The positions of the DNase I hypersensitive sites within the galactose gene cluster of the yeast *Saccharomyces cerevisiae* have been determined. When yeast nuclei were incubated with DNase I, the purified DNA restriction endonuclease digested and analyzed by Southern hybridization, five hypersensitive regions were observed in this cluster of three closely spaced genes. Areas between the 5' end of GAL 7 and the 3' end of GAL 10 and between the two 5' ends of the divergently transcribed GAL 10 and GAL 1 genes contain DNase I hypersensitive regions. DNase I hypersensitive sites were also observed in the areas near the 3' ends of the GAL 7 and GAL 1 genes. An additional site was observed within the GAL 10 structural gene. The region between GAL 10 and GAL 1 contains three tightly grouped cutting sites which map within a localized region of very high GC content. None of the five hypersensitive regions were observed when
isolated DNA was digested with DNase I. Hypersensitive sites were present irrespective of the level of transcription of the galactose genes. A deletion which eliminates the 5' end of GAL 10 and all of GAL 1 does not destroy the internal GAL 10 hypersensitive site.

The DNA of Saccharomyces cerevisiae has previously been reported to contain the modified base 5-methylcytosine. In order to determine if this methylation might occur in CCGG sequences, restriction endonuclease digestions were performed on DNA of strain B273-10B with the isoschizomer pair HpaII and MspI. These two enzymes differ in their ability to cut the sequence C(5MeC)GG. Ethidium bromide stained gel tracks of DNA restricted with these two enzymes appeared identical. The patterns of HpaII and MspI fragments within a 7.5 kb region about the gene for iso-2 cytochrome c and within the 6.3 kb two micron circle plasmid were also identical. Failing to have found any methylation in CCGG sequences, the genomic content of 5-methylcytosine was re-evaluated. HPLC analysis of highly purified yeast DNA showed 5-methylcytosine to be undetectable (less than 0.05% of total cytosine).
Gene Structure in Saccharomyces cerevisiae:
DNase I Hypersensitive Sites in Galactose Gene
Cluster and Non-Detection of 5-Methylcytosine

by

John Houston Proffitt

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Redacted for privacy

Professor of Biochemistry and Biophysics in charge of major

Redacted for privacy

Chairman of Department of Biochemistry and Biophysics

Redacted for privacy

Dean of Graduate School

Date thesis is presented December 14, 1982

Typed by Jeannette Emry for John Houston Proffitt
I would like to thank my major professor Dr. K. E. Van Holde for support during the course of this work. For supplying strains and plasmids I would like to thank Dr. Fred Sherman, Steve Bain, Dr. Ron Davis, Dr. Mark Johnston, Dr. Jim Yarger, Dr. Jim Hartley, Dr. Jim Hicks, and Dr. Gerry Fink. Dr. Jim Davie and Dr. Stanley Hattman are acknowledged for performance of HPLC analysis. Forest Ziemer provided instruction on in vitro radiolabeling by nick translation.

I would like to thank Dr. Rogers Yocum for supplying the unpublished sequence of the GAL10-GAL1 intergene region. Finally I would like to thank Dr. Fred Sherman, Dr. Gerry Fink, Dr. Jim Hicks and the entire Cold Spring Harbor yeast group for communicating their knowledge and enthusiasm.
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The concept that various structural features of eucaryotic chromosomes are related to the regulation of gene expression is not new. Such phenomena as X chromosome inactivation and associated chromosome condensation, lampbrush chromosome loops, and variegated position effects in Drosophila have long suggested a relationship between chromosome structure and gene activity. With the advent of recombinant DNA technology it has become possible to examine the chromatin structure of specific genes. Recently, using such techniques, two structural features have been associated with the active genes of higher eucaryotes. A DNase I hypersensitive region at or near the 5' end of a gene seems to be a necessary, but not sufficient, condition for transcription by RNA polymerase II. Also for many genes an inverse relationship has been demonstrated between the presence of the modified base 5-methylcytosine at specific loci and gene activation in the same region.

I have performed experiments to determine if these two structural features exist in the genome of the simple eucaryote Saccharomyces cerevisiae. Part I of this thesis describes experiments conducted to determine if DNase I hypersensitive sites exist in the yeast galactose gene cluster. Part II concerns the search for 5-methylcytosine in the yeast genome.
INTRODUCTION

DNase I Hypersensitive Sites

The technique of nuclease digestion has played a key role in the study of chromatin structure. The work of Weintraub and Groudine (1976) was the first such study to show that genes that are or have been transcriptionally active are preferentially digested by pancreatic deoxyribonuclease (DNase I). More recently, it was discovered that this same enzyme makes site specific double stranded cleavages of DNA near genes that are active or have the potential for becoming active (Wu et al., 1979).

The actual locations of these DNA double stranded breaks have been mapped for a number of genes. Wu (1980) first conducted such a mapping experiment for the Drosophila heat shock genes hsp 70 and hsp 83. By using the indirect end-labeling technique (described below), he showed the regions 5' to these two genes to be hypersensitive to DNase I digestion. When isolated Drosophila DNA was used as a substrate for DNase I digestion no such 5' hypersensitive regions were seen. Since these sites do not exist on naked DNA, they must be a result of chromatin structure. They represent, therefore, a type of DNA sequence specific chromatin structure.

Sequence specific DNase I cleavage sites have been reported in the region of genes for Drosophila histones (Samal et al., 1981), Drosophila heat shock proteins (Wu et al., 1979; Wu, 1980; Keene et al., 1981), Tetrahymena pyriformis ribosomal RNA (Borchsemius et al.,
1981), rat pre-proinsulin (Wu & Gilbert, 1981), chicken globins (Stalder et al., 1980; Weintraub et al., 1981; Groudine & Weintraub, 1981; McGhee et al., 1981; Weintraub et al., 1982), conalbumin (Kuo et al., 1979), Drosophila ribosomal protein 49 (Wong et al., 1981), endogenous avian retroviruses (Groudine et al., 1981) and in the Simian virus and polyoma minichromosomes (Scott & Wigmore, 1978; Waldeck et al., 1978; Saragosti et al., 1980; Herbomel et al., 1981).

Recently there was a report of a DNase I hypersensitive site near the two alcohol dehydrogenase genes in yeast (Sledziewski & Young, 1982). However, this study failed to include a control experiment of isolated DNA digested with DNase I. Therefore, it is not clear that the sites seen in this study are chromatin specific.

Also during the course of this thesis work it was learned that other DNase I hypersensitive sites have been observed in yeast. Dr. Kim Nasmyth (1981) has characterized hypersensitive regions within the yeast mating type loci HMR, HML, and MAT, which are described below.

In the above studies most DNase I hypersensitive sites were found to occur near the 5' ends of genes, but some were near the 3' ends, while others were some distance from the nearest known gene. In two cases hypersensitive sites were seen within a structural gene (Weintraub et al., 1981; Wu & Gilbert, 1981).

Wu has shown for heat shock gene hsp 70, an inducible gene, that hypersensitive sites are present both before and after induction. However, some of the 5' sites lose definition with induction (Wu, 1980). Studies have been done also with genes that show tissue-specific and developmental stage-specific expression.
Weintraub and coworkers reported a 5' hypersensitive site for the embryonic $\beta$-globin gene in chick embryonic red blood cells. This site is absent in adult red blood cells. In the adult cells two DNase I hypersensitive sites were observed 2 and 6 kb from the 5' end of the adult $\beta$2-globin gene. None of these sites were observed when brain cell nuclei were digested with DNase I (Stalder et al., 1980). The U gene in the $\beta$-globin gene cluster shows a similar behavior. A site is seen at its 5' terminus in 5 day old red blood cells, but not in those of an adult. Neither is this site seen in brain cell nuclei. The temporal pattern of hypersensitive site occurrence is identical to that of U gene transcription. There are however, sites in the globin cluster which occur in both 5 day old and more mature blood cells, but not brain cells. Also there is a DNase I hypersensitive site which exists in brain tissue as well as both types of blood cells. This persistent DNase I site is about three kb leftward of the U gene. The U gene is the leftmost member of the $\beta$-globin gene cluster (Weintraub et al., 1981). More recently, Weintraub and coworkers have used chicken bone marrow cells infected with a temperature-sensitive avian erythroblastosis virus to investigate the relationship between the acquisition of DNase I-hypersensitive sites and gene activation. These workers obtained two cell lines which would produce globin mRNA only upon a temperature shift to $42^\circ C$. The cells of one clone acquired DNase I hypersensitive sites upon the temperature shift as well. The other line, however, was found to already contain the globin hypersensitive sites before the temperature shift. This hypothetically more mature line would, nevertheless, not express the globin genes until the
temperature shift (Weintraub et al., 1982). Thus, the acquisition of
DNase I hypersensitive sites seems necessary for transcription to
occur, but whatever feature of chromatin structure they represent is
not sufficient to cause or allow transcription.

The most thorough study to date of the actual nature of a DNase I
hypersensitive site has been on the region at the 5' end of the
chicken adult $\beta^A$-globin gene (McGhee et al., 1981). An area
approximately 60 to 260 bp upstream from the gene was found to be
hypersensitive to attack by a number of different nucleases. The
restriction enzyme MspI cleaves at two sites, 115 bp apart, within
the nuclease sensitive region. MspI digestion of nuclei liberates
this fragment in high yield. Of the material so excised, approximately 35%
behaves as free DNA. This behavior suggests that
this region does not have the normal nucleosomal chromatin structure.
In addition this area of the chromosome is very GC rich (70%) and
contains a stretch of 18 consecutive deoxyguanosine residues. The
region has at its gene proximal terminus the consensus sequence
CCAAT. The TATA, or Goldberg-Hogness sequence, occurs between the
gene and the hypersensitive region.

In a recent review article a number of interesting unpublished
findings concerning DNase I hypersensitive regions were summarized
(Kolata, 1981). Mark Muskavitch, working at Stanford University, has
found that a small deletion 400 bp upstream from the Drosophila glue
protein Sgs 4 inactivates the gene. Steven Beckendorf (Berkeley)
showed that this deleted sequence coincided with the location of a
DNase I hypersensitive region. This suggests that the hypersensitive
region is necessary for transcription. In contrast to the work of
McGhee et al., which located the CCAAT sequence near the end of the hypersensitive region closest to the globin gene, the CCAAT consensus sequence upstream from the Sgs 4 gene is located in the middle of the hypersensitive region. Weintraub's lab (Seattle) finds the hypersensitive region near the active globin genes can be cut by S1 nuclease. Cleavage by this single strand specific enzyme suggests the DNA of the region may be unwound. This is in contrast to the published report of McGhee et al. (1981), who were unable to detect S1 cleavage in the DNaseI hypersensitive region at the 5' end of the chicken adult $\beta^A$-globin gene.

The author of this review, however, made a serious error when she described the work of Kim Nasmyth (Cold Spring Harbor). She stated that Nasmyth found the DNA of a hypersensitive region near a yeast mating type gene to be unwound by the action of the MAR genes, which is true. However, she further stated that when MAR protein bound in front of a mating type gene it is expressed, which is false. The four MAR or SIR gene products actually repress not only transcription of the silent mating type loci, but also prohibit the silent copies from acting as recipients of mating type DNA. MAR also causes the disappearance of a DNase I hypersensitive site within the silent loci. This same site is coincident with the site of in vivo cleavage by an endogenous yeast nuclease. Such cleavage is thought to be the initiating event in the mating type switch and to result from the action of the HO gene product. Since cleavage at this site is blocked by the action of MAR, this may represent a case of genetic regulation through limiting access to a DNA sequence. It is interesting to note that while Weintraub suggests that the appearance
of at least some DNase I hypersensitive sites might be associated with unwinding of the DNA double helix, the unwinding action of the yeast MAR protein upon the silent mating type loci coincides with the disappearance of a DNase I hypersensitive site rather than its appearance.

Although the function and nature of DNase I hypersensitive regions are just beginning to be understood, it seems clear from the yeast mating type phenomena that they are not solely associated with transcriptional activation. Also the hypersensitivity of the SV40 origin of replication suggests they might also be found at cellular origins of replication. The evidence to date implies that at least some of the hypersensitive regions are nucleosome free areas of the genome that occur at regulatory sequences and thereby allow increased accessibility of specific proteins to those DNA sequences. For reviews of DNase I hypersensitive sites and related topics see: (Igo-Kemenes et al., 1982; Weisbrod, 1982; Elgin, 1981; Kolata, 1981).

The Technique of Indirect End-Labeling

A scheme for mapping the DNase I hypersensitive sites near a hypothetical gene is shown in Figure 1. After DNase I digestion of nuclei, DNA is isolated and subjected to a secondary digestion with restriction endonuclease, in this case HindIII. The resultant DNA fragments are then separated by agarose gel electrophoresis, transferred to nitrocellulose paper by Southern blotting and hybridized to the $^{32}$P-DNA fragment probe shown. In the absence of any site specific DNase I cleavage, the only distinct band to appear
Figure 1. Mapping DNase I hypersensitive sites by indirect end-labeling. Shown is a hypothetical case to illustrate the method of indirect end-labeling.
Figure 1.
on the autoradiogram of the Southern pattern will be the HindIII to HindIII fragment H. Below fragment H will appear a smear of hybridizing material resulting from random cleavage by DNase I. However, if site specific cleavage by DNase I occurs at the positions indicated in Figure 1, two additional bands corresponding to fragments A and B will appear. The fragments distal (rightward) of the DNase I site will not appear on the Southern pattern as they lack homology to the probe. Because the fragments A and B are bounded by a DNase I site and the reference HindIII site, each of their sizes uniquely determines a DNase I hypersensitive site location. Techniques other than indirect end-labeling have been used for determining positions of DNase I hot spots by, among others, Weintraub and colleagues (e.g. Stalder et al., 1980). However, as indirect end-labeling is the easiest and most direct method it was used throughout this study.

Galactose Gene Cluster of Saccharomyces

The galactose gene cluster of Saccharomyces cerevisiae is composed of three lightly linked genes located near the centromere of chromosome II that are coordinately induced by galactose. These genes code for the three enzymes that catalyse the conversion of galactose to glucose-1-phosphate (Douglas & Condi, 1954; Douglas & Hawthorne, 1964; Adams, 1972; Broach, 1979). See Figure 2 for a summary of the reactions catalysed by the GAL 7 - GAL 10 - GAL 1 gene cluster. This figure also shows the physical arrangement of the genes of the GAL cluster. The galactose enzymes are translated as
separate polypeptides (Broach, 1979). Strains carrying chain-terminating mutations in the cluster show no polar effect (Broach, 1979).

The expression of the three gene products of the cluster are under the control of a positive regulatory locus, GAL 4 and a negative regulatory locus, GAL 80. These two regulatory loci are unlinked to both the galactose cluster and each other (Douglas and Hawthorne, 1966). Models have have been proposed in which GAL 4 protein, GAL 80 protein, and galactose interact to control the transcription of the inducible galactose genes (Perlman and Hopper, 1979; Matsumoto et al., 1980).

The transcription of the galactose gene cluster has recently been characterized in detail (St. John and Davis, 1981a; St. John and Davis, 1981b). It is estimated that each GAL RNA represents between 0.25 and 1.0% of the poly A-containing RNA of galactose grown cells. When cells are grown on glucose (GAL genes repressed), the steady state concentrations of the GAL mRNAs drop as much as 1000 fold. Repressed levels of GAL mRNAs may result from low level constitutive expression or the arising of GAL constitutive mutants at the rate of $10^{-6}$. An RNA species has been observed which contains both GAL 7 and GAL 10 messenger sequences. This eucaryotic "polycistronic message" is observed even though nonsense mutations in GAL 10 show no polar effect in GAL 7. This GAL 10 - GAL 7 transcript is not an obligate precursor to GAL 7 mRNA, as a deletion beginning within the 5' end of
Figure 2. Galactose gene cluster and reactions catalysed by gene products. Restriction map contains the sites for: EcoRI, R; Sali, S; and AvaI, A. Also shown are the locations of the hybridization probes.
GALACTOSE + ATP $\xrightarrow{\text{GAL1}}$ GALACTOSE 1-PHOSPHATE + ADP
galactokinase

GALACTOSE 1-PHOSPHATE + UDP-GLUCOSE $\xrightarrow{\text{GAL7}}$ GLUCOSE 1-PHOSPHATE + UDP-GALACTOSE
galactose 1-phosphate uridylyl transferase

UDP-GALACTOSE $\xrightarrow{\text{GAL10}}$ UDP-GLUCOSE
UDP-galactose-4-epimerase

Figure 2.
the GAL 10 structural gene and extending beyond GAL 1 results in a GAL 7, gal 10, gal 1 genotype. Although evidence suggests the presence of three promoters in the cluster, the site(s) for interaction of regulatory proteins remains unknown.

MATERIALS AND METHODS

List of Strains

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<tr>
<td>108-3C</td>
<td>a GAL trp 1 ura 1</td>
<td>H. Douglas</td>
</tr>
<tr>
<td>D585-11C</td>
<td>a GAL MAL 2 MEL lys 1</td>
<td>CSHYG*</td>
</tr>
<tr>
<td>YNN 27</td>
<td>a trp 1-289 ura 3-52 gal 2</td>
<td>R. Davis</td>
</tr>
<tr>
<td>YNN 70</td>
<td>YNN 27 gal 106</td>
<td>R. Davis</td>
</tr>
<tr>
<td><strong>Bacterial Strains</strong></td>
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<tr>
<td>BNN 45</td>
<td>hsr K- hsm K+</td>
<td>R. Davis</td>
</tr>
<tr>
<td>pNN 76</td>
<td>BNN 45[pBR322-SC4911]</td>
<td>R. Davis</td>
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*Cold Spring Harbor Yeast Genetics Course Strain Collection
Cell Growth and Isolation of Yeast Nuclei

Yeast cell growth was carried out in YP (1% yeast extract and 2% bactopeptone) containing either 2% glucose or 2% galactose. Cells were grown at 30°C on a New Brunswick rotatory shaker to mid-log phase (1 x 10⁷ to 1.5 x 10⁷ cells per ml). Bacterial cells were grown on a similar shaker but at 37°C and in LB media. LB media consists of per liter: 10g tryptone, 5g yeast extract, and 5g NaCl.

Yeast nuclei were prepared as described (Ide & Saunders, 1981) except the cells were first pretreated in 1M sorbitol, 1%(v/v) 2-mercaptoethanol and 25 mM EDTA, pH8 for 15 minutes at 34°C. Prior to DNase I digestion the nuclei were washed once in DNase I digestion buffer (10 mM PIPES pH6.5, 10mM NaCl, 2mM MgCl₂, and 1mM CaCl₂). The nuclei were then resuspended in the same buffer at a DNA concentration of 0.2 to 0.4 mg/ml and stored on ice for the brief period prior to their use. For all DNase I digestions only the upper "unlysed" fraction of nuclei from the percoll gradients employed in the isolation were used.

DNase I Digestion of Nuclei and DNA

Lyophilized bovine pancreatic DNase I (Worthington DPFF) was dissolved at a concentration of 1mg/ml in 10mM CaCl₂ and stored as frozen 100µl samples at -20°C. This storage, suggested by the supplier, inhibits the action of minor contaminating protease activity. Freshly thawed tubes of DNase I were diluted with DNase I digestion buffer and mixed by gentle inversion to give a final concentration of between 1 and 80U/ml. These dilutions served as stock solutions for the DNase I digestions.
Samples of nutlei were first incubated at 37°C for 3 minutes. Then DNase I was added to a final concentration of 0, 0.1, 0.4, 1.6, and 6.0 U/ml and the samples further incubated for 5 minutes. Digestions were terminated by the addition of EDTA to 0.05 M. Purified DNA of 0.25 mg/ml in DNase I digestion buffer was treated as above except DNase I concentrations were 0, 0.05, 0.1, and 0.3 U/ml.

Purification of DNA

Recently developed methods of rapid DNA isolation utilizing precipitation of potassium dodecyl sulfate-protein complexes in place of phenol extraction greatly simplify the preparation of DNA which is cleavable by restriction endonucleases.

DNA from yeast nuclei was prepared by a slight modification of the method of Davis et al. (1980). The procedure given here is for 1ml of nuclei which has been made 50mM in EDTA (pH8). 20μl of 10% (w/v) sodium dodecyl sulfate are added, the tube mixed and then heated at 65° to 70° C for 30 minutes. 100μl of 5M potassium acetate are added, the tube again mixed and allowed to sit on ice at least 30 minutes. The sample is then centrifuged at 15,000 RPM in a SS34 rotor for 15 minutes at 4°C. To the decanted supernate are added 2 volumes of room temperature 95% ethanol and the mixed solution spun at 15,000 RPM in the same rotor, but at room temperature, for 5 minutes. The supernate is discarded, the pellet briefly dried and then dissolved in 500μl TE (10 mM Tris, 1mM EDTA, pH 7.5). After centrifugation in an SS34 rotor for 10 minutes at 10,000 RPM to remove insoluble material, the solution is transferred to a new tube and incubated with RNAse (10μg/ml) RNAse A, 10 U/ml RNAse T1) for 30 minutes at 37°C.
Sodium acetate is added to 0.3M and DNA precipitated by addition of 3 volumes 95% ethanol and incubation at -20°C. The final pellet is redissolved in TE and the DNA concentration determined by absorbance at 260nm.

DNA for DNase I digestion controls was isolated from spheroplasts of log phase cells as described (Sherman et al., 1981). Then it was further purified by pronase digestion, twice extracting with phenol/chloroform/isoamylalcohol, 25:24:1 (vol/vol), twice with chloroform/isoamylalcohol, 24:1 (vol/vol), and three times with ether. To the final solution were added, 0.1 volume 3M sodium acetate and 3 volumes 95% ethanol and the DNA precipitated overnight at -20°C.

The recombinant plasmid pSC4911, containing the EcoRI to Sall fragment of the GAL 10 gene (St. John and Davis, 1981a), was prepared by CsCl-ethidium bromide density equilibrium centrifugation from crude bacterial lysates of BNN76 (Davis et al., 1980; St. John and Davis, 1981a).

Radiolabeling DNA by Nick Translation

The method described is based on the procedures of Forest Ziemer (personal communication) and R. Schleif and P. Wensink (1981). To a 1.4 ml microfuge tube are added: (1) 2μl of a mixture containing 1mM each dATP, dGTP, and dTTP; (2) 5μl of 500 mM Tris-HCl, pH 7.8, 90mM MgCl₂, 100mM 2-mercaptoethanol, and 50μg/ml bovine albumin; (3) 1μg DNA; (4) 100μCi[32P]-dCTP; (5) sterile distilled H₂O to make a volume of 47μl. After mixing, 1μl (4 units) of E.coli DNA Polymerase I (BRL) and 2μl of DNase I (Worthington, DPFF) freshly diluted by
1/10,000 from a frozen 1mg/ml stock, are added. The solution is gently mixed and then incubated at 15°C for 60 to 90 minutes. One tenth volume of both 250 mM EDTA and 10xSP buffer are then added. 10xSP buffer is 3M NaCl and 0.1M sodium acetate adjusted to pH 5 with acetic acid. The nick translated DNA is then separated from unincorporated deoxyribonucleotide triphosphates by passage over a small SP sephadex column.

Restriction Enzyme Digestion of Yeast DNA

Restriction enzyme digestions of yeast DNA were carried out using the appropriate low, medium, or high salt buffers listed in the Cold Spring Harbor Advanced Bacterial Genetics Manual (Davis et al., 1980). Enzyme concentrations were 4 to 5 units per μg DNA and digestion was at 37°C for 8 to 14 hours. Reactions were terminated by addition of excess EDTA. Ethanol precipitated DNA was resuspended in a small volume of TE appropriate for gel electrophoresis.

Electrophoresis and Southern Analysis

Agarose gel electrophoresis was conducted in a BRL model H4 horizontal electrophoresis apparatus using a tris-acetate buffer containing 0.5μg/ml ethidium bromide. Tris-acetate buffer is 40mM Tris OH, 20mM acetic acid, 2mM Na2EDTA and has a pH of 8.1.

Southern transfers were performed as described (Sherman et al., 1981) with slight modification. Neutralization of the gel was with 1M Tris pH 7, 0.6M NaCl instead of 0.25-0.5M Tris pH 7, 10xSSC. Also, rinsing of the nitrocellulose filter after transfer, but prior to baking, was omitted. Hybridization of filters with radioactive
nick translated probe and filter rinsing was conducted exactly as described. Rinsed filters were blotted with 3MM paper and wrapped while still damp in plastic wrap to prevent adhesion to the x-ray film. Kodak XAR-5 film and wrapped filters were placed together in film cassettes with double intensifying screens and exposure carried out at -80°C.

The probes used in this study were either the entire plasmid pSC4911 or an approximately 1400 bp fragment isolated from this plasmid. The fragment contains the 754bp PstI to EcoRI fragment of pBR322 and the approximately 538bp region of GAL 10 extending from the EcoRI site to the first HindIII site (see Figure 2 for regions of homology between probes and the GAL gene cluster).

Recovery of DNA Fragments from Agarose Gels

The method described is an unpublished method from Dr. Ron Reeder's laboratory. The DNA fragment to be recovered is located by UV light illumination, and a slot one to two mm wide is cut out in front of the fragment. Using forceps, a strip of DE-81 paper is inserted into the slot. Electrophoresis is carried out at 60 to 80 volts until all the ethidium bromide stained DNA has bound to the paper. The DE-81 paper is then placed into a 400μl microfuge tube from which the top has been removed and in which a number of small holes have been made at the bottom by a small gauge syringe needle. This tube is in turn placed into a 1.4 ml microfuge tube also with its top removed. The paper is washed by adding 100μl of 0.1 M NaCl, 0.1 mM EDTA, 10mM Tris, pH 8 to the paper and very briefly spinning in a microfuge. This step is repeated three times. No DNA is eluted
at this step. Next the paper is similarly washed, but with 1M NaCl, 0.1mM EDTA, 10mM Tris, pH 8. This DNA elution step is also repeated three times. The final combined 1M salt elutant is spun in the microfuge for a few minutes to remove any DE-81 fibers and transferred to another 1.4ml microfuge tube. Three volumes of ethanol are added and after chilling in a dry ice-ethanol bath for 10 minutes, the solution is spun in the microfuge for 10 minutes. Following removal of the supernate, the DNA pellet is dried briefly under vacuum and resuspended in TE buffer.

RESULTS

Choice of Hybridization Probe

Initially, radiolabeled plasmid pSC4911 was used to map the DNase I hypersensitive sites, by the method of indirect end-labeling, in the galactose gene cluster. This plasmid contains the SalI to EcoRI fragment of GAL 10 cloned into the bacterial vector pBR322 (Figure 2). The advantage of using this plasmid for mapping is two-fold. First, its central location in the GAL cluster allows mapping in both directions. Secondly, the relatively long distance leftward of the probe sequence to the next EcoRI site and rightward to the next SalI site (see Figure 2), allows this one probe to be used in mapping from 1.5 kb beyond the 3' end of GAL 7 to far beyond the 3' end of GAL 10. However, use of this probe was found to be inappropriate for such mapping for two reasons: (1) the EcoRI-SalI fragment of GAL 10
contains an internal DNase I hypersensitive site and (2) pSC4911 cross-hybridizes to the multicopy yeast plasmid, the two micron circle.

Isolated yeast nuclei from strain 108-3C grown on galactose were incubated with DNase I, DNA isolated from these nuclei and restricted with EcoRI. When the DNA fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with $^{32}$P-nick translated pSC4911, a 1.52 kb band below the 5.2 kb EcoRI fragment is seen (data not shown, see Figure 9 for equivalent experiment). Existence of this 1.52 kb band indicates a DNase I hypersensitive site 1.52 kb from the GAL 10 EcoRI site. Since the size of the EcoRI to SalI GAL 10 insert of pSC4911 is 1.57 kb, a hypersensitive site occurs within the region of the chromosome homologous to the probe. Implicit in the indirect end-labeling method is the assumption that hypersensitive regions cannot occur within the probe sequence. For if this happens, genomic DNA fragments that do not terminate at the reference restriction site will hybridize to the probe. Thus, the existence of the hypersensitive site within the SC4911 sequence make pSC4911 unsuitable as a probe for the indirect end-labeling technique.

Figure 3 shows the result of hybridizing pSC4911 to a Southern pattern of SalI cut (lane 1) and unrestricted (lane 2) 108-3C DNA. The lower faint band seen in both lanes is clearly artifactual since only one band should have homology to SC4911 in both unrestricted and SalI cut (see Figure 2) DNA. The existence of this artifactual band in unrestricted DNA suggests it results from hybridization to an extrachromosomal element since it does not co-electrophoreis with
Figure 3. Hybridization of pSC4911 to SalI cut and unrestricted DNA. Lane 1, SalI digestion; Lane 2, unrestricted DNA.
Hybridization of pSC4911 to a Southern pattern of EcoRI restricted DNA gave not only the 5.2 kb EcoRI GAL fragment, but faint doublets near 2 and 4 kb positions as well (data not shown). This is the EcoRI pattern expected of the yeast two micron circle plasmid (Scpl). It has been previously observed that the E. coli plasmid pBR322, the vector of pSC4911, can cross-hybridize to two micron DNA (Struhl et al., 1979). The authors of this paper suggested that stringent hybridization conditions would eliminate this aberrant cross-hybridization. However, when I used hybridization procedures described by these authors (Stinchomb et al., 1980) or by others (Wahl et al., 1979; Sherman et al., 1981) the artifactual hybridization was still obtained. This discrepancy between my results and those of Stinchcomb et al., may be due to the greater exposures of films I have used. It is generally necessary to grossly overexpose parent restriction bands in order to properly visualize DNase I hypersensitive bands. In any event, cross-hybridization of pSC4911 to two micron circle presents a serious problem because I have found the two micron circle itself to contain a number of DNase I hypersensitive sites (unpublished results). These sites could greatly interfere with mapping hypersensitive sites in the GAL cluster.

To solve the problem of the DNase I hypersensitive site within the probe sequence, a restriction site closer to the GAL 10 EcoRI site had to be used both as the leftward end of the probe and for restriction of the DNase I digested genomic DNA (see Figure 2). Figure 4 shows the positions of the restriction sites in the GAL 10 region determined by the laboratory of R. W. Davis (unpublished).
Figure 4. Restriction map of the GAL 10 gene. From (St. John and Davis, 1981a) and unpublished data from the laboratory of R. W. Davis.
Figure 4.
The selection of HpaI as the new left end of the probe was ruled out on the basis of cost and instability of the enzyme. KpnI initially was thought unsuitable because, for other members of our laboratory, it failed to give complete restriction of yeast DNA. The other enzymes were ruled out for similar reasons. Therefore, I began to screen for restriction sites by incubating various infrequent cutting restriction enzymes with the isolated SC4911 sequence. Of those tested, PstI, XhoI, BamHI, HindIII, and SmaI, only HindIII cleaved the SC4911 sequence. In fact, HindIII cuts the SC4911 sequence at two positions.

The HindIII restriction sites within SC4911 were mapped by incubating pSC4911 with each of the following restriction enzyme(s): HindIII, HindIII/EcoRI, HindIII/SalI, and electrophoresis of these digests on an agarose gel (Figure 5). Lane 3 of Figure 5 shows the EcoRI/HindIII digest produces two bands of low molecular weight. One is approximately 540 bp and the other a faint band at about 210 bp. As this 210 bp band is also seen when the plasmid is digested with HindIII alone (lane 2), this band must result from a HindIII fragment internal to the SC4911 region and the 540 bp fragment must abut the EcoRI site of GAL 10. SalI/HindIII restriction (lane 1) gave a 790 bp fragment as well as the small HindIII cleavage product. The 790 bp DNA segment must then terminate at the SalI site of pSC4911. For the positions of the HindIII sites of SC4911 relative to other restriction sites in the cluster see Figure 10. As the region between the EcoRI site of GAL 10 and the AvaI site of GAL 1 has recently been sequenced (R. Yocum, personal communication), it is important to know the distances between both the 5' HindIII and KpnI
Figure 5. Mapping the HindIII sites of SC4911. Shown is a 1.5% agarose gel of pSC4911 digested with: lane 1, SalI/HindIII; lane 2, Hind III; lane 3, EcoR I/Hind III; and lane 4, KpnI/EcoRI. Lane M contains HaeIII digested φX174 DNA size markers (see Appendix I for sizes).
Figure 5.
sites and the EcoRI position of GAL 10. This allows DNase I hypersensitive sites mapped from these positions to be properly aligned with the DNA sequence. Accurate determination of these distances was established from co-electrophoresis of HaeIII digested X174 DNA size markers with DNA equivalent to lanes 3 and 4 of Figure 5 (Appendix I).

In order to see how far DNase I hypersensitive site mapping could be extended from the GAL 10 5' HindIII site, the position of the next HindIII site rightward (as see in Figure 2) along chromosome II was determined. At the time of this experiment it was learned that the lab of R. W. Davis had carried out extensive transcription studies on the GAL cluster of strain D585-11C. Therefore, this strain was used in the remainder of the experiments on the GAL cluster.

DNA from strains 108-3C and D585-11C were restricted either with HindIII alone or with HindIII and SalI and fragments separated by agarose gel electrophoresis. The Southern blot pattern of this gel was hybridized with radioactive pSC4911. Two HindIII fragments were observed at approximately 2.4 and 2.5 kb. The 2.5 kb fragment was identified as the one extending rightward by its insensitivity to SalI cleavage. Lane 1, Figure 7 also shows this same HindIII fragment.

When a fragment of pSC4911 extending from the PstI site of pBR322 to the 5' GAL 10 HindIII site was used as a hybridization probe, only single bands were seen on a Southern blot of SalI, EcoRI, and unrestricted DNA (Figure 6). Thus, the two micron circle must not share homology with the small portion of pBR322 included in this probe. This HindIII to PstI fragment was therefore used in all
Figure 6. Hybridization of PstI to HindIII fragment of pSC4911 to unrestricted, SalI cut, and EcoRI cut DNA. Lane 1, unrestricted DNA, lane 2, SalI digestion; and lane 3, EcoRI digestion.
hybridizations to determine DNase I hypersensitive regions in the GAL cluster. The pBR322 sequence was included to increase the fragment size, which aids in its nick translation and hybridization. Also, inclusion of pBR322 sequences allows the same probe to hybridize to DNA size markers made from this plasmid. A DNA size marker set was constructed for these experiments and is described in Appendix II. It contains 7 fragments (from 754 to 4362 bp) all containing homology to the Pst I to EcoRI region of pBR322.

**DNase I Hypersensitive Region Between GAL 10 and GAL 1**

Yeast nuclei from strain D585-11C grown on galactose, GAL genes induced, were partially digested with DNase I. Isolated DNA from these nuclei was digested with HindIII, the resultant fragments separated on a 1.5% agarose gel and transferred to nitrocellulose paper. When the Southern blot was hybridized with a $^{32}$P-labeled DNA fragment containing the GAL 10 segment indicated in Figure 10 (also see previous section), additional bands were seen below the single 2.5 kb HindIII restriction fragment having homology to the probe (Figure 7, lanes 2-4). These additional smaller bands were absent when DNA of strain D585-11C (Figure 2, lane 1) and DNA or nuclei incubated in digestion buffer (data not shown) were restricted and analyzed. The size of each DNase I generated fragment corresponds to the distance between the GAL 10 5'-HindIII site and a DNase I hypersensitive cutting site (see Introduction). Two separate bands can be seen that co-migrate near the 939 bp DNA size marker. On the original autoradiogram, the upper broader band appears itself to be a closely spaced doublet.
Figure 7. Mapping hypersensitive regions between GAL 10 and GAL 1. Southern blot of a 1.5% agarose gel containing 10 micrograms DNA per lane was hybridized with the PstI to HindIII fragment of pSC4911. All samples except DNA size markers were digested with Hind III. Lane M, DNA size markers (Appendix II); lane 1, DNA; lanes 2-4, nuclei from cells grown on galactose digested with 0.1, 0.4, and 1.6 U/ml DNase I respectively; and lanes 5 and 6, DNA digested with 0.1 and 0.3 U/ml DNase I.
Figure 7.
When naked DNA was digested with DNase I and examined within the same HindIII fragment, the region hypersensitive to DNase I in nuclei is not observed (Figure 7, lanes 5 and 6). In fact, comparing lanes 4 and 5 of Figure 7, DNase I seems to have avoided the nuclear hypersensitive zone when isolated DNA was the substrate. It is also interesting to note that the DNase I digestion of DNA does give some banding, which oddly enough partially consists of a series of regularly spaced bands. Nevertheless, it is clear that the very strong DNase I hypersensitive region between the 5' ends of GAL 10 and GAL 1 (lanes 2-4) is dependent upon chromatin structure for its existence. Thus, this hypersensitive region is a site specific chromatin structure which occurs adjacent to the 5' ends of two active genes.

In order to accurately determine the location of these sites, an additional experiment was performed in which two adjacent gel lanes containing DNA identical to that of lane 3 of Figure 7 were analyzed, one of which itself contained DNA size markers (Figure 12). This procedure was followed to eliminate possible aberrant mobilities between lanes containing DNA size markers alone and those containing digested genomic DNA. Typically lanes of genomic DNA contain greater than 50,000 times the DNA loadings of DNA size marker lanes. Also, by running an adjacent lane of DNA without DNA size markers, mobilities of bands at or near the DNA size marker locations may be determined. Figure 12 also shows such mapping experiments with
Table 1. Mapping of DNase I Hypersensitive Region Between GAL 10 and GAL 1

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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</thead>
<tbody>
<tr>
<td>Band Centers</td>
<td>985</td>
<td>963</td>
</tr>
<tr>
<td></td>
<td>946</td>
<td></td>
</tr>
<tr>
<td></td>
<td>880</td>
<td>875</td>
</tr>
<tr>
<td>Limits of Region:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAL 10 side</td>
<td>851</td>
<td>833</td>
</tr>
<tr>
<td>GAL 1 side</td>
<td>1023</td>
<td>1043</td>
</tr>
<tr>
<td>Correlation Coefficient, DNA Size Markers</td>
<td>-0.9993</td>
<td>-0.9996</td>
</tr>
</tbody>
</table>

Distances given above are in base pairs from 5' HindIII site of GAL 10. Correlation coefficient is for least squares regression of DNA size markers (Appendix II).
internal DNA size markers for other DNase I sites in the cluster which are described below. The results from two completely independent mapping experiments from the HindIII site are given in Table 1.

Experiment 1 of Table 1 is data taken from Figure 12. The gel of Experiment 2 of Table 1 is not shown. While three hypersensitive bands could be detected in both Figure 2 and Figure 12 (Experiment 1 of Table 1), the two closely spaced upper bands were not resolved in Experiment 2. However, even with the poorer resolution of the gel of Experiment 2, the data of these two experiments agree well with each other. The positions of the hypersensitive site closest to GAL 10 differ by only 5 bp. Likewise, the 963 bp center of the one resolvable upper band of Experiment 2 varies only 2.5 bp from the midpoint between the two observed upper bands of Experiment 1 (965.5 bp). The variance seen between the determination of the limits of the whole hypersensitive region for the two experiments is larger than that seen for the center of the bands. This is at least partially due to the difficulty associated with trying to decide where, within a diffuse DNase I generated band, the region actually ends. A difference of approximately 20 bp is observed between the two experiments for the determination of each limit. The size of the region is different by about 40 bp for the two experiments.

Summarizing the data of Table 1, the region of DNase I hypersensitivity between the 5' ends of GAL 10 and GAL 1 extends for about 190 bp. The limits of the region are approximately 840 to 1030 bp from the GAL 10 5'-HindIII site. The centers of the three bands within this zone occur at approximately 880, 945, and 990 bp from the
same reference point (see Figure 10). The band at 945 appears to be the strongest of the three bands (lane 3 of Figure 7).

The DNA sequence between the 5' ends of the GAL 10 and GAL 1 genes has recently been determined (R. Yocum, personal communication). Examination of this DNA sequence show the 190 bp area containing the DNase I hypersensitive sites to be rich in GC content. This zone is 52% GC, while equivalent size regions immediately to the GAL 10 and GAL 1 sides of the hypersensitive area are 29% and 34% GC respectively.

It should also be noted that the area on the GAL 10 side of the major hypersensitive region does show some preferential sensitivity over the region to the GAL 1 side at the most extensive DNase I digestion (lane 4 of Figure 7).

Hypersensitive Region at 3' End of GAL 1

To extend the search of DNase I hypersensitive sites beyond the GAL 1 HindIII site, an experiment identical to that described above was performed except the restriction was performed with KpnI instead of HindIII. Hybridization of the probe to KpnI restricted DNA gave an approximately 6.4 kb band (lane 1 of Figure 8). The pattern for DNase I digested nuclei shows the presence of two regions of DNase I hypersensitivity (Figure 8, lanes 2-4). Again the hypersensitive region between GAL 10 and GAL 1 is seen, band near 1.0 kb, but is less well resolved here due to the lower percentage agarose gel used. An additional hypersensitive site is observed higher on the gel which corresponds to an area near the 3' end of GAL 10.
Figure 8. Mapping hypersensitive regions from the GAL 10 KpnI site. Southern blot of a 0.8% agarose gel containing 5 micrograms DNA per lane was hybridized with same probe as Figure 7. All DNA samples were digested with KpnI. Lane 1, DNA; Lanes 2-4, nuclei from cells grown on galactose digested with 0.1, 0.4, and 1.6 U/ml DNase I; lanes 5 and 6, DNA digested with 0.05 and 0.1 U/ml DNase I respectively.
Figure 8.
When these regions are mapped by the co-electrophoresis method described above (see Figure 12), the location of the two 5' GAL 10-GAL 1 bands observed differ by less than 15 bp from those determined by mapping from the HindIII site. Band centers were located at 999 and 916 bp from the KpnI site of GAL 10. The approximate GAL 10 5' HindIII site to KpnI is 48 bp (Appendix I). The location of the GAL 1 3' hypersensitive region was similarly determined to be 3.4 kb leftward of the GAL 10 KpnI site (see Figure 10). The width of this region is approximately 250 bp. DNase I digestion of isolated DNA gave a much more disperse pattern than nuclear digestion, but banding can be seen (Figure 8, lanes 5 and 6). As seen before in Figure 7, there is actually little hybridizing material in the areas of the naked DNase I digestions corresponding to the chromosomal hypersensitive region (compare lanes 5 and 6 to lane 4 of Figure 8).

Hypersensitive Regions Leftward of GAL 10 EcoRI Site

DNase I hypersensitive regions leftward, as seen in Figure 10, of the probe sequence were located as above, but the restriction step was performed with EcoRI. Such a mapping experiment is shown in Figure 9. DNase I hypersensitive regions were seen at approximately 1.5, 2.4, and 4.3 kb leftward from the GAL 10 EcoRI site (Figure 9, lanes 2-4). The positions of these bands relative to the galactose gene cluster are shown in Figure 10. Sites occur near the 3' end of GAL 7, between the 5' end of GAL 7 and 3' end of GAL 10, and within the GAL 10 structural gene near the SalI site. Widths of these regions
Figure 9. Mapping hypersensitive sites from the GAL 10 EcoRI site. Identical to Figure 8 except restriction was with EcoRI.
Figure 9.
Figure 10. Summary of DNase I hypersensitive regions in the yeast galactose gene cluster. Vertical arrows indicate centers of hypersensitive regions. Letters symbolize restriction sites for: EcoRI (R); SalI (S); HindIII (H); KpnI (K); and AvaI (A). Only a partial restriction map of the region is shown.
1 which appears to be closer to 300 bp wide. None of these three regions occur when isolated DNA is digested with DNase I (Figure 9, lanes 5 and 6).

All GAL Cluster Hypersensitive Regions Exist Prior to Induction

Nuclei from D585-11C cells grown on glucose were isolated and analyzed for DNase I hypersensitive regions as above. Nuclei from these cells, in which the genes of the galactose cluster are uninduced, contained all five regions of hypersensitivity seen within the cluster in fully induced cells. Lane 1 of Figure 11 shows a mapping experiment conducted leftward from the KpnI site. The hypersensitive region mapping experiment using the GAL 10 EcoRI site as its origin is shown in lane 2 of the same figure. These two lanes display hypersensitive bands at identical positions as those seen in Figures 8 and 9. Some bands within the cluster, however, do appear to be sharper before induction. This effect is most apparent for the region between 3' end of GAL 10 and 5' end of GAL 1. Comparison of the 2.4 kb bands of lane 3 of Figure 9 and lane 2 of Figure 11 demonstrates this effect.

Deletion of 5' Sequences of GAL 10 Does Not Eliminate Internal GAL 10 Hypersensitive Region

Yeast strain YNN 70 carries a deletion of chromosome II that extends from the EcoRI site of GAL 10 to the first EcoRI site beyond the 3' end of GAL 1 (see Figure 10). As a result of this deletion, this strain is incapable of transcribing the GAL 10 gene (St. John and Davis, 1981b). Strain YNN 70 was grown on glucose and analyzed
Figure 11. Hypersensitive regions in strains D585-11C and YNN 70 grown on dextrose. Southern blot of 0.8% agarose gel containing 5 micrograms of DNA per lane from nuclei digested with 0.4U/ml DNase I. Lane 1, D585-11C (KpnI) digestion; lane 2, D585-11C (EcoRI); lane 3, YNN 70 (KpnI); lane 4, YNN 70 (EcoRI). Probe is same as used in Figure 7.
Figure 12. Hypersensitive regions mapped with internal DNA size markers. DNA size markers used here are those described in Appendix II. Mapping is as described before, but duplicate lanes were run, one of which itself contained the DNA standards. Even numbered lanes contain standards, odd lanes do not. 12A: D585-11C (galactose)-HindIII. 12B: Lanes 1 and 2, D585-11C (gal)-KpnI; lanes 3 and 4, D585-11C (gal)-EcoRI; lanes 5 and 6, YNN 70-KpnI; and lanes 7 and 8, YNN 70-EcoRI.
Figure 12.
for DNase I hypersensitive regions as in the experiments above. Lane
3 of Figure 11 shows a mapping experiment from the KpnI site. The
dramatic reduction in the KpnI fragment size between lanes 1 and 3 of
the figure confirms that a large part of the region leftward of the
probe has been deleted. Lane 4 of Figure 11 is a mapping experiment
from the EcoRI end of the probe. The identical sizes of the EcoRI
fragments seen in lanes 2 and 4 confirm the deletion of YNN 70 does
not extend beyond the GAL 10 EcoRI site. All three of the undeleted
DNase I hypersensitive bands may be observed in lane 4. This
includes the band that corresponds to the hypersensitive region
within the GAL 10 gene. Therefore, the hypersensitivity to DNase I
digestion near the SalI site of GAL 10 occurs not only independently
of induction of the gene, but also is present when GAL 10 is
prevented from being transcribed by having sequences at its 5' end
removed. It is obvious that the hypersensitivity of this region is
independent of any structural features of the chromosome that occur
for 4 kb upstream of the 5' end of GAL 10.

DISCUSSION

The experiments presented here clearly demonstrate that despite a
number of differences that exist between the chromosomes of higher
eucaryotes and those of yeast, site specific regions of DNase I
hypersensitivity do exist within yeast chromatin. I have shown DNase
I hypersensitive regions to be located near both the 5' and 3' ends
of all three genes of the galactose cluster. An additional region of
DNase I hypersensitivity, having an unusual location, was also
discovered within the cluster. This region occurs inside the \textit{GAL} 10 gene about 500 bp from its 3’ end. Thus, the hypothesis put forth by Elgin (1981), among others, "that a DNase I hypersensitive site at or near the 5’ end of a gene is necessary, but not sufficient, for transcription by RNA polymerase II in vivo", appears to hold true not only for higher multicellular eucaryotes, but for the unicellular yeast as well.

DNase I digestion of isolated DNA showed none of the 5 hypersensitive areas in the cluster to be merely the result of DNA sequence preference by DNase I. Unfortunately such control experiments were omitted from the one yeast DNase I hypersensitivity study published to date (Sledziewski & Young, 1982). These areas of the galactose gene cluster must then be site specific chromatin structures that allow access of their DNA sequences to DNase I. For other observed DNase I hypersensitive regions, it has been proposed that such accessibility to DNase I may also extend to other DNA binding proteins involved in transcription, replication and other processes. This appears to be the case for the \textit{HO} gene product (Introduction).

The site(s) of action of the galactose regulatory proteins, \textit{GAL} 4 and \textit{GAL} 80, when determined, may well coincide with a DNase I hypersensitive region(s) identified in this study. Also hypersensitive areas 5’ to the galactosase cluster genes may provide access to the DNA for proteins that initiate transcription. Studies seeking to identify sites involved in initiation and regulation of transcription of the galactose cluster genes are currently in progress in the laboratories of M. Ptashne and R. Davis.
The hypersensitive region between GAL 10 and GAL 1 was studied in detail because it may contain sequences essential for transcription of two of the GAL genes. This region is also of special interest because it occurs in an area whose DNA base sequence has been determined (R. Yocum, personal communication). Within this approximately 190 bp hypersensitive region, three individual cutting sites have been observed. The centers of these three sites are all located within 100 bp of each other. This region extends to about within 160 bp of the 5' end of GAL 10 and to within 340 bp of the 5' end of GAL 1. Evidence suggests that the start point of the amino acid coding sequence and the mRNA initiation site may closely coincide for both GAL 10 and GAL 1 (St. John & Davis, 1981a; R. Yocum, personal communication). The most noticeable feature of the DNA sequence of this hypersensitive region is its high GC content. A hypersensitive region of similar length (200 bp) near the chicken adult β-globin gene has also been reported to be GC rich (McGhee et al., 1981). Elevated GC content may be a general characteristic of 5' DNase I hypersensitive regions. However, DNA sequence information must be obtained for many other hypersensitive regions in order to determine if this is indeed the case.

The other four regions of DNase I hypersensitivity, except one, are located near the 3' ends of cluster genes. The hypersensitive area near the 3' end of GAL 10 is also near the 5' end of GAL 7. This intergene region appears to be slightly broader than other hypersensitive areas observed. This effect is most pronounced when the genes of the cluster are fully induced. Although a function for any of the hypersensitive regions in the GAL cluster has not been
established, it is possible the observed 5' and 3' regions might be associated with transcription initiation and termination sites respectively. However, the possible function, if any, of the internal GAL 10 area remains more enigmatic. Wu and Gilbert (1981) did observe a hypersensitive region slightly within the 3' end of the rat pre-proinsulin II gene. Oddly enough, this site was absent in nuclei from an insulinoma in which the gene is actively transcribed. The hypersensitive region I have located in GAL 10 is present both before and after induction and is well removed, 500 bp, from the gene's 3' end.

I have shown all five regions of hypersensitivity to exist prior to induction of the cluster genes by galactose. However, more distinct boundaries were observed for at least some of the hypersensitive regions before induction. Similar behavior has been observed by Wu (1980) for the inducible Drosophila heat shock genes.

A study described here for the gal deletion strain YNN 70 shows the GAL 10 internal hypersensitive site to exist in spite of this mutation. The removal of about 4 kb of DNA sequence 5' to GAL 10 in this strain implies that such effects as low level transcription of GAL 10 and modulation of the chromatin structure by sequences 5' to this gene are not required to establish the GAL 10 internal hypersensitive site.
5-METHYLCYTOSINE IS UNDETECTABLE IN DNA OF SACCHAROMYCES
CEREOVISIAE

INTRODUCTION

5-methylcytosine (5MeC) occurs as a minor base in the DNA of many diverse organisms. All vertebrate and plant DNAs studied to date have been found to contain this modified base. 5MeC is known to function in some procaryotes, at least in part, in the host restriction-modification system. However, its presence in the DNA of higher eucaryotes has long remained cryptic. When total genomic 5MeC content is measured for various tissues of a vertebrate, only small differences are observed (Solage and Cedar, 1978; Singer et al., 1979). Despite this limited genomic variability, dramatic tissue specific differences are seen when specific loci within individual genes are examined for methylation. Within the last four years, workers utilizing modern molecular biological techniques have demonstrated an inverse correlation between the transcriptional activity of a gene and the presence of 5MeC in the vicinity of that gene. These findings have brought about a sudden increased interest in this modified base whose presence in calf thymus DNA was discovered over 30 years ago (Hotchkiss, 1948). There are a number of reviews covering recent advances in methylation of eucaryotic DNA (Weisbrod, 1982; Felsenfeld and Mcghee, 1982; Wigler, 1981; Ehrlich and Wang, 1981; Razin and Riggs, 1980).
The isoschizomer restriction endonuclease pair HpaII and MspI both recognize the sequence CCGG. However, HpaII cannot cleave the methylated sequence C(5MeC)GG. MspI cleavage is unaffected by such modification. When DNA from human lymphocytes is incubated with either HpaII or MspI and subjected to agarose gel electrophoresis a profound difference is seen between the gel tracks associated with each enzyme. The MspI lane displays a wide distribution of DNA fragment size, while virtually all the HpaII incubated material barely enters the gel (Ehrlich and Wang, 1981). Such resistance to HpaII digestion suggest the sequence C(5MeC)GG is much more common than is CCGG in human DNA. Waalwijk and Flavell (1978) were the first to combine such restriction enzyme procedures with Southern blot analysis to show tissue-specific methylation patterns for the rabbit β-globin gene. Here, as in other gene systems, undermethylation has been correlated with gene activity. Among the many instances in which this correspondence has been found are: chicken β-globin gene in red blood cells (McGhee and Ginder, 1979), ovalbumin, ovotransferin, and ovomucoid genes in hen oviduct (Mandel and Chambon, 1979), amplified ribosomal genes in Xenopus laevis (Bird and Southern, 1978), and various integrated viral genes (Sutter and Doerfler, 1980; Desrosiers et al., 1979; Groudine et al., 1981). Although these studies observed a reduction in methylation, the patterns of variation were complex and usually did not involve complete demethylation of extensive regions of DNA. There are some sites within genes that remain either methylated or unmethylated under all conditions irregardless of tissue specific gene expression (Mandel and Chambon, 1979). It should also be stressed that analysis
of cytosine methylation with HpaII and MspI is restricted to CCGG sequences and therefore examines only some of the possible modification sites.

Levels of methylation have been altered by both in vitro and in vivo manipulations to investigate possible effects on gene expression. HpaII methylase has been used to modify the herpes thymidine kinase (tk) gene prior to its transformation into mouse Ltk− cells (Pollack et al., 1980; Wigler et al., 1981; Stein et al., 1982). Transformants which expressed the tk gene were also observed to have partially demethylated tk sequences, even when another genetic marker was used to select transformants. The nucleoside analogue 5-azacytidine has been used to inhibit DNA methylation in vivo. Differentiation has been induced in cultured mouse embryo cells by use of this agent (Jones and Taylor, 1980). 5-azacytidine has been used as well to induce expression of inactive endogenous avian retroviral sequences (Groudine et al., 1981). Also Compere and Palmiter (1981) have used this inhibitor to cause expression of a normally uninducible metallothionein gene. For the latter two systems gene expression was associated with DNA demethylation.

In several systems investigators have failed to find a correlation between hypomethylation and gene activity. Two recent examples of such systems are the rDNA of Xenopus borealis (Macleod and Bird, 1982) and the chick 2(I) collagen gene (McKeon et al., 1982). The work of van der Ploeg and Flavell (1980) suggests that hypomethylation alone is insufficient to cause transcription of the human γ-globin genes. They found placenta and KB cells, which do not express the γ-globin genes, to be more hypomethylated at the locus
than expressing cells. CCGG and CGCG sequences in *Drosophila* do not contain detectable methylation (Rae and Steele, 1979; Bird and Taggart, 1980), yet this is a classical organism for the study of differentiation.

A model consistent with all the above information would postulate that the absence of methylation at specific residues is necessary, but not sufficient, for gene expression.

Although most recent research on 5MeC has been directed toward its relationship to gene activity, untranscribed satellite DNA sequences are more than twice as methylated as main band DNA (Solomon et al., 1969). Recently, the extent and location of methylation of bovine satellite DNA has been shown to be tissue specific (Sano and Sager, 1982).

It was reported in 1978 that yeast DNA contained 5MeC at a level of 0.3 to 1.0 mole percent of total cytosine (Hattman et al., 1978). As was noted recently (Fangan and Zakian, 1981) it has not been determined whether this reported 5MeC occurs at specific sites in the yeast genome. In order to determine if this methylation is present in CCGG sequences, I have compared gel patterns of HpaII and MspI digested yeast DNA. Total genomic DNA, the two micron circle, and the area about the gene for iso-2 cytochrome c all showed no evidence for cytosine methylation. In contrast to earlier reported results, HPLC analysis of highly purified yeast DNA showed 5MeC to be undetectable.
MATERIALS AND METHODS

Growth of cells, preparation of bacterial plasmids, nick translations, restriction of genomic DNA, Southern transfers, agarose gel electrophoresis, and hybridization of Southern filters were performed as described in Part I of this thesis.

Preparation of DNA

Yeast DNA for restriction endonuclease digestion was prepared as described by Sherman et al., (1981). DNA for HPLC analysis was prepared by the method described below.

Isolated yeast nuclei (Ide & Saunders, 1981) from either strain B273-10B or A364a were resuspended in the following solution: 75mM NaCl, 10mM Tris pH 7.4, 0.5% NP-40, and 1mM EDTA. After homogenizing with a motor driven Potter homogenizer, the material was pelleted by centrifugation for 10 minutes at 10,000 RPM in a SS34 rotor. Following two repetitions of the last step, the final pellet was suspended in 50mM EDTA, pH 8.5. DNA was then prepared by the method of Davis et al., (1981), except RNase digestion was at 50µg/ml RNase A and 50 U/ml RNase T1 for 2 hrs at 37°C. The resultant solution was subjected to pronase digestion, organic extraction and ethanol precipitation, as described in part I of this thesis. The DNA pellet was dissolved in TE buffer and again RNase and pronase digestions, organic extraction, and ethanol precipitation were performed. After dissolving the DNA pellet in TE buffer, BRL Ultrapure CsCl was added and the solution centrifuged in a 50 Ti rotor at 40,000 RPM for 48 hours. Fractions contained within the central portion of the one
peak observed in the gradient were pooled, diluted threefold with water, and ethanol precipitated. The final pellet, after having been washed three times with 70% ethanol, was prepared for HPLC analysis.

**Chicken DNA Controls**

To see if any of the steps of the relatively new DNA preparation procedures of Davis et al., (1981) or Sherman et al., (1981) might alter 5MeC content, control experiments were performed using chicken DNA, which is known to contain 5MeC. Using lysed nuclei from chicken erythrocytes as the starting material, chicken DNA was prepared essentially as described in the yeast method above starting at the EDTA resuspension step. The heating step of the Davis procedure was allowed to continue for 8 times the standard 30 minutes to maximize any possible effect this treatment might have. Chicken DNA was also prepared by the more conventional phenol extraction procedure. Neither of the chicken DNA samples were CsCl banded.

**Recombinant Plasmids**

p82-6B contains about 1.5 copies of the yeast two micron circle plasmid cloned into pMB9 by the poly (dA)-poly (dT) tailing technique (Hartley and Donelson, 1980). pAB 25 is composed of a 7.5 kb HindIII fragment containing the gene for iso-2 cytochrome c (CYC 7) cloned into the HindIII site of pBR322 (McKnight et al., 1981).
RESULTS

Restriction Enzyme Analysis

DNA from yeast strain B273-10B was incubated with either MspI or HpaII and then subjected to electrophoresis on a 1.5% agarose gel. The resultant patterns of ethidium bromide stained DNA fragments showed extensive digestion and were identical for both enzymes (Figure 13, lanes 1 and 2). This result implies that widespread methylation of the internal cytosine of the sequence CCGG does not occur in the yeast genome. It is also interesting to note in Figure 13 the many observable distinct DNA bands which are not commonly seen in genomic restriction patterns of many eucaryotes. These most likely result from multicopy DNA sequences such as the two micron circle and the rDNA repeat units. They are highly visible in yeast due to the relatively small genome size of approximately 14,000 kb.

In order to search for methylation within specific regions of the yeast genome, Southern blot patterns of HpaII and MspI digestions were probed with sequences homologous to either the two micron circle or the area about the gene for iso-2 cytochrome c.

When a blot is probed for two micron circle sequences with p82-6B, MspI and HpaII give identical fragments (lanes 2 and 3 of Figure 14A). Lane 1 of Figure 14A is a HindIII digestion hybridized with the same probe, which is used here as DNA size markers. All of the fragments expected from two micron DNA are not resolved from each other in Figure 14A. However, examination of the published two micron DNA sequence (Hartley and Donelson, 1980) for CCGG sequences reveals that failure to cut at any of these sites would result in a
Figure 13. HpaII and MspI digestions of yeast DNA. Lane 1, HirdIII; lane 2, MspI; and lane 3, HpaII.
fragment larger than the second smallest HindIII two micron fragment of 1314bp (lane 1, Figure 14A). As this is not observed, the sequence C(5MeC)GG must not occur in the yeast two micron circle plasmid. Since it has been reported that MspI cannot cleave (5MeC)GGG (Sneider, 1980), this sequence must also be absent from the yeast plasmid.

pAB25 was used to probe the 7.5 kb HindIII fragment of the yeast genome which contains the gene for iso-2 cytochrome c. Double digestions with either HindIII or HpaII and MspI gave identical fragment patterns within this region (Figure 14B, lanes 1 and 2). This implies that C(5MeC)GG sequences are absent in this region as well.

HPLC Analysis

Having failed to detect any evidence for cytosine methylation in yeast DNA by restriction enzyme analysis, I decided to re-evaluate the 5MeC content of yeast DNA. Yeast DNA purified as described in Materials and Methods was sent to the laboratory of Dr. Stanley Hattman, University of Rochester, HPLC determination of 5MeC content. The samples were analysed by HPLC for content of constituent deoxynucleotides by both conventional and reversed phase techniques. The results are shown in Table 2. 5MeC is undetectable in yeast DNA. While its presence cannot be totally ruled out, if present it must occur at a rate of less than 1 methylated base per 2,000 total cytosine residues.
Figure 14. HpaII and MspI cleavage in the two micron circle and near CYC 7. 14A: Probe is p82-6B (two micron), Lane 1, Hind III; lane 2, Msp I; and lane 3, HpaII. 14B: Lane 1 is MspI and lane 2 is HpaII cleavage. Probe is pAB25 (CYC 7).
Figure 14.
Table 2. HPLC Determination of 5-Methylcytosine Content

<table>
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<tr>
<th>Sample</th>
<th>% 5MeC</th>
<th>Method</th>
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<tr>
<td>Yeast A364a</td>
<td>&lt;0.05</td>
<td>H</td>
</tr>
<tr>
<td>Yeast B273-10B</td>
<td>&lt;0.05</td>
<td>H</td>
</tr>
<tr>
<td>Chicken (Davis)</td>
<td>3.25</td>
<td>D</td>
</tr>
<tr>
<td>Chicken (Phenol)</td>
<td>3.29</td>
<td>D</td>
</tr>
</tbody>
</table>

% 5MeC is the mole percent of 5MeC calculated on the basis of total cytosine residues.

H-HPLC analysis performed by the laboratory of Dr. Stanley Hattman, University of Rochester (Proffitt, Davie and Hattman, manuscript in preparation).

D-HPLC analysis by Dr. Jim Davie, O.S.U., by method previously reported.
Chicken DNA samples prepared either by a procedure which employed the protocol of Davis et al., (1981) or the more conventional phenol extraction procedure (see Materials and Methods) were given to Dr. Jim Davie, O.S.U., for HPLC determination of 5MeC content. Both DNA samples had the same 5MeC percentage (Table 2). These results suggest that 5MeC content is not altered by the DNA isolation procedure of Davis et al.

DISCUSSION

The extent of digestion and identical appearance of genomic HpaII and MspI digestions suggests that 5 MeC is absent from or occurs very rarely at CCGG sequences in yeast DNA. Methylation at external or internal cytosines block cleavage by MspI and HpaII respectively. The identical pattern seen for HpaII and MspI cleavage near the CYC 7 locus and within the two micron circle imply that the recognition sequences for the enzymes in these regions are free of cytosine methylation as well.

While analysis of 5MeC levels by HPLC cannot locate the positions of these modified residues, it has the great advantage that it "sees" all 5MeC residues. Detection of the presence of 5MeC is not limited to certain restriction enzyme recognition sequences. The HPLC data of Table 2 imply that 5MeC is either absent from the yeast genome or occurs at a frequency of less than one methylated residue per 2,000 total cytosines.
Although it has been previously reported that 5MeC was present at low levels in yeast (Hattman et al., 1978), the determinations made of 5MeC content in that work were not reproducible (0.3 and 1.0 mole % of total cytosine). The irreproducible nature of that data in the original study was limited to DNA from S. cerevisiae, while analysis of DNA samples from other simple eucaryotes did not show such fluctuations. This suggests that the component of yeast DNA measured in that study may not have been genomic 5MeC. The paper chromatography method used in the 1978 study also lacks the high resolution afforded by presently employed HPLC analytical techniques. Also, this original study analysed the 5MeC content by chromatography of the component bases of DNA. This current study, however, determined the mole percentage of 5MeC in yeast DNA by running samples as deoxynucleosides. The HPLC analysis can distinguish between deoxyribonucleosides and ribonudeosides. Thus, in the present study a modified base of RNA that might be present as a contaminant in the sample would not be mistaken for a component of yeast DNA. This is important because yeast cells contain much more RNA than DNA and also RNA is well known to be more heavily modified than is DNA. The yeast DNA used in this present investigation was made from detergent washed isolated yeast nuclei rather than spheroplasts as was the DNA of the original study. The use of such nuclei should also decrease the level of RNA and other potential contaminants from the cytoplasm.

The lack of extensive cytosine methylation in yeast DNA is interesting in view of the important role this modified base may play in determining chromatin structure and/or in regulating gene activity
in higher eucaryotes. While the macronuclear DNA of *Tetrahymena pyriformis* has been reported to lack detectable 5MeC, it was found to contain the modified base 6-methyladenine (6MeA) (Hattman *et al.*, 1978). This same study failed to detect 6MeA in yeast DNA. Thus, the results presented here suggest that the yeast *Saccharomyces cerevisiae* is the first eucaryote shown to have undetectable levels of both 6MeA and 5MeC. Clearly although 5MeC may fulfill an important function in some organisms, an appreciable amount of this base is not essential for life as a eucaryote.

Whether or not the lack of 5MeC and/or 6MeA is a general property of the fungi must await HPLC analysis of the DNA of other fungi. It will also be interesting to see if *Drosophila* DNA has 5MeC which is detectable by HPLC, since it has not been observed by HpaII and MspI restriction analysis (Rae and Steele, 1979). Unlike the fungi, *Drosophila* has significant amounts of satellite sequences and also is a highly differentiated organism. Therefore, if 5MeC plays some essential role in satellite DNA sequences or in differentiation, it should be present and detectable in this organism.
Bibliography


Appendix I. Accurate Determination of KpnI to EcoRI and HindIII (5') to EcoRI Distances in SC4911.

The plasmid pSC4911 (St. John and Davis, 1981a) was cut with EcoRI and then with KpnI or HindIII. Each double digest was run both by itself and with the inclusion of HaeIII cut φX174 DNA size markers on a 1.5% agarose gel. A least squares regression was performed on the distance of migration vs. log (base pairs) for the 5 largest X174 fragments. The resultant equation was used to determine the SC4911 sub-fragment sizes. The DNA sequence of φX174 has been completely determined (Sanger et al., 1978). Therefore, the sizes of the DNA length standards used here are known exactly. The data shown for EcoRI/HindIII digestion is for coelectrophoresis with size markers. That for EcoRI/KpnI is for electrophoresis of size marker in an adjacent well, as the 603 bp size marker is not well separated from the SC4911 sub-fragment.
<table>
<thead>
<tr>
<th>Migration (Centimeters)</th>
<th>Base Pairs</th>
<th>Correlation Coefficient</th>
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</thead>
<tbody>
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<td>Markers</td>
<td>11.25</td>
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<td></td>
<td>12.67</td>
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<td>872</td>
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<td>16.05</td>
<td>603</td>
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<td></td>
<td>19.60</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>-0.9995</td>
</tr>
<tr>
<td>KpnI/EcoRI</td>
<td>16.10</td>
<td>586</td>
</tr>
</tbody>
</table>

| Markers                | 11.17      | 1353                    |
|                        | 12.65      | 1078                    |
|                        | 13.97      | 872                     |
|                        | 16.1       | 603                     |
|                        | 19.65      | 310                     |
|                        |            | -0.9991                 |
| HindIII/EcoRI         | 16.63      | 537                     |
Appendix II. DNA Size Markers

Sizes of HaeIII digested \( \phi X174 \) DNA standards are given in Appendix I. DNA markers for DNase I hypersensitive site mapping experiments consisted of the sequenced plasmid pBR322 (Sutcliffe, 1978) cut with PstI/EcoRI; PstI/EcoRV; PstI/BamHI; PstI/SalI; PstI/AvaI; PstI/PvuII and PstI alone. DNA lengths of fragments having homology to the PstI to EcoRI fragment of pBR322 are: 754, 939, 1129, 1404, 2178, 2819, and 4362 base pairs.