Frog Virus 3 Deoxyribonucleic Acid

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SUMMARY

DNA extracted from frog virus 3 has a GC mol fraction of about 0.56. The mol. wt. of the DNA is about $100 \times 10^6$ (97 $\times 10^6$ by neutral sucrose gradient sedimentation; 102 $\times 10^6$ from renaturation kinetics). Analysis by neutral sucrose sedimentation indicated that the DNA exists as a single heteroduplex in the virus particle. Alkaline sucrose gradient sedimentation indicated that single strand alkali labile interruptions probably occur in both strands of the heteroduplex. Renaturation kinetic analysis also indicated that about 7% of the genome contains repeated sequences. Quantitative analyses of DNA–DNA homology showed no sequence homology between frog virus 3 DNA and the DNA extracted from iridescent virus types 2, 6 or 9. This lack of sequence homology reflects the markedly distinct profiles on acrylamide gels of the structural polypeptides of frog virus 3 and the iridescent viruses.

INTRODUCTION

Frog virus 3 (Granoff, 1969) is one of a number of viruses isolated from vertebrates, invertebrates or plants, which can be described as ‘icosahedral cytoplasmic deoxyriboviruses’ (Kelly & Robertson, 1973). Although frog virus 3 has been shown to contain DNA (Smith & McAuslan, 1969; Houts, Gravell & Darlington, 1970; Kucera, 1970), the virus particle DNA has not been extensively characterized. In this study we have attempted to determine the size and substructure of the frog virus 3 genome by neutral and alkaline sucrose density sedimentation and by analysis of DNA renaturation kinetics. From the results of the kinetic studies we have also determined the extent of sequence homology between frog virus 3 and selected small iridescent viruses (types 2, 6 and 9: Tinsley & Kelly, 1970; Kelly & Robertson, 1973) which are morphologically similar and are tentatively placed in the same pseudotaxonomic group: the so-called ‘Iridovirus’ genus (Wildy, 1971).

METHODS

Materials. [3H]-methyl-T-thymidine (19.7 Ci/mmol), [2-14C]-thymidine (60 Ci/mmol), [2-3H]-adenine (10 Ci/mmol), [32P]-orthophosphate and [35S]-L-methionine (200 Ci/mmol) were purchased from the Radiochemical Centre Ltd, Amersham. Bromelain was obtained from the Sigma London Chemical Co. Ltd, and Sarkosyl was obtained from B.D.H. Ltd.

Cells, bacteria and insects. Primary chick embryo fibroblasts, Escherichia coli B, and Galleria mellonella L. larvae were grown as previously described (Kelly & Tinsley, 1972; Kelly & Avery, 1974; Kelly & Dimmock, 1974).

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Viruses. Frog virus 3 was a gift from Dr B. R. McAuslan, Roche Institute of Molecular Biology, Nutley, New Jersey, U.S.A. The virus was grown and plaque assayed in chick embryo fibroblasts, and also purified, as described by McAuslan and colleagues (McAuslan & Smith, 1968; Smith & McAuslan, 1969; Tan & McAuslan, 1970). The virus was radioactively labelled with [3H]-thymidine, [3H]-adenine, [3S]-methionine, or [32P]-orthophosphate by continually labelling monolayers of cells infected at 10 p.f.u./cell (4 x 10⁷ cells/13 cm plastic Petri dish/15 ml medium) with 10 µCi isotope/ml for 48 h. Bacteriophage T4B was grown and radioactively labelled with [32P]-orthophosphate in *Escherichia coli* B, and iridescent viruses were grown and radioactively labelled with [2-14C]-thymidine in *Galleria mellonella* L. larvae; and the viruses were purified as previously described (Cowie, Avery & Champe, 1971; Kelly & Avery, 1974).

DNA was extracted from the viruses as previously described (Cowie et al. 1971; Kelly & Avery, 1974). Frog virus 3 was treated as for iridescent viruses.

Neutral sucrose gradient sedimentation. Radioactively labelled DNA was released from frog virus 3 and bacteriophage T4B by digestion of 10 µg virus with 10 µg of bromelain in 20 µl of 0·1 M-phosphate-buffered saline, pH 7·2, at 37 °C for 4 h. A vol. of 20 µl of 4 % (w/v) Sarkosyl, 4 % (w/v) SDS in 0·1 M-tris-HCl buffer, pH 7·4, was added to the digest and allowed to interact for 16 h at room temperature. The preparation was loaded on to linear 22 ml 5 to 20 % (w/v) sucrose gradients containing 0·015 % (w/v) Sarkosyl, 0·01 M-tris-HCl buffer, pH 7·4. Sedimentation was in a 3 x 23 ml swing out rotor at 25000 rev/min for 4 h using a MSE Superspeed ‘50’ ultracentrifuge. The gradients were sampled from the top with an Auto-Densiflow (Buchler Instruments, Chicago) and the fractions collected were emulsified in gel scintillant for radioactivity assay.

Alkaline sucrose gradient sedimentation. Radioactively labelled DNA was released from frog virus 3 and bacteriophage T4B by incubation of 10 µg of virus in 20 µl of 0·3 N-NaOH, 2 % (w/v) Sarkosyl, 2 % (w/v) SDS for 10 min at 24 °C. The preparation was then immediately loaded on to linear 22 ml 5 to 20 % (w/v) sucrose gradients containing 0·015 % (w/v) Sarkosyl, 0·01 M-tris-HCl buffer, pH 11·6. Sedimentation was at 30000 rev/min for 4 h as above.

DNA fragmentation. DNA preparations in 0·1 × SSC (0·15 M-NaCl, 0·015 M-tri-sodium citrate) were sheared by two passages through a French press (Aminco 40 ml French pressure cell) at 20000 lb/in². The fragmented DNA was then dialysed extensively against 0·1 × SSC or 0·14 M-neutral phosphate buffer (PB). The DNA fragments were homogeneous in alkaline sucrose ultracentrifugation and had a corrected sedimentation coefficient (s₂₀,₅₀) of 8·4 ± 0·1 determined by analytical ultracentrifugation (Kelly & Avery, 1974).

U.v. spectrophotometry. Unicam SP800A or Gilford 222A spectrophotometers equipped with external recorders was used to monitor temperature and absorbancy at 260 nm.

Melting profiles of DNA were obtained using a Unicam SP876 temperature programmer and an electrically heated block.

Renaturation studies were performed by heating the fragmented DNA in 0·1 × SSC in a closed tube to 100 °C for 15 min. The DNA was then adjusted to 1 × SSC by adding an appropriate vol. of 10 × SSC heated at 100 °C. The DNA was then placed immediately in a cuvette held at 25 °C below its Tm (temperature at which 50 % denaturation occurs in 1 × SSC) and the kinetics of the reaction were monitored to completion in terms of the altering extinction at 260 nm in the Gilford spectrophotometer. In these renaturation experiments the temperature was maintained using a water jacketed block and a Haake thermostat.

DNA–DNA hybridization. The extent of sequence homology between frog virus 3 DNA
Frog virus 3 DNA

and iridescent virus DNAs was determined by allowing \(^{3}H\)- or \(^{14}C\)-thymidine labelled DNA fragments from one virus to re-anneal in the presence of an excess of homologous or heterologous unlabelled DNA fragments. Experiments were designed so that a maximum of 10% of the labelled DNA fragments could have self annealed at a time when re-annealing of the unlabelled fragments was 95% complete. The extent of re-annealing was predicted by the formula of Britten & Kohne (1966):

\[
\frac{C}{C_0} = \frac{1}{1 + K(C_0 t)}
\]

where: \(C\) = concentration of unpaired DNA strands (mol nucleotides/l); \(C_0\) = total concentration of DNA (mol nucleotides/l); \(K\) = reaction rate constant (l/mol-s); \(C_0 t\) = concentration of DNA x time (mol-s/l).

Re-annealing of the DNA in 0.14 M-PB and analysis of the single- and double-stranded DNA by hydroxyapatite chromatography were described by Kelly & Avery (1974). Acrylamide gel electrophoresis was performed on 10% SDS gels as described by Kelly, Cooper & Walkey (1974).

RESULTS

Neutral sucrose gradient sedimentation

Preliminary experiments to estimate the mol. wt. of frog virus 3 DNA were performed (Fig. 1) by co-sedimentation of \(^{3}H\)-thymidine-labelled frog virus 3 DNA with \(^{32}P\)-labelled bacteriophage T4B DNA on neutral sucrose gradients. Frog virus 3 DNA sedimented less rapidly than bacteriophage T4B DNA, and both DNA preparations appeared as discrete and apparently homogeneous bands. Comparisons of frog virus 3 DNA and bacteriophage T4B DNA were made by assuming that bacteriophage T4B DNA has a \(s_{20,w}\) of 61.8 (Friedfelder, 1970), and that the relative rates of sedimentation of the DNA molecules, and estimates of their mol. wt. may be defined by the relationship devised by Burgi & Hershey (1963) as modified by Friedfelder (1970):

\[
\frac{d_1}{d_2} = \frac{s_1}{s_2} = \left(\frac{M_1}{M_2}\right)^{0.38}
\]

where \(d\) = distance sedimented, \(s\) = sedimentation coefficient, and \(M\) = mol. wt. of the DNA molecule. The sedimentation coefficient of frog virus 3 DNA was thus 55.8 ± 0.2S, and assuming the mol. wt. of bacteriophage T4B DNA to be 126 x 10^6 (Kelly & Avery, 1974), the mol. wt. of frog virus 3 DNA was 98 ± 2 x 10^6.
Co-sedimentation of[^H]-thymidine-labelled frog virus 3 DNA with DNA released from[^35S]-methionine-labelled frog virus 3 indicated that no labelled protein remained in association with frog virus DNA (Fig. 2).

**Alkaline sucrose gradient sedimentation**

These experiments were undertaken to detect interruptions in single strands of the frog virus 3 genome. Analysis of[^3H]-thymidine-labelled frog virus 3 DNA by co-sedimentation with[^32P]-labelled bacteriophage T4B DNA (Fig. 3) consistently indicated 5 or 6 peaks, whereas bacteriophage T4B DNA sedimented as a single discrete peak. Assuming that denatured bacteriophage T4B DNA has a sedimentation coefficient of 73S (Studier, 1965) and that there is a linear relationship between sedimentation coefficient and the distance migrated on a linear alkaline sucrose gradient (Abelson & Thomas, 1966), then the leading frog virus 3 DNA band corresponded to 67.5 ± 3S. This is equivalent to a mol. wt. of $48 \times 10^6$, if the relationship $s_{20,w} = 0.0528 M^{0.40}$ of Studier (1965) is valid: this result is equivalent to half the double-stranded mol. wt. and probably represents intact single strands. Less than 10% of the thymidine counts were found in this rapidly sedimenting band. Quantitatively similar results were obtained with [^3H]-adenine or[^32P]-labelled frog virus 3 DNA.
Fig. 4. Thermal denaturation profile of frog virus 3 DNA in 0.1 × SSC. Relative absorbance is the ratio of absorbance at 30 °C (A∞) to that at the elevated temperature (A1).

Fig. 5. Analysis of frog virus 3 DNA by equilibrium sedimentation of 3 μg of DNA in CsCl (1.7 g/ml 1 × SSC) in a Beckman model E ultra-centrifuge (44,000 rev/min at 25 °C for 20 h).

Table 1. Some physical properties of frog virus 3 DNA

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal denaturation temperature (Tm) in 0.1 × SSC*</td>
<td>77.0 ± 1.0 °C</td>
</tr>
<tr>
<td>Equivalent guanosine + cytosine mol fraction</td>
<td>0.547 ± 0.026</td>
</tr>
<tr>
<td>Buoyant density in caesium chloride*</td>
<td>1.7150 ± 0.0002 g/ml</td>
</tr>
<tr>
<td>Equivalent guanosine + cytosine mol fraction</td>
<td>0.561 ± 0.002</td>
</tr>
<tr>
<td>s20,w native DNA*</td>
<td>55.8 ± 2 × 10^-13 s^-1 g/ml</td>
</tr>
<tr>
<td>Equivalent mol. wt.</td>
<td>98 ± 2 × 10^6</td>
</tr>
<tr>
<td>½ (Cot) in 1 × SSC*</td>
<td>0.295 ± 0.004 mol·s/litre</td>
</tr>
<tr>
<td>Equivalent mol. wt.</td>
<td>102 ± 2 × 10^6</td>
</tr>
<tr>
<td>Redundancy*</td>
<td>7.0 ± 0.5%</td>
</tr>
</tbody>
</table>

* Standard deviation calculated from five determinations.

Determination of the GC mol fraction

As a preliminary to the study of the renaturation kinetics of frog virus 3 DNA (performed at 25 °C below the thermal denaturation temperature (Tm) in 1.0 × SSC) the Tm of the DNA in 0.1 × SSC was determined to be 77.0 ± 1.0 °C (Fig. 4). This is equivalent to a Tm of 91.7 °C in 1.0 × SSC and a GC mol fraction of 0.547 (Marmur & Doty, 1962; Mandel et al. 1970). The GC mol fraction was confirmed by buoyant density determinations in CsCl as shown in Fig. 5.

A single band of density 1.7150 ± 0.0002 g/ml was obtained, equivalent to a GC mol fraction of 0.561 (Schildkraut, Marmur & Doty, 1962). These results are summarized in Table 1.
Fig. 6. Reassociation kinetics of fragmented frog virus 3 DNA, (a) $C_0t$ plot, (b) reciprocal plot of initial reannealing where $A_t =$ absorbancy at a given time and $A_{oo} =$ absorbancy of native DNA. The amount of the genome containing repeated sequences is calculated from the difference of the two intersects in Fig. 6(b).

Table 2. Summary of the extent of DNA–DNA homology among DNA preparations from frog virus 3 and iridescent virus of types 2, 6 and 9

<table>
<thead>
<tr>
<th>Source of unlabelled DNA fragments</th>
<th>Source of labelled fragments</th>
<th>Percentage homology*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frog virus 3</td>
<td>Frog virus 3</td>
<td>100</td>
</tr>
<tr>
<td>Iridescent virus type 2</td>
<td>Frog virus 3</td>
<td>0</td>
</tr>
<tr>
<td>Iridescent virus type 6</td>
<td>Frog virus 3</td>
<td>0</td>
</tr>
<tr>
<td>Iridescent virus type 9</td>
<td>Frog virus 3</td>
<td>0</td>
</tr>
<tr>
<td>Frog virus 3</td>
<td>Iridescent virus type 2</td>
<td>1.5</td>
</tr>
<tr>
<td>Frog virus 3</td>
<td>Iridescent virus type 6</td>
<td>1.2</td>
</tr>
<tr>
<td>Frog virus 3</td>
<td>Iridescent virus type 9</td>
<td>0</td>
</tr>
</tbody>
</table>

* These experiments were designed so that 95% of the unlabelled DNA fragments re-annealed while the labelled DNA self annealed between 2 and 10% for preparations from different genomes. The values presented above are corrected accordingly. The homology between iridescent virus genomes have been presented elsewhere (Kelly & Avery, 1974). Experiments were performed in triplicate.

Renaturation of frog virus 3 DNA

Fragmented DNA preparations, previously denatured at 100 °C, were allowed to re- anneal at 67 °C. The fraction reassociated is shown (Fig. 6) as a function of log($C_0t$) (Britten & Kohne, 1968), and also in a second order rate plot (Wetmur & Davidson, 1968). The second order rate plot indicates that about 7% of the frog virus 3 genome re-anneals at approximately twice the rate of the remainder of the DNA. This indicates that the genome contains repeated sequences.

Assuming a linear relationship between $C_0t$ and genetic complexity (Britten & Kohne, 1968), and a $\frac{1}{2}C_0t$ value of 0.364 for bacteriophage T4B which has a genome size of $126 \times 10^6$ (Kelly & Avery, 1974), we estimate the genome size of frog virus 3 ($\frac{1}{2}C_0t = 0.295 \pm 0.004$) to be $102 \pm 2 \times 10^6$. Fig. 6 also indicates that the bulk of frog virus 3 DNA reanneals with kinetics approximating to ideal second order kinetics.
Quantitative DNA homology between frog virus 3 and iridescent viruses

Using the values of $\frac{1}{2}C_{60t}$ obtained for frog virus 3 and also our values for iridescent virus types 2, 6 and 9 (Kelly & Avery, 1974), we estimated the amount of sequence homology between frog virus 3 and the iridescent viruses. Our results are summarized in Table 2 and show no significant homology between frog virus 3 and the iridescent viruses.

Acrylamide gel electrophoresis of frog virus 3 and iridescent virus structural polypeptides

To further the comparison of frog virus 3 with the iridescent viruses, we analysed their structural polypeptides. The gels (Fig. 7) showed that the profile for frog virus 3 was consistently different.
DISCUSSION

Our results demonstrate that frog virus 3 virus particles contain high mol. wt. double-stranded DNA of GC mol fraction 0.55 to 0.56: this confirms the observations of Smith & McAuslan (1969) and Houts et al. (1970). Our estimate of $97 \times 10^6$ for the mol. wt. of frog virus 3 DNA was determined by co-sedimentation with bacteriophage T4B DNA and is appreciably lower than the estimate of $130 \times 10^6$ obtained independently by Smith & McAuslan (1969) and Houts et al. (1970) who claim that frog virus 3 DNA co-sediments with bacteriophage T4 DNA on neutral sucrose gradients. The discrepancy may be due to the failure of these workers to remove all of the protein associated with frog virus 3 DNA. In preliminary studies we found that inconsistent results were obtained unless frog virus 3 was freed by efficient proteolytic digestion from radioactively labelled protein; frog virus 3 DNA frequently co-sedimented with bacteriophage T4B DNA when associated with protein. Frog virus 3 DNA is smaller than bacteriophage T4B DNA and this was confirmed subsequently by our analyses of DNA renaturation kinetics.

The analysis of frog virus 3 DNA by alkaline sucrose gradient sedimentation demonstrated that, under conditions in which bacteriophage T4B DNA sedimented as intact single strands, only about 10% of the frog virus 3 DNA sediments as apparently intact single strands and the remainder as fragments. Quantitatively similar results were obtained with freshly prepared [$^{32}$P]-orthophosphate and [$^3$H]-adenine-labelled frog virus 3 DNA, and our estimate that about 10% of frog virus 3 DNA occurs as intact strands is probably valid and not a consequence of differences in the base composition of the two strands of the frog virus 3 DNA. As such it appears that frog virus 3 DNA contains alkali-labile interruptions in both strands of the duplex. Whether the intact 10% of the DNA represents both or either strand of the duplex is not resolved. The release of DNA under mild alkaline conditions is rapid, and it is likely that the DNA occurs with single strand interruptions in the virus particle. These interruptions may be created by the endodeoxyribonuclease activity associated with frog virus 3 (Kang & McAuslan, 1972; Palese & McAuslan, 1972). This may explain why both strands can contain interruptions though some strands are apparently still intact. In this respect the frog virus 3 genome differs from the genome of a variety of herpes type viruses which also contain alkali-labile interruptions in their DNA (Kieff, Bachenheimer & Roizman, 1971; Lee et al. 1971; Frenkel & Roizman, 1972; Mosmann & Hudson, 1973; Wilkie, 1973) in which about 50% of the DNA sediments as intact strands; though it is not resolved whether this represents single stranded DNA of one or both polarities (Wilkie, 1973). Since frog virus 3 occurs as a single heteroduplex on neutral gradients, it is unlikely that any of the single strand interruptions coincide in the two strands of the duplex.

The analysis of frog virus 3 DNA renaturation demonstrated that $93.0 \pm 0.5\%$ of the genome reassociated with kinetics approaching ideal second order kinetics and at a rate which indicated that these sequences are unique. $7.0 \pm 0.5\%$ of the DNA re-annealed at approximately twice the rate of the unique sequences and indicated that this fraction represents repeated sequences. The demonstration that the bulk of frog virus 3 DNA exists as unique sequences parallels that for human herpes virus 1 (Frenkel & Roizman, 1971) and iridescent viruses (Kelly & Avery, 1974).

Using $\frac{1}{2}C_{o}t$ values to estimate the genome size of frog virus 3 (using bacteriophage T4B as a standard) the value of $102 \pm 2 \times 10^6$ was obtained. This confirms the smaller size of frog virus 3 DNA and also indicates that the genome is smaller than that for the superficially similar iridescent viruses (genome sizes of 114 to $153 \times 10^6$: Kelly & Avery, 1974).
Studies of DNA–DNA homology also demonstrated that there was no sequence homology between the genomes of frog virus 3 and those of the iridescent viruses (types 2, 6 and 9). The iridescent viruses also show significantly different GC mol fractions (0·278 to 0·428: Kelly & Avery, 1974) compared to frog virus 3.

Analyses of the structural polypeptides contained by highly purified frog virus 3 and iridescent viruses, showed a quite distinct profile for each virus. All four viruses present complex patterns which probably reflect their large size (about 130 nm) and structural complexity (Wrigley, 1969; Kelly & Vance, 1973; Tripier & Kirn, 1973; Kelly & Tinsley, 1974; D. C. Kelly & N. F. Moore, unpublished observations). The differences between the pattern presented by frog virus 3 and those for the iridescent viruses reflect the lack of sequence homology between the viruses.

In conclusion, we consider that frog virus 3 is sufficiently different from the insect pathogenic iridescent viruses considered in this paper to warrant rejection of the proposal that frog virus 3 and these iridescent viruses should be classified together.

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REFERENCES


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