AN ENZYMATIC FACTOR FROM CALF THYMUS CAUSING HYPERCHROMICITY IN DNA WITHOUT STRAND SCISSION

A. P. Phillips

Medical Research Council,
Radiobiological Research Unit,
Harwell, Didcot,
Berkshire, England.

Received January 22, 1968

This paper contains evidence of the presence in calf thymus extracts of an enzymatic factor able to produce hyperchromicity in native DNA in vitro without causing fragmentation of the individual strands.

Methods

The extraction of the factor from calf thymus was performed by a method initially designed for the purification of DNA polymerase, in which a soluble extract was subjected successively to pH precipitation, protamine treatment, high speed centrifugation, and ammonium sulphate fractionation; details of the method will be published elsewhere. An ammonium sulphate fraction, fraction III ("Fr. III"), of protein content 8-15mg/ml, was used throughout this investigation. It was usually pre-treated by removal of the slight precipitate formed in the presence of low concentrations of Mg^{++}, EDTA, phosphate, and mercaptoethylamine. The deoxynucleoside triphosphates necessary for DNA polymerase action were never added.

The hyperchromicity-acid solubility assays were performed as follows

9.0ml of an incubation mixture (c.f. Table I, type A) containing 680γ calf thymus (CT) DNA [prepared according to Kay et al.(1952)]and either pancreatic DNase (0.14γ; Bovine, from "SIGMA LONDON Chemical Co.") or a volume of Fr. III containing 3mg protein, were incubated at 37°C, the absorption at 260mμ. (A_{260}) being automatically recorded on a UNICAM SP800 Recording Spectrophotometer. Periodically 0.5ml aliquots were removed and rapidly frozen. The incubation was stopped after a hyperchromic increase of 17% (17% "H"), i.e. about half maximal; this percentage was calculated as $\frac{(A_2 - A_1)}{A_1}100$, A_1 and A_2 being the initial and final A_{260} values. The aliquots from pancreatic DNase digests were later thawed and rapidly treated with 0.083ml 3mg/ml Bovine Plasma Albumin (BPA) and 0.33ml 2N Perchloric Acid (PCA); those from Fr. III incubations were treated with 0.05ml 800γ/ml CT DNA, 0.05ml 3mg/ml BPA, and 0.33ml 2N PCA. After standing in ice water they were spun in a bench centrifuge; the supernatants were removed by syringe and their A_{260} measured.

Solutions for chromatography (Table I, type B) contained 420γ of CT DNA, and were incubated with pancreatic DNase or Fr. III (0.22γ and 1.8mg protein respectively) to degrees of hyperchromicity varying from zero ("To") to 22½%; reaction was terminated by rapid freezing. Immediately before analysis the solutions were thawed rapidly and made at least 0.5M with respect to NaCl (this concentration completely inhibited both types of hyperchromic action). Finally 11-22γ of B. subtilis ^{14}C-DNA were added as a column marker. Chromatography was performed at 4°C on a Sephadex G-200 column (2.54 x 30.5 cm) in a Pharmacia glass assembly, flowed upwards at 13ml/hr with a buffer of 0.02M phosphate pH 7.2 containing 0.5M NaCl. Effluent fractions were assayed as follows. 0.1ml aliquots of each were pipetted onto Whatman No 1 paper dried, and counted for ^{14}C in a Beckman Liquid Scintillation Counter. The absorption of fractions of the Fr. III series were read in the UNICAM

SP800 at 260 and 280 mμ to allow calculation of nucleic acid concentrations (NA γ/ml) from a nomograph. [Adams, E., Calbiochem Ltd., after Warburg and Christian, (1942)]. Pancreatic DNase column fractions, being very low in protein content, were observed at 260 mμ only.

TABLE I

Incubation mixture	FINAL CONCENTRATION mM				
	EDTA/ mercaptoethylamine	Mg^{++}	Phosphate pH 7.2	TRIS pH7.0	TRIS pH8.0
Type A	0.67	5.0	6.7	8.3	1.7
Type B	0.33	5.0	4.2	5.0	6.7

Results

The performance of pancreatic DNase and Fr. III in the hyperchromicity acid solubility assay may be seen in Figure 1. It is clear that whereas pancreatic DNase digestion of DNA as far as 17% "H" is accompanied by

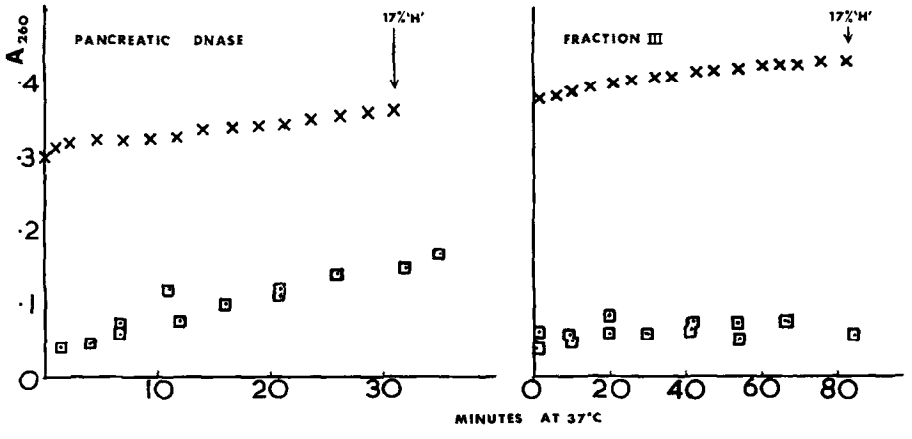

Figure 1 Hyperchromicity-acid solubility assays. Procedure as described in "Methods". Fr. III not pre-treated with Mg^{++} etc.
x - total A_{260} observed during incubation; □ - acid-soluble A_{260} of aliquots removed periodically. Measured in 2mm light path silica cell.

considerable release of acid-soluble material, Fr. III incubation to this level shows little or no increase in acid-soluble A_{260} above the background. It is concluded from Fig. 1 and from identical experiments that the achievement of 17% hyperchromicity is accompanied by the release of at least ten times less acid-soluble material when the enzymatic agent is Fr. III rather than pancreatic DNase.

To examine the degree of DNA fragmentation accompanying the hyperchromic action of our preparation, molecular sieve chromatography on a

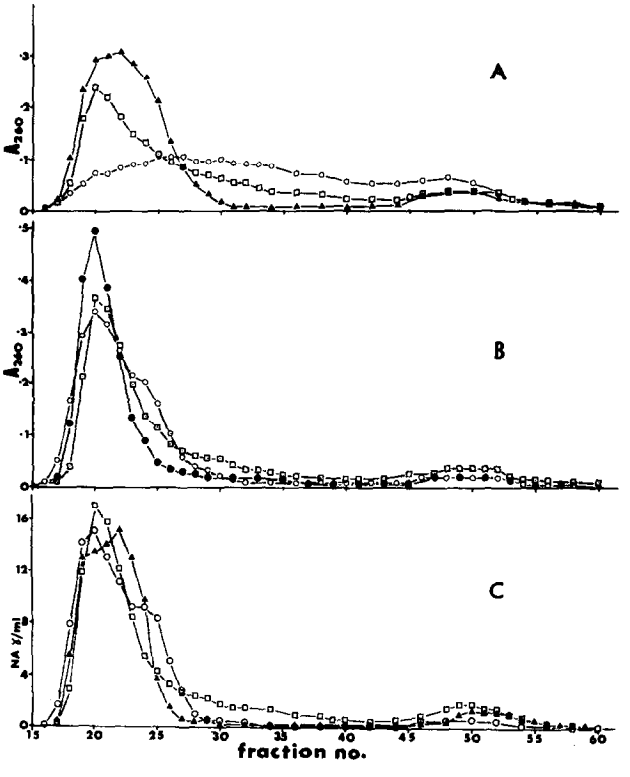

Figure 2 G-200 analyses of DNA incubated with Fr. III or pancreatic DNase to hyperchromic extents (%"H") varying from zero (To) to 22½%"H".

A:-▲-▲ - native DNA alone; □-□ - DNA plus DNase incubated to 6%"H" O—O - DNase 11%"H".

B:-O—O- DNase To; ●—● - DNase 2%"H"; □—□- DNase 4%"H".

C:-O—O- Fr. III To; ▲—▲- Fr. III 22½%"H" □—□- DNase 4%"H" followed by addition of Fr. III and freezing.

Sephadex G-200 column was carried out as described in "Methods" on Fr. III and pancreatic DNase series of DNA incubates. The inclusion of the ^{14}C-DNA marker was to check reproducibility. In fact the method was

found to be both stable and sensitive, and seemingly small differences between some of the profiles in Fig. 2 were reproduced exactly in analyses of duplicate incubations.

Initially the column exclusion volume was determined with Blue Dextran 2000 [Pharmacia (Great Britain) Ltd.]; this was first eluted in fraction 18, rose to a peak in fraction 20, and thereafter fell off quite sharply. By comparison the native DNA profile in Fig. 2A has a broad peak implying considerable diffusion into the gel matrix. However both the DNase and Fr. III To profiles show extensive movement of DNA into the first, "exclusion", half of this peak. Duplicate To analyses showed this migration to various extents, and indeed formed the only example of major variation between duplicates. This profile transition seems evidence of a primary reaction, long before any detectable hyperchromicity, occurring during the short time the To specimens were standing in ice water after addition of the enzyme and before freezing; the reaction may or may not have been inhibited by the addition of NaCl after thawing. The resulting effective increase in the size of the majority, if not all, of the DNA molecules may be a process of attachment of basic proteins to DNA, with or without the formation of multi-molecular aggregates.

The pancreatic DNase 2%"H" elution pattern (Fig. 2B) shows extreme sharpening of the "exclusion" peak relative to To, but by 4%"H" the peak is falling again with a simultaneous increase in the proportion of nucleic acid delayed in the gel (fraction 28 onwards). The 6%"H" and 11%"H" analyses are plotted in Fig. 2A to give some idea of the resolution of the column. In contrast with this series the Fr. III 22½% profile (Fig. 2C) shows incomplete sharpening of the exclusion peak relative to the Fr. III To, and the DNA, apparently in an intermediate state in the initial process of molecular enlargement, forms an unusual peak in fraction 22; the low absorption figures from fraction 30 onwards testify to the virtual absence of detectable hydrolysis products. It is concluded that

incubation with Fr. III to 22½%"H" causes far less DNA fragmentation than a pancreatic DNase 2%"H" digestion. A 22½%"H" incubation with Fr. III not purified by the Mg^{++} etc. precipitation described in "Methods", gave an elution profile almost identical in shape to that of DNase 2%"H"; the implication is that the precipitative pre-treatment of Fr. III had decreased the effective contamination with endonuclease(s).

The slight terminal peak occurring between fractions 46-56 seemed rather higher in the Fr. III 22½% columns than in comparable DNase profiles; such an excess of small fragments could result from an exonucleas contaminant in our preparation.

It was felt necessary to verify that the sole presence in the Fr. III incubates of a great excess of protein relative to the DNase digests does not interfere with the free migration of DNA. Consequently CT DNA was pre-incubated with pancreatic DNase to 4 or 6%"H", cooled in ice water, the usual volume of Fr. III added, and the mixture immediately frozen. G-200 analysis of the first of these (4%"H") is shown in Fig. 2C; the similarity to the simple DNase 4%"H" profile (Fig. 2B) is obvious. As a further check a pre-incubation with Fr. III to 22½%"H" was followed by the addition of an excess of pancreatic DNase and incubation to a further 6%"H"; the resulting G-200 analysis (not shown) was very like that of the simple 6%"H" DNase system.

Examination of the ultraviolet absorption spectrum showed that Fr. III, like pancreatic DNase, causes no shift of the absorption maximum during development of hyperchromicity at 260mμ.

Discussion

The initial hyperchromicity-acid solubility results are readily explicable in two ways. Assuming that hyperchromicity at 260mμ is evidence of DNA strand separation, the hyperchromic action of fraction III without simultaneous release of acid-soluble material would be con-

sistent with either the action of an endonuclease specific for double-stranded DNA, so that hydrolysis stopped when fragments produced were so short as to become single-stranded, or with the action of an "UNWIND-ASE" whose sole modus operandi was the rupture of DNA inter-strand hydrogen bonds. With these alternatives in mind the factor may be described at this stage as "HYPERCHROM-ASE".

When one remembers the well-documented progressive fragmentation of DNA mediated by endonucleases such as pancreatic DNase [reviewed by Laskowski, (1961)], our comparison of the G-200 molecular sieve analyses of pancreatic DNase and Fr. III incubates leads to the conclusion that there was very little fragmentation of DNA during considerable hyperchromic action by our Fr. III, and what little there was may be attributed to contaminating exo-and endo-nucleases. This clearly eliminates the double-strand-specific nuclease identity, but all our results remain consistent with the presence of "DNA UNWIND-ASE" in these calf thymus extracts.

In addition to the experiments described above, hyperchromicity-acid solubility studies were performed with B. subtilis ^{14}C-DNA, and hyperchromicity-associated DNA fragmentation was investigated by sucrose density gradient centrifugation. The results, which will be published later, entirely support the conclusions reached in this paper.

Acknowledgement

The author thanks Roger West for his thoughtful technical assistance.

References

Kay, E.R.M., Simmons, N.S., and Dounce, A.L., (1952) J. Am. Chem. Soc. 74, 1724.

Laskowski, M., Sr., in "The Enzymes", (Boyer, P.D., Lardy, H., and Myrbäck, K., eds.) 5, 123, Academic Press, New York.

Warburg, O., and Christian, W., (1942), Biochem. Z., 310, 384.