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Mechanism of Carcinogenesis by RNA Tumor Viruses, III.* Formation of RNA·DNA Complex and Duplex DNA Molecules by the DNA Polymerase(s) of Avian Myeloblastosis Virus†

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Abstract. DNA polymerase activity can be unmasked in avian myeloblastosis virus (AMV) by treatment with the nonionic detergent Nonidet P-40. Two products are formed: (1) RNA·DNA hybrid molecules and (2) duplex DNA molecules. The kinetics of dTTP incorporation into DNA are biphasic: an initial rapid reaction for 4 min at 37°C with a minimal polymerization rate of 10–20 nucleotides per sec, and a second reaction at about half the initial rate. Viral RNA·DNA complexes are detected as early as 30 sec after the initiation of DNA synthesis; DNA free of template is formed subsequently. Most of the free AMV DNA forms an RNA·DNA hybrid when annealed with viral RNA. Over half of the free AMV DNA product is inferred to be double-stranded, since it is retained on hydroxyapatite columns after elution with 0.12 M phosphate buffer, and is resistant to *Escherichia coli* exonuclease I. Adenovirus or calf-thymus DNA added to unmasked AMV stimulates DNA synthesis 4–16 times if there is no treatment with RNase, and 40–130 fold if RNase treatment precedes the enzyme assay. It is possible that two polymerases are present, or that a single enzyme forms both the RNA·DNA hybrid and the double-stranded product.

RNA-containing leukemia and sarcoma viruses from several animal species possess a DNA polymerase dependent upon viral RNA.[1–6] The initial enzymatic product is a viral RNA·DNA complex[3,4] whose subsequent fate and function are unknown. Here we report studies on the DNA polymerase enzyme activities of AMV (BAI strain A)** which show a rapid two-step synthesis of AMV DNA: the formation of RNA·DNA hybrid and duplex DNA molecules.

Materials and Methods. Virus from plasma of diseased birds was centrifuged for 30 min at 36,000 rpm onto 1 ml of 65% sucrose in D_2O overlaid with 0.5 ml of 15% sucrose in buffer (0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M EDTA) and was purified further by isopycnic centrifugation for 1 hr at 36,000 rpm[4] in 15–60% sucrose gradients in an SW41 rotor. The visible band at $\rho = 1.16$ was dialyzed for 3 hr against 0.01 M Tris buffer, pH 8.0.

DNA polymerase assay and conditions for zonal and equilibrium centrifugation of RNA and DNA in sucrose, CsCl, and Cs_2SO_4 gradients, and the sources of most materials have been described.[4]

TABLE 1. *Effect of NP-40 on AMV DNA polymerase activity.*

	NP-40 concentration (%)		Time of incubation at 37°C (min)	Incorporation of [^{3}H]dTTP (cpm)*
	Preincubation mixture	Incubation mixture		
Expt. 1†	0.50	0.10	0	58
	None	None	60	70
	0.10	0.020	60	4494
	0.50	0.10	60	4211
Expt. 3‡	...	0.020	0	73
	...	None	60	68
	...	0.010	60	42
	...	0.020	60	7017
	...	0.040	60	4200
	...	0.10	60	3254

* Average of duplicate enzyme assays under standard conditions with 10 μCi of [^{3}H]dTTP (11.4 Ci/mmol).

† Purified virus (4.1 μg of viral protein) was incubated with NP-40 for 15 min at 0°C, diluted 5-fold in substrate mixture, and incubated further at 37°C.

‡ Purified virus (4.3 μg of viral protein) was incubated at 37°C in substrate mixture containing NP-40.

DNA was treated with exonuclease I,[8] kindly provided by Dr. I. Lehman. Hydroxyapatite chromatography[9] of DNA was performed with Bio-gel HTP hydroxyapatite (Bio-Rad Laboratories, Richmond, Calif.).

Results. DNA polymerase activity of purified AMV: Considerable uptake of [^{3}H]dTTP into an acid-insoluble form was observed only after treatment of purified AMV with NP-40 (Table 1, Expt. 1). Treatment with 0.05–0.2% NP-40 (not shown), or inclusion of 0.02–0.04% NP-40 in the reaction mixture, gave maximal incorporation. Unmasking of viral polymerase activity is rapid with little dependence on either temperature or time of incubation (Table 2).

Requirements for DNA polymerase reaction: The AMV DNA polymerase has a pH optimum of 8.0 (Fig. 1*a*), similar to that of MSV.[4] Monovalent ions (Na^+ or K^+) are not essential for maximal enzyme activity (1*b*); however, 30 mM Na^+ was used in these experiments. Optimal activity occurred at 2–3 mM Mg^{++} or 1 mM Mn^{++} (1*c*); a slight increase was observed when both Mn^{++} and Mg^{++} were present (1*d*). DNA synthesis is completely dependent upon added deoxyribonucleoside triphosphates: the omission of a single deoxyribonucleoside triphosphate reduced incorporation 80–90%.

Kinetics of the AMV DNA polymerase reaction: In contrast to RNA-dependent DNA polymerase reactions described previously,[1–6] the reaction by

TABLE 2. *Effect of preincubation with NP-40 on AMV DNA polymerase activity.*

Preincubation		Time of incubation at 37°C (min)	Incorporation of [^{3}H]dTTP (cpm)*
Temperature (°C)	Time (min)		
0	15	0	64
	5	60	2255
	15	60	2141
	30	60	2582
37	5	60	3054
	15	60	2794
	30	60	2928

* As in Table 1, except 2.1 μg viral protein/assay.

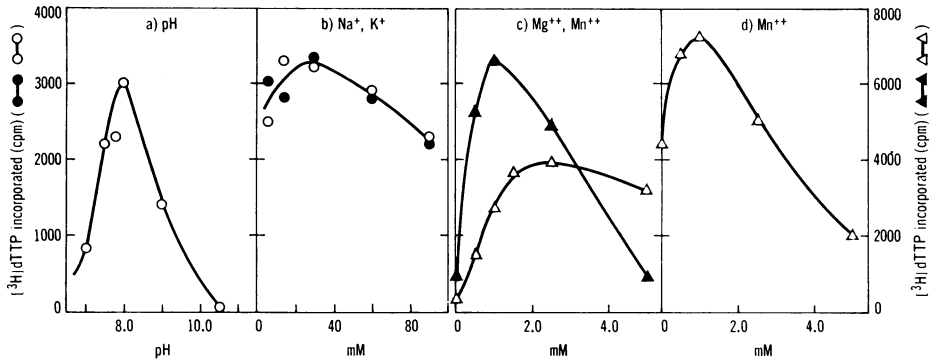

FIG. 1. pH optimum and monovalent and divalent ion requirements for the AMV DNA polymerase reaction. Each point represents the average of duplicate determinations. (*a*) pH optimum was determined with 3.2 μg of viral protein. (*b*) NaCl (○) or KCl (●) optima were determined with 3.2 μg of viral protein. (*c*) Mg^{++} optimum (△) was determined with 3.2-μg viral protein and Mn^{++} optimum (▲) with 5.0 μg of viral protein. (*d*) Mn^{++} optimum, in the presence of 2.5 mM Mg^{++}, was determined with 3.2 μg of viral protein.

AMV DNA polymerase is extremely rapid and shows two apparently distinct rates of polymerization. As shown in Fig. 2, *a–c*, the initial reaction is completed in 4 min at 37°C, and is followed by a less rapid reaction which levels off after 60–90 min (later time points not shown). Lowering the temperature to 25°C decreases the initial rate of polymerization 50–70% and the subsequent rate 30–40% (2*d*).

Formation of an RNA·DNA complex and template-free DNA: 30 sec after the reaction starts, most of the AMV DNA is present in two classes of RNA·DNA hybrid molecules, as shown by equilibrium centrifugation in Cs_2SO_4 gradients (Fig. 3): (1) "RNA-rich hybrids," which band at the position of free RNA, and contain viral RNA complexed with small amounts of DNA; and (2) "intermediate hybrids," which contain increased ratios of DNA to RNA, and band between the positions of free RNA and free DNA. After 7, 20, and 60 min of reaction, most of the AMV DNA is present free of viral RNA template, although significant quantities of intermediate hybrids are also present. The presence of RNA-rich hybrids after 10 min of reaction (Fig. 3, Expt. 2, 10 min) may indicate the reinitiation of RNA → DNA copying.

The intermediate hybrids in the Cs_2SO_4 gradient may consist of intact viral RNA molecules complexed with AMV DNA representing substantial portions of the viral genome. Alternatively, intermediate hybrids may consist of viral RNA complexes in which most of the RNA has been degraded by nuclease action, resulting in higher ratios of DNA to RNA. The latter alternative seems less likely, for the viral RNA template is stable during preincubation: no loss in DNA polymerase activity occurs when AMV is incubated for 30 min in a substrate mixture without [^{3}H]dTTP before incubation with [^{3}H]dTTP.

Hybridization of AMV DNA with AMV RNA: When AMV DNA was "annealed" with MSV RNA, no radioactivity was found in the hybrid position (Fig. 4*b*). In contrast, AMV DNA annealed efficiently with AMV RNA (4*a*)

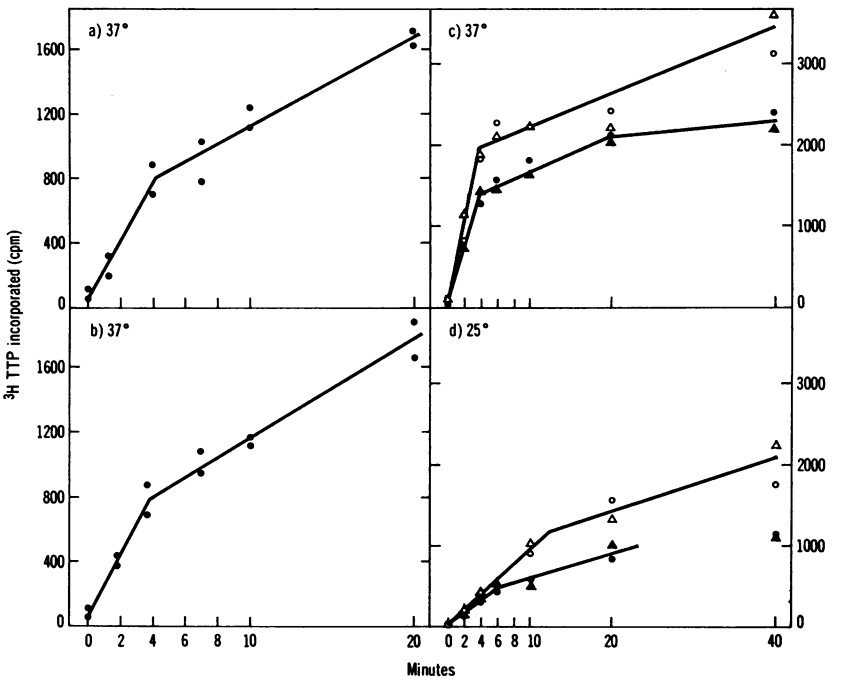

FIG. 2. Biphasic kinetics in the AMV DNA polymerase reaction. 0.2 vol of AMV was incubated with 0.2% NP-40 (*a, b*), 0.1% NP-40 (*c,d,* lower curve), or 0.02% NP-40 (*c, d,* upper curve) in the absence of substrate at the indicated temperature for at least 5 min, and mixed with 0.8 vol substrate mixture at the indicated temperature without NP-40 (*a,b; c,d,* lower curve) or with 0.02% NP-40 (*c,d,* upper curve). Duplicate 50-μl aliquots of the reaction mixture were processed[4] after stopping the reaction at the indicated times by the addition of 0.1 vol of 0.1 M EDTA (*a,b*) or 150 μl of 1 N $HClO_4$ (*c,d*). Each aliquot contained 2.1 μg (*a,b*) or 3.2 μg (*c,d*) of viral protein and 5 μCi of [^{3}H]dTTP. Experiments *a* and *b* are duplicate enzyme reactions; *c* and *d* used a different AMV preparation.

since 60–70% of [^{3}H]DNA bands at the RNA-rich and intermediate hybrid positions in the gradient.

Properties of the AMV DNA: Size, buoyant density, and strandedness: The size of AMV DNA formed after 30 sec, 7 min, and 60 min of reaction was determined by sedimentation in alkaline sucrose gradients. Calculated from the 34 S value of marker adenovirus DNA,[4] AMV DNA has a sedimentation value of 5–6, corresponding to a molecular weight of about 150,000. Alkali-treated, 60 min, AMV DNA forms a broad band after equilibrium centrifugation in CsCl with an average buoyant density of 1.721, consistent with DNA.

Hydroxyapatite chromatography discriminates between single and double stranded DNA. Native adenovirus DNA and adenovirus-transformed cell DNA are eluted by 0.4 M phosphate buffer but not by 0.12 M, while denatured viral and cellular DNA are eluted by 0.12 M buffer (Fig. 5). Most of the AMV DNA is duplex since 50–70% of 7 and 60 min AMV DNA is eluted by 0.40 M buffer. Surprisingly, 20–30% of alkali-denatured AMV DNA is not eluted by 0.12 M buffer, indicating that a portion of AMV DNA is apparently duplex even

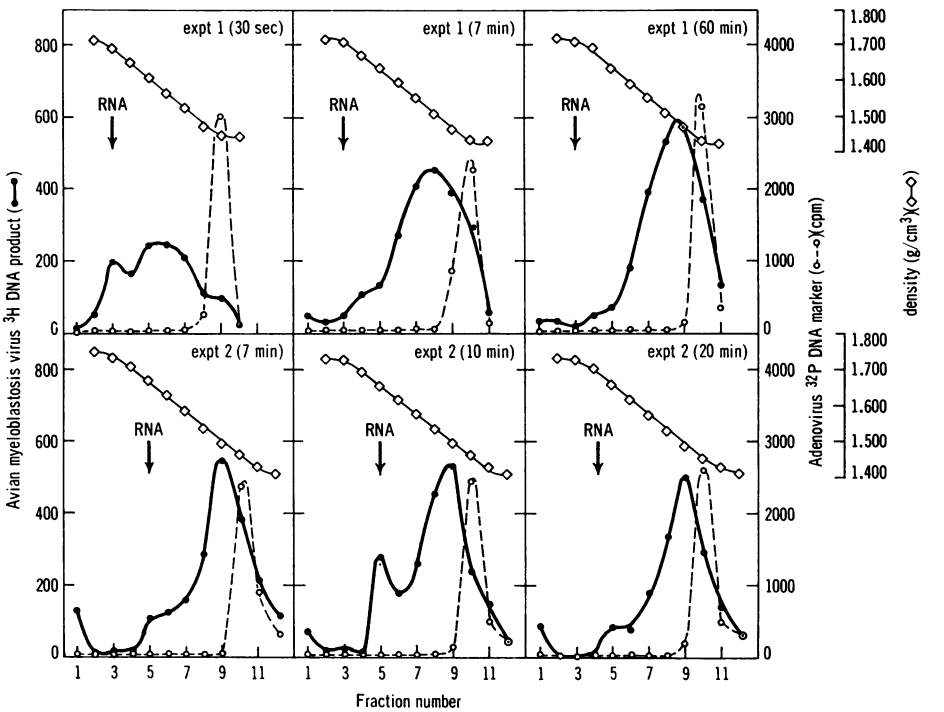

FIG. 3. Equilibrium sedimentation of AMV DNA in Cs_2SO_4 gradients. The DNA polymerase reaction (Expt. 1) as described in 2*c*, upper curve, with 6.4 μg of viral protein per 100 μl of reaction mixture; the reaction was stopped by 0.1 vol of 0.1 M EDTA. For Expt. 2, an aliquot of the reaction product of Fig. 2 (*b*) was used. Purified AMV DNA, 3.1 ml in 0.30 M NaCl–0.030 M Na citrate, containing unlabeled KB cell RNA (25 μg) and adenovirus type 7 [^{32}P]DNA markers, was centrifuged after adding 2.60 g of Cs_2SO_4 for 46 hr at 34,000 rpm (Expt. 1) or for 51 hr at 35,000 rpm (Expt. 2) in the SW50.1 rotor at 20°C. Fractions (0.375 ml) were collected and the refractive index, absorbance, and acid-precipitable radioactivity were determined.

after denaturation. This differs from the murine sarcoma and feline sarcoma DNA polymerase products which are quantitatively eluted by 0.12 M buffer after denaturation (unpublished data). Perhaps AMV DNA contains some hairpin structures or crosslinks.

E. coli exonuclease I degrades single-stranded, but not duplex, DNA molecules.[8] As shown in Table 3, adenovirus DNA is not digested by exonuclease but is completely digested after denaturation. The 60 min AMV DNA is 70% resistant to exonuclease I, thus is mainly double stranded. 30–35% of AMV DNA is resistant to exonuclease I even after denaturation, in agreement with the hydroxyapatite experiment.

DNA-dependent DNA polymerase activity in AMV: The biphasic reaction kinetics and the duplex properties of part of AMV DNA suggested the presence of a DNA-dependent DNA polymerase in the virion. We added adenovirus DNA or calf thymus DNA as an external template to AMV in the absence (1), or presence (2), of NP-40, and (3) after treatment of AMV with RNase +

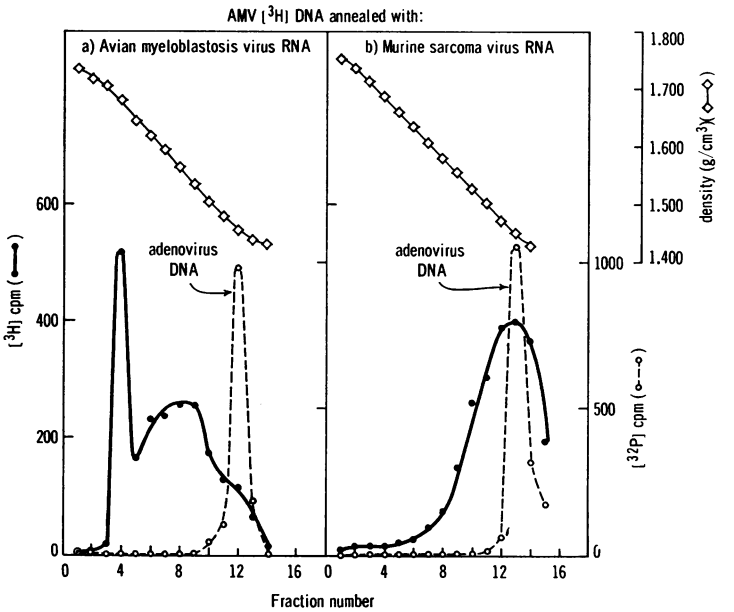

FIG. 4. Hybridization of AMV DNA with AMV viral RNA. A 60-min AMV DNA product (Fig. 3, Expt. 1) was purified, treated with 0.2 N NaOH for 30 min at 80°C, added to 2 μg of AMV RNA(*a*), or MSV RNA (*b*), and made up to a final volume of 300–500 μl in 0.30 M NaCl–0.030 M Na citrate. After annealing for 24 hr at 66°C, the products were analyzed as described in Fig. 3.

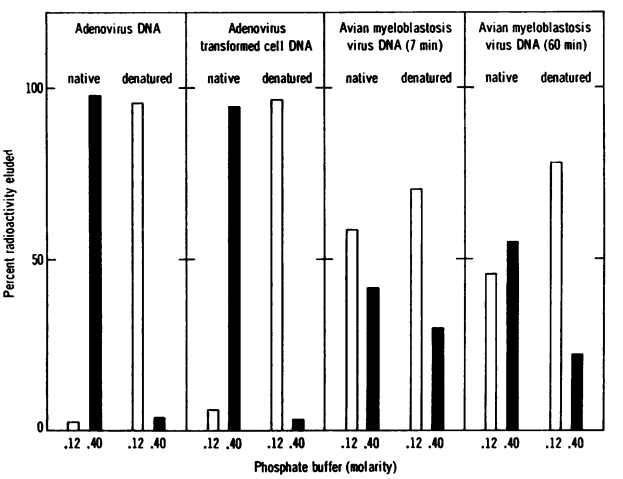

FIG. 5. Hydroxyapatite chromatography of AMV DNA. AMV DNA (Fig. 3, Expt. 1) was purified and a portion denatured with 0.2 N NaOH at 80°C for 20 min and neutralized. After sonication in the Raytheon DF-101 sonic oscillator at full power for 10 min, native or denatured DNA in 4 ml of 0.12 M phosphate buffer, pH 6.8, containing 0.4% sodium dodecylsulfate (SDS) was passed through a hydroxyapatite column maintained at 60°C.[8] The column was eluted with three 4-ml portions of 0.12 M phosphate containing 0.4% SDS and with three 4-ml portions of 0.40 M buffer. Acid-insoluble radioactivity of each fraction was determined.

TABLE 3. *Action of exonuclease I on the DNA product of the AMV DNA polymerase.**

DNA	Time of incubation at 37°C	Acid-insoluble DNA: Before denaturation (cpm)	Before denaturation (%)	After denaturation† (cpm)	After denaturation† (%)
AMV [^{3}H]DNA (60 min product)	0	1239	100	1208	100
	40	871	70	417	35
Adenovirus [^{32}P]DNA	0	2669	100	2248	100
	40	2592	97	112	5

* AMV DNA (Fig. 3, Expt. 1, 60 min), purified by the sodium dodecylsulfate–phenol method,[6] or adenovirus DNA[4] was treated with 10 units of exonuclease I in 67 mM glycine–NaOH buffer (pH 9.5)–6.7 mM $MgCl_2$–1.0 mM β-mercaptoethanol at 37°C for 40 min.

† DNA was treated with 0.2 N NaOH at 80°C for 20 min and neutralized before treatment with exonuclease I.

TABLE 4. *DNA-dependent DNA polymerase activity of AMV.*

Virus treated with	Added DNA (10 μg)	Incorporation of [^{3}H]dTTP (cpm)
None	None	70
	Adenovirus type 2 DNA	89
	Calf thymus DNA	79
0.02% NP-40	None	2,804
	Adenovirus type 2 DNA	12,189
	Calf thymus DNA	45,722
0.02% NP-40 + 100 μg/ml RNase*	None	231
	Adenovirus type 2 DNA	8,535
	Calf thymus DNA	30,356

* Virus was preincubated with 100 μg/ml of pancreatic RNase for 30 min in the presence of 0.02% NP-40 before the standard DNA polymerase assay.

NP-40 to destroy the viral RNA template. No activity was observed in the absence of NP-40 (Table 4). In the presence of NP-40, AMV incorporated 2800 cpm, but the addition of 10 μg of adenovirus or calf thymus DNA stimulated incorporation to 12,000 and 46,000 cpm. RNase abolishes RNA-dependent DNA polymerase activity (231 cpm) but the addition of adenovirus DNA or calf-thymus DNA to RNase-treated AMV stimulated incorporation to 8,000 and 30,000 cpm. The stimulation of DNA synthesis by added DNA template only after treatment with NP-40 is strong evidence for DNA-dependent DNA polymerase activity within the virion.

Discussion. The biphasic reaction, the formation of double-stranded DNA, and the large stimulation in DNA synthesis by added DNA indicate that in addition to RNA-dependent DNA polymerase activity, AMV possesses DNA-dependent DNA polymerase activity. Similar conclusions, based on the stimulation of DNA polymerase activity of Rous sarcoma virus and AMV by added DNA, were made by others (Mizutani and Temin, Spiegelman; personal communications). It is not clear whether one enzyme with several polymerase activities, or several different enzyme molecules, is present in the virus.

Our data suggest the initial rapid formation of an RNA·DNA hybrid, followed by the less rapid formation of duplex DNA molecules. We estimate a minimum polymerization rate of 10–20 nucleotides/sec, based on the detection of 5–6S DNA molecules at 30 sec. This rate compares favorably with the *in vivo* rate of DNA replication in mammalian cells (20–50 nucleotides/sec[10]).

The demonstration of a second activity in leukemia-sarcoma viruses, a DNA-dependent DNA polymerase, and the formation of duplex DNA molecules is consistent with the possibility that viral DNA is a transforming agent, and acts as a template for transcription of plus strands of viral RNA.[11]

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* Paper II in this series is Rokutanda, M., H. Rokutanda, M. Green, K. Fujinaga, R. K. Ray, and C. Gurgo, *Nature*, **227**, 1029 (1970).

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** Abbreviations used: AMV, BAI strain A (avian myeloblastosis virus[7]); NP-40, Nonidet P-40; MSV, murine sarcoma virus.

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