Monoclonal Antibodies That Recognize Transcription Unit Proteins on Newt Lampbrush Chromosomes

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Abstract. We prepared hybridoma cell lines from mice injected with newt germinal vesicle proteins. We tested culture supernates from these cell lines for antibodies that bound to specific morphological structures in lampbrush chromosome preparations (nucleoli, loops, chromomeres, etc.). Four mAbs that recognize antigens on the lateral transcription loops are described here. We suggest that these antigens are proteins associated with nascent RNA transcripts, although they are not among the 30–40-kD “core” heterogeneous nuclear ribonucleoproteins.

Although histones have been recognized for a long time as the major structural proteins of chromosomes, the characterization of nonhistone chromosomal proteins has posed several special problems. Some of these proteins may be only transiently associated with the chromosomes, because they are involved in discontinuous processes like transcription and replication. Others are rare because they are part of smaller structures like the centromere (Cox et al., 1983; Earnshaw and Rothfield, 1985) and telomere (Gottschling and Zakian, 1986). And still others are derived from biochemically defined “chromatin” fractions, which usually come from interphase nuclei and are thus difficult to correlate with morphologically defined chromosomes.

Chromosomal proteins can be identified and localized by the use of antibodies, an approach that has been especially valuable with the giant polytene chromosomes of Diptera (Jamrich et al., 1977; Saumweber et al., 1980; Howard et al., 1983; James and Elgin, 1986). Several groups have prepared antibodies against amphibian germinal vesicle proteins and have used either oocyte sections (Krohne and Franke, 1980; Dreyer et al., 1983) or isolated lampbrush chromosomes for intranuclear localization. The large size of the chromosomes, their ease of manipulation, and the wealth of morphological detail make them ideal for such studies.

Sommerville was the first to examine the localization of ribonucleoproteins on lampbrush chromosomes of the newt, Triturus, using various antisera prepared in rabbits (Scott and Sommerville, 1974; Sommerville et al., 1978; Sommerville, 1981). Scheer (quoted in Callan, 1986), Martin and Okamura (1981), and Moreau et al. (1986) studied the binding of several antibodies to lampbrush chromosomes, including antibodies against histone H2B, core heterogeneous nuclear ribonucleoproteins (hnRNPs), and nucleoplasmin. Scheer and his colleagues (Bona et al., 1981; Scheer et al., 1984) have also studied the effect on lampbrush chromosomes of antibodies injected into the oocyte. In a recent comprehensive study Lacroix and his colleagues (1985) identified potentially interesting mAbs by their ability to bind to lampbrush chromosomes. They prepared an mAb library against germinal vesicle proteins of the salamander, Pleurodeles waltl, and then selected antibodies that bound to various parts of the chromosomes (e.g., chromomeres, loops, spheres, etc.). We have carried out a similar study of mAbs raised against germinal vesicle proteins of the newt, Notophthalmus viridescens. We describe here four antibodies that recognize antigens on the lateral loops of the lampbrush chromosomes. We believe that these antigens are proteins associated with nascent RNA transcripts.

Materials and Methods

Cell Culture

The parent myeloma used in our studies was SP2/0, a cell line derived from BALB/c mice (Shulman et al., 1978). It was maintained in DME with 20% FCS and 10 μg/ml of 8-azaguanidine. 1 wk before fusion with spleen cells, SP2/0 cells were transferred to the same medium without 8-azaguanidine. After fusion the hybridoma cell lines were grown for 1 wk in DME with 20% FCS, supplemented with 2 mM L-glutamine, 10 μg/ml osaloacetic acid, 8 μg/ml insulin, 55 μg/ml sodium pyruvate, 68 μg/ml hypoxanthine, 1 μg/ml aminopterin, and 10 μg/ml thymidine (HAT medium). They were then maintained for 2 wk in this medium without aminopterin (HT medium), and finally without aminopterin, hypoxanthine, or thymidine.

Germinal Vesicles

Female newts, Notophthalmus viridescens, were purchased from Lee's Newt Farm, Oak Ridge, TN. They were kept in water at 8°C and fed approximately once a week on live Tubifex worms. Ovaries were dissected from anesthetized newts and stored at 4°C in OR2 medium (Wallace et al., 1973).

1. Abbreviations used in this paper: GV, germinal vesicle; hnRNP, heterogeneous nuclear ribonucleoprotein.

2. Although frequently referred to as waltl or waltii, the taxonomically accepted name of this species is walti (Frost, 1985, p. 61E).
Small pieces of ovary were transferred to a Ca²⁺-free medium consisting of 80 mM NaCl, 20 mM KCl, 10 mM Tris, 0.1 mM EDTA, pH 8.0. Under a dissecting microscope germinal vesicles (GVs) from large oocytes (>10 mm diameter) were hand isolated, washed free of cytoplasm by pipetting, and transferred to 100 mM Na acetate, 5 mM EDTA, pH 5.0. In this medium the nuclear contents immediately precipitated, thus reducing loss of proteins. Isolated nuclei were stored in a minimal volume of the same medium at -20°C.

**Immunization of Mice**

Three CAF/J female mice, 5-wk-old, were immunized according to the following schedule: (a) Day 1: i.p. injection of 100 GVs emulsified in Freund's complete adjuvant. (b) Days 7 and 14: i.p. injection of 100 GVs emulsified in Freund's incomplete adjuvant. (c) Day 17: Sample of blood taken from tail vein. Serum tested for antigen binding by solid phase RIA; all three mice were detectably positive at an antibody dilution of 10⁻⁵. (d) Days 24, 31, and 45: One mouse (No. 3) given an i.p. injection of 100 GVs emulsified in Freund's incomplete adjuvant. (e) Day 65: Mouse No. 3 given tail vein injection of 100 GVs. (f) Day 67: Spleen cells of mouse No. 3 fused with SP2/0 cells.

**Hybridoma Cell Lines**

Hybridoma cell lines were produced according to the general method developed by Koehler and Milstein (1975). Approximately 10⁷ spleen cells were mixed with 10⁷ SP2/0 cells and distributed into 2,000 wells in standard 96-well plates. Hybridoma colonies appeared in 90% of the wells; the average number of colonies per well was ~2.5, determined by visual inspection. 7 d after fusion the medium on the hybridoma cell lines was supplemented with 100 µl of HT medium. When the cells covered one-third of the bottom of the well (days 10–15), conditioned medium was removed and tested for mouse antibodies. Positive cell lines were transferred to a 24-well plate and grown for 4 d, at which time the medium was again tested for mouse antibodies. Positive cell lines were transferred to 25-cm² flasks. Before they entered the log phase of growth, a few cells were removed for conditioned medium from a cloned cell line.

**Solid Phase RIA**

The supernatant from each cell line was tested by RIA for the presence of mouse antibodies. For this test goat anti-mouse IgG antibody was placed in wells of a polyclonal antibody plate (1.5 µg of protein in 50 µl of PBS in each well) and left overnight to allow binding of protein to the plastic. Some lines were also tested for antibodies directed against GV proteins. In this test proteins from one GV (2–2.5 µg) were placed in each well. Blocking solution (10% horse serum, 0.1% Triton X-100 in PBS) was added to the wells for 2 h to saturate nonspecific binding sites. The wells were aspirated and 50 µl of conditioned medium was added. After 1 h this was replaced by 3 x 10⁶ cpm of [³²P]-labeled rabbit anti-mouse IgG antibody. Labeled antibody had a specific activity of 10⁻⁶ cpm/µg and was made according to Salacinski et al. (1981). Bound cpm was determined by scintillation counting or by fluorography (Roth, 1986).

**Immunofluorescence**

Lampbrush chromosome preparations were made as described by Gall et al. (1981). A GV was hand isolated in a medium containing 83 mM KCl, 17 mM NaCl, and 10 mM Na₂PO₄, pH 7.2. It was then dispersed in the same mixture diluted to 1/4 strength to which was added 0.1% paraformaldehyde. Dispersal took place in a specially prepared well slide, which was centrifuged at 2,500 g for ~45 min to attach the chromosomes to the slide. After centrifugation the slide was placed directly into 70% ethanol and held until used for immunofluorescence. Mitotic chromosome squashes were prepared from the gut epithelium of colchicine-treated newts as described by Macgregor and Andrews (1977). Paraffin sections were cut at 3 µm from newt ovaries that had been fixed by freeze substitution. Small pieces of ovary were placed in just-melted isopentane (~−71°C) for ~30 s and were then transferred to ethanol at ~−70°C for several days. After warming to room temperature the dehydrated tissue was passed through tertiary butyl alcohol into melted paraffin and embedded.

**Electrophoresis and Western Blots**

One- or two-dimensional PAGE was performed as described by Laemmli (1970) and O'Farrell (1975). Proteins were transferred from the gel to a cellulose nitrate filter and probed with antibody (Western blot) using a modification of the procedure described by Towbin et al. (1979).

**Results**

**Initial Screening and Stabilization of Hybridoma Cell Lines**

Before setting up hybridoma cell lines, we tested the sera from three mice that had been injected with germinal vesicle proteins. When lampbrush chromosome preparations were stained by indirect immunofluorescence using these polyclonal sera, we saw staining of everything on the slide, including all parts of the lampbrush chromosomes, nucleoli, various extrachromosomal granules, and precipitated nucleoplasm. At a serum concentration of 2.0 x 10⁻⁴ staining was still detectably stronger than with preimmune serum from the same mice at a concentration of 10⁻². These results gave us confidence that the hybridoma cell lines would produce antibodies against chromosomal proteins.

Initially we screened conditioned medium from 535 hybridoma cell lines from one mouse by indirect immunofluorescent staining of lampbrush chromosome preparations. Of the 66 cell lines that tested positive by this technique, 25 produced antibodies that bound to all components visible by phase-contrast microscopy. Of the remaining lines, 12 bound specifically to nucleoli, 8 to telomere and centromere granules, 10 to the majority of the lampbrush chromosome lateral loops, and 11 to specific sets of loops.

Because we were particularly interested in loop-specific proteins, we attempted to stabilize the 21 lines that bound to most loops or to specific sets of loops. We successfully stabilized seven cell lines by limiting dilution cloning, of which four will be described here in more detail.

**Antibodies That Label Most of the Lampbrush Chromosome Loops**

Antibodies SE5 and UA5 bound to most of the lateral transcription loops of newt lampbrush chromosomes (Fig. 1). Exceptions were the clusters of giant loops on chromosome 2, the major domain of the large "sequentially labeling loops" on chromosome 11, and a few other loops whose exact positions we did not try to identify. These antibodies did not stain the central chromatere axis of the chromosome, telomere and centromere granules, other types of axial granules, the histone spheres, or the extrachromosomal nucleoli (see Callan, 1986 for a general description of these components). However, there was detectable staining of precipitated nucleoplasm, even though the method used to prepare the chromosomes minimizes the amount of nucleoplasm on the slide. Both antibodies were qualitatively similar, but SE5 consistently showed brighter staining than UA5.

SE5 and UA5 reacted strongly with lampbrush chromosomes and somatic nuclei of urodeles from three different families (Triturus cristatus, Pleurodeles waltl, Ambystoma...
Figure 1. The left end of lambrush chromosome 6 of *N. viridescens* showing many typical loops extending from the chromomere axis. (a) Phase-contrast and (b) fluorescence images of the same region after treatment with mAb SE5 and a rhodamine-labeled second antibody. Note that most loops are labeled more or less in proportion to the intensity of their phase-contrast image, including the dense "lumpy loops" (l). However, an occasional loop is unlabeled (u). Also unlabeled are the extrachromosomal nucleoli (n) and the sphere (s). The sphere is characteristic of the histone locus (Gall et al., 1981). The chromomere axis is also unstained, although this fact is not evident at this magnification on chromosomes with such well developed loops. Bar, 30 μm.

mexicanum and *A. maculatum*, and *Plethodon cinereus*) but were negative with the anurans *Xenopus laevis* and *Rana temporaria*.

To determine the intracellular distribution of the antigens recognized by UA5 and SE5, we stained sections of ovary and kidney that had been fixed by freeze-substitution in ethanol. Fig. 2 shows binding of SE5 to oocytes and follicle cells in the ovary. With the exception of the nucleoli, the antigen is distributed throughout the germinal vesicle; it is not detectable in the oocyte cytoplasm. Bright staining of the surrounding follicle cell nuclei and the nuclear staining of kidney cells (not shown) indicate that this antigen is present in somatic nuclei. Again, the staining patterns of SE5 and UA5 were qualitatively similar except that SE5 was brighter.

Since both antibodies bound to actively transcribing regions of lambrush chromosomes, we wanted to know whether they also bound to transcriptionally inactive mitotic chromosomes. Fig. 3 shows binding of SE5 to a squash preparation from intestinal epithelium of the salamander, *Plethodon cinereus*. Interphase nuclei are well stained except for the nucleolus and for regions of high DNA concentration (condensed chromatin). The latter is shown by comparison of immunofluorescence and 4',6-diamidino-2-phenylindole (DAPI) staining on the same nucleus (Fig. 3, a and b). During prophase immunofluorescence is found throughout the nucleus except in the condensing chromosomes themselves. A similar picture is seen at full metaphase (Fig. 3, c and d), although now there is no nuclear envelope. Because most of the cytoplasm is lost in this type of preparation, one cannot determine how far the antigen extends away from the chromosome area. It is clear, however, that much if not all of the antigen persists throughout mitosis. The binding of UA5 at interphase and late prophase is similar to that seen with SE5, but at metaphase the overall staining is low (not shown). Whether this is due to more complete dispersal of the protein recognized by UA5 during metaphase or to its loss cannot be determined from these observations.

That antibodies SE5 and UA5 recognize different proteins...
was demonstrated by immunoblotting. Proteins from 40 GVs were subjected to electrophoresis on one-dimensional gels, transferred to nitrocellulose filters, and probed with the two antibodies (Fig. 4). SE5 bound to a single polypeptide with an apparent molecular mass of 90 kD. UA5 also bound to a single polypeptide, but with a mass of ~120 kD. On two-dimensional gels (not shown) the isoelectric points of the two proteins were 7.0 and 4.5, respectively.

**Antibodies That Label Specific Sets of Lateral Loops**

Although antibodies SE5 and UA5 bind to the majority of loops, they do not bind to the major domain of the sequentially labeling loops on chromosome 11 (Fig. 5, a and b) or to the giant loops on chromosome 2 (Fig. 6, a and b). During our screen we found two antibodies that bind almost specifically to these loops. One of these, UF6, binds to the
Figure 5. The giant "sequentially labeling loops" on chromosome II of *N. viridescens*. There are four loops altogether, two sister loops on each homologue. Each loop consists of a large gourd-shaped region, whose base lies next to the chromosome axis, and a thin strand returning from the neck of the gourd back to the axis. The gourd-shaped regions frequently fuse with each other, whereas the thin ends are always free. For more on these unusual loops, see Callan (1986). (a) Phase-contrast and (b) fluorescence images of the left end of chromosome II stained with mAb SE5 and rhodamine-labeled second antibody. This antibody labels almost all typical loops, including the thin portion of the sequentially labeling loops, but it does not stain the gourd-shaped regions. Arrows in b indicate the demarkation between stained and unstained regions of the loops. (c) Phase-contrast and (d) fluorescence images of a chromosome stained with mAb UF6 and rhodamine-labeled second antibody. This antibody stains the gourd-shaped part of the loops but not the thin returning strand. UF6 stains only a few other loops, none of which are nearly so large as the sequentially labeling loops. Note the many unstained typical loops in this field. Bar, 50 μm.

sequentially labeling loops but not to the giant loops, whereas the other, TH2, binds to both. UF6 and TH2 each bind to a few "typical" loops whose positions we have not mapped.

The binding of UF6 to the sequentially labeling loops is shown in Fig. 5, c and d. These loops are composed of two regions: a thin domain morphologically indistinguishable from the majority of loops, and a massive, gourd-shaped region. The two homologues of chromosome II each carry a pair of sequentially labeling loops; thus there may be four separate loops in a given nucleus. Often, however, two or more of the gourd-shaped regions are fused into a single mass. Careful examination shows that UF6 binds only to the thick domain of these loops, leaving the thin ends invisible after immunofluorescent staining. On the other hand, SE5 and U5, which do not bind to the thick domain, label the thin end of these loops distinctly (Fig. 5, a and b). Thus within the sequentially labeling loops the antigens detected by SE5 and U5 have a similar spatial distribution, but neither occurs in the region labeled by the UF6 antibody.

The second loop-specific antibody, TH2, resembles UF6 in that it binds to the major domain of the sequentially labeling loops. However, it binds equally strongly to the morphologically dissimilar giant loops on chromosome 2 (Fig. 6, c and d). Neither TH2 nor UF6 gave detectable binding on Western blots to GV proteins or proteins of a crude egg ex-
Figure 6. The giant loops near the centromere of chromosome 2 of *N. viridescens*. There are two or three clusters of extraordinarily long loops in this region, up to 200 μm long. (a) Phase-contrast and (b) fluorescence images of the giant loop region after staining with mAb SE5 and rhodamine-labeled second antibody. SE5 stains the great majority of typical loops but leaves the giant loops unstained except for a few short segments and for very faint staining of their axes (not evident here). (c) Phase-contrast and (d) fluorescence images of the giant loops after staining with mAb TH2 and rhodamine-labeled second antibody. TH2 stains the giant loops as shown here and the gourd-shaped portion of the sequentially labeling loops on chromosome 11 (not shown). Bar, 50 μm.

Discussion

The purpose of this study was to identify mAbs directed against chromosomal proteins, especially those associated with nascent RNA transcripts. We selected monoclonal lines by their ability to bind to specific regions of fixed lampbrush chromosomes. We found a variety of specific binding patterns, including antibodies that bound to nucleoli, to parts of the chromomere axis, and to lateral loops. Antibodies that bind to lateral loops most probably recognize antigens associated with the nascent RNA transcripts. The lateral loops are the major site of polymerase II activity (Schultz et al., 1981), and several lines of evidence, including electron microscopic observations on “Miller spread” chromosomes (Beyer et al., 1979), show that loops consist of one or a few transcription units (Gall et al., 1983). By far the greatest
fraction of their mass consists of nascent RNA chains and associated proteins (Callan, 1986). On the basis of the intense, generalized labeling of loops by the four antibodies described here, we believe that they recognize proteins associated with nascent transcripts. If these antibodies were directed against histones, RNA polymerase, or transcription factors associated with the DNA template, we would expect them to be localized along the axis of each loop and to give a much weaker immunofluorescence signal. For instance, Scheer has demonstrated localized binding of histone H2B antibody to the loop axis by immunogold labeling (unpublished observations quoted in Callan, 1986).

At this time we can make only a few inferences about the proteins recognized by our antibodies. SE5 and UA5, because they recognize proteins of 90 and 120 kD on Western blots, are certainly not directed against the major core hnRNPs, all of which have molecular masses in the range of 30–40 kD (reviewed in Dreyfuss, 1986). However, antibodies against the core hnRNPs do bind to lampbrush chromosome loops in a pattern similar to that of SE5 and UA5, as shown by Martin and Okamura (1981) using polyclonal sera. More recently Martin's group produced mAbs to hnRNPs (Leser et al., 1984) and these, too, bind to lampbrush loops (Scheer and Martin, unpublished observations quoted in Callan, 1986). Using one of these antibodies (ID2) we found binding to the majority of N. viridescens lampbrush loops with the exception of the major domain of the sequentially labeling loops and the giant loops on chromosome 2. We obtained essentially identical results with the anti-Sm mAb Y12, which recognizes several snRNP proteins (Lerner et al., 1981). In summary, the proteins recognized by SE5 and UA5 are found on almost all transcription units; they are associated with the whole of the loop, not just the loop axis; and their distribution is essentially identical to that of core hnRNPs and Sm snRNP proteins. We suggest, therefore, that the proteins recognized by these mAbs are involved in some common function such as processing, packaging, or transport of the nascent RNA transcripts. This function is not limited to the oocyte, since the proteins are also found in somatic nuclei. The proteins occur not only on the lampbrush loops but also in the nucleoplasm of the oocyte. This fact is evident from immunofluorescent labeling of oocyte sections in which the whole nucleus, exclusive of the nucleoli, is labeled, and from spread lampbrush chromosome preparations in which some precipitated nucleoplasm is always present and stained. We do not know whether the proteins in the nucleoplasm are free or associated with RNA transcripts that have been shed from the chromosomes.

It is more difficult to make inferences about the antigens recognized by UF6 and TH2. They are found primarily on the largest loops of the chromosome set, in regions that do not bind SE5 and UA5. Following the arguments presented above for UA5 and SE5, we believe they are proteins associated with nascent RNA. Because of their limited distribution they are unlikely to be core hnRNPs. However, we have not yet identified the corresponding antigens on Western blots.

Lampbrush chromosomes were used by Lacroix et al. (1985) to screen a monoclonal library made against GV proteins of the newt, Pleurodeles waltii. From 71 monoclonal lines they obtained 10 lines that showed specific patterns of chromosomal binding. One of their antibodies, A33, resembles SE5 and UA5 in that it bound strongly and uniformly to the majority of lateral loops, but not to all. In Pleurodeles it recognized a protein of 80 kD. Another of their antibodies, A1, bound strongly to several specific loops in Pleurodeles and to the giant loops on chromosome 2 of Notophthalmus. Whether this antibody recognizes the same protein as our TH2 remains to be determined.

In earlier studies the binding of polyclonal sera to lampbrush chromosomes was investigated. Scott and Sommerville (1974) immunized rabbits with various RNP fractions from newt oocytes and used the resulting antisera in studies on lampbrush chromosomes from Triturus cristatus. Several of their sera bound to the majority of lateral loops, although one reacted only with a specific set of about ten loop pairs. Sommerville et al. (1978) also prepared antisera against two polypeptides of 49 and 38 kD derived from a 40S cytoplasmic particle that contains both 5S RNA and tRNA. The antiserum against the 49-kD protein bound to a single pair of loops, whereas that against the 38-kD protein bound to several pairs of loops on different chromosomes. In view of the current interest in TFIIIa, the major protein associated with 5S RNA in oocytes (Engelke et al., 1980), the results of Sommerville et al., warrant further study.

The overall aim of our studies is to identify and characterize chromosomal proteins. In this paper we have concentrated on lateral loop antigens that are presumably associated with nascent transcripts, but during the screening we found antibodies that reacted with nucleoli, other parts of the chromosome, and "nucleoplasm." Lacroix's group found a similar variety. In addition to those that bound to loops they found antibodies that reacted specifically with chromonemes and the axial structures known as spheres. The morphological screen provides exquisite localization and allows at least some general deductions about possible functions of the antigens recognized. In principle the mAbs will allow further characterization of the proteins they recognize, either by Western blotting, affinity purification, or identification of the genes encoding the proteins in an expression library. However, the mAbs have potential disadvantages. They may not detect a protein in a standard Western blot, either because the protein is rare or because of technical factors that may be difficult to evaluate. For example, our antibodies SE5 and UA5 give strong reactions on Western blots but UF6 and TH2 do not. A similar problem may arise in using the mAbs to detect genes in an expression library. In preliminary experiments we have been unsuccessful in using our antibodies to isolate cDNA clones from a λgt11 library. A final disadvantage of the mAbs turns out to be their narrow species-specificity. We had originally hoped that we could use mAbs against newt GV proteins to recognize the corresponding proteins in Xenopus or more distantly related vertebrates. In an antigen binding study of about 200 antibodies directed against Notophthalmus and Xenopus GV proteins we found that fewer than 1% of the antibodies gave detectable cross-reactions. We believe that many if not most GV proteins are common to both species, but that the mouse immune system recognizes primarily those epitopes that are not shared by newts and frogs.

For these reasons we are currently trying another approach, the essence of which is to screen a cDNA expression library with polyclonal serum from an animal immunized against GV proteins. The positive clones are then used to
affinity-purify antibodies from the same polyclonal serum, and finally the purified antibodies are used in an immunofluorescence assay on chromosomes or tissue sections. This approach has so far yielded several affinity-purified antibodies that bind to specific morphological structures. It avoids the necessity for mAb lines, and it ensures that one has at least a putative gene probe for any antigen found to have an interesting morphological localization.

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