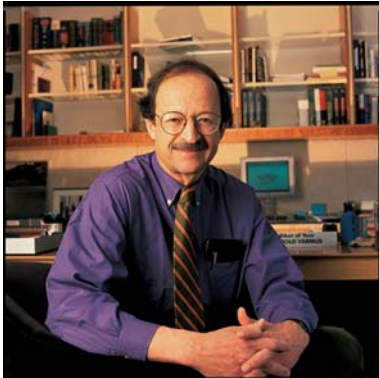


The Classic

Integration of Deoxyribonucleic Acid Specific for Rous Sarcoma Virus after Infection of Permissive and Nonpermissive Hosts (RNA tumor viruses/reassociation kinetics/duck cells)

Harold E. Varmus, Peter K. Vogt, J. Michael Bishop



Dr. Harold Varmus is shown. Photograph by Arnold Adler; provided courtesy of Memorial Sloan-Kettering Cancer Center Office of Public Affairs.

Embodying a renaissance of interests and aptitudes, Harold Varmus (b. 1939) has led the modern molecular genetic revolution, directed our national health research endeavor, and propelled Memorial Sloan-Kettering Cancer Center to new levels of accomplishment. As a physician and son of a physician, Dr. Varmus has always recognized that the patient is the reason for what we do. He has been a constant advocate for translational research. After studying philosophy and English literature he devoted himself to science, achieving the highest level of success. Dr. Varmus made important contributions to our understanding of glucocorticoid action, hemoglobinopathies, hepatitis B and, most importantly, retroviruses. This culminated in his receipt of the Nobel Prize for Medicine in 1979 with Michael Bishop for their fundamental observations that cancer is a disease of genes.

He spent two decades at UCSF before moving to Washington/Bethesda to spearhead the National Institutes

of Health. He was the Director of the NIH from 1993–1999 and started the historic doubling of the Institutes' budget.

Returning to academia, he was appointed President of Memorial Sloan-Kettering Cancer Center. Dr. Varmus continues an active research program focusing on the genetics of lung and other cancers, and increasingly provides expert commentary on issues of societal importance such as “What It Means to be Human”, the environment, and the role of religion in our society. He has championed open access to scientific articles as one of the most important strategies to accelerate medical progress.

This month's Classic article is unique in that it established a new paradigm for our understanding of sarcoma, and cancer in general. It was one of many important contributions that furthered our understanding of cancer and led to the Nobel Prize for Varmus and Bishop. Beyond the article's concrete scientific value, its historical significance was that it spawned the “west coast tumor virus cooperative.” This group exemplified how modern collaborative research could successfully probe the molecular genetics of cancer. In this particular instance, the collaboration led to the discovery of the *c-src*-proto-oncogene.

Orthopaedic surgery has not been blessed with its own Nobel Laureate. Potential recipients such as Sir John Charnley passed away before being recognized for contributions such as total hip replacement that made the greatest impact on patient function and quality of life. We are relegated to bask in the reflected glory of researchers in related fields. Sarcoma genetics, of strong orthopaedic interest, have been pivotal in understanding the action of retroviruses and reverse transcriptase. These basic observations have blossomed into our general understanding of cancer. This issue of Clinical Orthopaedics and Related Research deals with a

variety of important molecular genetic advances in musculoskeletal cancers using a variety of modern techniques. These investigations could only have been accomplished because of the groundbreaking, Nobel Prize-winning work by Varmus and Bishop. Reproduction of their article pays tribute to their accomplishment, highlights how important sarcoma biology has been to the elucidation of fundamental cancer mechanisms, and challenges orthopaedic investigators to aspire to the highest level of scientific accomplishment.

John H. Healey MD, FACS

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Communicated by Norman Davidson, July 11, 1973.

Abstract A relatively simple but stringent technique was developed to detect the integration of virus-specific DNA into the genomes of higher organisms. In both permissive (duck) and nonpermissive (mammalian) cells which normally contain no nucleotide sequences specific for Rous sarcoma virus, transformation by the virus results in the appearance of DNA specific for Rous sarcoma virus covalently integrated into strands of host-cell DNA containing reiterated sequences. Early after infection of mouse or duck cells by Rous sarcoma virus, unintegrated DNA specific for the virus can be demonstrated.

Abbreviations

RSV Rous sarcoma virus
C₀t Product of DNA concentration and time
gs Group-specific antigen
SV40 Simian virus 40

Introduction

Replication of and transformation by RNA tumor viruses probably proceed by way of a DNA intermediate [1]. Although the site of synthesis of oncornavirus DNA remains in dispute [2, 3], it is generally assumed that the DNA is synthesized immediately after infection by virion-associated RNA-directed DNA polymerase, integrated into the host-cell genome, and subsequently transcribed into viral RNA. Direct physicochemical observation of this sequence of events, however, has not been reported. Integration of genomes of DNA tumor viruses into host-cell DNA has been demonstrated by cosedimentation of viral DNA with high-molecular-weight cell DNA in alkaline sucrose gradients [4]. We have presented a preliminary report of a less cumbersome and more stringent method for

detecting integrated viral DNA in cells [5]. In this communication, we present details of this method and some examples of situations in which either integrated or unintegrated Rous sarcoma virus (RSV)-specific DNA is found.

The integration test is based upon the observation by Britten and coworkers that the vast majority of high-molecular-weight DNA extracted from higher organisms contains reiterated sequences [6, 7]. They showed that when unsheared cell DNA is incubated to C₀t values at which repeated, but not unique, sequences reassociate, “networks” of DNA are formed (Fig. 1). These networks can be separated from the remainder of the DNA by sedimentation. We now report that testing the DNA in networks for virus-specific nucleotide sequences by molecular hybridization constitutes a relatively simple assay for integration of viral DNA. The finding of virus-specific DNA in networks demonstrates covalent linkage of viral DNA to strands of cell DNA containing repeated sequences, and thus its integration into the host genome.

We used this approach to study RSV-specific DNA in two different host cells after infection by RSV: (i) duck-embryo fibroblasts, which contain no detectable endogenous RSV-specific nucleotide sequences, are readily transformed by

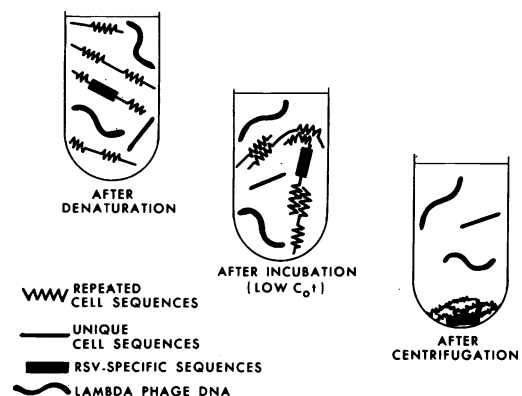


Fig. 1 Preparation of network DNA from unsheared cellular DNA.

RSV, and support its replication; and (ii) mammalian cells, particularly BALB/c 3T3 cells, which also possess no endogenous RSV-like genes, but are inefficiently transformed, and do not support replication of RSV. In both cases we show that under suitable circumstances unintegrated oncornavirus DNA can be demonstrated early after infection and that fully transformed cells have one or more copies of integrated virus-specific DNA.

Materials and Methods

Viruses and Cells

BALB/c 3T3 cells and 3T3 cells transformed by the B77 strain of avian sarcoma virus (B77/3T3) were described [8]. XC cells, derived from a tumor induced in a rat by the Prague strain of RSV [9], were kindly provided by Dr. Jay Levy. Normal Pekin duck cells prepared from 12- to 14-day embryonated eggs (purchased from Reichardt Duck Farm, Petaluma, Calif.) and RSV-transformed duck cells were grown in Medium 199 (Grand Island Biologicals Inc.) supplemented with 10% tryptose phosphate broth, 5% calf serum, and 1% heat-in-activated chick serum.

Prague strains (subgroups B and C) of RSV were grown in *gs*⁻ chick-embryo fibroblasts. B77 strain of avian sarcoma virus was generally grown in cultures of chick-embryo cells not tested for *gs* antigen. B77 virus was also grown in *gs*⁻ chick cells after rescue from B77-transformed 3T3 cells by cultivation with chick cells.

Short-Term Infections

Virus-containing medium from RSV-transformed chick-cell cultures was clarified by low-speed centrifugation (2000 rpm for 10 min), and 2–4 ml was added to each of several petri dishes (100 mm in diameter) containing about 3×10^6 normal duck or 3T3 cells. Polybrene (Aldrich) was present at 2 µg/ml, and multiplicities of infection varied from 0.1 to 5 focus-forming units (assayed with chick cells) per cell. After incubation at 38° for defined periods, the medium was removed by suction, and the cells were scraped into 4 ml of “DNA buffer” [containing 20 mM Tris · HCl (pH 8.0)–10 mM EDTA–0.1 M NaCl], collected by centrifugation (2000 rpm, 10 min), and resuspended in DNA buffer at 5 to 10×10^6 cells per ml.

DNA Purification

The technique has been described [8] and was designed to avoid loss of nonintegrated viral DNA and preserve high-molecular-weight DNA. In brief, cells were lysed with

0.5% Na dodecyl sulfate, incubated for at least 1 hr at 37° with 500 µg/ml of Pronase (digested for 2 hr at 37°), and gently extracted twice with phenol at room temperature. Two volumes of ethanol were added and, after 1–2 hr at –20°, the precipitated nucleic acids were centrifuged at 2000 rpm for 20 min, suspended in 10 mM Tris · HCl (pH 7.4)–10 mM EDTA, and treated for at least 3 hr at 37° with 100 µg/ml of pancreatic ribonuclease (Worthington; boiled for 10 min to inactivate DNase). Two phenol extractions at room temperature were followed by extensive dialysis against 15 mM NaCl–1.5 mM Na-citrate. After determination of absorbance at 260 and 280 nm, a portion of the DNA was sheared at 50,000 lbs/inch² for assay of virus-specific DNA and the remainder was used for preparation of networks.

Network Preparation

Unsheared cellular DNA in 15 mM NaCl–1.5 mM Na-citrate was denatured by heating at 100° for 3 min, then incubated at 2–4 Å units in a 68° bath in the presence of 0.6 M NaCl for 1 hr (Fig. 1). The solutions were chilled to 4° and centrifuged for 15 min at 40,000 rpm in a Spinco fixed-angle 40 rotor at 4°. The supernatant was removed and the visible pellet was vigorously suspended in 15 mM NaCl–1.5 mM Na-citrate. The amount of DNA entering networks was determined by reading the A_{260} of the supernatant and of the resuspended DNA pellet. These fractions were then sheared at 50,000 lbs/inch², precipitated with ethanol, and assayed for RSV-specific sequences. Occasionally, sheared supernatant DNA was freed of oligonucleotides by passage over a Sephadex G-50 column equilibrated with 0.6 M NaCl–10 mM EDTA–1 mM Tris · HCl (pH 7.4).

Assay for RSV-Specific DNA

We have described our procedure for determining the number of copies per diploid cell of RSV-specific DNA [5, 8, 10]. The method, originally devised by Gelb et al. for detection of SV40 DNA [11], is based upon the ability of virus-specific sequences present in unlabeled cell DNA to accelerate the reassociation of labeled, double stranded, virus-specific DNA synthesized by RSV polymerase. Reassociation reactions were performed in 100 µl containing heat-denatured, ³H-labeled polymerase product; denatured, sheared cell DNA; and 0.4 M phosphate buffer (equimolar NaH₂PO₄ and Na₂HPO₄). Mixtures were incubated at 68° for up to 80 hr and samples were periodically removed into 10 mM phosphate buffer for analysis of secondary structure by elution from hydroxyapatite (Biorad). Raw data were corrected according to the behavior of single- and double-stranded standards and

plotted against C_0t for the labeled DNA. Reduction in $C_0t_{1/2}$, caused by the unlabeled cell DNA is proportional to the amount of virus-specific DNA per cell. Determination of the number of complements of labeled virus-specific sequences and of diploid-cell genomes in each reaction mixture permits calculation of the copy number for those viral sequences per diploid cell. For example, if a reaction mixture containing one set of labeled viral sequences per genomic complement of cell DNA demonstrates a reduction of the $C_0t_{1/2}$, by half, the doubling of the rate indicates a doubling of the concentration of relevant sequences, or one copy per diploid cell. The accuracy of copy numbers is influenced by the moderate heterogeneity of polymerase products, by determination of the concentration of labeled DNA from its specific activity, and by uncertainties involved in estimating sequence complexity of polymerase products from their reassociation kinetics [8, 12]. In addition, redundancy or absence of a minor class of viral sequences in the cell may be overlooked with a technique that measures principally the $C_0t_{1/2}$; however, no data in support of this possibility have been obtained.

In experiments reported here, all assays were performed with the slowly reassociating fraction of double-stranded product synthesized by B77 virus polymerase (slowly reassociating DNA) [8, 12]. The $C_0t_{1/2}$ of this fraction is about 1×10^{-2} mol-sec/liter, and 10–90% of its reassociation occurs over two logarithmic units of C_0t . On the basis of comparison with DNAs of known composition, we estimate the complexity of this fraction to be about 6×10^6 daltons and thus representative of at least 30% of the 70S RNA genome of RSV [8, 12]. Because of our unpublished evidence that shared sequences comprise at least 85% of 70S RNAs and the slowly reassociating DNAs of B77, Schmidt-Ruppin, and Prague strains of RSV, we have used B77 slowly reassociating DNA in all experiments reported here.

Determination of Size of Cell DNA in Alkaline Sucrose Gradients

Cell DNA was denatured in 0.6 M NaOH at 37° for 15 min and layered on top of a 5–20% alkaline sucrose gradient (containing 0.9 M NaOH–1 M NaCl–10 mM EDTA). Sedimentation values were determined in relation to lambda bacteriophage DNA or SV40 DNA kindly provided by Dr. H. Boyer.

Results

Preparation of DNA Networks

Unsheared mammalian DNA prepared as outlined in *Methods* has a major high-molecular weight component

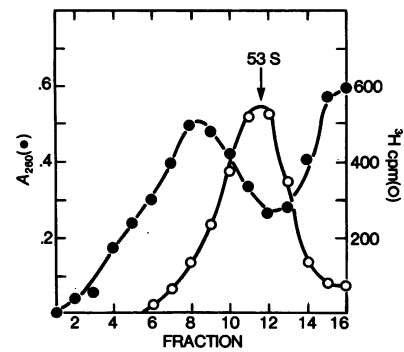


Fig. 2 Alkaline sucrose gradient of unsheared DNA from XC cells. 100 μ g of DNA extracted from XC cells was mixed with [3 H]DNA from lambda bacteriophage, incubated at 37° for 15 min in 0.6 M NaOH, and layered onto a 5–20% sucrose gradient containing 0.9 M NaOH–1 M NaCl–10 mM EDTA. After centrifugation at 64,000 rpm for 75 min at 15°, fractions were collected, measured for absorbance at 260 nm (●), and precipitated with trichloroacetic acid for determination of radioactivity (○).

sedimenting at 80–110 S in alkaline sucrose gradients (Fig. 2). This size corresponds to a molecular weight of about 30 to 50 $\times 10^6$ of single-stranded DNA. Similarly purified DNA from duck cells sediments at 40–60 S (data not shown). In accord with an earlier report [5], we found that generally 75–95% of DNA prepared from mammalian cells participates in network formation, whereas 60–75% of duck cell DNA forms networks. The variations for any one kind of DNA in the fraction forming networks are presumably due in part to random nicking of the DNA with production of fragments containing insufficient reiterated sequences. In addition, viscous preparations of high-molecular-weight DNA often retain appreciable amounts of oligonucleotides incapable of reassociating. These are apparent in alkaline sucrose gradients (Fig. 2) and can be considerably reduced by dialyzing the DNA against 150 mM NaCl–15 mM Na-citrate before network formation or removed at a later step by passing the fraction of DNA that does not form networks (supernatant DNA) through a Sephadex G-50 column.

The relative reduction in the fraction of duck DNA forming networks may also be influenced by the amount or distribution of repeated sequence DNA in the avian genome. Although estimates of repeated sequence fractions are approximate and readily affected by reaction conditions, comparison of published values for mammalian and avian genomes suggests that avian cells may contain as little as one-third as much reiterated DNA as mammalian cells [13]. In general, we observed about 20–25% of chick or duck DNA reassociating at low C_0t values (under 100 mol-sec/liter) at which 40% of mammalian DNA has reassociated, supporting the possibility that less extensive network formation with duck DNA may be due in part to a decrease in content of repeated sequence DNA.

We investigated the possibility that networks of high molecular-weight DNA might trap unintegrated viral DNA and cause it to appear in the pellet after centrifugation. Labeled DNA extracted from lambda bacteriophage (molecular weight 30×10^6) or SV40 virus (form II, molecular weight 3×10^6) OSC was added to unsheared DNA from B77/3T3 cells and networks were prepared. Although over 80% of the cell DNA (as measured by absorbance) was found in the pelleted network fraction, 85–90% of either species of viral DNA remained in the supernatant (Fig. 1). Therefore, the cell DNA remaining in the supernatant fraction was about 50-fold richer in viral DNA than was the network fraction. Since even a 2-fold enrichment of the supernatant fraction is detectable with the reassociation kinetics assay, it is clear that these minor degrees of trapping of viral DNA cannot account for results observed with RSV-infected cell DNA.

Integrated Viral DNA in Stably Transformed Cells

We used the network technique to assess integration of RSV-specific DNA into the genome of three types of cells transformed by RSV: (1) XC cells, widely-used progeny of a tumor produced in a rat line with Prague strain of RSV [9]; (2) B77/3T3 cells, derived from a single clone of BALB/c 3T3 cells transformed by the B77 virus [8]; and (3) duck-embryo fibroblasts transformed by infection with Prague C strain of RSV.

In contrast with other lines of RSV-transformed mammalian cells we have studied, the XC cell line has a large number of copies of DNA homologous to the sequences represented in the slowly reassociating fraction of RSV polymerase product. Sheared DNA from these cells

markedly accelerates the reassociation of labeled slowly reassociating DNA (Fig. 3A); the calculated copy number is 20 per diploid cell. When the preparation of unsheared XC cell DNA analyzed in the alkaline sucrose gradient in Fig. 2 was used to form networks, both the network DNA and the 25% of the DNA remaining in the supernatant possessed the same capacity as the total cell DNA to influence reassociation of slowly reassociating DNA (Fig. 3B). This result demonstrates that most, if not all, of the viral DNA present in the XC cells is covalently integrated into strands of cell DNA containing repeated sequences. It is probable that viral DNA remaining in the supernatant is integrated into strands of DNA that failed to join the networks on the basis of size or sequence composition. However, we cannot exclude the possibility that a small fraction of viral DNA is not integrated.

Similar results have been obtained with the B77/3T3 cell line. These cells, like most of the other RSV-transformed mouse, rat, or hamster cells we have studied [5, 8], contain one to two copies of RSV-specific DNA sequences per diploid cell. Network and supernatant DNA from these cells are equally efficient in accelerating the reassociation of RSV slowly reassociating DNA (Fig. 4). Their effect is similar to that of unfractionated DNA upon the reassociation of slowly reassociating DNA [5, 8], and the supernatant fraction shows no enrichment, as would be expected if unintegrated viral DNA were present. As for XC cells, we interpret these results to mean that most or all of the RSV-specific DNA is covalently integrated in B77/3T3 cells, and that the virus-specific DNA in the supernatant fraction is linked to randomly broken strands of cell DNA.

Duck cells, unlike other permissive avian cells we have tested [10], are free of endogenous RSV-specific nucleotide sequences, as tested by reassociation kinetics (Fig. 5) and

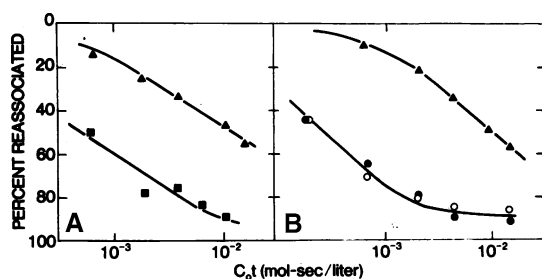


Fig. 3A–B Integration of RSV DNA in XC cells. Reassociation kinetics of slowly reassociating [^3H]DNA prepared with B77 virus polymerase (3.8 ng/ml, specific activity 5000 cpm/ng) were followed during incubation in the presence of calf-thymus DNA (\blacktriangle , $C_{0t_{1/2}} = 1 \times 10^{-2}$ mol-sec/liter), unfractionated XC DNA (\blacksquare , $C_{0t_{1/2}} = 5 \times 10^{-4}$), networks formed with XC DNA (\bullet , $C_{0t_{1/2}} = 2.8 \times 10^{-4}$), or DNA remaining in the supernatant after formation of networks with XC DNA (\circ , $C_{0t_{1/2}} = 2.8 \times 10^{-4}$). (A) 3.1 mg/ml of cell DNA; (B) 4.0 mg/ml of cell DNA. Reactions in B were correspondingly faster in the presence of XC DNA. Experiments not shown here demonstrate simple second-order kinetics throughout annealing in the presence of XC DNA.

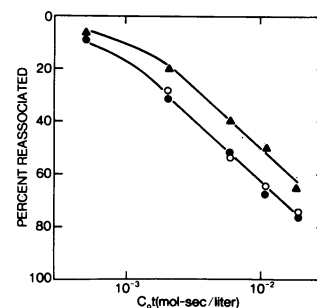


Fig. 4 Integration of RSV DNA in B77-transformed 3T3 cells. B77 slowly reassociating DNA (4.7 ng/ml) was reassociated in the presence of 3.1 mg/ml of calf-thymus DNA (\blacktriangle) or DNA from network (\bullet) and supernatant (\circ) fractions prepared from B77/3T3 cell DNA. $C_{0t_{1/2}}$ is reduced from 1×10^{-2} to 5×10^{-3} mol-sec/liter by DNA from B77/3T3 cells. Over 80% of the cell DNA entered networks.

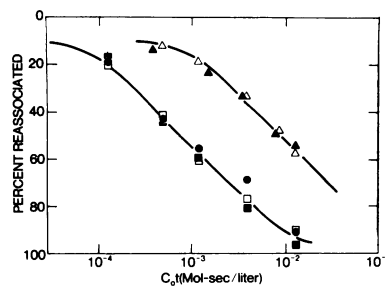


Fig. 5 Integration of RSV DNA in transformed duck cells. B77 slowly reassociating DNA (3.5 ng/ml) was reassociated in the presence of 2.4 mg/ml of calf-thymus DNA (▲); normal Pekin duck-embryo DNA (Δ); or unfractionated (●), network (■), or supernatant (□) DNA from duck cells transformed by Prague C strain of RSV. DNA from transformed cells reduced the $C_0t_{1/2}$, from 9×10^{-3} to 8×10^{-4} mol-sec/liter.

by hybridization of large excesses of duck DNA with labeled RSV 70S RNA (unpublished observations).

After transformation by B77 virus or by Prague C strain of RSV, duck cells contain four to six copies of RSV DNA sequences per diploid cell [14]. Figure 5 displays the equal effects of unfractionated, network, and supernatant DNA from RSV transformed duck cells upon the reassociation of slowly reassociating DNA, consistent with the presence of four copies of RSV sequences per diploid cell. Since only two-thirds of the duck DNA participates in network formation, we can conclude with certainty only that at least two-thirds of the viral DNA is covalently linked to cell DNA. It seems likely, however, that the remainder is integrated into strands incapable of forming networks.

Unintegrated DNA in Recently Infected Cells

The ease of testing for integration of viral DNA with the network assay facilitates study of the kinetics of integration. We therefore measured the appearance of newly synthesized virus-specific DNA in networks prepared from freshly infected cells. These experiments further validate the network test as a measure of integrated DNA, since they demonstrate that viral DNA may be present in a nonintegrated state soon after infection.

This situation is portrayed most dramatically in 3T3 cells 12 hr after infection with RSV (Fig. 6). Despite the relatively low efficiency with which mammalian cells are transformed by RSV [15], we discovered that readily detectable levels of RSV DNA may be observed in 3T3 cells within 12 hr after infection with RSV rescued from RSV-transformed mammalian cells [14]. No DNA synthesis is observed after infection by various RSV strains grown only in chick cells [14]. This phenomenon is probably related to the increased efficiency of transformation of rat cells by B77 virus observed with virus rescued from transformed rat cells [15]. In the experiment in Fig. 6, B77

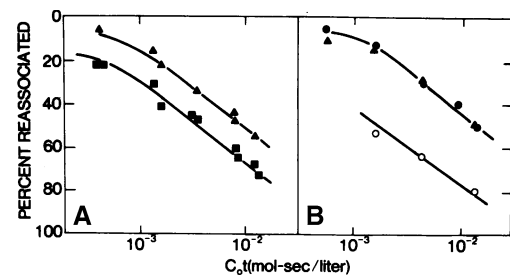


Fig. 6A–B Unintegrated RSV DNA in acutely infected 3T3 cells. Growing cultures of BALB/c 3T3 cells were infected with B77 virus rescued from B77-transformed 3T3 cells. 12 hr later, the DNA was extracted and a portion was used to prepare networks. B77 slowly reassociating DNA (3.6 ng/ml) was then reassociated in the presence of (A) unfractionated DNA (■, 4 mg/ml); (B) network DNA (●, 4 mg/ml), DNA remaining in the supernatant after network preparation (○, 3.1 mg/ml); (A and B) calf-thymus DNA (▲, 4 mg/ml). Unfractionated 3T3 DNA lowers the C_0t , from 1.0×10^{-2} to 3.5×10^{-3} mol-sec/liter; supernatant DNA reduces the $C_0t_{1/2}$, from 1.3×10^{-2} to 1.7×10^{-2} mol-sec/liter.

virus rescued from B77-transformed 3T3 cells was added to growing cultures of 3T3 cells at a multiplicity of about 1 focus-forming particle (as assayed on chick cells) per 3T3 cell. After 12 hr, cell DNA was extracted, a portion was sheared and tested for its content of RSV DNA (Fig. 6A), and the remainder was subjected to network formation. About 80% of the DNA formed networks. The network and supernatant DNAs were then also sheared and assayed for RSV DNA (Fig. 6B). The acceleration of reassociation observed in Fig. 6A indicates that an average of about 0.8 copies of RSV slowly reassociating DNA sequences are synthesized per diploid cell within 12 hr after infection. Since the efficiency of transformation of mammalian cells by RSV is low [10^{-3} – 10^{-5} relative to transformation of avian cells [15]], and the number of RSV DNA copies in transformed clones of mammalian cells is only one to two [5, 8], it is likely that a large percentage of cells are infected (as seen by synthesis of RSV DNA) but not transformed. Detection of RSV DNA in clones of infected nontransformed cells will be required to confirm this hypothesis. Networks prepared from this DNA are devoid of detectable RSV-specific sequences (Fig. 6B). The supernatant DNA however, contains 4.1 copies of slowly reassociating DNA per weight of DNA from one diploid cell. The supernatant DNA is, therefore, 5-fold enriched for RSV DNA compared to the unfractionated DNA. The degree of enrichment is consistent with the fraction of cell DNA (20%) remaining in the supernatant. We conclude that little, if any, RSV DNA is integrated 12 hr after infection of 3T3 cells. This conclusion is strengthened by the additional observations that integration of RSV DNA occurs in these cells (as shown by the appearance of RSV DNA in network fractions) within 22 hr after infection and

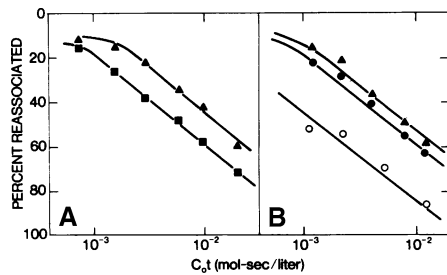


Fig. 7A–B Unintegrated DNA in acutely infected duck cells. Growing secondary cultures of duck-embryo fibroblasts were infected for 10 hr with Prague C strain of RSV. The influence of 2 mg/ml of unfraktionated DNA (■, A), network DNA (●, B), and supernatant DNA (○, B) upon the reassociation of B77 slowly reassociating DNA (5.1 ng/ml) was then determined. Normal duck-embryo DNA (▲) served as a control. Unfraktionated DNA reduces the $C_0t_{1/2}$ from 1.2×10^{-2} to 6×10^{-4} mol-sec/liter; the $C_0t_{1/2}$ is lowered from 1×10^{-2} to 8×10^{-3} by network DNA and to 2×10^{-3} mol-sec/liter by supernatant DNA.

that the viral DNA persists in an integrated state for at least six subsequent generations [14].

Similar results have been obtained with infected duck cells. Using either B77 virus or Prague C strain of RSV, we can detect virus-specific DNA within 3 hr after infection [14]. No viral DNA is found 16 hr after infection with Prague B strain of RSV to which duck cells are not susceptible [14, 16]. Using the network assay to follow integration of viral DNA, we observe increasing amounts of RSV DNA present in networks from 6–24 hr after infection at which point the networks contain the four to six copies found in fully transformed cultures (Fig. 5). 10 hr after infection of normal duck-embryo fibroblasts with Prague C strain of RSV, about 0.8 copies of slowly reassociating DNA sequences have been synthesized per diploid cell (Fig. 7A). 0.25 Copies have been integrated, as computed from the experiment in Fig. 7B, and the supernatant fraction is over 2-fold enriched for RSV DNA in comparison with unfraktionated DNA. These data indicate that about one-third of the RSV DNA synthesized during the first 10 hr of infection is integrated into the duck genome.

Discussion

The ease, efficiency, and stringency of the network technique recommend it for the study of integration of both DNA and RNA tumor virus genomes. The degree of trapping of nonintegrated DNA by the networks is small, as shown by trapping controls with labeled viral DNAs and by the detection of unintegrated viral DNA early after infection (Figs. 6 and 7). The single potential limitation of the technique resides in the possibility that viral DNA might, under some circumstances, be integrated specifically into

an uncommon region of the cell DNA that was devoid of reiterated sequences and did not form networks. In this case, integrated viral DNA would not be present in the networks, and the supernatant DNA would be enriched for viral sequences. However, in practice we have always found network DNA from transformed cells to be as rich in viral sequences as the unfraktionated DNA, and the supernatant DNA to be no further enriched (Figs. 3–5). It seems probable that the supernatant DNA is composed principally of a random selection of the cell genome that has been sufficiently nicked during extraction to prevent entry into networks.

In this report we illustrated the usefulness of the network assay for integrated viral DNA in mammalian and duck cells infected with Rous sarcoma virus. In contrast to studies of RSV-specific DNA in the natural host for RSV (chick cells), these experiments are not complicated by the presence of endogenous RSV genetic information [10]. Consequently, the viral DNA detected appears in the cell after infection, presumably as a product of the intracellular activity of virus-associated RNA-directed DNA polymerase. The assay, therefore, constitutes an assay for *in vivo* polymerase activity and may permit detailed analysis of the mechanism of RNA-directed DNA synthesis in the host cell.

Although the experiments reported here establish that RSV-specific DNA is integrated in transformed duck and mammalian cells and that the kinetics of integration can be studied in detail, the extent of representation of the viral genome in our hybridization probe, slowly reassociating DNA, may be as little as 30% [17]. Therefore, we cannot claim that the entire viral genome is integrated as DNA in the host cells we have studied. Moreover, we cannot describe the location or specificity of the integration sites. We conclude that there is now substantial support for the notion that the genomes of both RNA and DNA tumor viruses are integrated into host-cell DNA, but the sites and mechanism for integration remain unknown.

Acknowledgments Dr. Robin A. Weiss suggested the duck cell as a permissive host likely to be free of endogenous RSV-specific DNA. Ms. Suzanne Heasley and R. Howard provided excellent technical assistance. The work was supported in part by the American Cancer Society, California Division (Grant no. 608) and by NIH Contract 71-2147 of the Special Virus Cancer Program of the National Cancer Institute. H.E.V. is a recipient of Research Career Development Award (CA 70193) from the National Cancer Institute.

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