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Transcriptional properties of nucleoli isolated from Tetrahymena

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ABSTRACT

Nucleoli can be isolated from Tetrahymena in a yield of 30-60%. The isolated nucleoli contain rDNA (at least 90% pure) and have a protein to DNA ratio of 30:1. The endogenous RNA-polymerase activity of the r-chromatin has the following properties: (i) The in vitro transcript has a maximal size identical to the in vivo 35S rRNA precursor, demonstrating correct termination on the gene, (ii) 79% of the in vitro transcript is complementary to cDNA of 17S and 25S rRNA which is close to the theoretical maximum for the 35S rRNA precursor, (iii) the elongation rate of the endogenous RNA-polymerase molecules is 9-12 nucleotides/sec, (iv) an average of 4-16 active RNA polymerases are associated with each rDNA molecule depending upon the preparation.

INTRODUCTION

Recent developments in biochemical and cytochemical techniques have made it possible to investigate the transcription of specific gene sequences in cells, nuclei, and chromatin. However, a detailed understanding of the role of chromosomal proteins in gene expression will depend upon in vitro studies of the transcription of single genes in their chromatin state. This approach has been applied in the studies of transcriptionally active chromatin of small DNA viruses[1-3] and of nucleolar chromatin[4-10]. Among these systems nucleolar chromatin (r-chromatin) from Tetrahymena seems especially attractive since it contains only rDNA with a high percentage of coding sequences ($\geq$70%, cf. refs 11, 12).

We have previously described a procedure for isolation of the r-chromatin from Tetrahymena[5]. Considering the possible disadvantages associated with the procedures employed for solubilization of chromatin we have developed a procedure for purifica-

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tion of nucleoli in order to study the transcription of rDNA in a more intact chromatin state. The nucleoli can be isolated under extremely mild conditions probably because they are extrachromosomal and situated right under the nuclear membrane[12,13]. Each of the approximately 1000 nucleoli contains 5-10 rDNA molecules, which constitute a total of about 2% of the nuclear DNA[14,15]. The rDNA itself is a giant palindrome with a molecular weight of 13 $\times$ 10^6 daltons[16,17]. We will in this paper characterize the isolated nucleoli with respect to transcriptional properties.

MATERIALS AND METHODS

Preparation of nucleoli. Cultures of *Tetrahymena pyriformis*, strain GL (amicronucleate) were grown to a density of 60-80,000 cells/ml[5]. The cells were harvested by centrifugation at 300xg and washed with 30 volumes of nuclei buffer (0.1 M sucrose, 10 mM Tris pH 7.2, 3 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM NaCl) at 4^oC. The nuclei were prepared by lysis of the cells in 30 volumes of nuclei buffer containing 0.3% Nonidet P40. They were collected by centrifugation at 500xg and washed once with 30 volumes of nuclei buffer containing 0.1% Nonidet P40 and once with 30 volumes of nuclei buffer. The nuclei were resuspended in 10 volumes of extraction buffer (10 mM Tris pH 7.2, 140 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, and 1 mM mercaptoethanol) at 4^oC and exposed to gentle homogenization in a glass homogenizer. The extracted nuclei were removed by centrifugation at 500xg and the nucleoli in the supernatant layered on top of a 4.5 ml sucrose cushion (3.0 ml of 50% sucrose and 1.5 ml of 80% sucrose in 10 mM Tris pH 7.2, 140 mM NaCl, 1 mM EDTA, and 1 mM mercaptoethanol) in a SW27 centrifuge tube. After 30 min of centrifugation at 15,000xg nucleoli were collected in the bottom 3 ml of the cushion.

Analysis of DNA and RNA. The DNA composition of the isolated r-crhomatin was analysed on 0.7% agarose slap gels and the DNA was stained by ethidium bromide. RNA-size studies were done on 1.9% agarose gels containing 5 M urea by the method of Dudov *et al.*[18]. Control studies on formamide gels[19] gave results identical to those on the urea gels. The gels were calibrated with marker RNA from EMC (molw. 2.5 $\times$ 10^6 daltons), TMV (molw. 2.0 $\times$ 10^6 dal-

tons), and ribosomal RNAs from rat liver (18S and 28S) and *Eschericia coli* (16S and 23S). A semilogarithmic plot of the molecular weight versus the migration in gel gave a straight line. For quantitative studies the specific activity of DNA from ^{3}H-thymidine labelled cultures were determined by the method of Burton[20] and the amounts of DNA in the various fractions determined by counting acid-precipitated samples in a scintillation spectrometer.

Determinations of RNA-polymerase activity and elongation rates. The endogenous RNA-polymerase activity on the r-chromatin was determined by incubation with an assay mixture containing 4.9 mM $(NH_4)_2SO_4$, 8.2 mM $MgCl_2$, 3.8 mM 2-mercaptoethanol, 0.50 mM EDTA, 32 mM Tris-HCl pH 7.2, 2.1 mM KCl, 57 mM NaCl, 95 µM of ATP, GTP, and CTP, 7.5 µM of 5,6-^{3}H-labelled UTP (2 Ci/mmole). After incubation at 25°C acid-insoluble counts were determined as described[5]. For elongation-rate studies an incubation mixture similar to the above mentioned was used except that 30 µM cold UTP and 30 µM α^{32}P-labelled ATP (2 Ci/mmole) were used. One unit of RNA polymerase is defined as the amount of enzyme which can incorporate one pmole of UMP into RNA in 10 min at 25°C.

Preparation of DNA complementary to 17S and 25S rRNA. *Tetrahymena* ribosomes were dissociated with EDTA and 30S and 50S subunits separated in sucrose gradients. Peak fractions were extracted with phenol-chloroform and after alcohol precipitation the 17S and 25S rRNA was sedimented in sucrose gradients containing SDS. The 17S and 25S rRNA collected from these gradients were precipitated with alcohol. cDNA was synthesized from 17S and 25S rRNA after polyadenylation according to the method of Hell *et al.*[21]. 3-6S cDNA was collected from alkaline gradients. Back-titration with 17S and 25S rDNA has shown that all sequences in the 17S and 25S rRNA are equally represented in the complementary DNA (data not shown). Cross contaminations of 1.5% and 6% were found in the cDNA of 17S and 25S rRNA, respectively.

Hybridizations. *In vitro* transcription product for hybridization was synthesized from the nucleolar preparation under standard assay conditions except that the ATP concentration was 15 µM (4 Ci/mmole of α^{32}P-ATP) and UTP and Hg-UTP were present in concentrations of 6.0 µM each. The transcript was cleaved at pH 11

to an average size of 800 nucleotides and purified on Thiol Sepharose 4B (Pharmacia). Fixed amounts of transcript were titrated with cDNA from 17S and 25S rRNA, separately and combined. The samples were incubated to a Rot of 0.7 mole $\cdot$ liter^{-1} $\cdot$ sec^{-1}, which is sufficient to drive more than 95% of the complementary sequences into hybrid (the kinetic complexity of the precursor rRNA correspond to a Rot$_{\frac{1}{2}}$ of $2.3 \cdot 10^{-2}$ moles $\cdot$ liter^{-1} $\cdot$ sec^{-1}). Hybridizations and assays of hybrid resistant to S_1 nuclease were done according to the method of Hell et al.[21].

The amounts of cDNA and transcript were calculated from the specific activity of ^{3}H-dCTP and α^{32}P-ATP assuming equal representation of all four bases in cDNA and transcript. For control hybridization Tetrahymena ribosomes were labelled in vivo with ^{32}P-orthophosphate (specific activity ∿300.000 cpm/μg). The ribosomes were extracted with phenol-chloroform and total 17S + 25S rRNA collected from SDS-sucrose gradients without prior separation of subunits. The rRNA was nicked as above and challenged with cDNA of 17S and 25S rRNA in order to get estimates of the background caused by secondary structure, and the maximal efficiency of the hybridization.

RESULTS

Preparation of nucleolar bound r-chromatin. In exponentially growing cultures of Tetrahymena the nucleoli are situated directly under the macronuclear envelope. They can be extracted from nuclei in physiological ionic strength buffers and isolated as outlined in Figure 1(see Materials and Methods for details). The extracted nucleoli are separated from the nuclear complex by a 500xg centrifugation.Of the total nuclear RNA-polymerase activity, 50-80% is associated with the nucleoli in the supernatant. Further purification of the nucleoli is obtained by sedimentation into a 50-80% sucrose cushion. The bottom 3 ml of the sucrose cushion contains 20-50% of the RNA-polymerase activity applied. The purification table for a typical experiment is presented as Table 1.

More than 90% of the DNA in the nucleolar preparation is rDNA, as seen from the agarose gel in Figure 2. The identity of rDNA has been determined by its size, buoyant density, EcoR1 digestion

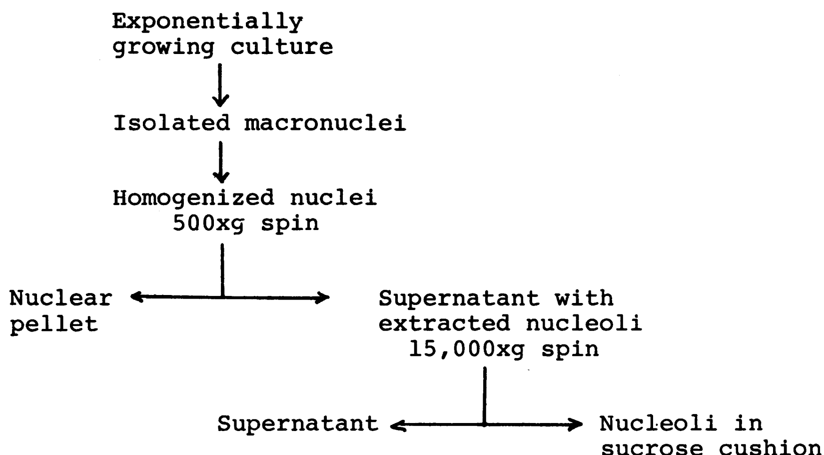

Figure 1. Flow diagram for isolation of nucleoli from *Tetrahymena*

Table 1. Purification table for an 18 liter culture containing 10^9 cells

Fraction	Protein (mg)	DNA (mg)	RNA polymerase units total	RNA polymerase units/mg DNA
cells	1050	10.9		
macronuclei	56	10.1	345,000	34,000
nucleoli	3.6	0.11	81,000	740,000

The culture was grown over night in the presence of ^{3}H-labelled thymidine. Protein, DNA, and RNA-polymerase activity were determined as described in Materials and Methods.

pattern and hybridization properties[5]. About 1% of the total DNA can be isolated as rDNA in nucleolar preparations. The isolated nucleoli are purified 300 fold with respect to cellular protein. The RNA-polymerase activity in the isolated nucleoli varies between 40,000-250,000 units/10^9 cells, depending upon the physiological state of the cells.

Electron micrographs of isolated nuclei show regular electron dense structures with a maximal diameter of approximately 0.3 µ. The dense, granular structure of the sectioned nucleoli as well as their size are very similar to what is seen in sections of whole cells[22].

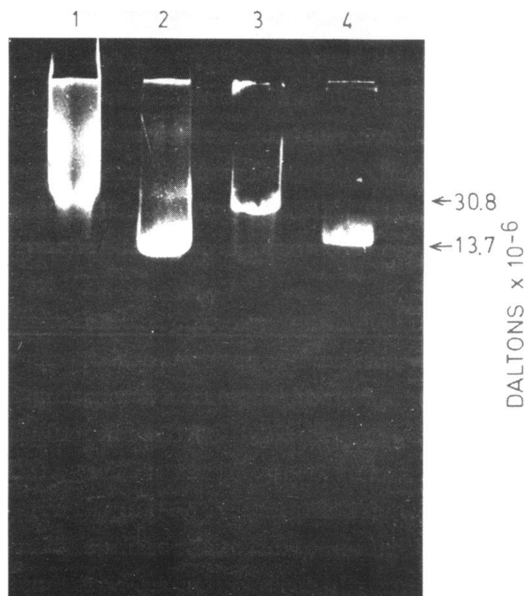

Figure 2. Agarose gel electrophoresis of DNA
Samples of 1) macronuclei (50 μl), 2) nucleoli (400 μl), 3) λDNA, and 4) EcoR1 digested λDNA were applied on a 0.7% agarose gel after incubation with 1% SDS at 45°C for 10 min.

Transcriptional properties of isolated nucleoli. The endogenous RNA-polymerase activity is constant for 5-10 min and, thereafter, levels off during the following 20-60 min. The reaction has an absolute requirement for divalent ions (with a slight preference for Mg^{2+}) and for all four ribonucleoside triphosphates.

The *in vitro* transcription is not inhibited by α-amanitin but it is inhibited more than 97% with 20 μg/ml actinomycin D. Hybridization of the *in vitro* synthesized RNA with 17S cDNA, and 17S and 25S cDNA combined gave saturation levels of 26% and 70%, respectively, with a background of 15% (Figure 3A+C). Parallel experiments with *in vitro* synthesized 17S plus 25S rRNA gave saturation levels of a 35% and 85% with a background of 12% (Figure 3D+F). The equivalence point determined from the titration curves shows the presence of about 50% endogenous rRNA in the transcript. A similar conclusion can be drawn from the RNA to cDNA ratio of the isolated hybrid (results not shown). The endo-

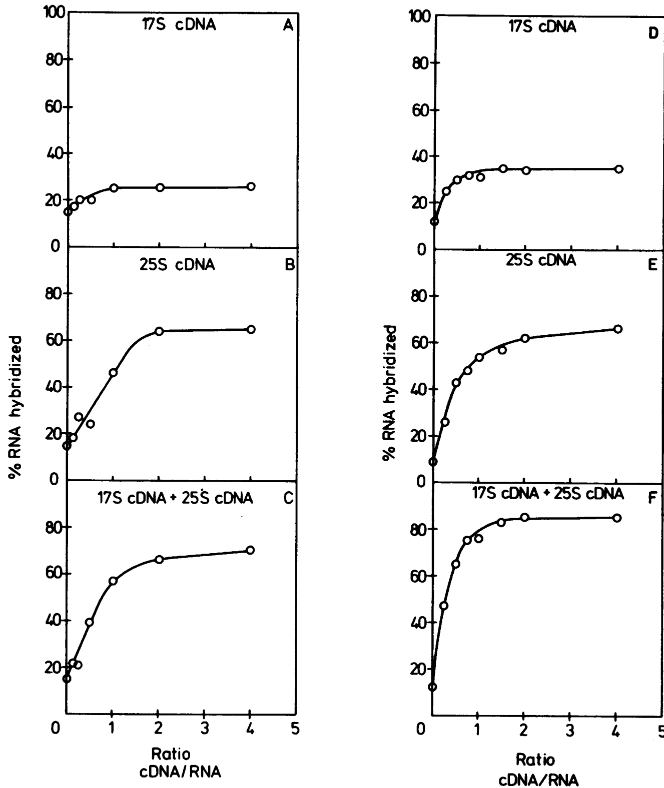

Figure 3. Hybridization studies on *in vitro* synthesized RNA and 17S and 25S rRNA

RNA (4 ng) transcribed *in vitro* by nucleoli (A-C) and 12 ng of the *in vivo* transcribed 17S+25S rRNA (D-F) were hybridized as described in the Materials and Methods with 17S cDNA (A and D), 25S cDNA (B and E), and 17S+25S cDNA (C and F). In C and F the abscissa gives the ratio of each of 17S and 25S cDNA to RNA.

genous rRNA has, however, no influence on the hybridization results since the experiments have been performed with excess of cDNA.

The *in vivo* synthesized precursor of ribosomal RNAs has a molecular weight of 2.3×10^6 daltons[11] and Figure 3 demonstrates that this precursor rRNA can be isolated from *in vivo* labelled nucleoli. The product synthesized *in vitro* has the same maximal size as seen from Figure 4. Isolated nucleoli were incubated with labelled triphosphate for 1½ min and then exposed to chase with high concentration of cold triphosphate for 2, 3, and

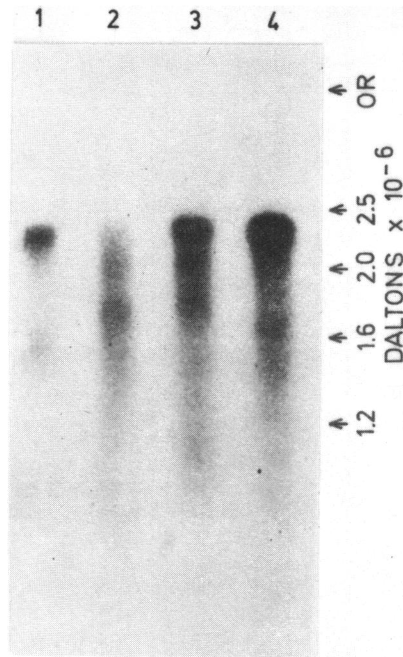

Figure 4. Size studies of in vivo and in vitro synthesized ribosomal RNA

Sample (1) represents in vivo labelled RNA associated with isolated nucleoli from cells grown for 1 hr in medium containing ^{32}P-orthophosphate (2 μCi/ml). Samples 2-4 represent aliquots of nucleoli incubated with ^{32}P-labelled assay mixture in a final volume of 1000 μl. After 1½ min of incubation the samples were chased with cold ATP (250 μM) for: (2) 2 min, (3) 3 min, and (4) 10 min. All samples were deproteinized and applied on a 1.9% agarose gel in 5 M urea. The positions of the RNA markers run in parallel are indicated.

10 min. After 10 min of incubation most of the in vivo synthesized RNA chains attached to the chromatin were elongated to a final size of 2.3×10^6 daltons.

In order to estimate the elongation rate for the RNA polymerase the r-chromatin was stripped for in vivo synthesized RNA chains by a 5 min incubation at 25°C with pancreatic RNase (2.5 ng/ml) followed by an additional sucrose cushion centrifugation. Gel studies with labelled RNA have demonstrated that no residual RNase could be detected after this additional sucrose cushion purifucation. The stripped nucleoli were incubated with labelled

triphosphate for the indicated periods of time, RNA isolated and the size estimated on 1.9% agarose gels. The time course of the reaction which is shown in Figure 5A+B demonstrates that short RNA chains (<4S) attached to the polymerase on the treated chromatin are elongated. The RNA polymerases elongate the chains with the rates given in Table 2.

Within the first 6 min of incubation we observe a nearly synchronous elongation of the small chains indicating that all RNA-polymerase molecules move on the gene with nearly the same rate. The reason for the gradual reduction in the elongation rate after the first 6 min of incubation is at the present unclear, but

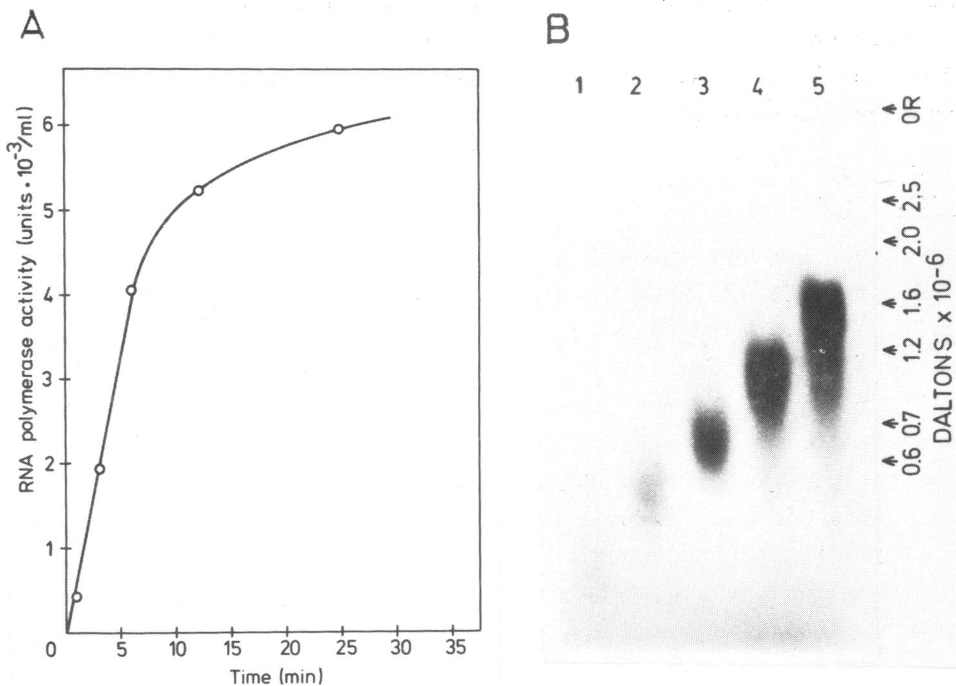

Figure 5. Elongation rates in isolated nucleoli

A. RNase-treated nucleoli (200 µl) were incubated with ^{3}H-UTP labelled assay mixture in a total volume of 500 µl. Aliquots of 75 µl were taken at the indicated time points.

B. RNase-treated nucleoli (200 µl) were incubated with ^{32}P-labelled ATP-assay mixture in a total volume of 500 µl. Aliquots of 75 µl were taken at the following time points: (1) 1 min, (2) 3 min, (3) 6 min, (4) 12 min, and (5) 25 min. All samples were deproteinized and applied on a 1.9% agarose gel in 5 M urea.

Table 2. Elongation rates on the r-chromatin

Incubations period (min)	Elongation (daltons $\times 10^{-3}$)	Elongation rate (nucleotides/sec)
1-3	210-640	12
3-6	640-1130	9.1
6-12	1130-1480	3.2
12-25	1480-1750	1.3

The elongation rates are calculated based on the position of the front of the spots in Figure 5B.

may be caused by shortage or instability of unknown factors. The elongation rates are calculated based on the position of the front of the spots, but nearly identical values are obtained, if the most intensively labelled areas are used for the calculation.

DISCUSSION

We have described a procedure for isolation and purification of nucleoli from *Tetrahymena*. The isolation procedure involves a series of differential centrifugation steps which are performed in the presence of physiological concentrations of salt. The yield of isolated r-chromatin in the final nucleoli preparation is between 30-60% of the reported *in vivo* amount[14,15]. The ratio of protein to DNA is about 30:1 while it is found to be 8:1 after a further purification of nucleoli by a metrizamide gradient centrifugation[23]. However, after the metrizamide purification the nucleoli have lost substantial amounts of RNA-polymerase activity and appear partially disintegrated in electron micrographs (unpublished result). Recent results by Jones[24] have demonstrated that r-chromatin isolated by a different procedure has a protein to DNA composition similar to what we find in the metrizamide purified nucleoli.

The hybridization experiments in Figure 3A-E demonstrate that a maximum of 70% of the *in vitro* transcript can be driven into hybrid with an excess of 17S and 25S cDNA. In parallel experiments with *in vivo* synthesized 17S plus 25S rRNA the maximal level of hybridization was 85%, Figure 3D-F. Using a background

of 15% and assuming that 85% is the maximal level of hybridization obtainable under the experimental conditions employed, it can be calculated that a minimum of 79% of the transcript is complementary to 17S plus 25S cDNA. Since we do not observe any RNA synthesis (<3%) in presence of actinomycin D and since the background level of hybrid formed with both *in vivo* and *in vitro* synthesized rRNA is 12-15%, the remaining 21% of the transcript which is not complementary to the 17S and 25S cDNAs represent transcribed spacers and not RNA-directed or nonsense DNA transcription.

From the results in Figure 3 the composition of the *in vitro* transcript can be estimated by calculations very similar to the above mentioned. The relative amounts of 17S RNA : 25S RNA : spacer are 16% : 63% : 21%. Using the molecular weights of the rRNAs determined from our gel studies (17S : 0.6×10^6 daltons; 25S : 1.2×10^6 daltons; precursor rRNA : 2.3×10^6 daltons) the composition of the precursor rRNA can be calculated to 25% : 52% : 22% in the same order as above. The exact arrangement of the spacers within the precursor rRNA is not known, but assuming that (1) the transcribed spacer is evenly divided between the 5'-end of the molecule and the region between the 17S and 25S sequences, (2) the RNA-polymerase molecules are randomly distributed throughout the transcription unit, and (3) no reinitiation occurs during the 15 min of incubation used for synthesizing the transcript, the approximate composition of the *in vitro* transcript can be calculated to be 13% : 75% : 12%. The underestimation of 17S and spacer according to this model indicates that at least one of the three assumptions is wrong. More detailed investigations will be required in order to elucidate this problem. Based on the elongation rates and the quantitative studies (Tables 2 and 1) we calculate that there is an average of 3 transcriptionally active RNA-polymerase molecules per gene in the isolated nucleoli. However, the number of RNA polymerases can vary between 2-8 in different preparations. In comparison, *in vivo* studies[25] demonstrate that there in exponentially growing cultures must be a minimum of 50 active RNA-polymerase molecules per gene. It is difficult to explain the difference between the *in vivo* and *in vitro* situation, but plausible explanations might

be that some of the polymerases fall off during the isolation and/or that some of the RNA-polymerase molecules are transcriptionally inactive and, therefore, arrest a large number of the active molecules.

The *in vivo* elongation rate for eukaryotic RNA polymerases has been estimated to about 25 nucleotides/sec at 25°C[26]. In the isolated r-chromatin we find an initial rate of 12 nucleotides/sec. This elongation rate is much faster than in other eukaryotic chromatin systems, in which the rates usually are found to be lower than 1 nucleotide/sec[1,27]. Even in isolated nuclei similar low rates are observed[28,29]. On purified SV40 DNA form I, Mandel and Chambon[30] found, however, chain elongation rates of 8-10 nucleotides/sec at 37°C with purified RNA polymerase I from calf thymus.

The *in vitro* transcript of the r-chromatin has a final size identical to that of the ribosomal RNA precursor, showing that the isolated nucleoli have preserved the correct termination properties. Recent studies on the termination signal have demonstrated that correct termination is lost when r-chromatin is released from the nucleolar complex and we observe transcription of rRNA molecules with a molecular weight of approximately 3×10^6 daltons (Leer, Gocke, Nielsen, and Westergaard, manuscript in preparation). This might suggest that the r-chromatin has lost a "ρ-like" factor during the dissociation from the nucleoli. The initial rate of transcription is unchanged by the dissociation.

In conclusion it is clear from the presented data that several of the transcriptional functions are preserved in the isolated nucleoli from *Tetrahymena*. The system transcribes faithfully with a rate which is half of the *in vivo* rate and the transcription terminates properly. Experiments are now in progress to investigate the termination process in details. In addition experiments are carried out to investigate to what extent reinitiation occurs in the system.

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