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Isolation of single stranded DNA related to the transcriptional activity of animal cells.

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ABSTRACT

Single stranded DNA (s.s.DNA) comprising I-2% of the total nuclear DNA was isolated by an improved method of hydroxyapatite chromatography from native nuclear DNA of embryonic chick cells, labeled for several cell generations with ^{3}H-thymidine. Small quantities of ^{3}H-DNA were annealed with a large excess of unlabeled DNA or polysomal RNA from chick embryos. Hybridization kinetics (monitored by the use of S_I nuclease digestion, hydroxyapatite chromatography and thermal fusion), indicated that s.s.DNA belongs to the non repetitious fraction of the cell genome. One third represents DNA sequences engaged in the transcription of messenger RNA's

INTRODUCTION

The first step in the synthesis of RNA, is the interaction of an RNA polymerase molecule with a specific promoter site on the DNA template. There are a number of possible mechanisms for activation of a DNA template for transcription; simple untangling of the DNA double helix, the insertion of single strand breaks in the double helix,[1] or a specific loclized structural change at a promoter.[2] Crick[3] has proposed that unpaired single stranded stretches of double stranded DNA, could be involved in the recognition sites needed for control purposes in higher organisms. Recently, we have shown that in cells of different animal species, a small fraction (I-2%) of native nuclear consists of single stranded sequences.[4,5] As presented in this communication, such sequences isolated from chick nuclear DNA were further analysed, and here we show that they apparently constitue an active transcribed fraction of the cellular genome.

MATERIALS AND METHODS

Cells and labeling procedures. Primary cell cultures were prepared from IO-day-old- chick embryos. The embryos were trypsinized and approx. $2xIO^8$ cells were seeded per Roux bottle containing IOO ml of Eagle's medium supplemented with IO% fetal calf serum and IO% TPB

(tryptose phosphate broth 2%). After incubating for two days at 37°C, the cells were transferred to Roux bottles ($2xIO^7$ cells per bottle) with the medium described above. After incubating for I2 hours at 37°C the medium was changed and 25 μC/ml of (^3H)-thymidine was added. The pH of the medium was maintainedat 7.4 during the course of incubation and the cells were grown for approximately three cell generations. In some chase experiments, after labeling the cells for I2 hours with (^3H)-thymidine, the radioactive medium was changed and the radioactive precursor omitted. The cells were then grown for one or two cell generations

Preparation of DNA. Cell nuclei were purified using non ionic detergent[6] resuspended in 0.0I M Tris-HCl pH 8.3, EDTA 0.0I M and lysed by incubating for 30 min. at 37°C in the presence of 0.I% sodium dodecyl sulfate (SDS) and I00 μg/ml of pronase. The concentration of SDS was increased to I% and the nuclei reincubated for I5 min. at 37°C. After addition of sodium perchlorate to I.0 M, the DNA was extracted 3 times with chloroform-isoamylic alcohol (99/I), precipitated with ethanol and redissolved in and dialysed against NaCl 0.0I M, Tris 0.0I M pH 8 EDTA 0.002 M. The DNA thus isolated was sheared by extruding 2 ml of the DNA solution (200 μg/ml) through a BD 26 gauge needle ten times by hand with a maximum pressure. The specific activity determined by counting aliquots in a liquid scintillation spectrometer was I-I.5xIO^6 cpm/μg DNA in different experiments.

Isolation of single stranded sequences. The improvement of the hydroxy apatite chromatography used in these studies has been already described (see ref. 5). Briefly, elution was carried out at 56°C with phosphate buffer (Na_2HPO_4-NaH_2PO_4) at pH 7.85. Instead of column chromatography, a batch procedure which facilitates the processing of relatively large numbers of samples was used. Under these conditions, the separation of the single and the double stranded DNA is greatly improved. When the pH of the phosphate buffer was carefully controled, reproducible result were obtained in all assays; 95% or more of the radioactivity was recovered.

Preparation of polysomal RNA. Cells of I0-day-old chick embryos were homogenized in Earle's medium, pelleted by low speed centrifugation, resuspended in 0.0I M Tris-HCl pH 7.4, 0.02 M $MgCl_2$, 0.2 M KCl, 0.25% sucrose containing 0.5% Nonidet P_{40} and incubated I0 min. at 4°C. After two successive centrifugations (I5 min. at 5000 rpm and 30 min.

at 10.000 rpm respectively), the supernatant was collected and $MgCl_2$ added to a final concentration of 0.2 M; it was then kept 12 hours at 4°C. The pellet obtained by centrifugation at 5000 rpm for 15 min. was resuspended in NaCl 0.01 M, Tris-HCl 0.01 M pH 7.2, EDTA 0.05 M containing pronase 100 µg/ml, 0.1% SDS and incubated one hour at 37°C. Sarcosyl to 1% was then added and the RNA was extracted 3 times with one volume of phenol plus one volume chloroform at 4°C. The last aqueous phase was precipitated once with LiCl 2.0 M and twice with ethanol. Polysomal RNA from liver of three-week-old chicks and mouse ascites tumor cells (FLS tumor) was prepared by similar procedures.

Fractionnation of RNA on poly(U)-sepharose. In some experiments, polysomal RNA was fractionnated on poly(U) sephrose.[7] The minor fraction retained on the column in high salt solution (2-2.5% of the total RNA) consists of messenger RNA containing terminal poly (A) chains (poly A mRNA's), whereas unbound RNA essentially represents ribosomal RNA (rRNA The fractionnation was monitored by the use of polysomal RNA from embryonnic chick cells, labeled with ^{32}P, the purity of each RNA fraction was ascertained by determining their sedimentation profile and base composition.[8]

DNA renaturation. Aliquots of s.s.DNA and d.s.DNA were mixed with an excess of unlabeled total nuclear DNA isolated from chick embryos. DNA was thereafter denatured by alkaline treatment: to 8 parts of the DNA solution 1 part of 1.0 M NaOH was added. After 15 min. of incubation at room temperature, the solution was cooled to 0°C and was neutralized by adding one volume of cold 2.0 M NaH_2PO_4. Renaturation of DNA was carried out by incubating at 66°C in 2xSSC for different time periods at a constant DNA concentration.[9] The rate of reassociation was followed by S_I nuclease digestion. Samples of reassociated DNA were divided into two fractions, one for S_I nuclease digestion, and the other submitted to the same treatment but without S_I nuclease.

The S_I nuclease was prepared by the method of Sutton,[10] and adjusted to 5000 units/ml as defined by Sutton. For the digestion procedure, aliquots of reassociated DNA were diluted at least 20 times in S_I buffer (NaCl 0.15 M, sodium acetate 0.03 M, $ZnSO_4$ $5x10^{-4}$ M pH 4.5). To each buffered samples containing 20-40 µg of DNA, 0.05 ml of S_I was added and the solution incubated at 40°C for 30 min. The reaction was stopped by adding cold trichloroacetic acid (TCA) to 5%. After a 10 min. incubation in an ice bath, the acid precipitable material was collected by

filtration on Whatman glass fibre paper GF/B the filters were dried and the radioactivity determined in a liquid scintillation spectrometer.

DNA-RNA hybridization. Total polysomal RNA (12 mg), rRNA (12 mg), or poly(A)-mRNA (0.6 mg) precipitated in 66% ethanol was centrifuged at 7000 rpm for 15 min. The pellet resuspended in 0.1xSSC containing 1 µg of (^{3}H-thymidine)-labeled DNA was boiled 10 min. and rapidly chilled on ice. The solution was adjusted to 2xSSC, 0.1% SDS and incubated at 66°C After different periods of time, samples were collected and treated by S_I nuclease followed by dialysis against NaCl 0.15 M, Tris-HCl 0.01 M pH 7.2, EDTA 0.002M and hydroxyapatite chromatography performed as described above.

RESULTS

Isolation and characterization of single stranded DNA. Chick nuclear DNA isolated as described in materials and methods and analysed by the improved method of hydroxyapatite chromatography contains two eluted fractions; a major one with a main peak of elution at 0.55 M phosphate buffer and a small one amounting to 1-2% of total DNA with a main peak of elution at 0.15 M like denatured DNA (fig. 1). Other evidence for identifying this minor fraction as s.s.DNA are as follows: in CsCl density gradients, it banded at a buoyant density somewhat higher than that of bulk d.s.DNA (fig. 2). As shown in table I, it is digested by deoxyribonuclease and S_I nuclease (the latter degrades single stranded nucleic acids under defined conditions), but it is resistant to alkaline hydrolysis, and its base composition analysed after labeling DNA with ^{32}P does not differ much from that of bulk DNA.[5]

Hydrolysis	ssDNA fraction cpm	dsDNA fraction cpm
none	4290	5500
Alkali	4145	5360
DNAse	28	80
S_I nuclease	295	5190

Table I. Further characterization of the ss DNA fraction.

ss-DNA and ds-DNA fraction isolated as shown in fig.1 were submitted to alkaline hydrolysis (by bringing the pH of the DNA solutions to 12.5 and incubating at 37°C for 18 hr.) and enzymatic digestion (for the DNAse digestion, $MgCl_2$ to 0.005 M, and 10 µg DNAse/ml were added: The S_I nuclease digestion was monitored as described in materials and methods). After hydrolysis, aliquots were processed for TCA precipitation and their radioactivity measured in liquid scintillation spectrometer.

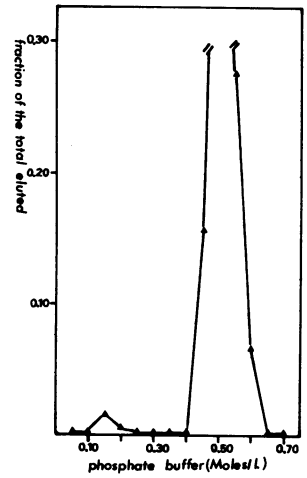

Fig. I Hydroxyapatite chromatography of native nuclear DNA. (^{3}H)-thymidine labeled nuclear DNA from chick fibroblastic cells was loaded on hydroxyapatite. The fractions were eluted at different phosphate buffer molarities (pH 7.85), and aliquots were processed for TCA precipitation as described in materials and methods.

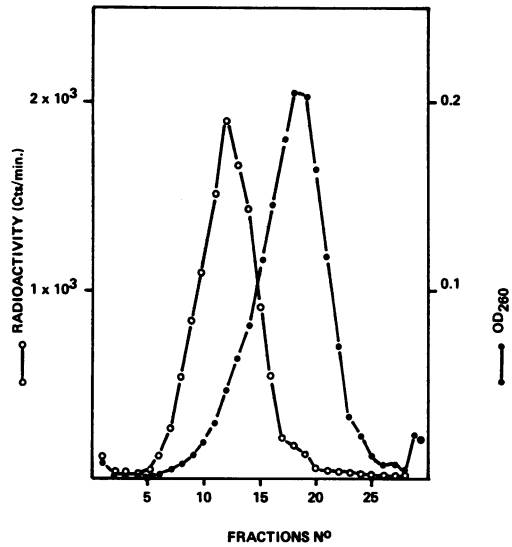

Fig. 2 Centrifugation in CsCl density gradient of (^{3}H)-thymidine ss-DNA fraction and unlabeled ds-DNA fraction (75 µg). Isolated ss-DNA fraction labeled with (^{3}H)-thymidine was mixed with unlabeled ds-DNA fraction, and dissolved in CsCl in 0.01 M Tris (pH 8.3) plus 2 mM EDTA to make the density approx. 1.7 g/ml. Placed in tubes suitable for the 50 Ti rotor of the model L ultracentrifuge, overlayed with paraffin oil, and centrifuged at 33000 rev.:min. at 18°C for 72 hours. The tubes were pierced at the bottom with a needle, and fractions were collected. Optical density as well as the TCA precipitable radioactivity were determined.

It is to note that the relative amounts of ss-DNA isolated from non labeled chick cells, were the same as those of radioactive ss-DNA after after labeling for 2-3 cell generations. Moreover this amount was also the same after long chase experiments. The sedimentation coefficient determined by alkaline sucrose gradient,[II] was comprised between I5 to I8 S for the ds-DNA and 8-I2 S for the ss-DNA. The renaturation kinetics illustrated by fig. 3 demonstrate that ss-DNA is an integral part of chick nuclear DNA, and mainly corresponds to its non repetitious sequences. Thus up to a C_0t value of 20, no significant amount of ss-DNA was reassociated in duplex structures (as compared with I0-I2% for ds-DNA), indicating that ss-DNA contains none of the highly reiterated sequences.

Hybridization between ss-DNA and homologous mRNA's. Each of the radioactive DNA fractions was hybridized with homologous polysomal RNA in excess, or with poly(A)-mRNA's and rRNA separated on poly(U)-sepharose.

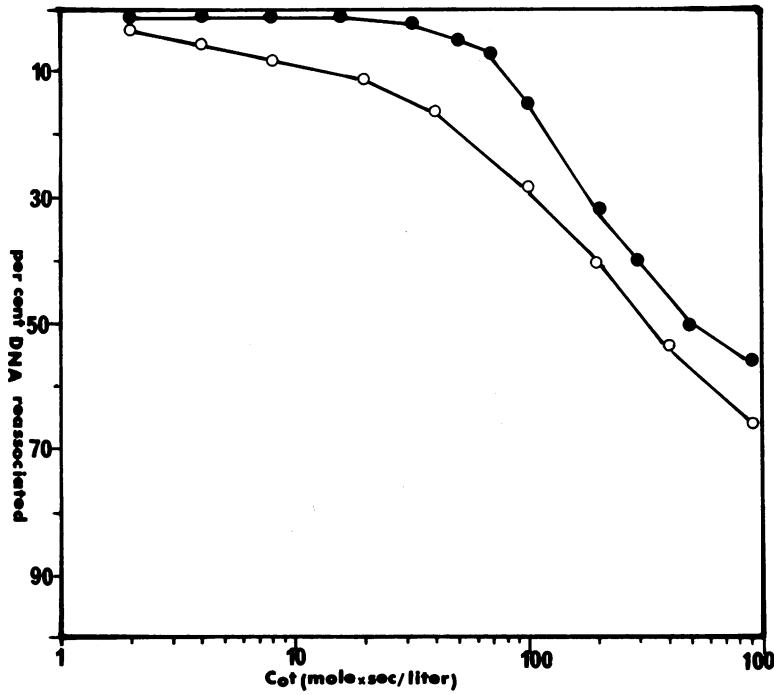

Fig. 3 Annealing experiments at different Cot values between (^{3}H)-thymidine ss-DNA fraction, ds-DNA fraction and unlabeled total nuclear DNA. (^{3}H)-thymidine labeled ss-DNA fraction (I µg) (●——●), and ds-DNA fraction (I µg) (○——○) were mixed with unlabeled total nuclear DNA (800 µg) and renatured at different Cot values. The rate of reassociation was measured as described in materials and methods.

Hybridization yields were calculated by measuring the fraction of radioactive DNA resistant to S_I nuclease digestion. Control DNA samples, were similarly processed before incubating (time zero), and after incubating for the same periods in the absence of RNA or in the presence of mouse polysomal RNA. Kinetics of hybridization with total polysomal RNA shown in fig. 4 were confirmed in two other experiments. Concerning ds-DNA, in control samples without RNA, the amounts that became double stranded increased with time (as the result of self annealing of repeated DNA sequences) and were not appreciably changed in the presence of mouse polysomal RNA. In the presence of polysomal chick RNA the percent of DNA found in duplex structures was significantly higher than that of self annealing but only after achieved high C_rt values. The maximum fraction hybridized with RNA was estimated as 8-9% of total ds-DNA. With ss-DNA, all of it remained single stranded at any time in control samples incubated without RNA. No more than 4% appeared to be hybridized with heterologous polysomal RNA, at the highest C_rt value achieved.

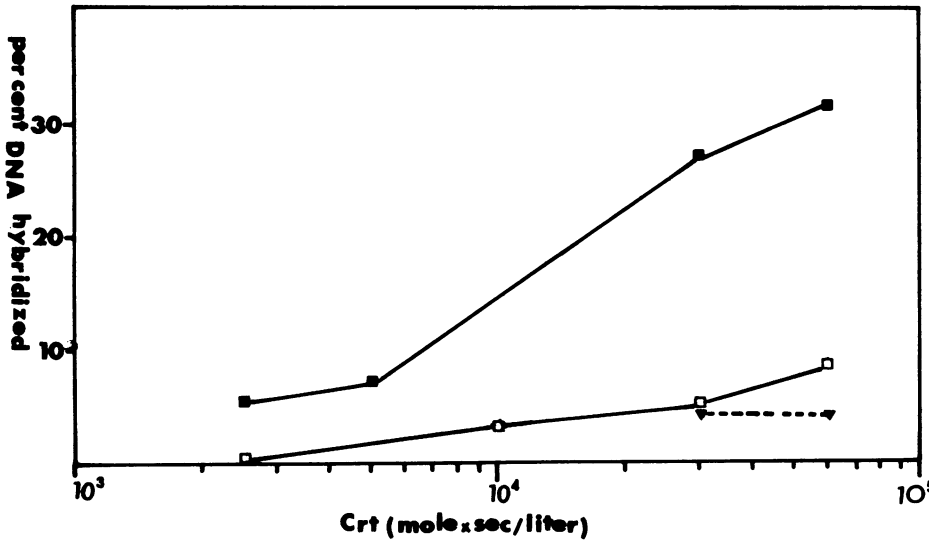

Fig. 4 Kinetics of hybridization between (^{3}H)-thymidine ss-DNA fraction and polysomal RNA isolated from chick embryos. Unlabeled polysomal RNA (I2 mg) from chick embryos was hybridized at different Crt values with I µg of (^{3}H)-thymidine labeled ss-DNA fraction (■——■), and ds-DNA fraction (□——□). Reassociation analysis was accomplished by allowing reassociation to occur for various periods of time, and determining the amount reassociated by measuring the resistance to S_I nuclease digestion as described in materials and methods. Control: unlabeled polysomal RNA (I2 mg) from mouse ascites tumor cells hybridized with labeled chick ss-DNA fraction (▽- - -▽).

This might reflect partial homology between the genome of chicken and mouse respectively. With homologous polysomal RNA 5-6% of ss-DNA was hybridized at relatively low C_rt values, and 30-33% at the highest C_rt value. Equivalent results were obtained using mRNA's from chicken liver purified on poly (U)-sepharose. After incubating DNA with a 1000 fold excess of RNA (up to C_rt value of 1500-3000) 25-26% of the ss-DNA was hybridized with poly(A)-mRNA's, as compared with 4-5% for ds-DNA and negligible amounts with rRNA.

Further characterization of hybrids. The double stranded structure of DNA-RNA hybrids was ascertained by other methods. When rechromatographed on hydroxyapatite, at least 80% of the S_I resistant ss-DNA annealed with chick polysomal RNA was now eluted as a double stranded structure. Furthermore the melting curve of the ss-DNA-RNA hybrid (fig. 5) shows that it was 6-7°C lower than that of the ds-DNA.

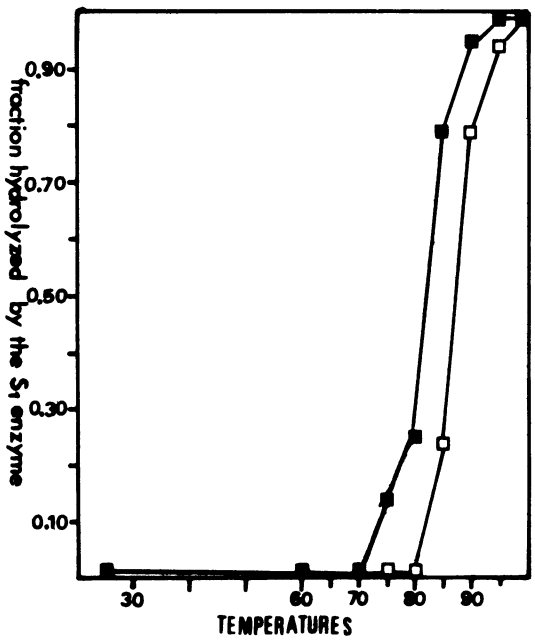

Fig. 5 Melting curve of ss-DNA-RNA hybrid. Aliquots of the ss-DNA-RNA hybrid (■——■) obtained as shown in fig. 4 and ds-DNA fraction (□——□) were denatured 5 min. at different temperatures in 0.15 M NaCl. Rapidly chilled in an ice bath, they were diluted at least 10 times in the S_I buffer and submitted to the S_I nuclease digestion as described in materials and methods. Controls (equivalent aliquots not submitted to the S_I nuclease digestion) as well as the digested aliquots were processed for TCA precipitation and their radioactivity measured in a liquid scintillation spectrometer.

DISCUSSION

We previously isolated single stranded DNA from the nuclear DNA of cells from various animal species.[4,5] The ss-DNA fraction represents 1-2% of the total DNA. Radioactive single stranded sequences had also been found by others in the **pulse** labeled DNA of eukaryotic cells [12-14] but these sequences were not further characterized. The isolation of ss-DNA raised several questions. In order to exclude the possibility that ss-DNA could simply result as an artifact of extraction, precautions were taken to avoid single strand breaks during purification. It was reported that the proportion of ss-DNA isolated from bacteria increased with combined heating and stirring. [15,16] In all our preparations heating and stirring were avoided. Furtermore the amount of ss-DNA was about the same in DNA isolated by centrifuging the nuclear lysate in CsCl density gradients, without any previous or further extraction.[4] Single strand DNA fragments are believed to be important intermediates in DNA synthesis in bacteria [17] as well as in animal cells. [18] However our previous studies on DNA replication with synchronized animal cells, revealed that the amount of ^{3}H-thymidine incorporated into ss-DNA did not increase after pulse labeling throughout the S phase.[19] Furtermore the long chase experiments indicated that the part of ss-DNA related to the newly replicated sequences was negligible. It is still less likely that the bulk of ss-DNA results of an accumulation of single strand breaks because of aging in cells. [20-21] Primary cell cultures derived from 10 day embryos were used in all experiments. Altogether none of the possible explanations for our findings can be totally excluded.

It was reported by others that a part of the nucleolar DNA consists of single stranded DNA sequences that were supposed to be the starting point for bulk destabilization away from the rDNA genes.[26] However in the present studies, the DNA renaturation kinetics (fig. 3) indicated that ss-DNA mainly if not exclusively, belongs to the non repetitious fraction of the animal cell genome, coding for the great majority of messenger RNA's.[22-25] The strongest evidence for the above was obtained with the DNA-RNA hybridization assays (fig. 4), showing that one third of the ss-DNA molecules recovered from chicken embryonic cells represent transcription sites. This conclusion is further strenghtened by recent studies of RNA synthesis in vitro. Using chromatin of chicken cells as template, it was indirectly demonstrated that the DNA duplex

is not maintained during transcription.[27]

Amongst various questions raised by the finding of ss-DNA, the most urgent is to know whether it originates from the bulk of transcribed DNA or represents a new molecular fraction. The present results support the former possibility. Since ss-DNA mainly belongs to the non repetitious part of the cell genome, hybridization of about one third of it must involve a great variety of RNA molecules. This is confirmed by hybridization kinetics. Assuming that despite hazardous factors (such as possible alterations of RNA in the course of incubation) saturation levels were obtained, and taking the mean DNA content of diploid chicken cells [28] as equivalent to $1.2\text{-}1.5 \times 10^{12}$ daltons, the annealed part of ss-DNA is equivalent to about 5×10^{9} daltons, which could correspond to 5000-10000 RNA species. This figure is compatible with available data on the number of gene transcripts in various animal cells (see general review by Lewin [29]).

Other controversial points cannot be clarified without additionnal evidence. For example, a model proposed by Crick postulates the existence of repeated unpaired DNA sequences that are probably not transcribed.[3] Whatever the implications of our own data, they do not obligatory signify that ss-DNA as evidenced here, does exist in vivo. It might represent a significant sort of artifact arising from duplex molecules somehow destabilized by transcription, and randomly split during isolation of DNA. In fact, experiments that are still in progress indicate that ss-DNA may contain both transcribed DNA sequences and their complementary strand. Assuming that only one strand of the DNA is transcribed it may be concluded that the greatest part, if not all of the ss-DNA represents an active fraction of the genome. Nevertheless the total amount of bulk double stranded DNA which is hybridizable with RNA from chicken embryos (grossly similar to that found in mouse embryos [22]), greatly exceeds the quantity of hybridized ss-DNA. At least two possibilities can account for this difference. First the embryos used for preparing RNA might synthesize a greater variety of mRNAs as compared with the cultured cells used for preparing radioactive DNA. Second, since there is no reason to admit that in any cell system, all the distinct RNA species are simultaneously and also continuously produced, ss-DNA might consist of DNA sequences partially or totally transcribed at a given time, whereas the hybridized part of bulk DNA represents the whole of active genome.

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