Appendix III
Chapter 4 Methods

General. Unless otherwise noted, reagents were purchased from the commercial suppliers Fisher (Fairlawn, NJ) and Sigma-Aldrich (St. Louis, MO) and were used without further purification. Protease and phosphatase inhibitors were purchased from Sigma-Aldrich or Alexis Biochemicals (San Diego, CA). Redivue L-[^{35}S]methionine (*in vitro* translation grade), and [α-^{32}P]rGTP and [γ-^{32}P]ATP were purchased from Amersham Biosciences (Piscataway, NJ). Restriction enzymes were obtained from Fisher, with the exception of NdeI (Promega) and DpnI (New England Biolabs, Beverly, MA).

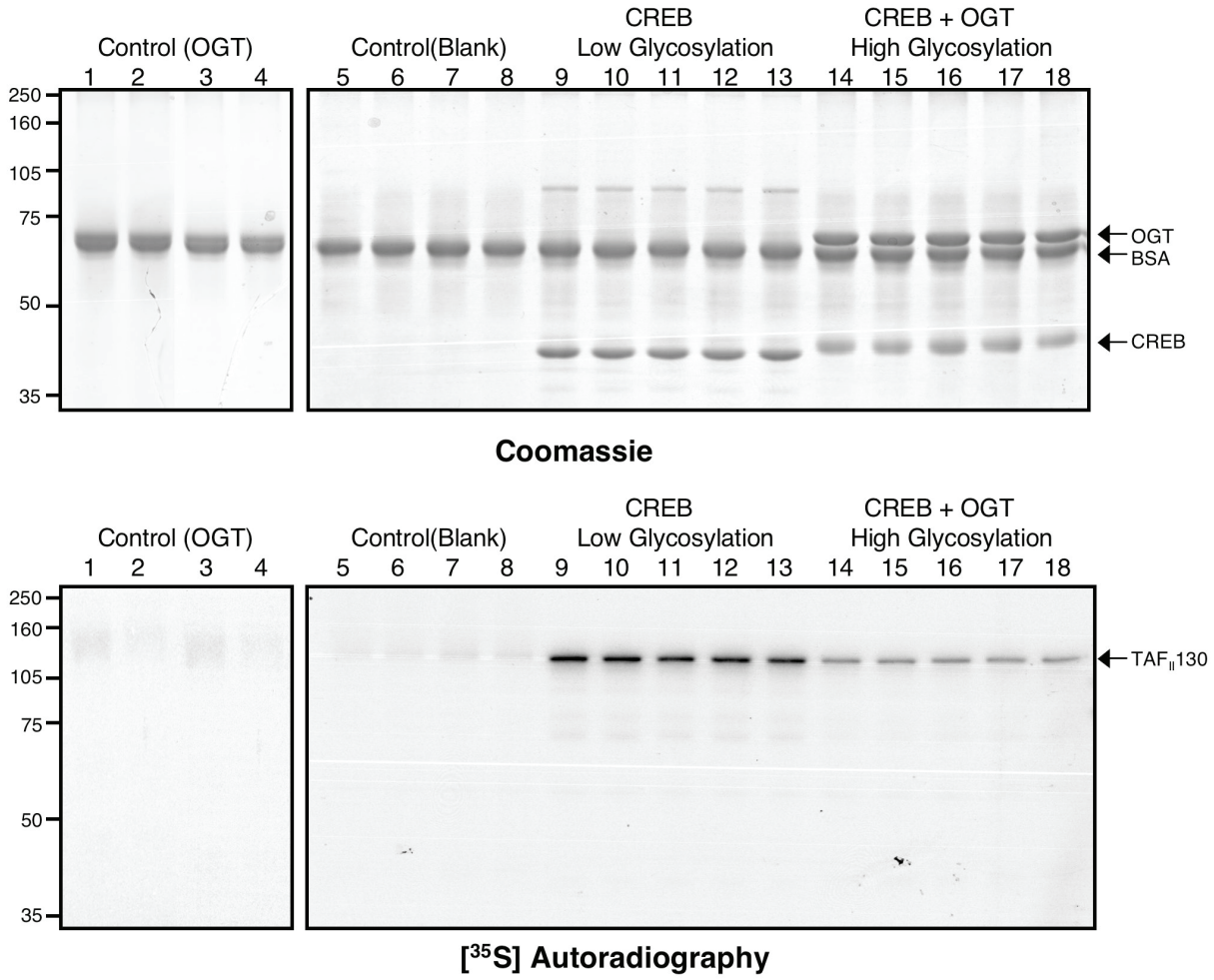
Purification of CREB and OGT from *Spodoptera frugiperda* (Sf9) cells. Rat CREB and human OGT were expressed and purified as described in Appendix II.

Electrophoretic Mobility Shift Assay (EMSA). EMSA assays have previously been used to analyze CREB DNA binding and the effects of Mg^{2+} concentrations on CREB:DNA binding affinity [1]. CRE and SP1 oligonucleotides were ordered from Qiagen (Valencia, CA): CRE1 5'- AGAGATTGCC**TGACGTCA**GAGACGTAG-3'; CRE2 5'-CTAGCTCTC**TGACGTCA**GGCAATCTCT-3'; SP1-1 5'-ATTCGAT**CGGGGCG**GGGCGAG-3'; SP1-2 5'-CTCGCCC**CGCCCCG**ATCGAAT-3'. Oligonucleotides were annealed to produce dsDNA, by combining equimolar amounts of each oligo for a final concentration of 8 pmol/μl in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and incubating 2 min 88^{o} C, 10 min 65^{o} C, 10 min 37^{o} C, 5 min 25^{o} C and stored at 4^{o} C.

[^{32}P]-labeled CRE dsDNA were prepared by first end labeling CRE1 and CRE2 oligonucleotides. Oligonucleotides (1 μl, 8 pmol) were combined with [γ-^{32}P]ATP (2.2 μl, 300 μl/mmol at 10 mCi/ml), Nuclease-free water (4.8 μl, Promega, Madison, WI), 10xPNK buffer (1 μl , Promega, Madison, WI) and T4 polynucleotide kinase (1 μl, 10 U, Promega, Madison, WI) and incubated at 37^{o} C for 30 min. Labeling reactions were diluted to 80 μl with Nuclease-free water then annealed as described above. Unincorporated [γ-^{32}P]ATP was removed using micro-BioSpin Biogel P-6 columns (Biorad, Hercules, CA).

EMSA reactions were set up by the combination of CREB (12 μl, 1.2 μg), poly(dI-dC)•poly(dI-dC) (12 μl, 1 mg/ml, Invitrogen, Carlsbad, CA) and 1x EMSA reaction buffer (204 μl, 10 mM HEPES pH 7.5, 3 mM $MgCl_2$, 0.25 mM EDTA, 100 mM KCl, 1 mM DTT, 1 mg/ml BSA, 4% glycerol). Three 38 μl aliquots were removed for analysis by SDS-PAGE and Coomassie staining. [^{32}P]-labeled CRE dsDNA (6 μl, 0.1 pmol/μl) was added to the remaining 114 μl reaction mixture. Reactions were aliquoted into four 24 μl samples and cold CRE dsDNA or cold SP1 dsDNA (1.5 μl, 8 pmol/μl) we added to one reaction each as specific and non-specific competition reactions. Samples were incubated for 30 min at 37^{o} C and then loaded onto native TBE mini-gels (Invitrogen, Carlsbad, CA) and run at 200 V for 20 min. Gels were dried and then exposed to film and visualized by autoradiography on Hyperfilm and quantified by analysis of scanned images using NIH Image 1.52 software. Values were normalized for the concentration of CREB in each experiment, as determined by Coomassie staining and densitometry. Data were analyzed by the Student's paired T-test using StatView 5.0.1 software, with significance defined as $p < 0.05$.

$TAF_{II}130$ Binding Assay. [^{35}S]-labeled $TAF_{II}130$ was synthesized using the TNT T7 Coupled Reticulocyte Lysate System (Promega) following the manufacturer's protocol. The $TAF_{II}130$ construct, pTβ-h$TAF_{II}130$, was kindly provided by Dr. N. Tanese (New York University School of Medicine) [2]. Binding assays were performed with minor modifications to published procedures [3, 4]. Proteins (50-100 μg CREB purified from Sf9 cells; 100-200 μg OGT purified from Sf9 cells as a control) were incubated with Ni-NTA agarose for 4-5 h at 4° C with mixing. Following centrifugation, the agarose was washed twice with buffer H (20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40, 20% glycerol, 0.1 mg/ml BSA) containing 50 mM imidazole and twice with buffer H [2]. The agarose was then incubated with [^{35}S]-labeled $TAF_{II}130$ lysate (0.13 volumes) in buffer H (2.5 volumes) for 4 h at 4° C with mixing [3, 4]. Following centrifugation, the agarose was washed five times with buffer H containing 50 mM imidazole. Samples were boiled in SDS-PAGE loading dye and resolved by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue, incubated with Amplify, and dried. The relative binding affinity of $TAF_{II}130$ was visualized by autoradiography on Hyperfilm and quantified by analysis of scanned images using NIH Image 1.52 software. Values were normalized for the concentration of CREB in each experiment, as determined by Coomassie staining and densitometry. Data were analyzed by the Student's paired T-test using StatView 5.0.1 software, with significance defined as $p < 0.05$. Appendix Figure 9 shows representative autoradiograms and Coomassie stained gels.



Appendix Figure 10. Representative Coomassie-stained gel (*upper panel*) and corresponding autoradiogram (*lower panel*) from the $TAF_{II}130$ binding assay. Lanes 1-4: controls with OGT alone; Lanes 5-8: controls in the absence of CREB; Lanes 9-13: CREB purified from Sf9 cells (low glycosylation stoichiometry); lanes 14-18: CREB co-expressed with OGT and purified from Sf9 cells (high glycosylation stoichiometry). Four or five duplicate runs are shown for each sample. Note that CREB co-expressed with OGT has a higher apparent molecular weight than CREB alone. BSA is present in all lanes due to its presence in incubation and wash buffers.

***In Vitro* Transcription Assay.** Assays were performed using HeLaScribe Nuclear Extract (Promega) according to the manufacturer's protocol with minor modifications. Linearized CRE template DNA was prepared by digesting the plasmid pCRE-Luc (Stratagene, La Jolla, CA) with NdeI and EcoRV. The resulting 2.1 kb fragment containing the CRE enhancer and partial luciferase ORF (~1.4 kb) was gel purified. CREB (0.3 - 0.5 μg) purified from Sf9 cells with or without OGT was combined with the

CRE template DNA (100 ng), 0.4 mM rATP, 0.4 mM rUTP, 0.4 mM rCTP, 0.016 mM rGTP and 10 μCi [α-^{32}P]rGTP (3000 Ci/mmol, Amersham Biosciences) in 7.3 mM HEPES pH 7.9, 6.1 mM $MgCl_2$, 37 mM KCl, 0.07 mM EDTA, 0.2 mM DTT, 5.5% glycerol. Transcription reactions were initiated by the addition of HeLaScribe nuclear extract (8 U). Run-off RNA transcripts were resolved on 7 M urea 6% TBE-PAGE gels, visualized by autoradiography using Hyperfilm MP film, and quantified by analysis of scanned images using NIH Image 1.52 software. Values for each reaction were corrected for CREB and [α-^{32}P] rGTP concentration, as determined by Coomassie staining and autoradiography, respectively. Data were analyzed by the Student's unpaired T-test using StatView 5.0.1 software, with significance defined as $p < 0.05$.

Bibliography

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