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Heidi Marie Groh

ENTITLED Screening Drosophila Lambda gt11 libraries with

Antibodies directed against microtubule associated proteins

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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Timothy L. Karr

Instructor in Charge

APPROVED: Robert L. Switz

HEAD OF DEPARTMENT OF Biochemistry

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SCREENING DROSOPHILA LAMBDA gt11 LIBRARIES WITH ANTIBODIES DIRECTED AGAINST MICROTUBULE ASSOCIATED PROTEINS

BY

HEIDI MARIE GROH

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ABSTRACT

Below I present a procedure for cloning genes. Several different antibody screening techniques were utilized, none of which involve the use of radioisotopes, to screen for certain genes from Drosophila melanogaster. The degree of sensitivity and rate of success varies depending on the method used.

INTRODUCTION

The lactose operon is an inducible system, turned on in the presence of lactose. Lac z is the gene in the lactose operon which encodes for the enzyme B-galactosidase (B-gal). B-gal cleaves lactose into galactose and glucose. λgt11 is a bacteriophage which contains a lactose operon. Fortuitously, there is an Eco R1 restriction enzyme site at the end of the lac z gene in λgt11. DNA fragments 0 to 4.8 kilobases (kb) can be inserted into λgt11 at this Eco R1 site. If the DNA insertion is in frame with the B-gal gene, then the encoded sequence will be expressed as a fusion protein with B-gal when lac z is induced. The lambdaphage is allowed to infect Escherichia coli (E. coli), and the resulting plaques can be screened with antibodies for the presence of the unknown peptide fused to B-gal.

Lambda is a good choice for a cloning vector, because about one-third of the phage genome is replaceable, or not necessary for the phage to undergo lytic growth. In lytic growth, the phage lyses the bacteria it has infected. In order for the phage to remain viable, it must be 78% to 105% of the length of its wild-type genome (1). There is a great deal of flexibility in allowable lengths of foreign DNA insertions. A good library of Drosophila melanogaster can therefore be obtained by taking advantage of these properties.

A Drosophila library is a population of recombinant DNA segments equally representative of all sequences in the Drosophila genome.

Theoretically, since all DNA sequences are equally represented in the library, screening a sufficient number of plaques should produce a plaque that contains the desired protein sequence. In this case, it is the epitope recognized by the antibody used for the screening. The screening was done with monoclonal Drosophila antibodies directed against microtubule associated proteins (MAPs) as primary antibodies.

MATERIAL AND METHODS

- λgt11 library obtained from John Tamkun, Univ. of Colorado, Boulder
- E. coli strain Y1090 obtained from John Tamkun, Univ. of Colorado, Boulder
- E. coli strain Y1089 obtained from John Tamkun, Univ. of Colorado, Boulder
- Primary tubulin antibody DM1A obtained from Sigma
- Primary polyvalent antibody 2L obtained from this lab
- Monoclonal antibodies P_1C5, P_2B9, P_1B2, P_1C11, P_2C11, P_1B12, and P_2B12 obtained from this lab
- goat-anti-mouse IgG conjugated to alkaline phosphatase (AP) obtained from Promega
- horse-anti-mouse biotinylated IgG obtained from Vector
- Streptavidin AP obtained from Chemicon International
- Vectstain ABC-AP Kit obtained from Vector
- Vectastain Elite ABC Kit obtained from Vector
- Alkaline Phosphatase Substrate Kits II and III obtained from Vector
- Isothiopropyl thiogalactoside (IPTG) obtained from Sigma
- Bovine serum albumin (BSA) obtained from Sigma
- agar from Difco
- agarose from Sigma
- nitrocellulose filters: 82mm filters from Bio-Rad, 137 mm

filters from Micron Separations Inc.

Recipes

LB (Luria Broth) (per liter)

10 grams (g) tryptone; 5g yeast; 5g NaCl; 1 ml 1N NaOH

autoclave 20 minutes

LB agar (per liter)

as above plus 15g agar; autoclave 20 mins.

LB top agarose (per liter)

10g tryptone; 5g yeast; 5g NaCl; 6.5g agarose

autoclave 20 mins; pour into test tubes in 3.5 or 8ml aliquots, autoclave with tops loosely on 20 mins.

Lambda dilution buffer (λ dil)

20 mM Tris-Cl, pH 8; 20 mM $MgCl_2$

autoclave 20 mins.

PBTK buffer (per 250 ml)

25 ml 10X PBS stock; 2.5 ml 10% NP-40; 1.25 ml 10% azide.

Growing E. coli Y1090 (2)

1. With a sterile inoculating loop, remove some E. coli suspended in gel and inoculate 1 ml of LB.

2. Incubate several hours at 37°C, with shaking, until turbid.

3. Using sterile technique, remove a loopful of culture from the tube.

4. Streak an LB + ampicillin (amp) (50 mcg/ml) plate for isolated colonies.

5. Incubate at 37°C overnight.

6. From the plate pick a single colony and inoculate 5-10 ml of LB.

7. Grow at 37°C, with shaking, until turbid; generally overnight.

8. Store refrigerated, 4°C, not longer than two weeks.

9. To check for contamination, prepare one or two LB + amp by spreading 100 ul culture from step 7 onto each plate.

10. Incubate at 37°C overnight. Only E. coli should be growing on the plates.

Bacteriophage Titer (3)

1. Set up sterile test tubes for a serial dilution of lambda.

2. To the first test tube, add 990 ul LB, add 900 ul LB to each remaining tube.

3. Add 10 ul of stock λgt11 to first tube; mix well (vortex): dilution factor 10^2.

4. Add 100 ul from test tube number one to test tube two; mix well: dilution factor 10^1, final dilution factor 10^3.

5. Repeat step four until a final dilution factor of 10^7 or 10^8 is reached.

6. Using sterile technique, prepare six or seven test tubes by adding 200 ul Y1090 to each.

7. Add 100 ul of the 10^2 dilution of lambda to one of the

Y1090 tubes; mix well.

8. Repeat step seven, putting 100 ul of each remaining lambda dilution into a separate Y1090 tube.

9. Allow phage to adsorb to E. coli by incubating at 37°C, 15 to 30 minutes.

10. After adsorption, add 3.5 ml LB top agarose (at about 50°C) to each and pour onto LB + amp plates.

11. Incubate at 37°C overnight.

12. To titer:

plaques x dilution factor/ .1 ml = p.f.u./ml

(plaque forming units/ml)

Preparing Plates for Antibody Screening (2)

1. For 100 mm plates, mix 200 ul Y1090 + 100 ul LB containing approximately 6 x 10^2 pfu; for 150 mm plates, mix .6 ml Y1090 + LB containing 4 to 5 x 10^3 pfu.

2. Allow phage to adsorb to cells for 15 - 30 minutes at 37°C.

3. For 100 mm plates, add 3.5 ml LB top agarose; for 150 mm plates, add 8.0 ml LB top agarose.

4. Pour onto LB + amp plates.

5. Incubate at 37°C for 8 hours or overnight. Do not let plates cool to below 37°C until nitrocellulose is removed.

6. Saturate nitrocellulose filters with 10 mM IPTG, allow to dry and then lay on top of plates. Filters should become evenly moist (no bubbles).

7. Incubate 2-3 hours at 37°C.

8. Remove plates from incubator, and orient the filters onto the plates by marking with a needle dipped in waterproof ink.

9. Carefully remove filters. Do not allow them to dry out in the subsequent washings and incubations.

10. Rinse filters in buffer for 30 minutes. Buffer is PBTK + .05% azide in all cases except when using Vectastain Elite Kit.

11. For 82 mm filters use 4-5 ml of solution; for 137 mm filters use 8-10 ml of solution.

12. After rinsing filters in buffer, block filters with .1% BSA by incubating, with gentle shaking, for 30 minutes.

13. Primary antibody: After blocking, remove BSA and add primary antibody diluted in buffer. Incubate, with shaking, 30 minutes.

14. Quickly rinse off filters 2 or 3 times with buffer, then let filters rinse in buffer 30 minutes.

15. This was followed with one of the screening procedures described below.

Antibody Screening with the Vectastain ABC-AP Kit (4, 5)

1. Secondary antibody: Mix one drop of stock biotinylated antibody for every 10 ml of buffer. Add to filters and incubate 30 minutes with shaking.

2. Rinse as in step 14 above.

3. AP reagent: Add 2 drops of Reagent A (avidin DH) in 10 ml

buffer. Then add 2 drops of Reagent B (biotinylated APH). Mix and let stand 30 minutes before using.

4. Incubate filters for 30 minutes with AP reagent.

5. Rinse as in step 14.

6. Proceed to directions under AP Substrate.

Antibody Screening with the Vectastain Elite ABC Kit (4, 5)

1. Secondary antibody: Mix one drop of stock biotinylated antibody for every 10 ml of buffer. Add to filters and incubate 30 minutes with shaking.

2. Rinse as in step 14 above.

3. Peroxidase reagent: Add 2 drops of Reagent A (avidin DH) in 5 ml buffer. Then add 2 drops of Reagent B (biotinylated horseradish peroxidase), and let stand 30 minutes before using.

4. Incubate filters for 30 minutes with peroxidase reagent.

5. Rinse as in step 14 above.

6. Peroxidase substrate: Mix equal volumes of .02% Hydrogen peroxide (made fresh in distilled water from 30% stock), and .1% (1 mg/ml) diaminobenzidine thc (DAB) made in .1 M Tris pH 7.2.

7. Use immediately.

8. Incubate with shaking until color develops.

Antibody Screening with the Streptavidin AP system

1. Secondary antibody: The secondary antibody is biotinylated horse-anti-mouse IgG diluted in buffer 1:500.

Incubate filters 30 minutes with shaking.

2. Rinse as in step 14 above.

3. Streptavidin AP (SA-AP): Dilute SA-AP 1:2500 in buffer, and incubate filters 30 min with shaking.

4. Rinse as in step 14 above.

5. Proceed to directions under AP Substrate.

Antibody Screening with the Goat-Anti-Mouse AP system

USING BUFFER

1. Secondary antibody: The secondary antibody is goat-anti-mouse IgG conjugated to AP. Dilute 1:5000 in buffer. Incubate filters 30 minutes with shaking.

2. Rinse as in step 14 above.

3. Proceed to directions under AP Substrate.

USING 5% NON-FAT DRY MILK (NFDM)

1. Blocking: Instead of BSA, block filters with 5% NFDM for 30 minutes with shaking.

2. Primary antibody: Same as above.

3. Rinse as in step 14 above.

4. Secondary antibody: Dilute goat-anti-mouse AP in 5% NFDM 1:5000. Incubate 30 minutes with shaking.

5. Rinse as in step 14 above.

6. Proceed to directions under AP Substrate.

7. NOTE: Some modifications in the above procedure were later implemented. Blocking in 5% NFDM for one hour, rinse for 30 minutes. Dilute primary and secondary antibodies in

buffer, not NFDM.

AP Substrate

1. Follow instruction sheet for Vector AP Substrate Kit, note appropriate buffer: for Kit II - 100 mM Tris-Cl pH 9.5
for Kit III - 100 mM Tris-Cl pH 8.2

2. To 10 ml buffer from step one add 2 drops Reagent 1 and mix; add 2 drops Reagent 2 and mix; add 2 drops Reagent 3 and mix. Use immediately.

3. Incubate filters with shaking until color develops.

Testing for a Positive Signal (2)

1. To test a positive signal, realign the filter with the proper plate. Use the ink marks to insure proper orientation.

2. If the spot lines up with a plaque, remove an agar plug containing that plaque.

3. Incubate the plug in 1 ml lambda dilution buffer for at least an hour.

4. Titer as outlined above.

5. Replate and repeat screening procedure until all plaques produce a positive signal.

RESULTS

Using the Vectastain ABC Kit, I obtained no positive results. This is possibly due to the age of the kit, or perhaps the reagents had gone bad. Using the Vectastain Elite Kit, I again saw no positive results. Upon further examination, I discovered that azide was present in the buffer used. Azide destroys the peroxidase reaction, thus no results. I repeated the experiment, but got no results.

At this point, I began to use the goat-anti-mouse AP system. I used DM1A (a tubulin antibody) as the primary antibody. One spot was identified, a plug was taken, and a rescreen produced several more positives. A spot of 2 ul purified tubulin on one filter before screening resulted in a positive spot. Convinced that the system was working, I moved on to screening unknown antibodies. For screening the unknown antibodies I switched from the goat-anti-mouse method to the biotinylated horse-anti-mouse / SA-AP method. Because the second system is more sensitive, we believed it would work better.

Using a mixture of P_1C5 + P_2B9 as a primary antibody, two positives were identified on each of two plates. A rescreen of two plaques produced several more positives. Attempts at further purification brought no result. The entire screening procedure was repeated from the beginning, using only P_1C5.

After several attempts, there were no positive results.

With P_2B9 as the primary antibody, the procedure was repeated. An initial screen produced one or two positives; a rescreen of those would produce five or six more. No further purification was possible. Thinking that perhaps the streptavidin system was too sensitive, I switched back to the goat-anti-mouse AP system. This time, I used 5% NFDM to block the filters and to dilute the secondary antibody. Screening the antibodies P_1C11, P_2C11, P_1B12, and P_2B12 yielded one very weak signal when P_1B12 was used as the primary antibody. Further attempts at amplification proved fruitless.

From this point on, NFDM was used as a blocking agent only; the antibodies were diluted in buffer, since it was determined that proteases in the milk were degrading the antibodies. A rescreen of P_1B12 with this procedural modification still brought no results.

Since the monoclonal antibodies were not rescreening with any great success, I attempted to screen the plaques with a polyvalent antibody. With the polyclonal antibody 2L, using the goat-anti-mouse AP system, positive spots were detected. A rescreen indicated more positives. Lack of time prevented me from carrying this line of experimentation further, but I believe this will prove successful when carried on by other students in the future.

DISCUSSION

Before attempting to discuss the actual experiments, it is first necessary to explain some of the minor details involved. Before laying the nitrocellulose filters onto the plate, the filter must be saturated with IPTG. IPTG has a structure similar to that of lactose. IPTG binds to the repressor of the lactose operon, preventing the repressor from binding to the operator. As a result, the lac operon is induced on. This means that the lac z gene is turned on, and its gene product, B-gal, is produced. Recall that the Drosophila DNA insertion is at the end of the lac z gene. By turning on lac z, the product of the in-frame DNA insertion is also transcribed. That protein fragment can then be recognized by and complexed to the antibody being used as the probe. The E. coli strain being used, Y1090, contains lac repressor, so IPTG is necessary to turn on, derepress, the operon.

Other considerations involve preserving the activity of the reagents. After use, it is a good idea to filter the primary antibody, secondary antibody, and the alkaline phosphatase reagent with either a .2um or .45 um Millipore filter before storage. This removes bits of agar, any bacteria or viruses that may be in the solutions, or any other unwanted "junk." The solutions then may be used over again 4 or 5 times. Another preventative measure that can be

implemented is the addition of .05% sodium azide to the solutions and reagents. Azide is a potent poison and, therefore, retards growth of bacteria and virus particles. Of course, azide cannot be used with the horseradish peroxidase system.

Understanding the steps involved in the screening procedure is as important as the ability to perform them. After incubating the plates with IPTG-saturated nitrocellulose and marking them with ink, the filters must be carefully removed and rinsed. Rinsing removes pieces of agar, and other dirt, and excess ink from the filters. After rinsing, the filters were blocked with either BSA or NFDM. Nitrocellulose has a very high affinity for binding proteins. While this makes the filters very useful for this procedure, it also presents some problems. The fusion protein made when lac z is turned on binds to the filters. Hopefully, the primary antibody, when added, will recognize part of that protein and bind to it. Realizing that the antibody itself is a protein, it will also bind to the nitrocellulose quite nicely. Rinsing the nitrocellulose first with proteins like BSA and NFDM allows these proteins to bind to the filter. Thus, the primary antibody can only bind to its proper epitope, not the entire filter. Failure to block the filter would result in a wasted experiment, because the entire filter would look like a positive screen.

During the course of experimentation, I alternated from using .1% BSA to 1% BSA to 5% NFDM. The reason I increased the BSA concentration 10-fold was to cut down on background noise on the filter. A greater concentration of protein should bind to the filter better. Non-fat dry milk binds even better to the filter than bovine serum albumin.

After blocking the filter, primary antibody can be applied. A monoclonal antibody recognizes a certain epitope or amino acid sequence. By screening several plates with the same antibody, eventually the proper epitope should be expressed. Ideally, the antibody will only bind to its specific sequence. Where it does not bind, it will be rinsed off in the next step.

After rinsing off excess primary antibody, secondary antibody can be applied. The secondary antibody is an anti-mouse antibody. Since the primary antibodies were obtained from a mouse spleen, an anti-mouse antibody will recognize this and form a complex with the primary antibody. The anti-mouse secondary antibody is biotinylated. This means that the antibody is conjugated with several molecules of biotin. Biotin is a small molecular weight vitamin, which binds irreversibly to avidin. Avidin has four binding sites for biotin, and most proteins can be conjugated with several molecules of biotin, as was the case here.

After the excess secondary antibody is rinsed off, the

next step in the screening process can be implemented. Depending on the screening procedure used, this step varies slightly. Generally, the next step involves the formation of an avidin biotin complex with the biotinylated antibody. The binding affinity of the avidin-biotin reaction is much greater than an antibody-antigen reaction, so this increases the sensitivity of the screening procedure. The avidin is also conjugated to biotinylated alkaline phosphatase. Alkaline phosphatase catalyzes the hydrolyses of phosphate containing substances such as those presented in the Substrate Kits manufactured by Vector.

Now we have a primary antibody (obtained from a mouse) bound to its epitope. This complex is bound to a biotinylated anti-mouse antibody, complexed by the avidin-biotin binding to an alkaline phosphatase and avidin complex. The avidin is complexed to the AP by an avidin-biotin reaction also. The final step of the screening process involves the reaction catalyzed by AP. The precise reaction is not revealed, but a hydrolytic reaction takes place yielding an insoluble colored product. Substrate kits II and III involve different reactions and result in different colored precipitates. A positive test, then, will yield a dark spot on the filter. This should line up with the plaque containing the correct DNA insertion.

In the goat-anti-mouse AP system, the alkaline phosphatase

is conjugated directly onto the secondary antibody, saving the avidin-biotin binding step. In the peroxidase system, the final substrate is avidin DH and a biotinylated horseradish peroxidase H reagent. The final reaction is a peroxidase reaction, not an alkaline phosphatase reaction, but the basic idea is the same.

The theory is sound, but the question remains as to why the results were so disappointing. Antibodies which produce strong signals in "Western" blots, as these antibodies did, should work well in this procedure (2). However, they are monoclonal antibodies. A monoclonal antibody recognizes only one specific site on the antigen called an antigenic determinant or epitope. By definition, a genomic library such as λgt11 is a homogeneous representation of all DNA sequences. It seems logical to assume that eventually the correct epitope should be found for the antibody in question. However, since the sequences were obtained by restriction enzyme digestion, a variety of factors may affect sequence representation (6). Factors such as rate of restriction enzyme digestion, overdigestion, and underdigestion to name a few.

As a last resort, a polyclonal antibody was used as the primary antibody. The advantage of using a polyclonal antibody as a probe is that they recognize more than one epitope. As a result, there is a greater probability of a positive signal, because the antibody can recognize multiple

peptide sequences. Continuing this screening with the polyvalent antibody 2L should eventually lead to success.

After the phage containing the "right" DNA sequence has been purified up so that all the plaques test positive, there are any number of steps that can be taken. First the Drosophila DNA must be removed from the phage. This DNA can then be radiolabeled and denatured into single strands. It can then be used as a probe to hybridize with denatured DNA from polytene chromosomes. An autoradiograph of this in situ hybridization (7, 8, 9) will "light up" the DNA sequences complimentary to the cloned DNA, and the cloned gene can be assigned a specific locus on the Drosophila chromosome.

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