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14. ABSTRACT Dysregulated estrogen receptor (ER) function underlies many forms of breast cancer. This proposal is aimed at understanding how ER α activates its target genes from distal enhancers in a Mediator-dependent fashion. We have hypothesized that ER-Mediator interactions would be critical for signal transduction at ER α target genes through establishment of chromatin loops that facilitate long-range enhancer-promoter communication. The main aims of the proposal are thus to establish both cell based and cell-free (in vitro) transcription systems to recapitulate and mechanistically dissect Mediator-dependent ER α function from a distal enhancer and further to develop peptidomimetic inhibitors of the ER α -MED1 interaction to disrupt enhancer-promoter communication. In this phase of the project we have established an assay system that supports rudimentary ER α function from distal binding sites. Using this as a baseline, future years will see further refinement of the in vitro systems so that contributions of Mediator and cohesin can be assessed. We have also begun generating the reagents we will require for our studies. Key reagents that we have obtained thus far include an active core of the Mediator complex that we have reconstituted through overexpression of its subunits using a baculovirus expression system. ER α -interacting MED1 and its mutant variants will be incorporated into this core Mediator for further ER α -based functional studies. We also have obtained GFP-derivatives of Mediator and TFIID complexes for proposed FRET analyses for looped chromatin. Peptidomimetic synthesis and analyses are also projected to commence shortly.					
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INTRODUCTION

Liganded estrogen receptor (ER α) activates transcription of ER α target genes in multiple steps entailing a series of coactivator exchanges that first make the chromatin more accessible and finally lead to the establishment of functional Pol II complexes at core promoters. This proposal is aimed at understanding mechanisms whereby ER α activates its target genes from distal enhancers in a Mediator-dependent manner. Recent studies have shown chromatin looping at ER α -dependent genes and for a role of cohesin, a chromosome segregation factor, in ER α -dependent gene expression. Studies of the Mediator coactivator complex, which has emerged as an integrative hub for transcriptional regulation, indicate that it also plays critical roles in generating a distinct chromatin architecture at active loci through interactions with activators and cohesin. Given previous demonstrations of ER α interaction and function through the MED1 subunit of the Mediator, we hypothesized that ER-Mediator interactions would be critical for signal transduction at ER α target genes through establishment of chromatin loops that facilitate long-range enhancer-promoter communication. The proposal is thus aimed at (i) establishing cell-free (in vitro) transcription systems to recapitulate and mechanistically dissect Mediator-dependent ER α function from a distal enhancer; (ii) establishing complementary cell-based assays to analyze putative ER α -Mediator stabilized chromatin loop in the context of living cells; and (iii) developing peptidomimetic inhibitors of the ER α -MED1 interaction to modulate ER α -dependent gene expression by targeting enhancer-promoter communication. These studies are expected to provide mechanistic insights into normal transcription regulatory processes that go awry in breast cancer. Furthermore, the proposed peptidomimetics, which are expected to target novel druggable targets in the ER α activation pathway, may prove to be useful in breast cancer therapy since, unlike the widely used SERMs, they may have minimal off-target effects.

KEYWORDS

Estrogen receptor; breast cancer; cohesin; FoxA1; Mediator coactivator complex; enhancers; chromatin looping, peptidomimetics.

OVERALL PROJECT SUMMARY (BODY)

As described below, this phase (Year 1) of the project has concentrated mainly on having in hand essential reagents that will be needed for the proposed biochemical mechanistic studies and on establishing key functional assays that will be the bases of much of the work.

Establishment of an in vitro transcription system for ER-dependent transcription activation from distal sites (SOW Task 1)

We previously published in vitro transcription assay reconstituted from purified components in which ER α activates a DNA template in a Mediator-dependent fashion (1). In contrast to these studies, in which the EREs were juxtaposed close to the core promoter, for our most recent studies that are aimed at establishing an experimental system to investigate activation by ER α over relatively long ranges, we have generated a series of templates in which the EREs were placed at progressively distant sites relative to the core promoter/TSS (Fig. 1A). After chromatinization using the ACF1/NAP1 assembly system (2), and ascertaining template quality by MNase1 digestion (Fig. 1B), the templates were transcribed in the presence or absence of ER α of unfractionated HeLa nuclear extract. Purified ER α for this purpose was expressed in Sf9 cells (Fig. 1C).

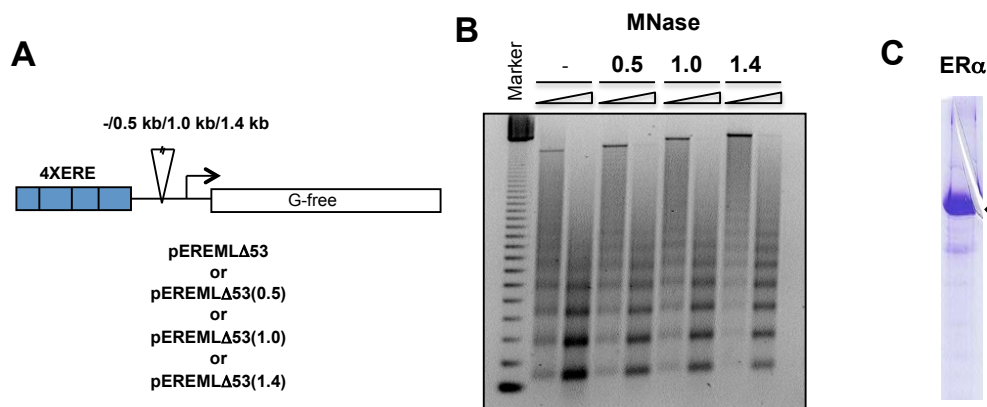


Fig. 1. Materials for ER α -dependent activation from distal sites. (A) Schematic of templates: parent vector pEREMLA53 and its derivatives in which ERE is 0.5, 1.0, or 1.4 kb away from core promoter. (B) MNase analysis of chromatinized templates. (C) Preparation of ER α following purification from baculovirus-infected Sf9 cells.

In these experiments, our standard template (1), in which the EREs are close to the core promoter, was efficiently activated in an ER α - and p300-dependent manner (Fig. 2). As expected, with the derivative templates we observed that there was a progressive decline in the ability of ER α to activate transcription as the distance between the EREs and the core promoter elements increased. Nonetheless, it was notable that even in the template (pEREMLA53(1.0)) in which the ER α is over 1.0 kb away from the core promoter, ER α -dependent transcription was readily detectable (lane 14). This indicates existence of long-range ER-PIC interactions that characterize physiological enhancer functions, a process that might be facilitated by chromatinization (data not shown) potentially involving higher-order/folded structures. At the same time, the several-fold diminution of signal relative to the parent template (lane 14 vs. lane 4) implies that certain critical factors or higher-order structures might yet be limiting.

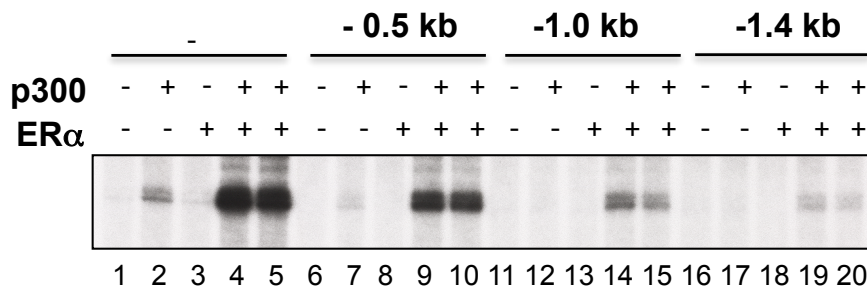


Fig. 2 ER-dependent activation from distal sites. In vitro transcription of chromatinized ERE templates (Fig 1B) in unfractionated HeLa nuclear extract with additions as indicated. Reactions in lanes 5, 10, 15, 20 are unrelated to the present issue.

These results establish that core factors for recapitulating ER α -dependent transcription activation from distal binding sites in cell-free systems are present in HeLa nuclear extract and thus open up avenues for further refinement of the system. In the immediate future, we expect these studies to develop along two main lines. First, and still working in the context of the unfractionated extract, we hope to identify factors necessary for optimal ER α -dependent activation of the pEREMLA53(1.0) template. Our immediate focus is on achieving the appropriate template

topology. For this purpose, expression and purification of cohesin components are underway and experiments are planned to assess how linker histone-induced higher order structures (which might affect chromatin looping) impact on transcription from this template. Given our focus on the Mediator complex, we are also planning experiments with extract from which Mediator has been immunodepleted (3). On a parallel track, we are proceeding with reconstituting long range ER α -dependent transcription with purified components (3) with the expectation that these systems will have to be supplemented with multiple additional factors that might specifically be needed for optimal ER α function from a distance.

Reconstitution of an active core of the Mediator complex for functional studies (SOW Task 1)

Although thus far we have successfully relied on Mediator preparations obtained from cultured mammalian cell lines that express selected epitope-tagged Mediator subunits (see below), isolation of the complex is cumbersome and suffers from limited yields. Furthermore, given that nearly all of the 30 Mediator subunits function only as part of the intact complex, mutational analysis of any given subunit is also greatly hampered. To facilitate our current studies on ER α , as well as others, we proceeded to reconstitute the active core of the Mediator complex. The large Mediator complex is composed of more or less discrete modules (“head”, “middle”, “tail” and “kinase”) (4). This modular organization in large part reflects the multiple layers of functions associated with the Mediator. Thus, whereas the head and middle modules are responsible for the essential core functions of the Mediator, such as interaction with RNA polymerase II (Pol II) and support of basal (activator-independent) transcription, the tail for the most part displays specialized functions dependent on specific activators. MED1, the subunit that interacts with ER α and other nuclear receptors and is essential for their activator function, is also dispensable for core Mediator functions. The kinase module is also required only conditionally.

To reconstitute the active Mediator core, we subcloned cDNAs encoding head and middle subunits into multiple baculovirus vectors for overexpression in the MultiBac system developed by Dr. T. Richmond and colleagues (5). Briefly, we were able to isolate a bi-modular complex containing the subunits of the head and middle modules (Fig. 3A). However, in functional tests for basal transcription in a system reconstituted from purified Pol II and general transcription factors, this reconstituted complex, unlike Mediator preparations obtained from epitope tagged stable cell lines was not active (Fig. 3C, lane 5 versus lanes 8 and 9). We therefore prepared reconstituted Mediator complexes in the presence of baculovirus vectors expressing the MED14 subunit. Although previously assigned to the tail module, we had reason to believe that MED14 might in fact be an integral component of the core complex. Indeed, we were finally able to isolate a head-middle-MED14 complex that contained stoichiometric amounts of all the input subunits (Fig. 3B). When tested for activity in supporting basal transcription, this complex was able to support basal transcription to the same extent as Mediator preparations isolated from HeLa cells (Fig. 3C, lane 6 versus lanes 8 and 9). A manuscript describing these results and other structural-functional studies of the Mediator is currently in preparation.

These results set the stage for incorporating MED1 and its mutant derivatives into the core complex for our ongoing studies on ER α function through the Mediator although preliminary indications are that signal transduction through MED1 might entail additional subunits not currently represented in the core complex (data not shown).

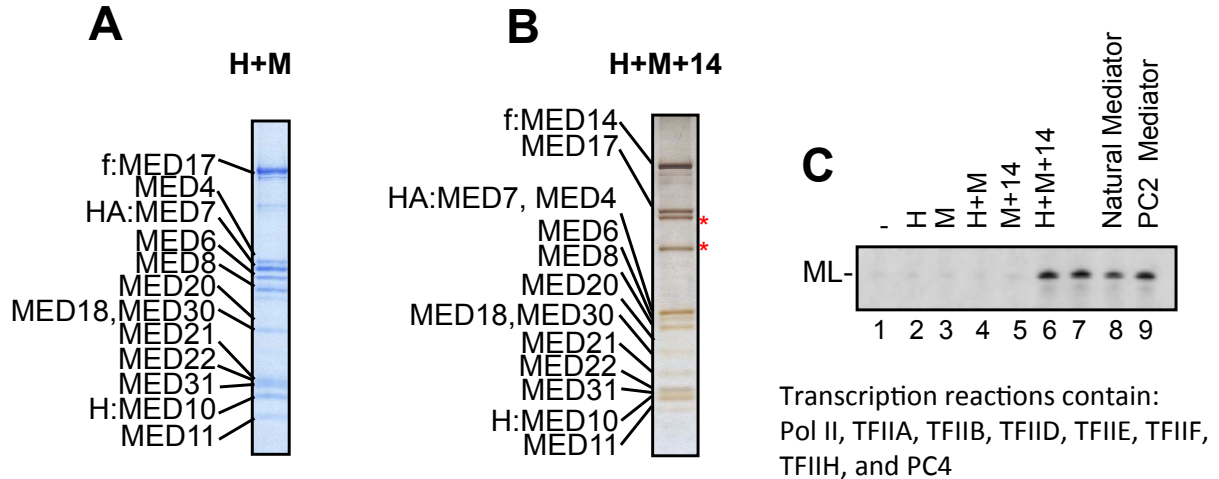


Fig. 3. Reconstitution of active Mediator core complex for functional studies of ER. (A) Mediator subunits of the head (H) and middle (M) modules were expressed via the Multibac system. A bi-module complex was purified by series of affinity and conventional chromatography steps (CBB staining). (B) Subunits of the Mediator head and middle modules were co-expressed with the MED14 subunit. Purification via the FLAG tag on MED14 and additional chromatography steps (including gel filtration) yielded the H+M+14 complex shown (silver staining). (C) In vitro transcription assay in a pure system demonstrating ability of the H+M+14 complex to support basal transcription. Reactions were reconstituted with Pol II, the indicated general transcription factors and PC4. Reaction in lane 7 is unrelated to the present issue.

Isolation of GFP-derivatives of Mediator and TFIID complexes for FRET studies (SOW Tasks 1,2)

An important FRET-based assay that we proposed to develop to monitor chromatin looping relies on suitable fluorescent probes for factors binding to distal enhancers and core promoter elements. Toward this, we have generated derivatives of the Mediator and TFIID complexes that carry GFP-derivative labels. For this purpose, we replaced the C-terminal half of MED1 (which is dispensable for its interactions with receptors with Cerulean-FP and stably expressed the resulting fusion protein with a FLAG tag. Following affinity purification on M2-agarose, we were able to obtain an intact Mediator complex that now bore a fluorescent tag (Fig. 4A). Similarly, we tagged the N-terminus of the TBP subunit of TFIID with Venus-FP and purified a TFIID complex bearing a fluorescent tag (Fig. 4B).

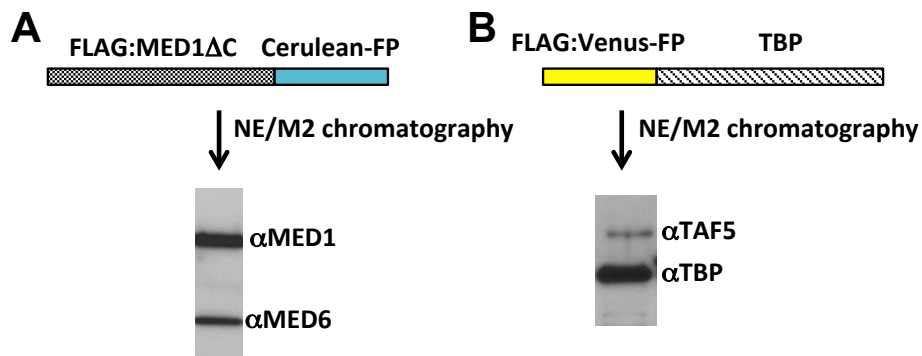


Fig. 4. Materials for FRET analyses of ER α - and Mediator-dependent chromatin looping. (A) Scheme for generating a Cerulean-FP tagged derivative of Mediator and immunoblot analysis of the purified complex with the indicated antibodies. (B) Scheme for generating a Venus-FP tagged derivative of TFIID and immunoblot analysis of the purified complex with the indicated TFIID antibodies.

Because Mediator is predicted to be recruited by enhancer-bound ER α and TFIID binds to core promoter sequences, we expect that these reagents will allow us to spectroscopically detect FRET between Cerulean-Mediator and Venus-TFIID if suitable conditions for generating a putative chromatin loop obtain in our in vitro systems. Given partitioning of Mediator between enhancer and core promoters, we are also currently working on a fluorescent ER α -derivative. Once fully optimized, a convenient FRET-based assay should allow rapid progress toward our main goal of making correlations between ER α - and Mediator-dependent looping and resultant transcriptional activation. Although our primary motivation for generating these derivatives is for use in biochemical experiments, cell lines expressing the tags will also be potentially useful in bioimaging studies for monitoring intracellular interactions.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of an ER α -dependent in vitro transcription system in which ER α activates a core promoter under its control from binding sites located at a significant distance.
- Reconstitution of an active core of the Mediator complex, into which ER α -interacting MED1 and its mutant variants will be incorporated for ER α -based functional studies.
- Isolation of GFP-derivatives of Mediator and TFIID complexes for FRET studies.

CONCLUSION

This phase of the proposal remains heavily focused on laying the groundwork for detailed mechanistic analyses that are projected for the next two years. Thus far we have focused on obtaining key reagents, including an active core of the Mediator complex that we have generated through overexpression of its subunits. This will serve as the “base” Mediator complex into which we will incorporate ER α -interacting MED1 and its mutant variants for ER α -based functional studies. Furthermore, we have obtained GFP-derivatives of Mediator and TFIID complexes for FRET analyses for looped chromatin. Most importantly, we have in place an assay that displays significant ER α -dependent activation from a distal location. Future years will see further refinement of the in vitro systems to begin to both optimize the effect and in the process evaluate the contributions of Mediator and ER α in orchestrating interplay with a variety of factors (e.g., cohesin or related proteins like CTCF, FoxA1/2, chromatin remodelers). Equally importantly, we are gearing up for peptidomimetic syntheses and analyses that will complement our mechanistic studies.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Nothing to report.

INVENTIONS, PATENTS, AND LICENSES

Nothing to report.

REPORTABLE OUTCOMES

Nothing to report.

OTHER ACHIEVEMENTS

- Cell lines that stably express epitope-tagged versions of Cerulean-MED1 and Venus-TBP.

SELECTED REFERENCES

1. Kang YK, Guermah M, Yuan CX, & Roeder RG (2002) The TRAP/Mediator coactivator complex interacts directly with estrogen receptors alpha and beta through the TRAP220 subunit and directly enhances estrogen receptor function in vitro. *Proceedings of the National Academy of Sciences of the United States of America* 99(5):2642-2647.
2. An W & Roeder RG (2004) Reconstitution and transcriptional analysis of chromatin in vitro. *Methods in Enzymology* 377:460-474.
3. Malik S & Roeder RG (2003) Isolation and functional characterization of the TRAP/Mediator complex. *Methods in Enzymology* 364:257-284.
4. Malik S & Roeder RG (2010) The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. *Nature Reviews Genetics* 11(11):761-772.
5. Bieniossek C, Richmond TJ, & Berger I (2008) MultiBac: multigene baculovirus-based eukaryotic protein complex production. *Current protocols in protein science / editorial board, John E. Coligan ... [et al.]* Chapter 5:Unit 5 20.