15. The vertical distribution of the DNA in the belt was examined through a confocal microscope (Meridian Insight, Okemos MI).
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17. Rabbit antibody was raised against COOH-terminus (22 amino acids) fused to β-galactosidase. Its Fab fragment [J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strober, in *Current Protocol in Immunology* (Wiley, New York, 1991), p. 2.8.1] was biotinylated [Instructions 20217X, Pierce] and coupled to a stoichiometric amount of rhodamine-avidin (*16*). Holoenzyme (*20*) was mixed with the labeled Fab and isolated through glycerol-gradient centrifugation [K. Igarashi, N. Fujita, A. Ishihama, *J. Mol. Biol.* **218**, 1 (1991)]. We used the immunolabeled enzyme (Fig. 2, Table 1) and chemically labeled enzyme (Fig. 3) (*16*). Samples on a plate maintained at 37°C were inspected through an Olympus RFLBHS microscope with a Hamamatsu C2400-08 camera.
18. S. A. Margarson and R. E. Glass, unpublished data.
19. A pair of aluminum electrodes with a gap of 80 to 100 μm was vacuum-deposited on a glass slide. Biotin was attached at both ends of T7 ΔD111 or λ*cI*857*S*am7 DNA (*20*), incubated with twice the stoichiometric amount of hen-egg avidin, and after dilution with water to give a 2 μg/ml DNA solution, 5 μl of the solution was placed near the electrode gap and covered with a cover slip. A 1-s pulse of 1 MHz at 20 kV/cm was applied at every 2 s for 10 to 30 min. The fixed DNA was washed with a working buffer [10 mM $MgCl_2$, 100 mM KCl, 0.1 mM dithiothreitol, tris-HCl (pH 8.0), and 0.4 mg of partially hydrolyzed casein per milliliter].
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21. We thank A. Ishihama, J. Tomizawa, and T. Yanagida for helpful discussions. Supported by grants from the Agency of Science and Technology of Japan and from the Ministry of Education, Science and Culture (N.S.) and by grants from the Medical Research Council, European Economic Commission, and British Council (R.E.G.).

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Nucleosome Disruption by Transcription Factor Binding in Yeast

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Studies in vivo and in vitro have shown that the packaging of DNA into chromatin can affect gene expression. Here, binding of the yeast transcriptional activator GAL4 to DNA in chromatin has been investigated in vivo with a yeast episome. A positioned nucleosome that is present in cells grown in glucose and contains a single GAL4 binding site is disrupted by GAL4 binding in galactose. GAL4 can also bind to DNA in chromatin when the carboxyl-terminal activation domain of GAL4 is either masked by GAL80 or is absent. These results show that a transcription factor can bind to its site in vivo in what would appear to be a repressive chromatin structure.

The incorporation of promoter elements into nucleosomes in vitro prevents transcription initiation, probably by occlusion or deformation of sequences recognized by specific trans-acting factors (*1*). Some factors, such as nuclear factor–1 (NF-1) and heat shock factor, do not bind well to nucleosomal DNA in vitro (*2*, *3*). However, other factors, such as the glucocorticoid receptor and GAL4, can bind and form ternary complexes with histone-bound DNA (*4*, *5*). The RNA polymerase III transcription factor TFIIIA can bind to a positioned nucleosome in vitro, but only when the histone NH_2-terminal regions are acetylated or absent (6).

Nucleosomes may also affect transcription initiation in vivo (*7*). In both the mouse mammary tumor virus promoter and the yeast *PHO5* gene, binding sites for transcriptional activators are located within positioned nucleosomes. In both cases, indirect evidence suggests that specific mechanisms are required to allow those factors access to their binding sites (*2*, *8*).

The well-characterized minichromosome TRP1ARS1 and its derivatives have been used to manipulate chromatin structure in yeast such that binding sites for trans-acting factors are included in or excluded from positioned nucleosomes (*9*–*11*). I have used this approach to investigate binding of the transcriptional activator GAL4 to its binding site when it is positioned within a nucleosome. A single copy of a near-consensus 17–base pair (bp) binding site for GAL4 was introduced into the DNA sequence normally located near the center of the stably positioned nucleosome I of a TRP1ARS1 derivative (*9*, *10*) to create the yeast episome TA17Δ80 (*12*). This plasmid was introduced into yeast cells, where it was maintained at >10 copies per cell. The chromatin structure of TA17Δ80 was assayed by the indirect end-label technique (*13*). In this technique, sites susceptible to micrococcal nuclease cleavage in chromatin and in naked DNA are mapped relative to a common restriction site. Regions that are protected in chromatin, but not in naked DNA, and which encompass 140 to 160 bp, are assumed to represent positioned nucleosomes (9).

When yeast cells harboring TA17Δ80 were grown in glucose, positioned nucleosomes were detected on the plasmid (Fig. 1, left panel, lanes labeled glucose). Nucleosome I contains the GAL4 binding site and is immediately adjacent to positioned nucleosome II. This structure was expected because GAL4 does not normally bind to its site in glucose, primarily because the *GAL4* gene is repressed (*14*). When cells harboring TA17Δ80 were grown in galactose, new micrococcal nuclease cleavage sites were observed in the nucleosome containing the GAL4 binding site, as well as in the neighboring nucleosome II (Fig. 1, left panel, lanes labeled galactose). This suggests that when cells harboring TA17Δ80 are grown in galactose, GAL4 is able to compete successfully with histones for occupancy of its binding site, resulting in the perturbation of a region of chromatin spanning two nucleosomes. This interpretation is supported by the finding that a control plasmid, TAΔ80, lacking the GAL4 binding site in nucleosome I, showed no change in the structure of this nucleosome between cells grown in glucose and galactose (Fig. 1, right panel). Nucleosome II appears less well positioned in TAΔ80 than in TA17Δ80, as evidenced by the incomplete protection of the cleavage in this region of TAΔ80; the reasons for this are not understood.

To determine whether GAL4 could activate transcription when its binding site is incorporated in a positioned nucleosome, I fused a 400-bp fragment encompassing nucleosomes I and II of TA17Δ80 to a β-galactosidase reporter gene (*15*). This construct, carried on a single-copy (CEN) plasmid, was introduced into yeast cells, which were assayed for β-galactosidase activity in the presence of glucose and galactose (Table 1). Activity was very low in glucose and strongly induced in galactose. The induced level of expression was not increased in the presence of a 2-μm-based plasmid containing the *GAL4* gene, indicating that maximal binding and transcription are achieved with normal wild-type levels of GAL4 protein. Micrococcal nuclease cleavage patterns of this plasmid were consistent with nucleosome I incorporating the same DNA sequence as in TA17Δ80 in cells grown in glucose; growth in galactose results in enhanced cleavage in this region (Fig. 2, arrow) as well as near the border of this

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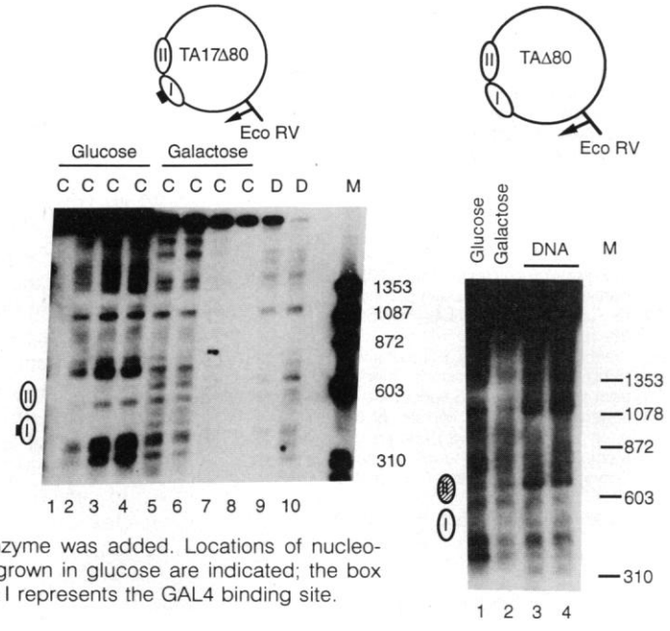

Fig. 1. Disruption of positioned nucleosomes in the yeast episome TA17Δ80 by GAL4 binding. Micrococcal nuclease cleavage sites in chromatin (C lanes) from cells grown in glucose or galactose medium, and in naked DNA (D lanes) were mapped in TA17Δ80 (left panel) and TAΔ80 (right panel) relative to the Eco RV site as indicated. Chromatin samples were digested with increasing amounts of micrococcal nuclease from the outer lanes towards the center, with lanes 1 and 8 being controls to which no enzyme was added. Locations of nucleosomes I and II in cells grown in glucose are indicated; the box attached to nucleosome I represents the GAL4 binding site.

Table 1. Transcriptional activation by GAL4 from a site within a positioned nucleosome. Yeast cells containing pRS314–17Δ80 were grown in the presence of glucose or galactose and assayed for β-galactosidase activity (*15*) in the presence or absence of the expression vector for GAL4 (*12*) or for GAL4(1–147)H (*19*). Activity is given in Miller units; numbers in parentheses indicate the number of independent determinations for each value.

Expression vector	Glucose	Galactose
–	<2	300 ± 20 (6)
GAL4	<5	290 ± 40 (4)
GAL4(1–147)H	10 ± 3 (4)	170 ± 50 (8)

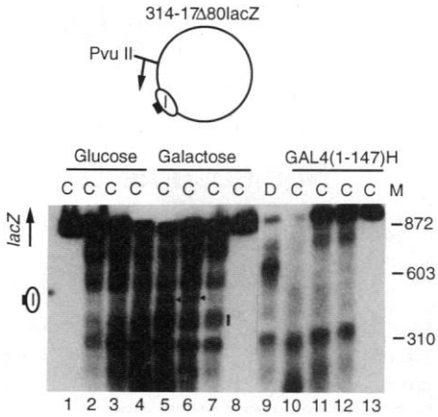

Fig. 2. Perturbation of positioned nucleosome I of pRS314-17Δ80lacZ by binding of GAL4 with or without its COOH-terminal activation domain. Micrococcal nuclease cleavage sites in pRS314-17Δ80lacZ from yeast cells grown in galactose, or in glucose in the presence or absence of GAL4(1–147)H were mapped relative to the Pvu II site as indicated. Samples in lanes 1 through 8 were digested with increasing concentrations of micrococcal nuclease (from the outer lanes towards the center), and samples in lanes 10 through 13 were digested with increasing concentrations of micrococcal nuclease (from right to left; lanes 1, 8, and 13 are control samples to which no nuclease was added). To the left of lane 1 is a Pvu II–Bgl II fragment that marks the border of the GAL4 binding site proximal to the *lacZ* gene. The location of nucleosome I in glucose is indicated; the box attached represents the GAL4 binding site.

nucleosome (Fig. 2, lanes 5 through 7, vertical bar). These results indicate that GAL4 can activate transcription with an accompanying perturbation of chromatin structure even when its site is incorporated into a positioned nucleosome in cells grown in glucose, consistent with the structural data shown in Fig. 1.

The DNA-binding domain of GAL4 is located in the NH_2-terminal 65 amino acids of the protein (*16*), whereas the transcriptional activation domain is located at the COOH-terminus. When cells are grown in medium lacking both glucose and galactose (for example, in raffinose), GAL4 occupies its normal binding sites, but the activation domain is masked by the repressor protein, GAL80, and transcription is not induced (*17*). To investigate whether the transcriptional activation domain of GAL4 functions in the protein's ability to bind to DNA in chromatin, I mapped the chromatin structure of TA17Δ80 from yeast cells grown in raffinose. The same cleavage sites seen in the regions of nucleosomes I and II in cells grown in galactose are seen in cells grown in raffinose, indicating that GAL4 is able to bind to TA17Δ80 and disrupt nucleosomes I and II even when its activation domain is masked by GAL80 (Fig. 3, left panel). Similar results were obtained when GAL4 was expressed at high levels in glucose in yeast cells harboring TA17Δ80 (Fig. 3, right panel) (*18*). A derivative of GAL4, GAL4(1–147)H, which includes only the first 147 amino acids of GAL4 fused to a shorter amino acid sequence including a hemagglutinin epitope (*19*), also binds to TA17Δ80 with the same effect on nucleosomes I and II (Fig. 4). Ectopic expression of this latter derivative in galactose interferes with transcriptional induction by GAL4 in the plasmid pRS314-17Δ80, as expected (Table 1), indicating that it is expressed at levels comparable to GAL4 in cells grown in galactose, and this derivative also perturbs the chromatin structure of pRS314-17Δ80 in cells grown in glucose medium (Fig. 2, lanes 10 through 13).

The nuclease cleavage pattern of TA17Δ80 differs in the presence of activating and nonactivating forms of GAL4 in the intensity of the cleavage site at the border of nucleosome II distal to nucleosome I. The intensity of this cleavage is decreased only when the activation domain of GAL4 is present and unmasked (Figs. 1, 3, and 4). This difference is under investigation. The strong cleavage site between nucleosome I of pRS314-17Δ80lacZ and the *lacZ* transcription start site that is present in cells grown in glucose or galactose is much reduced in the presence of GAL4(1–147)H (Fig. 2). It may be that this site is strongly cleaved in cells grown in glucose because it is at the border of nucleosome I (*9*), and in cells grown in galactose it may reflect the transcriptional activity of the *lacZ* gene (*20*). When GAL4(1–147)H is expressed in cells grown in glucose, nucleosome I is perturbed (Fig. 2, lanes 10 through 13) and the *lacZ* gene is not strongly transcribed (Table 1), and this may account for the weak cutting observed at this site.

Perturbation of nucleosomes I and II of TA17Δ80 corresponds exactly to conditions under which GAL4 is expected to bind (absence of glucose, Figs. 1 and 3, or ectopic expression of the GAL4 DNA-binding domain, Figs. 3 and 4) and depends on the presence of the GAL4 binding site in nucleosome I (Fig. 1). Moreover, this same binding site allows transcriptional induction of a reporter gene in galactose medium (Table 1) with an accompanying perturbation of chromatin structure (Fig. 2). Photofootprinting experiments also show changes within and near the GAL4 binding site of TA17Δ80 that correlate with the expected binding of GAL4 (*21*). Taken together, these results show that when cells are grown in galactose, GAL4

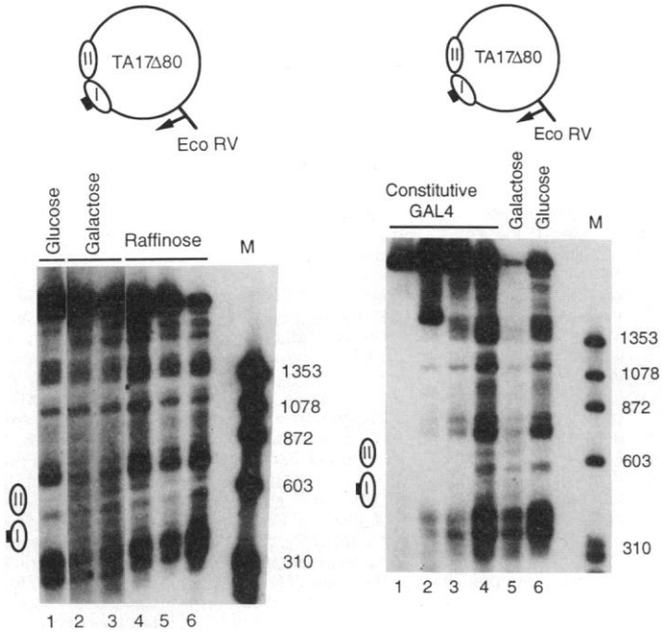

Fig. 3. Disruption of positioned nucleosomes in the yeast episome TA17Δ80 by GAL4 binding under noninducing conditions. Micrococcal nuclease cleavage sites in TA17Δ80 from yeast cells grown in raffinose, glucose, or galactose as indicated (left panel) or in glucose in the presence or absence of high levels of GAL4 (right panel) were mapped relative to the Eco RV site as indicated. Lanes 4 through 6, left panel, and lanes 1 through 4, right panel, contain samples digested with increasing amounts of micrococcal nuclease; lane 1, right panel, is a control to which no enzyme was added. Locations of nucleosomes I and II in cells grown in glucose are indicated; the box attached to nucleosome I represents the GAL4 binding site.

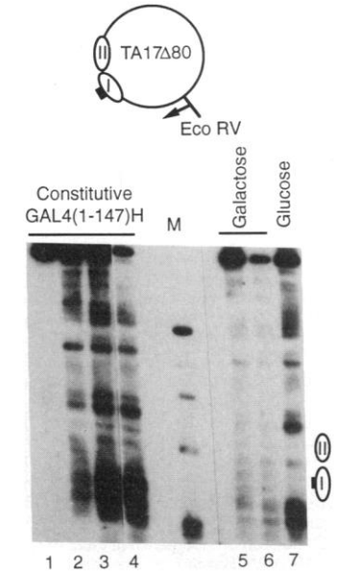

Fig. 4. Disruption of positioned nucleosomes in the yeast episome TA17Δ80 by a derivative of GAL4 lacking the COOH-terminal activation domain. Micrococcal nuclease cleavage sites in TA17Δ80 chromatin from yeast cells grown in glucose medium in the presence or absence of GAL4(1–147)H, or in galactose were mapped relative to the Eco RV site as indicated. Samples in lanes 1 through 4 were digested with increasing amounts of micrococcal nuclease, with lane 1 being a control to which no enzyme was added. Locations of nucleosomes I and II in cells grown in glucose are indicated; the box attached to nucleosome I represents the GAL4 binding site. The lane labeled M contains ϕX174 DNA digested with Hae III.

can bind to a near-consensus binding site that is incorporated within a positioned nucleosome during growth in glucose, giving rise to a "chromatin footprint." The ability of GAL4 to compete successfully with histones for occupancy of its binding site in yeast is consistent with results showing that GAL4 can bind to nucleosomal templates in vitro (*3*, *5*). GAL4 thus behaves like a factor (or factors) involved in tRNA transcription that interferes with nucleosome positioning in vivo (*11*). In contrast, incorporation of the ARS A sequence of the ARS1 element into a nucleosome interferes with its function (*10*). These results emphasize that different outcomes are possible in the competition between histones and trans-acting factors for a given DNA sequence in vivo.

Whether binding of GAL4 to its site in chromatin occurs by invasion into an already formed nucleosome, or takes place during replication when DNA must be at least transiently unoccupied by histone proteins, has not been addressed here. For the normal function of this transcription factor, this question may not be relevant, because induction is slow enough that at least one round of replication will occur before transcriptional activation occurs. A similar situation exists for induction of the *PHO5* gene in low-phosphate medium; in that case, a *ts* mutant of the negative regulatory factor PHO80 was used to demonstrate that PHO4 could bind between two positioned nucleosomes and disrupt chromatin structure in the absence of replication (*22*). I have also not addressed whether histones are displaced from TA17Δ80 by GAL4, or whether nucleosome structure is altered in some way to accommodate binding. However, since GAL4 contacts the major groove at two sites that are about 1.6 helical turns apart when it binds to DNA (*23*), it is likely that nucleosome structure is severely perturbed by GAL4 binding. The closely spaced micrococcal nuclease cleavage sites present in the regions of nucleosomes I and II in cells grown in galactose suggest that this is the case.

The *GAL1-10* promoter is activated by GAL4. Four sites for GAL4 are present in a short region between the divergently transcribed *GAL1* and *GAL10* genes, and this region is accessible to DNase I even when GAL4 is not bound (*24*). Activation of this promoter by GAL4 leads to changes in nearby chromatin structure, and these changes depend on an unmasked activation domain (*25*). This has led to the reasonable idea that this region of chromatin exists in an "open" configuration that allows binding and activation by GAL4. A binding site has also been located in this promoter for another protein, GRF2, which is capable of organizing positioned nucleosomes in vivo (*26*). Thus, it has been postulated that GRF2 is important in setting up a chromatin configuration that allows proper regulation of the *GAL1-10* promoter. The results presented here cast some doubt on this hypothesis. However, the binding site used in this work was closer to the consensus than any of the four sites present in the *GAL1-10* promoter; the ability of GAL4 to bind to its site in chromatin may be affected by the precise sequence of the binding site.

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12. TA17Δ80 was constructed from pRS104 (*11*), which contains TRP1ARS1 sequences from 615 to 1208 map units cloned into pUC18. The near-consensus 17-bp GAL4 binding site 5′-CGGAA-GACTCTCCTCCGATC-3′ [E. Giniger, S. M. Varnum, M. Ptashne, *Cell* **40**, 767 (1985)] was introduced into this plasmid between 857 and 858 map units with the polymerase chain reaction (PCR) [R. Higuchi, B. Krummel, R. K. Saiki, *Nucleic Acids Res.* **16**, 7351 (1988)]; the 80-bp deletion (from 872 to 951 map units), which leads to incorporation of the GAL4 binding site into nucleosome I (*10*), was also made by PCR. Digestion of the resulting plasmid, pRS104-17Δ80, along with pRS110, which bears the complementary part of the TRP1 gene and a copy of the ARS1 sequence (*11*), with Hind III and Sst I, followed by ligation yields TA17Δ80. This construction eliminates the GAL4 binding site from the *GAL3* promoter centered at 1415 map units in TRP1ARS1 [W. Bajwa, T. E. Torchia, J. E. Hopper, *Mol. Cell. Biol.* **8**, 3439 (1988)]. TA17Δ80 was introduced directly into the yeast strain FY24 (*matα*, *ura3-52, trp1Δ63, leu2Δ1*), which is isogenic to the strain S288C except that it is *GAL2*$^+$ (*27*), by a modification [J. Hill, K. A. Ian, G. Donald, D. E. Griffiths, *Nucleic Acids Res.* **19**, 5791 (1991)] of the method of Ito *et al.* [H. Ito, Y. Fukuda, K. Marata, A. Kimura, *J. Bacteriol.* **153**, 163 (1983)]. The resulting transformants were transformed with the GAL4 expression vector pRS425-GAL4, which comprises the 3.6-kb Bam HI fragment containing the *GAL4* gene from the plasmid pG525 [A. Laughon and R. F. Gesteland, *Mol. Cell. Biol.* **4**, 260 (1984)] carried on the 2-μm-based shuttle vector pRS425 (*28*). TAΔ80 was made identically to TA17Δ80 except that the initial introduction of the GAL4 binding site was omitted.
13. Yeast cells (400 ml) were grown at 30°C in media supplemented with 2% glucose or 2% galactose and amino acids plus uracil as appropriate [M. D. Rose, F. Winston, P. Hieter, *Methods in Yeast Genetics: A Laboratory Course Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990)] to an absorbance at 600 nm between 0.6 and 1.6. Spheroplast lysates were prepared according to Fedor *et al.* [in (*26*)], and 300-μl samples of the supernatant containing minichromosomes were incubated at 37°C for 5 min before treatment with micrococcal nuclease for 10 min at 37°C at concentrations varying from 0 to 20 U/ml. For digestion of naked DNA, samples were first treated with proteinase K, extracted with phenol and chloroform, then taken up in 300 μl of 150 mM NaCl, 5 mM KCl, 1 mM EDTA, 20 mM tris (pH 8.0) before digestion as above. Digestions were stopped by the addition of 55 μl of 5% SDS, proteinase K (5 mg/ml), and after >2 hours at 37°C, samples were extracted with phenol and chloroform, and the DNA was precipitated. The sedimented material was taken up in 100 μl of 10 mM tris acetate (pH 8.0), 0.05 mM EDTA, treated with RNase A (40 μg), and cleaned over 1-ml G50 spin columns (*29*). For analysis by indirect end-labeling [S. A. Nedospasov and G. P. Georgiev, *Biochem. Biophys. Res. Commun.* **92**, 532 (1980); C. Wu, *Nature* **286**, 854 (1980)], samples (25 to 40 μl) were digested with Eco RV for 3 to 4 hours, and the DNA was precipitated and then electrophoresed on 1.2% agarose gels at 4 V/cm for 5 to 6 hours. Bromophenol blue was omitted from sample buffers. DNA was transferred to nylon membranes (Duralon UV; Stratagene) by capillary action and hybridized with random-primer labeled probes (*29*). Hybridization and washing were as described [G. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984)]. Blots were probed with the 231-bp Eco RV–Hind III fragment from TRP1ARS1. Chromatin structure was mapped for at least two independent clones for all experiments described.
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15. A Pst I fragment encompassing the *lacZ* gene and *CYC1* TATA box from the plasmid pLG669-Z [L. Guarente and M. Ptashne, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2199 (1981)] was ligated into the Pst I site of pRS104 (*11*), at 823 map units of TRP1ARS1; the Kpn I–Hind III fragment of the resulting plasmid containing the *lacZ* gene and TRP1ARS1 sequences was then inserted into the polylinker of the CEN-containing shuttle vector pRS314 [R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989)], creating pRS314-17Δ80, and introduced into yeast cells. The GAL4 binding site is 300 bp upstream of the transcription start site for *lacZ* in this plasmid. Cells were grown in the presence of 2% glucose or 2% galactose, and assayed for β-galactosidase activity as described [J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972); M. D. Rose, F. Winston, P. Hieter, *Methods In Yeast Genetics: A Laboratory Course Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990)]. Samples for chromatin mapping were prepared as described (*11*) and analyzed by the indirect end-label technique (*13*). Digestion was with Pvu II, and the blot shown in Fig. 2 was hybridized with the 252-bp Pvu II–Cla I fragment from pRS314.
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18. The vector for constitutive expression of GAL4 is pCL1, a derivative of YCp50 bearing a *LEU2* marker and transcribing the GAL4 coding sequence from the *ADH1* promoter [S. Fields and O. Song, *Nature* **340**, 145 (1989)].
19. The expression vector for GAL4(1–147)H is derived from pAS1, a 2-μm-based plasmid in which the *ADH1* promoter drives expression of a fusion of the first 147 amino acids of GAL4 with the hemagglutinin epitope. The *TRP1* gene present on pAS1 was replaced by the *LEU2* gene from pRS425 (*13*) before transformation of *leu*$^-$ yeast cells.
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Tethering Ribozymes to a Retroviral Packaging Signal for Destruction of Viral RNA

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Cellular compartmentalization of RNAs is thought to influence their susceptibility to ribozyme cleavage. As a test of this idea, two retroviral vectors—one encoding a hammerhead ribozyme designed to cleave *lacZ* transcripts and another encoding the *lacZ* messenger RNA—were coexpressed inside retroviral packaging cells. Because of the retroviral packaging signal, the ribozyme would be expected to colocalize with the *lacZ*-containing viral genomic RNA but not with the *lacZ* messenger RNA. The ribozyme was found to reduce the titer of infectious virus containing *lacZ* by 90 percent, but had no effect on translation of *lacZ* messenger RNA. These results indicate that sorting gene inhibitors to appropriate intracellular sites may increase their effectiveness.

The ability to target ribozymes to cleave viral RNAs in vitro has led to speculation about their potential therapeutic value as antiviral agents in vivo (*1*). To develop ribozymes for this purpose, however, one must consider the characteristics that distinguish these two settings. In test tubes, ribozymes and their substrates diffuse freely, and trans-cleavage can proceed as fast as RNA duplex formation (*2*). In cells, RNAs do not appear to diffuse freely but rather are sorted to specific cellular locations (*3*). Such compartmentalization of viral RNAs in vivo may reduce their availability to ribozymes. We propose a strategy that takes advantage of the cell's propensity to compartmentalize biological molecules. We show that delivery of a ribozyme to the same cellular location as its target can substantially increase the effectiveness of the ribozyme.

Our experimental system exploits properties of retroviral replication as well as retroviral vector–mediated gene transfer. We used two types of Moloney murine leukemia virus

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