Evolutionary conserved mechanism of transcriptional repression by even-skipped

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ABSTRACT

Even-skipped (Eve) is a transcriptional repressor involved in segment formation in *Drosophila melanogaster*. In order to gain further insights into the mechanism of action of Eve we tested whether it would function as a transcriptional repressor in mammalian cells. We found that Eve was indeed a potent repressor in two different mammalian cell types and at several promoters. *In vitro* transcription assays confirmed that Eve directly represses transcription initiation when specifically targeted to a promoter. We also found that, unlike the case with transcriptional activators, Eve does not repress transcription synergistically. Analysis of the effect of Eve on preinitiation complex assembly in a crude HeLa cell nuclear extract demonstrated that the Eve repression domain functions by preventing the assembly of TFIID with the promoter. Our data support the hypothesis that Eve contains an active repression domain that functions specifically to prevent preinitiation complex formation.

INTRODUCTION

Transcription of a gene by RNA polymerase II (pol II) requires the assembly of the general transcription factors (GTFs) at the promoter to form a preinitiation complex (reviewed in 1,2). This begins with the recognition of the TATA element by the GTF TFIID, a complex of TATA-binding protein (TBP) and associated factors (TAFs). This provides a platform for the assembly of TFIIA and TFIIB. Pol II then enters the complex in association with TFIIF and the PIC is completed by TFIIE and TFIIH. An alternative pathway of PIC assembly involving GTFs pre-complexed with Pol II (a holoenzyme) has also been described (3).

Before PIC assembly can occur, the chromatin associated with the DNA must be remodelled in order to provide access to the promoter. Much progress has been made in our understanding of this process in the last few years (reviewed in 4). Of particular significance is the affect of histone acetylation on nucleosome structure. Acetylation of histone tails perturbs their tight association with DNA, thus providing access to the DNA by the transcription machinery (reviewed in 4–6). Several enzymes have been described that possess the ability to either acetylate or deacetylate histones. Furthermore, transcriptionally active

regions of the genome are generally associated with a high density of histone acetylation. In addition, other factors are able to modify nucleosome structure by ATP-dependent mechanisms (reviewed in 4).

The transcription rate of a gene is subject to tight control by factors that either stimulate transcription (activators) or inhibit transcription (repressors). Transcriptional regulation is believed to involve both the modulation of nucleosome structure and direct effects on the rate/extent of PIC assembly (4–10). Several transcriptional activator proteins have been shown to interact with proteins that possess intrinsic histone acetyl transferase activity, e.g. p300/CBP (11). Moreover, some transcriptional repressors have been found to associate with histone deacetylases (6,9).

With regard to direct effects on PIC formation, our understanding of how activators function is far more developed than that of repressors (7,8,10). Transcriptional activators facilitate PIC assembly by forming productive interactions (either directly or via coactivator proteins) with components of the general transcription machinery (7,8). Generally, several activator proteins are required at a promoter to elicit efficient transcription stimulation. This synergy is believed to arise from the requirement for activation domains to interact with several components of the transcription machinery.

Drosophila even-skipped (Eve), a transcriptional repressor protein, is involved in regulating genes that control segment formation in the early embryo (12). Eve can repress both basal transcription and the high levels of transcription mediated by activator proteins (e.g. VP16; 13). Several studies in *Drosophila* cells and using *Drosophila* nuclear extracts *in vitro* have demonstrated that Eve can inhibit PIC formation (14–17). Eve can prevent the association of purified TFIID with the TATA element, but has no effect on template-committed TFIID. The mechanism by which Eve prevents the association of TFIID with the TATA element has been a subject of debate. In one model, Eve associates with upstream promoter elements and prevents the association of TFIID with TATA via a direct interaction with the TBP subunit of TFIID (15). However, others have proposed that Eve indirectly prevents TFIID association with the TATA element by the interaction of Eve with low affinity DNA sites that flank the TATA element (16). In support of this hypothesis, Eve requires an intact DNA binding domain (homeodomain) to repress transcription, even when directed to the promoter via a heterologous DNA binding domain. Transcriptional repression by Eve is regulated by phosphorylation, which prevents Eve from associating with

TBP (18). Interestingly, the site of phosphorylation lies outside the region required for repression and TBP-binding, suggesting an allosteric mechanism of regulation of the repression domain.

In this study, we set out to determine if the Eve repression domain can function in mammalian cells. We found that this was indeed the case, suggesting that Eve functions by an evolutionary conserved mechanism. Both *in vivo* and *in vitro* experiments suggest that the Eve repression domain functions only when directed to a promoter by a DNA binding domain that associates with upstream promoter elements. We demonstrate that, unlike transcriptional activators, Eve does not function synergistically to activate transcription. Finally, we show that Eve can inhibit the association of TFIID with the promoter in a crude nuclear extract.

MATERIALS AND METHODS

Plasmids

GAL4-Eve was constructed by cloning a *Xmn*I fragment of Eve encoding residues 89–376 into the 6HIS GAL4-fusion expression vector pRJR1 (19). The GAL4-Eve was then cloned by PCR amplification into pCDNA3 for transient transfection assays. G0 and G5TKCAT were a kind gift of Andy Bannister and Tony Kouzarides. The HIV LTR CAT reporters have been described previously (20). Most of the E4 promoters for both *in vitro* transcription and transient transfection analysis have been described before (21). However, G1E4CAT was constructed by cloning the insert from G1E4T into the pCAT basic vector (Promega). Expression plasmids for TFIIB and human TBP (hTBP) were constructed by PCR of the coding sequence with primers that produced 5' *Bam*HI and 3' *Eco*RI termini. The products were then cloned into pET5a which placed a T7 epitope tag at the N-terminus.

Transfections

Human embryonic kidney 293 cells and mouse NIH 3T3 cells were cultured as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum, 5 mM L-glutamine, 100 mg/ml streptomycin and 100 U/ml penicillin. Cells were transfected in 90 mm dishes at 50% confluency using calcium phosphate as described previously (21). Forty-eight hours after transfection the cells were harvested and CAT assays were performed with cleared whole cell extracts. Assays were quantitated by phosphorimager and are presented relative to the activity of the reporter cotransfected with pCDNA3. The total amount of pCDNA3 backbone transfected is equal in all cases. For immunoblots, equal volumes of cells were lysed by the addition of SDS–PAGE loading buffer. After electrophoresis and transfer to immobilon P membrane (Millipore), immunoblotting was performed with anti-GAL4 antibody (raised against His-tagged GAL4 DNA binding domain by the Scottish antibody production unit). Detection was performed by chemiluminescence (ECL, Amersham). All transfections were performed at least three times.

In vitro transcription assays

6HIS GAL4 fusion proteins were prepared as described in Reece *et al.* (19). GAL4-AH was purified as described previously (22). *In vitro* transcription assays were performed as described

previously using HeLa cell nuclear extract (22; Computer Cell Culture Centre, Mons, Belgium).

Complex assembly and interaction assays

The Plasmid G5E4T, containing five GAL4 sites upstream of the promoter was cleaved with *Eco*RI and the ends filled in with Klenow in the presence of biotinylated dUTP. The plasmid was then cleaved with *Hin*dIII and the *Hin*dIII/*Eco*RI fragment isolated and purified. This biotinylated fragment was then immobilised on Streptavidin magnetic beads (Dynal) at a concentration of 20 ng G5E4T fragment/µl beads as described (23). Immobilised G5E4T in transcription buffer (12 mM HEPES pH 8, 12% glycerol, 60 mM KCl, 0.12 mM EDTA pH 8, 0.5 mM DTT and 0.05% NP-40) and 1 µg of either GAL4 or GAL4-Eve was incubated at 30°C for 30 min with constant agitation. Beads were washed three times in transcription buffer to remove the unbound GAL4-fusion. HeLa nuclear extract (100 µl) was added to the beads along with 0.5 ml transcription buffer, incubated at 30°C for 1 h with constant agitation and washed three times in transcription buffer. To determine if GAL4-Eve removed TFIID once bound, the above assay was repeated except the GAL4 fusions were added 30 min after the HeLa nuclear extract. Complexes were resolved by SDS–PAGE, transferred to Immobilon P and probed using α-TBP antibody.

Bacterial lysates containing T7-tagged human TFIIB and TBP were made by induction of 10 ml log-phase BL21 DE3 cultures with 1 mM IPTG for 3 h at 37°C. The bacteria were harvested and resuspended in 1 ml buffer D [20 mM HEPES pH 8, 20% (v/v) glycerol, 0.2 mM EDTA, 100 mM KCl, 1 mM DTT, 0.2 mM PMSF]. After sonication with a microtip, the debris was removed by centrifugation in a microfuge for 10 min. GST-fusion proteins were prepared as described previously (24). Bacterial lysate (2 µl) was incubated with 25 µl glutathione agarose beads containing 1 µg GST-fusion protein in 0.6 ml binding buffer [40 mM HEPES pH 8, 10% (v/v) glycerol, 150 mM KCl, 5 mM MgCl$_2$, 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF] for 2 h at 4°C. The beads were washed four times with binding buffer and bound proteins eluted with SDS–PAGE loading dye. Proteins were resolved by 12% SDS–PAGE and transferred to Immobilon P membrane (Millipore). Immunoblotting was performed with anti-T7 antibody (Novagen) and detected by chemiluminescence (ECL, Amersham).

RESULTS

We set out to determine if the repression domain of Eve could function in mammalian cells. The repression domain of Eve previously described as necessary and sufficient to direct maximal repression in *Drosophila* cells (15–17) was fused to the DNA binding domain of the yeast GAL4 protein (residues 1–93) under the control of a CMV promoter (Fig. 1A). This construct was transfected into human embryonic kidney 293 cells and expression monitored by immunoblotting whole cells extracts with an anti-GAL4 antibody (Fig. 1B). A GAL4-fusion protein of the expected molecular weight was detected in cells transfected with the GAL4-Eve construct. We first tested the effect of GAL4-Eve on the thymidine kinase (TK) promoter of Herpes simplex virus, which contains five GAL4 DNA-binding sites upstream of elements that drive a high intrinsic level of transcription (G5TKCAT; Fig. 1C, left
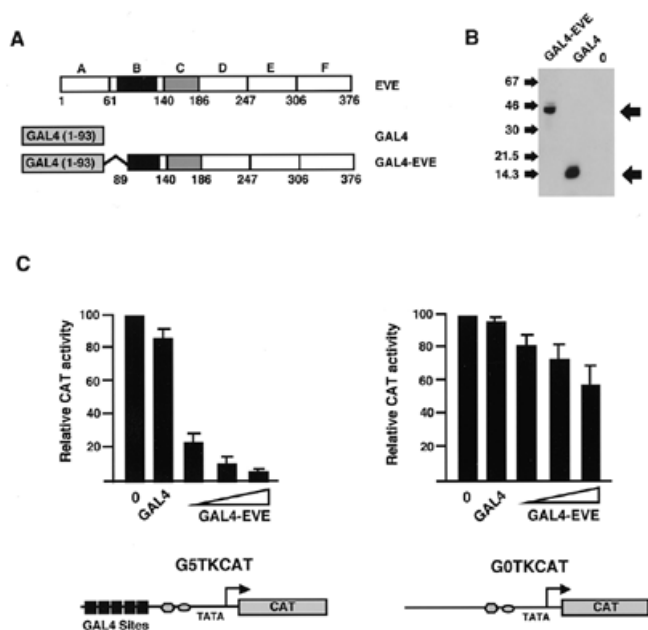
Figure 1. The Eve repression domain functions in mammalian cells. (**A**) Diagram of Eve showing the previously described subdomains (labelled A–F). The regions previously shown to be required for transcriptional repression include: B, homeodomain; C, alanine-rich region; D, proline-rich region (from 13). The GAL4-fusion constructs are shown below. (**B**) Expression vectors containing either GAL4 (1–93) alone or GAL4-Eve were transfected into 293 cells. Cells were harvested, lysed and the expression of GAL4 and GAL4-Eve detected by immunoblotting with anti-GAL4 antibody. Molecular weight markers (kDa) are shown at the left. (**C**) 0.5 µg of the reporters G5TKCAT (left) or G0TKCAT (right) were transfected into 293 cells with either empty expression vector or expression vectors containing either GAL4 (2.5 µg) or GAL4-Eve (0.1, 0.5 and 2.5 µg). Graphs shown as CAT activity relative to empty expression vector and are the average of three experiments.

Figure 2. Characterisation of Eve-mediated repression in mammalian cells. (**A**) 0.5 µg G5TKCAT was transfected into NIH 3T3 cells along with 1 µg empty expression vector or vector containing GAL4 or GAL4-Eve. The graph shows CAT activity relative to empty expression vector. (**B**) 293 cells were transfected with 0.5 µg G6HIVLTR along with 1 µg empty expression vector or vector containing GAL4 or GAL4-Eve. A diagram of the reporter construct is shown below. (**C**) 0.5 µg of the reporters E4CAT, G1E4CAT or G5E4CAT were transfected into 293 cells with either empty expression vector or expression vectors containing either GAL4 (1 µg) or GAL4-Eve (0.2 and 1 µg). Graphs shown as CAT activity relative to empty expression vector and are the average of three experiments.

panel). While transfection of a construct expressing the GAL4 (1–93) DNA binding domain alone had little effect on CAT activity, transfection of increasing amounts of the GAL4 (1–93)-Eve construct caused a dose-dependent repression of transcription. Figure 1C (right panel) shows a control experiment in which the transfection was performed as in the left panel, but using an identical TK core promoter that lacks the GAL4 DNA-binding sites. We observed a smaller dose dependent repression of transcription consistent with results obtained from *Drosophila* cells (15). Thus, the *Drosophila* Eve repression domain can function in mammalian cells when specifically targeted to an active core promoter.

We next tested whether the Eve repression domain was functional in another mammalian cell type. Mouse NIH 3T3 cells were transfected with the G5TKCAT reporter along with either the GAL4-Eve construct or the control GAL4 (Fig. 2A). As we had observed in 293 cells, GAL4-Eve effectively repressed the TK reporter in NIH 3T3 cells. We next tested another intrinsically active promoter, the HIV LTR, for repression by Eve. A CAT reporter construct containing the HIV LTR downstream of six GAL4 sites was transfected into 293 cells alone and with an expression construct containing GAL4 (1–93) or GAL4-Eve. GAL4-Eve was also able to repress this promoter
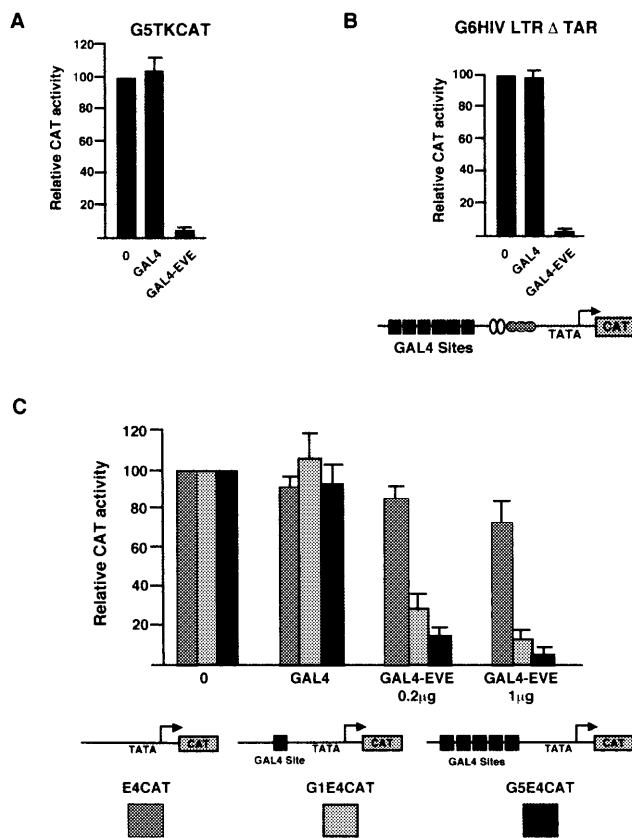
(Fig. 2B), but not an HIV LTR reporter construct that lacks GAL4 sites (data not shown). Thus, the Eve repression domain is functional in different mammalian cell types and also at different core promoters.

As mentioned above, the HSV TK and HIV LTR promoters exhibit an intrinsically high transcriptional activity, due to the presence of binding sites for cellular transcriptional activator proteins. Thus, the repression we observed may be due to either interference with these transcriptional activators or direct effects of the general transcription machinery. Studies in *Drosophila* suggest that Eve can repress basal activator-independent transcription (15–18). Furthermore, previous studies with Eve suggested that it might repress transcription synergistically (17). We next tested the effect of GAL4-Eve on

the minimal adenovirus E4 promoter, which does not contain any known binding sites for cellular transcriptional activators. Figure 2C shows the effects of transfection of increasing amounts of GAL4-Eve expression construct on the Ad E4 promoter (E4CAT) compared with the same promoter containing either one (G1E4CAT) or five (G5E4CAT) GAL4 DNA-binding sites. As we observed with the TK promoter, GAL4-Eve caused a low level of repression in the absence of GAL4 sites. However, there was a significant level of transcriptional repression when either a single or multiple GAL4 sites were present. Although the level of repression observed in the presence of five GAL4 sites was marginally greater than that observed with a single GAL4 site, these data suggest that the Eve repression domain does not function synergistically. Thus, the Eve repression domain represses a basal-level core promoter in mammalian cells in a non-synergistic manner.

The above studies demonstrated that the repression domain of Eve could function in mammalian cells. We next sought to determine if the repression domain of Eve could function directly on transcription initiation of a naked DNA template *in vitro*. Histidine-tagged fusions of GAL4 (1–93)-Eve and GAL4 (1–93) were expressed in bacteria and purified by nickel affinity chromatography. Figure 3A shows a Coomassie stained SDS–PAGE gel of the proteins, with the intact GAL4 derivatives indicated. GAL4 or GAL4-Eve was then incubated with a DNA template containing the E4 promoter downstream of five GAL4 sites and HeLa cell nuclear extract added followed by ribonucleotides. Transcripts were detected by primer extension with a radiolabelled oligonucleotide specific to the E4 RNA followed by denaturing electrophoresis. Figure 3B shows an autoradiograph of a representative assay. While the GAL4 DNA binding domain alone had marginal effects on basal transcription at the E4 promoter, GAL4-Eve significantly repressed transcription initiation. To confirm that the repression we observed involved specific promoter targeting of the GAL4-Eve construct, we compared the effect of GAL4 Eve on the E4 promoter in the absence and presence of GAL4 DNA binding sites. The transcriptional activator GAL4-AH was also included as a control for GAL4-mediated transcription effects. Neither GAL4-Eve nor GAL4 AH had any effect on transcription at the E4 promoter in the absence of GAL4 DNA-binding sites. However, in the presence of GAL4 sites GAL4-Eve completely repressed transcription and GAL4-AH activated transcription. Thus, Eve represses transcription of the E4 promoter *in vitro* in a manner dependent on specific recognition of upstream DNA-binding sites via the GAL4 domain.

Transcriptional activator proteins can be grouped in to those that are only able to function in a promoter proximal manner and those that can function when located at a distance from the TATA element (25). We therefore tested the activity of GAL4-Eve on the G5E4T promoter containing a 160 bp insert between the GAL4 sites and the TATA element (G5$_{I160}$E4T). Figure 4A shows that GAL4-Eve represses transcription even when located at a distance from the TATA box. In contrast, as has been reported before, the activator GAL4-AH was unable to function at a distance (22,26). Our initial studies *in vivo* suggested that GAL4-Eve can repress transcription of promoters that are intrinsically activated by cellular transcription factors. We were able to test this directly *in vitro* using a previously characterised E4 promoter derivative that contains a binding site for the cellular transcriptional activator ATF (G5$_{I160}$AE4T)
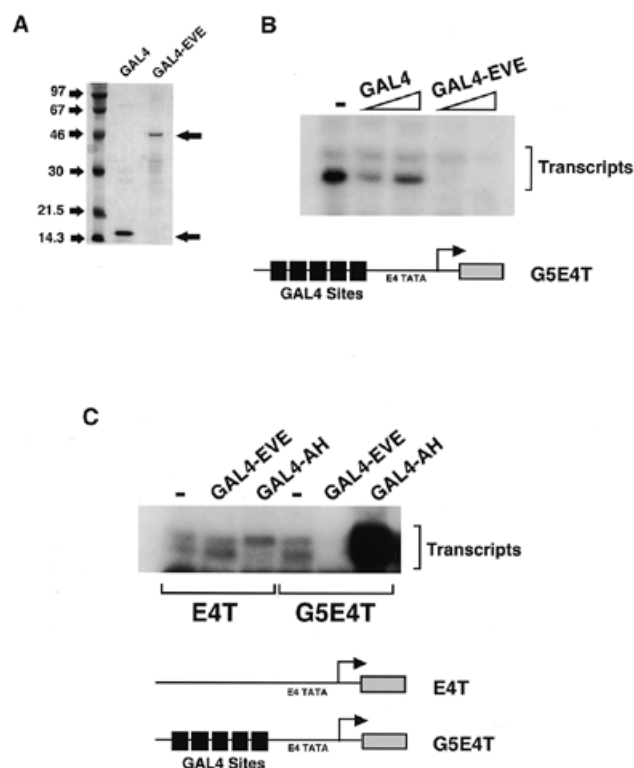

Figure 3. Repression by GAL4-Eve *in vitro*. (**A**) GAL4 and GAL4-Eve were expressed and purified from bacteria as His-tagged proteins and analysed by SDS–PAGE and Coomassie staining. Molecular weight markers (kDa) are shown at the left and an arrow at the right indicates the intact GAL4 derivatives. (**B**) GAL4 or GAL4-Eve (0.1 and 0.5 μg) were bound to the G5E4T promoter and HeLa nuclear extract with transcription buffer added to initiate transcription. Correctly initiated transcripts were detected by primer extension. A diagram of the promoter DNA template is shown below. (**C**) The indicated promoters were incubated with 0.5 μg of GAL4, GAL4-Eve or GAL4-AH and tested in transcription assays as in (B).

in addition to GAL4 DNA-binding sites (22). Figure 4B shows that transcription of G5$_{I160}$AE4T is efficiently repressed by GAL4-Eve in a dose-dependent manner. Thus, the Eve repression domain can inhibit both basal transcription and transcription under the control of cellular transcriptional activator proteins *in vivo* and *in vitro*.

Our transient transfection analysis suggested that GAL4-Eve requires only a single DNA-binding site to repress transcription. Previous studies with Eve *in vitro* suggested that it may repress transcription synergistically (17). However, as these studies used the native Eve protein, the authors were unable to rule out co-operative DNA binding as the source of the synergy rather that an effect mediated by the repression domain. The reporter constructs we have used in the *in vitro* transcription assays above contain multiple GAL4 sites. We therefore next tested if GAL4-Eve needs to work synergistically to repress transcription *in vitro*. Figure 5A shows transcription reactions comparing the effect of GAL4-Eve on the E4 promoter in absence or presence of either one or five GAL4-DNA binding sites. As before, GAL4-Eve had no effect on an E4 promoter construct that lacks GAL4 sites, but efficiently repressed transcription in the presence of five
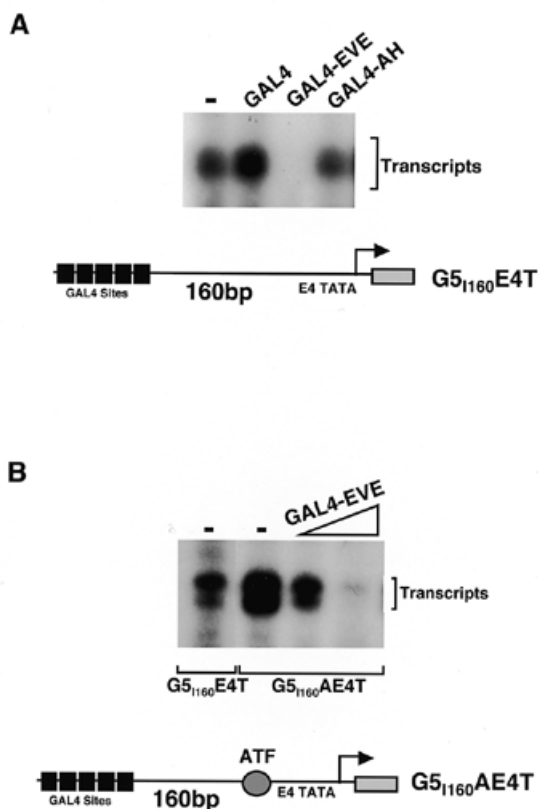
Figure 4. Characterisation of Eve repression *in vitro*. (**A**) The reporter G5$_{I160}$E4T was incubated with 0.5 µg of GAL4, GAL4-Eve or GAL4-AH and transcription assays performed as described in Figure 3. (**B**) The reporter G5$_{I160}$AE4T, containing an ATF-binding site was tested in a transcription assay as described in (A). G5$_{I160}$E4T was included as a control for basal transcription.

Figure 5. GAL4-Eve does not act synergistically. (**A**) The indicated reporter constructs were incubated with 0.5 µg GAL4-Eve and tested in *in vitro* transcription assays as described in Figure 3. (**B**) As in (A) except that 0.5 µg GAL4-AH was used.

GAL4 DNA binding sites. In agreement with our *in vivo* observations, GAL4-Eve also efficiently repressed transcription when only a single GAL4 site was present upstream of the E4 promoter. For comparison, the effects of GAL4-AH on the same promoter constructs are shown in Figure 5B. As is well documented, GAL4-AH requires multiple DNA binding sites to elicit strong transcriptional activation (22,23,27). Thus, the Eve repression domain does not need to function synergistically either *in vivo* or *in vitro* when directed to the promoter by a heterologous DNA binding domain.

Studies in *Drosophila* systems have demonstrated that Eve interacts with the TBP and prevents its interaction with the TATA element (15,17). These previous experiments used purified TFIID or TBP. We therefore next tested the effect of GAL4-Eve on the assembly of TFIID at the promoter in a system using crude HeLa cell nuclear extract. A DNA fragment containing G5E4T was immobilised on Streptavidin-magnetic beads via a biotin moiety and used in PIC assembly assays. GAL4 or GAL4-Eve was bound to the immobilised promoter DNA and the unbound portion washed away. The beads were then incubated with HeLa nuclear extract in transcription buffer in the absence of ribonucleotides. Complexes were purified by washing the beads in transcription buffer and the assembly of
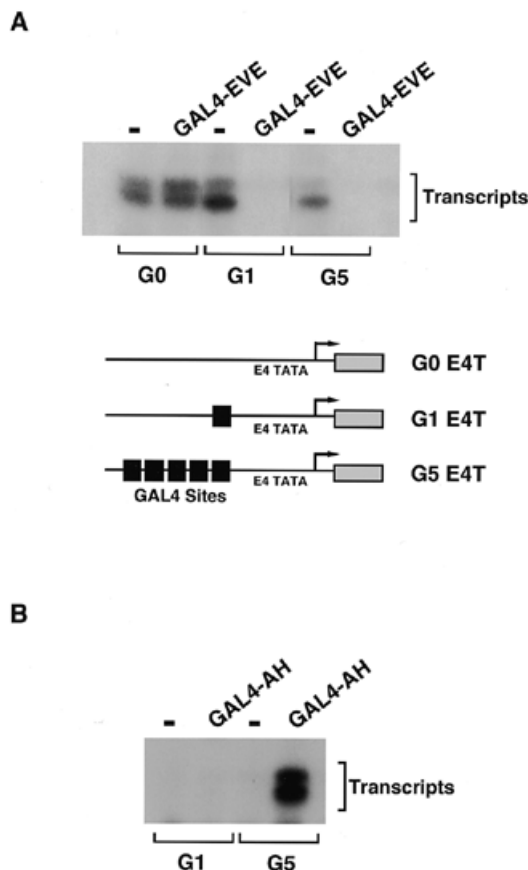
TFIID with the promoter was then assessed by immunoblotting with anti-TBP antibody (Fig. 6A). In the absence of any GAL4-derivative TFIID efficiently assembled with the immobilised G5E4T fragment, but not with magnetic beads lacking DNA (–). However, when GAL4-Eve was pre-bound to the promoter DNA there was a significant reduction in the assembly of TFIID that was not observed with the control GAL4 (1–93). Thus, GAL4-Eve prevents the association of TFIID with the promoter in a crude nuclear extract. We also tested the effect of GAL4-Eve on pre-formed PICs. Nuclear extract was incubated with the immobilised DNA template and PICs allowed to form, GAL4 or GAL4-Eve was then added and the incubation continued. Complexes were purified by washing the beads and then assessed for TFIID content. The data show that when we incubated the immobilised DNA with nuclear extract first and added GAL4-Eve after preinitiation complexes had formed GAL4-Eve had no effect on the amount of TFIID assembled at the promoter.

Because Eve can prevent the assembly of TFIID at the Ad E4 promoter we next tested in a GST pull down assay if the recombinant human GTFs TFIIB and TBP could interact with GST-EVE. Figure 6B shows that, consistent with previous results (15,17), GST-Eve bound hTBP. In comparison, TFIIB
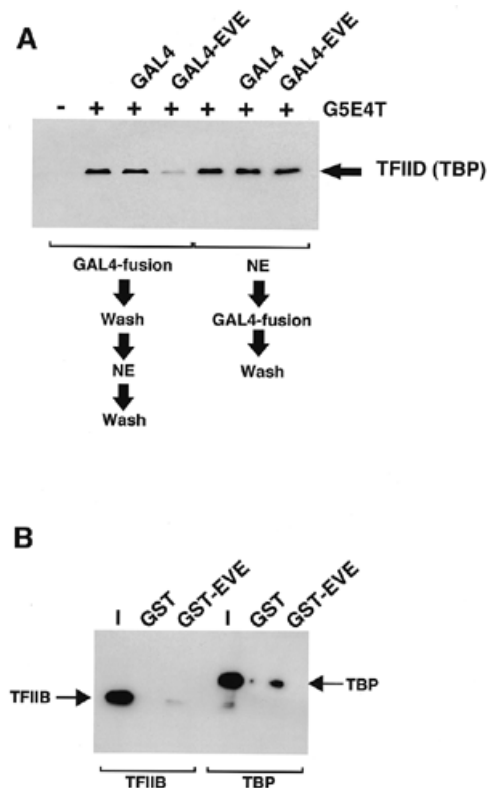
Figure 6. Eve prevents the assembly of TFIID with the promoter. (**A**) G5E4T was immobilised on magnetic beads as described in Materials and Methods and was incubated with the indicated GAL4-fusion proteins either before or after nuclear extract (as per schematic below autoradiogram). Complexes were purified, subjected to SDS–PAGE and immunoblotted with anti-TBP antibody to detect the presence of TFIID. (–) indicates magnetic beads that do not contain the promoter DNA fragment. (**B**) Bacterial lysates containing epitope tagged TFIIB or TBP were incubated with either GST or GST-Eve. After extensive washing the bound proteins were detected by immunoblotting with anti T7 antibody. I represents 10% of the input bacterial lysate.

bound very weakly to the GST-Eve beads. Thus, the Eve repression domain interacts specifically with TBP, consistent with the functional data we obtained showing that TFIID assembly is inhibited by Eve.

DISCUSSION

In this study we have demonstrated that *Drosophila* Eve represses transcription by a mechanism that is conserved in mammalian cells. Efficient repression by GAL4-Eve required the presence of GAL4 DNA-binding sites associated with the promoter and was also not distance sensitive. Eve repressed basal and activated transcription both in mammalian cells and nuclear extracts. We provide evidence that, unlike many transcriptional activators, Eve does not act synergistically. Furthermore, in a crude nuclear extract, the Eve repression domain inhibits the association of TFIID with the promoter, providing a molecular basis for transcriptional repression we observed.

Although the general transcription machinery is highly conserved between *Drosophila* and mammals there are some mechanistic differences. *Drosophila* and hTBP are interchangeable in *in vitro* transcription systems. However, human TFIIB can only substitute for *Drosophila* TFIIB at some promoters, but not others (28). We have found that Eve can repress transcription in different mammalian cell types and at several different core promoters. It is therefore likely that the target factor(s) of Eve is functionally conserved between *Drosophila* and mammalian cells. Our work and that of others suggests that TFIID assembly is a target of the Eve repression domain (15–17). As mentioned above TBP is functionally conserved between *Drosophila* and human cells. It is likely therefore that the TBP component of TFIID is the direct target contacted by Eve. Consistent with this we found that the Eve repression domain interacts with hTBP with a much greater affinity than with human TFIIB.

As mentioned previously, Eve has been suggested to function in a manner involving low affinity interactions with non-specific sites in the promoter region (16). Although we did observe a low level of repression by GAL4-Eve *in vivo* in the absence of GAL4 sites, there was a significant increase in repression in the presence of GAL4 sites. We did not observe this non-specific repression *in vitro*. Like other homeodomain proteins, Eve recognises a low consensus DNA-binding site, which is likely to be a significant contributor to repression at some promoters (29). However, our data support the idea that the Eve repression domain can function in a manner dependent upon upstream promoter elements.

Previous studies with Eve in transcription systems containing purified components have demonstrated that Eve blocks the interaction of TFIID (and TBP) with the TATA element (16,17). Studies of transcriptional activation have led to different requirements for cofactors in purified versus crude systems (reviewed in 30). These disparities probably arise from redundancies in the mechanisms by which transcription can be regulated. Purified TFIID exhibits a lower DNA-binding affinity compared to the TFIID in nuclear extracts. Indeed, activator-mediated recruitment of TFIID at the E4 promoter is observed in purified systems (31,32), but not in a crude nuclear extract (23,24,26). Thus, it is important to show that Eve can prevent the assembly of TFIID in the presence of other factors that can modulate the assembly of TFIID. Our experiments to determine the mechanism of action of Eve on PIC assembly were performed using crude nuclear extracts. In this system GAL4-Eve was able to prevent the association of TFIID with the TATA element, but had no effect when PICs were allowed to form first. Because our experiments were performed in a crude nuclear extract, we believe they strongly indicate that Eve-mediated inhibition of TFIID assembly is at least part of the mechanism by which Eve functions *in vivo*.

Our findings suggest that the Eve repression domain does not need to function synergistically. A previous study *in vitro* using intact Eve protein and Eve DNA binding sites found that the level of repression was dependent upon the number of Eve binding sites (17). In that study the authors could not rule out co-operative DNA binding rather than repression domain synergy as the effect observed. Indeed, Eve does bind DNA in a co-operative manner (29). The rationale behind activator synergy is that activation domains need to contact several components of the transcription machinery to enhance the assembly of several factors to the promoter (8,9). On the other hand, transcriptional repression may only require a block in

one step of PIC formation in order to elicit a negative effect. It is therefore possible that Eve needs only make one contact with the forming PIC in order to disrupt the process. TFIID assembly may indeed be this step. However, as our assays can only monitor the first step at which Eve can act, we cannot exclude the possibility that other steps in PIC assembly may also be targeted by Eve. Indeed, MDM2 has been proposed to repress transcription by engaging in contact with both TBP and TFIIF (33). Eve interactions with other components of the transcription machinery may be important for multiple rounds of transcription. After transcription initiation TFIID remains bound to the TATA element, and other GTFs are either released or travel with the elongating complex (26,34). As Eve can only prevent the assembly of TFIID and does not disrupt a pre-formed TFIID–TATA complex, repression of reinitiation must involve another step in PIC assembly. Significantly, Eve can repress transcription of TATA-less promoters which also suggests that Eve can function by mechanisms other than inhibition of the TBP–TATA interaction (13,35). Previous studies with purified GTFs indicated that TBP assembly was the only event blocked by Eve (17). Moreover, in such purified systems, pre-binding of TBP (or TFIID) alone to the TATA element blocks repression by Eve (15–17). It is therefore likely that the other target(s) of Eve are factors other than GTFs. Of interest, the transcriptional repressor E4BP4 mediates its effects by interacting with Dr1, a factor that interacts with TBP and blocks further PIC assembly (36). Perhaps Eve performs a similar function and can also block PIC formation post-TFIID assembly by the recruitment of a co-repressor that would not be present in a purified system. Thus, the inhibition of TFIID assembly may well represent only one facet of Eve function. Further experiments will be required to test these possibilities.

ACKNOWLEDGEMENTS

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