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Main properties of duck hepatitis B virus DNA polymerase: comparison with the human and woodchuck hepatitis B virus DNA polymerases

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Summary

The main properties of the duck hepatitis B virus (DHBV) DNA polymerase have been studied and compared with those of the human hepatitis B virus (HBV) and of the woodchuck hepatitis virus (WHV) DNA polymerases. All 3 enzymes are active under high salt conditions in the presence of high magnesium concentration. DHBV DNA polymerase was found less sensitive to ethanol and to operate at higher optimal pH than the HBV and WHV DNA polymerases. Like the other two viral endogenous DNA polymerases, the DHBV enzyme was strongly inhibited by phosphonoformic acid but not by aphidicolin, sulfhydryl group blockers or phosphonoacetic acid.

Inhibition of DHBV DNA polymerase by the triphosphate derivatives of several nucleoside analogs appeared similar to that reported for HBV or WHV endogenous polymerase. FIACTP was the most, and ACVTP the least effective inhibitor; BVdUTP was of intermediary potency; araCTP and araTTP had a greater inhibitory effect on DHBV DNA polymerase than HBV or WHV DNA polymerase. The similarities in the properties of DHBV and HBV DNA polymerase justify the use of the duck hepatitis B polymerase model for screening and evaluation of potentially active drugs against HBV infection.

Hepadnavirus; DNA polymerase; Inhibition antivirals

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Introduction

Following the identification of hepatitis B virus (HBV) (Tiollais et al., 1986) 3 HBV like viruses have been hitherto characterized: the woodchuck hepatitis virus (WHV) (Summers et al., 1978), the ground squirrel hepatitis virus (GSHV) (Marion et al., 1980) and the duck hepatitis B virus (DHBV) (Mason et al., 1980). All 4 viruses belong to the newly defined hepadna virus group (Robinson et al., 1981). They all share a similar morphology and genomic structure consisting of a circular partially double stranded DNA of 3000–3300 base pairs. The single stranded region of the genome can serve as a natural template for the viral endogenous DNA polymerase which repairs the single stranded gap during an in vitro reaction (Kaplan et al., 1973).

The molecular cloning (Charnay et al., 1979; Cummings et al., 1980) and nucleotide sequence analysis of hepadna viruses (Galibert et al., 1979, 1982) have improved our understanding of their biology. However, the absence of a cell culture system for the propagation of HBV- and HBV-like viruses has hampered studies on the mechanism of the viral replication and the search for antiviral agents active against HBV infection.

In order to look for suitable compounds which could inhibit HBV replication, the HBV DNA polymerase has been characterized. Inhibition of the enzymatic activity by intercalating agents (Hirschman and Garfinkel, 1978), pyrophosphate analogs (Nordenfelt et al., 1980) and nucleotide analogs (Hantz et al., 1984b; Hess et al., 1981) was demonstrated in vitro. Animal hepadna virus models may offer an alternative approach to the tissue culture systems in the development of a successful antiviral chemotherapy against HBV (Smee et al., 1985).

In this perspective, we have previously compared the principal properties of HBV and WHV endogenous DNA polymerases and their interaction with potential inhibitors (Hantz et al., 1984a,b). In the present report, we have compared the DHBV DNA polymerase with those of HBV and WHV and measured the inhibition of the enzyme by the triphosphate derivatives of several nucleoside analogs known to block herpes viruses DNA polymerases.

Materials and Methods

Chemicals and reagents

Unlabelled triphosphates (dATP, dCTP, dGTP and dTTP) as well as 9-β-arabinofuranosyladenine 5′-triphosphate (araATP) and 1-β-D-arabinofuranosylcytosine 5′-triphosphate (araCTP) were obtained from P.L. Biochemicals (Milwaukee, USA). 1-β-D-arabinofuranosylthymine 5′-triphosphate (araTTP) was a gift from Dr. G.A. Gentry (Mississippi Medical Center, Jackson, MS, U.S.A.). Acyclovir (ACV) was provided by Burroughs Wellcome (Research Triangle Park, NC, U.S.A.), *E*-5-(2-bromovinyl)-2′-deoxyuridine (BVdU) was kindly made available by Dr. E. De Clercq and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) was a gift from Dr. J.J. Fox (Sloan-Kettering Institute for Cancer Re-

search, NY, U.S.A.). The 3 nucleoside analogs were converted to their corresponding 5′-triphosphates, as described by Allaudeen et al. (1981). Tritiated deoxyribonucleoside triphosphates [^{3}H]dCTP (50 Ci/mmol), [^{3}H]dGTP (32 Ci/mmol) and [^{3}H]dTTP (50 Ci/mmol) were obtained from Amersham (France). Phosphonoformic acid and phosphonoacetic acid were purchased from Sigma Chemicals (St Louis, MO, U.S.A.) and aphidicolin was purchased from Boehringer Mannheim (France).

Ducks

The animals used in this study were obtained from a colony of congenitally DHBV-infected Pekin ducklings, established in our laboratory. The presence of DHBV in serum was tested by both the endogenous DNA polymerase assay and the hybridization spot test as described below.

Serum was obtained from 3 ducklings, which had been naturally infected by DHBV and which were bled by jugular puncture at 2 months of age.

Preparation of duck hepatitis B virus (DHBV)

Virions were pelleted from 50 ml of serum ultracentrifuged through a discontinuous sucrose gradient (10%;20%) at 25 000 rpm for 18 h in a Beckman SW 25 rotor. The pellets were resuspended in 3 ml of CsCl solution (d = 1.14 g/cm^3) containing Tris 10 mM, EDTA 10 mM (TE) plus 0.1% BSA and layered on the top of a continuous CsCl gradient (density (d) 1.09 g/cm^3 to 1.22 g/cm^3) in TE. After centrifugation at 35 000 rpm for 24 h in a Beckman SW 41 rotor, 0.6 ml fractions were collected from the top of the tubes and assayed for DNA polymerase activity and for DHBV DNA by the hybridization spot test (Fig. 1). Fractions containing DHBV DNA and the peak of DNA polymerase activity were pooled, diluted in TE and recentrifuged for 3 h at 40 000 rpm in a Beckman SW 41 rotor. The pellet was suspended in TE containing 0.1% BSA. This solution was divided in aliquots, and stored at −80°C. Three thousand cpm were obtained with 5 µl of the preparation in the standard DNA polymerase assay.

Preparation of woodchuck hepatitis virus (WHV) and human hepatitis B virus (HBV)

Purified HBV and WHV with high DNA polymerase activity were obtained as previously described (Hantz et al., 1984a).

DNA hybridization spot test

The DNA hybridization spot test was performed on 10 µl serum samples or on 5 µl of the CsCl fractions, diluted to 100 µl in 10 mM Tris, pH 7.4, 1 mM EDTA, 100 mM NaCl. Filter treatment, radiolabelling of DHBV DNA, and hybridization procedures were carried out as previously described (Cova et al., 1984). The relative amount of DHBV DNA circulating in duck serum was determined by cutting out pieces of a nitrocellulose filter containing a serum spot and counting them in a liquid scintillation counter (Cova et al., 1984).

DNA polymerase assay

DNA polymerase activity was carried out according to Kaplan et al. (1973) with minor modifications. The reaction was assayed in a 50 µl final reaction mixture containing Tris 50 mM, pH 8, $MgCl_2$ 40 mM, NH_4Cl 60 mM, Nonidet P40 0.5%, β-mercaptoethanol 10 mM, dATP, dCTP and dGTP 0.1 mM, [^{3}H]dTTP 0.4 µM (50 Ci/mmol) and inhibitors at different concentrations. The reaction was initiated by addition of the virus. After incubation at 37°C for 45 min, acid-precipitable radioactive material was collected on glass fiber filters (GF/C Whatman). Filters were washed 12 times with 5% trichloracetic acid containing 10 mM sodium pyrophosphate and once with 95% ethanol using a Millipore manifold. The filters were dried and counted in a liquid scintillation counter.

The demonstration of the specificity of the viral DNA polymerase assay was carried out by analysing the [^{32}P]DNA product of the standard polymerase reaction in agarose gel electrophoresis as described by Summers et al. (1978).

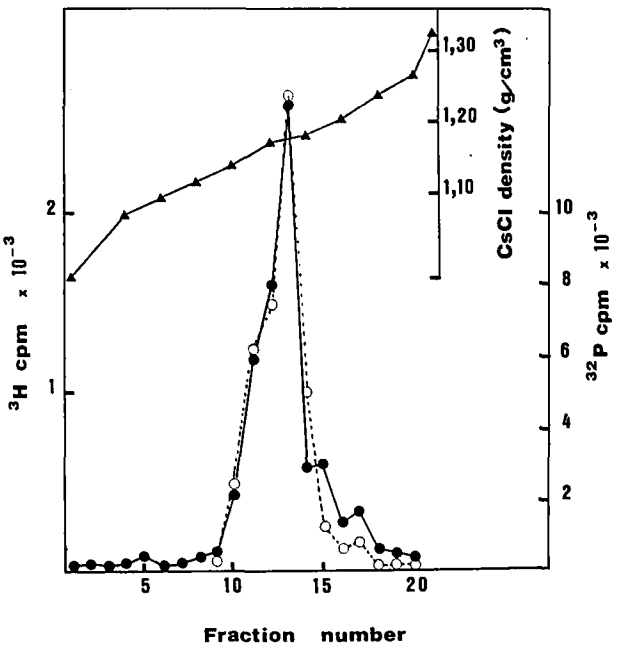

Fig. 1. Purification of duck hepatitis B virus by CsCl gradient centrifugation. Duck hepatitis B particles were concentrated from serum by pelleting through a discontinuous sucrose gradient. Pellets were suspended in CsCl solution (d = 1.14 g/cm^3) and layered on top of a continuous CsCl gradient (d = 1.09 g/cm^3 to d = 1.22 g/cm^3). After centrifugation, the fractions collected from the top of the tube were tested for DNA polymerase activity (●——●) and DHBV DNA by spot hybridization test (○——○). Fractions containing the peaks of DHBV DNA and DNA polymerase activity were pooled and used to study the characteristics of the DHBV-associated DNA polymerase.

Results

DHBV specificity of DNA polymerase activity

DHBV particles containing the DNA polymerase were isolated by CsCl gradient centrifugation as described in Fig. 1. Remarkably, endogenous DNA polymerase activity and precipitable DHBV DNA were found in the same peak corresponding to a density of 1.17 g/cm^3 of CsCl.

The endogenous DNA polymerase assay was carried out as described in Materials and Methods with [^{32}P]dCTP as labelled nucleotide. The product was then analysed by agarose gel electrophoresis and autoradiography. The results depicted in Fig. 2 show the synthesis of viral DNA after 15, 30, 45, 60 and 90 min. After 15 min, 2 major viral DNA bands of about 3 kb which correspond to double-stranded molecules in either linear or circular form (Mason et al., 1980) were observed together with smaller forms ranging from 1.8–2.8 kb representing the progressive duplication of the single-stranded region of viral DNA. Finally, it should be noticed that actinomycin D (100 μg/ml) inhibited only by 30% the maximal activity of the endogenous DNA polymerase of the viral particles used in this study. This indicated that in the following experiment, DNA synthesis occurred predominantly from a RNA template.

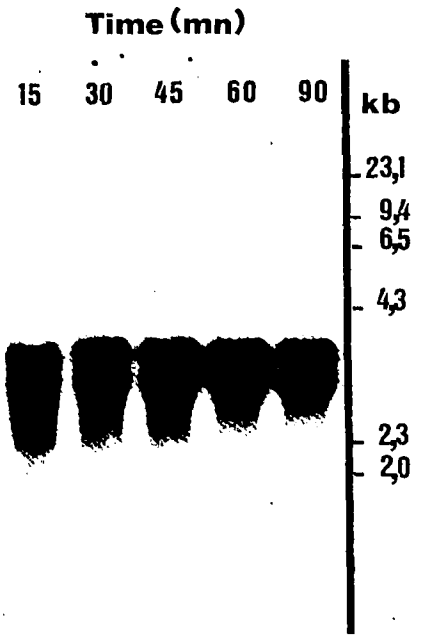

Fig. 2. Agarose gel electrophoresis of DHBV [^{32}P]DNA synthesized during the endogenous DNA polymerase reaction. The reaction was carried out in a reaction mixture containing [^{32}P]dCTP as the labelled nucleotide. At the indicated times, the reaction was stopped and the [^{32}P]DNA product analyzed by agarose gel electrophoresis and autoradiography. *Hin*dIII fragments obtained from λ DNA served as markers (kb = kilobases).

Principal properties of DHBV DNA polymerase

To determine the pH providing maximal DNA polymerase activity, the reaction mixture was assayed at various pH successively adjusted from 7 to 9. Optimal activity was found at pH 8.

DNA polymerase requires a divalent cation for activity. Maximal DNA polymerase activity was observed for $MgCl_2$ concentrations between 10 mM and 80 mM and decreased when the $MgCl_2$ concentration raised above 80 mM. Low activity (10%) was found in the presence of 1 mM $MnCl_2$ or 10 mM $ZnCl_2$. When $BaCl_2$, $CaCl_2$ or $CoCl_2$ were substituted for $MgCl_2$ in the reaction mixture no detectable activity was observed. The DHBV DNA polymerase was not found sensitive to high ionic strength (96% of activity at 0.1 M NH_4Cl concentration). The presence of N-ethylmaleimide (NEM) or N-methylmaleimide (NMM) (10 mM) in the reaction mixture did not affect the enzyme activity. Nevertheless at 20 mM NEM concentration, the activity decreased by half and became undetectable at a concentration of 50 mM. The activity was 75% and 55% of the maximum for ethanol concentrations of 10% or 25% respectively. When aphidicolin (100 μg/ml) was included in the reaction mixture, no inhibition was observed. The above data were compared with those previously reported for HBV and WHV DNA polymerase (Table I).

TABLE 1

Comparison of the principal properties of DHBV, WHV and HBV DNA polymerase.

Property	Endogenous DNA polymerase activity (%)		
	DHBV	WHV	HBV
pH 7.8	95	100	100
8	100	95	95
Bivalent cation			
$MgCl_2$ 10 mM	90	90	90
50 mM	100	100	100
100 mM	80	85	85
Ionic force			
NH_4Cl 10 mM	100	95	95
50 mM	96	100	100
100 mM	98	98	97
Inhibitors			
Aphidicolin (100 μg/ml)	97	100	98
NEM			
10 mM	98	95	96
20 mM	40	51	58
50 mM	13	20	22
Ethanol			
10%	75	71	78
25%	55	30	38

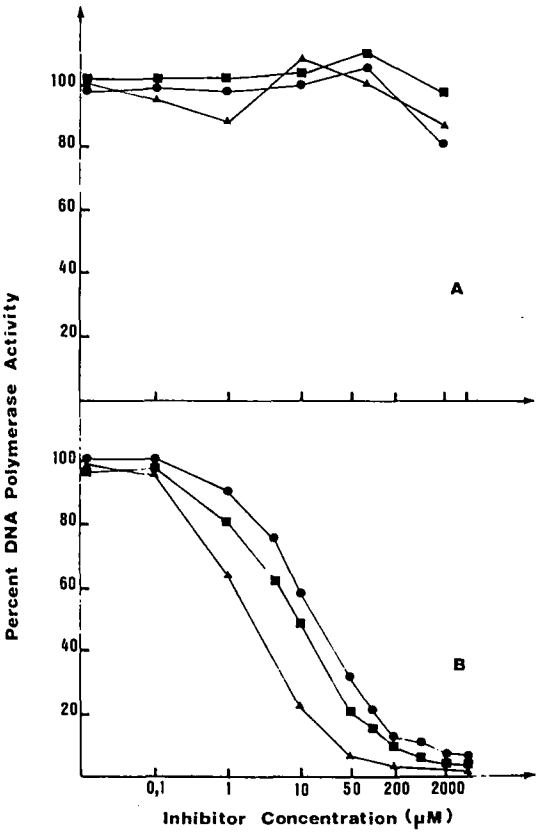

Fig. 3. Effects of phosphonoformic acid (PFA) and phosphonoacetic acid (PAA) on DHBV, HBV and WHV DNA polymerases. To determine the effect of PAA and PFA on hepadnaviruses associated DNA polymerases, the assay was carried out with different concentrations of PAA (panel A) and PFA (panel B). The experiment was performed with the DNA polymerases of DHBV (▲——▲), HBV (■——■) and WHV (●——●). Results are expressed as per cent activity of the control without inhibitor.

Like HBV and WHV DNA polymerase, DHBV DNA polymerase was found to be highly resistant to PAA (no inhibition at 1 mM PAA) but was inhibited by low concentration of PFA (75% inhibition at 10 μM PFA) (Fig. 3).

Effects of triphosphate analogs

To determine the sensitivity of the DHBV DNA polymerase to triphosphate analogs, the DNA polymerase reaction was measured in the presence of different concentrations of araATP, araCTP, araTTP, FIACTP, ACVTP or BVdUTP. Because all these analogs are known to be competitive inhibitors, the Km value of each radioactive nucleotide was determined and the inhibition studies were carried out for each with the corresponding natural triphosphate at a concentration of 2–3 times the Km value, while the 3 other nucleoside triphosphates were in excess (100 μM). The different inhibitory effects of these compounds are shown in Fig. 4.

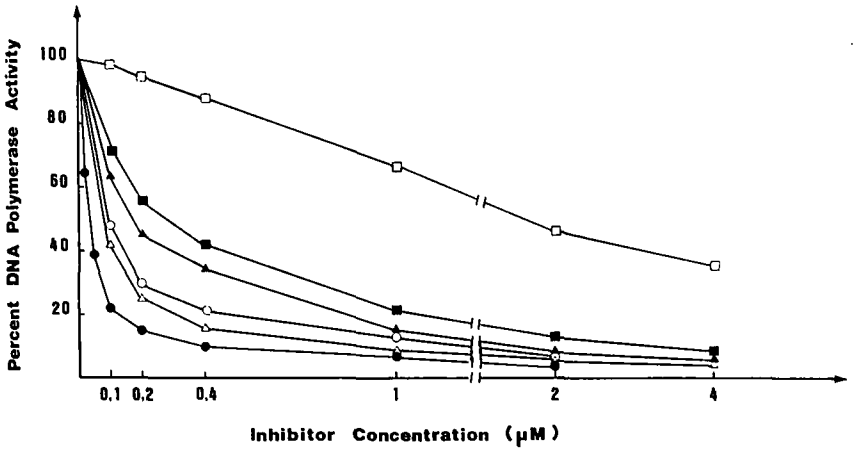

Fig. 4. Effect of triphosphate derivatives on the DHBV DNA polymerase. The DNA polymerase reaction (as described in Materials and Methods) was carried out with different concentrations of the following triphosphate analogues: araATP (□——□), araCTP (○——○), araTTP (△——△), FIACTP (●——●), ACVTP (■——■), BVdUTP (▲——▲). For each compound, the concentration of the corresponding labelled nucleotide was 2–3 times higher than the Km value. Results are expressed as per cent activity of the control without inhibitor.

FIACTP, araTTP and araCTP appeared to be the most efficient inhibitors; the least efficient was araATP, while BVdUTP and ACVTP had an intermediary effect. The ID_{50} values, which represent the molar concentration providing 50% inhibition, are presented in Table 2 together with those previously reported for HBV and WHV endogenous DNA polymerase. The lowest ID_{50} values were found for FIACTP, araTTP and araCTP, respectively.

TABLE 2

Comparative inhibitory activities of nucleotides analogs on DHBV, WHV and HBV DNA polymerases.

Inhibitor	ID_{50} (μM)		
	DHBV DNA polymerase	HBV DNA polymerase	WHV DNA polymerase
araATP	1.8	–	–
FIACTP	0.03	0.05	0.1
araCTP	0.09	1.10	1.2
ACVTP	0.35	0.9	0.7
araTTP	0.08	0.3	0.4
BVdUTP	0.17	0.25	0.3

ID_{50} represents the concentration of inhibitor decreasing DNA polymerase activity by 50%.

Discussion

In the absence of an in vitro culture system for HBV, DHBV infection of the Pekin duck represents together with the East American woodchuck and the ground squirrel a potentially useful animal model for in vivo evaluation of potential inhibitors of HBV replication. The Pekin duck is clearly the cheapest and easiest of these animal models to work with. However, among the hepadna viruses DHBV is the more distantly related from HBV. It was therefore crucial for the validation of this model to establish that the DHBV endogenous DNA polymerase did not significantly differ from that of HBV in its main properties, especially in its reactivity to inhibitors with antiviral potential. Our results confirm that the DHBV DNA polymerase indeed shares the same essential characteristics as the HBV and WHV endogenous DNA polymerases: need for high ionic strength and high magnesium concentration, insensitivity to a sulfhydryl group blockers, absence of inhibition by aphidicolin at a concentration which specifically inhibits the cellular DNA polymerases (Pedrali et al., 1979) and, as expected, no inhibition by PAA but inhibition by PFA (Hantz et al., 1984a). Only 2 minor differences were observed: DHBV endogenous DNA polymerase operates at a slightly higher pH and is less sensitive to ethanol than the HBV and WHV DNA polymerases. Viral DNA synthesis in the endogenous reaction was completed more rapidly for DHBV than for HBV or WHV (Hantz et al., 1984b). This indicates that the single stranded DNA portion of DHBV is smaller than that of HBV and WHV. These observations are in accord with those previously described by Mason et al. (1980).

These data are not surprising in spite of the fact that DHBV is phylogenetically more distant from HBV than are the two other mammalian hepadnaviruses: apparently the DNA polymerase-coding region is one of the most conserved regions of the hepadnaviral genome (Robinson et al., 1981). It should also be stressed that in our experiment the DHBV endogenous DNA synthesis was not completely inhibited by actinomycin D, an inhibitor of DNA-directed DNA synthesis. This indicates that the partially purified DHBV preparation probably contained immature virions with pregenomic RNA, as already reported for similar preparations of virus particles from DHBV and HBV (Cummings et al., 1980; Miller et al., 1984; Summers and Mason, 1982). As demonstrated by Summers and Mason (1982), hepadnaviruses replicate via the reverse transcription of a pregenomic RNA. Therefore the endogenous DNA polymerase found in viral particles is likely to be a reverse transcriptase. Indeed, nucleic acid homologies have been demonstrated between the *pol* gene of hepadnaviruses and the reverse transcriptase gene of several retroviruses (Tom et al., 1983) and cauliflower virus (Volovitch et al., 1984), a plant DNA virus which also replicates via a pregenomic RNA. Consistent with this finding, many properties of the DHBV, WHV and HBV endogenous DNA polymerases described in this report are indeed similar to those of the AMV reverse transcriptase (Sundquist and Oberg, 1975).

The inhibitory effects of the triphosphate analogs on DHBV DNA polymerase were similar to those reported for HBV and WHV DNA polymerases (Hantz et al., 1984b). FIACTP was the most efficient inhibitor while BVdUTP and ACVTP

demonstrated an intermediate to weak activity. Only araCTP and araTTP were found to be more potent inhibitors for DHBV DNA polymerase than for the HBV or WHV DNA polymerases.

Further studies are needed to determine the exact molecular mechanism of inhibition (i.e. competitive or not) of these analogs. It also remains to be ascertained whether they can serve as alternate substrates to their corresponding natural triphosphates, as demonstrated for BVdUTP and FIACTP in the case of herpes simplex virus-induced DNA polymerase (Allaudeen et al., 1981, 1982) or HBV and WHV DNA polymerases (Hantz et al., 1984b).

If ACV, BVdU and FIAC are therapeutically useful antiherpetic agents, this is so because (i) in vivo they are specifically phosphorylated within the cell by the virus-induced thymidine kinase, and (ii) their triphosphate derivatives inhibit more efficiently the viral DNA polymerase than the cellular DNA polymerases. These triphosphate derivatives are very efficient inhibitors of the hepadnavirus DNA polymerase. Whether the nucleosides can be phosphorylated in the HBV-infected cell in vivo remains unclear. Until now, no pyrimidine nucleoside kinase-encoding capacity has been ascribed to HBV, WHV or DHBV.

However, in vivo ACV inhibits HBV replication in patients suffering from chronic hepatitis (Weller et al., 1983). This indicates that phosphorylation of nucleoside analogs can occur even in the absence of a detectable viral thymidine kinase. Therefore, the nucleoside triphosphate analogs may be considered as potential anti-HBV agents which must be further evaluated in vivo. The DNA polymerase studies in the domestic Pekin duck and wild East American woodchuck are relevant for this purpose.

Acknowledgements

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