ALKALI UNWINDING KINETICS OF MAMMALIAN DNA IN A SIMULATED VISCOELASTOMETRY EXPERIMENT

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SUMMARY

When rat 9L brain tumor cells were lysed in test tubes under alkaline conditions identical to those used by Uhlenhopp to measure the viscoelastic properties of high-molecular-weight mammalian DNA (2), it took 4-6 hrs of alkaline lysis for the DNA to unwind so that it was completely susceptible to digestion by the single-strand-specific S1 nuclease. This time period corresponds exactly to the period during which the relative viscosity and the viscoelastic retardation time increased to a maximum in Uhlenhopp's experiments.

INTRODUCTION

Recently Uhlenhopp extended the technique of viscoelastometry to the study of single-stranded DNA molecules obtained from alkaline lysates of *Escherichia coli* and mouse 3T3 cells (1,2). In these studies he observed that the relative viscosity and the viscoelastic retardation time increased to a maximum during the first few hours of lysis. Once the maximum was attained, changes in the retardation time seemed to reflect events associated with the experimental cellular conditions investigated. Although there was indirect evidence to suggest that the initial increase in both the relative viscosity and the viscoelastic retardation time was due to unfolding and unwinding of the twin helical structure of the DNA into single strands, other possibilities existed, which, if true, would alter the interpretation of the results. Consequently, we simulated the viscoelastometry conditions used in the mouse 3T3 cell studies (2) and directly tested the single stranded-

ness of the alkali-released DNA as a function of storage time with the single-strand-specific S1 nuclease.

MATERIALS AND METHODS

Rat 9L brain tumor cells were grown for 24 hrs in 60-mm Falcon petri dishes as previously described (3,4) so all cultures were asynchronous when used. The DNA was unifilarly labeled by adding 0.03 μCi/ml of ^{14}C-thymidine (sp. act. 36 mCi/mmole) to each dish. 5 ml of saline (0.85% NaCl) were added to each of 3 dishes; the cells were scraped free with a rubber policeman and then combined in a 50-ml centrifuge tube. After addition of 25 ml of saline, the cells were pelleted by centrifugation at 1000 rpm for 10 min in an IEC-PR6 refrigerated centrifuge. The saline was decanted, the cells were resuspended in 10 ml of ice-cold 1 M NaCl by pipetting, and the solution was brought to a volume of 36 ml by adding ice-cold 1 M NaCl. The 10 mM Na_2PO_4 in Uhlenhopp's resuspension solution was not added since it can reduce the efficiency of the S1 nuclease (5). 3 ml of this suspension, containing approximately 2.5 x 10^5 cells, were added to each of 12 test tubes. Then 0.3 ml of a solution containing 4% sodium dodecyl sulfate, 0.16 M Na_4EDTA, and 0.2 N NaOH was gently layered in each tube.[1] The temperature was raised to 45°C for 5 min by placing the tubes in a hot water bath, after which the temperature was lowered to 25°C in another temperature-controlled water bath. Immediately after the temperature was lowered and again after ½, 1, 2, 4, and either 6 or 24 hrs, four 0.3-ml samples were removed with a wide-bore pipet from one of the tubes either with or without shaking the tube.

To determine the strandedness of the DNA, each 0.3-ml sample was added to 3.0 ml of S1 buffer (6) containing ≃ 20 μg of heat-denatured salmon sperm DNA. The buffer was adjusted with HCl so that the final pH was 3.9 - 4.0. To 2 of the tubes, 10 λ of a ^{3}H-thymidine-labeled, heat-denatured single-stranded DNA standard and 10 λ of the S1 enzyme were added. To the other 2 tubes, 10 λ of ^{3}H-thymidine-labeled double-stranded DNA standard and 10 λ of the S1 enzyme were added. The mixtures were incubated for 2 hrs at 50°C in a hot water bath. After incubation the tubes were placed in an ice bath to which 0.3 ml of carrier salmon sperm DNA (≃ 500 μg/ml) and 0.3 ml of 70% perchloric acid (PCA) were added. 30 min later the acid-insoluble material was precipitated by centrifuging the tubes at 3000 rpm for 20 min in an IEC-PR6 refrigerated centrifuge. The supernatant was removed and its radioactivity determined by liquid scintillation counting. The tubes were then thoroughly drained, 2 ml of 5% PCA were added, and the mixture was heated at 80°C for 30 min. The supernatant was then removed and its radioactivity determined as before. In all experiments a sample with no radioactive DNA was run as a blank. The percent of single-stranded DNA was determined by the ratio of the ^{14}C activity in the acid-soluble fraction to the total ^{14}C activity in both the acid-soluble and the acid-insoluble fractions (7).

RESULTS

Only complete experiments in which the S1 enzyme digested more than 95%

[1] All the compounds used in these experiments were obtained from Uhlenhopp and Zimm, so all conditions, including the pH, NaCl concentration, and DNA concentration, were identical to those used in the mouse 3T3 viscometry experiments (2).

of the single-stranded DNA standards and less than 15% of the double-stranded DNA standards were considered acceptable for analysis (Fig. 1). When samples of the DNA in the alkaline lysate were removed without shaking immediately after the temperature drop, approximately 50-60% of the DNA was single stranded. The percent of single strandedness of the DNA increased with increasing lytic storage time at 25°C until essentially all of the DNA was single stranded after 4 (Fig. 1a) to 6 (Fig. 1b) hrs. The DNA remained single stranded for lytic storage times up to 24 hrs (Fig. 1a). When the tubes were shaken before the samples were removed, the DNA was apparently 100% single stranded (Fig. 1), even though less than 10 sec elapsed between shaking and neutralization of the

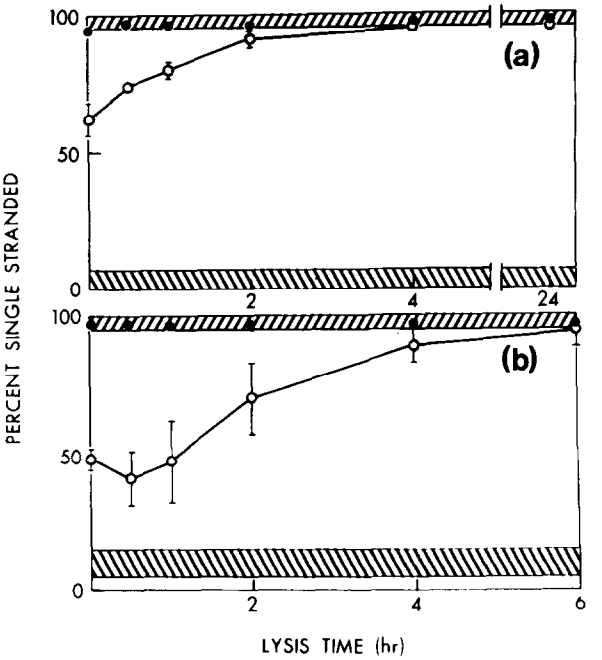

FIG. 1. Percent of rat 9L cell DNA rendered acid soluble by digestion with S1 nuclease as a function of alkaline lytic storage time in 2 separate simulated viscoelastometry experiments (a and b). Shaded areas at the top and the bottom of both a and b represent the maximum range of values obtained for the single-stranded and double-stranded DNA standards, respectively. ●, lytic solution shaken and the sample removed to enzyme buffer solution within 10 sec; ○, lytic solution not shaken before sample removed to enzyme buffer solution.

sample. Consequently, extreme care must be taken to avoid shearing the DNA while it is still in alkali.

DISCUSSION

As double-stranded DNA unfolds and unwinds to become single stranded, the relative viscosity and the viscoelastic retardation time are expected to increase (1). Our S1 nuclease assay indicates that the increase in these parameters observed by Uhlenhopp during the first 4-6 hrs of alkaline lysis (1,2) results from the unfolding and unwinding of the DNA structure. However, the S1 assay does not by itself give any information about the conformation or the configuration of this single-stranded structure. Indeed, evidence from other S1 digestion experiments suggests that it is possible to obtain extremely large single-stranded DNA molecules which do not have the sedimentation characteristics of linear chain polymers (K.T. Wheeler, in preparation). Therefore, the evidence we present here should not be construed as confirming the existence of single-stranded DNA species with molecular weights greater than 10^{10} daltons. Only when it is possible to determine the shape of these large single-stranded DNA molecules will we be able to determine their size.

In addition, since the S1 assay cannot determine the strandedness of the DNA with an accuracy greater than ±5% and since the population of molecules from which the large viscoelastic retardation times came was only 4% of the total DNA molecules in the solution (1,2), absolute proof that the viscoelastic signals observed by Uhlenhopp were from single-stranded DNA molecules and not from aggregates or gels is not possible. Nevertheless, the correspondence between the time period during which the relative viscosity and the viscoelastic retardation time of this small population increased and the period during which the unfolding and unwinding of chromosomal DNA to single-stranded DNA occurred is strong circumstantial evidence that these signals did come from single-stranded DNA.

Moreover, low doses of X-rays (< 100 rads) would be expected to introduce a small number of breaks either at specific linkages (8) or randomly throughout the DNA. In either case the introduction of a very small number of additional open sites would promote more rapid unfolding and unwinding of the DNA. This phenomenon would lead to a prediction that the viscoelastic retardation time should increase to a maximum more rapidly during lysis after low doses of X-rays than during the lysis of unirradiated cells. This is exactly what Uhlenhopp observed (2).

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