crease rapidly, not being detectable on day 8. This characteristic pattern was noted whether results were calculated as activity per spore or as activity per milligram of protein. In both instances enzyme activity was barely detected in the dry spores. During the first 24 hours of germination no change was noted in the level of malate synthase although isocitrate lyase activity increased. For the next 48 to 96 hours activity of both enzymes increased. Differences in the temporal pattern of increase in the activity of malate synthase and isocitrate lyase were observed. However, both enzymes displayed significant increases from day 2 to day 5 of spore germination. A rapid decline in activity took place between day 5 and day 7. These data indicate that the activity of glyoxylate cycle enzymes increases during the very early stages of spore germination before the spore coat ruptures and the rhizoid emerges. More importantly, enzyme activity is at its highest during the time lipids are being rapidly degraded. As the storage material is metabolized enzyme activity declines.

The correlation between lipid catabolism and glyoxylate enzyme activity is common to germinating "fatty" seeds. There the conversion of fat to carbohydrate is enhanced by increases in activity of glyoxylate cycle enzymes. By the time enzyme activity disappears, lipid reserves have been largely depleted and photosynthetic activity in the seedling is capable of providing a continuing source of energy and materials for subsequent growth.

In our study of germinating fern spores, isocitrate lyase and malate synthase activity could not be detected after 7 days of germination. By that time the prothallial cell had protruded from the spore and appeared to be photosynthetically competent. Mature chloroplasts with compact grana have been described in growing filaments of *D. filix-mas* and *P. vulgare*. Our evidence indicates that in fern spores—as in "fatty" seeds of higher plants—the glyoxylate cycle functions in the early stages of development. The decline of enzyme activity is coincident with the establishment of the prothallus as a free-living, autotrophic plant.

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Circular Forms of DNA Synthesized by Rous Sarcoma Virus in vitro

Abstract. *Electron microscopic analysis of the DNA product synthesized by detergent-disrupted preparations of Rous sarcoma virus in vitro revealed the presence of several interesting molecular forms including covalently closed circular DNA. The identification of such circular DNA indicates that virions of retroviruses contain all the components necessary to facilitate the complete synthesis of mature forms of viral DNA and therefore provide a useful system to delineate the molecular mechanisms involved in their synthesis.*

Information concerning the structure and mechanism of synthesis of retrovirus DNA has been obtained from studies conducted both in vitro and in vivo (*1*). Studies in vivo have contributed predominantly to a description of the structure of the intracellular forms of retrovirus DNA preceding integration, presumably because of logistical difficulties in obtaining sufficient amounts of viral DNA precursors for analysis. Studies in vitro, however, have provided considerable information that has guided our current thinking regarding the details of retrovirus DNA synthesis. For example, information concerning the nature of the primer molecule (*2–5*), site of initiation of DNA synthesis (*6–8*), genomic terminal redundancy (*8–12*), hydrolysis by ribonuclease H (*13, 14*), and elongation of 5′ initiated DNA at the 3′ end of the viral genome (*15, 16*) was obtained by studies in vitro in which investigators used detergent-disrupted preparations of Rous sarcoma virus (RSV) as well as reconstructed reactions containing purified retroviral genomic RNA and reverse transcriptase.

Recently, the DNA product synthesized by detergent-disrupted preparations of both murine (*17*) and avian retroviruses (*18*) has been demonstrated to exhibit infectivity. Studies on the structure of the DNA molecules present in the infectious preparations indicated the presence of molecules analogous to the linear forms identified in the cytoplasm of cells shortly after infection (*19, 20*). These molecules were double-stranded in nature, consisting of genome-length minus-strand and subgenomic fragments of plus-strand DNA, and represented virus-specific DNA molecules similar in structure to linear duplex DNA known to serve as the immediate precursor of the mature, covalently closed circular forms of retrovirus DNA identified in virus-infected cells (*17–22*). These findings indicated that the retrovirus reverse transcriptase was capable of transcribing the single-stranded viral RNA genome into the precursor of the mature, covalently closed circular forms of retrovirus DNA in vitro. In this report we demonstrate the presence of circular mature forms of retrovirus DNA in the DNA product synthesized by detergent-disrupted preparations of the avian retrovirus RSV in vitro, thereby suggesting that the virus particle contains all the components necessary to facilitate the synthesis of covalently closed circular DNA.

Viral-specific DNA was synthesized by detergent-disrupted preparations of RSV in vitro under enzymatic conditions that facilitate genome-length and infectious DNA synthesis as previously described (*18*). Single-stranded DNA molecules of approximately 7,500 to 10,000 nucleotides in length were isolated by

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rate-zonal sedimentation in alkaline sucrose. The viral-specific nature of these molecules was established by their ability to hybridize to RSV 70S RNA (*18*). When grids containing these viral-specific DNA preparations were analyzed by electron microscopy, several distinct species of DNA molecules were routinely observed (Fig. 1) (*23*). The most abundant species was single-stranded DNA of varying lengths, including molecules of approximately 7,500 and 10,000 nucleotides in length, representing the size of the genome from transformation-defective and transforming RSV, respectively, both of which are present in our purified virus preparations (Fig. 1A). Another group of molecules appeared circular and single-stranded in structure and were also found in both size classes (Fig. 1B). The curious feature of these circular molecules was that they contained a structural peculiarity resembling rabbit ears. The presence of such structures on these DNA molecules results from hydrogen bonding of two complementary or partially complementary regions of the molecules present at distances reflected in the size of the circles. This interpretation was supported by the fact that such structures disappeared from the preparations when they were spread under more stringent denaturing conditions (for example, with 80 percent formamide). The rabbit-ear structures themselves are reminiscent of panhandle structures, suggesting that inverted complementary repeated sequences are involved in their formation or maintenance, or both. A second form of circular DNA was also identified in these preparations (Fig. 1C). These circular molecules appeared single-stranded in nature in the electron microscope and, unlike the rabbit-ear-containing circular molecules described above, did not contain detectable interruptions, suggesting that they were covalently closed DNA structures.

In addition to single-stranded circular structures, molecules appearing double-stranded in nature were also observed in preparations of DNA synthesized in vitro by RSV (Fig. 2A). Direct proof of the nature of these molecules was obtained by subjecting the DNA preparations synthesized in vitro to equilibrium density centrifugation in CsCl containing propidium diiodide (*24*) and examining the DNA banding in the region of covalently closed circular molecules by electron microscopy. As shown in Fig. 2, B and C, molecules banding in this region of the CsCl-propidium diiodide gradients exhibited the structure characteristic of covalently closed circular DNA. Although slight variations in the size of these circular DNA's can be observed, these differences reflect the differences between nondefective and transformation-defective strains of RSV, both of which are present in our purified virus preparations. As was the case for the single-stranded circular molecules, these double-stranded structures were not detected when equivalent amounts of RSV were extracted under identical conditions in the absence of DNA synthesis. Furthermore, our contention that the single- and double-stranded covalently closed circular DNA molecules are indeed a product of our DNA synthetic

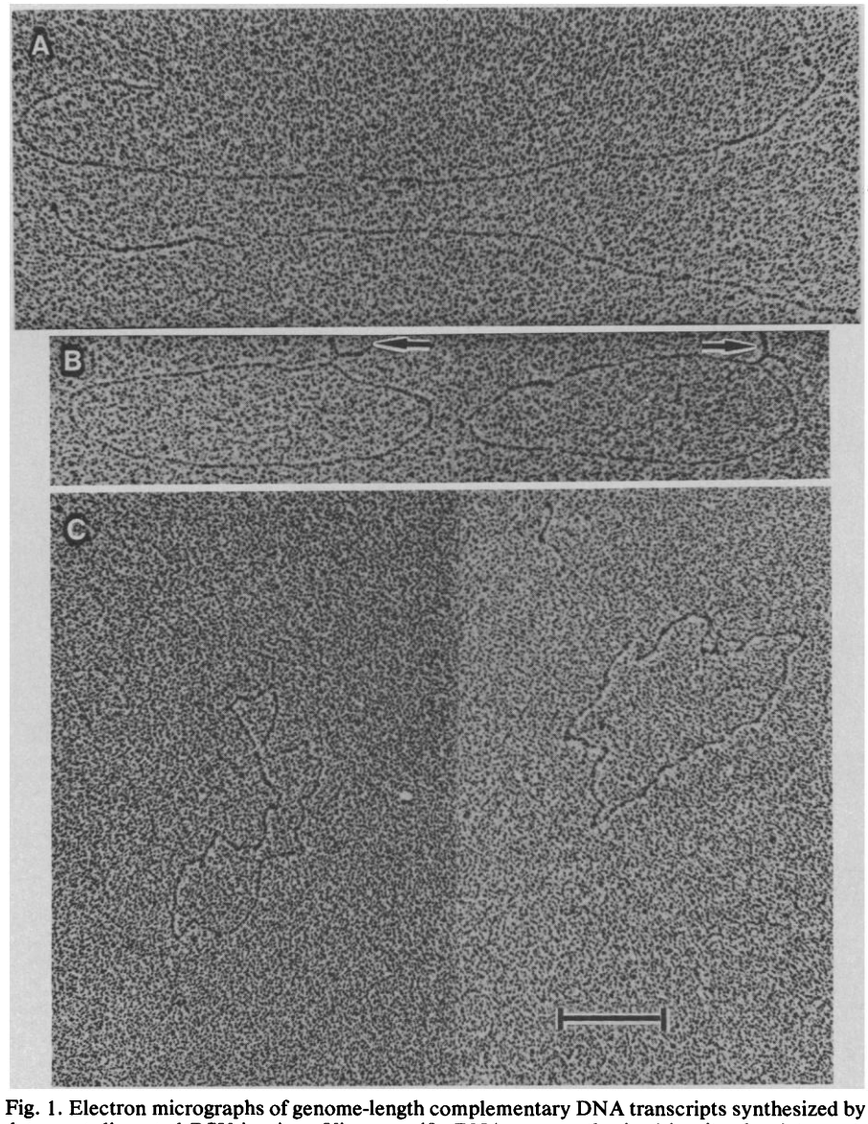

Fig. 1. Electron micrographs of genome-length complementary DNA transcripts synthesized by detergent-disrupted RSV in vitro. Virus-specific DNA was synthesized in vitro by detergent-disrupted RSV virions according to the method of Clayman *et al.* (*18*). The DNA product synthesized in vitro was then simultaneously freed of viral RNA and denatured by incubation in 0.3*N* NaOH for 4 hours at 37°C and subjected to rate-zonal sedimentation in alkaline sucrose as previously described (*18*). Alkaline sucrose gradient fractions containing DNA transcripts of approximately 7,500 to 10,000 nucleotides in length were then pooled, neutralized, and precipitated with ethanol. The genome-length DNA transcripts pooled from this region of the gradient have previously been demonstrated to be single-stranded in nature, of the minus-polarity, and to represent a complete, uniform transcript of the viral genomic RNA (*18*). Approximately 2 to 5 ng of DNA was dissolved in 0.01*M* tris-HCl, *p*H 8.5, 0.01*M* EDTA, and 40 to 45 percent formamide in the presence of 0.06 mg of cytochrome *c* per milliliter. The samples were then spread on water and after 1 minute were picked up on freshly prepared Parlodion-coated grids (3.5 percent Parlodion in amylacetate). The electron microscope was calibrated from micrographs of a carbon grating replica (54,800 lines per inch). Three distinct structural forms of genome-length DNA were observed: (*A*) single-stranded linear (7.5 to 9.5 kilobase pairs ± 0.5), (*B*) single-stranded circles with a rabbit-ear-like structure (5 to 20 percent of the total), denoted by the arrows (8.0 kb ± 0.5), and (C) covalently closed single-stranded circular forms (8.0 kb ± 0.5). The DNA molecules observed were photographed and the contour lengths measured. Polyoma DNA (*26*) was included as an internal length standard. The bar represents a length equivalent to 1 kb.

reactions in vitro is supported by the radioactive labeling of these molecules in vitro during enzymatic synthesis. Moreover, since the only covalently closed circular DNA detected in the cytoplasm of chick embryo fibroblasts is mitochondrial DNA, which is twice the size of the molecules we observed, we do not believe that our circular molecules represent contaminating circular DNA molecules of cellular origin. However, confirmation of this supposition must await the establishment of reaction conditions that facilitate the synthesis of sufficient quantities of these structures to perform restriction endonuclease mapping studies.

In this report we document the synthesis of mature forms of viral DNA by detergent-disrupted RSV virions under enzymatic conditions that facilitate the synthesis of both infectious and genome-length DNA in vitro. These findings indicate that virions of RSV contain all of the components necessary to synthesize covalently closed circular forms of viral DNA and therefore represent a much simpler system than infected cell cultures to delineate the precise steps in retrovirus proviral DNA synthesis and the possible involvement of virion components in addition to the viral reverse transcriptase in this process. It will be of interest to determine precisely which of the functions in the conversion of single-stranded genomic RNA to covalently closed form I DNA can be ascribed to the reverse transcriptase molecule itself. With regard to this issue we have recently identified a nicking-ligating enzyme contained within purified preparations of RSV (*25*), suggesting that at least one component distinct from the reverse transcriptase may participate in the synthesis of covalently closed circular viral DNA.

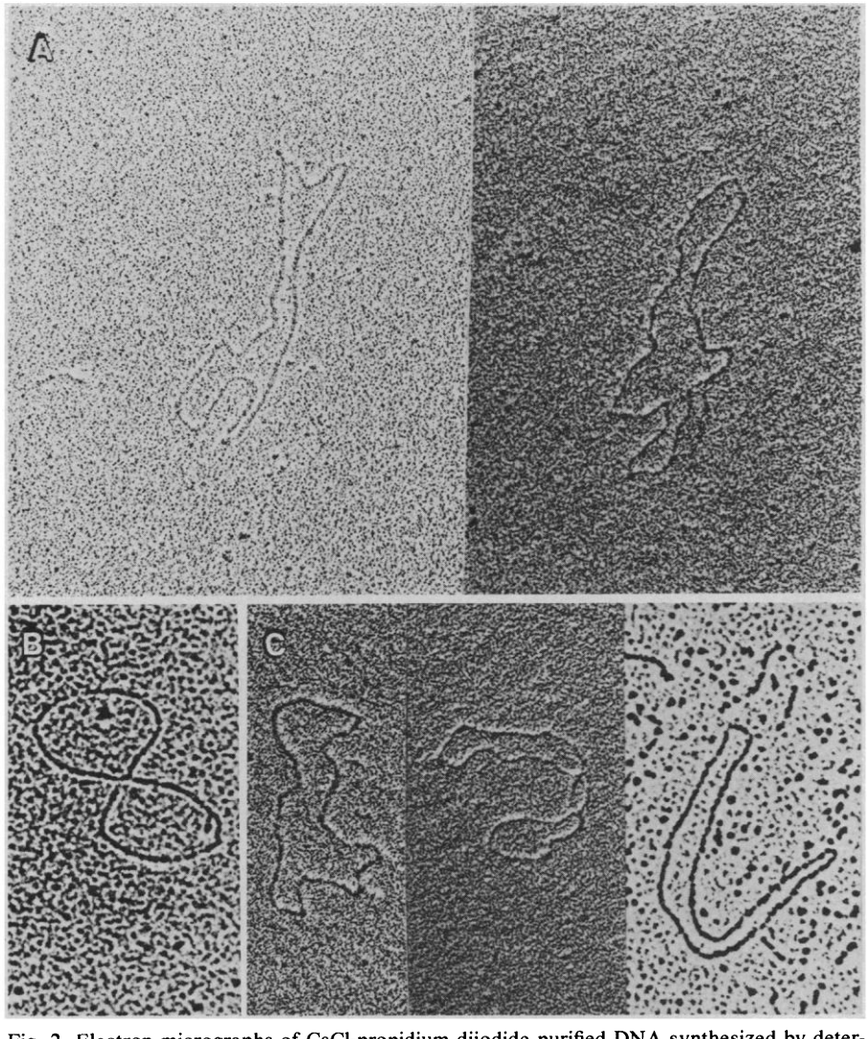

Fig. 2. Electron micrographs of CsCl-propidium diiodide purified DNA synthesized by detergent-disrupted RSV in vitro. Virus-specific DNA was synthesized by detergent-disrupted preparations of RSV according to the method of Clayman *et al.* (*18*). Endogenous proteins and RNA were removed by proteinase and ribonuclease treatments, respectively, as previously described (*18*). DNA samples were then dissolved in 5.3 ml of 0.02*M* tris-HCl, *p*H 7.4, and 0.01*M* EDTA to which 5.63 g of CsCl (optical grade) and 1.2 ml of propidium diiodide (2 mg/ml) were added to give a final density of 1.532 g/ml. Gradients were centrifuged for 60 to 70 hours at 33,000 rev/min at 20°C in a type-40 fixed angle rotor. DNA from the colicinogenic plasmid E1 (prepared by J. Weis of this laboratory) was centrifuged simultaneously in a parallel gradient and served as a marker for covalently closed circular DNA. Fractions within the region of the gradient containing covalently closed circular DNA were pooled, extracted five times with CsCl-saturated isopropyl alcohol, and subsequently dialyzed against 0.01*M* tris-HCl, *p*H 7.4, 0.01*M* NaCl, and 0.001*M* EDTA at 4°C for 36 hours. The DNA was then ethanol-precipitated and prepared for electron microscopy as described in the legend for Fig. 1. (A) Double-stranded appearing circular DNA molecules (9.5 kb ± 0.5). (B) and (C) DNA molecules obtained from the covalently closed circular DNA region of the CsCl-propidium diiodide density gradients (7.0 to 9.5 kb ± 0.5).

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