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Nucleosomes are positioned with base pair precision adjacent to the α2 operator in *Saccharomyces cerevisiae*

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Analysis of the chromatin structure of minichromosomes containing the binding site for the yeast α2 repressor protein by indirect end-labeling has previously indicated that nucleosomes are stably positioned over sequences adjacent to the α2 operator in the presence of the repressor. Development of a primer extension assay for nucleosome position now allows a more detailed examination of the location of these nucleosomes relative to the operator sequence, and indicates that nucleosomes are precisely and stably positioned both translationally and rotationally over sequences adjoining the operator. In addition, this assay enables analysis of the chromatin structure of single copy, genomic sequences. Chromatin structures determined for two genes regulated by α2, *STE6* and *BAR1*, are consistent with nucleosomes precisely positioned downstream of the operator sequence, incorporating promoter elements, in α cells but not in a-cells. The location of these nucleosomes relative to the operator sequence is highly analogous to that observed in the minichromosome. The stability of the nucleosomes adjacent to the operator together with the precision of their location suggests that they may play a role in repression of a-specific gene expression by α2. Further, the primer extension assay allows a comparison of the structure of these positioned nucleosomes formed *in vivo* to that previously described for core particles reconstituted *in vitro*.

Key words: chromatin/nucleosome positioning/repression/yeast mating type

Introduction

Positioning of nucleosomes (identical placement of histone octamers along a particular DNA sequence in all cells of a given population) has been suggested as one mechanism in regulation of the activity of DNA (reviewed in Simpson, 1991; Grunstein, 1990; Wolffe, 1990). The proposition is that *cis*-acting elements located in linker DNA between core particles are more available for interaction with *trans*-acting protein factors than the same sequences would be if sequestered within the core particle. Positioning appears to be influenced by a combination of several factors, including specific DNA–histone interactions, anisotropy of DNA structure, boundary formation, and chromatin folding (Ramsay *et al.*, 1984; Drew and Travers, 1985; Thoma and Simpson, 1985; Thoma, 1986; Fedor *et al.*, 1988; Thoma and Zatchej, 1988). Nucleosome positioning occurs for many eukaryotic genes, and several *cis*-acting elements are found near or within a positioned nucleosome (Almer *et al.*, 1986; Benezra *et al.*, 1986; Fedor *et al.*, 1988; Pina *et al.*, 1990; Simpson, 1991). Nucleosome positioning has recently been shown to affect the function of a *cis*-acting element involved in the replication of yeast TRP1/ARS1 plasmids *in vivo*. A remarkable decrease in copy number was observed when an essential ARS sequence was moved into the central region of a nucleosome core particle (Simpson, 1990). Similarly, Wolffe and Drew (1990) reported, using curved DNA to manipulate nucleosome position, that small changes in the location of a T7 RNA polymerase promoter within a nucleosome have large effects on transcription efficiency *in vitro*. Thus, positioning of nucleosomes may contribute to critical regulatory functions.

There are three different cell types in *S.cerevisiae*, haploid **a** or α cells and **a**/α diploid cells. The haploid cell types are determined by the α1 and α2 proteins transcribed from the MATα locus in α cells. α1 activates the transcription of α cell-specific genes, whereas α2 blocks transcription of **a** cell-specific genes (reviewed in Herskowitz, 1989; Dranginis, 1986). The α2 repressor binds to its operator co-operatively with another protein, MCM1, in α cells, and both α2 and MCM1 are required for repression of **a** cell-specific gene expression (Herskowitz, 1989; Keleher *et al.*, 1988, 1989; Sauer *et al.*, 1988). MCM1, which is non-cell type specific, is thought to bind to the α2 operator in **a** cells and to be an activator of **a** cell-specific genes (Jarvis *et al.*, 1988, 1989; Passmore *et al.*, 1988, 1989; Ammerer, 1990). The α2 operator consists of a largely symmetrical 31 bp sequence located approximately 200 bp upstream of five defined **a** cell-specific genes (Burkholder and Hartwell, 1985; Wilson and Herskowitz, 1986; Kronstad *et al.*, 1987; MacKay *et al.*, 1988). The operator can act at various distances to repress heterologous gene expression (CYC1) (Johnson and Herskowitz, 1985; Roth *et al.*, 1990); this suggested to us that repression by α2 might occur through changes in chromatin structure. Consistent with this idea was the finding that TRP1/ARS1 plasmids containing the α2 operator were packaged into stably positioned nucleosomes in cells which express α2 (Roth *et al.*, 1990).

We have studied the structural features of positioned nucleosomes adjacent to the α2 operator both in a minichromosome and in the yeast genome using a technique which allows base pair level resolution of nucleosome locations. This analysis can determine whether a nucleosome is translationally positioned over specific sequences and/or whether the rotational orientation of DNA on the surface of the histone octamer is uniquely fixed. We demonstrate

that nucleosomes are both translationally and rotationally positioned at the edges of the α2 operator in α cells. The precision of location of the nucleosomes adjacent to the α2 operator *in vivo* is far greater than anticipated for most postulated positioning mechanisms. Further, an array of nucleosomes is precisely and stably positioned downstream of the α2 operator, continuing into the coding regions of two **a** cell-specific genes examined, *STE6* and *BAR1*, in α cells. In **a** cells, these highly ordered structures are not observed, suggesting that they may be related to the repression of *STE6* and *BAR1* by α2. The precision and stability of nucleosome positioning adjacent to the operator in α cells reveals that the structural characteristics of these nucleosomes formed *in vivo* have both similarities and differences to those previously defined for core particles reconstituted *in vitro*; these are discussed in the context of the possible role of chromatin structure, specifically nucleosome positioning, in the function of chromatin DNA.

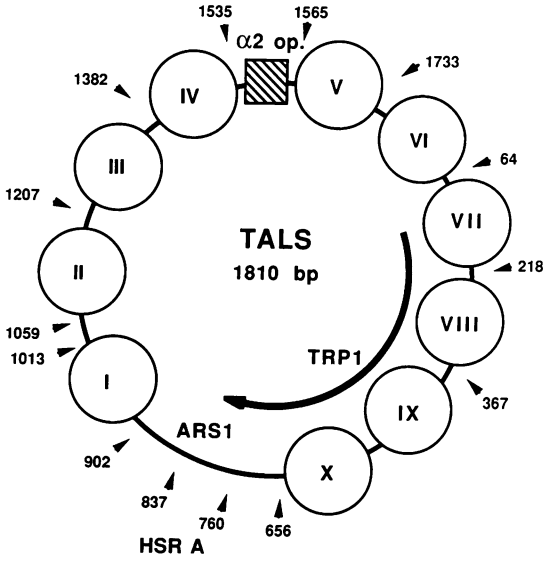

Fig. 1. Chromatin structure of the TALS plasmid in α cells. The positions of nucleosomes as determined by indirect end labelling are shown. (Roth *et al.*, 1990). Map unit positions begin at the *Eco*RI site (map unit 1) within nucleosome VI, and continue clockwise around the plasmid. The locations of characteristic micrococcal nuclease cut sites in chromatin are indicated by arrow heads. The α2 operator and *TRP1* gene are indicated by the shaded box and the curved arrow, respectively. The nuclease hypersensitive region (HSR A) includes sequences necessary for autonomous replication (ARS1 elements). The drawing is not to scale.

Results

High resolution micrococcal nuclease mapping of positioned nucleosomes in the TALS plasmid

The TALS plasmid contains the α2 operator from the *STE6* gene at the *Eco*RI site (map unit 1) of the TRP1/ARS1 plasmid (Roth *et al.*, 1990; Wilson and Herskowitz, 1986). Indirect end-labelling studies (using agarose gels) have shown that nucleosomes are precisely and stably positioned on this plasmid in α cells, but are more randomly located in **a** cells (Figure 1; Roth *et al.*, 1990). Such studies, however, are limited in the level at which they can localize nucleosome positions (±20 bp) and offer no information regarding the structure of individual nucleosomes. We have extended these studies by mapping the positions and structures of nucleosomes (IV and V) adjacent to the α2 operator in TALS using a primer extension assay (see Materials and methods). This assay allows base pair level resolution of cleavage sites in DNA induced by a variety of agents and is very useful in analysis of DNA–protein interactions (Gralla, 1985; Axelrod and Majors, 1989; Huibregtse and Engelke, 1989; Rahmouni and Wells, 1989). Use of *Taq* DNA polymerase

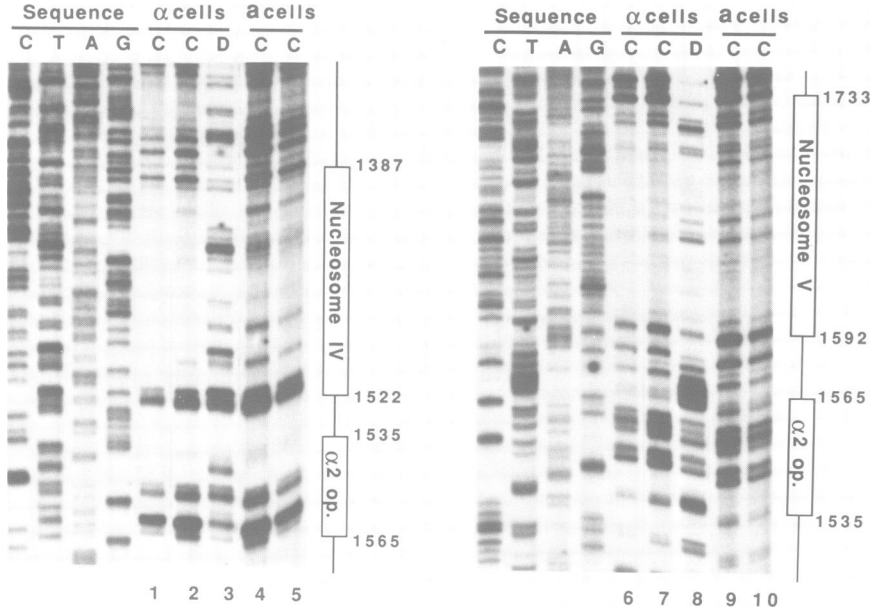

Fig. 2. Primer extension analysis of micrococcal nuclease cut sites in nucleosomes IV and V in the TALS minichromosome. The location of the α2 operator and the nucleosomes are shown to the right side of the gel. Each sequencing pattern (i.e. C) represents the pattern obtained when its complementary dideoxynucleotide (i.e. ddGTP) was added to the reaction. Each experiment was performed with a variety of nuclease concentrations as follows: 2.5 units/ml (lanes 1, 5, 6 and 10). 5 units/ml (lanes 2, 4, 7 and 9), 0.25 units/ml (lanes 3 and 8). C, digests for chromatin in isolated nuclei; D, digests of naked DNA. The oligonucleotide MS-1 was used in the analysis of the position of nucleosome IV; oligo MS-2 was used in the analysis of nucleosome V position.

allows extension at high temperature, increasing primer specificity. Moreover, multicycle linear primer extensions can be performed to amplify weak signals, allowing analysis of single copy genes (Axelrod and Majors, 1990).

Figure 2 shows high resolution mapping of micrococcal nuclease cleavage sites in the regions of nucleosomes IV and V in TALS chromatin in nuclei isolated from α and **a** cells. In α cells (lanes 1 and 2), a 135 bp region (from 1387 mu to 1522 mu) is largely protected from micrococcal nuclease digestion compared to digestion of naked DNA (lane 3), confirming a precise translational positioning of nucleosome IV in α cells. In contrast, the micrococcal nuclease cutting pattern of **a**-cell chromatin (lanes 4 and 5) is similar, but not identical, to that of naked DNA (lane 3), indicating that such positioning does not occur in **a** cells, in agreement with our previous results (Roth *et al.*, 1990). Assignment of the position of nucleosome IV in α cells is made possible by comparison of the location of nuclease hypersensitive sites at the edges of the protected region to sequencing reactions displayed on the same gel. We operationally define these hypersensitive sites to represent sequences exposed at the edges of the nucleosome, although we recognize, given the sequence selectivity of the nuclease, that some of these sites may actually reside within the periphery of the core particle. Our data indicate that nucleosome IV is positioned 13 bp (at 1522 mu) from the end of the α2 operator sequence (at 1535 mu) in the presence of α2. Furthermore, since this assay is extremely sensitive to both double and single strand cuts in the DNA, the extent of protection observed in the region of nucleosome IV indicates that this nucleosome is very stably positioned in a majority of the population of TALS molecules in α cells.

Sequences in the region of nucleosome V (1592−1733 mu; 141 bp) were also protected from micrococcal nuclease digestion in α cells (lanes 6 and 7). Again, cleavage of **a** cell chromatin (lanes 9 and 10) yielded a pattern similar to that of naked DNA (lane 8), consistent with a more stable positioning of nuclesome V in α cells than in **a** cells. The presence of specific hypersensitive sites in **a** cell chromatin, however, indicates that the structure of this region is not completely random, in **a** cells. In contrast to nucleosome IV, several cut sites are present between the edge of the protected region in α cells and the α2 operator sequence. The last of these (at 1592 mu) is 27 bp from the edge of the operator (at 1565 mu), twice the distance between the operator and nucleosome IV. These data indicate either that some flexibility is allowed in the positioning of nucleosomes adjacent to the operator in the presence of α2, or perhaps, that these nucleosomes are positioned by independent means (see Discussion).

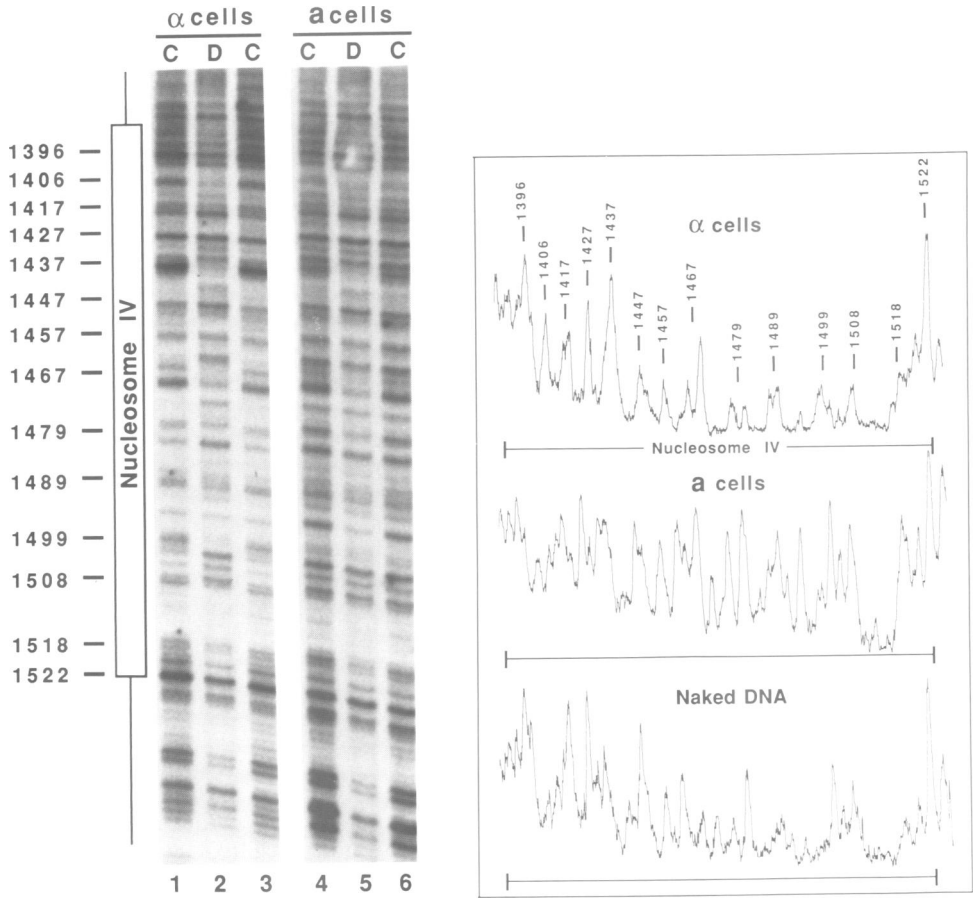

Fig. 3. Primer extension mapping of DNase I cutting sites in the nucleosome IV region in TALS. The translational location of nucleosome IV determined by analysis of micrococcal nuclease cutting sites (Figure 2) is shown to the left side of the gel. Chromatin (C) in isolated nuclei and naked DNA (D) were digested at 37°C for 10 min at different concentrations of nuclease as follows: 0.125 units/ml (lanes 1 and 4), 0.0625 units/ml (lanes 3 and 6), 0.001 units/ml (lanes 2 and 5). Oligonucleotide MS-4 was used as primer in these experiments. Densitometric profiles of the autoradiograms for α-cell chromatin (top), **a**-cell chromatin (middle), and naked DNA (bottom) are also shown to facilitate comparison of these digestion patterns.

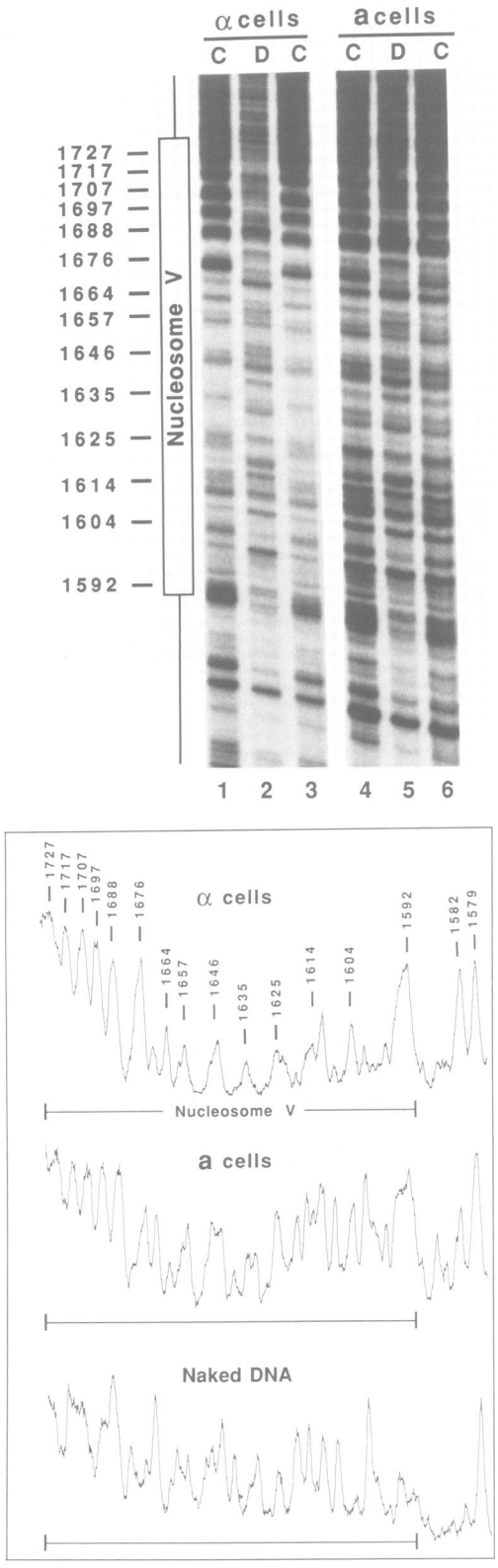

Fig. 4. Primer extension mapping of DNase I cutting sites in the nucleosome V region in TALS. The translational location of nucleosome V as determined by analysis of micrococcal nuclease cutting sites (Figure 2) is shown to the left side of the gel. Chromatin (C) in isolated nuclei and purified, naked DNA (D) were digested as in Figure 3. Oligonucleotide MS-5 was used as primer in these experiments. Densitometric profiles of the autoradiograms for α-cell chromatin (top), **a**-cell chromatin (middle), and the naked DNA (bottom) are also shown.

High resolution DNase I mapping of the TALS minichromosome

To analyze the structure of nucleosomes IV and V in more detail, DNase I mapping of TALS chromatin in isolated nuclei was performed. DNase I preferentially attacks sites in the minor groove of DNA which are exposed on the outer surface of a nucleosome (Simpson and Whitlock, 1976; Lutter, 1979). If a nucleosome is stably positioned in a rotational sense, DNase I cuts should occur with a 10 bp periodicity corresponding to the path of a given strand of DNA as it is alternately turned towards and then away from the surface of the nucleosome.

DNase I cutting sites within the region of nucleosome IV in α cell chromatin (lanes 1 and 3, Figure 3) are significantly different from those in naked DNA (lane 2, also see densitometric scan). Both nuclease hypersensitive sites and protected sites are observed. When isolated from **a** cells, cutting sites in this region of the minichromosome were much more similar to those of naked DNA (compare lanes 4 and 6 to lane 5). A ~10 bp spacing is observed between cutting sites in the region of nucleosome IV in α cell chromatin, but not in **a** cell chromatin or naked DNA. A ~10 bp periodicity is even more evident in α cell chromatin in the region of nucleosome V (Figure 4). Again, several sites exposed in naked DNA are protected in nucleosome V in α cells, while the pattern of cutting in **a** cell chromatin, in general, is more similar to that of naked DNA. Interestingly, some periodicity of DNase I cutting is observed in the region of 1664–1727 mu in **a** cell chromatin, indicating that the structure of this region is not entirely random in **a** cells.

The periodicity of DNase I cutting sites in α cells for both nucleosomes IV and V is consistent with a distinct rotational positioning of these nucleosomes in addition to the translational positioning observed above. Considering the high copy number of the TALS minichromosome (50–100 molecules per cell; Roth *et al.*, 1990) and the sensitivity of the primer extension assay, the micrococcal nuclease and DNase I mapping data taken together strongly indicate a very precise and stable positioning of nucleosomes IV and V in α cells, suggesting that the α2 repressor may be directly involved in the establishment or maintenance of this chromatin structure.

These nucleosomes have characteristics in common with, and some that differ from, those defined *in vitro* using reconstituted core particles positioned over specific sequences (Simpson and Stafford, 1983; Hayes *et al.*, 1990). The average helical repeat length, for example, for DNA wrapped around nucleosome IV (as determined by the average distance between DNase I cuts) is 10.2 bp/turn, in close agreement with that defined recently for a nucleosome reconstituted *in vitro*, 10.18 (Hayes *et al.*, 1990). The average repeat length for DNA associated with nucleosome V is 10.4 bp/turn. For both nucleosomes IV and V, cutting by DNase I occurs with 10.0 bp periodicity in the peripheral regions of the core particle. In the central ~30 bp, the cutting frame is shifted by 2 bp, similar to the shift in periodicity observed upon hydroxyl radical mapping of core particles reconstituted *in vitro* (Hayes *et al.*, 1990). Interestingly, cut sites in the interior of both nucleosomes IV and V are less frequently recognized than are cut sites at either end (notice the decreased amplitude of cutting sites in the center of the densitometric scans, Figures 3 and 4), consistent with a decreased accessibility of sequences near

the pseudodyad of a nucleosome core particle. This is in contrast to cutting site susceptibilities determined for isolated core particles (Simpson and Whitlock, 1976; Lutter, 1979; Simpson and Stafford, 1983). In conjunction with recent results that suggest limited accessibility of *cis*-acting DNA elements near the pseudodyad of the nucleosome (Simpson 1990), we suppose that the current data more accurately reflect the availability of nucleosomal DNA regions to proteins in solution *in vivo*.

Chromatin structure of genomic a-cell specific genes

The episomal (circular), amplified nature of the TALS minichromosome could contribute to its chromatin structure by limiting room for nucleosome formation or titration of specific DNA binding factors. To address this concern, we extended our studies to examine the genomic chromatin structure of two unique **a** cell-specific genes, *STE6* and *BAR1*. If the placement of nucleosomes adjacent to the α2 operator in TALS is a consequence of the binding of α2, then the chromatin structure of genes such as these regulated by α2 should be equivalent to that in the minichromosome.

The α2 operator is located approximately 200 bp upstream of the initiation codon of the five **a** cell-specific genes characterized to date, providing ample room for formation of a nucleosome in the promoter regions of these genes. We mapped micrococcal nuclease cut sites around the α2 operator in *STE6* and *BAR1* using the primer extension assay to determine whether nucleosomes were positioned adjacent to the operator in α cells or **a** cells. The nucleotide sequences of the promoter regions of both of these genes have been determined (MacKay *et al.*, 1988; Wilson and Herskowitz, 1988; McGrath and Varshavsky, 1989), facilitating assignment of nucleosome positions relative to the operator and other promoter elements.

Figures 5 and 6 show genomic maps of micrococcal nuclease cut sites in the *STE6* and *BAR1* genes, respectively, in isolated nuclei from both **a** and α cells. A summary of nucleosome positions inferred from these maps is depicted in Figure 7. In the studies shown here, cells which do not contain the TALS plasmid were used, to eliminate possible effects of the presence of multiple copies of the α2 binding site. Identical maps were subsequently obtained with cells containing the plasmid (data not shown).

The chromatin structure downstream of the α2 operator,

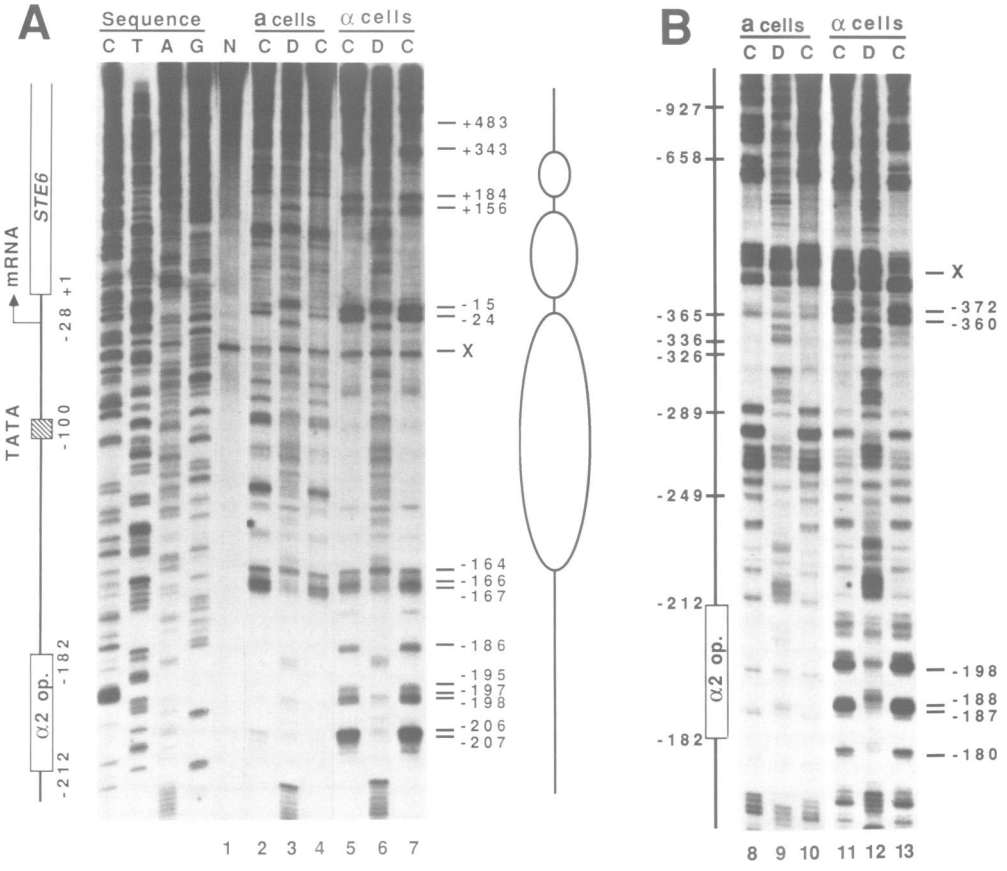

Fig. 5. Chromatin structure of the *STE6* gene. Micrococcal nuclease cleavage sites downstream (**A**) (oligonucleotide MS-7 used as primer) and upstream (**B**) (oligo MS-8 used as primer) of the α2 operator were mapped by primer extension. The α2 operator and the coding region of the *STE6* gene are shown by the open boxes to the left of the gel, and the TATA element is indicated by the shaded box. The arrow indicates the major transcriptional start site (Wilson and Hershowitz, 1986). The numbers to the right indicate the locations of micrococcal nuclease cut sites (relative to the ATG translational start site (Wilson and Herskowitz, 1986) determined by comparison to the sequencing reactions and to the mobility of DNA molecular weight markers (ΦX174 RF *Hae*III digests). Inferred positions of nucleosomes are shown to the right. Chromatin (in isolated nuclei) (C) and naked DNA (D) were digested at 37°C for 10 min with different concentrations of micrococcal nuclease as follows. 40 units/ml (lanes 2, 5, 8 and 11), 20 units/ml (lanes 4, 7, 10 and 13), 0.25 units/ml (lanes 3, 6, 9 and 12). Lane N is a primer extension analysis of undigested naked DNA. The band marked X represents an artefactual primer extension stop, not a micrococcal nuclease cleavage, since this band is present in the absence of digestion.

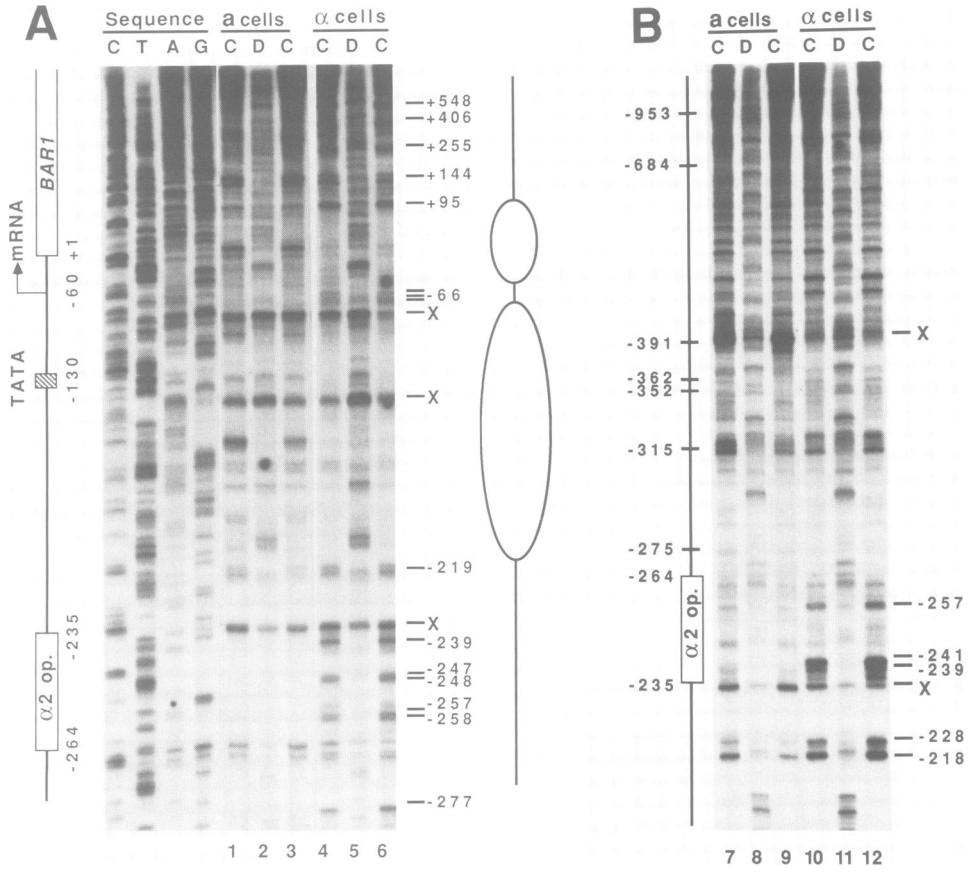

Fig.6. Chromatin structure of the *BAR1* gene. Micrococcal nuclease cleavage sites downstream (**A**) (oligonucleotide MS-9 used as primer) and upstream (**B**) (oligonucleotide MS-10 used as primer) of the α2 operator were mapped by primer extension. The α2 operator and the coding region of the *BAR1* gene are shown by the open boxes and the location of the TATA element is indicated by the shaded box. The arrow indicates the major transcriptional start site (Kronstad *et al.*, 1987). Locations of nuclease cleavage sites were determined by comparison to sequencing reactions and the mobilities of DNA size markers as in Figure 5 and numbers are relative to the position of the ATG translational start site (McGrath and Varshavsky, 1989). Inferred positions of nucleosomes are shown on right. Chromatin (in isolated nuclei) (C) and naked DNA (D) were digested at 37°C for 10 min with different concentrations of micrococcal nuclease as follows: 40 units/ml (lanes 1, 4, 7 and 10), 20 units/ml (lanes 3, 6, 9 and 12), 0.25 units/ml (lanes 2, 5, 8 and 11).

including the promoter and the beginning of the *STE6* coding region, is depicted in Figure 5A. Many micrococcal nuclease cleavage sites are present in these sequences in the control, naked DNA (lanes 3 and 6), and many of these are also exposed in chromatin isolated from **a** cells (lanes 2 and 4). A number of hypersensitive sites are also present in **a** cell chromatin (compare lanes 2, 3 and 4). In α cell chromatin, regions of protection spanning 140−170 bp are observed between hypersensitive sites at positions −164 through −24, −15 through +156, +184 through +343, and +343 through +483 (lanes 5 and 7). These data are consistent with an array of positioned nucleosomes formed adjacent to the α2 operator and extending into the *STE6* coding region (Figure 7). Interestingly, a hypersensitive site present in **a** cell chromatin (at about −100) which corresponds to the location of a TATA element in the *STE6* promoter is almost completely protected in α cell chromatin. Indeed, this sequence is located near the pseudodyad of a positioned nucleosome in α cells, a region predicted to be of low accessibility. The edge of this nucleosome, defined by the nuclease hypersensitive sites at −167, lies 15 bp from the edge of the α2 operator sequence, quite similar to the location of nucleosome IV relative to the operator in TALS chromatin.

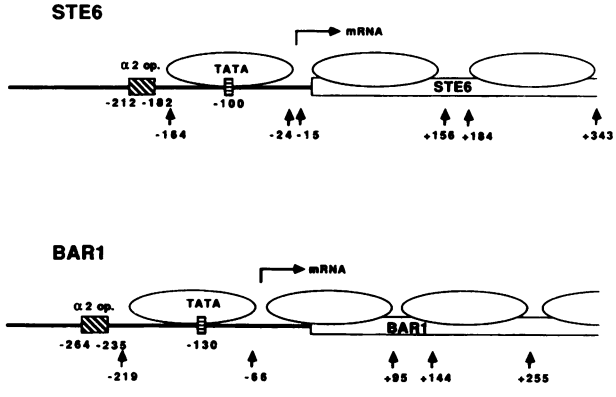

Fig. 7. Summary of *STE6* and *BAR1* chromatin structure. Inferred positions of nucleosomes adjacent to the α2 operator in α cells are shown. Locations of major micrococcal nuclease cut sites (arrowheads) which define these positions, as determined from experiments such as shown in Figures 5 and 6, are indicated, as are the locations of TATA elements, the α2 operator, and transcription start sites (bent arrows).

Upstream of the α2 operator in *STE6* (Figure 5B), the pattern of micrococcal nuclease cleavage is similar in α cell and **a** cell chromatin, although the intensities of some sites vary in the two cell types. Hypersensitive sites are located

at −360 and −372 in α cell chromatin as well as within the operator sequence, between −180 and −198 (lanes 11 and 13, Figure 5B). The importance of these sites is not understood at present, nor is the relative protection of these sites in **a** cells. The absence of an extended region of protection immediately upstream of the operator in either cell type, however, indicates that a nucleosome is not stably positioned over these sequences. In contrast to TALS chromatin, then, nucleosomes are positioned only downstream of the α2 operator in *STE6*. Nucleosomes may be positioned over sequences further upstream of *STE6* in both **a** and α cells, as evidenced by a periodic clustering of hypersensitive sites in chromatin relative to naked DNA.

An analogous chromatin structure was observed for the *BAR1* gene and its 5′ flanking sequences (Figure 6), although maps of this region were somewhat less clear than for *STE6*. Downstream of the α2 operator, regions protected from cleavage were observed between hypersensitive sites at −219 and −66, and between −66 and +95 in α cell chromatin (Figure 6A, lanes 4 and 6; Figure 7). These regions of protection and flanking hypersensitive sites are diminished or absent in **a**-cell chromatin (lanes 1 and 3). The triplet at −66, for example, appears to be completely absent in **a** cell chromatin, and sites between −219 and around −170 are more prevalent and more similar to naked DNA than in α cell chromatin. Interestingly, exposure of a minor cut site at −170 appears to be equal in chromatin from both cell types and in naked DNA. Perhaps the rotational setting of this site is such that it is recognized by the nuclease even within the core particle. As observed for *STE6*, cleavage sites upstream of the operator in *BAR1* were similar, but not identical, in both cell types and no nucleosome-sized region of protection was observed immediately upstream of the operator (Figure 6B). The overall consistency in the patterns of nuclease protection and hypersensitive sites surrounding the operator in *STE6* and *BAR1* indicate that these two genes share a similar chromatin structure. Our data are consistent with a positioned nucleosome located 16 bp from the edge of the α2 operator in *BAR1* between −219 and −66, incorporating the *BAR1* promoter region. The location of nucleosomes relative to the operator in *STE6* (15 bp downstream), *BAR1* (16 bp downstream), and nucleosome IV (13 bp downstream) in the TALS minichromosome (which corresponds to the promoter region of these genes) is quite consistent, suggesting that the precise location of these nucleosomes may be directed by α2.

Discussion

Previous studies indicated that nucleosomes are precisely and stably positioned over sequences adjacent to the α2 operator in minichromosomes in α cells (Roth *et al.*, 1990). In this paper, we extend these studies to base pair level resolution and demonstrate that nucleosomes are also positioned adjacent to the operator in the promoter regions of two chromosomal **a** cell-specific genes, *STE6* and *BAR1*. At this level of resolution, we can now define both translational and rotational positioning. Rotational positioning refers to the orientation of DNA on the surface of a histone octamer. Uniformly bent DNA might maintain a constant rotational orientation while having several translational locations. DNase I will detect this positioning, but micrococcal nuclease will not. In contrast, a nucleosome which is statistically positioned ±5 bp translationally will be totally unpositioned rotationally. This nucleosome will be scored by micrococcal nuclease but not by DNase I. Only when a nucleosome is precisely positioned both rotationally and translationally will both enzymes detect its location. Of course, all nuclease mapping data is limited by the sequence specificity of the nuclease. Nonetheless, differences in patterns between chromatin and naked DNA often allow assignment of nucleosome location. Our study indicates that nucleosomes abutting the α2 operator in α cells are both rotationally and translationally positioned. Positioning with this degree of precision for nucleosomes assembled *in vivo* has not, to our knowledge, been detected previously.

We now have at least five examples, three minichromosomes (Roth *et al.*, 1990; Simpson, 1990) and two single copy genomic sequences, wherein a specific positioning of nucleosomes over sequences adjacent to the α2 operator occurs in the presence of α2. The DNA sequences incorporated into the nucleosomes are different in these five situations, but high level resolution of the positions of three of these nucleosomes (this paper) indicate that they are placed within 13−16 bp of the edge of the operator sequence. The precise location of these nucleosomes exceeds that expected for a statistically positioned nucleosome (Kornberg and Stryer, 1988), suggesting that the α2 repressor may actively organize chromatin structure, perhaps through a direct interaction with a component of the core particle. In other studies, we have investigated the placement of nucleosomes around the α2 operator in cells containing mutations in the amino-terminal regions of specific histones (S.Roth, M.Shimizu, L.Johnson, M.Grunstein, and R.Simpson, submitted for publication). These studies have indicated that the amino-terminal region of histone H4 is required both for the positioning of nucleosomes adjacent to the operator in α cells and for the proper regulation of **a** cell-specific gene expression by α2.

Several mechanisms for the repression of **a** cell type-specific gene expression by α2 have been postulated (Herskowitz, 1989; Levene and Manley, 1989; Renkawitz, 1990, for review). Since α2 and MCM1 co-occupy the operator sequence in α cells, α2 might function by blocking the activation domains of MCM1 (Keleher *et al.*, 1988; Jarvis *et al.*, 1989; Passmore *et al.*, 1990). Alternatively, the α2/MCM1 complex might somehow interact with the transcription machinery in a way which blocks transcription initiation (Keleher *et al.*, 1988). However, mutations in MCM1 which affect activation of α cell-specific gene expression have only minimal effects on **a** cell-specific gene expression (Passmore *et al.*, 1988, 1989), indicating that MCM1 may play a limited role in the activation of **a** cell-specific genes. Also, α2 is one of the most short-lived eukaryotic proteins known, with a half life *in vivo* of only 5 min (Hochstrasser and Varshavsky, 1990). A long term interaction between α2 and a general transcription complex may not be consistent with this unstable property. We propose that positioning of nucleosomes by α2 in promoter regions may be involved in repression of **a** cell-specific gene expression. Our observation that TATA elements represent nuclease hypersensitive sites in **a** cell chromatin but are protected from digestion in α cells by the presence of a nucleosome is consistent with the sequestration of these sequences within the nucleosome. This limited accessibility of promoter elements to nucleases may reflect a limited

accessibility to various transcription factors as well. Once established, the organization of nucleosomes in these regions appears to be quite stable, capable of surviving nuclear isolation. Thus, although α2 appears to be required for the establishment of these repressive structures, it may not be required for their maintenance.

Although α2-dependent nucleosome positioning occurs in both the TALS minichromosome and the genome, differences in these chromatin structures are quite interesting. In the TALS minichromosome, two nucleosomes (IV and V) are positioned on either side of the α2 operator, whereas positioned nucleosomes are observed only downstream of the operator in *STE6* and *BAR1*. The orientation of the operator in TALS is such that nucleosome IV would be analogous to these nucleosomes (i.e. downstream of the operator), consistent with the conservation of the distance between the operator and these nucleosomes as described above. Several explanations are possible as to why nucleosomes are positioned on either side of the operator in the minichromosome but not in the genome. Firstly, nucleosome V in TALS may not be specifically positioned by α2, but might be fortuitously positioned by α2 binding to its operator and a length of DNA between the operator and the nuclease hypersensitive region of the ARS just sufficient to accomodate six nucleosomes. This would explain the greater distance between the operator and nucleosome V in α cells and may also explain the residual structure of this region in **a** cells. In addition, other *trans*-acting factors may bind to sequences upstream of the α2 operator in the *STE6* and *BAR1* loci, preventing nucleosome formation in these regions. The presence of specific DNA sequences (such as polypurine–polypyrimidine; Wilson and Herskowitz, 1986; MacKay *et al.*, 1988) in this region might also affect formation of a nucleosome. Differences in higher-order chromatin domain structure or in the direction of DNA replication might also explain the different organization of nucleosomes around the operator in the chromosomal genes and the minichromosome. These possibilities are the focus of present and future studies.

Differences in the susceptibility of the α2 operator to micrococcal nuclease were observed in the *STE6* and *BAR1* genes between α cells and **a** cells. Interestingly, the operator was equally susceptible in both cell types in the mini-chromosome. The basis for this difference is not understood, but again may reflect differences in surrounding sequences and/or association of other *trans*-acting factors in the genome versus the minichromosome. We are currently performing a more detailed analysis of protein–DNA interactions at the operator in intact cells and in isolated nuclei (S.Roth and M.Shimizu, unpublished observations). Understanding how *trans*-acting factors interact with *cis*-acting elements in the context of chromatin, and in the case of α2, how such a factor might manipulate chromatin structure, should advance our knowledge of the various processes involved in the regulation of gene expression.

Materials and methods

Yeast strains, plasmids and media

Strains Sc3 (MATα ura 3-52 trpl his3 gal2 gal10 cir^0) and E290 (MATa his4290 trp1) were obtained from L.W.Bergman and from Cold Spring Harbor Yeast Genetics Course collection, respectively.

Plasmid TALS is a TRP1/ARS1 derivative, which contains the *STE6* α2 operator as a 354 bp fragment inserted at the *Eco*RI site (Roth *et al.*, 1990). Stains without plasmids were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose). Strains containing plasmids were grown in 2% glucose, 0.67% nitrogen base without amino acids supplemented with 0.02% appropriate amino acids (except tryptophan) and uracil.

Isolation of yeast nuclei and nuclease digestions

Yeast nuclei were isolated as described previously (Szent-Gyorgyi and Isenberg, 1983; Szent-Gyorgyi *et al.*, 1987), except that oxalyticase (Enzogenetics) was used for spheroplast formation instead of lyticase.

Micrococcal nuclease (MNase) and DNase I digestions were performed as described by Szent-Gyorgyi *et al.* (1987). The nuclear pellet from 1-L culture (OD_{600} = 1.0) was gently suspended in 4.0 ml of the digestion buffer [10 mM HEPES (pH 7.5), 0.5 mM $MgCl_2$, 0.05 mM $CaCl_2$, 1 mM phenylmethanesulfonyl fluoride]. Suspended nuclei were divided into 200 μl portions and digested at 37°C for 10 min using successive 2-fold serial dilutions of MNase (40 to 1.25 units/ml) or DNase I (0.5 to 0.016 units/ml). Digestions were terminated by the addition of 0.1 vol of 0.1 M EDTA and chilled on ice. DNA was purified as described by Lohr *et al.* (1977). DNA purified from nuclei was above was digested with 10 to 60-fold lower concentrations of nucleases to provide naked DNA controls. The degree of digestion was confirmed by 1% agarose gel electrophoresis (Sambrook *et al.*, 1990).

Primer extension assay

MNase and DNase I cleavage sites were located by primer extension assay using *Taq* polymerase as described by Axelrod and Majors (1989) with minor modifications. The digested and purified DNA was dissolved in 100 μl of TE buffer (10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA), and passed through a 1 ml Sephadex G-50 (Pharmacia) spin column equilibrated with water. Oligonucleotide 35mers used as primers included:

MS-1: (1370–1404 mu in TALS) 5′-TCC GTT TAA CCG GAC CCT AGT GCA CTT ACC CCA CG-3′
MS-2: (1735–1701 mu in TALS) 5′-TTA GGC ACC CCA GGC TTT ACA CTT TAT GCT TCC GG-3′
MS-4: (1638–1604 mu in TALS) 5′-GAT TAC GCC AAG CTA GCT TGC ATG CCT GCA GGT CG-3′
MS-5: (1457–1492 mu in TALS) 5′-TCG AGC TCG GTC GAA AAT GAT GAA CGG CAA TAA TGC-3′
MS-7: (−338 to −304 bp in *STE6*) 5′-TGA TGG TCC TTG CAC TGA CAA ATA TGT TCC TTT CC-3′
MS-8: (−55 to −89 bp in *STE6*) 5′-TCT GTG TGA ACG TAA CAA CGG GAG ATA GTT CAG CC-3′
MS-9: (−391 to −357 bp in *BAR1*) 5′-TAT AGA TAA CGG CTC TTG CCG AAT TCA TAG GCT GC-3′
MS-10: (−81 to −116 in *BAR1*) 5′-TCA TTA AAG AAT TCA TCA TGA CAA GGC TCG ACG TGC-3′

Oligonucleotides used in individual experiments are indicated in the figure legends. The numbers given for *STE6* and *BAR1* sequences used as primers are relative to the ATG translation start site as defined by Wilson and Herskowitz (1986) and McGrath and Varshavsky (1989) for *STE6* and by MacKay *et al.*, (1988) for *BAR1*. For mapping of TALS multicopy-plasmid chromatin, one-cycle primer extension was performed as follows: 10 μl of the DNA (~10–50 ng of plasmid) was combined with 0.3 pmol of the ^{32}P-end labelled primer, 5 μl 5× *Taq* buffer (50 mM Tris–HCl (pH 8.3), 250 mM KCl, 15 mM $MgCl_2$, 0.25% NP-40, 0.25% Tween 20) (Innis *et al.*, 1988), and H_2O (total 24 μl). The sample was heated at 95°C for 5 min, and incubated at 48°C for 20 min. Then, 1.2 μl of a mixture (5 mM each) of dATP, dGTP, dCTP, and dTTP was added, followed by the addition of 1 unit of *Taq* polymerase in 5 μl of 1 × *Taq* buffer (10 mM Tris–HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.05% NP-40, 0.05% Tween 20) (Innis *et al.* 1988) and the incubation was continued at 70°C for 5 min. The reaction was terminated by chilling on ice and followed by ethanol precipitation. For mapping the chromatin structure of single copy genes (*STE6* and *BAR1*), linear primer extension was performed as follows: 10 μl of DNA (10–50 μg total DNA) was combined with 0.3 pmol of the ^{32}P-end labelled primer, 5× *Taq* buffer, and 1 unit of *Taq* polymerase (Cetus) (Total 25 μl) on ice and then overlayed with 25 μl of mineral oil. The DNA/primer mix was denatured at 94°C for 1 min, annealed at 55°C for 2 min, and then extended at 72°C for 2 min. This cycle was repeated 15 times. Dideoxy sequencing reactions using *Taq* polymerase were performed as described with undigested genomic DNA (Axelrod and Majors, 1989). After extension, the mineral oil was extracted by addition of an equal volume of $CHCl_3$ and the DNA was precipitated in 1/10 volume of 3.0 M sodium acetate and three vols of ethanol. DNA samples were electrophoresed on a 6% polyacrylamide sequencing gel containing 50% urea using standard techniques (Sambrook *et al.*, 1990).

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