Presence of Virus-specific DNA Sequences in Murine Type C Viruses

By M. J. BYERS,* R. J. AVERY, J. BOAZ† AND D. E. KOHNE‡
Department of Biological Sciences, University of Warwick, Coventry, U.K.,
†Scripps Institute of Oceanography, La Jolla, and the
‡La Jolla Cancer Research Foundation, La Jolla, Calif., U.S.A.

(Accepted 4 December 1978)

SUMMARY
Total nucleic acids prepared from a number of murine retroviruses have been shown to contain virus-specific DNA in addition to genomic RNA. This virus-specific DNA has been shown to be at least partially double stranded and to be present within the virus core particle. The DNA isolated from the virus is greatly enriched in virus-specific DNA relative to that from virus infected cells.

INTRODUCTION
Murine retroviruses contain, in common with other type C viruses, a complex RNA molecule about 70S in size which consists of at least two copies of the virus genetic information and specific host tRNAs (Erikson, 1969). DNA molecules with no known virus function are also present in these viruses. Retrovirus virion DNA has been detected in Rous sarcoma virus (RSV; Levinson et al. 1972; Darlix et al. 1977), avian myeloblastosis virus (AMV; Riman & Beaudreau, 1970; Biswal et al. 1971), Moloney murine leukaemia/sarcoma virus (Mo-MuLV/MuSV) and Rauscher leukaemia virus (RLV; Biswal et al. 1971) and the avian leukosis virus MC 29 (Weber et al. 1973). The virus DNA comprises from 0.5 to 2.5% of the total virus nucleic acid (Levinson et al. 1970; Biswal et al. 1971; Darlix et al. 1977) and in the case of AMV has been shown to be associated with the virus core and absent in the cell culture fluids (Deeney et al. 1976). The virion DNA (vDNA) present in RSV has been reported to consist of contaminating cellular DNA sequences and to have no role in the life cycle of the virus (Levinson et al. 1972). Other investigators have reported that Moloney murine leukaemia/sarcoma virus and RSV vDNAs significantly hybridize to their respective virus RNAs indicating that minus strand DNA sequences are present in these viruses (Biswal et al. 1971; Darlix et al. 1977).

We report here the detection of virus-specific plus strand DNA sequences in several murine retroviruses: the Moloney and Rauscher leukaemia viruses and the Kirsten leukaemia/sarcoma virus complex.

METHODS
Viruses. Moloney murine leukaemia virus (Mo-MuLV) and homologous DNA prepa- rations were a gift of H. Fan and R. Jaenisch, Salk Institute, San Diego, Calif. Scripps MuLV was purified from supernatant fluids of SCRF 60A continuously growing lymphoblastoid suspension cultures (Lerner et al. 1972). Rauscher MuLV was obtained from the National

* Present address: La Jolla Cancer Research Foundation, Box 1376, La Jolla, Calif., U.S.A.
Cancer Institute, Bethesda, Md. Kirsten leukaemia/sarcoma virus complex was purified from supernatant fluids of productively infected NRK cells. These fluids were harvested at four hour intervals, centrifuged briefly to clarify and pelleted from the supernatant fluids by ultracentrifugation. Virus pellets were resuspended and further purified by sedimentation through 15%, w/w, sucrose-STE (0.1 M- NaCl, 0.01 M-tris, pH 7.4, 0.001 M-EDTA) on to a 50%, w/w, sucrose-STE cushion. Cushioned virus was pooled and centrifuged to isopycnic density.

Virus cores were produced by treating a previously purified virus suspension with 1% sterox for 10 min at 0°C prior to banding to isopycnic density on 15 to 64%, w/w, sucrose-STE gradients.

Preparation of virus RNA. Virus RNA was initially prepared by lysis with SDS followed by deproteinizations with phenol and chloroform and ethanol precipitation (Method 1). Later, virus RNA was obtained by passage of a virus lysate (2 mg virus/ml in 0.3 M-LiCl, 0.01 M-tris, pH 7.4, 1% SDS, 1% β-mercaptoethanol) through 300 mg oligo(dT) cellulose (Collaborative Research Inc., Waltham, Mass.) in a jacketed column at 4°C. The column was washed with 0.3 M-LiCl, 0.01 M-tris, pH 7.4, 0.1% SDS until no further A260 absorbing material eluted. The bound material was then eluted with 0.005 M-sodium acetate, pH 6.0 at 30°C. A260 absorbing fractions were made to 0.3 M in LiCl and precipitated with ethanol (Method 2).

Preparation of total virus DNA (vDNA). Virus DNA was prepared by two methods. Originally, DNA was prepared by hydrolysis of total virus RNA prepared by Method 1 above. The hydrolysis conditions were 0.3 M-NaOH for 3 h at 60°C followed by the addition of tris to 20 mM and neutralization with HCl. As this method destroyed the virus RNA, an alternative procedure utilizing the unbound nucleic acid from the oligo(dT) cellulose columns in RNA preparation Method 2 (see above) was developed. The non-binding material was treated with Protease K (Sigma) at 20μg/ml for 30 min at 37°C, followed by deproteinization with phenol and chloroform and ethanol precipitation. The precipitate was redissolved and subjected to alkaline hydrolysis as above.

No virus-specific plus strand alkali stable sequences were detected in the oligo-dT-cellulose bound material (data not shown). Prior to use in hybridization experiments the hydrolysed nucleic acids were sonicated for 1 min at 4°C using a Branson Sonicator with microprobe at a power setting of 30 W.

DNA extraction. Cellular DNA was prepared by the urea-phosphate hydroxyapatite chromatography method (Britten et al. 1970) and fragmented to uniform piece size by sonication as described above.

cDNA preparation. cDNAs to the various virus RNAs were prepared by the appropriate endogenous reverse transcriptase reaction. The reaction mixes contained 2 mg/ml virus suspension, 0.1 mM each of dATP, dGTP and dCTP; 80 μCi/ml (2 μM) 3H-TTP (50 Ci/mmol, New England Nuclear, or 42 Ci/mmol, Amersham, Ltd.); 8 mM-dithiothreitol; 1 mM-MnCl₂; 0.02% NP-40 and 100 μg/ml actinomycin D. Following incubation at 37°C for 1 to 3 h the mixtures were deproteinized with phenol and chloroform and chromatographed on Sephadex G-100. Two cDNA preparations (RLV and Kirsten MuLV/MuSV) were further characterized as to numerical sequence representation (Duesberg & Canaani, 1970) and selected by hybridization to virus subunit RNA at low RNA:DNA ratios. Following selection, at least 60% of the cDNA preparations would hybridize with homologous RNAs at input ratios of 1:5:1. The specific activity of the resultant cDNAs was calculated as 2.1 x 10⁷ ct/min/μg.

Determination of virion DNA content. Total DNA content of a Kirsten MuLV/MuSV virion pool was determined as follows. Seven 150 cm² plastic dishes were each seeded with 2 x 10⁶ NRK cells productively infected with Kirsten MuLV/MuSV. Forty eight h later the
media were changed, fresh media supplemented with 10 μCi/ml ³H-thymidine being added. Following 48 h of incubation the spent media were discarded and 20 ml/plate fresh media plus ³H-thymidine again added. The supernatant fluids were harvested 24 h later and the labelled virus isolated as described above. The cells were scraped from the plates and the cellular DNA extracted by the urea-phosphate method described above. The specific activity of the cellular DNA was 265,000 ct/min/μg. Purified virions were sedimented through a 25 to 50% w/w, sucrose-STE gradient to isopycnic density. Fractions corresponding to a density of 1.16 g/ml were analysed for their absorbance at 260 nm and for radioactivity. As an example, in one case the peak fraction contained 0.85 A₂₆₀ units (approx. 100 μg virus protein as determined by the Lowry, 1951, procedure) and 2750 ct/min. Assuming uniform specific activity between the cellular and virus DNA, these experiments indicate a virus DNA content of 0.1 μg per mg virus protein (1% of virus nucleic acid). This value is close to the values obtained for other C-type viruses (Levinson et al. 1970; Biswal et al. 1971; Deeney et al. 1976).

**Hybridization.** Hybridization reactions were conducted in either 1.25 M-NaClO₄, 0.01 M-tris, pH 7.8, 7.5% phenol (v/v) or 0.48 M-sodium phosphate buffer (PB), pH 6.8 (the indicated molarity is for the phosphate moiety), 7.5% phenol (v/v) and shaken constantly to maintain an emulsion (Kohne et al. 1977). The reaction volume was either 1 or 5 ml and was constant within comparative kinetic experiments.

Detection of reassociated DNA:DNA and DNA:RNA duplexes (Britten & Kohne 1968) and thermal analysis of double stranded nucleic acids (Kohne, 1968) were by chromatography on hydroxyapatite in sodium phosphate buffer, pH 6.8.

**Ribonuclease treatment of reassociated duplexes.** Ribonuclease treatment at elevated temperatures was used to distinguish between DNA:DNA and DNA:RNA duplexes. At the time of assay two samples were taken, one being assayed directly on hydroxyapatite and the second dialysed against 0.14 M-PB. Thirty μg/ml RNase A and 15 units/ml RNase T₁ were added to the dialysed sample and incubated for 20 h at 59°C. This treatment is sufficient to destroy more than 90% of true duplexes formed between sonicated DNA and homologous RNA (Gelderman et al. 1971; Hough & Davidson, 1972; Kohne & Byers, 1973). Under the same conditions DNA:DNA duplexes remain double stranded. After incubation the mixture was passed over hydroxyapatite in 0.14 M-PB. Only those ct/min originally in DNA:DNA duplexes will bind to hydroxyapatite under these conditions, while any ct/min eluting in 0.14 M-PB represent DNA:RNA duplexes.

**RESULTS**

Total nucleic acids prepared from several murine retroviruses were used to prepare 70S RNA or vDNA as described in Methods. These preparations were then hybridized with homologous cDNAs. In all cases the cDNAs hybridized well with the homologous RNA preparations and, in addition, annealed significantly with alkali hydrolysed nucleic acid preparations to form stable duplexes (Table 1). The extent of annealing as shown in Table 1 may be variable since the degree of numerical sequence representation may not have been equal in the various cDNA preparations (see Methods). Thus the actual complexity of the virus-specific DNA cannot be determined from these data. Increasing the vDNA:cDNA ratio in the Rauscher and Kirsten virus preparations did not increase the extent of annealing (data not shown).

The ³H-cDNA was shown to be involved in DNA:DNA duplexes as follows: base hydrolysed Moloney MuLV nucleic acids and homologous cDNA were reassociated in the phenol system (phenol emulsion reassociation technique: PERT). After 3 h two samples were taken. One was assayed for duplex formation by hydroxyapatite chromatography, and
Table 1. Extent of annealing of cDNAs to virion nucleic acids*

<table>
<thead>
<tr>
<th>cDNA source</th>
<th>Virion nucleic acid source</th>
<th>% hybridization of (^{3}H)-cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moloney MuLV</td>
<td>Moloney MuLV</td>
<td>85</td>
</tr>
<tr>
<td>Rauscher MuLV</td>
<td>Rauscher MuLV</td>
<td>86</td>
</tr>
<tr>
<td>Rauscher MuLV</td>
<td>81</td>
<td>38</td>
</tr>
<tr>
<td>Scripps MuLV</td>
<td>73</td>
<td>20</td>
</tr>
<tr>
<td>Kirsten MuLV/MuSV</td>
<td>Kirsten MuLV/MuSV</td>
<td>84</td>
</tr>
</tbody>
</table>

* RNA hybridizations were done in 0.48 M-PB at 70 °C for a minimum COT of 0.5. Hybridizations with the hydrolysates (1 mg virus protein equivalent) were done in 1.25 M-NaClO₄, 0.10 M-tris, pH 7.8, 7.5% phenol (v/v) in 1 ml total vol. The actual reaction times for the data presented here varied from 5 to 12 h (the reactions were 90% complete in 2 h). All reactions were assayed on hydroxyapatite as described in Methods. Zero reaction time binding to hydroxyapatite was less than 0.5% for these cDNAs.

Fig. 1. Isopycnic densities of whole virus and of core preparations of Rauscher MuLV; 0.5 ml of virus suspension (1 mg/ml) was detergent disrupted as described in Methods and centrifuged to isopycnic density in a 15 to 64% (w/w) sucrose-STE gradient. A parallel gradient contained an equal amount of non-disrupted virus. Fractions were collected by tube puncture and A₂₆₀ absorbing fractions pooled and used for nucleic acid preparations. O--O, Disrupted virus; •--•, intact virus; ■--■, isopycnic density determined from refractive index measurements.

62% of the input ct/min were found to be in duplexes. The second was dialysed against 0.14 M-PB and treated with ribonuclease under conditions that digest over 90% of true DNA:RNA hybrids (see Methods).

This sample was then assayed on hydroxyapatite as above and 41% of the labelled material eluted as double stranded duplexes. Thus, at least 65% (0.41/0.62) of the duplexes found in the reassociation reaction were DNA:DNA duplexes. This is a minimum estimate, as some DNA:DNA duplexes may also be damaged to the extent that they no longer chromatograph on hydroxyapatite as double stranded molecules.

The Rauscher MuLV virus-specific DNA sequences do not co-purify with the virus by
Table 2. Reassociation analysis of alkali stable material from whole virus and cores*

<table>
<thead>
<tr>
<th>Reaction time (h)</th>
<th>Cores</th>
<th>Whole virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>8.6</td>
<td>10.8</td>
</tr>
<tr>
<td>0.3</td>
<td>18.0</td>
<td>15.7</td>
</tr>
<tr>
<td>0.5</td>
<td>19.2</td>
<td>18.3</td>
</tr>
<tr>
<td>1.0</td>
<td>20.8</td>
<td>17.7</td>
</tr>
</tbody>
</table>

* RNA prepared by Method 1 (see Methods) from the core and whole virus density peaks shown in Fig. 1 was base treated as described. Hybridization reactions in phenol were conducted at a concentration of material equivalent to 1 mg of whole virus protein and 1 x 10^-6 µg of cDNA per ml of reaction mix. Zero time binding of the cDNA was 0.5% and a parallel reaction in the absence of the hydrolysate reacted 3.5% in 1 h.

Table 3. Presence of complementary DNA strands in virus-specific DNA

<table>
<thead>
<tr>
<th>Hours reacted</th>
<th>% self reaction*</th>
<th>% hybridization of cDNA with Moloney MuLV vDNA†</th>
<th>% hybridization of cDNA with pre-annealed vDNA‡</th>
<th>% hybridization of cDNA with pre-annealed, then denatured, vDNA§</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>0.5</td>
<td>—</td>
<td>3.4</td>
</tr>
<tr>
<td>0.05</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>22.4</td>
</tr>
<tr>
<td>0.1</td>
<td>1.8</td>
<td>30.0</td>
<td>5.6</td>
<td>—</td>
</tr>
<tr>
<td>0.25</td>
<td>—</td>
<td>35.5</td>
<td>—</td>
<td>34.4</td>
</tr>
<tr>
<td>0.66</td>
<td>—</td>
<td>—</td>
<td>11.6</td>
<td>—</td>
</tr>
<tr>
<td>2.0</td>
<td>—</td>
<td>40.1</td>
<td>15.1</td>
<td>—</td>
</tr>
<tr>
<td>46.0</td>
<td>5.8</td>
<td>45.1</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Mo-MuLV 32P-cDNA was reassociated alone in 1.25 M-NaClO₄, 0.01 M-tris, pH 7.8, 7.5% phenol (v/v) at room temperature. When reacted with Moloney MuLV RNA, this cDNA preparation reacted to 84%.† An alkali hydrolysate of Moloney MuLV nucleic acid was reassociated with 32P-Moloney MuLV cDNA in the PERT system as above. Duplex formation was determined by assaying samples taken at the indicated times on hydroxyapatite.‡ Reaction as above except that the hydrolysed nucleic acids were pre-annealed in the PERT mixture for 12 h prior to the addition of the cDNA. The indicated time of reaction is from the addition of the cDNA.§ After 2 h, the reaction mixture described in (‡) above was heated to 70°C (approx. 20°C above the T_m in this solvent system) quickly cooled to room temperature and the reaction kinetics again determined.

adhesion to the outer surface, as virus cores prepared by detergent disruption (Fig. 1) contain amounts of virus-specific DNA equivalent to those found in whole virus (Table 2).

The presence of double stranded virus-specific DNA in Mo-MuLV is shown by the experiments presented in Table 3. Mo-MuLV 32P-cDNA was reacted with denatured alkali hydrolysed vDNA from Mo-MuLV. At 0.1 h of reaction, 30% of the cDNA was hybridized with the vDNA (cDNA alone reacted to 1.8% in 0.1 h). An equal amount of vDNA was reacted in the phenol system in the absence of 32P-cDNA for 12 h (or about five times longer than needed for essentially complete reaction with cDNA). 32P-cDNA was then added to the pre-reacted mixture and the kinetics of reaction of the cDNA monitored. Only 5% of the 32P-cDNA formed hybrids after 0.1 h of reaction and 15% of the cDNA reacted after 2 h incubation. Clearly, the amount of single strand virus-specific DNA capable of reacting with the cDNA was greatly reduced by the pre-hybridization step. After 2 h of reaction the reaction mixture was again denatured and incubated. The kinetics of this reaction were comparable to the original reaction where cDNA was mixed with denatured vDNA and reacted. These experiments indicate that both plus and minus virus-specific DNA strands are present in the vDNA.

The partial hybridization of the cDNA with the pre-annealed vDNA (Table 3) was
expected and is due to the basic nature of the hybridization process. Sonicated DNA is broken randomly so that the average complementary strand pair shares only one-half of its sequence when hybridized. This results in a situation where the first double strand molecules formed in a reaction are partially double stranded and partially single stranded. As the reaction proceeds the single strand portions of the double stranded molecules hybridize to form large concatenated molecules. As the reaction proceeds further, the single strand portions of larger and larger concatenates need to interact to ensure that no single strands are left in solution. The complete reaction of all of the single strand DNA never occurs. Thus there is some plus strand DNA still present in the pre-hybridized vDNA. The inability of this single strand DNA to react completely is probably due to steric inhibition caused by the large concatenated molecules. The slow rate of reaction of the eDNA with the pre-hybridized DNA (i.e. small cDNA reacting with the large concatenated vDNA) is due primarily to the low concentration of available virus specific plus strands in the pre-hybridized vDNA.

The Mo-MuLV used to prepare vDNA was grown in mouse cells. The DNA of these cells contain sequences which are similar to Mo-MuLV RNA sequences but not identical. Fig. 2 presents the thermal stability profile of Mo-MuLV cDNA:Balb/c uninfected cell DNA duplexes (□—◇). Balb/c cells do not normally contain Mo-MuLV. The one-half denaturation temperature (T_e90), as measured on hydroxyapatite, is about 78 °C for these duplexes. Also presented is the thermal stability profile (■—■) of the cDNA reacted with Mo-MuLV vDNA. The T_e90 of these duplexes is about 88 °C, ten degrees higher than the Balb/c:cDNA duplex T_e90. The large difference in the T_e90 indicates a considerable amount of base pair mismatching in the Balb/c:cDNA duplexes. The high vDNA:cDNA T_e90 indicates that the majority of these duplexes are close to being perfectly base pair matched and that the cDNA is reacting with DNA sequences which are not present in mouse DNA. Therefore the majority of the virus-specific DNA is Mo-MuLV DNA, not mouse DNA.

This raises an alternative possibility that the vDNA consists of a random selection of Mo-MuLV infected mouse DNA and that the high T_e90 is due to vDNA:Mo-MuLV proviral DNA duplexes. This is ruled out by the following considerations. The cDNA:vDNA reaction mixture contained 3 × 10^{-5} µg of cDNA and the equivalent of 10 µg/ml Mo-MuLV RNA. Since about 1% of the virus nucleic acid is DNA, this translates to 0.1 µg of virus derived DNA in the mixture. The vDNA:cDNA ratio in the mixture was then 3 × 10^{3}. At this ratio, 25% of the cDNA hybridized with the vDNA. This moderately large extent of reaction means that the 0.1 µg of vDNA contains Mo-MuLV plus stand DNA equivalent in mass to a quarter of the minus strand cDNA added to the mixture, or 7.5 × 10^{-6} µg of plus DNA. This amount of Mo-MuLV DNA is equivalent to 8 × 10^{5} full sized double strand virus DNA molecules each with a mol. wt. of 6 × 10^{8}. The mass equivalent of DNA from 1.6 × 10^{4} diploid mammalian cells is also 0.1 µg. If the vDNA is simply a random mixture of infected cell DNA, then the cellular DNA must contain at least 50 copies of Mo-MuLV proviral DNA per cell (8 × 10^{5} total virus DNA molecules/1.6 × 10^{4} cells). The Mo-MuLV infected mouse cell line which was used to provide the virus contains 6 to 8 proviral copies per cell (H. Fan, personal communication). Thus the presence of Mo-MuLV DNA in the vDNA cannot be solely attributed to the presence in the virus of a random mixture of Mo-MuLV infected mouse cell DNA.

A similar situation is seen for the vDNA isolated from Kirsten MuLV/MuSV. Ki-MuLV/ MuSV cDNA (3 × 10^{-5} µg) reacted 32% with 0.1 µg of vDNA from Kirsten MuLV/MuSV (see legend of Fig. 3). The vDNA contains the equivalent of at least 60 copies of Ki-MuLV/ MuSV per diploid cell. The Kirsten virus infected cells contain 4 to 8 proviral copies per cell (Fig. 3). Again the presence of Kirsten DNA in the vDNA cannot be solely attributed to the presence in the virus of a random mixture of infected cell DNA. In each case, at most, 10%
Virus-specific DNA in type C virions

Fig. 2. Thermal stabilities of cDNA duplexes. Reaction mixtures containing: □—□, ³²P-Moloney MuLV cDNA and Balb/c unlabelled DNA (labelled:unlabelled = 6 × 10⁻²); or ■—■, cDNA and a base hydrolysate of Moloney MuLV RNA (10 μg/ml RNA equivalent) were reassociated in phenol. The double strand duplexes found (24.5 and 55% of the input cDNA respectively) were analysed by thermal chromatography on hydroxyapatite. Those duplexes rendered single stranded at each 5°C temperature increment were eluted with 0.14 M-PB + 0.1% SDS. The T₉₀ of a parallel ³²P-Moloney MuLV cDNA reaction with Moloney MuLV RNA was 82.5°C.

of the virus-specific DNA can be due to the presence of a random selection of infected cell DNA. It is clear then that, somehow, the virus selectively includes virus-specific DNA into the virion.

Fig. 3 presents hybridization kinetic data which corroborate the above conclusion. These experiments utilized the phenol emulsion hybridization method to measure the concentration of Kirsten MuLV/MuSV-specific DNA in νDNA from the Kirsten virus complex and in Kirsten infected cells. The Kirsten cDNA (3 × 10⁻⁵ μg) was reacted with 0.1 μg of Kirsten νDNA. Chick DNA was added to bring the total DNA concentration to 10 μg/ml. This is necessary when using the PERT since the rate of hybridization is not directly proportional to the DNA concentration. The Ki-MuLV/MuSV cDNA (3 × 10⁻⁵ μg) was also hybridized with Kirsten infected NRK cell DNA (10 μg) obtained from the cells which...
were used to produce the virus. Radioactive DNA from infected NRK cells was mixed with 10 μg of non-radioactive NRK DNA and reacted to determine the rate of reaction of the single copy (per haploid cell) fraction of the NRK DNA. In all these reactions the total DNA concentration was 10 μg/ml. A comparison of the kinetic curves depicted in Fig. 3 shows that the Kirsten cDNA reacts about six times faster with infected cell DNA than the single copy fraction of the NRK cell DNA. Thus there are about six copies of proviral information per haploid cell (12 copies per diploid cell). The cDNA reacts with the vDNA at a rate about 30 times faster than with the infected cell DNA. Thus there are 360 copies of the proviral DNA per cell equivalent of vDNA. As concluded above, it is clear that the vDNA is greatly enriched in virus specific DNA sequence relative to the infected cell DNA.

**DISCUSSION**

We have examined several murine retroviruses and find that purified virions contain virus-specific DNA sequences. Data obtained from extent of hybridization, hybridization kinetic and thermal stability studies all indicate that vDNA is greatly enriched in these virus specific sequences relative to infected cell DNA.
Radioactive cDNA produced by the endogenous virus reverse transcriptase was used to detect these virus-specific DNA sequences. In several cases the cDNA preparations were reacted with purified virus RNA at low RNA:DNA ratios and the hybridizing fraction used to detect virus-specific DNA. This selected cDNA reacted to 85% or more with homologous virus RNA with the kinetics of hybridization expected for a virus RNA:cDNA reaction of type-C RNA complexity (data not shown). Thus it is highly unlikely that the cDNA reacting with the vDNA represents anything but virus-specific DNA.

The different cDNAs reacted 20 to 60% with their respective vDNA preparations. The reason for the considerable variation in the extent of reaction of the cDNA with the vDNA is not known. It is possible that each different extent of reaction is a consistent feature of each different virus type. The data presented here do not allow us to answer these points. The primary reason for this is that the numerical sequence representation of the different DNAs is not known. It is also possible that the variation in extent of reaction is at least partially due to a cDNA excess in some of the hybridization mixtures.

The radioactive cDNA used represents only the minus or non-message strand of the virus genome. Therefore it can directly detect only the presence of the plus strand DNA. Unfortunately the fraction of the virus genome which is present as minus strand DNA in the virus-specific DNA cannot be determined from the data presented here since it is not known whether the minus strand represents the entire virus genome or if each of the cDNA sequences is present at equal frequency. In addition, the degree of representation and the frequency of representation of each minus strand sequence present in the virus-specific DNA is not known. Therefore the most that can be said at present is that a portion of the virus genome is represented in the virus-specific DNA fraction.

The actual amount of total virus-specific DNA present in the viruses is also unknown. A minimum estimate of the amount of plus strand virus specific DNA can be calculated. As mentioned earlier 1 mg of MuLV yields 10 µg of virus RNA, 0.1 µg of vDNA and about 8 x 10^-6 µg of virus-specific plus strand DNA. This amount of plus strand DNA is equivalent to 8 x 10^5 full sized single strand virus DNA plus strands. Since 1 mg of virus equals 7 x 10^11 particles this is enough plus strand DNA to have one full sized DNA per 10^6 viruses. Alternatively, if only 10% of the virus is represented or the whole plus strand is represented but only 10% is present in any virus, there would be one DNA piece per 10^5 viruses. Roughly similar values are seen for Ki-MuLV/MuSV and RLV. The minimum amount of minus strand present in Mo-MuLV can be calculated by using the results in Table 3. These experiments indicated that 50 to 90% of the virus-specific plus strand DNA has a complementary sequence present in the vDNA. This dictates that the minimum frequency of minus strands is similar to that of plus strand DNA. The actual amount of plus and minus strand virus-specific DNA present in these viruses can be much higher and still be consistent with the results presented here. Biswal et al. (1971) have reported that a large fraction of the vDNA from Moloney MuLV/MuSV hybridizes to the virus RNA. Similarly Darlix et al. (1977) have reported that about 10% of the vDNA present in Rous sarcoma virus (RSV) hybridizes to RSV 70S RNA and, further, that 40 to 90% of purified RSV 70S RNA molecules have one or two small virus-specific DNA molecules hybridized to them. No effort was made to determine the amount of double strand virus-specific DNA in these viruses. Our data do not rule out the existence of a similar situation in the murine viruses. The cDNA probe has minus strand polarity and would not detect the presence of large amounts of minus strand virus-specific DNA. The reaction of Ki-MuLV/MuSV cDNA with Kirsten vDNA was done at several different vDNA/cDNA ratios varying over a factor of three with no change in the extent of reaction (see legend to Fig. 3). This indicates that the vDNA virus-specific plus strand DNA is in considerable excess over the input radioactive
minus strand cDNA at all the vDNA/cDNA ratios used. However, since the cDNA is asymmetric in its representation of the virus genome there may be significant amounts of plus strand virus-specific DNA which cannot be detected by the cDNA. In addition there may be virus-specific plus strand DNA sequences present for which there is a large excess of minus strand present also. Thus, even if the cDNA contained the proper minus strand sequence, the large excess of minus strands in the non-radioactive DNA would greatly dilute the radioactive cDNA sequence and make it undetectable.

The origin of the virus-specific DNA present in the vDNA is not known. Proviral DNA sequences may somehow be preferentially included into the virus during the assembly of the virus in the cell. Alternatively the virus-specific DNA may be synthesized after virus assembly by the action of reverse transcriptase on the virion RNA. No differences were seen in the extent of reaction of Kirsten MuLV/MuSV cDNA with vDNA isolated from Kirsten virus harvested at 4 h or at 24 h intervals. This would indicate that if endogenous virus-specific DNA synthesis is occurring in the intact virion it must take place within 4 h after the virus is released from the cell.

Virus-specific DNA, either plus or minus strand, has been detected in a variety of murine and avian retroviruses. There is as yet no known biological role for this DNA, but the potential biological significance of the virus-specific DNA warrants further study. One interesting speculation is that this DNA is somehow involved in the process of cell transformation.

The authors would like to thank H. Fan, R. Jaenisch, F. Jensen, A. Puga and T. Borras for generous donation of materials. Part of this work was done at the Scripps Clinic and Research Foundation, La Jolla, Calif., U.S.A.

REFERENCES


(Received 13 July 1978)