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Genetic analysis of P-TEFb function via heterologous nucleic acid tethering systems

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Abstract

Recent global organismal analyses demonstrated that transcription elongation control is a common feature in eukaryotes. One of the factors central to this type of control is the positive transcription elongation factor P-TEFb. P-TEFb is a cyclin-dependent kinase, which controls the fraction of RNA polymerase II (RNAP II) that can enter productive elongation. While the biochemical properties of P-TEFb and its associated factors have been characterized extensively *in vitro*, its function *in vivo* remains less well understood. In this manuscript, we describe various heterologous nucleic acid tethering systems that can be used to examine transcription factors that function via P-TEFb.

1. Introduction

Originally identified in HIV (1) and heat shock genes (2), transcription elongation control is now recognized as an important control step in eukaryotic gene expression (3). After synthesizing short nascent transcripts, RNAP II pauses in the promoter-proximal region due to the restriction by the negative transcription elongation factor (N-TEF), which includes DSIF (DRB sensitivity-inducing factor) and NELF (negative elongation factor). The kinase activity of P-TEFb is then required to overcome effects of N-TEF, and cause the transition to productive elongation (4). In support of this notion, global analyses of RNAP II distribution demonstrated that paused RNAP II can be found even at most silent promoters (3). This finding was supported by deep sequencing, which revealed the prevalence of short transcripts from these promoter-proximal regions (5). Moreover, analyses of DSIF and NELF colocalized them with the paused RNAP II (3). These observations and those on co-transcriptional processing of nascent transcripts suggest that the expression of most mRNA-coding genes are regulated at the step of elongation (4).

P-TEFb is a cyclin-dependent kinase that controls the transition of RNAP II from abortive to productive elongation (6). It is composed of one of two isoforms of Cdk9 and cyclin T1, T2 (7) or K (8,9) in humans. P-TEFb phosphorylates serine 2 in the heptapeptide repeats present in the carboxyl terminal domain (CTD) of the largest subunit of RNAP II, the Spt5 subunit of DSIF and RD in NELF, alleviating the effect of N-TEF (4). While half of endogenous P-TEFb is regulated by reversible inhibition via its association with the 7SK ribonucleoprotein complex (7SK snRNP), the other half can be recruited by various transcription factors to activate transcription (10). Indeed, P-TEFb must be recruited to transcription units to activate transcription. This can be achieved through DNA- or RNA-bound factors. The clearest example

of recruitment of P-TEFb is at the HIV long terminal repeat (LTR). HIV transcription initiates but does not elongate past the LTR, generating a short transcript called the transactivation response (TAR) element. Efficient elongation occurs only when the viral transactivator Tat binds the bulge in TAR via its arginine-rich motif, and recruits P-TEFb, whose CycT1 subunit binds both Tat and TAR (4). Thus, RNA-tethering can bring P-TEFb to RNAPII.

Several genetic assays based on the principle of yeast one- and two-hybrid systems have been developed and utilized widely to assay interactions between proteins as well as proteins and nucleic acids in mammalian cells. In the classic yeast one-hybrid system, specific DNA sequences are used to attract interacting proteins or peptides fused to heterologous transcription activation domains (TADs). In the two-hybrid system, a bait protein is fused to the DNA binding domain (DBD) of Gal4 or LexA proteins, and a prey protein is fused to a TAD. The physical interaction between these interacting species results in the activation of a reporter gene in a measurable output.

In this manuscript, we describe a nucleic acid tethering system that is based on the yeast one-hybrid system, and exploits the property of the HIV LTR, that is, the transcription from the HIV LTR is tightly controlled at the step of elongation. Thus, this system can be utilized to examine effects of factors on transcription elongation *in vivo*. A DNA-tethering system was initially developed by fusing subunits of P-TEFb to the Gal4-DBD. Gal4 DNA-binding sites (UASs) upstream of the HIV LTR recruit these fusion proteins. This localization brings P-TEFb to the proximity to the paused RNAP II, thus bypassing the recruitment of Tat and TAR. The outcome is the activation of the chloramphenicol acetyltransferase (CAT) reporter gene downstream of the HIV LTR. A derivative of this DNA-tethering system was subsequently developed to tether factors via a modified TAR RNA. First, the operator of R17/MS2 was grafted onto the double stranded stem in TAR (Reviewed in 4). Second, the high affinity-binding site for the viral regulator of expression of virion proteins (Rev) (stem loop IIB or SLIIB) was placed in the same position (11). In both cases, CAT again is the reporter gene. Since effects on RNAPII via TAR RNA represent the transition from abortive to productive elongation, these systems measure directly effects on transcription elongation of factors tethered to this heterologous RNA. With additional constructions of target plasmids, these nucleic acid tethering systems represents a genetic assay for activators and repressors that recruit or decoy P-TEFb for their effects.

2. Methods

2.1. A DNA-tethering system to study transcription elongation in vivo

The DNA-tethering system was initially developed to examine the function of Cdk9 and CycT1. Cdk9 or CycT1 was fused to the Gal4-DBD for its recruitment to 6 repeated UASs, localized upstream of the HIV LTR. When tethered this way, both Cdk9 and CycT1 were able to stimulate transcription from the HIV LTR, indicating that DNA-bound transcriptional activators may function through P-TEFb. Indeed, subsequent studies demonstrated that several activators, when tethered to DNA, are able to stimulate transcription by recruiting P-TEFb.

This system was further modified to include six additional DNA binding sites of LexA protein (6 repeated SOSs), 442-bp downstream of the Gal4 DNA binding sites, to examine the action of transcriptional repressors. They are fused to the LexA-DBD. By doing so, they will be recruited to the same target plasmid as DNA-tethered P-TEFb. Tethering both factors to the same region allows us to examine directly their effects on transcription.

2.1.1. Generation of effector and target plasmids—To generate a generic plasmid target (pG6L6CAT), 6 UASs and 6 SOSs were placed upstream of the HIV LTR, followed by the CAT reporter gene (14). To construct the plasmid effector, the gene of interest was fused

to the C-terminus of the DNA binding domain of Gal4 (positions 1-147) and/or LexA (positions 1-87) (Fig. 3).

2.1.2. Transfection and the CAT assay—The CAT assay is carried out with the following procedure in triplicate for each sample.

1. On the day of transfection, cells in 6-well plates are around 50 % confluent. 0.1 µg of target plasmid together with different combinations of plasmid effectors up to 4 µg are used to transfect cells in each well with Lipofectamine 2000 following the manufacturer's instruction.
2. 24–48 hours post transfection, medium is aspirated from the plate, each well is washed with 1 ml of PBS, cells are scraped and transferred to microcentrifuge tubes.
3. Cells are spun for 5 minutes at 14K rpm.
4. 200 µl of lysis buffer (250 mM Tris, pH 7.5, 0.1% Triton) is added and tubes are vortexed.
5. Cells are spun for 10 minutes at 4°C and 14K rpm.
6. Supernatants are collected and transferred to a fresh tube.
7. Cells are heat inactivated at 65°C for 5 minutes.
8. The spin is repeated and supernatants are transferred (about 200 µl) to a fresh tube.
9. 100 µl are transferred to scintillation vials. The rest is saved for protein concentration analysis and as normalization standards.
10. Chloramphenicol is dissolved in ddH₂O water to a final concentration of 1.66 mg/ml.
11. The following reagents are added: 0.5 µl of ³H-acetyl CoA (250 µCi, Amersham), 30 µl of chloramphenicol, 5 µl of 250 mM Tris (pH 7.5), 14.5 µl ddH₂O.
12. 3 ml of Econofluor is added in a chemical fume hood to each sample.
13. The activity of CAT is measured with a scintillation counter.

2.1.3. An example of analysis of repressor function with the DNA-tethering system—Pharynx and intestine in excess protein 1 (PIE-1), which represses transcription globally in early embryonic germ cell precursors in *C. elegans* (15,16), contains a CTD-like heptapeptide repeat, in which the serines at positions 2 and 5 are substituted by alanines (17). When these alanines are mutated to serines to resemble the wild-type CTD or acidic residues to resemble the phosphorylated CTD, PIE-1 only partially or no longer represses transcription. Moreover, *in vivo* disruption of this CTD-like repeat does not influence the stability or localization of PIE-1, but impairs its transcriptional silencing function. These observations suggested that the PIE-1 repression domain might target a protein complex that can phosphorylate the CTD of RNAP II (17).

We hypothesized that PIE-1 may exert its repressive function by inhibiting P-TEFb via a substrate-mimicking mechanism. Various plasmid effectors expressing Lex.PIE-1 fusion proteins were generated to test their effects on transcription activated by P-TEFb (Fig. 4). The wild-type PIE-1 protein (Lex.PIE-1), its C terminus (Lex.PIE-1C), mutant C-terminal serine-substituted [Lex.PIE-1C(STS)], and acidic [Lex.PIE-1C(DQEQ)] heptapeptide repeats were linked to the LexA DNA binding domain. First, pG6L6CAT and Gal.CycT1 or Gal.Cdk9 fusion proteins were co-expressed in HeLa cells. As expected, DNA-tethered CycT1 and Cdk9 increased this CAT activity more than 45-fold over basal levels. Next, we additionally co-expressed Lex.PIE-1 or Lex.PIE-1C with these plasmid target and effectors. Both Lex.PIE-1

and Lex.PIE-1C chimeras repressed the activation by DNA-tethered CycT1 (Fig. 4A) and Cdk9 proteins (Fig. 4B) by about 80%. However, co-expression of serine substituted mutant Lex.PIE-1C (STS) protein, which resembles the wild-type CTD, decreased this activity by only 50%. Importantly, acidic heptapeptide repeat mutant Lex.PIE-1C (DQEQ) protein, which resembles the phosphorylated CTD, did not decrease this activation at all (Fig. 4). The expression levels of LexA fusion proteins were comparable. In addition, as the deletion of SOSs from pG6L6CAT target plasmid abrogated their effects, the repression of PIE-1 chimeras depended on their recruitment to the promoter. Thus, whereas the wild-type Lex.PIE-1C chimera repressed P-TEFb, the substitution of aspartate and glutamate in the heptapeptide repeat abrogated this effect. Thus, PIE-1 appears to be a cellular repressor that functions by mimicking the substrate of P-TEFb.

The interaction between PIE-1 and P-TEFb was also analyzed by other standard methods including immunoprecipitation and GST-pulldown assays (14), which are not topics of this manuscript.

2.2. A RNA-tethering system to study transcription elongation in vivo

A derivative of the DNA-tethering system was subsequently developed to tether factors via a modified TAR RNA. The synthesis of this modified TAR RNA represents the transition point from abortive to productive elongation. Thus this system measures directly the effect on transcription elongation of factors tethered to this modified TAR RNA.

In the better of the two systems, the gene of interest is fused to the Rev protein. The chimera is then recruited to SLIIB, which was grafted onto the bottom stem in TAR. If the gene of interest can interact with P-TEFb either directly or indirectly, the chimera will stimulate transcription elongation because P-TEFb is brought into close proximity to RNAP II to overcome the effect of N-TEF (4).

2.2.1. Generation of effector and target plasmids—To generate a generic target plasmid (pSLIIBCAT), the critical TAR sequence in HIV LTR was replaced by SLIIB, derived from the HIV-1 Rev response element (RRE), and fused to the CAT reporter gene (11). To construct the plasmid effector, the gene of interest was fused to the C-terminus of Rev in a suitable mammalian expression vector (Fig. 1). This RNA tethering system is chosen over other systems because one SLIIB site can bind multiple Rev molecules, thus amplifying the effect of fused proteins. Plasmid effectors containing Rev alone or the Rev-Tat chimera can serve as negative or positive controls respectively.

2.2.2. Transfection and CAT assay—Transfection and the CAT assay are carried out in the same way as described in 2.1.2.

2.2.3. An example of analysis of activator function with the RNA-tethering system—For Tat transactivation to occur, Tat has to be synthesized first. Early studies suggested that P-TEFb could be recruited by artificially tethered factors on DNA to activate initial rounds of elongation from the HIV LTR. Because NF- κ B contributes to the activation of the HIV LTR and synergizes with Tat (12), its p65 (RelA) subunit was examined (13).

To determine if RelA could stimulate the elongation of pSLIIBCAT transcription, cDNAs of RelA (RevRelA), its N-terminal Rel homology domain (RevRelA-RHD), and C-terminal RelA transactivation domain (RevRelA-TD) were fused to Rev as plasmid effectors. When pSLIIBCAT alone or together with plasmid effectors expressing Rev or RelA were co-transfected into cells, there was no increase in CAT gene expression (Fig. 2). In contrast, RevRelA and RevRelA-TD chimeras activated pSLIIBCAT 32-fold and 14-fold over basal levels, respectively. Similarly, the hybrid RevTat protein, which served as a positive control,

activated transcription 48-fold over basal levels (Fig. 2). However, the RevRelA-RHD chimera was not active on pSLIIBCAT, suggesting that the trans-activation domain of RelA is critical for its activity (Fig. 2). Importantly, since the presence of increasing amounts of the dominant-negative Cdk9 protein reduced this activity in a dose-dependent manner, effects of the hybrid RevRelA protein depended on P-TEFb. We conclude that the transactivation domain of RelA is sufficient for activating transcription via RNA and that P-TEFb is critical for this effect (13).

The interaction between RelA and P-TEFb was also analyzed by other standard methods including immunoprecipitation and GST-pulldown assays (13), which are not topics of this manuscript.

3. Summary

Given that the recruitment of P-TEFb to transcription units results in the elongation of transcription of target genes, we speculate that one mechanism by which transcriptional activators work is by recruiting P-TEFb. Similarly, active repression can be simply blocking the substrate recognition of P-TEFb (4).

These heterologous nucleic acid tethering systems represent genetic assays for activators that recruit P-TEFb for their effects. Armed with this system, in combination with other assays, we and others have shown that c-Myc (18), the class II transactivator (CIITA) (19), MyoD (20), steroid hormone receptors (21), and VP16 (22), all interact with P-TEFb, leading to the modification of RNAPII for elongation. We expect that more and more factors will be discovered with this system that activate transcription by recruiting P-TEFb. Of note, histone acetyl transferases (HATs) such as p300/CBP and P-CAF, histone deacetylases (HDACs) such as HDAC1-11 and SirT1 as well as several other BAF, Swi/Snf proteins, when tethered via DNA or RNA, did not increase CAT activity (data not presented), consistent with their roles in transcription initiation and chromatin remodeling. Besides activators, this system can also be used to analyze repressors that block P-TEFb. It appears that the global repression of RNAP II transcription is a general strategy used by lower eukaryotes to repress somatic programs in early germ cell specification (23). This goal is achieved by blocking P-TEFb function. Similar to PIE-1 in worms (14), *Drosophila* protein Pgc represses P-TEFb globally during early embryogenesis (24). Strikingly, PIE-1 and Pgc do not share any detectable sequence homology. Although germ cell specification in mammals is different from that in worms and flies (25), a transient, global loss of RNAP II CTD Ser2 phosphorylation is observed (26). It will be of great interest to determine if there is a functional equivalent of PIE-1 and Pgc in mammals. The tethering system present here will certainly facilitate the identification and validation of such factor(s). For example, one can establish a germ cell specific cDNA library with factors tethered to LexA DBD. The TK gene can replace the CAT reporter on the target plasmid to facilitate screening. A stable 293T cell line can be generated to contain this target plasmid and Gal4-CycT1. Into these stably expressing cells, the cDNA library can be introduced. In the presence of gancyclovir, which will kill any cells that express TK polymerase, only cells with repressors that block the action of Gal4-CycT1 will survive. These cells will grow and the repressors will be identified. Similar screening efforts can be carried out with any tissue-specific cDNA libraries to identify tissue-specific P-TEFb inhibitors.

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Fig. 1.

Schematic representation of plasmid targets and effector for the DNA-tethering system.

Plasmids with different promoters should be chosen for the expression of the Gal4-activator and LexA-repressor chimeras to avoid impaired gene expression due to promoter competition. In pG6L6CAT, six Gal4- and LexA-binding sites with a linker of 442 bps were engineered upstream of the HIV LTR to achieve optimal activation or repression, respectively. Expression of CAT is under the control of a Gal4-linked activator and/or a LexA-linked repressor. Other genetic elements necessary for the expression in mammalian cells are omitted for simplicity.

Fig. 2.

PIE-1 requires the CTD-like repeat to repress transcriptional activation by P-TEFb. Cells were transfected with pG6L6CAT reporter plasmid. In addition, indicated repressor plasmid effectors were co-transfected with those encoding either Gal4-linked CycT1 (A) or Cdk9 (B) proteins. CAT activity was measured 48 hours later. Activity remaining was normalized to that of the control cells with mock transfection.

Fig. 3.

Schematic representation of the plasmid target and effector for the RNA-tethering system. The expression of the fused protein between Rev and gene of interest (G.I.) in the plasmid effector can be driven by any suitable promoters (CMV promoter is given in this example). In pSLIIBCAT, the Rev-binding site SLIIB was grafted onto TAR for binding of the chimera. Activation of CAT gene depends on the ability of the chimera to interact with P-TEFb. Other genetic elements necessary for expression in mammalian cells are omitted for simplicity.

Fig. 4.

RelA activates transcription via RNA. Cells in 6-well plates were transfected with the plasmid reporter pSLIIBCAT (0.1 μ g), together with indicated plasmid effectors (0.5 μ g of each). CAT activity was measured 48 hours later. Fold transactivation was normalized to that of the control cells with mock transfection.