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ARTICLES

Helix 12 in the Human Estrogen Receptor (hER) Is Essential for the hER Function by Overcoming Nucleosome Repression in Yeast

Xiaohong Gu*

Department of Pharmacology and Cancer Biology, Medical Center, Duke University, Durham, North Carolina 27710

Abstract When exogenous human estrogen receptor (hER) binds with estrogen, it can activate transcription of target genes in yeast cells. The estrogen dose-response expression patterns in yeast are very similar to those in human cells. This implies that hER may function in yeast cells via mechanisms similar to those in human cells. In this study, *Saccharomyces cerevisiae* was used to dissect mechanisms of hER-activated transcription in yeast. The hER contains two transcription activation domains: ER-AF-1 and ER-AF-2 (LBD or HBD). In both human and wild-type yeast cells, hER must bind with estrogen in order to activate transcription. In those cells, ER-AF-2 is independently active upon hormone binding, but ER-AF-1 by itself is inactive. In a mutagenesis screen, we found a mutant strain in which the ER-AF-1 was independently active. It was determined that this mutant strain carried a Tup1 mutation. More interestingly, a small hER fragment ER-AF-0, containing neither ER-AF-1 nor ER-AF-2, was also fully active in the ΔTup1 cells. This suggests that in this strain, hormone binding is not required for transcription activation by hER. It is known that the Tup1/Ssn6 complex plays an important role in general transcription repression by protecting histone acetylation sites thus stabilizing nucleosomes. In the ΔTup1 cells, nucleosomes are known to be unstable because histones can be easily accessed by acetylase and cause nucleosome disassociation. Two point mutations in helix 12 (H12) in ER-AF-2, which abolished hER function in human cells, also completely abolished hER function in the wild-type yeast cells. This suggested that H12 is essential for hER transcription activation function. However, hER with the H12 mutation is able to activate transcription in ΔTup1 cells. This indicates that the normal function of H12 is required for transcription activation by hER only if nucleosomes are not acetylated and are therefore stable. The results of this work suggest that there is a close relationship between hER function and nucleosome remodeling. It also provides insight about H12 activity and its functional relationship with other domains in hER. We propose here that H12 is essential for hER function by recruiting strong nucleosome remodeling proteins to the promoter region thus overcoming nucleosome repression. J. Cell. Biochem. 86: 224–238, 2002.

Key words: transcription; histone; Tup1-Ssn6; nuclear-receptor; estrogen

The human estrogen receptor (hER) is a DNA-binding transcription activator with 595 amino acids, which responds to estrogen induction and regulates the development and reproduction function in the ovary and breast. This receptor also actively regulates target gene expression in the bone and cardiovascular systems. Apparently, hER activity is associated with the cause of breast cancer. It is important to understand how hER activates transcription at the promoter sites of its target genes.

It has been determined that the receptor has three major function domains: two transcription activation domains (ER-AF-1, 1–179 a.a. and ER-AF-2, 352–595 a.a.) and a DNA binding domain (DBD, 180–262 a.a.) [McDonnell et al., 1995]. ER-AF-2 is also referred to as the hormone-binding domain (HBD) or the ligand-binding domain (LBD). There is a hinge region between DBD and HBD. A dispensable activation domain, ER-AF-2a (262–351 a.a.), is located in the hinge region [Norris et al., 1997]. There are 12 major α-helixes and one β-sheet in ER-AF-2, named according to the structure of hPR and hTR before the ER-AF-2

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*Correspondence to: Xiaohong Gu, PhD, GlaxoSmithKline, 1002 Willow Drive, Suite 62, Chapel Hill, NC 27514.
E-mail: xiaohong_gu@gsk.com

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structure of hER was published [Tanenbaum et al., 1998]. In helix 12 (H12), there are two LLXXL (Leu Leu a.a. a.a. Leu) motifs arranged in opposite directions. The sequence of one of the LLXXL motifs is LLEML (Leu Leu Glu Met Leu) that has shown functional importance in human cells [Nichols et al., 1998]. The most significant progress in the field is the recent solution of the estrogen-ER, tamoxifen-crystallographic ER, and raloxifen-ER crystallographic structures [Brzozowski et al., 1997; Tanenbaum et al., 1998]. By comparing these three crystallographic structures, we can see that the most significant difference is the position of helix 12. In the estradiol-ER crystallographic structure, H12 is fold back in one orientation, while in either tamoxifen-ER or raloxifen-ER structure, H12 is folded back in another orientation. The current model proposed that this structure change altered protein–protein interaction between the receptor and cofactors [Shiau et al., 1998].

It has been shown that transcription activity of each activation domain is highly dependent on the cell type and promoter context [McDonnell et al., 1993; Tzukerman et al., 1994]. In most human cell types, even if estrodial-ER-AF-2 alone is able to activate transcription of the target genes, ER-AF-1 is required for maximal hER-transcription activity. However, isolated ER-AF-1 fragment is inactive in most cell types. This suggests that ER-AF-1 activity is dependent on ER-AF-2 with hormone binding. In some types of cells, either ER-AF-1 or ligand-ER-AF-2 is sufficient to activate transcription. This indicates that ER-AF-1 is capable of activating transcription by itself but the activity is dependent on the context of hER complex and the transcription initiation complex. While the function of the ER-AF-1 is still unknown, ER-AF-2 is much better studied in human cells because it can independently activate transcription with hormone binding in most cell lines. Several human protein factors have been found to bind to ER-AF-2. However, because of the complexity of the hER-signaling-pathway in human cells, how hER activates the silent transcription initiation complexes at the promoter site of its targeted genes is still unclear. It is desirable to find a simple cellular system to probe hER action.

The yeast genome is only less than 5% of human genome size. There is no estrogen-receptor gene in the yeast genome and most hER-associated human transcription co-activators do not exist in yeast cells. Interestingly, hER expressed from the transformed expression vector is able to respond strongly to estrogen and fully activate transcription initiation of inserted target genes in yeast cells in a manner very similar to that in most human cells [McDonnell et al., 1991]. In a yeast cell, ER-AF-1 is not independently active, while isolated ER-AF-2 is as active as the full-length hER. It was shown that in a yeast strain with a SSN6 deletion, ER-AF-1 was constitutively active [McDonnell et al., 1992]. Due to extreme sickness of the ssn6 mutant cells, the investigation did not go further so that the mechanism was still not clear. While very few yeast proteins were reported to bind to hER, Ichinose has shown that a yeast protein complex SWI/SNF2 binds to the LLEML motif in the H12 of hER in the ligand-dependent manner [Ichinose et al., 1997b]. SWI/SNF2 (human BRG1 homologue) is a strong ATP-driven nucleosome remodeling protein. Single-point mutations in this motif abolished the SWI/hER binding [Ichinose et al., 1997b], which are the same mutation abolished hER activity in the human cells. Interestingly, both SSN6 and SWI/SNF were reported to be involved in chromosome remodeling [Hirschhorn et al., 1992; Peterson, 1998; Pollard and Peterson, 1998]. Several researchers have reported that SSN6 forms a complex with another protein TUP1 to stabilize nucleosomes [Williams and Trumbly, 1990; Trumbly, 1992; Varanasi et al., 1996; Braun and Johnson, 1997; Magee, 1997; Redd et al., 1997]. More interestingly, Gavin reported that SWI/SNF2 destabilized nucleosomes and appeared to be a TUP1/SSN6 antagonist by targeting the terminal of histone, which is not protected by TUP1/SSN6 [Gavin and Simpson, 1997]. Many researchers have demonstrated that chromosome remodeling plays a key role in general transcription initiation [Edmondson and Roth, 1996a; Grunstein, 1997]. Therefore, it is possible that chromosome remodeling is a major event for hER-activated transcription initiation in yeast.

Recent results from other scientists have shown that transcription regulation by nuclear receptors in human and mouse cells are associated with nucleosome remodeling [Aranda, 2001; Lizcano, 2001; Sheldon, 2001; Wang, 2001; Zheng, 2001]. However, due to the complexity of the human cells, the details of the nucleosomes-remodeling process in human cells

are not yet clear. Considering that yeast is a simple and well-studied system, and its estrogen response is so similar to human cells, we decided to use yeast as a probe to dissect hER action.

This work investigates how hER activates transcription initiation at the targeted genes in yeast cells, as a potential model to understand how hER works in human cells. *S. cerevisiae* was used as a simplified model system to study the mechanisms of transcription activation by the human estrogen receptor. In this work, we used the construct hER, ER-AF-1, ER-AF-2, and ER-AF-0 (a small fragment contains neither ER-AF-1 nor ER-AF-2). Our results indicated that hER-activity is strongly associated with nucleosome remodeling and the major function of H12 in hER might be to release nucleosome repression.

MATERIALS AND METHODS

Plasmids

The constructs containing hER and its variants are shown in Figure 1A. The ERwt (1–595 a.a.), ERN282 (1–282 a.a.), and ER179C (179–595 a.a.) constructs are in plasmids pYEPE10 (hER, Trp), pERN-282g (ER-AF-1, Trp), and YEP-28 (ER-AF-2, Trp). Those plasmids have been described in previous publications [Tzukerman et al., 1994]. The ER179-321 (ER-AF-0, 179–321 a.a.) construct was made by deleting a fragment between the restriction sites CelII (322 a.a.) and SstI (595 a.a.) from the ER179C

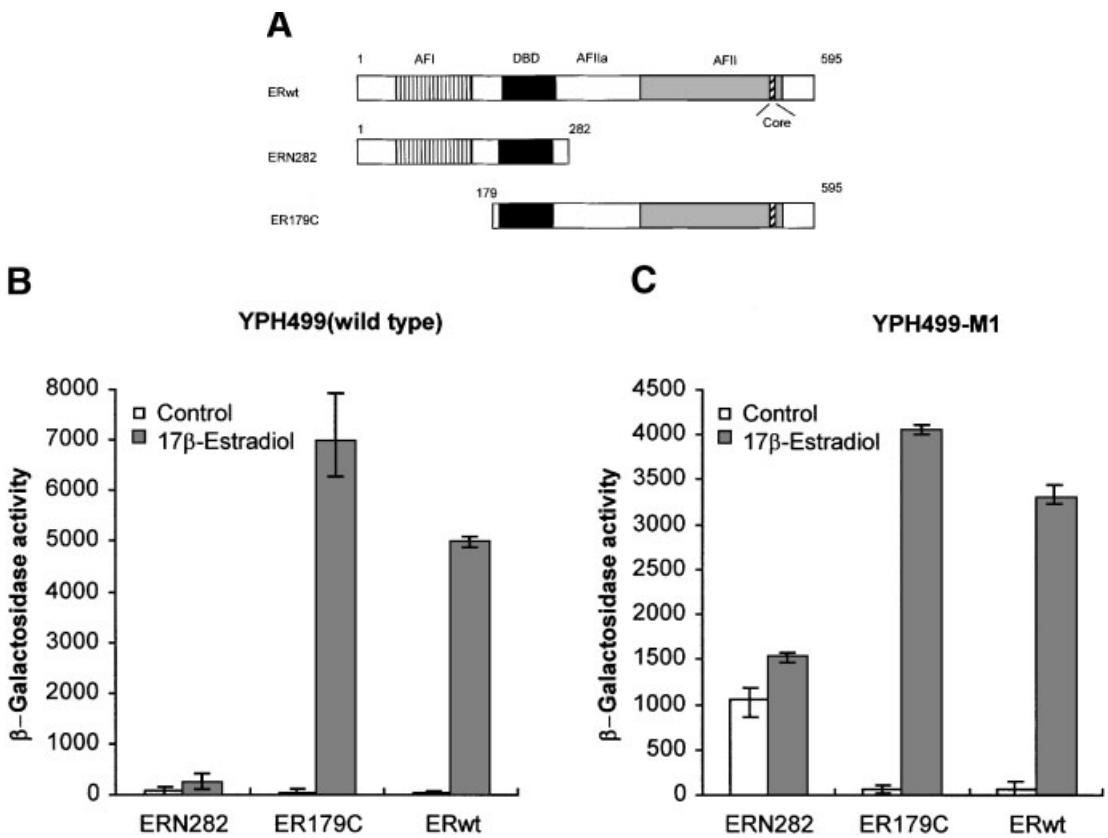

Fig. 1. Transcription activation by ERwt, ER-AF-1, and ER-AF-2 in wild-type and mutant yeast cells. (**A**) The constructs of hER, ER-AF-1, and ER-AF-2. ERwt is the full-length hER containing 595 amino acids. There are three major functional domains: trans-activation domain I (ER-AF-1, 1–179 a.a.), trans-activation domain II (ER-AF-2, 351–595 a.a.), and DNA-binding domain (DBD, 180–262 a.a.). A minor trans-activation domain ER-AF-2a (262–351 a.a.) is dispensable for hER activity. ERN282 is the ER-AF-1 construct (1–282 a.a.) and contains both ER-AF-1 and DBD. ER179C is the ER-AF-2 construct (179–595 a.a.) and contains ER-AF-2 and DBD. The core element was located in ER-AF-2, which has been shown to be important for hER function. (**B**) β-Galactosidase assay in the wild-type YPH499 cells. The expression vector ERN282 (Trp, 2μ), ER179C (Trp, 2μ), and ERwt (Trp, 2μ) were transformed into YPH499 cells along with the 2×ERE-LacZ (Leu, 2μ) reporter. The transformed cells were incubated 12 h in the media with and without 10^{-6} M of 17β-estradiol. The lighter bars are the activity levels without hormone induction while the darker bars are with hormone induction. (**C**) The mutant strain YPH499-M1 was selected from the screen. The same assay was conducted as in (B) with YPH499-M1 cells. The most significant difference between (B) and (C) was that ERN282 was inactive in the YPH499wt cells (B) but it was constitutively active in the YPH499-M1 cell (C).

construct. The ERLL mutant construct pYEPE10LL contains two point mutations at 539 and 540 a.a. (Leu Leu-> Ala Ala) in the ERwt constructs pYEPE10. The TUP1-deletion plasmid pCK36 and the TUP1-expression vectors were kindly provided by Dr. Robert Thumbly (Fig. 5A) [Williams and Trumbly, 1990]. The SSN6 deletion plasmid pJS90 was a gift from Dr. J. Schultz [Schultz et al., 1990]. The GCN5 plasmids were the generous gift from Dr. Leonard Guarente.

Yeast Strains

The yeast strains *S. cerevisiae* YPH499 (MATa, ura3-52, lys2-801amber, Ade2-101ochre, trp1-D1, his3-D200, leu2-D1,) and YPH500 (MATa, ura3-52, lys2-801amber, ade2-101ochre, trp1-D1, his3-D200, leu2-D1,) were used for transformation and mutagenesis. The mutant strain YPH499-M1 was created by EMS mutagenesis. The wild-type cells YPH499 containing pERN-282g (ER-AF-1, Trp) and YRPE2 (2XERE-LacZ, Leu) were treated with the alkylating agent EMS (ethylmethane sulfonate of methanesulfonic acid ethyl ester), according to the standard procedure. The cells were then plated on X-gal phytoagar plates containing synthetic media and 20 μg/ml of X-gal. The plates were incubated at room temperature for 5 days. The blue colony was selected against white colony background.

The TUP1 deletion strain YPH499ΔTup1 was created by inserting pCK36 (PstI/HindIII, Leu) into the genome of YPH499 (ER-AF-1-Trp and 2 ×ERE-LacZ, Leu). The transformants were plated on a phytoagar plate containing a synthetic medium. Each colony was tested by β-galactosidase assay. All the colonies tested had constitutive ER-AF-1 activity with a growth phenotype and morphology similar to YPH499-M1. The Ssn6 deletion strain YPH500ΔSsn6 was created by inserting PJS90 (PstI, Ura) into the genome of YPH500 containing ER-AF-1-Trp and 2 ×ERE-LacZ-Leu, following the same procedure as the same as that for the Tup1 deletion. The cells were then transformed with YRPE2 (2 ×ERE-LacZ, Leu) and one of the following: pYEPE10 (ERwt, Trp), pERN-282g (ER-AF-1, Trp), and YEP-28 (ER-AF-2, Trp).

β-Galactosidase Assay

The yeast cells were incubated in a 50-ml tube containing 10-ml synthetic medium with the appropriate amino acid added. The OD_{600} for the cell concentration started with 0.01. The cells were incubated at 30°C for 12 h. The cell cultures were diluted to $OD_{600} = 0.01$ and 200 ml of dilution was transferred to each well of a 96-well plate. The plates were incubated in a 30°C incubator for about 12 h. OD_{600} was measured again before the assay. One hundred milliliters of Z buffer (60 mM $NaHPO_4$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 0.2% ONPG, 0.1% SDS, 50 mM β-mercaptoethanol, and 200 U/ml oxalyticase) was added to each well. The plates were incubated in a 30°C incubator. After the color turned to yellow, the incubation time was recorded and 50 ml of stop buffer (2 M $NaCO_2$) was added to the well. OD_{550} and OD_{420} were read to calculate the activity of β-galactosidase activity.

RESULTS

ER-AF-1 Became Constitutively Active in a Mutant *S. cerevisiae* Strain YPH499-M1

The hER contains two transcriptional activation domains: the N-terminal activation domain ER-AF-1 and the C-terminal activation domain ER-AF-2. The N-terminal activation function of hER (ER-AF-1) is not able to function as an independent transcriptional regulator in most human cells or in wild-type *S. cerevisiae* cells. To explore the potential regulatory mechanism, we reconstituted the hER-responsive transcription system in *S. cerevisiae*. This was accomplished by introducing vectors expressing hER (ERwt), ER-AF-2 (ER179C), or ER-AF-1 (ERN282) (Fig. 1A) into an YPH499 yeast strain which contained a 2 ×ERE-CYC1-β-galactosidase reporter plasmid. The activity of the various ER-mutants was assayed, following induction with the ER-agonist 17-β-estradiol. Although ER-AF-1 does not contain the ER-ligand binding domain, we measured its activity in the presence and absence of 17-β-estradiol to control for potential non-specific effects of this hormonal treatment on transcription in the host cells. In this yeast strain, ERwt functions as an efficient, ligand-dependent activator of transcription. Interestingly, in this background, the transcriptional activity of the ER179C (ER-AF-2) is responsive to hormone induction at least as much as the ERwt (Fig. 1B), suggesting that ER-AF-2 contained the element(s) that is essential and sufficient for the full-length hER function in yeast. In contrast, as we observed before, the ERN282 (ER-AF-1) was unable to

activate ERE-mediated transcription in this environment (Fig. 1B). This indicated that ER-AF-1 is not able to activate transcription without the presence of ER-AF-2. This result is consistent with the result from human cells [McDonnell et al., 1995].

Having established the activities of the individual activation domains, we next focused on identifying mutations in yeast that could permit ER-AF-1 activity to be manifest. To this end, we subjected the strain containing ERN282 cognate reporter to EMS-mediated chemical mutagenesis. The treated cells were then plated onto selective media containing the β-galactosidase substrate X-gal. Of the 30,000 colonies recovered in this manner, we identified a single colony (YPH499-M1) that displayed a significant level of β-galactosidase activity (blue in a white background). This mutant colony was purified by two successive plattings and subsequently subjected to further analysis. As a initial step, we confirmed that the constitutive ER-AF-1 activity observed was due to a stable genomic mutation by evicting the expression and reporter plasmids, and demonstrating that the mutant phenotype was restored following retransformation with new aliquots of the same plasmids. Subsequently, the quantitative characteristics of the mutant identified were assessed by measuring the β-galactosidase activity of ER-AF-1 (ERN282), ER-AF-2 (ER179C), and hER (ERwt) in the YPH499-M1 mutant background (Fig. 1C). Interestingly, in this mutant strain, the activity levels of ERwt and ER-AF-2 were not altered significantly, whereas the ER-AF-1 activity was observed to be constitutively active. Western-immunoblot analysis demonstrated that the ERN282 derived protein was expressed at the same level in the mutant and wild-type strains (data not shown). Finally, it was determined that the mutation within YPH499-M1 was recessive, since we were able to show that ER-AF-1 activity was suppressed in diploid which resulted from the cross of YPH499-M1 and the isogenic strain YPH500 (data not shown). Thus, we proceeded to identify the mutant locus and directly access its role in ER action.

Complementation Analysis Indicated That YPH499-M1 Bore an Inactivating Mutation in TUP1

Our attempts to identify the mutated gene by using cDNA genomic libraries to complement the phenotype in YPH499-M1 were unsuccessful. Initially, we considered that this might indicate that YPH499-M1 bore a complex multigenic mutation. However, we now believe it may be due to the fact that the transformation efficiency of the mutant strain is very low for large size genes. During the course of these investigations, we observed that the growth characteristics (extreme flocculation) of YPH499-M1 were very similar to that observed in strains of yeast that contain inactivating mutations of the SSN6 transcriptional repressor. However, the YPH499-M1 cells grew faster, were larger, and showed less sickness than the SSN6 mutant cells. Indeed, we had demonstrated previously that ER-AF-1 could function as an autonomous activator in yeast strains in which SSN6 was disrupted. We introduced a SSN6 expression plasmid into YPH499-M1 but it did not suppress ER-AF-1 activity. It has been determined that SSN6 forms a large complex with TUP1 protein and that both proteins are required for transcriptional repression to certain genes in yeast. Given the similarity of the phenotype of YPH499-M1 and that observed previously in the SSN6 mutant strain, we considered whether the constitutive ER-AF-1 activity in YPH499-M1 strain might result from a mutation in the TUP1 gene. To test this possibility, we examined the ability of a TUPI expression plasmid to suppress the constitutive activity of ER-AF-1 observed in YPH499-M1. As shown in Figure 2, introduction of a low-copy centromeric expression vector (1–2 copies/cell) containing the intact TUPI cDNA was able to completely suppress the constitutive ER-AF-1 activity manifest in YPH499-M1. This result suggested that in YPH499-M1 strain, the TUP 1 gene carried an inactivating mutation that jeopardizes TUP1 normal transcription repression function.

ER-AF-1 Constitutive Transcriptional Activity Was Repressed by Yeast TUP1 Protein in Wild-Type Yeast Cells

To confirm a direct link between the constitutive activity of ER-AF-1 and a defect in the TUP I locus, we created a yeast strain (YPH499ΔTup1) by homologous recombination in which the endogenous copy of TUPI was disrupted using insertional mutagenesis. For comparative purposes, a similar approach was used to create a yeast strain (YPH500ΔSsn6) that contained a deletion of the SSN6 gene. In

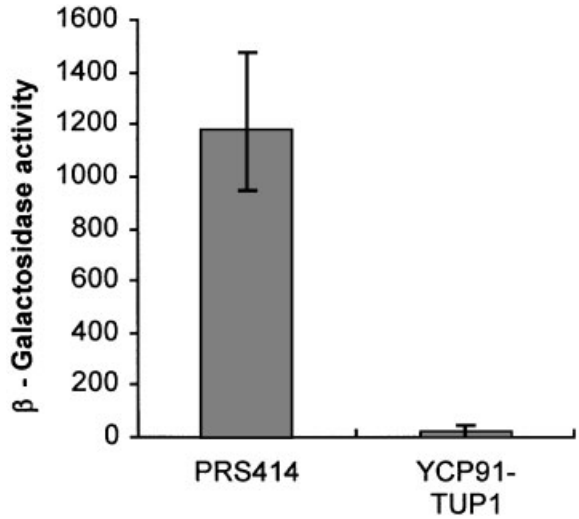

Fig. 2. The β-galactosidase assay for complementary test to determine the mutated gene in YPH499-M1. YPH499-M1 (ER282-Ura, 2 ×ERE-lacZ-Leu) cells were transformed with either control empty vector PRS414 (Trp) or Tup1 expression vector YCP91-TUP1 (Tup1-Trp). The cells were plated on the synthetic media plates with histidine, and incubated at 30°C for 4 days. The colonies were collected and subjected to β-galactosidase assay. The result showed that while the cells with PRS414 still have ER-AF-1 constitutive activity, the cells with YCP91-TUP1 totally lost ER-AF-1 activity.

the latter case, the YPH500 strain (isogenic to YPH499) was used, as repeated attempts to perform an SSN6 disruption in YPH499 were not successful. A 2 ×ERE-CYC1-reporter and vectors expressing ERwt, ERN282, and ER 179C were introduced into the wild-type cells (YPH499, YPH500), the YPH499ΔTup1 and the YPH500ΔSsn6 strains. The results of this analysis are shown in Figure 3. Importantly, as in the original YPH499-M1 strain (Fig. 3A), we observed that in the YPH499ΔTup1 stain activity of the ERwt and ER179C (ER-AF-2) were not significantly affected by the deletion of TUP1, whereas the activity of ERN282 (ER-AF-1) was increased by over 100-fold (Fig. 3B). The growth patterns and the cell shapes are also extremely similar between these two strains. Thus the ability of ER-AF-1 to manifest independent transcriptional activity mapped to a defect in the TUPI locus. Interestingly, we observed that in the TUP1 deletion, the activity of ER-AF-1 (ERN282) was comparable to that of ERwt and ER179C. This is in contrast with the result observed in YPH499-M1 where the activity of ERN282 was significantly less that the ERwt. This suggests that the original YPH499-M1 strain did not contain a TUP1 null allele, but rather bore a mutation in TUP1which reduced its activity.

Several genetic and biochemical studies have suggested that SSN6 and TUP1 are co-dependent and that the phenotypic consequence of a TUP1 and SSN6 mutation are very similar. The previous report that ER-AF-1 was an independent activator in strains in which SSN6 was deleted supported this latter association. However, it was important to show that within the same genetic background, deletion of the SSN6 gene would be phenotypically equivalent to that observed when TUP1 was deleted. The effect of deleting SSN6 on ER function in YPH500 (isogenic to YPH499) is shown in Figure 3C,D. Similar to that observed in the YPH499ΔTup1 strain, we observed that deletion of SSN6 in the YPH500 background had the specific effect of permitting independent ER-AF-1 activity. These data suggest that the presence of both SSN6 and TUP1 is required to block ER-AF-1 constitutive activity in yeast wild-type cells. This is consistent with the results shown by other researchers that SSN6 and TUP1 form a protein complex, and co-dependently perform the transcription repression function [Williams and Trumbly, 1990; Trumbly, 1992; Varanasi et al., 1996; Braun and Johnson, 1997; Magee, 1997; Redd et al., 1997].

TUP1 Provides a Link Between hER and Chromatin Remodeling in Yeast

Current models suggest that the TUPI/SSN6 repress transcription initiation by interacting with histones in the nucleosomes around promoter regions. Edmondson et al. [1996b] has shown that the repression domain of TUP1 can specifically associate with underactylated histone H3 and H4 at the acetylation sites of the histones. Interestingly, the TUP1-binding sites of H3 and H4 are responsible to nucleosome repression [Ling et al., 1996, Huang et al., 1997]. It has been shown that the stable nucleosomes at promoter sites usually inhibit transcription initiation, and they can be disassociated after histones are acetylated by histone acetylase [Grunstein, 1997]. It is most likely that SSN6/TUP1 complex repress transcription activation by stabilizing nucleosomes.

Many transcription co-factors regulate transcription through direct interacting with transcription activators. However, in our protein–protein interaction assays, we did not find any evidence to show that hER and SSN6/TUP1 had

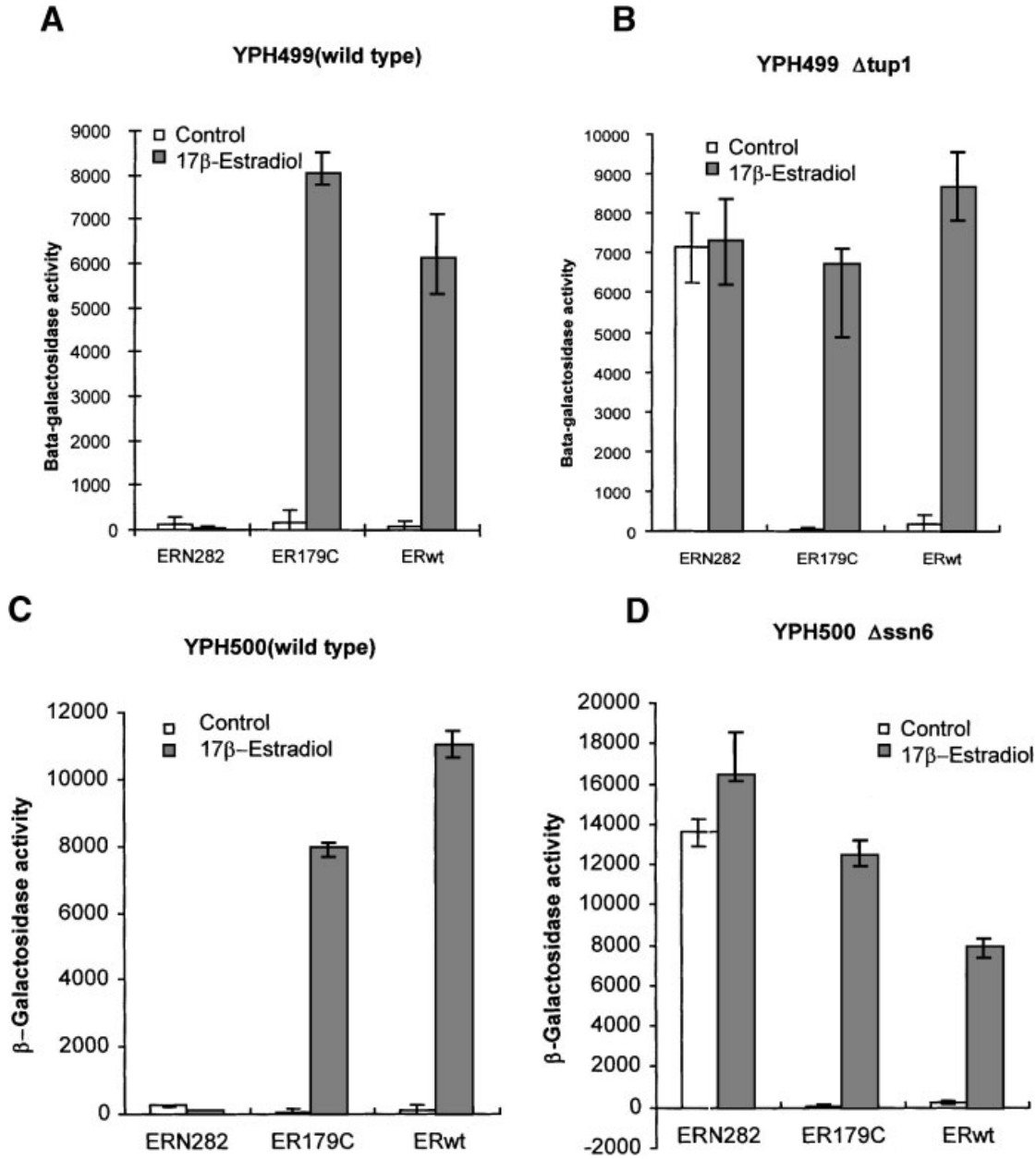

Fig. 3. The β-galactosidase assays for the Tup1 deleted cells (YPH499ΔTup1) and the Ssn6 deleted cells (YPH500ΔSsn6). (**A**) The β-galactosidase assays for the wild-type cells. YPH499 cells were transformed with 2 ×ERE-LacZ-Ura and then transformed with ERN282-Trp, ER179C-Trp, or ERwt-Trp. The transformants were incubated at 30°C for 4 h with or without 10^{-6} M 17β-estradiol, and then subjected to β-galactosidase assay. (**B**) The β-galactosidase assays performed at the same time as in (A) for the YPH499ΔTup1 cells. The Tup1 deletion plasmid pCK36 (Leu) was digested with PstI and HindIII, and transformed into the wild-type strains YPH499 (ERN282-Trp, 2 ×ERE-Ura), YPH499 (ER179C-Trp, 2 ×ERE-Ura), and YPH499 (ERwt-Trp, 2 ×ERE-Ura). The transformants were plated on the synthetic media plates with histidine, and incubated at 30°C for 4 days. The cells were incubated in the synthetic media with either absence or presence of 10^{-6} M 17β-estradiol at 30°C for 4 h, and then subjected to β-galactosidase assay. (**C**) The β-galactosidase assay for the wild-type cells. YPH499 (2 ×ERE-Leu) cells with ERN282-Trp, ER179C-Trp, and ERwt-Trp were incubated at 30°C for 4 h with or without 10^{-6} M 17β-estradiol and then subjected to β-galactosidase assay. (**D**) The β-galactosidase assay performed at the same time as in C for the YPH500ΔSsn6 cells. The Ssn6 deletion plasmid pJS90 (Ura) was digested with HindIII/PstI and transformed into the wild-type strains YPH499 (ERN282-Trp, 2 ×ERE-Leu), YPH499 (ER179C-Trp, 2 ×ERE-Leu), and YPH499 (ERwt-Trp, 2 ×ERE-Leu).

direct interaction (data not shown). Thus, we decided to investigate the mechanism by which the TUPI/SSN6 complex interfaces with hER in yeast through function assays. One of the first steps in this investigation was to determine if the functional domains within TUP1 believed to be responsible for its ability to function as a transcriptional repressor in yeast were also required for suppression of ER-AF-1 transcriptional activity. The functional domains within TUP1 responsible for SSN6 binding, as well as for H3 and H4 binding have been mapped previously [Williams and Trumbly, 1990; Edmondson et al., 1996b]. It was reported that TUP1 has three distinguishable domains: SSN6 binding domain (1–72 a.a.), histone binding domain (72–385 a.a.), and WD-40 repeats (Fig. 4A) [Magee, 1997]. The SSN6-binding domain is required to form the TUP1/SSN6 complex. The histone-binding domain binds to the under-acetylated histone H3 and H4 at the N-terminal acetylation sites of H3/H4. The histone-binding domain is also required for general transcription repression. It was shown that the WD-40-repeats of TUP1 are required for interaction between TUP1and alpha2 [Komachi and Johnson, 1997]. It was important for us to know which functional domain in the TUP1 protein was important for repression of the ER-AF-1 activity. The full-length TUP1 and its different deletions (as shown in Fig. 4A) were transformed into the YPH499-M1 cells. As expected, the full-length TUP1 is able to suppress ER-AF-1 constitutive activity (Fig. 4B). However, we were surprised that none of the deletions is able to reverse the ER-AF-1 constitutive activity in the TUP1 mutant cells (Fig. 5B). These results indicate that all of the known functional domains of TUP1, such as SSN6, histone H3 and H4 binding domains, are required for TUP1 to repress ER-AF-1 activity, suggesting that SSN6, histone H3 or H4 are all involved in the ER-AF-1 repression by TUP1. In other words, to prevent ER-AF-1 being active, TUP1 needs to bind to SSN6 to form the complex and to bind to histone to repress transcription. Thus deletion of TUP1 protein revealed a link between ER-AF-1 function and nucleosome remodeling.

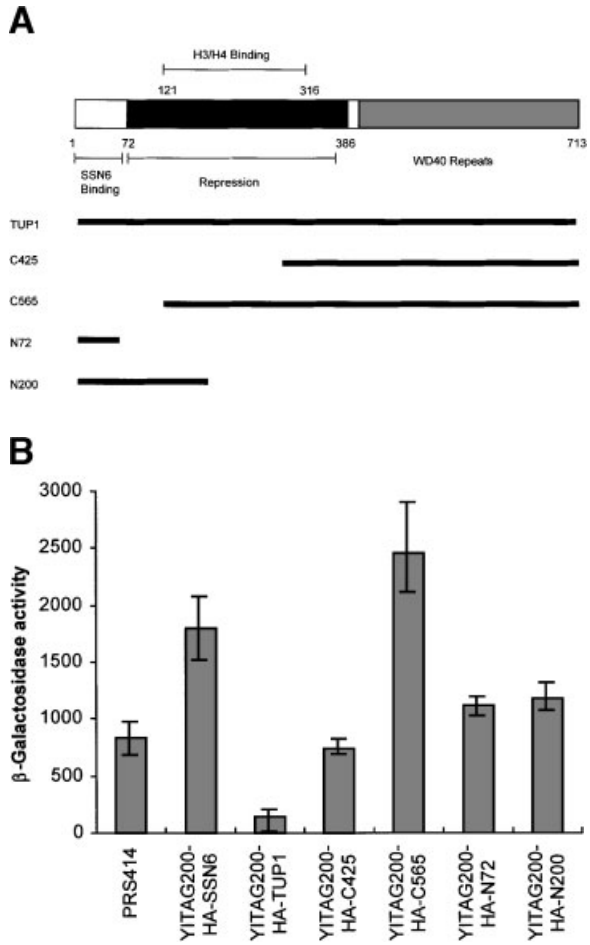

Fig. 4. The β-galactosidase assays to identify the required fragments for repressing ER-AF-1 constitutive activity. (**A**) Tup1 function domains and its deletions. The Tup1 protein has 713 a.a. and contains a Ssn6 binding domain (1–72 a.a.), a histone H3/H4 binding domain (73–385 a.a.), and WD40 repeats (400–713 a.a.). C425 contains 425 a.a. at the C-terminal of Tup1, which covers all of WD40 repeats. C565 contains 565 a.a. from the C-terminal of Tup1, which covers WD40 repeats and part of the histone-binding region. N72 contains 72 a.a. at the N-terminal, which is the SSN6 binding domain. N200 contains 200 amino acids at the N-terminal, which covers the Ssn6 binding domain and part of the histone-binding domain. (**B**) The β-galactosidase assay for complementary test in YPH499-M1 cells. YPH499-M1 (ER282-Ura, 2 ×ERE-lacZ-Leu) was transformed with an empty control vector PRS414, an expression vector YITAG200-HA-Ssn6 (Ssn6-Trp), or an expression vector YITAG200-HA-Tup1 (Tup1-Trp), or one of the Tup1 deletion expression vectors: C425 (C425-Trp), C565 (C565-Trp), N72 (N72-Trp) and C200 (C200-Trp). The cells were plated on the synthetic media plates with histidine, and incubated at 30°C for our days. The colonies were collected and subjected to β-galactosidase assay. It was shown that only the full-length Tup1 is able to significantly reduce the ER-AF-1 constitutive activity.

ER-AF-0 (179–321 a.a.) Containing Neither ER-AF-1 nor ER-AF-2 Is Able to Fully Activate Transcription in the YPH499ΔTup1 Cells

As we showed above, ER-AF-1 is constitutively active in the TUP1 deletion cells. The question was whether the ER-AF-1 constitutive activity was caused by specific activity of ER-AF-1 or was solely caused by a lack of TUP1 repression. To find the answer, we constructed

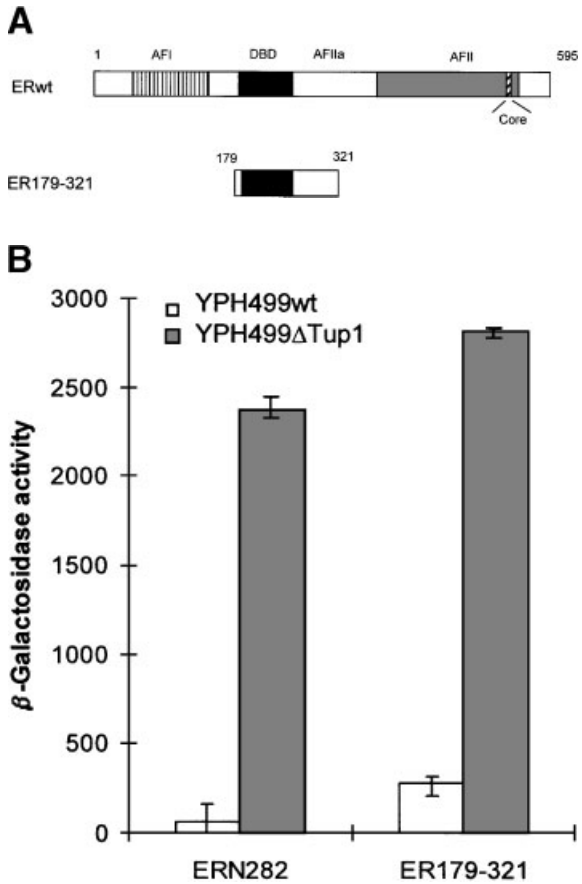

Fig. 5. The β-galactosidase assays to determine transcription activity of ER-AF-0. (**A**) The construct of ER-AF-0 (ER179–321-Trp) contains amino acid 179–321 in ERwt. (**B**) The β-galactosidase assays. The plasmids containing ER-AF-1 (ERN282-Trp) or ER-AF-0 (ER179–321-Trp) were transformed into both YPH499 (2 ×ERE-LacZ-Ura) and YPH499ΔTup1 (2 ×ERE-LacZ-Ura). The β-galactosidase assay was performed for both the wild-type YPH499 cells and the YPH499ΔTup1 cells. The result showed that ER-AF-0 has full constitutive activity in the YPH499ΔTup1 cells.

plasmid, containing the fragment 179–321 a.a. and named it as ER-AF-0 (Fig. 5A). This fragment contains DBD (179–262 a.a.) and a 60-a.a. fragment as a part of hinge region, but it contains neither ER-AF-1 nor ER-AF-2. The plasmid was transformed into both of the YPH499 and YPH499ΔTup1 cells containing 2 ×ERE-LacZ-reporter plasmid. The cells were then subjected to the β-galactosidase assay. The result showed that this fragment was inactive in the wild-type yeast cells as expected. To our surprise, this ER-AF-0 fragment was constitutively active in the YPH499ΔTup1 cells (Fig. 5B), suggesting that in the cell in which the TUP1 repression is lacking, neither ER-AF-1 nor ER-AF-2 is required for transcription initiation. Why this ER-AF-0 fragment was able activate transcription was not yet understood. However, this result clearly stated that constitutive activity in the YPH499ΔTup1 cells was not ER-AF-1 specific. While ER-AF-1 and ER-AF-0 did not change no matter they are in the wild-type cells or in the ΔTup1 cells, the only difference between these cell lines is whether TUP1 is present or not. Removing of TUP1 repression may create a favorable condition for transcription initiation in the ΔTup1 cells. In such a condition, many proteins that are inactive in the present of TUP1 repression may be able to activate transcription initiation. Therefore, removing TUP1 repression from the transcription initiation complex is likely to be one of the key events in transcription initiation activation.

Functional Helix 12 (H12) in hER Is Essential for hER Function in Wild-Type Yeast, But in the YPH499ΔTup1 Cells, the H12 Function Can be Bypassed

Our data indicate that in the cells lacking the TUPl/SSN6 represses, all ERwt, ER-AF-1, ER-AF-2, and even ER-AF-0 can activate transcription. However, in the wild-type cells, where nucleosomal histones H3/H4 are protected by TUP1/SSN6, only ERwt and ER-AF-2 can activate transcription. The question is why the TUPl/SSN6 complex does not repress ERwt or ER-AF-2 activity in the wild-type cells. It was possible that the intact hER or ER-AF-2 contained a functional element which is able to overcome the repressor activity of the TUPI/SSN6 complex. To test this hypothesis directly, we examined the transcriptional activity of an ER-mutant ERLL in the wild-type YPH499 strain and the derivative YPH499ΔTup1 strain. ERLL was created by converting two required hydrophobic residues (Leu539 and Leu 540) to Alanine (Fig. 6A). It was demonstrated that this mutation completely blocks ER-AF-2 function and prevents the interaction of hER with most of the known hER associated co-activators including SWI/SNF2.

The results of this analysis indicate that ERwt acts as a ligand dependent transcription factor in both the wild-type and TUP 1 disrupted strain of yeast. In contrast, we observed significant differences in the transcriptional activity of ERLL when assayed in the same manner (Fig. 6B). The mutated hER (ERLL) was almost completely inactive even with hormone binding in the wild-type cells, which was consistent with

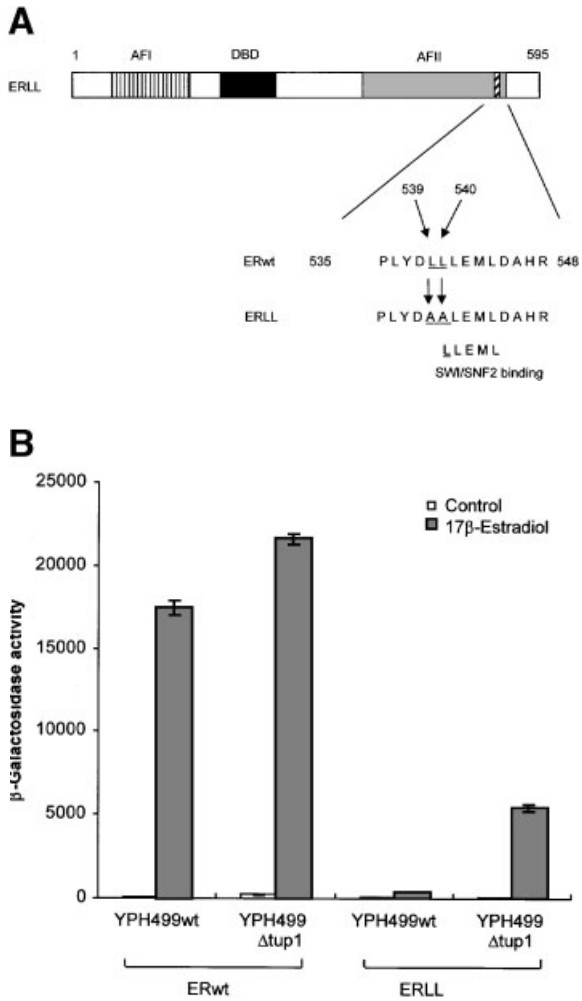

Fig. 6. The β-galactosidase assays to determine transcription activity of ERLL. (**A**) ERLL contains two point mutations in H12 of ERwt. The mutation replaces both Leu 539 and 540 with Ala. The sequence of the wild-type and the mutated H12 are both shown. (**B**) The β-galactosidase assays for the wild-type hER. The plasmids YEPE10 (ERwt-Trp) or YEPE10LL (ERLL-Trp) were transformed into both the YPH499 strain (2 ×ERE-LacZ-Ura) and the YPH499ΔTup1 (2 ×ERE-LacZ-Ura) strain. The cells were incubated in 0–10^{-6} M of estradiol at 30°C for 4 h, and the β-galactosidase assay was performed. The β-galactosidase assay for the YPH499ΔTup1 cells was performed at the same time as in (A). The plasmids YEPE10 (ERwt-Trp) were transformed into the tup1 strain YPH499ΔTup1 (2 ×ERE-LacZ-Ura). The cells were incubated in 0–10^{-6} M of estradiol at 30°C for 4 h, and then the β-galactosidase assay was performed. The data showed that ERLL was not active in the YPH499wt cells but it was active in the YPH499ΔTup1 cells.

the result from human cells. Significantly however, when assessed in YPH499ΔTupl, it was observed that ERLL was able to function as an efficient hormone-dependent activator (Fig. 6B). The activity level of ERLL in the YPH499ΔTup1 cells is over 10-fold higher than that in the wild-type cells. Thus, by disrupting the TUPI/SSN6 complex, the need for a functional ER-AF-2 for ER-mediated transcriptional activity is bypassed. Since the mutations in ERLL were only located in H12, this result strongly supported our hypothesis that H12 was essential for hER function in the wild-type yeast cells because of its ability to overcome TUP1 repression.

DISCUSSION

To understand how the human estrogen receptor activates transcription, we overviewed the whole process of the transcription initiation process, from silence to active. The transcription initiation complex of the human estrogen receptor targeted genes is inactive prior to ligand-receptor binding. It was interesting to see how the transcription initiation complex was kept in silence.

Accumulated evidence showed that nucleosomes at the transcription initiation site were the major inhibitor for transcription initiation [Edmondson and Roth, 1996a; Grunstein, 1997; Krumm et al., 1998]. Under-acetylated histones form octamer nucleosomes that are wrapped by DNA. The C-terminal of histone H3 and H4 strongly bind to the subunits of TBP to block transcription initiation. The histone proteins in the nucleosome, therefore, are the ultimate factor implementing the repression function. To activate transcription, the nucleosomes must be removed from the promoter region. One of the efficient ways to remove the nucleosomes from the promoter region is to acetylate histone so that nucleosomes become very unstable and tend to disassociate.

The nucleosomal histone acetylase in yeast is GNC5 which targets the histone H3 and H4 in nucleosomes [Kuo et al., 1996; Owen-Hughes et al., 1996; Pollard and Peterson, 1997b; Roberts and Winston, 1997; Wang et al., 1997; Tanenbaum et al., 1998; Zhang et al., 1998]. To test the role of GCN5 in hER activated transcription initiation process, we deleted the GCN5 from the wild-type cells as well as overexpressed GCN5. We were surprised that, in the above experiments, GNC5 did not have any significant effect on transcription level activated by hER. The only effect we observed was that the basal activity was completely cleared when the GCN5 was deleted (data not shown). This result supported the observation by other groups, as they showed that GCN5 was only able to enhance weak activators but not strong activators [Pollard et al., 1997a]. As in our case,

hER was obviously a strong activator so that it was not supposed to be enhanced by GCN5. Based on the above observation, we ruled out the possibility that histone acetylase might be the primary transcription co-activator for hER in yeast. However, we cannot role out the possibility that histone acetylase may play a secondary role in transcription activation by hER in yeast.

The possible reason that the acetylase is not the strong co-activator for hER is that at the promoter site, the Tup1/Ssn6 complex binds to the N-terminal of histone H3 and H4 to prevent access of acetylase. The transcription repression region of Tup1 overlaps with its H3/H4 binding site [Edmondson et al., 1996b]. More interestingly, the H3/H4 N-terminals are acetylation sites that are also responsible for nucleosome repression [Durrin et al., 1991; Mann and Grunstein, 1992; Johnson et al., 1992; Roth, 1995; Lenfant et al., 1996; Ling et al., 1996; Huang et al., 1997]. Therefore, transcription repression activity of the Tup1/Ssn6 complex was actually indirect. It was clear that the Tup1/Ssn6 complex repressed the transcription initiation by binding to H3/H4 to stabilize nucleosomes. It was the only mechanism found so far for Tup1/Ssn6 transcription repression activity. When the Tup1/Ssn6 complex binds to nucleosomes, the histone acetylase GCN5 cannot access the nucleosomes, unless the Tup1/Ssn6 complex is removed. In the wild-type cells, hER is able to activate transcription in the presence of Tup1/Ssn6, indicating that hER was able to overcome Tup1/Ssn6 repression to destabilize nucleosomes. There was a possibility that hER was able to kick off Tup1/Ssn6 from the promoter site. However, although we have tried different approaches, there was no evidence to show that there was any direct physical interaction between hER and the Tup1/Ssn6 complex. Therefore, we do not have evidence to show that hER is able to remove the Tup1/Ssn6 complex from the promoter.

We have shown that H12 is essential for the function of hER as the mutation in H12 almost completely abolished hER activity. We also showed that if the Tup1/Ssn6 complex is not present, hER function could be partially recovered. It showed that in the absence of the Tup1/Ssn6 complex, hER was able to activate transcription initiation without its key element, helix 12. It gave us a strong hint that hER and the Tup1/Ssn6 complex might target the same protein, the histone in nucleosomes. While Tup1/Ssn6 is stabilizing nucleosomes, hER can indirectly destabilize them. This hypothesis was supported by the report that H12 in hER binds to SWI/SNF2 protein [ichinose et al., 1997a]. SWI/SNF2 is an ATP driven DNA helicase and a key protein in nucleosome remodeling [Richmond and Peterson, 1996]. The SWI/SNF2 complex can be recruited by transcription activators to the promoter region [Yudkovsky et al., 1999]. In yeast, SWI/SNF2 functions as an antagonist of Tup1/Ssn6 [Gavin and Simpson, 1997]. SWI/SNF2 is able to directly dissociate histone H2 and DNA in an ATP driven manner [Hirschhorn et al., 1995]. Importantly, SWI/SNF2 was able to facilitate DNA-binding proteins to bind to DNA. SWI/SNF2 binds to H12 at sequence of L (540) LEML [Ichinose et al.,

Fig. 7. (overleaf) The proposed model for the possible mechanism of transcription activation by hER and its function domains in yeast cells. (**A**) ER-AF-1, (**B**) ER-AF-2, (**C**) ER, and (**D**) ERLL in the wild-type cells (a,b,c) and the ΔTup1 cells (d,e,f). Without hER activation, nucleosomes are located close to the TBP (Aa,d, Ba,d, Ca,d, Da,d). The histones interacted with TBP to block transcription initiation. The nucleosomes are composed with under-acetylated histones so that they are stable. The TUP1/SSN6 complex binds to the acetylation site of the histones to stabilize the nucleosome. Transcription initiation complex was in silence. In the wild-type cells, nucleosomal histones H3/H4 are protected by TUP1/SSN6 so that acetylase (HAT) brought by ER-AF-1 is not able to access to the acetylation sites (Aa,b,c). Stable nucleosomes keep the transcription initiation complex in silence. However, if TUP1/SSN6 is absent, ER-AF-1 is able to activate transcription because HAT is able to access to histones (Ad,e,f). (B) ER-AF-2 brings SWI/SNF2 to the promoter region. Since histones are not protected by Tup1/Ssn6 from SWI/SNF2 access for releasing nucleosomes from DNA, ER-AF-2 is able to activate transcription in both wild-type cells and Tup1 deletion cells. In case of the intact hER, the process is the combination of the two ER-Afs (Ca,b,c). The estrogen-bound receptor brought both TUP1/SSN6 and SWI/SNF2 to the promoter region. After hER binds to DNA, SWI/SNF2 knocks off the Tup1/nucleosome complex by disassociating H2a/H2b/DNA. Without the nucleosome repression, transcription can start. Only in the environment TUP1/SSN6 repression is absent, hER associated histone acetylase (HAT) can target H3 and H4 in the nucleosomes and make contribution to transcription activation (Cd,e,f). Therefore, in the ΔTUP1/SSN6 cells, either histone acetylase or SWI/SNF2 is able to remove nucleosome repression. When H12 is mutated, SWI/SNF2 is not able to bind to hERLL (Da,b,c). Even if hERLL can still bring HAT to the promoter, it is not able to active transcription because the TUP1/SSN6 repression and the absence of SWI/SNF2 activity. However, if TUP1/SSN6 does not exist, acetylase associated with hERLL is able to activate transcription (Dd,e,f).

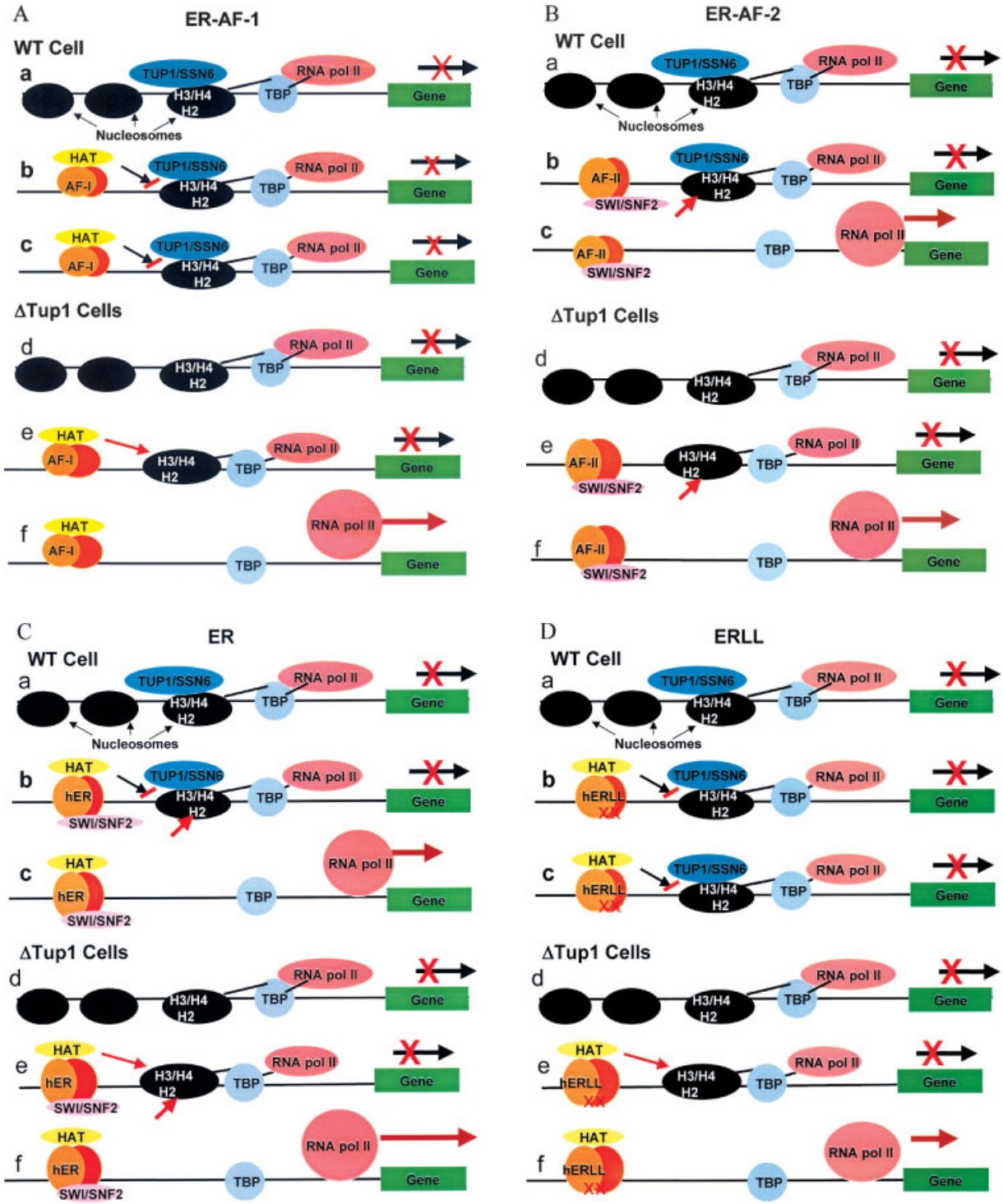

Fig. 7.

1997a]. In the mutant ERLL, Leu (540) was mutated so that ERLL was not able to bind to SWI/SNF2. This might explain why ERLL was completely inactive in wild-type cells. Here we proposed a possible model to explain our results and the estrogen receptor action in yeast (Fig. 7). In the wild-type cells, nucleosomal histones H3/H4 are protected by TUP1/SSN6 so that acetylase are not able to access to the acetylation sites. Stable nucleosomes keep the transcription initiation complex in silence (Fig. 7Aa, Ba,Ca, Da), ER-AF-1 brings acetylase (HAT) to

the promoter. Transcription cannot be activated because histones are protected by TUP1/SSN6 (Fig. 7Ab,c). However, if TUP1/SSN6 is absent, ER-AF-1 is able to activate transcription because HAT is able to access to histones (Fig. 7Ad,e,f). ER-AF-2 brings SWI/SNF2 to the promoter region. Since histones are not protected from SWI/SNF2 access for releasing nucleosomes from DNA, ER-AF-2 is able to activate transcription in both wild-type cells and Tup1 deletion cells (Fig. 7B). In case of the intact hER, the process is the combination of the two ER-AFs. Before estrogen binds to the estrogen receptor, the receptor is inactive because H12 is hidden and not able to contact with co-activators. After estrogen binds to the estrogen receptor, the receptor changes its conformation and H12 is available for co-activators. Thus, the SWI/SNF2 complex can bind to H12 of hER. The estrogen-bound receptor brought SWI/SNF2 to the promoter region. SWI/SNF2 is able to promote DNA accessibility by hER. After hER binds to DNA, SWI/SNF2 knocks off the Tup1/nucleosome complex by disassociating H2a/H2b/DNA (Fig. 7Cb). Without the nucleosome repression, transcription can start (Fig. 7Cd). Only in the environment, TUP1/SSN6 repression is absent (Fig. 7Cd,e), hER-associated histone acetylase (HAT) can target H3 and H4 in the nucleosomes and make contribution to transcription activation. Therefore, in the ΔTUP1/SSN6 cells, either histone acetylase or SWI/SNF2 is able to remove nucleosome repression (Fig. 7Ce,f). This explains why H12 is sufficient but not necessary for transcription activation by hER in the ΔTUP1/SSN6 cells. When H12 is mutated, SWI/SNF2 is not able to bind to hERLL. Even if hERLL can still bring HAT to the promoter, it is not able to activate transcription because the TUP1/SSN6 repression and the absence of SWI/SNF2 activity (Fig. 7Da,b,c). However, if TUP1/SSN6 does not exist, acetylase associated with hERLL is able to activate transcription (Fig. 7Dd,e,f).

Although this work is based on yeast system, most proteins involved are either identical or highly homologues to that in the human cells. For example, hER is the human estrogen receptor and histone H2/H3/H4 are identical to those in human cells. SWI/SNF2 (human BRG1homologue) and GCN5 (human P/CAF homologue) are highly homologous to those in the human cells. It was also shown that TLE and UTY/X might play the same functional role in mammalian cells as Tup1-Ssn6 in the yeast cells [Grbavec et al., 1999]. Since Tup1 does not physically interact with hER, functional homologues are very important. While we have extensive study and discussion about Tup1/Ssn6 in this work, Tup1/Ssn6 is just a protein complex protecting histone acetylation sites. The protein protects histone acetylation sites in human cells might be a different protein from Tup1. However, Tup1 provide us a hint to link hER activity with nucleosome remodeling. Based our results from ER-AF-1 and ER-AF-0, it is possible that when the acetylation sites of nucleosomal histone proteins are not protected, any protein able to recruit acetylase to the promoter sites might be able to activate transcription. If the histone acetylation sites are protected, acetylase is not able to activate transcription even if they sit at the promoter region. The significance of this work is to link H12 and ER-AF-2 to histone remodeling. We think that one of the major functions of hER is to recruit nucleosome-remodeling proteins, such as SWI or HAT, to the promoter sites of the target genes. Once hER binds to DNA at the promoter region, the transcription activation function might be similar to many other DNA binding transcription factors, such as releasing nucleosomes from the promoter region. We think that the result of this work provides one explanation about the basic mechanism, how hER activates transcription at the promoter sites, even if the situation in the human cells is much more complex. We hope that the information we provided here could help us better understand human estrogen receptor action in the human cells.

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