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Transcription complexes that program *Xenopus* 5S RNA genes are stable *in vivo*

(oocyte injection/chromatin/transcription factor TFIIIA)

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ABSTRACT **The long-term stability of transcription complexes on 5S RNA genes has been demonstrated *in vivo*. Complexes on oocyte and somatic-type 5S RNA genes injected into *Xenopus laevis* oocyte nuclei are stable for at least 4 days. Tissue culture cells and mature erythrocytes have equivalent numbers of somatic 5S RNA genes programed into transcription complexes, yet the former cell type has a >50-fold higher cellular content of transcription factor IIIA (TFIIIA). Functional transcription complexes on somatic 5S RNA genes in nucleated erythrocytes of *Xenopus* are stable for weeks, perhaps months, even though a mature erythrocyte has less than two molecules of TFIIIA for each somatic 5S RNA gene. These findings strengthen our proposal that stable transcription complexes are a means of maintaining the differentiated state.**

The expression of *Xenopus* 5S RNA genes, as it has been studied *in vitro*, is controlled by the interaction of multiple proteins that interact with a region of DNA within the gene called the internal control region (1–5). This transcription complex is characterized by a remarkable stability in some extracts such that it survives many rounds of passage by RNA polymerase III (6). We have argued that a transcription complex can account at least in part for the maintenance of the differentiated state (6, 7). This is the lasting epigenetic imprint that causes nondividing terminally differentiated cells to express one set of genes and to repress another set. Stable transcription complexes have been demonstrated *in vitro* for genes transcribed by all three classes of RNA polymerase (8–10) and on isolated chromatin for class III genes (11–13). In this paper we present experiments to show that transcription complexes that control the accurate initiation of 5S RNA genes are stable *in vivo*.

METHODS

Plasmids. All recombinant DNA constructs containing one 5S RNA gene have been previously described: the *Xenopus laevis* oocyte 5S RNA gene pXlobs (14); the *Xenopus borealis* 5S RNA genes pXbs115/105, pXbs115/77, and pXbs3′Δ+124 (15); a *X. laevis* methionine transfer RNA gene, $pXlt^{met}$ (16). Head-to-tail multimers were prepared by two methods. The *Ava* I/pARA technique (17) was used to generate pmXbs115/77 and pmTM10 (a multimer of $pXlt^{met}$). The multimer pmXbsΔ124 was constructed from a monomer with a *Bgl* II site at one end and a *Bam*HI site on the other. Ligation in the presence of the two restriction enzymes ensures the generation of head-to-tail multimers (18). The multimer was cloned into the *Bam*HI site of pUC9.

Oocyte Injections. Stage VI (Dumont) oocytes were injected essentially as described by Colman (19). Oocytes were released from surrounding follicle cells by gently shaking in 0.2% collagenase (Sigma, type II) in Ca^{2+}-free OR2 medium (20) for ≈90 min at room temperature. Oocytes were generally maintained in OR2 medium unless they were used for incubations longer than 48 hr, in which case an L15 (Liebowitz)-based medium was used (21). DNA was dissolved in 88 mM NaCl/10 mM Tris·HCl, pH 7.5, before injection.

Transcription of Nuclei. Following injection and incubation at 19°C, germinal vesicles (nuclei) were manually dissected from oocytes under J buffer (22). Individual nuclei were transferred in 1 μl of J buffer to 0.4-ml reaction vials and then transcribed immediately or frozen rapidly in liquid nitrogen and stored at −80°C.

Isolated nuclei were transcribed in a total volume of 5 μl of J buffer containing 0.2 mM ATP, 0.2 mM CTP, 0.2 mM UTP, 0.02 mM GTP, and 7 units of RNasin (Boehringer Mannheim). Transcripts were labeled for 2 hr at 22°C by addition of 10 μCi (1 Ci = 37 GBq) of [α-^{32}P]GTP (New England Nuclear) and separated by electrophoresis on polyacrylamide gels under denaturing conditions (1).

Chromatin Preparation and Electrophoretic Transfer Blotting. Chromatin was prepared from purified tissue culture cell nuclei as described by Schlissel and Brown (11), from liver by the method of Woll *et al.* (23), and from mature erythrocytes as described by Cozzarelli *et al.* (24). Transcription of chromatin was performed *in vitro* as described by Andrews and Brown (25). DNA content was quantitated by fluorimetry (26). Quantitative electrophoretic transfer blotting of somatic cell extracts was performed with a rabbit antibody directed against transcription factor IIIA (TFIIIA), followed by ^{125}I-labeled protein A (Amersham) (25). Tissues were extracted directly into a $NaDodSO_4$-containing gel loading buffer and alkylated with iodoacetamide, and the proteins were separated by electrophoresis according to Ryrie and Gallagher (27). The efficiency of extraction of TFIIIA from the tissues was determined by adding known amounts of TFIIIA to duplicate extracts of the same tissues and electrophoresing these samples and blotting them alongside the control samples. TFIIIA was quantitated by liquid scintillation spectroscopy of the radioactive protein band bound to nitrocellulose.

Methods for the preparation of oocyte nuclear extract for *in vitro* transcription (22) and the purification of TFIIIA (28) and RNA polymerase III (24) have been described.

RESULTS

Transcription Complexes Assembled on 5S RNA Genes Are Stable in Living Oocyte Nuclei. *Xenopus* oocytes whose nuclei have been injected with cloned 5S RNA genes can be maintained in culture for days and will express the exogenous genes throughout this period (29). Since relatively large quantities of exogenous DNA can be introduced into an oocyte nucleus and assembled into chromatin, this experi-

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Abbreviation: TFIIIA, transcription factor IIIA.

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mental system was used to test for complex stability by the "template exclusion" method (6). In this assay, limitation of one or more transcription complex components, other than RNA polymerase, is achieved with an excess of gene template. The stability with which the transcription factors are sequestered by that gene is assayed by transcription from a subsequently added gene.

The quantity of 5S DNA that was required to be injected into the nucleus to sequester a limiting transcription factor was determined empirically as the amount that saturates the rate of transcription. About 100 ng of a 4-kilobase (kb) plasmid containing one copy of a 5S RNA gene is needed to saturate the transcription rate of 5S RNA (30), an amount that compromises oocyte survival in culture. To reduce the DNA requirement, head-to-tail multimers of 5S RNA genes were constructed. The two multimers used in this study, pmXbsΔ124 and pmXbs115/77, each contain about 10 copies of previously described somatic-type 5S RNA genes (see *Methods*). Titration of the injected multimers indicated that transcription rates did not increase when >20–30 ng of multimer DNA was injected per nucleus (data not shown).

Our scheme to determine the stability of complexes was to inject 30 ng of a 5S DNA multimer into the nucleus of an oocyte to sequester completely a limiting transcription factor. The transcription complex associated with this gene was then challenged by injection of a second 5S RNA gene (the "reporter gene"). The reporter gene monitors the availability of functional transcription factor(s). At various times after the second injection, between 2 hr and 6 days, the germinal vesicle was dissected from the oocyte and the activity of the two 5S RNA genes was measured by incubation with radioactive precursor. Individual oocytes rather than pooled samples were analyzed for each experiment because successful template exclusion of the reporter gene results in the absence of RNA synthesis from that gene, and hence the physical presence of both genes in the nucleus must be demonstrated. Therefore, half of the *in vitro* labeling reaction mixture from a single nucleus was used for RNA analysis by denaturing polyacrylamide gel electrophoresis and the remainder was used to determine DNA content by Southern blotting.

Fig. 1*A* illustrates the transcription pattern obtained for sequential injection of the 5S DNA multimer, pmXbs115/77, and the monomer plasmid, pXbs115/105, which encode electrophoretically distinct 5S RNA transcripts. Template exclusion of the reporter gene occurs only when another 5S RNA gene has been injected earlier, in which case labeled transcripts derive solely from the first gene (lane 3). If the first DNA is plasmid DNA lacking 5S RNA genes, then the reporter gene (pXbs115/105) is transcribed (lane 4) with the same efficiency as when it is injected alone (lane 2). A coinjection of the two 5S RNA genes is shown in lane 1 and illustrates the ratio of transcripts that would be expected if the two genes competed for the factors. Southern blots confirm the physical presence in the nuclei of both injected genes that were used for transcription (data not shown).

Transcription factors are stably and specifically bound to the first 5S RNA gene for 24 hr *in vivo* (Fig. 1*A*, lanes 5 and 6). The viability of oocytes in culture enabled us to monitor template exclusion for several days. Fig. 1*B* shows that the complexes assembled on the 5S DNA multimer are stable for at least 4 days.

The 5S DNA multimer does not exclude a second 5S RNA gene by limitation of RNA polymerase in the nucleus. This was revealed in an experiment designed to determine which factor was being limited in the oocyte nucleus. tRNA genes are transcribed by polymerase III, and fractionation of cell extracts indicates that some transcription initiation factors are shared with the 5S RNA genes (31). Thus we asked whether a 5S DNA multimer is able to exclude a tRNA gene from transcription by sequestration of these common factors.

The results presented in Fig. 2 show clearly that a 5S RNA gene can readily exclude a second 5S RNA gene but not a tRNA gene when the latter two are coinjected as reporter genes (compare lanes 3 and 4). Thus, RNA polymerase III cannot be limiting.

This result implies that a transcription complex component specific to the 5S RNA gene is stably sequestered. This factor appears to be TFIIIA, since coinjection of purified TFIIIA with a reporter 5S RNA gene enables expression of a gene that would otherwise be excluded (data not shown).

Stable Transcription Complexes Are Assembled on Oocyte and Somatic-Type 5S RNA Genes in Oocyte Nuclei *in Vivo*. The relative stability of complexes assembled on oocyte and somatic 5S RNA genes was determined by a competition protocol (Fig. 3). A comixture of oocyte and somatic 5S RNA genes was injected into an oocyte nucleus, and the oocytes were incubated at 19°C to allow complex assembly. The 10:1 ratio of oocyte to somatic 5S RNA genes injected resulted in the expected 2:1 ratio of transcripts (14). The same oocyte nuclei were then injected with a large amount of somatic 5S DNA multimer, an amount already shown to exclude 5S RNA genes if injected as the first template. The multimer thus acts as a sink for free or dissociating transcription factors. The number of monomer genes injected first is insufficient to exclude completely the somatic multimer. The

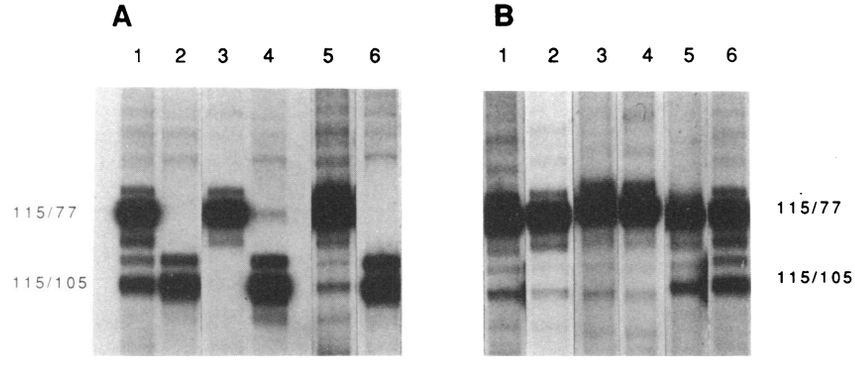

FIG. 1. Transcription complexes of 5S RNA genes are stable in oocytes. (*A*) Autoradiograph of transcripts labeled by *in vitro* incubation of isolated oocyte nuclei with [α-^{32}P]GTP for 2 hr. Oocyte nuclei were injected with the following: lane 1, a comixture of 30 ng of pmXbs115/77 and 20 ng of pXbs115/105; lane 2, 20 ng of pXbs115/105; lane 3, 30 ng of pmXbs115/77 followed after 2 hr by 20 ng of pXbs115/105 (individual nuclei were isolated for transcription *in vitro* 2 hr later); lane 4, as in lane 3, except that the first injection was 30 ng of pARA, the vector DNA; lane 5, as in lane 3, except that nuclei were isolated for labeling after 24 hr; lane 6, as in lane 4, but nuclei were isolated for labeling after 24 hr. (*B*) Oocytes were injected with 30 ng of pmXbs115/77 and 2 hr later with 20 ng of pXbs115/105. Nuclei were isolated and labeled after 24 hr (lane 1), 48 hr (lane 2), 72 hr (lane 3), 96 hr (lane 4), and 108 hr (lane 5). Lane 6, coinjection of pmXbs115/77 and pXbs115/105.

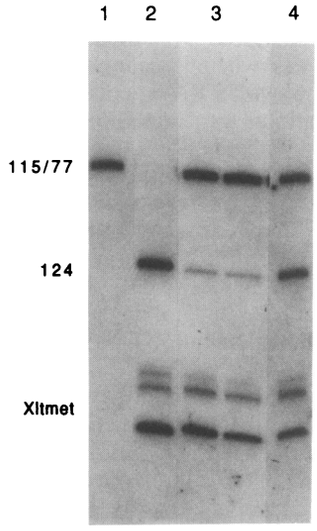

FIG. 2. A 5S RNA gene excludes another 5S RNA gene but not a tRNA gene *in vivo*. Oocytes were injected with genes as described below. Transcripts were labeled by injection of 0.5 μCi of [α-^{32}P]GTP and incubated for 2 hr at 19°C. Lane 1, 30 ng of pmXbs115/77; lane 2, 20 ng of pmXbsΔ124 and 2 ng of pmTM10; lane 3, pmXbs115/77 followed by a comixture of pmXbsΔ124 and pmTM10 4 hr later (duplicates); lane 4, comixture of pmXbs115/77, pmTM10, and pmXbsΔ124.

ratio of transcripts initiating from the oocyte and somatic 5S RNA genes does not change upon challenge by injection of the multimer, indicating that the transcription complexes assembled on each gene have comparable stability in the oocyte nucleus. Transcript initiation from the tRNA gene is also unaffected by the somatic 5S DNA multimer but, as described above, this is due to the fact that excess 5S RNA genes do not readily exclude tRNA genes.

Stable Transcription Complexes on Endogenous 5S RNA Genes. Having demonstrated the presence of stable transcription complexes *in vivo* in an experimentally manipulable system such as the oocyte, we then asked whether stable complexes could be directly demonstrated on endogenous 5S RNA genes. We reasoned that we would need to demonstrate the presence of functional transcription complexes in the chromatin of a cell that was devoid of at least one of the transcription complex components. Purified chromatin from various cell types can be transcribed *in vitro* by the addition of purified RNA polymerase III to generate specific transcripts (24). Under conditions of polymerase excess, the relative abundance of transcripts from different preparations of chromatin indicates the relative number of genes programed into transcription complexes, since RNA-processing activities are absent from these preparations. The cellular content of one component of the transcription complex, TFIIIA, can be measured by electrophoretic transfer blotting (25).

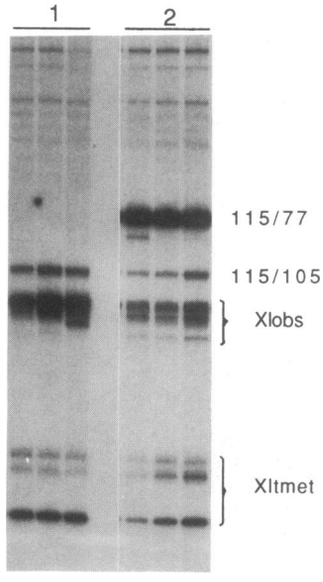

FIG. 3. Transcription complexes on oocyte and somatic-type 5S RNA genes are stable in the oocyte. A mixture of genes, pXlobs, pXbs115/105, and pXlt^{met} (10 ng, as a 10:1:1 gene ratio) was injected into oocytes. The oocytes were incubated for 16 hr at 19°C in OR2 medium, and nuclei were dissected and labeled *in vitro* for 2 hr with [α-^{32}P]GTP (lanes 1, triplicate). A second group was injected with the gene mixture followed 1 hr later by 30 ng of pmXbs115/77 before incubation and labeling as above (lanes 2, triplicate).

We compared the transcriptional properties of chromatin preparations and the TFIIIA content of three cell types having widely varying metabolic activities: cardiac-derived tissue culture cells that divide about once a day, adult liver, and mature, transcriptionally inactive, circulating erythrocytes. The amphibian erythrocyte synthesizes 5S RNA along with other housekeeping products of the cytoplasm early in its life cycle. It then accumulates hemoglobin and discards cytoplasmic structures such as ribosomes. A mature nucleated erythrocyte is transcriptionally silent for months; it has no detectable endogenous RNA polymerase III (32, 33).

The chromatin transcription patterns of the three cell types are shown in Fig. 4*A*. All three chromatin preparations transcribed their endogenous 5S RNA genes with approximately equal efficiency, varying by at most 2-fold, indicating that each kind of chromatin was programed with similar numbers of transcription complexes. In each case, the 5S RNA transcripts were of the somatic type as determined by a hybridization assay [data not shown (11)]. In contrast to the similar transcription levels of the three kinds of cells, their TFIIIA contents are widely different (Fig. 4*B* and quantitated in Table 1). The three kinds of cells contained predominantly the larger form of TFIIIA (TFIIIA′) (34). Only tissue culture cells had detectable amounts of the 38.5-kDa form of TFIIIA that is so abundant in oocytes. It should be noted here that the amount of TFIIIA and TFIIIA′ in tissue culture cell extracts is higher than those previously reported (34, 35). The difference is that the measurements reported in Table 1 were made on total extracts, whereas those made previously were carried out on supernatant fractions. The TFIIIA′ in erythrocytes can barely be detected, and the value we estimate is a maximal one since contamination from leukocytes or immature erythrocytes cannot be excluded completely.

Though we are unable to make the statement that there is no free nuclear TFIIIA in circulating erythrocytes, the cellular content of TFIIIA is unrelated to the state of programing of 5S RNA genes in the three somatic cell types. In mature erythrocytes there are at most two molecules of TFIIIA per somatic 5S RNA gene. We infer from these data that the complexes programed into erythroblasts are subsequently stable for the lifetime of the cell.

DISCUSSION

The demonstration of stable transcription complexes previously shown *in vitro* as a property of certain cell-free extracts (6) has been extended to living cells. Complexes formed with either oocyte or somatic 5S RNA genes injected into oocyte nuclei remain stable for as long as 4 days (Fig. 1*B*). Beyond that, we were unable to determine if complexes dissociate since the oocytes deteriorated, and the experiment could not be extended for longer periods.

Oocyte and somatic 5S RNA genes form stable transcription complexes in extracts of oocyte nuclei *in vitro* (6).

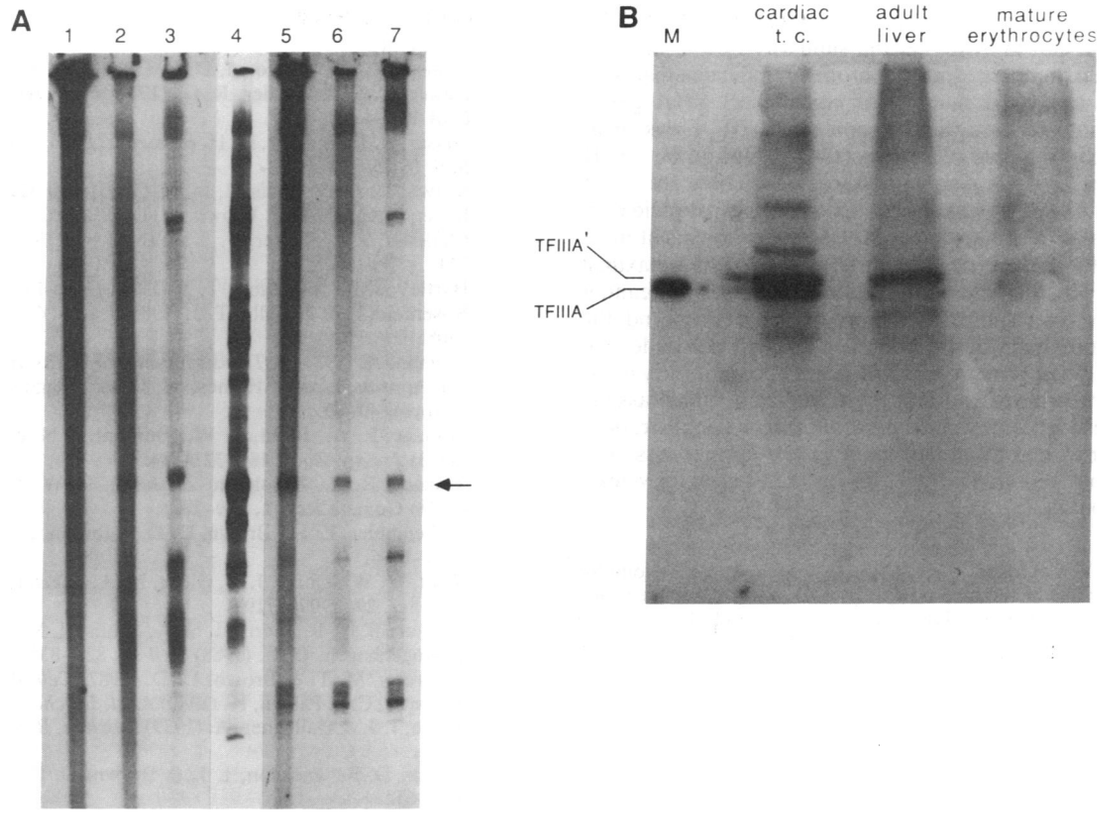

FIG. 4. Comparison of chromatin transcription and TFIIIA content of somatic cells. (*A*) Chromatin preparations from tissue culture cells (lanes 1 and 5), adult liver (lanes 2 and 6), and mature erythrocytes (lanes 3 and 7) were transcribed *in vitro* with either purified *Xenopus* RNA polymerase III (lanes 1–3) or an oocyte nuclear extract (lanes 5–7). Purified, genomic *X. laevis* DNA transcribed in oocyte nuclear extract is shown in lane 4 for comparison. The arrow marks the position of 5S RNA. (*B*) Electrophoretic transfer blot of tissue homogenates separated by $NaDodSO_4$/polyacrylamide gel electrophoresis. M, marker lane containing 2.5 ng of purified TFIIIA; t.c., tissue culture.

However, complexes with oocyte 5S RNA genes are destabilized in extracts of unfertilized eggs under conditions where complexes with somatic 5S RNA genes are stable (36). This differential stability of transcription complexes has been proposed to play a major role in the differential control of the two genes in embryogenesis, accounting for the selective repression of oocyte 5S RNA genes in somatic cells. It seems probable that the transcription complex on the oocyte 5S RNA gene, which from these studies is stable in the oocyte nucleus, is destabilized during meiotic maturation, since injection of DNA into unfertilized eggs or *in vitro* matured oocytes shows the preferential somatic gene transcription characteristic of the same genes added to an egg extract (14, 36).

A second way of demonstrating stable complexes comes from an analysis of three kinds of somatic cell types—cells in culture, liver, and nucleated erythrocytes. These cells are known to be highly active, moderately active, and inactive, respectively, with regard to their transcriptional activity *in vivo*. Yet there is very little difference in the ability of their isolated chromatin to support somatic 5S RNA and transfer RNA synthesis. The oocyte 5S RNA genes are stably repressed in all three types of chromatin. Only purified RNA polymerase III need be added to the chromatin to transcribe the somatic 5S RNA genes. Equivalent transcription is therefore evidence that about the same number of RNA polymerase III genes are programed into active transcription complexes. The interesting feature of nucleated erythrocytes is that they have little, if any, detectable TFIIIA (Fig. 4), no endogenous RNA polymerase III, or, for that matter, the other transcription factors required for tRNA and 5S RNA gene transcription. This latter fact is deduced from the inactivity of erythrocyte extracts in transcription assays (unpublished data). Maximal transcription rate for a 5S RNA gene has been estimated to be several hundred RNA molecules synthesized per gene per hour (37). There are many possible reasons for the inefficient

Table 1. Comparison of somatic 5S RNA gene transcription rates with the cellular TFIIIA content in three chromatin preparations

Chromatin source	Transcription rate*		TFIIIA concentration	
	Oocyte nuclear extract†	RNA polymerase III‡	Molecules, no. per cell × 10^{-3}	Molecules, no. per somatic 5S gene
Tissue culture	5	6	90	110
Liver	3	3	6	8
Erythrocytes	6	7	<2	<2

*Number of transcripts per somatic 5S RNA gene per hour.
†Transcription of chromatin in oocyte nuclear extract.
‡Transcription of chromatin with purified RNA polymerase III. These numbers are the result of a single experiment.

rates using isolated chromatin as a template (Table 1), but the explanation is not known. Yet the similarity in transcription rates between the three kinds of chromatin indicates that they have about the same number of somatic 5S RNA genes programed into transcription complexes. TFIIIA was measured directly in whole cell extracts and found to be <0.04 molecule per 5S RNA gene in erythrocytes. There are about 40,000 oocyte and 800 somatic 5S RNA genes in a diploid cell. The figure of 0.04 molecule per 5S RNA gene means at most two copies for each somatic 5S RNA gene. This is a maximal value for TFIIIA in these cells. This level of sensitivity cannot be increased without fractionation of the extract and the associated problems of loss of material. We conclude that programing of the somatic 5S RNA genes occurred early in the life of an erythrocyte and that the complexes remain assembled on these genes even though free factor concentrations have been reduced to an extremely low level, perhaps even eliminated altogether. The cell remains in this state for weeks, perhaps months.

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