Synthesis of Long Complementary DNA in the Endogenous Reaction by Equine Infectious Anemia Virus

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In the endogenous reverse transcriptase reaction, equine infectious anemia virus is able to synthesize complementary DNA (cDNA) of 8,000 nucleotides in high yield. After 2 h in 50 μ M dNTP, about 2.8 μ g of cDNA per mg of protein is produced, almost 30% of which is long cDNA. The system thus compares favorably with the other two well-characterized endogenous reaction systems, Moloney murine leukemia virus and avian sarcoma virus. Elongation rates of 100 to 150 nucleotides per min have been observed; these rates are comparable to those seen with purified avian myeloblastosis virus reverse transcriptase and significantly higher than those observed *in vivo*. In the absence of actinomycin D, equine infectious anemia virus does not require high dNTP levels for either optimal incorporation or long cDNA synthesis. The amount of long cDNA synthesized is maximal at 2 h in 50 μ M dNTP; neither longer time nor higher dNTP levels (through 1.8 mM) increased this yield. Half-maximum yield in 2 h was achieved at about 15 μ M dNTP, which is very similar to the published K_M 's for isolated avian and murine reverse transcriptases. Total incorporation, on the other hand, continues to rise slowly through 1 mM dNTP; the half-maximum was 30 to 50 μ M dNTP. In the presence of 100 μ g of actinomycin D per ml, however, higher dNTP levels are required for long cDNA synthesis. We conclude that equine infectious anemia virus is exceptionally well-suited to studies of the physical organization of the retrovirus genome and to investigations of the mechanism of synthesis of the double-standard cDNA endogenous reaction product.

Cleavage and fractionation of DNA complementary to the retrovirus genome (cDNA) into subclasses of specific fragments would facilitate the detailed characterization attainable through mapping and sequencing. The ability to generate these fragments, however, depends on the availability of at least one full-length strand of cDNA. Similarly, investigation of the mechanism of double-stranded cDNA synthesis, which is so difficult to pursue *in vivo* because of low yields, depends on the ability of the virus to mimic the *in vivo* process *in vitro*. At the very least this means the virus must be able to synthesize one full-length cDNA strand. Until recently, however, cDNA's synthesized in the endogenous reaction were found to be quite small, usually several hundred nucleotides. Although these have been very useful for detection of relatedness to other cDNA's and RNA's, they cannot be employed for the studies mentioned above.

Genome-length cDNA's can now be synthesized by Moloney murine leukemia-virus (M-MuLV) (22, 23, 27), avian sarcoma virus (ASV) (13, 14, 27), and by the endogenous langur virus

(3) when dNTP levels are high and Mg^{2+} is limiting, and/or when the detergent concentration is carefully controlled. We report here that equine infectious anemia virus (EIAV), recently shown to be a retrovirus (1, 6, 18, 20), is also able to synthesize long cDNA *in vitro*. With M-MuLV and Rous sarcoma virus (RSV) it shares the virtue of being able to produce this cDNA in relatively high yield.

MATERIALS AND METHODS

Virus. Viruses were produced and purified in the Viral Resources Laboratory of the Frederick Cancer Research Center. The Malmquist strain of tissue culture-adapted EIAV (16) was propagated in roller bottle cultures of equine fetal kidney cells (EFK-2 line, more than 20 passages postinfection) grown in RPMI-1640 containing 15% fetal bovine serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. Culture fluid was collected at 5-day intervals and clarified, and virus was purified by double banding in sucrose gradients as described by Benton et al. (2). Pelleted virus was suspended in 0.01 M Tris (pH 7.2)–0.1 M sodium chloride–0.001 M EDTA (TNE) at 10,000-fold concentration (4.4 to 5 mg of

protein per ml) relative to the culture fluid. Two different lots of virus were used in these experiments; results were qualitatively the same in all aspects tested, but lot 808 had only about one-third the endogenous reverse transcriptase activity (per milligram of protein) of lot 877.

Rauscher MuLV (R-MuLV) was grown in the JLSV-9 BALB/c bone marrow cell line in Hanks minimum essential medium containing 10% fetal bovine serum, 2 mM glutamine, 200 U of penicillin per ml, and 200 μ g of streptomycin per ml. Virus was harvested at 24-h intervals. The endogenous baboon virus M7 was grown in a suspension culture of the A204 human rhabdomyosarcoma line in McCoy 5A medium supplemented as above. Cells were seeded at 2×10^5 per ml, and virus was harvested at 96-h intervals. Virus was purified as described by Benton et al. (2).

Synthesis of cDNA. The endogenous reaction mixture consists of 0.1 M Tris (pH 8), 0.005 M magnesium acetate, 0.05 M sodium chloride, 0.01 M dithiothreitol, and EIAV at approximately 0.9 mg/ml. Triton X-100 concentration was 0.02% for EIAV lot 877 and 0.04% for lot 808. [3 H]dTTP (17 to 50 Ci/mm) was usually 30 to 50 μ M. Both nonradioactive dNTP concentration and incubation time at 41°C varied with the experiment; details are provided in figure legends. Incorporation was stopped by addition of sodium dodecyl sulfate (SDS) to a final concentration of 1%.

Conditions for R-MuLV and M7 cDNA synthesis were identical to those for EIAV, except that NP-40 was employed instead of Triton X-100 and that R-MuLV and M7 were present at 4 and 5 mg/ml, respectively.

Centrifugation. The size of cDNA was estimated from its sedimentation in alkaline sucrose gradients (5 to 20% sucrose in 0.8 M sodium chloride, 0.2 N sodium hydroxide, 0.001 M EDTA, 0.01% Sarcosyl), which were centrifuged in the SW40 or SW41 rotor at 34,000 to 40,000 rpm at 10°C for 15 to 20 h. For routine determinations of approximate size, samples included a [14 C]DNA of an average single-strand length of 400 nucleotides; size calculations were performed by the method of Studier (24). For more precise size determinations of long molecules, simian virus 40 (SV40) [14 C]DNA (form III) (Bethesda Research Laboratories) was included in the sample; we have taken the genome length of SV40 to be 5,225 bases (11, 19).

Hybridization and S_1 nuclease digestion. EIAV 70S RNA was prepared as previously described (20). Hybridizations of about 0.2 ng cDNA with about 0.3 μ g of RNA were carried out in 25 μ l of 0.04 M Tris-0.2 M sodium chloride at 63°C for about 1 h ($C_t = 0.19$ mol·s/liter). Samples to be S_1 -treated were diluted into 0.8 ml of 0.1 M sodium acetate (pH 5)-0.2 M sodium chloride- 10^{-4} M zinc chloride and treated with S_1 nuclease at 41°C for 1 h. Trichloroacetic acid-precipitable radioactivity was then determined.

RESULTS

Time course of cDNA synthesis. Net synthesis of EIAV cDNA in the endogenous reaction proceeds approximately linearly with time for about 1 h, then slows and reaches a plateau by about 6 h (Fig. 1). No further incorporation

FIG. 1. Time course of incorporation. A 5- μ l amount (22 μ g total protein) of EIAV lot 877 was incubated in a 25- μ l endogenous reaction mixture containing (A) 200 μ M dNTP and 38 μ M [3 H]dTTP (40 Ci/mm) (●); (B) same as A, but containing 100 μ g of actinomycin D per ml (Δ); (C) 1 mM dNTP, 38 μ M [3 H]dTTP, 100 μ g of actinomycin D per ml (○). At the indicated times, 2- μ l samples were withdrawn and assayed for trichloroacetic acid-precipitable radioactivity.

was seen at 10 or 27 h. In this experiment, the initial rate of synthesis was about 2,300 pmol of dTTP/mg of viral protein per h; at the plateau, about 6.5 μ g of cDNA/mg of viral protein had been synthesized. This time course was performed with dATP, dGTP, and dCTP each present at 200 μ M ("200 μ M dNTP") and [3 H]dTTP at 38 μ M, conditions which are close to optimal for incorporation (see below). At 50 μ M dNTP, the plateau is somewhat reduced, but the general shape of the curve is the same.

Actinomycin D at 100 μ g/ml reduced both the initial rate of synthesis and the final extent. With dNTP present at 200 μ M ([3 H]dTTP at 38 μ M), only about one-third as much cDNA was synthesized as in the absence of actinomycin D. Raising dNTP to 1 (or 2) mM in the presence of actinomycin D increased the yield to approximately two-thirds the control level (Fig. 1).

Optimization of dNTP concentration. To determine optimal conditions for total incorporation, the concentration of dNTP was varied from 10 μ M to 1 mM while holding [3 H]dTTP constant at 30 μ M. It was found that incorporation increased as dNTP was raised from 10 μ M to about 200 μ M, but that little further increase resulted from incubation in 1 mM dNTP (Fig. 2A). Half-maximum incorporation was achieved at about 50 μ M dNTP. In contrast, two other mammalian retroviruses we have examined (R-MuLV and the endogenous baboon virus) require significantly higher nucleoside triphosphate levels (150 to 200 μ M) to achieve half-maximal incorporation rate (Table 1). In addition, EIAV preparations tend to be about 20

FIG. 2. Variation of nucleoside triphosphate concentration in the endogenous reaction. (A) A 2.5- μ l amount of ELAV lot 877 was incubated in a series of 12.5- μ l endogenous reaction mixes containing 30 μ M [3 H]TTP (●), or 90 μ M [3 H]dTTP (○) (both at 40 Ci/mm), and various concentrations of dATP, dCTP, and dGTP. After 30 min at 41°C, duplicate 3- μ l samples were withdrawn and assayed for trichloroacetic acid-precipitable radioactivity; (B) same as A, except dATP was at 3 mM throughout, [3 H]dTTP was at 30 μ M, and dGTP and dCTP concentrations were varied. Duplicate 3- μ l samples were withdrawn after 20 min and assayed for trichloroacetic acid-precipitable radioactivity.

times more active (per milligram of protein) than our best M7 lots and at least as active as our best R-MuLV lots.

To minimize use of radioactivity, optimization curves are often performed at low dTTP concentration, as above. With ELAV, raising [3 H]dTTP to 90 μ M made little or no difference in either the shape of the optimization curve or in its plateau level (Fig. 2A). Similarly, maintaining the three nonradioactive triphosphates at 100 μ M while varying the [3 H]dTTP concentration resulted in little or no increase in incorporation beyond [3 H]dTTP $\approx$ 30 μ M (data not shown).

These observations are consistent with the optimization curve's being primarily determined by the affinities of the ELAV reverse transcriptase for the various triphosphates. It is possible, however, that the shape of the curve is determined by secondary phenomena (e.g., salt introduced by the triphosphates, chelation of Mg^{2+} , inhibition of RNase). Were that the case, maintaining one of the nonradioactive substrates at 3 mM while varying the other two (from 3 μ M

to 500 μ M) would be expected to give a substantially different result from that seen in Fig. 2A. As shown in Fig. 2B, the resulting curve does appear to be somewhat steeper and to plateau at lower dNTP, but these effects are not marked. The maximum incorporation rate and the concentration of precursors required to achieve half-maximum rate ($\sim$ 30 μ M) are quite similar to those observed in Fig. 2A.

Incorporation is fairly insensitive to changes in the other components of the reaction mixture. The above experiments were performed at a Triton X-100 concentration of 0.02%, for example, but 0.03% gave very similar results. Omitting NaCl had no appreciable effect at dNTP concentrations of either 50 μ M or 1 mM; increasing Mg^{2+} from 5 mM to 9 mM had no effect at dNTP levels of 200 μ M, 1.2 mM, or 2 mM.

Size of the cDNA. Having established the dependence of total incorporation on time and nucleotide concentration, we next determined the effect of these parameters on maximum size of the newly synthesized cDNA. Centrifugation of partially purified reaction products in alkaline sucrose gradients revealed a time-dependent increase in their size (Fig. 3). After 15 min in 50

TABLE 1. Dependence of incorporation on nucleoside triphosphate concentration^a

dNTP	% of incorporation at 1 mM dNTP		
	R-MuLV	M7	ELAV
25 μ M	NT	NT	31
50	14	13	49
100	36	28	60
200	62	50	78
500	78	NT	NT
1 mM	100	100	100
1.5	98	104	NT

^a R-MuLV (4 mg/ml) and M7 (5 mg/ml) were incubated in the standard endogenous reaction mixture containing NP-40 at the previously determined optimal concentration (0.03% and 0.035%, respectively). [3 H]dTTP concentration was 40 μ M, and dNTP concentration varied as indicated. After 30 min, the mixtures were brought to 1% in SDS, and trichloroacetic acid-precipitable radioactivity was determined for the R-MuLV samples. Absolute incorporation at 1 mM dNTP was 340 pmol/mg of protein per 30 min. The M7 samples were sedimented for 150 min at 40,000 rpm in the SW41 rotor through 15 to 30% sucrose gradients, and fractions were assayed for trichloroacetic acid-precipitable radioactivity. Activity in the top 25% of the gradients was not included in incorporation calculations. Absolute incorporation at 1 mM dNTP was 70 pmol/mg per 30 min. Results were unaffected by lowering R-MuLV and M7 concentration 5- to 10-fold. ELAV data are taken from Fig. 2A; absolute incorporation at 1 mM was 1,800 pmol/mg per 30 min. NT, Not tested.

FIG. 3. Time-dependent increase in size of cDNA. In three separate experiments, EIAV lot 877 was incubated in the standard endogenous reaction mixture containing 50 μ M dNTP and 30 μ M [3 H]dTTP (50 Ci/mm) at 41°C for 15 min, 1 h, or 2 h. After addition of SDS, reaction mixtures were centrifuged for 150 min at 40,000 rpm in the SW41 rotor through neutral 15 to 30% sucrose gradients made up in TNE and 0.01% SDS. Portions of the fractions were assayed for trichloroacetic acid-precipitable radioactivity, and peak fractions were then recentrifuged through alkaline sucrose gradients in the presence of a [14 C]DNA marker of average-size 400 nucleotides. Fractions were assayed for trichloroacetic acid-precipitable radioactivity. Arrows indicate the calculated position of DNA of about 8,000 nucleotides.

μ M dNTP, the largest cDNA is about 1,500 nucleotides; after 1 h, 5,800 nucleotides; and after 2 h, about 8,000 nucleotides. Beyond 2 h of incubation, there is no further increase in size.

Newly synthesized cDNA, even after incubation for 2 h or longer, exists in a broad range of sizes, from a few hundred to about 8,000 nucleotides. This can be seen by centrifuging the entire reaction product directly through an alkaline sucrose gradient (Fig. 4A). Again, the longest cDNA synthesized in 100 min was found to be about 8,000 bases, based on results of its cosedimentation with linear SV40 DNA (Fig. 4B). No increase in maximum size was observed in products of 3- or 4-h incubations, nor did the amount of long cDNA increase beyond 2 h of incubation, though total incorporation continued to rise gradually. By 4 h, the amount of long cDNA had actually declined slightly.

As the data in Fig. 4A make clear, the yield of 8,000-nucleotide cDNA is quite high. About 16% of the total counts per minute are in fractions 1 to 7, and about 26% are in fractions 1 to 8. Since at least half of the material in fraction 8 was about 8,000 nucleotides long (based on results of its cosedimentation with SV40 [14 C]DNA), the yield of maximum-size cDNA in this experiment is at least 20% of the total incorporation. This result is not atypical. In 10 such experiments performed over the course of several months, we observed the yield of 8,000-nucleotide-long cDNA to range from 13 to 23% of the total when the dNTP concentration in the reaction mix was 200 μ M.

Raising dNTP above 200 μ M did not increase either the size of the largest cDNA or its yield. A series of endogenous reaction mixtures, identical except for dNTP concentration, were incubated for 2 h, lysed with SDS, and centrifuged through alkaline sucrose gradients in the pres-

FIG. 4. Sedimentation of total reaction product through an alkaline sucrose gradient. A 60- μ l amount of EIAV lot 808 was incubated in 250 μ l of the standard reaction mixture containing 200 μ M dNTP and 48 μ M [3 H]dTTP (17 Ci/mm) for 100 min at 41°C. After bringing to 1% SDS, the entire mixture was applied to an alkaline sucrose gradient and centrifuged at 34,000 rpm at 10°C for 16 h. (A) Portions of the fractions (5 μ l each) were assayed for trichloroacetic acid-precipitable radioactivity. Judging from identical gradients spun at the same time, SV40 [14 C]DNA would appear in about fraction 9; (B) a mixture of a 10- μ l portion of fraction 7 and SV40 [14 C]DNA was recentrifuged through a second alkaline gradient, as above. Trichloroacetic acid-precipitable counts per minute were determined in all fractions. Calculated size of cDNA in the 3 H peak is 8,000 nucleotides.

ence of linear SV40 [14 C]DNA. The absolute amount of long cDNA synthesized in the presence of 50 or 500 μ M dNTP is the same (Fig. 5); this same amount is observed after incubation

FIG. 5. Effect of dNTP concentration on size of cDNA. A 2.5- μ l amount of EIAV lot 808 was incubated in 12.5 μ l of the standard reaction mixture containing 48 μ M [3 H]dTTP (17 Ci/mm) and either 50 μ M dNTP (●) or 500 μ M dNTP (○) for 150 min at 41°C. After bringing to 1% SDS, the samples were sedimented through alkaline sucrose gradients at 34,000 rpm at 10°C for 16 h. Trichloroacetic acid-precipitable counts per minute were determined for all fractions. SV40 [14 C]DNA was mixed with the 50 μ M dNTP sample before centrifugation; its sedimentation is indicated by the arrow.

in 1, 1.4, or 1.8 mM dNTP. Thus, the total incorporation stimulation that results from raising dNTP about 50 μ M (Fig. 2 and 5) appears to be due to increased synthesis of fragments that are only 400 to 1,500 nucleotides long. Judging from their inability to reassociate with viral RNA, these appear to be predominantly positive strands. The relative yield of long cDNA is therefore highest at 50 μ M dNTP, and, as in the experiment shown in Fig. 5, it may amount to nearly 30% of the total incorporation.

The lower the dNTP concentration below 50 μ M, the less 8,000-nucleotide-long cDNA is synthesized in 2 h. There is significant long cDNA synthesized even at 10 μ M dNTP, although it amounted to only about 30% of that made at 50 μ M dNTP (Fig. 6). In contrast to incubation in 50 or 200 μ M dNTP, in which the yield of long cDNA stops increasing after 2 h, extending the incubation time of these low dNTP reactions to 3 or 4 h increased the yield proportionately, up to the maximum observed at 2 h in 50 μ M dNTP. However, for a 2-h incubation, half-maximum yield was achieved at about 15 μ M dNTP.

Finally, actinomycin D at 100 μ g/ml significantly reduced the size of cDNA synthesized in 2 h. At 200 μ M dNTP, the average product was about 1,000 nucleotides long, and there was little or no material of 8,000 nucleotides. At 1 mM dNTP, the average size remained about the same, but about 5% of the total appeared to be about 8,000 long.

Long cDNA is complementary to viral RNA. To show that viral RNA was the template for synthesis of long cDNA, the latter was isolated from alkaline sucrose gradients and annealed with purified 70S EIAV RNA. It was found that essentially 100% of the cDNA became resistant to S_1 nuclease digestion after incubation to C:t 0.19, whereas cDNA alone, whether annealed or not, was at least 96% digestible (Table 2). It can be concluded that essentially all long cDNA is complementary to viral RNA, that long positive strands, if they exist, amount to less than 2% of the total, and that the length of a self-complementary hairpin region, if it exists, can be no longer than about 150 nucleotides.

DISCUSSION

The experiments reported here demonstrate the synthesis in high yield of cDNA of 8,000 nucleotides by EIAV. This long cDNA constituted nearly 30% of the total product made in 2 h in 50 μ M dNTP, a higher yield than has been reported heretofore for the endogenous reverse transcriptase reaction of any retrovirus. In previous studies with both avian and mouse viruses, full-length cDNA yields ranging from about 4 to

FIG. 6. Effect of dNTP concentration on the yield of long cDNA. All reaction mixtures, incubations, and centrifugations were as described for Fig. 5, except that dNTP varied as indicated. All alkaline sucrose gradient profiles resembled those in Fig. 5 in having a broad size distribution with a clearly defined peak at a DNA position of about 8,000 nucleotides. The absolute yield of long cDNA was calculated as the total counts per minute from the bottom of the gradient up to and including this peak; the results were then normalized to the absolute yield at 50 μ M dNTP. For comparison, we have included the data from Fig. 2A (●), showing the effect of dNTP concentration on total incorporation. Measuring incorporation after 2 h or (as shown here) after 30 min yielded the same curve.

TABLE 2. *S₁ nuclease sensitivity of long cDNA^a*

Expt no.	cDNA prep	Substrate	<i>S₁</i> nuclease	Trichloroacetic acid-precipitable cpm
1	1	cDNA, not annealed	—	2,065
		cDNA, not annealed	—	2,002
		cDNA, not annealed	+	69
		cDNA-EIAV RNA hybrid	+	2,153
2	1	cDNA, annealed	+	93
		cDNA, not annealed	—	2,188
		cDNA, not annealed	+	90
3	2	Boiled cDNA, not annealed	—	3,320
		Boiled cDNA, not annealed	+	125

^a Neutralized portions of maximum-size cDNA from alkaline sucrose gradients were incubated at 41°C for 1 h with or without *S₁* nuclease, as indicated above. Two samples in experiment 1 were first annealed with or without EIAV viral RNA (C:t 0.19). Trichloroacetic acid-precipitable counts per minute were measured.

15% have been reported (13, 14, 22, 27). The EIAV endogenous reaction is still considerably less efficient than the reconstituted avian myeloblastosis virus (AMV) system described by Myers et al. (17), in which full-size cDNA totaled 60 to 70% of the product.

The absolute amounts of both long cDNA and total cDNA synthesized by EIAV also compare favorably with other systems. After 2 h in 50 μ M dNTP, about 2.8 μ g of cDNA per mg of protein has been synthesized, up to 30% of which is 8,000-nucleotide cDNA. If the RNA content of the virion is 1 to 2% of the total mass, and protein is 60%, long cDNA yield amounts to 2 to 5% of input RNA. Total cDNA yield is, of course, considerably higher; at 1 mM dNTP, about 6.5 μ g of cDNA per mg of protein was synthesized in 6 h, a yield of 20 to 40% of input RNA. Similar yields have been observed at high nucleotide concentration in the endogenous reactions of M-MuLV (21, 23) and RSV (14). Junghans et al. (13), using RSV, and Myers et al. (17) and Darlix et al. (8), using reconstituted systems with AMV reverse transcriptase, have obtained even higher yields (>60%). In contrast, two other mammalian retroviruses that we have employed are much less efficient at synthesizing long cDNA in the endogenous reaction. R-MuLV gives a high yield of cDNA, but an insignificant amount of it has been >6,000 nucleotides. And, at best, M7 has produced (per milligram of protein) only 5% of the cDNA of a typical EIAV lot.

The elongation rate of cDNA strands by EIAV is also among the highest reported to date. A 5,800-nucleotide transcript was synthesized in 1 h, giving a polymerization rate of about 100

bases per min (Fig. 3). Another virus lot synthesized a 4,500-nucleotide-long transcript in 30 min, thus proceeding at about 150 bases per min. This is comparable to the rate of copying by purified avian reverse transcriptase of AMV RNA (2.6×10^6 dalton cDNA synthesized in 1 h, or at least 140 bases per minute) (17) or of homopolymeric templates or isolated mRNAs (80 to 400 bases per min) (5, 9, 25). It is significantly higher than the elongation rate of ASV cDNA in vivo (about 30 nucleotides per min) (26).

In the absence of actinomycin D, EIAV did not require high dNTP levels for either optimal incorporation rate or long cDNA synthesis. Raising three triphosphates from 10 to 100 μ M each (dTTP constant at 30 μ M) resulted in a 2.5-fold increase in incorporation (Fig. 2). Raising dNTP to 1 mM produced an additional 1.5-fold stimulation. Half-maximum incorporation occurred at 30 to 50 μ M dNTP (Fig. 2), which is slightly higher than the K_M values of 10 to 20 μ M reported for isolated RSV, AMV, and RLV reverse transcriptases (10, 12, 15). Much the same data have been reported by Junghans et al. (13) with RSV and by Darlix et al. (8) with a reconstituted system. In contrast, the effect of increasing dNTP was much more marked in the endogenous reactions of both R-MuLV and M7, in which half-maximal incorporation was not achieved until 150 to 200 μ M dNTP (Table 1).

Although incorporation continues to increase through 6 h of incubation and as dNTP is raised to about 1 mM, long cDNA synthesis is already maximal at 2 h in 50 μ M dNTP. Longer time or higher dNTP concentration had no effect on the absolute amount of 8,000-nucleotide cDNA synthesized. Recently, Buell et al. (5) also reported that the yield of full-size ovalbumin cDNA synthesized by AMV reverse transcriptase is maximal at 50 μ M dNTP, although total incorporation rises through 1 mM dNTP. Below 50 μ M dNTP, EIAV long cDNA increases with increasing dNTP. Half-maximal yield at 2 h is achieved at about 15 μ M dNTP, remarkably similar to the reported reverse transcriptase K_M 's cited above. It seems possible that the curve of total incorporation versus dNTP is made up of at least two separate reactions (synthesis of negative and positive strands, respectively) which differ in half-maxima. Some support for this view comes from our observation that the short cDNA fragments whose synthesis is stimulated at 500 μ M dNTP (Fig. 5) fail to hybridize with viral RNA, but will hybridize with long cDNA; they are thus positive strands. Similarly, we have observed that the higher the dNTP concentration, the greater the percentage of the

reaction product that exists as double-stranded DNA (N. R. Rice and L. Coggins, manuscript in preparation).

When the endogenous ELAV reaction is carried out in the presence of 100 μ g of actinomycin D per ml, elevated triphosphate levels are required both for high incorporation and for long cDNA synthesis. Raising dNTP from 200 μ M to 1 mM doubled incorporation (Fig. 1) and increased the yield of long cDNA from essentially 0 to about 5% of the total. Similar results have been reported for MuLV (4, 21).

We do not know that the long cDNA we observe in these experiments is genome length. Since the molecular weight of ELAV viral RNA, as determined by velocity sedimentation and by electrophoretic mobility in nondenaturing gels, has been reported to be 2.7 to 2.9×10^6 (7), a genome size of 8,000 bases is reasonable. Nevertheless, since we have not tested the infectivity of the long cDNA product, it is possible that it is less than full size. The possibility also exists that these transcripts are longer than full size. However, since they are 96% S_1 digestible, the length of a hairpin region could be at most about 150 nucleotides.

In summary, in vitro synthesis of cDNA by ELAV compares favorably in total yield, yield of long transcript, and elongation rate with the other two well-studied endogenous reaction systems, M-MuLV and ASV. ELAV is therefore a highly useful model with which to investigate problems common to all retroviruses. For example, studies of the physical organization of the ELAV genome are possible, given the high yield of long cDNA. In addition, we are pursuing the mechanism of synthesis of double-stranded cDNA in the endogenous reaction, since there are good indications with other viruses that this process is very similar to that observed in vivo (26). Finally, the ELAV reverse transcriptase itself may be of interest. We would like to know whether it has special characteristics that account for the activity and efficiency of the endogenous reaction and, if so, whether it could be used to generate long cDNA from other, less active viruses, particularly those of primates.

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