

Analysis of the in vivo interaction between a basic repressor and an acidic activator

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Abstract The artificial basic repressor SSB24 represses transcription of a reporter construct activated by GCN4. We show that the positively charged SSB24 and the negatively charged acidic activator GCN4 interact in vitro and in vivo. However, deleting the interaction domain from the GCN4 activator does not result in loss of repression by SSB24. Similarly, transcription activated by the holoenzyme component SRB2 is repressed, although SSB24 and SRB2 do not interact. Repression by SSB24 therefore does not depend on the observed protein-protein interaction between SSB24 and GCN4.

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Key words: Repression; SSB24; GCN4; Split ubiquitin assay

1. Introduction

Regulation of mRNA synthesis involves a complex interplay of activation and repression at the transcriptional level. Transcriptional activators are thought to recruit components of the transcription complex to the promoter and/or stabilize the assembled compounds. Many contacts between different activators and general transcription factors or components of the RNA polymerase II holoenzyme have been demonstrated [1–5]. Disruption of the chromatin structure may also be an important functional aspect of transcriptional activation. In particular, several activator proteins have been shown to require histone acetyltransferase activities for activation [6–8]. Conversely, some transcriptional repressors appear to recruit histone deacetylases [9,10]. In addition, repressors can also act directly on the transcriptional machinery. For example, the general corepressors Ssn6/Tup1 in yeast fail to give full repression in the absence of SRB10, a component of the RNA polymerase II holoenzyme [11–13]. Alternatively, repressors may impair activator function. One possibility is to interfere with DNA binding of the activator. For example, the giant repressor, a gap gene expressed during the blastoderm stage of the *Drosophila* embryo, is proposed to prevent DNA binding of the morphogen bicoid in the even-skipped promoter [14]. Another possibility is to interact with functional domains of the DNA bound activator, thereby masking them from their targets. Such a mechanism would allow a local repression without affecting neighboring segments in complex promoters [15].

The small basic repressor SSB24 has been isolated from a

library of random fragments fused to the Gal4 DNA binding domain (aa 1–147) in a screen for peptides repressing GCN4 activated transcription. SSB24 can repress transcription of a reporter with Gal4 sites upstream of the GCN4 site [16]. Because SSB24 is a highly basic protein (91 amino acids, net charge +25), we asked whether transcriptional repression might be due to a direct interaction with the acidic activator GCN4. Although SSB24 does indeed interact with GCN4 in vitro and in vivo, we show in a deletion analysis that the interacting domain in GCN4 is dispensable for repression by SSB24. This observation suggests that SSB24 repression does not depend on interaction with the activator but might be due to non-specific DNA binding of the positively charged factor.

2. Materials and methods

2.1. Strains and plasmids

All β -galactosidase assays were performed in strain NLY2 [16] with the reporter SS38G4 [16] or the derivative SS38 Δ GL, where the GCN4 site had been replaced by a LexA site, integrated at the Ura3 locus. LexA-activator fusions were on the Trp1 marked ARS-CEN plasmid Y201. Y201 was derived from Y1 [17], with the Gal4(1–147) sequences replaced by LexA(1–202). The Gal4(1–147)-SSB24 fusion was on a 2 μ m plasmid based on the His3 marked vector MA424 [18]. GST fusion proteins were expressed from derivatives of pGEX-5X-1 (Pharmacia); in vitro transcription/translation reactions with T7 polymerase used constructs based on pBluescript KS (Stratagene) as templates. HA-tagged SSB24 was cloned into pET11a (Invitrogen) and expressed in *E. coli*. The constructs for the split ubiquitin assay were cloned by replacing SOD in SODCubDHFRha-Cup314 (a gift from N. Johnsson) or by cloning into NubIpACNX. NubIpACNX is a derivative of pADNS [19] where the 2 μ m origin has been replaced by an ARS-CEN element and the N-terminal half of ubiquitin is expressed under the control of the ADH1 promoter. The assays were performed in strain JD55 [20].

2.2. β -galactosidase assays

Yeast strains transformed with the indicated plasmids were grown in liquid culture and assayed for β -galactosidase activity as described [21]. All values shown represent at least three independent measurements.

2.3. In vitro binding assays

In vitro binding experiments to investigate protein-protein interactions were done essentially as described [22]. Bacterial lysates containing approximately 50 μ g of GST fusion protein were coupled to glutathione sepharose beads (Pharmacia). Beads were incubated with 20 μ l of 35 S-methionine labelled protein from a TNT T7 Coupled Reticulocyte Lysate in vitro transcription/translation reaction according to the manufacturer's instructions (Promega). After extensive washing, bound proteins were released by boiling with SDS sample buffer, resolved on a 15% SDS-PAGE gel and analyzed by autoradiography. For the deletion analysis of GCN4, equal amounts of GST fusion proteins coupled to glutathione sepharose beads were incubated with 100 μ l bacterial extracts containing HA-tagged SSB24 for 1 h at 4°C. Beads were washed five times with PBS, boiled with SDS sample buffer to release bound proteins, and analyzed by Western blotting with anti-HA antibody (BAbCO).

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2.4. Far Western blotting

For far Western blotting, SSB24 and SSB24C were expressed as GST fusions in *E. coli*. Bacterial extracts were coupled to glutathione sepharose beads and cleaved for 2 h at room temperature with Factor Xa in the buffer recommended by the supplier (New England Biolabs). Aliquots of the supernatant were resolved on a 15% SDS-PAGE gel and blotted onto nitro-cellulose. The blot was blocked in HBB (20 mM HEPES pH 7.7, 75 mM KCl, 2.5 mM MgCl₂) + 5% skim milk, incubated overnight at 4°C in HBB + 5% skim milk + 1 mM DTT with 1 µg/ml full length GCN4 protein fused to GST (purified from *E. coli*) and the indicated NaCl or NP40 concentrations. Bound protein was detected using anti-GST antibody. Washing steps were performed with PBS + 5% skim milk + 0.1% Triton X-100.

2.5. Split ubiquitin assays

Yeast strain JD55 was transformed with plasmids expressing the indicated Cub and Nub fusions. Ten ml cultures in minimal medium were induced with 50 µM copper for 2 h to activate expression of the Cub fusion from the CUP1 promoter, harvested and either an aliquot was boiled with sample buffer and directly loaded on an SDS-PAGE gel or extracts were prepared as described [23], but with only 80–150 µl total volume of lysis buffer lacking Triton X-100. HA-tagged DHFR from the Cub fusion was detected by Western blotting using anti-HA antibody (BAbCO). Quantitations were performed using a lumiimager (Boehringer).

3. Results

The small basic peptide SSB24 represses transcription when it is tethered to the promoter by means of a fusion to Gal4(1–147) [16]. A reporter construct containing five Gal4 sites upstream of a single GCN4 site is repressed approximately 5- to 6-fold by Gal4(1–147)-SSB24 compared to Gal4(1–147) alone (Fig. 1). The C-terminal half of SSB24 (SSB24C, 45 amino acids, net charge +15) is sufficient for the repression (not shown).

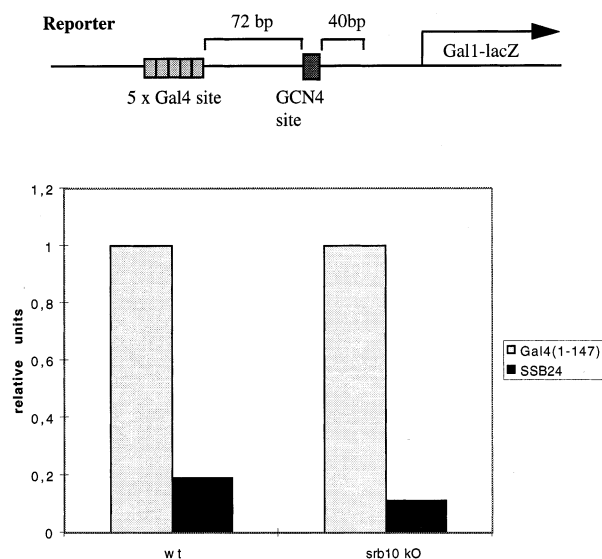
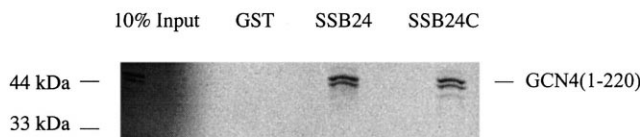


Fig. 1. Repression by SSB24 in wt and *srb10* knockout strains. β -galactosidase activity was assayed from strains with an integrated reporter containing five Gal4 sites upstream of a single GCN4 site (depicted at the top). Gal4(1–147)-SSB24 (black columns) or Gal4(1–147) alone (grey columns) were expressed from a plasmid. Results are shown for wt (left) and *srb10* knockout (right) genetic backgrounds. For better comparison, values for the Gal4(1–147) were set to 1.

A



B

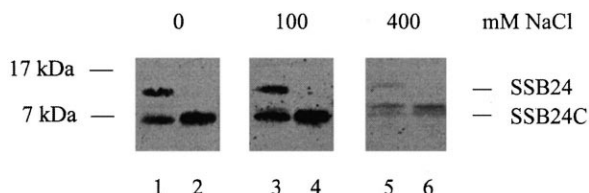


Fig. 2. SSB24 and GCN4 interact in vitro. A: Interaction of SSB24 and GCN4(1–220) in an in vitro binding assay. In vitro translated ³⁵S labelled GCN4(1–220) was incubated with GST fusion proteins coupled to glutathione sepharose beads. Bound protein was resolved on an SDS-PAGE gel and analyzed by autoradiography. GST fusion proteins are indicated at the top. GCN4(1–220) (24 kDa) runs with an apparent molecular weight of approximately 50 kDa in SDS-PAGE gels. B: Interaction of SSB24 and GCN4 in a far Western blot. SSB24 (lanes 1, 3, 5) and SSB24C (lanes 2, 4, 6) were resolved on an SDS-PAGE gel and blotted onto nitro-cellulose. The blot was incubated with purified full length GCN4 protein fused to GST at 1 mg/ml. The binding buffer contained 75 mM KCl plus the NaCl concentrations indicated at the top. Bound protein was detected using an anti-GST antibody. The SSB24 preparation contained degradation products which interact with GCN4, too.

3.1. Repression by SSB24 does not require SRB10

The yeast repressor $\alpha 2$ works by recruiting the general corepressors Ssn6/Tup1 to the promoter [24]. Since $\alpha 2$ can no longer repress transcription in a yeast strain lacking SRB10 [11] and repression by Ssn6/Tup1 is reduced in a Δ *srb10* strain [12], this protein, which is associated with the holoenzyme [13], may be the final target of the $\alpha 2$ repression pathway. While SSB24 was shown to repress transcription independently of Ssn6 [16], it might still target Tup1 or, by another pathway, SRB10. To test this hypothesis, we investigated SSB24 repression in an *srb10* knock out strain. As shown in Fig. 1, SSB24 is able to repress transcription independently of SRB10.

3.2. SSB24 directly interacts with the activator GCN4

Since SSB24 is a highly basic protein, we asked whether transcriptional repression might be due to a direct interaction with the acidic activator GCN4, which may potentially mask the activator's function. In an in vitro binding assay, the two proteins do indeed interact (Fig. 2A). In vitro translated GCN4(1–220) binds to GST-SSB24 and GST-SSB24C, but not to the GST control. SSB24C is a derivative of SSB24 consisting of only the C-terminal half. As a Gal4(1–147) fusion, SSB24C is sufficient for transcriptional repression (not shown). In the in vitro binding assay, a GCN4 derivative lacking the DNA binding domain (aa 1–220) was used to avoid possible DNA bridging in the binding reaction, since DNA was present in the in vitro translated protein preparation. In order to demonstrate the interaction with an independent method, a far Western blot was performed (Fig. 2B). This method uses purified proteins, showing that the

interaction must be direct. As seen in the *in vitro* binding assay (Fig. 2A), the C-terminal half of SSB24 (SSB24C) is also sufficient for interaction with GCN4 in the far Western blot (compare lanes 1 and 2). When the salt concentration in the binding buffer was enhanced to 400 mM NaCl, the binding became significantly weaker (Fig. 2B). Conversely, including 0.5% or 1% of the non-ionic detergent NP40 in the binding buffer enhanced the association between SSB24 and GCN4 to some extent (not shown). This observation indicates that the interaction is of an ionic nature, as would be expected for two highly charged proteins.

To investigate whether the SSB24 and GCN4 proteins could also interact *in vivo*, we used the split ubiquitin assay [25]. Unlike the 2-hybrid assay, this method is independent of a transcriptional readout and can therefore also be used to analyze transcription factors. The two proteins tested are fused to the N-terminal and the C-terminal half of ubiquitin, respectively. One of the fusions carries as a reporter an HA-tagged protein that can be detected in a Western blot. If the two tested proteins interact, the reassociated halves of the ubiquitin moiety are recognized by a ubiquitin specific protease and the reporter is cleaved C-terminal of the ubiquitin (Fig. 3A). The cleavage efficiency can be monitored by Western blotting, because the full length and cleaved reporter yields bands of distinct size. As shown in Fig. 3B, expression of a fusion of SSB24C and the N-terminal half of ubiquitin (Nub) significantly enhances cleavage efficiency with a reporter fused to GCN4(1–220) and the C-terminal half of ubiquitin (Cub). The same result is seen with an Nub fusion of SSB24 (not shown). When we controlled the expression level of Nub and Nub-SSB24C in a Western blot, we found that Nub alone is expressed at least 10-fold more than the Nub-SSB24C fusion (Fig. 3C). The expression of Nub alone is already sufficient to give substantial cleavage. However, relative cleavage efficiency was always markedly increased in the presence of the Nub-SSB24C, despite the much lower expression level of the fusion protein.

3.3. Deletion analysis of GCN4

Next, we sought to determine whether SSB24 interacts with the acidic activation domain of GCN4, located between amino acids 87 and 154 [26], as would be suggested by the finding that the interaction is ionic in nature. Surprisingly, an *in vitro* binding assay with different deletion derivatives of GCN4 (shown schematically in Fig. 4A) revealed that the interaction domain resides C-terminal of the activation domain instead. This domain, aa 153–220, is both necessary and sufficient for the *in vitro* interaction with SSB24 (Fig. 4B, compare lanes 4 and 7).

In an approach aiming to elucidate the relevance of the defined interaction domain for *in vivo* repression, we used the different GCN4 constructs in repression assays. To avoid interference by endogenous GCN4, a LexA fusion was used in combination with a reporter containing five Gal4 sites upstream of a single LexA site (Fig. 4C). In contrast to wild-type GCN4, whose DNA binding domain is at the C-terminus, the heterologous DNA binding domain was fused to the N-terminus in these constructs. This may explain the weak activation potential of the constructs containing the region C-terminal of the activation domain (aa 153–220). The different GCN4 constructs show a high variation in their ability to activate transcription (2.2 β -galactosidase units for GCN4 87–

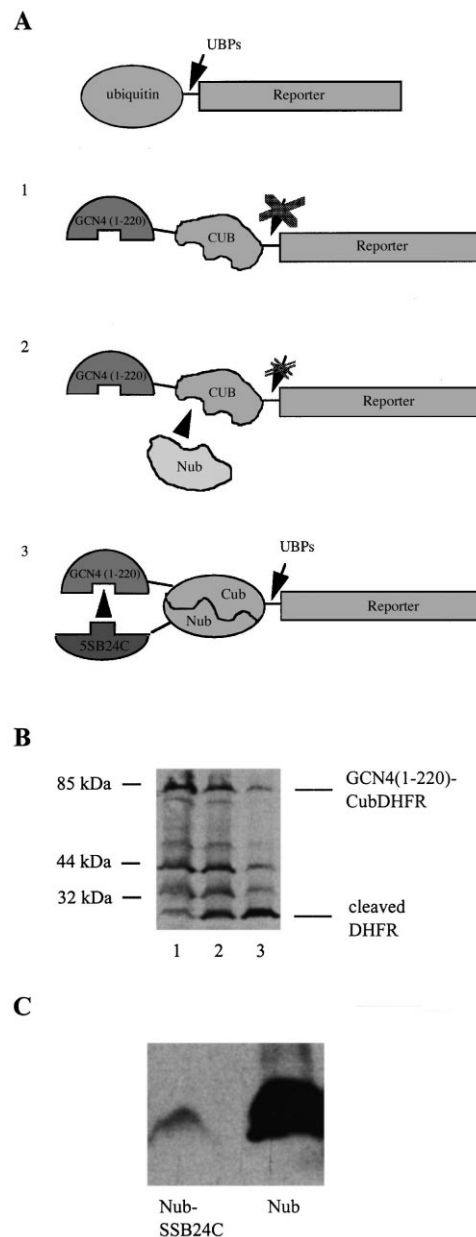


Fig. 3. SSB24C and GCN4 interact *in vivo*. A: Schematic representation of the split ubiquitin assay: When a protein is marked with ubiquitin, *ubiquitin specific protease* will cleave C-terminal of the ubiquitin moiety. If only the C-terminal half of ubiquitin (Cub) is fused to the protein, there will be hardly any cleavage (lane 1). When the N-terminal half (Nub) of ubiquitin is coexpressed, some molecules will reassociate, leading to a slightly enhanced cleavage rate (lane 2). If, however, the Nub moiety is fused to a protein that interacts with a protein fused to the Cub reporter construct, this interaction will bring the two halves of ubiquitin together, resulting in more prominent cleavage. B: Western blot showing interaction between SSB24C and GCN4(1–220). Yeast cells expressing GCN4(1–220)-Cub-DHFR (lane 1) or GCN4(1–220)-Cub-DHFR in combination with Nub alone (lane 2) or Nub-SSB24C (lane 3) were boiled with SDS sample buffer and loaded on a gel for Western blotting with anti-HA antibody. The positions of the full length Cub fusion and the cleaved HA-DHFR product are marked. C: Western blot with anti-HA antibody showing the expression level of Nub-SSB24C and Nub in strain JD55.

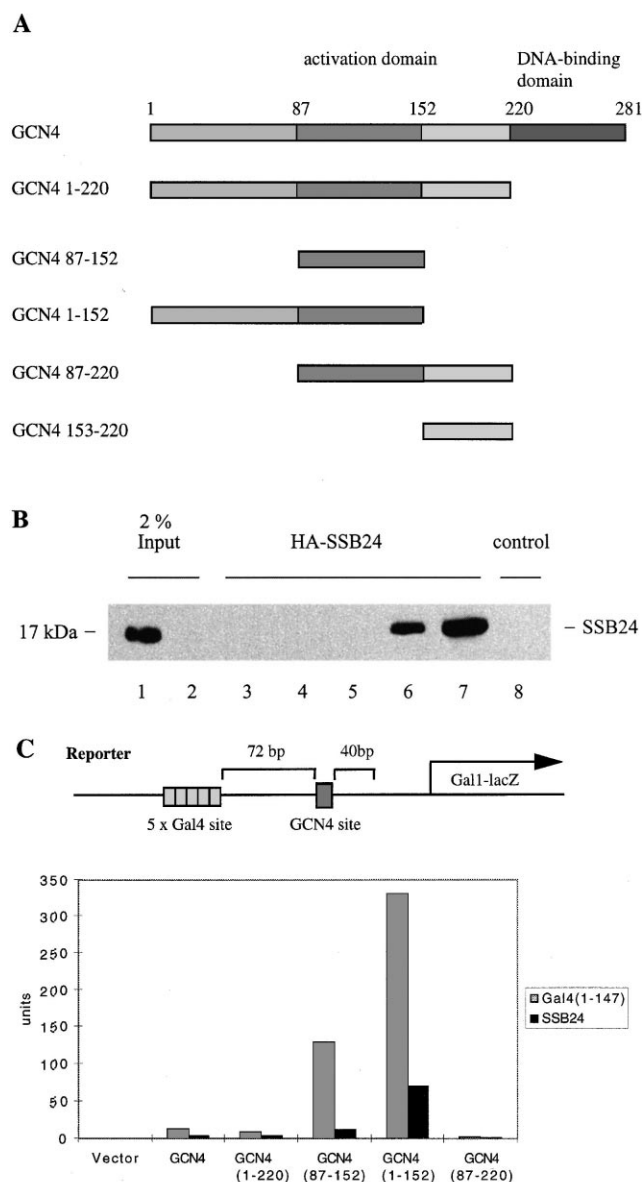


Fig. 4. Deletion analysis of GCN4. A: Schematic representation of the GCN4 derivatives used. B: In vitro interaction of SSB24 with GCN4 requires the domain C-terminal of the activation domain. Bacterial lysates containing HA-tagged SSB24 (lanes 3–7) or control lysate (lane 8) were incubated with GST fusion proteins coupled to glutathione sepharose beads. Bound protein was detected by Western blotting using anti-HA antibody. Lanes 1 and 2 show 2% input of the HA-SSB24 and control extracts, respectively. The GST fusions used are as follows: Lane 3, GST; lane 4, GST-GCN4(1–152); lane 5, GST-GCN4(87–152); lane 6, GST-GCN4(87–220); lanes 7, 8, GST-GCN4(153–220). C: Repression of different GCN4 derivatives by SSB24. β -galactosidase activity was assayed from a strain with an integrated reporter containing five Gal4 sites upstream of a single LexA site (depicted at the top). Gal4(1–147)-SSB24 (black columns) or Gal4(1–147) alone (grey columns) and the indicated LexA-GCN4 fusions (compare Fig. 5A) were expressed from plasmids.

220 versus 330 units for GCN4 1–152, Fig. 4C). However, all constructs are repressed by SSB24. Therefore, interaction with the activator does not seem to be important for repression by SSB24, because a GCN4 derivative lacking the SSB24 interaction domain (GCN4 1–152) is still repressed efficiently.

3.4. SSB24 also represses transcription activated by SRB2

While we could not detect interaction between SSB24 and the activation domain of GCN4 in our in vitro assay, it seemed possible that a relevant weak interaction exists when the two proteins are placed in close proximity by binding to the promoter region together. Indeed, we observed a weak interaction between SSB24C and the activation domain of GCN4 alone (aa 87–152) in the split ubiquitin in vivo assay (data not shown). In order to test if such a weak interaction might be responsible for the repression by SSB24, we sought to repeat the repression experiments using an activator completely deficient in SSB24 interaction. Typical yeast activator proteins have an acidic activation domain and may thus, like GCN4, interact weakly with the positively charged SSB24. We therefore used a LexA fusion of SRB2 which, like nearly all holoenzyme components tested, enhances transcription when tethered to a promoter, presumably by recruiting the holoenzyme complex [27–29]. As shown in Fig. 5A, SRB2 does not interact with SSB24 in the split ubiquitin in vivo assay. None-

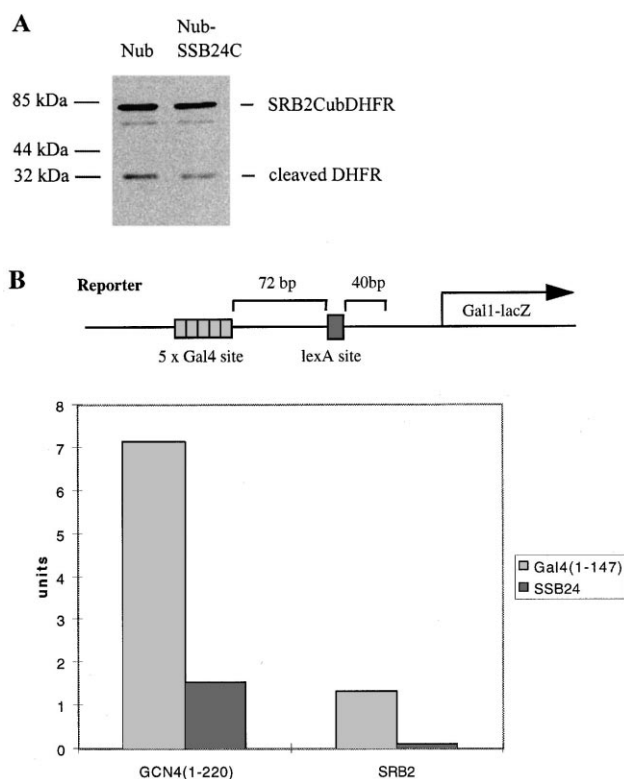


Fig. 5. SSB24 represses transcription activated by SRB2. A: SSB24 does not interact with SRB2 in the split ubiquitin assay. Extracts were prepared from cells expressing SRB2-Cub-DHFR in combination with Nub alone (left) or Nub-SSB24 (right). Aliquots were loaded on a gel for Western blotting with an anti-HA antibody. The position of the full length Cub fusion and the cleaved product is indicated. The percentage of cleavage in the presence of Nub-SSB24 is not enhanced compared to Nub alone (19 and 17% cleaved, respectively, as quantitated with a lumimager (Boehringer)). B: Transcription activated by LexA-SRB2 is efficiently repressed by SSB24. A yeast strain with the integrated reporter indicated at the top was transformed with LexA fusions of GCN4(1–220) or SRB2 as indicated and the Gal4(1–147) alone (grey columns) or fused to SSB24 (black columns), and β -galactosidase activity was determined. Transcription activated by LexA-SRB2 was at least as efficiently repressed by SSB24 as the Lex-GCN4(1–220) control.

theless transcription activated by a LexA-SRB2 fusion is repressed by SSB24 (Fig. 5B). We conclude that SSB24 repression is not triggered by interaction with the activator. An alternative mechanism would be non-specific binding to the negatively charged DNA backbone, which may interfere with the binding and/or function of (an)other factor(s).

4. Discussion

Protein-protein interactions play a key role in transcriptional regulation. Activators interact with their target proteins to open up chromatin or to transmit signals to the transcriptional machinery. However, interaction with another protein can also impair a transcriptional activator's ability to enhance mRNA synthesis. For example, such a scenario has been reported for pairs of activators which can, when bound together to specific promoters, cancel each other out. In particular, the glucocorticoid receptor can impair AP-1 as well as NFκB activation function in certain cases [30,31].

A mechanism involving direct interaction with the activator protein has also been proposed for the *Drosophila* repressor snail. This was based on the observation that efficient repression by snail requires the binding sites to be located within 100 bp of the activator sites [15]. Snail does indeed interact with dorsal, one of the activators of the rhomboid enhancer which is repressed by snail, in vitro (A.W. and N.L., unpublished observation). However, snail can also repress transcription from a promoter proximal position independent of activator sites [32]. It has recently been established that snail can interact with the corepressor dCtBP [33].

In a similar way as snail, the artificial repressor SSB24 had also been shown to lose its repression potential when the binding sites were removed far away from the activator sites [16]. Its high positive charge suggested that it might interact with the negatively charged activator GCN4, which is repressed by SSB24. We show that SSB24 and GCN4 do interact in vitro and in vivo and that the interaction is ionic in nature. However, a deletion analysis of GCN4 revealed unexpectedly that the interacting domain is not the highly charged activation domain but the adjacent domain towards the C-terminus. Consequently, it was possible to construct a GCN4 derivative lacking the interaction domain but retaining the ability to activate transcription. Because this activator construct was still repressed efficiently by SSB24, the interaction between SSB24 and GCN4 cannot be the mechanistic background of repression by SSB24. Similarly, transcription activated by a LexA fusion of SRB2, a holoenzyme component unable to interact with SSB24, was repressed, which suggests that SSB24 repression is not specific to acidic activators.

While SSB24 interaction with the activator was shown not to be relevant for its repression function, it does not target either the general corepressors Ssn6/Tup1 or the holoenzyme component SRB10 ([16] and this study). We cannot rule out the possibility that SSB24 makes contact to other proteins which may in turn affect transcriptional activity. However, SSB24 may act by a different mechanism, namely by interacting with the negatively charged phosphate backbone of promoter DNA. Such a mechanism would explain why a large spacing between activator and repressor sites results in loss of repression. Interestingly, a lac-repressor fusion of the basic peptide LysAla, which had been designed to mimic SSB24 [16], resulted in enhanced repression by the dimeric form of

lac repressor [34]. This was explained by the ability of the mutant lac repressor to make non-specific contacts to DNA via the basic tail. SSB24 may therefore simply block binding of the activator or other factors to the promoter region by binding and distorting the DNA helix.

While we could show interaction between GCN4 and SSB24 with a variety of in vitro and in vivo methods, our transcription assays indicate that this interaction is irrelevant for the observed function of SSB24. We conclude that demonstration of protein-protein interactions has to be treated with care until a functional assay for the in vivo situation is available.

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