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The Differential Expression of Ribosomal RNA Genes

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The differential expression of ribosomal RNA genes

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[Plates 1 and 2]

Ribosomal RNA genes are localized at chromosomal sites termed nucleolus organizers because nucleoli form around transcribed ribosomal RNA genes. The relative activities of arrays of ribosomal RNA genes can be estimated cytologically by comparing the sizes of nucleoli in the same cell. Also, active nucleolus organizers give rise to visible constrictions in metaphase chromosomes whereas inactive nucleolus organizers do not. With these assays the differential expression of nucleolus organizers and ribosomal RNA genes has been observed frequently, especially in interspecies hybrids. Studies on wheat have revealed that differences in gene expression are associated with differences in chromatin structure and cytosine methylation. Active loci have higher proportions of their genes decondensed and accessible to proteins and also higher proportions with a non-methylated cytosine residue at a CCGG site in the region of the promoter. Short, related sequences with dyad symmetry have been noted between −140 and −70 base pairs from where transcription is initiated in a wheat ribosomal RNA gene. Similar sequences are reiterated upstream of the promoter over 2000 base pairs. From comparison of this gene structure with that of *Xenopus* ribosomal RNA genes it can be concluded that these short sequences are likely to act as enhancers of transcription by binding to specific regulatory proteins that function to stimulate the attachment of polymerase I complexes. Differential expression of arrays of ribosomal RNA genes results when genes have different numbers of enhancer repeats or a higher affinity for the regulatory protein(s). This model to explain differential gene expression and the origins of genetic variation affecting ribosomal RNA gene expression are discussed.

Introduction

Nucleolar dominance is an example of a phenomenon now recognized to be the differential expression of ribosomal RNA genes that was first reported in plants in 1934 by Navashin. Ribosomal RNA (rRNA) genes are clustered in tandem arrays at nucleolus organizers, so called because in interphase cells nucleoli form around this chromosomal site. Within the nucleoli the chromatin containing the rRNA genes is dispersed and transcribed (Stahl 1982; Chouinard 1975) (figure 1*a*, plate 1). The ribosomal RNA products become associated with proteins and ribonucleoprotein particles accumulate before being exported to the cytoplasm to sustain protein synthesis. During prophase of the cell cycle, rRNA synthesis declines and ceases at metaphase. The nucleolus is dismantled. The chromatin that was dispersed in the nucleolus condenses but remains associated with specific nucleolar proteins and stains poorly compared with the rest of the metaphase chromosome (Goessens & Lepoint 1974). This differential

staining produces an apparent 'gap' or secondary constriction in the stained chromosome at the site of the nucleolar organizer (figure 1*b*). The 'gap' is evidence of rRNA genes that were active in the preceding interphase.

Navashin (1934) was studying interspecies hybrids in the genus *Crepis* and noted that in certain hybrids the secondary constriction was visible in the relevant chromosome from only one of the parents – not both as expected. It was inferred that the nucleolus organizer activity from the other parent was suppressed in the hybrid. McClintock extended observations on the phenomenon of nucleolar dominance as did Wallace & Langridge (1971), who substantiated the existence of a species ranking in which a nucleolus would be suppressed in an interspecies hybrid with any species ranked above it. The phenomenon has been observed in many species hybrids including *Hordeum* (Kasha & Sadasivaiah 1971; Jessop & Subrahmanyam 1984), *Ribes* (Keep 1962), *Solanum* (Yeh & Peloquin 1965), and triticale (wheat × rye hybrid) (Darvey & Driscoll 1972; Lacadena *et al.* 1984; Appels *et al.* 1986).

The absence of a secondary constriction in metaphase chromosomes at the site of rRNA genes implies that the specific proteins found in the constriction do not bind to inactive genes and also that inactive genes reside in a different chromatin configuration from active genes. These conclusions are supported by the presence of silver-binding proteins in active, but not inactive, nucleolus organizers (Schwarzacher *et al.* 1978; Orellana *et al.* 1983; Lacadena *et al.* 1984) and the demonstration that rye rRNA genes are preferentially condensed and less dispersed in interphase nucleoli in wheat × rye hybrids where the rye nucleolus organizer is suppressed (Appels *et al.* 1986).

The differential binding of proteins and differential DNA condensation between rRNA gene loci, characteristic of nucleolar dominance, also occurs between genes at a single locus. This is because rRNA genes are in excess over the number required to sustain ribosome synthesis in plants (Ingle *et al.* 1975) and only a proportion of the genes at each locus are maintained in an active state; some remain condensed in heterochromatin during interphase and are not dispersed in the nucleolus (figure 1*a*, *c*). This has been particularly well documented in maize where a major proportion of the ribosomal DNA has been shown to reside in heterochromatin adjacent to the nucleolar constriction (Givens & Phillips 1976; Phillips 1978). However, in many plant species, heterochromatin is found on one or both sides of the nucleolar constriction. Figure 1*c* shows the organization of ribosomal RNA genes in a wheat nucleus, revealed by hybridization of ^{3}H-labelled rDNA to the cell (Flavell & Martini 1982). Some hybridization occurs over the nucleolus and is assumed to be associated with the rRNA genes dispersed in the nucleolus whereas other major foci of hybridization are present around the periphery of the nucleolus. These probably represent the aggregates of genes in condensed chromatin (see also Appels *et al.* 1986). Thus for the rRNA genes differential expression occurs within a locus as well as between loci. It is probable that the same regulatory mechanism(s) are operative between genes whether they reside in the same or different loci.

The cytological observations described above indicate that active and inactive genes are organized differently. Inactive genes lie in supercoiled chromatin outside nucleoli whereas active genes are dispersed and gather nucleolar material around themselves (Stahl 1982; Chouinard 1975; Ashraf & Godward 1980; Scheer *et al.* 1982; Flavell 1986). However, electron microscopical observations also show that within the nucleolus some genes lie in a condensed conformation and have specific (silver binding) nucleolar proteins bound to them (Busch *et al.* 1982). The genes in these so-called fibrillar centres (Stahl 1982) are not transcribed but are available to be activated rapidly, uncoiled and transcribed – in contrast to the genes

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condensed in heterochromatin outside the nucleolus. The proportion of nucleolar rRNA genes in the fibrillar centres varies with the activity of the cell. More active cells contain less fibrillar centre material (Deltour *et al.* 1979) presumably because the rRNA genes are uncoiled.

From the knowledge summarized in this introduction, it is apparent that the differential expression of rRNA genes involves the organization of genes into different chromatin configurations. To understand the differential expression observed it is necessary to explain (1) what determines which genes, and how many, are not condensed in heterochromatin but become included in a nucleolus, and (2) which genes, and how many, of those in a nucleolus are uncoiled out of the fibrillar centres and transcribed. These questions are addressed in this paper from our studies on rRNA genes in wheat.

Examples of differential rRNA gene expression in wheat

The phenomenon of differential nucleolar organizer expression is not confined to interspecies hybrids but occurs also within species, though to a more limited extent. In hexaploid wheat, nucleolus organizers are localized on chromosomes 1B, 6B, 5D and 1A. In the variety Chinese Spring, much larger nucleoli form at the 1B and 6B loci. These loci also have more rRNA genes than the 5D and 1A loci (Flavell & O'Dell 1979). The much greater number of rRNA genes at the 1B and 6B loci is undoubtedly part of the explanation for the much larger nucleoli formed at the 1B and 6B loci, i.e. differential expression is determined in part by variation in gene number at the locus.

The number of rRNA genes at homologous loci differs between plants within the species (Flavell & Smith 1974) and this similarly contributes in part to the observed differential activity between these homologous loci. For example, studies some years ago showed that replacement of a 1A chromosome possessing few rRNA genes with one possessing a large number, resulted in a much larger nucleolus at the locus on chromosome 1A (Flavell & O'Dell 1979). Studies on a range of genotypes varying only in the source of chromosome 1A showed that the visibility of the additional nucleolus, i.e. its activity, was related to the number of rRNA genes at the nucleolus organizer introduced (see figure 2). However, variation in the number of genes at the locus is not the only molecular basis for the differential expression of the locus, especially in interspecies hybrids. In the wheat variety Chinese Spring, the number of rRNA genes at the nucleolus organizer on chromosome 6B is twice that on chromosome 1B (Flavell & O'Dell 1979) but the volume of the nucleolus formed and the length of the nucleolar constriction at metaphase is only half that at the 1B locus. Thus the 1B set of rRNA genes is semi-dominant over the 6B set (Martini & Flavell 1985).

When a nucleolus organizer-bearing chromosome from *Aegilops umbellulata* is crossed into Chinese Spring wheat, the wheat nucleolus organizers are suppressed and a large proportion of the rRNA gene activity is at the *Ae. umbellulata* locus (Martini *et al.* 1982). This is illustrated in figure 3, plate 2. The four nucleolar constrictions visible in metaphase chromsomes of Chinese Spring wheat are not visible in the presence of the *Aegilops umbellulata* chromosome; instead only one pair of constrictions is visible and these are in the *Aegilops umbellulata* chromosome. In interphase cells the major nucleoli formed at the 1B and 6B loci of Chinese Spring wheat are much reduced and the much larger nucleoli are formed at the nucleolus organizer of *Aegilops umbellulata*. This strong dominance of the *Ae. umbellulata* rRNA genes over the Chinese Spring wheat genes is another example of interspecies nucleolar dominance.

Studies on aneuploid derivatives of Chinese Spring, where individual chromosomes carrying

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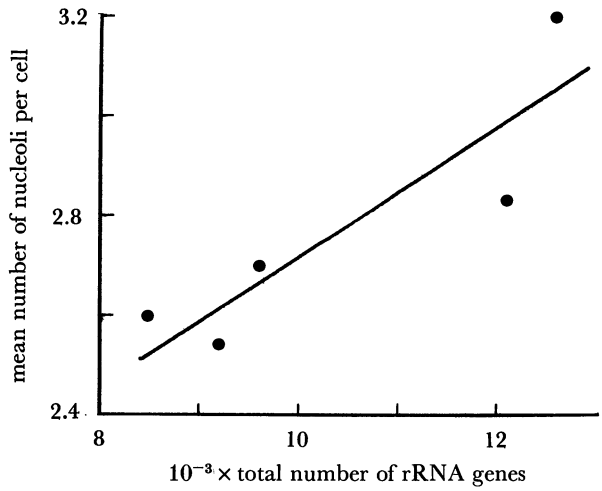

FIGURE 2. Relation between the number of easily visible nucleoli and variation in the number of rRNA genes at the 1A locus. The plants used were Chinese Spring wheat with 1A chromosomes from different varieties substituted in place of the 1A chromosome of Chinese Spring. Numbers of rRNA genes in each variety were determined by hybridization studies. Nucleoli were scored as described in Flavell & O'Dell (1979). Further details of these results in Flavell & O'Dell (1979).

nucleolus organizers have been deleted or duplicated, have shown that organizers compete with each other. When a dominant organizer is deleted, the recessive organizer become more active to compensate (Longwell & Svihla 1960; Martini & Flavell 1985). It is important to appreciate this competition phenomenon because, as described later, it is central in the models to explain the differential expression of rRNA genes.

THE STRUCTURE OF RIBOSOMAL RNA GENES

To uncover part of the explanation for differential gene expression in wheat it was considered essential to study the structure of the genes. Ribosomal RNA genes are organized in tandem arrays; a segment of an array is shown in figure 4. The 18 *S*, 5.8 *S* and 25 *S* sequences are transcribed together into a single RNA, which is then processed to form the mature RNAs. Treatment of duplexes formed *in vitro* between a single rDNA repeat unit and the rRNA precursor transcript with S_1 nuclease suggests that transcription begins about 1130 base pairs before the beginning of the 18 *S* rRNA sequence (figure 4). Extension *in vitro* of a short DNA primer by using the rRNA precursor as template supports this conclusion (M. Vincentz & R. B. Flavell, unpublished data).

Upstream of the site of rRNA initiation is a series of repeats (Appels & Dvorak 1982*a*) distinguishable into two sets. Each of the A set is about 135 base pairs except for the first and last, which are shorter, whereas those in the B set are longer, about 150 base pairs (R. F. Barker & R. B. Flavell, unpublished data).

Comparison between rRNA genes within and between plants has shown that there is considerable variation in this upstream region containing the A and B repeats (Appels & Dvorak 1982*a*, *b*; Flavell 1985; Flavell *et al.* 1986). This variation contrasts with the relative invariance of the sequences specifying the rRNAs, which are highly conserved between species. It is therefore on the variable region – the promoter and sequences to the 5′ side of the

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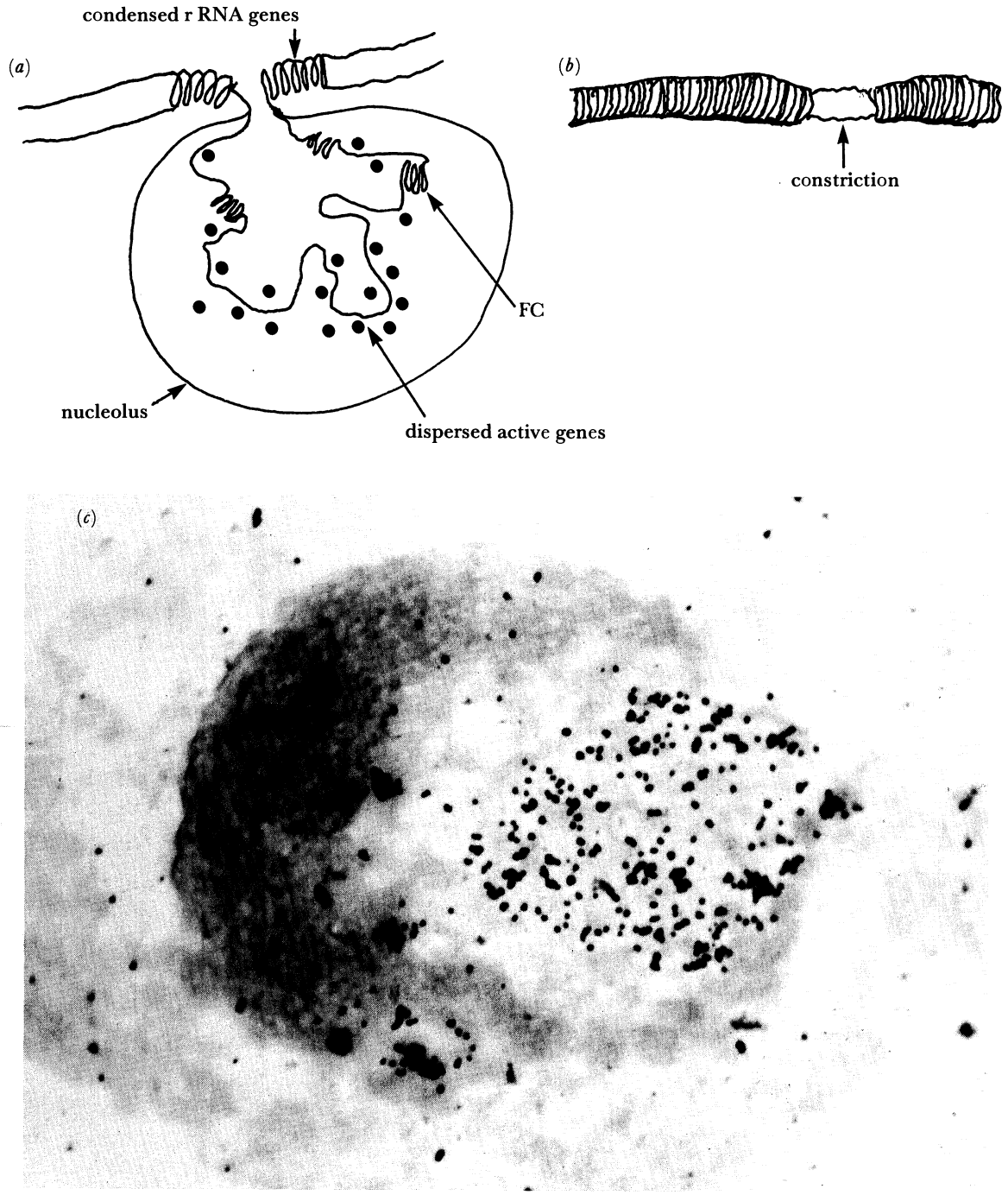

FIGURE 1. Organization of ribosomal RNA genes in the cell cycle. (*a*) A schematic representation of a nucleolus at interphase. Only some of the ribosomal RNA genes are dispersed in the nucleolus. Others remain condensed outside the nucleolus. FC, fibrillar centre. (*b*) A representation of a metaphase chromosome showing the 'constriction' or 'gap' due to the condensation of ribosomal RNA genes that were active in the preceding interphase. (*c*) Organization of rRNA genes in an interphase cell revealed by *in situ* hybridization of ^{3}H-labelled rDNA with a squashed interphase root-tip cell. The sites of hybridization of the probe with the rRNA genes were revealed by autoradiography (Flavell & Martini 1982). The position of the nucleolus is marked by the scattered silver grains on the right side of the nucleus. The major foci of hybridization on the left side of the nucleus are presumed to be condensed rRNA genes.

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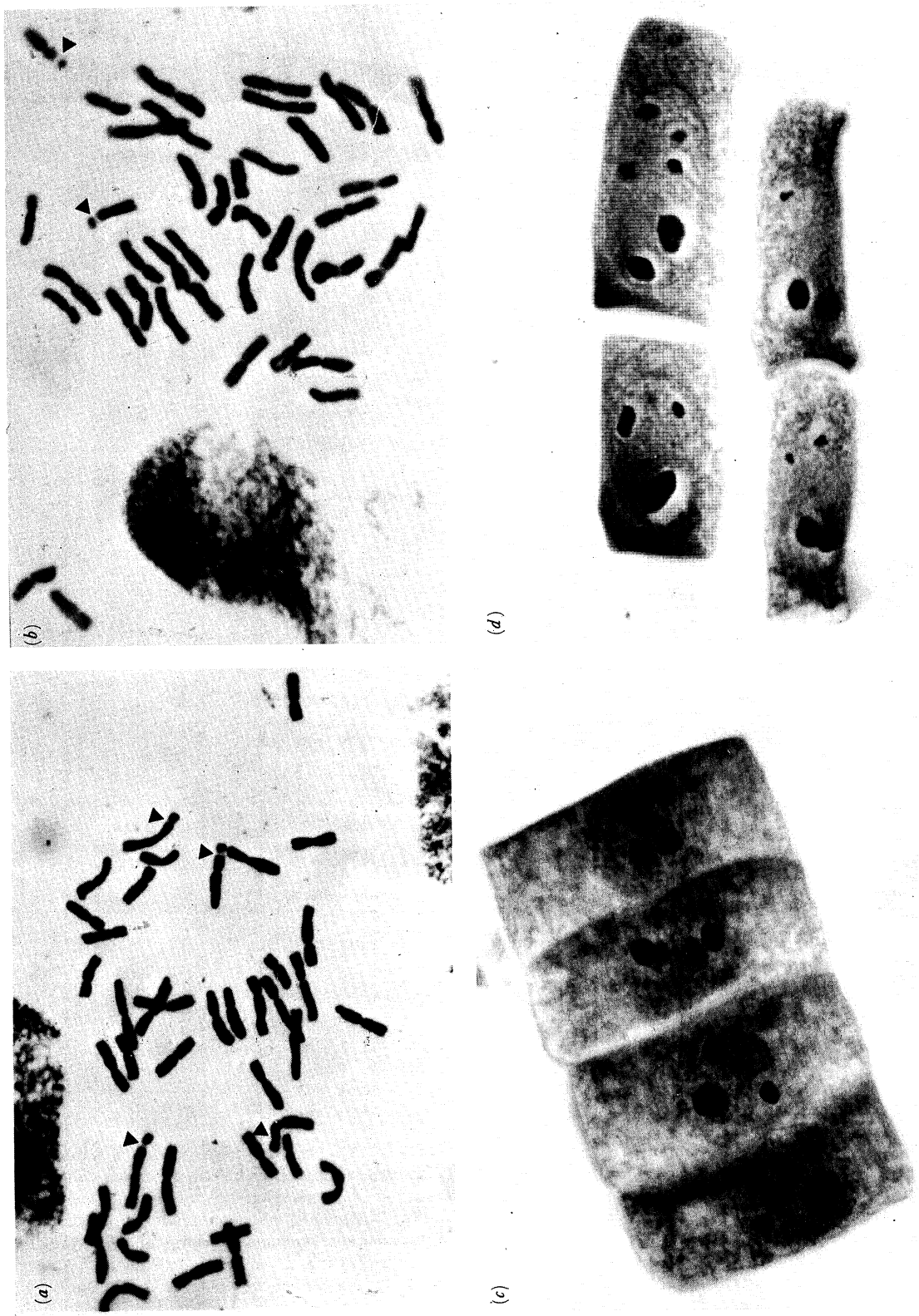

FIGURE 3. Suppression of wheat nucleolus organizers by a chromosome carrying a nucleolus organizer from *Aegilops umbellulata*. (*a*, *b*) Metaphase chromosomes of Chinese Spring (CS) wheat (*a*) and Chinese Spring + chromosome 1U from *Aegilops umbellulata* (*b*). The nucleolar constrictions are marked ▼. (*c*, *d*) Nucleoli stained in root-tip cells of Chinese Spring (CS) wheat and Chinese Spring + chromosome 1U from *Aegilops umbellulata*.

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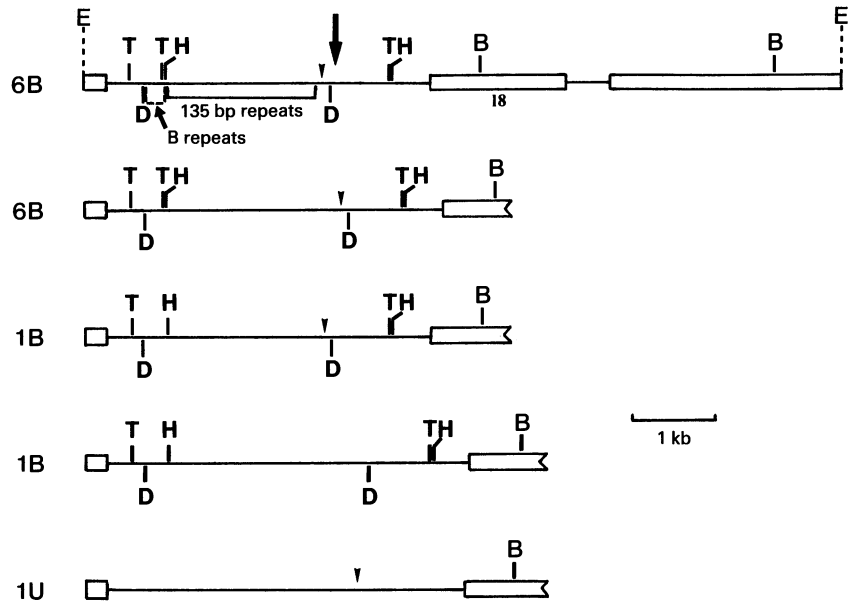

FIGURE 4. A map of a ribosomal RNA gene repeat unit from wheat and variation in the length of intergenic DNA in the genes at specific loci. The map drawn from sequence information (R. F. Barker & R. B. Flavell, unpublished data) shows the A (135 base pair) and B repeats in the spacer between transcription units, the proposed site of initiation of transcription (↓) (M. Vincentz & R. B. Flavell, unpublished data) and the site of the CCGG sequence that is preferentially unmethylated in active genes (▾) (Flavell *et al.* 1983). Also shown are the intergenic regions of the major genes at the chromosomal 6B and 1B loci of Chinese Spring wheat and the 1U locus from *Aegilops umbellulata*. The restriction enzyme sites used to characterize these regions are as marked: T, *Taq*I; D, *Dde*I; H, *Hinf*I; B, *Bam*HI; E, *Eco*RI. Other sites for *Taq*I, *Dde*I and *Hinf*I exist outside the array of A and B repeats but have been omitted for clarity.

promoter – that attention has been focused to uncover part of the molecular explanation for nucleolar dominance.

Variation uncovered by restriction endonuclease mapping for the genes at the 6B and 1B loci of Chinese Spring wheat and the 1U locus of *Aegilops umbellulata* is shown in figure 4. Two major variants occur at the 6B locus, differing by approximately 140 base pairs – the length of one A repeat. Most of the genes at the 1B locus are similar in length to the shorter of the types at the 6B locus. However, a small percentage of genes have a much longer intergenic region consisting of A repeats. The genes at the 1U locus also have a long intergenic region. These results have been gained by studying plants with each locus deleted or added in turn and noting the characteristics of the genes subtracted or added.

Some analysis of the rRNA gene promoter of wheat can be performed by reference to the well-analysed, equivalent promoter from *Xenopus* species. In *Xenopus laevis* there is a sequence residing at −100 to −75 with respect to where transcription is initiated that is conserved in different species. It is required for high rates of transcription because deletion of it reduces transcription considerably (for review see Moss *et al.* 1985). In the region −156 to −70 of wheat, there are four related sequences 12–16 base pairs long residing between blocks of A residues (figure 5*a*). Each of the sequences displays some dyad symmetry. The one residing −90 to −75 is duplicated at −130 to −114. The wheat and *Xenopus* sequences with some dyad symmetry at −90 to −75 and −102 to −87 respectively are surprisingly related (see figure 5*B*).

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Upstream of the promoter in *Xenopus* lie many short repeats of either 60 or 81 base pairs which are closely related to each other and to the region of the promoter from about −115 to −70, i.e. they include the conserved sequence highlighted above and in figure 5*b*. They function as enhancers of transcription, i.e. when deleted, transcription from genes on the same DNA molecule is reduced and when more are added the relative rate of transcription is

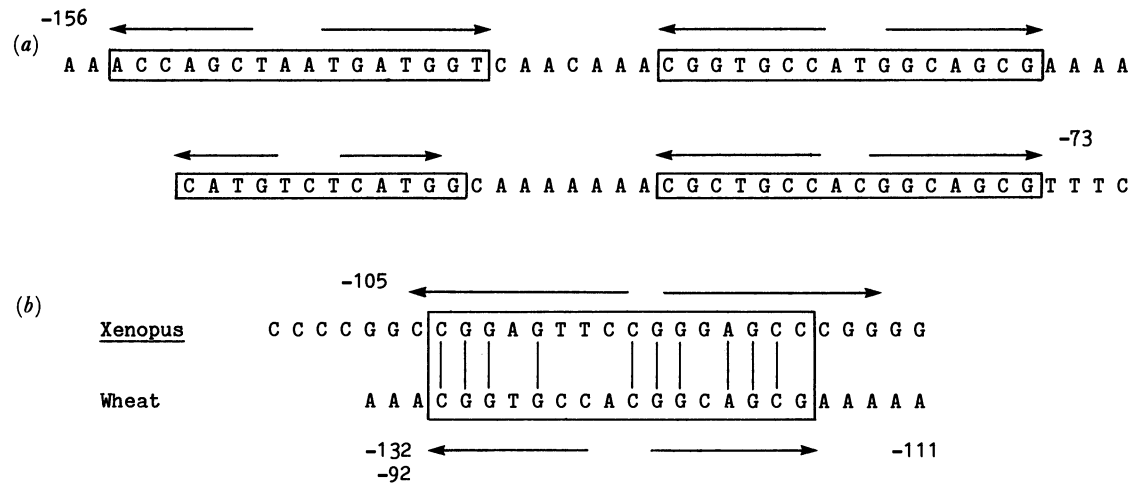

FIGURE 5. (*a*) Related sequences with dyad symmetry in the promoter of a wheat rRNA gene. (*b*) Relation between sequences with dyad symmetry in the promoters of wheat and *Xenopus* rRNA genes. The arrows mark the sequences with dyad symmetry. The numbers indicate the number of bases before the first base of the pre-ribosomal RNA transcript.

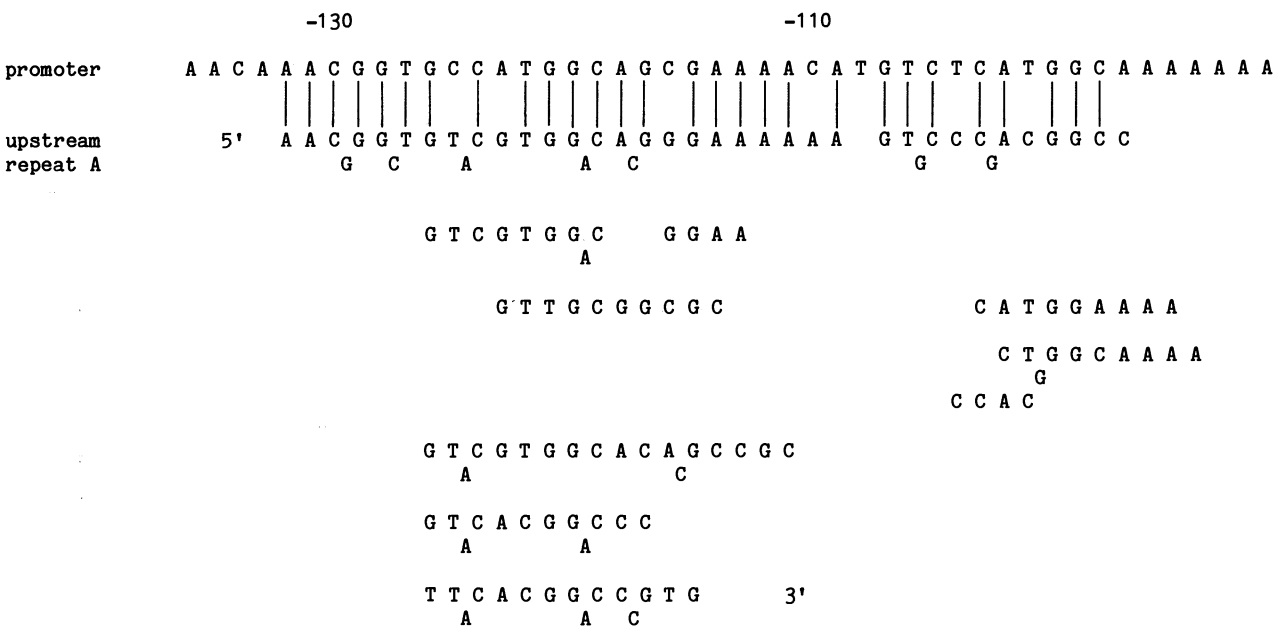

FIGURE 6. Homology between promoter and upstream A repeat sequences in a wheat rRNA gene. The numbers in the promoter sequence indicate the number of bases before the first base of the pre-ribosomal RNA transcript. The sequence of the 135 base pair A repeat (Appels & Dvorak 1982; R. F. Barker & R. B. Flavell, unpublished data) is written in a fragmented form to emphasize the segment related to the promoter sequence and the composite nature of the repeat.

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increased (Reeder 1984). This information implies that the sequences in the promoter at −115 to −70 similarly act to enhance transcription. Variants of the wheat promoter sequence are similarly reiterated in the A and B repeats upstream from the promoter (see figure 6). Thus it is reasonable to hypothesize that the upstream repeats in wheat also act as enhancers of rRNA gene transcription. However, the A and B repeats are complex. Each A repeat, for example, consists of reiterated fragments of short sequences related to, but not identical with, the promoter sequence −130 to −110 and −93 to −75 (figure 6). There is considerable variation between repeats and between genes for the number and precise sequence of the reiterated fragments (figure 4) (Appels & Dvorak 1982*a*, *b*; R. F. Barker & R. B. Flavell, unpublished data). It therefore appears that there are a very large number of short sequences related to the promoter sequence −150 to −70 which occupy a further 2000–3000 base pairs upstream from the promoter (figures 4 and 6). These upstream repeats, postulated to serve as enhancers, are related to several other enhancer sequences in animals. Because of their diversity all the upstream repeats are unlikely to have the same efficiency as enhancers; many are probably too aberrant to function well.

A MODEL TO EXPLAIN DIFFERENTIAL GENE EXPRESSION AND NUCLEOLAR DOMINANCE

When *Xenopus* rRNA genes differing in the number of enhancers are injected together into *Xenopus* oocytes in high copy number, those with the more enhancers are transcribed more efficiently (Reeder *et al.* 1983; Moss *et al.* 1985). It has therefore been postulated that rRNA genes compete for a protein that is in limiting concentration and binds to the enhancer sequence. The differential transcription results because the protein (1) helps binding of the transcription complex, (2) is part of the transcription complex or (3) stimulates movement of the transcription complex to the transcription initiation site. At least it can be concluded that the presence of more enhancer-binding protein on a gene increases the concentration of transcription complexes on the gene (figure 7). The model provides an explanation for nucleolar dominance

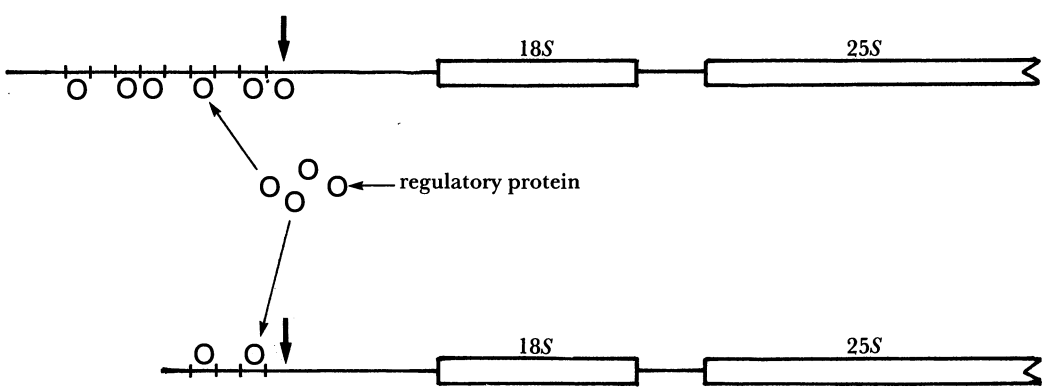

FIGURE 7. A model to explain differential expression of rRNA genes. The model, described in the text, is a simplified version of that of Moss (1983) and Reeder & Roan (1984). It illustrates that a gene with more upstream 'enhancer' elements can bind more regulatory proteins in limiting concentration and/or transcription complexes than a gene with fewer 'enhancer' elements. Genes with more transcription complexes bound in their 5′ upstream regions are transcribed more rapidly than genes with fewer transcription complexes bound. Alternatively, the accumulation of more regulatory proteins and/or transcription complexes on a gene occurs because the binding affinity between the protein and the DNA is greater.

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(Reeder & Roan 1984). It postulates that genes at a dominant nucleolus organizer have a higher affinity for a regulatory protein that is in limiting supply. The increased affinity is due either to there being more copies of the enhancer (binding site) or to each binding site's having a higher affinity for the protein.

If this model is also relevant to nucleolar dominance in plants, then genes with more 'enhancer'-type sequences or with higher affinity for a regulatory protein should be dominant over those with fewer. The genes from *Ae. umbellulata* that are dominant over those in Chinese Spring wheat have a longer upstream region and contain more repeat units (figure 4) (Martini *et al.* 1982). Furthermore, there is a subset of genes at the 1B locus that have more A repeats than those on chromosome 6B (figure 4) (R. B. Flavell & M. O'Dell, unpublished data). However, the subset contains relatively few genes and may be insufficient to account for the larger nucleolus at the 1B locus compared with the 6B locus. Most of the genes at the 1B locus have the same spacer length as those at the 6B locus (figure 4) (R. B. Flavell & M. O'Dell, unpublished data). However, it is possible that the sequences of the repeats differ between the two kinds of gene so that the postulated regulatory protein has a differential binding affinity. This needs to be studied. As described below, promoter and upstream regions of the genes at the 1B locus can be distinguished from those at the 6B locus on the basis of other properties correlated with activity. Thus the data available so far for wheat are consistent with the explanation that differential gene expression is due to variation in regulatory DNA sequences that lie upstream from the promoter. However, more data are required.

The model can be linked to the cytological observations during the cell cycle as follows: the postulated regulatory protein is induced at telophase and binds to rRNA genes. The number of genes bound will be determined by the limiting amount of the protein. Which genes are bound will be determined on a competitive basis by their affinity for the protein. Genes associated with the proteins are able to uncoil, become associated with polymerase I and other specific transcription factors and are responsible for development of a nucleolus. Genes not associated with the protein remain condensed in heterochromatin. When the genes are replicated, those associated with the regulatory protein and the transcription machinery maintain their conformation while those outside the nucleolus become associated with the protein complement characteristic of heterochromatin. At prophase, when nucleolar activity decreases and the nucleolus is disassembled, the regulatory protein remains attached to a subset of the genes to maintain the differential condensation. Thus the subset of genes selected by the regulatory protein is maintained through the cell generations (Flavell 1986).

The model is built upon observations that non-expressed genes are condensed in heterochromatin whereas the active or potentially active genes lie in nucleoli with the sequences in their upstream regions accessible to the protein(s) of the transcription complexes. This was tested by studying the accessibility of genes in nuclei of Chinese Spring wheat with and without the *Aegilops umbelluluta* 1U gene to the protein DNase I. Nuclei were isolated from young leaves, treated with low doses of DNase I, the reaction terminated and the DNA purified. After cleavage of the Chinese Spring DNA with a suitable restriction enzyme, fractionation of the fragments on an agarose gel, transfer to nitrocellulose and hybridization with rDNA, it was concluded that the DNase preferentially attacked the genes of chromosome 1B and those of chromosome 6B with more upstream repeats (figure 4) (W. F. Thompson & R. B. Flavell, unpublished data). In nuclei from Chinese Spring plants containing the dominant 1U locus, the genes at the 1U locus were preferentially cleaved by DNase I (figure 8). Thus the

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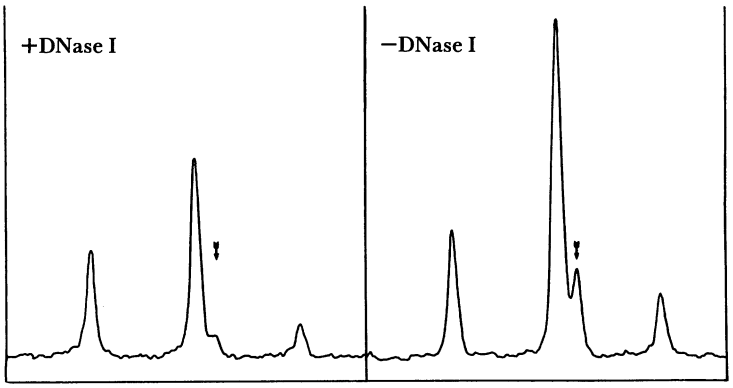

FIGURE 8. Differential susceptibility of rRNA genes in nuclei to DNase I. Nuclei were isolated from young seedlings of Chinese Spring wheat containing chromosome 1U from *Aegilops umbellulata*. One sample (left) was exposed to a brief treatment with DNase I. Another (right) was incubated without DNase I. The DNAs were then isolated, treated with *Bam*HI, fractionated through agarose by electrophoresis, transferred to nitrocellulose and hybridized with ^{32}P-labelled ribosomal DNA. Ribosomal DNA fragments were revealed by autoradiography. The figure illustrates scans of the resulting autoradiographs. The arrows represent fragments exclusively from the *Aegilops umbellulata* rRNA genes that are preferentially degraded by the DNase I compared with the adjacent fragments, which are the equivalent fragments exclusively from the wheat rRNA genes.

accessibility of the genes to DNase I is consistent with the model in that a higher proportion of the genes of the most active locus is accessible to the protein. Also of the two sorts of gene at the less active 6B locus, the one with the greater number of spacer repeats is more accessible.

Subsequent experiments to determine where the genes are most accessible to cleavage by DNase I have shown that there is a preferential cleavage site at each of the A and B repeats as well as around the promotor (W. F. Thompson & R. B. Flavell, unpublished data). This finding strongly supports the hypothesis that the upstream repeats, in the active subset of rDNA genes, are maintained in a configuration that makes them accessible to proteins such as polymerase I and transcription factors.

CYTOSINE METHYLATION AND DIFFERENTIAL GENE EXPRESSION

Wheat rRNA genes in active as opposed to inactive loci are differentially methylated on specific cytosine residues and this is likely to be a part of the system regulating transcription. The status of cytosine methylation has been studied by using the restriction enzymes *Msp* I and *Hpa* II, which both recognize CCGG sites but *Hpa* II does not cleave DNA when the internal cytosine is methylated. There are a large number of CCGG sites in rDNA, in many of which the internal cytosine is methylated. One site is preferentially unmethylated in a subset of the genes. It lies between the upstream A repeats and the promoter (figure 4). In Chinese Spring wheat carrying the dominant 1U locus, most of the genes not methylated at this site lie in the 1U locus (Flavell *et al.* 1983). This contrasts with plants without the 1U locus where many genes at both the 1B and 6B loci are not methylated at this site. In Chinese Spring the genes from the more active 1B locus and the genes with the longer spacer from the 6B locus (figure 4) are preferentially cleaved by *Hpa* II at the site between the A repeats and the promoter compared with the genes with the shorter spacer DNA at the 6B locus. The results parallel closely the susceptibility of the genes to DNase I. Thus differential methylation at one site in

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the spacer DNA is correlated with differential activity of the locus. From a study of a large number of genotypes it has been found that genes with more repeats upstream from the promoter (longer intergenic spacers) are preferentially cleaved by *Hpa* II in comparison with other genes in the same plant. This is consistent with the hypothesis that genes with more intergenic repeats are more active.

The number of rRNA genes at a locus that are not methylated at CCGG sites changes with the genetic background in a manner that correlates with the activity of the locus determined by nucleolar volumes. For example, in a plant where the 6B loci are deleted, the 1B locus becomes more active to compensate and the proportion of 1B genes not methylated at all CCGG sites increases (Flavell *et al.* 1983). Also, as noted above, the number of genes at the 1B and 6B loci that are methylated at all CCGG sites increases when the dominant 1U locus is introduced into the plant.

Cytosine methylation has been shown in many genes to be correlated with non-expression of a gene (Razin & Riggs 1980; Cedar 1984). The location of the site particularly sensitive to changes in methylation for these rRNA genes, immediately upstream from the promoter, suggests that if this sequence is methylated in a wheat rRNA gene, transcription does not occur. Methylation may exert its effect by influencing chromatin structure, by binding a protein, by preventing binding of a protein, or by preventing passage of the transcription complexes from the upstream enhancers to the promoter (Keshet *et al.* 1986). The correlation of the non-methylation pattern with the DNAse I accessibility and activity of rDNA locus implies that the regulatory mechanism determining cytosine methylation is correlated with the mechanisms determining the other properties. In the model outlined earlier, selection of the genes to be maintained in a configuration for transcription was determined by binding of specific nucleolar proteins to the upstream regions and promoters of a subset of the genes. These proteins could interfere with the methylation process and thereby ensure that the subset of genes selected for active use were not incorporated into heterochromatin but kept capable of being transcribed efficiently.

Nucleolar dominance and ribosomal RNA gene evolution

The results summarized in this paper illustrate that the differential expression of rRNA genes within and between wheat loci involves differences in chromatin conformation and cytosine methylation at a specific site in the promoter. These differences are postulated to result from the differential binding of regulatory proteins to reiterated sequences upstream from the promoter. The differential binding between genes occurs because rRNA genes differ in the number and sequence of the upstream sequences postulated to bind proteins. These differences occur between genes within a locus but are present to a greater extent between the arrays of genes at different loci and especially between loci in separate species. This is why nucleolar dominance is stronger and more readily observed in interspecies hybrids. The relative homogeneity of genes within a locus compared with between loci results from the turnover mechanisms such as unequal crossing over and gene conversion, which are responsible for the rapid homogenization of repeats in a tandem array (Smith 1976; Dover 1982; Coen *et al.* 1982; Flavell 1985). These processes frequently lead to the elimination of new variants within a locus and so the genes are maintained relatively homogeneous. Occasionally, however, a new variant is spread through a locus by these mechanisms. Opportunities for gene conversion between loci

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reduce the probability of major divergences between loci within a species. However, for loci in separate populations or species there is no opportunity for unequal crossing over or gene conversion between the loci. It is then highly likely that divergent variants will be fixed in the different loci, as is observed (Appels & Dvorak, 1982*a*, *b*; R. B. Flavell & M. O'Dell, unpublished data).

The activity of a locus relative to another depends not only on the qualities of the genes present but may also depend on the relative numbers of genes at the loci. Results illustrating this were referred to earlier (figure 2). The number of genes at a locus is also altered by unequal crossing over and such changes occur relatively frequently. It is therefore interesting that rRNA genes and their control sequences have organizational features that promote loss, gain and turnover, which can lead to rapid change in evolution. There must be advantages to this sort of organization of genes and regulatory sequences. The organization of genes in tandem arrays offers the opportunity for polymerase molecules to move through one gene to the next to make transcription more efficient. Some evidence for this has been reported for *Xenopus* (Labhart & Reeder 1986) and *Drosophila* rRNA genes (Tautz & Dover 1986). However, the major advantage of turnover mechanisms working at such a high rate is that the very large number of genes and control sequences can be maintained relatively homogeneous and many deleterious mutant forms can be eliminated. However, as stated above, new variants will occasionally be spread by the turnover processes. Before a deleterious variant is eliminated by natural selection it must presumably be amplified to a reasonable proportion of the genes at a locus, otherwise it will have little effect on the phenotype of the organism (Dover & Flavell 1984). This implies that many ill-adapted rRNA genes probably occur within a species but have little consequence on the individual because there are many additional genes to be used to sustain overall rRNA synthesis in the cell. This accumulation of deleterious genes, amplified by the same turnover processes that also serve to maintain optimal genes, is the price that is paid to be able to keep adapted sequences in a high proportion of the thousands of rRNA genes in each individual. The differential expression of different loci and of different groups of genes within a locus is a direct consequence of this system of gene gain, loss and turnover. Because of the large numbers of rRNA genes and the particularly high frequency of molecular events that result in sequence turnover of rRNA genes, the features behind differential rRNA gene expression may not be readily applicable to other genes present in only one or a few copies. However, it is surely likely that differential expression between active genes or alleles is often similarly due to variation in the regulatory sequences that lie upstream from or within promoters.

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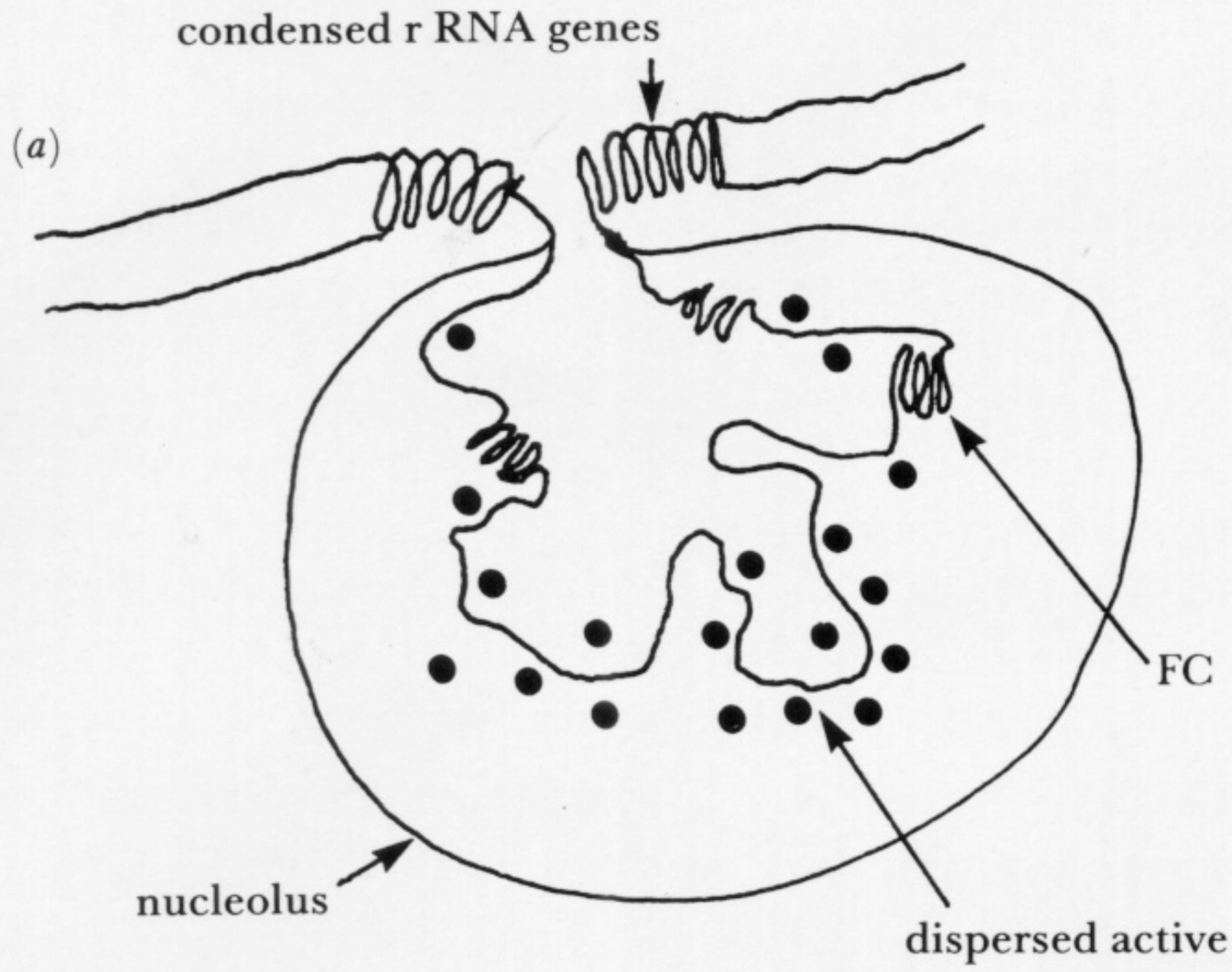

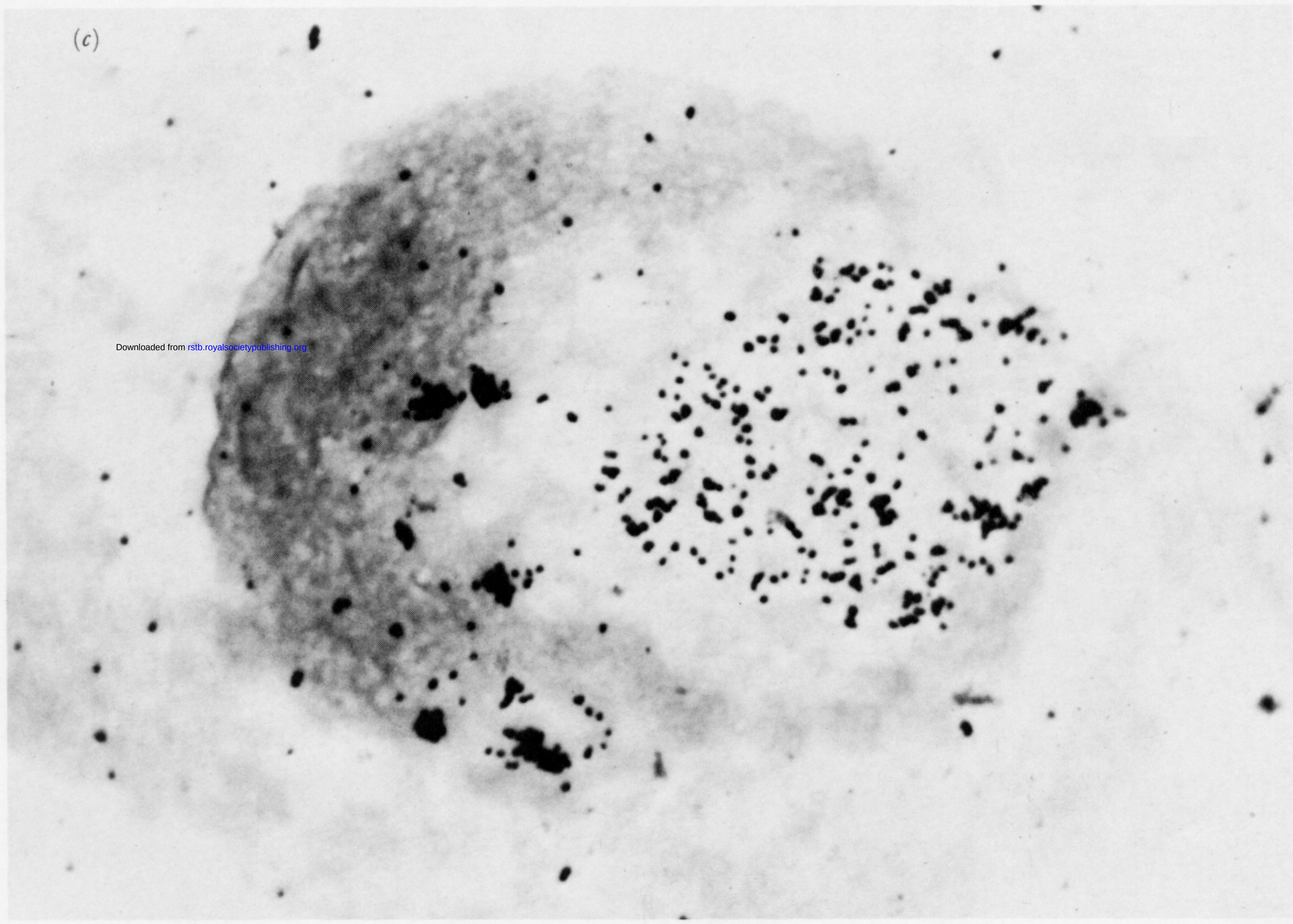

FIGURE 1. Organization of ribosomal RNA genes in the cell cycle. (*a*) A schematic representation of a nucleolus at interphase. Only some of the ribosomal RNA genes are dispersed in the nucleolus. Others remain condensed outside the nucleolus. FC, fibrillar centre. (*b*) A representation of a metaphase chromosome showing the 'constriction' or 'gap' due to the condensation of ribosomal RNA genes that were active in the preceding interphase. (*c*) Organization of rRNA genes in an interphase cell revealed by *in situ* hybridization of ^{3}H-labelled rDNA with a squashed interphase root-tip cell. The sites of hybridization of the probe with the rRNA genes were revealed by autoradiography (Flavell & Martini 1982). The position of the nucleolus is marked by the scattered silver grains on the right side of the nucleus. The major foci of hybridization on the left side of the nucleus are presumed to be condensed rRNA genes.

FIGURE 3. Suppression of wheat nucleolus organizers by a chromosome carrying a nucleolus organizer from *Aegilops umbellulata*. (*a*, *b*) Metaphase chromosomes of Chinese Spring (CS) wheat (*a*) and Chinese Spring + chromosome 1U from *Aegilops umbellulata* (*b*). The nucleolar constrictions are marked ▼. (*c*, *d*) Nucleoli stained in root-tip cells of Chinese Spring (CS) wheat and Chinese Spring + chromosome 1U from *Aegilops umbellulata*.