A Monoclonal Antibody That Recognizes a Phosphorylated Epitope Stains Lampbrush Chromosome Loops and Small Granules in the Amphibian Germinal Vesicle

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Abstract. An mAb library was produced against proteins from the germinal vesicle (GV) of the frog *Xenopus laevis*; mAb 104 was selected from this library on the basis of its immunofluorescent staining of lampbrush chromosome loops. Chromosomes from several species of frogs and salamanders stained equally well. The antibody also stained the surface of numerous small granules in the GV nucleoplasm. The interior of the same granules was stained by antibodies against small nuclear ribonucleoproteins (snRNPs). mAb 104 also stained somatic nuclei from many vertebrate and invertebrate species, usually in a finely punctate pattern similar to that described for anti-snRNP and other antinuclear antibodies. The staining of somatic nuclei was much stronger during the mitotic stages than during interphase. Immunoblot analysis showed that mAb 104 recognizes a phosphorylated epitope.

LAMPBRUSH chromosomes from amphibian oocytes provide a useful system for studying proteins associated with nascent RNA transcripts. Each chromosome has a central axis consisting of transcriptionally inactive chromatin (chromomeres) from which loops of active chromatin extend laterally. The bulk of a loop consists of nascent transcripts with associated ribonucleoprotein (RNP)[1] still attached to the DNA template. The RNP matrix of a loop is so abundant that individual transcription units are readily visible by light optical microscopy (Scheer et al., 1976; Gall et al., 1983). Some loops consist of a single transcription unit, whereas many contain two or more units in various orientations.

Antibodies have been used to identify proteins associated with the nascent transcripts on lampbrush loops (Scott and Sommerville, 1974; Sommerville et al., 1978; Martin and Okamura, 1981; Lacroix et al., 1985; Roth and Gall, 1987; Gall and Callan, 1989; Piñol-Roma et al., 1989). The majority of loops are morphologically similar and contain a set of common proteins, including both heterogeneous nuclear RNPs (hnRNPs) and small nuclear RNPs (snRNPs). A few "landmark" loops are morphologically distinct (reviewed in Callan, 1986), and in several cases their protein composition is known to be unusual.

We have produced a number of mAbs from mice injected with germinal vesicle (GV) proteins from the frog *Xenopus laevis*, and the newt *Notophthalmus viridescens*. Several of these bind strongly to lampbrush chromosome loops (Roth and Gall, 1987) or to other intranuclear structures. Most are relatively species-specific; for instance, most mAbs raised against *Xenopus* react with *Xenopus* and other anurans (e.g., *Rana*) but not with urodeles or other vertebrates. One mAb, designated 104, has proved of unusual interest because it cross-reacts with a wide variety of vertebrate and invertebrate species. In immunofluorescence assays mAb 104 stains most lampbrush loops and numerous small granules in the GV nucleoplasm. In somatic cells it stains similar but smaller nuclear granules in a strongly cell cycle–dependent manner. Immunoblot analysis shows that the epitope recognized by the antibody contains a phosphate residue. Here we describe our studies on mAb 104, with emphasis on its reaction with lampbrush loops and its usefulness in defining a population of intranuclear granules.

1. *Abbreviations used in this paper*: GV, germinal vesicle; hn, heterogeneous nuclear; RNP, ribonucleoprotein; sn, small nuclear.

Materials and Methods

mAbs

The production of mAbs and their use in immunoblot analysis and immunofluorescence are described in Roth and Gall (1987).

Lampbrush Chromosome Preparations

GVs were isolated by hand from oocytes of *X. laevis* and *N. viridescens*. Lampbrush chromosome preparations were made essentially as described in Roth and Gall (1987) and Callan et al. (1987), except that 1 mM Mg^{++} was included in both the nuclear isolation and dispersal media. Mg^{++} is essential for good morphological preservation of nucleoli, spheres, and small granules, especially in the dispersal medium. After centrifugation to attach the nuclear contents to the slide, preparations were placed directly into 70% ethanol.

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Immunofluorescence

Lampbush chromosome preparations were transferred from ethanol to PBS, and then into a "blocking" solution consisting of 10% horse serum in PBS with 0.02% NaN_3 as preservative. After a few minutes, the blocking solution was replaced by the primary antibody, usually undiluted cultured supernate from a hybridoma line. After 1 h the antibody was washed away with 10% horse serum in PBS and the secondary antibody was applied for 1 h. For mAb 104 this was rhodamine-conjugated goat anti-mouse IgM (Cappel 2,211-0201); in other cases rhodamine-conjugated goat anti-mouse IgG (Cappel 2,611-0081) or goat anti-human IgG (Cappel 2,201-0081) was used. Preparations were mounted in 50% glycerol containing 1 mg/ml phenylenediamine to prevent fluorescence fading.

Phosphatase Treatment of Proteins

Proteins were treated with phosphatase either before or after electrophoresis and transfer to nitrocellulose membranes. 20 GVs were isolated in 20 μl of a buffer consisting of 64 mM KCl, 16 mM NaCl, 10 mM $MgCl_2$, 10 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM DTT, and 100 μM PMSF. 10 U of calf intestinal alkaline phosphatase (Boehringer Mannheim Diagnostics, Inc., Houston, TX) were added and the solution was incubated at 37°C for 1 h. Cytological preparations and immunoblots were treated with phosphatase by placing them directly into a solution of phosphatase (0.5 U/μl) in 50 mM Tris, pH 8.0, 1 mM EDTA for 1 h at room temperature.

Results

Production of mAbs

To obtain antibodies against various nuclear antigens, we immunized mice with proteins from *Xenopus* GVs according to the procedure used previously for *Notophthalmus* (Roth and Gall, 1987). Hybridoma cell lines were produced from the spleens of the mice, and conditioned medium from 1,300 lines was tested against GV proteins in a solid-phase radioimmune assay. All positive media were then tested for their ability to bind to *Xenopus* lampbrush chromosomes by indirect immunofluorescence.

Immunofluorescent Staining of GV Contents by mAb 104

In our first experiments with mAb 104, we detected binding on *Xenopus* lampbrush chromosomes with a fluorescent secondary antibody directed against mouse IgG. The intensity of staining was quite variable, and we thought that binding was primarily to a small subset of prominent landmark loops near the middle of chromosome 14 (Callan et al., 1987). After learning that mAb 104 was an IgM, we carried out subsequent experiments with a secondary antibody against mouse IgM. We still found staining of the landmark loops, but now we saw staining of nearly all other loops, as well as numerous small granules in the nucleoplasm.

Because mAb 104 cross-reacted with proteins from many different species, we studied the staining pattern in GVs of the newt *N. viridescens*, whose lampbrush chromosomes are far superior to those of *Xenopus* for detailed morphological analysis. In the newt, mAb 104 stained almost all typical lampbrush loops (Fig. 1). Typical lampbrush loops consist of one or more "thin-to-thick" regions that represent individual transcription units (reviewed in Gall et al., 1983; Callan, 1986). The same thin-to-thick pattern was visible by immunofluorescence and by phase contrast or differential in-

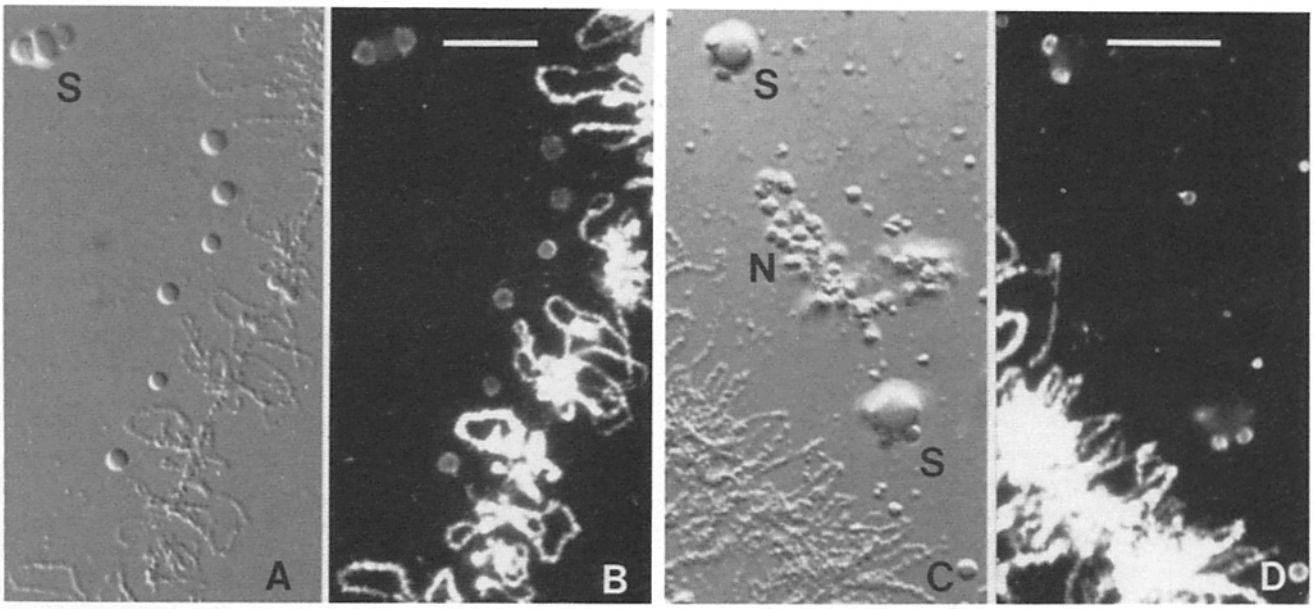

Figure 1. (*A*) Segment of a lampbrush chromosome, six B granules and a small sphere (*S*) with two B granules on its surface, from a GV of the newt, *N. viridescens*. Differential interference contrast shows loops extending laterally from the chromomere axis; the loops consist primarily of nascent RNA transcripts and associated protein, whereas the chromomeres are regions of condensed chromatin. The loops and B granules are known from an earlier study to contain snRNPs (Gall and Callan, 1989). (*B*) Immunofluorescence of the same area after staining with mAb 104 and rhodamine-labeled goat anti-mouse IgM. The lampbrush loops stain uniformly, but the chromomeres are unstained. Only the periphery of the B granules is stained. The body of the sphere is at background level. (*C*) A short segment of a lampbrush chromosome, an extrachromosomal nucleolus (*N*) and two spheres (*S*) with attached B granules, from a GV of *N. viridescens*. Differential interference contrast. (*D*) Immunofluorescence image of the same region after staining with mAb 104. The chromosome loops and the periphery of the B granules are stained; the nucleolus and the sphere bodies are unstained. Bars, 10 μm.

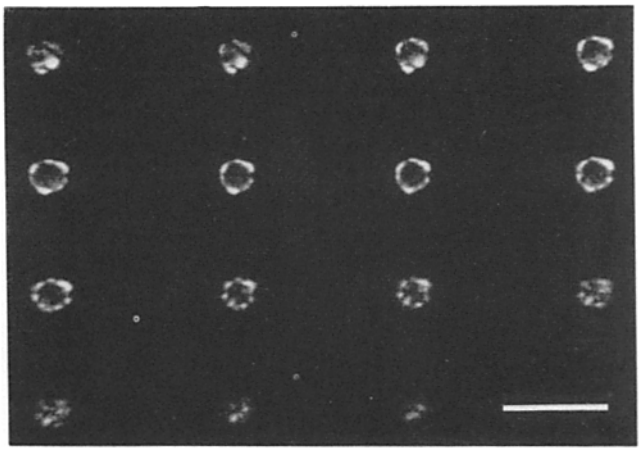

Figure 2. A single B granule from a GV of the frog *Rana temporaria*, stained with mAb 104 and rhodamine-labeled goat anti-mouse IgM. A through-focus series taken with a confocal laser scan microscope at 0.5-μm intervals. Stain is limited to irregular patches on the surface of the granule. Bar, 10 μm.

terference contrast, suggesting that the antigen recognized by mAb 104 is uniformly distributed over the transcription unit. Among the few unstained or weakly stained loops were the "sequentially labeling" loops on chromosome 11 and the giant loops on chromosome 2 (Callan, 1986). These landmark loops fail to stain with other antibodies that stain most loops (Roth and Gall, 1987; Gall and Callan, 1989), including mAb iD2, which is directed against several hnRNPs (Leser et al., 1984), and mAb Y12, which recognizes the Sm epitope common to the major snRNPs (Lerner et al., 1981). The chromomere axis of the chromosomes (Fig. 1, *A* and *B*) and the multiple extrachromosomal nucleoli (Figs. 1, *C* and *D*; 3, *A* and *B*) also failed to stain.

In addition to the chromosome loops, mAb 104 stained thousands of small granules, ~1–4 μm in diameter, scattered throughout the preparation (Figs. 1–3). These "B" granules, as we call them, were suspended in the nucleoplasm before the GV was spread for cytological analysis. Staining was limited to minute patches that cover the surface of the granules, as seen most convincingly by confocal laser scan microscopy (Fig. 2). B granules contain snRNPs, as shown by their reaction with antibodies against the Sm antigen and against the trimethylguanosine cap of the major snRNAs (Gall and Callan, 1989). Additional evidence for snRNPs in the B granules is provided in Fig. 3, *C* and *D*, which shows staining with serum 361, a human autoimmune serum that immunoprecipitates U1 snRNPs (David Wassarman, unpublished observations). It is clear that the snRNP antigen(s) detected by serum 361 are in the center of the B granules, in contrast to the peripheral localization of 104 antigen.

Although mAb 104 stains thousands of small granules in the nucleoplasm, others remain unstained. Many of the unstained granules have a distinctive "doughnut" appearance (Fig. 3, *A* and *C*). This second type, which we call "A" granule, stains intensely with serum 361 (Fig. 3 *D*).

One other prominent nuclear structure stained by mAb 104 is the so-called sphere organelle. A GV from a medium-sized oocyte of the newt contains several dozen spheres, of which the largest are 8–10 μm in diameter (Gall and Callan,

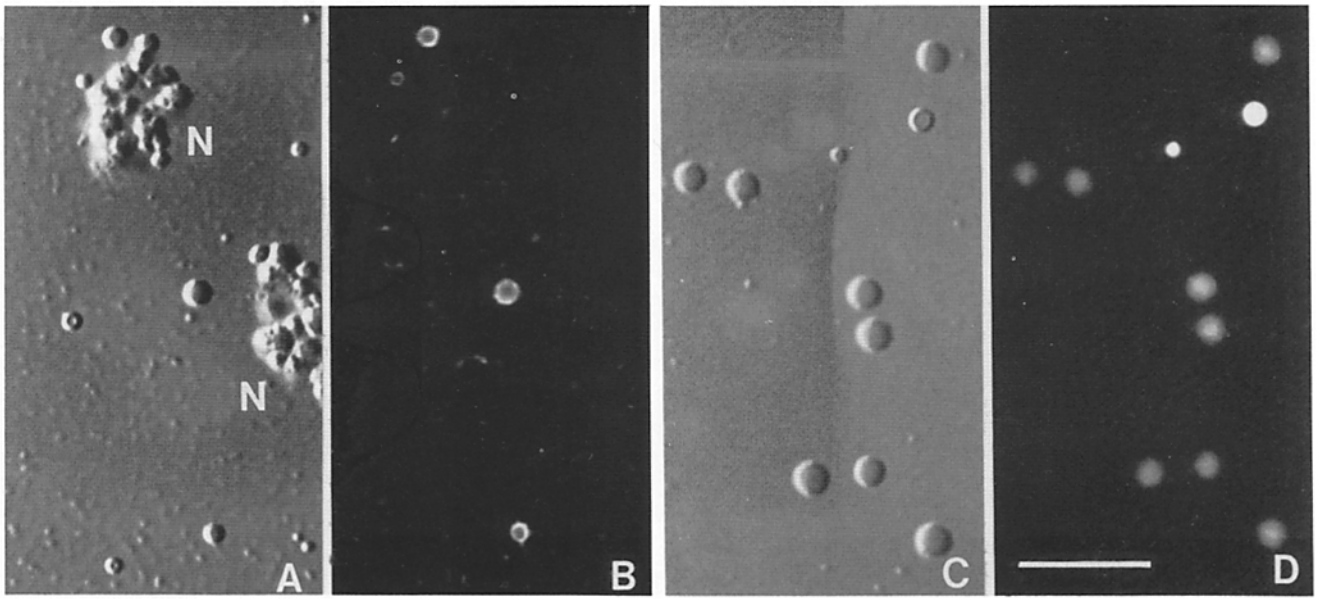

Figure 3. (*A*) A field containing A and B granules and two extrachromosomal nucleoli from a GV of *N. viridescens*. Differential interference contrast. Each nucleolus (*N*) consists of an irregular clump of material. Two A granules are distinguishable by their "hollow" appearance, whereas the B granules are more homogeneous. (*B*) The same area after staining with mAb 104 and rhodamine-labeled goat anti-mouse IgM. The surface of each B granule is stained. Nucleoli and A granules are unstained. (*C*) Two A and eight B granules from a GV of *N. viridescens*. Differential interference contrast. (*D*) The same area after staining with human serum 361, which is specific for U1 snRNPs, and rhodamine-labeled goat anti-human IgG. The B granules stain more or less homogeneously with serum 361, in contrast to their peripheral staining with mAB 104. The A granules stain intensely with this antibody. Bar, 10 μm.

1989). They usually have smaller spherical or subspherical protuberances on their surface (Fig. 1, *A* and *C*). The body of the sphere is not stained by mAb 104, but the protuberances stain exactly like the B granules just described; that is, staining is in minute patches on the surface of the protuberances (Fig. 1, *B* and *D*).

Antigens Recognized by mAb 104 Do Not Coprecipitate with hnRNPs

A few other antibodies stain the periphery of the B granules in the same pattern as mAb 104 (data not shown). Of particular interest is mAb iD2, which recognizes several abundant hnRNPs (Leser et al., 1984). We asked whether the antigen(s) recognized by mAb 104 might be coprecipitated with hnRNPs. hnRNPs were immunoprecipitated from HeLa cell supernatant using mAb 4F4 (Piñol-Roma et al., 1988), electrophoresed on a polyacrylamide gel, and transferred to a nitrocellulose filter. mAb 104 did not react with any protein in the immunoprecipitate, although it readily recognized a protein when total HeLa cell proteins were tested at the same time (data not shown).

Staining of Other Cell Types

We tested for cross-reaction of mAb 104 with cells of other organisms. Formaldehyde-fixed tissue culture cells from several animals were stained by indirect immunofluorescence; these included human (HeLa), African green monkey (Vero), mouse (L cells and NIH 3T3), *Drosophila* (Schneider), and *Xenopus* (Xla). We also tested tissues from a variety of organisms, including nematode (*Caenorhabditis*), earthworm, grasshopper (*Melanoplus*), mouse, and amphibians (Rana, *Plethodon*). In all cases punctate nuclear staining was detected. Fig. 4, *A–C* shows labeling of a single mouse L cell. There was no detectable labeling of nucleoli or of condensed chromatin, as can be seen by comparing the immunofluorescence and DAPI-stained images. The major differences among the different organisms and cell types were in the number and size of the punctate regions in the nucleus.

Mitosis

We noted that the intensity of staining was strongly dependent on the cell cycle (Fig. 4, *D–F*). As cells entered

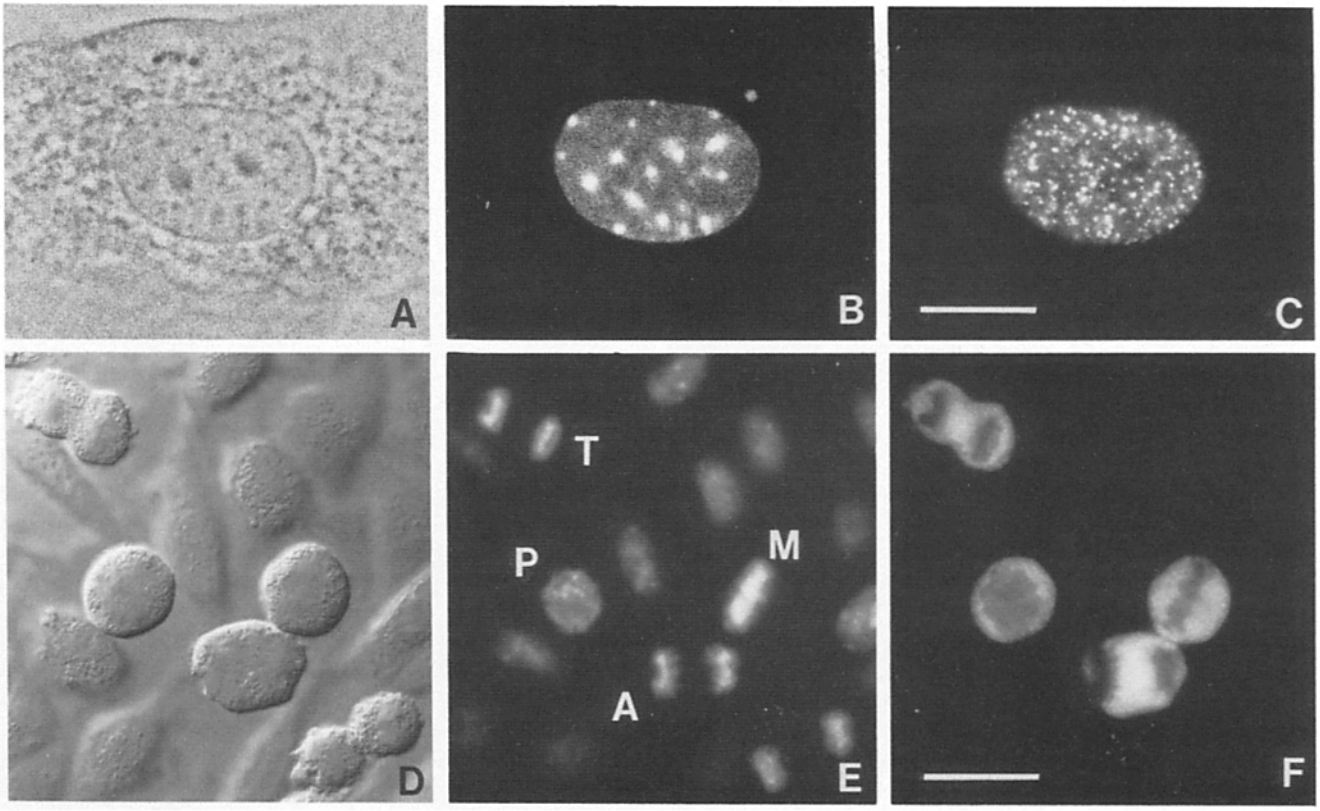

Figure 4. (*A*) Phase-contrast view of a mouse L cell, fixed briefly with formaldehyde and double stained with mAb 104 (rhodamine-labeled second antibody) and the DNA-specific dye 4′, 6-diamidino-2-phenylindole (DAPI). (*B*) DAPI stain shows heterochromatic masses (primarily satellite DNA) against a generalized background stain. (*C*) Immunofluorescence shows numerous bright dots against a lightly stained background. The clumps of antigen do not correspond to the heterochromatic regions. (*D*) Differential interference contrast view of cultured mouse L cells. Dividing cells have rounded up. (*E*) DAPI stain of the same cells. P, M, A, and T indicate cells in prophase, metaphase, anaphase, and telophase, respectively. (*F*) The same cells after immunofluorescent staining with mAb 104 and rhodamine-labeled goat anti-mouse IgM. Staining is intense in cells in mitosis but drops off abruptly at the beginning of interphase (compare the well-stained telophase cell at upper left with the unstained early interphase at lower right). Stain is excluded from the chromosome area. Many but not all of the interphase nuclei in the background are actually stained as in *C*, but a photographic exposure long enough to show this fact would obliterate all detail in the intensely stained mitotic cells. Bars: (*C*) 10 μm; (*F*) 20 μm.

prophase, nuclear staining with mAb 104 became more prominent. After breakdown of the nuclear envelope, intense staining was seen throughout the cytoplasm, and this persisted through metaphase and anaphase. Early in telophase cytoplasmic staining disappeared and granules reappeared in the newly formed daughter nuclei. At the same time, the general intensity of nuclear staining decreased, reaching a minimum during interphase.

Sedimentation of Granules Reacting with mAb 104

Indirect immunofluorescence of many different animal cells suggested that much of the antigen recognized by mAb 104 was organized into microscopically visible granules. To determine whether this property could be used to enrich the corresponding antigen(s), we sonicated hand-isolated GVs and centrifuged them at 12,000 *g* for 2 min. Analysis of the proteins in the pellet and supernate by immunoblotting showed that all of the immunoreactive material was in the pellet. Magnesium (10 mM) in the isolation medium was essential for this effect; without it the antigen remained suspended, even after centrifugation at 70,000 *g* for 1 h. We examined some of the sedimented material by centrifuging sonicated GV contents onto microscope slides, which were then fixed in formaldehyde and stained with mAb 104. The antibody stained an array of minute granules, many of which were smaller than the B granules in our cytological preparations.

mAb 104 Recognizes a Phosphorylated Epitope

To further characterize the antigen(s) recognized by mAb 104, we used the antibody on immunoblots of *Xenopus* GV proteins. The antibody bound to a sharp band at 43 kD and to a poorly resolved smear above 100 kD (Fig. 5, lane *1*). On immunoblots of proteins extracted from *Drosophila* tissue culture cells, the antibody bound strongly to a band at 55 kD and weakly to a few other bands.

Because mAb 104 stained mitotic cells more intensely than interphase cells, we reasoned that either the protein was turning over during the cell cycle, or the epitope recognized by the antibody was altered by a posttranslational modification in a cell cycle–dependent manner. Since many proteins are transiently phosphorylated during mitosis, we tested for antibody binding after dephosphorylation. Nitrocellulose strips that carried GV proteins were incubated with calf intestinal alkaline phosphatase before immunostaining with mAb 104. After incubation, antibody binding could not be detected (Fig. 5, lane *2*). β-glycerophosphate, a strong competitive inhibitor of phosphatase, prevented the loss of antigen activity (Fig. 5, lane *3*). Similar results were obtained by treating GV proteins with phosphatase before electrophoresis and transfer to nitrocellulose membranes. Immunofluorescent staining of formaldehyde-fixed tissue culture cells and granule preparations was likewise greatly reduced or eliminated by phosphatase treatment (data not shown). These results suggest that dephosphorylation of the antigen inhibits antibody binding, and that the epitope recognized by mAb 104 involves a phosphate residue. The epitope probably includes other residues, since many phosphoproteins do not bind this antibody. For instance, nucleoplasmin is the most abundant GV protein and it is heavily phosphorylated (Krohne and Franke, 1980), yet it does not bind mAb 104.

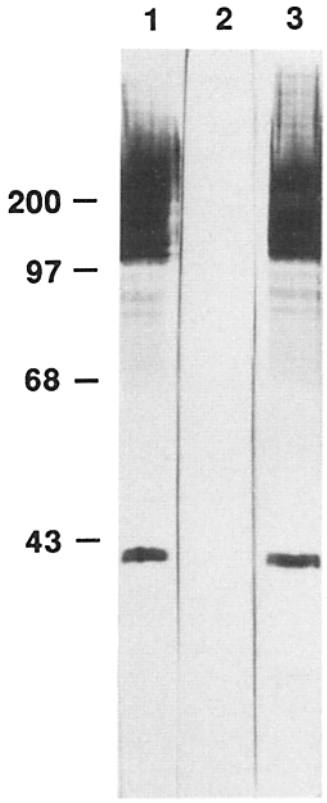

Figure 5. Immunoblot of *X. laevis* GV proteins stained with mAb 104. (lane *1*) Control lane containing proteins from 25 GVs separated by SDS-PAGE and immunostained with mAb 104. Detection was by HRP. A band is visible at ~43 kD, as well as a smear in the range above 100 kD. Positions of molecular mass standards are given in kilodaltons. (lane *2*) A similar immunoblot, but the filter was treated with calf intestinal alkaline phosphatase before immunostaining. No stain is visible. (lane *3*) Similar to lane *2* except that the filter was treated simultaneously with calf intestinal alkaline phosphatase and the competitive inhibitor β-glycerophosphate.

Discussion

We began our immunofluorescence studies on amphibian GVs with the aim of identifying proteins associated with the nascent transcripts on lampbrush chromosome loops (Roth and Gall, 1987). In our first studies we used a Mg^{++}-free solution of relatively low ionic strength (20 mM K^+ and Na^+) for spreading the GV contents. In such a solution the matrix of the loops expands, and the multiple nucleoli partially dissolve. Both features are helpful when attention is directed specifically to the chromosomes. In order to preserve nonchromosomal elements of the GV in our more recent experiments, we have added 1 mM Mg^{++} to the solutions in which GVs are isolated and in which the nuclear contents are spread for cytological observation (in Mg^{++} concentrations of 5 mM or more, GV contents will not spread). In 1 mM Mg^{++}, the nucleoli are preserved intact, as are thousands of smaller granules present in the nucleoplasm. Spheres are retained in spread preparations either with or without Mg^{++}, but the protuberances on their surface are better preserved in Mg^{++}. With the good morphological preservation attained in Mg^{++} preparations, we now find that several antigens associated with nascent transcripts are also present in the spheres and granules. This first became clear when we studied two antibodies specific for snRNPs: mAb Y12 that recognizes the Sm epitope (Lerner et al., 1981) and mAb K121 that recognizes the trimethylguanosine cap of snRNAs (Krainer, 1988). These two antibodies stain the spheres and granules (both A and B types), but they also stain most lampbrush loops (Gall and Callan, 1989). In the present study we found that mAb 104, detected originally on the basis of its loop staining, also stains B granules and the

protuberances on spheres; it does not stain A granules. The detailed staining pattern of mAB 104, however, is quite different from that of mAbs Y12 and K121. mAb 104 stains the periphery of the B granules and the periphery of the protuberances on the spheres (Figs. 1–3), whereas Y12 and K121 stain the body of the sphere and the interior of the granules and protuberances. Other anti-snRNP antibodies, such as human serum 361 (Fig. 3, *C* and *D*), similarly stain the interior of the B granules and protuberances. The granules stained by mAb 104 are abundant in the GV, but as already noted, there are many granules that do not stain with this antibody. Partly on the basis of morphology (Fig. 3, *A* and *C*) and partly on the basis of composition, we recognize two types of granule: A granules, which often have a doughnut appearance and fail to stain with mAb 104, and B granules, which are homogeneous in appearance and stain peripherally with mAb 104. In GVs of *N. viridescens* these two categories probably account for the majority of granules with diameters in the 1–4-μm range, although additional types may be recognized as we test more antibodies. The protuberances on spheres are identical in all respects to B granules.

Two other antibodies that we have studied in detail stain like mAb 104. These are mAb iD2, which recognizes several abundant hnRNPs in a variety of vertebrate species (Leser et al., 1984), and mAb SE5, which recognizes a 90-kD loop protein from the newt (Roth and Gall, 1987). The staining of B granules (both free and on the spheres) by mAb iD2 is particularly interesting, because it suggests that one or more of the major hnRNPs are associated with snRNPs, not only on the nascent transcripts, but in extrachromosomal structures as well. On the basis of their identical subnuclear localization, we might expect that the antigens recognized by mAbs 104 and SE5 would also be hnRNPs. We immunoprecipitated the hnRNP complex from HeLa cells (Piñol-Roma et al., 1988), but mAb 104 did not react with any protein in this group. Because mAb SE5 does not cross-react with mammalian proteins, we did not carry out a comparable test with it. Further study will be required before we can classify the antigen(s) recognized by mAB 104 and assess the relationship between proteins in the B granules and those on the nascent transcripts.

The staining pattern on the chromosome loops is quite similar for all the antibodies we have discussed (mAbs 104, SE5, iD2, Y12, K121, and serum 361). All stain the majority of typical loops, but leave certain prominent landmark loops unstained; chief among the latter are the "sequentially labeling loops" on chromosome 11 and the giant loops on chromosome 2. Staining of typical loops is more or less proportional to the mass of RNP matrix, as shown by the general similarity of the immunofluorescent and phase contrast or differential interference contrast images (Fig. 1). Certain morphologically identifiable loops may be stained relatively more intensely by one antibody, but we have not seen cases where antibody staining was limited to one part of a transcription unit. A possible interpretation of these observations is that most nascent transcripts are associated along their whole length with a common set of hnRNP and snRNP proteins. Certain loops lack these proteins and a few have them in altered relative proportions, but within a given transcription unit their distribution is uniform. Our observations are less compatible with models in which hnRNP and snRNP proteins or protein complexes go on and off the nascent transcripts independently.

mAb 104 has been useful in characterizing various components in the GV, particularly the granules that contain snRNPs. mAb 104 also stains somatic nuclei from a variety of vertebrate and invertebrate species. The staining pattern in somatic nuclei is similar to that described as speckled or punctate for several anti-snRNP antibodies (Lerner et al., 1981; Reuter et al., 1984; Nyman et al., 1986; Spector and Smith, 1986; Spector, 1990) and a non-snRNP splicing factor (Fu and Maniatis, 1990).

Staining by mAb 104 is strongly dependent on the cell cycle. Although weak staining is evident in most nuclei that we have observed, the intensity of stain is dramatically enhanced from prophase through late telophase of the cell cycle (Fig. 4, *D–F*). After breakdown of the nuclear envelope, staining occurs throughout the cell, as is common for many nuclear antigens.

Immunoblots of proteins from several sources (mouse, human, newt, and *Drosophila*) show a strong immunoreactive band in the range of 40–55 kD, suggesting that the immunofluorescence assay may recognize related proteins in these different species. We have shown that the epitope recognized by mAb 104 includes a phosphate residue. We do not know whether the 104 protein fluctuates in amount during the cell cycle or is phosphorylated and dephosphorylated in a cell cycle–dependent fashion (or both). Many proteins are phosphorylated during mitosis including lamins (Ottaviano and Gerace, 1985), pp60$^{c\text{-}src}$ (Chackalaparampil and Shalloway, 1988), and proteins involved in the control of mitosis (Draetta and Beach, 1988). Some antibodies raised against mitotic cells react with phosphorylated groups on a variety of proteins only during mitosis (Davis et al., 1983). Whether the antigen(s) recognized by mAb 104 play an essential role in mitosis, or merely undergo secondary changes during the cell cycle remains to be determined.

We have used mAb 104 to screen cDNA expression libraries of *Xenopus* and *Notophthalmus* ovary RNA, but so far have failed to recover positive clones. A likely problem is that the phosphorylated epitope recognized by the antibody is not present on the protein produced by lambda phage. As an alternative approach we have purified enough of the 55-kD protein from *Drosophila* cells to begin microsequencing. We hope eventually to study the production of the 104 antigen in oocytes and somatic cells, and to examine its regulation during the cell cycle.

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