

EXPRESSION, PURIFICATION, AND BINDING ACTIVITY OF DmPBP: A PROTEIN REQUIRED FOR TRANSCRIPTION OF *DROSOPHILA* *MELANOGASTER* GENES CODING FOR U1 AND U6 SMALL NUCLEAR RNAS.

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U1 and U6 small nuclear RNA (snRNA) genes represent a good model to study gene expression because the genes have similar promoter sequences but different RNA polymerase specificity. *Drosophila melanogaster* proximal sequence element-binding protein (DmPBP) regulates transcription of snRNA genes. DmPBP binds a DNA sequence, the proximal sequence element (PSEA), found before the transcription start site of U1 and U6 snRNA genes. The long-term goal is to discover how the three DmPBP protein subunits interact with each other and additional proteins to promote transcription of U1 and U6 snRNA genes by different RNA polymerases. DmPBP bacterial expression, protein purification and electrophoretic mobility shift assay (EMSA) experiments have been conducted. A standard purification protocol for bacterially expressed DmPBP is still in its developmental stages. Various protocols involving nickel-chelating chromatography have been executed and effective purification steps have been identified. Collected protein fractions have demonstrated DNA-binding activity via EMSA. The observed band shift from bacterially expressed DmPBP however, is slightly lower than the band shift produced by DmPBP purified from *Drosophila* embryos that serves as a positive control. The difference in mobility could arise from a variety of reasons, such as partial degradation of DmPBP in bacteria, or the possible absence of additional interacting *Drosophila* proteins. Experiments have been performed to determine the specificity of the band shifts. The results indicated the band shifts are due to a non-specific DNA-binding activity. Consequently, a coupled *in vitro* transcription/translation system was tested for expression of DmPBP in bacterial extracts. An autoradiograph of ³⁵S incorporation revealed the 43 and 50 subunits were synthesized, but expression of the 190 subunit of DmPBP could not be detected.

The sequencing of entire genomes from a growing number of organisms is requiring biology, more than ever, to elucidate how individual genes are expressed and how this expression is regulated. Elucidating gene expression in general is important to understand normal and abnormal biological process and how potentially to counter disease. A large part of gene expression is regulated at the transcriptional level. Many proteins that regulate gene expression at the transcriptional level, and other levels, have been discovered in different organisms. The mechanism of action of many gene regulatory proteins however, is not well understood.

Drosophila U1 and U6 small nuclear RNA (snRNA) genes represent a good model to study gene expression because the genes have similar promoter sequences but different RNA polymerase specificity. The *Drosophila melanogaster* (fruit fly) proximal sequence element-binding protein (DmPBP) is a protein that regulates transcription of small nuclear RNA genes. DmPBP is made up of three protein subunits called DmPBP 43, DmPBP 50 and DmPBP 190. Each DmPBP protein subunit is named based on its approximate molecular weight in kilodaltons. DmPBP binds a DNA sequence, called the proximal sequence element (PSEA), found upstream the transcription start site of *Drosophila* U1 and U6 snRNA genes (6, 10). Once bound to the PSEA, DmPBP promotes transcription of U1 and U6 snRNA genes by recruiting other transcription proteins. DmPBP recruits RNA polymerase II (RNAP II) to transcribe the U1 snRNA gene (6). DmPBP however, recruits RNA polymerase III (RNAP III) to transcribe the U6 snRNA gene (1, 2, 3, 4, 5, 7, 8, 9). How DmPBP recruits RNA polymerase II and RNA polymerase III to carry out transcription of U1 and U6 snRNA genes, respectively, is unknown.

This study has several goals. The long-term goal is to answer the question: "How do the three DmPBP protein subunits interact with each other and additional transcription proteins to promote transcription of U1 and U6 snRNA genes?" In other words, how does DmPBP recruit RNAP II in the case of the U1 gene, but RNAP III in the case of the U6 gene? In support of the long-term research goal, bacterial expression of DmPBP, protein purification and electrophoretic mobility shift assay (EMSA) experiments have been conducted. Collected protein fractions have demonstrated DNA-binding activity via EMSA. The observed band shift from bacterially expressed DmPBP however, is slightly lower than the band shift produced by DmPBP purified from *Drosophila* embryos that serves as a positive control. The difference in mobility could arise from a variety of reasons, such as partial degradation of DmPBP in bacteria, or the possible absence of additional interacting *Drosophila* proteins. DNA competition experiments have been conducted to determine the specificity of the observed band shifts. Results from the DNA competition experiments indicate the band shifts are due to non-specific protein binding. Consequently, a coupled

in vitro transcription/translation system was tested for expression of DmPBP in bacterial extracts. An autoradiograph of ^{35}S incorporation revealed the 43 and 50 subunits were synthesized, but expression of the 190 subunit of DmPBP could not be detected.

Materials and Methods

Protein expression and purification of bacterially expressed DmPBP

Both the 43 and 50 protein subunits of DmPBP were simultaneously expressed in *Escherichia coli* from a single plasmid using the pCMT7/CT-TOPO (Invitrogen) system. The 43 and 50 subunits were not tagged. The 190 protein subunit of DmPBP was expressed in *E. coli* using the pET (Novagen) system. The 190 subunit was tagged at the C terminus with six histidines. DmPBP was purified by affinity chromatography on nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen). To reconstitute and purify DmPBP, 190 first was bound to the agarose beads under denaturing conditions (1 M Urea, 1.72 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 18.3 mM Na_2HPO_4 , 500 mM NaCl, pH 7.8). The beads, with the 190 subunit bound, were then equilibrated in non-denaturing buffer (1X BCZ without EDTA, 20% glycerol, 100 mM KCl, 0.5 mM PMSF, pH 8.0). A cell lysate containing 43 and 50 was then applied to the agarose beads under native conditions. DmPBP was eluted from the Ni-NTA agarose beads in non-denaturing buffer containing 750 mM imidazole.

Electrophoretic mobility shift assays (EMSAs)

The binding reactions were performed in a total volume of 18 μl for band shift experiments and 20 μl for DNA-competitor experiments. A binding reaction contained either 1.5 μl of DmPBP HA300 fraction from fruit fly embryos (9) or 15 μl of DmPBP purified from *E. coli* by nickel-chelating chromatography. The final buffer composition was approximately 20 mM HEPES (pH 7.9), 100 mM KCl, 5 mM MgCl_2 , 0.01 mM ZnCl_2 , 0.2 mM EDTA, 3 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 20% (by volume) glycerol. Reaction mixtures also contained 1 μg of poly(dI-dC) · poly(dI-dC) and 1 μg of poly(dG-dC) · poly(dG-dC). Competitor DNA, when included, was added at the beginning of the incubation to a final concentration of 0.025 ng/ μl , 0.125 ng/ μl , or 0.625 $\mu\text{g}/\mu\text{l}$. The radioactively labeled DNA that was used, had the sequence 5'GTTTCGTTGCAATTCCTCCCAACTGGTTTTAGCTGCTCAGCCATGGAAACC3', in which the U1 PSEA is underlined. When competitor DNA was included, the probe was added last into each binding reaction. The specific-competitor had the sequence 5'AATTCATTCTTATAAATTCCTCCCAACTGGTTTTAGCTGATTGCTGCAC3', in which the U1 PSEA is underlined. The competitor DNA was not radiolabeled. The non-specific-competitor had the sequence 5'CGACTACAAGGATGACGATGACAAGGGCGAGCAGAAGCTGATCTCCGAGGAAGATCTGA3'. The binding reactions were incubated at 20°C for 30 min prior to being loaded and fractionated on a 5% polyacrylamide gel (29:1 acrylamide-bisacrylamide) in non-circulation buffer (0.25 M Tris, 1.9 M glycine, 10 mM EDTA, pH 8.3).

Expression of DmPBP subunits via a coupled transcription/translation system

The ExpresswayTM Plus Expression System (Invitrogen) was used to express DmPBP subunits *in vitro*. Protein synthesis was monitored by ^{35}S incorporation. The radioactive methionine was purchased from PerkinElmer Life Sciences with a specific activity of 1175.0 Ci/mmol and a concentration of 10.2 mCi/ml. A 10% polyacrylamide gel was used for SDS-PAGE separation of proteins before autoradiography.

Results

Electrophoretic mobility shift assays (EMSA) reveal a DNA binding activity in fractions from a Ni-NTA column expected to contain bacterially expressed DmPBP subunits

EMSA is a sensitive technique used to assay for specific DNA-binding activity in protein extracts or chromatography column fractions. It is based upon the principle that a radioactive DNA probe, when complexed with a protein, will migrate more slowly through a native gel than the free unbound DNA, thus creating a band of shifted mobility. To detect the DNA-binding activity of bacterially expressed DmPBP, fractions obtained from Ni-NTA column chromatography (see Materials and Methods) were incubated with a DNA probe containing a PSEA

sequence. A heparin-agarose chromatography fraction (HA-300) from *Drosophila* embryo nuclear extracts was used as a positive control for native DmPBP DNA-binding activity (9). Figure 1 reveals the band shift results.

In Fig. 1 the free probe is seen to migrate near to the bottom of the gel. Since the probe is not bound by protein, its migration through the gel is not retarded in any way and it migrates rapidly through the gel. The DmPBP purified from fruit fly embryos generate two band shifts in the HA-300 lanes. The upper band represents the native DmPBP band shift. The lower band is believed to arise from a partial degradation product of DmPBP during long-term storage. In lanes E, F and G the 190 protein subunit by itself does not produce a band shift. When all three subunits of PBP are present in lanes I, J, K, EF1, EF2, EF3 and EF4, however, a band shift results. The observed band shift from bacterially expressed DmPBP, however, was slightly lower than the upper band shift produced by the positive control.

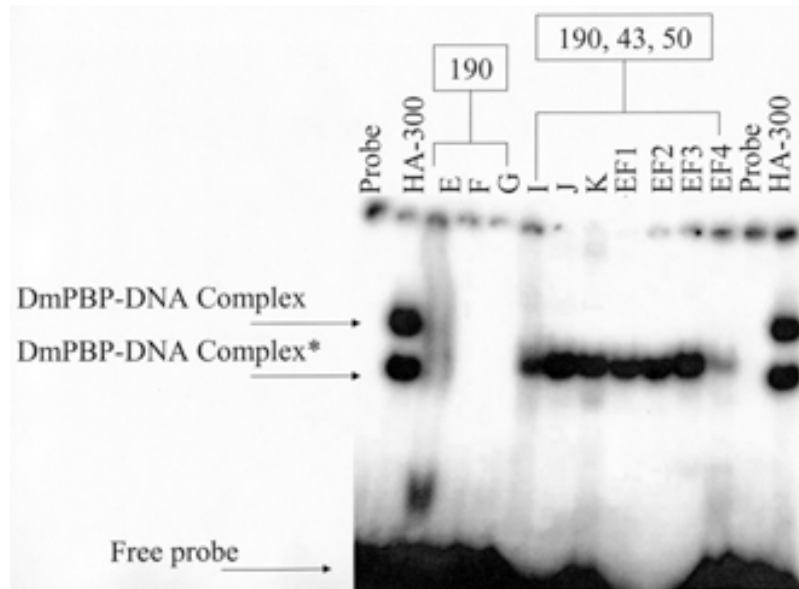


Figure 1. Electrophoretic mobility shift assays carried out by using Ni-NTA column fractions from bacterially expressed DmPBP and a probe that contains the U1 PSEA. The probe lanes were used to monitor the migration of radioactive DNA that is not mixed with any protein. The HA-300 lanes use DmPBP prepared from fruit fly embryos as a positive control. The asterisk indicates a complex believed to result from partially degraded DmPBP. Lanes E, F and G use protein fractions that were collected after the 190 subunit of DmPBP was bound to the Ni-NTA column. Lanes I, J, K, EF1, EF2, EF3 and EF4 use protein fractions that were collected after the 43 and 50 subunits of DmPBP were added to the same column where 190 was initially bound. Only the protein fractions labeled as EF represent fractions eluted with imidazole. The earlier fractions represent wash fractions.

Competition with specific and non-specific DNA oligos suggest that the binding observed in the band shift assay is non-specific

If the shifted band observed in Fig. 1 is due to the specific binding of DmPBP to the PSEA, then an unlabeled double-stranded oligonucleotide that contains the PSEA sequence should compete away the signal. On the other hand a non-specific oligo that does not contain a PSEA should not compete. The results of such an experiment are shown in Fig. 2. The band shift due to DmPBP purified from fruit fly embryos in lane 1 was specifically competed when 12.5 ng competitor PSEA was used (lane 2). The non-specific oligo in contrast, did not compete (lane 3). Lanes 4-10 examines bacterially expressed DmPBP. Lane 4 shows the band shift without any competitor. The intensity of the band shift remained unchanged in lanes 5-10 where specific and non-specific competitor DNA was used. Because the band shift was not competed by the PSEA oligo, the result suggests that the band shift was due to non-specific DNA-binding.

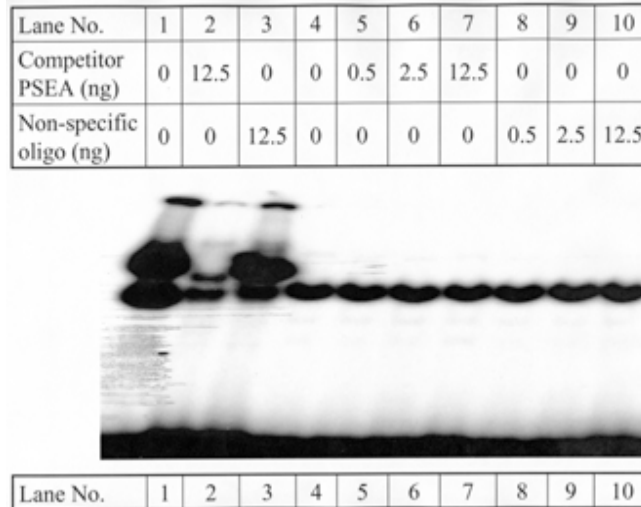


Figure 2. EMSA competition experiments with unlabeled competitor oligos. Two unlabeled competitor oligos were used, one that contained a PSEA and one that lacked a PSEA. Lanes 1-3 used an HA-300 sample of DmPBP purified from fruit fly embryos. Lanes 4-10 employed a Ni-NTA column fraction of extract from bacteria expressing the subunits of DmPBP. Reactions in Lanes 1 and 4 contained no competitor. Lanes 2 and 3 contained 12.5 ng PSEA and non-specific competitor respectively. Lanes 5-7 and 8-10 contained increasing amounts of PSEA or non-specific competitor respectively, as indicated above each lane.

In vitro expression of DmPBP subunits in bacterial extracts

As an alternative approach, expression of DmPBP subunits was carried out using the Invitrogen Expressway™ Plus Expression System. The newly synthesized proteins were labeled by incorporation of ³⁵S-methionine. Protein products were separated via SDS-PAGE and an autoradiograph was obtained (Fig. 3). Lanes 2 and 3 demonstrate that the 43 and 50 subunits were expressed with reasonable efficiency. In addition, the chloramphenicol acetyltransferase (CAT) and β-galactosidase positive controls were easily detected in lanes 5 and 6 respectively. However, in the reaction with the 190 template, no detectable 190 protein product was observed above the background of a reaction that lacked template (compare lanes 1 and 7). When an attempt was made to express all three subunits in a single reaction, expression of the 50 and 43 subunits could be observed, but no expression of 190 was detectable (lane 4).

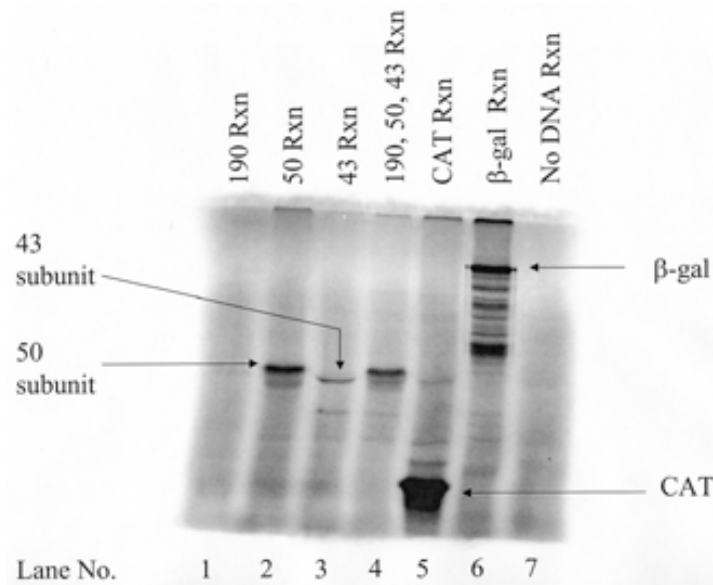


Figure 3. Autoradiography of protein products obtained from a coupled bacterial transcription/translation system. DmPBP and positive controls (β -galactosidase and CAT) genes were expressed using the ExpresswayTM Plus Expression System (Invitrogen), and ³⁵S-labeled protein products were separated by SDS-PAGE. Each lane is labeled with the DNA template that was included in the respective reaction.

Discussion

The specific aim of this study was to reconstitute the DNA binding activity of DmPBP for the PSEA from bacterially-expressed proteins. Although DmPBP has been successfully overexpressed in *Drosophila* S2 tissue culture cells, expression in *E.coli* has proven difficult. Although DmPBP 50 can be efficiently expressed in *E.coli*, expression of DmPBP 43 has always resulted in a mixture of the full length protein and a degradation product (western blot data not shown). However, the real challenge has been to express the 190 subunit. DmPBP 190 is synthesized at a very low level in *E.coli*, and numerous degradation products are always observed. Only a very small fraction of the 190 product is full length protein (western blot data not shown). We expect that the low level of 190 expression and its degradation is responsible for our inability to reconstitute the DNA-binding activity of DmPBP from the bacterially expressed subunits. Initially, the band shifts presented in Fig. 1 appeared very promising because the strength of the signal was unprecedented. Unfortunately, the competition experiments demonstrated that the band shifts are not specifically due to DmPBP.

In response to this result, we turned to an *in vitro* coupled transcription/translation system for expression of DmPBP. Unfortunately, the results were similar to the results obtained from expression of the proteins in living bacteria. Most notably, no expression of 190 was detectable in the *in vitro* system. From these cumulative results, efficient expression of DmPBP in bacteria may not be feasible. Thus the higher costs and expenses of producing wild type and mutant forms of DmPBP in eukaryotic expression systems may be unavoidable.

Acknowledgments

This work was supported by National Science Foundation grant MCB-0131151 and in part by the California Metabolic Research Foundation. In addition, I want to thank the SDSU McNair Scholars Program for funding my exciting and rewarding research experience. I am also thankful for the activities McNair provided for the students such as GRE preparation. I thank Veronica Bejar, Sara Boquin, and Robert Pozos, Ph.D. from the SDSU McNair Scholars Program. Finally, I want to thank my mentor William E. Stumph, Ph.D. for contributing to my scientific maturation over approximately two years.

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