A Conserved Family of Nuclear Phosphoproteins Localized to Sites of Polymerase II Transcription

Mark B. Roth, Alan M. Zahler, and John A. Stolk

Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

Abstract. An antibody was identified previously that recognizes sites of polymerase II transcription on lampbrush chromosomes, puffs on polytene chromosomes, and many small granules in the nucleoplasm of all cells tested. This antibody binds a conserved family of phosphorylated polypeptides in vertebrate and invertebrate cells. We developed a method for purifying these proteins that involves differential solubility in $MgCl_2$. We isolated a *Drosophila* cDNA encoding one of the proteins using information obtained from microsequencing. In vivo expression studies show that this protein is concentrated on sites of polymerase II transcription and that it is highly phosphorylated. The protein shares a high degree of homology with proteins involved in alternative splicing of pre-mRNA suggesting the possibility that this protein plays a role in pre-mRNA splicing.

NASCENT transcripts are bound by proteins, concomitant with synthesis by polymerase II. This was demonstrated in observations of ribonuclease treated amphibian lampbrush chromosomes (Gall and Callan, 1962) where it is possible to see nascent protein-bound transcripts by light microscopy, and was later confirmed by a number of studies in other systems (Malcolm and Sommerville, 1974; Lamb and Daneholt, 1979). Electron microscopic studies of transcription units using spreading techniques developed by Miller and colleagues confirmed this observation (Miller and Bakken, 1972).

Because of their role in the elaboration of genetic information, there has been a major effort to identify transcript-binding proteins. To date, two sets have been identified: hnRNP and snRNP proteins. The hnRNP proteins, which number at least 24, have been defined by biochemical cofractionation with radiolabeled hnRNA (Samarina et al., 1966; Beyer et al., 1977) and more recently by immunochemical analysis (Piñol-Roma et al., 1988). Several genes encoding hnRNP proteins have been cloned and at least one protein has been shown to interact directly with RNA in vitro (Merrill et al., 1988). Twelve snRNP proteins were identified by co-immunoprecipitation with snRNAs using an antibody that recognized a common epitope found on these proteins (Lerner and Steitz, 1979).

We show here that a mAb 104 (mAb104), described as binding to lateral loops on amphibian lampbrush chromosomes (Roth et al., 1990) and to puffs on *Drosophila* polytene chromosomes (unpublished observation) binds a previously undescribed conserved family of nuclear phosphoproteins. Antigens recognized by mAb104 do not coprecipitate with anti-hnRNP antibodies (Roth et al., 1990). We have cloned a gene that encodes a mAb104 immunoreactive protein from *Drosophila melanogaster*. The encoded protein shares sequence homology with proteins involved in pre-mRNA splic-

ing. We show that heterogeneously-sized RNA—but not snRNAs—coprecipitate using mAb104. Based on these experiments as well as in vivo expression studies in *Xenopus laevis*, we suggest mAb104 binds an epitope found on a family of non-snRNA-binding proteins involved in pre-mRNA splicing.

Materials and Methods

Antibodies

We used three mouse mAbs in this work. Antibody 104 (mAb104) is an IgM produced by Roth et al. (1990). Antibody 9E10 (mAb9E10) is an IgG produced by Evan et al. (1985). mAb9E10 binds to a 12 amino acid sequence contained in the human *c-myc* protein; the single letter amino acid designation of the minimal sequence defined that contains this epitope is MEQKLISEEDL. The third mAb, mAbK121, is an IgG that recognizes the 5' trimethylguanosine cap found on most U-snRNAs (Krainer, 1988).

Protein Purification and Microsequencing

Approximately 1×10^9 *Drosophila* (Schneider or Kc), or human (HeLa) cultured cells, or immature *Xenopus* ovary was washed and resuspended in 10 ml of lysis buffer (65 mM KCl, 15 mM NaCl, 10 mM MOPS, pH 7.5, 10 mM EDTA, 5 mM betaglycerophosphate, 5 mM DTT, 5 mM KF, and 200 μM PMSF). Cells were lysed by several passes through a Dounce homogenizer and the lysate spun at 70,000 g for 1 h. A 60 to 90% ammonium sulfate precipitate was prepared and the resulting precipitate resuspended in and dialyzed extensively against lysis buffer containing 1 mM EDTA. The dialysate was centrifuged at 13,000 g for 20 min to remove insoluble protein and then adjusted to 20 mM $MgCl_2$ with 100 mM $MgCl_2$. After 30 min the suspension was centrifuged at 13,000 g for 20 min to recover the precipitated proteins.

After SDS-PAGE, and electrotransfer to PVDF membrane (Matsudiara, 1987) the intact protein was sequenced from the amino terminus by successive cycles of Edman degradation (model 477A; Applied Biosystems, Forest City, CA), followed by amino acid analysis using a reverse phase C18 column (model 120A; Applied Biosystems Inc.). Protein sequence obtained from internal fragments generated by tryptic cleavage of the protein, separa-

tion of these fragments, and microsequencing was done by Harvard Microchemistry.

PCR Amplification, Cloning, Sequencing, and In Situ Hybridization to Polytene Chromosomes

Degenerate 14-base oligonucleotides corresponding to short stretches of amino acids on either end of fragment C, not including the central seven nucleotides, were used to amplify the genomic DNA sequence encoding the protein (Gil et al., 1988). The reactions contained 0.1 μg of each oligonucleotide (one of two was labeled at the 5' end with [^{32}P]), 1 μg of Drosophila genomic DNA, 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM MgCl$_2$, 200 μM each of dATP, dTTP, dCTP, and dGTP, and 2.5 U of AmpliTaq polymerase (United States Biochemical) in a total volume of 25 μl. After initial denaturation at 94°C for 4 min the reaction was carried out for 30 cycles at 94°C for 1 min, 50°C for 2 min, and 70°C for 1 min. The reaction products were separated on an 18% denaturing polyacrylamide gel. The expected 35-bp band was excised and used in a second round of amplification. The second round products yielded sufficient amounts of each strand to use in Maxim-Gilbert sequencing (Bencini et al., 1984).

Two 34-base oligonucleotide generated using the PCR sequence information was used to screen 1.5 × 10⁵ plaques from an EcoRI linker oligo dT primed embryo cDNA library in the vector lambda ZAP (Stratagene, La Jolla, CA). Filters were washed using tetramethylammonium chloride (Wood et al., 1985). Seven positive phage were plaque purified, the inserts transferred to Bluescript, and sized after EcoRI digestion. The largest cDNA (1,380 bp) was sequenced on both ends and found to contain a short poly A sequence of 19 residues on one end and an open reading frame on the other that could encode amino acid sequence obtained from microsequence analysis of the intact protein.

The sequence of this cDNA was obtained on both strands using sequenase (United States Biochemical, Cleveland, OH) and several deletions prepared by exonuclease III and S1 nuclease. Protein sequence similarity was observed using PATMAT (Wallace, unpublished results; Henikoff et al., 1988), and MOTIF (Smith et al., 1990) with modifications (Henikoff and Henikoff, unpublished results).

In situ hybridization was done according to previously described methods (Simon et al., 1985).

Construction of Tagged Proteins

Fusion of the 13 amino acid myc tag (MT) coding sequence (Munro and Pelham, 1984) to the amino terminus of the SR55 open reading frame was accomplished by cloning a 17-bp adapter into the amino terminus of SR55 changing the start methionine to an alanine but otherwise not altering any other residues. This construct was then cloned into the EcoRI site of Bluescript MT1, a vector that was constructed by transferring a HindIII/EcoRI 60-bp DNA fragment from the human myc gene into the HindIII/EcoRI sites of BS KS+. The myc tag binding mAb9E10 was originally isolated by Evan et al. (1985). Nomenclature used to describe tagged constructs is MT (number of tags)/gene designation.

The six tag concatemer construct was prepared by annealing and ligating two staggered partially overlapping 39-bp oligonucleotides encoding the 13 amino acid myc tag. The ligation products were separated on a 6% polyacrylamide gel. Concatemers of various sizes were pooled, eluted from the acrylamide, the ends filled in using the Klenow fragment of DNA polymerase, and inserted into the HindIII site of MT1/SR55. The desired plasmids were obtained by screening transformants using the mAb9E10 after IPTG induction. Concatemer bearing fusion proteins were slower migrating on SDS-PAGE and more easily detectable than the parental MT1/SR55. Subsequent analysis of the plasmic DNA confirmed the number of concatemers obtained in independent clones.

Expression and Analysis of Tagged Proteins in Xenopus oocytes

Capped runoff in vitro transcripts encoding fusion proteins were prepared according to previously described methods (Roth and Gall, 1989). Injection of synthetic mRNAs was done by methods described by Gurdon et al. (1971). Phosphatase treatment and Western binding analysis of pools of 20 to 30 hand isolated germinal vesicles 48 h after injection of RNA with or without 25 nCi of [gamma-^{32}P] ATP was done according to Roth et al. (1990). Radioactively labeled proteins were detected by autoradiography af-

ter the proteins were separated by SDS-10% PAGE, and electrotransferred to nitrocellulose. Xenopus lampbrush chromosomes were prepared according to Callan et al. (1987); indirect immunofluorescence staining was done according to Roth and Gall (1987).

In Vivo ^{32}P-labeling of Drosophila Tissue Culture Cells

Drosophila Kc tissue culture cells were grown in D22 media (Sigma Chemical Co., St. Louis, MO). Drosophila SL2 tissue culture cells were grown in Schneider's media (Sigma Chemical Co.) supplemented with 10% FBS (Gibco Laboratories, Grand Island, NY). Cells were grown to a density of 5 × 10⁶/ml. Cells were washed three times with TBS and then resuspended at 5 × 10⁶/ml in phosphate free media supplemented with 3% FBS. ^{32}P orthophosphate was added to 0.4 mCi/ml and cells were grown at room temperature for 1 to 4 h. Cells were harvested by centrifugation followed by three washes with TBS.

Immunoprecipitation of SRp55 from ^{32}P-labeled Cells

^{32}P-labeled tissue culture cell pellets were resuspended in Net-2 buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% NP-40) which also contained 20 mM β-glycerophosphate and 500 U/ml ribonuclease inhibitor (Promega Biotec, Madison, WI). Cells were lysed by sonication in a cuphorn sonicator and insoluble material was removed by centrifugation. Cell lysates were then added to protein G sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) which had antibodies bound to them, and these were incubated at 4°C with shaking for 1 h.

mAb104 is an IgM and does not bind to protein G. A goat anti–mouse IgM antibody was used to link mAb104 to protein G sepharose. Protein G sepharose beads were incubated with goat anti–mouse IgM (Tago Inc., Burlingame, CA) in Net-2 buffer on a shaker at room temperature for 30 min. Beads were washed three times in Net-2 buffer and then resuspended in Net-2. mAb104 from ascites fluid was added to the beads, and these were incubated for 30 min with shaking at room temperature. Beads were then washed three times with Net-2 buffer and cell lysate was added to the bead pellet.

After a 1-h incubation with shaking at 4°C, beads were washed three times with Net-2 buffer. For protein immunoprecipitation studies, beads were resuspended in SDS protein sample buffer and separated by SDS-PAGE. For RNA coprecipitation studies, beads were resuspended in 300 μl of Net-2. Then, 2 μl of 1 mg/ml Escherichia coli tRNA, 30 μl of 10% SDS, 150 μl phenol, and 150 μl of chloroform were added to each tube. Tubes were vortexed and incubated at 37°C for 15 min. Tubes were centrifuged, the aqueous layer moved to a new tube, and the nucleic acids in this tube were ethanol precipitated. Nucleic acids were resuspended in formamide loading buffer and the samples were loaded onto a 5% acrylamide (19:1) denaturing gel. RNAse digestion of the pelleted beads containing the immunocomplexes was done with 100 μg/ml RNAse A, or oxidized RNAse A in Net-2 buffer for 15 min at 15°C.

Results

mAb104 Binds A Conserved Family of Nuclear Phosphoproteins

It has been demonstrated that mAb104 shows punctate nuclear staining in cells from all metazoans tests, including: human (HeLa), African green monkey (Vero), mouse (3T3 and L), Drosophila (Schneider), and Xenopus (Xla) tissue culture cells, and tissues from nematode (Caenorhabditis), earthworm, grasshopper (Melanoplus), mouse, and amphibians (Rana and Plethodon) (Roth et al., 1990). It was also shown previously that mAb104 binding on immunoblots, and fixed cytological preparations could be eliminated by phosphatase. The action of phosphatase to eliminate mAb104 binding could be suppressed if the proteins were incubated with beta glycerophosphate. These results demonstrated that mAb104 antigens are phosphorylated, and that the epitope recognized by mAb104 involves phosphate (Roth et al., 1990).

To further characterize the antigens recognized by mAb-

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1. Abbreviation used in this paper: MT, myc T.

104, we used the antibody for immunoblotting studies of proteins from human (HeLa), African Green Monkey (Vero), *Xenopus* (Xla), and *Drosophila* (Kc) tissue culture cells. Live cells were washed in isotonic medium and placed directly into protein sample buffer containing SDS. The results showed that mAb104 binds several different proteins; the staining, however, is barely detectable even when large amounts of protein are used (data not shown). We found the same proteins can be detected much more easily if they are first purified from whole cell extracts. The purification scheme was based on three observations. First, all mAb104 immunoreactive proteins are soluble in magnesium-free solutions containing 60% of saturation ammonium sulfate even after centrifugation at 70,000 g for 1 h. Second, all mAb104 immunoreactive proteins are insoluble in solutions containing 90% of saturation ammonium sulfate. These observations suggest that mAb104-binding proteins are very hydrophilic. The third observation is that all mAb104 immunoreactive proteins precipitate and pellet if centrifuged at 10,000 g for 20 min in buffer containing 20 mM $MgCl_2$. Insolubility in $MgCl_2$ remains a characteristic of these proteins even after they are purified by SDS-PAGE and eluted from the polyacrylamide. This suggests that differential solubility in $MgCl_2$ is intrinsic to these proteins and not a consequence of noncovalent interactions with RNA or other macromolecules.

Fig. 1, lanes *1*, *2*, and *3* show immunoblotting of mAb104 to ammonium sulfate and magnesium chloride precipitated proteins from *Drosophila* (Kc) and Human (HeLa) tissue culture cells, and *Xenopus* immature ovary, respectively. Several proteins with apparent molecular sizes of $\sim$28–30, 40, 55, 70, and 180 kD are observable. All three samples contain bands of 28 and/or 30, and 40 kD. *Xenopus* immature ovary has broad weak staining between 100 and 130 kD and no detectable 55- and 70-kD proteins. The 180-kD protein is only detectable in *Drosophila* cells. The bands just below the *Drosophila* 55-kD protein are not consistently observed. The relative intensity of staining in any one sample is reproducible; the 55-, 30-, and 43-kD proteins are the most abundant in Kc, HeLa, and immature ovary, respectively. We estimate the relative degree of purification achieved using this method is well over several hundredfold and that the percentage recovery is near quantitative. mAb104 binding to all proteins shown in Fig. 1 can be eliminated by phosphatase treatment, and the action of phosphatase can be suppressed by incubation with beta glycerophosphate (data not shown), therefore, they are all phosphoproteins.

Figure 1. Western binding of mAb104 to purified cell proteins. Because mAb104 antigens are relatively rare, they were first purified by ammonium sulfate and magnesium precipitation (see Materials and Methods). Lanes *1*, *2*, and *3* contain purified proteins from *Drosophila* (Kc), human (HeLa), and *Xenopus* immature ovary, respectively. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and stained with mAb104. Several bands with approximate molecular sizes of 28–30, 40, 55, and 75 kD are common to Kc and HeLa cells. A 180–kD band is present only in Kc cells. *Xenopus* immature ovary has bands with approximate molecular sizes of 28 and 40 kD, and broad weak staining between 100 and 130 kD. The series of bands below the 55-kD band in Kc cells is not consistently observed.

Purification of Drosophila SRp55

To begin to characterize the molecular structure of the epitope recognized by mAb104 as well as the function of the phosphoproteins recognized by the antibody, we purified a mAb104 immunoreactive protein to homogeneity. This was accomplished using the method just described with *Drosophila* Schneider tissue culture cells as starting material. When proteins obtained after ammonium sulfate and magnesium precipitation were separated by SDS-PAGE a prominent mAb104 immunoreactive 55-kD polypeptide was detectable by Coomassie brilliant blue R staining (data not shown). This protein was used to obtain partial amino acid sequence.
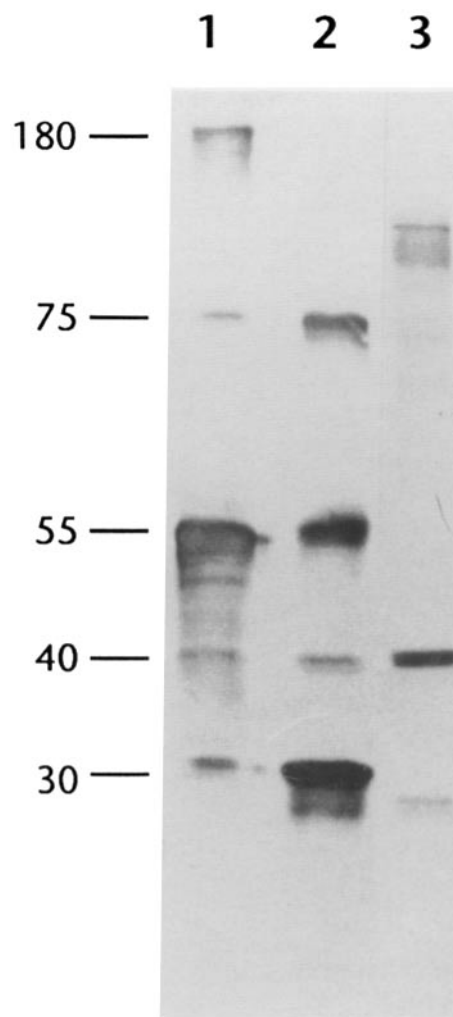
Based on the predicted protein sequence of the purified protein we refer to it hereafter as SRp55: the S and R stand for serine and arginine, the two most abundant amino acids in the protein; p55 denotes the apparent molecular size. The gene designation we use is SR55.

Isolation of the Gene Encoding SRp55

Because mAb104 does not recognize the dephosphorylated form of the antigen (Roth et al., 1990) we were unable to use it to screen an expression library. As an alternative, we microsequenced several peptides from SRp55 by sequencing either the purified protein (Fig. 2, line *A*), or by sequencing

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                                      CCT GCT CCA GAT ACG TAA GGA ACC GTT ATC
1/1 ___ ___ ___ ___ ___ _____A_____
ATG GTG GGA TCT CGA GTG TAT GTG GGC GGT CTG CCC TAC GGA GTG CGC GAG CGC GAT TTG
Met val gly ser arg val tyr val gly gly leu pro tyr gly val arg glu arg asp leu
61/21                         RNP2
GAG CGC TTT TTC AAA GGC TAC GGC CGC ACA CGC GAC ATC CTC ATC AAA AAT GGC TAC GGC
glu arg phe phe lys gly tyr gly arg thr arg asp ile leu ile lys asn gly tyr gly
121/41                                                              RNP1
TTT GTG GAA TTC GAA GAC TAT CGT GAT GCC GAC GAT GCC GTC TAT GAA CTG AAT GGC AAA
phe val glu phe glu asp tyr arg asp ala asp asp ala val tyr glu leu asn gly lys
181/61 RNP1
GAG CTG CTT GGC GAA CGT GTG GTT GTT GAA CCC GCC AGG GGA TCC GCT CGT GGC AGC AAC
glu leu leu gly glu arg val val val glu pro ala arg gly ser ala arg gly ser asn
241/81
CGC GAC CGC TAC GAC GAT CGA TAT GGT GGT CGG CGG GGG GGC GGG GGC GGT CGT TAC AAC
arg asp arg tyr asp asp arg tyr gly gly arg arg gly gly gly gly gly arg tyr asn
301/101
GAA AAA TCA TCC TCT CGT TAT GGC CCA CCG TTG CGC ACT GAG TAC CGA CTG ATT GTG GAG
glu lys ser ser ser arg tyr gly pro pro leu arg thr glu tyr arg leu ile val glu
361/121                    _____B_____                      _____C_____
AAT TTG TCT AGC CGC GTT AGC TGG CAG GAT CTC AAG GAT TAC ATG CGC CAG GCT GGC GAG
asn leu ser ser arg val ser trp gln asp leu lys asp tyr met arg gln ala gly glu
421/141        C
GTC ACC TAT GCC GAT GCC CAC AAG CAG CGT CGC AAT GAG GGC GTG GTT GAG TTC GCC TCG
val thr tyr ala asp ala his lys gln arg arg asn glu gly val val glu phe ala ser
481/161
TTG TCG GAC ATG AAG ACG GCC ATT GAG AAG TTG GAT GAC ACC GAG CTA AAC GGC AGG CGG
leu ser asp met lys thr ala ile glu lys leu asp asp thr glu leu asn gly arg arg
541/181
ATC CAC TTG GTC GAG GAT CGT CGC GGA GGA CGC AGC GGT GGT GGC GGC GGC AGC GGA CGT
ile his leu val glu asp arg arg gly gly arg ser gly gly gly gly gly ser gly arg
601/201
GGA CGC TCC CGT TCG TCC AGC TCC CGA TCG CGC TCC CGA TCC CGC AGG CGT TCT CGC TCT
gly arg ser arg ser ser ser arg ser arg ser arg ser arg arg arg ser arg ser
661/221
CGC CGC TCG TCG CAC TCG CGC TCC AAG TCT CGC AGC CGC TCC AAA TCT CGT GGC GGA CGC
arg arg ser ser his ser arg ser lys ser arg ser arg ser lys ser arg gly gly arg
721/241
TCC AAG TCC AAG TCG CCA GTC AAG TCT CGT TCT CGC TCC CGC TCG CGA TCC AAC AAA TCG
ser lys ser lys ser pro val lys ser arg ser arg ser arg ser arg ser asn lys ser
781/261
CGT GAC GTG TCC AAG TCC AAG TCA AAG TCC CAC TCC CGC ACC CGC TCC CGT TCG CCC AAA
arg asp val ser lys ser lys ser lys ser his ser arg thr arg ser arg ser pro lys
841/281
CGT GAG CGT GAC TCC CGT TCC CGT ACG CGG TCC GTC TCG AAG CGC GAG TCC CGT TCC CGC
arg glu arg asp ser arg ser arg thr arg ser val ser lys arg glu ser arg ser arg
901/301
TCA CGT TCC AAG TCC ATC CAC CGC GAC TCC CGT TCA CGC GAT CGA TCC GCA TCG GCT GAA
ser arg ser lys ser ile his arg asp ser arg ser arg asp arg ser ala ser ala glu
961/321
AAC AAG TCG CGT TCG CGC TCA CGC TCC CGT TCG GCC TCG CCC AAA AAT GGA AAC GCC TCA
asn lys ser arg ser arg ser arg ser arg ser ala ser pro lys asn gly asn ala ser
1021/341
CCG GAC CGC AAT AAC GAG AGC ATG GAC GAT TAG ATT AGT TAG CTG TCC CAT TAA TAT TAA
pro asp arg asn asn glu ser met asp asp AMB
1081
AAC ACT TGA TTT TAG ACT TCT ACT ATA ACT ATT CTA CAA ACA TCA ACA CTC AGA CAC CCC
1141
GAC TAC AAC CAA AAC TTA ATG AAT ATG AAA TCA TAC CAA TCC ACC ACT GTC GGG CCT CGT
1201
CAT TTT CTT TTA AAT ATA TAA CGA CAA TTT GTC CTA AGT CTT GTT GTG GAC ATG GGA CAT
1261
TGG CTG GAA GAT GAC AGG GAT CGG CTG AAT GTG CCG ATA TTT TTT ATA AGA ATA TTA TTA
1321
ATA TAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA
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Figure 2. Nucleotide sequence and conceptual translation of cDNA SR55. Lines labeled *A*, *B*, and *C* indicate peptide sequences derived from purified SRP55 protein. Lines labeled RNP-1 and RNP-2 indicate homology with a conserved RNA recognition motif. These sequence data are available from EMBL/GenBank/DDBJ under accession number X58720.

isolated fragments obtained after digestion with trypsin (Fig. 2, lines *B* and *C*). we found that for the intact protein the amino acid identified in the first Edman cycle was valine at position 2, followed by serine and glycine at positions 3 and 4. We did not obtain a signal at Edman cycle 4. Cycles 5 through 14 revealed the remainder of peptide A. Oligonucleotides were synthesized that could encode either the first five or the reverse complement of the last five amino acids from peptide C. Because of degeneracy of the genetic code, these were both mixtures of 64 oligonucleotides. These oligonu-
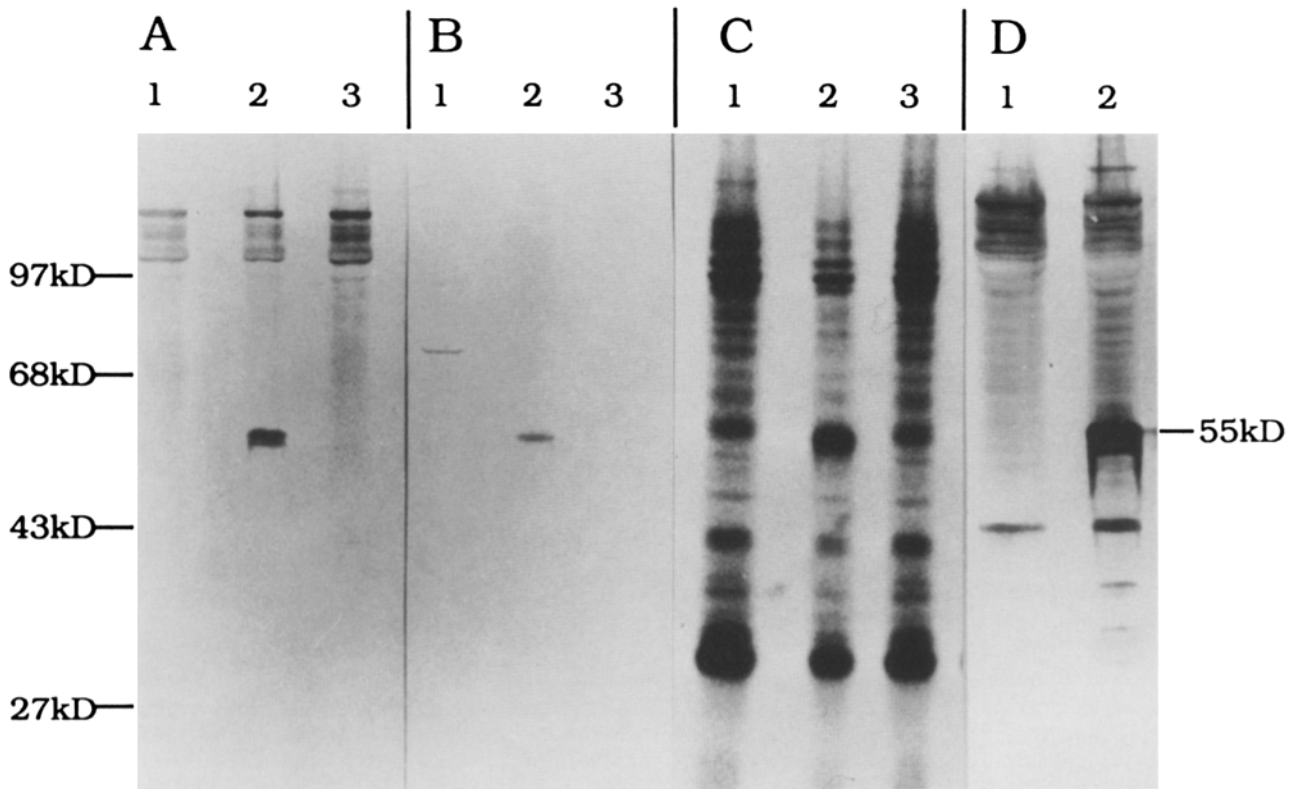
Figure 3. In vivo formation of mAb104 epitope on SRp55 expressed in *Xenopus* oocytes. Oocytes were injected with transcripts encoding tagged SRp55 (MT1/SRp55) or a control tagged protein SE5 (MT1/SE5), and germinal vesicles dissected from them after 48 h. These nuclear proteins were separated by SDS-PAGE, transferred to nitrocellulose and stained with either mAb104 (*A* and *D*) or an antibody specific for the tag, mAb9E10 (*B*). Lane *1* in each panel contains germinal vesicle proteins obtained after injection of oocytes with synthetic transcripts encoding MT1/SE5. Lane *2* in each panel contains germinal vesicle proteins from oocytes injected with MT1/SRp55. Lanes *3* contain germinal vesicle proteins from uninjected oocytes. *A*, lane *2* shows a 55-kD band detectable with mAb104 that is not detectable in MT1/SE5 injected or uninjected oocytes (*A*, lanes *1* and *3*). To be certain the staining of the 55-kD band in lane *2* was above the background of endogenous bands detectable with mAb104 we stained a filter identical to *A*, lanes *1* and *2* for a longer time (*D*, lanes *1* and *2*). *B*, lane *2* shows a band detectable with the tag antibody (mAb9E10) that comigrates with the 55-kD band in *A*, lane *2*. The 70-kD band *B*, lane *1* is the tagged protein expressed from MT1/SE5 transcript. As expected, germinal vesicle proteins from uninjected oocytes do not stain with the tag antibody (*B*, lane *3*). In a parallel experiment oocytes were injected with gamma ^{32}P ATP and transcripts. Radioactive proteins were detected by autoradiography. Lanes *1* and *2* in *C* contain germinal vesicle proteins from oocytes injected with the same transcripts as those in *A* and *B*. An enhanced labeling is detectable at a position of 55-kD (*C*, lane *2*) that is reduced in either control MT1/SE5 or buffer-injected oocytes (lanes *1* and *3*).

cleotide pools and *Drosophila* genomic DNA were used in a polymerase chain reaction to amplify the 36-bp genomic sequence. Both strands of the amplified DNA were sequenced and found to contain a unique sequence that could encode the entire C peptide.

Two 35-base oligonucleotides corresponding to these two strands of unique sequence were used to screen 1×10^5 clones from a lambda library of *Drosophila* cDNAs. Approximately 80 positive clones were obtained, several were plaque purified, and one was completely sequenced. This 1380-bp clone (SR55) contains the coding sequence for SRp55 because conceptual translation reveals all three peptides obtained from microsequencing (Fig. 2).

Sequence Analysis of SR55 and In Situ Hybridization to Salivary Polytene Chromosomes

Analysis of the 39-kD open reading frame revealed two obvious domains in the protein SRp55. Amino acids 1-45 contain a sequence highly similar to proteins that interact with RNA.

The RNA binding consensus sequence can be split into two short blocks, termed RNP-1 and RNP-2 (Bandziulis et al., 1989). By searching the 17,000 member SWISS-PROT data base (version 15) together with SRp55, we found that all known RNA binding proteins and SRp55 scored in the top 35 when either of the two blocks was used. The identified RNP-2 and RNP-1 sequences have the appropriate orientation in SRp55 (Fig. 2). The spacing between RNP-2 and RNP-1 in 32 known RNA-binding proteins ranges between 26 and 34 amino acids; the spacing between RNP-2 and RNP-1 in SRp55 is 25 amino acids.

A second domain present in SRp55 is a long stretch of alternating arginine and serine residues in the carboxy terminal half of the protein. Over this 131 amino acid sequence 42% are arginine and 30% are serine. In some positions lysine—and in one case, histidine—is found instead of arginine, suggesting that an important feature of this sequence may be alternating basic/serine amino acids.

In situ hybridization of the SR55 cDNA to *Drosophila* polytene chromosomes showed binding to one locus on chro-

mosome 3R. The specific map position was between 87F4 and 87F11 (data not shown).

In Vivo Reconstitution of the mAb104 Phosphorylated Epitope

To show that DNA clone SR55 can encode a protein that contains the mAb104 phosphorylated epitope, we expressed SRp55 protein in *Xenopus* oocytes. We distinguished newly synthesized protein from endogenous mAb104 immunoreactive proteins by fusing a piece (12 amino acid) of the *myc* gene to the 5' end of the SR55 open reading frame. In this construction we changed the SR55 start methionine codon to alanine. This fusion protein was detected using an antibody (mAb9E10) specific for the 12 amino acid *myc* sequence. In vitro synthesized transcripts encoding *myc*-tagged SRp55 (MT1/SRp55) and another nuclear localized tagged protein, MT1/SE5 (Roth and Gall, 1989), were injected into stage V-VI oocytes. Germinal vesicle nuclei were hand dissected from the oocytes after 48 h.

Fig. 3 shows the results of staining of germinal vesicle proteins with either mAb104 (*A* and *D*) or mAb9E10 (*B*). Each lane in all experiments contains ~25 germinal vesicles. Lanes 3 in *A* and *B* contain nuclei from uninjected oocytes. As expected, *Xenopus* nuclear proteins are detectable with mAb104 (*A*, lanes *1* and *3*, and *D*, lane *1*), but not with mAb9E10 (*B*, lane *3*). Lanes 1 in *A* and *B* contain nuclei from MT1/SE5 transcript-injected oocytes. MT1/SE5 encodes a 70-kD nuclear localized protein detectable with mAb9E10 (*B*, lane *1*), but not mAb104 (*A*, lane *1*). Lanes 2 in *A* and *B* contain nuclei from MT1/SR55 injected oocytes. Both 9E10 and 104 antibodies detect a protein with an apparent molecular size of 55 kD. *D* is the same as lanes *1* and *2* of *A* except it was stained for a longer period of time. Although mAb104 binds many different phosphoproteins (*D*, lane *1*), SRp55 is easily detectable above this background (*D*, lane *2*). These results demonstrate that transcripts of clone MT1/SR55 encode a protein in *Xenopus* oocytes that contains the mAb104 epitope.

To analyze the structure of the epitope on SRp55 that is recognized by mAb104, we injected gamma ^{32}P-labeled ATP and transcripts into oocytes. Fig. 3 *C* shows an autoradiograph of an SDS protein gel after electrophoresis of hand dissected nuclei from these labeled oocytes. Both MT1/SE5 injected (*C*, lane *1*) and control nuclei (*C*, lane *3*) show the same pattern of labeled proteins; however, after injection of MT1/SR55 transcripts (*C*, lane 2), a 55-kD protein is detectable. Densitometric comparison of the band which migrates slightly slower than SRp55 in lanes *1* or *3* with the 55-kD MT1/SRp55 protein shows that there was five times more radioactivity in the 55-kD band in *C*, lane 2 than in corresponding regions of lanes *1* or *3*. These results suggest that SRp55 is phosphorylated.

Further evidence that the MT1/SRp55 protein contains phosphate is shown in Fig. 4. Nuclei from oocytes injected with MT1/SR55 transcripts were untreated (*A* and *B*, lanes *1*), treated with phosphatase (*A* and *B*, lanes *2*), or treated with phosphatase and β-glycerophosphate (*A* and *B*, lanes *3*). The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and stained with either mAb104 (*A*), or mAb-9E10 (*B*). *A*, lane *2* shows that pretreatment with phosphatase eliminates the ability to detect all proteins containing the mAb104 phosphorylated epitope, including the 55-kD
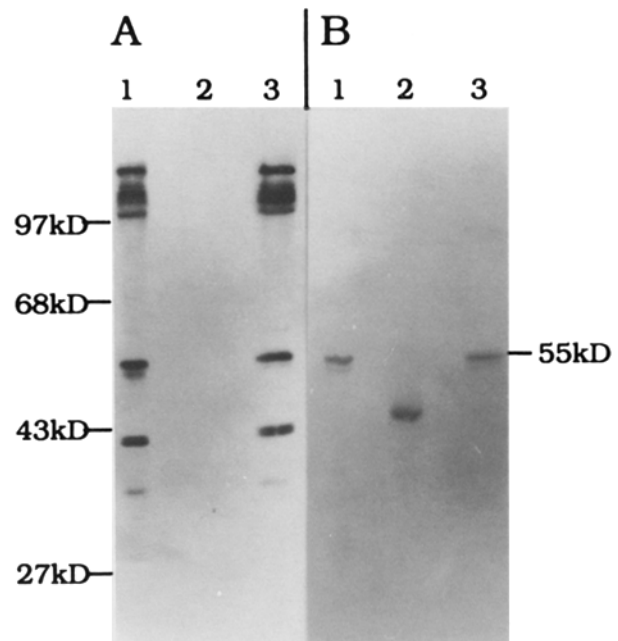

Figure 4. Phosphatase sensitivity of mAb104 binding to MT1/SRp55. Transfers of germinal vesicle proteins from MT1/SR55 transcript injected oocytes stained with either mAb104 (*A*), or mAb-9E10 (*B*). Lanes *1* in each case contain untreated proteins. Lanes *2* in each case contain proteins treated with calf intestinal alkaline phosphatase before electrophoresis. Lanes labeled *3* were treated the same as lanes *2* except β-glycerophosphate was added during phosphatase treatment. Pretreatment with phosphatase eliminated the ability of mAb104 to detect any germinal vesicle proteins including 55-kD MT1/SRp55 (*A*, lane *2*). This treatment also resulted in an increase in the migration of MT1/SRp55 (*B*, lane *2*).

protein encoded by MT1/SR55. *B*, lane 2 shows that phosphatase treated MT1/SRp55 protein has an increased mobility; the new molecular size is near 39 kD (the expected molecular size of the unphosphorylated protein) β-glycerophosphate, a strong competitive inhibitor of phosphatase, prevents the loss of antigen recognition (*A* and *B*, lanes *3*).

In Vivo Targeting of SRp55 Expressed Protein to Polymerase II Transcription Units

Indirect immunofluorescent staining shows that mAb104 labels the lateral loops of lampbrush chromosomes. A lateral loop contains one or more transcription units (reviewed by Callan, 1986). This labeling pattern suggests that phosphoproteins recognized by mAb104 interact with the nascent transcripts being produced at these loci by RNA polymerase II. In addition to staining the lateral loops, mAb104 also stains thousands of small (1-4 μm) granules, referred to as B granules (Roth et al., 1990; Wu et al., 1991), that are present throughout the nucleus.

To begin to define the molecular interactions that the phosphoprotein SRp55 makes in vivo with lateral loops and B granules, we studied the subnuclear distribution of SRp55 in oocytes injected with MT/SR55 transcripts. As described above, we used mAb9E10 to distinguish MT1/SRp55 tagged protein from the endogenous mAb104-detectable protein. Initially, we found that even after injecting large amounts of synthetic RNA (200 nanograms per oocyte) and incubating
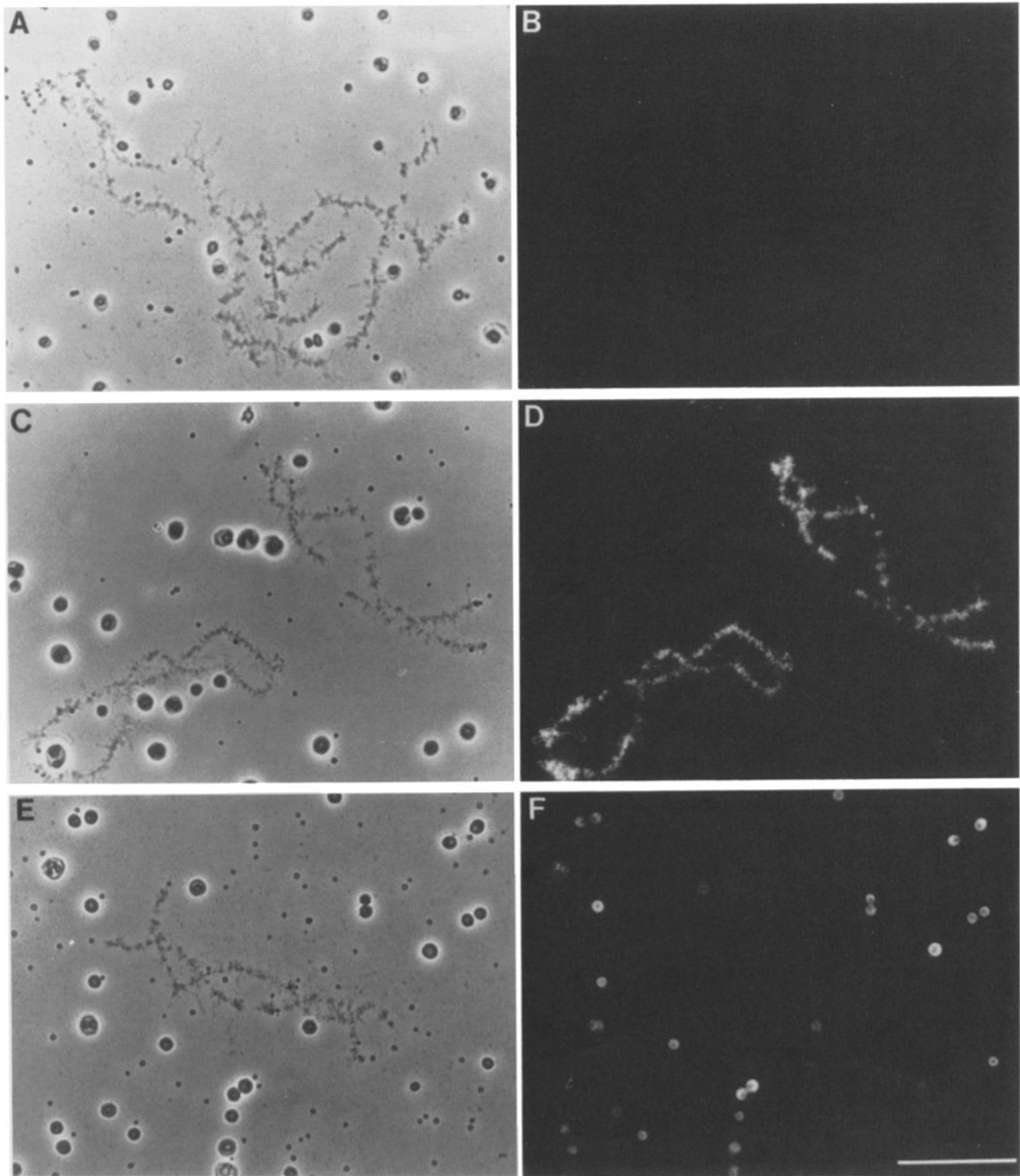
Figure 5. Spread nuclear preparations stained with mAb9E10. Phase contrast image (*left*) and immunofluorescence image (*right*) from oocytes injected with MT1/SR55 containing only one tag (*A* and *B*), MT6/SR55 containing a concatemer of six tags (*C* and *D*), and MT6/164 encoding a nucleolar localized protein with six tags (*E* and *F*). Note that the fusion protein containing six tags and SRp55 is localized to the lateral loops. This is particularly evident in the loops shown in the lower left corner of *C* and *D*. Tags do not appear to influence protein localization because a nucleolar protein fused to the same six tags was localized to nucleoli (*E* and *F*). Bar, 40 µM.

for 48 h we were unable to detect the MT1/SRp55 tagged fusion protein by indirect immunofluorescence (Fig. 5, *A* and *B*).

Because MT1/SRp55 is expressed and localized to the nu-

cleus, we reasoned that our inability to detect the protein was because of a lack of sensitivity of detection. As an alternative, we used synthetic oligonucleotides to generate in-frame amino terminal concatemers of the 13 amino acid *myc* epi-
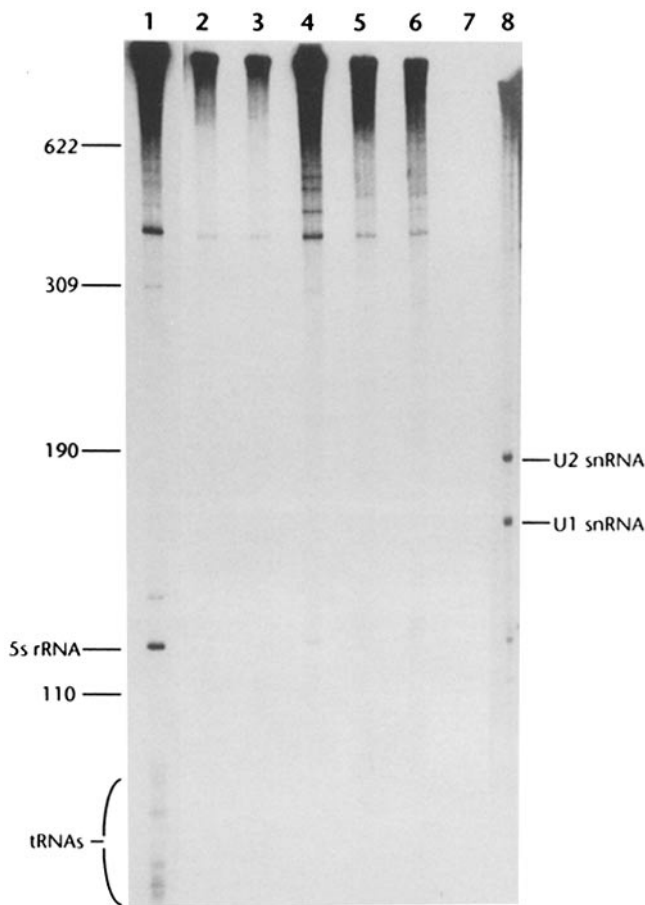
Figure 6. Coprecipitation of in vivo–labeled RNA using mAb104. Labeled nucleic acids from immunoprecipitates were separated on polyacrylamide and observed by autoradiography. Lane 1 contains whole cell nucleic acids. Lanes 2, 3, and 4 contain immunoprecipitates recovered from equivalent amounts of cell extract. mAb104 was coupled to protein G sepharose beads by incubating the beads with mAb104 and anti-mouse IgM before precipitation (lane 4). Immunoprecipitation with beads incubated with anti-mouse IgM alone (lane 2), or mAb104 alone (lane 3) served as negative controls. Comparison of the control lanes (2 and 3) with lane 4 shows that the mAb104 precipitate is relatively enriched in nucleic acids that are >300 bases long. Lanes 5, 6, and 7 contain equivalent amounts of the immunoprecipitate incubated with buffer (lane 5), buffer containing oxidized RNAse A (lane 6), or buffer containing RNAse A (lane 7). Degradation of all radioactive material with RNAse A, but not with oxidized RNAse suggests that mAb104 precipitates contain predominantly RNA. Lane 8 shows labeled U-snRNAs recovered after immunoprecipitation with an α-tri-methylcap antibody (mAbK121).

tope coding sequence on the SR55 open reading frame (see Materials and Methods). Concatemers of three, four, and six tags were constructed. The DNAs were all stable in *E. coli*, and although each tag in the concatemer contains a methionine, the most amino terminal one appears to be used preferentially for translation. We used the six tag SR55 construct (MT6/SR55) to study the localization of SRp55. As early as 38 h after injection of MT6/SR55 transcripts, we detected mAb9E10 staining of the lateral loops of the lampbrush chromosomes (Fig. 5, C and D). We also observed weak staining of the B granules present in the injected oocytes. Fu-

sion of the same six tags to the amino terminus of an open reading frame encoding a nucleolar protein (MT6/164) (Peculis and Gall, 1991) resulted in nucleolar localization (Fig. 5, E and F). The localization of MT6/SRp55 to lateral loops, therefore, is dependent on the SRp55 amino acid sequence and not the tag concatemer.

Coprecipitation of RNA with mAb104

In an attempt to understand the function of the family of phosphoproteins recognized by mAb104, we began to analyze the macromolecules that interact with these proteins. We suspected that SRp55—and possibly other mAb104 antigens—bind RNA because mAb104 stains the lateral loops on lampbrush chromosomes, the predicted protein sequence contains conserved RNA binding sequences RNP2 and RNP1, and MT6/SRp55 is localized on the lateral loops. To test this possibility we attempted to precipitate RNA with mAb104. mAb104 does not bind RNA directly, therefore, if mAb104 could precipitate RNA it would indicate a direct or indirect association of mAb104 antigens with RNA. *Drosophila* (Kc) cell extracts labeled with [^{32}P]orthophosphate were prepared and used for immunoprecipitation. Because mAb104 is an IgM and does not bind directly to protein G, we incubated protein G sepharose with goat anti-mouse IgM antibodies and then incubated the bound sepharose with mAb104. We found that excess mAb104 bound beads can clear an extract of the Kc cell antigens (data not shown).

The labeled nucleic acids that co-precipitated with the mAb104 antigens were separated on polyacrylamide and detected by autoradiography (Fig. 6). Lane 1 contains whole extract before precipitation. Nucleic acids recovered from precipitates of equivalent amounts of extract are shown in lanes 2, 3, and 4. The sample in lane 4 was recovered from extract precipitated with mAb104 bound beads; lanes 2 and 3 served as negative controls and had either beads and anti-IgM antibody, or beads and mAb104. Lane 4 contains approximately tenfold more radioactive material than either of the two control lanes (2 and 3), suggesting that mAb104 precipitated nucleic acids. Several bands that run between the 309 and 622 base markers are enriched relative to 5S and tRNA in the whole extract (lane 1). Lane 8 shows immunoprecipitation with a mAb against the trimethylguanosine cap found on the 5' ends of all abundant snRNAs except U6. None of these known small nuclear RNAs is enriched in lane 4. The mAb104 precipitated nucleic acid was sensitive to RNAse treatment (lane 7), but not to oxidized RNAse (lane 6) suggesting that mAb104 binding proteins interact directly or indirectly with RNA.

Discussion

mAb104 was first identified because it bound to active sites of polymerase II transcription and to small B granules in the nucleoplasm of the amphibian germinal vesicle. Indirect immunofluorescence studies of several tissue culture cells and tissues from various organisms showed that proteins recognized by this antibody are located in small granules in the nucleus. It was difficult, however, to characterize the antigens on immunoblots, presumably because of the low abundance of these phosphoproteins. Making use of the unusual solubility in ammonium sulfate and magnesium, it is now possible to detect the antigens in immunoblots of vertebrate

and invertebrate cell proteins. The molecular sizes of several of the proteins (30, 40, 55, and 75 kD) are apparently identical between vertebrates and invertebrates suggesting a high degree of conservation.

All family members are highly soluble in ammonium sulfate and insoluble in magnesium, raising the possibility that the mAb104 epitope itself (or some other shared structural feature) is important for their unusual biochemical properties. SDS-PAGE purified protein can form aggregates, suggesting the ability to precipitate in magnesium is an intrinsic property of these phosphoproteins. The mAb104 epitope probably includes a phosphate residue, therefore, it is possible that phosphopeptides on these proteins interact with magnesium ions to form large insoluble aggregates. By analyzing the amount of $MgCl_2$ required to sediment mAb-104 antigens we found that as little as 1 mM can be used. Because earlier studies of mAb104 antigen containing B granules show that they are stabilized by the same concentrations of $MgCl_2$ that are required to form the insoluble aggregates in vitro, we hypothesize that $MgCl_2$ aggregates of mAb104 antigens may form in vivo and that this contributes to the formation and stability of B granules.

Possible explanations for the biochemical properties of SRp55 are suggested from analysis of the cDNA sequence. The protein contains over 41% charged residues which, in part, accounts for its hydrophilicity. Another factor that contributes to this property is phosphorylation. Two results suggest that the phosphorylation level is high. First, co-injection of synthetic SR55 transcripts with gamma-^{32}P ATP results in labeled SRp55. The degree of phosphorylation of SRp55 during the synthesis period is comparable to nucleoplasmin (30 kD), the most abundant germinal vesicle protein. Although it has been shown that nucleoplasmin incorporates more ^{32}P than any other germinal vesicle protein, quantitative data concerning the number of phosphates incorporated per molecule are not available (Krohne and Francke, 1980). Second, there is approximately a 15 kD (27%) decrease in the apparent molecular size on SDS-PAGE of MT1/SRp55 protein after treatment with calf intestinal alkaline phosphatase. Altered mobility of phosphoproteins after phosphatase treatment is not uncommon. Modified forms of the large subunit of polymerase II (Dahmus, 1981), and *fos* oncoprotein (Barber and Verma, 1987) both show a 25% decrease in apparent molecular size after phosphatase treatment, and both have been shown to contain multiple sites of phosphorylation. We know of no examples of single sites of phosphorylation causing such large changes in apparent molecular size. There is a long sequence of alternating basic and serine residues in the COOH-terminal half of SRp55. From position 202 to 333, 40% of the amino acids are serine, and 52% are basic (30% arginine, 10% lysine, and 2% histidine). Phosphoamino acid analysis shows that this protein contains only phosphoserine (no phosphothreonine or phosphotyrosine) (data not shown). We favor the possibility that mAb104 binds the phosphorylated form of the basic/serine domain, however, it is conceivable that the antibody binds elsewhere. We have expressed subfragments of *myc*-tagged SRp55 to map this epitope but have not been able to detect mAb104 binding. Further work will be required to resolve this issue. Another domain in the amino terminus is a consensus sequence common to several RNA-binding proteins (Bandziulis et al., 1989). This sequence has been shown to

fold into an RNA-binding domain and may account for the RNA coprecipitation with mAb104, and for the accumulation of these antigens on lateral loops of amphibian lampbrush chromosomes, and puffs on *Drosophila* polytene chromosomes.

SRp55 is similar in structure to three *Drosophila* proteins: transformer (Boggs et al., 1987), transformer-2 (Goralski et al., 1989), and suppressor of white apricot (Chou et al., 1987). They contain sequences of alternating arginine and serine, and transformer-2 and suppressor of white apricot have consensus binding sites for RNA. Analysis of these gene products suggest that they are important in regulating gene expression by alternative splicing.

SRp55 also shares a high degree of sequence homology throughout its entire length with SF2; an essential pre-mRNA splicing factor isolated from HeLa cell nuclear extracts (Krainer et al., 1991). SF2 has also been shown to be involved in alternative splicing; the concentration of this protein can affect 5' splice site selection (Krainer and Maniatis, 1985; Krainer et al., 1990a, b). Purified SF2 contains two closely spaced bands of ∼33 kD; similar in apparent molecular size to two HeLa cell proteins that are immunoreactive with mAb104. The purification of SF2 and all mAb104 immunoreactive proteins begins with a similar ammonium sulphate precipitation; 50–80% for SF2, and 60–90% for proteins that bind mAb104. Furthermore, extracts depleted in SF2 activity are prepared by spinning protein extracts containing ∼4.5 mM $MgCl_2$ at 10^5 g for 1 h. This would quantitatively precipitate SRp55, as well as all antigens recognized by mAb104. Finally, Krainer et al. (1990a) note that while optimizing the purification of SF2 (33 kD) they observed a 55-kD polypeptide that co-purifies with the 33-kD polypeptides. Based on the expression studies of tagged SRp55 and the sequence similarity with other proteins involved in pre-mRNA splicing we hypothesize that mAb104 recognizes a family of nuclear phosphoproteins involved in pre-mRNA splicing, and that SF2 and SRp55 are members of this family.

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