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Studies on the Replication of Reticuloendotheliosis Virus: Detection of Viral-Specific DNA Sequences in Infected Chick Cells

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Infection of stationary chick embryo fibroblasts by reticuloendotheliosis virus (REV) is sensitive to cytosine arabinoside, an inhibitor of DNA synthesis. Furthermore, the majority of the nucleic acid sequences contained in the REV genome can be detected in infected cells in the form of DNA by RNA-DNA hybridization techniques. A small portion (ca. 5%) of the REV-specific sequences can be detected in uninfected chick embryo fibroblasts suggesting that these cells contain at least part of the REV genome as endogenous DNA sequences. These observations are consistent with an involvement of REV-specific proviral DNA as an intermediate in the replication of REV in chick embryo fibroblasts.

INTRODUCTION

The reticuloendotheliosis viruses (REV) are a group of avian RNA-containing viruses that exhibit several morphological and biochemical features similar to the avian leukosis-sarcoma virus (ALSV) complex (Zeigel *et al.*, 1966; Baxter-Gabbard *et al.*, 1971; Maldonado and Bose, 1971; Halpern *et al.*, 1973; Maldonado and Bose, 1973; Kang and Temin, 1973). They do not however, share any serological relationship or genetic homology with the avian RNA tumor viruses and are therefore considered a separate group of avian viruses distinct from the ALSV complex (Theilen *et al.*, 1966; Maldonado and Bose, 1971, 1973; Halpern *et al.*, 1973; Kang and Temin, 1973; Mizutani and Temin, 1973; Purchase *et al.*, 1973). Infection of chick or duck embryo fibroblasts with REV results in some cell death; however, chronically infected cells, exhibiting no demonstrable cytopathological effects, can be established in cell culture (Bose and Levine, 1967; Temin and Kassner, 1974; Faras, A. J., unpublished observations). Furthermore, recent studies have demonstrated that REV can transform bone marrow cells *in vivo* (Franklin *et al.*, 1974).

The precise mechanism of infection of avian cells by REV has yet to be elucidated. The establishment of chronically infected carrier cultures *in vitro* and transformation of bone marrow cells *in vivo* suggest that these viruses may replicate through the agency of a DNA provirus similar to members of the ALSV complex (Temin, 1971a). However, we and others have been unable thus far to detect, in purified preparations of REV, an endogenous DNA-polymerase reaction in which the 70 S virion RNA acts as a template for the synthesis of DNA (Kang and Temin, 1974; Kieras and Faras, 1975). It was of considerable interest therefore to determine whether the REV group replicates through a DNA intermediate.

In this communication we report that REV replication is sensitive to cytosine arabinoside, an inhibitor of DNA synthesis, and actinomycin D, an inhibitor of DNA-dependent RNA synthesis. These results are analogous to those obtained with

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members of the ALSV complex (Temin, 1963; Temin, 1967) and another member of the REV group, Trager duck spleen necrosis virus (Temin and Kassner, 1974). Furthermore, employing RNA–DNA hybridization with DNA in vast excess (Bishop, 1972; Melli *et al.*, 1971), we have directly detected DNA sequences in REV-infected cells that are complementary to a large majority of the nucleotide sequences of the REV 70 S RNA genome. We conclude that the replication of REV in chronically infected chick cells proceeds through a DNA provirus intermediate. While this manuscript was in preparation, Kang and Temin reported similar observations with Trager duck spleen necrosis virus (TDSNV) (Kang and Temin, 1974).

MATERIALS AND METHODS

Cells and virus. Embryonated chick eggs, negative for avian leukosis virus group-specific antigen, were obtained from SPAFAS, Roanoke, IL; embryonated Pekin duck eggs were obtained from the Tulip City Duck Farm, Holland, MI. B77 strain (subgroup C) of Rous sarcoma virus (RSV) was propagated in chick embryo fibroblasts and purified as previously described (Faras and Dibble, 1975). The Cook strain of REV, presumably derived from the original virus isolate of Twiehaus (strain T; Theilen, 1966) was a kind gift from Dr. G. Purchase. REV was propagated in chick embryo fibroblasts. Although infection of chick cells with REV resulted in some cell death, many of the infected cells exhibited no apparent cytopathic effects and were maintained through several passages. The cells appeared to be chronically infected with REV since they continually produced virus. These cells were the source of REV-infected cell DNA.

Preparation of REV and RSV 70 S RNA. ^{32}P-labeling of RSV and REV-RNA was accomplished by previously published procedures (Dahlberg *et al.*, 1974; Faras and Dibble, 1975). Briefly, infected cultures were washed with phosphate-free medium and then labeled with 1 mCi of carrier-free [^{32}P]orthophosphate per ml. Virus was harvested at 24-hr intervals and stored at $-70°$. Virus was purified by pelleting through a 25% sucrose cushion in an SW 27 rotor at 27,000 rpm for 2.5 hr at 4°. The virus pellets were resuspended in STE (0.1 *M* NaCl, 0.02 *M* Tris–HCl, pH 7.4, and 0.01 *M* EDTA), treated with 0.5% sodium dodecyl sulfate (SDS) and 500 μg/ml of Pronase for 30 min at 37°, and extracted three times with cold STE-saturated phenol. Viral 70 S RNA was further fractionated from the low molecular weight RNAs by rate–zonal sedimentation in 15–30% sucrose in an SW 41 rotor at 41,000 rpm for 3 hr at 4°. The specific activity of the ^{32}P-labeled 70 S RNA was approximately 3–4 $\times$ 10^{6} cpm/μg and was estimated either directly by determining the A_{260} of the P^{32}-labeled 70 S RNA preparation or indirectly by determining the specific activity of total cytoplasmic RNA extracted from the virus-producing cells. In our hands the specific activities obtained by either method were comparable.

All preparations of ^{32}P-labeled REV-RNA were assayed for the presence of avian leukosis virus-specific sequences by measuring the extent of homology between the REV genome and the genomes of the ALSV complex by molecular hybridization. This was accomplished by reacting DNA complementary to either the RSV or RAV-2 viral genome (cDNA) with ^{32}P-labeled REV 70 S RNA under conditions that permit saturation of most, if not all, of the complementary nucleotide sequences in the RNA (Garapin *et al.*, 1973). The extent of hybridization (sequence homology) was obtained by determining the proportion of the labeled viral RNA which became resistant to pancreatic ribonuclease (Duesberg and Canaani, 1970; Garapin *et al.*, 1973). The cDNA utilized in these experiments was prepared as described previously (Garapin *et al.*, 1973). The reaction mixtures contained 300 μg/ml of virus protein, 0.1 *M* Tris–HCl, pH 8.1; dGTP, dCTP, dATP, and [^{3}H]TTP (300 cpm/pmole), each at 5 $\times$ 10^{-5} *M*; 0.01 M MgCl$_2$; 2% β-mercaptoethanol; 0.01% (v/v) Nonidet-P40; and actinomycin D (100 μg/ml) to promote a more uniform transcription of the template RNA into DNA. Reactions were carried out at 37° for 4 hr, then extracted with

SDS-phenol (Faras *et al.*, 1972). Nucleic acids were recovered by precipitation with ethanol, treated with 0.3 *M* NaOH at 37° for 16 hr to remove viral RNA, neutralized, and precipitated again with ethanol. Conditions for hybridization are given in the legend to Fig. 1. As depicted in Fig. 1, no measurable ribonuclease-resistant hybrids could be detected between RSV or RAV-2 cDNA and REV 70 S RNA indicating the lack of ALSV-specific sequences in our preparations of REV-RNA. These results establish the purity of the REV 70 S RNA employed in the hybridization experiments

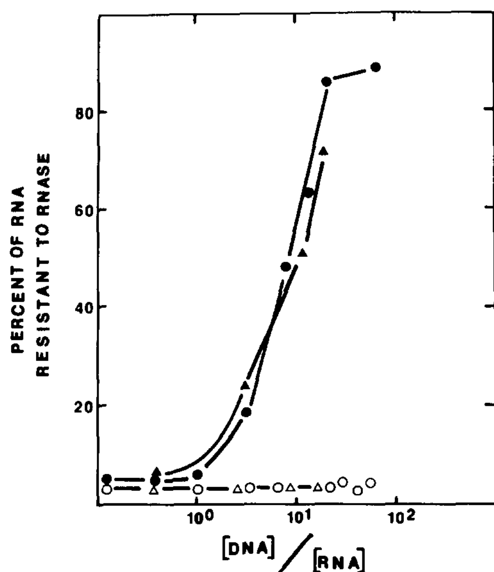

FIG. 1. Sequence homology between the 70 S RNA genomes of REV and RSV. ^{3}H-labeled cDNA, prepared from detergent-disrupted RSV (Schmidt-Ruppin strain) and Rous-associated virus-2(RAV-2) under reaction conditions described in Materials and Methods, was hybridized to ^{32}P-labeled 70 S RNA of REV and their respective ^{32}P-labeled 70 S RNA's as follows. Approximately 0.5–1 ng of ^{32}P-labeled RNA and varying amounts of ^{3}H-labeled DNA in 10 μl of annealing buffer (0.6 *M* NaCl, 0.01 *M* EDTA, 0.02 *M* Tris-HCl, pH, 7.4) were sealed in 30-μl glass capillary tubes and incubated for 68 hr at 68°. The extent of hybridization was measured by hydrolysis with pancreatic ribonuclease as described in Materials and Methods. The results are plotted as a function of the ratio of DNA and RNA present in each sample (Garapin *et al.*, 1973). Symbols: ●, RSV-specific cDNA hybridized to RSV 70 S RNA; ○, RSV-specific cDNA hybridized to REV 70 S RNA; ▲, RAV-2-specific cDNA hybridized to RAV-2 70 S RNA; △, RAV-2-specific cDNA hybridized to REV 70 S RNA.

presented in this communication (Tables 1 and 2 and Fig. 4) and further document the lack of sequence homology between these two groups of avian RNA-containing viruses (Kang and Temin, 1973).

Preparation of cell DNA. Cellular DNA was prepared by previously published methods (Varmus *et al.*, 1973). Cells were lysed with 0.5% SDS and incubated for 2 hr at 37° with 500 μg/ml of Pronase (self-digested for 2 hr at 37° prior to use). The disrupted cells were extracted with phenol twice at room temperature, and the nucleic acids were precipitated with 2 vol of ethanol. The nucleic acids were recovered by centrifugation, resuspended in 0.02 *M* Tris-HCl, pH 7.4, 0.01 *M* EDTA, and treated for 4 hr at 37° with 100 μg/ml of pancreatic ribonuclease (boiled for 10 min to inactivate deoxyribonuclease). The sample was further incubated with 100 μg/ml of Pronase for 30 min at 37° and then subjected to two phenol extractions at room temperature. At this stage the DNA was either precipitated with ethanol or extensively dialyzed against 1 × SSC (0.15 *M* NaCl plus 0.015 *M* sodium citrate). The A_{260}/A_{280} ratio of DNA prepared in this manner was invariably greater than 1.85. Prior to hybridization all DNA was fragmented to a length of 300–400 nucleotides by limited depurination as described previously (McConaughy and McCarthy, 1967; Neiman, 1972). The concentration of DNA was adjusted to 2 mg/ml in 0.1 × SSC and the pH adjusted to 4.3 with sodium acetate. The DNA was then incubated at 70° for 90 min, the pH was adjusted to 11.0 with NaOH, and the sample was further incubated at 50° for 10 min before neutralizing to pH 7.0. DNA was precipitated with ethanol and resuspended in 1 m*M* EDTA.

Hybridization conditions. Details of the hybridization conditions are presented in the legends to the tables and figures. Annealing is generally performed in 0.6 *M* NaCl, 0.02 *M* Tris-HCl (pH 7.4), 0.002 *M* EDTA and 0.05% SDS at 68°. Volumes of 30 μl or less were incubated in sealed capillary pipettes, and larger volumes were incubated in glass tubes under a layer of mineral oil. Extent of hybridization was determined by treatment of the samples

with 50 μg of pancreatic ribonuclease per ml in 2 × SSC at 37° for 45 min.

RESULTS

Effects of an Inhibitor of DNA Synthesis on Replication of REV in Chick Embryo Fibroblasts

We have investigated the effects of an inhibitor of DNA synthesis on the replication of REV in an effort to determine whether virus-specific DNA synthesis is required for REV infection. It has been previously reported that the rate of multiplication of chick embryo fibroblasts *in vitro* is directly determined by the amount of serum in the medium (Temin, 1969) and that, in the absence of serum, the cells do not divide but remain stationary (Temin, 1971b). These stationary cell cultures remain susceptible to infection with members of both the ALSV and REV groups of viruses (Temin, 1971a; Temin and Baltimore, 1972; Temin and Kassner, 1974; Fig. 2). The efficiency of infection of stationary chick cells with REV or RSV was similar to the infection of the same number of normally dividing chick embryo fibroblasts indicating that the stationary cultures were not inhibitory to REV or RSV replication (data not shown). If these stationary chick cultures are treated with 2×10^{-4} *M* cytosine arabinoside and infected with either REV or RSV, considerable inhibition of virus production is observed (Figs. 2a and b), suggesting that viral-specific DNA synthesis is required for the replication and establishment of infection of chick cells with both REV and RSV.

Virus production by REV-infected cells was also dramatically inhibited (>99%)

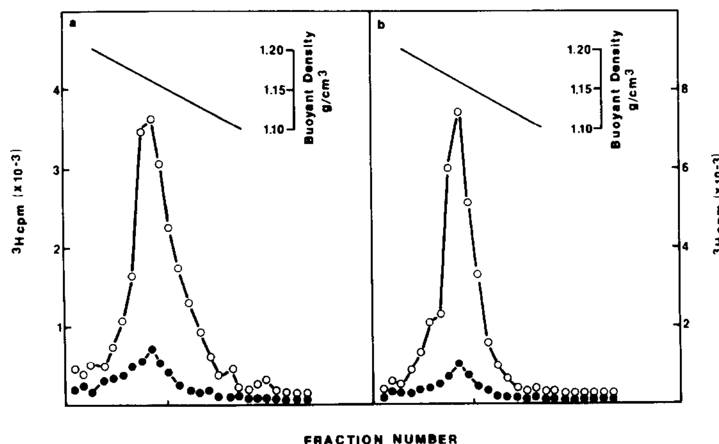

FIG. 2. Effect of cytosine arabinoside upon infection of chick embryo fibroblasts by REV and RSV. Cultures of stationary chick embryo fibroblasts (Temin, 1971) were exposed to REV or RSV and incubated for 1 hr at 37°. The inoculum was removed and 5 ml of medium 199, devoid of calf serum but containing 2×10^{-4} *M* cytosine arabinoside, was added to the cultures. After 18 hr at 37° the medium was removed and replaced with medium containing 4% calf serum but lacking cytosine arabinoside. After 48 hr of incubation at 37°, cultures were labeled with 30 μCi/ml of [^{3}H]uridine and incubated an additional 24 hr at 37°. Control experiments consisted of infecting cells with REV or RSV under identical conditions, described above, except that cytosine arabinoside was omitted from the medium. Cytosine arabinoside appeared to be somewhat toxic (60%) to cells at the concentrations employed (Temin and Kassner, 1974). However, the drug had no effect on the production of REV from chronically infected, nondividing cells. Furthermore, virus production was normalized to the number of viable cells remaining in control and treated cultures. Virus production was assayed by isopycnic centrifugation. Exactly 5 ml of [^{3}H]uridine-labeled medium was layered onto a 2-ml solution of 15% sucrose which overlayed a 5-ml 25–55% sucrose gradient. The virus was centrifuged at 41,000 rpm in an SW 41 rotor for 2.5 hr at 4°. Fractions were collected, analyzed for refractive indexes, and precipitated with 10% trichloroacetic acid. Acid-precipitable radiolabel was collected onto glass-fiber filters and counted in a Beckman scintillation spectrometer. Data are presented as ^{3}H cpm per 2×10^5 cells. (a), RSV-infected chick embryo fibroblasts; (b), REV-infected chick embryo fibroblasts. Symbols: ○, cells infected with virus in the absence of cytosine arabinoside; ●, cells infected with virus in the presence of 2×10^{-4} *M* cytosine arabinoside.

when actinomycin D was included in the medium suggesting that transcription of DNA is also required for the replication of REV (Fig. 3).

Detection of REV-Specific DNA Sequences in Infected Chick Cells

We have employed hybridization techniques, which allow nearly complete hybridization of complementary RNA with DNA from eukaryotes (Bishop *et al.*, 1972; Melli *et al.*, 1971), in an attempt to detect REV-specific DNA sequences in chick embryo fibroblasts subsequent to infection of cell cultures with REV. The extent of hybridization between an excess of single-stranded DNA fragments prepared from uninfected chick embryo fibroblasts, REV-infected chick embryo fibroblasts, and uninfected duck embryo fibroblasts and

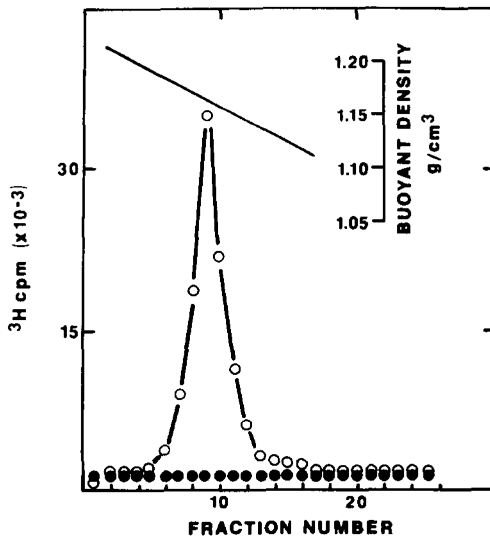

FIG. 3. Effect of actinomycin D upon production of REV by infected chick embryo fibroblasts. REV-infected and producing cultures of chick embryo fibroblasts were exposed to 2 μg/ml of actinomycin D at 37°. After 2 hr, 20 μCi/ml of [^{3}H]uridine was added to the medium and the cultures were incubated for an additional 24 hr. The [^{3}H]uridine-containing medium was harvested and 5 ml were assayed for virus by isopycnic centrifugation as described in Fig. 2. Control cultures were subjected to identical manipulations except that actinomycin D was omitted from the medium. Symbols: O, control cultures; ●, REV-infected cells incubated in the presence of 2 μg/ml of actinomycin D.

radioactive REV 70 S RNA is presented in Table 1.

Whereas no more than 10% of the REV viral RNA formed ribonuclease-resistant hybrids when the hybridization was performed in the presence of a large excess of DNA from uninfected chick embryo fibroblasts, approximately 78% of the REV viral RNA sequences became ribonuclease-resistant when hybridization was performed in the presence of DNA isolated from REV-infected chick embryo fibroblasts. Under similar hybridization conditions, 75% of the RSV 70 S RNA genome forms ribonuclease-resistant hybrids with DNA from RSV-transformed chick cells (Neiman, 1972; Varmus *et al.*, 1974; Table 1), whereas only 30% of the RSV sequences can be detected in uninfected chick cells (Neiman, 1972, 1973; Varmus *et al.*, 1974; Table 1). Therefore, as is the case with the avian RNA tumor viruses, most, if not all, of the genetic sequences contained in the REV-RNA genome are present in the form of DNA subsequent to infection of chick cells with the virus.

The detection of appreciable (ca. 30%)

TABLE 1

HYBRIDIZATION OF REV AND RSV 70 S RNA TO UNINFECTED AND INFECTED AVIAN CELL DNA[a]

70 S RNA	Source of cellular DNA	Percent hybridization[b]
REV	Uninfected duck	3.6
REV	Uninfected chick	9.7
REV	REV-infected chick	78.0
RSV	Uninfected duck	1.7
RSV	RSV-infected duck	78.5
RSV	Uninfected chick	28.0
RSV	RSV-infected chick	75.0

[a] ^{32}P-labeled 70 S RNA (2,000 cpm, 0.5 ng) from either REV or RSV was incubated at 68° in a solution containing 0.6 *M* NaCl and a vast excess of fragmented, denatured cellular DNA (10 mg) to C_ot values of approximately 3×10^4 mole sec/liter. Extent of hybridization was determined by resistance to pancreatic ribonuclease as described in Materials and Methods.

[b] The percent hybridization was corrected for the intrinsic ribonuclease-resistance of the RNA (5%) and nonspecific interaction of viral RNA with calf thymus DNA (6%). Data are presented as the averages of three or more separate determinations.

hybridization of the RSV genome to uninfected chick cell DNA is consistent with reports from other laboratories (Neiman, 1972; Neiman, 1973; Schincariol and Joklik, 1973; Varmus *et al.*, 1974) and further documents the presence of RSV-specific endogenous sequences in uninfected chick embryo fibroblasts. In contrast to RSV, only a small portion (ca. 10%) of the REV-RNA genome forms ribonuclease-resistant duplexes with uninfected chick cell DNA. In view of the low levels of duplex formation observed between REV 70 S RNA and uninfected chick cell DNA, the specificity of hybridization was determined by competition experiments. ^{32}P-labeled REV 70 S RNA was hybridized to uninfected chick cell DNA at C_0t(C_0 is the concentration of DNA nucleotides in moles/liter, and t is time in seconds) (Britten and Kohne, 1968) values of approximately 2×10^4 mole sec/liter in the presence of a 10,000-fold excess of either unlabeled REV 70 S RNA, RSV 70 S RNA, or HeLa cell ribosomal RNA. From the results presented in Table 2, it appears that both unlabeled RSV 70 S RNA and HeLa cell ribosomal RNA reduce duplex formation between ^{32}P-labeled REV 70 S RNA and uninfected chick cell DNA to 4–5%, indicating that at least part of the ribonuclease-resistant, ^{32}P-labeled REV-RNA observed was probably a result of nonspecific interaction between the nucleic acids present in the hybridization mixtures. However, complete competition of ^{32}P-labeled REV-RNA was observed when unlabeled REV 70 S RNA was included in the hybridization mixtures indicating that approximately 4–5% of the ribonuclease-resistant hybrids formed, when radioactive REV 70 S RNA is reacted with uninfected chick DNA, are REV-specific. It therefore appears that uninfected chick cells contain approximately 5% of the REV genome as REV-specific endogenous DNA sequences.

No ribonuclease-resistant hybrids were observed when radioactive RSV 70 S RNA was hybridized to uninfected duck DNA, a result consistent with recent reports from other laboratories (Varmus *et al.*, 1973, 1974; Kang and Temin, 1974). However we have routinely observed low levels (2–4%) of duplex formation between REV 70 S RNA and uninfected duck DNA (Table 1). Further studies employing competition–hybridization experiments will be required before the nature of these duplex structures can be determined.

TABLE 2

COMPETITION HYBRIDIZATION BETWEEN ^{32}P-LABELED REV 70 S RNA AND UNLABELED REV, RSV OR RIBOSOMAL RNA FOR DUPLEX FORMATION WITH UNINFECTED CHICK CELL DNA[a]

DNA	Competing RNA[b]	Percent hybridization
Uninfected chick	None	10.0
Uninfected chick	REV 70 S RNA	0
Uninfected chick	RSV 70 S RNA	5.0
Uninfected chick	HeLa ribosomal RNA	4.0

[a] Hybridization between ^{32}P-labeled REV 70 S RNA and uninfected chick DNA in vast excess was performed essentially as described in Table 1. C_0t values of 2×10^4 mole sec/liter were achieved in all cases. The percent hybridization was corrected for the intrinsic ribonuclease-resistance of the RNA and nonspecific interaction of viral RNA with calf thymus DNA (11%). Each experiment represents an average of duplicate samples.

[b] Competition–hybridization was performed by including 5 μg of unlabeled REV 70 S RNA, RSV 70 S RNA, or HeLa ribosomal RNA in the hybridization mixtures described in footnote *a*.

Estimation of the Number of REV-Specific DNA Genome Equivalents in Infected Cells

Figure 4 illustrates the kinetics of hybridization between an excess of uninfected and REV-infected chick cell DNA and radioactive REV 70 S RNA plotted as a function of C_0t. Approximately 75% of the viral RNA formed ribonuclease-resistant hybrids with REV-infected chick cell DNA at C_0t values of 3×10^4 mole sec/liter. Similarly, relatively high C_0t values are also required for the reassociation of unique-sequence chick cell DNA presumably present at a frequency of one copy per haploid genome (Britton and Kohne, 1968; Neiman, 1972). Although one must take into account the apparent differences between the rates of RNA–DNA hybridiza-

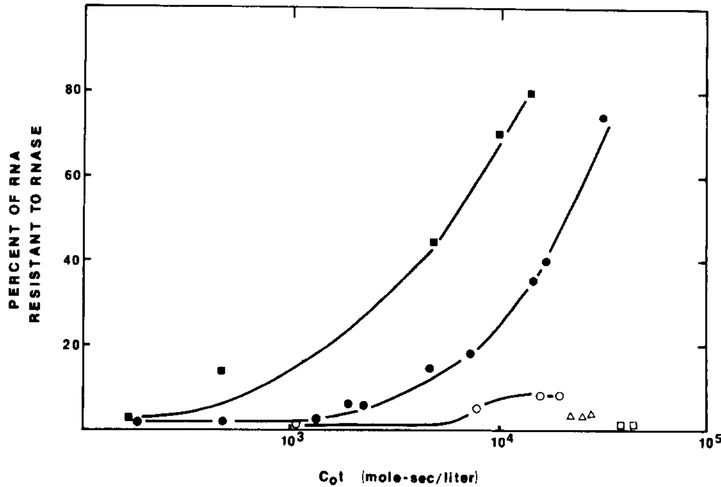

FIG. 4. Kinetics of hybridization of ^{32}P-labeled REV 70 S RNA with uninfected and REV-infected chick embryo fibroblast DNA. ^{32}P-labeled 70 S RNA from either REV or RSV (2,000 cpm, 0.5 ng) was incubated at 68° in a solution containing 0.6 *M* NaCl and various concentrations of an excess of DNA (160 μg/ml–1lmg/ml) to achieve the desired C_0t values for a 50 hr incubation period. Extent of hybridization was determined by pancreatic ribonuclease digestion as described in Table 1. The extent of hybridization was corrected for background ribonuclease-resistance and nonspecific interaction of viral RNA with calf thymus DNA (11%). Symbols: ●, REV 70 S RNA hybridized with REV-infected chick embryo fibroblast DNA; ○, REV 70 S RNA hybridized with uninfected chick embryo fibroblast DNA; △, REV 70 S RNA hybridized with uninfected duck embryo fibroblast DNA; ■, RSV 70 S RNA hybridized with RSV-infected duck embryo fibroblast DNA; □, RSV 70 S RNA hybridized with uninfected duck embryo fibroblast DNA.

tion and DNA–DNA reassociation (Bishop, 1972; Melli *et al.*, 1972), the observed rate of hybridization of REV-RNA and infected chick DNA suggests that the REV-specific DNA sequences are present at a low frequency per cell. To directly test this possibility we have compared the rate of hybridization of REV 70 S RNA and REV-infected chick cell DNA with that of RSV 70 S RNA and RSV-transformed duck cell DNA which contains approximately four to six copies of RSV-specific DNA sequences per diploid genome (Varmus, 1973). The data in Fig. 4 indicate that REV 70 S RNA hybridizes more slowly to REV-infected chick cell DNA than does RSV 70 S RNA to RSV-infected duck cell DNA, suggesting that the REV sequences are present in relatively low copy number. An approximate estimate of the frequency of REV-specific DNA sequences present in REV-infected chick cells is one to two copies per diploid cell.

DISCUSSION

We have previously demonstrated that virions of REV lack an endogenous RNA-directed DNA-polymerase activity characteristic of most, if not all, avian RNA tumor viruses (Kieras and Faras, 1975). Apparently the virion-associated DNA polymerase is unable to utilize the viral 70 S RNA as template for the *in vitro* synthesis of DNA. In view of these observations it was of considerable importance to determine whether reverse transcription of the REV genome occurs *in vivo* during infection of chick cells with REV.

In this report we present data indicating that DNA synthesis, presumably REV-specific, is required for the establishment of infection of chick cells with REV. Furthermore, we have directly detected REV-specific sequences in DNA extracted from chick embryo fibroblasts infected with REV. These results are analogous to those recently reported on another member of the REV group by Temin and co-workers (Temin and Kassner, 1974; Kang and Temin 1974) and lend support to the involvement of a DNA intermediate in the replication of REV. Additional support was obtained by the demonstration that DNA extracted from REV-infected cells was in-

fectious (Cooper and Temin, 1974).

Although the specific hybridization techniques employed in our studies provide one with the best minimal estimate to date of the fraction of the viral genome present in cell DNA (Bishop, 1972; Melli *et al.*, 1972; Neiman, 1972, 1973; Varmus *et al.*, 1974), we have been unable to anneal more than 75–80% of the REV-RNA to infected-cell DNA. This limitation is apparently a function of several factors including the unfavorable rate of RNA–DNA as opposed to DNA–DNA annealing (Bishop, 1972; Melli *et al.*, 1972), the thermal lability of the RNA, and the prolonged incubation times required to achieve high C_0t values (Melli *et al.*, 1972). We have also attempted to determine the relative frequency at which the REV-specific DNA sequences are present in infected cells by comparing the rates of hybridization of REV and RSV 70 S RNA to REV-infected chick and RSV-infected duck cell DNA, respectively. These studies suggested that REV-specific DNA sequences are present in relatively low frequency (one to two copies) in infected cells. This is a rough estimate since the precise determinations of frequency number by such procedures are difficult because of the possible variability of annealing rates among RNA species (Strauss and Bonner, 1972). Furthermore, we cannot exclude the possibility at this time that the low estimate of copy number in REV-infected cells reflects the inability to infect every cell with virus. Therefore, a more extensive analysis of the quantitation of REV-specific DNA sequences is currently under investigation in our laboratory.

Although most of the REV genome can be detected in infected cells, only 4–5% of REV-specific sequences can be detected in uninfected chick cells. This is in contrast to members of the ALSV complex which appear to share approximately 30–40% of their genome with uninfected chick cell DNA under similar conditions of hybridization (Neiman, 1972, 1973; Schincariol and Joklik, 1973; Varmus *et al.*, 1974; Table 1). Nevertheless, these low levels of REV-specific endogenous DNA sequences present in uninfected chick cell DNA have been observed consistently with several different preparations of chick DNA. Since the bulk of the REV 70 S RNA genome utilized in these experiments exhibits no detectable sequence homology with either avian leukosis or sarcoma virus 70 S RNA (Fig. 1) and no competition of REV-RNA for duplex formation was observed with RSV 70 S RNA (Table 2), it is unlikely that the REV-specific sequences found in uninfected chick cell DNA are related to avian RNA tumor viruses. Kang and Temin have observed slightly more homology between the TDSNV genome and uninfected chick cell DNA (Kang and Temin, 1974). Although we presently cannot explain the reason for these differences, they may at most, reflect minor sequence differences between the genomes of REV(T) and TDSNV.

We do not, as yet, know the status of these REV proviral DNA sequences in infected cells. We are currently attempting to determine whether they are integrated into the chick cell genome DNA, as is the case with the avian RNA tumor viruses (Varmus *et al.*, 1973). Although the REV DNA-polymerase is incapable of transcribing the REV genome *in vitro*, it appears from these studies that the process of reverse transcription does in fact occur *in vivo*. It will be of considerable interest to determine if a host cell function is required to facilitate the transcription of the REV genome subsequent to infection of cells with REV.

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