The Kinetics of Synthesis of Infectious Unintegrated Virus DNA in Rous Sarcoma Virus Infected Chicken Cells

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SUMMARY

Infectious virus nucleic acids were extracted from the Hirt supernatant of chicken embryo fibroblasts infected for different times with the Schmidt–Ruppin strain of Rous sarcoma virus (RSV). Infectious DNA and DNA–RNA hybrid molecules could be recovered from 6 h after infection in experiments using $1.5 \times 10^9$ infected cells. Only small amounts of infectious virus DNA could be purified 6 h after infection whereas at 24 h approximately one infectious DNA molecule could be recovered for each input virus infectious unit. At 24 h, both infectious supercoiled and non-supercoiled molecules were found. The specific infectivity of the supercoiled fraction was less than that of the non-supercoiled fraction. Infectious supercoiled DNA could be recovered from 16 h after infection. Evidence is presented that both forms of unintegrated virus DNA may rest unintegrated for at least 8 days in the cell, though chronically infected cells were shown to contain less than one unintegrated molecule per $10^5$ to $10^8$ cells.

Type C retroviruses synthesize, in infected cells, a DNA copy of virus RNA. This DNA may be found in a linear or closed circular conformation (see Weinberg, 1977, for review). Both molecular forms are infectious (Smotkin et al. 1975; Guntaka et al. 1976; Fritsch & Temin, 1977) and are considered to be precursors of the integrated provirus. In avian sarcoma virus-infected quail cells both forms persist for at least 25 days post-infection (Guntaka et al. 1976). The technique of molecular hybridization used here cannot distinguish between biologically active and inactive molecules. More recently, Fritsch & Temin (1977) reported the kinetics of formation of infectious DNA of a lytic avian virus. Unintegrated DNA of this virus was found in chicken cells as late as 14 days post-infection. The kinetics of synthesis of the two forms of unintegrated virus DNA was not, however, discussed. In this paper we describe our results concerning the kinetics of synthesis of supercoiled and non-supercoiled virus DNA in Rous sarcoma virus (RSV)-infected chicken cells. In addition we provide evidence suggesting that a minor fraction of infectious molecules synthesized early in virus-infected cells is composed of DNA–RNA hybrids. A preliminary account of this work has been presented elsewhere (Hill et al. 1978).

Chicken embryo fibroblasts (CEF) were obtained from 11-day-old embryos of brown Leghorns, phenotype C/E, positive for chick helper factor (Murphy, 1977). Cells were grown in modified Eagle’s medium (Macpherson & Stoker, 1962) supplemented with 10% (v/v) tryptose phosphate broth (TPB) and 5% heated calf serum unless otherwise stated. The Schmidt–Ruppin strain of RSV, subgroup D (SR-D) No. 304 and its transformation defective derivative (td) No. 300 were isolated following transfection of chicken cells with a DNA of a focus-cloned SR-D as described elsewhere (Hillova et al. 1974). The technique for the preparation of the virus stocks has been described previously (Ono et al. 1976). The titre of
transforming virus was determined by focus-formation assay and of non-transforming virus by end-point dilution.

Secondary cultures of CEF were infected 24 h after seeding with SR-D at a multiplicity of infection (m.o.i.) of 1 to 5 focus-forming units (f.f.u.)/cell and with the tdSR-D at a m.o.i. of 1 to 5 infectious units (i.u.)/cell in the presence of 2 μg/ml Polybrene. At different times after infection the cells were lysed and subjected to Hirt's fractionation procedure (Hirt, 1967). Nucleic acids were extracted from the Hirt supernatant as described (Hill et al. 1978). This extraction included two protein removal steps using phenol and chloroform-isoamylalcohol and, when stated, treatment with RNase A (Worthington, 50 μg/ml, pre-heated at 80 °C for 10 min) in 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0 (SSC) at 37 °C for 1 h. Purified nucleic acids were precipitated with ethanol and stored at −20 °C. Contamination of the Hirt supernatant fraction by chromosomal DNA was estimated to be less than 5% of the total cell DNA content. Infectivity of this contaminating chromosomal DNA during the first 24 h post infection was found to be negligible (results in preparation).

The transfection technique has been extensively described previously (Hill & Hillova, 1976). The calcium phosphate technique of Graham & van der Eb (1973) was used as described by Hillova et al. (1975). DNA samples were diluted 10× serially in HEPES buffered saline and the final DNA concentration was adjusted to 10 μg/ml by the addition of rat thymus DNA. Five cultures were treated with DNA at each dilution. After the DNA treatment the cells were subcultured three times during 3 weeks and examined both for foci of transformed cells and by reverse transcriptase assay (Hillova et al. 1976) for non-transforming virus. The specific infectivity, in terms of i.u. per 10⁶ infected cells, was determined at the end-point dilution from the fraction of virus positive cultures (see Hill & Hillova, 1976). In experiments using sarcoma virus DNA, transforming and non-transforming viruses are recovered at approximately equal frequency (Hillova et al. 1974) at the end-point dilution.

For nucleic acid hybridization, the DNA, in 0.1× SSC, was denatured with 0.3 M-NaOH, heated at 80 °C for 2 h, cooled, neutralized with 2 M-HCl, 1 M-tris, and precipitated with 2.5 vol. ethanol at −20 °C. The DNA precipitate was then spun down by centrifugation and dissolved in 50 μl of 0.6 M-NaCl, 0.02 M-tris-HCl (pH 7.5), 0.01 M-EDTA containing 2000 ct/min of ¹²⁵I-labelled PR-RSV-B genomic RNA (sp. act. 20 to 80×10⁶ ct/min/μg) prepared according to Commerford (1971) with modifications (Woo et al. 1975). Hybridization was carried out at 68 °C for 72 h in a sealed 50 μl Corning micropipette. The samples were then mixed with 0.5 ml of 2× SSC solution containing 100 μg/ml RNase A (Worthington, pre-heated as above), incubated at 37 °C for 60 min, precipitated with 10% TCA at 0 °C, and the amount of acid-insoluble ¹²⁵I-RNA–DNA hybrids (resistant to RNase digestion) measured in a Packard gamma scintillation spectrometer, Model 578. Background counts (approx. 60 ct/min) determined in control samples containing ¹²⁵I-RNA and carrier DNA alone were subtracted.

The first experiments were based on the suggestion that the first step in the synthesis of an infectious virus DNA leads to an RNA–DNA hybrid molecule composed of virus RNA and complementary DNA (Junghans et al. 1977; see also Taylor, 1977, for review). Such hybrids and RNA–DNA covalent hybrids have been found by other authors at 1 to 2 h after infection (Sveda et al. 1974; 1976; Takano & Hatanaka, 1975; Leis et al. 1975). We have therefore examined RSV-infected chicken embryo fibroblasts for infectious hybrid molecules at different times after infection. The deproteinized Hirt supernatant (without RNase step) was centrifuged, together with radioactively labelled ribosomal normal rat kidney (NRK) cell RNA and mouse cell DNA density markers, in a Cs₂SO₄ gradient prepared from 10 ml
Table 1. Infectivity of the nucleic acids purified from the Hirt supernatant of chicken embryo fibroblasts infected by RSV and centrifuged to equilibrium in caesium sulphate gradients*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Number of infected cells</th>
<th>Time after infection (h)</th>
<th>Number of infectious units in the Hirt supernatant† (I.U./10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>td SR-D</td>
<td>5 × 10⁷</td>
<td>3</td>
<td>&lt; 0.02, &lt; 0.02, &lt; 0.02</td>
</tr>
<tr>
<td>td SR-D</td>
<td>5 × 10⁷</td>
<td>6</td>
<td>&lt; 0.02, &lt; 0.02, &lt; 0.02</td>
</tr>
<tr>
<td>td SR-D</td>
<td>1.5 × 10⁸</td>
<td>6</td>
<td>&lt; 0.002, 0.006, &lt; 0.002</td>
</tr>
<tr>
<td>td SR-D</td>
<td>1.5 × 10⁹</td>
<td>6</td>
<td>0.01, &lt; 0.002, &lt; 0.002</td>
</tr>
<tr>
<td>td SR-D</td>
<td>1.5 × 10⁹</td>
<td>24</td>
<td>&gt; 1, 0.01, &lt; 0.002</td>
</tr>
<tr>
<td>td SR-D</td>
<td>1.5 × 10⁹</td>
<td>6</td>
<td>0.05, 0.002, &lt; 0.002</td>
</tr>
<tr>
<td>td SR-D</td>
<td>1.5 × 10⁹</td>
<td>24</td>
<td>5.2, 0.016, &lt; 0.002</td>
</tr>
</tbody>
</table>

* The nucleic acids were extracted from the Hirt supernatant of CEF infected by RSV and centrifuged to equilibrium in a Cs₂SO₄ gradient. The material present at the densities of references (DNA and RNA) and also at the intermediate density (DNA–RNA hybrid) was pooled and dialysed against 0.1 × SSC for 48 h at 4 °C and then against the buffer HEPES-saline for 24 h. The infectivity in the three samples was titrated by transfection to end-point dilution.

† In these experiments the recovery of infectious DNA from the Hirt supernatant was considered to be 100%.
‡ No infectivity was recovered. In these cases, therefore, the infectivity was less than the value given after the symbol (<).
§ The symbol (>) indicates that all DNA treated cultures were positive at the dilution given and that the titration was not continued as far as the end-point dilution.

It was not possible to recover infectious unintegrated DNA from small amounts (5 × 10⁷) of infected cells during the first 6 h of infection. Using 1.5 × 10⁹ infected cells, infectious virus nucleic acids can be found from 6 h after infection, for both the td and the sarcoma virus. At this time from 0 to 75 I.U. could be recovered in the DNA fraction and from 0 to 9 I.U. in the hybrid fraction. Twenty-four hours after infection much larger amounts of infectious unintegrated DNA can be recovered from the Hirt supernatant. More than 1500 I.U. of td virus DNA and 7800 I.U. of sarcoma virus DNA were recovered. The hybrid fraction was also infectious 24 h after infection and 15 and 24 I.U. were recovered for the td and sarcoma virus, respectively.

The infectivity at the DNA–RNA position increases only two or more times for the td virus and eight times for the sarcoma virus between 6 and 24 h, whereas the infectivity at the DNA position increases at least 100 times for both viruses. If the infectivity found in the hybrid fraction were due to contamination of the DNA peak we would expect the infectivity recovered in the two fractions to increase in parallel. The fact that they do not increase in proportion and the fact that in one experiment infectious hybrids were found in a gradient in which the DNA fraction was uninfected provide evidence for a true infectious DNA–RNA hybrid molecule. Further experiments are necessary to confirm this point.

Fractionation of supercoiled DNA was performed by equilibrium density gradient centrifugation in caesium chloride-ethidium bromide (CsCl-EthBr) gradients, as described in the legend of Fig. 1(a). Virus DNA sequences hybridizing to a 125I-RNA probe were found.
in two peaks corresponding to supercoiled and non-supercoiled DNAs. Pooled fractions corresponding to these two peaks both contained infectious virus DNA, though because the infectivity at the position of supercoiled DNA was less (see below) than that of the non-supercoiled DNA (Fig. 1b), it was not a priori certain that the supercoiled DNA was infectious; contaminating non-supercoiled DNA could explain the infectivity found at the position of supercoiled DNA. Proof that two separate infectious species of virus DNA exist was provided by re-banding in a second CsCl-EthBr gradient and titrating the infectivity of each fraction. Such a gradient is shown in Fig. 1(a). Two peaks of infectious DNA are clearly seen corresponding to the two peaks of virus sequences detected with an \(^{125}\)I-RNA probe. Both peaks were found at densities slightly heavier than the corresponding form I SV40 and mouse cell DNAs. This is explained by the difference in G+C content between RSV (51\%\,; Rosenthal & Zamecnik, 1973) and mouse cell (40\%) and SV40 (41\%) DNAs (Sober, 1970).
It should also be noted that the supercoiled DNA is less infectious than the non-supercoiled DNA; for the same quantity of virus sequences detected by hybridization in Fig. 1(a) there are 3.5 times less i.u. In view of the results of other authors (Guntaka et al. 1976) this may be explained by the presence of uninfectious defective circular molecules in the DNA preparation.

The kinetics of synthesis of the two forms of infectious td virus DNA are shown in Fig. 1(b). Infectious non-supercoiled DNA is first found 6 h after infection. The amount of infectious non-supercoiled DNA increases rapidly to a maximum between 24 and 48 h after infection. This result, which has been amply confirmed in numerous experiments and for other viruses including RAV-1 and SR-E after infection of chick and quail cells respectively (results not shown), differs from those obtained using metabolic inhibitors and molecular hybridization, which suggest that virus DNA synthesis is complete from 6 to 8 h after infection (Varmus et al. 1974; see also Weinberg, 1977, for references). Though this result may be significant, it may just reflect the difference in virus and host cell, or the fact that in our experiments the cells were infected in a semi-confluent state a long time (24 h) after seeding.

If the specific infectivity of unintegrated virus DNA is the same as that of integrated duplex DNA (Hill & Hilleva, 1976) it may be estimated that at 24 h there are from 0.5 to 5 infectious non-supercoiled DNA molecules per cell. This corresponds to the multiplicity of infection which was from 1 to 5 f.f.u./cell. Our results therefore complement those of others (Khoury & Hanafusa, 1976) who obtained much larger amounts of virus DNA; up to 200 molecules per cell. These authors used the molecular hybridization technique which does not distinguish between defective and complete virus DNA molecules. Such results may witness the presence of defective DNAs (synthesized perhaps from defective virions always present in the virus stocks) that would not be detected in our assay.

Infectious supercoiled DNA was at first detected 16 h after infection with the td virus. The quantities of this form of virus DNA were maximum also between 24 and 48 h after infection. Approximately 8, 6 and 25% of the infectious DNA molecules in the Hirt supernatant have a supercoiled conformation at 1, 2 and 10 days after infection.

Fig. 1(b) also shows that an infectious unintegrated virus DNA can be recovered 10 days after infection by the tdSR-D virus at a multiplicity of infection of from 1 to 5 i.u./cell. Interference tests have shown that under identical conditions to those used for the preparation of unintegrated DNA, td virus-infected cells are completely resistant to re-infection by a virus of the same subgroup 48 h after infection (N. S. Stedman, unpublished data). Hence the DNA recovered at 10 days cannot be a product of re-infecting virus. It may either be a product of reverse transcriptase and virus RNA in the infected cell as suggested by Varmus & Shank (1976) or have remained unintegrated in the cell for at least 8 days. We favour this second hypothesis for two reasons. Unintegrated virus DNA cannot be recovered from chronically infected cells. We have tested both chicken cells infected with tdSR-D and subcultured for 4 weeks and quail cells infected for 6 months with SR-E virus (results not shown) for infectious unintegrated DNA. The sensitivity of the transfection assay allows us to confirm that less than 1 cell in 10^8 to 10^9 chronically infected cells contains an unintegrated infectious DNA molecule. Secondly, Canaani & Duesberg (1972) have shown that the tRNA primer binds to the template after budding of the progeny virus from the infected cell. If unintegrated DNA can be synthesized before budding, the method of priming must be different from that of the in vitro reaction.
Short communications

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REFERENCES


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