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The Stem-Loop Binding Protein (SLBP1) Is Present in Coiled Bodies of the *Xenopus* Germinal Vesicle

Jennifer Abbott,* William F. Marzluff,† and Joseph G. Gall*‡

*Department of Embryology, Carnegie Institution, Baltimore, Maryland 21210; and †Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina, 27599

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The stem-loop binding protein (SLBP1) binds the 3′ stem-loop of histone pre-mRNA and is required for efficient processing of histone transcripts in the nucleus. We examined the localization of SLBP1 in the germinal vesicle of *Xenopus laevis* oocytes. In spread preparations of germinal vesicle contents, an anti-SLBP1 antibody stained coiled bodies and specific chromosomal loci, including terminal granules, axial granules, and some loops. After injection of *myc*-tagged SLBP1 transcripts into the oocyte cytoplasm, newly translated *myc*-SLBP1 protein was detectable in coiled bodies within 4 h and in terminal and axial granules by 8 h. To identify the region(s) of SLBP1 necessary for subnuclear localization, we subcloned various parts of the SLBP1 cDNA and injected transcripts of these into the cytoplasm of oocytes. We determined that 113 amino acids at the carboxy terminus of SLBP1 are sufficient for coiled body localization and that disruption of a previously defined RNA-binding domain did not alter this localization. Coiled bodies also contain the U7 small nuclear ribonucleoprotein particle (snRNP), which participates in cleavage of the 3′ end of histone pre-mRNA. The colocalization of SLBP1 and the U7 snRNP in the coiled body suggests coordinated control of their functions, perhaps through a larger histone-processing particle. Some coiled bodies are attached to the lampbrush chromosomes at the histone gene loci, consistent with the view that coiled bodies in the oocyte recruit histone-processing factors to the sites of histone pre-mRNA transcription. The non-histone chromosomal sites at which SLBP1 is found include the genes coding for 5 S rRNA, U1 snRNA, and U2 snRNA, suggesting a wider role for SLBP1 in the biosynthesis of small non-spliced RNAs.

INTRODUCTION

Before being transported to the cytoplasm, histone pre-mRNAs in higher eukaryotes undergo a unique processing reaction involving a single endonucleolytic cleavage to form the 3′ end of the histone mRNA (Birchmeier *et al.*, 1984; Krieg and Melton, 1984; Gick *et al.*, 1986). This cleavage is directed by two conserved sequences within the pre-mRNA, one upstream and one downstream of the cleavage site. The downstream region binds the U7 small nuclear ribonucleoprotein particle (snRNP)[1] via complementarity to part of the U7 snRNA (Strub *et al.*, 1984; Schaufele *et al.*, 1986; Bond *et al.*, 1991). The upstream sequence is a stem-loop that binds an additional factor or factors required for processing (Mowry and Steitz, 1987; Vasserot *et al.*, 1989; Melin *et al.*, 1992). Recently the yeast three-hybrid system (SenGupta *et al.*, 1996) has been used to identify the human stem-loop binding protein (SLBP1) (Wang *et al.*, 1996) or the hairpin-binding protein (HBP) (Martin *et al.*, 1997). Human, mouse, and *Xenopus* SLBP1 are closely related in sequence but are not similar to other known proteins (Wang *et al.*, 1996). A novel RNA-binding domain has been identified in SLBP1 by deletion analysis. SLBP1 binds to the histone pre-mRNA in the nucleus, probably during transcription, and accompanies the mature histone mRNA to

‡ Corresponding author: Department of Embryology, Carnegie Institution, 115 West University Parkway, Baltimore, MD 21210. E-mail address: gall@mail1.ciwemb.edu.

[1] Abbreviations used: DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast; GV, germinal vesicle; NLS, nuclear localization signal; PBS, phosphate-buffered saline; SLBP1, stem-loop binding protein; snRNP, small nuclear ribonucleoprotein particle; TMG, trimethylguanosine.

the cytoplasm, where the complex associates with polyribosomes (Dominski *et al.*, 1995; Hanson *et al.*, 1996). In the *Xenopus* oocyte, unlike in somatic cells, histone mRNA is stored in the cytoplasm and not translated during the long growth phase (Woodland and Adamson, 1977; Woodland, 1980). In the stored form of histone mRNA, SLBP1 is replaced by another stem-loop binding protein, SLBP2, which is oocyte-specific (Wang *et al.*, 1999). The RNA-binding domains of SLBP1 and SLBP2 are similar, but otherwise the two proteins are not obviously related in sequence.

We have examined the localization of SLBP1 in the oocyte nucleus or germinal vesicle (GV) of *Xenopus*. Because of its large size, up to 400 μm in diameter, the GV has been particularly useful for both cytological and molecular analyses of RNA transcription and processing. The major structural components in a GV are the giant lampbrush chromosomes, ~1500 extrachromosomal nucleoli, several thousand small granules (1–4 μm in diameter) known as B-snurposomes, and 50–100 coiled bodies (Wu *et al.*, 1991). Each of these components is involved in some aspect of RNA transcription or processing. Coiled bodies are of particular interest in the present context, because of their association with histone pre-mRNA processing. In both oocytes (Wu and Gall, 1993; Wu *et al.*, 1996) and somatic nuclei (Bauer *et al.*, 1994; Frey and Matera, 1995), coiled bodies are the only structures in which U7 snRNA has been demonstrated. Furthermore, in the GV some coiled bodies are attached to the lampbrush chromosomes at the histone gene loci (Gall *et al.*, 1981; Callan *et al.*, 1991), and a similar association is true in interphase HeLa nuclei (Frey and Matera, 1995). These facts suggest that coiled bodies are involved in the recruitment of processing factors to the sites of histone pre-mRNA transcription and perhaps in the assembly of processing complexes (Wu *et al.*, 1996; Bellini and Gall, 1998). In support of this hypothesis, we find that an antibody against SLBP1 stains coiled bodies in GVs of *Xenopus*. The distribution of SLBP1 within the coiled body is almost identical to that of the U7 snRNP. We have confirmed the localization by demonstrating that epitope-tagged SLBP1 is targeted to coiled bodies, and we have shown that 113 amino acids from the carboxy terminus of SLBP1 are sufficient for targeting to coiled bodies, even though the RNA-binding domain is disrupted.

MATERIALS AND METHODS

Oocytes and GV Spreads

A piece of ovary was surgically removed from a female *Xenopus laevis* anesthetized in 0.15% tricaine methane sulfonate or MS222 (A5040; Sigma, St. Louis, MO). The isolated ovary was held in Ca^{2+}-free OR2 saline (Wallace *et al.*, 1973) containing 0.2% collagenase, type II from *Clostridium histolyticum* (C6885; Sigma), to defolliculate and separate individual oocytes. The defolliculated oocytes were held at 18°C in OR2 saline until used for injections or GV spreads. GV spreads were made as described previously (Gall, 1998). Centrifuged preparations were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.0) for 1 h or longer. GV spreads used for in situ hybridization were held in 70% ethanol for at least 1 h.

Immunofluorescence

GV spreads were rinsed in PBS and blocked with 10% horse serum in PBS for 15 min. Spreads were incubated with primary antibody for 1 h at room temperature and then washed in three changes of PBS for a total of 15 min. Secondary antibody was applied for 1 h at room temperature, and slides were washed again in three changes of PBS. mAbs were used as undiluted culture supernate or diluted 1:3 in 10% horse serum (mAb 9E10), whereas rabbit sera were diluted 1:200 to 1:1000 with 10% horse serum. Secondary antibodies were Cy3- or fluorescein-conjugated donkey anti-rabbit or donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) used at a dilution of 1:500 or 1:250, respectively. In double-labeling experiments, the primary antibodies and their corresponding secondaries were applied successively. Preparations were mounted in 50% glycerol containing phenylenediamine at 1 mg/ml and 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg/ml. Antibodies used for immunofluorescence included X16C, a rabbit polyclonal serum raised against a 17-amino acid peptide from the carboxy terminus of SLBP1 and purified by selection with protein A (Wang *et al.*, 1996), mAb H1 against *Xenopus* coilin (also called SPH-1) (Tuma *et al.*, 1993), mAb K121 against the trimethylguanosine (TMG) cap of snRNAs (Krainer, 1988), mAb H14 against RNA polymerase II (Bregman *et al.*, 1995), and mAb 9E10 against the c-*myc* epitope (Evan *et al.*, 1985). Peptide-1, used for production of antibody X16C, was KDAFDLEPCFIEEELLS; peptide-2, derived from SLBP2 and used here as a nonspecific competitor, was KGHTNDYTYPHWIGL.

Subcloning of SLBP1 and Deletions

Xenopus SLBP1 was amplified by PCR from the original pGad10 cDNA clone described by Wang *et al.* (1996) and subcloned into a derivative of the MT6 vector that contains six copies of the c-*myc* epitope (Roth *et al.*, 1991) and the SV40 nuclear localization signal (NLS) (Wu *et al.*, 1994). PCR primers were designed to amplify the coding region and ~300 nucleotides of the 3′ untranslated region. The primers were JA31 and JA36. Deletions of SLBP1 were amplified with PCR primers and subcloned into the same vector. The deletions were as follows (see Figure 7): *myc*-SLBP1-N: amino acids 1–122 (primers JA31 and JA39); *myc*-SLBP1-NR: amino acids 1–195 (primers JA31 and JA40); *myc*-SLBP1-RC: amino acids 123–253 (primers JA38 and JA36); *myc*-SLBP1-RCΔ: amino acids 140–253 (primers JA32 and JA36); *myc*-SLBP1-R: amino acids 123–195 (primers JA38 and JA40); *myc*-SLBP1-C: amino acids 196–253 (primers JA41 and JA36); *myc*-SLBP1-NC: constructed by subcloning *myc*-SLBP1-N and *myc*-SLBP1-C together at the in-frame *Bam*HI site created by the primers.

The primers were as follows: JA31: 5′-GC + *Bam*HI + amino acids −5–8; JA32: 5′-GC + *Bam*HI + G + amino acids 140–146; JA36: 5′-GC + *Xba*I + GAAAAAGGCTTATACCGTGCC (~300 nucleotides beyond the SLBP1 translation stop codon); JA38: 5′-GC + *Bam*HI + amino acids 123–129; JA39: 5′-GC + *Bam*HI + CCTCCATGTGTAGATGAATC; JA40: 5′-GGC + *Xba*I + GTCCCACTGATGTAGAGCAATTCTCC; JA41: 5′-GC + *Bam*HI + amino acids 196–206.

Injection of myc*-tagged SLBP1 Transcripts*

Capped sense-strand transcripts were synthesized with T3 RNA polymerase (Stratagene, La Jolla, CA) from 1 μg of the appropriate subclone after linearization with *Xba*I. The RNA was analyzed for size on a 1% agarose, 1.3% formaldehyde gel. Aliquots were pre-

cipitated and resuspended at 1 μg/μl; 23 nl of RNA solution was injected into the cytoplasm of defolliculated oocytes using a Drummond Nanoject injection apparatus (Drummond Scientific, Broomall, PA). Injected oocytes were transferred to OR2 saline and stored at 18°C until used for GV spreads or Western analysis. *myc*-tagged SLBP1 was detected on GV spreads and Western blots with mAb 9E10, which is specific for the c-*myc* epitope (Evan *et al.*, 1985).

Western Blotting

Isolated GVs were centrifuged to separate organelles from the nucleoplasm. All samples were boiled with the appropriate amount of 2× gel buffer for 5–10 min and electrophoresed on a 10% polyacrylamide-SDS gel (Laemmli, 1970). Proteins were electroeluted from the gel onto polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA). Membranes were blocked in 5% dried milk plus 0.05% Tween in PBS and incubated with the appropriate primary antibody. Detection of signal was by chemifluorescence (ECF kit; Amersham, Arlington Heights, IL) with the Storm 860 Scanner (Molecular Dynamics, Sunnyvale, CA).

RESULTS

SLBP1 Is Present in Coiled Bodies and Specific Chromosomal Loci

Cytological studies of SLBP1 in the *Xenopus* GV were performed primarily with antibody X16C, a rabbit polyclonal serum raised against a 17-amino acid peptide from the carboxy terminus of SLBP1 and purified by selection with protein A (Wang *et al.*, 1996). The antibody was used to stain organelles isolated from single GVs. The nuclear envelope of a manually isolated GV was removed with forceps, and the gelatinous contents were allowed to spread on a standard microscope slide (Gall, 1998). After centrifugation of the slide, the soluble nucleoplasm was washed away, leaving the chromosomes, nucleoli, B-snurposomes, and coiled bodies attached to the slide (Wu *et al.*, 1991). Coiled bodies in the oocyte are large structures up to 10 μm in diameter, divided into three morphological compartments (Figure 1). Preparations immunostained with serum X16C showed a strong reaction in coiled bodies. The stain was clearly present in the matrix but excluded from both the B-snurposomes on the surface and the internal B-like inclusions (Figure 2, A–H). The unstained B-snurposomes and inclusions were visible in the differential interference contrast (DIC) image (Figure 2, A, E, and I). All coiled bodies in the GV stained with SLBP1, including those attached to the lampbrush chromosomes at the histone gene loci (Figure 2M). To identify coiled bodies unambiguously, we costained GV spreads with serum X16C and mAb H1 against *Xenopus* coilin (also called SPH1) (Tuma *et al.*, 1993). The two antibodies stained with essentially the same pattern (Figure 2, A–H). GV spreads were also costained with serum X16C and mAb K121, which is specific for the TMG cap of snRNAs (Krainer, 1988). Earlier experiments demonstrated that mAb K121 stains U7 snRNA in the matrix

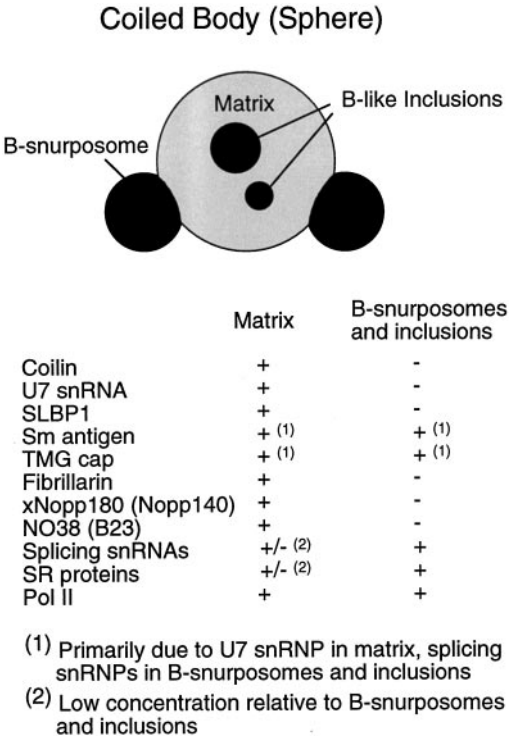
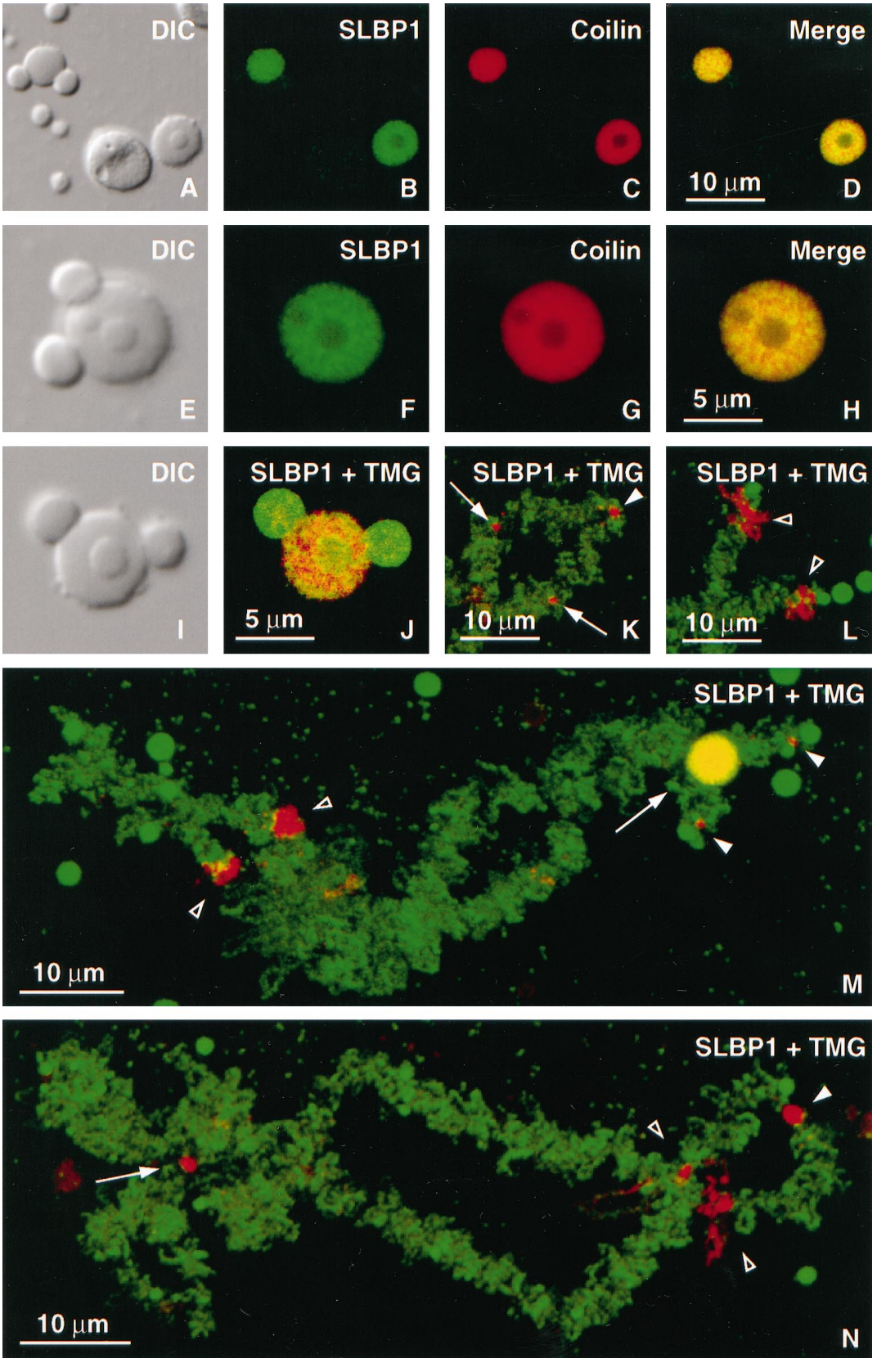


Figure 1. Diagram of a coiled body from a *Xenopus* GV and a list of some of its molecular components. The coiled body consists of three parts: a matrix, B-snurposomes attached to the surface, and B-like inclusions. The number of attached B-snurposomes and inclusions is variable, and they may be absent. The attached B-snurposomes are identical in all respects to the hundreds of free B-snurposomes in the nucleoplasm. The inclusions are generally smaller than B-snurposomes but are otherwise identical. The terms coiled body and sphere are used synonymously. In some previous publications (Wu *et al.*, 1996), the term C-snurposome referred to the matrix and inclusions only.

of the coiled body and splicing snRNAs in the B-snurposomes and inclusions (Wu *et al.*, 1991; Wu and Gall, 1993; Bellini and Gall, 1998). Figure 2, I and J, shows that X16C and K121 both stain the matrix of the coiled body, whereas only K121 stains the B-snurposomes and the inclusion.

Because X16C is a polyclonal rabbit serum that might cross-react with proteins of unknown nature in the GV, we tested its specificity by immunostaining in the presence of two peptides: peptide-1, the 17-amino acid sequence used for immunization, and peptide-2, an unrelated sequence derived from SLBP2. Peptide-2 had no effect on the staining of X16C. By contrast, peptide-1 caused a marked reduction in the staining of coiled bodies, the extent of which was determined by measuring the intensity of stain with a charge-coupled device (CCD) camera. Figure 3A is a graph of fluorescence intensity versus coiled body volume for preparations stained with secondary antibody only or with X16C in the presence of peptide-1 or peptide-2. Pep-


DIC
A
SLBP1
B
Coilin
C
Merge
10 μm
D
DIC
E
SLBP1
F
Coilin
G
Merge
5 μm
H
DIC
I
SLBP1 + TMG
5 μm
J
SLBP1 + TMG
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L
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M
SLBP1 + TMG
10 μm
N

Figure 2.

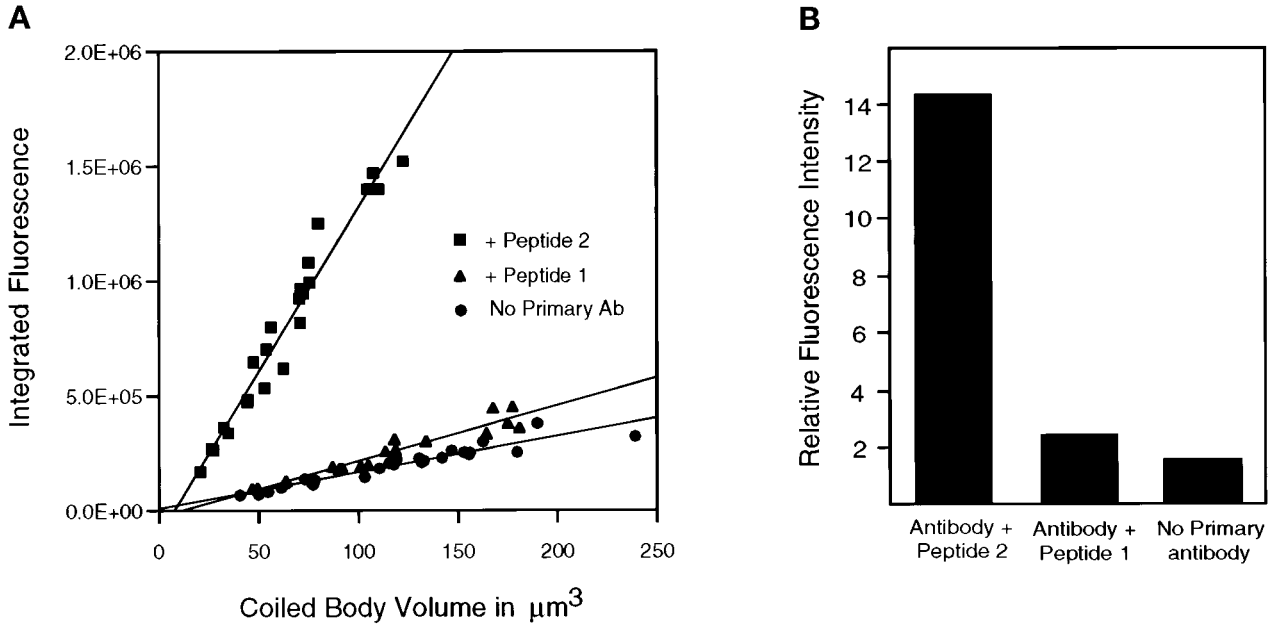

Figure 3. Staining of coiled bodies with anti-SLBP1 serum X16C is reduced by treatment with peptide-1, against which the antibody was raised. (A) Images of stained coiled bodies were taken with a charge-coupled device (CCD) camera; the total fluorescence from individual coiled bodies was then measured as the sum of pixel values and plotted as a function of coiled body volume. The linear relationship shows that the amount of SLBP1 in a coiled body is proportional to its volume. Staining was markedly reduced by previous treatment of the antibody with peptide-1, whereas the unrelated peptide-2 had no effect. (B) The slopes of the curves in A were determined and compared. The slope represents the amount of stain per unit volume and is assumed to be a measure of stain per unit of SLBP1.

tide-1 reduced the staining of X16C approximately sevenfold, to a level only slightly above the background because of secondary antibody alone (Figure 3B). These data support the assumption that X16C staining in coiled bodies is caused by SLBP1. In summary, immunostaining with X16C demonstrates that SLBP1 occurs in the matrix of oocyte coiled bodies, where it is colocalized with coilin and the U7 snRNP. This finding strengthens the view that coiled bodies are involved in some aspect of histone pre-mRNA processing. Because some coiled bodies are attached to the lampbrush chromosomes at the histone gene loci, an attractive hypothesis is that coiled bodies recruit processing components to the site of histone gene transcription, where they may be assembled into functional processing complexes (Wu *et al.*, 1996; Bellini and Gall, 1998).

Serum X16C also stained several chromosomal loci: all terminal granules (Figure 2, K, M, and N), several so-called axial granules (Figure 2, K and N), and approximately a dozen sets of specific transcription loops (Figure 2, L–N). In each case, staining was essentially abolished by pretreatment of the antibody with peptide-1 against which it was raised. Although it was easy to rationalize the colocalization of SLBP1 with the U7 snRNP in coiled bodies, staining of ter-

Figure 2 (facing page). Localization of SLBP1 in the *Xenopus* GV by immunofluorescence. Stained images were taken with a Leica (Heidelberg, Germany) TCS NT confocal microscope. (A–D) DIC and immunofluorescence images of two coiled bodies, a nucleolus, and several B-snurposomes double-stained with serum X16C against SLBP1 (fluorescein) and mAb H1 against *Xenopus* coilin (Cy3). SLBP1 and coilin are both limited to the matrix of the coiled body, being excluded from the B-snurposomes on the surface (left coiled body) and the internal B-like inclusion (right coiled body). (E–H) Higher magnification of a similar coiled body that has two B-snurposomes on the surface and two inclusions. Note the patchy distribution of SLBP1 staining and the lack of complete correspondence between the SLBP1 and coilin stains in the merged image. (I and J) Coiled body double-stained with serum X16C for SLBP1 (Cy3) and mAb K121 for the TMG cap of snRNAs (fluorescein). mAb K121 detects U7 snRNA in the matrix of the coiled body (Bellini and Gall, 1998) and splicing snRNAs in the B-snurposomes and inclusions (Wu *et al.*, 1991). (K–N) Lampbrush chromosomes double-stained with serum X16C for SLBP1 (Cy3) and mAb K121 for TMG (fluorescein). The nascent transcripts on almost all chromosome loops are associated with splicing snRNAs and hence stain strongly for TMG. Those few loops that stain red with serum X16C do not stain with mAb K121, presumably because they lack splicing snRNAs (open arrowheads in L–N). Terminal granules stain only with X16C (filled arrowheads in K, M, and N); the same is true of axial granules (arrows in K and N). M shows chromosome 9, which has a coiled body attached at the histone gene locus (arrow). In this case the single coiled body joins the two homologous chromosomes; in other cases each homologue may have its own coiled body. The coiled body is yellow, because it stains with both antibodies.

minal granules, axial granules, and specific loops came as a surprise, because these structures have no known association with histone genes or histone pre-mRNA transcription and processing. Terminal and axial granules were originally described on the basis of their morphology and staining characteristics (Callan, 1986, pp. 92–95; Pyne *et al.*, 1995; Hock *et al.*, 1996). They consist of small masses of DAPI-negative material attached to DAPI-positive chromomeres; terminal granules occur at the very tip of the chromosome, whereas axial granules occupy interstitial positions. Both terminal and axial granules are recognizable in *Xenopus* lampbrush chromosomes by their intense staining with antibodies against polymerase II (Gall and Murphy, 1998). Terminal granules are found at the end of the long arm on 15 of the 18 *Xenopus* lampbrush bivalents, and all of these sites stain with serum X16C. Previously these 15 sites were shown by in situ hybridization to be associated with oocyte-type 5S rRNA genes (Pardue *et al.*, 1973; Callan *et al.*, 1988).

For the most part, the genes at the sites of axial granules are unknown, but we have found that both the U1 and U2 snRNA genes are associated with axial granules. In *Xenopus* the U1 and U2 genes are arranged in tandem arrays. There are ~500 copies of the U1 snRNA genes per haploid genome (Lund *et al.*, 1984) and ~500–1000 copies of U2 (Mattaj and Zeller, 1983). The highly repetitive nature of these genes greatly facilitated their detection on lampbrush chromosomes. Fluorescence in situ hybridization with a biotinylated probe revealed that U1 genes occur at an axial granule on the long arm of chromosome 2 (our unpublished data). As shown by immunofluorescence, this axial granule also stains with serum X16C against SLBP1 (Figure 2K, arrows). The U2 genes are similarly situated at a single site associated with an SLBP1(+) axial granule on the long arm of chromosome 11. In this case SLBP1(+) loops also occur at the site (Figure 2N, open arrowheads). X16C stains several other axial granules, about which we have no molecular information; an example is that near the centromere of chromosome 11 (Figure 2N, arrow). Interestingly, coiled bodies are attached at the U1 and U2 gene sites in HeLa and HEp-2 cell nuclei (Frey and Matera, 1995; Smith *et al.*, 1995; Matera, 1998). Although coiled bodies as such are not present at these loci in the *Xenopus* GV (Figure 2, K and N), the common factor here may be SLBP1. That is, the association of coiled bodies with the U1 and U2 genes in somatic nuclei may be a mechanism for bringing SLBP1 (and other molecules) to these sites.

A strong reaction with serum X16C was also observed on specific loops on several lampbrush chromosomes. Staining was limited to a small number of loops at each site and was usually detectable on both homologues (Figure 2, L–N). Although morphologically unremarkable, these loops share an unusual

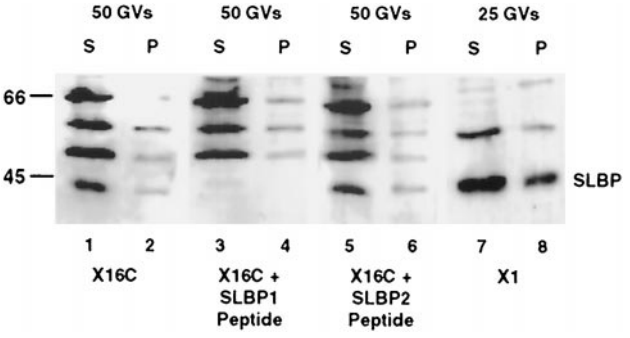

Figure 4. Western blot of GV proteins probed with antibodies against *Xenopus* SLBP1. GV contents were centrifuged to separate the soluble nucleoplasm (S) from the pellet (P), which contains chromosomes, nucleoli, B-snurposomes, and coiled bodies. (Lanes 1 and 2) Proteins from 50 GVs probed with antibody X16C. (Lanes 3 and 4) Proteins from 50 GVs probed with X16C in the presence of peptide-1, against which the antibody was raised. A single band with a mobility corresponding to SLBP1 (~40 kDa) is specifically competed. (Lanes 5 and 6) Proteins from 50 GVs probed with X16C in the presence of the unrelated peptide-2. (Lanes 7 and 8) Proteins from 25 GVs probed with antibody X1, which is more specific for SLBP1 on Western blots but gives poor immunofluorescent staining. Molecular weight standards are on the left.

characteristic; they do not have splicing factors associated with their nascent transcripts. This is shown by their failure to stain with mAb Y12, mAb SC35, and mAb K121, which recognize, respectively, the Sm proteins, members of the SR group of non-snRNP splicing factors, and the TMG cap found on splicing snRNAs (Figure 2, L–N). By contrast, the overwhelming majority of lampbrush loops contain splicing machinery and react strongly with these three antibodies (Figure 2, K–N) (Wu *et al.*, 1991).

Of several antibodies against SLBP1 available to us, X16C gave the strongest immunofluorescent signal on coiled bodies and chromosomes and had a particularly low background on other nuclear organelles. However, it reacted with several bands on Western blots of GV proteins. Isolated GVs were centrifuged to separate insoluble organelles (chromosomes, nucleoli, B-snurposomes, and coiled bodies) from the soluble nucleoplasm, and the fractions were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to an Immobilon membrane for immunostaining. Antibody X16C reacted with several bands, one of which had the mobility expected of SLBP1 (~40 kDa) (Figure 4, lanes 1 and 2). The 40 kDa band, but none of the other bands, was competed by the SLBP1 peptide-1 (Figure 4, lanes 3 and 4), whereas the unrelated peptide-2 had no effect on any bands (Figure 4, lanes 5 and 6). Most SLBP1 was in the soluble nucleoplasm, although a detectable signal was seen in the insoluble pellet. It is presumably this small amount of insoluble SLBP1 that is detected by immunofluorescent staining in coiled bodies and chromosomes. We do not know

the nature of the other bands that cross-react with serum X16. Although most of the cross-reacting material is in the soluble fraction and would be washed out of the cytological preparations, some is in the insoluble pellet and must be on the stained slides. It presumably contributes to the general background but does not interfere with detection of SLBP1 itself. A much cleaner Western blot was given by antibody X1 (Wang *et al.*, 1999), an affinity-purified rabbit polyclonal serum that was raised against SLBP1 expressed in *Baculovirus* (Figure 4, lanes 7 and 8). Unfortunately, this antibody gave a high level of nonspecific staining when used for immunofluorescence.

myc-*tagged SLBP1 Is Targeted to Coiled Bodies and to Terminal and Axial Granules of Chromosomes*

To confirm the localizations derived from immunofluorescence, we examined the intranuclear localization of epitope-tagged SLBP1. A full-length SLBP1 cDNA was subcloned into a vector that carried the T3 RNA polymerase promoter, six copies of the c-*myc* epitope (Roth *et al.*, 1991), and the SV40 NLS (Wu *et al.*, 1994). Capped sense-strand transcripts were synthesized with T3 RNA polymerase and injected into the cytoplasm of stage V–VI *Xenopus* oocytes. After incubation for 24 h, oocytes were separated into GV and cytoplasmic fractions for Western analysis with mAb 9E10, which is specific for the c-*myc* epitope. A single protein product migrating at ~50 kDa was present in GVs of injected oocytes (Figure 5, lanes 1 and 2). The same Western blot, when reprobed with antibody X1 against SLBP1, demonstrated both the endogenous 40-kDa SLBP1 and newly synthesized 50-kDa *myc*-SLBP1 (Figure 5, lanes 3 and 4). There is somewhat more newly synthesized than endogenous SLBP1 in the GV at this time point.

GV spreads were prepared at various times from 2 to 24 h after injection of *myc*-SLBP1 transcripts. Within 4 h the matrix of coiled bodies was detectably stained by anti-*myc* antibody, and at later times the staining became increasingly strong (Figure 6, A and B). B-snurposomes attached to the surface of the coiled bodies, which are visible in the DIC image of Figure 6B, did not stain. Thus, newly synthesized SLBP1 is rapidly and specifically targeted to the matrix of coiled bodies. The anti-*myc* staining pattern in coiled bodies of injected oocytes was essentially the same as that seen with the X16C antibody in uninjected oocytes, supporting the conclusion that X16C accurately detects endogenous SLBP1 in coiled bodies.

During the first few hours after injection, staining with anti-*myc* antibody was limited to the coiled bodies. At later time points, all structures in the GV began to show detectable label, especially the nucleoli and the loops of the lampbrush chromosomes. The staining of loops was generalized and relatively weak. At

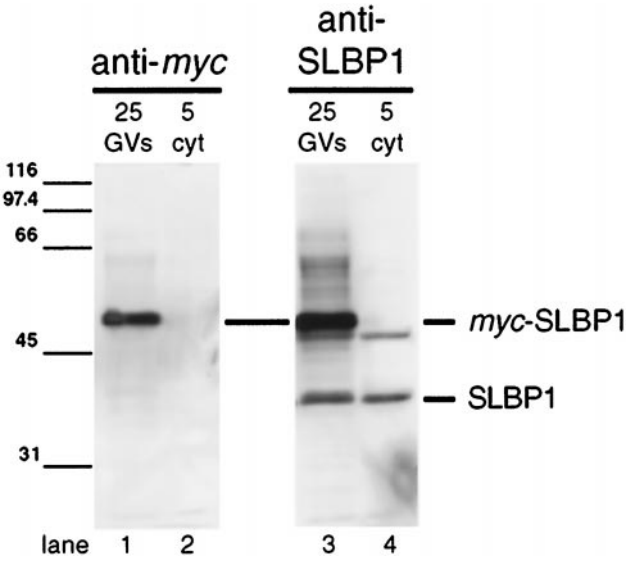

Figure 5. Transcripts of full-length *myc* plus NLS–tagged SLBP1 were injected into the oocyte cytoplasm. Sixteen hours later, GVs and cytoplasms (cyt) were manually isolated, and their proteins were separated on an SDS-polyacrylamide gel. Western blots were made with mAb 9E10 against the c-*myc* tag (lanes 1 and 2) or with antibody X1 against SLBP1 (lanes 3 and 4). All *myc*-tagged SLBP1 is found in the nucleus and is recognized by both antibodies at a mobility corresponding to ~50 kDa. Antibody X1 also recognizes endogenous SLBP1 in both nucleus and cytoplasm at a mobility of ~40 kDa. It also stains a minor cross-reacting band in both nucleus and cytoplasm just below the heavy band of *myc*-SLBP1.

no time did we see highlighting of specific loops, which was such a prominent feature of the staining with antibody X16C. On the other hand, terminal and axial granules did stand out above the increased level of overall staining. An example is shown in Figure 6, C and D. In this case two B-snurposomes are attached to a chromosome very near a terminal granule. These structures appear similar in the DIC image (Figure 6D), but the terminal granule is brighter than the B-snurposomes after staining with anti-*myc* antibody (Figure 6C). To provide certain identification of these otherwise inconspicuous structures, some preparations were counterstained with mAb H14 against RNA polymerase II, which gives an intense stain in axial and terminal granules.

We interpret the strong staining of coiled bodies at early time points after injection and the weak but specific staining of terminal and axial granules at later time points as indicative of the true intranuclear distribution of SLBP1. On the other hand, the high generalized staining of nucleoli and chromosome loops at later time points is more problematic. Although it is possible that newly expressed SLBP1 normally transits these structures, it may be that nonspecific "sticking" of *myc*-SLBP1 occurs during specimen preparation. An advantage of the oocyte system, as compared with

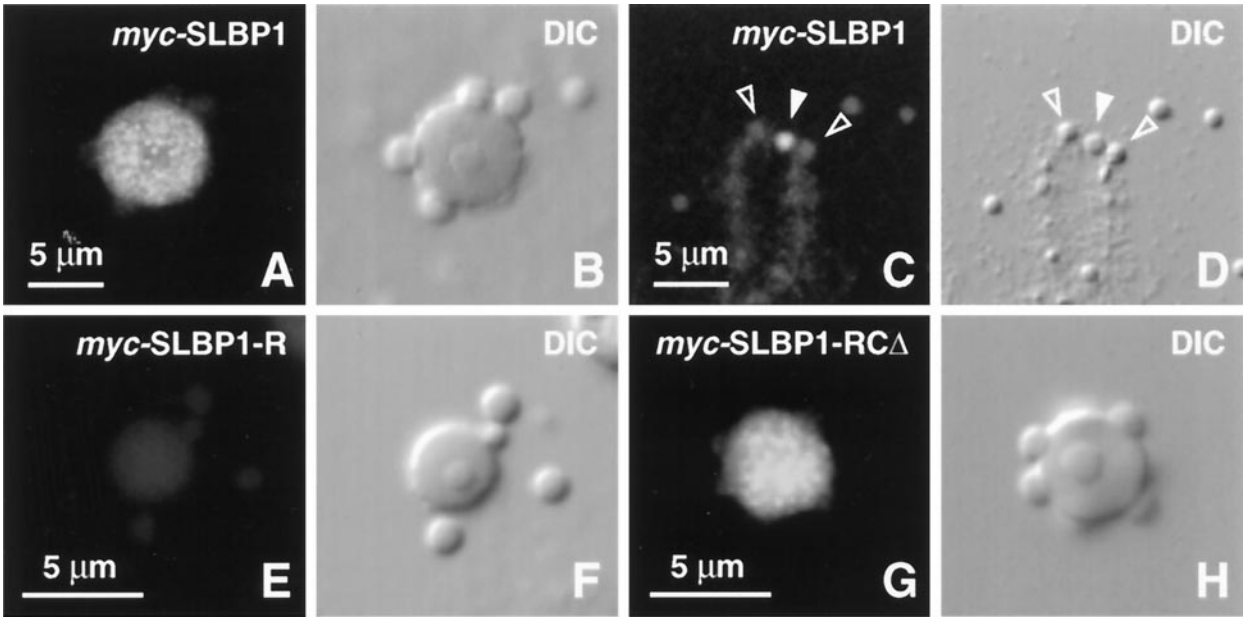

Figure 6. Localization of *myc* plus NLS–tagged SLBP1 in GV spreads. Each pair of panels shows a DIC image and the corresponding immunofluorescent image after staining with mAb 9E10 against the c-*myc* tag. (A and B) A single coiled body from an oocyte injected 8 h previously with transcripts of full-length *myc*-SLBP1. The tagged protein accumulates in the matrix of the coiled body in a distinctly granular pattern. (C and D) End of a chromosome 8 h after a similar injection. The solid arrowhead points to a stained terminal granule; the open arrowheads point to two adjacent unstained B-snurposomes. (E and F) A single coiled body and three B-snurposomes 8 h after injection of transcripts of *myc*-SLBP1-R, which consists of the RNA-binding domain alone. Staining is at background level. (G and H) A single coiled body 8 h after injection of transcripts of *myc*-SLBP1-RCΔ. This protein accumulates in the matrix of the coiled body despite lacking a functional RNA-binding domain.

transient transfections, is that the amount of newly translated protein can be regulated to avoid high levels of overexpression and consequent mislocalization. Nevertheless, even in these experiments, in which the total amount of new protein produced in 24 h is only slightly more than the endogenous level, the rate of new synthesis is well above normal levels and may lead to spurious localization.

Amino Acids 140–253 Are Sufficient to Target SLBP1 to Coiled Bodies

We examined deletion constructs of SLBP1 to determine which region(s) of the protein is required for targeting to coiled bodies. In the original description of SLBP1, Wang *et al.* (1996) identified a 73-amino acid RNA-binding domain (amino acids 123–195), which was defined by its ability to cause a gel shift of the stem-loop structure found at the 3′ end of histone pre-mRNA. Otherwise, there are no known motifs with specific functions or similarities to other proteins in the data bases. For this reason our analysis was based on constructs that divided the molecule into three segments: an amino-terminal fragment (amino acids 1–122), the RNA-binding domain (amino acids 123–195), and a carboxy-terminal fragment (amino acids 196–253). Appropriate fragments were amplified by PCR from the original pGAD10 clone and subcloned into the MT6 vector plus the SV40 NLS. Because the NLS of SLBP1 has not been identified, the addition of the SV40 NLS ensured that all deletion constructs would be transported to the nucleus. The constructs used for injection are shown in Figure 7A. Capped, *myc*-tagged RNA was transcribed in vitro and injected into the oocyte cytoplasm. GVs and cytoplasms were isolated after 8 h and prepared for Western blotting with mAb 9E10 against the c-*myc* epitope. As shown in Figure 7B, all constructs were expressed at readily detectable levels in the oocyte and were localized primarily to the GV.

GV spreads were also prepared 8 h after injection and stained with mAb 9E10. Constructs that contained the amino-terminal fragment (*myc*-SLBP1-N, amino acids 1–122), the amino terminus plus the RNA-binding domain (*myc*-SLBP1-NR, amino acids 1–195), or the RNA-binding domain alone (*myc*-SLBP1-R, amino acids 123–195) failed to accumulate in any specific structure (Figure 6, E and F). These results demonstrated that the first two-thirds of the molecule did not contain sufficient information for targeting to the coiled bodies. However, constructs that contained only the carboxy terminus (*myc*-SLBP1-C, amino acids 196–253) or the carboxy and amino termini together

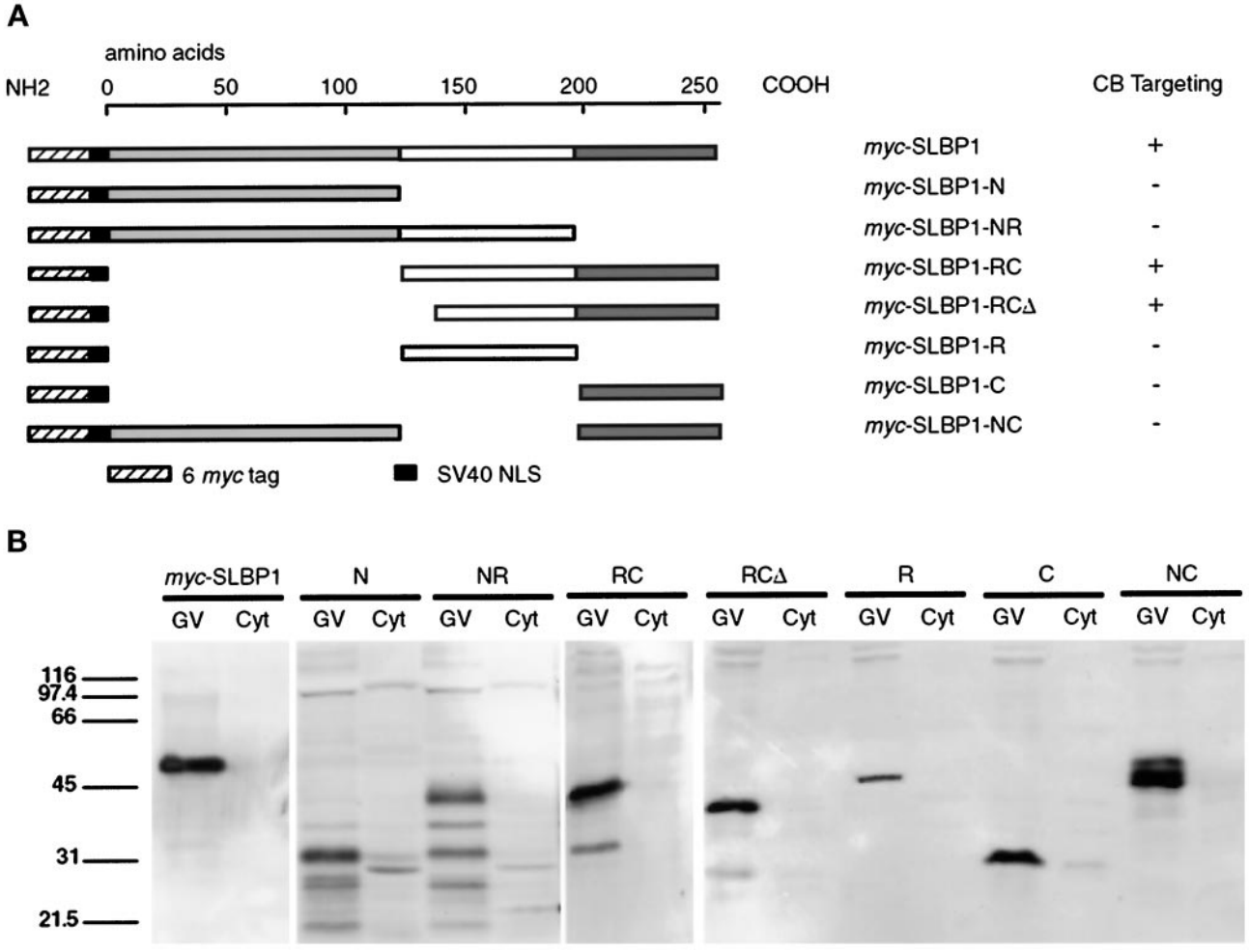

Figure 7. (A) Full-length *myc* plus NLS–tagged SLBP1 and constructs were derived by deleting the amino terminus (amino acids 1–122), the RNA-binding region (amino acids 123–195), or the carboxy terminus (amino acids 196–253), either singly or in pairs. Construct *myc*-SLBP1-RCΔ had a further deletion of 17 amino acids from the left end of the RNA-binding region. The table on the right shows which constructs were targeted to coiled bodies (CB). (B) In vitro transcripts were produced from clones encoding these constructs and were injected into the cytoplasm of stage V–VI oocytes. GVs and cytoplasm (Cyt) were isolated 10 h later, and expressed proteins were detected on Western blots with mAb 9E10 against the c-*myc* epitope. In each case a protein was detected in the GV, whereas little reactivity was seen in the cytoplasm. All constructs included an SV40 NLS to ensure import into the GV. Construct *myc*-SLBP1-R has an anomalously low mobility.

(*myc*-SLBP1-NC, amino acids 1–122 + 196–253) also failed to accumulate in any specific structure, suggesting that either the entire protein was required for targeting to coiled bodies or the necessary sequences overlapped two or more regions covered by the deletions. The latter turned out to be the case, because a construct consisting of the RNA-binding domain and the carboxy terminus (*myc*-SLBP1-RC, amino acids 123–253) accumulated within coiled bodies at levels comparable with the full-length protein. As with full-length *myc*-SLBP1, the staining pattern of *myc*-SLBP1-RC was limited to the matrix of coiled bodies, being excluded from the B-snurposomes on the surface and the inclusions.

To determine whether a functional RNA-binding domain was necessary for coiled body localization, we deleted 17 additional amino acids from the RNA-binding domain (*myc*-SLBP1-RCΔ, amino acids 140–253). This construct also accumulated in coiled bodies at a level similar to that of the full-length protein (Figure 6, G and H). In their original study, Wang *et al.* (1996) found that a deletion of just seven amino acids from the amino end of the RNA-binding domain eliminated in vitro binding to the stem-loop structure. Thus, targeting of SLBP1 to coiled bodies is not dependent on an intact RNA-binding domain.

We looked for targeting of the deletion constructs to terminal and axial granules, especially in the case of

myc-SLBP1-RC and *myc*-SLBP1-RCΔ, which accumulate in coiled bodies. In no case did we see convincing staining of the chromosomal granules. It is possible that chromosomal targeting requires protein interactions different from those involved in coiled body localization. However, because high background staining made targeting of even the full-length protein to the chromosomes somewhat difficult to demonstrate, failure to detect the deletion constructs may have been attributable to insufficient sensitivity.

DISCUSSION

A polyclonal serum (X16C) raised against a carboxy-terminal peptide from *Xenopus* SLBP1 showed strong immunofluorescent staining of coiled bodies in spread preparations of *Xenopus* GV contents. Staining was markedly reduced by pretreatment of the antibody with the peptide antigen against which it was raised. Furthermore, when a *myc*-tagged SLBP1 transcript was injected into the oocyte cytoplasm, newly translated SLBP1 was imported into the nucleus and targeted to the coiled bodies. Other minor sites were recognized by the antibody and by the tagged protein (see below), but coiled bodies are the most prominent structures in the GV that contain SLBP1. Coiled bodies in the GV are variable in size, ranging up to 10 μm or more in diameter, and they exhibit considerable substructure that is not detectable in the much smaller coiled bodies of somatic nuclei (Figure 1). Specifically, coiled bodies in the GV often have one or more smaller granules on their surface that are identical in structure and composition to the many hundreds or thousands of B-snurposomes present throughout the GV. In addition, they may have one to many B-like inclusions (Wu *et al.*, 1991). Previous immunofluorescence and in situ hybridization studies demonstrated sharp differences in the composition of these components. The B-snurposomes and B-like inclusions contain splicing factors (all five splicing snRNPs plus SR proteins), whereas the body or matrix of the coiled body contains the U7 snRNP and the coiled body–specific marker protein p80-coilin (originally called SPH-1 in *Xenopus*) (Wu *et al.*, 1991, 1994; Tuma *et al.*, 1993; Wu and Gall, 1993; Gall *et al.*, 1995; Bellini and Gall, 1998).

Both the staining results and the targeting of *myc*-tagged SLBP1 show that SLBP1 is colocalized with the U7 snRNP and coilin in the matrix of coiled bodies. Thus, the two major factors known to be necessary for histone pre-mRNA processing occur together within the same subcompartment of the coiled body. However, as can be seen in Figure 2, H and J, staining with anti-SLBP1 serum has a distinctly mottled appearance that is not apparent with either mAb Y12 (against Sm proteins) or mAb K121 (against TMG), both of which are markers for the U7 snRNP in the matrix of coiled bodies (Bellini and Gall, 1998). Similarly, *myc*-SLBP1 that has been targeted to the matrix of the coiled body has an inhomogeneous distribution, as visualized by anti-*myc* antibody (Figure 6, A and G). Thus, it is possible that SLBP1 and the U7 snRNP are not strictly colocalized. On the other hand, electron micrographs of the matrix (see Gall *et al.* [1995], their Figure 3) show a relatively homogeneous population of 30- to 50-nm particles, without much suggestion of higher order substructure that would be resolvable by light microscopy.

Not only do coiled bodies contain the two major components required for histone pre-mRNA processing, but some coiled bodies are attached to lampbrush chromosomes at the histone gene loci (Gall *et al.*, 1981; Callan *et al.*, 1991). A similar preferential association of coiled bodies with histone genes has been demonstrated in HeLa cell nuclei (Frey and Matera, 1995). Histone gene transcription in the GV takes place on loops of the lampbrush chromosomes that are immediately adjacent to the attached coiled bodies (Gall *et al.*, 1983; Diaz and Gall, 1985), but there is no evidence that transcription occurs within the coiled bodies themselves. These relationships suggest two possible, nonexclusive functions for coiled bodies. The first is that coiled bodies recruit SLBP1 and the U7 snRNP to the sites of histone gene transcription. Such a role for coiled bodies was first suggested by the targeting of U7 snRNA to coiled bodies within minutes after injection into the cytoplasm of oocytes (Wu *et al.*, 1996). The targeting of *myc*-SLBP1 in the similar experiments reported here is not so rapid, although this may reflect the time required for translation of sufficient protein from the injected transcripts or the sensitivity of the assay. Another possible function for coiled bodies, suggested by the presence of both SLBP1 and the U7 snRNP in the matrix, is the formation of a bifunctional complex that would simultaneously engage the stem loop and the U7-binding sequence of histone pre-mRNA. In preliminary experiments we were unable to demonstrate an interaction between SLBP1 and the U7 snRNP in nuclear extracts by coimmunoprecipitation with anti-SLBP1 antibodies. Such a complex might represent only a small fraction of the total SLBP1 in the nucleus, or it might involve weak interactions that are difficult to demonstrate. Both complications were encountered in a recent demonstration of interaction between coilin and the U7 snRNP (Bellini and Gall, 1998). Alternatively, a stable complex between SLBP1 and the U7 snRNP may occur only in the presence of histone pre-mRNA (Dominski, personal communication).

Only the carboxy-terminal third of the SLBP1 molecule is required for targeting to coiled bodies. Of particular interest is the fact that a functional RNA-binding domain is not needed. That is, an SLBP1 frag-

ment that cannot interact with the stem loop of histone pre-mRNA is targeted to the coiled body as well as the full-length protein. Thus, SLBP1 does not need to engage the histone pre-mRNA before it goes to the coiled body. A similar situation exists for U7 snRNA, which does not require its histone pre-mRNA–binding region for targeting to the coiled body (Wu *et al.*, 1996). It is possible, therefore, that both major factors involved in histone pre-mRNA processing, SLBP1 and U7 snRNA, may go to the coiled body before binding to histone pre-mRNA.

The discussion to this point has focused on SLBP1 in coiled bodies, especially those coiled bodies attached to the chromosomes at the histone gene loci. A complete account of SLBP1 in the GV must accommodate two additional facts: first, that most of the 50–100 coiled bodies in the GV are free in the nucleoplasm (not associated with histone genes) and, second, that most SLBP1 in the GV is not in coiled bodies but in the "soluble" phase of the nucleoplasm. The most probable explanation is that much of the SLBP1 in the GV is not functional in the sense of being actively engaged in histone pre-mRNA processing but rather is being stored in the oocyte for later use during embryogenesis (Wang *et al.*, 1999). Many other molecules in the GV, including components of coiled bodies such as coilin (Bauer and Gall, 1997), the Sm proteins (Forbes *et al.*, 1983; Zeller *et al.*, 1983; Fritz *et al.*, 1984), and U7 snRNA (our unpublished data), are in this category. Our interpretation is that the small amount of SLBP1 in coiled bodies at the histone gene loci represents active molecules about to be used for histone pre-mRNA processing. A minute fraction of the rest might represent SLBP1 associated with processed transcripts that have yet to be exported, but a major fraction of the SLBP1 in the nucleus is probably there simply for storage.

An unexpected finding of this study was the staining of terminal granules, axial granules, and a few chromosomal loops with the anti-SLBP1 antibody. That the staining of terminal and axial granules is caused by SLBP1 and not by cross-reaction or by contaminating antibodies is supported by the elimination of staining by the SLBP1 peptide and the targeting of *myc*-tagged SLBP1 to these same loci. The staining of chromosomal loops is more difficult to interpret. Loop staining was eliminated by the peptide, but *myc*-tagged SLBP1 was not specifically targeted to these loops. One possibility is that the positive loops do not contain SLBP1 but another protein that shares an epitope recognized by antibody X16C. Alternatively, the *myc*-tagged SLBP1 protein may assume a conformation that prevents it from being targeted appropriately to the loops. In any case, the positive loops represent a rare category of nascent transcripts that are not stained by antibodies against splicing factors. The terminal granules were shown previously to be the sites of the 5S rRNA genes, and we know that the U1 and U2 snRNA genes are located at X16C(+) axial granules. All of these X16C(+) loci (the special loops as well as the terminal and axial granules) represent genes that produce unspliced transcripts. This finding suggests that SLBP1 may have a role in RNA processing unrelated to histone pre-mRNA. It will be interesting to determine whether SLBP1 can bind to any of these other RNA molecules and, if so, whether binding is mediated by a stem loop.

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