated by homologous recombination (Fig. 4). The targeted disruption of NASP was achieved by deletion of six exons encoding amino acid residues 100–605 in the TNASP sequence and amino acid residues 74–253 in the sNASP sequence (15), knocking out all of the histone-binding sites. Homologous recombination of the targeting vector into the NASP gene introduces a neomycin resistance cassette containing a PstI site and eliminates a SacI site (Fig. 4A). These sites were subsequently used for screening ES cell colonies and mouse tail DNA by Southern blot analysis (Fig. 4B), and their genotypes were confirmed by PCR (Fig. 4C).

Among 333 F₂ progeny, no mice with the null mutation NASP<sup>−/−</sup> were born. NASP<sup>−/−</sup> mice appeared normal in development, gross anatomy, fertility, and behavior and lived normally for at least 1 year. Although quantitation of NASP mRNA in homozygous NASP<sup>−/−</sup>-disrupted cells was not possible because of early embryonic lethality, NASP mRNA isolated from the testes of NASP<sup>−/−</sup> mice was reduced to approximately half of the levels expressed by NASP<sup>+/−</sup> littermates (Fig. 5). There were no truncated mRNA species expressed in the NASP<sup>−/−</sup> mice. Despite the fact that NASP mRNA decreased by half in heterozygotes, the amount of NASP protein remained the same in both NASP<sup>−/−</sup> and NASP<sup>+/−</sup> mice as judged by protein titration on Western blots (data not shown).

**NASP<sup>−/−</sup> Embryos Develop to the Blastocyst Stage**—To determine when embryonic lethality occurred, embryos were examined in utero by serial sections at 3.5, 4.5, 5.5, 6.5, and 8.5 dpc. At 5.5, 6.5, and 8.5 dpc, no implanted embryos were found that were negative for NASP mRNA and/or protein, and therefore there were no truncated NASP<sup>−/−</sup> embryos (n = 16). To determine whether blastocyst stage embryos were viable, embryos were collected at 3.5 dpc and cultured in vitro. After 3–5 days in culture, NASP<sup>−/−</sup> blastocysts appeared smaller than the wild-type or heterozygotes and contained fewer cells (Fig. 6A). Throughout the period of culture, no differences
could be seen in the growth of NASP<sup>+/−</sup> versus NASP<sup>+/+</sup> blastocyst colonies. By 10 days in culture, the NASP<sup>−/−</sup> blastocysts had completely failed to develop (Fig. 6A). As determined by PCR (Fig. 6B), NASP<sup>−/−</sup> mice transmitted the targeted allele to 86% of their blastocysts (n = 56, NASP<sup>+/−</sup> = 8, NASP<sup>+/+</sup> = 33, NASP<sup>−/−</sup> = 15). In situ hybridization of serial sections of 4.5 dpc embryos (n = 22) with a NASP oligonucleotide probe revealed an embryo that was negative for NASP mRNA (and therefore NASP<sup>−/−</sup>; Fig. 7, A–C). An adjacent embryo in the same serial section of uterus was considerably larger in size and positive for NASP mRNA (and therefore NASP<sup>+/−</sup> or NASP<sup>+/+</sup>; Fig. 7, E and G, arrows). Control sections of embryos treated with the in situ probe in the presence of 100× unlabeled probe were negative (Fig. 7L). Serial sections of the NASP<sup>−/−</sup> and the NASP<sup>+/−</sup> embryos at 4.5 dpc were both positive for NASP protein (Fig. 7, D and H, respectively, arrows).

The death of NASP<sup>−/−</sup> embryos most likely coincides with the depletion of the maternally derived NASP protein reservoir. To determine whether a reservoir of stored maternal NASP protein exists, normal embryos were examined for NASP protein (and therefore NASP mRNA; and therefore NASP<sup>−/−</sup> or NASP<sup>+/−</sup>; Fig. 7, A–C). An adjacent embryo in the same serial section of uterus was considerably larger in size and positive for NASP mRNA (and therefore NASP<sup>+/−</sup> or NASP<sup>+/+</sup>; Fig. 7, E and G, arrows). Control sections of embryos treated with the in situ probe in the presence of 100× unlabeled probe were negative (Fig. 7L). Serial sections of the NASP<sup>−/−</sup> and the NASP<sup>+/−</sup> embryos at 4.5 dpc were both positive for NASP protein (Fig. 7, D and H, respectively, arrows).

The cellular requirement for NASP to complete the cell cycle (Figs. 1–3) and consequently the implicit requirement for H1 histones (2, 3, 29) reflect the fact that both NASP (12) and H1 (30, 31) are in a dynamic equilibrium within the nucleus. The equilibrium of NASP with H1 and H1 with DNA appears critical for nucleosome spacing and transcriptional activation. Embryos lacking several H1 variants die in midgestation (2), and embryos lacking NASP protein in the cytoplasm of oocytes (Fig. 7). We do not know whether stored NASP has bound histones in its three histone-binding sites (16) or whether it is associated with HSP90, which facilitates histone binding to NASP (11).

FIGURE 5. NASP<sup>+/−</sup> mice express decreased levels of <i>tN</i>ASP mRNA in their testes. Northern blot analysis was performed using 5, 2, 1, and 0.5 μg of poly(A)<sup>+</sup> RNA from either wild-type (WT) or heterozygous (Het) mice followed by probing with a cDNA that recognizes both testis and somatic NASP. Heterozygous to wild-type ratios of the PhosphorImager volume quantitation of pixel intensities are shown below the blot.
and NASP$^{+/−}$ cells were indistinguishable, suggesting that the production of excess NASP protein may ensure the binding of all non-DNA-bound H1 to NASP and the degradation of any noncomplexed NASP. Consequently, the balance between supply and demand for NASP must be regulated, perhaps in concert with the other proteins in the chaperone complex. This hypothesis is supported by our observation that loss of NASP delays cells in G$_1$ (Figs. 1D and 3B) and by our previous studies on NASP (12), which determined that overexpression of NASP delayed progression through the G$_1$/S border.

The lack of cell proliferation in the absence of NASP, which leads to abnormal development, has been ascribed to the lack of linker histone availability because NASP has been shown to bind linker histones in the cytoplasm, transport them into the nucleus, and transfer them to DNA (11, 12). However, one alternative explanation might be that NASP has a critical role in

FIGURE 7. NASP mRNA and protein in blastocysts. A–D and E–H, serial sections of two blastocysts (4.5 dpc) from the same uterine section. A and C demonstrate by in situ hybridization a blastocyst lacking NASP mRNA (NASP$^{−/−}$), whereas E and G demonstrate NASP mRNA (arrows) in blastocyst cytoplasm (NASP$^{+/−}$). L, in situ control, probed in the presence of 100-fold excess unlabeled probe. B and F, staining with hematoxylin and eosin. D and H demonstrate by anti-NASP antibody staining the presence of NASP in the cytoplasm and nuclei of the NASP$^{+/−}$ blastocyst and in the cytoplasm of the NASP$^{−/−}$ blastocyst (arrows). M, antibody control embryo (6.5 dpc), probed with anti-NASP antibody absorbed with recombinant NASP. I–K demonstrate by anti-NASP antibody staining the presence of NASP (arrows) in the cytoplasm of a NASP$^{+/−}$ oocyte (I, 1$^{\text{st}}$ meiotic division), a one-cell embryo (J) and a three-cell embryo (K). A–C, E–H, L–M, ×40 objective; bar, 20 µm. D, ×100 objective; bar, 10 µm. I–K, ×100 objective; bar, 20 µm.
the multichaperone protein complex that is independent of H1 transport into the nucleus. If the lack of NASP activated a G1/S checkpoint (Chk2) similar to that observed in a dominant-negative mutant of CAF-1 (6), then DNA replication might be delayed independently of the availability of linker histones. In HeLa cells, NASP is a binding partner of the heterodimer Ku70/ Ku80 and its catalytic subunit DNA-PK (13). Ku70/Ku80 and DNA-PK are necessary for DNA double-strand break repair by nonhomologous end joining (4, 32), and H1 histones have an important role in protecting DNA degradation during end joining (33). Thus NASP could have a role in nucleosome remodeling during DNA repair by mediating H1-DNA binding.

Several characteristics of the NASP protein strengthen the hypothesis that NASP has a role in nucleosome remodeling. Mouse sNASP is an acidic protein (pI = 4.23) of 45.8 kDa, which is identical to the tNASP form (pI = 4.16; 83.9 kDa), except that two internal segments of the protein have been deleted (14). Both forms have a leucine zipper, coiled-coil domains, and tetratricopeptide repeat domains, all of which may contribute to the interaction of NASP with other proteins, including HSP90 (11). Whether NASP binds DNA directly remains to be determined. Interestingly, motifs for binding Chk2 and docking the coactivator p300 exist within the NASP sequence and may prove important in future investigations of function.

The multichaperone protein complexes (9) that coordinate the assembly of new nucleosomes in both DNA-dependent (CAF-1) and DNA-independent (HIRA) pathways contain several proteins in common, including Asf1a, HAT1, and NASP (8, 34). The multichaperone complexes containing CAF-1 interact with the DNA replication machinery via proliferating cell nuclear antigen (35), which is the sliding clamp of the replication fork (5, 36). A multiprotein complex containing HAT1 and NASP interacts with the DNA replication machinery via proliferating cell nuclear antigen (35), which is the sliding clamp of the replication fork (5, 36). A multiprotein complex containing HAT1 and NASP-like protein has been reported in yeast; the histone chaperone Hif1p associates with the Hat1p/Hat2p complex following entry into the nucleus and participates in histone-DNA interaction (37). Hif1p and NASP share sequence similarities with the Xenopus protein N1/N2 (37, 38). Consequently NASP appears to be a conserved and critical member of the multichaperone complexes that participate in nucleosome remodeling. We conclude from this study that NASP and, by implication, the linker histones are key players in the assembly of chromatin after DNA is newly replicated and that both NASP and H1 are required to ensure a faithful transition of chromatin from one cell cycle to the next.

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