Appendix I
Chapter 3 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). Bovine GalT and ovalbumin were obtained from Sigma-Aldrich. Uridine diphospho-D-[6-^{3}H]galactose was 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).

Preparation of Rat Brain Nuclear Extracts. Nuclear extracts were prepared as previously reported by Andrews and Faller with minor modifications [1]. The forebrains of Sprague Dawley rats (Charles River Laboratories, Kingston, MA) were dissected on ice and homogenized in 10 volumes of ice-cold buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) containing protease inhibitors (5 μg/ml pepstatin, 5 μg/ml chymostatin, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml antipain, 0.2 mM PMSF), phosphatase inhibitors (20 mM NaF, 1 mM Na_3VO_4, 50 μM Na_2MoO_4) and hexosaminidase inhibitors (50 mM GlcNAc, 10 μM streptozotocin). The resulting lysate was centrifuged at 1,000 xg at 4°C for 10 min, and the crude nuclear pellet was washed with buffer A. The pellet was resuspended at 2 mg/ml in buffer B (20 mM HEPES pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 50 mM GlcNAc, 0.2 mM PMSF, 25% (v/v) glycerol) by stirring at 4° C for 40 min. Following centrifugation at 10,000 xg for 30 min, the supernatant was defined as the nuclear extract. The nuclear extract was dialyzed against buffer D (20 mM HEPES pH 7.9, 0.1 M KCl, 0.5 mM DTT, 0.2 mM EDTA, 50 mM GlcNAc, 20% (v/v)

glycerol) for 6 h at 4°C, and proteins were precipitated using $(NH_4)_2SO_4$ (38% final concentration). The protein pellet obtained after centrifugation at 21,500 xg for 10 min was solubilized in buffer C (0.3 volumes; 25 mM Tris pH7.5, 1 mM EDTA, 1 mM DTT containing protease inhibitors) by gentle mixing for 1 h at 4° C. Following clarification at 21,500 xg for 5 min, the supernatant was dialyzed into buffer G (20 mM HEPES pH 7.3, 0.1 M KCl, 0.5 mM DTT, 0.2 mM EDTA, 5 mM $MnCl_2$, 0.2 mM PMSF) overnight at 4° C. The protein concentration was determined using the BCA assay (Pierce/Endogen Biotech, Rockford, IL).

2-D Gel Electrophoresis. Nuclear extracts were prepared and trichloroacetic acid (TCA)-acetone precipitated with the addition of 10% (v/v) TCA-acetone (1g TCA, 454 μl acetone, 7 μl 2-mercaptoethanol (BME)), and incubated at –20° C for +20 min. Precipitated protein was centrifuged for 10 min at 21,500 xg. The supernatant was removed, the pellet rinsed two times with 0.07% BME acetone, and then lyophilized to dryness. The pellet was resuspended in 200 μl of thiourea buffer (2 M thiourea, 7 M urea, 4% CHAPS, 0.5% Biolytes (Biorad, Hercules, CA), 2 mM tributylphosphine). The sample was centrifuged for 5 min at 5000 xg. The protein concentration of the sample was calculated using the Bradford Assay (Biorad), and the desired amount of protein was loaded onto IEF strips, diluted to 185 μl with thiourea buffer plus 1 μl of bromophenol blue. The first dimension was run for 60,000 V/h. The IEF strip was equilibrated in DTT buffer (6 M urea, 50 mM Tris pH 8.8, 2% SDS, 30% glycerol, 1% DTT) for 15 min with rocking at room temperature. The IEF strip was transferred to iodoacetamide buffer (6 M urea, 50 mM Tris pH 8.8, 2% SDS, 30% glycerol, 2.5% iodoacetamide) and incubated for

15 min at room temperature with rocking. The IEF strip was set in a SDS-PAGE gel with low-melt agarose and run at 15 mA for 15 min, after which the amperage was increased to 30 mA.

Labeling With β-1,4-galactosyltransferase (GalT). GalT labeling was performed using minor modifications to published procedures [2]. Nuclear extracts (200-400 μg) were combined with autogalactosylated bovine GalT (100 mU) and 5'-adenosine diphosphate (1.25 mM final concentration). Samples were transferred to centrifuge tubes containing uridine diphospho-D-[6-^{3}H]galactose (0.03 μCi/μl final concentration). Following incubation at 37° C for 90 min, the labeling reaction was stopped by the addition of 100 mM EDTA (0.1 volumes). Ovalbumin controls were treated in parallel as a positive control.

To label recombinant CREB from Sf9 cells, CREB (2-4 μg) was boiled in 0.1% SDS for 5 min, cooled, and then incubated with GalT (75 mU) and 400 μM UDP-Gal in CREB storage buffer (20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 15% glycerol) plus 5 mM $MnCl_2$, 1.25 mM 5'-adenosine diphosphate and 1% Triton X-100 overnight at 4° C. Labeling reactions were stopped by the addition of 4x SDS-PAGE loading dye and boiled for 5 min.

PNGase F Treatment. An equal volume of 2xPNGase buffer (150 mM Na_2HPO_4 pH 8.6, 15 mM EDTA, 5% NP-40) containing protease inhibitors was added to the eluted protein or to the ovalbumin control (boiled in 1% SDS, 1% BME). Samples were incubated in the presence or absence of PNGase F (2 U; New England Biolabs) for 5 h with rocking at 25ºC.

Additional PNGase F (1 U) was added, and the incubation continued for 5 h [2]. Samples were then boiled for 5 min in SDS-PAGE loading dye and resolved by SDS-PAGE [3]. Gels were stained in 0.1% Coomassie Brilliant Blue, 50% MeOH and 5% AcOH, for 15 min, and destained in 50% MeOH, 5% AcOH. Gels were treated with Amplify solution (Amersham Biosciences) for 30 min, rinsed with 2% glycerol and dried. Tritium-labeling was visualized using Hyperfilm™ MP (Amersham Biosciences) exposed at -80° C.

Immunoprecipitation of CREB. Radiolabeled nuclear extracts, in Buffer G, were precleared by incubation with protein A sepharose (Pierce/Endogen Biotech) for 2 h at 4°C. Samples were centrifuged for 30 s at 2,000 xg, and the supernatant was transferred to a fresh centrifuge tube. Samples were then incubated with an antibody selective for CREB (5 μg; Upstate Biotechnology, Lake Placid, NY) or CREB immunoprecipitation (IP) buffer (10 mM HEPES pH 7.9, 100 mM KCl, .2% Triton X-100 and 1 mM EDTA) (negative control) with gentle inversion at 4°C for 7 h. After 2 h, the samples were supplemented with additional leupeptin (20 μg/ml) and antipain (20 μg/ml) protease inhibitors. Following the incubation period, protein A sepharose was added, and the samples were incubated for 2 h at 4°C. Samples were then centrifuged for 30 s at 2,000 xg, and the sepharose was washed twice with CREB IP buffer and four times with PBS (137 mM NaCl, 2.7 mM KCl, 5 mM Na_2HPO_4, 1.7 mM KH_2PO_4). Proteins were eluted with boiling in 1% SDS, 1% β-mercaptoethanol (BME) for 10 min. Immunoprecipitated samples were resolved by 10% SDS-PAGE. The efficiency of the immunoprecipitation was measured by immunoblotting using an anti-CREB antibody (Chemicon, Temecula, CA) and [^{3}H]-labeling was measured by fluorography.

Plasmids. *E. coli* expression vector pET23b-CREB was subcloned by amplifying full length rat $CREB_{341}$ cDNA from pRcRSV-CREB, a gift of R. Goodman (Oregon Health and Sciences University), using primers with 5' Nde1 and 3' HindIII restriction sites and inserted into pET23b+ (Novagen) to afford pET23b-CREB.

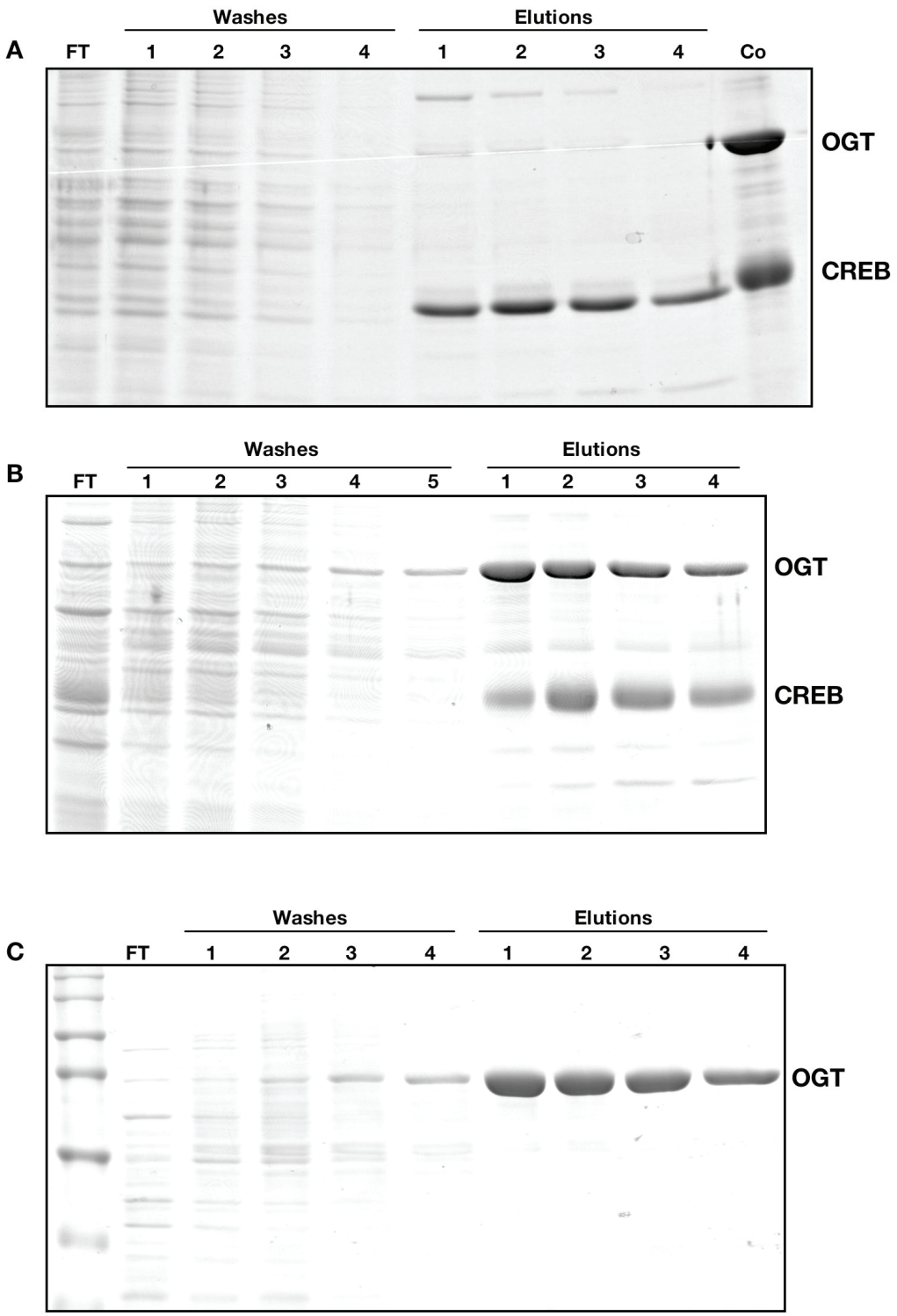
***E. coli* Expression of CREB.** Electrocompetent BL21(DE3) cells were combined with plasmid DNA and transferred to an electrocuvette (Invitrogen). Cells were electroporated with 1500 V, 50 mAmps, and 100 W (Invitrogen). Cells were immediately resuspended in warm Luria Broth (LB), transferred to 15 ml culture tubes and incubated at 37° C for 45 min. Cells were plated on LB/ampicillin (100 μg/ml) and incubated over night at 37° C. Colonies from the plate were sampled and grown in LB/Amp (100 μg/ml) to an $A_{600} = 0.6$.

Overexpression of BL21(DE3) cultures transformed with pET23b(+)CREB was induced at $A_{600} = 0.3$ with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Alexis Biochemicals). Cells were grown for ~3 h after induction at 37° C. Cultured cells were pelleted and stored at -20° C.

Purification of OGT from *Spodoptera frugiperda* (Sf9) and Hi-5 Insect Cells and CREB from *E. coli*. OGT cDNA clones was generously provided by Dr. J. A. Hanover (NIDDK, National Institutes of Health), and were cloned into baculovirus expression vectors in frame with a histidine tag [4, 5]. Baculovirus preparation and protein expression in Sf9 cells were performed by Dr. P. Snow at the Beckman Institute Protein Expression Facility at

the California Institute of Technology [6]. Proteins were purified using Ni-NTA agarose (Qiagen, Valencia, CA) with the following modifications to the manufacturer's protocol. For optimal solubility, the lysis, wash and elution buffers were supplemented with 10 mM BME and 0.1% Triton X-100. Purified CREB and OGT were dialyzed into CREB storage buffer or OGT storage buffer (50 mM Tris-HCl pH 7.5, 12.5 mM $MgCl_2$, 40% glycerol) and stored at -80°C. Purity was assayed by resolving analytical samples (1-5 μg/well) by SDS-PAGE and visualizing protein by Coomassie staining following general procedures (Appendix Fig. 1) [3].

***In Vitro* OGT Labeling.** A master OGT-labeling mix (OLM) containing ~0.046 μg/μl recombinant OGT, purified from Sf9 or Hi5 insect cells, and ~0.023 μCi/μl uridine diphospho-*N*-acetyl-D-[6-^{3}H]glucosamine (UDP-[^{3}H]GlcNAc; GE Healthcare Bio-Sciences Corp., Piscataway, NJ) in 50 mM Tris-Hcl pH 7.5, 12.5 mM $MgCl_2$, 1 mM DTT was prepared. ~1 μg of recombinant CREB, purified from BL21(DE3) *E. coli*, was combined with 86 μl of OLM and the total volume was corrected to 100 μl with CREB storage buffer (20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 15% glycerol). Reactions were incubated overnight at ~5-7° C. Reactions were quenched by the addition of 4x loading dye (200 mM Tris-Hcl pH 6.8, 400 mM DTT, 8% SDS, 0.2% bromophenol blue, 40% glycerol) and boiling for 5 min. Samples were resolved by SDS-PAGE, and relative CREB concentrations were measured by Coomassie staining and [^{3}H]GlcNAc-labeling by autoradiography.



Appendix Figure 1. Ni-NTA His6 purification of CREB and OGT expressed in insect cells. (**A)** Coomassie gel of recombinant CREB purified from insect cells. (**B**) Coomassie gel of recombinant CREB and OGT purified from insect cells. **(C)** Coomassie gel of recombinant OGT purified from insect cells

LC-MS/MS Analysis of Recombinant and Native CREB. Liquid chromatography and tandem mass spectrometry (LC-MS/MS) were performed on an LCQ Classic ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) that was fitted with a laboratory-built nanospray source and interfaced with a Surveyor HPLC system (ThermoFinnigan, San Jose, CA). Tryptic digests of recombinant CREB were analyzed using a PicoFrit™ capillary column (0.075 mm i.d. x 50 mm; New Objective, Woburn, MA) packed with 5 μm 100 Å C18 reverse phase (RP) particles (Magic C18, Michrom BioResources, Auburn, CA). All other digests were analyzed using a Michrom BioResources capillary column (0.3 mm i.d. x 150 mm) pre-packed with 5 μm 200 Å C18 RP particles. To achieve microflow rates compatible with capillary chromatography (~1 μL/min), the flow from the HPLC was reduced with a splitting tee and a 150 mm section of fused silica tubing (0.05 i.d.). Approximately 2 pmoles of the tryptic digests were loaded on the microcolumns using the Surveyor Autosampler (ThermoFinnigan), and separated by RP chromatography. The LC buffers used were A: 2% CH_3CN, 0.1% AcOH, 0.005% heptafluorobutyric acid (HFBA), 97.9% H_2O and B: 90% CH_3CN, 0.1% AcOH, 0.005% HFBA, 9.9% H_2O. The gradient, which was optimized for separation of the glycosylated peptides, consisted of: 0-5 min, 0% B; 5-10 min, 0-18% B; 10-50 min, 18-40% B; 50-55 min, 40-100% B; 55-60 min, 100% B.

The LCQ was operated in automated mode using the Xcalibur™ software of the LCQ. The nanospray voltage was 1.8 kV and the heated capillary was 170-180˚ C. Automatic gain control was active, and the ion injection time was set at 200 ms for full scan mode of operation (3 scans per scan) or 400 ms (5 scans per scan) for MS/MS mode. Dynamic exclusion was used during data acquisition to ensure that the majority of co-eluting

peaks would be selected for collision-induced dissociation (CID). In this mode of analysis, the ion trap acquires full scan MS spectra until an ion is present in a scan above a specified threshold. This triggers the ion trap to isolate that ion and generate a product ion spectrum (MS/MS). The ion trap returns to full scan operation when the ion intensity drops below the specified threshold. For native CREB, a parent ion of 1512.8 *m/z*, corresponding to $[M+GlcNAc]^{2+}$ was selected for MS/MS analysis. Relative collision energy for CID was preset to 35% and a default charge state of +2 was selected to calculate the scan range for acquiring tandem MS spectra. The precursor ion isolation window was set at 2.5 for maximum sensitivity.

To calculate the stoichiometry of glycosylation within region 256-284, the on-line LC-MS peaks assigned to the four distinct forms of peptide 256-284 were integrated using the Xcalibur software. CREB co-expressed in the presence and absence of OGT exhibited 55.4% and 9.6% glycosylation, respectively.

Bibliography.

1. Andrews, D.V. and N.C. Faller, Nucleic Acids Research, 1991. **19**: p. 2499.

2. Roquemore, E.P., T.Y. Chow, and G.W. Hart, Methods of Enzymology, 1994. **230**: p. 443-461.

3. Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, ed. N. Ford and C. Nolan. Vol. 3. 1989, New York: Cold Spring Harbor Laboratory Press. 18.53.

4. Loriaux, M.M., et al., Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**: p. 9046-9050.

5. Lubas, W.S., et al., *O-Linked GlcNAc Transferase Is a Conserved Nucleocytoplasmic Protein Containing Tetratricopeptide Repeats*. The Journal of Biological Chemistry, 1997. **272**(14): p. 9316-9324.

6. Strong, R.K., et al., Journal of Molecular Biology, 1994. **241**: p. 483-487.