THE TRANSCRIPTIONAL ACTIVITY OF INDIVIDUAL RIBOSOMAL DNA GENE CLUSTERS IS MODULATED BY SERUM CONCENTRATION*

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SUMMARY
The activity of ribosomal gene clusters has been studied by cytological methods in human cultured cells grown in different amounts of serum under controlled experimental conditions. It has been shown that increasing amounts of serum induce an increase in ribosomal RNA synthesis at the single cell level. Furthermore, the concomitant identification of individual rRNA gene clusters by fluorescent techniques allowed us to demonstrate: (1) that individual gene clusters have differential transcriptional activity and differential frequency of activation; (2) that ribosomal gene activity is closely associated with the amount of silver-positive gene product and; (3) that environmental variations modulate rRNA synthesis by repressing or derepressing specific gene clusters.

INTRODUCTION
Ribosomal RNA gene activity has been shown to be easily and promptly regulated by environmental modifications in a series of living organisms. Bacterial cells respond to amino acid starvation by reducing rRNA synthesis (Gallant & Lazzarini, 1978), as do more complex eukaryotic cells (Hersko, Mamont, Shields & Tomkins, 1971; Grummt, Smith & Grummt, 1976). In starved eukaryotes the activity of rRNA polymerase I is reduced (Franze-Fernandez & Pogo, 1971) due to the lowering of the initiation frequency at the rRNA cistrons (Grummt et al. 1976). In higher organisms the rate of rRNA synthesis is tightly coordinated with the proliferation rate of cells: stimulation of cell growth induces rapid and dramatic increases in rRNA and protein synthesis (Nowell, 1960; Barry & Gorski, 1971). Also, in cultured human cells the quantity and composition of animal serum in the culture medium are known to influence the rate of cell growth significantly (Zakai & Mellman, 1974).

We have studied the effects of different amounts of serum on the ribosomal gene

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activity of human cultured cells under controlled experimental conditions. The cytological approach that was chosen – cytochemical staining (Goodpasture & Bloom, 1975; Bloom & Goodpasture, 1976) of the chromosomal sites (nucleolus organizers, NOs) of active rRNA cistrons (Miller, Dev, Tantravahi & Miller, 1976; Miller, Tantravahi, Dev & Croce, 1976; Croce, Talavera, Basilico & Miller, 1977) – has already been proved to visualize at the cellular level the onset of rRNA synthesis in embryonic systems (Engel, Zenzes & Schmid, 1977; de Capoa et al. 1983) as well as the increase of rRNA synthesis in the erythroleukaemic cell line K562(S) after hemin induction (de Capoa et al. 1985a). The silver-stainable material has been shown to be an acidic protein (Howell, 1977; Hubbell, Rothblum & Hsu, 1979; Williams, Kleinschmidt, Krohne & Franke, 1982) rich in SH groups (Buys & Osinga, 1980; de Capoa et al. 1982) or in both carboxyl and SH groups (Buys & Osinga, 1984). The amount of silver-stainable material is in most cases related to the ribosomal DNA content (Warburton, Atwood & Henderson, 1976; Warburton & Henderson, 1979). However, observed exceptions to this rule (D. A. Miller et al. 1978) demonstrate that rRNA gene activity – expressed as the frequency of involvement in satellite association – is more closely associated with the amount of silver-positive material than with the amount of rDNA (Miller, Tantravahi, Dev & Miller, 1977).

MATERIALS AND METHODS

Leucocyte and fibroblast cultures from the same donor have been set up with standard methods under controlled experimental conditions. The donor, a normal female, has several fluorescent variants of the short arm and centromeric region of acrocentric chromosomes, thereby allowing the individual recognition of all the D- and G-group chromosomes.

Leucocyte cultures were set up from three different blood samples at different times. Fibroblast cultures from a primary explant were analysed at three different passages (3rd, 4th and 5th). In each experiment three different percentages of foetal bovine serum: 5, 10 and 20 %, were used. Leucocytes were grown in Ham F10 supplemented with antibiotics and different quantities of serum from the same batch (FCS; Gibco). Phytohaemagglutinin (PHA; Wellcome) in the concentration of 0.15 ml in 10 ml of complete medium was added to the cultures. Twenty drops of heparinized (Liquemin, Roche) whole blood were seeded in each 10 ml vial. Cultures were arrested after 72 h by adding colchicine (Fisher; 10^{-7} M) for the last 1.5 h of culture, and harvested by standard methods.

Fibroblast cultures were set up in RPMI 1640 (Flow) supplemented with antibiotics and different amounts of serum from the same batch (FCS; Gibco). In situ preparations from logarithmically growing cells were obtained by adding colchicine (final concentration 0.5 μg/ml) for 5 h, 48 h after seeding.

In all the experiments the same batches of serum, medium, phytohaemagglutinin, colchicine and the same growth and harvesting conditions were used.

Staining methods: individual identification of each NO-bearing chromosome was achieved by sequential Chromomycin A3 (Boehringer) staining (identification of homologues 15p, 21p and 22p) followed by Quinachrine mustard (Sigma) staining (identification of 13c and 14s) and by treatment with AgNO3 solution according to the technique of Howell & Black (1980) for the identification of silver-positive NOs.

Experiments were performed according to the following protocol: (1) Evaluation of the total number of silver-positive NOs per cell was performed on cell populations from both leucocyte and fibroblast cultures by counting the number of silver-positive NOs (active rDNA clusters) on the D- and G-group chromosomes in metaphase spreads. Counts were done under oil immersion by at least two independent observers. (2) Identification of individual silver-positive NOs in single
cells was performed on subsamples of the general leucocyte and fibroblast populations cultured in 10 and 20% serum concentration, after sequential Chromomycin A3/Quinachrine mustard/silver staining, by three independent observers on photographic prints.

Fig. 1. Distribution of silver-positive NOs per cell in leucocyte cultures grown in 5 (A), 10 (B) and 20% (C) FCS. A, \( \bar{x} = 6.73, n = 116; \) B, \( \bar{x} = 7.15, n = 219; \) C, \( \bar{x} = 7.56, n = 210. \)
RESULTS AND DISCUSSION

Total number of silver-positive NOs in cell populations

The three leucocyte cultures set up at different times with the same batch of FCS gave very similar results. Data have therefore been pooled in the final elaboration (Fig. 1). For fibroblast cultures, data obtained at different passages with the same serum used for leucocyte experiments are shown in Fig. 2. In Fig. 3 data from these experiments (A) are compared with data from previous experiments (B, C), in which serum from different sources was used. The consistency of repeat leucocyte experiments at the same serum concentration is shown in Fig. 4.

As shown in Figs 1 and 2, increasing percentages of serum result in increased numbers of Ag-positive NOs per cell and in a shift to the right of the distribution of silver-positive NOs per cell in both the leucocyte (Fig. 1) and the fibroblast (Fig. 2) populations. The average values increase from 6.71 to 7.15 and 7.56 in leucocytes and from 7.37 to 7.65 and 7.88 in fibroblasts. The increases are statistically significant (Table 1). The modal values shift from 7 to 8 in leucocytes (5 to 20% FCS), while in fibroblasts the number of cells in the modal class increases from 52 to 85% (Table 1). Data shown in Figs 1 and 2 also show that in both tissues the number of cells with 8 and 9 Ag-positive NOs increases progressively with increasing serum concentrations. Recent data from our laboratory (de Capoa et al. 1984b) have shown that the cells with 8 Ag-positive NOs have 6 D- and 2 G-group chromosomes (the 21p and the 22), while those with nine have an additional G-group (either the 21 or the 22p) silver-positive chromosome. These data demonstrate that in this material all gene clusters are potentially active and can all be induced — though with different frequencies — under permissive environmental conditions.

Results analogous to those for the increase in the number of silver-positive NOs per cell at higher serum concentrations were obtained from fibroblast cultures grown in medium supplemented with FCS from different sources (Fig. 3). It can be seen from Fig. 3 that all the experiments show a very good internal consistency. Repeat leucocyte cultures from the same donor performed at different time intervals and grown under the same experimental conditions (Fig. 4) also gave quite consistent results. The homogeneity of the response obtained from the two tissues suggests that the small amount of autologous serum in leucocyte cultures does not exert a critical influence on the results. Results obtained in repeat experiments on lymphocyte cultures from the same donor further support this assumption.

Identification of individual active rRNA clusters

Several fluorescence variants of the NO-bearing chromosomes that allow intrapair recognition of homologues can be identified in this donor (Fig. 5). By sequential Chromomycin A3/Quinacrine mustard/silver-staining (Fig. 6) the frequency of activation of individual rDNA gene clusters can be determined. The amount of silver precipitate on individual NOs can also be roughly evaluated (Fig. 6).

The detailed analysis at the single gene cluster level summarized in Fig. 7
Modulation of rDNA clusters activity

Fig. 2. Distribution of silver-positive NOs per cell in fibroblast cultures grown in 5 (A), 10 (B) and 20% (C) FCS. A, \( \bar{x} = 7.37, n = 165; \) B, \( \bar{x} = 7.65, n = 159; \) C, \( \bar{x} = 7.88, n = 186. \)

demonstrates that in cells grown under more restrictive conditions three gene clusters in leucocytes (those on chromosomes 14s, 15 and 22) and four in fibroblasts (those on 14s, 15, 15p and 22) are preferentially silver-stained in more than 90% of cells. Two
others, those on chromosomes 21 and 22p, are preferentially inactive, showing very low staining frequencies in both tissues at both serum concentrations. All the other

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Fig. 3.** Fibroblast cultures: distribution of silver-positive NOs per cell in repeat experiments on cells from the same donor grown in serum from three different sources (A, B, C). In A the same data as presented in Fig. 2 are shown for comparison. (-----) 20%, (---) 10%, (---) 5% FCS.
Modulation of rDNA clusters activity

Table 1. Average and modal values of silver-positive NOs per cell in leucocyte and fibroblast cultures grown with different amounts of serum

<table>
<thead>
<tr>
<th>Serum concentration</th>
<th>Leucocytes</th>
<th></th>
<th>Fibroblasts</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average value</td>
<td>Modal class</td>
<td>Average value</td>
<td>Modal class</td>
</tr>
<tr>
<td>5%</td>
<td>6.71±0.98</td>
<td>7</td>
<td>7.37±0.78</td>
<td>8(52%)</td>
</tr>
<tr>
<td>10%</td>
<td>7.15±0.88</td>
<td>7(38%)</td>
<td>7.65±0.68</td>
<td>8(74%)</td>
</tr>
<tr>
<td>20%</td>
<td>7.56±0.69</td>
<td>8(38%)</td>
<td>7.88±0.53</td>
<td>8(85%)</td>
</tr>
</tbody>
</table>

Significant activity levels with the t-test (2nd and 3rd rows) are also shown. Numbers in parenthesis indicate the percentages of cells in modal class.

NOs, about half of the total number, show increased frequency of selective staining at the higher serum concentration, with the possible exception of chromosome 21p in leucocytes (Fig. 7). A comparative cytological analysis of gene activation frequency in the two tissues has been presented elsewhere (de Capoa et al. 1985a).

Fig. 4. Leucocyte cultures. Distribution of silver-positive NOs per cell in three repeat experiments (····, ---, ----) on cells from the same donor grown in medium supplemented with the same serum. Serum concentrations are shown above.
It can be concluded that of the 10 potentially active rRNA clusters, some are preferentially stained—constitutively transcribing—even under restrictive conditions while the majority is induced and its activity is modulated under more permissive environmental conditions. The remaining two (21 and 22p), mostly observed as silver-positive in cells with nine positive NOs, appear to be induced under the most favourable conditions.

These findings could be interpreted as the result of clonal selection, favouring under permissive conditions clones having higher rRNA transcriptional activity. However, the analysis of coincident silver positivity of the different NOs ('silver-staining patterns') in cells from a subsample of the two populations (Table 2) shows that at 10% several different patterns (10 in leucocytes and 8 in fibroblasts) are observed, many of which have high numbers (7 and 8) of positive NOs per cell, while none has nine. At the higher serum concentration the number of patterns is reduced to 7 and 3, respectively, while patterns with 9 positive NOs, which further confirm data shown in Figs 1 and 2, can also be observed. Also, some of the higher patterns observed at 10% serum were not to be observed at 20% and, conversely, some of the lower
Fig. 6. Leucocyte cultures; 10% FCS. Sequential Chromomycin A3 (A) /Quinachrine mustard (B) /silver staining (C) of the same metaphase. In A chromosomes 15, 21p and 22p (large arrowheads) can be identified by short-arm fluorescence. Small arrowheads indicate the homologues. In B chromosomes 13c and 14s are identified by bright fluorescence of the centromeric region (13c) and of the satellites (14s). Small arrowheads indicate the homologues. In C note large amounts of silver on chromosomes 14s, 15 and 22 (arrowheads). Chromosomal NOs 21 and 22p are silver-negative.
patterns seen with 20% serum could not be observed at 10%. Furthermore, some of the patterns with high numbers of Ag-positive NOs are not the same at both serum concentrations. These data do not support the idea of clonal selection favouring cells with higher transcriptional activity. Pattern analysis is then indicative of generalized gene activation involving several gene clusters, with different frequencies. This interpretation is in agreement with recent data on generalized rRNA gene activation observed 48 h after induction in erythroid-induced K562(S) cells (de Capoa et al. 1985a).

In the course of these studies it was also observed that some chromosomal NOs show much larger amounts of silver-stainable material than others. The identification of the chromosomal location of the NOs by sequential fluorochrome/silver-staining (Fig. 6) demonstrates that the largest amount of silver precipitate is located over the preferentially (over 90% frequency) stained NOs. This observation, which confirms our previously published data on preferential silver-staining of some NO variants (de Capoa et al. 1978), suggests a strict correlation between preferential silver-staining, i.e. preferential transcriptional activity of some gene clusters, and large quantities of silver-stainable material. Conversely, clusters having very low frequency of activation (Fig. 7), also show very low amounts of silver-positive material (Figs 6, 8). Analogous results on the low frequency of activation of some weakly Ag-positive rRNA clusters have recently been obtained in hemin-induced K562(S) cells (de Capoa et al. 1985a).

It can then be concluded that high frequency of activation is usually associated with large amounts of silver-stainable material located on specific chromosomal NOs. Published data (D. A. Miller et al. 1977) demonstrating that large quantities of silver positive material are associated with a high frequency of satellite associations further support this conclusion.

The whole of our results demonstrate that increasing levels of serum in the culture medium activate rRNA synthesis and induce higher rates of transcription by acting on individual gene clusters in individual cells. These findings are in agreement with data from the general literature showing that the quantity of serum influences cell growth and division, and that rapidly growing cells are accumulating rRNA (Emerson, 1971; Learned, Small, Haltiner & Tjian, 1983); in fact, all the cultures analysed were in the logarithmic phase of growth. Conversely, our data also agree with published data on decreased rRNA transcription under conditions of starvation for amino acid, glucose, phosphate or serum (Hersko et al. 1971; Grummt et al. 1976; Learned et al. 1983), as well as with cytological findings on nucleolar alterations in starved human fibroblasts (Jordan & Mc Govern, 1981) and on the reduction of silver-positive material in inactive cells such as unstimulated lymphocytes or prematurely condensed chromosomes (for a review, see Schwarzacher & Wachtler, 1983).

Fig. 7. Frequency of activation (%) of individual ribosomal gene clusters expressed as silver positivity of the NOs at 10% (left) and 20% (right) serum concentrations in leucocyte (A) and fibroblast (B) cultures. The fluorescent variants of marker chromosomes (c, s, p) are shown in black.
Modulation of rDNA clusters activity

Fig. 7
<table>
<thead>
<tr>
<th>Silver staining pattern</th>
<th>Leucocytes</th>
<th>Fibroblasts</th>
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<tbody>
<tr>
<td></td>
<td>No. of cells</td>
<td>D-group</td>
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<tr>
<td><strong>10 % serum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>+</td>
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<tr>
<td>U</td>
<td>1</td>
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<td>P</td>
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<td>V</td>
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<td>Q</td>
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<td>T</td>
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<tr>
<td><strong>20 % serum</strong></td>
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<tr>
<td>A</td>
<td>8</td>
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<tr>
<td>B</td>
<td>3</td>
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<td>C</td>
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<td>D</td>
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<tr>
<td>E</td>
<td>1</td>
<td>+</td>
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<td>F</td>
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<tr>
<td>G</td>
<td>1</td>
<td>+</td>
</tr>
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</table>
The following conclusions can be drawn: (1) All the rDNA clusters are transcriptionally active in this material, though with different activation frequencies. (2) The transcriptional activity of several rDNA clusters in two different tissues appears to be regulated by simple quantitative changes in the nutrient mixture, thereby suggesting a critical influence of some serum constituent(s). This in vitro situation may mimic environmental variations occurring in vivo. (3) In each cell the activation of individual ribosomal gene clusters seems to follow a precise hierarchy, clusters being sequentially activated in order of decreasing efficiency in their transcriptional activity. Environmental factors are then acting on a genetically determined background by sorting out in sequence clusters having higher transcriptional activity. (4) High frequency (preferential) gene activation is related to large quantities of silver-positive material: in fact, the chromosomal sites of preferentially active, constitutively transcribing rDNA clusters are heavily silver stained, those of high-frequency inducible clusters are less heavily stained, while seldom induced clusters are weakly stained. It may be assumed that under restrictive conditions it is more convenient for cell economy to 'switch on' the most active — either in terms of gene number or of higher activity — transcriptional units, while under more permissive conditions additional, less active units can in turn be switched on when necessary. (5) The cytological approach, which analyses in fine detail the transcriptional activity of ribosomal genes allows to demonstrate that: (a) the transcriptional activity of these genes is regulated in individual cells at the single gene cluster level; (b) the rate of transcription is modulated by repression or derepression of individual clusters according to the metabolic requirements of cells; and (c) rRNA gene activity is modulated by environmental factors.
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REFERENCES


Modulation of rDNA clusters activity


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