IN VITRO SYNTHESIS OF A 2.1 X 10^6 DALTON DNA IN THE ENDOGENOUS RETROVIRUS REVERSE TRANSCRIPTASE REACTION

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SUMMARY: A discrete, homogeneous population of single-stranded DNA molecules complementary to virion RNA has been synthesized in a modified RNA directed DNA polymerase reaction involving the endogenous retrovirus produced by the mouse cell line JLS V-6. A molecular weight of 2.1 X 10^6 dalton for the DNA was assigned from the sedimentation coeffecient in alkaline sucrose gradients, the electrophoretic mobility in formamide gels, and the lengths of the molecules in electron micrographs.

INTRODUCTION: It was realized soon after the discovery of RNA directed DNA polymerase (RDDP;1,2) that the polydeoxyribonucleotide products of the *in vitro* reaction were much shorter than the 30-40S RNA template of the virion. It was hypothesized (3) that the short DNA fragments were made double-stranded by the virion associated DNA directed DNA polymerase, ligated by polynucleotide ligase, and inserted into the host cell genome to form the provirus. This scheme was unlikely, due to the limited number of primer sites on the retrovirus genome (4,5) and the lack of a known mechanism for correctly ordering the fragments prior to ligation. It has recently been shown (6) that at least the minus DNA strand is transcribed into one length of DNA in the infected cell, and that RNA directed DNA synthesis is not necessarily discontinuous.

The *in vitro* synthesis of DNA transcripts equivalent to 60-100 percent of a retrovirus 30-40S RNA subunit have recently been reported (5,7,8), although the yeild of large DNA in these experiments was very low and the size distribution very heterodisperse. Complete DNA transcripts of various RNAs have been obtained in experiments involving purified enzymes and templates (9,10), indicating that host cell factors do not necessarily play a crucial role in RNA directed DNA synthesis. Nonetheless, long DNA transcripts of a discrete size have not previously been obtained in significant yeild from the endogenous RDDP reaction. We report here the synthesis of such a DNA, using the endogenous virus from the Balb/c mouse cell line JLS V-6.

MATERIALS AND METHODS

Cells and Virus: The constitutively producing Balb/c mouse cell line JLS V-6 was the source of virus (11). Virus was collected from the growth medium (MEM with 10 percent tryptose phosphate and 5 percent fetal bovine serum) at 3 or 24 hour intervals as described (12). Following purification, the virus was suspended in .01 M Tris-HCl, pH 8.1; .01 M dithiothreitol; .3 M sucrose; and 50 ug/ml

dextran sulfate. Virus preparations were used immediately and never frozen.

Endogenous RNA Directed DNA Synthesis: A modification of the method described previously was used (13). The standard reaction mixture contained .1 M Tris-HCl, pH 8.1; .06 M KCl; .002 M $MnCl_2$; .007 M dithiothreitol; .02 percent Triton-X-100; .009 M creatine phosphate; 100 ug/ml creatine phosphokinase; 200 uCi ^{3}H-TTP (New England Nuclear; 19 Ci/mmole), 50 ug/ml actinomycin D (Calbiochem) and four dNTP's were added to the concentration indicated in the figure legends. Purified virus was added at a concentration of 1 mg of protein per ml.

RESULTS: The rate of deoxyribonucleotide incorporation into trichloroacetic acid (TCA) insoluble material is greatly dependent upon the concentration of the four dNTP's in the reaction mixture, as shown in Fig. 1. For reactions terminated

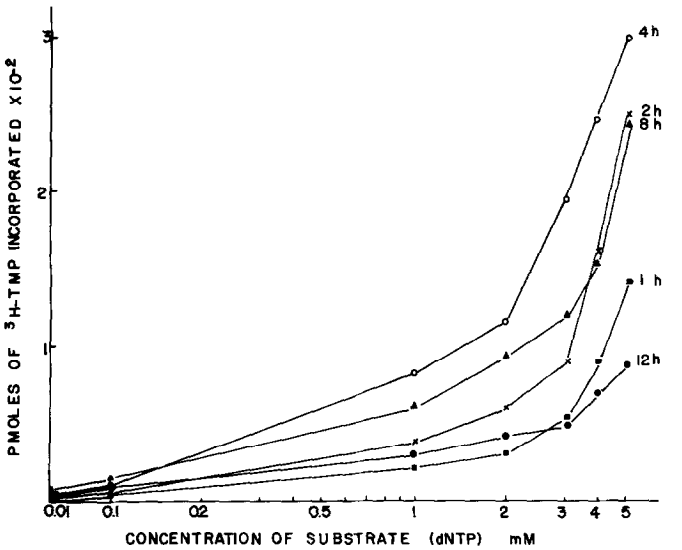

Figure 1. Effect of dNTP Concentration on Rate of DNA Synthesis. Standard .1 ml reaction mixtures were prepared containing equimolar concentrations of the 4 dNTP's as indicated, and the polymerization reactions terminated as indicated at right. The virus in the reaction mixtures had been collected at 24 hr intervals. To terminate the reaction, 0.1 ml of 0.1 M sodium pyrophosphate, 100 umoles TTP, and 2 ml of cold 5% TCA were added. After 30 min. at 0°C, the samples were collected on Gelman triacetate filters (GA-6) and filters were placed in a counting vial, and mixed with 1 ml of 1 M NH_4OH and 10 ml of Triton-X-toluene containing counting fluid.

after 2 or 4 hr of incubation, incorporation increases approximately 30 fold when the dNTP concentration is raised from .01 to 2 mM, and a further 2 to 3 fold when raised from 2 to 5 mM. Even at 5 mM, no evidence of saturation is seen. These data are in agreement with the findings of others (5,7,8).

We found that while the polymerization reaction continued for at least 12 hr,

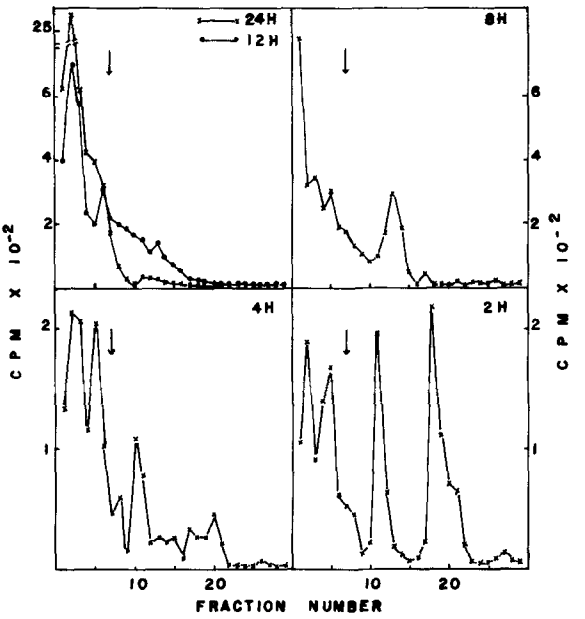

Figure 2. Effect of Reaction Time on Size of DNA Transcript. DNA was extracted as previously described (23) from reactions terminated at the indicated times and layered on linear 5 to 25 percent alkaline sucrose gradients, which were spun at 24,000 rpm for 24 hr at 2°C in the small (17 ml) buckets of the SW 27 rotor. Unlabeled marker DNA of 880 nucleotides was run in the same tube, and located spectrophotometrically. The virus used in the synthesis of the DNA had been collected at 24 hr intervals. Sedimentation is from left to right. Samples were treated and counted as in Fig. 1.

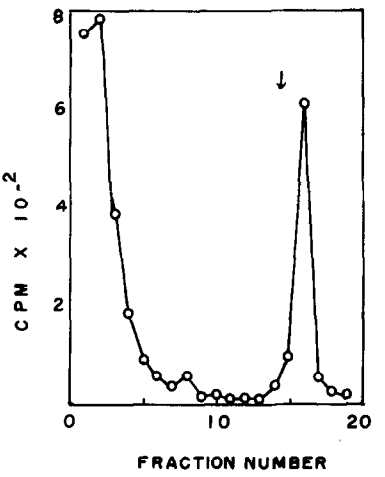

Figure 3. Effect of Age of Virus on Size of DNA Transcript. All conditions are identical to those of Fig. 2, except that the DNA was synthesized in reaction containing virus collected at 3 hr intervals, and linear SV 40 DNA was used as marker.

the DNA products of the long reactions were much shorter than those obtained from reactions terminated after 2 hr, as can be seen from Fig. 2. The short reaction time probably serves to minimize hydrolysis of the DNA by the virion associated nucleases. An incubation time of 2 hr was found to give the best combination of yeild and product size. Fig. 3 shows that virus harvested at 3 hr intervals was capable of synthesizing larger DNA in higher proportional yeilds than virus harvested at 24 hr intervals when incubated for 2 hr. This is probably due to the template RNA in the virions harvested at 3 hr intervals is less degraded than that from "older" virions (14,15, our unpublished data).

After alkali treatment, all TCA insoluble material banded at a density of 1.44 g/cc in neutral cesium sulfate, indicating that the product was DNA. S_1 nuclease digestion and hydroxyapatite chromatography were used to assess the effectiveness of actinomycin D in preventing the synthesis of duplex DNA. More than 90 percent of the radioactivity was solubilized by digestion with S_1 nuclease and less than 10 percent bound to hydroxyapatite, indicating that the majority of the transcript was single-stranded. The single stranded nature of the DNA transcript was confirmed by electron microscopy after staining with T_4 gene 32 protein (Fig. 4). Gene 32 protein binds selectively and cooperatively to single stranded polynucleotides, providing a method for differentiating single stranded

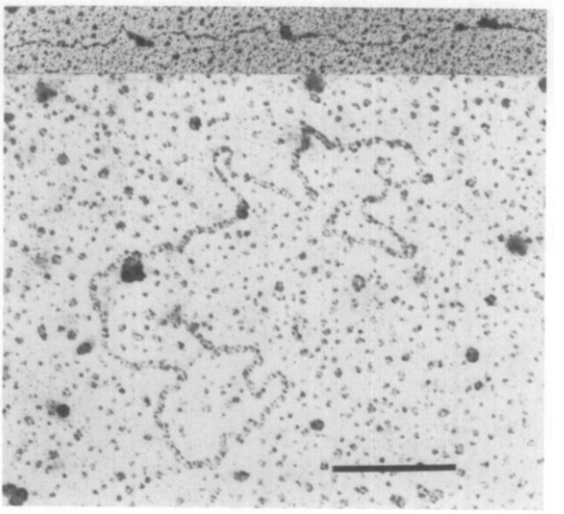

Figure 4. T_4 Gene 32 Protein Electron Microscopy of DNA Transcript. The DNA Transcript was taken from the peak fraction shown in Fig. 3 and was prepared by the method of Wu and Davidson (16). Inset shows "gapped" DNA prepared identically. The thick regions are single-stranded; duplex region are thin and difficult to visualize. The DNA transcript can be seen to be single stranded over its entire length. Marker is .25 um.

from duplex DNA by electron microscopy (16). Variability in length of molecules prepared by this technique prevent the determination of molecular weights from these micrographs.

83 percent of the DNA transcript can be protected from S_1 nuclease digestion by hybridization to virion 60-70S RNA, indicating that the DNA is complementary to virion RNA. It is not known why 17 percent of the DNA remained sensitive to S_1 nuclease. When ^{32}P labeled 60-70S virion RNA was hybridized to the larger DNA, similar (although much more variable) amounts of ^{32}P labeled 60-70S virion RNA also showed sensitive to S_1 nuclease. This prevented us from determining by this method whether or not the DNA was a complete transcript of the genome. These difficulties are probably due to problems inherent in RNA-DNA hybridization (17).

A molecular weight of 2.1 X 10^6 dalton was assigned to the large DNA transcript by comparing the sedimentation behavior (Fig. 5A) of the transcript to that of linear and open circular SV 40 DNA (MW = 1.8 X 10^6 per strand; 18), by estimating it's electrophoretic mobility in formamide gel electrophoresis using linear SV 40 DNA as marker (Fig. 5B), and by measuring the lengths of molecules

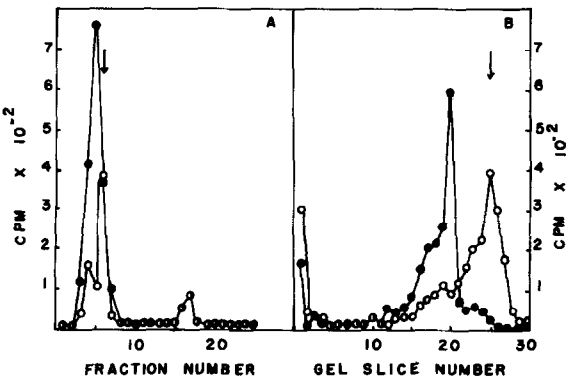

Figure 5. The Size of the DNA Transcript. Panel A: Sedimentation profile of ^3H-labeled DNA transcript and ^{14}C SV 40 DNA. The DNA obtained from ethanol precipitation of fractions 20 to 26 in Fig. 3 was mixed with SV 40 DNA and layered on 5 to 25% alkaline sucrose gradients as described in Fig. 2. DNA transcript is denoted by closed circles (●——●), SV 40 DNA by open circles (o——o). Centrifugation was for 4.5 hr at 45,000 rpm in the SW 50.1 rotor at 2°C. Sedimentation was from right to left; fractions were taken from the bottom of the tube. Arrow indicates linear form of SV 40 DNA. Samples were treated and counted as in Fig. 1. Panel B: Formamide gel electrophoresis of ^3H-DNA transcript and linear ^{14}C-SV 40 DNA. The ^3H-DNA was mixed with SV 40 DNA (as in panel A) and layered on a 3.5% acrylamide-formamide gel (5 X 50 mm). The electrophoresis was run in 40 mM Tris-acetate buffer, pH 7.9 at 10 ma for 6 hr. The gel was then frozen, cut, solubilized, and counted as described (19,22). Closed circles (●——●) identify ^3H-DNA transcript and open circles (o——o) represent the ^{14}C-SV 40 DNA marker.

in electron micrographs mounted by the cytochrome c spreading technique, with ϕX 174 DNA as a length standard (unpublished data). The DNA was homogeneous by all criteria. The variation in the molecular determinations was ± .2 X 10^6 daltons.

DISCUSSION: A few of the factors controlling polydeoxyribonucleotide chain length in the products of the endogenous RDDP reaction can be identified. One important factor involves the nucleases known to be present in the virions of retroviruses (14,15). We have shown that the effects of these nucleases can be circumvented, at least in part, by the use of rapid harvest virus to minimize RNA template degradation and by employing short reaction times to reduce DNA transcript breakdown. One effect of high dNTP concentrations is to increase the lengths of the DNA transcripts, as has been shown before (5,7,8, 10,20). Part of this effect is probably due to a stimulation of the rate of transcription while the rate of hydrolysis remains essentially constant; synthesis outstrips degradation under these conditions. It has also been shown that dNTP may function as an allosteric effector for the RNA directed DNA polymerase (21); one manifestation of this control may be a reduced tendancy of the DNA polymerase to terminate transcription in the presence of high concentrations of dNTP.

While 2.1 X 10^6 daltons (about 6,000 nucleotides) is smaller than the 30-40S subunits of most retroviruses, it is well within the range of the molecular weights (2.0 - 2.4 X 10^6 daltons) calculated for the 30 - 40S RNA of Moloney murine sarcoma virus (22). Our preliminary formamide gel electrophoresis data indicate that the molecular weight of the 30 - 40S subunits may be in the same range as that of Moloney murine sarcoma virus (unpublished observation). The non-linear relationship between the electrophoretic mobility of large polynucleotides and molecular weight in formamide gel has been noted by Duesberg and Vogt (19). The molecular size of the subunits will have to be determined by other means.

The biological significance of the large DNA described in this report has yet to be determined. However, the large DNA will almost certainly be of use in genome fractionation studies and should serve as a superior probe for molecular hybridization purpose.

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