Molecular Relationship of the DNA and RNA of Intracytoplasmic A Particles to Mouse and Mammary Tumour Virus Genomes

By TIMOTHY J. HENRY AND GILBERT H. SMITH

Laboratory of Molecular Biology, National Cancer Institute, Bethesda, Maryland 20205, U.S.A.

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SUMMARY

We have directly tested the hypothesis that single-stranded cytoplasmic A particle-associated DNA (ss CAP DNA) is a murine mammary tumour virus (MMTV) proviral intermediate by hybridizing $^{125}$I-labelled ss CAP DNA to MMTV RNA or to MMTV complementary DNA (cDNA). $^{125}$I-labelled CAP DNA did not form duplexes with either MMTV RNA or MMTV cDNA. In contrast, CAP RNA hybridized readily with MMTV cDNA. CAP RNA contained all the MMTV virus sequences, but at lower concentrations than in MMTV virus particles.

Single-stranded CAP DNA hybridized readily with mouse DNA from several sources. A study of the rate of hybridization of CAP DNA to cell DNA at various driver to probe ratios showed that its rate of hybridization is similar to that of tumour cell DNA reassociation. Further, in reassociation studies accelerated by using the phenol emulsion reassociation technique (PERT), CAP DNA originally isolated as single-stranded DNA was shown to reanneal (70%), to protect $^{125}$I-cell DNA to the same extent (67%), and to do so with kinetics of reassociation equivalent to that of mouse DNA. Although CAP DNA isolates were slightly enriched for MMTV specific sequences when compared to total cellular DNA, we conclude that the majority of ss CAP-associated DNA is equivalent to a random sample of total tumour cell DNA.

INTRODUCTION

Cytoplasmic A particles (CAP) are virus-like particles found within the cytoplasm of murine mammary tumours, oestrogen-induced Leydig cell tumours and ML+ leukaemias. Their occurrence and isolation have been described in previous publications (Bernhard, 1958; Kerckaert et al. 1970; Tanaka et al. 1972; Smith & Wivel, 1972, 1973). CAP are antigenically related to the murine mammary tumour virus (MMTV; Tanaka et al. 1972; Smith, 1978) and it has been shown that the major proteins in CAP are polyproteins which, when appropriately cleaved, yield proteins of a size typical of those of MMTV cores (Smith & Lee, 1975; Tanaka, 1977; Smith, 1978). It is, therefore, a tenable hypothesis that CAP are normal intermediates in the production of MMTV; a direct test of this precursor-product relationship has not yet been carried out.

We have shown in previous work that the CAP which we isolate have associated with them a relatively large amount of DNA (Smith et al. 1974; Longfellow & Smith, 1976). This DNA was shown to be single-stranded (ss) by its behaviour on hydroxylapatite, its buoyant density in CsCl, and its unequal content of deoxyadenosine and deoxythymidine. Because of the ss nature of the DNA and because attempts to 'contaminate' CAP with
exogenously added $^3$H-labelled DNA were successful, we were led to suggest that CAP DNA might be a MMTV proviral intermediate similar to that seen in other retrovirus systems (Smith et al. 1974; Longfellow & Smith, 1976). Since our CAP are isolated from tumours which do not shed virus, we conjectured that the long-term exposure of CAP to the deoxynucleoside triphosphate-containing cellular milieu might lead to at least partial activation of the endogenous, reverse transcriptase catalysed reaction and, thus, to the synthesis of an intraparticle DNA related to MMTV [CAP do contain reverse transcriptase (Kohno & Tanaka, 1977; Michalides et al. 1977) and also MMTV RNA (Michalides et al. 1977; this work)].

A limited test of this proviral DNA hypothesis was carried out previously on one sample of CAP DNA; it showed that CAP DNA was apparently not related to either the plus or minus strand of MMTV (Michalides et al. 1977). It was, however, still possible that CAP DNA might represent only a small portion of MMTV genome and as such would have been difficult to detect in the experiments previously reported. We have labelled CAP DNA with $^{125}$I so that we could overcome this sensitivity limitation and could directly follow the hybridization of CAP DNA to DNA or RNA related to MMTV (or MuLV) and could study the kinetics of hybridization of CAP DNA to cellular DNA. These hybridizations are the subject of this paper; they show that the majority of CAP-associated DNA is not related to MMTV or MuLV and that CAP DNA appears to be, rather, a uniform sample of cellular DNA.

**METHODS**

*Isolation of CAP. CAP* were isolated from Leydig cell tumours which arose in oestrogen-treated male BALB/c mice and which are carried by subcutaneous transplantation in BALB/c females, or they were isolated from mammary tumours arising in C3H/StfC3H/He mice. Tumour tissue was disrupted in a Polytron homogenizer (Brinkman Instruments, Westbury, N.Y. 11590) and CAP were isolated as previously described (Smith et al. 1974). CAP isolated from several tumours and tumours at different levels of passage were pooled to provide enough material for nucleic acid extraction.

The tissue origin of CAP nucleic acids is indicated by a letter in parentheses following CAP; of those used in this paper CAP(I)DNA and CAP(II)DNA were from CAP isolated from BALB/c ICT-53 Leydig cell tumours; CAP(M)DNA was from CAP isolated from C3HStMMTV + mammary tumours; CAP(N)DNA was from CAP isolated from BALB/c NIV Leydig cell tumours (which were induced and carried in BALB/c MMTV-L + mice); CAP(G)DNA was from a pool of CAP from BALB/c NIV and BALB/c ICT-53 tumours.

*Isolation of CAP nucleic acids. Two slightly different techniques were used to isolate CAP nucleic acids. In the first, CAP were disrupted in 1% SDS, 0.05 M-$\beta$-mercaptoethanol, extracted repeatedly with phenol-chloroform (50:50 v/v) and then precipitated with ethanol (as described by Smith et al. 1974). In the second method, CAP in TNE (0.05 M-tris, pH 7.4, 0.1 M-NaCl, 0.001 M-EDTA) were made 500 µg/ml in protease K, 1% in SDS and incubated 30 to 60 min at 37 °C. The digest was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, v/v) and three or more times with chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated with 2 vol. of ethanol from solutions made 0.3 M in sodium acetate. CAP nucleic acids were fractionated in Cs$_2$SO$_4$ gradients (Szybalski, 1968).

*Isolation of DNA from tissues.* Recently excised tissue was disrupted in a Polytron homogenizer in 5 to 10 vol. of TNE containing 1% SDS. Protease K was added to a level of 1 mg/ml and the mixture was stirred at 25 to 30 °C overnight. Occasionally, tissue was quick frozen in liquid nitrogen, then shattered into a powder by striking the frozen tissue with a hammer; the powder was dissolved in TNE, SDS, protease K and digested as given...
above. The solution was extracted twice with an equal vol. of phenol-chloroform-isoamyl alcohol (25:24:1) and three times with chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated with ethanol, resuspended in water, made 0·3 m in NaOH and digested overnight at 37 °C to remove RNA. The base digest was neutralized with acetic acid and the DNA precipitated out with ethanol. The DNA was resuspended in water prior to base catalysed fragmentation.

Fragmentation and sizing of DNA. We used base catalysed scission of the DNA chain to obtain pieces of a size suitable for hybridization. Two variations of the procedure were used. In the first (Varmus et al. 1974), the DNA was dissolved in 0·3 N-NaOH, 0·005 M-EDTA and heated in a boiling water bath for 20 min. It was neutralized with acetic acid, the DNA was precipitated out by ethanol and the size of the DNA fragments determined as described below. In the second technique the DNA is first subjected to a limited acid depuration followed by base treatment at milder temperatures than in method 1. (We would like to thank Dr Philip Andersen for conveying his optimized conditions for this technique to us.) A 2 mg/ml solution of DNA in 0·1 m-Na-acetate, pH 4·3, was heated for 90 min at 70 °C, then NaOH added to bring the pH to 11·0, the solution was reheated 5 min at 70 °C, neutralized with acetic acid, the DNA precipitated with ethanol and sized as given below.

Sizing of the fragmented DNA was determined by flat bed agarose gel electrophoresis under denaturing conditions (0·03 M-NaOH, 0·002 M-EDTA) as described by McDonell et al. (1977). Agarose gels (1·4%) were run overnight at about 2 V/cm or until the bromocresol green dye had migrated about 60% of the gel length. The DNA bands were stained with ethidium bromide (0·5 μg/ml in 0·05 m-tris-Cl, pH 7·0, for 1 h) and their size determined by comparison with fragments of known size (HaeIII restricted φX-174 RFI fragments from Bethesda Research Labs, Rockville, Md. 20850).

Generation of 3H-DNA complementary to MMTV RNA. 3H-DNA complementary to MMTV RNA was made by the calf thymus DNA fragment primed endogenous reverse transcriptase reaction utilizing whole MMTV produced by the Mm5mt/c1 cell line (Taylor et al. 1976). Pelleted MMTV (provided by the Frederick Cancer Research Center through the office of Dr Jack Gruber, Viral Oncology Program, NCI) was resuspended in 0·05 m-tris-Cl, pH 8·3. This was made 0·3% in NP40 and 0·005 M in dithiothreitol and, after standing 30 min at 0 °C, was added to an equal vol. of solution to give final concentrations of 0·05 m-tris-Cl, pH 8·3 (pH measured at 25 °C), 200 μM in dTTP, dGTP, and dATP, 50 μg/ml actinomycin D, 0·3% NP40, 0·005 M-dithiotreitol, 0·01 M-MgCl₂, 300 μg/ml calf thymus fragments, 40 μM-3H-dCTP and 2·5 mg/ml virus. The reaction was carried out at 37°C for 10 h, stopped by adding protease K to 200 μg/ml and SDS to 1% and digesting at 37 °C for an additional 30 min. Carrier salmon sperm DNA (50 μg) was added to the protease-treated reaction and nucleic acids were isolated by phenol/chloroform extraction and ethanol precipitation followed by base digestion to remove RNA. The product was further purified on a Sephadex G-25 column. Peak fractions from this column were pooled, ethanol precipitated, resuspended in H₂O and stored at -20 °C. In some preparations it was necessary to remove a self-annealing fraction (15 to 20% of the total) by hydroxylapatite chromatography. To do this, the probe was self-annealed in 0·6 M-phosphate buffer (PB) at 68 °C to a Cₒ₅ of 0·1, diluted to 0·03 M-PB and applied to a hydroxylapatite column maintained at 60 °C. After washing with 0·05 M-PB (this removes the remaining, unincorporated ³H-dCTP), the single-stranded probe was eluted with 0·14 M-PB.

³²P labelling. DNA or RNA samples were labelled with ³²P under the optimum reaction conditions described by Commerford (1971) and as adapted to microgram amounts by Prensky (1976). Briefly, 0·3 to 2 μg of nucleic acid in 5 μl of water was mixed, in a siliconized tube, with 3 μl of 0·001 M-thallium chloride dissolved in 1 m-Na-acetate, pH 4·9.
To this was added 1 to 2 mCi of low pH Na\textsuperscript{125}I (3 µl; New England Nuclear, Boston, Mass.) and the mix was taken up in the tip of a Natelson blood collecting pipette. The pipette was inserted into a close-fitting, siliconized Wintrobe tube and incubated in a 60 °C water bath for 15 min (RNA samples) or 30 min (DNA samples). The reaction was stopped by expelling the mixture into 0.5 ml of TNE containing 0.05 M-β-mercaptoethanol. Unstable intermediates were removed by reheating the stopped reaction mixture for 30 min at 60 °C. It was cooled, 50 µg of denatured salmon sperm DNA was added as carrier and the labelled product was separated from free iodide by passage through a small G-25 column equilibrated with TNE. The peak fractions were pooled, precipitated with ethanol, the iodinated probe was resuspended in water and stored at -20 °C. The typical probe had a specific activity of 1 × 10⁶ cts/min/µg.

Hybridizations. Hybridizations were generally carried out in sealed capillary tubes containing 0.6 M-NaCl, 0.025 M-tris-Cl, pH 8.3, at 25 °C, 0.001 M-EDTA and an appropriate amount of driver and probe (with added salmon sperm DNA to make at least 500 µg/ml nucleic acid in those cases where the amount of driver was low). The reactions were started by immersing the capillaries in a boiling water bath for 5 min followed by transfer directly into a 68 °C water bath. At suitable times capillaries were chilled in ice-water, opened and expelled into 2.2 ml of S1 buffer (0.15 M-NaCl, 0.03 M-Na-acetate buffer, pH 4.5, 0.001 M-ZnCl₂ and 20 µg/ml denatured salmon sperm DNA; Vogt, 1973). The sample was split into two 1.0 ml fractions and enough S1 enzyme was added to degrade all of the single-stranded DNA (approx. 800 units of S1 from Miles Biochemicals, Elkhart, Indiana, U.S.A.) within a 2 h period at 37 °C. The enzyme-treated and control samples were chilled, 200 µg of denatured salmon sperm carrier DNA was added and the contents precipitated by adding 1 ml of 10% trichloroacetic acid. After 15 min at 0 °C, the precipitates were collected on 0.45 µm Millipore filters, washed with 5% TCA, dried and then counted in a Beckman gamma counter (for \textsuperscript{125}I samples) or with scintillation fluid in a scintillation counter (for \textsuperscript{3}H and \textsuperscript{3}H/\textsuperscript{125}I double-label experiments). Percentage hybrid was calculated as the number of S1-resistant counts divided by the counts in the untreated control. C₀t values were plotted and corrected for salt concentration to equivalent C₀t as described by Britten et al. (1974). Except for the PERT hybridizations (below), all C₀t values in the text are actually equivalent or EC₀t's.

Phenol emulsion reassociation technique (PERT) hybridizations were carried out as described by Kohn et al. (1977). Since the rates of reassociation in different salts and at different salt concentrations are not readily interconvertible, one set of standard conditions has been used throughout this work. The procedure was to combine, in a 3 ml V-shaped vial containing a V-shaped magnetic stirrer (Reacti-vials, Pierce Chemical Co., Rockford, Illinois 61105, U.S.A.), sample and salt in a vol. of 1.7 ml to give, based upon a final vol. of 2.0 ml, 1.25 M-NaCl, 0.1 M-tris-Cl, pH 7.4 and about 4 µg/ml driver DNA. The vial was closed tightly, heated in a boiling water bath for 5 min, then chilled and allowed to come to room temperature. The vial was opened, placed on a magnetic stirrer and 0.3 ml of 90% phenol, 10% tris-Cl, pH 7.4, was added; this time was taken as the start of the hybridization. At appropriate times, 50 µl or 100 µl samples were removed and diluted into 0.6 ml of 0.6 M-NaCl, 0.03 M-Na-acetate, pH 4.5, 0.001 M-ZnCl₂, 20 µg/ml denatured salmon sperm DNA as suggested by Kohn et al. (1977). These were later diluted with 1.8 ml of 0.03 M-Na-acetate, pH 4.5, 0.001 M-ZnCl₂, 20 µg/ml denatured salmon sperm DNA to give the usual S1 assay conditions. One ml aliquots of each sample were assayed with or without S1 enzyme as described above and the % S1 resistant, TCA precipitable hybrid determined. We found that under these conditions, the reassociation rates for \textit{Escherichia coli} DNA and total mouse DNA were accelerated about 6000-fold whereas \textsuperscript{3}H-labelled, single-stranded \textit{φX-174} virus DNA did not become S1 resistant.
Table 1. Hybridization of MMTV ³H-cDNA to CAP DNA and CAP RNA

<table>
<thead>
<tr>
<th>Driver</th>
<th>C₀⁺</th>
<th>S₁ resistant hybrid</th>
<th>Copy number†</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMTV RNA (740)*</td>
<td>0.1</td>
<td>80 (ER₉₀₄ = 0.02) ‰</td>
<td>—</td>
</tr>
<tr>
<td>CAP(G)RNA 700)</td>
<td>0.5</td>
<td>80 (ER₉₀₄ = 0.10) ‰</td>
<td>—</td>
</tr>
<tr>
<td>CAP(M)DNA (2500)</td>
<td>0.2</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>CAP(N)DNA (5000)</td>
<td>0.1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>CAP(I)DNA (15000)</td>
<td>0.8</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>218</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Leydig tumour DNA (15000)</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>104</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* Ratio of driver to probe.
† Number of MMTV genome equivalents per 4 × 10⁶ pg of CAP DNA (equivalent to one haploid amount of mouse DNA).

RESULTS

Hybridization of CAP DNA and CAP RNA to MMTV ³H-cDNA

Because CAP proteins are related to MMTV, the hypothesis that CAP DNA was a proviral intermediate was tested first by examining the ability of CAP DNA to protect MMTV ³H-cDNA. As shown in Table 1, our cDNA hybridized well (80%) to MMTV RNA with a R₀⁺ value (2 × 10⁻²) typical of a polynucleotide with a single-strand complexity of 2.9 × 10⁶ mol. wt. (Leong et al. 1972). However, three different preparations of CAP DNA failed to give appreciable protection of the probe at C₀⁺ values 100 times that expected for virus-type hybridization. When similar hybridizations were carried out under probe excess conditions (Heilmann et al. 1977), the amount of MMTV-like sequences present in CAP DNA was equivalent to three to 16 MMTV provirus copies per haploid amount of mouse DNA. In contrast to CAP DNA, RNA isolated from Leydig cell CAP protected MMTV cDNA to the same final extent (80%, Table 1) as did MMTV RNA, but at a R₀⁺ value five times that of MMTV RNA. Thus, CAP RNA contained all of the MMTV sequences, but at concentrations only 20% that of purified MMTV RNA.

Lack of protection of CAP ¹²⁵I-DNA by virus RNA or MMTV cDNA

One advantage of using ¹²⁵I-labelled CAP DNA was to be able to test the hypothesis that CAP DNA represented only a small portion of the provirus genome. If, for example, CAP DNA consisted of minus strand virus sequences representing only 5% of the MMTV genome, then attempts to protect labelled MMTV RNA with CAP DNA would protect only 5% of the RNA probe; this value could easily be lost in the background. On the other hand, we would expect virus RNA (in excess) to protect all of the ¹²⁵I-labelled CAP DNA even if CAP DNA represented only a minor fraction of the virus sequences. Similarly, if CAP DNA were composed of plus strand sequences, then CAP ¹²⁵I-DNA should be well protected by MMTV cDNA. However, as seen in Table 2, CAP ¹²⁵I-DNA was not
Table 2. Hybridization of $^{125}$I-labelled CAP(I)DNA to Virus RNA or MMTV cDNA

<table>
<thead>
<tr>
<th>Probe</th>
<th>Driver</th>
<th>$R_{at}$ or $C_{at}$</th>
<th>5S-resistant hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP $^{125}$I-DNA</td>
<td>Yeast RNA (2000)*</td>
<td>0.2</td>
<td>15</td>
</tr>
<tr>
<td>CAP $^{125}$I-DNA</td>
<td>MMTV RNA (10)</td>
<td>0.3</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.3</td>
<td>13</td>
</tr>
<tr>
<td>CAP $^{125}$I-DNA</td>
<td>MMTV RNA (100)</td>
<td>0.26</td>
<td>3</td>
</tr>
<tr>
<td>CAP $^{125}$I-DNA</td>
<td>MuLV RNA† (40)</td>
<td>1.3</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>$^3$H-cDNA MuLV†</td>
<td>MuLV RNA (100)</td>
<td>0.02</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.3</td>
<td>91</td>
</tr>
<tr>
<td>$^3$H-cDNA MMTV</td>
<td>MMTV RNA (700)</td>
<td>0.02</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>80</td>
</tr>
<tr>
<td>CAP $^{125}$I-DNA</td>
<td>MMTV cDNA (0.5)</td>
<td>0.19</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.19</td>
<td>5</td>
</tr>
<tr>
<td>MMTV 70S $^{15}$I-RNA</td>
<td>MMTV cDNA (11)</td>
<td>0.001</td>
<td>10†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMTV cDNA (4)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMTV cDNA (11)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMTV cDNA (36)</td>
<td>0.23</td>
</tr>
</tbody>
</table>

* Ratio of driver to probe.
† Rauscher murine leukaemia virus.
‡ RNase sensitivity in 2X SSC.

protected any better by MMTV or MuLV RNA than it was by yeast RNA. Nor was CAP $^{125}$I-DNA protected by MMTV cDNA (at ratios of cDNA/CAP DNA five times that necessary to maximally protect labelled 70S MMTV RNA). Thus we conclude that CAP DNA is not enriched for even a small portion of either proviral strand.

Hybridization of CAP DNA to cell DNA

A positive control for the ability of our iodinated CAP DNA to actually function as a hybridization probe was to hybridize it to DNA from those cells in which the CAP-containing tumours arose. When we attempted to determine the amount of cell DNA necessary to satisfy the requirement for hybridization in DNA excess, we found that complete hybridization of the probe is obtained at driver to probe ratios as low as $5 \times 10^9$. Assuming that to obtain 70% hybridization of a probe one needs a driver to probe ratio of at least 4, it can be calculated that the complexity (mol. wt.) of CAP RNA must be at least $2 \times 10^9$. DNA of this complexity could be present as one unique sequence or as multiple copies of a sequence or sequences with lower complexity. If CAP DNA were a pure species with a complexity similar to the MMTV genome ($2.9 \times 10^9$; Dion et al. 1977), there would have to be at least 500 copies per cell of this sequence. This multiple copy hypothesis predicts that CAP $^{125}$I-DNA would hybridize to cell DNA at a rate 500 times the single copy rate. A hybridization between CAP DNA and cell DNA is shown in Fig. 1. The rate at which CAP DNA hybridizes to cell DNA is exactly the same as the reassociation rate of cell DNA. Thus, CAP DNA cannot be significantly enriched for any species present in cell DNA in multiple copies and probably has a complexity approaching that of cell DNA. Similar results have been obtained with CAP DNA probes from two different CAP DNA preparations from Leydig cell tumours, both of which behaved like ssDNA on hydroxylapatite chromatography.
Intracytoplasmic A particle DNA

Fig. 1. Hybridization of Leydig cell tumour ICT-53 total DNA with either \(^{125}\text{I}-\text{labelled total tumour DNA (O-O)}\) or with \(^{125}\text{I}-\text{labelled CAP(II)DNA (A-A)}\). Samples of 20 \(\mu\)l containing 80 \(\mu\)g of tumour DNA and 50 \(\mu\)g of probe in 0.6 M-NaCl, 0.05 M-tris-Cl, pH 8.4, at 25 °C, 0.001 M-EDTA, were sealed in 40 \(\mu\)l capillaries, boiled 5 min and then incubated at 68 °C. At appropriate times, samples were assayed for S\(1\) resistance as described in Methods. Values have been normalized to 100 %; the actual final extents of hybridization were: 69 % for total tumour probe at \(C_{ot} = 40000\) and 58 % for the CAP DNA probe at \(C_{ot} = 20000\). The iodinated probe sizes were 430 bases for the total tumour DNA and 180 for the CAP(II)DNA.

Fig. 2. PERT hybridizations of CAP DNA. Nucleic acid levels were as described in the Methods; probe levels were adjusted to give 1000 ct/min/100 \(\mu\)l sample. Hybridization values have been normalized to 100 %; the uncorrected, final levels of hybridization are given in parentheses. \(\nabla\), \(^{125}\text{I}-\text{CAP(I)DNA to CAP(I)DNA (67 %)}\); O, \(^{125}\text{I}-\text{CAP(G)DNA to CAP(I)DNA (87 %)}\); \(\triangle\), \(^{125}\text{I}-\text{total tumour DNA to CAP(I)DNA (65 %)}\); \(\square\), \(^{125}\text{I}-\text{total tumour DNA to total tumour DNA (57 %)}\).

Reassociation of CAP DNA

While the majority of the early isolations (26 out of 43) of DNA from CAP yielded a product which was definitely single-stranded as shown by its behaviour on hydroxylapatite and its buoyant density of CsCl (Longfellow & Smith, 1976), later preparations gave either double-stranded or single-stranded material (never both) even though these DNA isolations were performed on pools of tumour tissues. Thus, it seemed appropriate to ask whether our ss CAP DNA might have arisen as a denaturation artifact during isolation; if it had, then it should be capable of reassociation. Because of the limited amount of CAP DNA...
(20 to 100 μg/isolation) it normally would have been difficult to answer that question, but by utilizing the PERT technique (Kohne et al. 1977) we have been able to show (Fig. 2, ▽) that ss CAP DNA can reassociate. Further, the kinetics of reassociation are, as in the conventional hybridization of Fig. 1, exactly the same as the reassociation kinetics of total cellular DNA (Fig. 2, □). Fig. 2 (△) shows that CAP DNA can protect labelled cellular DNA to the same extent and with the same kinetics as does cellular DNA. Thus, CAP DNA contains all the cell DNA sequences and at the same relative concentration. The last set of data points (Fig. 2, ○) show that different preparations of CAP DNA are fully related. These results show that CAP DNA is very heterodisperse and indistinguishable from cellular DNA.

**DISCUSSION**

The hybridization experiments between CAP DNA and ³H-labelled virus cDNA (Table 1) show that CAP DNA is not enriched for MMTV plus strand sequences beyond that expected for DNA from mammary tumours (6 to 30 copies per diploid cell; Morris et al. 1977; McGrath et al. 1978). That CAP DNA is neither the plus nor minus strand MMTV provirus DNA is demonstrated by the CAP ¹²⁵I-DNA hybridizations in Table 2 as is the point that CAP DNA cannot be a minor portion of the MMTV provirus. Thus, it is clear that CAP DNA is not a MMTV provirus intermediate.

What CAP DNA is can be deduced from Fig. 1 and 2. Since we were able to calculate a minimum complexity for CAP DNA (as presented in Results), it was clear that if a single set of sequences of RNA tumour virus complexity were present, the rate of CAP DNA hybridization to cell DNA would be more rapid than that observed. In fact, the rate of hybridization of CAP DNA to cell DNA is exactly the same as the rate of reassociation of cell DNA. Thus, not only is CAP DNA not related to MMTV, as shown in the last section, but also it cannot be any unknown type of virus DNA with a complexity near that of the RNA tumour viruses. The telling hybridization in which CAP DNA completely protects labelled cellular DNA (Fig. 2, △) shows that CAP DNA contains all of the cell DNA sequences. Since the rate and shape of that curve are the same as that of the cell DNA reassociation, we conclude, on the basis of these hybridization experiments, that CAP DNA is indistinguishable from cellular DNA.

How ss cellular DNA comes to be associated with CAP is a puzzling question. Our previous inability to 'contaminate' CAP preparations with exogenously added ³H-labelled cell DNA (Smith et al. 1974) and the presence of autoradiographic grains from ³H-thymidine over CAP inclusion in situ (Smith et al. 1975) suggest that CAP DNA association occurs in situ and/or very rapidly after tissue homogenization. The perinuclear location of CAP inclusions might predispose them to DNA contamination. However, there were reports in other systems which seemed to support our hypothesis that CAP-associated DNA was or could be MMTV-specific: Leibovitch et al. (1977) showed that avian myeloblastosis virus-infected cells contained ssDNA enriched for virus sequences; Levinson et al. (1970) showed that DNA was incorporated into retrovirus particles; and Ringold et al. (1975, 1977, 1978) have demonstrated that unintegrated MMTV provirus DNA appears in the infected cell cytoplasm under certain conditions in infected rat tissue (though not in mouse tissue). Moreover, we knew that the major CAP structural protein, the precursor polyprotein p82, contained the MMTV ssDNA-binding protein, p14 (Arthur et al. 1978). We felt that this protein might possess the specificity necessary for the binding and encapsulation of MMTV DNA. To us, the most persuasive observation which suggested that CAP DNA might be unique was the persistent appearance of ssDNA as the major component (90%) in nucleic acid isolates of pooled or separate CAP preparations from either BALB/c mouse Leydig cell tumours or C3H/He mammary tumours. Yet it is clear from our
Intracytoplasmic A particle DNA

PERT analysis that the CAP-associated ssDNA can renature and is indistinguishable from mouse cellular DNA.

Accepting the finding that CAP DNA is actually a random sample of cellular DNA, we are endeavouring to solve the conundrum of how it becomes single-stranded. Since all mock CAP DNA isolations processed identically, but in the absence of CAP, have yielded only double-stranded mouse DNA, we are attempting to determine whether or not CAP proteins have dsDNA unwinding activity. The denaturation of cellular DNA may be related to the CAP DNA-binding protein or to the associated reverse transcriptase activity since avian myeloblastosis virus reverse transcriptase has been shown to have unwinding capacity (Collett et al. 1978) as does yeast RNase H1 (Dezelee et al. 1978).

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