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GAL4 disrupts a repressing nucleosome during activation of *GAL1* transcription in vivo

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Photofootprinting in vivo of *GAL1* reveals an activation- dependent pattern between the UAS_G and the TATA box, in a sequence not required for transcriptional activation by GAL4. The pattern results from a nucleosome whose position depends on sequences within the UAS_G. In the wild-type gene, activation by GAL4 and derivatives disrupts this nucleosome. This activity is independent of interactions with DNA-bound core transcription factors and is proportional to the strength of the activator. Presence of the nucleosome correlates with low basal transcription levels under various conditions, suggesting a role in limiting basal expression. We propose a role for the GAL4 activation domain in displacing a nucleosome and suggest that this is part of the mechanism by which GAL4 activates transcription in vivo.

[*Key Words:* Transcription; nucleosomes; footprinting in vivo; GAL4]

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Tight regulation of gene expression in eukaryotic cells requires mechanisms both to activate transcription in expressing cells and to repress transcription in nonexpressing cells. These regulatory mechanisms act on genes that are packaged into chromatin. A large body of literature documents changes in chromatin structure in vivo that accompany activation of genes (Eissenberg et al. 1985; Gross and Garrard 1987), and accumulating evidence supports important and direct roles for chromatin subunits, that is, nucleosomes, both in preventing expression from repressed genes and in permitting derepression of activated genes (Kornberg and Lorch 1991). When promoter sequences are first assembled into chromatin-like structures, their response in vitro to transcriptional activator proteins most closely resembles that observed in vivo (Knezetic and Luse 1986; Workman and Roeder 1987; Knezetic et al. 1988; Workman et al. 1990, 1991; Croston et al. 1991; Laybourn and Kadonaga 1991; Straka and Horz 1991). In this context, nucleosome assembly results in increased fold activation by suppressing basal expression rather than by augmenting induced levels. Further support for a suppressing role for nucleosomes comes from experimental manipulation of histone protein expression in yeast, which shows that reduction of histone levels and disruption of normal nucleosome structures result in elevated expression of selected genes (Clark-Adams et al. 1988; Han and Grunstein 1988; Han et al 1988). That nucleosomes play a role in allowing full-level expression has been demonstrated in at least one example: Mutations in the amino terminus of yeast histone H4 prevent activation of *GAL1* and selected other genes to wild-type levels (Durrin et al. 1991). This result was interpreted to indicate that relief of nucleosome-mediated repression failed in these mutants, thereby not allowing full activation. Taken together, these results imply that nucleosomes that suppress basal transcription must be displaced by transcriptional activators in vivo to effect activation.

Systems for examining transcription in vitro, whether the template is reconstituted with nucleosomes or not, depend on observing functional transcription. This process depends on the ordered assembly of general transcription factors, TFIID, TFIIB, polymerase II, TFIIE, and TFIIF, into a complex at transcriptional initiation sites (for review, see Roeder 1991). Sequence-specific transcriptional activators enhance assembly of this complex. Both TFIID, the TATA-binding factor (Abmayr et al. 1988; Horikoshi et al. 1988a,b; Workman et al. 1988; Stringer et al. 1990; for review, see Ptashne and Gann 1990), and TFIIB (Lin and Green 1991) have been implicated as potential targets for acidic activator proteins, and less well-defined coactivators have also been postulated to mediate or facilitate the activation step (Berger et al. 1990, 1992; Kelleher et al. 1990; Meisterernst et al. 1990; see also Hoey et al. 1990; Pugh and Tjian 1990). In these systems, binding of TFIID (or TFIIB) has been suggested to be the rate-limiting step.

In the reconstituted chromatin transcription experi-

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ments, it might be presumed that nucleosomes were disrupted upon activation; however, their status was not directly examined nor could it be determined whether nucleosome disruption resulted from interaction of the activator with basal transcription factors or whether it was a direct effect of the activator on the nuclesome. In light of the observation that transcriptional activators produce greater fold activation in these systems, it is likely that the rate-limiting step has been altered and that conditions more closely approximate those existing in vivo.

In the present studies, we address these questions by examining the promoter of the *GAL1* gene of the yeast, *Saccharomyces cerevisiae*. This well-studied promoter (Johnston 1987) is regulated by an upstream activating sequence (UAS_G) that contains multiple 17-bp-binding sites (Bram and Kornberg 1985; Giniger et al. 1985; Selleck and Majors, 1987b) for the transcriptional activator GAL4 protein. In cells grown in noninducing carbon sources, such as glycerol or raffinose, GAL4 protein is bound to the UAS_G (Giniger et al. 1985; Selleck and Majors 1987b) but is kept inactive by associated GAL80 protein (Lue et al. 1987). Transfer of these cells to the inducing carbon source galactose activates expression (about 1000-fold) by blocking the effects of *GAL80* (Johnston et al. 1987; Lue et al. 1987; Ma and Ptashne 1987b). In cells grown in glucose (or galactose and glucose), the promoter is repressed and GAL4 binding to the UAS_G is lost (Giniger et al. 1985; Selleck and Majors 1987b); decreased GAL4 binding results at least in part from decreased GAL4 expression (Griggs and Johnston 1991). Here, we use a primer extension-based photofootprinting method (Axelrod and Majors 1989; Axelrod et al. 1991) and additional studies to show that under conditions where the *GAL1* promoter is inactive, a nucleosome is positioned between the UAS_G and its TATA element. We show further that (1) the nucleosome is displaced by activation, (2) its displacement is dependent on the acidic activation domain of GAL4 or derivatives, (3) its displacement is probably an effect of activator function but does not depend on interaction with DNA-bound basal transcription factors, and (4) the nucleosome suppresses basal transcription levels in the uninduced state.

Results

An activation-dependent photofootprint between UAS_G and TATA of GAL1

In previous studies, a photofootprinting procedure was used to analyze the association of proteins in vivo with the entire *GAL1–10* regulatory region (Selleck and Majors 1987a,b,1988). Photofootprinting methods rely on the observation that formation of UV-light-induced photoproducts (for a review of DNA photochemistry, see Wang 1976) is sensitive to DNA comformation, which can be altered by bound proteins (Becker and Wang 1984; Selleck and Majors 1987a). In the earlier work, a transcription-dependent footprint at both the *GAL1* and *GAL10* TATA elements was observed (Selleck and Majors 1987a) and regulated binding of GAL4 to the UAS_G was demonstrated (Selleck and Majors 1987a,b). In addition, changes in photoproduct patterns in the region between the UAS_G and the *GAL1* TATA box [referred to here as the interposed sequence (IS)] could be correlated with *GAL1* promoter activity (Selleck and Majors 1988). These studies used a chemical cleavage/blot hybridization protocol to detect photoproducts. In this study we use a primer extension protocol in which photoproducts are detected on the basis of their ability to stall or arrest an elongating polymerase. We first showed that similar to what was seen with the chemical photofootprinting procedure (Selleck and Majors 1988), the primer extension protocol detects IS photoproduct patterns that are altered in response to promoter activation. Cultures of a strain bearing wild-type *GAL* genes were grown either under noninducing conditions (raffinose), inducing conditions (galactose), inducing/repressing conditions (galactose plus glucose), or repressing conditions (glucose); and the patterns shown in Figure 1A were obtained. In the wild type, an induced alteration in the pattern is apparent on the top strand ~60 bp upstream of the *GAL1* TATA box, at the same position reported previously (Selleck and Majors 1988). The data dissociate the altered pattern from GAL4 binding alone, because growth on raffinose (GAL4 bound) and growth on glucose (GAL4 not bound, as verified by photofootprinting; data not shown) both produce the "off" pattern. To assess the dependence of the "on" pattern on galactose or *GAL80*, a wild-type and a *gal80*Δ strain were both grown on raffinose and the photoproduct patterns were compared (Fig. 1B). The on pattern is observed in the actively transcribing *gal80*Δ strain but not in the wild-type, uninduced strain, indicating that the change in pattern depends on activation but not specifically on galactose or *GAL80*. As was observed previously (Selleck and Majors 1988), photoproduct formation at several additional sites within the IS also varies with promoter activity (data not shown). Notably, chemical footprint analysis of this region with dimethylsulfate (DMS) showed no differences between induced and uninduced samples (data not shown).

Although photofootprinting cannot directly identify states in which proteins are bound to DNA, several observations support the view that the patterns observed in these experiments reveal protein–DNA contacts that exist only in the off state. First, the pattern seen in the on state closely resembles that seen with naked DNA. Second, the same is true when the region is analyzed with a different probe: High-resolution DNase I footprinting shows protections and enhancements relative to naked DNA only in the off state, not in the on state (M.S. Reagan and J. Majors, unpubl.). Finally, micrococcal nuclease protection studies show sites that are protected in the off state and sensitive in the on state (see below). We therefore favor the view that the IS DNA is protein bound in the off state. Note that this interpretation differs from that proposed in earlier studies (Selleck and Majors 1988).

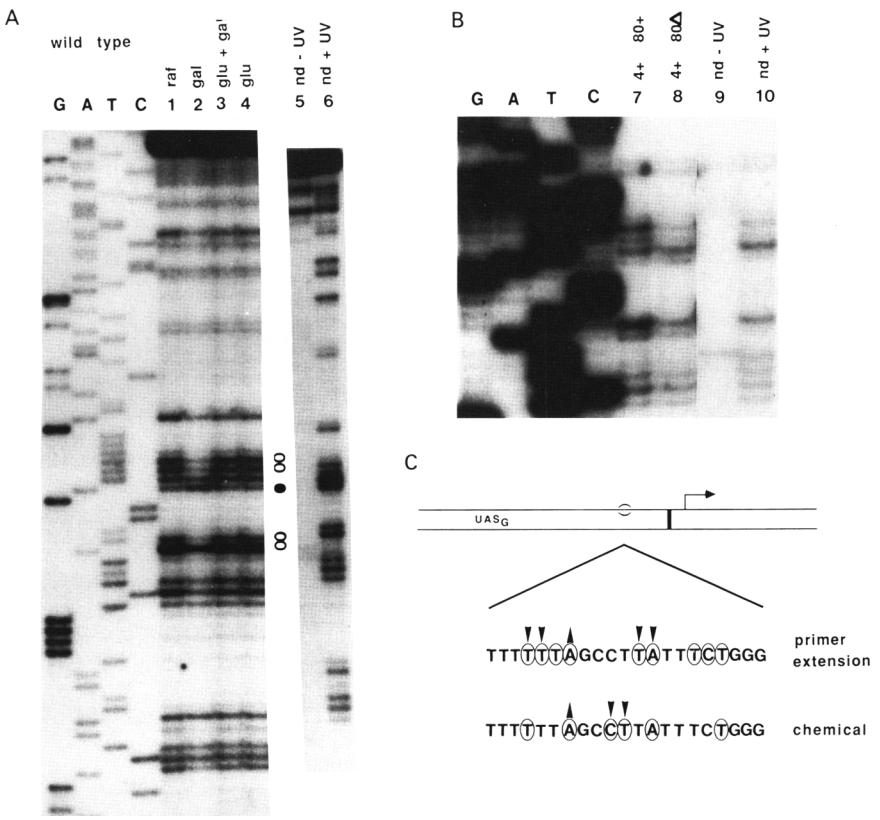

Figure 1. Photofootprint in the *GAL1* IS. (*A*) A wild-type strain (YM262) was photofootprinted after growth on raffinose (Raf = uninduced), galactose (Gal = induced) glucose + galactose (Glu + Gal = induced/repressed) or glucose (Glu = repressed) as described previously. The IS photofootprint was obtained by primer extension with oligonucleotide 2114. (○) Sites of GAL4-dependent repressions; (●) a GAL4-dependent enhancement. (Lanes 5,6) Unirradiated and in vitro-irradiated naked DNA controls. The original controls for this experiment were run with insufficient template DNA, so a more typical example is shown (see also Figs. 3, 4, and 6) Using insuficient template DNA consistently led to results similar to those for nd + UV shown in Figs. 1B and 3B. (*B*) A similar photofootprint of a *GAL4*$^+$–*GAL80*$^+$ strain (YM262) compared with a *GAL4*$^+$–*gal80*Δ strain (YM654), both uninduced by growth on raffinose. (*C*) Comparison of photofootprinting results obtained from chemical (Selleck and Majors 1988) and primer extension photofootprinting methods. Circled nucleotides are sites of photoproduct detection. Upward and downward arrowheads denote GAL4-dependent enhancements and repressions, respectively. The location of the footprint site is schematically shown. The vertical solid bar represents the TATA box; the arrow indicates the site and direction of *GAL1* transcription initiation. Note that although the precise nucleotides at which enhancements and repressions are seen do not coincide, the footprints are qualitatively similar in that they appear in response to the same regulatory conditions. This difference between the two methods is consistent with previous studies of the GAL4-binding sites, in which the two methods also detected different, overlapping sets of photoproducts and photoproduct enhancements and repressions (Axelrod and Majors 1989).

Sequences at the IS photofootprint site are not required for normal GAL1 *activation*

We set as our goal a better understanding of the nature of the protein–DNA interactions responsible for the altered photopatterns and of their role in regulation of the promoter. Previous work by West et al. (1984) showed that deletion of portions of the IS that encompass this target had little if any effect on regulation of *GAL1* expression. However, in those constructs, the UAS$_G$ was brought closer to the TATA element than in the wild type. To test the requirement for this sequence without significantly altering the wild-type spacing, several recombinant genes were constructed. (1) We placed a linker substitution at the site of the photofootprint and placed the UAS$_G$ within 25 bp of its wild-type location (YAX28). (2) We made eight constructions in which the entire IS was replaced by random *Drosophila* DNA fragments that left the wild-type spacing essentially unchanged (YAX29n; Fig. 2). Each modified promoter was fused to *HIS3*-coding sequences and was integrated into the genome in single copy at the *LEU2* locus, as was a control plasmid bearing a wild-type *GAL1* regulatory region fused to *HIS3* (YAX29c). Cultures were grown on raffinose; half of each culture was harvested for RNA preparation prior to induction (uninduced); the remainder was induced with galactose for 2 hr prior to harvest. Northern blots (Fig. 2) demonstrate that the linker-substituted promoter and seven of the eight IS-substituted promoters show essentially wild-type induced and uninduced levels of expression. Two conclusions may be drawn. First, normal regulation is retained upon integration at this site (as is the wild-type photofootprint pattern; see Fig. 4B). Second, the sequence within the IS whose light sensitivity we are monitoring is not required for normal *GAL1* activation. [The IS, however, does mediate a part of the glucose repression pathway (Flick and Johnston 1990, 1992).]

Because this sequence was not essential for correct expression of *GAL1*, we hypothesized that we were detecting a sequence-independent protein–DNA contact that responds to elements located elsewhere in the regulatory region.

The IS footprinting structure is nucleosome dependent

Several lines of reasoning suggested that we were detecting the unfolding of a nucleosome. First, low resolution data from nuclease protection studies (Lohr 1984; Fedor

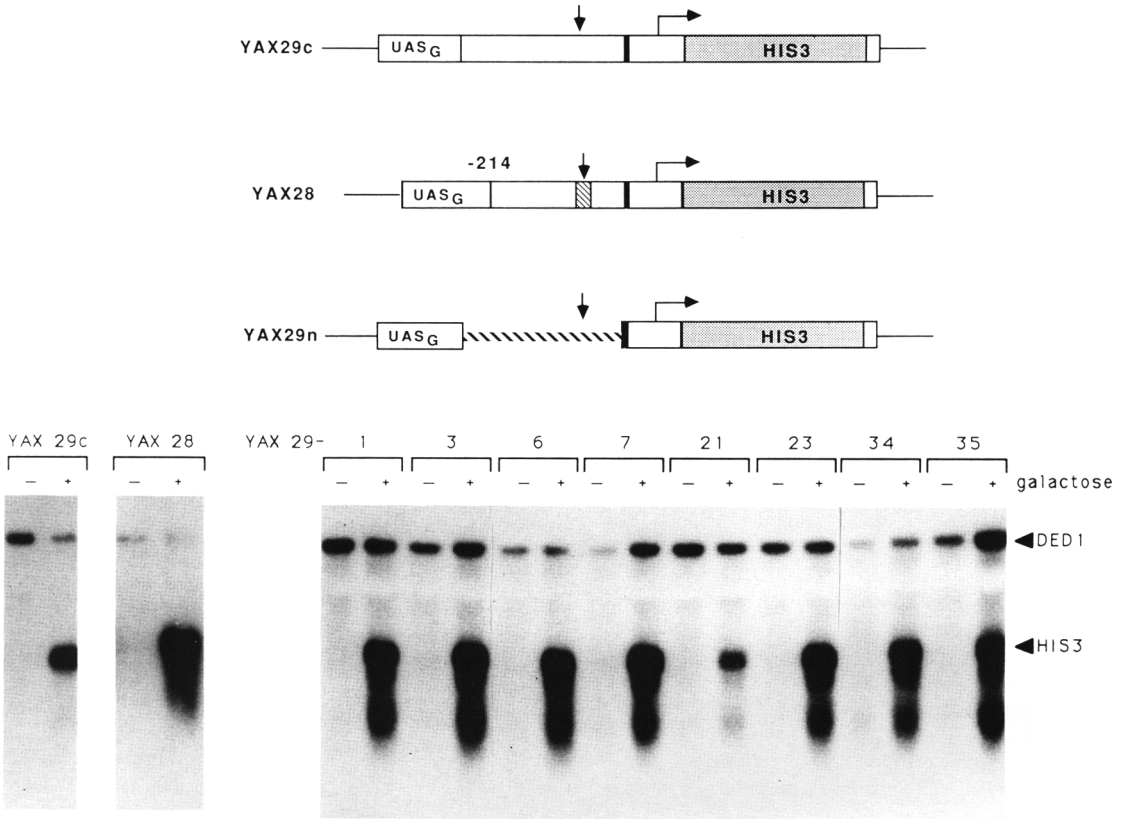

Figure 2. Northern blots of wild-type and IS-substituted *GAL1–HIS3* fusions. The constructions are depicted schematically. The hatched box in YAX28 represents the linker substitution; the broken line in YAX29-n represents *Drosophila* DNA. The open boxes are *GAL1* sequences or a small piece of YIp5 downstream of *HIS3* (shaded; not drawn to scale). Plasmids were integrated, and the cultures were grown on raffinose. Half of each culture was harvested for uninduced RNA preparation (−) while the remainder was induced for 2 hr by the addition of 2% galactose (+) prior to harvest. Northern blotting was as described previously. *HIS3* and *DED1* mRNAs are shown. *DED1* served as an internal control, as it is not regulated. The YAX29c (+) sample is underloaded compared with most of the others (see also Fig. 4B).

and Kornberg 1989) are consistent with the positioning of a nucleosome at this site. Second, we carried out DNase I protection experiments with isolated nuclei from repressed or noninduced cells; these studies revealed alternating protections and enhancements in the IS region, with a periodicity of ~10 bp, a pattern considered diagnostic for nucleosomal DNA (M.S. Reagan and J. Majors, unpubl.). Third, Kornberg and co-workers showed that for the wild-type gene on minichromosomes, a nucleosome was positioned to include the IS photofootprint site, adjacent to a nucleosome-free region of ~230 bp including the UAS$_G$. They identified a sequence overlapping GAL4-binding site II that is a binding site for a protein named GRF2 (Fedor et al. 1988; Chasman et al. 1990; see Brandl and Struhl 1990), and they proposed that GRF2 forms a boundary that positions nucleosomes in adjacent sequences (Fedor et al. 1988).

We tested the hypothesis that the altered pattern is generated by a nucleosome in two ways. We first asked whether micrococcal nuclease protection patterns observed under various conditions were consistent with this model (Fig. 3). Nuclei from cells bearing wild-type or modified *GAL1* genes were isolated and digested with micrococcal nuclease. Protection in the IS region was probed by Southern blotting. In the wild type, the IS region is protected from digestion when the cells are grown on glucose, but not on galactose, consistent with the observations of others that a nucleosome appears to occupy this site in the inactive but not the active gene (lanes 1–4). We note that with DNA from cells grown on glucose, but not on galactose, ordered protection is also observed in the *HIS3*-coding region, suggesting that nucleosome phasing spans from the IS into the coding region.

We then examined the role of upstream sequences in establishing the IS photopattern by comparing the photofootprint and micrococcal nuclease patterns on several templates (Fig. 4A). Modified genes were introduced into yeast, their DNAs were photofootprinted, and their activities were assayed by Northern blotting. Integration and photofootprinting of two constructs placing UAS$_G$ closer to TATA (at position −214) in the forward (YAX26) and reverse (YAX27) orientation (Fig. 4B,D) re-

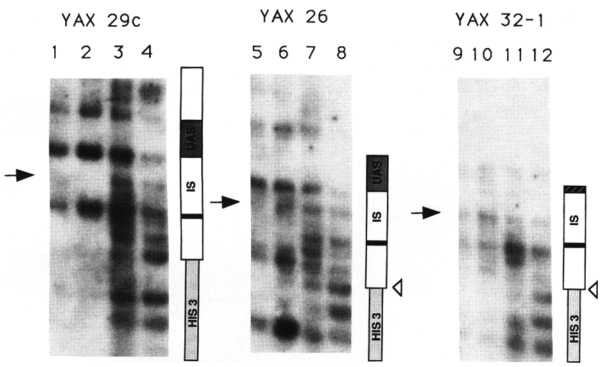

Figure 3. Comparison of micrococcal nuclease cleavage patterns of constructs with altered upstream elements. (Lanes *2,5,9*) Nuclei from glucose-grown cells exposed to Micrococcal nuclease for 2 min. (Lanes *1,6,10*) Nuclei from glucose-grown cells exposed to Micrococcal nuclease for 5 min. (Lanes *3,7,11*) Nuclei from galactose-induced cells exposed to Micrococcal nuclease for 30 sec. (Lanes *4,8,12*) are micrococcal nuclease digestions of naked DNA. Ethidium bromide staining of total digested DNA confirmed that the extent of digestion was equivalent in all lanes (data not shown). The solid arrow indicates the equivalent position in all three constructs that is protected from Micrococcal nuclease digestion in YAX29c glucose-grown cells. In the maps of the constructs (see also Fig. 4A), UAS is UAS_G, the solid bar is the *GAL1* TATA box, the dark striped box in YAX32-1 is the consensus GAL4-binding site, and the open triangles in YAX26 and YAX32-1 indicate a deletion of 37 bp at the *GAL1–HIS3* fusion junction.

vealed that in uninduced cultures, movement of the UAS_G closer to TATA partially relieved the off pattern. Induction of either construct generated the full on pattern. When the UAS_G was replaced entirely, the off pattern was lost: substitution of either one (YAX32-1) or two (YAX32-2) consensus GAL4 sites at −214 produced galactose-dependent expression, but both induced and uninduced footprints gave the on pattern (Fig. 4A,C). Substitution of two GCN4-binding sites at −214 resulted in histidine starvation-dependent transcription (Hinnebusch 1984), but, again, both induced and uninduced photofootprints gave the on pattern (YAX31; Fig. 4A,C). Chimeric genes having *LEXO* or the *PHO5* UAS at −214 responded to induction by a *LEXA/GAL4* fusion (Brent and Ptashne 1985) or *pho80Δ* (Oshima 1982), respectively, but these also only produced the on footprint pattern (data not shown).

If the wild-type photopatterns result from a nucleosome, we reasoned that the rearrangements and substitutions that alter the photopattern would alter the micrococcal nuclease patterns in a corresponding fashion. Accordingly, two of the above constructs were examined in the micrococcal nuclease assay (Fig. 3). Digestion of YAX26, in which the UAS_G is repositioned closer to the TATA box (see Fig. 4A), shows that the IS protection is altered such that the protected region is observed in a position downstream of that seen in the wild type (lanes 5–8). In YAX32-1, the UAS_G has been replaced with a synthetic GAL4-binding site (see Fig. 4A). Digestion of this construction shows no protection at the IS (lanes 9–12). These observations are consistent with the photofootprint results for these strains, in which the photofootprint is diminished by moving UAS_Gdownstream (YAX26) or abolished by replacing UAS_G with a synthetic GAL4-binding site (YAX32-1). The photopattern therefore correlates with nuclease protection in these constructs. The position of micrococcal nuclease-protected sites and the presence of the IS photofootprint appear to depend on the spacing between the UAS_G and the IS target site, suggesting that the position of the nucleosome depends on sequences within UAS_G. It is possible that repositioning of GRF2 binding accounts for these altered footprints and protections (Fedor et al. 1988; Chasman et al. 1990); however, a mutation of the GRF2 site that abolishes GRF2 binding in a wild-type gene fails to alter the IS nuclease protection pattern (M.S. Reagan and J. Majors, unpubl.).

Three conclusions may be drawn from this set of experiments. First, the off photopattern is only evident in constructs containing UAS_G. Second, the off pattern is sensitive to the spacing between UAS_G and the TATA element; as the UAS_G is moved closer to TATA, the off pattern is diminished in uninduced cultures. Third, we see that the photopatterns observed here correlate with patterns of micrococcal nuclease protection in the three constructs tested (cf. Figs. 3 and 4), suggesting that they result from packaging of the IS region into a nucleosome.

Our second approach to demonstrate that the photopattern results from a positioned nucleosome used a yeast strain in which nucleosomes can be depleted by regulated expression of histone H4 (Kim et al. 1988). If the off photopattern is generated by a nucleosome, then nucleosome depletion should lead to its partial loss when cells are grown on raffinose or glucose. In this strain a single *H4* gene is controlled by the *GAL1* promoter on a centromere plasmid. H4 is produced when the cells are grown on galactose. When the cells are shifted to media containing raffinose or glucose, H4 is not expressed and nucleosomes are partially depleted prior to growth arrest. Figure 5, A and B, shows the result of such an experiment. When compared with wild type (lanes 1,2), DNA from cells partially depleted of nucleosomes by growth on raffinose or glucose loses the off photopattern and, instead, looks more like on (lanes 3 and 4 are more similar to lane 5 than is lane 1 to lane 2). Laser densitometric scans of the patterns are shown to facilitate comparison. The off pattern is diminished but not abolished in these conditions, consistent with the expected halving of nucleosome content. This result demonstrates that the off photopattern is nucleosome dependent. As a control, photofootprinting of a wild-type strain bearing a centromere plasmid carrying *GAL1–10* regulatory sequences but with coding regions deleted demonstrates that there is no effect of extra copies on the footprint (data not shown).

On the basis of these results, we propose that our altered photopatterns are generated by a nucleosome and that this nucleosome is disrupted by GAL4 activation of *GAL1*.

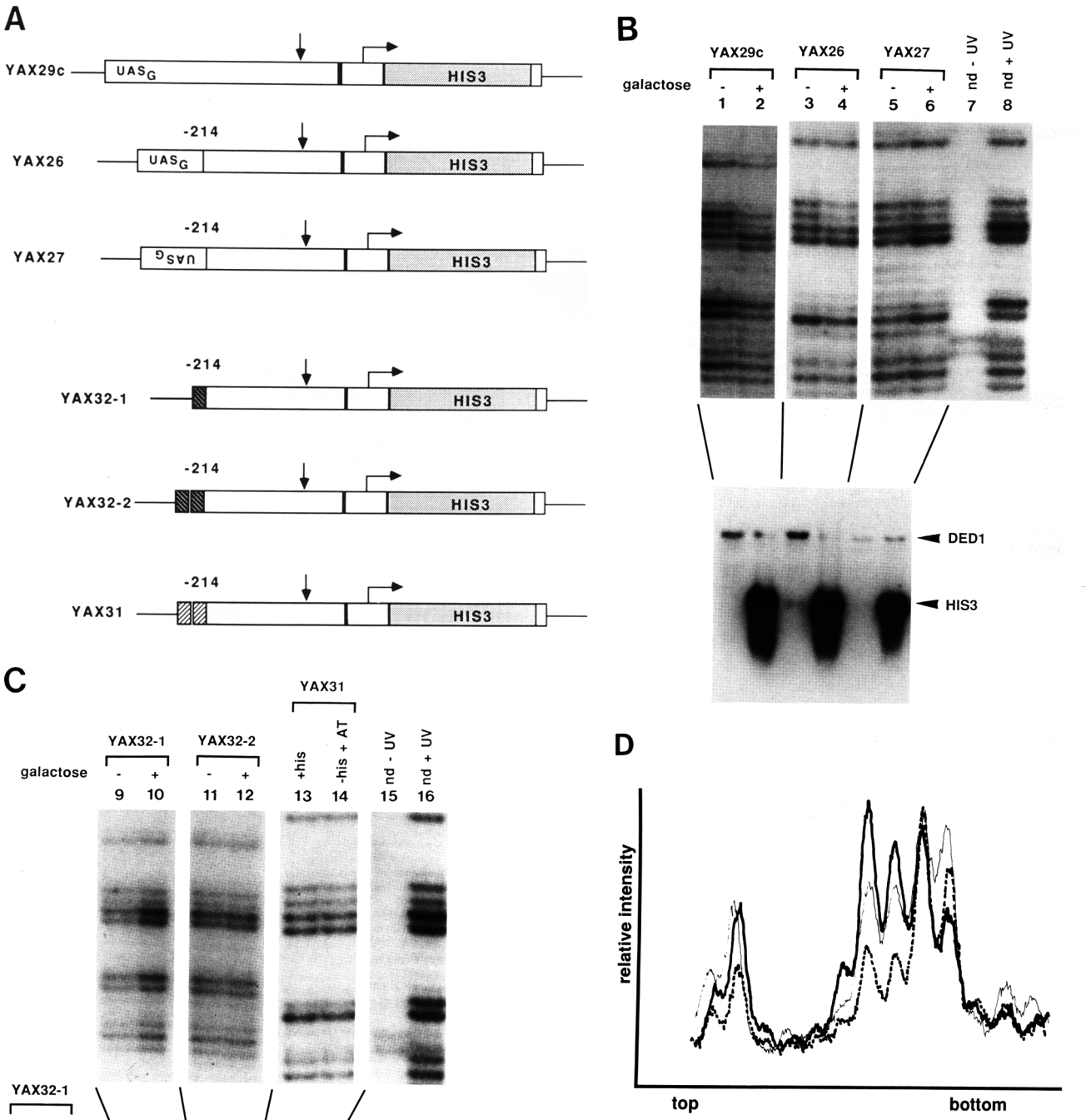

Figure 4. Effects of position and substitution of the UAS_G on the IS photofootprint. (*A*) Schematics of the constructions altering UAS_G position or substituting a synthetic UAS. The dark hatched boxes are GAL4-consensus-site oligonucleotides; the light hatched boxes are GCN4 site oligonucleotides. (*B*) Photofootprints of wild type and constructs moving UAS_G closer to TATA. Strains were grown on raffinose and either induced with galactose (+) or not (−). The corresponding Northern blots are also shown. (*C*) Photofootprints of UAS-substituted constructs. YAX32-1 and 32-2 were grown as in *B*. Uninduced YAX31 was grown in minimal medium plus raffinose containing histidine (+His). Induction of YAX31 was accomplished by growth in the same medium lacking histidine, followed by addition of 10 mM aminotriazole 2 hr before harvest (−His + AT). The corresponding Northern blots are shown. For comparison of uninduced and catabolite-repressed levels, YAX32-1 was also grown on glucose (lane *17*, glucose repressed) for comparison to lane *9* (uninduced). (*D*) Densitometric scans of lanes from *B*. Uninduced YAX29c (broken line), induced YAX29c (heavy line), and uninduced YAX26 (fine line) are superimposed to facilitate comparison.

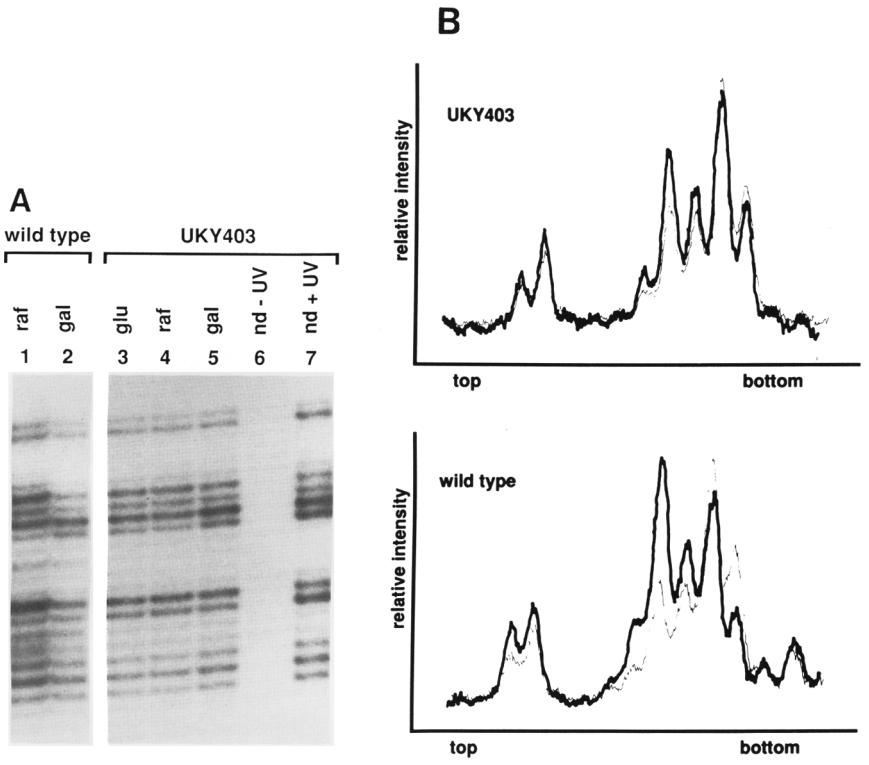

Figure 5. Dependence of the IS photofootprint on nucleosome depletion. (*A*) Strain UKY403 was footprinted after growth on the carbon source shown and compared with wild type (YM262). The UKY403 samples are underexposed relative to the YM262 samples, because UKY403 bears extra copies of the *GAL1–10* control region driving the *H4* gene. Extra copies of the *GAL1–10* control region have no effect on the IS photofootprint: A wild-type strain with a plasmid bearing the *GAL1–10* control region, but not expressing H4, produces a footprint indistinguishable from wild type (data not shown). In this experiment all strains were grown in synthetic medium − Trp + Gal and shifted to alternate media as appropriate, because UKY403 cannot sustain multiple doublings in carbon sources other than galactose. (*B*) A region containing the cluster of four bands in the photofootprint was scanned with a laser densitometer, and for each strain the results for growth on raffinose and galactose are superimposed. Samples from cells grown on galactose (fine lines) and on raffinose (heavy lines) are shown. When superimposed, the patterns for UKY403 and YM262 on galactose are nearly identical (not shown).

The off pattern correlates with low basal expression levels

Examination of the noninduced, nonrepressed expression levels from the wild-type, the two UAS_G fusions at −214, and the consensus GAL4 site fusions at −214 shows a correlation between relief of the off photofootprint and increased uninduced expression levels. Northern blots were run and exposed to reveal uninduced RNA levels (Fig. 6). RNA levels were quantitated by scanning with a laser densitometer, and the *HIS3* transcript was normalized to the *DED1* internal control. Tabular results (Table 1) show that *HIS3* mRNA increases in uninduced cultures when the UAS_G is moved closer to TATA, inverted, and deleted and replaced with two consensus GAL4 sites. The expression levels correlate with the degree to which the off photopattern is disrupted in this series of constructs. A simple model to explain these observations is that the IS nucleosome is responsible for limiting basal transcriptional activity to low levels. Activation by GAL4 disrupts or displaces this nucleosome.

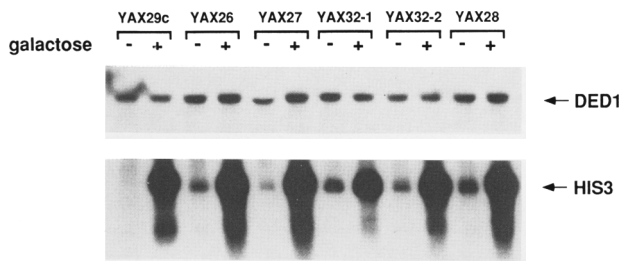

Figure 6. Northern blots demonstrating loss of suppression as a function of spacing. Repeat Northern blots of wild type and strains moving UAS_G downstream, or replacing UAS_G with synthetic GAL4 sites, are shown. The *HIS3* portion of the blot was overexposed to reveal basal expression levels.

Table 1. *Relationship between basal expression and off photofootprint*

Strain	Uninduced *HIS3* mRNA[a]/normalized[b]	Off photofootprint[c]
YAX29c	1	+ +
YAX26	22	+
YAX27	22	+
YAX28	40	+
YAX32-1	35	−
YAX32-2	34	−

[a]*HIS3* transcript is made from the *GAL1–HIS3* fusions. The endogenous *HIS3* gene has been deleted.
[b]Lanes from the Northern blot shown in Fig. 6 were scanned by laser densitometry, and the areas under the peaks determined. Relative *HIS3* RNA levels were determined after normalization to the level of *DED1* RNA. *DED1* expression is not affected by carbon source.
[c]The photofootprints shown in the appropriate figures were assessed as to the degree the pattern resembles the wild-type off footprint. (+ +) Wild-type off pattern; (−) wild-type on pattern; (+) intermediate result.

Its position and repressing function in noninducing, nonrepressing conditions are also affected by manipulations that either alter the spacing of the wild-type gene or delete UAS_G. This is consistent with in vitro studies showing suppression of basal transcription when the template is preassembled into nucleosomes (Knezetic and Luse 1986; Workman and Roeder 1987; Knezetic et al. 1988; Workman et al. 1990, 1991; Straka and Horz, 1991) or with the chromatin component histone H1 (Croston et al. 1991; Laybourn and Kadonaga 1991).

The IS photofootprint does not depend on transcription or on sequences downstream of the IS

Previous studies showed that the IS patterns were unaffected by a 3-bp substitution in the *GAL1* TATA box (TATATAAA → TCGCTAAAT) that severely diminished transcription (Selleck and Majors 1988). We confirmed this result using the primer extension assay (Fig. 7), and we conclude that disruption of the IS nucleosome requires neither a functional TATA element nor active transcription. It was still possible, however, that the photofootprint depended on a part of the downstream transcription complex other than TFIID or on sequences surrounding the TATA element or the initiation site. To test this, we made a deletion spanning from just upstream of the TATA box to just upstream of the *HIS3* AUG in the fusion gene and integrated and photofootprinted this construct (YAX41). The removal of these sequences had no effect on the IS photofootprint (Fig. 7). Because all *GAL1* sequences from the TATA box downstream are absent in the deleted *GAL1–HIS3* fusion, disruption of the nucleosome requires an activator but is independent of interactions with downstream sequences or with basal transcription factors that are bound there.

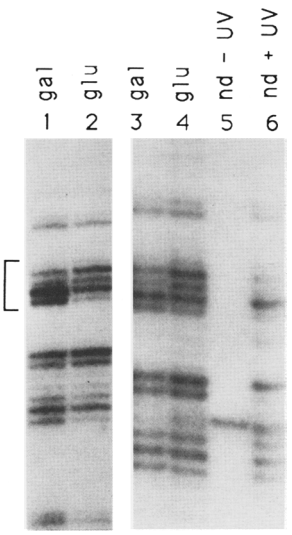

Figure 7. Photofootprint of *GAL1* bearing a mutant or deleted TATA element. (Lanes *1,2*) Photofootprint of *GAL1–HIS3* fusion gene bearing a deletion spanning from upstream of the TATA box through the start site of the *HIS3*-coding region. YAX41 was footprinted at the IS site, and the pattern is identical to the wild-type footprint. (Lanes *3,4*) The strain described previously bearing a 3-bp mutation in the *GAL1* TATA element (Selleck and Majors 1988) was photofootprinted at the IS photofootprint site using the primer extension method. (Lanes *5,6*) Naked DNA controls. The footprint patterns are not different from that seen in the wild-type gene. In both constructs, >99% of full-length transcription is abolished (Selleck and Majors 1988; data not shown).

GAL4 derivatives disrupt the IS nucleosome in proportion to their strength as transcriptional activators

Whether the transcriptional activation function of GAL4 protein is responsible for disrupting the IS nucleosome, and altering the IS photofootprint, we reasoned that GAL4 derivatives that are weaker activators should show lesser effects on the photopattern. The structures of several such derivatives are schematized in Figure 8A (the plasmids were generous gifts of J. Ma, E. Giniger, and M. Ptashne, Harvard University, Cambridge, MA). The relative strength of the GAL4 derivatives in activating GAL1–LacZ expression, when expressed from a 2μ plasmid, is shown (data derived from Giniger and Ptashne 1987; Ma and Plashne 1987a). pMA236 and pMA238 (Ma and Ptashne 1987a) express the GAL4 DNA-binding domain fused either to the acidic carboxy-terminal activation domain (pMA236) or to a fragment of the carboxyl terminus lacking many of the acidic residues (pMA238). pMA236 activates about half as well as wild-type GAL4, whereas pMA238 has almost no activity. pEG50 expresses the GAL4 DNA-binding domain fused to an amphipathic helix, whereas pEG52 expresses the DNA-binding domain fused to the same amino acids in scrambled order (Giniger and Ptashne 1987). pEG50 has 17% of wild-type activity, whereas pEG52 has essentially none (Giniger and Ptashne 1987). Plasmids expressing the GAL4 derivatives were transformed into a *gal4Δ gal80Δ* strain, grown on raffinose to activate expression, and photofootprinted at the IS and the UAS_G. The IS footprint results are shown in Figure 8B. When compared with the wild type, the nonfunctional GAL4 derivatives (pMA238 and pEG52) leave the off footprint pattern unchanged, whereas the functional derivatives produce the on pattern to a degree that corresponds roughly with their strength as transcriptional activators. pMA236 disrupts the IS nucleosome and alters the off footprint, but to a lesser extent than wild type; pEG50 is less active and appears to be less effective at altering the pattern. To our surprise, the pMA210 transformant used in this experiment (expressing intact GAL4) failed to produce the on footprint. However, photofootprints of the UAS_G revealed that the GAL4-binding sites were unoccupied, suggesting that functional GAL4 protein was not being produced in this isolate. The other transformants had fully occupied GAL4-binding sites (data not shown). A repeat experiment for several of the GAL4 derivatives is shown in Figure 8B. These results support the hypothesis that the activating function of GAL4 is responsible

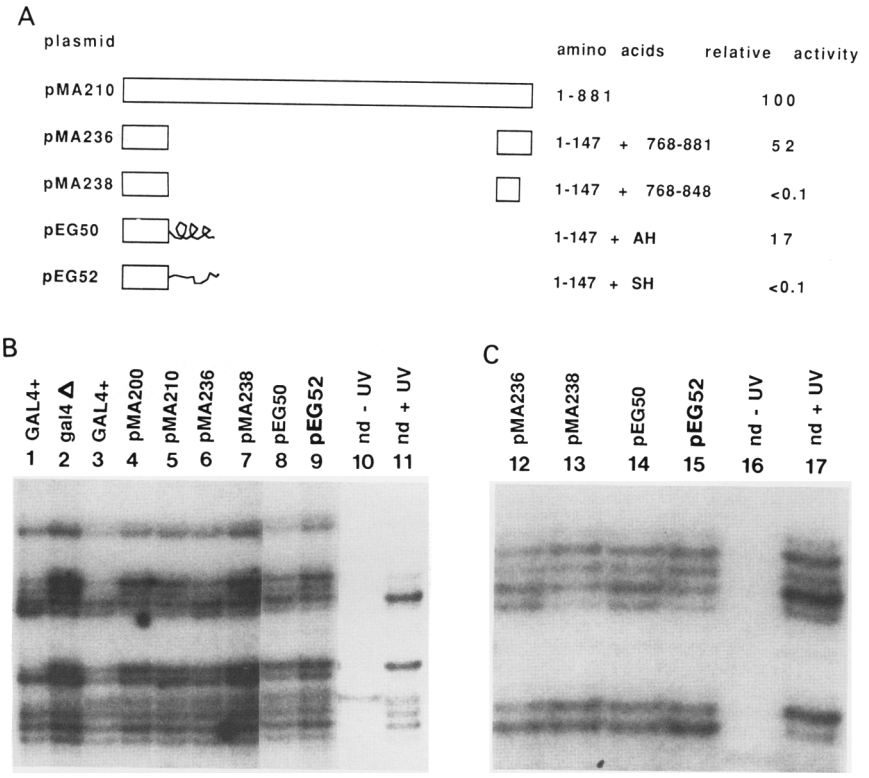

Figure 8. IS photofootprints from strains bearing GAL4 derivatives. YM709 (*gal4*$^-$–*gal80*$^-$) was transformed with 2μ plasmids expressing GAL4 or the derivatives shown in (*A*), or a control plasmid (pMA200). Their activities as reported in Ma and Ptashne (1987a) and Giniger and Ptashne (1987), relative to pMA210, are shown. (*B*) The samples were grown in minimal medium containing raffinose and photofootprinted (lanes 4-9). Controls included YM654 (*GAL4*$^+$–*gal80*$^-$) and YM709 grown on complete medium plus 5% glycerol and 0.1% glucose (uninduced, lanes *1,2*), and YM654 grown in minimal medium plus raffinose (lane 3). (*C*) A second experiment with several samples, as in B.

for disrupting the nucleosome positioned on the IS, thus altering the IS off footprint.

Discussion

A photofootprint within the IS of the *GAL1* gene is altered when transcription is stimulated by GAL4. For several reasons we believe that the alterations result from displacement of a nucleosome. First, conventional nucleosome mapping studies (Lohr 1984; Fedor and Kornberg 1989) and our micrococcal nuclease protection results reported here, as well as high-resolution DNase I footprinting data (M.S. Reagan and J. Majors, unpubl.), show patterns consistent with the presence of a nucleosome at the IS when the promoter is inactive. Second, the footprint depends on normal histone H4 expression; diminished H4 expression (Han and Grunstein 1988; Han et al. 1988) results in partial loss of the footprint. Third, in various in vitro transcription systems, nucleosomes, as well as histone H1, either separately or additively, can inhibit basal transcription in vitro when assembled on the template before the addition of transcription factors (Knezetic and Luse 1986; Workman and Roeder 1987; Knezetic et al. 1988; Workman et al. 1988, 1990, 1991; Pina et al. 1990; Croston et al. 1991; Laybourn and Kadonaga 1991; Straka and Horz 1991), and we see increased basal transcription in vivo when the footprint-inducing structure is altered (note, however, that yeast strains do not have histone H1). Finally, DMS protection experiments failed to detect protein binding to the IS; it is thought that nucleosomes fail to protect DNA from methylation at G residues because of a lack of intimate contacts in the major groove. In contrast, evidence exists for the modulation of photoproduct formation by nucleosomes (Gale and Smerdon 1988).

Loss of both the photofootprint and micrococcal nuclease protection indicate that the IS nucleosome is displaced by activation. Its displacement is a result of the GAL4 activation signal: The efficiency of this event is proportional to activator strength, and downstream sequences or factors are not required. Altering the spacing between the $UAS_G$and the IS sequence repositions or disrupts the nucleosome, as reflected both by the loss of the photofootprint and by altered micrococcal nuclease protection. These alterations result in increased expression from the uninduced promoter, indicating that the nucleosome functions to suppress transcription in the absence of activation.

Our results hint that disruption of suppression by the nucleosome is a rate-limiting step in a two-step activation process in vivo. For constructions in which the suppressing effect of the nucleosome is lessened (e.g., YAX32-1; Fig. 4C, lane 9), increased basal expression still depends on GAL4. This implies that during growth on raffinose, a modest amount of GAL4 activity escapes inhibition by GAL80. This "leaky" activity is insufficient to activate transcription in wild-type cells grown under the same conditions (YAX29c; Fig. 4B, lane 1). We suggest that displacement of the nucleosome requires a stronger or more sustained activation signal than does the downstream target of GAL4 . This view is consistent

with the observation that the degree of footprint relief correlates with the strength of activator derivatives.

What is the downstream target of GAL4? The acidic carboxy-terminal domain has been implicated in transcriptional activation (Brent and Ptashne 1985; Ma and Ptashne 1987a). We show here that GAL4, acting through this acidic carboxyl terminus, displaces a nucleosome from the *GAL1* promoter in vivo. In addition, acidic activators appear to interact directly in vitro with both TFIIB and with the TATA-binding factor component of TFIID (Horikoshi et al. 1988a; Lin and Green 1991; Stringer et al. 1990; for review, see Greenblatt 1991). In addition, still poorly characterized adaptor proteins have been proposed to mediate activator–core complex interactions (Berger et al. 1990, 1992; Kelleher et al. 1990; Meisterernst et al. 1990; see also Hoey et al. 1990; Pugh and Tjian 1990). Resolution of these issues awaits the generation and use of more highly purified components for study in vitro, and further biochemical and genetic tests of the functional importance of these interactions.

How does GAL4 protein disrupt the nucleosome? Previous studies in vitro implied that promoter-bound nucleosomes are disrupted by activation but failed to distinguish between two possibilities: (1) that an activator–core complex interaction is sufficiently strong to displace the nucleosome or (2) that the activator can displace the nucleosome independent of any interaction with the core transcription complex. We have demonstrated by TATA mutation and by deletion of the core complex-binding region that interaction of the activator with DNA-bound core complex is most likely not necessary for nucleosome disruption. However, we cannot rule out the formal possibility that the 1% of wild-type transcription seen in the TATA mutant results from residual core complex assembly that is sufficient to allow mechanism 1 to occur, nor can we rule out the possibility that the activator interacts with components of the core complex not bound to DNA. In addition, the demonstration in this paper that the activation strength of several GAL4 derivatives, including one bearing a synthetic acidic domain, correlates with IS nucleosome disruption, suggests that the same feature of the activator protein that activates transcription also disrupts the suppressing nucleosome. Experiments reported by Durrin et al. (1991) identified a potential target for GAL4 on the nucleosome. Mutations in the histone H4 amino-terminal residues 4–23 inhibit activation of *GAL1* in vivo. Interestingly, these mutations had variable effects on other genes. The authors mentioned above suggest that the activation step requiring this region of H4 is necessary only for genes whose promoters are tightly folded into nucleosomes that suppress basal transcription. Additional factors may be required to disrupt the nucleosome. Hirschhorn et al. (1992) have recently demonstrated a requirement for *SNF2/SWI2* and *SNF5* in activation-dependent nucleosome rearrangement on the *SUC2* promoter, and mutations in these same genes prevent activation mediated by GAL4 (Laurent and Carlson 1992; for review, see Winston and Carlson 1992). A more complete picture of transcriptional activator function will require a better understanding of the interactions between activators and nucleosomes.

Materials and methods

Plasmids

The plasmids used to create the various *GAL1–HIS3* fusions in this study are all derived from pJD16 or pJD19. pJD16 was constructed from pBM1436, which has been described in detail (Flick and Johnston 1990). In brief, pBM1436 contains two short sequences from immediately downstream of the *LYS2* terminator. They flank the *LEU2* UAS fused to the *GAL1* IS followed by the *HIS3*-coding sequences, and a small piece of YIp5. Outside the *LYS2* sequences is a fragment from YIp5 containing the yeast *URA3* gene, *ori*, and the *amp* gene. Yeast transformation of this plasmid or its derivatives after cleavage with *PvuII* directs integration near *LYS2*, and selection of the resulting transformants against *URA3* results in some colonies that retain just the UAS–IS–*HIS3* fragment inserted downstream of the *LYS2* terminator. The resulting integrants show no transcription that begins upstream of the inserted sequences (data not shown).

pJD16 was derived from pBM1436 by deletion of the *LEU2* UAS. All plasmids bearing UAS elements fused at −214 of the *GAL1* IS were made by inserting the appropriate sequences into the *Eco*RI site in pJD16, except for pJD23. The UAS_G *Eco*RI fragment is a 143-bp *Rsa*I (−393) to *Alu*I (−250) fragment modified with *Eco*RI linkers (from pBM1499; Flick and Johnston 1990). When inserted into pJD16, the resulting plasmids were pJD18 (wild-type orientation) and pJD18R (reversed orientation). For pJD23, nucleotides −146/−127 of the *GAL1* IS were replaced with a 10-bp *Xba*I linker (from pBM1635; Flick and Johnston 1990) followed by insertion of the UAS_G fragment. The consensus GAL4 site was synthesized as an oligonucleotide (5′-AATTATCTAGACGGAGGACAGTCCTCCG-3′) and inserted into pJD16. The resulting plasmids were pJD32-1 (one copy of consensus GAL4 site) and pJD32-2 (two copies). pBM1626 is similar to pJD32-2 except that it contains two GCN4 binding sites embedded in the sequence 5′-AATTCA-GTGACTCACGTCAGTGACTCACG-3′ (Hinnebusch 1988).

The remaining plasmids were derivatives of pJD19, which was constructed as follows: pBM261 (Johnston and Davis 1984), containing the entire *GAL1–10* regulatory region fused to *HIS3*-coding sequences, was site specifically mutagenized according to a published procedure (Morinaga et al. 1984), changing 2 bp immediately upstream of the TATA box to create a *Cla*I site (5′-TTAACAGATATA → TTAATCGATATA). The *Eco*RI–*Kpn*I fragment of pJD16 was replaced with the *Eco*RI–*Kpn*I fragment containing the *GAL* sequences and most of *HIS3* from the altered pBM261, resulting in pJD19. pJD35 results from replacement of the *Cla*I–*Kpn*I fragment of pJD19 with a *Sac*I (immediately upstream of the HIS3 AUG)–*Kpn*I fragment from pJD16, reconstructing pJD19 except for a deletion from upstream of TATA to the AUG. Substitution of the IS with random *Drosophila* DNA fragments was accomplished by adapting the 3′*Eco*RI site of pJD16 with a *Sac*I and a *Cla*I site and inserting the resulting *Eco*RI–*Cla*I UAS_G fragment into pJD19, from which the GAL1 sequences from *Eco*RI–*Cla*I had been removed. This plasmid was then linearized at *Sac*I and *Cla*I, and random, size-fractionated *Sac*I–*Cla*I *Drosophila* DNA fragments were inserted. The resulting pJD29-series plasmids were screened for *Drosophila* inserts of a size approximating the wild-type spacing. pMA200, pMA210, pMA236, pMA238 (Ma and Ptashne 1987a), and pEG50 and pEG52 (Giniger and Ptashne 1987) were

gifts of J. Ma, E. Giniger, and M. Ptashne and are multicopy plasmids expressing GAL4 or its derivatives as depicted in Figure 3. All strains having the pBM designation were the generous gifts of J. Flick and M. Johnston (1992). The structures of the integrated plasmids are schematized in the appropriate figures. *GAL1* map positions are relative to the major *GAL1* transcription start site (+1).

Yeast strains and media

The isogenic yeast strains used in this study are descendants of S288C, and include YM262 (α *ura3-52, his3Δ200, ade2-101, lys2-801, tyr1-501*), YM599 (**a,** *ura3-52, ade2-101, lys2-801, trp1Δ*), YM654 (α *ura3-52, ade2-101, his3Δ200, tyr1-501, lys2-801, gal80Δ538*), YM709 (**a** *ura3-52, his3Δ200, ade2-101, lys2-801, trp1Δ, tyr1, met, can*^{r}, *gal4Δ542, gal80Δ538*), YAX22 (**a** *galΔ112, his3Δ200, lys2-801, tyr1-501, trp1-289, ura3-52*), and YAX24 (**a** *galΔ112, his3Δ200, lys2-801, trp1-289, leu2, ura3-52*). YAX22 was transformed by the LiAc procedure (Ito et al. 1983), and single integrants were obtained (Flick and Johnston 1990), yielding the following strains (followed by the integrating plasmid used for each): YAX26(pJD18), YAX27(pJD18R), YAX28-(pJD23), YAX29c(pJD19), YAX29-series(pJD29-series), YAX32-1(pJD32-4), YAX32-2(pJD32-2), YAX41(pJD35), YAX43(pJD16), YAX44(pJD36), YAX45(pJD37), YAX47(pJD16-36), and YAX48-(pJD16-37). YAX31 was created by transformation of YAX24 with pBM1626. Strains designated YM were gifts of the M. Johnston laboratory (Washington University, St. Louis, MO).

Strain UKY403 (α *ade2-101 his3-Δ200 leu2-3,112 lys-801 trp1-Δ901 ura3-52 GAL*$^+$ *thr tyr arg4-1 Δh4-1[HIS3*$^+$*] Δh4-2*[LEU2$^+$]/pUK421 [*CEN TRP1*$^+$ *GAL–H4-2*$^+$]](Kim et al. 1988) was a generous gift of M. Grunstein (University of California at Los Angeles). As a control for copy number on effects on the IS photofootprint, YM599 was transformed with pBM753, a *CEN-TRP* plasmid bearing the *GAL1–10* control region (but no histone H4-coding region). The strain bearing the 3-bp TATA mutation has been described (Selleck and Majors 1988).

In most experiments yeast cultures were grown in 1% yeast extract, 2% Bacto-peptone, and either 2% glucose, 2% raffinose, 2% galactose, or 5% glycerol + 0.1% glucose, as indicated in the figure legends. In all strains derived from YAX22 or YAX24, induction by galactose was accomplished by growing the cultures in 2% raffinose and, 2 hr prior to harvest, adding 2% galactose. YAX31 and transformants carrying the 2μ plasmids expressing GAL4 or derivatives were grown in 0.17% yeast nitrogen base, 0.5% NH_4SO_4, supplemented with uracil, adenine, lysine, tryptophan, tyrosine, methionine, and the carbon source shown in the figure. UKY403 as well as the control strains in Figure 5 was grown in synthetic medium minus tryptophan, containing galactose, washed in water, and shifted to YP containing the appropriate carbon source for 5–6 hr prior to harvest. All cultures were harvested for footprinting or RNA analysis at an A_{600} of 1.5–2.0.

Photofootprinting

The photofootprinting procedure has been described (Axelrod and Majors 1989) and was followed with only minor modifications. In brief, yeast cultures were grown in the appropriate media, harvested by centrifugation, and resuspended in phosphate buffered saline (PBS). They were then exposed to UV light, and DNA was isolated as described previously. The DNA was cut with *Hae*III, and adjusted to 0.5 mg/ml. DNA (3.5 mg) was used per primer extension reaction, in which photoproducts were detected by arrest of *Taq* polymerase. Samples were electrophoresed, and most of the sequencing gels were fixed and dried prior to autoradiography. All footprints except that of YAX41 were visualized using oligo nucleotide 2114 (5′-CAAACCGAAAATGTTGAA-3′) complementary to *GAL1* position −28 to −45. YAX41 was footprinted using oligo nucleotide 6037 (5′-CGCAATCTGAATCTTGGT-3′) complementary to a proximal segment of the *HIS3*-coding region (Struhl 1985).

Analysis of nucleosome positioning by micrococcal nuclease protection

Five hundred milliliters of yeast was grown in the appropriate medium to OD_{595} of 2.0, and spheroplasts were made and lysed with modifications of a published procedure (Lue and Kornberg 1987). The cells were pelleted at room temperature at 5000*g* and resuspended in 30 ml of 40 mM EDTA, 100 mM β-mercaptoethanol, and incubated at 30°C for 30 min. The cells were then pelleted at room temperature at 5000*g* and resuspended in 5 ml of growth medium with 1 M sorbitol and treated with lyticase. Digestion proceeded until the OD_{595} of an aliquot of cells diluted 20-fold into 1% SDS was <10% of the starting value. Spheroplasts were collected by centrifugation at 3000*g* for 5 min at room temperature and lysed by resuspension in 1.5 ml of 18% (wt/vol) Ficoll, 20 mM Tris-HCl (pH 8.0), 20 mMKCl, 5 mM $MgCl_2$, 3 mM dithiothreitol (DTT), 1 mM EDTA, 10 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM pepstatin A, 0.6 mM leupeptin followed by treatment with 10 strokes of a hand-held Dounce homogenizer. Micrococcal nuclease digestion of the exposed chromatin was effected by the addition of 10 units of enzyme (Sigma) and incubation at 30°C for the indicated time. The reaction was terminated by addition of an equal volume of 2% SDS, 1 M NaCl, 20 mM EGTA, 50 mM Tris-HCl (pH 7.4), with 0.2 mg of proteinase K, and the mixture was incubated at 55°C for 30 min. The DNA was then isolated by sequential extraction with phenol and chloroform, precipitation with isopropanol, digestion with RNase A, and subsequent isolation as described (Axelrod and Majors 1989). For control samples, deproteinized genomic DNA was isolated as described (Hoffman and Winston 1987). Approximately 10 μg of DNA was suspended in 50 ml of lysis buffer, and 0.1 units of micrococcal nuclease was added and allowed to digest the DNA for 1 min at room temperature. The digest was stopped with 20 mM EGTA, and the DNA was isolated by extraction with phenol/chloroform and precipitation with isopropanol. All samples were restricted with *Bgl*II and electrophoresed. Equivalent extents of digestion were confirmed for all nuclear and control samples by ethidium bromide staining in an agarose gel. We speculate that global changes in nuclear structure account for the varying digestion times required to achieve equal digestion in different growth media. Hybridizing size markers were made from YAX29c and YAX32-1. The DNA was transferred onto Genescreen (New England Nuclear) UV-cross-linked, and visualized by indirect end-labeling using a riboprobe. The riboprobe was generated by insertion of a *Bgl*II–*Hind*III fragment (extending from +422 to +331 in the *HIS3* sequences of the constructs) between the *Bam*HI and *Hind*III sites of the phagemid BluescriptII SK(+) (Stratagene). The plasmid was linearized with *Xba*I, and the probe was synthesized as described (Selleck and Majors 1987a). The membranes were hybridized and washed at 60°C as described (Church and Gilbert 1984).

RNA analysis

RNA was isolated, electrophoresed, and blotted according to the method described in Flick and Johnston (1990). When appropriate, cultures for RNA isolation were taken from the same cul-

tures used for photofootprintng. The *HIS3* riboprobe was made from linearized pBM1034 (Flick and Johnston 1990), and the *DED1* riboprobe was transcribed from a similar plasmid containing the *Xho*I–*Bam*HI fragment from the *DED1* gene (Struhl 1985), according to the method described in Selleck and Majors (1987a). Blots were hybridized in 50% formamide, 0.5% bovine serum albumin, 0.5 mM EDTA, 0.25 M Na_2PH_4 adjusted to pH 7.2, and 3.5% SDS at 60°. Blots were washed according to Church and Gilbert (1984), except that the first step was omitted and the temperature was gradually raised until background was low as measured with a hand-held monitor.

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