THE LENGTH OF REPETITIVE SEQUENCES OF MONKEY CELL AND SALMON SPERM DNA

György Berencsi*

Institut für Virusforschung,
Deutsches Krebsforschungszentrum,
Heidelberg

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SUMMARY: Reassociated DNA duplexes were isolated from both CV-1 and salmon sperm DNA using the S_1 endonuclease. Sequences reiterated 1000-50,000 times were not found in the CV-1 cell DNA. The mean size of reassociated, double-stranded repetitive molecules, which reassociate at $C_o t$ values less than 40, was found to be 500 base pairs in the case of CV-1 cell DNA and 300 base pairs in the case of salmon sperm DNA.

INTRODUCTION: Eukaryotic cells have been found to contain DNA of different frequencies of reiteration (1). The average length of the repetitive elements was estimated to range between 50 and 300 base pairs for Drosophila and 300±100 base pairs for Xenopus DNA (2-3). On the other hand, a mean length of 715 base pairs has been calculated for the tandemly repeated mouse main band DNA examined in ring formation experiments (4-6).

The utilization of the single-strand specific S_1 endonuclease (7) will be described in this paper for the examination of the size of "low $C_o t$** DNA fractions" (1-3) present in CV-1 cells and salmon sperm. It will be shown that the S_1 resistant double-stranded reassociation products isolated from CV-1 DNA (reiteration frequency higher than 50,000) have a mean length of about 500 base pairs, while those of salmon sperm DNA (reiteration frequency higher than 100) comprize about 300 base pairs as determined by velocity sedimentation analysis.

MATERIALS AND METHODS

1 - DNA preparations: Permanent monkey kidney cells (CV-1), free of mycoplasma, were labeled with [^{3}H]Thymidine (25 Ci/mole, 6 μCi/ml, Amersham, England) for 48 hours in Eagle's minimum essential medium

*Present address: Institute for Virus Research, National Institute of Public Health, H-1966 Budapest, Hungary.

**$C_o t$ - quantitation of reassociation (mole x sec x liter^{-1})

supplemented with 10% calf serum. DNA purification was performed according to Waldeck et al. (8), but a phenol extraction step at room temperature was also included between the SDS and chlorophorm-isoamyl-alcohol treatments. Simian virus 40 (SV-40) DNA was prepared by a modified Hirt procedure (8) from CV-1 cells infected with SV-40 strain Rh 911. Unlabeled salmon sperm DNA and calf thymus DNA were purchased from Serva, Heidelberg.

2 - DNA reassociation: DNA samples, except those of salmon sperm, were heat-denatured in 0.1xSSC (0.015 M NaCl and 0.0015 M Na-citrate, pH 7.0) at 100°C for 7 or 10 min followed by immediate quenching in an ice bath. Salmon sperm DNA was denatured in 1 N NaOH at room temperature for 30 min and the solution was subsequently adjusted to pH 7.0 with 5 N HCl at 0°C. To avoid loss of single-stranded material due to adsorption, siliconized glass vessels or hydrophobic plastics were used throughout. Denatured DNA solutions of 100, 677 and 935 µg/ml were adjusted to 0.25-0.77 M with 5 M NaCl and allowed to reassociate at 60°C. Samples were stored at -20°C until S_1 treatment or hydroxylapatite fractionation. $C_o t$ values were corrected for salt concentration (9).

3 - Conditions for assay of S_1 nuclease: The S_1 nuclease was purified by A. Fried and I. Maichle until the DEAE-cellulose step, following the procedure of Vogt (10). The assay mixtures contained 30 mM Na-acetate, 50-100 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol at pH 4.6 and were adjusted to have a final DNA concentration of 15 µg/ml with unlabeled, denatured calf thymus DNA. The treatment proceeded at 44°C for 2 or 3 hours in the presence of 10 µl of enzyme per ml. Degradation of single-stranded DNA was measured by the reduction of acid-precipitable radioactivity on Sartorius filters (pore size 0.45 µ). Under the conditions used at least 95% of single-stranded, but no double-stranded DNA was rendered acid soluble.

4 - Hydroxylapatite chromatography: DNA samples reassociated to different $C_o t$ values were passed through 1 ml hydroxylapatite columns

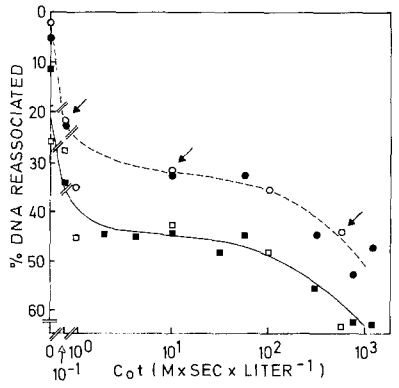

Fig. 1: Reassociation of CV-1 cell DNA estimated by S_1 treatment and hydroxylapatite chromatography. Filled symbols: DNA concentration 935 µg/ml. Open symbols: DNA concentration 100 µg/ml. Circles: per cent double-stranded material determined by S_1 treatment. Squares: per cent reassociated DNA determined by hydroxylapatite chromatography. Arrows: samples, which were used for neutral sedimentation analysis shown in Fig. 2c.

(Biorad HT) at 60°C in 0.12 M phosphate buffer (equimolar mixture of NaH_2PO_4 and Na_2HPO_4, pH 7.0). The single-stranded DNA and the S_1 digestion products were washed out with 4 ml of the same buffer. The double-stranded material was eluted with 4 ml of 0.44 M phosphate buffer. The eluates were quantitated by the determination of the acid precipitable radioactivity or by the determination of E_{260} and E_{320} values. Eluates used for velocity or equilibrium centrifugation were dialysed against 4000 volumes of 0.1xSSC, except for the 0.12 M eluates of S_1 treated samples, which were not dialysed.

5 - Gradient centrifugation of native and reassociated DNA: Velocity sedimentation was carried out in the Spinco SW 65 TI rotor at 20°C, 55,000 revs/min for 250 min using 5-20% (w/v) linear sucrose gradients containing 1 M NaCl, 20 mM EDTA and 20 mM TRIS (pH 7.2).

RESULTS

1 - Evidence for the absence of one main group of reiterated sequences from CV-1 DNA: Results of two reassociation experiments are shown in Fig. 1. No fractionation of the CV-1 DNA preceeded reassociation and no "zero time correction" (3) was used. The amount of S_1 resistant

reassociation products increased rapidly up to a level of 33% before a $C_o t$ value of one was reached. The lack of measurable duplex formation between a $C_o t$ value of one and a $C_o t$ value of 50 shows that sequences, similar to those of Xenopus, composing the bulk of repetitive DNA of this animal species (3), are absent from the CV-1 cells. At $C_o t$ values higher than 50 a further increase of the amount of S_1 resistant material occurred suggesting the presence of sequences reiterated less than 1000 times.

It is concluded that the distribution of repetitive DNA in CV-1 cells is similar to that of calf thymus DNA (1). It contains sequences of a frequency of reiteration higher than 50,000 and a second group of sequences, which are reiterated less than 1000 times.

2 - Alteration of the length of DNA during denaturation, reassociation and S_1 treatment: There is a significant amount of heat induced hydrolysis of phosphodiester bonds of single-stranded DNA at the temperatures used for denaturation and reassociation (11). The other factor, which may influence the size of the reassociation products isolated after S_1 digestion, is the ability of the enzyme to produce nicks in double-stranded DNA (12) and also to attack native DNA at the sites of single-strand scissions, thus producing double-strand breaks (13). To test these effects, the native CV-1 DNA was incubated with S_1 enzyme. Sedimentation of the digestion products through neutral sucrose gradients shows (Fig. 2a) that the native DNA (larger than 18 S) was reduced in its size to a relatively homogeneous population of molecules having a mean sedimentation coefficient of 10.3 S, corresponding to about 1000 base pairs (14). Therefore, the measurement of the isolated reassociation products may lead to underestimation of the actual length of the repetitive sequences, if the DNA degradative effects were not taken into consideration.

3 - Size of reassociated CV-1 DNA duplexes: The reassociation products

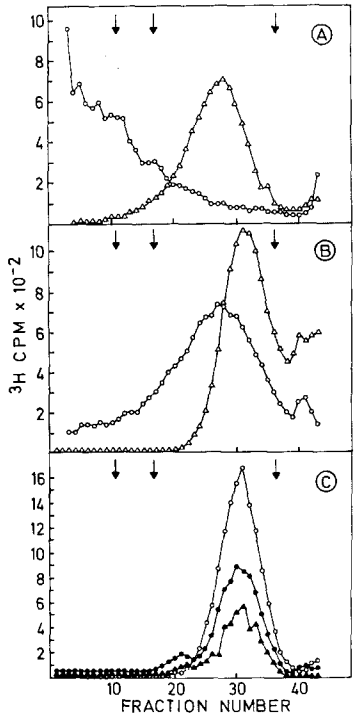

Fig. 2: Neutral velocity sedimentation analysis of the CV-1 DNA and reassociation products. Conditions of reassociation and centrifugation are described in Materials and Methods. Five-drop fractions were collected in scintillation vials and counted in PPO-toluene scintillation fluid. Direction of sedimentation from right to left. Panel A: (o—o—o), native, untreated CV-1 cell DNA; (Δ—Δ—Δ), S_1 treated native CV-1 cell DNA. Panel B: Hydroxylapatite isolated double-stranded reassociation products formed until a $C_o t$ value of 767 at 100 µg/ml DNA concentration. (o—o—o), without S_1 treatment; (Δ—Δ—Δ), after S_1 treatment. Panel C: S_1 resistant reassociation products isolated by hydroxylapatite chromatography. (▲—▲—▲), $C_o t$ 0.1; (●—●—●), $C_o t$ 10; (o—o—o), $C_o t$ 767. Arrows: Positions of SV-40 form I (21 S), SV-40 form II (16 S) and tRNA (4 S) markers from left to right, respectively.

were examined by velocity sedimentation analysis in neutral sucrose gradients before and after S_1 treatment (Fig. 2b-c). Before S_1 treatment the hydroxylapatite isolated duplexes formed a broad peak at a mean of 10.5 S. S_1 digestion converted a certain part (presumably the single-stranded regions) of the material to low molecular weight fragments, which were unable to penetrate into the gradients. In addition a relative homogeneous peak with a mean sedimentation constant of 8.0 S appeared (Fig. 2b). All S_1 resistant duplexes, isolated by hydroxyl-

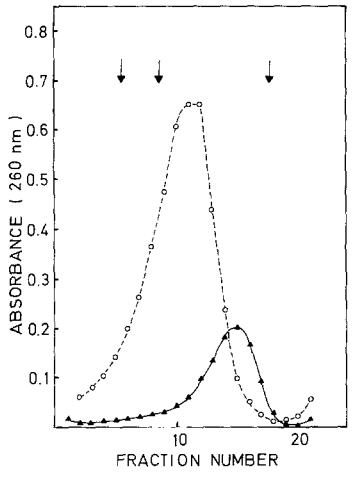

Fig. 3: Neutral velocity sedimentation analysis of S_1 treated salmon sperm DNA and reassociation products. DNA was reassociated at a concentration of 677 µg/ml until a $C_o t$ value of 40. Ten-drop fractions were collected and the difference of E_{260} (o----o) and E_{320} values was measured after the addition of 0.3 ml distilled water. (o----o), S_1 treated native DNA; (▲—▲—▲), S_1 resistant reassociation products isolated by hydroxylapatite chromatography. Arrows: Positions of markers as described in the legend of Fig. 2.

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apatite chromatography and tested by velocity sedimentation analysis, gave symmetrical peaks with mean sedimentation coefficients of 7.6, 8.0, 8.3 and 8.3 S. No significant differences could be observed between the size and distribution of duplexes isolated from reassociation products at $C_o t$ values 0.1, 10 and 767 (Fig. 2c). Nevertheless, at $C_o t$ values of 0.1 and 10 a small amount of larger material (Fig. 2c, fractions 18-23) can be observed, which disappears during the incubation at 60°C for 7 days. This material corresponds probably to satellite DNA consisting of long streches of tandemly repeated short oligonucleotide sequences (15-16). The presence of this group of molecules shows that the DNA degradation is not so great that it will prevent the formation of reassociation products larger than 8 S at least at the beginning of the incubation. Thus, the limiting factor seems to be the actual length of the repetitive elements, calculated to be about 500 base pairs on the basis of the mean sedimentation coefficient of 8.0 S (14).

4 - Size of reassociated salmon sperm DNA duplexes: The size analysis of double-stranded regions present in reassociated salmon sperm DNA was also

done. The reiterated fraction of this DNA has been found to be very heterogeneous (1). To reduce degradation due to heat induced hydrolysis alkali denaturation and short reassociation time were used ($C_o t$ 40 at DNA concentration of 677 µg/ml). As shown in Fig. 3 the size of the S_1 treated native molecules was determined to be 13 S (about 2000 base pairs). In spite of this the renatured DNA molecules, which were resistant to the enzyme, sedimented at 7.4 S. This mean sedimentation coefficient corresponds to about 300 base pairs (14).

DISCUSSION

The mean length of the S_1 resistant reassociation products was found to be about 500 base pairs in the case of CV-1 DNA and about 300 base pairs in the case of salmon sperm DNA. The presence of a small amount of significantly larger duplexes in the CV-1 DNA reassociation mixtures indicates that DNA degradation due to the double-strand scissions produced by S_1 and heat induced hydrolysis during incubation at 60°C for less than 3 hours do not preclude the formation of longer duplexes. The reassociation products isolated from the salmon sperm DNA proved to be even smaller than those of the CV-1 cell DNA in spite of the fact that the S_1 digested native molecules were twice as large (2000 base pairs) as those of the CV-1 cell DNA (1000 base pairs). Thus, both of the estimated lengths of the reassociated S_1 resistant structures seem to correlate with the actual size of the continuous reiterated elements present in the DNA preparations examined.

It is an additional result of these experiments that the group of repetitive sequences, which reassociate between $C_o t$ values of 1 and 50, is absent from the CV-1 cell DNA.

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