Published in final edited form as:

Methods Mol Biol. 2009 ; 590: 209–221. doi:10.1007/978-1-60327-378-7_13.

Isolation of Proteins Associated with the DNA-Bound Estrogen Receptor α

Jennifer R. Schultz-Norton, Yvonne S. Ziegler, Varsha S. Likhite, and Ann M. Nardulli

Abstract

Regulating gene expression is a complex process requiring the interaction of multiple transcription factors with their cognate recognition sequences. While these DNA-bound transcription factors are the primary drivers of gene expression, the capacity of a transcription factor to alter gene expression is tempered by its association with a host of coregulatory proteins that are recruited to the DNA-bound transcription factor. We have developed a novel approach to isolate large complexes of proteins associated with the DNA-bound estrogen receptor α (ER α) using an agarose-based electrophoretic mobility shift assay (EMSA). This method should be readily adapted to a variety of cultured cell lines, DNA sequences, and transcription factors and has the potential to provide valuable information about a wide variety of regulatory proteins involved in influencing gene expression.

Keywords

Transcription factor; estrogen receptor; complex isolation; agarose-based electrophoretic mobility shift assay; protein–DNA complex

1. Introduction

Estrogen receptor α (ER α also referred to as ESR1) is a member of a large superfamily of nuclear receptors that function as ligand-inducible transcription factors (1). Upon binding hormone, ER α undergoes a conformational change, binds to estrogen response elements (EREs) residing in estrogen-responsive genes, and initiates changes in gene expression. We and others have demonstrated that, in addition to conformational change induced by hormone binding, individual ERE sequences function as allosteric modulators of ER α conformation (2–7). Thus, both hormone and DNA influence ER α structure.

The ERE-bound ER α recruits a multitude of coregulatory proteins to estrogen-responsive genes and requires the participation of these proteins to effectively modulate transcription (reviewed in 8, 9). The identification of coregulatory proteins has been extremely useful in helping to define mechanisms involved in regulating transcription. Initially, the majority of coregulatory proteins identified were isolated through their interaction with a discrete functional domain of the receptor, most commonly the ligand binding domain.

To better understand how ER α regulates transcription of estrogen-responsive genes, we have developed a method of isolating novel proteins associated with the estrogen- and DNA-bound ER α . This agarose-based electrophoretic mobility shift assay (EMSA) utilizes full-length purified ER α and endogenously expressed nuclear proteins and takes into consideration ligand- and DNA-induced changes in receptor conformation, which we feel more faithfully represents the transcriptionally active DNA-bound receptor than the free

receptor or individual receptor domains. Using this method, we have isolated and identified more than 100 proteins associated with the ERE-bound ER α and shown that more than a dozen of these proteins interact with ER α and influence estrogen-responsive gene expression (*see* Table 13.1, **refs.** 10–18 and unpublished work). Thus, our agarose-based EMSA has been very effective in isolating novel regulatory proteins that associate with the ERE-bound ER α and influence receptor activity. This method could be applicable in isolating proteins that influence the activity of other DNA-bound transcription factors.

2. Materials

2.1. HeLa Cell Culture, Lysis, and Nuclear Extract Purification

1. Dulbecco's Modified Eagle Medium: Nutrient Mix F-12, pH 7.4 containing 15 mM HEPES and L-glutamine (Invitrogen, Carlsbad, CA) (*see* Note 1).
2. 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA).
3. 100 U/ml penicillin, 100 μ g/ml streptomycin, 25 μ g/ml gentamycin
4. Hank's Balanced Salt Solution (HBSS): 10 mM HEPES pH 7.6, 0.075% sodium bicarbonate.
5. HBSS-EDTA (HE): 10 mM HEPES pH 7.6, 0.075% sodium bicarbonate, 1.2 mM EDTA pH 7.4.
6. TEG: 50 mM Tris pH 7.4, 0.1 mM EDTA, 10% glycerol.
7. TEG-500: 50 mM Tris pH 7.4, 0.1 mM EDTA, 10% glycerol, 0.5 M KCl.
8. Protease Inhibitor Cocktail (PIC, P8340, Sigma, St. Louis, MO) containing 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin. Dilute the 100x stock 1:100 into the sample as needed.
9. Trypan blue.
10. Tissue grinder with B pestle (Kontes, Vineland, NJ).

2.2. Protein Assay

1. BioRad Protein Assay Kit II, containing 5x Protein Assay Solution and Bovine Serum Albumin (BioRad, Hercules, CA). 5x Protein Assay Solution is diluted 1:5 in deionized H₂O (dH₂O) and filtered through a Whatman filter (#1001-150, Fisher Scientific).
2. Disposable glass culture tubes (Fisher Scientific).

2.3. Preparation of ³²P-Labeled Oligos

1. Oligos containing the *Xenopus laevis* vitellogenin A2 estrogen response element: 5'-CTA GAT TAC AGG TCA CAG TGA CCT TAC TCA-3' and 5'-GAT CTG AGT AAG GTC ACT GTG ACC TGT AAT CTA G-3' (IDT, Coralville, IA).
2. Oligos containing a nonspecific DNA sequence: 5'-CTA GAT TAC TTC TCA TGT TAG ACA TAC TCA-3' and 5'-GAT CTG AGT ATG TCT AAC ATG AGA AGT AAT CTA G-3'.
3. 10x Annealing Buffer: 10 mM Tris pH 7.8, 10 mM MgCl₂, 50 mM KCl, 1 mM EDTA, 1 mM EGTA.

¹All cell culture reagents are from Invitrogen, Carlsbad, CA, unless otherwise stated.

4. ^{32}P -ATP with a specific activity of 7000 Ci/mmol (MP Biomedicals, Solon, OH).
5. T4 Kinase (10U/ μL , Invitrogen).

2.4. DNA Binding Reactions

1. 10x Reaction Buffer: 150 mM Tris pH 7.9, 2 mM EDTA, 800 mM KCl, 500 μM ZnCl_2 , 50 mM MgOAc, 40 mM DTT, 0.5 μM 17 β -estradiol. Make fresh from stock solutions. 1 M DTT stock solution, which is stored at -20°C , should not be refrozen. 17 β -estradiol should be prepared in ethanol.
2. Salmon sperm DNA: 10 mg/ml stock diluted to 1 mg/ml in dH_2O (Invitrogen).
3. BSA: 50 mg/ml stock diluted to 1 mg/ml in dH_2O (Invitrogen) or ovalbumin, 20 mg/ml stock diluted to 1 mg/ml in dH_2O (Roche Diagnostics Corp., Indianapolis, IN).
4. Poly-deoxyinosine/deoxycytidine: resuspended to 1 mg/ml in dH_2O (Amersham Biosciences, Piscataway, NJ).
5. 50% glycerol.
6. HeLa nuclear extracts prepared as described in **Section 3.1**.
7. Purified ER α can be prepared as described (2, 19) or purchased from a commercial supplier.
8. ER α -specific antibody (sc-8002, Santa Cruz Biotechnology, Santa Cruz, CA).
9. Nonspecific antibody (YY1-specific, sc-7341, Santa Cruz Biotechnology).

2.5. Agarose Gel Electrophoresis for Initial Protein/DNA Complex Characterization

1. 10x Running Buffer: 5.4 g Tris base, 27.4 g boric acid, 11.2 g MgOAc $\cdot$ 4 H_2O , 20 ml 0.5 M EDTA. Bring to 1L with dH_2O .
2. Molecular biology grade agarose (BioRad) (*see* Note 2).
3. DE81 ion exchange cellulose acetate (Whatman, Florham Park, NJ).
4. Biomax XAR autoradiography film (Carestream Health, Inc, Rochester, NY).

2.6. Agarose Gel Complex Isolation Electrophoresis for and Protein Extraction

1. 10x Running Buffer as described in **Section 2.5.1**.
2. Molecular biology grade agarose (BioRad).
3. Biomax XAR autoradiography film (Carestream Health, Inc).
4. Montage gel extraction kit (Millipore, Billerica, MA).
5. Microcon YM-10 size exclusion columns (Millipore).

3. Methods

Small agarose gels should be used initially to determine the conditions required for protein–DNA complex formation. ^{32}P -labeled ERE-containing oligos, HeLa nuclear extracts, which contain full-length endogenously expressed coregulatory proteins, and purified ER α are used for these small-scale experiments. To ensure that a protein–DNA complex formed is specific, a number of steps should be taken. First, ^{32}P -labeled oligos containing a

²We have successfully used low-melt, megabase, and SeaKem agarose to isolate protein–DNA complexes.

nonspecific DNA sequence should be run in parallel with the ^{32}P -labeled ERE-containing oligos. A specific protein–DNA complex will be formed with the ERE-containing oligos, but not with oligos containing a nonspecific DNA sequence (data not shown). Second, the receptor and HeLa nuclear extract can be added alone and in combination to demonstrate that protein–DNA complex formation requires the addition of both the receptor and the nuclear proteins (Fig. 13.1, Lane 4) and that neither the receptor (Lane 2) nor the nuclear proteins (Lane 3) alone are sufficient for complex formation. Third, competition assays should be carried out with an excess (50–100x) of unlabeled ERE-containing oligos and unlabeled oligos lacking an ERE to demonstrate that ERE-containing oligos decrease protein–DNA complex formation (Lanes 5–6), but that the same molar excess of oligos containing a nonspecific DNA sequence does not affect complex formation (Lanes 7–8). Fourth, an ER α -specific antibody can be added to the binding reaction to demonstrate that the protein–DNA complex is supershifted and that ER α is present in the complex (Fig. 13.2, Lane 5). In contrast, a nonspecific antibody is unable to alter the migration of the protein–DNA complex (Fig. 13.2, Lane 6).

Once the gel conditions required for formation of a specific protein–DNA complex have been identified, the reactions can be scaled up and run on larger gels to isolate sufficient amounts of protein for further analysis. Mass spectrometry analysis can be used to identify novel proteins associated with the DNA-bound ER α and Western blots can be performed to confirm that a protein is present in the protein–DNA complex when the ERE-containing oligos are utilized, but not present when oligos containing a nonspecific DNA sequence are utilized. This method has been extremely useful in identifying proteins associated with the DNA-bound ER α using HeLa nuclear extracts and purified ER α as well as MCF-7 nuclear extracts, which contain endogenously expressed ER α (13–18, 21–23) and should be amenable for use with other cell types and systems.

3.1. HeLa Cell Culture, Lysis, and Nuclear Extract Purification

1. HeLa cells are grown in T175 flasks at 37°C until 80–90% confluent. Approximately 20 flasks are used for a typical preparation. The media should be changed the day before harvest (*see* Note 3).
2. Wash each flask with 10 ml HBSS.
3. Harvest the cells by adding 2 ml HE to each flask and incubate at 37°C for 2 min. Tap the flasks to release the cells. Add 25 ml HBSS to the first flask. Collect the cells and transfer to the next flask. Repeat this procedure for 8–10 flasks and transfer the cells to a 250 ml centrifuge bottle on ice. Add 10 ml HBSS to a flask to remove any cells still adhering to the flask and transfer the remaining cells sequentially to 4 flasks. Repeat this process until all of the flasks have been rinsed. Transfer the remaining cells to the centrifuge bottle.
4. Centrifuge the cells for 5 min at 4°C at 150g.
5. Aspirate the media. From this point forward, keep the cells on ice and work quickly to limit protein degradation.
6. Resuspend the cell pellet in 200 μl of TEG with 1x PIC at 4°C for each T175 flask harvested. Loosen the cell pellet by tapping the centrifuge bottle. Transfer the resuspended cells to a chilled tissue grinder and homogenize on ice with 25 shallow strokes of a B pestle. Check at this point to ensure that the cells have been lysed and that the nuclei are intact. Combine 10 μl cell lysate with 1 μl Trypan blue and

³HeLa cells can be grown in spinner flasks, which is useful if larger numbers of cells are needed. For spinner flask cultures, maintain the cells at a density of 2×10^6 cells/ml. Pour cells into 250-ml centrifuge bottles and skip to step 3.1.4 of the protocol.

examine under a microscope. The nuclei, but not the intact cells, will appear blue. If all cells are not lysed, homogenize the cells with an additional 10 shallow strokes of a B pestle.

7. Transfer the homogenate to a chilled 11 ml polyallomer high speed centrifuge tube on ice. Rinse the homogenizer with 200 μ l of TEG containing 1x PIC and add to the polyallomer tube.
8. Pellet the homogenate for 10 s at 4°C at 10,200g
9. Quickly place the pelleted nuclei on ice and remove the supernatant.
10. Resuspend the nuclei in 75 μ l of TEG-500 with 1x PIC at 4°C for each T175 flask harvested by pipetting the nuclei through a 1-ml pipette tip 5–7 times. For example, 1.5 ml of TEG-500 will be needed for 20 flasks of cells. Incubate on ice for 20 min and vortex every 5 min.
11. Centrifuge the nuclear extract for 30 min at 4°C at 142,000g. 12. Transfer the supernatant to a clean microcentrifuge tube and aliquot. Freeze aliquots quickly on dry ice and store at –80°C.

3.2. Protein Assay

1. Dissolve 12.5 μ g BSA per ml dH₂O (12.5 μ g/ml) and store at 4°C. Prepare standards to generate a standard curve by diluting the BSA to 10 μ g/ml, 7.5 μ g/ml, 5 μ g/ml, and 2.5 μ g/ml in dH₂O.
2. Aliquot 1 ml of 1x Protein Assay Solution into each of 12 disposable glass culture tubes. Reserve 2 tubes for blanks. Add 10 μ l of each diluted BSA standard in duplicate to the remaining 10 tubes. The final concentrations of BSA in the tubes will be 0, 25, 50, 75, 100, and 125 ng/ml. Vortex.
3. Incubate at room temperature for 5 min.
4. Determine the absorbance of each standard at 595 nm using a spectrophotometer. Plot the standards on a curve with the concentration on the *x*-axis and absorbance on the *y*-axis.
5. Determine the concentration of the nuclear extract prepared in Section 3.1 by diluting 1–2 μ l of the extract in 10 μ l dH₂O and add to 1 ml of 1x Protein Assay Solution in a disposable test tube. Prepare duplicates for each sample. Vortex. The same 1x Protein Assay Solution should be used for samples and standards, but samples should be prepared after the standard curve is determined to ensure that the amount of sample used falls on the standard curve.
6. Incubate at room temperature for 5 min.
7. Measure the absorbance at 595 nm and use the standard curve to determine the concentration of protein in the sample.

3.3. Preparation of ³²P-Labeled Oligos

1. Anneal 2 nmol of complimentary oligos in 1x Annealing Buffer in a thermocycler. The annealing mixture should be brought to 85°C for 2 min and the temperature reduced 1°C per minute for 60 min followed by incubation on ice. Alternatively, the mixture could be boiled in a hot block and allowed to cool in the hot block at room temperature for 1 h. The annealed mixture should be kept on ice until ready to end-label.

2. End label 40 pmol of the annealed oligos with 1 μ l γ 32 P ATP using 2 μ l of T4 Kinase (10 U/ μ l, Invitrogen) according to manufacturer's directions.

3.4. DNA Binding Reactions

1. For initial optimization and characterization of protein–DNA complexes, prepare Small-scale Reaction Mixes. On ice, combine 20,000–40,000 cpm 32 P-labeled oligos, 400 fmol purified ER α , 1 μ l of salmon sperm DNA (1 mg/ml), 1 μ l of BSA (1 mg/ml) or ovalbumin (1 mg/ml), 1 μ l of poly-deoxyinosine/deoxycytidine (1 mg/ml), 1.2 μ l of 10x Reaction Buffer, and 2.4 μ l of glycerol (50%). Bring the reaction to 12 μ l of final volume with dH₂O. Incubate for 10 min at room temperature once all reagents are added.
2. For isolation of protein–DNA complexes, prepare Large-scale Reaction Mixes. On ice, combine 50 pmol unlabeled ERE-containing oligos, 18 pmol ER α , 262 μ g HeLa nuclear extract, 16 μ l of salmon sperm DNA (1 mg/ml), 16 μ l of BSA (1 mg/ml) or ovalbumin (1 mg/ml), 16 μ l of polydeoxyinosine/deoxycytidine (1 mg/ml), 20 μ l of 10x Reaction Buffer, and 40 μ l of glycerol (50%). Bring the reaction to 200 μ l with dH₂O. ER α -specific antibody can be added to help stabilize the protein–DNA complex.

3.5. Agarose Gel Electrophoresis for Initial Protein/DNA Complex Characterization

1. Prepare a 6.5 $\times$ 10 cm 1–1.25% agarose gel in 25 ml of 1x Running Buffer. Allow the gel to solidify at room temperature. Chill the gel at 4°C for 15 min prior to removing the comb (*see* Note 4).
2. Set up the 4°C gel with 1x Running Buffer at 4°C in a cold room and chill thoroughly (*see* Note 5).
3. While the gel is chilling, prepare the Small-scale Reaction Mix (~10 μ l) described in Section 3.4.1 on ice. Unlabeled oligos containing an ERE or nonspecific DNA sequence (50–100-fold excess) and ER α -specific and nonspecific antibodies can also be added at this point. Incubate for 10 min at room temperature.
4. Add 10 μ g HeLa nuclear extract to the Reaction Mix for a final volume of 12.5 μ l and incubate for 20 min at room temperature (*see* Note 6).
5. Load the samples onto a horizontal gel and electrophorese at 100 V for 1–2 h at 4°C (*see* Note 7).
6. Place the gel on a piece of DE81 cellulose acetate with two pieces of Whatman blotting paper underneath. Dry the gel at 65°C for 30 min on a vacuum gel dryer. Expose the dried gel to autoradiography film overnight to determine the position of the protein–DNA complexes and the free oligos.

⁴If more wells are needed, use 1–1.25% agarose in 50 ml of Running Buffer for an 18-well gel using the BioRad Wide Mini Sub Cell gel system. If a nebulizer will be used for extracting complexes, do not exceed 1.25% agarose as it will clog the filter.

⁵Some protein complexes may be more stable at lower temperatures. In this case the gel box can be placed in an ice bath to maintain it at a lower temperature throughout the run.

⁶This protocol can be adapted to use extracts from multiple cell lines. We have successfully formed specific protein–DNA complexes using nuclear extracts from HeLa and MDA-MB-231 cell nuclear extracts in combination with purified ER α and with MCF-7 cell nuclear extracts, which contain endogenously expressed ER α .

⁷The duration of electrophoresis will depend on the size of the protein–DNA complex and the length of the oligos and should be determined empirically. No loading buffer is needed since the glycerol in the reaction mix will allow the sample to sink to the bottom of the well. Dyes should generally not be used since they may interfere with protein–DNA complex formation.

3.6. Agarose Gel Electrophoresis for Complex Isolation and Protein Extraction

1. For isolation of proteins, prepare a 15 × 10 cm 1–1.25% agarose gel in 50 ml of 1x Running Buffer using a prep-style comb. Alternatively, the teeth of a comb can be taped with electrical tape to increase the well size, as shown in Fig. 13.3A. Chill the gel at 4°C for 15 min prior to removing the comb.
2. While the gel is chilling, prepare the Large-scale Reaction Mix described in Section 3.4.2 on ice. ERα-specific antibody can be added to help stabilize the protein–DNA complex. Prepare Small-scale Reaction Mixes described in Section 3.4.1 with ³²P-labeled ERE-containing oligos, which are run adjacent to the large-scale reactions with the unlabeled ERE-containing oligos to identify the location of each protein–DNA complex (*see* Note 8).
3. Load the samples onto the horizontal gel and separate the complexes at 100 V at 4°C for the time required to separate the protein–DNA complex from the free DNA.
4. After fractionation, the wet gel is wrapped in plastic and exposed to autoradiography film overnight at 4°C. The position of each protein–DNA complex will be defined by the radiolabeled protein–DNA complexes, which are run in parallel (*see* Fig. 13.3B). Draw 2 lines across the autoradiogram to define the position of the unlabeled protein–DNA complexes (arrowheads). Using a single-edge razor, cut a 3-sided flap in the autoradiogram where each unlabeled protein–DNA complex has migrated (dashed lines). Place the auto-radiogram directly over the agarose gel. Pull back the 3-sided flap, fold it back along the solid line shown in Fig. 13.3B, and excise the gel region containing the protein–DNA complex. Recover the proteins from the gel with a Montage gel extraction kit according to manufacturer's directions (*see* Note 9).
5. The extracted proteins can be concentrated with Microcon size-exclusion columns according to manufacturer's directions. An appropriate molecular weight cutoff should be used to ensure that low molecular weight proteins of interest are not lost (*see* Note 10). The identity of the proteins present in the complexes can be determined by mass spectrometry analysis and the presence of a particular protein can be verified by Western blot analysis.

Acknowledgments

We are indebted to J. Yates and coworkers for their mass spectrometry analysis of the proteins isolated. This work was supported by NIH grant R01 DK 53884 (to AMN). JRS-N was supported by an NIH Reproductive Biology Program Training Grant (T32 HD07028).

References

1. Beato M, Herrlich P, Schutz G. Steroid hormone receptors: Many actors in search of a plot. *Cell*. 1995; 83:851–57. [PubMed: 8521509]

⁸Unlabeled oligos are utilized for large-scale reaction mixes to prevent contamination of mass spectrometry equipment with the ³²P-labeled probe. Large-scale reaction mixes should be kept in as small a volume as possible.

⁹We have found that the Montage gel extraction kit is far more efficient in isolating ERα and its associated proteins than acetone or trichloroacetic acid precipitation.

¹⁰We typically use a filter with a 10 kDa molecular weight cutoff. A small amount of Triton X-100 (0.2 mM) can be added during the concentration step to minimize the amount of protein that sticks to the plastic or filter (20). Micro-liquid chromatography electrospray ionization tandem mass spectrometry combined with database searches can be used to identify proteins by correlating experimental spectra to protein sequences in the human sequence database. Mass spectrometry analysis can be performed at a number of commercial and academic facilities on a fee for service basis. The presence of a protein in the protein–DNA complex can be confirmed by Western analysis of the eluted proteins.

2. Wood JR, Greene GL, Nardulli AM. Estrogen response elements function as allosteric modulators of estrogen receptor conformation. *Mol Cell Biol.* 1998; 18:1927–34. [PubMed: 9528764]
3. Wood JR, Likhite VS, Loven MA, Nardulli AM. Allosteric modulation of estrogen receptor conformation by different estrogen response elements. *Mol Endocrinol.* 2001; 15:1114–26. [PubMed: 11435612]
4. Loven MA, Likhite VS, Choi I, Nardulli AM. Estrogen response elements alter coactivator recruitment through allosteric modulation of estrogen receptor beta conformation. *J Biol Chem.* 2001; 276:45282–88. [PubMed: 11574541]
5. Loven MA, Wood JA, Nardulli AM. Interaction of estrogen receptors alpha and beta with estrogen response elements. *Mol Cell Endocrinol.* 2001; 181:151–63. [PubMed: 11476949]
6. Klinge CM. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.* 2001; 29:2905–19. [PubMed: 11452016]
7. Hall JM, McDonnell DP, Korach KS. Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. *Mol Endocrinol.* 2002; 16:469–86. [PubMed: 11875105]
8. Robyr D, Wolffe AP, Wahli W. Nuclear hormone receptor coregulators in action: Diversity for shared tasks. *Mol Endocrinol.* 2000; 14:329–47. [PubMed: 10707952]
9. Torchia J, Glass C, Rosenfeld MG. Co-activators and co-repressors in the integration of transcriptional responses. *Curr Opin Cell Biol.* 1998; 10:373–83. [PubMed: 9640539]
10. Loven MA, Muster N, Yates JR, Nardulli AM. A novel estrogen receptor alpha associated protein, template activating factor I beta, inhibits acetylation and transactivation. *Mol Endocrinol.* 2003; 17:67–78. [PubMed: 12511607]
11. Likhite VS, Cass EI, Anderson SD, Yates JR, Nardulli AM. Interaction of estrogen receptor alpha with 3-methyladenine DNA glycosylase modulates transcription and DNA repair. *J Biol Chem.* 2004; 279:16875–82. [PubMed: 14761960]
12. Loven MA, Davis RE, Curtis CD, Muster N, Yates JR, Nardulli AM. A novel estrogen receptor alpha-associated protein alters receptor-deoxyribonucleic acid interactions and represses receptor-mediated transcription. *Mol Endocrinol.* 2004; 18:2649–59. [PubMed: 15308690]
13. Schultz-Norton JR, Gabisi VA, Ziegler YS, McLeod IX, Yates JR, Nardulli AM. Interaction of estrogen receptor alpha with proliferating cell nuclear antigen. *Nucleic Acids Res.* 2007; 35:5028–38. [PubMed: 17636311]
14. Schultz-Norton JR, McDonald WH, Yates JR, Nardulli AM. Protein disulfide isomerase serves as a molecular chaperone to maintain estrogen receptor {alpha} structure and function. *Mol Endocrinol.* 2006; 20:1982–95. [PubMed: 16690750]
15. Schultz-Norton JR, Walt KA, Ziegler YS, McLeod IX, Yates JR, Raetzman LT, Nardulli AM. The deoxyribonucleic acid repair protein flap endonuclease-1 modulates estrogen-responsive gene expression. *Mol Endocrinol.* 2007; 21:1569–80. [PubMed: 17488975]
16. El Marzouk S, Schultz-Norton JR, Likhite VS, McLeod IX, Yates JR, Nardulli AM. Rho GDP dissociation inhibitor alpha interacts with estrogen receptor alpha and influences estrogen responsiveness. *J Mol Endocrinol.* 2007; 39:249–59. [PubMed: 17909265]
17. Curtis CD, Likhite VS, McLeod IX, Yates JR, Nardulli AM. Interaction of the tumor metastasis suppressor nonmetastatic protein 23 homologue H1 and estrogen receptor alpha alters estrogen-responsive gene expression. *Cancer Res.* 2007; 67:10600–7. [PubMed: 17975005]
18. Rao AK, Ziegler YS, McLeod IX, Yates JR, Nardulli AM. Effects of Cu/Zn superoxide dismutase on estrogen responsiveness and oxidative stress in human breast cancer cells. *Molecular Endocrinology.* 2008; 22:1113–24.
19. Kraus WL, Kadonaga JT. p300 and estrogen receptor cooperatively activate transcription via differential enhancement of initiation and reinitiation. *Genes Dev.* 1998; 12:331–42. [PubMed: 9450928]
20. Suelter CH, DeLuca M. How to prevent losses of protein by adsorption to glass and plastic. *Anal Biochem.* 1983; 135:112–9. [PubMed: 6670734]
21. Creekmore AL, Walt KJ, Schultz-Norton JR, Ziegler YS, McLeod IX, Yates JR, Nardulli AM. The role of retinoblastoma associated proteins 46 and 48 in estrogen receptor α mediated gene expression. *Mol Cell Endocrinol.* 291:79–86. [PubMed: 18577416]

22. Curtis CD, Thorngren DL, Ziegler YS, Sarkeshik A, Yates JR, Nardulli AM. Apurinic/aprimidinic endonuclease 1 alters estrogen receptor activity and estrogen responsive gene expression. *Mol Endocrinol.* 2009; 23:1346–59. [PubMed: 19460860]
23. Rao A, Ziegler YS, McLeod IX, Yates JR, Nardulli AM. Thioredoxin and thioredoxin reductase influence estrogen receptor α mediated gene expression in human breast cancer cells. *J Mol Endocrinol.* 2009 In Press.

Fig 13.1.

Small-scale agarose-based gel mobility shift assay with unlabeled competitor DNA. ^{32}P -labeled, ERE-containing oligos were incubated without (lanes 1–2) or with HeLa nuclear extracts (NX, lanes 3–8). Purified ER α (lanes 2, 4–8) was included as indicated. Increasing amounts of unlabeled oligos containing ERE (lanes 5–6) or non-specific DNA sequence (lanes 7–8) were included in the binding reaction as indicated. Complexes were resolved on an agarose gel and visualized by autoradiography.

Fig 13.2. Small-scale agarose-based gel mobility shift assay with antibody supershift. ^{32}P -labeled, ERE-containing oligos were incubated without (lanes 1–2) or with HeLa nuclear extracts (lanes 3–6). Purified ER α (lanes 2, 4–6) was included as indicated. ER α -specific (lane 5) or non-specific (lane 6) antibody was included as indicated. Complexes were resolved on an agarose gel and visualized by autoradiography.

Fig 13.3. Large-scale agarose-based gel mobility shift assay. **(A)** Schematic drawing of a comb with taped teeth utilized for large-scale isolation of ER α -containing protein–DNA complexes. **(B)** Unlabeled ERE-containing oligos were incubated with HeLa nuclear extracts in the absence or in the presence of purified ER α . Small-scale reactions containing HeLa nuclear extract, ER α and ^{32}P -labeled, ERE-containing oligos were run on the outside lanes (*) to indicate where the protein–DNA complexes were located. The underlying gel was excised from the regions beneath the boxed areas. Proteins were extracted from the gel regions from control (HeLa) or protein–DNA complex (ER α +HeLa) lanes and were identified by mass spectrometry analysis.

Table 13.1

ER α -interacting proteins isolated from agarose gel complexes. Proteins associated with the DNA-bound ER α were identified by mass spectrometry analysis. The effects of some of these ER α -associated proteins on the ER α -ERE interaction and ER α -mediated transcription are indicated. $\uparrow/\downarrow$ represents gene-specific effects

Protein	ER α -ERE interaction	ER α -mediated Transcription	References
APE1	$\uparrow$	$\uparrow/\downarrow$	(22)
FEN1	$\uparrow$	$\uparrow/\downarrow$	(15)
MPG	$\uparrow$	$\downarrow$	(11)
NM23-H1	$\uparrow$	$\downarrow$	(17)
PCNA	$\uparrow$	$\uparrow$ basal	(13)
PDI	$\uparrow$	$\uparrow/\downarrow$	(14)
pp32	$\uparrow$	$\downarrow$	(12)
RbAp46	not done	$\uparrow/\downarrow$	(21)
RbAp48	not done	$\downarrow$	(21)
Rho-GDI	$\uparrow$	$\uparrow/\downarrow$	(16)
SOD1	$\uparrow$	$\uparrow$	(18)
TAF-I β	$\uparrow$	$\downarrow$	(10)
Trx	–	$\uparrow/\downarrow$	(23)
TrxR	$\uparrow$	$\uparrow/\downarrow$	(23)