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PREPARATIONS FOR TRANSFORMATION OF DROSOPHILA MELANOGASTER WITH GREEN FLUORESCENT PROTEIN-TAGGED DMNOPP140 AND DMNOPP140-RGG BY P-ELEMENT TRANSFORMATION

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PREPARATIONS FOR TRANSFORMATION OF *DROSOPHILA MELANOGASTER* WITH GREEN FLUORESCENT PROTEIN-TAGGED DMNOPP140 AND DMNOPP140-RGG BY P-ELEMENT TRANSFORMATION

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Abstract

The nucleolus is a large nuclear organelle dedicated primarily to the orchestration of ribosome biosynthesis. Nopp140 is a non-ribosomal nucleolar protein initially identified in rats as a nuclear localization signal-binding protein. Nopp140 is thought to shuttle on curvilinear tracks between the nucleolus and the cytoplasm, and may also shuttle between the nucleolus and Cajal bodies. There is a homolog of rat Nopp140 in *Drosophila* called DmNopp140. *Drosophila* also has a splice variant of DmNopp140 in which the carboxy tail has been replaced by a glycine-arginine rich domain. Understanding the role of Nopp140 in nucleolar function is especially important because this single protein may be involved in several nucleolar tasks by trafficking essential molecules to and from nucleoli. This work prepares for and develops techniques for transforming *Drosophila* embryos with DNAs that encode GFP-tagged DmNopp140 and its splice variant. A DNA segment encoding a GFP-tagged DmNopp140 protein was cloned into a P-element expression vector, pCaSpeR hs-act. In addition techniques were developed for injecting the DNA into embryos including embryo preparations and needle preparations.

Introduction

Eukaryotic ribosome biosynthesis is rather complicated. Ribosomes are made up of many smaller molecules including four RNA molecules transcribed in the nucleus (18S, 5.8S, 28S, and 5S ribosomal RNA molecules) and as many as eighty proteins that must be transported from the cytoplasm. The nucleolus, a large nuclear organelle, forms around tandemly repeated pre-ribosomal RNA genes and is dedicated primarily to the orchestration of ribosome biosynthesis. In the nucleolus, one 45S rRNA molecule is transcribed by RNA polymerase I and is cleaved into 18S, 5.8S, and 28S rRNA molecules. The 5S rRNA is transcribed outside of the nucleolus but then enters the nucleolus where it assembles with the other ribosome components (Hadjiolov, 1985).

Nucleolar structure varies among species, cell types, and developmental stages, but in general, nucleoli can be divided into three regions: the fibrillar center (FC), the surrounding dense fibrillar region (DFR), and the granular region (GR) which surrounds the dense fibrillar region (Scheer and Hook, 1999). The FC contains rDNA and RNA polymerase I which transcribes pre-rRNA, and it has been proposed that actual transcription occurs on the border of the FC and the DFR (Hozák et al, 1994). The DFR contains small nucleolar RNAs (snoRNAs) which are involved in cleavage, methylation, and pseudouridination of the pre-rRNA transcript (Weinstein and Steitz, 1999). Ribosome assembly begins within the DFR. As ribosomal proteins are added the ribosomal subunits move to the GR where assembly continues. Ribosomal subunits are then exported to the cytoplasm where a few final ribosomal proteins are added (Hadjiolov, 1985).

The nucleolus disappears during prometaphase of mitosis. Maturation Promoting Factor (a complex of $p34^{cdc}$ kinase and its regulatory protein, cyclin B) (or MPF) has kinase activity which is involved in triggering mitosis. Many nucleolar proteins have MPF phosphorylation sites, thus the same signal that triggers mitosis may trigger nucleolar disassembly. The nucleolus reassembles at the beginning of interphase around rRNA genes. These areas within genomes are called nucleolar organizers. The mechanisms by which nucleoli assemble and disassemble are not well known. Non-ribosomal nucleolar proteins have been implicated in every nucleolar task including disassembly and reassembly of the nucleolus, the importation of ribosomal proteins, the transcription and processing of pre-rRNA, and the assembly and export of ribosomal subunits (DiMario, 1999).

The nucleolar protein Nopp140 (nucleolar phosphoprotein of 140 kDa) is present at its highest level in the DFR of the nucleolus. Rat Nopp140 was the first identified version of the protein (Meier and Blobel, 1990). Homologs have been identified including SRP40 in yeast (Meier, 1996), xNopp180 in *Xenopus* (Cairns and McStay, 1995), p130 in humans (Pai et al, 1995), and most recently, DmNopp140 in *Drosophila* (Waggener and DiMario, submitted for publication). Rat Nopp140 was originally identified as a nuclear localization signal (NLS) binding protein (Meier and Blobel, 1990). Nuclear proteins are directed to the nucleus by NLS motifs within their structures. NLS-receptor proteins bind the NLS domain of a nuclear protein and carry it through pores in the nuclear membrane (Schwab and Dreyel, 1997). Nopp140 may be one of these carriers.

Nopp140 has three domains. The carboxy terminus and the amino terminus are highly conserved among organisms. The majority of the protein, the central region, consist of alternating basic and acidic regions. These alternating basic and acid regions may neutralize charges on proteins and other molecules that are bound to Nopp140 (Meier and Blobel, 1992). For this reason, and because Nopp140 binds NLS-sequences, the current belief is that Nopp140 acts as a chaperone for other molecules. Curvilinear tracks have been identified that extend from the DFR of the nucleolus to the cytoplasm. Nopp140 is thought to shuttle on these tracks between the nucleolus and the cytoplasm, possibly carrying molecules essential for ribosomal subunit biosynthesis or the pre-ribosomal subunits themselves (Meier and Blobel, 1992).

Besides its localization within the nucleolus, Nopp140 is found at relatively high levels in Cajal bodies. Cajal bodies are nuclear organelles that are closely associated with nucleoli, but whose function is unclear. A popular theory is that Cajal bodies are storehouses for nuclear proteins and RNAs, including small nucleolar RNAs involved in pre-rRNA processing. Nopp140 may also be responsible for shuttling molecules between the nucleolus and Cajal bodies (Isaac et al, 1998).

The acidic domains of Nopp140 contain phosphorylation sites for casein kinase II, and the basic domains have MPF phosphorylation sites (Meier, 1996). In fact, Nopp140 is one of the most heavily phosphorylated proteins in the cell (Meier, 1996). When the acidic domains of Nopp140 are phosphorylated, Nopp140 binds NLS-sequences (Meier and Blobel, 1990). Many nucleolar proteins have casein kinase and MPF phosphorylation sites, and it is believed that nucleolar tasks are regulated by phosphorylation of nucleolar proteins (DiMario, 1999).

Unlike many other nucleolar proteins, Nopp140 has no identifiable RNA binding domain (Isaac et al, 1998). Therefore, Nopp140 function probably depends more on interactions with other proteins. The carboxy terminus of Nopp140 contains several NLS-sequences. The central repeat region seems to be important for localization to the nucleolus and Cajal bodies. The amino terminus contains a potential nuclear export signal similar to the HIV Rev protein. According to a theory proposed by Isaac et al. (1998), the amino and carboxy termini are important for specific Nopp140/protein interactions, while the internal repeat segment neutralizes charges on proteins and RNP molecules for transport through the nuclear pore.

It has been shown that expressing Nopp140 mutants that are missing any one of its three domains causes mislocation of several nucleolar proteins. For instance, when a Nopp140 mutant which is missing its amino terminal domain is expressed in COS-1 cells (African monkey kidney cell line CV-1 transformed with origin deficient SV40 virus), fibrillarin, a protein that plays important roles in pre-rRNA processing, UBF, a transcription factor, and NAP57, another nucleolar protein probably important in pre-rRNA processing, all fail to localize to the nucleolus (Isaac et al, 1998). This suggests that Nopp140 interacts with these proteins, and it supports the theory that Nopp140 shuttles molecules important for ribosome biosynthesis. Understanding the role of Nopp140 in nucleolar function is especially important because this single protein may directly or indirectly participate in several nucleolar tasks by trafficking essential molecules.

As previously mentioned, a homolog of rat Nopp140 has been identified in *Drosophila melanogaster*, DmNopp140. A splice variant has also been observed in

which most of the carboxy tail is replaced with a very different sequence containing a glycine and arginine rich domain (referred to as either the GAR or RGG domain) (Waggener and DiMario, submitted for publication). RGG domains are relatively common in proteins that bind RNA, and they are present in several nucleolar proteins. The splice variant is called DmNopp140-RGG. The RGG domain in DmNopp140-RGG is very similar to that found in the carboxy terminus of nucleolin, an RNA-binding nucleolar protein involved in rRNA cleavage and perhaps in other processing events (Ginisty et al, 1999). The function of RGG domains is unclear, but in nucleolin it is necessary but not sufficient for nucleolar localization (Heine et al, 1993).

Both DmNopp140 and DmNopp140-RGG localize to Cajal bodies in human HeLa cells, but only DmNopp140 localizes to Cajal bodies in *Drosophila* Schneider II cells (Waggener and DiMario, submitted for publication). Thus Waggener and DiMario have proposed that the RGG domain of DmNopp140-RGG may act as a strong nucleolar localization signal or as a nucleolar retention signal. More experimentation is needed to determine if DmNopp140 and DmNopp140-RGG are differentially expressed in different cell types at different developmental stages. Eventually, our goal is to characterize the expression and localization of each variant in *Drosophila* cells.

In order to observe DmNopp140 and DmNopp140-RGG in several *Drosophila* tissues, *Drosophila* embryos will be transformed by P-element mediated transposition. This technique employs P-transposable elements found naturally in "P-strains" of *Drosophila*. Though stable within the genomes of P-strain flies, P-elements will insert themselves into the genomes, and then continue to excise themselves and reinsert at high frequency when introduced into non-P-strains of *Drosophila*. Non-autonomous P-

elements can be engineered by deletions to be stable and immobile by themselves, but to transpose in the presence of functional P-element transposons (Pirrotta, 1988).

DmNopp140/GFP DNA will be inserted into a non-autonomous P-element vector, pCaSpeR hs-act, and will be injected into *Drosophila* embryos along with a helper plasmid, pUChsΔ2-3. This P-element helper plasmid has been engineered so that it has a functional transposase activity but is unable to insert itself into the *Drosophila* genome. Thus, it is present in P1 flies to provide transpose activity but is absent from generation 1 flies so the insert segment can no longer transpose.

The pCaSpeR hs-act vector contains the *white* gene. Expression of the *white* gene is controlled by the minimal *white* gene promoter. DmNopp140-GFP expression will be controlled by the hsp70 promoter. There is an actin 5C trailer sequence and poly A addition site at the 3' end of the insert DNA so that the transcript is stable under non-heat shock conditions. White-eyed flies that have been injected with the DNA will be crossed to white-eyed, non-injected flies of the opposite sex. The progeny of transformed flies will have pigmented eyes. Eye color will range from pale yellow to red. The color depends on where the P-element DmNopp140 cDNA was inserted into the genome (Pirrotta, 1988).

Drosophila eggs are about half a millimeter long. The ventral side is concave; the dorsal side is flat (Bownes, 1982). (See **Figure 1a.**) They are encased in a chorion shell. The anterior end of the chorion has two appendages (not shown). The chorion can be removed with a 50% Clorox bleach solution. Removal can be gauged by the disappearance of these chorionic appendages (Pirrotta, 1988). Dechorionated embryos are extremely fragile. The anterior end of the dechorionated embryo can be identified by

looking for the micropylar. (See **Figure 1**. The micropylar is denoted by blue arrows.) This relatively large, thorn-shaped structure is the site of sperm entry (Bownes, 1982). The opposite pole, the posterior pole, is the target for P-element DNA injections.

Fertilization of *Drosophila* eggs occurs within the uterus by a single sperm (Mahowald and Hardy, 1985). The male and female pronuclei fuse within a few minutes of sperm entry and the nuclei divide syncytially (without forming cell membranes) at 9-10 minute intervals. After 7-8 divisions, 10-20 nuclei migrate to the posterior pole of the embryo and become cellularized (enclosed in cellular membranes) (Bownes, 1982). These cells are called pole cells, and the adult gametes ultimately derive from these cells. (See **Figure 1b**. In this embryo, pole cells have begun to form.) The entire process from fertilization to cellularization takes about 90 minutes (Pirotta, 1988). The object is to inject the P-element clone into the posterior end of the embryo within 90 minutes of fertilization and have the injected DNA integrate within the genome of forming pole cells such that the adult gametes are transformed. (See **Figure 1a**. In this embryo pole cells have not yet formed).

Female flies will not lay their fertilized eggs until the embryo has reached an advanced developmental stage, except under better than average laying conditions that include an abundant food source and a good laying surface (Bownes, 1982). Embryos can be collected on plates of grape juice and agar covered in yeast. To obtain the youngest embryos possible, embryos must be collected twice with the first collection (a pre-lay) discarded (Pirrotta, 1988). The pre-lay usually contains well advanced embryos.

Transformation efficiencies depend on many factors: the fly strain, the size of the insert, the experience of the injector, and the size of the needle (Pirrotta, 1988).

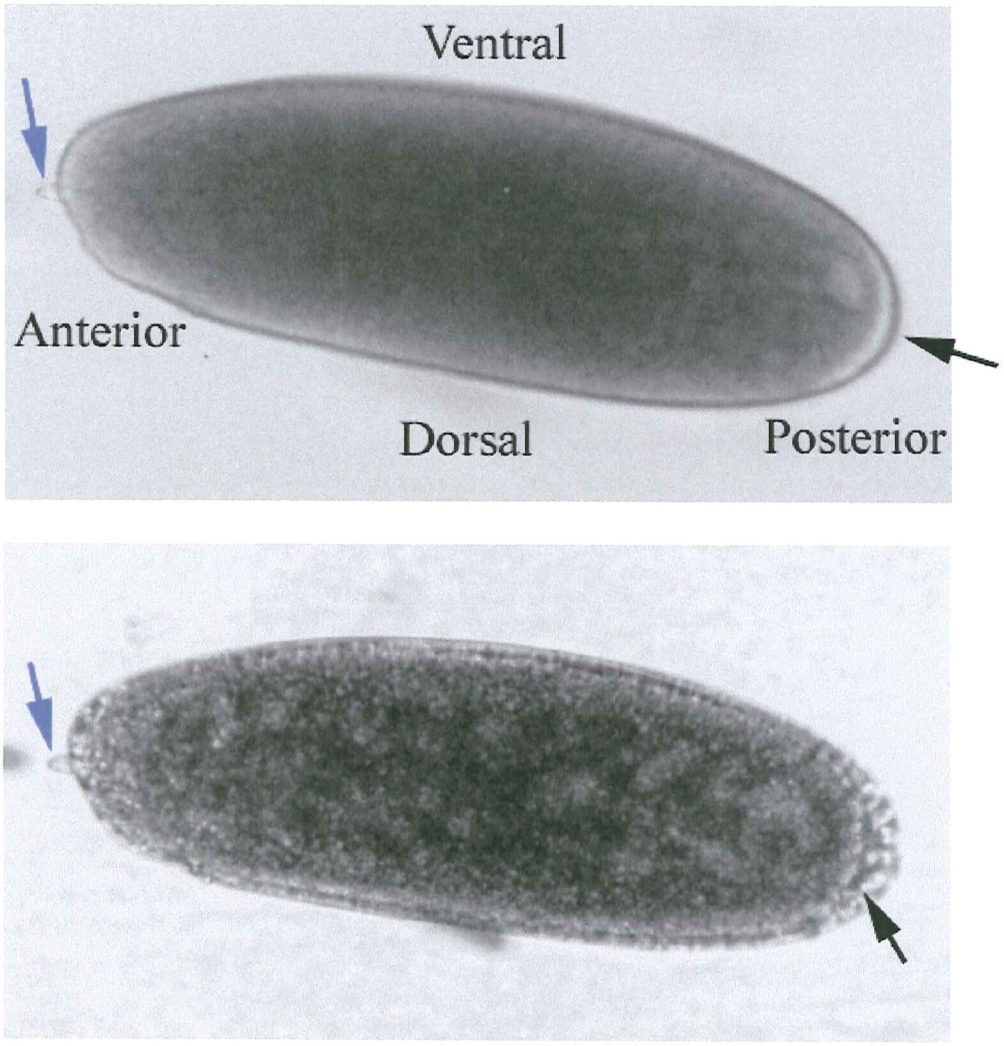


Figure 1. Dechorionated *Drosophila* embryo. **(a)** We will attempt to transform embryos of this age. The pole cells have not yet started to form at the posterior pole. The blue arrow points to the micropylar. The black arrow points to the injection site. **(b)** An advanced stage embryo. Here the embryo is too old to inject. Pole cells have already formed at the posterior pole (black arrow). The blue arrow at the opposite pole points to the micropylar structure.

Successful transformation to produce the desired fly line will require injecting 100 to 200 embryos (Pirrotta, 1988).

Our final goal is to transform *Drosophila* germ cells to establish lines of flies expressing the green florescent protein (GFP) fused to either DmNopp140 or DmNopp140-RGG. The purpose of this thesis work, however, is only to prepare materials for the transformation. The DmNopp140-RGG expression vector was prepared previously. The DmNopp140/GFP expression vector will be prepared by removing a DmNopp140/GFP DNA fragment from EGFP-C3/Nopp140 plasmid and cloning it into pCaSpeR hs-act, a *Drosophila* P-element expression vector. Techniques will be tested for preparing beveled needles from capillaries small enough for injecting *Drosophila* embryos.

Materials and Methods

Part I. Cloning

All restriction enzymes and DNA ligase were purchased from New England Biolabs (Beverly, MA). EGFP-C3/DmNopp140 plasmid was the starting point vector for this thesis project. It was prepared prior to the beginning of this work. The pCaSpeR hs-act vector was obtained from the laboratory of Carl Thummel at the Howard Hughes Medical Institute at the University of Utah. Cloning methods were adapted from Current Protocols in Molecular Biology (Ausubel et al., 1987-1995).

DmNopp140/GFP was cut out of the EGFP-C3/DmNopp140 plasmid using restriction enzymes *Nhe*I and *Bam*HI. Nopp140 has an internal *Bam*HI site, so a partial digest was necessary. The EGFP-C3/DmNopp140 DNA was cut first with *Nhe*I. See, the restriction maps for pEGFP-C3/DmNopp140 and pCaSpeR hs-act, **Figure 2a and 2b** respectively. The restriction sites to be digested are denoted by blue arrows pointing down. The internal *Bam*HI site is denoted by "*". The *Nhe*I cut DNA was ethanol precipitated and resuspended in H_2O. The mixture was separated into five different tubes with five different concentrations (a serial dilution) of *Bam*HI. The digestion proceeded for 15 minutes (a fraction of the time used for a complete digest). The mixture was then put on ice to inactivate *Bam*HI. The tube in which *Bam*HI cut only one site per DNA molecule contained DNA cut at the internal *Bam*HI site and DNA cut at the desired *Bam*HI site.

pCaSpeR hs-act was cut with *Bam*HI and *Xba*I to produce identical sticky ends for a ligation reaction (*Nhe*I and *Xba*I create identical sticky ends). The digested pCaSpeR hs-act DNA and each of the five DmNopp140/GFP digests were separated on a

Figure 2a. pEGFP-C3/DmNopp140 restriction map. Blue arrows mark target restriction sites. The asterisk marks the internal *Bam*HI site.

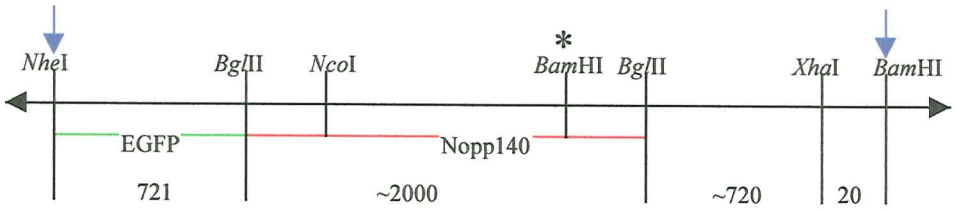


Figure 2b. pCaSpeR hs-act restriction map. Blue arrows mark target restriction sites.

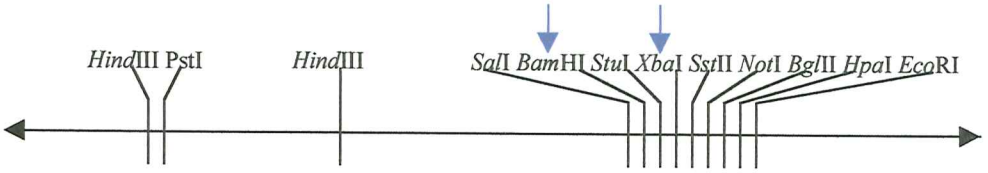
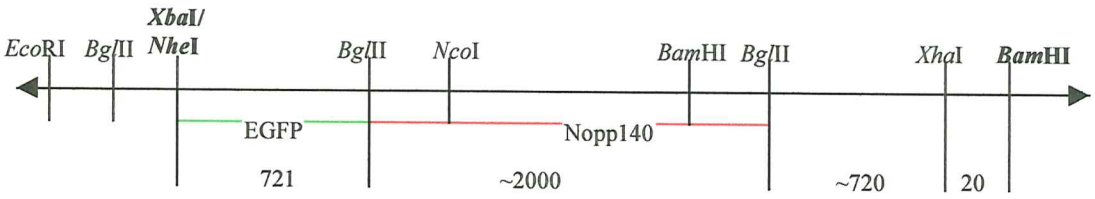


Figure 2c. pCaSpeR/DmNopp140-GFP restriction map. The sites with bold labels represent points of ligation.



low melting agarose gel. The appropriate bands were cut out, and the DNA was ligated together using T4 DNA ligase.

Part II. Preparation of Glass Needles for Embryo Injections

The needle-puller used was the Narishige PB-7. It pulls needles from capillary tubes using a hot filament and gravity. Glass needles were pulled from Kwik-fil™ borosilicate glass capillary tubes (manufacture's ID#: M1B100F-6). The needles had to be small enough to inject *Drosophila* embryos which are about 0.15 mm wide. Several settings on the puller were used to determine the ideal temperature and weight to produce a needle with a small enough tip. The settings are described in the results section.

To create a bevel-tipped needle, the closed needles were opened using a sand/water slurry that was stirred on a stir plate. The sand used was a silicon carbide powder (grit 120, No. 40-6400-120-016 from Buehler, Lake Bluff, IL). This technique was initially developed in preparing needles for injecting mouse embryos (Gundersen et al, 1993), and was first adapted for *Drosophila* embryos by John Powers at Indiana University. The slurry was stirred to create a slight vortex. When stirring, the surface of the vortex had to be smooth and unchanging. The needle was held in place by a syringe clamped to a ring stand. The needle was positioned at an angle to the surface of the water such that the tip barely touched the surface of the water. The needle was not be submerged. After one minute the needle was removed. To test if the needle was open, the end of the needle was submerged in water, and then examined under a dissecting scope. Opened needles had water drawn into their tips by capillary action.

Part III. Collection and Dechorionation of Embryos and Preliminary Injections

To collect fly embryos, w^{1118} flies were transferred to a plastic beaker. The beaker was capped with a grape juice and agar plate covered with a yeast paste. The beaker was inverted and holes were punched in the top for air exchange. A pre-lay was collected for 2 hrs. A new plate replaced the pre-lay plate and embryos were collected for 30 min. The embryos from the second plate were scraped from the plate and put into a 50% Clorox ® bleach/water solution. When the chorionic appendages had dissolved the bleach was removed, and the embryos were rinsed with water six times.

The dechorionated embryos were attached to a microscope slide with double-stick tape. They were desiccated with Drierite for 1 min and covered in oil. Two different oils were used and both worked well, Sigma Halocarbon Oil 700 H-8898 and Halocarbon 27 Oil 8C-00-159, made by Halocarbon Products Corp. Later, a DNA solution with between 50-100 μL/mL of the pUChsΔ2-3 helper plasmid DNA and 200-500 μL/mL pCaSpeR/DmNopp140-GFP DNA will be injected into the embryos using an oil-filled Drummond microinjector and a Zeiss micromanipulator. For the purpose of this thesis only water was injected. After injection, the slide with the embryo was put in a petri dish along with a wet piece of filter paper. They embryos were left to develop overnight.

Results

Part I: Cloning

Cutting out the DmNopp140/GFP insert:

The EGFP-C3/DmNopp140 plasmid DNA was cut first with *Nhe*I. A 4 μL volume of DNA was cut with 2 μL of *Nhe*I in 2 μL of 10 x digestion buffer no. 2 from New England Biolabs, 2 μL of 10 x BSA, and 10 μL of H_2O. This mixture was placed at 37° C for 2 hours. The *Nhe*I digest was then ethanol precipitated. The pellet was re-suspended in 90 μL of H_2O. Eighty (80) μL of the re-suspended DNA was combined with 10 μL of 10 x *Bam*HI reaction buffer and 10 μL of 10 x BSA. The mixture was divided into 5 tubes: 30 μL was put into tube 1, 20 μL each into 2-4, and 10 μL into tube 5. One (1) μL of *Bam*HI restriction enzyme was added to tube 1. The contents of tube 1 were mixed well and 10 μL was transferred to tube 2. The contents of tube 2 were mixed and 10 μL were transferred to tube 3 (this was continued on through tube 5).

The reaction was allowed to continue for 15 min at 37° C. The DNA samples were then separated by agarose gel electrophoresis (see **Figure 3).** It appeared that *Bam*HI cut to completion in every tube and there was no partial digestion (lanes 1-5). Also, the *Nhe*I digest alone (lane 6) did not cut completely.

A second attempt was made to cut out the DmNopp140/GFP segment. The EGFP-C3/DmNopp140 plasmid was again cut with *Nhe*I. Since *Nhe*I did not cut completely the first time in 2 hrs, the reaction was sealed in an eppendorf tube using wax and placed in a 37° C incubator overnight. The digest was ethanol precipitated and again partially cut with *Bam*HI. For the partial digest only 0.5 μL of *Bam*HI were used compared to 1 μL used previously. The digested DNA samples were separated on an

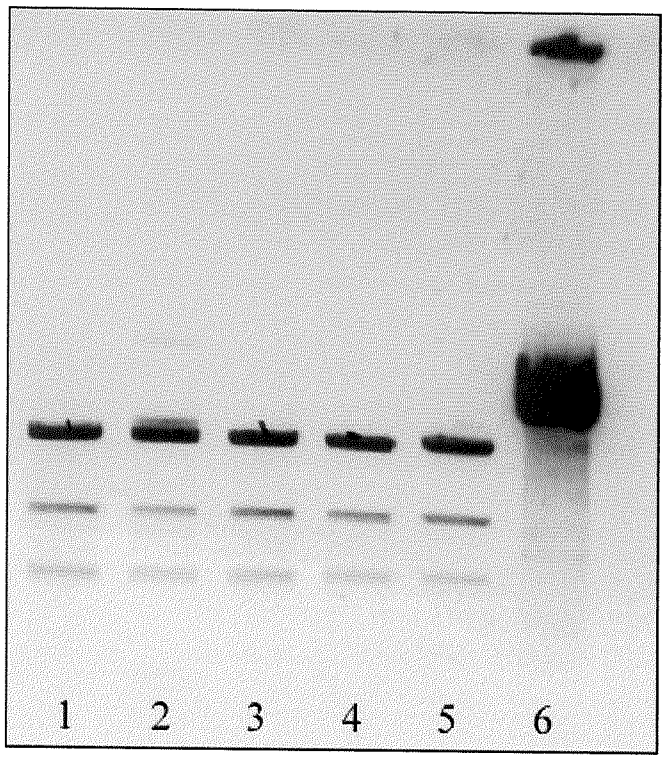


Figure 3. First attempt at partial *Bam*HI digestion of the *Nhe*I-cut pEGFP-C3/DmNopp140. Lanes 1-5 contain a serial dilution of *Bam*HI *Nhe*I-digest of the EGFP-C3/DmNopp140 plasmid. No partial digest was observed. Instead, the DNA was cut completely by *Bam*HI. Lane 6: *Nhe*I digest of the EGFP-C3/DmNopp140 plasmid.

agarose gel (see **Figure 4**). (See also **Figure 2a**, the restriction map of the pEGFP/DmNopp140 construct.) The second partial digest worked well. The bands containing the full-length DmNopp140 insert (band 4 in lanes 5 and 6) were cut from the gel and saved for use in the ligation reaction. This procedure was followed each time the insert was needed for additional attempts at the ligation reaction.

Amplifying and isolating the vector DNA:

The pCaSpeR hs-act vector has the ampicillin resistance gene for selection in *E. coli*. The vector was amplified from frozen glycerol stocks in 5 mL of LB with 50 μg/mL ampicillin. Cells were isolated by centrifugation and re-suspended in a solution called Alk I (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl, pH 8.0). Alk II (0.2 N NaOH and 1% SDS) and Alk III (acetic acid and 3 M potassium acetate solution, pH 5.5) combined with Alk I were used to lyse and precipitate cell membranes, respectively. The EGFP-C3/DmNopp140 construct had been previously amplified and purified.

Cutting the pCaSpeR hs-act vector:

The pCaSpeR hs-act vector is 9.1 kbp. The purified vector was cut with *Bam*HI and *Xba*I. A 5 μL volume of DNA was cut with 1 μL of *Xba*I, 1 μL of *Bam*HI in 2 μL of 10 x BSA, 2 μL of 10 x buffer no. 2, and 9 μL of H_2O. The agarose gel (not shown) showed no bands, but contained large patches of RNA at the bottom of the gel.

The DNA was cut again, this time with 20 μL of DNA, 2 μL of *Xba*I and 2 μL of *Bam*HI (compared to 1 μL of each enzyme used previously), 1 μL of RNase, 5 μL of 10 x buffer no. 2 and 5 μL of 10 x BSA in 15 μL of H_2O. The reaction mixture was separated on an agarose gel. The DNA sample was run over three lanes (see **Figure 5**). Some of the DNA did not leave the wells. This has been observed occasionally in the lab when

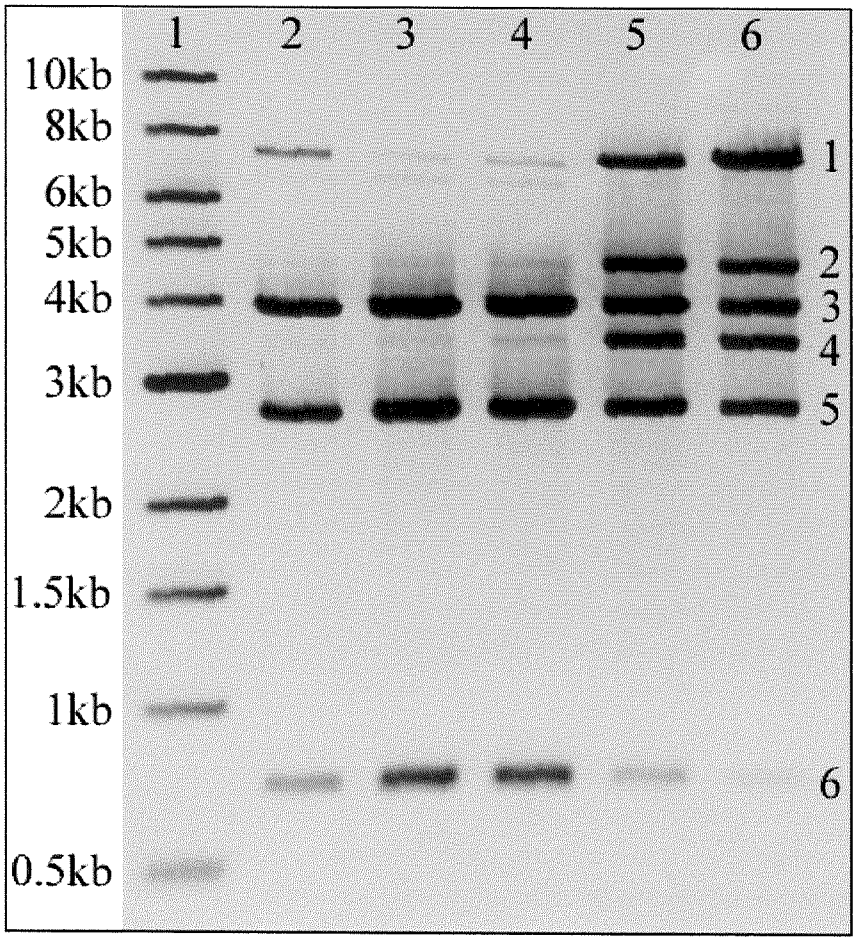


Figure 4. Partial *Bam*HI digestion of *Nhe*I-cut EGFP-C3/DmNopp140 plasmid. Samples are compared to the 1 kb ladder from New England Biolabs (lane 1). Bands 3, 5, and 6 represent segments produced by complete *Bam*HI digestion of the *Nhe*I-cut plasmid (bands in lane 6 are numbered). **Band 5** (about 2.8 kbp) represents the DNA between the *Nhe*I site and the internal *Bam*HI site. **Band 6** (about 750 bp) represents the DNA between the *Bam*HI sites. **Band 3** (about 4 kbp) is the rest of the plasmid (from the *Bam*HI site to the *Nhe*I site). Lanes 4 and 5 show three extra bands. These three bands represent the partial digests. **Band 1,** between 7 and 8 kb, represents the entire plasmid cut only by *Nhe*I. **Band 2**, about 3.5 kbp, represents the portion of the plasmid from the internal *Bam*HI site around to the *Nhe*I site. **Band 4**, about 3.6 kbp, represents the section between the *Nhe*I site and the second *Bam*HI site (the full length DmNopp140 insert plus the GFP tag).

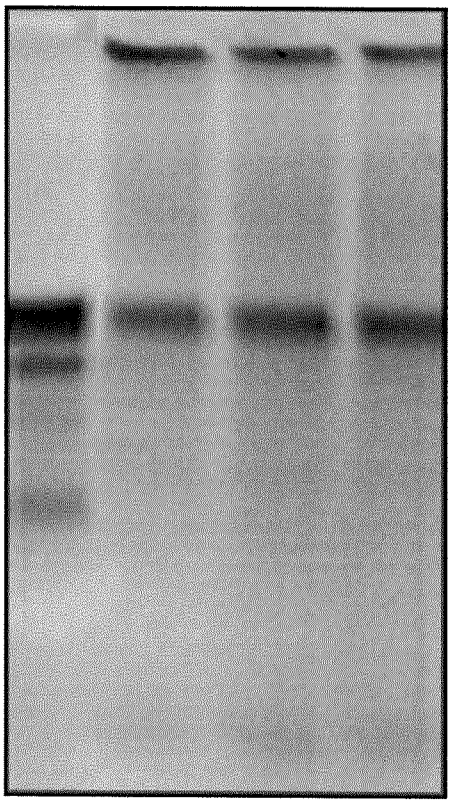


Figure 5. The pCaSpeR hs-act vector cut with *Bam*HI and *Xba*I. Sample is compared to the lambda phage *Hind*III digest markers. The reaction contained RNase. Some of the DNA did not leave the wells. This may be caused by RNase interference. The bands near the middle of the gel were cut out for the ligation reaction.

RNase is used. It may be caused by contaminated or decaying RNase binding to DNA, or binding to a restriction enzyme and preventing it from cleaving the DNA or separating from the DNA. Other labs have observed this effect only in reactions where BSA is added. The bands (about 9 kbp) were cut out.

The Ligation Reaction:

To ligate the DmNopp140/GFP insert into the pCaSpeR hs-act vector, 2 μL of the digested vector and 7 μL of the insert DNA were combined with 8 μL of H_2O at 70° C for 10 min. The tube was then transferred to 37° C, and 2 μL of 10 x T4 ligase buffer and 1 μL of T4 ligase were added. The ligation reaction continued in a 16° C water-bath overnight.

Amplification of the Construct by Transformation in *E.coli*:

Five (5) μL of the ligation mixture were added to 200 μL of XL1-blue competent *E. coli* cells. This mixture sat on ice for 1 hr. The cells were then heat shocked for 1.5 min at 42° C, and 1 mL of LB was added. After 45 min of shaking at 37° C, the cells were transferred to ampicillin plates and incubated overnight at 37° C. No colonies grew on the plates in this first attempt.

Repeat attempts:

The experiment was repeated starting from the frozen pCaSpeR hs-act vector stocks. Still no colonies grew from the transformation.

The experiment was repeated a third time. This time the digested pCaSpeR hs-act DNA was removed from the agarose gel using phenol and phenol/chloroform extractions. An aliquot was run on an agarose gel to estimate the concentration (not shown). There

was only a faint band near the 9.4 kbp band of the lambda phage *Hind*III digest markers. It appeared that too much of the vector DNA was lost in the purification steps.

The pCaSpeR hs-act was digested a fourth time, this time starting with 1 L of culture instead of a 5 mL. RNase was omitted from the digest to rule out possible interferences. Still, the vector DNA was not present in high enough concentrations to perform the ligation (see **Figure 6**).

The original pCaSpeR hs-act DNA samples used to make the frozen stocks came from researchers at the University of Utah. More DNA was requested and received. The new pCaSpeR hs-act DNA was transformed to XL1-blue competent *E. coli* cells. New glycerol stocks were frozen away, and CaSpeR hs-act plasmid DNA was extracted from cells. A 5 μL aliquot was run on a gel to estimate the DNA concentration (see **Figure 7**).

One (1) μL of plasmid DNA was cut with 2 μL of *Xba*I, 2 μL of *Bam*HI in 4.5 μL of 10 x buffer no. 2, 4.5 μL of 10 x BSA, and 29 μL of H_2O. New RNase was obtained and 2 μL was added to the reaction to remove RNA. This was run over 4 lanes of an agarose gel (not shown). The bands were cut out and combined with the insert in a ligation reaction as described previously. Following transformation in XL1-blue *E. coli* there were again no colonies.

The experiment was repeated a fifth time. The pCaSpeR/DmNopp140-GFP construct is a relatively large plasmid (9.1 kbp). In order to increase the likelihood of getting successful transformation, the ligation mixture was divided in half. Half of the mixture was used in a conventional transformation as done in the first four trials, the other half was subjected to electroporation. In electroporation, DNA is introduced into cells by "zapping" the cells with high voltage and low amperage in the ligation solution.

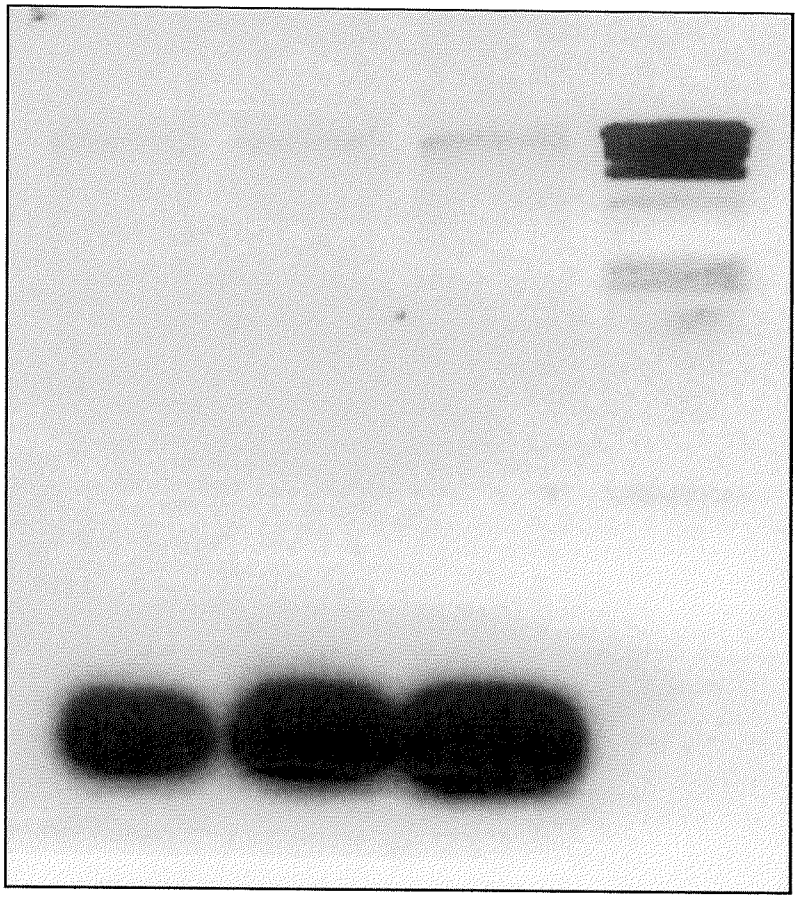


Figure 6. Double digestion of pCaSpeR hs-act with *Xba*I and *Bam*HI. The sample is compared to lambda phage *Hind*III digest markers. RNase was omitted from the reaction. There is not enough vector DNA for a successful ligation.

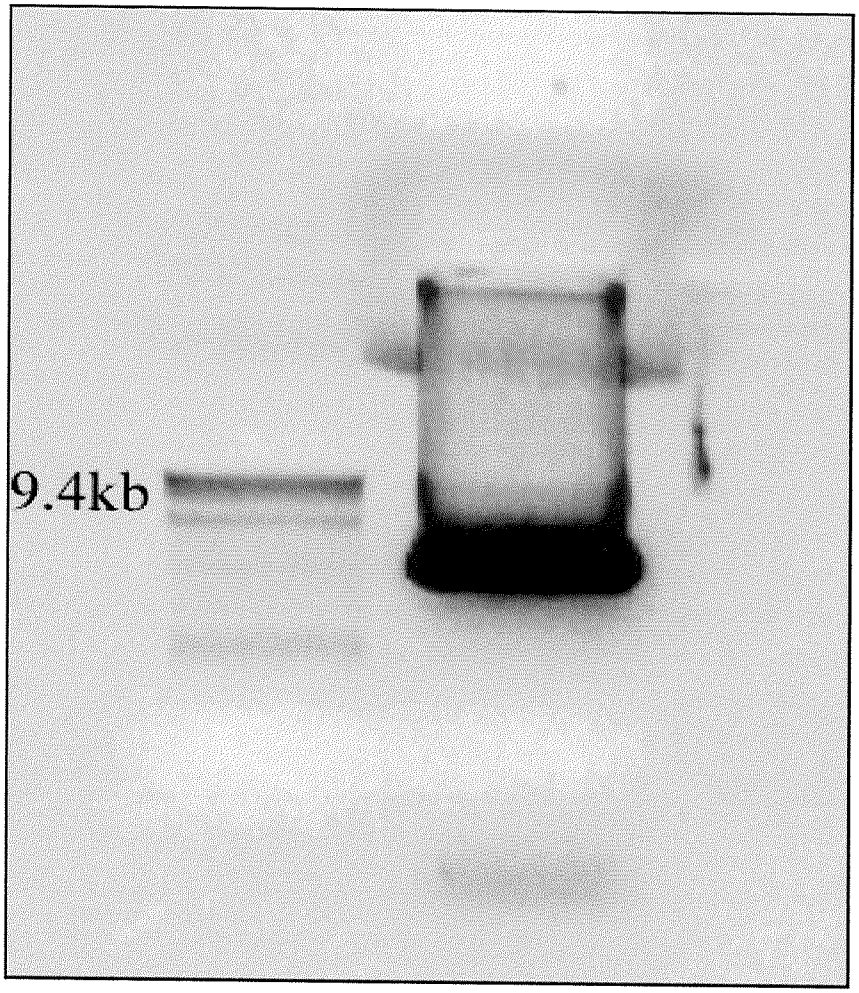


Figure 7. Uncut pCaSpeR hs-act. The band migrated farther than the 9.4 kbp band of lambda *Hind*III digest marker as would be expected for circularized, supercoiled DNA of 9.1 kbp. This gel verifies transformation of the newly aquired pCaSpeR hs-act vector was successful.

A quick, high pulse of electricity moves the negatively charged DNA across the plasma membrane. No colonies were observed on ampicillin plates containing cells from the conventional transformation. The electroporated cells produced 22 colonies.

Screening colonies:

One (1) mL overnight cultures were started from 16 of the 22 colonies that arose from the electroporated cells. The plasmid DNA was isolated and cut with the restriction enzyme *Bgl*II. A 5 μL volume of DNA was cut with 1 μL of *Bgl*II in 2 μL of 10 x buffer, 0.5 μL of RNase, and 11.5 μL of H_2O. The samples were separated by electrophoresis and compared to lambda phage *Hind*III digest markers (see **Figure 8**).

The pCaSpeR/DmNopp140-GFP construct should have three *Bgl*II sites (see **Figure 2c**), and three bands are expected. **Band 1** should be about 720 bp; it represents the segment between the first two *Bgl*II sites. **Band 2** should be about 2 kbp, and it represents the segment between the second and third *Bgl*II sites. **Band 3** should be about 10 kbp, and it represents the rest of the plasmid. An incomplete digest should produce three additional bands. **Band 4** should be about 12.7 kbp, and it represents the plasmid cut only once. **Band 5** should be about 2.7 kbp, and it represents the segment between *Bgl*II sites 1 and 3. **Band 6** should be about 10.7 kbp, and it represents the portion of the plasmid between the second *Bgl*II site back around to the first.

*Bgl*II digests of colonies 9 and 13 contained expected bands 1, 2, 5, and at least two bands between 9.4 kbp and 23 kbp. There was an extra band a bit shorter than 9.4 kbp, and it is probably uncut, circularized DNA. These latter bands could be the expected bands 3, 4, or 6. The colony 9 plasmid looked the best, and it was amplified, isolated, and then purified twice by CsCl density ultracentrifugation.

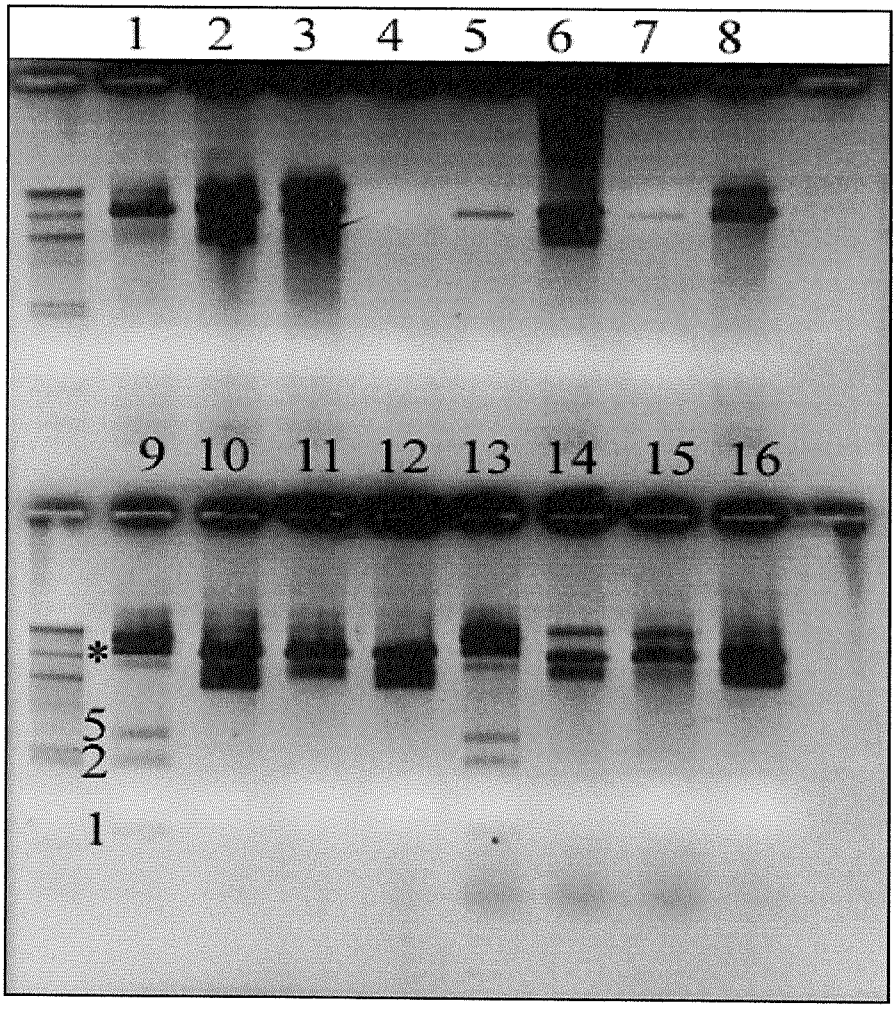


Figure 8. *Bgl*II digestions of 16 colonies grown from cells transformed by electroporation. The samples are compared to the lambda phage HindIII digest markers. Colonies 9 and 13 (lanes 9 and 13) contain expected restriction fragments. **Band 1** (about 720 bp) represents the segment between the first two *Bgl*II sites. **Band 2** (about 2 kbp) represents the segment between the second and third *Bgl*II sites. **Band 5** (about 2.7 kbp) represents the segment between the first and the third *Bgl*II sites. The asterisk marks at least two bands between 23 and 9.4 kilobases. These bands may represent any of three DNA segments: a 12.7 kbp fragment representing the linear plasmid (cut only once), a 10 kbp fragment representing the segment from the third *Bgl*II site back to the first, or a 10.7 kbp fragment from the third *Bgl*II site back to the second *Bgl*II site.

Because some of the bands in the *Bgl*II digest of the colony 9 plasmid were not conclusively identified, the CsCl purified sample was digested with *Bam*HI, *Eco*RI, and a combination of both enzymes (see **Figure 9** and **Figure 2c**). The double digest (lanes 1, 2, and 3) produced the expected bands. **Band 1** (about 9 kbp) is a complete digest, and it represents the segment from the second *Bam*HI site to the *Eco*RI site. **Band 2** (about 3.7 kbp) is a partial digest, and it represents the segment between the *Eco*RI site and the second *Bam*HI site. **Band 3** (about 2.8 kbp) represents the segment from *Eco*RI to the first *Bam*HI site. **Band 4** (about 750 bp) represents the segment between the two *Bam*HI sites. The separate *Bam*HI and *Eco*RI digests, however, showed unexpected bands. The *Bam*HI digest (lanes 4, 5, and 6) has the expected bands of about 12 kbp and about 750 bp, but there are extra bands near 3.5 kbp and 2.1 kbp. The *Eco*RI digest (lanes 7, 8, and 9) shows the expected band near 12 kbp, but there is an extra band at about 3 kbp.

To determine if these bands resulted from cleavage at unmapped restriction sites in the pCaSpeR hs-act vector or in the DmNopp140/GFP insert, pEGFP/DmNopp140 and pCaSpeR hs-act were cut separately with *Bam*HI, *Eco*RI, and a combination of both enzymes. The restriction patterns were compared to the 1 kbp ladder (see **Figure 10**).

The pCaSpeR hs-act vector has only one *Bam*HI and one *Eco*RI site. The two sites are separated by only a few bases. The pCaSpeR hs-act digestions (see **Figure 10**) with *Bam*HI and *Eco*RI (lane 7), *Bam*HI alone (lane 8), and *Eco*RI alone (lane 9) did not account for the extra restriction fragments. All three digests migrate to positions between the 8 and 10 kbp markers. EGFP-C3/DmNopp140 has two *Bam*HI sites and no *Eco*RI sites. The EGFP-C3/DmNopp140 plasmid digestions (see **Figure 10**) with *Bam*HI and *Eco*RI (lane 4), *Bam*HI alone (lane 5), and *Eco*RI alone (lane 6) did not account for the

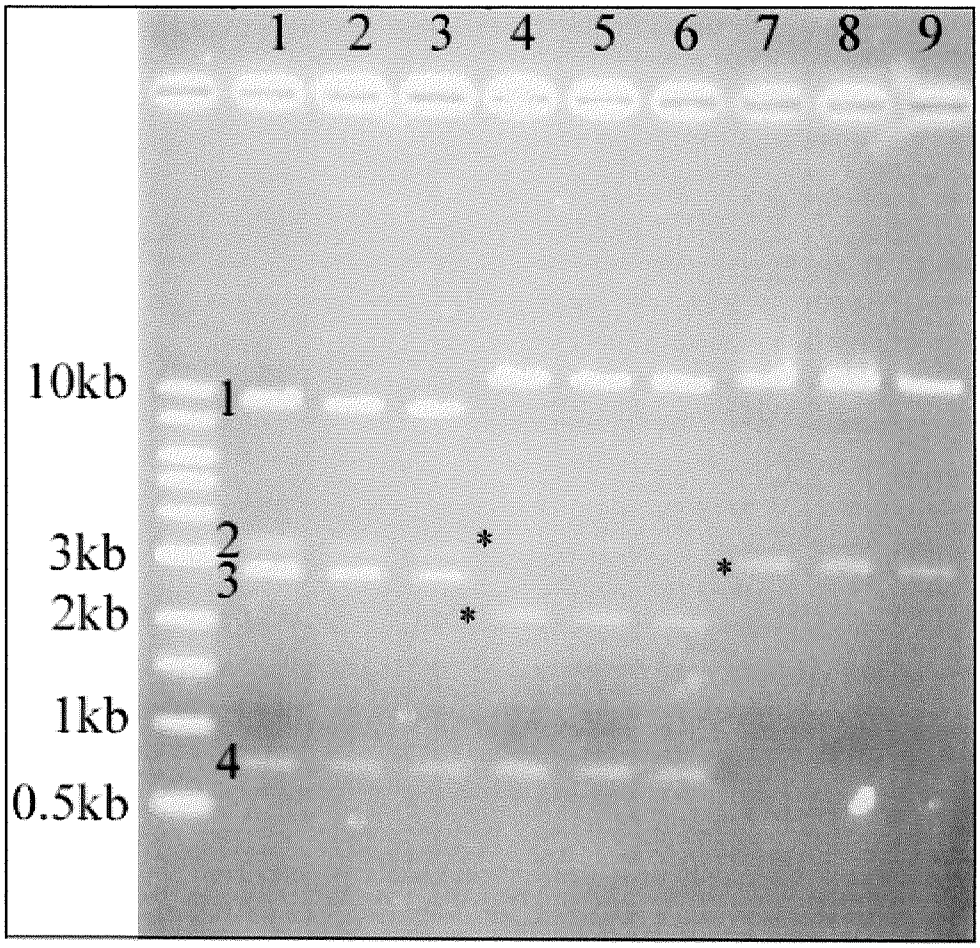


Figure 9. Screening of the CsCl purified plasmid from colony 9. The restriction patterns are compared to the I kb ladder from New England Biolabs. Lanes 1-3 are double digests of pCaSpeR/DmNopp140-GFP with *Bam*HI and *Eco*RI. See Figure 1c for the restriction map. **Band 1** represents the segment between the second *Bam*HI site and the *Eco*RI site. **Band 2** is a partial digest, and it represents the segment between the *Eco*RI site and the second *Bam*HI site. **Band 3** represents the segment between the *Eco*RI site and the first *Bam*HI site. **Band 4** represents the segment between the *Bam*HI sites. Lanes 4-6 are *Bam*HI digests. There are unexpected bands near 3.5 kbp and 2.1 kbp (denoted by asterisks). Lanes 7-9 are *Eco*RI digests. There is an extra band near 3 kbp (also denoted by an asterisk).

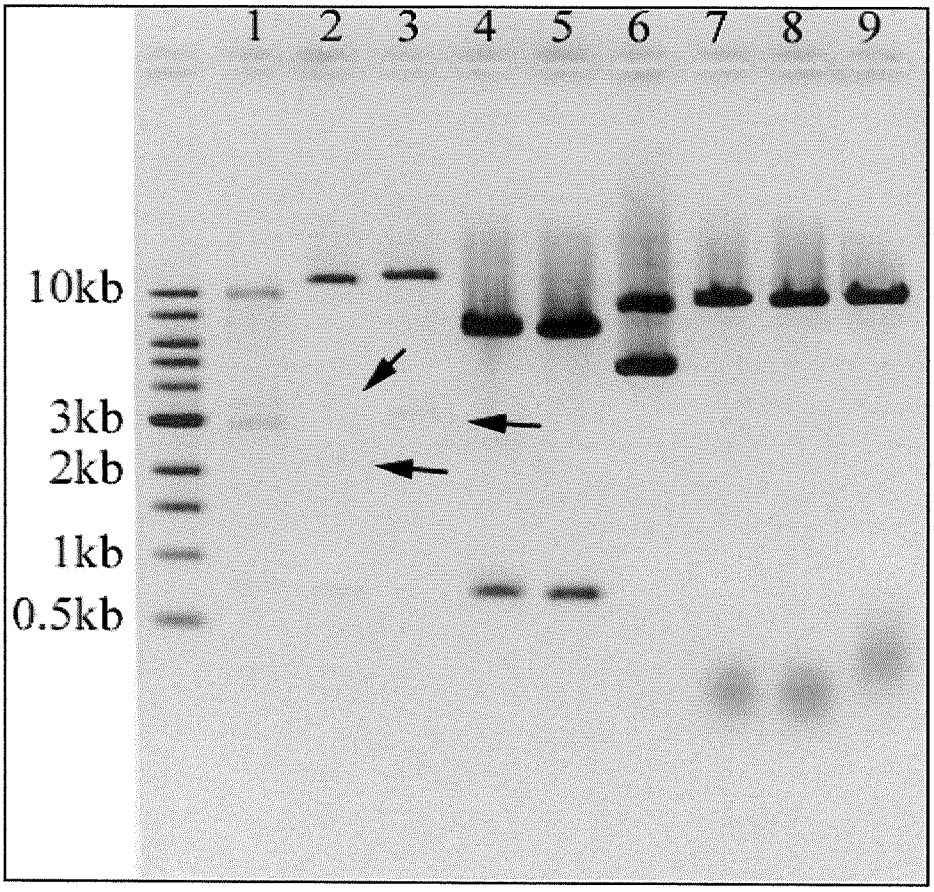


Figure 10. Colony 9 plasmid, pEGFP/DmNopp140, and pCaSpeR hs-act DNA digested with *Eco*RI and *Bam*HI. The samples are compared to the 1 kb ladder from New England Biolabs. Lane 1 contains an *Eco*RI and *Bam*HI digest of the colony 9 plasmid. Lane 2 is a digest of colony 9 with *Bam*HI. Lane 3 is a digest of colony 9 with *Eco*RI. There are still extra bands in lanes 2 and 3 (denoted by black arrows). Lane 4 is a digest of pEGFP-C3/DmNopp140 with *Eco*RI and *Bam*HI. Lane 5 is a digest of pEGFP-C3/DmNopp140 with only *Bam*HI. Lane 6 is a digest of pEGFP-C3/DmNopp140 with *Eco*RI. Lane 7 is a digest of pCaSpeR hs-act with *Eco*RI and *Bam*HI. Lane 8 is a digest of pCaSpeR hs-act with *Bam*HI. Lane 9 is a digest of pCaSpeR hs-act with *Eco*RI. All the pCaSpeR hs-act and pEGFP/DmNopp140 digest bands were expected.

extra restriction fragments. The *Bam*HI digests show bands at about 750 bp and another representing the rest of the plasmid. The *Eco*RI digest shows two bands of uncut DNA (supercoiled and nicked but closed circle plasmid). The colony 9 plasmid (see **Figure 10**) digested with *Bam*HI and *Eco*RI (lane 1), *Bam*HI alone (lane 2), and *Eco*RI alone (lane 3) had the same restriction patterns as before (as in **Figure 8**). The *Bam*HI and *Eco*RI digests of the construct still show extra bands. These bands may have come from a contaminating plasmid in the electroporation cells. Because the bands could not be explained, the experiment was repeated.

Nopp140/GFP was again cut from the pEGFP/Nopp140 construct. pCaSpeR hs-act was again cut with *Bam*HI and *Xba*I. The pieces were ligated together, this time 1 μL of the vector digest (versus 2 μL used in previous attempts) and 7 μL of the insert where combined with 8 μL H_2O at 70° C for 10 minutes. The tube was transferred to 37° C and 2 μL of 10 x T4 ligase buffer and 1 μL of T4 ligase were added. The reaction continued at 16° C overnight. The ligated DNA was transformed, using a conventional transformation, into XL1-blue *E. coli* cells. Colonies grew and 16 were selected for screening. Plasmids from each of the 16 colonies were amplified, isolated and cut with *Bgl*II (see **Figure 11**). This time all but one (colony number 13) potentially contained the pCaSpeR hs-act vector.

DNA from colony 10 was CsCl purified. The purified DNA was cut with *Bam*HI alone, *Eco*RI alone, and a combination of both enzymes (see **Figure 12**). The double digest (lanes 1 and 2) has the expected bands. **Band 1** (about 9 kbp) represents the segment from the second *Bam*HI site to the *Eco*RI site. **Band 2** (about 2.8 kbp) represents

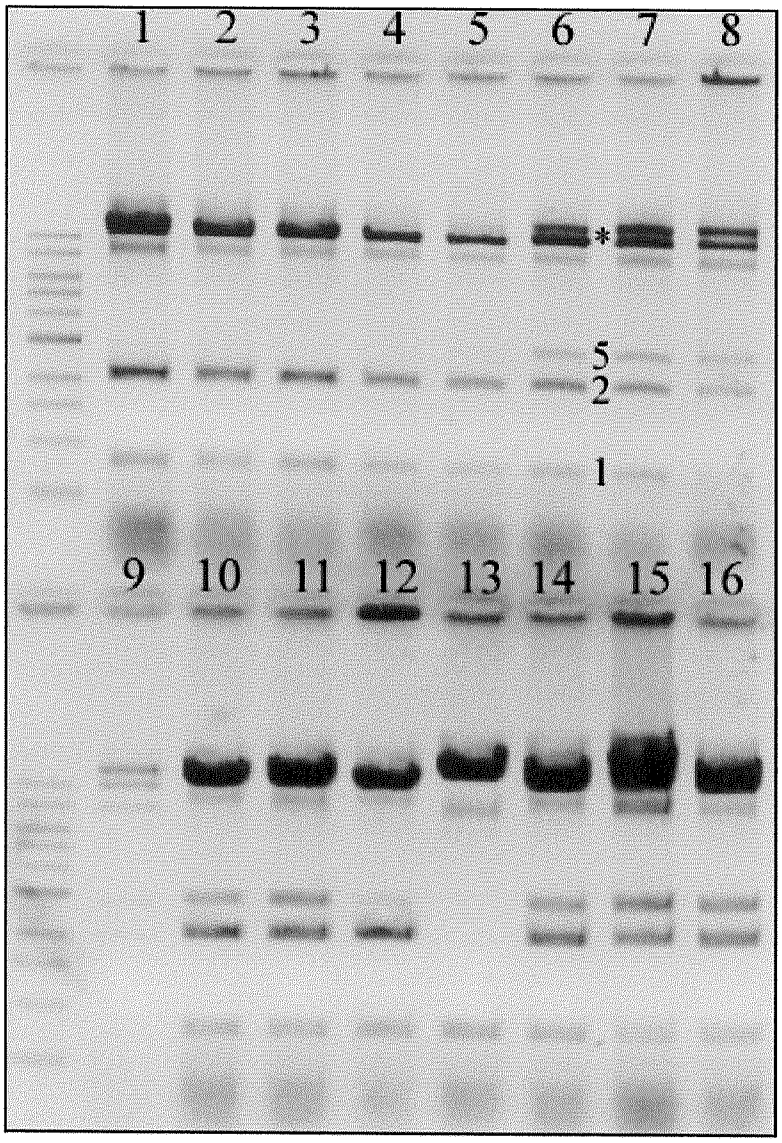


Figure 11. *Bgl*II digestions of plasmids from 16 colonies. Samples are compared to the 1 kb ladder from New England biolabs. Colony 7 (lane 7) contains band of expected sizes. **Band 1** (about 720 bp) represents the segment between the first two *Bgl*II sites. **Band 2** (about 2 kbp) represents the segment between the second and third *Bgl*II sites. **Band 5** (about 2.7 kbp) represents the segment between the first and the third *Bgl*II sites. The asterisk marks at least two bands near the 10 kbp marker. These bands may represent any of three DNA segments: a 12.7 kbp fragment representing the linear plasmid (cut only once), a 10 kbp fragment representing the segment between the third *Bgl*II site back to the first, or a 10.7 kbp fragment representing the segment between the third *Bgl*II site back to the second *Bgl*II site.

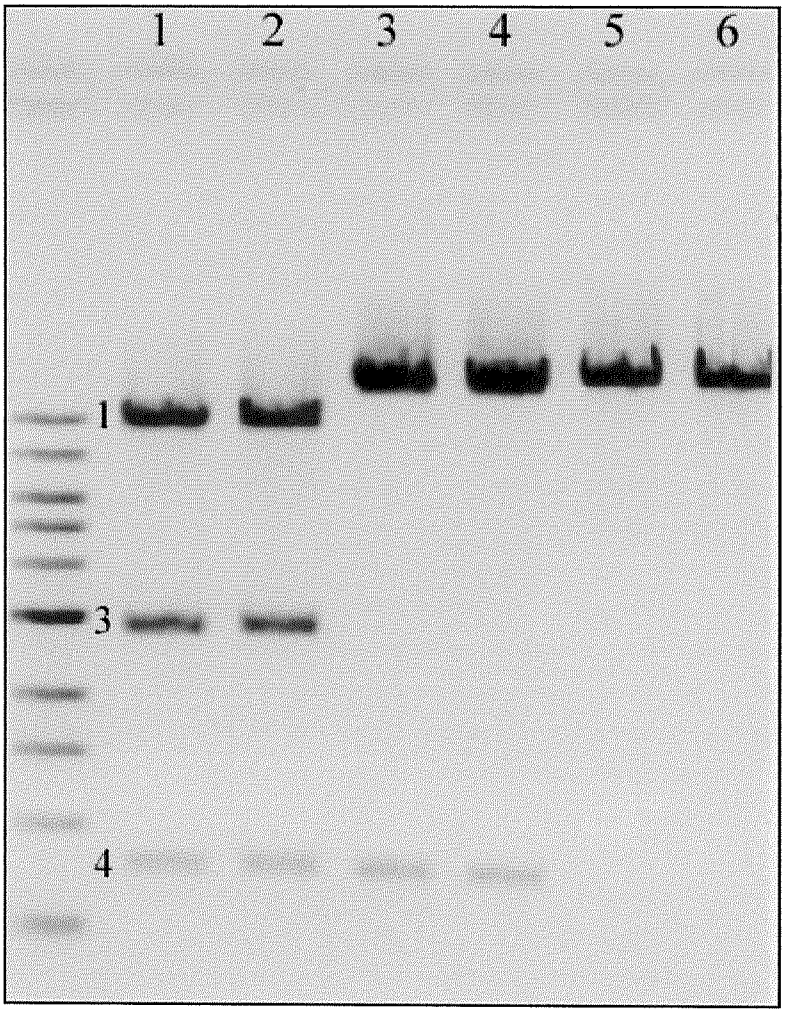


Figure 12. Screening of the CsCl purified plasmid from colony 10. Samples are compared to the 1 kb ladder from New England Biolabs. Lanes 1 and 2 (compare to lanes 1-3 of Figure 8) are double digests with *Eco*RI and *Bam*HI of the colony 10 pCaSpeR/DmNopp140-GFP construct. **Band 1** represents the segment between the second *Bam*HI site and the *Eco*RI site. **Band 3** represents the segment between the *Eco*RI site and the first *Bam*HI site. **Band 4** represents the segment between the *Bam*HI sites. Lanes 3 and 4 (compare to lanes 4-6 in figure 8) are *Bam*HI digests (there are no extra bands near 3.5 kbp and 2.1 kbp). Lanes 4 and 6 (compare to lanes 7-9 in Figure 8) are *Eco*RI digests. There is no extra band near 3 kbp.

the segment from *Eco*RI to the first *Bam*HI site. The separate *Bam*HI and *Eco*RI digests show expected bands only (compare to DNA in **Figures 8** and **9**). The pCaSpeR hs-act/DmNopp140-GFP plasmid was successfully constructed and isolated without contaminating plasmids. It will be stored for *Drosophila* embryo injections.

Part II: Needle Preparations

Table 1. Heater settings and number of weights used for the Narishige PB-7 needle-puller along with a description of needle point.

Heater setting	Number of weights	Narrowness of tip	Length of tip
37.32	4	Ideal	Ideal
37.32	3	Good	Short
47.32		Okay	Short
47.32	3	Good	Long
37.32	2	Good	Long
27.32	3	Okay	Short
27.32	4	Okay	Too short
38.32	4	Okay	Short
38.32	3	Good	Too long
36.32	4	Good	Too short

The size of the needles were compared to the size of *Drosophila* embryos using a dissecting scope. The ideal settings for the needle-puller heater was 37.32 with three weights in place. A needle with a sufficiently narrow, but longer tip was obtained with a heater setting of 47.32 with three weights. A needle with a shorter tip was obtained by decreasing the heater settings and using fewer weights.

Part III. Collection and Dechorionation of Embryos and Preliminary Injections

Our initial injection attempts have been only to familiarize ourselves with dechorionation techniques and to learn to use the injection equipment. We have only injected water, and we have not yet injected embryos young enough for a successful transformation.

Discussion

Having successfully prepared the DmNopp140-GFP plasmid, having prepared several needles with beveled tips small enough to inject *Drosophila* embryos, and having practiced the injections using water only, we are prepared to begin transforming flies.

The collection and dechorionation processes are very important. In order to obtain a transformed cell line we will have to inject between 100 and 200 flies. For the transformation to occur, injections must take place within the first 90 min after the embyros are conceived. The most difficult part of the transformation, once we begin injecting embryos, will be getting enough embryos on the slides and injecting them before the pole cells form. Just collecting the embryos in itself will be a challenge. Several factors affect whether of not the flies will lay eggs. Successful collections require weeks of planning and careful adhesion to a strict timetable. Flies must be of the proper age (14-16 days), and they lay more eggs in the early morning hours (Sisson, 2000). Also it is imperative that the flies are well fed exactly two days before an attempt to collect embryos. Once collected and dechorionated, the embryos are extremely fragile. Dechorionated embryos must be lined up on slides and secured with double-stick tape. The posterior ends of the embryos must face outward so that they may be approached by the DNA-filled needle mounted on the injection apparatus. Though it is important to transfer embryos from rinsing wells onto the tape on the microscope slides quickly, great care must be taken as they are being transferred so as not to kill the embryos.

Once transformed, our goal is to map the fluorescent Nopp140 variants within different cell types. Data obtained from transformed flies, combined with data obtained from experiments using antibodies for p80 coillin (a Cajal body protein), DmNopp140,

DmNopp140-RGG, and other nucleolar proteins may significantly add to our understanding of interactions between nucleolar proteins.

As mentioned previously, DmNopp140-RGG localizes to Cajal bodies in human HeLa cells, but not in *Drosophila* Schnieder II cells (Waggener and DiMario, submitted for publication). We are interested in determining how each protein will behave in different tissues at different developmental stages in *Drosophila.* This data may provide important information about the different functions of the two proteins.

RGG domains are found in several RNA binding proteins. Their function has not been determined. Waggener and DiMario (submitted for publication) have proposed that the RGG domain of DmNopp140-RGG acts as a strong nucleolar retention signal in *Drosophila* Schneider II cells. By comparing localization data from the two GFP-tagged proteins, we may be able to learn more about the purpose of RGG domains in general.

We can also use the GFP-tagged proteins to learn more about the differences in nucleoli in different cell types. Of special interest are nurse cell nucleoli. *Drosophila* nurse cells have large nucleoli and are good cells for observation of these proteins. Nurse cells are found within *Drosophila* egg chambers, and they make maternal products, including ribosomes, for the developing oocyte. Near the end of oogenesis the nucleolus disassembles, nurse cells are destroyed by apoptosis, and the contents of the cells flow into the oocyte through cytoplasmic bridges (Bownes and Dale, 1982). In transformed flies we can use the GFP-tagged proteins to follow the fates of nucleolar components when the nucleolus disassembles. Following what happens to the nucleolar proteins will tell us how they are stored in the oocyte and embryo, and how they are reutilized in the formation of new nucleoli (nucleogenesis) in the *Drosophila* embryo.

Very little is known about the *Drosophila* Nopp140 proteins. We feel that research with Nopp140 proteins is important because, as a shuttling protein, Nopp140 may be indirectly involved in many nucleolar tasks. In *Drosophila*, with two versions of the protein, nature provides us with a tool to further our understanding of Nopp140 in general and its role in nucleolar processes.

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