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The Yeast *GAL1-10* UAS Region Readily Accepts Nucleosomes in Vitro†

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ABSTRACT: To test if the absence of nucleosomes on the UAS region of the yeast *GAL1-10* genes in vivo could be due to a low inherent affinity of this DNA for histones, DNA fragments containing the UAS and various amounts of flanking DNA were reconstituted into chromatin. Restriction enzyme and DNase I digestion analyses show that DNA in the UAS becomes protected against digestion in the reconstitutes. Thus, nucleosomes can assemble on the UAS region in vitro. The level of protection of the UAS and of the flanking DNA regions is comparable and remains so at various levels of nucleosome loading, suggesting that the UAS DNA has no tendency to exclude nucleosomes. In fact, DNase I results suggest that the UAS elements themselves preferentially bind histones.

Hypersensitive regions are a common feature of eukaryotic chromatin [cf. Gross and Garrard (1988)]. In many cases, these regions correspond to nucleosome-free gaps in the chromatin structure. Gene-regulating DNA elements often lie in these gaps.

It has been shown that histones and transcriptional regulatory proteins can bind to the same piece of regulatory DNA [cf. Gottesfeld (1987) and Workman et al. (1988)]. Generally, for gene expression to occur, regulatory factor assembly must prevail over nucleosome assembly. Thus, maintaining nucleosome-free gaps around DNA regulatory elements could be one aspect of eukaryotic transcriptional regulation.

The UAS,[1] or upstream activation sequences, are the DNA elements which mediate induction of gene expression of the yeast *GAL1-10* genes (Guarente et al., 1982). During growth in galactose or glycerol/ethanol, but not in glucose, the regulatory factor GAL4 binds to these small (~17 bp) elements (Giniger et al., 1985; Lohr & Hopper, 1985). In galactose, the bound GAL4 promotes expression of the genes. There is a large (~170 bp) nucleosome-free gap around the UAS elements under all growth conditions (Lohr, 1984; Lohr & Hopper, 1985). It is of interest to know how the cell maintains

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[1] Abbreviations: bp, base pair(s); DNase I, deoxyribonuclease I (EC 3.1.4.5); UAS, upstream activation sequence(s).

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this nonnucleosomal region, particularly in glucose-grown cells.

One way a cell can maintain a nucleosome-free region is to allow other protein factors which exclude histones to bind to the region. For *GAL1-10*, Fedor et al. (1988) have suggested a protein which could carry out this function. This factor, "Y", binds in vitro to a portion of the UAS elements (Fedor et al., 1988). Alternatively or additionally, the DNA sequence in a nonnucleosomal region could have a low intrinsic affinity for histones. This has been suggested to produce the nucleosome-free region upstream of the *Ded1* gene (Chen et al., 1987). We have tested by in vitro reconstitution whether the DNA sequence on and around the *GAL1-10* UAS has any tendency to exclude nucleosomes. We find that it does not.

Materials and Methods

Preparation of Histone H1–H5-Depleted Chicken Chromatin. Chicken blood was obtained from Pel-Freeze Biochemicals. Erythrocytes were washed 3–4 times in 1× SSC (150 mM NaCl/15 mM sodium citrate), aliquoted, and stored as 2-mL packed pellets at −80 °C until use.

The chromatin preparation follows that described by Yager et al. (1989). Four milliliters of packed cells was quick-thawed in NB (150 mM NaCl/15 mM sodium cacodylate, pH 7.2, plus PMSF added to 0.1 mM just before use) at 37 °C. The cells were washed once in NB, twice in NB + 0.5% NP-40 to lyse the cells, and twice in NB again. All centrifugations were for 5 min at 750*g* in a Sorvall SS34. The nuclei were diluted to [DNA] ≤ 10 mg/mL, adjusted to 1 mM in $CaCl_2$, and digested with 120 units/mL micrococcal nuclease at 37 °C for various lengths of time. The reactions were stopped with Na_2EDTA (2.5 mM). Samples with the desired degree of digestion were centrifuged as above; the soft nuclear pellet were resuspended in lysis buffer (0.25 mM EDTA, pH 7.5, plus 0.1 mM PMSF), stirred on ice for 1 h, and then centrifuged at 8000*g* for 20 min to pellet unwanted debris. The supernatant was adjusted to 10 mM Tris, pH 8.0, and to 350 mM NaCl by the slow addition of 4 M NaCl, with stirring.

Histones H1 and H5 were removed by stirring with 30 mg/mL Sephadex C25-120 beads on ice for 2–3 h. The beads were removed by centrifugation at 8000*g* for 30 min. The chromatin-containing supernatant was concentrated and then loaded onto 10–30% sucrose step gradients in a buffer of 10 mM Tris, pH 8.0, 0.2 mM EDTA, and 300 mM NaCl. After centrifugation for 15 h at 23 000 rpm in a Beckman SW28 rotor, the gradients were fractionated by bottom puncture. Individual fractions were analyzed by A_{260}, to determine the DNA concentration, by electrophoresis on minigels, to determine the mean DNA length, and by analysis on SDS gels, to characterize the histone content and verify the absence of H1 and H5. Fractions were pooled appropriately and then concentrated for use in the reconstitution experiments.

Preparation of End-Labeled Template DNA. Two yeast DNA fragments were used in the reconstitution experiments. One is a 907 bp fragment which includes the entire *GAL1-10* intergenic region plus some of the coding regions of the *GAL10* and *GAL1* genes (Figure 1). This fragment was cut out of pSc4816 (St. John & Davis, 1981) with *Eco*RI, gel purified (Maxam & Gilbert, 1981), and then end-labeled by filling in the sticky ends with [α-^{32}P]dATP using reverse transcriptase.

A 558 bp fragment which includes the UAS and sequences around the 5′ end of *GAL1* was produced from the 907 bp DNA by *Dde*I digestion (Figure 1). Only the *GAL10* proximal end of this fragment was labeled by carrying out the reverse transcriptase fill-in reaction with [α-^{32}P]dATP and unlabeled dTTP. (To label the other sticky *Dde*I end would require dGTP also.) The labeled fragment was gel-isolated as above.

Reconstitution. Radiolabeled DNA was incubated at 37 °C for 30 min in 10 mM Tris, pH 8.0, 1 mM EGTA, and 2 M NaCl with chicken chromatin at a final concentration of at least 3.5 mg/mL. Step dialysis was carried out against a buffer of 10 mM Tris, pH 8.0, and 1 mM EGTA plus 1 M NaCl for 4–6 h, then against buffer plus 0.5 M NaCl for at least 12 h, and then against buffer only for 4–6 h.

The reconstituted samples were layered onto 5–25% sucrose gradients in 10 mM Tris, pH 8.0, and 0.1 mM EGTA. The gradients were centrifuged at 4 °C for 24–26 h at 28 000 rpm in a Beckman SW28.1 rotor and fractionated by bottom puncture, and the fractions were analyzed by liquid scintillation counting.

Reconstitute Analysis. Individual or pooled fractions from the gradient-isolated reconstitutes were digested with *Rsa*I, *Hin*fI, *Hin*PI, or *Msp*I using a large excess of enzyme (100–200 units of enzyme per digest) in TMDSSE buffer (12 mM Tris, pH 7.5, 6 mM $MgCl_2$, 0.4 mM dithiothreitol, 0.15 mM spermine, 0.5 mM spermidine, and 0.1 mM EGTA) and 100 μg/mL BSA, at 32 °C for 1–4 h. For DNase I analysis, $CaCl_2$ to 0.5 mM and TMDSSE buffer to 1× were added to the fractions. Digestion was at 37 °C with 16 units/mL for the reconstituted chromatin or at 0.64 unit/mL for naked DNA control material. In all cases, digestions were terminated by the addition of EDTA to 10 mM and chilling on ice.

DNA was purified by phenol and/or IAC extraction and ethanol precipitation. Purified DNA was electrophoresed at 2 V/cm on 5.0% polyacrylamide gels for 20–24 h and autoradiographed on Kodak XAR film with an intensifying screen at −80 °C.

Results and Discussion

End-labeled DNA containing the UAS region plus surrounding DNA was reconstituted into chromatin by nucleosome exchange with chicken nucleosomal donor fragments in stepwise dialysis from 2 M NaCl. The reconstitute was purified on a sucrose gradient. Naked DNA template to use for digestion controls was isolated in parallel using the same dialysis and centrifugation regimen. The reconstituted material always runs well ahead of the naked DNA in the parallel gradient, verifying its reconstitution into chromatin. Material from the peak fraction(s) was digested with restriction enzymes which cut at various sites in and around the UAS or with DNase I.

Figure 1 shows a map of the region and locates the restriction sites used in this analysis: *Msp*I, a single site at 438 bp; *Hin*PI, a site in the UAS, at 470 bp, and one outside, at 870 bp; *Rsa*I, one UAS site, at 365 bp, and two in the flanking regions, at 112 and 851 bp; *Hin*fI, one site in the UAS, at 410 bp, one in the in vivo nonnucleosomal region, at 495 bp, and one in the flanking region, at 573 bp.

In the restriction analysis, each chromatin digest is accompanied by a parallel digest of naked DNA. The naked digest will show one (the 558 bp template) or two (the 907 bp template) bands. These arise from cutting at the restriction site(s) closest to the labeled end(s). If a restriction site lies within a nucleosome in the reconstitute, that site will be inaccessible to the enzyme and the band corresponding to digestion at that site will be reduced or absent in the autoradiogram. Thus, nucleosome protection will result in a loss of intensity from the band(s) corresponding to the end-proximal site(s) and an increase in intensity of band(s) corresponding to more internal sites and/or to the intact fragment.

Nucleosomes Can Assemble on the UAS. Figure 2 shows restriction data for the two templates when reconstituted under our most favorable conditions for chromatin assembly, small

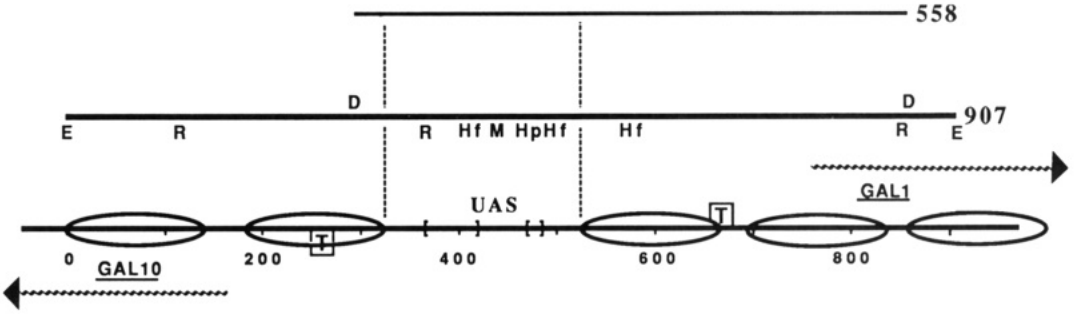

FIGURE 1: *GAL1-10* intergenic region. The middle line ("907") shows the 907 bp cloned *Eco*RI fragment used in many of the reconstitutions. Locations of restriction enzyme sites used in this work are shown: D = *Dde*I, E = *Eco*RI, Hf = *Hin*fI, Hp = *Hin*PI, M = *Msp*I, R = *Rsa*I. These same symbols are used throughout the paper. Digestion of the 907 bp fragment with DdeI yields the 558 bp fragment shown at the top. The bottom line with ovals diagrams the chromatin structure of the intergenic region in nuclei (Lohr et al., 1987). Ovals depict nucleosomes. The region which is nonnucleosomal lies between the dotted lines. Positions on the DNA are referred to in bp. Zero lies at the *Eco*RI site in *GAL10*. Tic marks are shown every 100 bp, with numbers every 200 bp. Wavy lines and arrowheads show the start sites and directions of transcription of the *GAL10* and *GAL1* genes, T's denote TATA boxes, and brackets show the location of the UAS elements.

(mono- and dinucleosome) chromatin donors at a concentration of ~8 mg/mL.

(*A*) *The Single End-Labeled 558 bp Template* (*Right Side, Figure 2*). (1) The *Msp*I digest of naked DNA shows a 141 bp band corresponding to cutting at the single *Msp*I site. The reconstitute shows clear protection of this site, with only a small amount of label appearing as the 141 bp band. Most of the counts remain as the uncut 558 bp fragment.

(2) For *Hin*PI, the reconstitute again shows only a weak band at the position (173) corresponding to cutting at the 470 bp *Hin*PI site, and most of the counts remain in the uncut fragment. This again indicates considerable protection of this UAS site from digestion. The naked DNA control digest showed some loss of DNA. Nevertheless, no uncut 558 bp DNA remains in the naked digest.

(3) *Rsa*I shows similar results, protection of the 365 bp site in the reconstitute, as judged by the low intensity of the 68 bp band in this digest and the strong band remaining at the position of uncut DNA.

(4) There is strong protection of all three *Hin*fI sites, at 410, 495, and 573 bp, in the reconstitute. There is slightly greater representation of the band (198), which results from digestion at the 495 bp site. The 113 bp band is almost undetectable, suggesting that protection at the 410 bp site in the UAS is particularly strong. The 573 bp site (276 band) lies outside the in vivo nonnucleosomal region. One in vivo nonnucleosomal site (495 bp) is less strongly protected, and one (410 bp) is more strongly protected than this flanking site.

(*B*) *The Double End-Labeled 907 bp Template* (*Left Side, Figure 2*). These results parallel those obtained with the 558 bp fragment. For example, the reconstitute *Msp*I digest shows only weak intensity in the bands which arise from cutting at the *Msp*I site; most of the counts remain as the uncut 907 bp template (Figure 2, M). The *Hin*fI digest again shows chromatin protection of all three sites. The relative intensities in the three bands are similar to those from the 558 bp template (495 bp > 573 bp > 410 bp). The *Rsa*I digest is particularly interesting because it allows comparison of a UAS site and sites quite far from the UAS. All of the bands in the reconstitute show only weak intensity, indicating protection of both the UAS and flanking regions, at a more or less similar level.

A trivial explanation of the chromatin results is that the restriction digestion was simply not complete in the reconstitutes. The parallel digests of gradient-isolated naked DNA provide one control for efficiency of restriction digestion.

We also performed internal controls, digestions of reconstitutes with heterologous naked DNA fragments also present

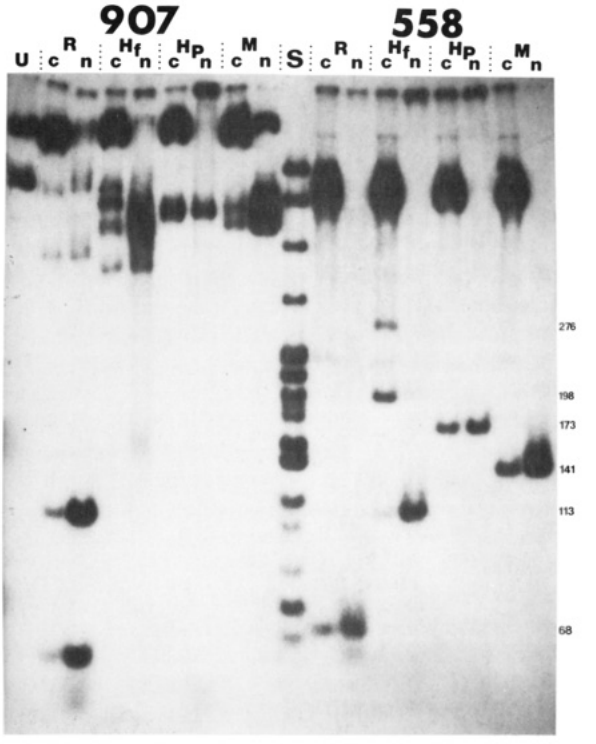

FIGURE 2: Restriction enzyme digestion. Isolated, reconstituted chromatin and naked DNA control material were digested with restriction enzymes and the products analyzed on 5.0% polyacrylamide gels. The left side shows results for the 907 bp fragment; the right side shows results for the 558 bp fragment. Restriction enzymes are designated by letter symbols as in Figure 1 (R = *Rsa*I, Hf = *Hin*fI, Hp = *Hin*PI, M = *Msp*I). Digests of reconstituted chromatin are indicated by c and digests of naked DNA by "n". Lane U shows undigested 907 and 558 bp DNA. Lane S shows molecular weight markers, end-labeled pBR322/*Msp*I DNA. The numbers to the right are the sizes of the bands in digests from the 558 bp reconstitutes.

in the same tube (in smaller quantities). Figure 3 shows three such digests on a 558 bp reconstituted chromatin template with naked 907 bp DNA present. The sizes of bands expected from digestion of the 907 bp naked DNA are shown. The bands from the 558 bp reconstitute are noted by small letters. In all cases, the internal control digest appears complete; the 907 bp fragment completely disappears, and the requisite fragments appear. Although the heterologous naked DNA is completely cut in these digests, the 558 bp chromatin fragment shows the usual patterns of protection. Thus, inefficient restriction digestion does not appear to explain the low intensity of the bands in the chromatin digests. Furthermore, if low efficiency

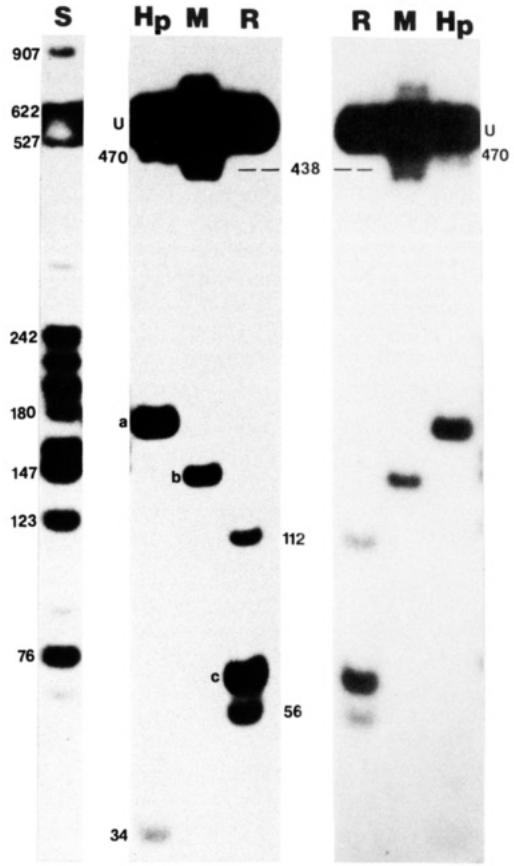

FIGURE 3: Restriction enzyme analysis of the 558 bp reconstitute with 907 bp naked DNA as an internal control. The 558 bp template was reconstituted, isolated, and digested with restriction enzymes as in Figure 2. A smaller number of cpm of naked 907 bp DNA was included in the chromatin digest as an internal control. The fragments expected from the 907 bp naked DNA internal control are located by numbers (their size in bp). The sizes of the bands from the 558 bp reconstitute are noted by letters and are the same as in Figure 2. "U" locates the center of the uncut 558 bp DNA band. Lane S is labeled pBR322/*Msp*I molecular weight markers plus 907 bp DNA. The sizes of some of these marker fragments are shown to the left. The three tracks to the right are a lighter print to show the region around the 558 bp band more clearly.

of restriction were the cause for the low band intensities in the chromatin digests, one might expect significant variation in band intensity from experiment to experiment or from enzyme to enzyme due to variation in the efficiency of restriction. This is not observed. The intensity of bands in the reconstitute digests does depend on the extent of reconstitution (see below), again suggesting that the assay measures the level of template protection.

From the above studies, we conclude that the UAS sequence can accept nucleosomes in vitro. From the *Rsa*I and *Hin*fI results, the sites in the UAS appear no less protected, and thus no less able to accept nucleosomes, than sites outside the UAS.

Does the UAS Exclude Nucleosomes under Less Favorable Reconstitution Conditions? The above conditions, small donor fragments at high concentrations, are quite favorable for reconstitution of nucleosomes onto the radiolabeled acceptor. The high level of nucleosome loading on the template under these conditions might be able to overcome any weak tendency for nucleosome exclusion and force nucleosomes onto the UAS. Reconstitution conditions which produce lower nucleosome loading might allow the UAS to show such a weak tendency, if it has one. To check this possibility, reconstitutions were carried out at lower concentrations (3.6 mg/mL) and using several lengths of longer donor chromatin fragments. Both

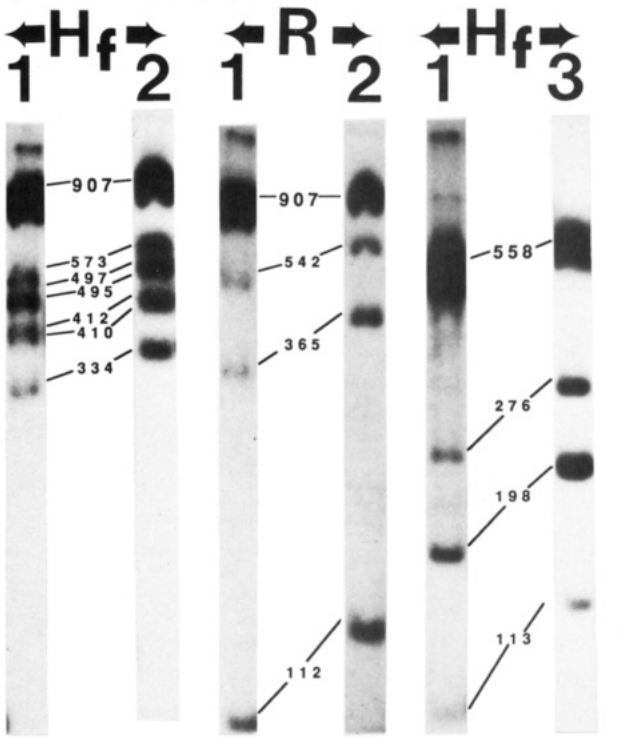

FIGURE 4: Comparison of different levels of reconstitution. The 907 and 558 bp templates were reconstituted under conditions producing different nucleosome loading, i.e., various lengths of donor fragments at various concentrations. The reconstitutes were isolated and digested with restriction enzymes as described above. The lanes labeled "1" are taken directly from Figure 2 and used mono- and dinucleosome donor fragments at 7.7 mg/mL. Reconstitute "2" used polynucleosome donor fragments (>15–20 nucleosomes per fragment) at 3.6 mg/mL; "3" used donors with 6–10 nucleosomes per fragment at 3.6 mg/mL. Corresponding bands are connected by lines, and the fragment lengths in base pairs are shown in the middle of each of the three comparisons. From left to right, the digests compared are *Hin*fI and *Rsa*I digests of the 907 bp template and a *Hin*fI digest of the 558 bp reconstitute.

of these will produce lower nucleosome loading on the radiolabeled acceptor. In all cases, the labeled templates efficiently reconstituted into chromatin, as judged by the sucrose gradient isolation. However, the reconstituted templates did appear to be less heavily loaded with nucleosomes because they did not sediment as rapidly as the reconstitutes analyzed in Figures 2 and 3 (not shown).

Figure 4 shows three sets of digests comparing various reconstitutions. The more efficient reconstitution is on the left ("1") while the less efficient is on the right ("2" or "3"). Two features are apparent. In the less efficient reconstitutes, the intensities of the bands arising from chromatin cutting are still small compared to the amounts of the template remaining uncut. Thus, there is still significant protection of the various sites in the less efficient reconstitutes. However, in these reconstitutes, the bands arising from cutting in chromatin are relatively stronger, and there is relatively less intensity remaining in the uncut fragment than in the more efficient reconstitutions. This is expected from digestion of a template which is less fully loaded with nucleosomes. Most importantly, the increased intensity occurs in bands from both UAS and flanking region sites. In fact, the relative intensities of the various bands in the *Rsa*I and *Hin*fI profiles are quite similar in the digests from different reconstitutions. Thus, the UAS still shows no tendency to exclude nucleosomes compared to flanking regions; the lower level of nucleosome loading is spread over all regions of the fragment. These results also suggest a random location of nucleosomes on the reconstituted template; the fraction of sites which fall outside a nucleosome

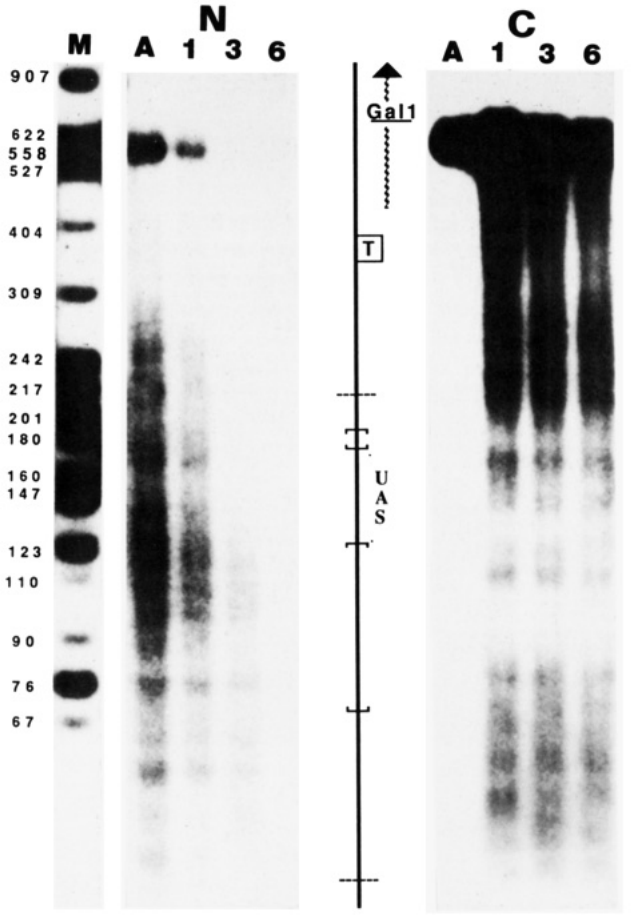

FIGURE 5: DNase I analysis of reconstitutes. The 558 bp template was reconstituted as for Figure 2 and digested with DNase I ("C"). The numbers above the tracks refer to times of digestion, i.e., after the addition of enzyme (A = 20 s; 1, 3, and 6 min). DNase I digests were also performed on gradient-isolated naked DNA ("N"). The digested DNA was reisolated and electrophoresed on 5.0% polyacrylamide gels, and autoradiography was performed. M denotes the pBR322/*Msp*I molecular weight markers plus some 907 bp DNA. The map in the middle is drawn to scale and locates various regulatory features of this DNA region: the UAS elements in brackets; the *GAL1* TATA box, "T"; the *GAL1* transcription start site (wavy line); the nonnucleosomal region determined in nuclei (dotted lines).

region depends on the nucleosome density.

DNase I Studies. Restriction enzymes analyze the accessibility of a very small region of DNA. To obtain a more global view of nucleosome protection on the reconstitute, we performed DNase I digestions. Figure 5 shows the results of such an analysis of the reconstituted 558 bp template, reconstituted as in Figures 2 and 3. The 907 bp fragment has also been analyzed and shows similar results (not shown).

These digests suggest that in the reconstitutes the UAS is relatively more protected from DNase I than the flanking regions; there is a region of very low intensity on the UAS elements themselves. The protection is a chromatin feature for it is not observed in naked DNA digests (Figure 5, "N"). This may be the explanation for some of the variations in restriction site accessibility noted above. For example, the weakly cut *Hin*fI site at 410 bp lies in the strongly protected region while the more strongly cut site at 495 bp is outside of it. Thus, the in vivo nonnucleosomal region appears in vitro to bind nucleosomes more strongly than the flanking regions. This reinforces the conclusion that the DNA of the UAS is unable by itself to exclude nucleosomes. The observation that in vitro the UAS can bind nucleosomes more avidly than the flanking regions makes the absence of nucleosomes in vivo even more remarkable.

Thus, it appears that the nucleosome-free region on the UAS in vivo is mainly due to the action of cellular factors keeping histones off the region. The results of Fedor et al. (1988) suggest one such factor. It binds in vitro to the center of the same region which by DNase I protection seems to bind nucleosomes most strongly in vitro.

One reservation concerning our results is that the system is heterologous; yeast histones were not used. We have tried to get clean yeast nucleosomes but so far have not been successful. To our knowledge no one has ever used yeast histones in a reconstitution. We cannot exclude the possibility that yeast histones would give different results. However, a number of other reconstitution studies have used heterologous systems (Chao et al., 1979; Simpson & Stafford, 1983; Ramsey et al., 1984; Linxweiler & Hörz, 1984; Poljak & Gralla, 1987; Workman et al., 1988). Many of these heterologous systems have detected specific nucleosome positioning, including systems using prokaryotic DNA. Thus, the information for specific nucleosome placement, and by inference nonplacement, seems to reside largely in the DNA sequence [see also Linxweiler and Hörz (1985)]. The use of a heterologous system to study the type of question we pose thus seems appropriate.

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