Chapter 13

In Vitro Transcription to Study WT1 Function

Stefan G.E. Roberts

Abstract

In vitro transcription methods using mammalian nuclear extracts have been available for over 30 years and have allowed sophisticated biochemical analyses of the transcription process. This method has been extensively used to study the basic mechanisms of transcription, allowing the identification of the general transcription factors and elucidation of their mechanisms of action. Gene-specific transcriptional regulators have also been studied using in vitro transcription. This has facilitated the identification of their cofactors and provided information on their function that is invaluable to facilitate their study in a more physiological setting. Here we describe the application of in vitro transcription methods to study the mechanism of action of WT1. Coupling transcription assays with methods to purify transcription complexes, and protein affinity chromatography, has provided insights into how WT1 can both positively and negatively regulate transcription.

Key words WT1, BASP1, Transcription, Nuclear, Promoter, Histone, Affinity chromatography

1 Introduction

WT1 is a zinc-finger protein that plays a central role in development and tumorigenesis (reviewed in ref. 1). The four zinc fingers of WT1 can interact with DNA sequence elements with a consensus 5′-GCGGGGGCG-3′ and thus a major function of WT1 is to act as a transcription factor [2]. Alternative splicing of WT1 generates several isoforms and one of the alternative forms contains an additional three amino acids (KTS) between the third and fourth zinc fingers. This +KTS form of WT1 can also bind to RNA and in addition form complexes with several splicing factors (reviewed in ref. 3). Thus, WT1 likely has roles at multiple events in the gene expression process. Although there is still substantial evidence for a role for the +KTS form of WT1 in transcription, experimental analysis of transcription has traditionally focused on the –KTS WT1 isoforms.

WT1 has been shown to regulate the expression of several genes involved in cell growth, apoptosis, and differentiation (reviewed in ref. 1). WT1 can activate or repress the transcription rate of several genes and the actual outcome is dependent on both

Nicholas Hastie (ed.), *The Wilms' Tumor (WT1) Gene: Methods and Protocols*, Methods in Molecular Biology, vol. 1467,
DOI 10.1007/978-1-4939-4023-3_13,

cell type and cell context. Early studies showed that WT1 contains both transcriptional repression (R, residues 71–180) and activation (A, residues 180–245) domains [4–6], and that within the repression domain a specific region, the suppression domain (SD; residues 71–101), acts to silence the transcriptional activation domain ([7]; Fig. 1a). WT1 also contains a second transcriptional activation domain that is specific to the splice isoforms containing the 17 amino acids encoded by exon 5 ([8]; Fig. 1a).

WT1 exhibits complex transcriptional regulatory functions. Indeed, in addition to acting through its own DNA-binding

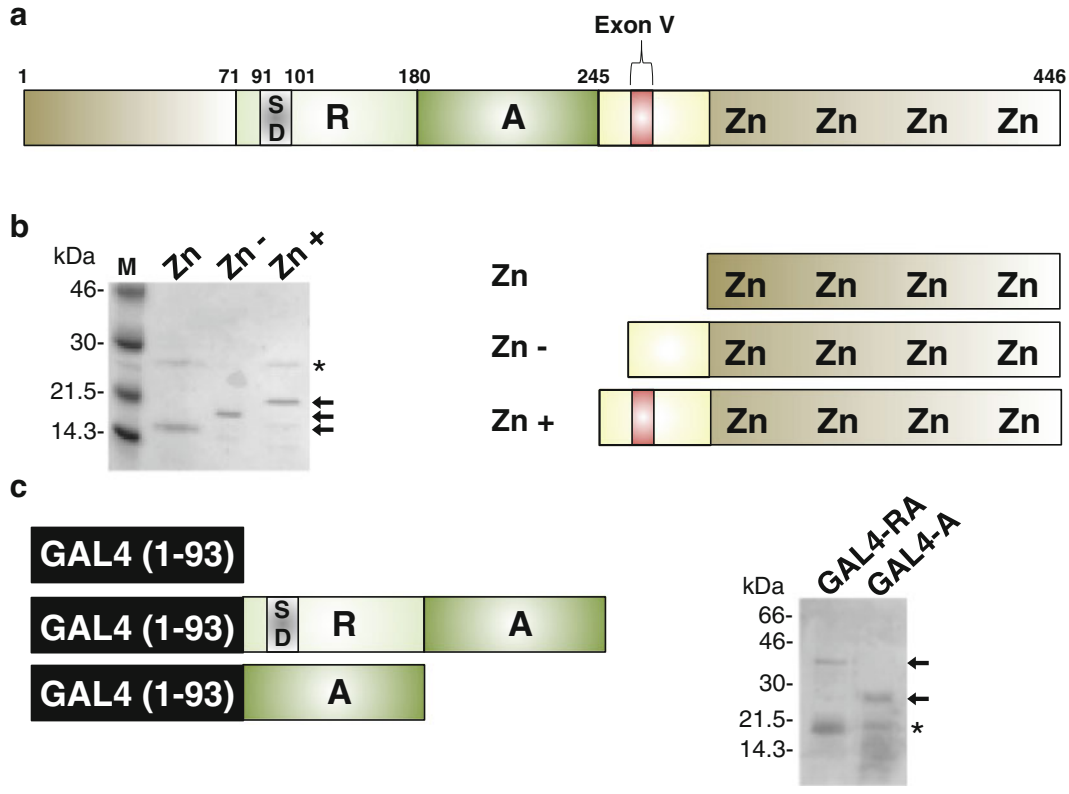

Fig. 1 Preparation of 6XHIS-tagged WT1 proteins. (**a**) Schematic of WT1. Specifically, the isoforms that lack the KTS insertion are shown for simplification. R is the transcriptional repression domain, A is the transcriptional activation domain, SD is the suppression domain. The zinc fingers are indicated (Zn). The 17-amino-acid region encoded by exon V and alternatively spliced is indicated. Amino acid numbering denotes the WT1 form that contains exon V but lacks the KTS insertion. (**b**) At right a schematic of WT1 derivatives containing the zinc finger region alone, and the zinc finger region in addition to N-terminal sequences that either lacks or contains the 17AA insertion. At left a Coomassie-stained gel is shown that contains these purified fusion proteins. *Arrows* denote the specific WT1 derivatives and contaminants are indicated by an *asterisk*. Molecular weight markers (kDa) are shown at left. These data are from Richard et al. [8]. (**c**) At left a schematic of GAL4 (residues 1–93)-WT1 fusion proteins is shown that contain the transcriptional repression (R) and transcriptional activation (A) regions. At right a Coomassie-stained gel is shown of purified GAL4-RA and GAL4-A fusion proteins. *Arrows* denote the specific WT1 derivatives and contaminants are indicated by an *asterisk*. Molecular weight markers (kDa) are shown at left. These data are from McKay et al. [7]

domain, WT1 can also be recruited to the promoter of a gene by interacting with other DNA-binding proteins, for example steroidogenic factor 1 [9] and the tumor suppressor p53 [10]. The intricate transcriptional regulatory activities, acting as either a transcriptional activator or repressor, suggest that WT1 likely also interacts with different cofactors to elicit distinct outcomes. Indeed, WT1 can interact with several other proteins to direct the final transcriptional outcome. WT1 binds to the histone acetyl transferase CBP [11] to effect acetylation of histone tails at the promoter. In contrast, when WT1 associates with the transcriptional corepressor BASP1 it can recruit histone deacetylases [12], and/or recruit the CTCF protein to modulate chromatin architecture [13]. Moreover, WT1 can act with menin and recruit the DNA methyltransferase DNMT1, which leads to modification of both histone tails and DNA through methylation [14]. Thus, the disparate transcriptional regulatory activities of WT1 can lead to multiple effects on the local environment surrounding gene promoters and ultimately in the regulation of transcriptional activity. These functions are further complicated by the multiple forms of WT1 that arise from alternative splicing which alters the WT1 interactome and results in distinct transcriptional outcomes (reviewed in ref. 1).

While the analysis of the function of transcription factors in cell culture systems yielded much information, the use of in vitro transcription methods has the potential to allow a more mechanistic analysis of function and the identification of cofactors functionally rather than through interaction alone. RNA interference and chromatin immunoprecipitation (ChIP) have greatly facilitated the analysis of transcription factors in living cells, but there are still aspects of in vitro transcription analysis that can be exploited to dissect the mechanism of action of WT1. In this chapter I will provide methods for in vitro transcription analysis and demonstrate how it can be exploited to shed light on WT1 function.

2 Materials

2.1 Nuclear Extract Preparation

1. 10× Phosphate-buffered saline (PBS): 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4.
2. Hypotonic buffer: 10 mM *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonyl fluoride (PMSF).
3. Dounce Homogenizer (ThermoFisher Scientific, Waltham, MA).
4. Nuclear lysis buffer: 20 mM HEPES pH 7.6, 25% (v/v) glycerol, 0.42 M NaCl, 5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, and 1 mM PMSF.
5. Buffer D: 20 mM HEPES pH 8, 100 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 mM DTT, 0.2 mM PMSF.

2.2 Preparation of His-Tagged Proteins

1. H-buffer: 20 mM Tris(hydroxymethyl)aminomethane (TRIS)–HCl pH 8.0, 1 M NaCl, 10 mM β-mercaptoethanol, 20 mM Imidazole, 10% (v/v) glycerol, 0.1% (v/v) Nonidet-P40 (NP40).
2. Misonix S-4000 Sonicator with the ¼ inch horn (Misonix, Farmingdale, NY).
3. Ni-NTA agarose (Qiagen, Valencia, CA).
4. H-buffer with imidazole: 20 mM Tris(hydroxymethyl)aminomethane (TRIS)–HCl pH 8.0, 1 M NaCl, 10 mM β-mercaptoethanol, 20 mM imidazole, 10% (v/v) glycerol, 0.1% (v/v) Nonidet-P40 (NP40), 0.3 M imidazole.
5. Buffer D: 20 mM HEPES pH 8, 100 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 mM DTT, 0.2 mM PMSF.

2.3 In Vitro Transcription

1. 100 mM $MgCl_2$.
2. 10 mM rNTP mix (containing 10 mM each of ATP, CTP, GTP, and UTP). Store in aliquots at –20 °C for several months.
3. Stop solution: 125 mM Tris–HCl pH 7.5, 12.5 mM EDTA, 150 mM NaCl, 1% (w/v) SDS.
4. Proteinase K (2 mg/ml in H_2O): Can be stored in aliquots for >1 year at –20 °C.
5. Phenol/chloroform (2:1 v:v).
6. 3 M Sodium acetate pH 5.2: Dissolve sodium acetate in H_2O and adjust the pH with glacial acetic acid.
7. Propan-2-ol.
8. 80% Ethanol (v/v in H_2O).
9. 5× Hybridization buffer: 0.2 M Piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES) pH 6.4, 5 mM EDTA, 2 M NaCl.
10. γ32P-ATP (3000 Ci/mmol; Perkin Elmer, Waltham, MA).
11. 0.5 M Ammonium acetate in H_2O.
12. 5× RT buffer: 250 mM Tris–HCl pH 8.3, 370 mM KCl, 20 mM $MgCl_2$.
13. 10 mM dNTP mix (10 mM each of dATP, dTTP, dCTP, and dGTP): Store in aliquots at –20 °C for several months.
14. 0.1 M DTT (in H_2O): Store in aliquots at –20 °C for several weeks.
15. AMV reverse transcriptase.
16. Loading dye: 95% (v/v) Formamide, 0.09% (w/v) bromophenol blue, and 0.09% (w/v) xylene cyanol.
17. 10% Denaturing polyacrylamide gel (20 cm × 20 cm, 0.4 mm thick, gel).

2.4 Immobilized DNA Template

1. Immobilization buffer: 1 M NaCl, 10 mM Tris–HCl pH 8.0, 0.005 % (v/v) Tween.
2. Streptavidin magnetic beads (Life Technologies, Grand Island, NY).
3. Transcription buffer: 12 mM HEPES pH 8.0, 12 % (v/v) glycerol, 60 mM KCl, 0.12 mM EDTA pH 8.0, 7.5 mM $MgCl_2$, and 0.5 mM DTT.
4. Magnetic separation rack (Life Technologies, Grand Island, NY).
5. Standard SDS-PAGE protein mini-gel system.

2.5 Functional Identification of WT1 Cofactors

1. Buffer D: 20 mM HEPES pH 8, 100 mM KCl, 0.2 mM EDTA, 20 % (v/v) glycerol, 0.5 mM DTT, 0.2 mM PMSF.
2. 2 ml Disposable plastic columns (ThermoFisher Scientific, Waltham, MA).
3. Protein precipitation solution: 100 % (w/v) Trichloroacetic acid/0.4 % (w/v) sodium deoxycholate.
4. Acetone.
5. 4× SDS-PAGE loading buffer: 200 mM Tris–HCl (pH 6.8), 400 mM DTT, 8 % (w/v) SDS, 40 % (v/v) glycerol, 0.4 % (w/v) bromophenol blue.

3 Methods

3.1 Preparation of Nuclear Extracts That Are Competent for In Vitro Transcription

The standard method for the preparation of transcription-competent nuclear extracts was developed by Dignam et al. [15]. A non-adherent HeLa cell line is used to grow cells in bulk, typically yielding greater than 1 g of cells. All steps are performed with chilled buffers on ice.

1. The cells are harvested by mild centrifugation at 2300 × *g* for 2 min. Remove the media and estimate the packed cell volume (PCV).
2. The cells are washed two consecutive times in 1× PBS to remove the remains of the growth medium. To do this, resuspend the cells in 20× PCV of 1× PBS, fully (but gently) dispersing the cells, then centrifuge at 2300 × *g* for 20 min, and discard the supernatant. Repeat.
3. The cells are resuspended in 5× packed cell volumes (PCV) of hypotonic buffer. The cells are left to swell on ice for 20 min and then harvested at 2000 × *g* for 10 min.
4. The swollen cells are resuspended in 2× PCV of hypotonic buffer and transferred to a Dounce homogenizer. After five strokes in the Dounce homogenizer using the tight pestle the disrupted cells are immediately centrifuged at 2300 × *g* for 10 min (*see* **Note 1**).

5. The upper cytoplasmic layer is discarded (but can also be used for other purposes). The nuclear pellet is resuspended in 1.5 volumes of nuclear lysis buffer. Use a pipette tip with the end clipped to widen the bore and take care not to cause frothing (*see* **Note 2**). The tube is placed on a rocker at 4 °C for 30 min.
6. Remove the nuclear debris by centrifugation at 16,000 × *g*, 4 °C, for 30 min. The supernatant is then dialyzed against two changes of Buffer D over 3 h.
7. The nuclear extract is then centrifuged at 16,000 × *g*, 4 °C, for 30 min to remove any precipitate that has formed during dialysis. Aliquot and store at −80 °C. Nuclear extracts can be stored at −80 °C for several months without losing significant activity. After removing from the −80 °C freezer, the nuclear extract should be centrifuged at 16,000 × *g* for 20 min before use (*see* **Note 3**).

The above protocol is useful for large-scale preparation of nuclear extracts, but frequently it is either necessary or convenient to prepare nuclear extracts from cells grown on a smaller scale. For preparation of nuclear extracts from smaller volumes of non-adherent cells, or adherent cells grown on tissue culture plates, a modified method can be employed [16].

1. After harvesting the cells and washing them in PBS twice to remove the culture medium the cells are resuspended in 1× PCV of hypotonic buffer, transferred to a microtube, and placed on ice for 15 min.
2. Pre-wash a 1 ml syringe with hypotonic buffer and then draw the suspended cells into the syringe. Place a 23 Gauge needle onto the syringe and force the cells through the needle into a microtube. Draw the cells back into the syringe and repeat a further four times. Then, immediately microfuge the cells at 15,000 × *g* for 20 s (*see* **Note 1**).
3. Discard the supernatant and resuspend the nuclear pellet in 2/3 PCV of nuclear lysis buffer. The method then follows the remainder of the method for large-scale preparations.

For transcriptional competence it is important to obtain nuclear extracts with a high protein concentration. Routinely we aim for 10 mg/ml, but we have been able to obtain transcription signals with extracts as low as 1 mg/ml protein concentration.

3.2 Preparation of Recombinant WT1 Proteins

Full-length WT1 proteins have proved difficult to produce. One way around this problem has been to produce sections of WT1 either alone or as fusion proteins. We took an approach to prepare histidine-tagged WT1 derivatives that lack regions at the N-terminus of WT1 [7]. Several vectors are available commercially to produce histidine-tagged proteins that can be rapidly

purified using nickel-agarose beads. The N-terminal region of WT1 is largely responsible for difficulties in purifying the intact protein or fragments. However, the C-terminal region does not pose significant problems and purification of WT1 derivatives composed of C-terminal regions is straightforward. For example, we have previously purified WT1 sections encompassing the C-terminal region that either lack or contain the 17 additional amino acids encoded by exon 5 that are present only in specific isoforms of WT1 ([8]; Fig. 1b). These derivatives contain the zinc finger region of WT1 (and thus possess intrinsic DNA-binding activity) in addition to more central regions of the protein which harbor transcriptional regulatory function.

The N-terminal region of WT1 is more problematic in purification from *E. coli*, but the solubility can be enhanced by generating fusion proteins. For example, we have purified 6XHIS-tagged GAL4 (1-93)-fusion proteins that contain the repression (R) and activation (A) regions of WT1 (Fig. 1c). The study of GAL4-fusion proteins that contain the transcriptional regulatory regions of WT1 can facilitate mechanistic studies of the function of specific motifs. Although such studies are highly derivatized, subsequent experiments using full-length WT1 in cells can then be used to validate the results. The 6XHIS-tagged WT1 proteins (both native and GAL4-fusion) were purified by Nickel-NTA affinity chromatography using a method based on Reece et al. [17]. The protocol uses a 1 l culture of bacteria in which expression of the His-tagged protein has been induced. Intact WT1 and GAL4 are zinc-finger proteins and thus it is important to induce protein synthesis after the addition of $ZnCl_2$ to the growth media to a final concentration of 100 μM. Solubility of the proteins is also aided by limiting the production using short induction times (typically 2 h) at 30 °C.

1. Resuspend the *E. coli* pellet in 10 ml of H-buffer. Ensure that the bacteria are fully resuspended with no clumps and then sonicate the suspended bacteria to rupture the cells (*see* **Note 4**). We use a Misonix S-4000 with the ¼ in. horn with a medium setting, using 6 × 30 s sonication procedure (*see* **Note 5**). The cell debris are then pelleted at 16,000 × *g* for 20 min.
2. The supernatant which contains the soluble proteins from the *E. coli* is transferred to a fresh tube. Add 0.5 ml Ni-NTA-agarose (preequilibrated in H-buffer). Place the sample on a rocking table at 4 °C for 30 min to allow the His-tagged protein to bind to the Ni-NTA agarose.
3. Collect the Ni-NTA agarose by centrifugation at 1000 × *g* for 1 min. Wash briefly with 12 ml of H-buffer and collect the beads as above. Wash for 10 min in H-buffer at 4 °C. Repeat.
4. Load the Ni-NTA agarose into a small disposable column (*see* **Note 6**). Wash the column with 10 ml H-buffer.

5. Elute the His-tagged protein in 2 ml of H-buffer containing 0.3 M imidazole (*see* **Note 7**).
6. The eluted protein is dialyzed into 1 l of buffer D to remove the imidazole. The purified protein is aliquoted and can be stored at −80 °C for over 1 year.

3.3 In Vitro Transcription Method Using Primer Extension

In vitro transcription methods involve incubating nuclear extract with a DNA template containing a promoter sequence and ribonucleotides, followed by analysis of the transcripts that are produced. Detection of transcripts can be achieved either by using radioactive UTP in the transcription reaction or by using a ^{32}P-labeled primer and primer extension to detect the transcripts. We have employed the latter technique because the reaction conditions are less complex and it also provides the additional benefit of transcription start site mapping [18, 19].

1. A typical in vitro transcription reaction contains

   25 μl nuclear extract (optimally at a concentration of 10 mg/ml)

   3 μl 100 mM $MgCl_2$

   1 μl DNA template (200 ng)

   3 μl rNTP mix (containing 10 mM each of ATP, CTP, GTP, and UTP)

   8 μl H_2O

   Further proteins can be added to the reaction (for example, activators or repressors), reducing the water accordingly (*see* **Note 8**).
2. The transcription reaction is incubated at 30 °C for 1 h. 160 μl of stop solution is then added and then 5 μl of 2 mg/ml Proteinase K. Place the reaction at 55 °C for 15 min.
3. Add 60 μl of phenol/chloroform (2:1), briefly vortex, and microfuge at 15,000g for 5 min. Keep the supernatant (*see* **Note 9**).
4. Add 60 μl of chloroform, briefly vortex, and then microfuge at 15,000g for 3 min.
5. The final supernatant is added to 10 μl of 3 M sodium acetate pH 5.2 and then 200 μl of propan-2-ol. Vortex briefly, then incubate on dry ice or in the −80 °C freezer for 10 min, and collect the nucleic acid pellet in a microfuge at 15,000g for 10 min.
6. Wash the pellet with 100 μl of 80 % ethanol. Allow the pellet to air-dry for 2 min.
7. The nucleic acid pellet is resuspended in 20 μl of 1× hybridization buffer. 2 ng of ^{32}P-labeled primer is added. Annealing of the primer to the RNA is performed at 37 °C overnight (*see* **Note 10**).

8. 160 µl of 0.5 M ammonium acetate is added and then 200 µl of propan-2-ol. Vortex briefly and then incubate on dry ice (or at −80 °C) for 10 min to precipitate the nucleic acid. Collect the precipitate in the microfuge at 15,000g for 10 min.
9. Wash the pellet in 80% ethanol and then air-dry. The pellet is resuspended in 6 µl of nuclease-free H_2O, 2 µl of 5xRT buffer, 1 µl of dNTPs (containing 10 mM each of dATP, dCTP, dTTP, dGTP), 1 µl of 0.1 M DTT, and 1 µl (1 unit) of AMV reverse transcriptase. Place the reaction at 42 °C for 1 h to allow primer extension to take place.
10. Add 10 µl of loading dye. Heat the samples at 95 °C for 2 min and resolve on a 10% denaturing polyacrylamide gel (we typically use a 20 cm × 20 cm, 0.4 mm thick, gel). Run the bromophenol blue 2/3 of the way along the gel and this will retain the unincorporated oligonucleotide on the gel, sufficiently spaced from the reverse-transcribed products. After drying the gel, the ^{32}P-labeled products are visualized by autoradiography or phosphorimaging.

3.4 Transcriptional Regulation by WT1 In Vitro

The DNA template used in the in vitro transcription assays needs to contain a promoter region that encompasses a transcriptional start site that is recognized by RNA polymerase II and the general transcription factors. Frequently, viral promoters are used because of the robust signals that are generated in transcription assays, but we have found that cellular promoters can also be effectively used in such assays [19, 20]. The adenovirus E4 (AdE4) promoter contains a functional TATA motif and initiator sequence and functions robustly in in vitro transcription, producing two distinct clusters of transcripts generated from tandem initiation sites [19]. Figure 2a shows a schematic of the AdE4 core promoter linked to a group of five GAL4 DNA recognition sites (G5E4T). Multiple GAL4 sites will facilitate the binding of several transcriptional activator proteins to produce the synergy required for robust transcriptional activation [21]. The transcript from this DNA template will ultimately be analyzed using a specific primer that will anneal to the RNA and correspond to approximately 40 bases downstream of the transcription initiator.

Incubation of the G5E4T template with HeLa cell nuclear extract as outlined above produces a low level of transcription (Fig. 2b). When GAL4 protein is included in the reaction, transcription initiated from the AdE4 promoter is unchanged. GAL4 (residues 1–93) is able to dimerize and bind to a consensus GAL4 DNA-binding sequence, but it lacks any domains that can regulate transcription. A GAL4 fusion protein that contains both the repression and activation domains of WT1 (GAL4-RA) was also unable to elicit a transcriptional response at the G5E4T DNA template [7]. In contrast, a GAL4-fusion protein containing the WT1 activation domain alone

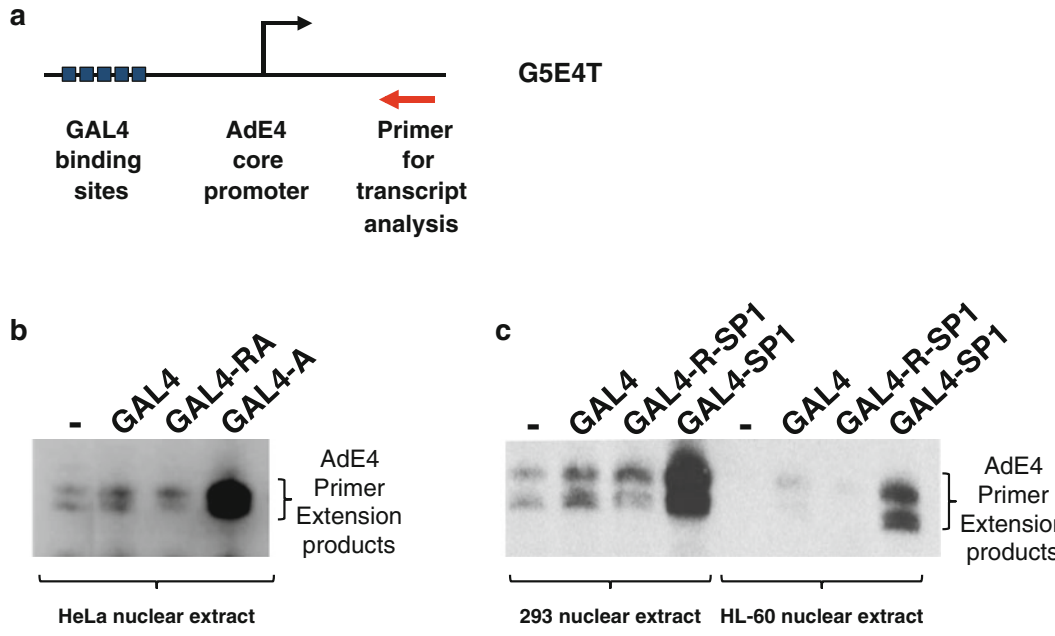

Fig. 2 In vitro transcription analysis with GAL4-WT1 fusion proteins. (**a**) A schematic of the AdE4 core promoter linked to five GAL4 DNA-binding sites (G5E4T). The *red arrow* indicates the site containing the sequence recognized by the primer used to perform primer extension analysis. (**b**) Section of a gel to analyze E4 transcripts. In vitro transcription assays were performed with the G5E4T DNA template, HeLa nuclear extract, and the GAL4 derivatives indicated (and shown in Fig. 1c). E4 transcripts form a doublet. These data are from McKay et al. [7]. (**c**) Section of a gel to analyze E4 transcripts. In vitro transcription assays were performed with the G5E4T DNA template. Nuclear extract prepared from either HEK293 or HL-60 cells was used in in vitro transcription with a GAL4, a GAL4-fusion protein containing GAL4 (1-93) linked to the WT1 repression domain linked to the SP1 transcriptional activation domain, or a GAL4-fusion protein linked to the SP1 transcriptional activation domain

(GAL4-A) was able to stimulate transcription of the G5E4T template. Thus, even though GAL4-RA contains a transcriptional activation domain, it is unable to elicit transcriptional activation. This is due to an inhibitory effect of the repression (R) domain. Indeed, the WT1 repression domain has the capacity to inhibit a heterologous transcriptional activation domain [7, 22]. In Fig. 2c we show transcription assays performed with the G5E4T DNA template in nuclear extracts prepared from two different cell types (human embryonic kidney 293 cells and leukemic HL-60 cells). These nuclear extracts were prepared using the small-scale method as described above. While a GAL4-fusion protein containing the SP1 transcriptional activation domain (GAL4-SP1) is able to stimulate transcription, when the WT1 repression domain (R) is fused to the SP1 activation domain (GAL4-R-SP1), the activity of the SP1 activation domain is repressed. These data, in combination with others, led to the proposal that the WT1 repression domain acts to suppress transcriptional activation by recruiting a transcriptional corepressor [6, 7, 22].

The above analysis of WT1 transcription function employed a GAL4-fusion approach to overcome solubility problems with the WT1 N-terminal regions when proteins are prepared from *E. coli*. We have also studied WT1 derivatives that contain the WT1 DNA-binding region and also segments of the transcriptional regulatory domains of WT1 ([8]; Fig. 1b). For these experiments, the GAL4 DNA-binding sites in the G5E4T DNA template were replaced with five consensus WT1 recognition elements (5′-GCGGGGGCG-3′) to produce W5E4T. While mammalian extracts do not contain any DNA-binding proteins that recognize the GAL4 DNA-binding site, there are several mammalian factors that recognize a DNA-binding site similar to that bound by WT1. Thus, transcription assays comparing the baseline activity of G5E4T and W5E4T DNA templates in a HeLa cell nuclear extract show that W5E4T has a high transcriptional activity (Fig. 3a). HeLa cells do not contain detectable WT1 and so the high activity of W5E4T in HeLa nuclear extract is likely due to other transcriptional activators that can bind to the WT1 recognition motif. This high activity will interfere with analysis and we therefore took the approach to remove these factors from the HeLa nuclear extract by fractionation over a column containing immobilized DNA motifs of the WT1 site. These extracts were devoid of high baseline activity. Using the fractionated HeLa nuclear extract it is now possible to analyze the transcriptional activity of WT1 derivatives that contain the natural WT1 DNA-binding region. Such studies allowed the identification of a second transcriptional activation domain that requires the 17AA encoded by exon V of the WT1 gene (compare WT1 Zn+ and WT1 Zn– that contain and lack the 17AA, respectively; [8]; Fig. 3b).

3.5 Purification of Transcription Complexes

In vitro transcription measures RNA production from a promoter construct. This functional measure is useful to assess the effect of proteins on transcription rate. It is also possible to explore some of the underlying mechanisms that are involved in the transcription changes. This is achieved by purifying the transcription complexes and requires the immobilization of the promoter DNA template [23–25]. The promoter region and regulatory sites of G5E4T or W5E4T (or other promoters) are amplified by PCR in which one of the primers is biotinylated at the 5′ end. Such primers are commercially available from several sources. The biotinylated DNA fragment can then be attached via the biotin moiety to streptavidin-coated beads as follows:

1. Incubate the biotinylated DNA with magnetic beads in 1 ml of immobilization buffer for 2 h at room temperature. Use 0.08 pmol of promoter DNA per 2 µl of magnetic streptavidin beads (*see* **Note 11**).
2. Wash the beads three times in immobilization buffer by placing the tube in a magnetic rack, allowing the beads to collect

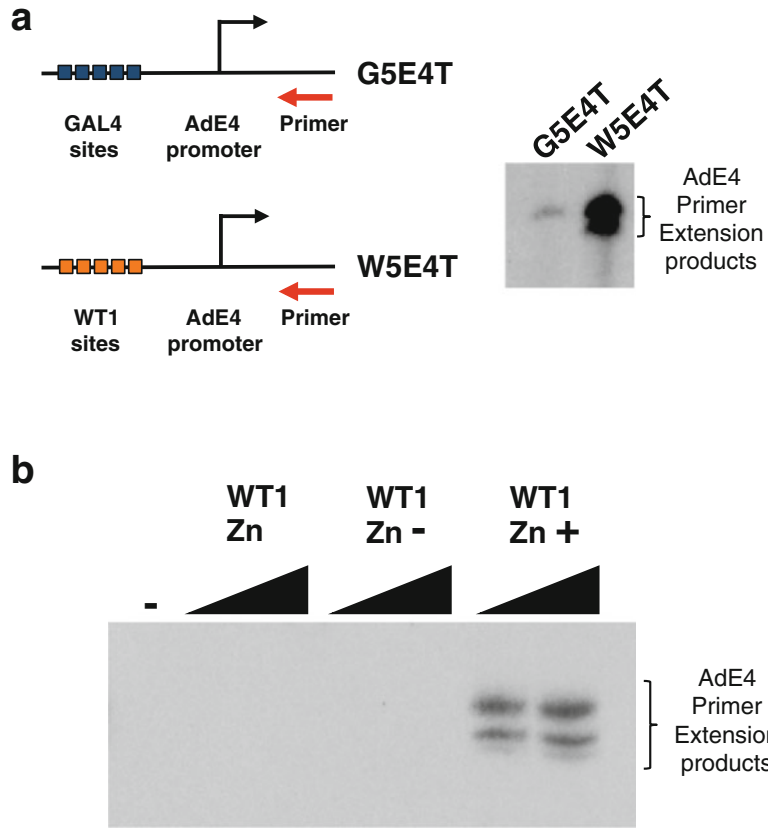

Fig. 3 Analysis of transcriptional regulation by WT1 derivatives. (**a**) At left, a schematic of the G5E4T DNA template (as shown in Fig. 2a), and the same DNA template in which the five GAL4 DNA-binding sites have been replaced with five WT1 DNA-binding sites (W5E4T). At right, an in vitro transcription reaction was performed with G5E4T and W5E4T DNA templates and HeLa cell nuclear extracts. These data are from Richard et al. [8]. (**b**) An in vitro transcription reaction using HeLa cell nuclear extract that had first been depleted of factors that interact with the WT1 DNA-recognition site. The assays contained 6XHIS-tagged WT1 proteins as indicated and also shown schematically in Fig. 1b. AdE4 extension products are indicated. These data are from Richard et al. [8]

and aspirating the supernatant (*see* **Note 11**). Resuspend the beads in fresh immobilization buffer by gentle pipetting.

3. After the final wash, resuspend the beads in 1× PBS such that 2 µl of the solution contains approximately 0.08 pmol of DNA fragment (*see* **Note 12**).
4. To form transcription complexes assemble the following components:

   25 µl Nuclear extract (optimally at a concentration of 10 mg/ml)

   3 µl 100 mM $MgCl_2$

   2 µl Magnetic bead/DNA template

   10 µl H_2O

5. Incubate at 30 °C for 1 h, keeping the beads in suspension by gentle pipetting every 10 min.
6. Add 1 ml of transcription buffer containing 0.0003% NP40 (*see* **Note 13**). The beads are then harvested using a magnetic rack.
7. Wash the beads three times in 1 ml of transcription buffer containing 0.0003% NP40.
8. Resuspend the beads containing purified complexes in 40 µl of transcription buffer.

Such purified transcription complexes are functional, and will undergo transcription when rNTPs are added. Alternatively, the purified complexes can be resolved by SDS-PAGE and immunoblotted to detect the level of specific factors and therefore monitor their recruitment to the promoter.

Figure 4 shows an immunoblot of purified transcriptional complexes formed on an immobilized promoter DNA template in the absence or presence of a transcriptional activator protein. The purified complexes were immunoblotted with antibodies against the TBP subunit of TFIID, TFIIB, or RNA polymerase II. The data show that the activator protein stimulates the recruitment of TFIIB and RNA polymerase II, but has only a modest effect on TFIID recruitment. Analysis of complexes in this way can yield valuable information on the effects of transcriptional regulators on the recruitment of other factors to the promoter and therefore on their mechanism of action. Indeed, the transcriptional activation function of WT1 has been associated with both TFIID [8] and TFIIB [7], suggesting a role in their recruitment to the promoter during the stimulation of transcription.

3.6 Functional Identification of WT1 Cofactors

In vitro transcription assays coupled with analysis in cells allows the identification of the domains within a transcription factor that play a role in regulating the transcription machinery. Using these methods, we identified a discrete region of WT1 (residues 71–101) that is responsible for inhibiting the transcriptional activation domain [7]. We termed this region of WT1 the suppression domain (SD), because it specifically suppressed transcriptional activation domains rather than directly repressing the RNA polymerase II complex. Site-directed mutagenesis of the WT1 suppression domain coupled with in vitro transcription assays identified key phenylalanine residues (F92 and F100) that are critical to mediate inhibition of a transcriptional activation domain [22]. Due to the mechanism of action it was most likely that the suppression domain functioned by interacting with a nuclear protein to mediate its effects.

Protein affinity chromatography coupled with SDS-PAGE and protein stains can be used to identify interaction partners for a protein of interest. The availability of mutant-derivative proteins can provide a correlation between the ability of a protein to bind and a functional effect that is affected by the mutation. In addition, protein affinity

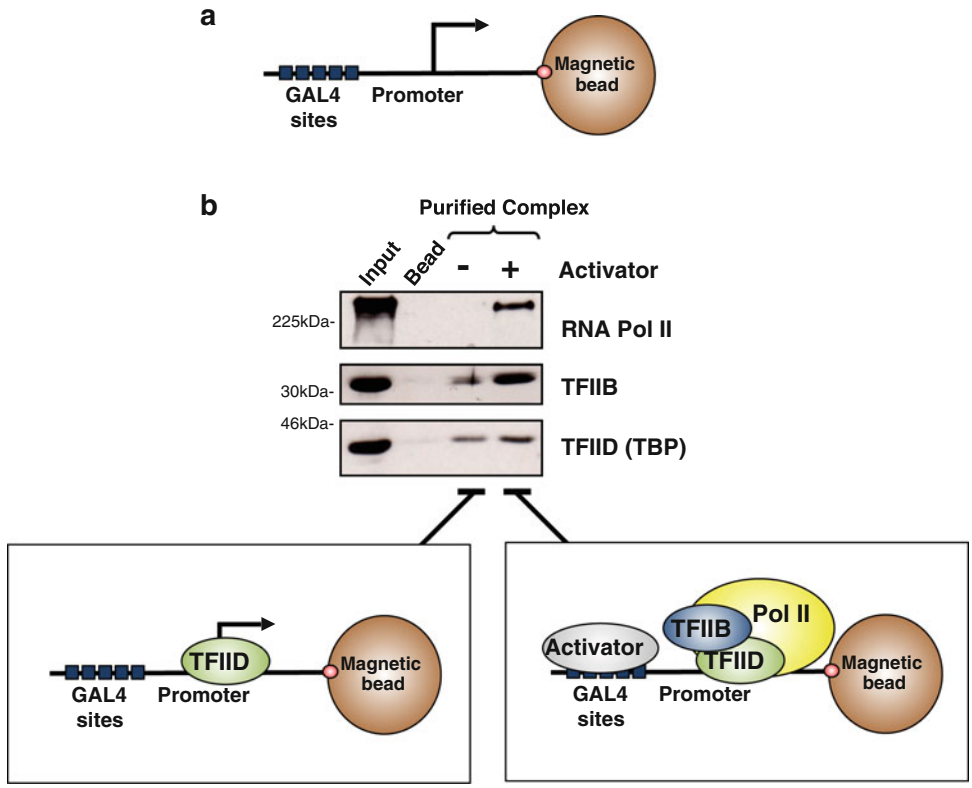

Fig. 4 Experiments with immobilized DNA templates can be used to analyze recruitment of the transcriptional machinery. (**a**) A promoter DNA template is immobilized onto streptavidin-coated magnetic beads via a biotin moiety. The beads can then be incubated with nuclear extract, and then washed to remove unbound factors. (**b**) The content of the complexes is analyzed here by immunoblotting against candidate factors. Magnetic beads containing nonspecific DNA is used as a control (bead). Example immunoblots are shown exploring the effect of an activator protein (GAL4) in the recruitment of RNA polymerase II (pol II), TFIIB, and TFIID (through the TBP subunit) to the promoter. Below the autoradiograms the content of the complexes is shown diagrammatically

chromatography can be used to specifically deplete factors from a nuclear extract that can then be tested in transcription analysis to determine if depletion of such factors has an effect on transcription.

We used protein affinity chromatography to analyze the WT1 suppression domain [22]. Large-scale preparation of GST or GST linked to the WT1 suppression domain (residues 71–101) allowed the assembly of affinity columns containing 2 mg of purified protein linked to 1 ml of glutathione agarose (Fig. 5). In addition we produced a GST fusion protein containing a WT1 suppression domain in which the phenylalanine residues at positions 92 and 100 were substituted with alanine (F92A/F100A). Immobilized GST-fusion proteins were prepared as described [18]. In vitro transcription assays had demonstrated that F92A/F100A mutations rendered the suppression domain as nonfunctional [22]. Nuclear proteins that interact with the WT1 suppression domain were identified as follows;

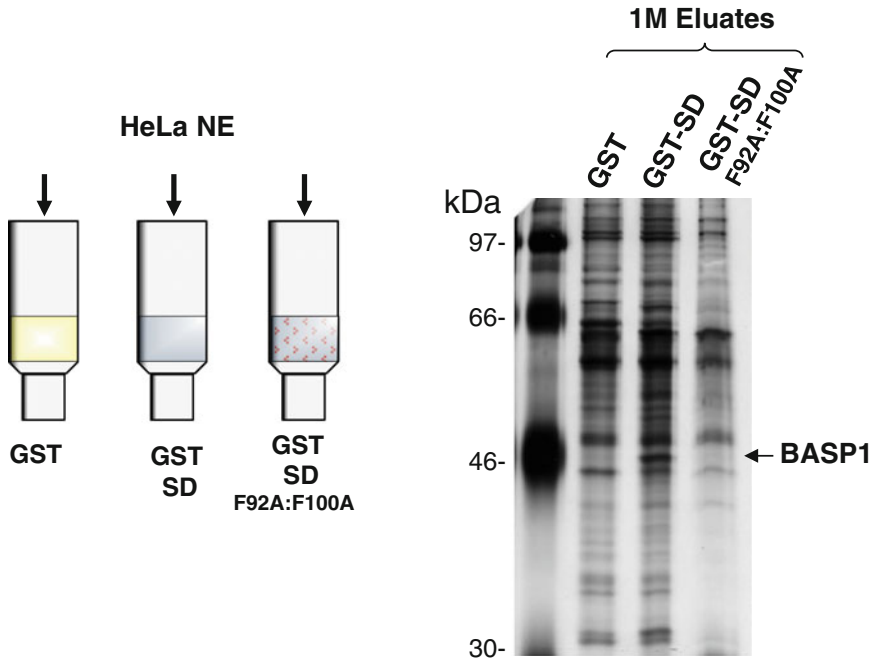

Fig. 5 Identification of WT1 cofactors by protein affinity chromatography. GST, GST-SD (containing the suppression domain of WT1, residues 71–101), and GST-SD F92A:F100A (containing residues 71–101 with F92A and F100A substitutions) were prepared from *E. coli* and retained as GST-fusion proteins bound to glutathione agarose. The glutathione agarose containing the fusion proteins was used to prepare affinity columns. 100 mg of HeLa nuclear proteins was fractionated over each column, and then the columns were washed with 20 column volumes of Buffer D. The stably bound proteins were eluted with Buffer D containing 1 M KCl. After precipitation of the proteins, they were resolved by SDS-PAGE and the gel was subjected to silver stain. The GST-SD, but not GST-SD F92A:F100A, column showed that a 50 kDa protein specifically interacted with the wild-type functional repression domain but not the F92A:F100A mutant derivative. This 50 kDa protein was subsequently identified as BASP1 [22, 26]

1. HeLa nuclear extract prepared as described in Subheading 3.1 is fractionated over the columns. The flow through is re-applied three times to allow the columns to capture as much interacting proteins as possible.
2. Wash the column with 20 column volumes of Buffer D.
3. Elute stably bound proteins with 3 ml Buffer D containing 1 M KCl.
4. The eluted proteins are then precipitated by adding 0.25 volumes of a solution containing 100% (w/v) trichloroacetic acid/0.4% (w/v) sodium deoxycholate. Briefly vortex and place on ice for 15 min.
5. Collect the precipitated proteins by microfuge at 15,000g for 15 min.
6. Add 1 ml of acetone, vortex, and microfuge at 15,000g for 5 min.

7. After removing the acetone by pipetting the pellet is then dried under vacuum for 5 min and resuspended in SDS-PAGE loading dye (*see* **Note 14**).
8. The proteins were resolved by standard SDS-PAGE and the gel was silver-stained using a commercially available kit.

Eluates from all three columns contain a number of proteins that were retained by the column and are common in all three samples (Fig. 5). The eluate from the column that contains the functional WT1 suppression domain (GST-SD) contains a specific band of approximately 50 kDa that later analysis was revealed to be brain acid-soluble protein 1 (BASP1). The band corresponding to BASP1 is not present in the eluate from the column containing GST-SD F92A:F100A, providing a functional link between the interaction of the WT1 suppression domain with BASP1. This analysis coupled with in vitro transcription methodology allowed the functional identification of BASP1 as a transcriptional corepressor of WT1 [22, 26].

4 Notes

1. It is critical that centrifugation is performed immediately after the homogenization because the nuclei will leak essential proteins.
2. Frothing will denature the nuclear proteins and reduce the activity of the nuclear extract in transcription.
3. This is important because each time the nuclear extract is thawed, precipitate will form and this will interfere with subsequent experiments.
4. A French Press can also be used to disrupt the *E. coli*.
5. It is important to leave sufficient gaps between each 30-s pulse to ensure that the sample does not become warm. This could denature the proteins.
6. We use Pierce scientific 2 ml disposable columns. The kits for these columns also provide a reservoir to facilitate the washes.
7. We find it helpful for the release of the His-tagged protein to cap the column after 1 ml has flowed through, then wait for 5 min, and continue the elution. If the column has a high flow rate, this step will help to ensure optimal elution of the His-tagged protein.
8. Ensure that the water used is ultrapure and guaranteed free of nuclease and protease activity.
9. There is usually considerable material at the interface, so be careful to avoid it.

10. These conditions facilitate priming of the RNA specifically and do not allow annealing of the primer to the DNA template.
11. Alternatively, streptavidin agarose beads can be used. If an agarose bead is used then collect the beads by allowing them to settle to the bottom of the tube.
12. The beads containing immobilized DNA can be stored at 4 °C for several days.
13. The low level of NP40 is sufficient to prevent coagulation of the beads but does not inhibit transcription.
14. Generally it is necessary to add 1–3 μl of 1 M Tris–HCl (pH 8) to neutralize the acidity of the sample.

Acknowledgements

Work in the author's laboratory is supported by the BBSRC (BB/K000446/1), and MRC (MR/K001027/1). I would like to thank Brian Carpenter, Derek Richard, and Yuming Wang for providing the data that are included in this work.

References

1. Toska E, Roberts SGE (2014) Mechanisms of transcriptional regulation by WT1 (Wilms' tumour 1). Biochem J 461:15–32
2. Nakagama H, Heinrich G, Pelletier J et al (1995) Sequence and structural requirements for high-affinity DNA binding by the WT1 gene product. Mol Cell Biol 15:1489–1498
3. Davies R, Moore A, Schedl A et al (1999) Multiple roles for the Wilms' tumor suppressor, WT1. Cancer Res 59:1747s–1750s
4. Wang ZY, Qiu QQ, Deuel TF (1993) The Wilms' tumor gene product WT1 activates or suppresses transcription through separate functional domains. J Biol Chem 268:9172–9175
5. Madden SL, Cook DM, Rauscher FJ 3rd (1993) A structure-function analysis of transcriptional repression mediated by the WT1, Wilms' tumor suppressor protein. Oncogene 8:1713–1720
6. Wang ZY, Qiu QQ, Gurrieri M et al (1995) WT1, the Wilms' tumor suppressor gene product, represses transcription through an interactive nuclear protein. Oncogene 10:1243–1247
7. McKay LM, Carpenter B, Roberts SGE (1999) Regulation of the Wilms' tumour suppressor protein transcriptional activation domain. Oncogene 18:6546–6554
8. Richard DJ, Schumacher V, Royer-Pokora B et al (2001) Par 4 is a coactivator for a splice-isoform specific transcriptional activation domain in WT1. Genes Dev 15:328–339
9. Nachtigal MW, Hirokawa Y, Enyeart-VanHouten DL et al (1998) Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. Cell 93:445–454
10. Maheswaran S, Park S, Bernard A et al (1993) Physical and functional interaction between WT1 and p53 proteins. Proc Natl Acad Sci U S A 90:5100–5104
11. Wang W, Lee SB, Palmer R et al (2001) A functional interaction with CBP contributes to transcriptional activation by the Wilms' tumor suppressor WT1. J Biol Chem 276:16810–16816
12. Toska E, Campbell HA, Shandilya J et al (2012) Repression of transcription by WT1-BASP1 requires the myristoylation of BASP1 and the PIP2-dependent recruitment of histone deacetylase. Cell Rep 2:462–469
13. Essafi A, Webb A, Berry RL et al (2011) A Wt1-controlled chromatin switching mechanism underpins tissue-specific wnt4 activation and repression. Dev Cell 21:559–574
14. Xu B, Zeng DQ, Wu Y et al (2011) Tumor suppressor menin represses paired box gene 2 expression via Wilms' tumor suppressor protein–polycomb group complex. J Biol Chem 286:13937–13944

15. Dignam JD, Lebovitz RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489
16. Lee KA, Bindereif A, Green MR (1988) A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. Gene Anal Tech 5:22–31
17. Reece RJ, Rickles RJ, Ptashne M (1993) Overproduction and single-step purification of GAL4 fusion proteins from Escherichia coli. Gene 126:105–107
18. Lin YS, Green MR (1991) Mechanism of action of an acidic transcriptional activator in vitro. Cell 64:971–981
19. Fairley JA, Evans R, Hawkes NA et al (2002) Core promoter-dependent TFIIB conformation and a role for TFIIB conformation in transcription start site selection. Mol Cell Biol 22:6697–6705
20. Deng W, Roberts SGE (2005) A core promoter element downstream of the TATA box that is recognized by TFIIB. Genes Dev 19:2418–2423
21. Lin YS, Carey MF, Ptashne M et al (1988) GAL4 derivatives function alone and synergistically with mammalian activators in vitro. Cell 54:659–664
22. Carpenter B, Hill KJ, Charalambous M et al (2004) BASP1 is a transcriptional cosuppressor for the Wilms' tumor suppressor protein WT1. Mol Cell Biol 24:537–549
23. Choy B, Green MR (1993) Eukaryotic activators function during multiple steps of preinitiation complex assembly. Nature 366:531–536
24. Roberts SGE, Choy B, Walker SS et al (1995) A role for activator-mediated TFIIB recruitment in diverse aspects of transcriptional regulation. Curr Biol 5:508–516
25. Roberts SGE, Green MR (1996) Purification and analysis of functional preinitiation complexes. Methods Enzymol 273:110–118
26. Green LM, Wagner KJ, Campbell HA et al (2009) Dynamic interaction between WT1 and BASP1 in transcriptional regulation during differentiation. Nucleic Acids Res 37:431–440