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Transcription in the Yeast rRNA Gene Locus: Distribution of the Active Gene Copies and Chromatin Structure of Their Flanking Regulatory Sequences

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In growing yeast cells, about half of the 150 tandemly repeated rRNA genes are transcriptionally active and devoid of nucleosomes. By using the intercalating drug psoralen as a tool to mark accessible sites along chromatin DNA in vivo, we found that the active rRNA gene copies are rather randomly distributed along the ribosomal rRNA gene locus. Moreover, results from the analysis of a single, tagged transcription unit in the tandem array are not consistent with the presence of a specific subset of active genes that is stably maintained throughout cell divisions. In the rRNA intergenic spacers of yeast cells, an enhancer is located at the 3′ end of each transcription unit, 2 kb upstream of the next promoter. Analysis of the chromatin structure along the tandem array revealed a structural link between transcription units and adjacent, 3′ flanking enhancer sequences: each transcriptionally active gene is flanked by a nonnucleosomal enhancer, whereas inactive, nucleosome-packed gene copies are followed by enhancers regularly packaged in nucleosomes. From the fact that nucleosome-free enhancers were also detected in an RNA polymerase I mutant strain, we interpret these open chromatin structures as being the result of specific protein-DNA interactions that can occur before the onset of transcription. In contrast, in this mutant strain, all of the rRNA coding sequences are packaged in nucleosomal arrays. This finding indicates that the establishment of the open chromatin conformation on the activated gene copies requires elongating RNA polymerase I molecules advancing through the template.

In most eukaryotic organisms, the rRNA genes occur in the form of tandem repeats that are clustered at one or several chromosomal sites (31). Whereas the rRNA coding sequences are strongly conserved among disparate species, both size and DNA sequence of the intergenic spacers differ greatly. Despite these differences, recent studies revealed a consensus in both structure and function of the intergenic spacer elements that regulate expression of these genes (for reviews, see references 43 and 44). Enhancer sequences that strongly stimulate transcription from the rRNA gene promoter were originally discovered in frogs (*Xenopus laevis*) (42); later, their counterparts in insects (*Drosophila melanogaster*) (14) and mammals (mice) (22, 41) were identified. The recent identification of similar elements in the plant *Arabidopsis thaliana* further confirms their widespread occurrence (10). Common features of these RNA polymerase I enhancers are their location just upstream of the rRNA gene promoter, their repetitive character, and the fact that they can be transcribed from one or several spacer promoters (43, 44).

Spacer sequences exhibiting all the attributes of a transcriptional enhancer have also been identified in the rRNA genes (rDNA) of the yeast *Saccharomyces cerevisiae* (11, 12, 18). However, this element does not seem to possess any of the common structural features of the generic RNA polymerase I enhancers described above. In fact, besides the absence of spacer promoters (44), sequences that can augment transcription of the yeast rRNA genes are not repetitive in structure and are confined to an approximately 300-bp-long spacer region located close to the 3′ end of the transcription unit (12, 18, 23, 39, 45). Furthermore, unlike in other eukaryotes, additional functional elements are colocalized in this short transcriptional regulatory region. This spacer region contains a transcription terminator (29, 53) and a polar replication fork barrier (RFB) that prevents the replication machinery from entering the rRNA transcription unit in the opposite direction of transcription (2, 3, 21, 30).

The proximity of enhancer and termination activity in the rDNA spacer of yeast cells has inspired models proposing a functional link between these two processes (18, 20, 23). In these models, enhancer and promoter elements of different rDNA repeats are physically associated, forming a complex from which coding and spacer regions are looping out. In this scenario, the enhancer would facilitate the direct loading of terminating polymerases to an adjacent promoter, allowing RNA polymerase I molecules to pass from repeat to repeat without being released to the free pool. However, results arguing against this type of recycling of RNA polymerases have also been presented (4).

Earlier work from our laboratory demonstrated that in a growing yeast cell, only a fraction of the approximately 150 tandemly repeated rRNA genes are transcriptionally active and devoid of nucleosomes (8). The remaining gene copies are not transcribed and regularly packaged in nucleosomal arrays as inactive bulk chromatin. In the present study, we wished to analyze how yeast cells select the fraction of genes to be activated. One could imagine that the existence of regulatory mechanisms taking advantage of the tandem, head-to-tail arrangement of these repeated genes will be reflected by most of the transcriptionally active gene copies being organized in one or a few large clusters. In contrast, here we demonstrate that transcriptionally active genes are rather randomly distributed along the yeast rDNA locus.

In an attempt to gain more insight into the mode of action of the yeast rDNA enhancer, the chromatin structure of enhancer regions flanking transcriptionally active and inactive

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gene copies was analyzed. It is shown that each transcriptionally active rRNA gene has a nonnucleosomal enhancer at its 3′ end, whereas inactive gene copies are followed by nucleosome-packed enhancers. By analyzing an RNA polymerase I mutant strain, we show that the disruption of chromatin structure over these regulatory sequences can occur before the onset of transcription. The significance of these results with respect to enhancer, transcription termination, and replication termination functions is discussed.

MATERIALS AND METHODS

Strains, media, and plasmids. *S. cerevisiae* SC3 (*MAT***a** *ura3-52 his3-1 trp1 gal2 gal10*) was used for most analyses described in this work (47). The cells were grown at 30°C in complex medium (YPD) or in minimal medium (SD) to a density of about 10^7 or 3×10^6 cells per ml, respectively, supplemented with the appropriate amino acids as described previously (46). *S. cerevisiae* W303-1a (*MAT***a** *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100*) and its derivative NOY408-1a (*MAT***a** *rpa135*::*LEU2 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100* [pNOY102] [40]) were grown in a complex medium with 2% galactose instead of dextrose to a density of about 10^7 cells per ml. To construct plasmid pRD2T, rDNA sequences comprising the 3′ half of the yeast rRNA transcription unit (from the *Kpn*I site to the *Hpa*I site) were cloned into the polylinker of the shuttle vector pRS304 (48). A sequence tag comprising a 200-bp fragment from the multiple cloning site and the *lacZ′* region of plasmid YEp352 (16) was then inserted at the *Nar*I site in the 25S rRNA coding region. To obtain transformants with a single, tagged rRNA transcription unit, plasmid pRD2T was linearized with *Bgl*II and integrated into the rDNA locus of SC3 cells (17). Transformants with a single integration were selected by comparing the *TRP1* marker on the plasmid with the single-copy chromosomal *TRP1* gene.

Psoralen cross-linking. Yeast cells were irradiated in the presence of 4,5′,8-trimethylpsoralen with a 366-nm UV lamp (model B-100 A; Ultra Violet Products, Inc., San Gabriel, Calif.) at a distance of 6 cm as previously described (8). Psoralen stock solution in ethanol (0.05 volume of 200 μg/ml) was added five times at intervals of 5 min for a total irradiation time of 25 min.

DNA isolation, gel electrophoresis, transfer, and hybridization. DNA isolation and purification of rDNA on CsCl gradients was done exactly as previously described (8). Electrophoretic separation of the 9.1-kb repeats was performed in 1% agarose gels at 2 V/cm for 48 h. DNA was recovered from low-gelling-temperature agarose gels (SeaPlaque; FMC BioProducts) by using the GELase protocol (Epicentre Technologies). Small DNA fragments were separated on 1.8 or 2% MetaPhor agarose gels (FMC BioProducts) at 4 V/cm for 7 h according to the manufacturer's recommendations. Alkaline Southern blotting and hybridizations were performed as previously described (34). The probes used are shown in the rDNA maps in the relevant figures. Probes A and D and probes B and E represent rDNA fragments derived from subclones of plasmids pSZ26 and pSZ1, respectively (51). Probe C detecting the tagged rRNA transcription unit derives from plasmid YEp352 (16). DNA bands on Southern blots were quantitated by using a Molecular Dynamics PhosphorImager or a Shimadzu TLC Scanner.

Electron microscopy. Purified rDNA was prepared for electron microscopy exactly as described previously (49). DNA contour length measurements were made with a Hewlett-Packard digitizer on photographic prints. To calibrate the length, DNA from psoralen-cross-linked simian virus 40 minichromosomes was coprepared (50).

RESULTS

Transcriptionally active rRNA gene copies are randomly distributed along the rDNA locus. In earlier work from our laboratory, a method which allows the accurate determination of the fraction of transcriptionally active rRNA gene copies present in a population of eukaryotic cells was developed (7). It has been possible to distinguish the active from the inactive gene copies by their different chromatin structures and accessibility in vivo to the intercalating drug psoralen, which, upon UV light irradiation, can introduce cross-links into DNA sites that are not protected by nucleosomes (15, 49). The presence of these two distinct populations of rRNA genes can be visualized by separating restriction fragments of DNA isolated from photoreacted cells on native agarose gels by taking advantage of the fact that the more a fragment is cross-linked, the more slowly it migrates. In this psoralen-gel retardation assay, each rRNA coding fragment is separated into two distinct bands: a slowly migrating band representing heavily cross-linked DNA derived from transcriptionally active, nonnucleosomal genes (s band) and a fast-migrating band containing slightly cross-linked DNA originating from the inactive, nucleosome-packed gene copies (f band). The relative intensities of these two bands reflect the ratio of active to inactive gene copies of the cell population analyzed (7, 8, 34).

In this study, we wished to use the psoralen-gel retardation assay to examine the arrangement of the transcriptionally active rRNA gene copies along the single rDNA locus in the yeast *S. cerevisiae*. We reasoned that the analysis of the cross-linking pattern of restriction fragments containing portions of two adjacent rRNA genes should allow the determination of the frequency of neighboring active and inactive genes. This frequency can be used to determine the average size of the clusters containing the active gene copies. The outline of this experiment is presented in Fig. 1. DNA purified from psoralen-cross-linked cells is digested with *Bgl*I, which cuts only once per rDNA repeating unit (see also Fig. 2a). The released 9.1-kb rDNA repeats comprise about 80% of rRNA coding sequences of one gene, the intergenic spacer, and about 20% of coding sequences of the adjacent, downstream gene. According to the four possible combinations of nearest neighbors present in the linear succession of active and inactive genes, these repeats fall into four classes characterized by distinct cross-linking patterns (type 1 to 4 molecules in Fig. 1). As a considerable portion of the *Bgl*I repeat represents coding sequences of the upstream gene, molecules in which the upstream gene was active (i.e., types 1 and 2) migrate more slowly in a gel retardation assay than molecules in which the upstream gene is inactive (i.e., types 3 and 4). In contrast, variations in the transcriptional activity of the downstream gene are expected to be reflected in only minor changes in the total extent of cross-linking of the molecules, as the portion of coding sequences of the downstream gene represents only about 15% of the total length of the repeat. Therefore, fractionation of these 9.1-kb rDNA repeats in a first psoralen-gel retardation assay will separate them into two classes (s and f bands) according to the extent of cross-linking (i.e., transcriptional activity) of the upstream gene. The relative intensities of these two bands will represent the proportion of active and inactive gene copies of the cell population under study. Separate elution of the s and f bands, redigestion with *Sma*I, and subsequent gel electrophoretic analysis of the small excised fragment comprising rRNA coding sequences of the downstream gene will allow quantitation of the fraction of each type of nearest neighbor. The average number of active genes per cluster is given by the ratio of the percentage of active genes to the percentage of neighboring 5′ active-3′ inactive (or 5′ inactive-3′ active) gene copies. As shown in Fig. 1, in a situation in which half of the 100 tandemly repeated rRNA genes are transcriptionally active, the average cluster size can vary from 2 to 50 copies, depending on whether the active genes are either randomly distributed or grouped together in a single cluster.

The results of this analysis are presented in Fig. 2. As expected, the 9.1-kb *Bgl*I repeats originating from psoralen-treated cells are separated into two distinct bands (s and f bands in Fig. 2b, lane 2). To confirm that these rDNA repeats had been indeed separated according to the extent of cross-linking of the upstream gene, s and f band DNA eluted separately from the gel was redigested with *Eco*RI. The 2.85-kb *Eco*RI fragment excised from the coding portion of the upstream gene (Fig. 2a) was then analyzed in a second psoralen-gel retardation assay. As shown in Fig. 2c, lanes 3 and 4, analysis of the 2.85-kb fragments originating from s and f band DNA shows only one slowly migrating and one fast-migrating band, respectively, confirming our anticipation. A different result is obtained from the analysis of the 1.5-kb *Sma*I-*Bgl*I frag-

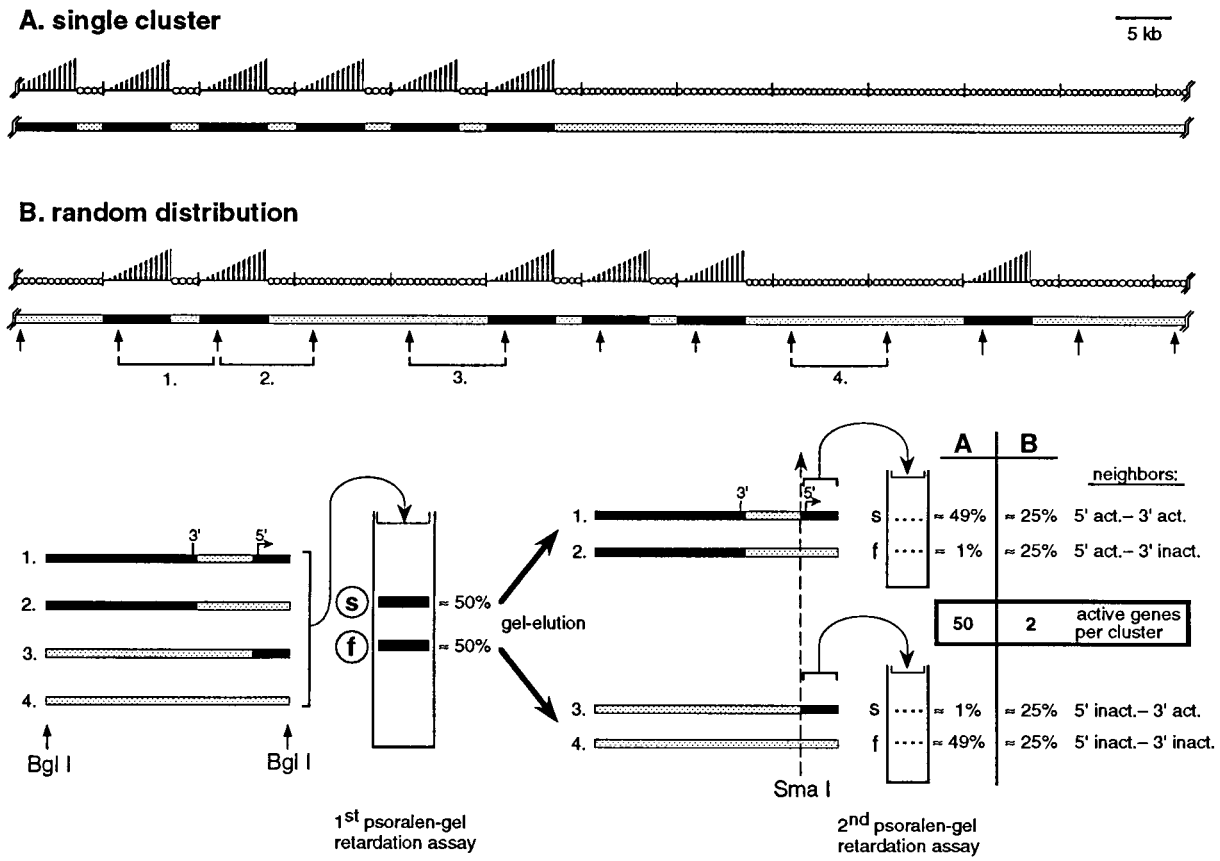

FIG. 1. Outline of the experiment used for analysis of the arrangement of the transcriptionally active rRNA gene copies. Two extreme situations in which the active genes are either grouped together in a single cluster (A) or randomly distributed (B) are represented. The schematic drawings at the top show the structural organization in vivo. The nonnucleosomal, transcriptionally active copies are represented by the characteristic gradient of nascent transcripts (vertical lines increasing in length from left to right). The intergenic spacers and the silent gene copies are organized in nucleosomes (rows of open circles). The horizontal lines represent rDNA isolated from cells photoreacted in the presence of psoralen; patches of highly cross-linked DNA sequences originating from the active gene copies are shown in black, whereas slightly cross-linked regions originating from nucleosomal chromatin are shadowed. The small vertical arrows indicate the unique *Bgl*I cutting sites in each rDNA repeat. *Bgl*I digestion of the cross-linked rDNA releases four different types of repeats characterized by distinct cross-linking patterns. These molecules correspond to the four possible combinations of nearest neighbors present in the linear succession of active (act.) and inactive (inact.) gene copies. The relative frequency of each type of molecule can be determined by two subsequent psoralen-gel retardation assays (see text for explanation). The frequencies shown in columns A and B are those expected for single-cluster and random distribution, respectively, in a situation in which 50% of the 100 tandemly repeated rRNA genes are transcriptionally active. The percentage of active genes divided by the percentage of neighboring 5′ active-3′ inactive (or 5′ inactive-3′ active) gene copies gives the average number of active genes per cluster.

ment containing rRNA coding sequences of the downstream, nearest-neighbor gene (Fig. 2a). This fragment was always separated into two distinct bands, independently of whether it was excised from s or f band DNA of fractionated *Bgl*I repeats (Fig. 2d, lanes 3 and 4). This result points to the presence of a fraction of nearest-neighbor genes showing different transcriptional states. Similar results were obtained by the separate analysis of s and f band DNA of fractionated *Nar*I repeats (Fig. 2a). In this case, the 9.1-kb rDNA repeats are first separated according to the extent of cross-linking of the downstream gene (Fig. 2c, lanes 5 and 6). Analysis of the adjacent upstream gene is then performed by separating the 1.3-kb *Nar*I-*Hpa*I fragment in the second gel retardation assay (Fig. 2e, lanes 3 and 4). Quantitative data obtained from nine individual experiments with cells growing either in complex medium or in minimal medium are summarized in Table 1. In both cases, the calculated average number of active genes per cluster was very similar to the value expected for a random distribution. This result is consistent with most of the transcriptionally active rRNA gene copies being rather randomly distributed along the entire rDNA locus.

The enhancer at the 3′ end of each transcriptionally active rRNA gene copy is free of nucleosomes. Intergenic spacer sequences which have been reported to show enhancement activity have been localized just downstream of the 35S rRNA transcription unit (11, 12, 45). Although there is no consensus yet about the exact boundaries of this enhancer, all reported sequences are contained within the approximately 300-bp-long *Eco*RI-*Hpa*I fragment spanning intergenic spacer sequences from 96 to 413 bp downstream of the 3′ end of the 25S rRNA coding region (Fig. 3a) (for a review, see reference 45). To study the chromatin structure of the enhancer, we analyzed the 203-bp *Hpa*II fragment located in this region by using the psoralen-gel retardation assay (Fig. 3a). As shown in Fig. 3b, lane 2, this enhancer fragment is resolved in the two characteristic s and f bands showing proportional intensities similar to those obtained from restriction fragments containing rRNA coding sequences (for comparison, see Fig. 2c, lane 2). This result indicates that there are as many nucleosome-free enhancers as transcriptionally active, nonnucleosomal genes.

To study the distribution of the open enhancers with respect to transcriptionally active and inactive gene copies, we frac-

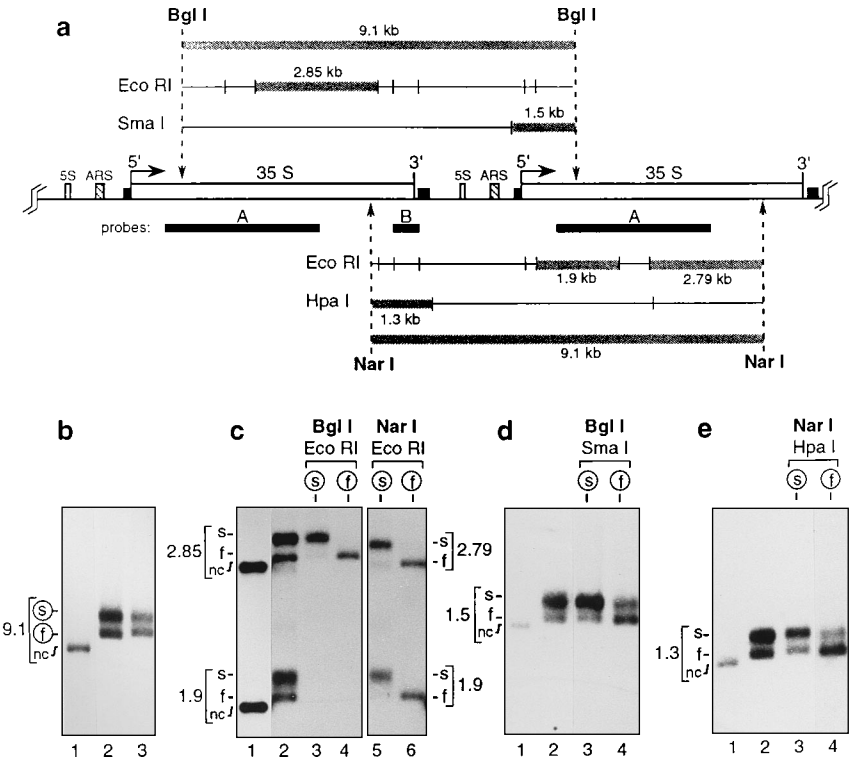

FIG. 2. Psoralen-gel retardation analysis of the arrangement of the transcriptionally active rRNA gene copies. (a) Map of two adjacent rDNA repeats. The 35S rRNA transcription units are represented by large open boxes. The transcription initiation site (5′) and the 3′ end of the 25S rRNA are indicated. The small filled boxes near the 5′ and 3′ ends correspond to the promoter and the enhancer element, respectively. The 5S gene and the autonomous replicating sequences (ARS) located in the intergenic spacer are also shown. The positions of the hybridization probes used are indicated below the map (filled boxes labelled A and B). The dashed vertical arrows point to *Bgl*I and *Nar*I cutting sites. Restriction maps for *Eco*RI, *Sma*I, and *Hpa*I are also indicated. The shaded boxes show the locations and lengths of the restriction fragments analyzed. (b) Yeast cells growing exponentially in complex medium were photoreacted with psoralen. The purified DNA was digested with *Bgl*I (lane 2) or *Nar*I (lane 3) and separated on a 1% agarose gel alongside non-cross-linked control DNA (lane 1). The 9.1-kb rDNA repeats were visualized after blotting and hybridization with probe A. The positions of the s and the f bands and of the non-cross-linked control DNA (nc) are indicated. (c) The 9.1-kb *Bgl*I repeats derived from psoralen-cross-linked cells were first separated on a 1% agarose gel as shown in panel b, lane 2. DNA migrating in the s and f bands was eluted separately from the gel, digested with *Eco*RI, and reelectrophoresed on a 1.2% gel (lanes 3 and 4, respectively). The same procedure was applied to fractionated s and f band DNA of *Nar*I repeats (lanes 5 and 6, respectively). Lane 1, *Eco*RI-digested, non-cross-linked control DNA; lane 2, *Eco*RI-digested DNA purified from psoralen-cross-linked cells. The gel was blotted and hybridized with probe A. (d) s and f band DNA from fractionated *Bgl*I repeats was digested with *Sma*I, reelectrophoresed on a 1.5% agarose gel, blotted, and hybridized with probe A (lanes 3 and 4, respectively). Lanes 1 and 2 represent *Bgl*I-*Sma*I-digested DNA isolated from untreated and cross-linked cells, respectively. (e) s and f band DNA from fractionated *Nar*I repeats was digested with *Hpa*I and reelectrophoresed as in panel d (lanes 3 and 4, respectively). Lanes 1 and 2, control DNA and DNA from psoralen-treated cells, respectively, both digested with *Nar*I and *Hpa*I. The gel was blotted and hybridized with probe B.

tionated *Bgl*I repeats into s and f bands in a first psoralen-gel retardation assay as in Fig. 2b, lane 2. Subsequent gel electrophoretic analysis of the *Hpa*II enhancer fragment excised from the eluted s and f band DNA enabled us to examine independently the chromatin structure of enhancers located downstream of active and inactive gene copies, respectively. From the fact that *Hpa*II fragments originating from s and f band DNA of *Bgl*I repeats showed only one slowly migrating and one fast-migrating band, respectively (Fig. 3b, lanes 3 and 4), we conclude that each transcriptionally active gene is followed by a nucleosome-free enhancer, whereas the inactive gene copies are followed by nucleosome-packed enhancers. The result described here was confirmed by the analysis of the cross-linking pattern of the 534-bp *Hph*I-*Hin*dIII fragment containing the same amount of coding and enhancer sequences (Fig. 3a). We reasoned that if an active gene is followed by a nucleosomal enhancer (or if an inactive, nucleosome-packed gene is followed by an open enhancer), this overlapping fragment would be half heavily and half slightly cross-linked and would show an intermediate electrophoretic mobility (i.e., be-

TABLE 1. Quantitative analysis of the arrangement of the transcriptionally active gene copies[a]

Growth medium	Active gene copies (%)	Neighbors (%)				Avg no. of active gene copies/cluster[b]
		5′ active-3′ active	5′ active-3′ inactive	5′ inactive-3′ active	5′ inactive-3′ inactive	
Complex	52 ± 5	33 ± 4 (27)[c]	19 ± 1 (25)	20 ± 2 (25)	28 ± 3 (23)	2.6 ± 0.3 (2.1)
Minimal	40 ± 7	20 ± 6 (16)	19 ± 1 (24)	20 ± 3 (24)	40 ± 7 (36)	2.0 ± 0.4 (1.8)

[a] Data (means ± standard deviations) are from five and four experiments performed with cells growing in complex and minimal media, respectively.
[b] Calculated by dividing the percentage of active genes by the average between 5′ active-3′ inactive and 5′ inactive-3′ active neighbors.
[c] Numbers in parentheses represent theoretical values expected for random distribution.

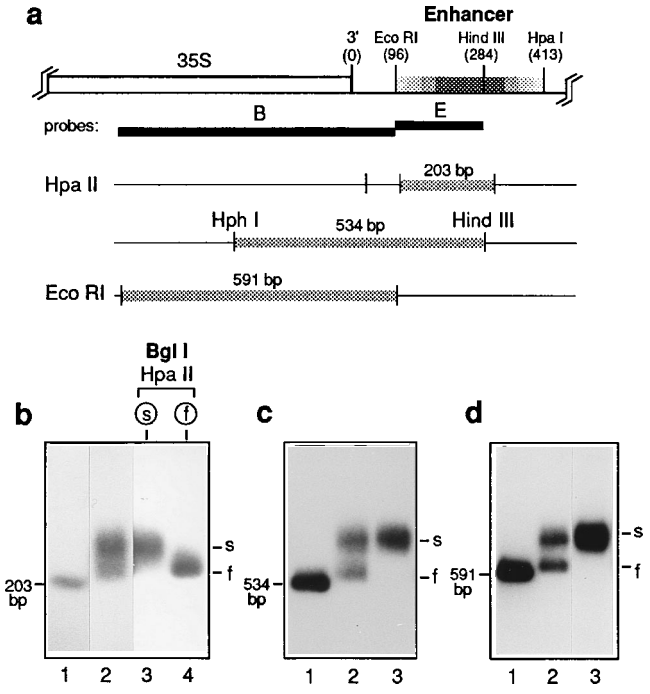

FIG. 3. Chromatin structure of the rDNA enhancer. (a) Map showing rDNA sequences around the 3′ end of the yeast rRNA transcription unit. Relevant restriction sites are indicated; the numbers in parentheses indicate their positions with respect to the 3′ end of the 25S rRNA coding region. Enhancer sequences are indicated by the shaded box. The locations of additional restriction sites are indicated below the map. Shaded boxes represent restriction fragments analyzed below. (b) The 9.1-kb *Bgl*I repeats (see map in Fig. 2a) derived from psoralen-cross-linked cells were first separated on a 1% agarose gel as shown in Fig. 2b. DNA migrating in the s and f bands was eluted separately from the gel, digested with *Hpa*II, and reelectrophoresed on a 2% MetaPhor agarose gel (lanes 3 and 4, respectively). Lanes 1 and 2 represent *Hpa*II-digested DNA isolated from untreated and cross-linked cells, respectively. The gel was blotted and hybridized with probe E. (c) DNA isolated from psoralen cross-linked cells was digested with *Hph*I and *Hin*dIII, separated on a 1.8% MetaPhor agarose gel, blotted, and hybridized with probe E (lane 2). Lanes 1 and 3 are controls representing untreated and extensively cross-linked DNA, respectively. (d) Same experiment as in panel c, except that the DNA was digested with *Eco*RI and hybridized with probe B.

tween the mobilities of the s and f bands; see Fig. 5 of reference 34). When this fragment was analyzed in the gel retardation assay, we detected only two distinct bands, showing mobility shifts similar to those of the s and f bands of the 591-bp *Eco*RI fragment containing only coding sequences (compare lanes 2 in Fig. 3c and d). This finding indicates that adjacent coding and enhancer regions have similar chromatin structures which can be of one of two distinct types, either fully or slightly accessible to psoralen.

We then analyzed the extent of cross-linking of coding and adjacent spacer sequences on single rDNA repeats which had been prepared for electron microscopy under denaturing conditions. This approach allows visualization of nucleosomes as single-stranded bubbles of 140 to 160 nucleotides, whereas highly cross-linked DNA derived from nonnucleosomal chromatin regions is prevented from denaturing and has a double-stranded appearance (8, 32, 49). The electron micrograph in Fig. 4a represent a 9.1-kb *Bgl*I repeating unit showing neighboring 5′ active-3′ inactive gene copies. A stretch of fairly continuously cross-linked DNA corresponding to the portion of the upstream gene is followed by a region organized in single-stranded bubbles comprising the intergenic spacer and the portion of the downstream gene (Fig. 4b). It is apparent that the heavily cross-linked domain extends slightly beyond the position calculated for the 3′ end of the upstream gene (arrowhead in Fig. 4a). By analyzing a shorter rDNA restriction fragment spanning this region (not shown), the boundary between active and inactive chromatin was mapped to 388 ± 170 bp downstream of the 3′ end of the 25S rRNA coding region (116 molecules analyzed). This result is fully consistent with our previous gel electrophoretic analyses indicating that transcriptionally active, nonnucleosomal genes are followed by enhancer sequences devoid of nucleosomes.

Chromatin structure of adjacent coding and spacer sequences was also examined on cross-linked *Nar*I rDNA repeats showing neighboring 5′ inactive-3′ active gene copies (Fig. 4d). In these molecules, a region organized in single-stranded bubbles comprising a portion of the upstream gene and the intergenic spacer is followed by a domain of heavily cross-linked DNA representing the portion of the downstream gene. The boundary between these two distinct sections maps to −165 ± 365 bp with respect to the transcription initiation site, indicating that the nonnucleosomal chromatin domain includes the gene promoter (104 molecules analyzed). This result was confirmed by analyzing *Bgl*I repeats showing neighboring 5′ inactive-3′ active gene copies originating from the f band (data not shown). Mapping of the position of short stretches of heavily cross-linked, double-stranded DNA larger than 150 bp that occasionally occur in the domain organized in single-stranded bubbles revealed a random distribution (Fig. 4c). In contrast, the same analysis performed with *Bgl*I repeats of the type shown in Fig. 4a revealed a preference for these stretches to be located near the 5′ end of the downstream gene (star in Fig. 4b). This finding indicates that promoter sequences of some inactive gene copies might be devoid of nucleosomes. These "open" promoters have been found to occur predominantly on newly replicated genes and have been classified as potentially active (36). On the other hand, the absence of nonnucleosomal enhancers downstream of inactive, nucleosome-packed gene copies (brackets in Fig. 4c and d) confirms again our previous results.

The open chromatin structure of the enhancer is independent of transcription. Recent in vitro transcriptional studies have identified an RNA polymerase I terminator near the Reb1p binding site located 108 bp downstream of the 3′ end of the 25S rRNA coding region (28, 29). The presence of this terminator should prevent most of the enhancer and downstream spacer sequences from being transcribed. However, since transcription terminates so close to the enhancer element, it is possible that the open chromatin structure that we detect on these regulatory sequences is simply a direct consequence of the act of transcription. To study the chromatin structure of the enhancer in the absence of transcription, we used a mutant strain that has a deletion in the gene coding for the second-largest subunit of RNA polymerase I (40). In this strain, this lethal mutation is suppressed by the presence of a multicopy plasmid containing 35S rRNA coding sequences that are transcribed by RNA polymerase II from a *GAL7* promoter (Fig. 5b).

We first analyzed the chromatin structure of rRNA coding sequences of the RNA polymerase I mutant strain. To be able to distinguish the chromosomal from the plasmid gene copies, we chose restriction fragments including small portions of the adjacent intergenic spacer (Fig. 5a). As shown in Fig. 5c and d, the 2.6- and 2.4-kb fragments containing rRNA coding sequences of the tandemly repeated gene copies are resolved in the slightly retarded f band. This finding is consistent with all of these chromosomal copies not being transcribed and packaged in nucleosomes. In contrast, in the wild-type strain grown

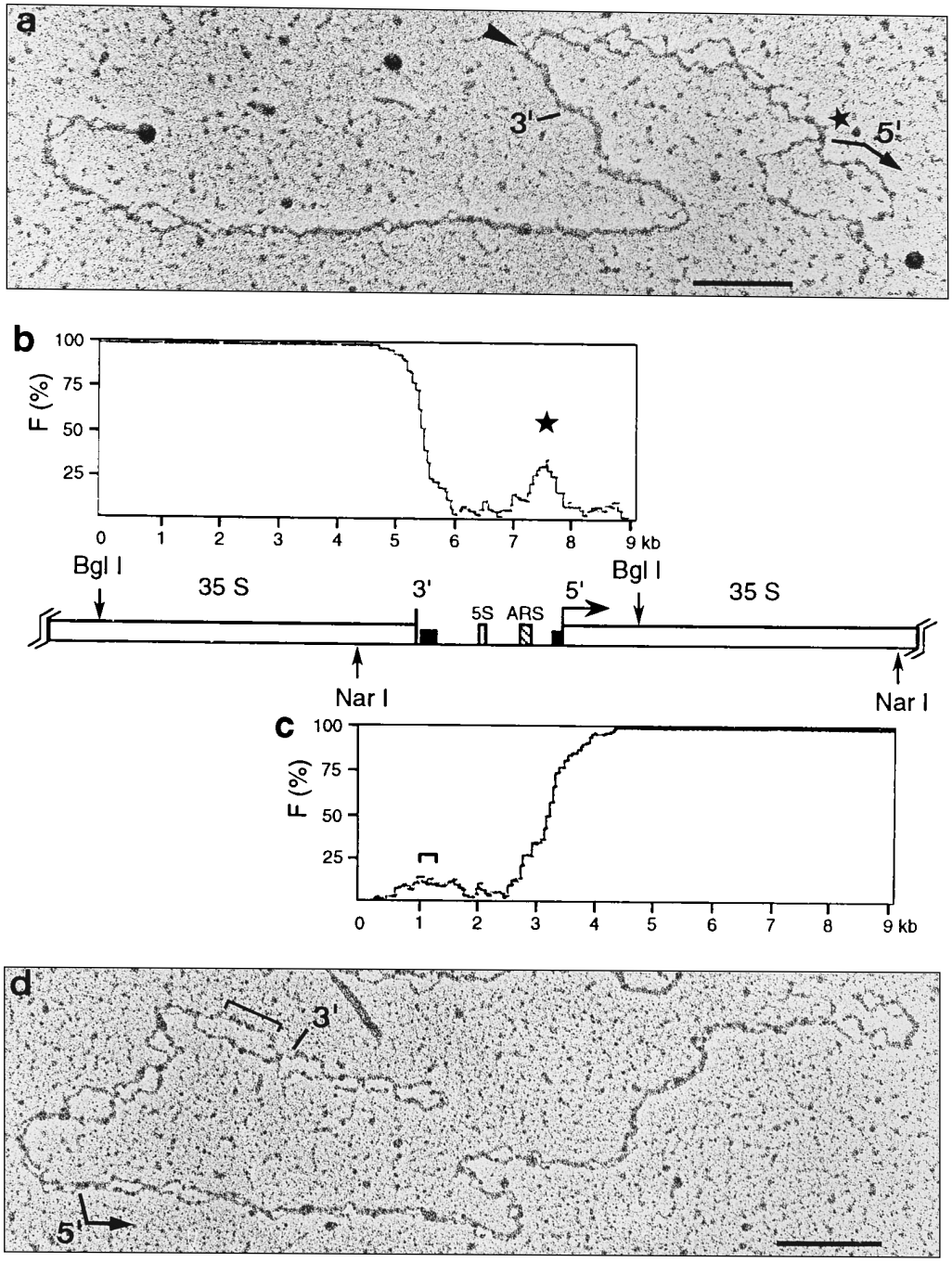

FIG. 4. Electron microscopy of rDNA molecules showing neighboring active and inactive genes. CsCl gradient-enriched rDNA derived from psoralen-treated cells was digested with *Bgl*I or *Nar*I (see map in panel b) and separated on a 1% agarose gel. DNA migrating in the s band was eluted from the gel and spread for electron microscopy under denaturing conditions. (a) Electron micrograph showing a 9.1-kb *Bgl*I repeat unit in which the rRNA coding portion of the upstream gene appears fairly continuously cross-linked, whereas the intergenic spacer and the 5′ portion of the downstream gene are organized in single-stranded bubbles. The arrowhead indicates the boundary between heavily cross-linked and slightly cross-linked DNA. The putative 3′ and 5′ ends of the two adjacent genes are also shown. The star indicates a short, heavily cross-linked, double-stranded DNA stretch near the 5′ end of the downstream gene. The bars in panels a and d represent 0.5 kb. (b) Histogram of the location of the fairly continuously cross-linked region and of double-stranded DNA stretches larger than 150 bp along *Bgl*I-digested molecules similar to the one presented in panel a. The lengths of the 70 molecules analyzed were normalized to 9.1 kb. The value F on the ordinate indicates the percentage of the molecules that showed heavily cross-linked DNA in a given length increment of 50 bp. The star marks a peak occurring at the position of the promoter. ARS, autonomous replicating sequence. (c) One hundred four *Nar*I repeats in which the 3′ portion of the upstream gene was organized in single-stranded bubbles were analyzed as in panel b. One molecule of this type is represented in the micrograph in panel d. The bracket indicates the position of the enhancer.

under the same conditions (i.e., in complex medium containing galactose), about 20% of the rRNA gene copies were transcriptionally active and devoid of nucleosomes (data not shown). Gel electrophoretic analysis of the 3.1-kb fragment derived from the plasmid copies also revealed only slightly cross-linked DNA, confirming that DNA sequences transcribed by RNA polymerase II are packaged in nucleosomes (Fig. 5c, lane 2; see references 5 and 9).

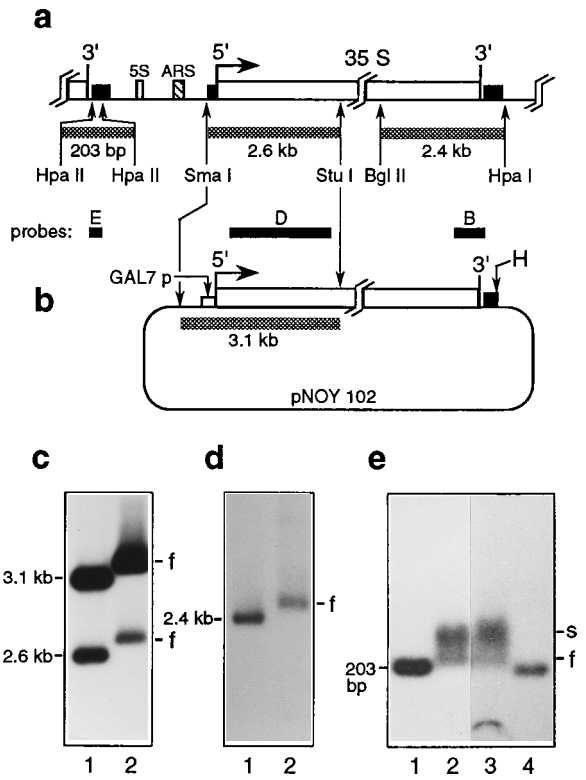

FIG. 5. Chromatin structure of rDNA sequences in the RNA polymerase I mutant strain NOY408-1a (40). (a) Map of an rDNA repeat unit. Sequences in the middle of the 35S rRNA coding region were deleted. Notation is as for Fig. 2a. Relevant restriction sites and fragments are indicated. (b) Map of plasmid pNOY102 (40) containing a yeast rRNA gene under the control of the *GAL7* promoter. This plasmid contains rDNA sequences spanning from the RNA polymerase I initiation site (5′) to the *Hin*dIII site located 284 bp downstream of the 3′ end of the 25S rRNA (H). (c) Log-phase cells of the RNA polymerase I mutant strain grown in YPG were photoreacted in the presence of psoralen. The purified DNA was digested with *Sma*I and *Stu*I, separated on a 1.2% agarose gel, blotted, and hybridized with probe D (lane 2). Lane 1, non-cross-linked control DNA. (d) Same experiment as in panel c, except that the DNA was digested with *Bgl*II and *Hpa*I and hybridized with probe B. (e) DNA from psoralen cross-linked and untreated mutant cells was digested with *Hpa*II and separated on a 2% MetaPhor gel (lanes 3 and 4, respectively). Lanes 1 and 2, control DNA and DNA from psoralen-treated wild-type SC3 cells grown in YPD, respectively, both digested with *Hpa*II. The gel was blotted and hybridized with probe E.

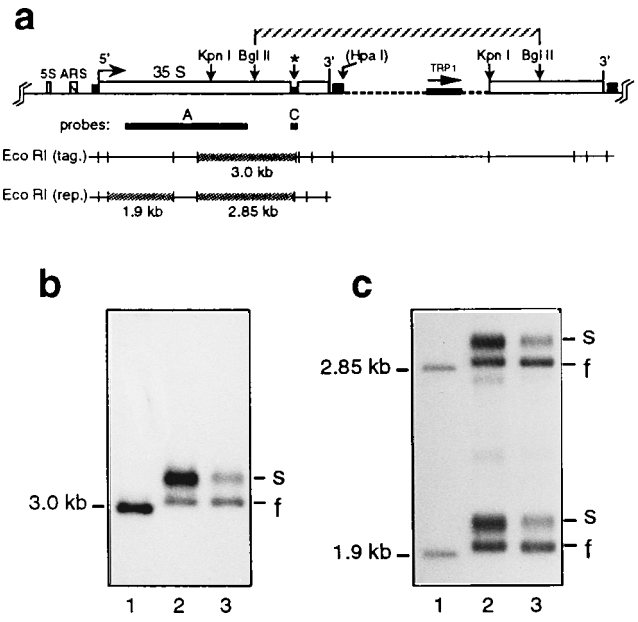

FIG. 6. Analysis of a single, tagged rRNA transcription unit. (a) Map showing the rDNA region around the tagged gene copy. This transformant was obtained by a single integration of plasmid pRD2T (striped area) into the rDNA locus. Vector sequences are indicated by the dashed line (the *TRP1* marker gene is also shown). The sequence tag is labelled by an asterisk. *Eco*RI restriction maps of the tagged gene copy (tag.) and of the bulk of the tandemly repeated copies (rep.) are indicated. ARS, autonomous replicating sequence. (b) Cells growing exponentially in complex or minimal medium were cross-linked with psoralen, and the DNA was isolated, digested with *Eco*RI, and separated on a 1.2% agarose gel (lanes 2 and 3, respectively). Lane 1 shows non-cross-linked, control DNA. The gel was blotted and hybridized with probe C. (c) Experiment similar to that in panel b, except that the DNA was hybridized with probe A.

A different result was obtained from the analysis of the *Hpa*II enhancer fragment originating from the chromosomal copies. This fragment was separated in the two distinct s and f bands, indicating the presence of nucleosome-free and nucleosome-packed enhancers, respectively (Fig. 5e, lane 3). The detection of nonnucleosomal enhancers in the absence of transcription points to the presence of factors other than the RNA polymerase I itself that can exclude nucleosomes from these regulatory sequences.

The subset of active rRNA gene copies is not stably inherited during cell division. From the nearest-neighbor analysis, we concluded that transcriptionally active rRNA gene copies are randomly distributed along the yeast rDNA locus. The next question that we wanted to address was whether there is a specific subset of active genes which is stably maintained throughout cell divisions or whether every time the cells duplicate, the subset of the genes to be activated is chosen at random. To answer this question, we analyzed single, tagged rRNA transcription units in several populations of cells derived from single transformants. We reasoned that if active and inactive gene copies are stably inherited, all cells of a clone will have the tagged transcription unit either in an active or in an inactive transcriptional state. In contrast, if during subsequent cell divisions transcription occurs at randomly chosen genes, the percentage of cells having the tagged transcription unit in an active state will be equal to the percentage of active, nontagged rRNA gene copies of the cell population analyzed.

To generate transformants with a tagged rRNA transcription unit, an insertion vector containing 25S rRNA coding sequences with an approximately 200-bp-long insert of foreign DNA was integrated by homologous recombination into the rDNA locus (see Materials and Methods; the map of the region around the integration site is shown in Fig. 6a). Seven transformants in which a single integration occurred were grown separately in complex and minimal media and were photoreacted in the presence of psoralen. DNA was isolated, digested with *Eco*RI, and analyzed in a gel retardation assay after blotting and hybridization with probes specific for the tagged or for the tandemly repeated rRNA gene copies (Fig. 6b and c, respectively). In all of the transformants analyzed, the 3-kb *Eco*RI fragment originating from the tagged transcription unit was resolved in the two distinct s and f bands, indicating the presence of both active and inactive genes, respectively (Fig. 6b, lanes 2 and 3). From the intensities of the two bands, we calculated that 67% ± 7% and 47% ± 6% of the cells grown in complex and minimal media, respectively, had the tagged gene copy in a transcriptionally active, nonnucleosomal state. Since these values are not significantly different from the fractions of transcriptionally active gene copies present in the cell populations analyzed (58% ± 3% and 40% ± 3% for cells

growing in complex and minimal media, respectively), we conclude that the subset of the transcriptionally active rRNA gene copies is not stably propagated during cell division.

DISCUSSION

Organization of the transcriptionally active rRNA gene copies. The clustering of tandemly repeated rRNA genes in one or few chromosomal sites can be interpreted as one of the most efficient strategies that allow cells to ensure high levels of rRNA synthesis. It has been postulated that the head-to-tail arrangement of these genes might facilitate recruitment of RNA polymerase I by mechanisms that allow direct transfer of this enzyme from repeat to repeat (23, 26, 37).

In this study, by using psoralen cross-linking as a tool to mark transcriptionally active DNA in vivo, we analyzed the linear distribution of the active gene copies along the yeast rDNA locus. We show that in growing yeast cells, in which only about half of the genes are expressed, the average number of active genes per cluster ranges from two to three copies. These data are consistent with most of the active gene copies being rather randomly distributed along the entire rDNA locus. This observation argues against the existence of major transcriptional regulatory mechanisms taking advantage of the tandem head-to-tail nature of the rRNA genes. Models envisioning that efficient expression of these genes relies on direct transfer of RNA polymerases from one repeat to the next would demand a consecutive arrangement of active genes in the form of one or few large clusters. However, our findings do not rule out models proposing direct passage of terminating RNA polymerases back to the promoter of the same transcription unit (20) or, alternatively, to promoters of distal, nonadjacent rRNA genes (18).

Mechanisms for efficient recycling of RNA polymerase I molecules from repeat to repeat have been postulated for the tandemly repeated rRNA genes of the frog *Xenopus laevis* (26, 37). However, later experiments in the same system showed that high levels of transcription at the rRNA gene promoter are not dependent on RNA polymerases coming from upstream (27, 33).

Additional evidence arguing against the notion that tandem, head-to-tail repetition may play a role in optimizing rRNA transcription comes from experiments in which insertions of single rDNA repeats at different locations in the *Drosophila* chromosome resulted in high levels of expression (19). However, it should be pointed out that these analyses were performed on polytene chromosomes, in which the lateral repetition of the rRNA transcription units might have facilitated expression by increasing the local concentration of transcriptional regulatory factors. In this context, it is worth mentioning that from our analysis, we cannot exclude the possibility that in the nucleolus, transcriptionally active rRNA gene copies, even though randomly distributed along the entire locus, are pulled together into a three-dimensional structure.

Recent studies by our group have shown that in yeast cells, during chromosome duplication, the replication machinery moving into transcriptionally active rRNA genes always generates two daughter strands regularly packed in nucleosomal arrays characteristic of inactive chromatin (36). From these findings, it is evident that the subset of active, nonnucleosomal rRNA gene copies has to be established anew after each round of replication. However, the question remained open as to whether there is a specific subset of active genes which is always selected every cell cycle or whether the active genes are chosen at random after DNA replication. The result that we obtained by analyzing the activity state of single, tagged rDNA transcription units in the tandem array argues against the existence of a subset of active genes that is stably maintained throughout cell divisions (Fig. 6). Instead, our findings are consistent with a scenario in which all the rRNA genes have no "memory" and have the same probability of being selected by the transcription machinery, irrespective of whether they have been transcribed in the preceding G_1 phase.

Once the decision to activate an rRNA gene is made, how is the active chromatin structure established? From our failure to detect nonnucleosomal rRNA gene copies in the RNA polymerase I mutant strain, we conclude that generation of the open chromatin structure on the rRNA coding region needs the presence of functional RNA polymerase I molecules. From this result and from the finding of newly activated rRNA genes with only a 5′ portion of the coding region highly accessible to psoralen (36), it is reasonable to propose that disruption of the chromatin structure is mediated by RNA polymerase I molecules advancing through the rDNA template. It is still not understood why nucleosomes remain on templates transcribed by RNA polymerase II (5, 9) (Fig. 5c), whereas they seem to be lost or drastically modified on RNA polymerase I genes (7, 8, 32, 34, 49).

Structures and functions of the enhancer elements in the tandem array. In the rDNA of higher eukaryotes, the repetitive enhancer elements occupy 1 to 3 kb of spacer sequences located just upstream of the gene promoter (43, 44). From this type of arrangement and from the fact that in some species, the intergenic spacers can reach a length of about 30 kb (31), it is reasonable to propose that in the tandem array, the primary target of the enhancer is represented by the adjacent, downstream promoter.

In the tandemly repeated rDNA of *S. cerevisiae*, the RNA polymerase I enhancer lies immediately downstream of one transcription unit and about 2 kb upstream of the next promoter. This peculiar arrangement raises the question of whether this transcriptional regulatory element exerts its function on the upstream or on the downstream gene. What is intriguing is that each rDNA enhancer contains two additional types of regulatory elements whose functions are clearly dedicated to the preceding transcription unit, namely, a transcription terminator and an RFB (2, 29, 30). Functional analysis within the tandem array showed that deletion of a single enhancer effects transcription of both the preceding and the downstream flanking gene (23). However, it must be emphasized that in those experiments, the transcription terminator was also eliminated, and therefore RNA polymerase I molecules transcribing through the intergenic spacer might have disrupted the stable promoter complex of the downstream unit by the mechanism termed promoter occlusion (1).

In the present study, in an effort to detect which enhancer is exerting its activation function, we analyzed the chromatin structure of the enhancer elements flanking transcriptionally active and inactive gene copies. We reasoned that regulatory sequences at which factor-DNA interactions have occurred would be organized in a disturbed chromatin structure. Indeed, transcription factor binding to reconstituted chromatin and concomitant disruption of nucleosomes at the DNA binding site have recently been demonstrated in vitro (25, 52). Here we show that enhancer elements located at the 3′ end of transcriptionally active rRNA genes are always organized in a chromatin structure highly accessible to psoralen, consistent with the absence of nucleosomes. In contrast, inactive gene copies have nucleosome-packed enhancers at their 3′ ends. The fact that we could detect nonnucleosomal enhancers in the RNA polymerase I mutant strain is not consistent with these open chromatin structures being simply a direct consequence of the act

of transcription. Therefore, we interpret the disrupted chromatin structure on these transcriptional regulatory sequences as being the result of specific protein-DNA interactions that can occur before the onset of transcription. It is possible that the yeast rDNA enhancer represents the primary target of a key regulatory factor whose concentration in the cell determines the fraction of the genes to be activated. In fact, we have recently shown that yeast cells can regulate rRNA gene transcription by modulating the proportion of active and inactive gene copies (8).

Do our results mean that every enhancer element in the tandem array is committed to activating only a single gene, namely, the adjacent upstream one? It is difficult to draw definitive conclusions regarding enhancer function from the particular structural link that we observed between coding and adjacent spacer regions. In fact, it is possible that the nonnucleosomal structure at the 3′ end of each transcriptionally active gene might also be related to transcription termination and replication termination functions. Indeed, factor-DNA interactions have been postulated for both of these activities (3, 28, 29). Furthermore, it is known that the Reb1p factor (24, 38) implicated in transcription termination (28) corresponds to factor GRF2, a general transcription factor which has been shown to be able to create a nucleosome-free space around its binding site (6, 13). Therefore, the strict correlation between nucleosome-free coding and enhancer regions along the tandem array can also be interpreted as the absolute requirement for each activated gene to have a functional transcription terminator as well as a functional RFB at its 3′ end.

How do cells manage to establish these functions only at the 3′ end of the expressed gene copies? It is possible that at the transcription units chosen to be activated, these two important functions are established before the onset of transcription, in concert with formation of the stable preinitiation complex at the gene promoter. The detection of replication forks arrested at the 3′ end of rRNA genes in the RNA polymerase I mutant strain confirms that the RFB function can be established before the onset of transcription (3). Furthermore, the fact that, in the wild-type strain, arrested forks were found mostly at the 3′ end of the active transcription units is consistent with these functional RFB complexes being absent at the 3′ end of the inactive gene copies (35). Formation of functional transcription terminators might also occur at the beginning of the activation process. This is quite reasonable, as specific protein-DNA complexes capable of arresting transcription elongation have to be established at the 3′ end of the activated gene before arrival of the first RNA polymerases.

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