*GENOMIC FUNCTION DURING THE LAMPBRUSH CHROMOSOME STAGE OF AMPHIBIAN OÖGENESIS**

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Throughout its lengthy developmental history the disposition of the genetic material in the amphibian oöcyte nucleus differs from that in other cell types. The chromosomes in the oöcyte nucleus, arrested for the whole of oögenesis at the prophase of the first meiotic division, are known to contain at least the tetraploid amount of DNA.[1, 2] Oögenesis in amphibia requires months or even years to complete, depending on the species. By the mid-stages of the process, amphibian oöcyte chromosomes have assumed the paired, lateral loop "lampbrush" structure characteristic of oögenesis in many other classes of animal as well.[3] Early in oögenesis hundreds of independent, extrachromosomal nucleoli are also elaborated, each of which contains its own segment of DNA,[1, 4, 5] and by the onset of the lampbrush stage these nucleoli have begun to carry out an independent synthesis of ribosomal RNA.[6, 7] RNA synthesis is active in the lampbrush chromosome itself,[8, 4] but the rate of this synthesis is very low compared to that in the nucleoli. As the long process of growth and differentiation nears completion, the lampbrush structures disappear, and at the termination of oögenesis the contents of the nuclear sap are released to the cytoplasm by the dissolution of the nuclear membrane.

To date, the only class of gene product synthesized in the lampbrush stage oöcyte whose ultimate disposition is known is ribosomal RNA (rRNA). We have shown previously that over 98 per cent of the RNA synthesis in the lampbrush nucleus per unit time is rRNA synthesis[6] and that this RNA is conserved throughout oögenesis.[9] It is clear that during cleavage, blastulation, and much of gastrulation the amphibian embryo is obligatorily dependent on the rRNA formed in the course of oögenesis,[9–12] and this also appears to be true for transfer RNA.[14, 11] In the unfertilized egg and early embryos of the sea urchin, a class of template-active RNA's synthesized at some time in oögenesis is known to exist, according to an impressive variety of studies,[15–18] and Gross has shown directly that RNA of this type is synthesized during the last week of ovarian oögenesis.[19] Much less evidence exists to support similar assumptions regarding the presence of "maternal messenger" in amphibian eggs, however. It is known that enucleate frog eggs can synthesize protein, after activation, at the same rate as normal fertilized eggs,[20] exactly as in the sea urchin. There is also a brief report to the effect that template-active RNA is present in unfertilized eggs of the frog.[21]

In this paper we present studies designed to estimate the amounts of lampbrush chromosome-synthesized RNA other than ribosomal and soluble RNA which are present in the oöcyte, at lampbrush and later stages, and to measure the fraction of the genome active during the lampbrush stage.

Methods.—Extraction of P^{32}-labeled RNA from oöcytes: *Xenopus laevis* females were labeled by intraperitoneal injection of carrier-free P^{32}-phosphate as described by Brown and Littna.[10] Lamp-

brush-stage oöcytes (1000–3000) (stage 4 of Duryee[22]) or mature oöcytes (stage 6) were manually removed from their follicles, and the RNA was extracted as described by us previously,[9] except for the following variations: The pH 9.0 and pH 7.6 fractions were pooled and further deproteinized with the phenol-cresol mixture described by Kirby.[23] In preparing RNA from stage 6 oöcytes tri-isopropyl naphthalenesulfonate was utilized.[23] After ethanol precipitation the RNA solution was passed over a Sephadex G100 column running in 0.30 *M* NaCl–0.03 *M* NaCitrate (2xSC) and only the exclusion peak was harvested, thus purifying the preparation of all 4*S* and 5*S* RNA. The RNA was treated with preautodigested pronase B in the same solution at 500 μg/ml final concentration for $1^1/_2$ hr at 37°C. The preparation was again deproteinized with phenol, and the RNA was twice more precipitated in ethanol. Figure 1 shows the UV-absorption spectrum of a typical preparation. RNA extracted by this method is extremely stable to freezing, thawing, heating, etc., over long periods of time: it contains no spurious, adsorbed P^{32} counts; and it is 100% labile to ribonuclease.

Extraction of H^3-labeled DNA: DNA was obtained from *Xenopus* testes, which were minced and suspended in "Solution X"[24] containing 10 mc/ml thymidine-H^3, 12,800 mc/mM, for 5 hr at 18°C. The extraction was then carried out by a method similar to method B of Dawid,[25] which is essentially a combination of the pronase method of Berns and Thomas[26] with that of Marmur.[27] The DNA was subjected to ribonuclease treatment, and was ultimately spooled from isopropyl alcohol,[27] before being stored sterilely in 0.015 *M* NaCl–0.0015 *M* NaCitrate (SC/10). Figure 2 shows the banding profile in a CsCl density gradient, the UV spectrum, and the hyperchromicity curve for a typical preparation. Specific activity of the preparations varied from about 900 to 1300 H^3 cpm/μg.

RNA-DNA hybridization: For these experiments we used a liquid-liquid method modified from that described by Gillespie and Spiegelman.[28] The procedure was scaled down to annealing volumes of 200 μl and the hybrids were trapped on filter discs cut to 7 mm in diameter. Hybridization was carried out in 6xSC. After annealing for 13 hr, by which time maximum levels of hybridization were obtained, purified ribonuclease A[29] (an important point) and tris buffer were added to final concentrations of 10 μg/ml ribonuclease and 0.025 *M* tris, and the samples were incubated at 25°C for 60 min. Each filter was washed with 8 cc 6xSC. This procedure resulted in the total elimination of P^{32} "noise" from nonhybridized RNA. An alternate method for the analysis of hybrid frequency was the use of a 0.9 × 48-cm G200 Sephadex column over which the annealing mixture was poured at the conclusion of the ribonuclease treatment. The exclusion peak contained all the DNA and RNA-DNA hybrids, while nonhybridized RNA was retarded. This method always yielded the same results as the filter method.

Template activity assay: A cell-free *E. coli* ribosome and supernatant system similar to that first described by Nirenberg and Matthaei[30] was utilized in these studies. The methods used were those of Schwartz,[31] with the filter assay technique of Mans and Novelli.[32]

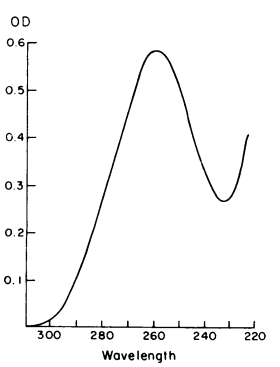


FIG. 1.—UV-absorption spectrum of a typical stage 4 oöcyte RNA preparation.

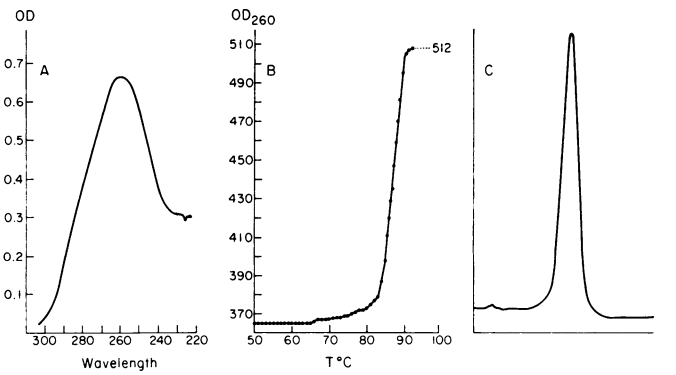


FIG. 2.—Characteristics of *Xenopus* DNA extracted from testes: (*A*) UV spectrum; (*B*) hyperchromicity plot showing a T_m of 86.8°C; (*C*) densitometer tracing of a DNA sample banded in a CsCl gradient.

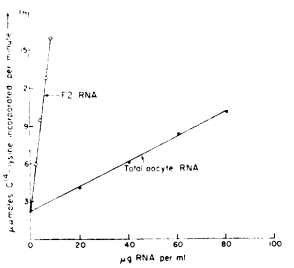


FIG. 3.—Response of the cell-free protein synthesis system to exogenous template-active RNA. Reaction mixture[31] (200 μl) containing 1.11 mg S-30 protein was present in each reaction tube. At 2-min intervals, 20-μl samples were adsorbed on Whatman 3 MM filter pads and assayed for hot TCA-insoluble C^{14}-lysine counts.[32] The rate of synthesis obtained directly from the time course of incorporation is a linear function of the RNA concentration. Each slope is proportional to the amount of template-active RNA present.

Direct Evidence for the Accretion of Large Amounts of Template-Active RNA during Oögenesis.—In order to estimate the amount of oöcyte RNA capable of displaying template activity, the stimulation of protein synthesis in the cell-free system obtained with varying amounts of oöcyte RNA was compared quantitatively to that obtained with a known messenger RNA standard. The RNA standard chosen, F2 coliphage RNA, consists of template for phage-specific proteins.[33] We have sought to minimize the difficulties inherent in comparisons between heterologous template active RNA's[34] by dealing with low concentrations of RNA, by testing only RNA's of high molecular weight, and by operating under optimal conditions with respect to significant variables affecting the activity of the cell-free system.[35] As shown in Figure 3, the rate of protein synthesis obtained under our conditions with total oöcyte RNA is a linear function of concentration for oöcyte RNA, just as it is for F2 RNA.

In Table 1, which summarizes these experiments, it can be seen that RNA preparations from the stage 4 oöcytes of different individual animals contain approximately equal amounts of template-active RNA. The amounts are surprisingly large. Stage 4, the maximal lampbrush stage of oögenesis, lasts for an unknown amount of time in *Xenopus*, and lampbrush chromosomes are also present during stage 3; it is clear that by maximum lampbrush a considerable quantity of template-active RNA has already been synthesized and stored in the oöcyte. In the course of stage 5, the extreme lampbrush condition slowly recedes, and by stage 6 the oöcyte contains a slightly greater amount of template-active RNA than is present in stage 4, taking the average value 41.6 mμg/oöcyte as representative of stage 4. Since there is almost no RNA synthesis in mature, resting, stage 6 oöcytes, the large stockpile of template-active RNA in these oöcytes must have been synthesized earlier, during the preceding lampbrush phases.

Measurement of the Percentage of the Genome Active in the Lampbrush State Oöcyte.—By direct measurement of the amount of *Xenopus* DNA which is complementary

TABLE 1

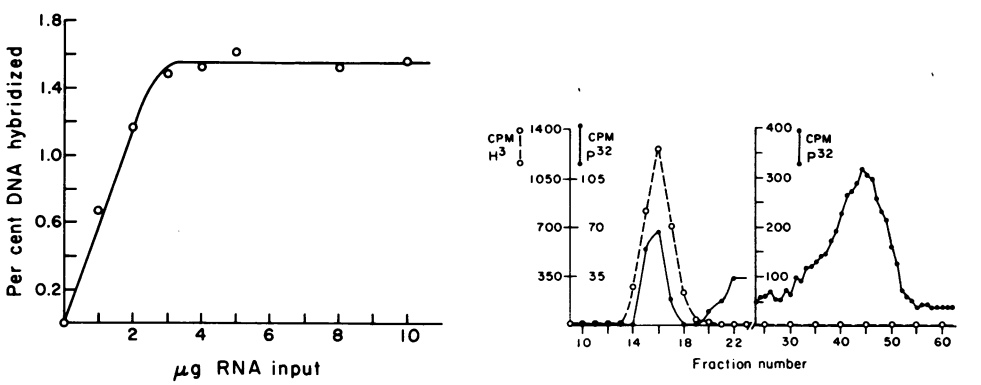
AMOUNTS OF TEMPLATE-ACTIVE RNA IN *Xenopus* OÖCYTES ESTIMATED BY COMPARISON WITH THE TEMPLATE ACTIVITY OF KNOWN AMOUNTS OF F2 RNA*

Oöcyte stage	RNA preparation	Template-active RNA (%)	Template-active RNA per oöcyte (mμg)		mμg Template-active RNA/mμg DNA/tetraploid chromosome
4	A†	2.9	54.1		4510
4	B†	2.2	41.8	Av.	3490
4	C†	1.9	37.1	41.6	3090
4	D	1.8	33.3		2770
6	E†	1.9	46.8		3900

* Values are corrected for the different lysine contents estimated for F2- and oöcyte RNA-coded protein.[35] RNA preparations marked with a dagger have been pronase-treated. In our experience nonpronased samples are frequently inactive. Total RNA content of stage 4 and stage 6 oöcytes has been reported, see reference 9.

to newly synthesized (labeled) stage 4 oöcyte RNA, it is possible to obtain an estimate of how much of the genome is active in the lampbrush chromosome. This experiment, however, requires that one know the specific activity of that particular fraction of the oöcyte RNA which hybridizes with the DNA. If the nonribosomal gene products of the lampbrush chromosome are mainly stored, as is rRNA, rather than being used and degraded, then irrespective of the duration of labeling, their specific P^{32} activity should be the same as that of rRNA synthesized at the same time. Soon after a single i.p. injection of $Na_2HP^{32}O_4$, the stage 4 oöcyte precursor pool arrives at its maximum level, which is the same in each individual stage 4 oöcyte of a given ovary. Label accumulates in the oöcyte RNA at a linear rate for more than 4 days thereafter. Four independent lines of evidence indicate that the newly synthesized, nonribosomal, high-molecular-weight RNA of the oöcyte is indeed conserved, at least over the 4-day labeling period we have used for the experiments to follow: (*a*) we have never been able to obtain direct evidence for rapidly labeling and rapidly degrading RNA fractions, even in analyses of RNA extracted after very short *in vitro* labeling periods (e.g., 30 min); (*b*) as noted above, the large quantities of template-active RNA present in stage 4 and stage 6 oöcytes indicate extensive conservation of gene products throughout this phase of oögenesis. To this we may add (*c*) an experiment described below which demonstrates that most of the nonribosomal RNA found in the stage 6 oöcyte is qualitatively the same as that made during stage 4. Furthermore, (*d*) the *in vivo* radioautograph studies of Gall and Callan[8] have shown that newly synthesized RNA remains present in the chromosome loops themselves for at least 14 days. For these reasons we conclude that all classes of RNA present in each preparation may be considered to possess the same specific activity at the end of a 4-day labeling period.

In Figure 4 we present two hybridization experiments with somatic H^3-DNA



(*a*) (*b*)

FIG. 4.—(*a*) Typical saturation curve for the formation of ribonuclease-resistant hybrids between testis H^3-DNA and lampbrush stage P^{32}-RNA. Each 200 μl of annealing mixture contained 5 μg DNA and the amounts of RNA plotted on the abscissa. Specific activity of the RNA was 914 cpm/μg. After RNase treatment the hybrids were trapped on filters and washed (see *Methods*). Each filter was then hydrolyzed in 150 μl 20% TCA, and the acid extract was assayed for P^{32} and H^3 in a scintillation counter. From the known specific activities of the input nucleic acids the amount of DNA retained on the filter and the amount of RNA hybridized could be calculated.[28] (*b*) G200 Isolation of stage 4 RNA-DNA hybrids formed under the same conditions as in (*a*).

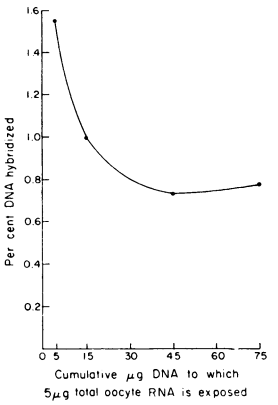


FIG. 5.—Rehybridization experiment in which the per cent of DNA hybridizing with lampbrush-synthesized RNA is measured after each of three successive exposures of a single sample of RNA to excess DNA. RNA (25 μg) in 1.6 ml 6xSC were annealed with 75 μg DNA to begin the experiment. After annealing for 4 hr, the annealing mixture was passed over a nitrocellulose filter to remove the RNA-DNA hybrids and the remaining DNA. Recovery of RNA and removal of DNA were then checked by counting the filtrate, and fresh DNA was added to a ratio (DNA/RNA) of 6. The first point plotted (5 μg of DNA) represents the control hybridization frequency obtained in the presence of excess RNA.

and P^{32}-labeled stage 4 oöcyte RNA. Table 2 summarizes the results of other experiments, with three different RNA preparations. Both methods of hybrid assay show that about 1.5 per cent of the DNA is complementary to the newly synthesized lampbrush stage RNA. The results are constant from preparation to preparation, and, since stage 4 oöcytes of every gradation in maturity have contributed to each RNA preparation, it is clear that the 1.5 per cent value is not peculiar to particular animals or particular substages of lampbrush development.

Only one strand of the DNA is expected to be active in RNA synthesis *in vivo*. Thus our data show that at the maximal lampbrush stage about 3 per cent of the organism's total genome is functional.

Evidence for the Existence of a Class of Nonribosomal Gene Products Present in Large Amount in the Lampbrush State Oöcyte.—As described in detail in the legend, the experiment of Figure 5 is a "cascade" experiment in which a single sample of total stage 4, P^{32}-RNA is subjected to three successive cycles of hybridization with excess DNA. RNA representing 32 per cent of the DNA which is *active* in the lampbrush stage nucleus is removed from the preparation after a total exposure of the sample to 15 μg of DNA, and a smaller group of gene products is exhausted during the second cycle of the experiment. However, the third step, by the end of which the RNA has been exposed to a total of 75 μg of DNA, fails to lower the hybridization frequency further. Figure 5 therefore demonstrates the presence of relatively large quantities of a class of gene products representing about 0.66 per cent of the DNA beyond that complementary to the bulk ribosomal RNA of the preparation.[13]

TABLE 2

PLATEAU HYBRIDIZATION LEVELS WITH *Xenopus* LAMPBRUSH-STAGE RNA

RNA preparation	Hybrid assay methods	Plateau frequency: % DNA hybridized
A	G 200	1.40
A	G 200	1.35
A	Filter	1.34
A	"	1.40
A	"	1.44
B	"	1.61
B	"	1.52
C	"	1.56
C	"	1.53

Direct Evidence for Retention of Nonribosomal Lampbrush Stage RNA in Resting Stage 6 Oöcytes.—Figure 6 describes a hybridization competition experiment in which unlabeled stage 6 RNA is used to compete with P^{32}-labeled stage 4 RNA for DNA binding sites. The experiment shows that 60 per cent of the species of RNA in synthesis at mid-lampbrush stage are also present, stockpiled in the mature oöcyte. Other experiments have yielded up to 74 per cent homology between gene products present in stage 6 and the gene products in synthesis at stage 4. This means that lampbrush chromosome-synthesized RNA's are retained throughout oögenesis. The remaining 30 or 40 per cent of the nonribosomal stage 6 RNA species may have been produced earlier in oögenesis, perhaps during stage 3, when it is possible that a somewhat different spectrum of genes is active than in stage 4.

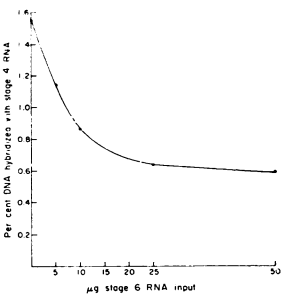


FIG. 6.—Competition experiment in which DNA binding sites complementary to P^{32}-stage 4 RNA are competed for by unlabeled RNA from mature stage 6 oöcytes. Each tube contained 5 μg DNA, 5 μg stage 4 RNA, and the amounts of stage 6 RNA shown on the abscissa.

Discussion.—The experiments we have described show that during the lampbrush stage of oögenesis maternal gene products needed for embryogenesis are synthesized and in some way sequestered for prolonged storage during the remainder of oögenesis. Previously, we demonstrated that the *ribosomal* RNA synthesized at the lampbrush stage follows this pattern of events.[6, 9] We now find that massive stores of template-active, *nonribosomal* RNA have already accumulated by the maximum lampbrush stage and that on the basis of both quantitative and qualitative evidence, *these stored RNA's are still present in the completed stage 6 oöcyte.* In the lampbrush-stage nucleus almost all the RNA synthesis is ribosomal RNA synthesis (something over 98% per unit time[6]). In the presence of this avalanche of ribosomal RNA synthesis it is difficult with chemical techniques to distinguish the relatively small amount of nonribosomal, high-molecular-weight RNA synthesis occurring simultaneously. Our experiments nevertheless demonstrate that by the end of oögenesis the oöcyte contains template-active, high-molecular-weight RNA equal in mass to almost 3900× the quantity of DNA in the tetraploid chromosomes upon which it was formed (Table 1). However, this amount of RNA constitutes only 1.9 per cent of the total oöcyte RNA. Even by mid-lampbrush stage some 42 mμg of template-active RNA have accumulated according to our average stage 4 figure. We have shown here that about 2.7 per cent of the genes in the stage 4 nucleus are active, excluding the ribosomal RNA genes. We can then calculate that on the average each such gene, assuming the size of that coding for the β chain of the hemoglobin molecule, could be represented 263,000 times in the oöcyte population of template-active RNA's.[36] This is not to say that all of the RNA synthesized at the lampbrush stage is retained, for the immature oöcyte carries on an active *de novo* protein synthesis[37, 8] and for this some messenger RNA is certainly required. Nonetheless, the over-all picture is one of massive RNA synthesis and accretion, indicating a specialized genomic activity in preparation for embryogenesis. Brown[10, 14] has found that a considerable amount of new "DNA-like" RNA synthesis occurs in the final 12 hr following hormonal stimulation and ovulation. The total quantity of this RNA, however, is less than $1/40$ of that already

stored in the mature stage 6 oöcyte, according to our minimum estimates. It is therefore unlikely that as Brown has suggested,[14] the only maternal template-active RNA present in the newly shed egg is made during the 12-hr ovulation period.

Only 3 per cent of the genes appear to be active in the lampbrush chromosome (Table 2). This value agrees well with existing estimates of the proportion of DNA present at any one time in the lampbrush loops of *Triturus*.[38] Comparing the total length of the lampbrush chromosome loops to that calculated for the total DNA complement of the chromosomes, the loops are found to constitute only 5 per cent of the total expected DNA length and thus 5 per cent of the genome. In fact this may be an overestimate, since Izawa *et al.*[1] have reported the presence of almost 2× as much DNA in the lampbrush chromosomes than the tetraploid amount previously assumed.[38] Excluding the 0.11 per cent of the DNA coding for the ribosomal RNA's,[13] if each gene active were to be the size of that coding for a hemoglobin β chain, then the amount of the *Xenopus* genome active would comprise 756,000 such genes. Since this large number is unlikely to represent the number of diverse sites which are functional, it is not improbable that, like the ribosomal RNA's, some of the nonribosomal RNA's synthesized in the oöcyte represent highly multiple loci for the same gene product.

The lampbrush chromosome is unique, not in the proportion of its genome which is active, but in its role as a site for the elaboration of large quantities of extremely long-lived gene products which must ultimately be utilized in embryogenesis. Possibly its unusual structure is related to this specialized role. Thus the newly synthesized RNA is perhaps packaged *in situ*, on the loops themselves, within newly synthesized protector proteins. This might explain the presence of the astonishingly high protein content of the chromosomes[1] and of the intense protein synthesis occurring continuously on the lampbrush loops.[8] The sequential labeling patterns and the accumulation of labeled RNA at one end of some loops reported by Gall and Callan[8] can be interpreted in terms of a polarized RNA packaging phenomenon occurring on each loop.

Summary.—About 2.2 per cent by mass of the lampbrush-stage oöcyte RNA is template-active RNA. This RNA is synthesized during the lampbrush stage through the activity of about 2.7 per cent of the genome. It is retained throughout oögenesis and is present in the mature oöcyte. At maturity the oöcyte contains about 47 mμg of this type of RNA, which is almost 3900× the mass of the chromosomal DNA. At least 60 per cent of this RNA is the product of the genes which are active during the mid-lampbrush stage.

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[36] A structural gene for the 146 amino acid hemoglobin β chain would have a mass of $(438 \times 302/6.02 \times 10^{23}) \times 2 = 4.39 \times 10^{-19}$ gm DNA, assuming 3:1 coding ratio and considering that the DNA is double-stranded. Since 1.38% by weight hybridizes, excluding rRNA hybridization, this is $0.0138 \times 12 \times 10^{-12} = 1.66 \times 10^{-13}$ gm DNA, calculating the tetrapoloid DNA content from the 2C value found by Dawid for *Xenopus*.[25] Since only one strand hybridizes, 3.32×10^{-13} gm DNA is in the active genes and the number of β chain-sized genes active is 3.32×10^{-13} gm/4.39×10^{-19} gm $= 7.56 \times 10^{5}$. Similarly, 47 mμg template-active RNA could contain $47 \times 10^{-9}/2.36 \times 10^{-19}$ gm/mRNA $= 19.9 \times 10^{10}$ mRNA molecules. Thus, every gene active could be represented $19.9 \times 10^{10}/7.56 \times 10^{5} = 2.63 \times 10^{5}$ times.

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