Mechanism of transcriptional antirepression by GAL4–VP16

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Promoter- and enhancer-binding factors appear to function by facilitating the transcription reaction as well as by counteracting chromatin-mediated repression (antirepression). We have examined the mechanism by which a hybrid activator, GAL4–VP16, is able to counteract histone H1-mediated repression by using both H1–DNA complexes and reconstituted H1-containing chromatin templates. The GAL4 DNA binding domain alone was sufficient to disrupt local H1–DNA interactions, but a transcriptional activation region was additionally necessary for antirepression. GAL4–VP16-mediated antirepression required an auxiliary factor, denoted as a co-antirepressor, which was partially purified from *Drosophila* embryos. We have found that the co-antirepressor activity was sensitive to digestion with RNase A. Moreover, total RNA from *Drosophila* embryos could partially substitute for the co-antirepressor fraction, which indicated that the co-antirepressor may function as a histone acceptor (“histone sink”). These findings suggest a model for gene activation in which sequence-specific transcription factors disrupt H1–DNA interactions at the promoter to facilitate transfer of H1 to a histone acceptor, which then allows access of the basal transcription factors to the DNA template.

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The proper control of gene expression is essential for the development, growth, and sustenance of eukaryotic organisms, yet the strategies and mechanisms by which genes are regulated remain to be clarified. An early step in the pathway leading to gene expression is initiation of transcription. Synthesis of mRNA is carried out by the RNA polymerase II transcriptional machinery, which comprises RNA polymerase II and several auxiliary factors that are commonly referred to as general factors (for recent reviews, see Saltzman and Weinmann 1989; Sawadogo and Sentenac 1990; Conaway and Conaway 1991; Zawel and Reinberg 1992). Transcription by the basal transcriptional apparatus is regulated by sequence-specific DNA-binding factors that interact with promoter and enhancer elements (for review, see Johnson and McKnight 1989; Mitchell and Tjian 1989), and it presently appears that many of these promoter- and enhancer-binding proteins may stimulate transcription by acting in conjunction with another class of factors that are referred to as coactivators, mediators, adaptors, or intermediary factors (for review, see Lewin 1990; Ptashne and Gann 1990; Pugh and Tjian 1992). Transcriptional activity is also affected by chromatin structure (for review, see Weintraub 1985; Elgin 1988; Gross and Garrard 1988; van Holde 1989; Grunstein 1990; Wolffe 1990, 1992; Kornberg and Lorch 1991; Simpson

1991; Felsenfeld 1992); thus, it is important to consider the function of the general transcriptional machinery, the promoter- and enhancer-binding factors, and the co-activators with the chromatin template.

To study the relationship between chromatin structure and transcriptional activity, we have been examining the biochemical properties of promoter- and enhancer-binding factors in the context of two different models. In the first model, the sequence-specific factors facilitate the inherent transcription reaction, which is referred to as “true activation.” In the alternate model, the promoter- and enhancer-binding factors counteract a general repression of basal transcription that is mediated by a nonspecific DNA-binding entity (i.e., chromatin), which is designated as “antirepression.” In our initial studies (Kerrigan et al. 1991), we found that the GAGA factor (a promoter-binding factor from *Drosophila*) and a GAL4–VP16 fusion protein [which contains the DNA-binding domain and a transcriptional activation region of the yeast GAL4 protein and the transcriptional activation region of the herpesvirus protein VP16 (Sadowski et al. 1988; Chasman et al. 1989)] were capable of either antirepression only (GAGA factor) or both true activation and antirepression (GAL4–VP16). In those experiments, however, it was determined that transcriptional repression was mediated by a nonspecific DNA-binding factor under conditions in which chromatin assembly did not occur. The DNA-binding repressor was purified,

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and the protein, which is present in most standard nuclear extracts employed for *in vitro* transcription (Dignam et al. 1983; Soeller et al. 1988), was identified as histone H1 (Croston et al. 1991a,b). Hence, these data suggested that promoter- and enhancer-binding factors function to facilitate the transcription process as well as to counteract H1-mediated repression. The interpretation of the experiments with H1-repressed templates in the absence of nucleosomes (referred to in this work as H1–DNA complexes) is dependent, however, on the validity of the use of the H1–DNA complexes as a model for H1-containing chromatin. This issue was addressed by the reconstitution and analysis of H1-containing chromatin (Laybourn and Kadonaga 1991), and the transcriptional properties of H1–DNA complexes were found to be similar to those of H1-containing chromatin. Thus, in some instances, H1–DNA complexes may serve as a simplified model for H1-containing chromatin.

Histone H1 is a central component of chromatin, and it is present at a stoichiometry of approximately one molecule per nucleosome (Bates and Thomas 1981). It is believed that the globular domain of the protein binds at the nucleosomal pseudodyad, whereas the lysine-rich tails of H1 interact with the linker DNA between nucleosomal cores (Allan et al. 1980). H1 has an important role in the structure of chromatin and is required for the compaction of chromatin into the 30-nm filament (Finch and Klug 1976; Renz et al. 1977; Thoma et al. 1979; Butler and Thomas 1980). In addition, a number of studies have suggested that H1 is involved in transcriptional repression and is depleted from the promoter regions of transcriptionally active genes (Schlissel and Brown 1984; Nacheva et al. 1989; Shimamura et al. 1989; Wolffe 1989; Kamakaka and Thomas 1990; Postnikov et al. 1991; Bresnick et al. 1992). Thus, the finding that sequence-specific factors are able to counteract H1-mediated transcriptional repression (Croston et al. 1991a; Laybourn and Kadonaga 1991) was consistent with previous results that have implicated H1 as a repressor of basal transcription. In this study we examine the mechanistic basis of transcriptional antirepression with both H1–DNA complexes and H1-containing chromatin by using a derivative of the yeast GAL4 protein as a representative member of the class of transcription factors with acidic activation regions (Ptashne 1988).

Results and discussion

A transcriptional activation domain is required for antirepression

To examine the functional domains of the transcription factors that are required for antirepression, we first compared the properties of GAL4–VP16 to that of GAL4(1–94), a truncated GAL4 derivative that contains the DNA-binding and dimerization motifs in the amino-terminal 94 amino acids of the protein but lacks a transcriptional activation domain (Workman et al. 1991). In particular, we sought to test the requirement of a transcriptional activation domain for antirepression. Previously, Work-

man et al. (1991) had shown that GAL4–VP16, but not GAL4(1–94), was able to prevent transcriptional repression resulting from chromatin that was reconstituted from core histones and heat-treated *Xenopus* egg extracts. In those experiments, however, it was not known whether the transcription repression was the result of nucleosomal cores, H1 or an H1-related species, or some other component in the *Xenopus* extract.

To investigate the specific functional interactions between the transcription factors and H1, we performed a series of reactions with either GAL4–VP16 or GAL4(1–94) by using both H1–DNA complexes and reconstituted chromatin templates. With the H1–DNA complexes (Fig. 1), the absolute levels of transcription were reduced upon the addition of histone H1 to the reactions (Fig. 1A), but GAL4–VP16 prevented H1-mediated repression more efficiently than GAL4(1–94). This effect is displayed in Figure 1B as the ratio of the amount of transcription in the presence versus the absence of GAL4 derivative (fold activation) at different concentrations of H1. In the absence of histone H1, the amount of true activation by GAL4–VP16 was 4.6-fold, whereas that of GAL4(1–94) was 1.7-fold. In the presence of H1, the fold activation (a combination of true activation and antirepression) by GAL4–VP16 progressively increased to a factor of 40, whereas that by GAL4(1–94) remained somewhat constant at a factor of 5. We then examined the properties of the GAL4 derivatives with chromatin templates that were reconstituted from purified components in a stepwise process (Laybourn and Kadonaga 1991). Briefly, nucleosomal cores were deposited onto circular plasmid template DNAs with polyglutamic acid, and the resulting chromatin was purified by sucrose gradient sedimentation. Histone H1 and the sequence-specific transcription factors were then simultaneously incorporated into the purified chromatin by salt gradient dialysis. As shown in Figure 2, transcriptional antirepression with chromatin templates was observed with GAL4–VP16, but not GAL4(1–94). These data suggest that an activation region is not only required for true activation but also for efficient antirepression.

It was possible, however, that GAL4–VP16 was more effective than GAL4(1–94) for transcription antirepression because of a difference in the DNA-binding properties of the GAL4 derivatives rather than a transcription activation function in the GAL4–VP16 protein. To test this hypothesis, we conducted a DNase I footprint analysis of GAL4(1–94) and GAL4–VP16 binding to promoter DNA in the presence or absence of H1. The reaction conditions, the template DNA, the quantities of GAL4 derivatives, and the amounts of histone H1 in these experiments were all identical to those in the transcription reactions, except for the absence of ribonucleoside triphosphates and the basal transcription factors. In particular, the DNase I footprints were performed with supercoiled plasmid DNA (Gralla 1985) rather than restriction fragments to recreate the conditions that were employed in the transcription reactions. The DNase I footprints of GAL4(1–94) and GAL4–VP16 were identical in both the absence and presence of H1 (Fig. 3). Identical

Figure 1. A transcriptional activation domain of GAL4-VP16 is required for antirepression. pG₅E4T template DNA (100 ng) was incubated at 4°C for 30 min with GAL4(1-94) protein (128 ng), GAL4-VP16 protein (32 ng), or buffer only (as a control). Transcription was initiated by the simultaneous addition of SNF (32 μg) [Kamakaka et al. 1991], H1 (as indicated; 1 unit of H1 corresponds to 140 ng of protein) [Croston et al. 1991a], and ribonucleoside 5'-triphosphates, and the reactions were incubated at 25°C for 30 min. The resulting transcripts were then subjected to primer extension analysis, and the amount of transcription in each reaction was quantitated by liquid scintillation counting of the appropriate gel slices containing the reverse transcription products. (A) The levels of transcription in the presence or absence of GAL4 derivatives. The hatched bars represent reactions performed with GAL4-VP16; the stippled bars denote reactions performed with GAL4(1-94); the solid bars indicate reactions performed in the absence of a GAL4 derivative. (B) The levels of factor-mediated increase in transcription at different concentrations of H1. The amounts of transcription in the presence of either GAL4(1-94) or GAL4-VP16 were divided by the corresponding amounts of basal transcription (in the absence of a GAL4 derivative) at each of the indicated concentrations of H1. The resulting ratios, which are the levels of factor-mediated increase in transcription by either GAL4(1-94) (stippled bars) or GAL4-VP16 (hatched bars) are shown.

results were obtained with H1-containing chromatin (data not shown). Thus, the binding of GAL4(1-94) to DNA is not sufficient for transcriptional antirepression, which appears to require the binding of the transcription factor to the template [Croston et al. 1991a], as well as an activation region.

DNA binding by GAL4 derivatives causes a local alteration in the structure of H1-DNA complexes

The mechanism of antirepression may involve a transcription factor-mediated disruption of H1 binding to the template DNA. To probe for such interactions, we investigated the ability of the GAL4 derivatives to induce DNase I hypersensitivity with either naked DNA or H1-DNA complexes. In the presence or absence of GAL4 derivatives, the plasmid pG₅E4T [Lin et al. 1988], which contains five tandem GAL4 binding sites, was digested with DNase I. The DNA was then deproteinized, digested with *Bgl*I (which cleaves pG₅E4T at a unique site ~1.6 kbp from the GAL4-binding sites), and analyzed by agarose gel electrophoresis. In the absence of H1, the GAL4 derivatives did not induce DNase I hypersensitivity (Fig. 4, lanes 2-4). In the presence of H1, however,

both GAL4(1-94) and GAL4-VP16 specifically induced DNase I hypersensitivity at the boundaries of the GAL4-binding sites (Fig. 4, lanes 5-13). Hence, these data indicate that the DNA-binding domain of the GAL4 derivatives is sufficient for localized disruption of the interaction of H1 with the DNA. Note also that there were DNase hypersensitive sites both upstream and downstream of the GAL4-binding sites; thus, restriction digestion of the DNase-treated samples with *Bgl*I resulted in two sets of closely spaced doublets, which were not well resolved in the agarose gel shown in Figure 4. The specific locations of these hypersensitive sites can be seen at higher resolution in the DNase I footprinting studies with the GAL4 derivatives (Fig. 3). These findings suggest that the DNA-binding domain of the GAL4 derivatives is able to modify the H1-DNA interactions in the immediate vicinity of the GAL4-binding sites to increase the accessibility of proteins to the template DNA.

In general, a strong correlation exists between gene activity (or potential gene activity) and hypersensitivity of the DNA to nucleases [Weintraub and Groudine 1976; Elgin 1988; Gross and Garrard 1988]. Nuclease hypersensitivity probably reflects greater accessibility of factors

Figure 2. Transcriptional antirepression by GAL4-VP16 with chromatin templates requires a transcriptional activation domain. Chromatin was reconstituted onto pG₅E4T (Lin et al. 1988) with the indicated amounts of H1 in the presence or absence of either GAL4-VP16 (A) or GAL4(1-94) (B), which contains only the amino-terminal 94-amino-acid residues of GAL4 that comprise the DNA-binding domain. The resulting chromatin (50 ng of DNA) was transcribed in vitro with the SNF (Kamakaka et al. 1991). (Lanes 1,2) Naked DNA template (50 ng); (lanes 3,4) chromatin in the absence of H1; (lanes 5–10) chromatin with the indicated amounts of H1 (given in molecules of H1 per nucleosome = 200 bp DNA). The levels of transcriptional activation mediated by the GAL4 derivatives are given at the bottom. The reverse transcription products of adenovirus E4 RNA are shown.

to DNA as a consequence of such phenomena as chromatin decondensation, binding of sequence-specific factors, and reconfiguration or removal of nucleosomal cores and H1. We have observed nuclease hypersensitivity at specific locations where the structure of the H1–DNA complexes was disrupted by binding of the GAL4 derivatives. These experiments mimic, to a limited extent, the nuclease hypersensitivity that is observed in chromatin. Nuclease hypersensitivity in vivo often indicates a preactivated or transcriptionally competent state rather than the transcriptionally active state of a gene (Weintraub and Groudine 1976; Elgin 1988; Gross and Garrard 1988). In our studies, DNA binding by GAL4(1-94), which lacks an activation domain that is necessary for transcriptional antirepression (Figs. 1 and 2), was sufficient for induction of nuclease hypersensitivity with H1–DNA complexes (Fig. 4). It is possible that DNA binding by the GAL4 derivatives results in an alteration of the H1–DNA complexes that is necessary, but not sufficient, for antirepression.

A novel activity is required for GAL4-VP16 mediated antirepression with H1–DNA complexes

To explore further the requirements for transcriptional antirepression by GAL4-VP16, we performed transcription experiments by using either the soluble nuclear fraction (SNF) (Kamakaka et al. 1991) or partially purified factors (Wampler et al. 1990) as the source of the general transcription machinery. We had found previously that GAL4-VP16-mediated activation and antirepression occurred with the SNF (Croston et al. 1991a; Laybourn and Kadonaga 1991); therefore, we examined the possibility of GAL4-VP16 counteracting H1-mediated repression when the transcription reactions were carried out with the partially purified general factors. In the absence of H1, the magnitude of GAL4-VP16-mediated activation was 3.6-fold with the SNF (Fig. 5, lanes

1,2) and 1.6-fold with the fractionated system (Fig. 5, lanes 9,10). Then, as H1 was included in the transcription reactions, antirepression was observed with the SNF (>50-fold activation; Fig. 5, lanes 1–8) but not with the fractionated general factors (0.5-fold activation; Fig. 5, lanes 9–16). Hence, the DNA binding and activation domains of GAL4-VP16 and the basal transcription factors are not sufficient for antirepression. In addition, the basal levels of transcription by the SNF and fractionated factors in the absence of both H1 and GAL4-VP16 were similar (Fig. 5, cf. lanes 1 and 9). It thus appears that the requirements for transcriptional antirepression by GAL4-VP16 extend beyond the sequence-specific factor and the basal activity of the general transcriptional machinery.

These results suggested a few possible mechanisms for GAL4-VP16-mediated antirepression. For example, an auxiliary factor that is present in the SNF but absent in the partially purified general factors may be required to mediate antirepression by GAL4-VP16. Recent studies of transcriptional activation by GAL4-VP16 have suggested that an additional factor, which has been termed a mediator, adaptor, or intermediary factor (Berger et al. 1990; Kelleher et al. 1990; Flanagan et al. 1991; White et al. 1991), is not a component of the basal transcription apparatus but is required for GAL4-VP16-mediated activation. The mechanism by which such a factor might function could be direct (e.g., by providing a link between GAL4-VP16 and the general factors with protein–protein interactions) or indirect (e.g., by altering the binding of H1 to DNA or by modifying the activity of the transcription factors). Alternatively, the data are consistent with models for antirepression that do not invoke additional factors. For instance, it is possible that an auxiliary activation/antirepression activity is inherent in the general transcription factors and is functional in the crude nuclear extract but not in the fractionated and partially purified transcription factors.

Figure 3. Sequence-specific DNA binding by GAL4(1-94) and GAL4-VP16 in the presence or absence of H1. DNase I footprinting was performed with supercoiled plasmid DNA by the primer extension method (Gralla 1985). Supercoiled pG₅E4T DNA (100 ng) (Lin et al. 1988) was incubated with either a GAL4 derivative [32 ng of GAL4-VP16; 128 ng of GAL4(1-94)] or buffer only (as a control) at 25°C for 30 min in a medium that was identical to the buffer used for preincubation of transcription reactions. Histone H1 (amount as indicated) was added, and the incubation was continued at 25°C for 30 min. The protein-DNA complexes were then digested with DNase I for 1 min, and the samples were subjected to primer extension analysis as described in Materials and methods. The region protected by the GAL4 derivatives is indicated by a bracket. The arrows indicate the positions of factor-induced DNase I hypersensitivity relative to the transcription start site.

To investigate these hypotheses, we examined whether there existed a complementary activity that enabled GAL4-VP16 to counteract H1-mediated repression when transcription reactions were performed with the fractionated general transcription factors. We have partially purified such an activity from the SNF (Fig. 6) and have tentatively denoted this new activity as a co-antirepressor to minimize potential confusion with the mediator/adaptor/intermediary factor (Berger et al. 1990; Kelleher et al. 1990; Flanagan et al. 1991; White et al. 1991). In the absence of GAL4-VP16, a partially purified preparation of the co-antirepressor (Q Sepharose fraction) did not significantly affect the overall levels of either basal transcription in the absence of H1 (Fig. 6, cf. lanes 1 and 3) or H1-repressed transcription (Fig. 6, cf. lanes 5 and 7). The co-antirepressor did, however, enable GAL4-

VP16 to counteract H1-mediated repression (Fig. 6, cf. lanes 5 and 6 with lanes 7 and 8).

We examined whether the co-antirepressor is a component of the basal transcriptional machinery. Transcription reactions with reconstituted factors and the co-antirepressor fraction revealed that there is no detectable TFIIB, TFIID, TFIIE/F, or RNA polymerase II activity in the co-antirepressor fraction. In addition, when the fractionated general factors (TFIIB, TFIID, TFIIE/F, and RNA polymerase II) were each added individually in several-fold excess to reconstituted transcription reactions in the absence of the co-antirepressor fraction, GAL4-VP16-mediated antirepression was not observed (data not shown). The co-antirepressor is also distinct from the TATA-binding protein-associated factors (TAFs), which are required for transcriptional activation by Sp1

Figure 4. Analysis of GAL4(1-94)- and GAL4-VP16-induced DNase I hypersensitivity with H1-DNA complexes. pG₅E4T DNA (400 ng) (Lin et al. 1988) and a GAL4 derivative [either 128 ng of GAL4-VP16 or 512 ng of GAL4(1-94)] or buffer only, as a control, were incubated at 21°C for 30 min in a total volume of 120 μ l in a medium identical to that used for preincubation of transcription reactions. These conditions are identical to a four-fold scale up of a standard transcription reaction. Histone H1 (amounts are given in units per 100 ng of DNA) was added, and the mixture was incubated at 21°C for an additional 30 min. An appropriate quantity of DNase I (8 μ l volume, containing from 24 to 200 ng of DNase per reaction) was added to the protein-DNA complexes, and the sample was digested for 2 min. The sample was then digested with *Bgl*II, which cuts the pG₅E4T at a unique site ~1.6 kbp from the GAL4- binding sites, and analyzed by electrophoresis in a 1% agarose gel. The positions of linear plasmid DNA and DNA fragments resulting from factor-induced, hypersensitive DNase I digestion are indicated by brackets. Lanes 1 and 14 contain the 1-kb ladder (BRL) as molecular mass markers.

Figure 5. GAL4-VP16 and the general transcription factors are not sufficient for transcriptional antirepression with H1-DNA complexes. pG₅E4T template DNA (200 ng) was incubated with GAL4-VP16 protein (either 0 or 64 ng) at 4°C for 20 min, and transcription was initiated by the addition of ribonucleoside triphosphates, histone H1 (at 0, 0.6, 0.8, or 1.0 units per 100 ng of DNA), and general transcription factors [either SNF (Kamakaka et al. 1991) or fractionated factors (Wampler et al. 1990), as indicated]. Reactions were carried out at 21°C for 30 min, and the resulting transcripts were subjected to primer extension analysis. The reverse transcription products of AdE4 RNA are shown.

(Dynlacht et al. 1991). The TAFs are present in the TFIID fraction employed in these experiments (Wampler et al. 1990; Dynlacht et al. 1991), and transcription reactions performed with this TAF-containing fraction support Sp1-mediated activation/antirepression (Dynlacht et al. 1991; data not shown) but not GAL4-VP16-mediated activation/antirepression.

The co-antirepressor activity is required for transcriptional antirepression with reconstituted chromatin templates

Although the use of H1-DNA complexes was expedient for the initial identification and characterization of the co-antirepressor activity, it was important to examine the biochemical activity of the co-antirepressor with H1-containing chromatin, which is a more physiological model for the state of the template in vivo. We first examined whether binding of GAL4-VP16 to chromatin was sufficient to allow access of the basal transcriptional machinery to the template DNA. To address this question, histone H1-containing chromatin templates were reconstituted in the presence or absence of GAL4-VP16, and the resulting samples were divided into two equal portions that were transcribed with either the SNF or fractionated basal transcription factors. With the SNF, we observed a normal antirepression effect (Fig. 7A); but with the fractionated basal factors, GAL4-VP16 did not counteract the histone H1-mediated repression (Fig. 7B). In the reactions with the fractionated system (Fig. 7B), GAL4-VP16 mildly repressed transcription, possibly owing to sequestration of basal transcription factors by the activator ("squelching") (Gill and Ptashne 1988; Ptashne

1988). Notwithstanding, binding of GAL4-VP16 to the H1-containing chromatin templates was not sufficient for transcriptional antirepression.

Next, we characterized the properties of the co-antirepressor with the H1-containing chromatin templates (Fig. 8). First, the co-antirepressor did not affect the levels of basal transcription in the absence of GAL4-VP16 with chromatin templates (Fig. 8, cf. lanes 1 and 3, lanes 5 and 7, and lanes 9 and 11) but mildly increased the ability of GAL4-VP16 to activate transcription with chromatin templates that do not contain H1 (Fig. 8, cf. lanes 1 and 2 with lanes 3 and 4). With the H1-containing chromatin, however, the co-antirepressor was required for transcriptional antirepression by GAL4-VP16 (Fig. 8, cf. lanes 9 and 10 with lanes 11 and 12). Hence, antirepression by GAL4-VP16 with chromatin templates minimally involves binding of the factor to the template along with the co-antirepressor and the basal transcriptional machinery. These data are consistent with the results obtained with the H1-DNA complexes and support the hypothesis that transcriptional antirepression by GAL4-VP16 requires an additional activity beyond that of the basal transcriptional machinery.

The co-antirepressor may function as a histone acceptor

To investigate the function of the co-antirepressor, we sought to purify the activity as well as to characterize its

Figure 6. Identification of a co-antirepressor activity that is required to mediate transcriptional antirepression by GAL4-VP16 with H1-DNA complexes. The assays for co-antirepressor activity were carried out as follows. pG₅E4T template DNA (200 ng) (Lin et al. 1988) was incubated with GAL4-VP16 protein (either 0 or 64 ng) (Chasman et al. 1989) at 4°C for 20 min, and transcription was initiated by the addition of ribonucleoside triphosphates, histone H1 (0 or 0.8 units per 100 ng of DNA), a Q-Sepharose fraction containing the co-antirepressor (0 or 2 μg) and fractionated basal transcription factors (Wampler et al. 1990). Reactions were carried out at 21°C for 30 min, and the resulting transcripts were subjected to primer extension analysis. The reverse transcription products of AdE4 RNA are shown. The levels of transcription relative to that in lane 1 are given at the bottom.

Figure 7. Fractionated basal transcription factors are deficient in an activity that is required for antirepression by GAL4-VP16 with H1-containing chromatin templates. Chromatin was reconstituted on pG₅E4T in the presence or absence of GAL4-VP16, and one-half of each sample (containing 50 ng of DNA) was transcribed with the SNF (A) while the other half of each sample was transcribed with fractionated basal transcription factors (B). (Lanes 1,2) Naked DNA template (50 ng); (lanes 3,4) chromatin in the absence of H1; (lanes 5–10) chromatin with the indicated amounts of H1 (given in molecules of H1 per nucleosome = 200 bp DNA). The levels of transcriptional activation mediated by the sequence-specific factors are given at the bottom. The reverse transcription products of *in vitro*-synthesized RNAs are shown.

biochemical properties. Our attempts to purify the factor by ion exchange and gel filtration chromatography did not yield a significant increase in the specific activity, and the chromatographic properties of the factor suggested that it may be a large, heterogeneous complex. The co-antirepressor remained active after incubation at 60°C for 15 min but was inactivated by incubation at 90°C for 15 min. In addition, the co-antirepressor was insensitive to treatment with 8 mM *N*-ethylmaleimide. These results suggested that RNA may be a component

Figure 8. Transcriptional antirepression by GAL4-VP16 with H1-containing chromatin requires an auxiliary factor that is not a component of the basal transcription machinery. Chromatin was reconstituted onto pG₅E4T with the indicated amounts of histone H1 (given in molecules of H1 per nucleosome = 200 bp DNA) in the presence or absence of GAL4-VP16, and the resulting chromatin (50 ng of DNA) was transcribed *in vitro* with the fractionated basal transcription factors. Where indicated, the co-antirepressor fraction was included in the reactions. The levels of transcriptional activation mediated by GAL4-VP16 are given at the bottom. The reverse transcription products of AdE4 RNA are shown.

of the activity in the co-antirepressor fraction, which contained 0.5 mg/ml of protein and 0.6 mg/ml of RNA. We thus examined the effect of treatment of the co-antirepressor with RNase A (Fig. 9). In these experiments, the co-antirepressor fraction was incubated with RNase A, the RNase was inactivated with RNase inhibitor, and the resulting sample was tested for co-antirepressor activity. Treatment of the co-antirepressor with RNase A results in a significant loss of activity (Fig. 9, cf. lanes 3 and 4 with lanes 7 and 8). As a control, RNase A that was inactivated by pretreatment with an RNase inhibitor did not affect co-antirepressor activity (Fig. 9, cf. lanes 3 and 4 with lanes 9 and 10). This RNase sensitivity suggests that RNA may be an important component of the co-antirepressor.

To examine further the relationship between RNA and the co-antirepressor, we carried out a series of experiments in which we compared the properties of the co-antirepressor fraction with that of purified, total RNA from *Drosophila* embryos. Transcription reactions were performed with the template DNA as naked DNA, H1-DNA complexes, chromatin containing only nucleosomal cores, or H1-containing chromatin (Fig. 10). In these experiments, the reactions included buffer only (as a reference; Fig. 10, lanes 1,2), co-antirepressor fraction (1, 2, or 4 μ l; Fig. 10, lanes 3–8), or purified *Drosophila* embryo RNA (100, 200, or 400 ng; Fig. 10, lanes 9–14). The biochemical properties of the purified RNA were similar, but not identical to, that of the co-antirepressor fraction. With naked DNA or nucleosomal templates that did not contain H1, the effect of RNA on transcription was similar to that of the co-antirepressor, although mild repression of transcription was observed with the RNA (Fig. 10A,C; for discussion of RNA content in co-antirepressor fraction, see Materials and methods). With the H1-DNA complexes, both the RNA and the co-antirepressor appeared to mediate antirepression by GAL4-VP16, although the variation of activity with the concentration

Figure 9. Co-antirepressor activity is sensitive to RNase A. Co-antirepressor fraction (20 μ g of Q-Sepharose fraction; 40 μ l) or buffer only (as a control) was incubated with RNase A (2 μ l of a 100-ng/ μ l solution, where indicated) for 20 min at 30°C. RNase A was inactivated by the addition of RNase inhibitor (4 μ l of 0.5 units of Inhibit-Ace/ μ l; 5 Prime-3 Prime, Inc.) followed by incubation on ice for 10 min. The fractionated transcription factors were then added, the samples were incubated on ice for 20 min, and transcription was initiated by the addition of pG₅E4 template DNA (200 ng; preincubated for 20 min on ice with 64 ng of GAL4-VP16, where indicated), histone H1 (1.6 units per 200 ng of template DNA, where indicated), and ribonucleoside 5'-triphosphates. The transcription reactions were carried out at 21°C for 30 min. The relative amounts of reverse transcription products of the *in vitro*-synthesized RNA were quantitated with a PhosphorImager (Molecular Dynamics), and the data are displayed as a bar graph. (Lanes 1–4) No RNase A added (control); (lanes 5–8) co-antirepressor fraction or buffer treated with RNase A before the addition of RNase inhibitor; (lanes 9,10) RNase inhibitor preincubated with RNase A before addition to the co-antirepressor fraction, as a control.

of co-antirepressor was distinct from that of purified RNA (Fig. 10B). With the H1-containing chromatin, however, differences between the co-antirepressor and purified RNA were apparent (Fig. 10D). These experiments, which were carried out under conditions similar to those employed previously in Figure 8 (lanes 5–8), revealed that the co-antirepressor fraction was able to mediate antirepression more effectively than RNA with H1-containing chromatin templates. Notwithstanding, the partial substitution of RNA, a polyanion, for co-antirepressor suggests that both RNA and the co-antirepressor may function as a negatively charged histone acceptor, or “sink.”

We interpret these findings to suggest that histone acceptors may be involved in the sequence-specific factor-mediated relief of transcriptional repression by chromatin. From a simple point of view, it is sensible that histone acceptors are required for the removal or reconfiguration of histones during transcriptional activation. We do not presume, however, that the active species in our co-antirepressor fraction, which may be ribonucleoprotein particles, are necessarily the histone ac-

ceptors that are used *in vivo*. It is possible that other factors, such as acidic proteins or DNA, may also serve as histone acceptors in the nucleus. In addition, it is important to note that the histone acceptor function that we have observed is not simply a nonspecific removal of histones from the template DNA. First, the action of the co-antirepressor in the soluble nuclear fraction was dependent on a transcriptional activation domain in the sequence-specific factor (Figs. 1 and 2). Second, the co-antirepressor fraction did not activate basal transcription (in the absence of GAL4-VP16) under conditions in which it was able to mediate antirepression by GAL4-VP16 with either H1-DNA complexes (Figs. 6 and 10B) or H1-containing chromatin (Figs. 8 and 10D), although an increase of basal transcription by the co-antirepressor has been observed occasionally with H1-repressed templates (Fig. 9). These data suggest a mechanism for antirepression in which GAL4-VP16, but not GAL4(1–94), destabilizes the interaction of H1 with the promoter, and then the weakly bound H1 is transferred to the co-antirepressor/histone acceptor to allow access of the basal transcription factors to the template DNA.

Summary and perspectives

We have examined the requirements for transcriptional antirepression by GAL4-VP16 by using both H1-DNA complexes and H1-containing chromatin templates. Nuclease sensitivity experiments indicated that DNA binding is sufficient to disrupt local H1-DNA interactions, whereas *in vitro* transcription studies revealed that a transcriptional activation region is additionally necessary for antirepression. Furthermore, GAL4-VP16-mediated antirepression required an auxiliary factor, designated as a co-antirepressor, that may function as a histone sink or acceptor.

The mechanism by which GAL4-VP16 activates transcription by RNA polymerase II has been the subject of considerable investigation (for review, see Ptashne 1988; Ptashne and Gann 1990). Current data suggest that GAL4-VP16 can interact directly with TFIID (Stringer et al. 1990; Ingles et al. 1991) and TFIIB (Lin and Green 1991; Lin et al. 1991), and it appears that activation of transcription by GAL4-VP16 is dependent on an auxiliary activity that has been referred to as a mediator, adaptor, or intermediary factor (Berger et al. 1990; Kelleher et al. 1990; Flanagan et al. 1991; White et al. 1991). In addition, it has been shown that binding of GAL4-VP16 to naked DNA can prevent inhibition of transcription that occurs upon treatment of the DNA with core histones and chromatin reconstitution factors (Workman et al. 1991). In this study we have employed both H1-DNA complexes and H1-containing chromatin to examine the ability of GAL4-VP16 to counteract histone H1-mediated repression of transcription. In the experiments with chromatin templates, the nucleosomal cores were first deposited onto plasmid DNA and the resulting chromatin was purified by sucrose gradient sedimentation before the addition of GAL4 derivatives and histone H1 to the template. Then, by using either a crude extract (sol-

Figure 10. RNA possesses activity that is similar, but not identical to that of the co-antirepressor fraction. Transcription reactions were performed with fractionated basal transcription factors, with naked DNA as the template (A), H1-DNA complexes (B), chromatin containing nucleosomal cores only (no H1) (C), or H1-containing chromatin (with ~ 0.5 – 1.0 molecules of H1 per nucleosome) (D). The indicated amounts of co-antirepressor (Q-Sepharose fraction; 0.5 mg/ml of protein) and total RNA from 0- to 12-hr *Drosophila* embryos were incubated with the fractionated transcription factors on ice for 20 min to give the transcription factor mixture. For reactions in A and B, pG₅E4 template DNA (200 ng; preincubated for 20 min on ice with 64 ng of GAL4-VP16, where indicated) was added to the transcription factor mixture followed by histone H1 (for B only; 1.6 units per 200 ng of DNA) and ribonucleoside 5'-triphosphates to initiate transcription, which was carried out at 21°C for 30 min. For reactions in C and D, chromatin was reconstituted with pG₅E4 template DNA in the presence or absence of histone H1 and GAL4-VP16, as described in Materials and methods. The chromatin templates were incubated with the transcription factor mixture at 21°C for 30 min before the addition of ribonucleoside 5'-triphosphates. The transcription reactions were performed at 21°C for 30 min. The relative amounts of reverse transcription products of the in vitro-synthesized RNA were quantitated with a PhosphorImager (Molecular Dynamics), and the data are presented as bar graphs.

uble nuclear fraction; Kamakaka et al. 1991) or partially purified and fractionated transcription factors (Wampler et al. 1990) as the source of the basal transcriptional machinery, we identified and characterized the requirement for a co-antirepressor/histone acceptor for antirepression by GAL4-VP16. The relation between the co-antirepressor and the mediator/adaptor/intermediary factor is not known presently, but we have not yet observed a requirement for an auxiliary activity beyond that of the co-antirepressor. On the basis of the current data, a specula-

tive model for transcriptional activation by GAL4-VP16 is that GAL4-VP16 directly interacts with TFIID and TFIIB to facilitate the assembly of the transcription initiation complex and to reconfigure chromatin structure by a mechanism involving interactions between histones and histone acceptors.

The use of H1-DNA complexes versus H1-containing chromatin has distinct merits and shortcomings. H1-DNA complexes lack nucleosomes but are probably a better model for repressed chromatin than naked DNA.

With H1-DNA complexes, the identity of H1 as the transcriptional repressor is unambiguous, whereas with crude chromatin prepared from cell extracts, the identity of the species that repress transcription *in vitro* is not known. In addition, H1-DNA complexes can be prepared rapidly and easily, whereas the reconstitution of H1-containing chromatin from purified components is technically difficult and time consuming. Nevertheless, the H1-containing chromatin prepared from purified components has proven to be useful in the analysis of transcriptional regulation. For example, long-distance (1300 bp from activator-binding sites to the TATA box) activation of transcription as well as threshold phenomena have been reconstituted *in vitro* with H1-containing chromatin (Laybourn and Kadonaga 1992). It may be a wise strategy to perform preliminary studies with H1-DNA complexes and then to carry out subsequent work with H1-containing chromatin templates. For instance, in this study, H1-DNA complexes were used to identify the co-antirepressor activity, and then reconstituted chromatin was employed to characterize the biochemical properties of the co-antirepressor.

An emerging picture for the function of promoter- and enhancer-binding factors involves their ability to counteract chromatin-mediated repression, and further studies with transcription factors in the context of well-defined, transcriptionally repressed templates should continue to provide new insights into the mechanisms by which genes are regulated.

Materials and methods

Preparation of factors

GAL4 derivatives were purified to ~75% homogeneity by the procedure of Chasman et al. (1989). The DNA-binding activity of the GAL4 derivatives was determined by primer extension footprint analysis (Gralla 1985) with supercoiled pG₅E4T DNA. These titration experiments revealed that 32 ng of GAL4-VP16 and 128 ng of GAL4[1-94] were required to give complete protection of the five GAL4-binding sites in 100 ng of pG₅E4T. When corrected for the purity of the protein fractions, these data indicated that complete protection in the footprint experiments required 1.8 molecules of GAL4-VP16 dimers per binding site and 11 molecules of GAL4[1-94] dimers per binding site.

Partial purification and characterization of the co-antirepressor

The co-antirepressor activity was partially purified from *Drosophila* embryos as follows. First, the soluble nuclear fraction was prepared from *Drosophila* embryos as described previously (Kamakaka et al. 1991), except that 0.1 M KCl was used in the extraction buffer instead of 0.4 M potassium glutamate. This extract was subjected to chromatography in HEMG buffer (Wampler et al. 1990) with DEAE-Sepharose Fast Flow (Pharmacia-LKB). The 0.2–0.5 M KCl eluate was dialyzed into HEMG buffer containing 0.1 M KCl and 5 mM DTT and then applied to a Q-Sepharose Fast Flow resin (Pharmacia-LKB) equilibrated with HEMG buffer containing 0.1 M KCl and 5 mM DTT. The co-antirepressor activity eluted in a 0.4–0.5 M fraction. This fraction was dialyzed into HEMG buffer containing 0.1 M KCl

and 5 mM DTT and incubated at 60°C for 15 min. The sample was immediately chilled to 4°C and then stored at –100°C.

A comparative study of the Q-Sepharose co-antirepressor fraction (1, 2, and 4 μ l) and purified, total RNA from *Drosophila* embryos (100, 200, and 400 ng) is shown in Figure 10. Analysis of the RNA content of the Q-Sepharose fraction revealed that 1 μ l of the fraction contained ~600 ng of RNA. Thus, in Figure 10, there was approximately six times more RNA present in the co-antirepressor reactions (lanes 3–8) than in the corresponding reactions performed with purified RNA (lanes 9–14). Because the addition of ~1000 ng of purified RNA to the transcription reactions results in potent inhibition of transcription, it is likely that the RNA species in the co-antirepressor fraction were not present as free RNA but, rather, as ribonucleoprotein complexes. This hypothesis is also supported by the observation that the nucleic acid component of the co-antirepressor fraction (prepared by extraction with phenol-chloroform and precipitation with ethanol) strongly inhibited transcription under conditions where the corresponding amount of the original fraction did not affect basal transcription.

DNase I footprinting

DNase I footprinting was performed by the primer extension method (Gralla 1985) as follows. Supercoiled pG5E4T DNA (100 ng) (Lin et al. 1988) was incubated with a GAL4 derivative [either 32 ng of GAL4-VP16 or 128 ng of GAL4[1-94]] or buffer only, as a control, and histone H1 (amount as indicated) at 21°C in a total volume of 30 μ l in a medium that was identical to the buffer used for preincubation of transcription reactions. An appropriate amount of DNase I (2 μ l volume, containing from 12.5 to 333 ng of DNase per reaction; Worthington Biochemicals, DPF grade) was added to the protein-DNA complexes, and the sample was digested for 1 min. (Histone H1 inhibits DNase I digestion; thus, the amount of DNase I that was required for the different samples varied with the amount of H1 that was added to the DNA.) The DNase I digestion was terminated by the addition of a solution (90 μ l) of 20 mM EDTA (pH 8), 0.2 M NaCl, 1% (wt/vol) SDS and 0.25 mg/ml of glycogen (Sigma cat. no. G-0885). The samples were then digested with proteinase K, extracted with phenol-chloroform, and precipitated with ethanol. One-fifth of the sample (100 μ l in TE), which contains ~20 ng of DNA, was then used for the primer extension reaction as follows. First, 2 M NaOH (12 μ l) was added to the 100 μ l sample, and the mixture was incubated at room temperature for 5–10 min to denature the DNA. Next, 0.13 pmole of 5'-³²P-labeled adenovirus E4 (AdE4) primer (4 μ l of 0.033 pmole/ μ l; this primer is identical to the AdE4 primer used for primer extension of *in vitro*-synthesized E4 transcripts) (Wampler et al. 1990; Kerrigan et al. 1991), 12 μ g of glycogen (2 μ l of 6 μ g/ μ l), and 2.5 M NH₄OAc (90 μ l) were added, and the DNA was precipitated with ethanol (600 μ l). The resulting DNA was washed with 75% ethanol, dried in a Speedvac rotary concentrator, and dissolved in 10 μ l of 1 $\times$ Taq polymerase buffer (Stratagene). The sample was then incubated at 58°C for 10 min. Next, 1.3 unit (0.5 μ l) of Taq polymerase (Stratagene) and deoxyribonucleoside triphosphates (0.5 μ l of a stock containing 10 mM in each) was added, and the primer extension reaction was carried out at 70°C for 10 min. The DNA was precipitated with ethanol, suspended in formamide loading buffer, boiled for 3 min in a water bath, and applied to an 8% polyacrylamide-urea DNA sequencing gel.

Reconstitution of chromatin

Reconstitution of chromatin and *in vitro* transcription analysis was performed as described previously (Laybourn and Kadonaga

1991), and specific conditions were as follows. Core histone octamers were deposited onto circular template DNA with polyglutamic acid at a histone to DNA mass ratio of 0.8, and the resulting chromatin was purified by sucrose gradient centrifugation. The concentration of the reconstituted chromatin was estimated by extraction of the samples with phenol-chloroform followed by agarose gel electrophoresis and ethidium bromide staining with DNA standards. The purified chromatin was then subjected to salt gradient dialysis from 0.6 to 0.05 M KCl in the absence or presence of a GAL4 derivative with variable amounts of purified histone H1 from *Drosophila* embryos. GAL4-VP16 was used at a concentration of 1.5 dimers per binding site, and GAL4(1-94) was used at a concentration of 11 dimers per binding site. The samples were subjected to in vitro transcription analysis with either the SNF [prepared by extraction of nuclei with 0.1 M KCl instead of the previously recommended 0.4 M potassium glutamate (Kamakaka et al. 1991)] or fractionated basal transcription factors from *Drosophila* embryos (Wampler et al. 1990). Synthesis of RNA was assayed by primer extension analysis and quantitated by liquid scintillation counting of the appropriate gel slices. With different chromatin preparations, we observed some variation in the relative amounts of transcription with the naked DNA templates compared with the nucleosomal templates. This variation was the result of inaccuracy in the determination of the concentration of the reconstituted chromatin after sucrose gradient purification and does not affect the conclusions of experiments based on the magnitude of activation by GAL4-VP16.

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