Vol. 83, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1978 Pages 763-770

IN VITRO SYNTHESIS OF DOUBLE STRANDED RNA BY DROSOPHILA X VIRUS PURIFIED VIRIONS

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Received March 3,1978

Summary: Purified Drosophila X virus (DXV) presents a virion associated RNA polymerase activity. The RNA product is not spontaneously released from the virion and behaves like the virion-borne RNA in velocity gradients. Both product and template are RNase resistant at high ionic strength all over the synthesis. Reannealing of the denatured purified RNAs is faster when an excess template is added. This may be the first published case of a virion-associated replicase.

DXV was discovered as a contaminant in Drosophila melanogaster. It can be grown either in injected adult flies, or at a low yield, as a persistant infection in established Drosophila cell cultures.(4). The 64 nm diameter viral particles contain two segments of 94% RNase resistant RNA(5). The present paper evidences that an RNA polymerase is associated with the purified virions, and that both product and template remain RNase resistant all over the synthesis.

METHODS

The growth and purification of DXV are described elsewhere (4). Shortly, the infected flies are blended in saline; the extract was treated with freon to remove host membranes; the virions were concentrated and purified on cesium chloride gradients, then resuspended in low salt buffer (LSB: NaCl, 0.15M; Tris-HCl pH 7.4, 0.1M) and stored at -80º until used. The infectivity was retained up to 20% after purification (5). When labelled RNA was needed, the virus was diluted 1/2 with ^{32}P-trisodic orthophosphate (200mCi/mM; Amersham) before inoculation. The conditions for the *in vitro* assay of the polymerase activity are given in the legends.

0006-291X/78/0833-0763$01.00/0

When RNAs were to be purified, the reaction was stopped by adding two drops/ml of DEPC and two volumes of absolute ethanol. The samples were maintained overnight at -20º, then centrifuged in the HB4 Sorval rotor. The pellet was dissolved in K buffer (NaCl, 0.01M; Tris-HCl pH8.0, 0.01M; SDS, 0.5%) containing 1mg/ml of proteinase K (3) and incubated for 3hrs at 25º. The solution was then layered on top of a preformed 5-30% (v/v) sucrose gradient in T buffer (Tris-HCl pH7.6, 0.15M; NaCl, 0.15M; EDTA, 1%), and centrifuged as described in the legend. Denaturation and reannealing in 70% formamide were as described in the legends.

RESULTS

GENERAL CHARACTERISTICS OF THE REACTION: When all conditions were optimized, the polymerization of the radioactive precursor into an acid-precipitable product could be observed for 26hrs (fig.1) with variations from one batch of virus to another. The optimum pH was 7.4 and the optimum $MgCl_2$ molarity 4mM(not shown). Mn^{++} could not replace Mg^{++} (table 1); when mercaptoethanol (ME) was used in place of DTE at a concentration of 0.5mM, the activity was reduced to 39% of the control and 1mM inhibited it completely; the need for Triton N101 varied from one batch of virus to another, optimum concentration being 1 µl/ml. Omission of UTP, ATP, or CTP reduced the incorporation respectively to 3.5, 63, and 88% of the control, but the commercial stocks were not repurified before use. Dactinomycin had no inhibitory effect. All four phosphoribonucleosides could be incorporated and A+G/C+U = 1.19 (table 2). Since blending the flies releases nucleases and proteases, especially from the digestive tract, we did not use the virus before purification.

ANALYSIS OF THE POLYMERASE PRODUCT: When deproteinization was omitted, most of the radioactivity was pelleted at the bottom of the velocity gradients with the virions (not shown). Treatment with 1% SDS or 2M LiCl or DEPC alone were not sufficient to release the RNAs. Phenol-cresol-chloroform was not suitable either since most of the radioactivity was irreversibly trapped in the interface.

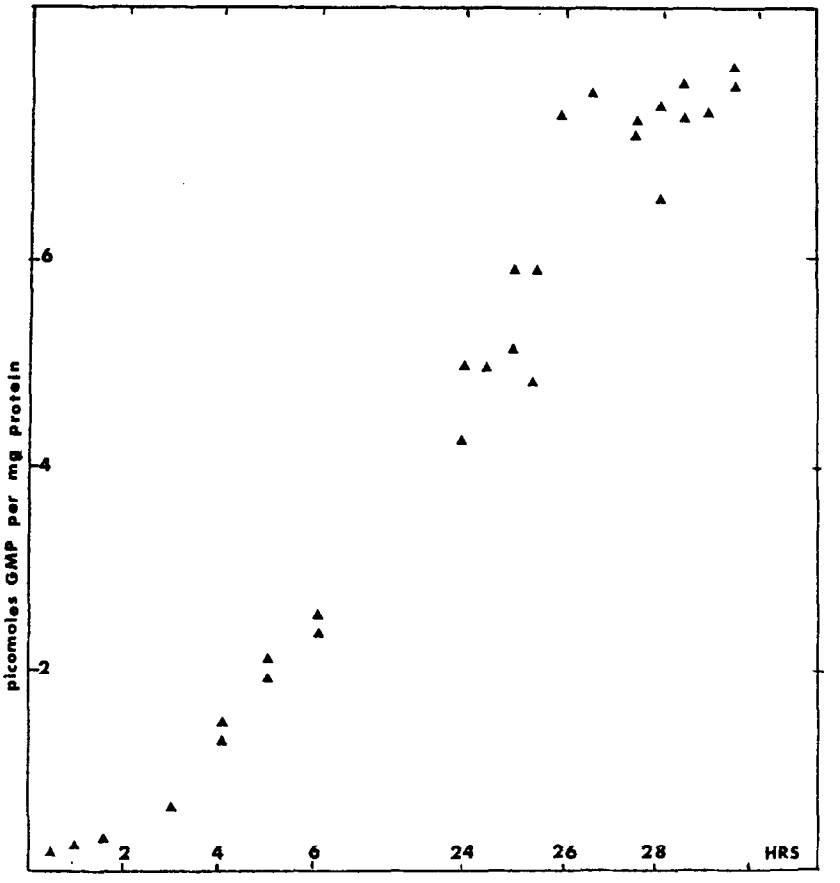

Fig.1: KINETIC OF INCORPORATION OF GMP: Two 1ml reaction mixtures were prepared (Tris-HCl pH7.4, 0.1mM; ATP, UTP, CTP, 0.7mM each; 3H-GTP (specific activity 11Ci/mM, Amersham), 4.5µM; MgCl2, 4mM; dithioerythritol (DTE,Sigma) 3mM; Triton N101 (Sigma), 0.08% (v/v). The mixture was maintained in ice until the addition of virions, then incubated at 25º. Aliquots 50µl (25µg proteins) were withdrawn from both samples at each indicated time to be precipitated with cold TCA, filtered on Whatmann CF/c glass fiber discs, washed with 30ml cold 5% TCA, and counted in Bray's scintillation mixture.

After 2.5hrs synthesis, then deproteinization with proteinase K, two peaks of radioactivity could be observed, both for the ^{32}P-labelled template and the ^{3}H-GMP labelled product (fig.2). Thesmaller peak was 4-5s and both product (A) and template (B) were RNase sensitive. Both product and template were also found as a 14s peak, and both were 100% RNase resistant in 0.1 and 0.4M NaCl.

dpm - t_o	control	modified	%inhibition
- Mg^{++},+ Mn^{++} 2.1 to 16.8mM	9,733	0	100
- DTE	20,125	627	97
- Triton	5,353 4,894	1,544 2,360	71 52
- DTE + 0.5mM ME	11,196	4,413	60
- A - C - U	11,615	7,365 10,221 411	37 12 96.5
+ Dactinomycin 25 µg/ml	7,943	7,728	2.7

TABLE I- Replacement or omission of various components: Incubation was for one hour at 25ºC, with 3H-GTP (specific activity 11 Ci/mM, Amersham) as a labelled precursor. t_o was a non incubated sample. Virus batches varied from one experiment to another.

	AMP	CMP	UMP	GMP
dpm - t_o	12,002	8,745	8,138	8,091
% Total	32.2	23.8	21.9	21.9

TABLE 2- Nucleotides ratio: Four aliquots were prepared. For each of them, unlabelled ATP, CTP, UTP or GTP were successively reduced to 0.35mM, and 168 pM (5 µCi) of the corresponding 32P-labelled triphosphoribonucleoside (150 Ci/mM) was added. 40 µl aliquots, containing 5 µg of virus were drawn in duplicate before and after 1 hour incubation at 25ºC. The values represent the average calculated on each duplicate measure.

After 7.5hrs synthesis, the neosynthesized 14s molecules were RNase A+T1 resistant in 1 X SSC, but could be completely digested by the enzymes in 0.01 x SSC (table3).

DENATURATION AND REANNEALING: We first verified (fig.3) that the purified ^{32}P-labelled 14s genopic RNA could be denatured when heated for 30min at 60º in 70% formamide in LSB. Then the 14s RNAs from a 26hrs incubation experiment were resuspended in the formamide mixture and 20µl aliquots were heated at 60º for different lengths of time (fig.4). Denaturation of ^{3}H-GMP labelled molecules was shown to be progressive and

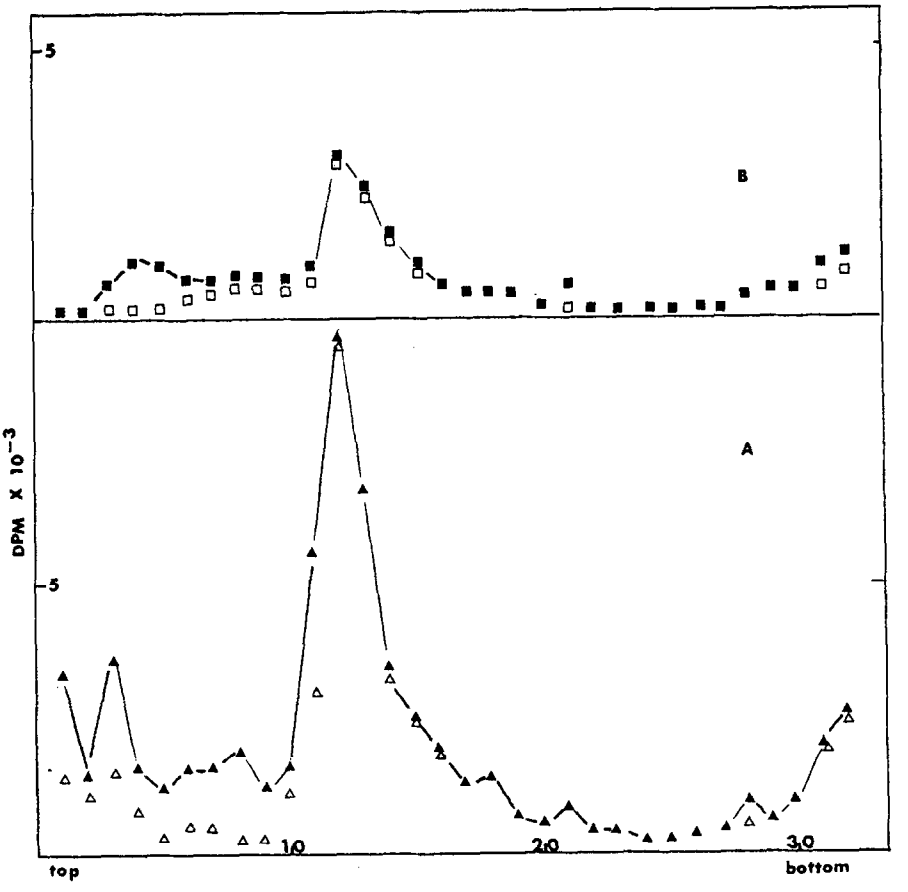

Fig.2: SUCROSE VELOCITY GRADIENT ANALYSIS OF THE TEMPLATE AND PRODUCT after 2.5hrs reaction: the template was labelled with 32P and the product with 3H. After deproteinization with proteinase K, the RNAs were centrifuged for 18hrs at 150,000 x g in a SW41 Beckmann rotor. Fractions 0.4ml were collected from the top of the gradient. Aliquots 100µl were withdrawn from each fraction either as control or to be treated with 10µg/ml RNase A, in 0.1 and 0.4M NaCl. The values for both ionic strengths were identical

■ ^{32}P □ + RNase A ▲ ^{3}H △ +RNase A

30% was still RNase A resistant after 30min. Complete denaturation was obtained after 1 hr (not shown).

In an independant experiment, the 14s RNAs of a 7hrs reaction were resuspended in the formamide mixture and divided into 10 aliquots, 20µl each; five aliquots were diluted to 40µl with the formamide mixture alone, and the other five received 40µl of unlabelled purified 14s genomic RNA. The

3H-cpm	control	+ T1, + A	% resistance
0.01X SSC	819	38	4%
1X SSC	734	679	92%

TABLE 3- RNase resistance at different salt concentrations: The purified 14s RNAs were resuspended in either 0.01 or 1 X SSC (SSC: NaCl, 0.15M; sodium citrate, 0.015M) The samples were divided in two equal aliquots, and to one of them, 10 µg/ml RNase A and 10 units/ml RNase T1 were added. The samples were TCA precipitated after 30min incubation at 37ºC.

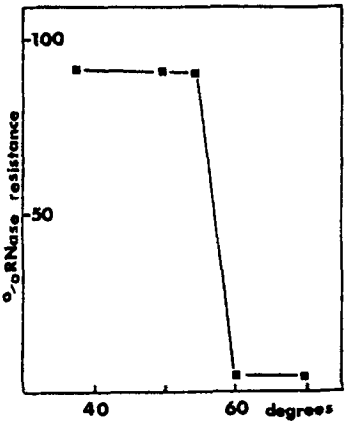

Fig.3: DENATURATION OF THE GENOMIC RNA: The purified 14s RNA from the virions was heated for 30min at the indictaed temperature in 70% formamide in LSB, then chilled in ice and diluted with LSB.

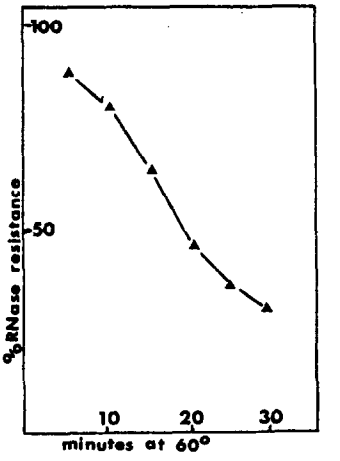

Fig.4: DENATURATION OF THE PRODUCT RNA: The purified 14s RNAs from a 26hrs reaction were heated in the formamide mixture for the indicated time, then diluted and treated with RNase.

samples were denatured for 30min at 60º to 30% RNase resistance as above, then transferred to 37º for different lengths of time. Complete renaturation occured in both samples, but

100% RNase A resistance was obtained in 5 hrs when excess viral RNA was added (fig.5), while more than 10hrs were necessary for complete self-annealing.

DISCUSSION

Purified DXV particles contain an RNA polymerase which

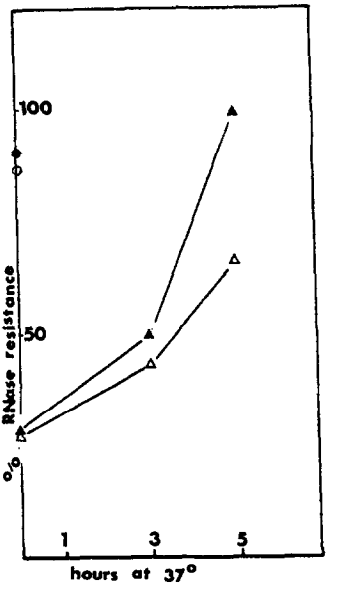

Fig.5: ANNEALING OF THE POLYMERASE PRODUCT: The purified 14s RNAs of a 7hrs reaction were denatured for 30min at 60º as described, then transfered at 37º for the indicated time. Circles: undenatured; black triangles: excess genomic RNA added.

synthezises RNA in vitro. Since the virions were extracted from the host tissues, we don't know wether or not the polymerase has to be activated, and slight variations in removal of the host-borne material could account for variations in the need for Triton N101.

Since the product could not be separated from the template, the denatured samples contained both. Thus, the progressive denaturation we observed (fig.4) may be due to competitive reannealing. On the other hand, the product is complementary to the template, since when genomic RNA was added, hybridization was faster then self-annealing (fig.5).

Since in the experiment presented on fig.2, the template was labelled with ^{32}P of a specific activity 200mCi/mM, 3.000 dpm in fraction 12 are equivalent to 6.8×10^{-6} µM ^{32}P incorporated into the template 14s RNA. Since the product was labelled with ^{3}H-GMP of a specific activity 11 Ci/mM and GMP represents 22% of the incorporated nucleosides, 10,000 dpm in the same fraction are representative of 1.89×10^{-6} µM nucleosides in the product RNA. Since in a polynucleotide there is one phosphate per nucleoside, thus 2.5 hrs after the beginning of the synthesis, the product with this virus stock represents 27.8% of the template. Similar experiments are in progress with longer incubation lengths

of time. It should be noted that variations in the rate of synthesis were observed with different batches of virus, and as a function of time at higher protein contents, because a phosphohydrolase activity was then demasked (to be published).

The most important results are that both 14s template and product were RNase resistant whichever the length of the reaction was, and the polymerase activity was found in purified infectious particles. Thus the mechanism of the synthesis may be different from what has been described for PsV-S virus particles (2), since a single virus band with no single strand RNA was isolated during purification on CsCl gradients, and for intracytoplasmic reovirus replication intermediates (1,6), since in our case the mature particle was active.

REFERENCES

1- ACS, G.; H.KLETT; M.SCHONBERG; J.CHRISTMAN; D.H.LEVIN; S.C.SILVERSTEIN. J.of Virol 8, 684-689, 1976.
2- BUCK,K.W. Nucleic Acid Research 2, 1889-1902, 1975.
3- DOBOS, P. Nucleic Acid Research 3, 1903-1924, 1976.
4- TENINGES, D. et al Submitted to publication, J.of Gen.Virol.
5- Teninges, D. Submitted to publication.
6- SAKUMA, S.; Y.WATANABE. J.of Virol.10, 628-638, 1972.