Interaction of Sp1 transcription factor with HIV-1 Tat protein: looking for cellular partners

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Abstract The Tat protein of human immunodeficiency virus type 1 (HIV-1) *trans*-activates HIV-1 transcription by functionally interacting with a number of cellular proteins, among which the Sp1 transcription factor. We recently demonstrated that Tat does not directly interact with Sp1 either in vitro or in vivo, and we suggested that other protein(s) could indirectly mediate Tat–Sp1 interaction. In keeping, here we showed that addition of HeLa cell nuclear extracts to purified Tat and Sp1 proteins allows the formation of a Tat/Sp1 complex in in vitro binding assays. In an attempt to identify the partner(s) that bridge Tat and Sp1, we developed a yeast multi-protein system, in which cellular proteins recently shown to play a relevant role in Tat function, namely TATA box-binding protein, cyclin T1, CDK9, and cyclin T1/CDK9 complex, were coexpressed, individually or in pair-wise combination, with Tat and Sp1 hybrids. We demonstrated that none of these candidate partners bridges Tat and Sp1. However, our yeast multi-protein system, which allows simple and rapid detection of interactions among up to four proteins, will be most helpful to further dissect the interaction of Tat and Sp1 with other candidate partners that participate in the assembly of transcriptionally active complexes at the HIV-1 LTR.

Key words: Human immunodeficiency virus type 1 transcription; Tat; Sp1; TATA-box binding protein; Cyclin T1/CDK9; Yeast two-hybrid system

1. Introduction

The Tat protein of the human immunodeficiency virus type 1 (HIV-1) is a powerful *trans*-activator of viral gene expression and is essential for HIV-1 replication [1]. Tat acts by binding the Tat-responsive region (TAR) of HIV-1 long terminal repeat (LTR) and by functionally interacting with a number of proteins, among which the Sp1 cellular transcription factor [2]. Sp1 is a 95–105-kDa protein, ubiquitously expressed in mammalian cells, that modulates transcription initiation through interaction with other proteins, called coactivators [3]. Several reports suggest that interaction between Sp1 and Tat is required for Tat-mediated HIV-1 LTR *trans*-activation [4–7]; however, the mechanistic puzzle of how Tat–Sp1 functional interaction enhances HIV-1 transcription has not yet been explained.

Exploiting both biochemical and genetic techniques that allow detection of direct protein–protein interactions, we recently demonstrated that HIV-1 Tat protein does not physically interact with Sp1 both in vitro and in vivo, and we suggested that other protein(s) could indirectly mediate Tat–Sp1 interaction [8]. A number of cellular proteins have been reported to interact with Tat, including Tat-binding protein 1 [9], TATA box-binding protein (TBP) [10,11], TBP-associated factor TAFII55 [12], HT2A [13], Tip60 [14], cyclin T1 (CycT1) [15], Puralpha [16], and many others. Also Sp1 has been shown to interact with several proteins, including TBP [17], dTAFII110/hTAFII130 [18], YY1 [19], Oct-1 [20], E2F [21] and the retinoblastoma-related protein p107 [22]. Any of these proteins, other cellular protein(s) or HIV-1-encoded protein(s) could indirectly mediate Tat–Sp1 interaction.

In this report, we investigated whether a component of mammalian nuclei could indeed mediate the interaction of Tat with Sp1 in in vitro assays with HeLa cell nuclear extracts. Moreover, by means of the yeast two-hybrid system we assayed the interaction of Tat and Sp1 with cellular proteins shown to play a relevant role in Tat function, namely TBP, CycT1, cyclin-dependent kinase 9 (CDK9) and the CycT1/CDK9 complex. Finally, to test whether any of these proteins might be able to bridge Tat and Sp1, we developed a yeast multi-protein system that allows detection of interactions among up to four protein partners. Our experiments dissect some key protein–protein interactions involved in the assembly of transcription complexes at the HIV-1 LTR and thus could contribute to a better understanding of the puzzling mechanism of HIV-1 replication.

2. Materials and methods

2.1. Constructs

The construction of LexA-Tat, LexA-Sp1, GAD-Tat, GAD-Sp1, Tat-LexA, Sp1-LexA, LexA-TBP, GAD-TBP, LexA-CycT1, and GAD-CycT1 fusion proteins (encoded by pBTM-Tat, pBTM-Sp1, pACT-Tat, pACT-Sp1, pNLexA-Tat, pNLexA-Sp1, pBTM-TBP, pACT-TBP, pBTM-CycT1, and pACT-CycT1 plasmid, respectively) has been previously described [8]. To create pBTM-CDK9 and pACT-

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Abbreviations: CDK9, cyclin-dependent kinase 9; CycT1, cyclin T1; GAD, GAL4 protein-activating domain; HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; MBP, maltose-binding protein; RAP1, repressor-activator protein 1; rhSp1, recombinant human Sp1; RIF1, RAP1 interacting factor 1; TAR, Tat-responsive region; TBP, TATA box-binding protein

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CDK9 plasmids, expressing the LexA-CDK9 and GAD-CDK9 hybrids, respectively, the CDK9 coding sequence was amplified from pBS-CDK9 (kindly supplied by X. Grana, Temple University, Philadelphia, PA, USA) with primers 5′-AACGCAGGATCCAAGTGGCAAAGCAGTACGAC-3′ (forward) and 5′-ACACTAGTCGACTCAGAAGACGCGCTCAAA-3′ (reverse), and cloned into *Bam*HI/*Sal*I sites of pBTM116 [23] and into *Bam*HI/*Xho*I sites of pACTII (gift of D. Shore, Columbia University, New York, NY, USA), respectively. TBP, CycT1 and CDK9 coding sequences were also cloned into the yeast expression vector pMYK (a gift from A. Pallavicini, University of Padua, Italy), a derivative of pHybLex/Zeo (Invitrogen), where the *Hin*dIII fragment, expressing LexA, was excised. The pMYK vector contains a strong, constitutive alcohol dehydrogenase (ADH1) promoter and transcription terminator driving the expression of downstream cloned genes, and the zeocin resistance gene allowing selection of both bacterial and yeast transformed strains. pMYK-TBP was created by amplifying TBP coding sequence from pKB104 (provided by A.J. Berk, Molecular Biology Institute, University of California, Los Angeles, CA, USA) with primers 5′-AACGAAGGATCCATGGATCAGAACAACAGC-3′ (forward) and 5′-TCTCTAATGCATGTCGACTTACGTCGTCTTCCTGAA-3′ (reverse), and cloning the *Bam*HI/*Sal*I fragment into *Bgl*II/*Sal*I sites of pMYK. pMYK-CycT1 was generated by amplifying the CycT1 coding sequence from pGEX2T-cyclin T (a gift from L. Lania, Department of Genetics, General and Molecular Biology, University of Naples 'Federico II', Naples, Italy) with primers 5′-TGAATCGGATCCATGGAGGGAGAGAGGAAG-3′ (forward) and 5′-TTCATCGTCGACTTACTTAGGAAGGGGTGG-3′ (reverse), and cloning the *Bam*HI/*Sal*I fragment into *Bgl*II/*Sal*I sites of pMYK. pMYK-CDK9 was constructed by amplifying the CDK9 coding sequence from pBS-CDK9 with primers 5′-ACAACCGGATCCATGGCAAAGCAGTACGAC-3′ (forward) and 5′-ACACTAGTCGACTCAGAAGACGCGCTCAAA-3′ (reverse), and cloning the *Bam*HI/*Sal*I fragment into *Bgl*II/*Sal*I sites of pMYK. Finally, the pAL-CDK9 plasmid was created by amplifying the *ade2* marker gene under its own promoter (nucleotides 2170–4686) from pMET A (Invitrogen) with primers 5′-GTCACAGGATCCTTCGGATTCATGCTTATG-3′ (forward) and 5′-CATCACGGATCCGATCTTATGTATGAAATTC-3′ (reverse), and cloning the *Bam*HI fragment into the *Bam*HI site of pMYK-CDK9. All recombinant plasmids were sequenced with an ABI Prism™ 310 Genetic Analyzer (Perkin Elmer, Boston, MA, USA).

2.2. Yeast manipulation and β-galactosidase assays

The yeast strain *Saccharomyces cerevisiae* CTY10-5d (*MAT*a *ade*2-1 *trp*1-901 *leu*2-3,112 *his*3-200 *gal*4 *gal*80 *URA*3::*lexA op-lacZ*) contains the *lacZ* reporter gene, integrated at the *URA3* locus, with eight binding sites for LexA dimers upstream of the transcription start site [23]. Growth media and standard methods for manipulating yeast cells were as described by Rose et al. [24]. *S. cerevisiae* CTY10-5d was transformed with plasmid DNA by the lithium acetate method of Schiestl and Giest [25]. Transformed cells were assayed for expression of the *lacZ* reporter gene by X-Gal filter assays as described [26]. Reporter gene activity was tested on all transformed clones.

2.3. Yeast multi-protein assays

To test multi-protein interactions, the yeast strain CTY10-5d containing Tat and Sp1 two-hybrid plasmids was grown in media lacking leucine and tryptophan or histidine, and transformed with a third plasmid, derived from pMYK. Transformed yeast cells were selected in the presence of zeocin (300 μg/ml, from Cayla) on plates lacking leucine, tryptophan or histidine. In addition, triple transformants expressing Tat and Sp1 hybrids plus CycT1 in a non-fused form were transformed with a fourth plasmid, pAL-CDK9, and selected in the presence of zeocin onto synthetic minimal media lacking leucine, tryptophan or histidine, and adenine. Transformed yeast cells were tested for expression of the *lacZ* reporter gene as described above.

2.4. In vitro binding assays

In vitro binding assays between Tat and Sp1 were performed as described [8]. The maltose-binding protein (MBP)-Tat fusion protein (MBP-Tat) used in these assays was purified as reported [27]. Recombinant human Sp1 was purchased from Promega. In some samples, nuclear extracts from HeLa cells, prepared as previously described [28], were added.

3. Results

3.1. Analysis of HIV-1 Tat and Sp1 interaction in vitro in the presence of cell nuclear extracts

We recently reported that purified HIV-1 Tat protein does not physically interact with human recombinant Sp1 (rhSp1) in vitro, and we suggested that other cellular protein(s) could indirectly mediate Tat-Sp1 interaction in vivo [8]. To test this hypothesis, an in vitro binding assay between Tat and Sp1 in the presence of nuclear extracts from HeLa cells was performed. Purified MBP-Tat fusion protein was incubated with rhSp1, in the presence or in the absence of HeLa cell nuclear extracts. Samples were retrieved by an amylose resin and analysed for the presence of Tat and Sp1 by Western blot analysis. As shown in Fig. 1, MBP-Tat did not bind rhSp1 in the absence of nuclear extracts, as we previously reported [8]. In contrast, a complex between Tat and Sp1 could be detected when HeLa cell nuclear extracts were incubated with purified MBP-Tat and rhSp1, as revealed by Western blot analysis

Table 1
Analysis of the interaction of Tat and Sp1 with cellular proteins by the yeast two-hybrid system

Hybrid		β-Gal expression[a]
DNA-binding domain fusion	Activation domain fusion	
LexA-TBP		−[b]
	GAD-TBP	−[b]
LexA-TBP	GAD-TBP	−
LexA-Tat	GAD-TBP	+[b]
Tat-LexA	GAD-TBP	+[b]
LexA-TBP	GAD-Tat	+[b]
LexA-Sp1	GAD-TBP	+[b]
Sp1-LexA	GAD-TBP	+[b]
LexA-TBP	GAD-Sp1	+[b]
LexA-CycT1		−[b]
	GAD-CycT1	−[b]
LexA-CycT1	GAD-CycT1	+
LexA-Tat	GAD-CycT1	+[b]
Tat-LexA	GAD-CycT1	+[b]
LexA-CycT1	GAD-Tat	+[b]
LexA-Sp1	GAD-CycT1	−
Sp1-LexA	GAD-CycT1	−
LexA-CycT1	GAD-Sp1	−
LexA-CDK9		−
	GAD-CDK9	−
LexA-CDK9	GAD-CDK9	−
LexA-Tat	GAD-CDK9	−
Tat-LexA	GAD-CDK9	±
LexA-CDK9	GAD-Tat	−
LexA-Sp1	GAD-CDK9	−
Sp1-LexA	GAD-CDK9	−
LexA-CDK9	GAD-Sp1	−
LexA-RAP1		−
	GAD-RIF1	−
LexA-RAP1	GAD-RIF1	+

TBP, CycT1 or CDK9 coding sequences were fused to the C-terminus of LexA (DNA-binding domain) or of GAL4 activation domain (GAD), and assayed for interaction with Tat and Sp1 hybrids by β-Gal assays as described in Section 2. As a positive control for protein–protein interaction, yeast cells were transformed with plasmids encoding the LexA-RAP1 (repressor-activator protein 1) and GAD-RIF1 (RAP1 interacting factor 1) hybrid proteins, that were previously shown to interact [36].

[a](+) Strong blue colour detected after 3–5 h incubation; (±) light blue colour detected after 8–12 h incubation; (−) no signal detected after 24 h incubation.

[b]Data previously reported [8].

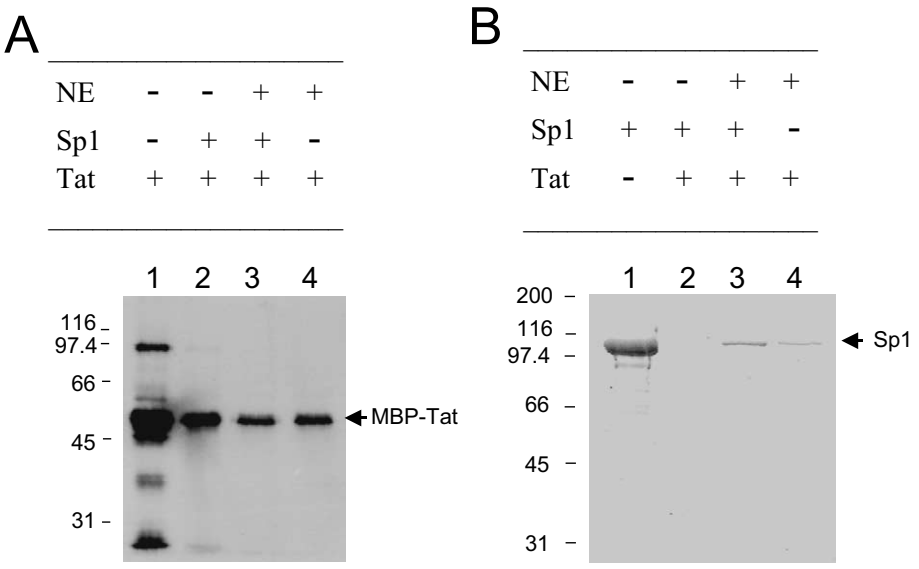

Fig. 1. In vitro binding assay between MBP-Tat and recombinant human Sp1 in the presence of HeLa cell nuclear extracts (NE). Supernatants derived from the incubation of purified MBP-Tat and Sp1 (lane 2), MBP-Tat and Sp1 with NE (lane 3), or MBP-Tat alone with NE (lane 4) were analysed by Western blot using anti-Tat (A) or anti-Sp1 antibodies (B). As internal controls for Western blot, MBP-Tat or Sp1 were also included (lane 1). The positions of MBP-Tat and Sp1 proteins and the molecular masses (in kDa) of the markers are indicated on the right and left, respectively.

with specific anti-Tat (Fig. 1A) and anti-Sp1 antibodies (Fig. 1B). Moreover, a complex between Tat and endogenous Sp1 could also be detected when HeLa cell nuclear extracts were incubated with purified MBP-Tat alone, as previously reported [28].

These data provide further evidence that Sp1 does not directly contact Tat, and that a molecule(s) present in mammalian nuclei indeed mediates Tat–Sp1 interaction.

3.2. *Analysis of the interaction of Tat and Sp1 with candidate cellular partners by yeast two-hybrid and three-hybrid systems*

The above results suggest that one or more cellular protein(s) could mediate Tat–Sp1 interaction in a multi-protein complex. We previously demonstrated, by fusing TBP to LexA and GAD and testing its interaction with Tat and Sp1 by means of the yeast two-hybrid system, that TBP binds both Tat and Sp1 ([8]; see also Table 1), confirming previous reports [10,11,17]. Therefore, to determine whether TBP may promote interaction between Tat and Sp1, we developed a yeast three-protein system allowing detection of Z-protein-mediated X/Y interactions. TBP was cloned in the pMYK yeast expression vector, to allow its expression in a non-fused form, and transfected into yeast clones containing the Tat and Sp1 two-hybrid plasmids. As shown in Table 2, all the Tat/TBP/Sp1 combinations yielded negative results, suggesting that TBP alone does not bridge Tat–Sp1 interaction. A slight expression of β-Gal was observed in yeast clones producing Tat-LexA and GAD-Sp1 hybrid proteins; however, as we previously reported [8], this expression was comparable to that of control cells producing the Tat-LexA fusion protein alone.

Other cellular proteins that may bridge Tat and Sp1 are CycT1 and CDK9, since the CycT1/CDK9 complex was recently shown to play a key role in Tat-mediated *trans*-activation of HIV-1 LTR [15,29]. Therefore, we tested whether CycT1 and/or CDK9 interact with Tat and Sp1 by means of the yeast two- and three-hybrid systems. The results of the two-hybrid system indicate that CycT1 interacts with Tat, as we and others previously reported [8,15], but not with Sp1, whereas CDK9 does not bind either Tat or Sp1 (Table 1). The slight expression of β-Gal observed in yeast clones producing Tat-LexA and GAD-CDK9 fusion proteins was again due to background *trans*-activating activity of the Tat-LexA hybrid. Interestingly, we also observed that CycT1 dimerises.

The results of the three-protein system with CycT1 and CDK9 were negative (Table 2), indicating that neither CycT1 nor CDK9 alone mediates Tat-Sp1 interaction. In contrast, expression of CycT1 in an unfused form, but not of TBP (not shown), allowed the specific interaction between Tat and CDK9 (Table 2), as shown previously [15], demonstrating that the experimental system is capable of detecting interaction of three proteins. However, the possibility existed that a quaternary complex is formed, where Sp1 does not directly bind CycT1 (bound to Tat) or CDK9, but contacts the composite contour created by the Tat/CycT1/CDK9 complex. To test this hypothesis, the yeast three-protein system was expanded to include a fourth protein for detecting quaternary complex formation. Still, after transformation of yeast cells with plasmids encoding Tat and Sp1 hybrids plus CycT1 and CDK9 in a non-fused form, no expression of the β-Gal reporter gene was detected (Table 2). Therefore, we conclude that also the CycT1/CDK9 complex is not able to mediate Tat–Sp1 interaction.

4. Discussion

We recently provided evidence that the HIV-1 Tat protein and the Sp1 cellular transcription factor do not directly interact either in vitro or in vivo [8], suggesting that the synergy between Tat and Sp1 cannot be simply explained by a phys-

Table 2
Analysis of Tat–Sp1 interaction in the presence of TBP, CycT1, CDK9 and CycT1/CDK9 complex by the yeast multi-protein system

Hybrid		Unfused protein(s)	β-Gal expression[a]
DNA-binding domain fusion	Activation domain fusion		
LexA-Tat	GAD-Sp1		−
Tat-LexA	GAD-Sp1		±
LexA-Sp1	GAD-Tat		−
Sp1-LexA	GAD-Tat		−
LexA-Tat	GAD-Sp1	TBP	−
Tat-LexA	GAD-Sp1	TBP	±
LexA-Sp1	GAD-Tat	TBP	−
Sp1-LexA	GAD-Tat	TBP	−
LexA-Tat	GAD-Sp1	CycT1	−
Tat-LexA	GAD-Sp1	CycT1	±
LexA-Sp1	GAD-Tat	CycT1	−
Sp1-LexA	GAD-Tat	CycT1	−
LexA-Tat	GAD-Sp1	CDK9	−
Tat-LexA	GAD-Sp1	CDK9	±
LexA-Sp1	GAD-Tat	CDK9	−
Sp1-LexA	GAD-Tat	CDK9	−
LexA-Tat	GAD-Sp1	CycT1/CDK9	−
Tat-LexA	GAD-Sp1	CycT1/CDK9	±
LexA-Sp1	GAD-Tat	CycT1/CDK9	−
Sp1-LexA	GAD-Tat	CycT1/CDK9	−
LexA-Tat	GAD-CDK9	CycT1	+
Tat-LexA	GAD-CDK9	CycT1	+
LexA-CDK9	GAD-Tat	CycT1	+

The yeast multi-protein assay was used to determine whether the interaction between Tat and Sp1 requires TBP, CycT1, CDK9 or CycT1/CDK9 complex as a bridging partner, as described in Section 2. Ternary interaction among Tat/CycT1/CDK9 was assayed as a positive control.
[a](+) Strong blue colour detected after 3–5 h incubation; (±) light blue colour detected after 8–12 h incubation; (−) no signal detected after 24 h incubation.

ical interaction. Most likely, the functional interaction between Tat and Sp1 relies on a more complex interplay between viral and cellular factors. Indeed, a number of cellular proteins have been reported to interact with Tat and/or Sp1 [9–22]. Any of these proteins or other unknown cellular protein(s) could indirectly mediate Tat–Sp1 interaction. Moreover, the possibility also existed that binding of HIV-1 Tat with Sp1 could be promoted by other virus-encoded molecule(s), i.e. TAR RNA or other HIV-1 protein(s). The HIV-1 virion-associated protein (Vpr), for example, has been shown to interact both with Tat [30] and with Sp1 [31].

Therefore, to investigate the latter possibility, in vitro binding assays between Tat and Sp1 in the presence of nuclear extracts from uninfected cells were performed. We showed that addition of HeLa cell nuclear extracts to purified Tat and Sp1 proteins allows the formation of a Tat/Sp1 complex, demonstrating that a molecule(s) present in mammalian nuclei is indeed necessary and sufficient to mediate Tat–Sp1 interaction.

Looking for a cellular partner(s) which could bridge Tat and Sp1, TBP appeared particularly interesting as a candidate. Some reports have shown that Tat directly binds, through its activation domain, human TBP [10,11]. The functional relevance of Tat–TBP interaction for *trans*-activation of HIV-1 gene expression still remains unclear, even though it has been reported that Tat activity is stimulated when TBP is overexpressed [11]. Noteworthy, Sp1 also interacts with TBP [8,17], making conceivable the idea that TBP may mediate the interaction between Tat and Sp1. Supporting this hypothesis, it has recently been shown that the correct spatial arrangement among TAR, Sp1-binding sites and TATA motif crucially influences HIV-1 expression, suggesting that Tat, Sp1 and TBP must contact each other for optimal expression and replication of HIV-1 [32]. However, TBP is not able to bridge Tat and Sp1, as shown by the yeast three-protein experiments. The inability of TBP to interact simultaneously with both Tat and Sp1 could be explained by the fact that both Tat and Sp1 bind the C-terminal conserved domain of human TBP [10,17], raising the possibility that they may compete in TBP binding. Noteworthy, Sp1 does not bind to yeast TBP [17] and does not stimulate transcription in *S. cerevisiae* [33]. In addition, yeast TBP does not physically or functionally interact with HIV-1 Tat [10], suggesting that yeast TBP does not interfere with human TBP in these experiments.

Another potential candidate for bridging Tat and Sp1 could be the CycT1/CDK9 complex. CycT1 was recently reported to interact with the activation domain of Tat and with the protein kinase CDK9, enhancing the affinity of Tat for TAR RNA [15]. The interaction of Tat with CycT1/CDK9 and TAR ensures hyperphosphorylation of the carboxy-terminal domain of RNA polymerase II holoenzyme by CDK9, thus promoting transcriptional elongation of the nascent viral mRNA [15,29]. Since recent results showed that Sp1 interacts with a cyclin (cyclin A) and can be phosphorylated by a cyclin-associated kinase, which modulates its *trans*-activating activity [34], one could assume that the interaction between Tat and CDK9, or Tat and the CycT1/CDK9 complex, has the role to bring the protein kinase in proximity of Sp1, bound to the HIV-1 LTR. This could allow Sp1 phosphorylation without direct contact between Tat and Sp1. Analyses of Tat interaction with the CycT1/CDK9 complex by means of the yeast two- and three-hybrid system show that this interaction occurs through a physical contact only between Tat and the cyclin T1 component, as reported [15,29], since CDK9 does not interact with Tat. Interestingly, we observed that CycT1 dimerises. This finding implies that the Tat/CycT1/

CDK9 complex is not ternary, as previously suggested [15], but quaternary or even of higher order, since also Tat can dimerise [35].

Our results with the yeast multi-protein system indicate that neither CycT1 and CDK9 alone or the CycT1/CDK9 complex can mediate Tat–Sp1 interaction. Thus, the protein(s) that bridge Tat and Sp1 are still to be identified. However, the yeast multi-protein system we developed allows simple and rapid detection of interactions among up to four protein partners, and therefore will be a useful tool to further dissect the protein–protein interactions involved in the assembly of transcription complexes at the HIV-1 LTR or, in general, any relevant protein–protein interaction in multi-protein complexes.

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