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Characterization of RNA Polymerase Products of Nebraska Calf Diarrhea Virus and SA11 Rotavirus

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The endogenous RNA polymerase of the calf rotavirus was shown to synthesize single-stranded RNA transcripts of one polarity which were identical in size to the denatured parental double-stranded RNA segments. The transcripts were notable in their absence of polyadenylate sequences. The polymerase activity associated with the cores of calf rotavirus was minimally altered by the reaction, since calf rotavirus cores could be reused after purification, whereas the RNA polymerase products appeared to be released from the virion.

Rotaviruses have been identified as the cause of acute gastroenteritis in numerous animal species as well as in infants and young children (5, 9). These viruses are classified in the Reoviridae family because they contain a segmented genome comprised of double-stranded (ds) RNA. Like the reoviruses, the human rotavirus, Nebraska calf diarrhea virus (NCDV), and the simian rotavirus (SA11) possess a magnesium-dependent RNA polymerase which synthesizes single-stranded RNA that is complementary to the viral genome (6, 12, 16, 17). Transcripts of both NCDV and SA11 seem to be synthesized from all their respective genome segments and are active in stimulating protein synthesis in a cell-free system (7, 16, 19). This work characterizes the transcripts of the NCDV and SA11 RNA polymerases by CsCl centrifugation and by electrophoresis under dissociating conditions in methylmercury-agarose gels in order to compare the size of the transcripts with that of the original viral genome. In addition, the transcripts were examined for the presence of polyadenylate sequences by affinity chromatography.

The product of the NCDV RNA polymerase reaction was released from virion cores produced by treatment with EDTA (8) (Fig. 1). The product of the reaction appeared at the top of the gradient, and labeling of the NCDV "core" antigen peak did not occur. In addition, in comparison with controls, the "core" particle density (specific gravity, 1.38) did not change after polymerase reaction. Thus, in undergoing the reaction, the native NCDV. ds RNA is apparently conserved and does not permanently combine with the new RNA product, since the newly formed RNA is found separate from the virion after centrifugation in a CsCl gradient.

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The NCDV and SA11 RNA polymerases appeared to function in an almost identical manner to that of the reovirus transcriptase, synthesizing complementary copies from only one strand of the ds genome (11). This was shown by demonstrating the inability of the transcripts to self-hybridize (Fig. 2). Polymerase products were prepared in vitro from NCDV cores and purified by a combination of ultracentrifugation and precipitation with 2 M LiCl (2) in order to eliminate any virion ds RNA which might be released attendant on breakdown of a small fraction of the cores. Unlabeled ds virion RNA was extracted from band-purified virus by digestion of the virus with proteinase K for 30 min in the presence of 1% sodium dodecyl sulfate. The [^{3}H]-uridine-labeled single-stranded product was hybridized both with itself and with dissociated (ds) viral RNA at 65°C (4). Self-annealing of 0.5 to 1 μg of labeled product resulted in background levels of RNase resistance (10%), implying that most of it remained unhybridized. Control HEp-2-cell rRNA prepared by *Tritirachium* alkaline proteinase also demonstrated a 10% level of RNase resistance. By contrast, annealing of the NCDV products with the 2 to 10 μg of parental ds genome rapidly produced nuclease-resistant hybrids. Experiments with SA11 (obtained from H. Malherbe [15]) yielded virtually identical results. The conditions for the self-annealing were such that 50% hybridization should have occurred at 24 h according to the C_0t value empirically calculated from the reannealing experiments. Thus, it appears that the NCDV and SA11 polymerases synthesize single-stranded RNA strands of only one polarity to the parental ds RNA template.

The virion-associated RNA polymerase of NCDV synthesized copies of all the genome segments (Fig. 3). Comparison of the size of the transcripts with the dissociated parental single-

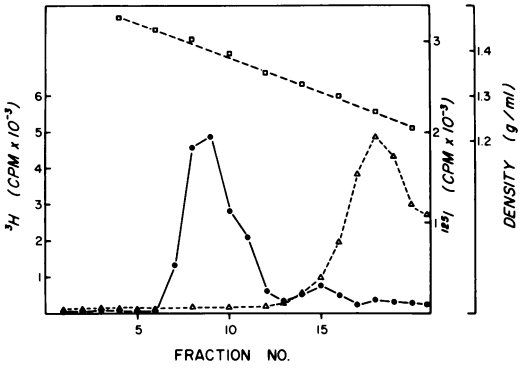

FIG. 1. *Release of the RNA polymerase product from the virion. The product of the RNA polymerase reaction was examined to determine whether it was released from the virion or sequestered within it. Also examined was the possibility of a shift in viral density after the polymerase reaction. An aliquot of purified NCDV "cores" was subjected to the standard RNA polymerase reaction for 2 h at 37°C. As controls, the following were used: one aliquot was incubated under standard conditions save for the addition of [^{3}H]UTP. Another aliquot was stored at 4°C during the incubation period and was not subjected to the polymerase reaction mixture. After 2 h, each aliquot was layered over a preformed CsCl gradient and centrifuged as described in the text. Fractions were collected and measured for refractive index (□), NCDV antigens (●), and radioactive product (△).*

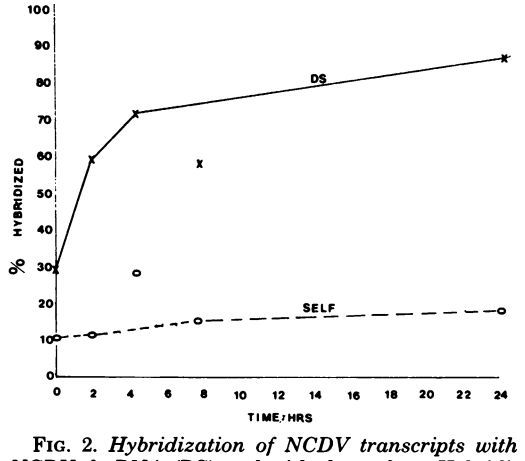

FIG. 2. *Hybridization of NCDV transcripts with NCDV ds RNA (DS) and with themselves. Hybridization was carried out as outlined in the text, and RNase resistance was assayed at selected times.*

stranded genome was done by electrophoresis on 2% agarose with 5 mM methylmercury (1) followed by fluorography (3). Figure 3A is a 4-h fluorograph of the transcripts, and Fig. 3B is a composite of a 4-h exposure of the smaller segments and a 72-h exposure of segment 1 of NDCV and segments 1 through 4 of SA11. As demonstrated in lanes 3_A and 3_B, the NCDV polymerase synthesized copies of all genomes, although segments 1, 2, and 4 seemed to be produced at a lower frequency. Similarly, the larger-molecular-weight transcripts of SA11 (lanes 2_A and 2_B) appeared to be synthesized in lesser amounts; however, as the composite fluorograph of the SA11 RNA transcripts demonstrated, all of the SA11 genome segments were also synthesized.

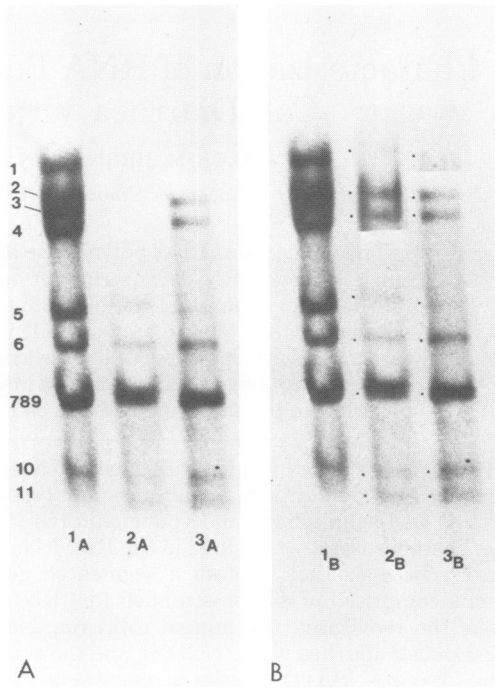

FIG. 3. *Methylmercury-agarose gel electrophoresis of NCDV and SA11 transcripts. NCDV and SA11 transcripts were synthesized with [^{3}H]UTP as a label. [^{3}H]uridine-labeled NCDV ds RNA was prepared as outlined in the text. These were electrophoresed in parallel on a 2% agarose gel containing 5 mM methylmercury, and fluorography was performed. (1) NCDV ds RNA; (2) SA11 transcripts; (3) NCDV transcripts. (A) Four-hour fluorograph; (B) composite of a 4-h exposure of the lower-molecular-weight segments and a 72-h fluorograph of segments 1 through 4 of SA11 and segment 1 of NCDV.*

Because of the decreased quantity of transcripts of genome segments 1, 2, and 4, we examined the rate of transcript synthesis as a function of time. Total RNA synthesis proceeded linearly with time as measured by trichloroacetic acid-precipitable counts. Synthesis of each of the size classes of transcripts was also linear as assessed by measuring the density of

each transcript's fluorographic image (data not shown).

The molecular weights of the rotavirus transcripts are shown in Table 1. Since our system did not resolve genome segments 2 and 3 or 7, 8, and 9, they are expressed together. *Escherichia coli* and HEp-2 rRNA standards were used (21). These determinations, the first done under denaturing conditions, generally agree with those previously reported (13), although our molecular weights are slightly smaller for NCDV and slightly larger for SA11.

Quantitative analysis of the fluorographs of the rotavirus transcripts substantiated that varying amounts of each genome segment were synthesized (Table 2). Transcripts 1, 2, 3, and 5 were apparently not synthesized as rapidly as the other segments, since there was no evidence of partially digested transcripts or low-molecular-weight degradation in the fluorographs. This situation differs from that which is seen in reoviruses, where the rates of synthesis of the RNAs of each of the genome segments, as measured in bases/time, are essentially equivalent (18).

Finally, the transcripts were examined by chromatography on an oligodeoxythymidylate column (Collaborative Research) for the presence of 3'-terminal polyadenylate sequences, since most mammalian mRNA's and many viral RNAs possess them (10, 14). NCDV transcripts did not bind to the oligodeoxythymidylate column, in contrast to polyadenylate-containing controls (Rous sarcoma virus RNA and [^{3}H]-polyadenylate) (Fig. 4). Thus, the NCDV transcripts do not appear to have any detectable polyadenylate sequences.

It appears that the RNA-dependent RNA polymerase of the rotaviruses has many of the same characteristics as the reovirus transcriptase. Both produce single-stranded transcripts which are of one polarity, do not contain poly-

TABLE 1. *Molecular weights of rotavirus transcripts*[a]

Genome segment	Mol. wt. ($\times 10^6$)	
	NCDV	SA11
1	1.4 ± 0.2	1.7 ± 0.2
2, 3	1.2 ± 0.1	1.3 ± 0.2
4	1.1 ± 0.1	1.2 ± 0.1
5	0.8 ± 0.1	0.88 ± 0.10
6	0.66 ± 0.02	0.69 ± 0.01
7, 8, 9	0.52–0.54 ± 0.01	0.55–0.57 ± 0.02
10	0.42 ± 0.03	0.43 ± 0.05
11	0.39 ± 0.02	0.40 ± 0.04

[a] Rotavirus polymerase products were electrophoresed on CH_3HgOH gels in parallel with 16, 18, 23, and 28S rRNA. The molecular weights of the RNA polymerase products were calculated by comparing their migration distances with those of known rRNA standards. Migration distance was proportional to 1/log molecular weight.

TABLE 2. *Production of rotavirus RNA transcripts*

Gene segment	[^{3}H]UTP-labeled NCDV transcript (cpm)[a]	[^{3}H]uridine-labeled NCDV RNA (cpm)[a]	Transcript ratio[b]	Relative RNA synthesis rate[c]
1	55	1,097	0.06	0.16
2, 3	570	3,995	0.18	0.41
4	610	1,370	0.56	0.86
5	345	1,840	0.24	0.36
6	882	1,340	0.83	1.03
7, 8, 9	1,971	2,500	1.00	1.00
10	547	640	1.08	0.86
11	490	515	1.21	0.88

[a] Rotavirus polymerase products were electrophoresed in parallel with ^{3}H-labeled purified NCDV ds RNA, and fluorography was performed to locate the RNA. The transcripts or ds RNA were excised from the dried gel, solubilized, and counted in a scintillation counter.

[b] Transcript ratio = transcript counts per minute/virus counts per minute, assuming that gene 789 transcript ^{3}H counts per minute/gene 789 virus ^{3}H counts per minute = 1.

[c] Relative RNA synthesis rats (bases/time) = transcript ratio × (molecular weight × 10^{-6}), assuming that the relative RNA synthesis rate of genome segment 789 = 1.0.

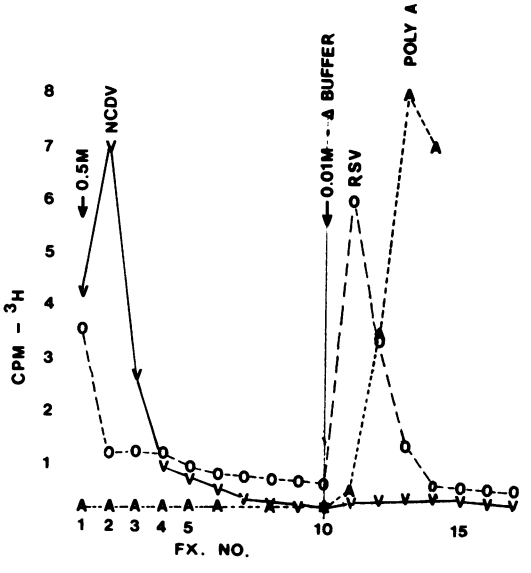

FIG. 4. *Oligodeoxythymidylate chromatograph of NCDV transcripts. NCDV transcripts, Rous sarcoma virus RNA, and commercial [^{3}H]polyadenylate were chromatographed on oligodeoxythymidylate-cellulose, and fractions (FX.) collected and counted in a scintillation counter as described in the text. (V) NCDV transcripts; (○) Rous sarcoma virus RNA; (A) [^{3}H]polyadenylate.*

adenylate sequences (20), and stimulate in vitro protein synthesis. The major difference seems to be in the efficiency of transcription of the higher-molecular-weight species, but then this differs between the reoviruses and the rotavirus as well as among the rotaviruses themselves.

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