Transcribed and non-transcribed regions of *Tetrahymena* ribosomal gene chromatin have different accessibilities to micrococcal nuclease

Ted E. Palen and Thomas R. Cech

Department of Chemistry, University of Colorado, Boulder, CO 80309, USA

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ABSTRACT

DNA renaturation kinetics was used to examine the relative accessibility of various regions of the *Tetrahymena* ribosomal RNA gene (rDNA) chromatin to micrococcal nuclease. In nuclei from cells active in rRNA transcription, the transcribed region of the rDNA chromatin was as much as 5-fold more accessible than the average of the total chromatin. As few as 20% inactive genes in the population could have accounted for all of the hybridization, so the transcribed region of the active units may be totally unprotected from nuclease degradation. The terminal non-transcribed spacer downstream from the transcription unit was also preferentially digested, but to a smaller degree. The central non-transcribed spacer was degraded to the same extent as total chromatin after a high degree of nuclease digestion. In nuclei from starved cells, which have 96% reduced rRNA transcription, the transcribed and terminal spacer regions of the rDNA were again more accessible than the total chromatin from the same nuclei, but the difference did not exceed 2-fold. We conclude that transcriptional activation is accompanied by major changes in the structure of the ribosomal gene chromatin, and that the extent and/or type of structural alteration differs in each functionally defined region of the rDNA.

INTRODUCTION

In previous studies of the organization of the rDNA chromatin of *Tetrahymena*, micrococcal nuclease digestion and DNA blot hybridization were used to examine the structure of transcribed and non-transcribed regions of the gene (1,2). When a transcribed region probe was hybridized to micrococcal nuclease digests of DNA from log-phase cells (high rRNA transcriptional activity) and from starved cells (96% reduced activity; ref. 3-4), the amount of hybridization seemed to be significantly less with the DNA from log-phase cells (2). There are many difficulties inherent in the quantitation of blot hybridization data. We have therefore examined the kinetics of DNA renaturation in solution to determine the amount of nuclease-resistant DNA in various sections of the rDNA in log-phase and starved cells.

MATERIALS AND METHODS

All materials and methods not described below are the same as those described previously (2).

Digestion of Nuclei with Micrococcal Nuclease

Nuclei were isolated from log-phase cells (grown to a density of $1.0-1.2 \times 10^5$ /ml) and from starved cells (incubated 24 hr in 50 mM Tris-HCl, pH 7.5). The isolated nuclei were resuspended in TMS buffer (250 mM sucrose, 10 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5) at $5-7 \times 10^7$ nuclei/ml (200-300 μ g DNA per reaction). For the "low" degree of digestion, the nuclei were treated with 0.1 U micrococcal nuclease (Sigma No. N-3755) for 20 or 30 sec at 37°C. This gave samples with TCA (trichloroacetic acid) solubilities of 0.5-2.0%, based on scintillation counting of the 3H -labeled DNA. For the "high" digestion samples, nuclei were treated with 0.2 U for 4 or 8 min or with 0.36 U for 16 min. These treatments produced 35-50% TCA solubility of DNA in log-phase nuclei and 25-40% TCA solubility of DNA in starved cell nuclei.

Preparation of Total DNA Samples

Isolated nuclei were incubated in TMS buffer at 37°C for 2 min without added nuclease, and their DNA was purified. The deproteinized, high molecular weight DNA was then digested with 0.01 or 0.05 U micrococcal nuclease for 20 sec at 37°C in 3 mM $CaCl_2$, 10 mM $MgCl_2$, resulting in 1-3% TCA solubility. This DNA is referred to as total DNA digested with nuclease. DNA was also purified directly from Tetrahymena cells to give the total undigested DNA samples.

Shearing of DNA

High molecular weight DNA samples (DNA from log-phase and starved nuclei that had been digested with nuclease to a low extent, total undigested Tetrahymena DNA, and total DNA digested with nuclease) were sheared by sonication to reduce their average size to that of the DNA from nuclei that had been digested to a high extent with nuclease. The DNA was dissolved in 50 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.8 and sonicated 10 times (15 sec pulses, power setting #2, 60% duty cycle, microtip of the Branson Sonifier Cell Disruptor 200). The samples were kept on ice through the procedure, and 45 sec were allowed for cooling between each pulse. DNA was then adjusted to 0.15 M NaCl and precipitated with 2.5 vol ethanol.

Preparation of Probes

The gene region probe was the 2.1 kb (kilobase) Hind III fragment of the rDNA, obtained by digestion of pRP4 (5). The terminal spacer probe was the 1.175 kb Hind III fragment of the rDNA, obtained by digestion of pRP7 (5).

The promoter-region probe was the 0.8 kb Taq I-Xba I fragment of the rDNA, obtained by digestion of pRP59; it included 750 bp of DNA upstream from the initiation point of pre-rRNA transcription (6) and 50 bp downstream (transcribed spacer sequences). These three plasmids were a gift from Ron Pearlman. The central spacer probe was the 0.65 kb Taq I-Xba I fragment of the rDNA, obtained by digestion of pTtr1, a gift from Peter Challoner and Liz Blackburn. Restriction fragments were purified by gel electrophoresis prior to being labeled by nick translation.

Assay of the Kinetics of DNA Renaturation

The hybridization buffer consisted of 50% deionized formamide, 0.05 M sodium phosphate, pH 6.8, 5 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate). Each reaction mixture contained 0.3, 1.0, 5.0 or 10.0 μg *Tetrahymena* DNA, 1.5 μg degraded herring sperm DNA (Sigma No. D-2251, Type IV) and 10,000-12,000 cpm of the ^{32}P -labeled probe DNA ($0.23\text{--}1.7 \times 10^8$ cpm/ μg) in 13-15 μl of hybridization buffer. Control reaction mixtures containing only the probe and the sperm DNA were included to ascertain the amount of self-renaturation of the probe. Samples were sealed in siliclad 25 μl glass micropipets and boiled for 8 min to denature the DNA. The mixtures were then allowed to renature at 42°C (or 37°C) for 0, 0.1, 1, 10, 24 or 48 hr. Reactions were stopped by dilution into enough S_1 nuclease buffer (150 mM NaCl, 25 mM sodium acetate, 1 mM ZnCl_2 , pH 4.55) to give 100 μl final volume. The 100 μl was divided into 4 portions in siliclad 0.5 ml polypropylene tubes (Eppendorf). Two of the portions were precipitated by the addition of 30 μl 10% TCA for 30 min on ice. The other two were treated with 62.5 U S_1 nuclease for 15 min at 37°C; the reactions were stopped by addition of sodium phosphate, pH 6.8 to 100 mM; 3 μg of sperm DNA was added as a carrier; and the DNA was acid precipitated as above. Acid precipitates were collected by centrifugation for 15 min in the Eppendorf 5414 centrifuge. The supernatants were dissolved in Triton X-100-containing fluor and counted directly. The pellets were incubated with 100 μl Protosol (New England Nuclear) at 37°C for 6-20 hr, then cooled, neutralized with 12.5 μl acetic acid, and counted in Triton X-100 fluor. The counts per minute of each duplicate pair of samples were averaged. The fraction renatured was calculated from the cpm in the supernatants as $f_s(t) = [S(0) - S(t)]/S(0)$, where $S(t)$ is the cpm in the supernatant of the S_1 -treated sample minus the cpm in the supernatant of the untreated sample for each renaturation time t . Fraction renatured was calculated independently from the cpm in the pellets as $f_p(t) = [P(t) - P(0)]/\bar{P}$, where $P(t)$ is the cpm in the pellet of the S_1 -treated sample after renatura-

tion for time t , and $\bar{P}$ is the average of the cpm in the untreated samples from all time points. Finally, the fraction renatured was taken as $f(t) = [f_g(t) + f_p(t)]/2$.

RESULTS

Experimental Design

An excess of ^3H -labeled Tetrahymena DNA, digested in nuclei with micrococcal nuclease, was used to drive the renaturation of a trace amount of cloned, ^{32}P -labeled "probe" DNA specific for a certain region of the rDNA. In parallel reactions, renaturation of the probe was driven by total undigested DNA or by total DNA that had been subjected to limited nuclease digestion after deproteinization. If a region of chromatin were preferentially degraded by the nuclease, the DNA probe for that region would renature more slowly (with a higher $\text{Cot}_{1/2}$) when driven by DNA from micrococcal nuclease-digested nuclei than when driven by total DNA. [$\text{Cot}_{1/2}$ is the product of initial driver DNA concentration (moles DNA $\text{PO}_4/1$) and time (sec) required for renaturation to proceed 50% to completion.]

Driver DNA

DNA samples from nuclei digested with micrococcal nuclease for 20 and 30 sec were combined to give the low digestion level driver DNAs. Most of the DNA still had a high molecular weight. The small proportion that had been degraded formed a faint nucleosome ladder (Fig. 1A, lanes 1 and 2). With these limited digestion conditions, DNA samples from log-phase and starved cell nuclei had very similar TCA solubilities and very similar gel electrophoresis patterns.

The samples digested for 4, 8 and 16 min were combined to give the high digestion level driver DNAs. Under these digestion conditions, the chromatin from log-phase cells was digested to 35-50% TCA solubility, approximately 10% more than the chromatin from starved cells (25-40% acid solubility). The log-phase driver therefore had a larger proportion of the DNA in monomer and dimer size nucleosome fragments (Fig. 1B, lanes 1-3) than did the starved cell driver (lanes 4-6).

A sample of total Tetrahymena DNA was used as a driver that contained all sequences of the cell in their naturally occurring proportions. Another sample of DNA was isolated from nuclei that had been self-digested for 2 min; then, following deproteinization, the DNA was treated to a limited extent with micrococcal nuclease (samples with 1% and 3% TCA solubility were combined). This driver should be depleted both in any sequences that are susceptible to

Figure 1. Driver DNAs analyzed by electrophoresis on 1.2% agarose gels and stained with ethidium bromide.

(A) Unsheared DNA from the "low level" micrococcal nuclease digests of nuclei from (Lane 1) log-phase cells and (Lane 2) starved cells (both 0.5-2.0% TCA solubility). (Lane 3) Total DNA digested with micrococcal nuclease after deproteinization (1.0-3.0% TCA solubility). (Lane 4) Total undigested DNA. (B) "High" digestion level drivers. (Lanes 1-3) Nuclei from log-phase cells were digested with micrococcal nuclease for 16 min with 0.36 unit (Lane 1), 8 min with 0.2 unit (Lane 2), or 4 min with 0.2 unit (Lane 3), and the DNA was isolated. (Lanes 4-6) The same as lanes 1-3 except the DNA was derived from nuclei of starved cells.

(C) The same DNA samples as in A after being sheared by sonication (see Materials and Methods). (M) Marker DNA fragments (λ -Hind III DNA, ϕ X174-Hae III DNA, or a mixture of both), sizes given in kb.

endogenous nucleases in the nuclei and in any DNA that has intrinsically high susceptibility to micrococcal nuclease by virtue of its sequence (7,8). The molecular weight distributions of these driver DNAs prior to shearing can be seen in Fig. 1A (lanes 3 and 4). All high molecular weight drivers were reduced to an average molecular weight of 600-700 bp by sonication prior to their use (Fig. 1C).

Specificity of DNA Probes

Total Tetrahymena DNA was cleaved with various restriction endonucleases, blotted onto nitrocellulose, and hybridized with the DNA probe. As shown in Fig. 2, the probes for the transcribed region, promoter region, and central spacer hybridized only to the expected rDNA fragments. The specificity of the terminal spacer probe had been shown previously (2). We concluded that all DNA probes hybridized specifically to the region of the rDNA from which they were derived.

DNA Renaturation Analysis

Each probe was renatured in the presence of total Tetrahymena DNA and DNA from micrococcal nuclease-digested nuclei from both log-phase and starved cells. The hybridization conditions were the same as used in the earlier blot hybridization study (2), so the quantitative data could be compared directly to the earlier qualitative data. Samples of the primary data are given in Fig. 3, and the results of all the experiments are summarized in Fig. 4. In none of the experiments was there a significant change in the final extent of hybridization of the probe, as might be seen if a specific subset of the sequences being investigated was degraded to the point that it could no longer drive hybridization of the probe. The $Cot_{1/2}$ values therefore provide an adequate summary of the data. The interpretation of the results is given in the Discussion.

Figure 2. Probe specificity.

(A) A partial map of the rDNA (5) showing the locations of the probes. The horizontal lines with arrowheads correspond to the bands in the autoradiograms that show hybridization of (B) the central spacer probe; (C) the promoter region probe; and (D) the gene region probe. Alongside the lines in (A) are the sizes of the hybridizing bands in base pairs and the restriction endonucleases used to generate the bands. The δ sign before the name of an enzyme indicates that a partial digest was performed with that enzyme. All of the restriction endonuclease digests were performed on total cellular DNA from T. thermophila. The digests were electrophoresed on agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P -labeled probes. Each marker lane (M) contained a Hind III - Eco RI double digest of λ DNA which had been end-labeled with γ - ^{32}P -ATP and polynucleotide kinase. All sizes in B, C, and D are given in kb.

Figure 3. Cot Curves.

The gene region probe was hybridized with (A) the "low" digestion drivers and (B) the "high" digestion drivers. The central spacer probe was hybridized with (C) the "low" digestion drivers and (D) the "high" digestion drivers.

(O) Probe self-renaturation with only sperm DNA as carrier.

Driver DNAs in A and C:

(—□—) 0.5-2.0% TCA soluble DNA from log-phase cells

(---+---) 0.5-2.0% TCA soluble DNA from starved cells

(···Δ···) Total nuclear deproteinized DNA digested to 1-3% TCA solubility by micrococcal nuclease

Driver DNAs in B and D:

(—□—) 35-50% TCA soluble DNA from log-phase cells

(---+---) 25-40% TCA soluble DNA from starved cells

(···Δ···) Total undigested cellular DNA

The renaturation of the 3H -labeled driver DNA was also monitored in each experiment. The renaturation profile of each driver DNA was highly reproducible from experiment to experiment. This reproducibility gave us confidence that the 2-fold differences in $Cot_{1/2}$ observed in some of the ^{32}P -labeled probe renaturation experiments were significant. The total undigested DNA and the low digestion level driver DNAs all renatured with

Figure 4. Summary of DNA renaturation data.

(A) A map of half of the rDNA molecule, showing the locations of all the probes.

(B) A table of the $Cot_{1/2}$ values for all the probed regions and the different low and high digestion level DNA drivers. Unless otherwise indicated, all renaturation was performed at 42°C. The T_m 's of DNA•DNA hybrids in the renaturation buffer were calculated (9) as 60.3°, 62.6°, and 60°C for the promoter region, gene region, and terminal spacer, respectively. Because of the similarity of these T_m 's, renaturation at 42°C provided similar hybridization stringencies for these 3 probes. The central spacer had a calculated T_m of 55.4°C, so renaturation was also performed at 37°C to maintain the same stringency as used for the other regions. T_m calculations were based on % G+C from DNA sequencing studies (ref. 6, 10, and R. Pearlman and A. Amin, personal communication).

approximately the same kinetics, $Cot_{1/2} = 70 \pm 20$. This value is in close agreement with the $Cot_{1/2}$ for total *T. thermophila* macronuclear sequences reported by Yao and Gorovsky (11) when the differences in salt concentration (12) and the effect of formamide on the solution viscosity (13) are taken into account. The high digestion level driver DNAs renatured with retarded kinetics, which reflected their increased state of digestion. The more highly digested the sample (the higher the % TCA solubility) the more slowly it renatured. This implies that specific sequences in the *Tetrahymena* genome are depleted preferentially upon micrococcal nuclease digestion of the nuclei.

DISCUSSION

We have used micrococcal nuclease digestion and DNA renaturation kinetics to examine the accessibility of four functionally distinct regions of the Tetrahymena rDNA as it exists in isolated nuclei. Based on other work, we know that at least 3 of these regions also have distinct patterns of fragmentation by micrococcal nuclease. The gene region has a 184 bp nucleosome repeat, the central spacer a highly ordered structure with a 200 bp nucleosome repeat, and the promoter region a complex pattern of altered nucleosome spacings and other areas of increased protection from nucleases (ref. 2 and our unpublished results).

During micrococcal nuclease treatment of isolated nuclei, the DNA is also susceptible to degradation by endogenous nucleases. Mathis et al. (14) have pointed out that an endogenous DNase I-like activity could make active genes appear to be micrococcal nuclease sensitive, and Vavra et al. (15) have found that such an endogenous nuclease is active in their isolated Tetrahymena nuclei incubated at room temperature. We are confident, however, that the sequence depletion we observe is due to the exogenous micrococcal nuclease. First, each probe was renatured both with total DNA driver and with a driver DNA isolated from nuclei that had been incubated under nuclease digestion conditions without added nuclease. No significant differences in $Cot_{1/2}$ were observed. (The small difference seen with the gene region probe is in the opposite direction to that expected for preferential digestion of gene-region sequences by an endogenous nuclease.) Second, we have incubated nuclei without added nuclease for 1 hr at 37°C, isolated the DNA, cleaved it with a restriction nuclease, and analyzed it by gel blot hybridization with various rDNA probes. The DNA isolated from these nuclei shows a similar hybridization pattern to that obtained with DNA isolated from unincubated nuclei; in neither case was very much degradation of the DNA detected (unpublished results). Our nuclei isolation procedure is much different than that used by Vavra et al. (15), and the degree to which endogenous nucleases are leached out of the nuclei during isolation may be quite different for the two methods.

Transcribed Region

In log-phase cells, the transcribed region of the rDNA was the most accessible to micrococcal nuclease attack. After extensive digestion, 5-fold more transcribed region DNA than total genomic DNA had been degraded to non-hybridizable fragments. In starved cells, the difference was only ~2-fold. The preferential sensitivity of the transcribed region in log-phase

cells seems more dramatic when one considers that all 20,000 copies of the gene in the cell might not be active. If 20% of the genes in log-phase cells had the same sensitivity as the total chromatin (or if 40% had the sensitivity of the average ribosomal gene in starved cells), then these inactive genes would account for all of the hybridization. The transcribed regions of the active majority of the genes would then have to be completely digested by the nuclease. This interpretation is strongly supported by the blot hybridization data (2), which showed that the gene region of the rDNA chromatin in log-phase cells had the same nucleosomal pattern as that in starved cells, but the former gave a hybridization signal only 20% as intense.

We therefore propose that the transcriptionally active region of rChromatin has an altered nucleosome structure that is preferentially sensitive to micrococcal nuclease and, at high levels of digestion, is completely degraded by the nuclease. This picture differs sharply from that originally proposed by Mathis and Gorovsky (16) and Piper et al. (17), who concluded that the transcribed region of rChromatin was protected from micrococcal nuclease cleavage to the same extent as total chromatin. In these earlier studies, rDNA was preferentially labeled with tritium during refeeding of a starved culture of Tetrahymena. When the nuclei were isolated and treated with micrococcal nuclease, it was found that the ^3H -labeled, rDNA-enriched chromatin was digested to TCA-soluble fragments at the same rate and to the same final extent as the ^{14}C -labeled bulk chromatin (16, 17). These studies have been critically reviewed by Vavra et al. (15), who point out that in such an experiment only 25-50% of the tritium radioactivity represents the transcribed portion of the rDNA; the remainder represents non-transcribed regions of the rDNA and contaminating bulk DNA. Therefore the preferential labeling approach will be much less sensitive to depletion of gene-region sequences than DNA hybridization with a gene-region probe, the approach of the present study. Nevertheless, based on the magnitude of the sequence depletion that we observe, one would expect the preferential labeling experiments to have shown some effect. This discrepancy could be explained if the gene-region sequences were being degraded to fragment sizes in the 10-30 base pair range, which might be too small to form stable hybrids but large enough to be TCA precipitable.

Preferential accessibility of transcribed sequences to micrococcal nuclease has been seen in other systems. 40% of the transcribed sequences of the rDNA chromatin of Xenopus are unprotected from micrococcal nuclease attack when the genes are in a state of near-maximal activity (stage 46 tadpoles of a

0/+ nu heterozygote), and up to 63% of the sequences were eliminated in the highly active stage II and III oocytes (18, 19). The gene region of the rDNA chromatin of Physarum is degraded by micrococcal nuclease to monomer-size particles much more quickly than the non-transcribed central spacer or the total chromatin (20, 21). Stalder et al. (21) also present evidence that much of the transcribed region of the Physarum rDNA chromatin is not organized in a 200 base pair repeat, in agreement with our findings for Tetrahymena (2). An increased sensitivity of transcribed sequences to micrococcal nuclease has also been reported for the hen ovalbumin gene (22, 23), the Drosophila heat shock genes (24), and trout testis mRNA genes (25).

Terminal Spacer

The terminal non-transcribed spacer was preferentially sensitive to micrococcal nuclease, but less so than the transcribed region of the rDNA. The ratio of nuclease sensitivity between log-phase and starved cells and between high and low levels of digestion was similar for the two regions. A simple explanation of the data would be that changes in chromatin structure necessary for transcription extend some distance into the downstream non-transcribed region. Unfortunately, we have no information about the structural homogeneity of the chromatin in this region, because the micrococcal nuclease digestion pattern is dominated by DNA sequences that are highly susceptible to cleavage (2). [The nuclease hot spots do not affect the DNA renaturation kinetics, as evidenced by the $Cot_{1/2}$ of the terminal spacer probe being the same when driven by total DNA or by total DNA digested with nuclease (Fig. 4). This result seems reasonable; although the renaturation rate should be affected if sequences are degraded to non-hybridizable fragments, it should not be affected very much by a few specific endonucleolytic cleavages.]

Promoter Region

The promoter region probe included ~750 bp upstream and 50 bp downstream from the initiation point of transcription. This region showed no preferential sensitivity in starved cells, but 2-fold more sensitivity than total chromatin in log-phase cells. While it is clear that some form of chromatin activation is taking place, the probe covered too large a region to make a more detailed interpretation meaningful. We are in the process of mapping specific structural changes in the vicinity of the transcription initiation site by the indirect end-labeling technique of Wu (26).

Central Spacer

The portion of the central spacer included in the probe has a regular

nucleosomal chromatin structure with the same 200 bp repeat as total chromatin (ref. 2 and our unpublished results). This region does not include the origins of replication (27, 28) and, in fact, may have no function in the vegetative growth cycle.

At a high level of nuclease digestion, the central spacer DNA had been degraded to the same extent as the total chromatin. We interpret this to reflect the normal histone-DNA interaction of transcriptionally inactive nucleosomes. The failure to observe a nucleosomal pattern on Southern blots at high levels of nuclease digestion (2) may be due to additional intranucleosomal cleavages as a consequence of the extreme A+T-richness of this region. [Micrococcal nuclease prefers A+T-rich sequences (7, 8, 29).] Such additional cuts could obliterate a nucleosomal pattern without appreciably depleting the sequences left available for solution renaturation.

However, at a low level of nuclease digestion, the central spacer did show a preferential sensitivity to the nuclease when compared to total chromatin. The ability of central spacer sequences to form snap-back molecules during renaturation could result in the decrease of the effective concentration of central spacer sequences in the driver, artificially increasing the $Cot_{1/2}$ values. However, since the total DNA control drivers were sheared and renatured in the same way as the log-phase and starved cell DNA drivers, the control drivers had the same opportunity to form snap-back molecules. The end result is that the log/total and starved/total $Cot_{1/2}$ ratios should be independent of any loss of DNA due to intramolecular renaturation. By the same argument, the extreme A+T-richness of this region may increase the $Cot_{1/2}$ values, but the effect should be the same for the total DNA digested with nuclease as for the log-phase and starved cell DNA drivers. Preferential degradation at low levels but not at high levels of nuclease treatment occurred with no other region of the rDNA. At the present time, we have no satisfactory explanation for this observation.

In summary, we have found that the chromatin structure varies between functionally-defined regions of the rDNA molecule. The order of accessibility of the various regions to micrococcal nuclease was the following: transcribed region > terminal spacer > promoter region > central spacer. This order was independent of the state of transcriptional activity. When probed by trimethylpsoralen crosslinking, the transcribed region was also found to be very accessible and the central spacer very protected (30; see also ref. 31), so these results do not appear to be highly dependent on the nature of the probe. We have also found that major structural alterations occur in the rDNA

chromatin when the cell enters a transcriptionally active state. The increase in accessibility was most dramatic for the transcribed region, where the degree of sequence depletion was consistent with the transcribed regions of the active rDNA units being completely unprotected from micrococcal nuclease degradation.

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