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Characterization of an Endogenous Primate Virus: Extent of Nucleic Acid Homology with Various DNAs

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Abstract

A [^{3}H]cDNA viral probe has been prepared from the baboon endogenous virus isolate designated M7 via the endogenous reverse transcriptase reaction. The M7 [^{3}H]cDNA viral probe reassociated extensively and with high thermal stability to both its original viral genomic RNA template and DNA of baboon origin. Hybridization of this M7cDNA probe with baboon (*Papio cynocephalus*) cellular DNAs obtained from established cell lines and frozen tissues detected approximately 40 to 95 copies of viral-related DNA sequences per haploid genome. Approximately 10 to 20 times fewer copies of viral-related sequences were detected in the DNAs extracted from cell lines exogenously infected with a baboon endogenous virus than were detected in DNAs of *P. cynocephalus* origin. No correlation was found to exist between the level of active virus production by established baboon cell lines and the number of viral-related sequences detected in their cellular DNAs. In addition, less than one copy of viral-related sequences per haploid genome was detected in cellular DNA of mouse, normal human, and leukemic human origin. All hybridization reactions were conducted under conditions of low stringency in order to detect even distantly related sequences. The ratio of cellular DNA to M7cDNA used in all experiments was sufficient to detect at least one copy of viral-related sequences per haploid genome.

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I. Introduction

A variety of environmental agents are known to cause abnormalities in normal cells. When these abnormalities affect a change in normal cellular heredity patterns, the daughter cells produced may be less responsive to normal regulatory controls. The resulting progeny do not behave as highly integrated and differentiated members of parental tissue and are considered to be transformed cells. Uncontrolled growth of these transformed cells within a tissue or an organ results in either a benign or a malignant neoplasia. Benign neoplasms or tumors consist of highly differentiated cells which differ only slightly in morphology and behavior from their tissue of origin. Those tumors showing a marked deviation in cellular morphology and deficiency of differentiation are termed malignant.

The mechanisms by which these environmental agents induce and/or increase the frequency of cellular transformation remain unknown. One major common feature of all transformed cells is that all progeny are transformed, *as though* there has been a stable heritable change in the cell's genetic information. This observation provided a basis for the theory that environmental agents act on a molecular level to alter the genetic information carried within a normal cell.

The isolation and identification of a group of RNA-containing viruses from naturally occurring tumors in animals provided an opportunity to study tumor virus and host cell interactions on a molecular level. These RNA tumor viruses possess the ability to cause cellular transformation leading to malignancy *in vivo* and to cellular de-differentiation *in vitro*. Studies of the viral replication cycle indicate definite interaction with cellular DNAs. Both the quality and quantity of interaction between virus

and host cell DNAs have been explored through nucleic acid hybridization techniques. As a model system, the RNA tumor viruses have proven invaluable in the study of how viral genetic information could be introduced into normal cell populations to promote a stable, heritable change in cellular genetic material.

A mechanism by which these RNA tumor viruses multiply in exogenously infected cells has been proposed (Temin, 1964). Viral replication via a DNA intermediate or provirus which then becomes integrated into the host cell's chromosomal DNA would explain the stable, heritable change observed in cell lines exogenously infected with this group of viruses. Some mammalian species have also been identified as normally carrying viral-specific genetic information within their cellular DNA (Lieber et al., 1973). That is, viral genetic information is present or endogenous in the cellular DNA of all members of the species regardless of an obvious lack of overt virus production.

In this thesis, nucleic acid hybridization techniques were employed to examine the extent of baboon endogenous virus (M7) proviral information contained in various cellular DNAs. The amount of baboon endogenous proviral information carried in cellular DNA of baboon origin will be compared to the amount found in heterologous host cells intentionally infected with and producing a baboon endogenous virus isolate. Secondly, the number of sequences of proviral DNA present in an active virus-producing endogenous cell system will be compared to the number found in non-virus producing baboon cells. Lastly, hybridization reactions were conducted to explore the extent of homology, if any, between an endogenous baboon virus isolate and cellular DNAs obtained from normal and leukemic humans.

II. Literature Review

The history of RNA tumor virus etiology began at the turn of the century when two Danish scientists, Ellerman and Bang (1908), demonstrated that an agent smaller than bacteria could cause leukemia in chickens. An additional three years were to pass before Rous (1911) published his now classic paper describing a virus as the causative agent for production of a malignant sarcoma in a chicken. Despite independent confirmations of these results, the discovery of both leukosis and sarcoma inducing viruses in an avian system had little impact on the scientific community. It was not until Bittner (1942), a biologist working with inbred strains of mice, observed the first transmission of a virus contained in mother's milk and correlated it with the increased incidence of mouse mammary gland carcinomas in offspring that a number of scientists were drawn by both greater publicity and aroused curiosity to characterize these viruses and the diseases which they caused. Nine years later, Gross (1951) provided the evidence for a mammalian counterpart to the avian model for leukemia with his successful passage of the condition from one mouse to another via a cell free extract. The positive association of these viruses with malignant disease finally prompted an all out search for both new tumor viruses and for new techniques in tissue culture, electron microscopy, immunology, and molecular biology with which to characterize them and their modes of action.

Physical and biochemical characterization of mammalian retraviruses

The RNA-containing tumor viruses have been subjected to a number of classifications and are variously described in the literature as leukoviruses, oncornaviruses, B-type, and C-type RNA viruses. A much broader family name, retraviruses, based on their mode of replication via a

reverse transcriptase, has recently been proposed (Parks et al., 1973) and will be utilized for this discussion. The retraviruses are commonly sub-divided according to their morphology as determined by electron microscopy and are referred to as A, B, C, and D particles (Bernhard, 1960; de Harven, 1968; Dalton et al., 1975; Schidlovsky, 1978). Although all essential features of replication appear to be the same throughout the retravirus group, within the limits of present knowledge only some of the viruses having B- or C-type morphology demonstrate oncogenic potential when inoculated into recipient animals. Since the retravirus to be characterized in this paper is a mammalian C-type, the remaining features to be presented will concentrate on this sub-group.

According to the model recently proposed by Bolognesi (1978), uncleaved viral structural precursor molecules, possessing specific recognition sites for both viral envelope constituents and viral RNA, align themselves at a budding site along the interior cell membrane. Particle maturation is initiated through proteolytic cleavage of these viral components with concomitant association of molecules into virion substructures. The virus particles appear to complete their formation while budding through the plasma membrane (de Harven, 1968). The resultant retravirus C-type particles are essentially spherical, have diameters ranging from 100-150 nm, and possess a centrally located spherical nucleoid (Gross, 1970; Sarkar et al., 1972). The mature virion structure consists of an outer envelope composed of a single membrane lined with glycoproteins. This outer envelope surrounds an inner core of icosahedral symmetry bounded by a major internal viral protein. A ribonucleoprotein nucleoid is contained within this internal core (Nermut et al., 1972).

Concentrated preparations of mature virions may be made by centrifugation of virus-containing fluids to equilibrium in a sucrose density gradient. Virion banding occurs at a density of 1.16-1.18 g/cc. (Robinson et al., 1965; Valentine and Bader, 1968). The overall chemical compositions of the C-type retraviruses are quite similar. Mature virions are composed of approximately 30% lipid, 62% protein, 6% hexose and less than 2% RNA (Bonar and Beard, 1959; Quigley et al., 1971). Lipid constituents are present almost exclusively in the viral envelope and are quite similar to those of the host cell plasma membrane from which the viral envelope is derived (Quigley et al., 1971). Two high molecular weight glycoproteins are associated with the envelope of mature virions and are responsible for the type-specific antigenicity of these particles. Several smaller polypeptides which are associated with the nucleoid are responsible for two classes of group-specific antigenic activity: (a) an intraspecies activity specific to all virus isolates from a single-species, and (b) an interspecies activity which is common to viruses isolated from different species (Oroszlan et al., 1971).

These C-type retraviruses possess a 60-70S single-stranded RNA (m.w. ~6 X 10^6 daltons) which is presumed to be the viral genome (Robinson et al., 1967). As determined by electron microscopy (Kung et al., 1975) and under denaturing conditions (Duesberg, 1968), the actual structure of this 60-70S RNA molecule consists of two identical, non-covalently linked, 35S subunits. In addition to this apparently diploid genome, several smaller species of RNA appear complexed in the 70S molecule (Bishop et al., 1970a; Bishop et al., 1970b; Emanoil-Ravicovitch et al., 1973; Erikson and Erikson, 1970). Some of these include varying species of 4S RNAs, attached by hydrogen bonds, that serve as functional transfer

RNAs (Erikson and Erikson, 1971; Dahlberg et al., 1974). Recently, a number of laboratories have reported that some retraviruses contain small amounts of DNA which sediments at 7S and which by hybridization experiments appears to be homologous to cellular DNA (Levinson et al., 1970; Rinan and Beaudreau, 1970; Rokutanda et al., 1970; Biswal et al., 1971; Varmus et al., 1971). The function of this DNA is as yet unknown, and its presence is considered bymany investigators to be a contaminant introduced during virion isolation and purification. A variety of enzymes with associated activities ranging from nucleic acid synthesis to carbohydrate metabolism have been isolated from preparations of purified virions (Temin and Baltimore, 1972; Mizutani and Temin, 1971). The majority of these enzymes, with one well-documented exception, appear to be cellular contaminants either introduced during particle maturation by adsorption onto viral nucleoids and envelopes, or trapped within vesicles which co-purify with complete virions (Bader, 1975). The single exception, an RNA-dependent DNA-polymerase (reverse transcriptase) is known to be viral specific (Temin and Mizuntani, 1970; Baltimore, 1970).

Reverse transcriptase activity is located within the virus particle and has been isolated from core fractions of detergent treated virions (Coffin and Temin, 1971). The reverse transcriptase of avian C-type viruses is composed of two polypeptide subunits of 65,000 and 105,000 daltons (Kacian et al., 1971). A single polypeptide, possessing a molecular weight of approximately 70,000 daltons, has been identified as the reverse transcriptase of mammalian viruses (Tronick et al., 1972). The presence of inhibitors of DNA polymerase activity in crude viral lysates makes accurate determinations of overall purification and yield of reverse transcriptase from virion samples difficult. Kacian et al. (1971)

reported recovering approximately .3-1% of the starting protein and estimated 5-17 enzyme molecules per avian myeloblastosis virion. A second activity, ribonucleaseH, which degrades the RNA moiety from DNA-RNA hybrids but which does not degrade either single or double-stranded RNA, has been found associated with reverse transcriptase in purified preparations (Mölling et al., 1971). Since ribonuclease H activity copurifies with AMV reverse transcriptase, both activities are presumed to be located on a single molecule having a molecular weight of 65,000 (Grandgenett et al., 1973). The failure to find an RNA-dependent DNA polymerase in uninfected, non-virus producing cells seems to indicate that this enzyme is coded for by a viral, not a cellular gene (Baltimore et al., 1973; Weissbach et al., 1972). Further, defective virions which are incapable of infecting any known cell, lack reverse transcriptase suggesting both a viral origin for this enzyme and an important role for its activity during infection (Hanafusa and Hanafusa, 1971). Additional support for the viral origin of reverse transcriptase and its essential role in virus replication is provided by the studies of Linial and Mason (1973). Their characterization of temperature-sensitive mutants of Rous sarcoma virus indicated that the defective viral gene function was required to initiate but not maintain virus replication, and that the genetic lesion involved the primary structure of the reverse transcriptase molecule.

Replication of mammalian C-type retraviruses

The first step in reproduction of these viruses, as with any virus, involves the interaction of viral and host cell membranes leading to attachment. Recent evidence suggests that this attachment is due to viral envelope glycoproteins which are capable of binding to specific receptor sites either on or in the cell membrane (Rand et al., 1975).

Once attached, the virion must somehow penetrate the cell membrane and shed its protective coat thereby introducing its own genetic material into the host cell. Studies conducted on mammalian viruses (Sarkar et al., 1970; Miyamoto and Gilden, 1971) provided evidence for three possible mechanisms by which viral penetration may occur: (1) an actual fusion of viral and cellular membranes releasing the viral core into the cytoplasm, (2) passage of intact virions into the cytoplasm, and (3) engulfment of virions into vesicles which may later coalesce with vesicle membranes or combine with lysosomes. The exact mechanism or combination of mechanisms remains unknown. However, within one to two hours post infection, intact parental viral RNA and whole virions have been located in the peri-nuclear cytoplasm (Dales and Hanafusa, 1972). Autoradiography studies conducted on newly infected cells also indicated an increased level of DNA synthesis within the cytoplasm (Hatanaka et al., 1971). Further isolation and characterization of pulse-labeled cytoplasmic DNA at 2 hrs. post-infection revealed that the newly synthesized DNA could hybridize to viral 70S RNA with an efficiency of 60% (Robin et al., 1974). The mechanism by which this viral specific DNA is synthesized, and the proposed model for its integration into the cellular genome to insure the vertical transmission of viral information to all progeny, have been two of the major accomplishments in the research conducted on retraviruses.

Early data had established that cell cultures having a minimal rate of DNA synthesis due to contact inhibition, incubation in serum-free medium, exposure to x-irradiation, or treatment with chemical inhibitors of DNA synthesis (e.g. cytosine arabinoside) routinely exhibited resistance to infection by retraviruses (Temin and Rubin, 1959; Bader, 1966; Nakata and Bader, 1968; Temin, 1968; Temin, 1964; Bader, 1965). One

particular drug, Actinomycin D, was proven effective both at blocking initial infections and at decreasing virion production in retravirus producing cells (Temin, 1963; Vigier and Golde, 1964). In contrast, Actinomycin D had no effect on other RNA-containing viruses which were previously characterized as reproducing via an RNA replicative form (Reich et al., 1961). Since Actinomycin D was known to act specifically to inhibit DNA transcription (Temin, 1963; Vigier and Golde, 1964), Howard Temin (1964) proposed an hypothesis which called for a DNA intermediate form in the replication cycle of retraviruses. The provirus, a DNA copy of the viral RNA genome, would explain the inhibition of viral replication caused by a sensitivity to both inhibitors of DNA synthesis and DNA transcription. Furthermore, if the DNA provirus became integrated into host cell DNA as bacterial prophages do, future progeny would inherit viral information at mitosis. This hypothesis would therefore account for the stable vertical transmission of retraviruses within a host cell population.

The existence of a DNA intermediate form pre-supposes the synthesis of a DNA copy by an enzyme which utilizes the viral RNA genome as a template. In order to function during an initial infection, this enzyme would have to pre-exist in the host cell or be an actual component of mature, extracellular virions. The discovery of an RNA-dependent DNA polymerase, now commonly referred to as a reverse transcriptase, occurred about six years later. Howard Temin (1970) and David Baltimore (1970), researchers in two independent laboratories, announced that virus particles subjected to treatment with low concentrations of nonionic detergent in the presence of deoxynucleoside triphosphates were capable of incorporating these DNA precursors into a DNA product. The requirements for

this endogenous reaction include the presence of all four deoxynucleoside triphosphates, manganese or magnesium ions, a reducing agent, and a pH of 7 to 8 (Temin and Baltimore, 1972). That the newly synthesized complementary DNA (cDNA) represents a negative strand or copy of the positive 70S RNA template was demonstrated by (1) the sensitivity of the endogenous reaction to ribonuclease treatment, (2) the close association of the newly-formed DNA with the viral genome, and (3) the positive hybridizations of nascent DNA to 70S RNA isolated from virions (Spiegelman et al., 1970; Manly et al., 1971).

DNA polymerases in general are known to be primer-dependent, that is, capable of chain elongation but incapable of initiating new chains of DNA (Goulian et al., 1968). The viral RNA-dependent DNA polymerase is no exception in that in the endogenous reaction it elongates a primer RNA molecule through incorporation of available deoxynucleotides to copy the viral 70S RNA template (Smoler et al., 1971; Hurwitz and Leis, 1972). Denaturation of the product of this endogenous reaction and centrifugation to equilibrium in cesium sulfate gradients reveals that the early DNA product remains covalently attached to a 4S transfer-like RNA which serves as the primer molecule (Verma et al., 1971). Recent investigations have identified one such primer to be a virion tryptophan transfer RNA molecule (Taylor and Illmensee, 1975). As reviewed by Temin and Baltimore (1972), in addition to copying the endogenous viral genome, the viral DNA polymerase will also copy a variety of exogenous templates. With the exception of divalent cation preferences, maximum synthesis of a product from exogenous natural DNA was found to have the same requirements as reported for the endogenous reaction. Spiegelman et al. (1970) were first to demonstrate that synthetic RNA and DNA homopolymers could

act as exogenous templates for the viral DNA polymerase. When oligodeoxyribonucleotides, which function as primers, were complexed to either synthetic DNA or RNA homopolymer duplexes, both the initial rate of synthesis and overall yield of DNA were found to exceed those observed with natural DNA templates. Since other known DNA polymerases are relatively inactive with poly(c)-oligo(dG), this template-primer combination has proven most effective and specific in detecting viral DNA polymerase activity.

Identification of a primer site near the 5' end of the viral 70S RNA aided in the formulation of a model for DNA synthesis from this endogenous template. Since DNA synthesis has been characterized as proceeding from the 5' to 3' direction, with RNA transcription occurring in the 3' to 5' direction, a contact between the 5' and 3' ends of the genomic 35S RNA subunits has been postulated (Weinberg, 1977). Identical sequences have been found at both the 5' and 3' ends of the viral 35S subunits (Schwartz _et al_., 1977; Coffin and Haseltine, 1977). This discovery favored the existence of a circular DNA intermediate since transcription initiated at the primer site and proceeding toward the 5' end might continue at the 3' end of the same or other 35S RNA subunit due to the presence of complementary bases on the newly synthesized cDNA strand. The cDNA synthesized would therefore be associated with both ends of the 35S RNA template, linking these ends to produce a transient circular form.

Although the actual mechanism of formation of a double-stranded DNA molecule is unknown, its presence as a product of the endogenous reaction discussed above is well-documented (Taylor _et al_., 1972). Presumably these DNA duplex molecules could evolve via digestion of the template

RNA strand in the initial RNA-cDNA hybrid by the reverse transcriptase associated ribonuclease H activity (Haseltine and Baltimore, 1976). This action might expose a free 3'-hydroxy end group of RNA molecules hydrogen bonded to the cDNA. The complex could serve as an effective primer site for additional DNA synthesis. Whether DNA second strand formation occurs through the action of reverse transcriptase and/or another enzyme remains unknown. However, evidence for the synthesis of a proviral DNA duplex molecule existing in the cytoplasm of infected host cells has been reported (Gianni et al., 1975; Guntaka et al., 1976; Ringold et al., 1977).

Closed circular forms of double-stranded DNA were first demonstrated in Rous sarcoma virus infected cells (Varmus et al., 1975) and more recently have been detected in nuclei of mouse embryo fibroblasts shortly after infection by Rauscher murine leukemia virus (Kakefuda et al., 1977). These circular DNA forms were found only in the nuclear portion of cell extracts, were not detected in uninfected cells, and were viral in origin as demonstrated by hybridization to viral specific cDNA probes. Time studies involving the detection of cytoplasmic double-stranded DNA molecules and nuclear circular forms have been conducted in newly infected cells (Guntaka et al., 1976). The kinetics of accumulation of these proviral DNA forms suggest that the non-closed cytoplasmic double-stranded DNA may be a precursor to the circular nuclear form. The mechanism of transport of proviral DNA forms from cytoplasm to nucleus, where integration could occur is not known.

A number of studies provide evidence that the integration of virus-specific DNA sequences into cellular DNA in newly infected cells does occur. Highly repeated sequences contained in the DNA of higher organisms reassociate quite rapidly after total cellular DNA preparations have been denatured (Britten and Smith, 1969). These reannealed sequences of

high molecule weight DNA are readily sedimented and isolated from the total DNA preparation by centrifugation. Hybridization data reveals that up to 12 hours post infection of 3T3 cells with Rous sarcoma virus, the majority of viral specific sequences are not sedimentable. At later time periods, the viral specific sequences are found associated with the pellet of high-molecular weight DNA and are thus presumably integrated (Varmus et al., 1973). Additional studies conducted on virus-producing chick embryo cells provided evidence for an alkali-stable, covalent linkage between viral and cellular DNA (Markham and Baluda, 1973). Also, in situ hybridizations performed by Loni and Green (in press) showed a marked increase of autoradiographic grains associated with the interphase nuclei over a 24 hour period post synchronous infection of mouse and rat cells with the Harvey strain of MSV(MLV). The mechanism of integration of proviral DNA into host cell chromosomes remains as yet unknown, but the nucleases and ligases which would be required are certainly present in the host cell.

Once integration of proviral DNA has been achieved, the remaining steps in the replication cycle can occur. Proviral DNA sequences are transcribed by existing RNA polymerases during normal cellular replication. The newly synthesized positive (+) strand of viral RNA, acting as a messenger RNA, attaches to polyribosomes to initiate synthesis of viral structural precursor proteins. Viral RNA and protein precursors are then sequestered near the cell membrane where assembly occurs as the particle buds from the cytoplasmic membrane. The complete virion is now capable of infecting new host cells to initiate another replicative cycle. The infected host cell, from which the virion was released, and all of its progeny, will continue to replicate new virions as long as a minimal rate of DNA synthesis is maintained.

Endogenous C-type retraviruses

Up to now this discussion has centered upon the events following infection of a host cell by a retravirus. Type C virogenes (DNA sequences proven homologous by hybridization to the RNA genome of known retraviruses) have been detected in somatic cell DNA of supposedly "virus-free" cell lines derived from chicken, mouse, hamster, rat, pig, cat, and baboon tissues (Baluda, 1972; Lieber and Todaro, 1975). Production of complete infectious virus particles from these lines may be achieved by treatment with certain chemical inducing agents, co-cultivation with permissive cell lines derived from heterologous species, or may appear to be a totally spontaneous event. The sudden production of these "endogenous" viruses has been attributed to the activation and/or derepression of viral sequences which are an integral part of the host species chromosomal DNA (Todaro, 1976). In general, cells from all the animals within each of these species carry the endogenous viral specific sequences in their DNA, and when placed in tissue culture will resist an exogenous infection by the homologous endogenous C-type virus. As yet, the role of these endogenous viruses in inducing neoplasia *in vivo* or cellular transformation *in vitro* remains unclear. However, when an endogenous virus is used to infect cells of a heterologous species, viral replication proceeds as previously described.

To date, type-C retraviruses have been isolated from the mammalian, avian, and reptilian classes of vertebrates. The spontaneous appearance of complete, infectious particles in animals of certain mammalian species and in cultured cells derived from these animals as well as the heritable nature of some murine leukemia viruses demonstrated by *in vivo* studies, led Huebner and Todaro (1969) to formulate a general theory of carcinogenesis. The virogene-oncogene hypothesis suggests that the information

required for production of type-C retraviruses is transmitted genetically from parent to progeny along with other cellular genes (Todaro and Huebner, 1972). The virogene is thought to be normally repressed, but may with activation lead to partial or total viral expression. This concept differs from Temin's provirus theory in that it requires preexisting discrete oncogenic sequences rather than integration of viral information into cell DNA via infection by these viruses. The end effect remains the same in that once integrated, the expression of the viral sequences falls under cellular control. Subsequent experimentation has lent support to both theories as mechanisms by which type-C retraviruses may be maintained in animal populations and contribute to the natural occurrence of cancer.

Immunological relatedness of mammalian C-type retraviruses

The establishment of sub-groups within the mammalian C-type retraviruses has been based primarily on studies of immunological relatedness of viral proteins. Mammalian viruses isolated to date have been classified on the basis of a major protein associated with their nucleoid. This structural protein has a molecular weight of approximately 30,000 daltons (p30) and has proven to be a genetically stable marker showing no alterations with passage in heterologous hosts (Gilden *et al.*, 1974). The mammalian p30's possess antigenic determinants which demonstrate both interspecies reactivities (cross-reactivities between species) and species-specific reactivities (cross-reactivities within species). Type specificity within groups has also been established (Gilden, 1975). The six groups established through species determinants are: (1) murine, (2) rat, (3) hamster, (4) feline leukemia, (5) gibbon ape, woolly monkey, Mus caroli, and pig endogenous virus, and (6) feline endogenous (RD114) and baboon endogenous virus (Gilden, 1977). Cross-reactivities have

recently been observed within groups (5) and (6) demonstrating that two distinct species are able to share "species" determinants.

Specific antisera prepared against the virion associated reverse transcriptase has been utilized in enzyme inhibition studies to group these viruses via relatedness of reverse transcriptase. In general, this data supports previous groupings based on p30 antigenic determinants (Gilden, 1977). Antisera prepared against murine viral reverse transcriptase inhibits mouse, feline, hamster, and rat viral polymerase activities. A second cross-reactive group is composed of the gibbon ape, woolly monkey, and endogenous pig viral enzymes which appear unrelated to other viral reverse transcriptases. The third group is represented by the RD114 and endogenous baboon isolates. None of the above groups of mammalian C-type viral reverse transcriptases have demonstrated any cross-reactivity with other known retraviruses. However, it has recently been reported that anti-woolly monkey reverse transcriptase antisera inhibited an enzyme activity purified from human leukemic cells (Todaro and Gallo, 1973). Biochemically, this enzyme activity resembles a viral reverse transcriptase and has not been isolated from normal blood lymphocytes even after phytohemagglutinin stimulation. These results, suggestive of viral information associated with a human leukemic condition, await confirmation by other researchers.

The discovery of endogenous viruses presented a unique model system in which to study discrete sets of cellular genes which code for viral specific products. Efficient propagation of endogenous viruses has been best demonstrated in host cells of a heterologous species, thus making the detection of homologous viral sequences most feasible. Detection and quantitation of homologous type-C viral sequences and determinations

of taxonomic relationships are currently being conducted using nucleic acid hybridization technology. Most recently these techniques have been utilized to characterize an endogenous virus isolate from a baboon.

Baboon endogenous virus isolates

Electron microscopy of placentas obtained from normal baboons (*P. cynocephalus*) at various stages of pregnancy first revealed the presence of C-type particles in this primate species (Kalter *et al.*, 1973). Subsequent direct co-cultivation of first trimester placental tissue, obtained by Caesarian section from an 11-year-old *P. cynocephalus*, was performed with various mammalian cell lines known to be permissive for endogenous mouse and cat retraviruses (Benveniste *et al.*, 1974b). Culture fluids from indicator cell lines of dog, rhesus, bat, and human origin were screened for viral reverse transcriptase activity. Viral replication was detected in all cultures between 5 and 13 weeks post co-cultivation. Indicator cultures examined by electron microscopy showed C-type viral particles present in intracytoplasmic vacuoles and budding from the cellular membranes. Designated M7, the baboon virus isolate was grown in quantity for further biochemical characterization.

The M7 virus isolate has met all criteria for classification as a C-type retravirus. Morphologically M7 exhibits the typical budding pattern and spherical central nucleoid characteristic of C-type virions (Benveniste *et al.*, 1973). Isopycnic centrifugation of M7 virions yields particle banding at a density of 1.16-1.18 g/cm^{-3} (Sherr *et al.*, 1974). M7 reverse transcriptase activity elutes from G-100 Sephadex at the position characteristic for a 70,000 molecular weight mammalian C-type polymerase (Sherr *et al.*, 1974; Ross *et al.*, 1971). On phosphocellulose, this polymerase activity elutes at 0.3 M KCl and demonstrates the

characteristic divalent cation preference for Mn^{++} over Mg^{++} (Sherr et al., 1974; Scolnick et al., 1970). When the M7 reverse transcriptase was tested for cross-reactivity with enzymes isolated from known C-type retraviruses, only the antiserum against RD-114 polymerase was inhibitory (Sherr et al., 1974). Antisera prepared against the viral polymerases of a woolly monkey virus or Rauscher murine leukemia virus, although showing a partial cross-reaction with each other, demonstrated no inhibition of the M7 viral polymerase. This data indicated that the M7 and RD-114 reverse transcriptases possess cross-reactive sites not detectable in other mammalian viral polymerases.

Competitive radioimmunoassay systems were established for analysis of the M7 p30 viral protein (Sherr et al., 1974; Sherr and Todaro, 1974a). Both the M7 and RD-114 viral lysates failed to cross-react with the p30s from Rauscher murine leukemia virus and woolly monkey virus. However, the M7 viral lysate was quite efficient in displacing the test antigen in an assay system specific for RD-114 p30. This cross-reactivity indicates that although not identical, the viral p30 proteins of M7 and RD-114 may share some common sites. Secondly, although both the M7 and woolly monkey viruses were isolated from primates, their p30 viral proteins demonstrate little relationship to one another.

Additional studies were conducted to explore the relationship of the RD-114 virus to M7. RD-114 was originally isolated from a human rhabdomyosarcoma cell line (RD) which had been passaged in a cat (McAllister et al., 1972). This virus has been demonstrated to be closely related to other endogenous cat viruses, CCC group, by nucleic acid hybridization and competitive radioimmunoassay techniques (Benveniste and Todaro, 1973). In a competitive radioimmunoassay system, the M7 virus competes less

effectively for the RD-114 test antigen than these endogenous cat viruses and is therefore easily distinguished (Sherr et al., 1974). The baboon virus may also be distinguished from the RD-114/CCC group of endogenous cat viruses by the inability of M7 virus to replicate in a fetal, feline cell strain FFc60WF.

Nucleic acid homology comparisons using DNA-RNA hybridization techniques were also conducted to establish the relatedness of the M7 virus to other mammalian C-type viruses (Benveniste et al., 1974a). A single-stranded ^{3}H-cDNA copy of the M7 viral RNA was prepared using the endogenous reverse transcriptase activity. The ^{3}H-cDNA product was annealed to viral RNAs of murine, rat, feline, hamster, pig, woolly monkey, and gibbon ape origin. S_1 digests of the RNA-DNA hybrids revealed that all viruses tested, with the exception of RD-114, had less than 2% homology to the ^{3}H-cDNA probe prepared from M7. A 10% homology was observed with RD-114 viral RNA. These results thus supported the earlier immunological data.

Further hybridization studies confirmed M7 as an endogenous virus of the baboon, vertically transmitted from generation to generation. When an M7 ^{3}H-cDNA probe was annealed to DNAs extracted from a normal baboon liver and from the original baboon placenta, complete (100%) homology was observed in both cases (Benveniste et al., 1974a). Obtaining this extent of homology between a viral probe and normal DNA means that viral genomic information must be present or endogenous in all cellular DNA preparations. On the other hand, DNA preparations obtained from dog and pig livers, a murine cell culture producing a mouse C-type virus, and an uninfected fetal canine cell line showed less than 6% homology to the M7 ^{3}H-cDNA viral probe. A feline liver DNA preparation yielded a 15% homology

thereby reflecting relatedness of M7 to the endogenous cat virus, RD-114. In sum, this data indicates that the M7 virus is genetically different from previous mammalian C-type virus isolates and represents the first C-type endogenous virus to be identified in any primate.

DNA sequences related but not identical to M7 have also been found in primates closely related evolutionarily to the baboon (Benveniste et al., 1974a). The Old World monkeys such as the patas, African green, rhesus, and stumptail contain viral-related sequences in their DNA as demonstrated by hybridizing 14 to 34% of the M7 ^{3}H-cDNA probe. Despite a great deal of effort, only one other C-type virus has been isolated from any of these monkeys (Todaro et al., 1978). Using the same probe, <5% of M7 related viral-specific information has been detected in DNA extracted from tissues of more distantly related primates - apes (with the possible exception of chimpanzees), humans, or New World monkeys (spider, woolly, marmoset, and capuchin). The inability to detect more than a 5% homology has been attributed to either the high degree of evolutionary divergence between the baboon type C genetic information and that of distantly related primates, or the introduction of an M7 related virus into the primate stock after the divergence of the Old World monkeys from the apes.

Subsequent to the isolation and characterization of the baboon M7 virus, four additional type C viruses were isolated from tissues of four different animals of the genus Papio (Todaro et al., 1974). These new C-type virus particles have been isolated from baboon lung, kidney, and testicular cells by co-cultivation with indicator cell lines, and they have been shown to be closely related to the initial M7 isolate and to each other by immunological and hybridization criteria, and by viral

host range. The baboon type C viruses therefore represent a distinct new class of endogenous primate retraviruses. As such, this group provides a primate model in which to examine both endogenous viral information expression and transfer. Information gained by application of nucleic acid hybridization technology in this system has proven directly applicable in screening for potential retravirus-host cell interactions in man.

Most recently, a positive association between the baboon endogenous virus group and a human malignant condition (acute myelogenous leukemia) was described (Reitz et al., 1976; Wong-Staal et al., 1976). Initially, an infectious woolly monkey-related type C virus was observed being released from cultured leukemic blood cells (Gallagher and Gallo, 1974; Gallagher et al., 1975). This new virus, HL23V, was successfully transmitted to secondary cells (Teich et al., 1975). Some of the HL23V produced by these infected cells contained a reverse transcriptase and poly(A)-containing 70S RNA closely related to those of the woolly monkey virus. However, some of the secondary virus isolates contained components related to the endogenous baboon virus (Reitz et al., 1976; Teich et al., 1975; Okabe et al., 1976). Previous reports (Strand and August, 1974; Sherr and Todaro, 1974b) described the detection of baboon endogenous virus related proteins in human tissues, but did not detect any nucleic acid relationships. Recent screening of various tissues from leukemic patients by molecular hybridization utilizing a baboon endogenous viral probe has detected the presence of related proviral sequences in both fresh blood cell and spleen cell samples (Wong-Staal et al., 1976). Hybrid analysis based on melting temperature data and reaction extents suggest an evolutionary basis for relationship. Additional studies are

required to detect the existence of a potential human endogenous virus, its role in development of human neoplasia, and its relationship to the established groups of C-type retraviruses.

Nucleic acid hybridization technology

Molecular hybridization experiments may be specifically designed to detect nucleotide sequence homology between nucleic acids from viruses and cells, relatedness among nucleic acids from various viruses, and differences between nucleic acids from normal vs. transformed cells. Detection of hybrid formation between a viral nucleic acid and cellular DNA suggests the presence of virus specific sequences in the cellular genome. Specific viral sequences may be demonstrated by using stringent hybridization conditions and measurements of the thermal stability of the duplex detected. This technique has been used repeatedly, e.g. the M7 characterization previously described, in the detection of endogenous viruses.

The formation of a duplex between specific ^{3}H-cDNA probes and viral nucleic acids obtained from different viruses has provided information regarding their genetic relatedness. Since differences observed in final reaction extents and thermal stabilities of the hybrids aided in delineating evolutionary divergence patterns, discrimination of viruses by molecular hybridization provides a powerful tool for their classification. Nucleic acids from newly isolated viruses may be easily screened and quickly categorized based on possession of nucleotide sequences related to known viruses. Detection of hybrid viruses, i.e. viruses possessing one species of nucleic acid but composed of another species protein coat, may also be accomplished by this technique.

The detection of differences between nucleic acids from normal vs. transformed cells is most commonly explored by hybridizing an excess of

cellular nucleic acids to radioactively labeled viral nucleic acids. The increase in hybridization values observed with RNA or DNA from infected cells over that of normal cells, and the high melting temperature of the hybrids formed with nucleic acids from these infected cells is suggestive of virus specific duplex formation. Host cells expressing endogenous viral information may also be assayed for the number of copies of the integrated viral genome and in some cases for their specific chromosomal location.

Although proven a useful tool in the detection and molecular analysis of endogenous viruses, both the conditions of nucleic acid hybrid formation and the technique utilized for hybrid detection should be carefully selected since misuse of either contributes directly to experimental error. For example, incubation of a reaction mixture at open criteria for hybrid formation (conditions which would allow reannealing of imperfect complementary strand pairs) followed by a detection technique based on stringent criteria (detection of only highly base-paired duplexes) may not yield the same data as an identical incubation conducted under stringent conditions.

Stringent hybridization conditions (moderate ionic strength, high temperature) dictate that only the more stable complexes be formed. Stringent conditions of hybrid formation and detection should be utilized if data on exact complementarity between two nucleic acids is desired. For confirmation of data suggestive of genetic similarity between distant species, open criteria for both hybrid formation and detection would allow the greatest potential for identification. In all cases, the purity of the nucleic acids utilized in the reactions and the inclusion of all appropriate controls relative to the specificity of reactions must be considered.

Hybridization performed in solution may be any of three types: DNA with RNA, DNA with DNA, or RNA with RNA. One or both nucleic acids may be radioactively labeled for ease of detection. Briefly, single-stranded nucleic acids are incubated under conditions which promote specific annealing of complementary polynucleotide strands (neutral pH, moderate ionic strength, 25°C below the melting temperature, T_m, of the initial duplex molecule). Because reassociation of the polynucleotide strands is a bi-molecular reaction, the kinetics of reannealing are second order, and the rate of the reaction is dependent upon the concentration of strands, their size, their complexity, and the salt concentration of the solution (Wetmur and Davidson, 1968; Britten and Kohne, 1968). The interaction of complementary single-stranded polynucleotide chains to form double-stranded structures is most frequently assayed by hydroxylapatite chromatography or by the use of specific nucleases which differentiate between single- or double-stranded nucleic acids (Kohne and Britten, 1971). A brief discussion of the theory behind the methods utilized for work reported in this thesis follows.

RNA Methodology

Several hybridization methods exist which are useful in searching for C-type viral nucleic acid sequences in cellular DNA preparations. Conventional hybridizations of labeled viral RNA with an excess of cellular DNA have yielded reasonable estimates of the number of viral DNA copies in both normal and transformed cells (Neiman, 1972). Competitive hybridizations between viral nucleic acids yield the fraction of sequences in the radioactive preparation similar to sequences in the unlabeled competing RNA (Gillespie *et al.*, 1973). Most recently a technique employing saturation-competition analyses in RNA-DNA hybridizations has been described (Gillespie *et al.*, 1976).

The technique of RNA-DNA hybridization in solution has the important advantage that the entire viral genome is represented by the labeled probe, but is hampered by the unfavorable rate of reaction of RNA with DNA as compared to DNA with DNA, by requiring large amounts of DNA, by the relative instability of RNA, and most often by the inability to obtain from these viruses the quantities of labeled viral RNA required to perform extensive studies. The most stable subset of complexes formed during an RNA-DNA hybridization reaction may be detected by resistance to specific nucleases and by chromatography on hydroxylapatite. All RNA-DNA complexes formed, regardless of stability, may be detected by retention on nitrocellulose filters. The most meaningful results are frequently obtained using a combination of these detection methods.

Although an initial attempt was made to utilize RNA-DNA hybridizations in parallel to support DNA-DNA studies, both the inability to obtain adequate amounts of labeled viral RNA and its relative instability once obtained made this approach unfeasible. The majority of the studies conducted in this thesis utilized DNA-DNA hybridization in solution.

DNA Methodology

The DNA-DNA hybridization reactions conducted employed a single-stranded ^{3}H-cDNA transcript of the viral RNA and preparations of native DNA isolated from various cell lines. The complementary DNA (cDNA) negative (-) strand is synthesized in an endogenous reaction. Whole virus preparations are exposed to detergent in the presence of all four deoxynucleoside triphosphates, one of which is radioactively labeled. Transcription of the viral genome occurs by the action of the virion reverse transcriptase. The principal limitation of this approach is that the cDNA products of the endogenous reaction may not represent the viral

genome in a 1:1 fashion. This limitation has been somewhat circumvented by addition of Actinomycin D to the synthesis reaction mixture. Reports indicate that the cDNA synthesized under these conditions is a complete and almost uniform copy of the entire viral genome (Garapin et al., 1973).

The ^{3}H-cDNA product is usually isolated by co-sedimentation with 70S RNA to assure both specificity and freedom from contaminating nucleic acid sequences. The specificity of the ^{3}H-cDNA in a hybridization reaction is assured by conducting back hybridizations to purified viral RNA and by checking the thermal stability of the duplex formed. Well matched RNA-DNA hybrids dissociate sharply at a T_m of 80-88° C in a solution of approximately 0.2 M salt. Only the formation of a duplex involving this viral specific ^{3}H-cDNA probe and a single strand of cellular DNA containing complementary viral nucleic acid sequences would be detected within the total population of cellular DNA sequences present during the reaction. The method chosen for detection of these viral specific hybrids was hydroxylapatite chromatography.

Hydroxylapatite (HAP) is an insoluble form of calcium phosphate. The mechanism of nucleic acid binding to HAP is not well understood, but it has the property of separating nucleic acids on the basis of single- or double-strandedness. HAP is frequently used to assay DNA reassociation (Britten and Kohne, 1968). At low phosphate ion concentrations (10-30 mM PB) both single- and double-stranded nucleic acids are absorbed to HAP, but at intermediate concentrations (0.12-0.14 M PB) single strands are eluted. Therefore, if a cDNA-cell DNA reaction mixture is loaded onto an HAP column at 50° C in 0.14 M phosphate buffer, only the hybrids formed during the reaction will be retained on the column. Hybrid formation may then be detected by a stepwise thermal elution to

95° C or by stepwise salt elution to 0.4 M PB, and by monitoring the fractions obtained for the presence of the ^{3}H-cDNA viral probe.

The HAP system offers the advantages of easy detection, separation, and recovery of reassociated and nonreassociated fractions plus the additional characterization and fractionation of the hybrids formed by a thermal chromatography analysis. One may also monitor total DNA reassociation and RNA-DNA hybridization with this single technique. This proves quite valuable in thermal stability analysis profiles of viral RNA-cDNA hybrids since an internal standard (usually an unrelated, alternatively labeled, well-matched hybrid) may be introduced into the reaction mixture prior to column loading as a check on column performance.

Once the presence of sequences complementary to viral ^{3}H-cDNA has been detected by this method, the extent of proviral representation in cellular DNA preparations may be determined by an analysis of the nucleic acid reassociation kinetics. The reassociation rate of complementary sequences has been proven dependent upon (Kohne, 1970): (1) the monovalent cation (+) concentration, an increase in which decreases the intermolecular repulsion of the negatively charged DNA strands; (2) the reassociation reaction temperature, the optimal being about 25-30° C below the T_m of the DNA reassociation; (3) the size of the DNA fragments, with the rate of reassociation varying directly with the square root of the size; (4) the DNA concentration, which determines the frequency of collisions between reassociating fragments; and (5) the base composition, with higher G + C DNAs reassociating faster.

The reassociation of polynucleotide strands follows bimolecular reaction kinetics (Wetmur and Davidson, 1968; Britten and Kohne, 1968; Kohne and Britten, 1971). The rate of disappearance of single strands

is expressed as,

$$-\frac{dC}{dt} = kC^2 \qquad \text{or} \qquad -\frac{dC}{C^2} = kdt$$

where C is the concentration of single-stranded DNA in moles of nucleotide per liter, t is time in seconds, and k (liters/mole seconds) is a second-order rate constant. The value of k is dependent upon cation concentration, temperature, fragment size, and the sequence complexity of the DNA population. When this term is integrated with the initial conditions of t = 0 and $C = C_0$,

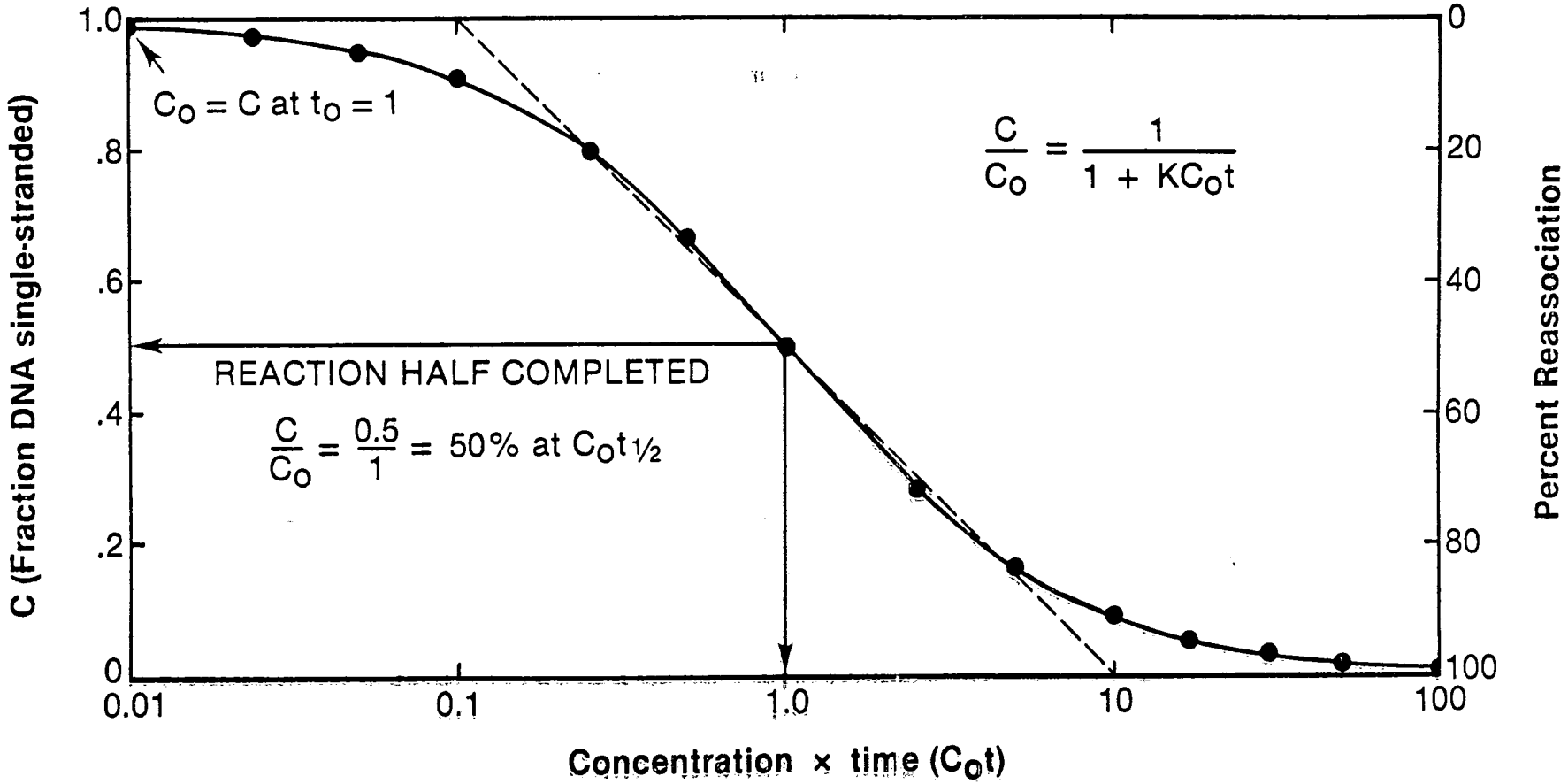
$$\frac{C}{C_0} = \frac{1}{1 + kC_0t}$$

since at time zero all the DNA is single-stranded, C_0 equals the total DNA concentration. It follows therefore, that the fraction of single-stranded DNA remaining in a reassociation reaction ($\frac{C}{C_0}$) is a function of the product of the initial concentration and the time of reaction. Results of a series of timed reactions are frequently plotted to generate a C_0t curve (Figure 1). The $C_0t1/2$ represents the value of C_0t yielding half completion ($C/C_0 = 1/2$) of the reaction. This experimentally derived value is the reciprocal of the second-order rate constant $C_0t_{1/2} = 1/k$. The generation of C_0t curves from experimental data reveals no information on nucleotide sequence arrangements within the genome or on the degree of complementary pairing of two strands, but may reveal the presence of repeated sequences within the DNA population under study.

All prokaryotic genomes studied to date have been shown to contain mostly simple DNA, that is, a DNA containing few repeated sequences. The reassociation of simple DNA approximates the second-order kinetics expected when the DNA contains no repetitive regions in its sequence. Approximately 80% of the DNA reassociation occurs over a two log interval

Figure 1. C_0t curve of an ideal second-order reassociation reaction. Note that 80% of the reaction occurs over a 2-log interval of C_0t. (From R. Britten and D. Kohne, Science 161:529, 1968).

FIGURE 1
IDEAL REASSOCIATION TIME COURSE



in C_0t (Britten and Kohne, 1968). In contrast, reassociation of sheared eukaryotic DNA occurs over a range much greater than two logs. During a fixed reaction time, the repeated sequences (i.e. sequences occuring more frequently within the population) would reassociate faster than unique or non-repeated sequences. The C_0t curves generated in this manner are frequently biphasic or multiphasic, indicative of the presence of sequences occuring at varying frequencies within the total DNA population. A DNA preparation from eukaryotic cells may be fractionated on an HAP column to obtain a class of DNA fragments highly enriched in simple or unique sequences. An independent reassociation of this unique population would result in an essentially single phase or simple C_0t curve.

Since the ^{3}H-cDNA viral probe represents a simple sequence DNA, a calculation of the average number of proviral DNA copies per cell may be achieved by direct comparison of reassociation kinetics observed with unique sequence cellular DNAs. If all hybrid formation and detection methods are made identical, the $C_0t_{1/2's}$ of each simple C_0t curve may be compared. For example, (1) a ^{3}H unique DNA preparation is isolated by HAP chromatography and reacted with a total cellular DNA preparation to yield a $C_0t_{1/2}$ of 3,000 and, (2) a ^{3}H-cDNA viral probe is reacted with the total cellular DNA preparation of viral infected cells to yield a $C_0t_{1/2}$ of 1500. Since the DNA present at a single copy level per haploid genome reassociated with a $C_0t_{1/2}$ of 3,000, the increase in reassociation rate observed with the viral probe ($C_0t_{1/2}$ 1500) suggests the presence of two copies of the provirus per haploid genome. This technique has been used with much success to quantitate proviral representation and was the method chosen to conduct the following studies.

Thesis objectives

The purpose of this study is threefold: (1) An M7 ^{3}H-cDNA viral probe will be utilized to quantitate the extent of proviral representation in cells transmitting endogenous viral information. Both producer and nonproducer cell lines will be examined to determine the relationship, if any, between proviral representation and virus production in an endogenous cell system. (2) The M7 ^{3}H-cDNA viral probe will also be utilized to determine the extent of proviral representation in cells transmitting endogenous viral information vs. an exogenously infected cell system. (3) An attempt will be made to determine the extent of nucleic acid homology between both normal and leukemic human DNA samples and the M7 ^{3}H-cDNA viral specific probe.

III. Materials and Methods

Cells. Virus-infected cells are designated according to an earlier proposal (Gillespie et al., 1975). The abbreviations utilized for the cell lines in this study are: A204, a cultured human rhabdomyosarcoma line developed by Giard et al. (1973) and A204(M7), infected with and producing the baboon endogenous (placental) virus isolate designated M7; 8155(M7), a dog thymus cell line (Benveniste et al., 1974b) infected with and producing M7 virus; HOS(M7), a human osteosarcoma cell line (Rhim et al., 1975) infected with and producing M7 virus; BEF-1, a non-virus producing baboon (P. cynocephalus) embryo fibroblast cell line; BEF-2, a baboon (P. cynocephalus) endogenous virus-producing embryo fibroblast cell line; and a human rhabdomyosarcoma cell line A673 (Giard et al., 1973) infected with and producing a baboon (P. anubis) endogenous virus BAB455-K (Todaro et al., 1974). Cell packs representative of each of the above cell lines were the gift of Mr. Charles Benton, Head, Viral Resources Section, Frederick Cancer Research Center.

Tissue. Approximately 15 grams of liver from a West African baboon (P. papio) was the gift of Dr. John R. Stephenson, RNA Tumor Virus Section, National Cancer Institue at Frederick Cancer Research Center.

Virus. Virus concentrates utilized in this study were provided by the Viral Resources Section, Frederick Cancer Research Center. The baboon endogenous virus, M7, was propagated in and isolated from the A204 cell line. A204(M7) cells (passage 17) were seeded into Corning T150 flasks and grown in RPMI-1640 medium containing 10% fetal calf serum, 2 mM glutamine, 100 units penicillin, and 100 μg/ml streptomycin. Culture fluids were collected at 24 hour intervals, starting at day 8 post seeding, and were clarified. M7 virus was concentrated from clarified supernatants

by continuous flow centrifugation and was banded on sucrose gradients according to the procedure described by Johnson et al. (1976). Pelleted virus was resuspended in TNE (0.01 M Tris pH 7.2 - 0.1 M sodium chloride - 0.001 M ethylenediaminetetraacetic acid) at a 5,000-fold concentration relative to the culture fluid. Virus concentrates were held at 4°C prior to use in this study.

Electron Microscopy. Cell pellet preparations and thin-sectioning were conducted by personnel in the Electron Microscopy Section, Frederick Cancer Research Center. Photomicrographs presented here are courtesy of Mr. Matt Gonda, Head, Electron Microscopy Section. Cell pellets were prepared from monolayers of A204(M7) cells. Cells were fixed in situ with 2.5% glutaraldehyde in 0.5 X Dulbecco's phosphate buffered saline and 0.1 M cacodylate buffer, scraped from flasks with a rubber policeman, and sedimented immediately by centrifugation at 10,000 x g for 10 minutes. The resultant cell pellet was fixed for 1 hour in 1% osmium tetroxide in 0.2 M cacodylate buffer and then en bloc stained for 24 hours in 0.25% uranyl acetate (UA) in 4.5% sucrose buffer. The cells were then dehydrated in graded ethanols, infiltrated with and embedded in Epon 812, and polymerized at 50°C for 24 hours followed by 70°C for 48 hours. Thin sections were cut with a diamond knife on an LKB Utratome III, mounted naked on 300 mesh copper grids, and double-stained with a saturated solution of UA (Gibbons and Grimstone, 1960) and Reynolds' lead citrate (Reynolds, 1963). The mounted sections were then stabilized by evaporating a thin layer of carbon onto the grids. Observations and micrographs of the thin sections were made with an Hitachi HU-12A or HU-12 operated at 75 kV.

SPAGE profile of M7 viral proteins. This work was conducted in the Immunochemistry Section under the direct supervision of Dr. Allen Schultz and Mr. G. Michael Bowling. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SPAGE) was performed according to the method described by Maizel (1971). The polyacrylamide slab gel was prepared using the buffer system of Laemmli (1970). Virus samples were diluted in 2x sample buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.1 M Tris buffer (pH 6.8), 0.1 M 2-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue. This solution was boiled for three minutes. The running gel (20 x 20 cm slab gel) consisted of 13% acrylamide, 0.1 M Tris (pH 8.8), 0.1% SDS, 15 μl of tetramethylenediamine, and 0.18 mg of ammonium persulfate under a stacking gel of 5% acrylamide, 0.1 M Tris (pH 6.8), 0.1% SDS, 5 μl of tetramethylenediamine, and 0.1 mg of ammonium persulfate. The electrophoresis running buffer contained .05 M Tris-glycine (pH 8.8) with 0.1% SDS. Electrophoresis was conducted using a Bio-Rad Model 220 unit operated at 40V (12 mA/gel) for 16 hours at room temperature.

The protein molecular weight markers used were: bovine serum albumin, 68,000; carbonic anhydrase, 28,000; myoglobin, 17,000; and cytochrome C, 11,700. Proteins were located by staining the gel for 1 hour with 0.2% Coomassie brilliant blue in 50% methanol - 7% acetic acid. Gels were destained overnight in 5% methanol - 7% acetic acid. Photographs of this protein gel were made by the personnel of the Photography Section, Frederick Cancer Research Center.

Preparation of radioactively labeled viral RNA. Four Corning T150 flasks containing 70% confluent A204(M7) cells in 20 ml of RPMI-1640 medium supplemented with 5% fetal calf serum, 2 mM glutamine, 100 units penicillin, 100 μg/ml streptomycin were pulsed for 2 hours with 50 μCi/ml

[5,6-^{3}H]-uridine (New England Nuclear (NEN), Boston, Massachusetts, specific activity 44.9 Ci/mmole), 50 μCi/ml [5-^{3}H]-cytidine (NEN, specific activity 24.8 Ci/mmole), and 19 μCi/ml [2,8-^{3}H]-adenosine (NEN, specific activity 30.9 Ci/mmole). An additional 20 ml of cold RPMI-1640 medium were added to each flask at the end of the 2 hour pulse, and all cultures were held overnight in a 37°C walk-in incubator. At 16 hours post pulse, tissue culture fluids were harvested, clarified by centrifugation at 3,000 rpm for 10 minutes in an IEC PR-6 centrifuge, and virus was pelleted by further centrifugation at 100,000 x g for 90 minutes at 4°C in a Beckman SW27 rotor. The viral pellets were resuspended in 1.0 ml of TNE. Approximately 10 ml of clarified tissue culture fluid was retained at 4°C for reference. The four T150 flasks were subjected to an additional pulse as described above, followed by an 8 hour chase with cold medium, and all tissue culture fluids harvested were processed as previously described.

The 10 ml reference samples from pulse 1, pulse 2, and the 8 hour chase were pooled, pelleted as above, resuspended in 0.3 ml of TNE, layered onto a 10-50% sucrose gradient, and centrifuged at 100,000 x g for 16 hours at 4°C in an SW41 rotor. Fractions of 0.5 ml were collected by gravity from the bottom of the gradient by using a Buchler piercing unit. Samples of 20 μl were taken from each fraction and assayed for trichloroacetic acid (TCA)-precipitable radioactivity by filtration through 0.45 μm membranes (Millipore HA). Filters were counted in 5.0 ml of Aquasol II (NEN) in an Intertechnique liquid scintillation spectrophotometer.

The fractions containing the radioactive virus peak were pooled, diluted twofold with TNE, made 1% in sodium dodecyl sulfate (SDS) and 750 μg/ml in Pronase (Calbiochem, B-grade, self-digested for 2.0 hours at

37°C), and incubated at 37°C for 30 minutes. This sample was extracted with a 5:1:5 mixture of chloroform, m-cresol, and water-saturated phenol. The aqueous phase was made 0.3 M in sodium chloride, 67% in ethanol, and was held at -20°C overnight. The precipitate was recovered by centrifugation at 10,000 rpm for 15 minutes in a Sorvall RC-2B centrifuge, redissolved in 0.5 ml TNE and sedimented for 2.5 hours through a 15-30% sucrose gradient at 40,000 rpm in an SW41 rotor run at 10°C. Gradients were fractionated and fractions were screened for acid-precipitable radioactivity as previously described. Fractions in 70S region were pooled, reprecipitated in ethanol, resuspended in TNE, and held for further analysis.

The viral pellet suspensions obtained from pulse 1, pulse 2, and the 8 hour chase were extracted in an identical manner and run on separate sucrose gradients. Fractions in the 70S region from all three gradients were pooled, reprecipitated in ethanol, resuspended in TNE and held for further analysis.

Synthesis of cDNA. A reaction mixture of 1.0 ml contained 0.30 ml of a 5,000-fold concentrate of M7 virus. Fifty μM each of dATP, dGTP, and dCTP, 20.0 μM [^{3}H]TTP (NEN, specific activity 52.6 Ci/mM), 0.025% NP40 (Nonident P-40), 0.1 M Tris (pH 8.0), 5 mM magnesium acetate, 50 mM sodium chloride, and 0.1 M dithiothreitol (DTT). After incubation at 37°C for 1 hour, the reaction was made 1% in SDS and the sample was split onto two 15-30% sucrose gradients made up in TNE and 0.01% SDS. The gradients were centrifuged for 2.5 hours at 40,000 rpm at 10°C in an SW41 rotor. Fractions were screened for acid-precipitable radioactivity as previously described. The peak areas from both gradients were pooled, made 0.3 N in NaOH and incubated at 37°C for 4 hours. The prep was made

.05 M in Tris, 0.3 N in HCl to neutralize (pH 8.0), extracted with an equal volume of chloroform; octanol (24:1), and precipitated with two volumes of ethanol at -20°C. The precipitate was redissolved in TNE, checked for radioactivity, and reprecipitated with two volumes of ethanol. The precipitate was resuspended in 0.6 ml of TNE and an aliquot was taken for sizing by sedimentation through alkaline sucrose gradients (5-20% sucrose in 0.8 M sodium chloride - 0.2 N sodium hydroxide; sedimentation overnight in an SW41 rotor at 40,000 rpm at 10°C. The yield from this reaction was approximately 3.17×10^5 cpm. Assuming 25% thymidine in the cDNA, the specific activity of this probe was 3.4×10^7 cpm/μg.

DNA preparations. *P. papio* liver DNA and cellular DNAs were prepared according to the urea-phosphate method (Britten *et al.*, 1974). DNA was also prepared from BEF-2 cells by the Hirt (1967) procedure. The Hirt pelleted DNA was purified further by the urea-phosphate method. Before use in hybridization experiments, all final DNA preparations were sonicated using a Branson sonifier at a setting previously determined to yield fragments suitable for reassociation experiments (Hoyer *et al.*, 1973). The average size of DNA fragments yielded by this treatment was approximately 450 nucleotides as determined by sedimentation through alkaline sucrose gradients (previously described) which included a radioactively labeled DNA marker of known size. Actual size calculations were made according to the method of Studier (1965).

Four additional DNA preparations, RD(BAB8-K) #137471, D17(BAB8-K) #137470, placenta #7502027 and #7601124, were received from Dr. Robert McAllister, Children's Hospital of Los Angeles. RD(BAB8-K) is a human cell line (McAllister *et al.*, 1969) infected with and producing an endogenous baboon virus (kidney) isolate. D17(BAB8-K) is a dog osteosarcoma

cell line (Riggs et al., 1974) also infected with and producing the BAB8-K virus. Both DNA preparations were treated by the urea-phosphate method, sonicated, and sized as described above. Placental DNA preparations (P. cynocephalus) were sonicated and sized as described above.

Preparations of ^{3}H HeLa DNA (enriched in unique sequences), normal and leukemic human DNAs, and mouse (Mus musculus) DNA, were the generous gifts of Dr. Nancy Rice.

Hybridization reactions and hybrid detection. [^{3}H]M7cDNA (300-400 cpm, approximately 0.1 ng) was incubated with cellular DNA (300-400 μg per time point) in a sodium-phosphate buffer based reaction mix. Hybridizations were initiated by boiling the reaction mixture for 2 minutes, adding sodium-phosphate buffer (PB) to a 1.0 M final buffer concentration, and incubating the reaction vials (Wheaton 1.0 ml conical vials with teflon-lined caps) in a 63°C water bath.

At the end of the hybridization, reactions were diluted to 0.14 M PB containing 0.02% SDS. Samples were applied at 50°C to pre-washed HAP (2.0 ml bed volume) contained in water-jacketed glass columns connected to a circulating water bath (Haake, Type FE). Fractions of 6.0 or 7.0 mls were collected at 10°C increments in temperature up to and including several fractions at 95°C. The optical density (260 nm) of each fraction was read to verify that a normal pattern of cellular DNA reassociation was achieved during the course of the reaction. Column fractions were then assayed for total radioactivity by addition of 2 volumes of Aquasol II and counting an average of 5 sets per reaction in a liquid scintillation spectrophotometer. The final percent of reassociation was taken as the percentage of total radioactivity eluting from HAP above 50°C. All C_0t curves shown reflect C_0t values corrected to the standard condition of 0.12 M PB as defined by Britten et al. (1974).

T_m determinations. Thermal melting (T_m) experiments were performed in the following manner. Reaction mixtures containing 100 μg-1 mg of cellular DNA and 900-2000 cpm of [^{3}H]M7cDNA were constructed as previously described and incubated to a C_0t of about 10,000. Hybridization reactions were terminated as before and a native HeLa [^{14}C]DNA marker (about 450 base pairs) was added to the sample prior to column loading. An HAP (8.0 ml bed volume) column was set up as previously described, prewashed, and the sample was loaded at 50°C in 0.14 M PB - 0.02% SDS. The column was washed again, and a linear temperature gradient was initiated to elute the bound material. Column operation and elution conditions were monitored via the HeLa [^{14}C]DNA marker. Optical densities (260 nm) of fractions were again recorded to check on total cellular DNA reassociation. Fractions received 2 volumes of Aquasol II and were assayed for total radioactivity. Final reassociation values were taken as that percent of total radioactivity eluting above 50°C. T_m values are reported as that temperature at which 50% of the material initially bound to the HAP has been eluted (Te_{50}).

Data analysis. Separate reaction mixtures were incubated for varying time periods to generate a series of C_0t values. HAP column fractions for each reaction point were counted at least 5 times to yield an average percent reassociation for each C_0t value. The percent single-stranded DNA was calculated for each C_0t value, and both of these figures along with an estimate of the $C_0t1/2$, and initial point and final extent of the reaction, were used in a computer program designed to generate the best fit by least squares with equal weight for each measured value (Britten *et al.*, 1974).

An additional program, one devised to plot a histogram for assays containing single or dual labels, was utilized for construction of the thermal elution profiles presented within this text. Histogram plots of ^{3}HcDNA:DNA melts were converted to integral plots by a program designed by Mr. Melvin Bostian, Computer Services Division, Frederick Cancer Research Center.

IV. Results

Virus characterization. Morphologically, the virus grown in cultures of human A204 cells resembles a typical type C virus. Figure 2 shows the stages in the budding process of M7 virions. Particle formation is initiated at the cell membrane, with budding particles exhibiting typical type C crescent-shaped nucleoids. Extracellular virions matured to an average particle diameter of 100 nanometers, with an electron-lucent core of approximately 50 nanometers. No cytoplasmic A particles, characteristic of non-type C retraviruses of primates, or primate type D retravirus particles were observed in the A204(M7) infected cell line.

Samples taken from the purified preparations of M7(A204) virus utilized in this study were subjected to sodium dodecyl sulfate-polyacrylamide gel analysis. Figure 3 shows the similarity of protein patterns between the C type viral proteins of Rauscher murine leukemia virus (wells #2, 4, 6) and the M7(A204) virus (wells #16, 18). Molecular weight markers are present in wells #8, 10, 12, and 14, and are discussed in the legend to Figure 4. The viral envelope associated glycoproteins (gp70), the internal core shell protein (p30), the virion surface associated protein (p15E), an internal protein (p15), the viral surface protein (p12), and the nucleoprotein (p10) are all quite evident in the gel profile (lanes 2, 4, and 6) of the Rauscher virus lysate. The M7 virus lysate gel profile (lanes 16 and 18) possesses distinct gp70, p30 and p10 bands corresponding to those seen in the Rauscher virus profile. A series of three bands migrating to a position above that observed for the Rauscher p15E and p15 proteins, plus an additional distinct band corresponding to the 11,700 molecular weight marker were also evident in the M7 gel profile. The dense staining band corresponding to the p30 viral protein is

Figure 2. Electron micrographs of thin sections made through an A204(M7) cell pellet. The initial formation of a crescent-shaped nucleoid under the cell plasma membrane is typical of the morphogenesis of type C retraviruses (a). A transitional form with the membrane further enveloping a horseshoe-shaped nucleoid and resulting in a stalked particle (b). A completed, immature extracellular particle possessing a doughnut-shaped nucleoid (c). Mature extracellular type C particles containing spherical centric nucleoids (d). Magnification 150,000 X.

FIGURE 2
BUDDING PROCESS OF M7 VIRIONS

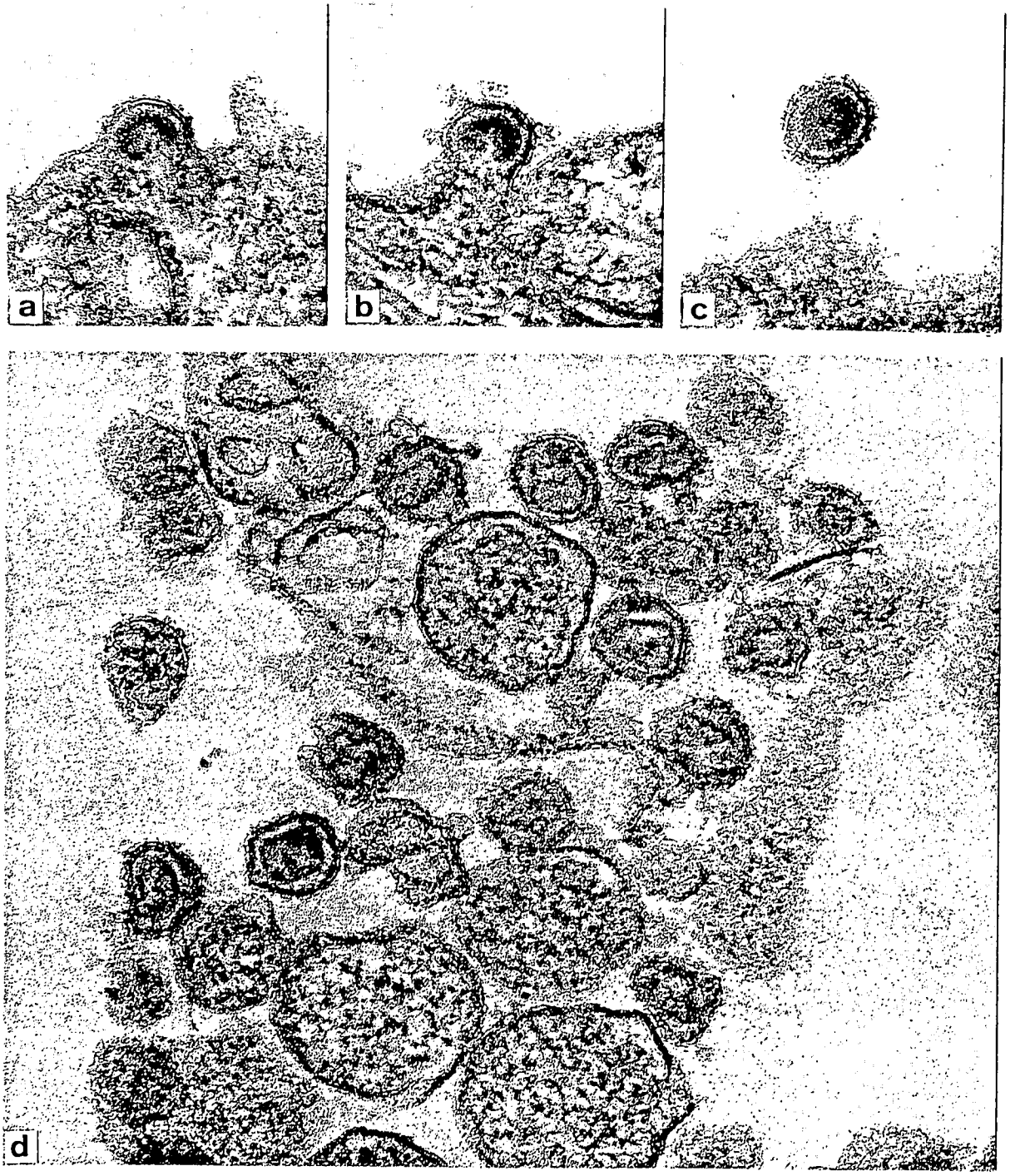
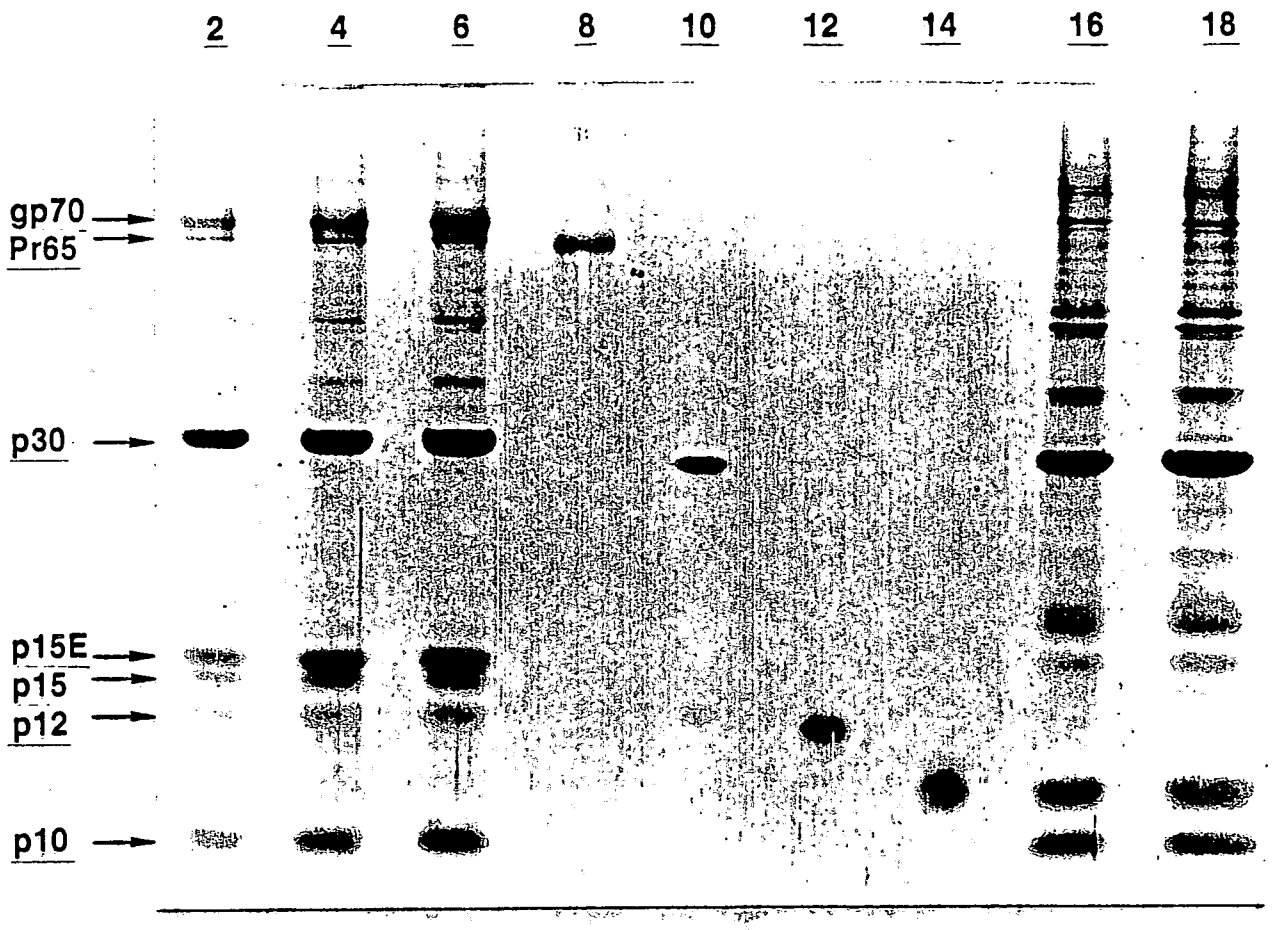


Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of M7 viral proteins: photograph of a stained 13% polyacrylamide-SDS slab gel. Lanes 2, 4, and 6 contain increasing amounts of a Rauscher murine leukemia virus lysate. Lane 8 contains the 68,000 molecular weight marker, bovine serum albumin. The 28,000 molecular weight marker, carbonic anhydrase, was run in lane 10. A 17,000 molecular weight marker, myoglobin, was run in lane 12. Lane 14 contains cytochrome C, the 11,700 molecular weight marker. Increasing amounts of an M7 virus lysate were run in lanes 16 and 18. All samples were subjected to electrophoresis for 16 hours at 40V (12 mA/gel).

FIGURE 3
SPAGE ANALYSIS OF M7 VIRION PROTEINS



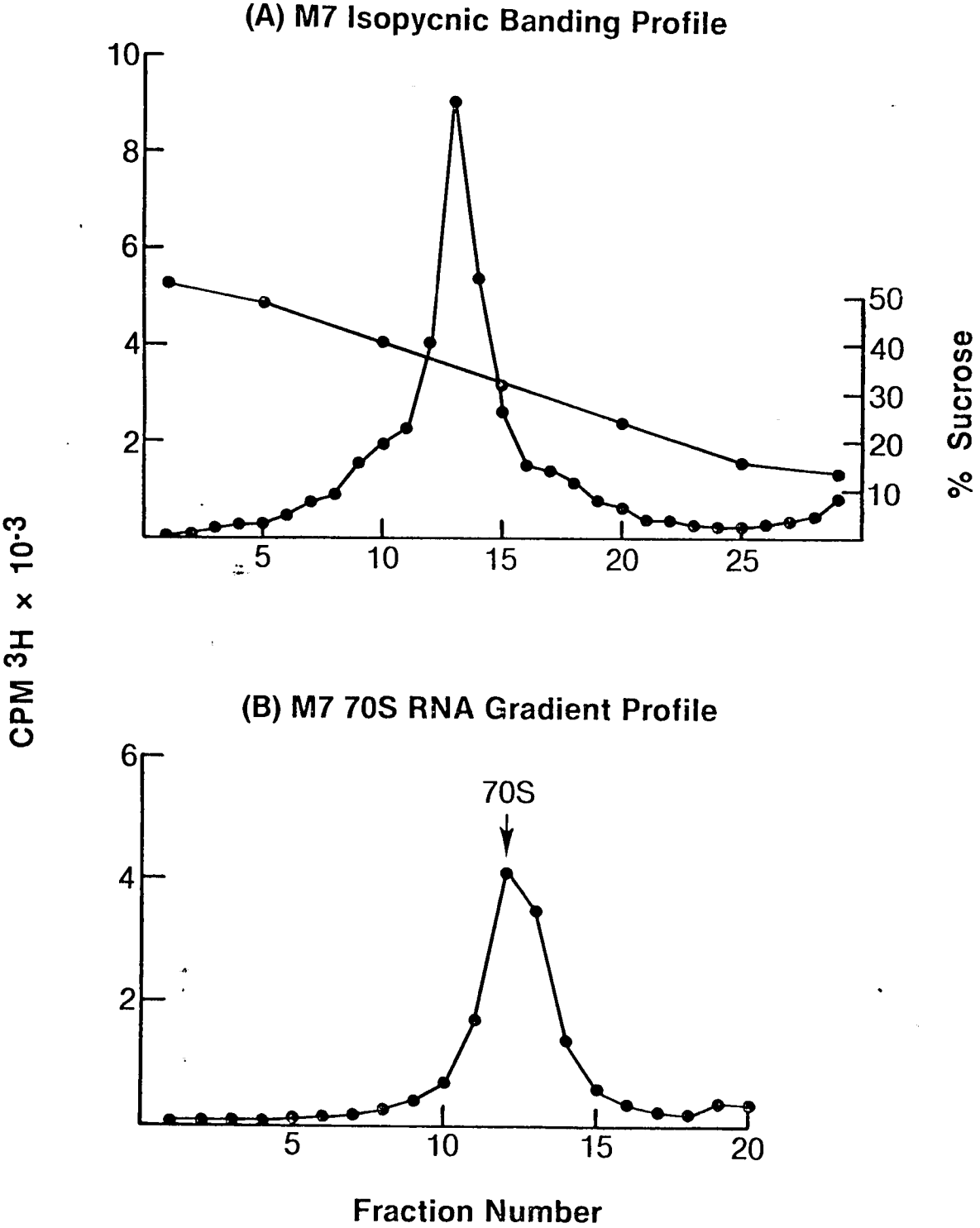
indicative of its representing approximately 25% of the total virion protein (Oroszlan et al., 1970).

M7 virions were directly pelleted from clarified supernatant fluids of [^{3}H]-uridine, [^{3}H]-cytidine, and [^{3}H]-adenosine labeled monolayer cultures of A204(M7) cells, and subjected to isopycnic banding on sucrose gradients. Figure 4A shows the profile of one such gradient. The virion density, as located by the peak of acid-precipitable radioactivity, is at 1.14-1.16 g/cm^3. This density is typical for purified preparations of mature, extracellular C-type retraviruses.

[^{3}H] labeled preparations of isopycnically banded M7 virions were further characterized by isolation of a high molecular weight virion component, presumed to be genomic RNA. Figure 4B shows a velocity sedimentation gradient profile of the high molecular weight component isolated from these virions. Subjecting this [^{3}H] component to boiling for 2 minutes and overnight incubation at 63°C did not effect the recovery of total acid-precipitable counts present in the sample tested. The [^{3}H] component was shown to be 70S RNA by its resistance to treatment with DNase, its sensitivity to digestion by RNase, and its hydrolysis upon treatment with sodium hydroxide. Initial attempts made to utilize this [^{3}H]70S RNA in molecular hybridization experiments with various cellular DNAs yielded incomplete data. Although 90 to 100% of the input cpm were recovered from control reactions containing only the [^{3}H]70S RNA, approximately 40% of the input cpm could be recovered in reactions containing test DNAs. This phenomenon was consistent regardless of the amount of DNA added to the reaction mixtures, was not due to a pH differential, and was not a result of RNase contamination of test DNAs or reaction buffers. Therefore, all of the data representing these hybridization attempts has been omitted from this thesis.

Figure 4. Isopycnic banding of M7 virions and sedimentation of M7 viral 70S RNA in neutral sucrose gradients. (A) [^{3}H] labeled M7 virus was centrifuged to equilibrium in a 10 to 50% sucrose gradient using an SW41 rotor run at 100,000 x g for 16 hours at 4°C. The virion density, as determined by the location of a peak of acid-precipitable radioactivity within the gradient, falls at 1.14-1.16 g/cm^3 which is typical for C-type retraviruses. (B) Velocity sedimentation of [^{3}H] viral RNA through a 15 to 30% sucrose gradient in an SW41 rotor run at 40,000 cpm for 2.5 hours at 10°C. Aliquots (0.02 ml) were taken from each 0.5 ml fraction and assayed for TCA precipitable counts by membrane filtration. Sedimentation of viral RNA samples under these conditions consistently yields a peak of viral 70S RNA in fractions number 10 through 14.

FIGURE 4

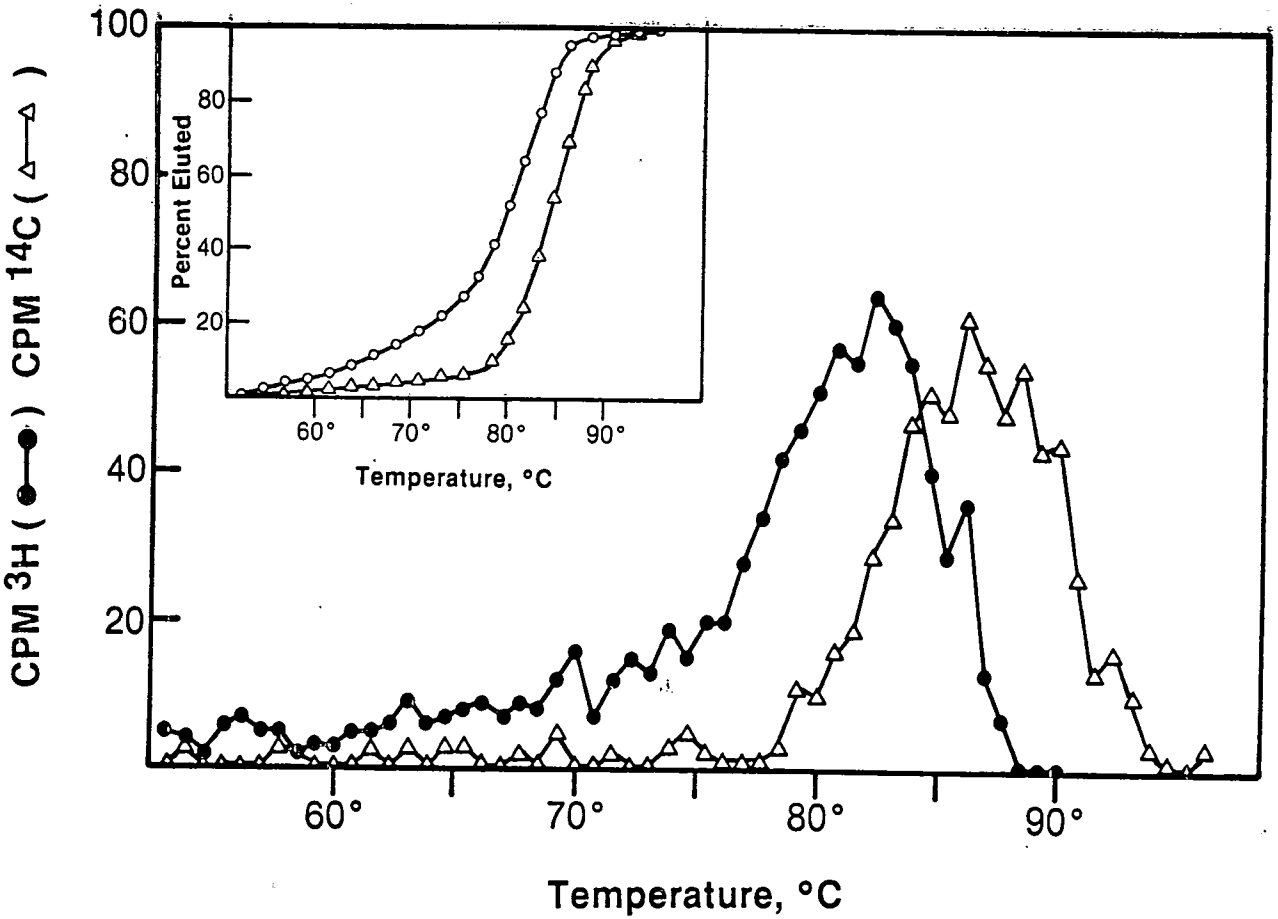

(A) M7 Isopycnic Banding Profile
10
8
6
4
2
5
10
15
20
25
50
40
30
20
10
% Sucrose
CPM 3H x 10-3
(B) M7 70S RNA Gradient Profile
6
4
2
70S
5
10
15
20
Fraction Number

Characterization of the [^{3}H]cDNA probe. A single-stranded, virus specific [^{3}H]cDNA probe was synthesized in an endogenous reverse transcriptase reaction containing detergent treated preparations of the baboon virus, M7. The M7[^{3}H]cDNA synthesized by this reaction was isolated from sucrose gradients, treated with sodium hydroxide to hydrolyze any viral genomic RNA still associated with the cDNA product, neutralized with HCl, extracted with chloroform:octanol (24:1), precipitated in ethanol, and sized on alkaline sucrose gradients. After this purification, the M7 viral probe was about 160 nucleotides in length, had a specific activity of 3.4 x 10^7 cpm/μg, was more than 96% single-stranded as measured by HAP column chromatography, and demonstrated sensitivity to treatment with DNase.

In order to determine the extent of complementarity between the newly synthesized cDNA transcript and its original RNA template, the [^{3}H]cDNA viral probe was hybridized to M7 70S RNA. In the presence of an excess amount of viral 70S RNA (0.3 μg), 81% of the newly synthesized cDNA probe was hybridized. Therefore, at least 81% of the M7[^{3}H]cDNA probe used in these studies was complementary to M7 viral 70S RNA. The thermal stability of the duplex formed between the viral probe and viral 70S RNA was also examined as an additional check on complementarity. The thermal dissociation profile of the M7[^{3}H]cDNA-M7 70S RNA hybrid is depicted in Figure 5. The temperature at which 50% of the bound [^{3}H]cDNA eluted from the hydroxylapatite column was 80.1°C. By comparison, the [^{14}C]HeLa native DNA marker eluted at a Tem_{50} of 84.4°C. As previously cited and as represented here by the thermal elution of the native HeLa DNA, well-matched DNA hybrids dissociate at temperatures above 80°C. Since the difference observed in the thermal elutions of the [^{3}H] labeled

Figure 5. Thermal elution profile of the hybrid formed between M7[^{3}H] cDNA and M7 viral RNA. A reaction mixture containing 3.5 x 10^{-2} ng (1,190 cpm) of M7[^{3}H]cDNA with an average size of 160 nucleotides and 0.75 μg of M7 viral RNA was incubated to a C_rt of 1.1 and applied to an HAP column at 50°C. A total of 77% of the ^{3}H counts per minute bound. A 50 μl sample of native HeLa [^{14}C]DNA was also applied to the column to serve as an internal marker on column performance. The material bound to the column was eluted during a linear temperature gradient (0.8°C per fraction), and fractions were assayed for radioactivity after addition of two volumes of Aquasol II. o, ^{3}H counts per minute in each fraction; Δ, ^{14}C counts per minute per fraction. In the inset is the integral plot of the data, showing the cumulative percentage of the bound material eluted versus temperature. Temperature at which 50% of bound ^{3}H eluted = 80.1°C; temperature at which 50% of bound ^{14}C eluted = 84.4°C.

FIGURE 5
THERMAL STABILITY OF THE M7cDNA-70SRNA HYBRID



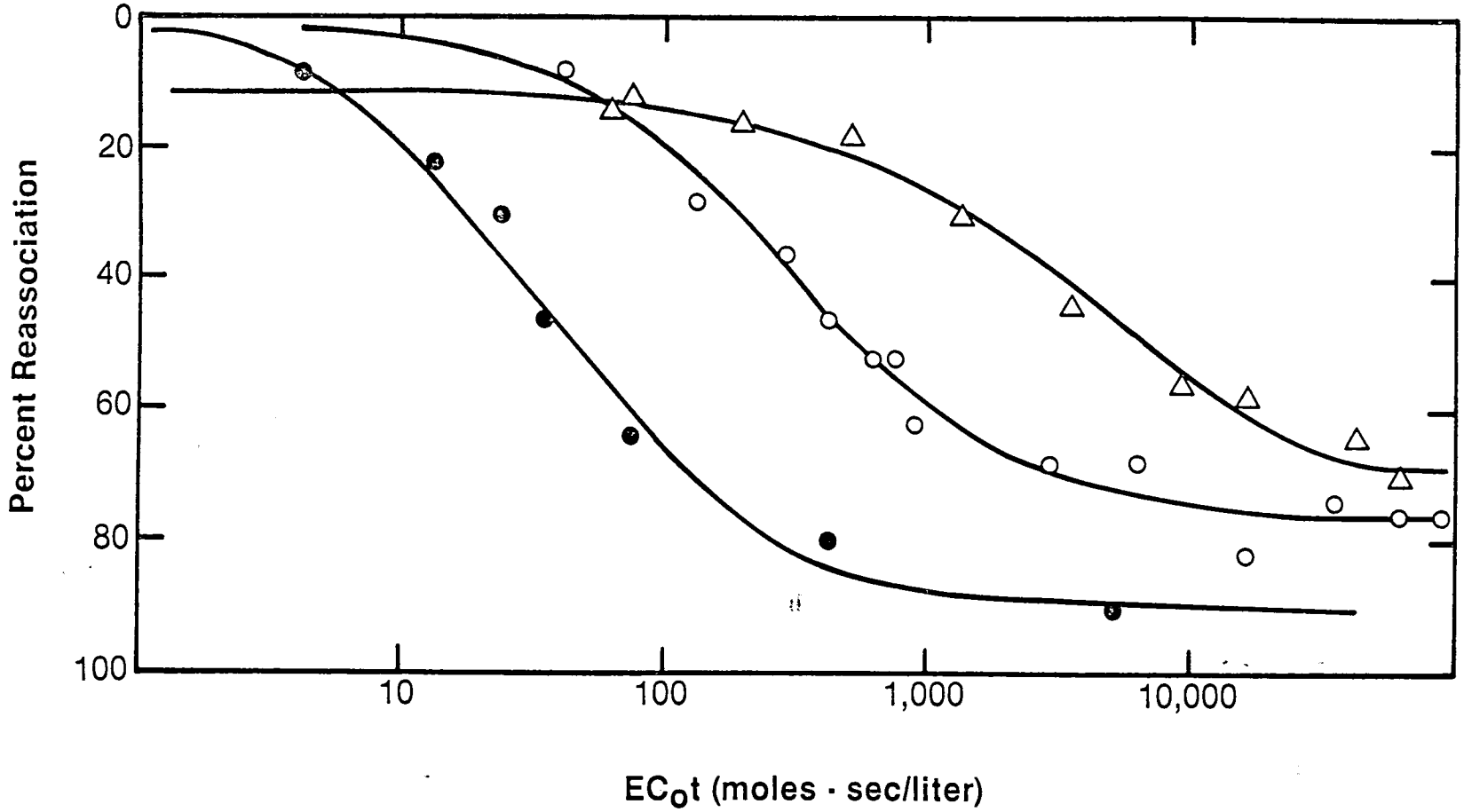
hybrid and the [^{14}C] labeled native DNA preparation (ΔT_m ^{3}H-^{14}C) was only 4.3°C, the hybrid formed between the viral probe and the viral RNA must have exhibited an extensive degree of complementarity.

The limits of detection for the M7[^{3}H]cDNA probe were defined by generating a ratio curve of cell DNA to viral probe. Reaction mixtures containing a constant amount of [^{3}H] probe and increasing amounts of cell DNA were incubated to extended C_0ts. Results from this experiment indicated that the M7[^{3}H]cDNA probe required 200 μg DNA per 1.0 μl probe to reach 80% of its final extent of reassociation if incubated to an extended C_0t. Since the cellular DNA tested, RD(BAB8-K), was known to contain 5 copies (viral related sequences) per haploid genome, a quantity of 1.0 mg/μl probe would be required in order to detect a single viral related sequence per haploid genome. The hybridization reactions conducted for this study always contained a cell DNA:cDNA ratio well within the limits of detection. The average ratio was approximately 300-400 μg DNA per 0.5 μl probe.

Detection of viral-related sequences in the DNA of baboon embryo fibroblasts. Once the M7[^{3}H]cDNA probe was proven complementary to its original RNA template and the limits for detection of viral-related sequences in denatured cellular DNA preparations were established, the M7 viral probe was reacted with DNA obtained from the endogenous system, i.e., baboon cellular DNA. Since the M7 virus was originally isolated from a baboon (*P. cynocephalus*) placenta, the baboon cellular DNA utilized for this set of hybridization reactions was extracted from a baboon (*P. cynocephalus*) embryo fibroblast cell line, herein designated BEF-2. The association kinetics of the M7[^{3}H]cDNA probe and DNA extracted from BEF-2 cells are presented in Figure 6. The C_0t curve generated by this series

Figure 6. Comparison of reassociation kinetics of the M7[^{3}H]cDNA probe with DNA from A204(M7)-infected cells (o——o) to the reassociation kinetics of nonrepeated [^{3}H]HeLa DNA with the same cellular DNA preparation (Δ——Δ). The C_0t curve representing reassociation kinetics of M7[^{3}H] cDNA probe with DNA from BEF-2 cells is included for reference (⊙——o). For each reaction point on the M7[^{3}H]cDNA-A204(M7) cellular DNA C_0t curve, 0.01 ng (350 cpm) of viral probe was incubated with 320 μg of denatured DNA (average size 890 bases). For each point on the [^{3}H]HeLa DNA-A204 (M7) C_0t curve, 5 ng (2025 cpm) of nonrepeated DNA (average size 150 nucleotides) was incubated with 170 μg of A204(M7) denatured DNA. Hybridization conditions for these reactions and hybrid detection by HAP column chromatography were identical and as previously reported in Materials and Methods. [^{3}H]HeLa nonrepeated DNA reassociated with A204(M7)DNA with an $EC_0t_{1/2}$ of 3300. M7[^{3}H]cDNA reassociated with A204(M7)DNA with $EC_0t_{1/2}$ of 300. This increase in reassociation rate ($k = \frac{1}{C_0t_{1/2}}$) observed with the viral probe suggests the presence of approximately 10 viral DNA copies per haploid genome in the A204(M7) infected cells. The reassociation kinetics of BEF-2 cellular DNA with the viral probe are indicative of approximately 100 viral DNA copies present per haploid genome.

FIGURE 6
COMPARISON OF ASSOCIATION KINETICS
COPY NUMBER DETERMINATION



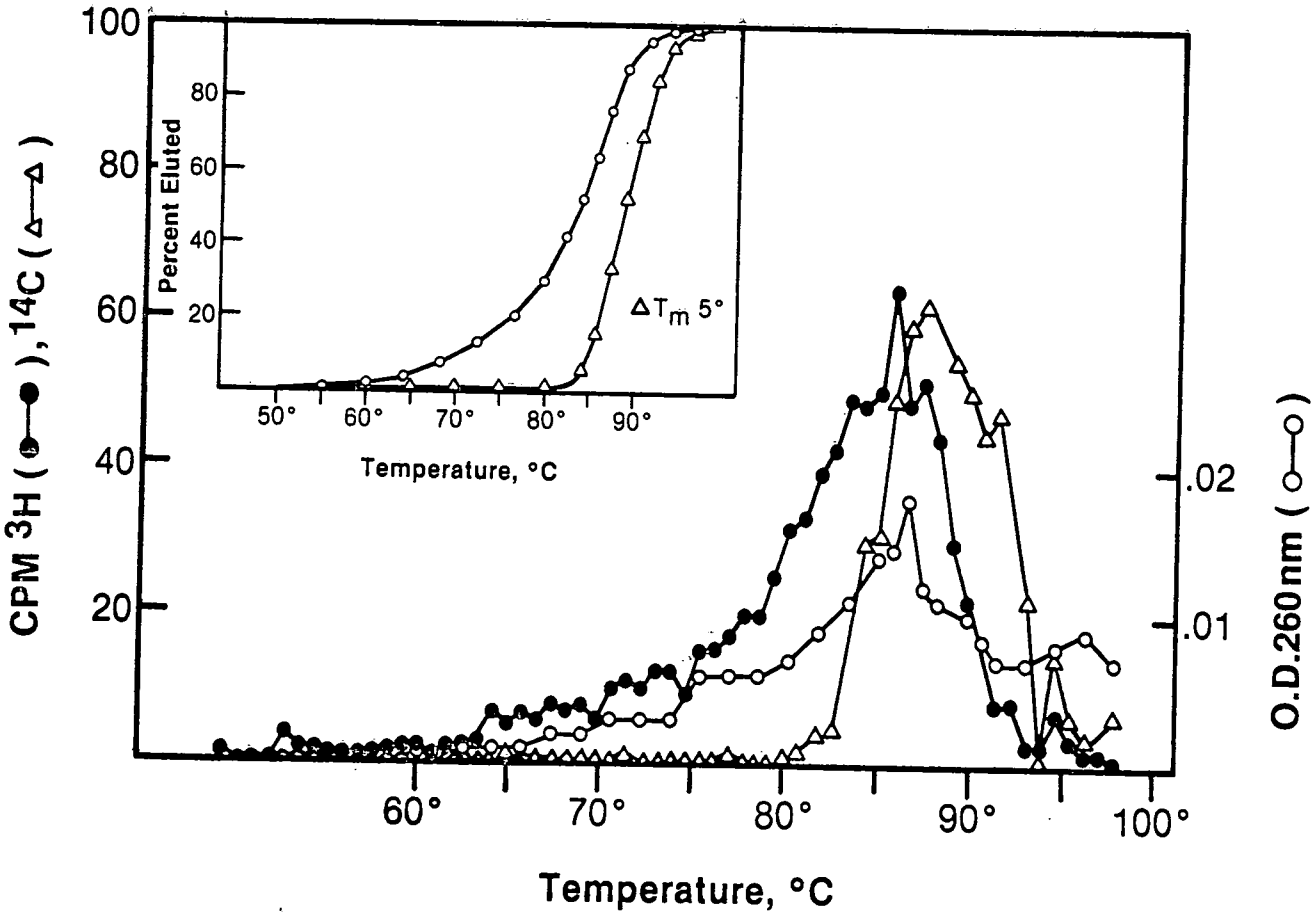
of reactions approximates the ideal reassociation time course previously discussed. Up to 90% (EC_0t 5,200) of the [^{3}H] viral probe formed a duplex with BEF-2 cellular DNA. Approximately 5% (EC_0t 124,000) of the [^{3}H]-viral probe formed a duplex with mouse liver DNA under identical reaction conditions. This amount of duplex formation was considered negligible since an average of 5% association was observed on the numerous self-reactions (incubation of probe alone under identical reaction conditions) conducted during this study.

An attempt was also made to determine whether or not the M7 viral-related sequences detected in this baboon cellular DNA exist in a free (cytoplasmic) form or in an integrated (nuclear) form. Two time points appearing on this baboon C_0t curve represent association of the [^{3}H] viral probe with DNA extracted from BEF-2 cells by the Hirt procedure. Extraction of DNA from only the Hirt pellet fraction preferentially isolates high molecular weight (nuclear) DNA molecules. The lack of any observable difference in association kinetics between the [^{3}H]cDNA probe and cellular DNAs extracted by either the urea-phosphate method or the Hirt procedure, suggests that the viral associated DNA sequences detected exist in an integrated proviral state within the BEF-2 nuclear DNA.

The stability of the duplex formed between the M7 viral probe and the viral-related sequences contained in the BEF-2 cellular DNA was assessed by subjecting the hybrid population to thermal elution on hydroxylapatite. Figure 7 contains the thermal denaturation profile of the duplex formed between the M7[^{3}H]-labeled probe and BEF-2 cellular DNA. Again, dissociation of DNA hybrids occurring at temperatures greater than 80°C implies complementarity. The [^{3}H]cDNA viral probe was half-eluted at 83.7°C, whereas the native [^{14}C]Hela DNA marker eluted with a Tem_{50} of 80,7°C.

Figure 7. Thermal elution profile of the hybrid formed between M7[^{3}H] cDNA and BEF-2 DNA. A reaction mixture containing 2.6 x 10^{-2} ng (875 cpm) of M7[^{3}H]cDNA was incubated with 100 μg of BEF-2 denatured cellular DNA with an average size of 500 nucleotides to a C_0t of 1200 and applied to an HAP column at 50°C. A total of 83% of the ^{3}H counts per minute bound. A 50 μl sample of native HeLa[^{14}C]DNA was applied to the column to serve as an internal marker on column performance. The material bound to the column was eluted during a linear temperature gradient (0.8°C per fraction). Each fraction was measured for absorbance at 260 nm to determine if dissociation of cellular DNA had proceeded appropriately, and was assayed for radioactivity after addition of two volumes of Aquasol II. The insert shows an integral plot of the data. Temperature at which 50% of bound ^{3}H eluted = 83.7°C; temperature at which 50% of bound ^{14}C eluted = 88.7°C. ●—●, ^{3}H counts per minute in each fraction, △—△, ^{14}C counts per minute per fraction; o—o, O.D.$_{260}$ nm.

FIGURE 7
THERMAL STABILITY OF THE M7cDNA-BEF-2 DNA HYBRID



A high degree of complementarity between the BEF-2 cellular DNA and the [^{3}H]cDNA probe is indicated by the high temperature of thermal elution exhibited by this hybrid population as observed in this melt profile.

Analysis of the number of viral-specific sequences present in the haploid genome of M7 producing BEF-2 cells was made by comparison of the $C_0t_{1/2}$ value of the BEF-2 C_0t curve with the $C_0t_{1/2}$ value of a C_0t curve representing the association kinetics of non-repeated cellular DNA sequences. The latter curve was obtained by hybridizing a preparation of DNA enriched in human unique sequences ([^{3}H]Hela native DNA) to human DNA extracted from A204(M7) cells. For ease of comparison, both C_0t curves appear on Figure 6. The reassociation of [^{3}H]Hela nonrepeated DNA with A204(M7) cellular DNA gave an equivalent $C_0t_{1/2}$ of 3300. The equivalent $C_0t_{1/2}$ value observed for reassociation of BEF-2 cellular DNA with the [^{3}H] viral probe was 35. Since all hybridization conditions, including size of the reactants, were identical, one may conclude that the increase in reaction rate observed in the BEF-2 C_0t curve ($k = \frac{1}{35}$) over that observed with sequences present only once ($k = \frac{1}{3300}$) must be due to the presence of additional viral-related DNA sequences in the BEF-2 DNA. The average number of viral copies present in BEF-2 DNA could then be calculated to be approximately 3300 ÷ 35, or 95 copies of viral related sequences present in BEF-2 DNA.

In sum, the results obtained with the M7[^{3}H]cDNA viral probe and cellular DNA representative of the endogenous baboon system indicate that: (1) the M7 viral probe is capable of detecting integrated related sequences in baboon cellular DNA preparations; (2) the duplexes formed in these hybridization reactions demonstrate a high degree of thermal stability (and therefore, complementarity) when subjected to thermal

denaturation on hydroxylapatite; and (3) on comparative analysis with unique cellular DNA sequences, the association kinetics of the M7[^{3}H]cDNA viral probe with BEF-2 denatured cellular DNA indicate that approximately 95 copies of M7-related sequences per haploid genome are present in baboon cells grown in culture.

Detection of viral-related sequences in the DNA of human cells exogenously infected with M7 virus. As soon as the extent of proviral representation in cells transmitting endogenous viral information was known, an attempt was made to determine the extent of proviral information contained in the DNA extracted from an exogenously infected cell system. Cellular DNA extracted from A204(M7), a human cell line exogenously infected and actively producing the baboon endogenous virus isolate M7 was hybridized to the M7[^{3}H]cDNA viral probe. The C_0t curve generated from this set of hybridization reactions is presented in Figure 6. Under hybridization conditions identical to those previously described, the reactants achieved a final reaction extent of 75%, and an equivalent $C_0t_{1/2}$ of 300. When this same A204(M7) cellular DNA was allowed to associate with [^{3}H]HeLa native human DNA, the $EC_0t_{1/2}$ of this reaction, specific for detection of human single copy or unique sequences, was achieved at 3300. As shown in Figure 6, the increase in association kinetics of M7[^{3}H]cDNA over those observed for [^{3}H]Hela human single-copy sequences with A204(M7) human DNA was sufficient to shift the entire C_0t curve approximately one log to the left. Only the detection of nucleic acid sequences complementary to the M7[^{3}H]cDNA viral probe would cause the acceleration of duplex formation indicated by a shift of this magnitude.

Since the A204(M7) cell line was actively producing the M7 virus when cellular DNA preparations were made, it was also possible to assume that

the shift in association kinetics observed with this DNA preparation might be due to the presence of a contaminating viral RNA species. That is the M7[^{3}H]cDNA might actually be reassociating with a contaminating viral RNA which was not entirely eliminated in the DNA purification scheme, rather than reassociating with integrated proviral information as contained in the cellular DNA. In order to eliminate this as a possible source of error, a sample of A204(M7) cellular DNA was alkali digested prior to hybridization with the M7[^{3}H] viral probe. Two of the reaction points on the M7[^{3}H]cDNA-A204(M7) cellular DNA C_0t curve were obtained using this alkali-treated DNA preparation. The position of these two C_0t points relative to these reaction points obtained with the routine A204 (M7)DNA preparation suggests that the cellular DNA purification scheme employed for sample preparation eliminated any viral-specific RNA contaminants, and that all of the reassociation scored involved the M7[^{3}H] viral probe and viral-related sequences present in cellular DNA.

Based on a direct comparison of the M7[^{3}H]cDNA-A204(M7) cellular DNA $EC_0t_{1/2}$ with that $EC_0t_{1/2}$ observed for association of human single-copy sequences, approximately 11 copies of viral-related sequences were detected in the A204(M7) cellular DNA tested. Since the endogenous BEF-2 cell system was shown to contain 95 copies of viral-related sequences present in baboon cellular DNA, there appears to be a real and quantitative difference in the extent of proviral information contained in an endogenous vs. an exogenous cell system.

Cellular DNA reassociation during hybridization reactions. As previously mentioned, the bulk (microgram quantities) of the DNA present in these hybridization reactions is denatured, cellular DNA. In comparison, only trace amounts (nanogram quantities) of the M7 viral probe are contained

in these reaction mixtures. Therefore, the association of the M7[^{3}H]cDNA viral probe with denatured cellular DNA strands could only be monitored by virtue of its radioactive [^{3}H]-label. Since the reaction conditions employed throughout this study allow the reassociation of denatured, cellular DNA strands, and because cellular DNA reassociation increases with an increase in C_0t, the total cellular DNA reassociation achieved for each reaction point was easily measured by monitoring the optical density (absorbance 260 nm) of fractions obtained by hybrid detection on HAP columns. The percent total cellular DNA reassociation was scored as that fraction of total cellular DNA binding to hydroxylapatite at 50°C in 0.14 M phosphate buffer - 0.02% SDS. The results obtained by monitoring the total cellular DNA reassociation in this manner served as an internal control on the validity of the hybridization reactions involving the M7 [^{3}H]cDNA viral probe.

A direct comparison of the cellular DNA reassociation data from experiments involving the M7[^{3}H]cDNA viral probe with DNA reassociation C_0t curve data representative of most mammalian DNAs provides a basis for determining whether or not the total cellular DNA reassociation is proceeding normally. If total cellular DNA reassociation proceeds normally, then the association between the M7[^{3}H] viral probe and the fraction of viral-related sequences it detects within the bulk of the cellular DNA should be proceeding normally. C_0t curve data obtained from reassociation of mammalian DNAs indicates that approximately 30-50% of the total cellular DNA sequences have reassociated by C_0t 100, with approximately 50% of the remaining frequency classes of DNA reassociating with a $C_0t_{1/2}$ of several thousand. Total cellular DNA reassociation may be approached at C_0t values exceeding 10,000. In these experiments, if at a given C_0t

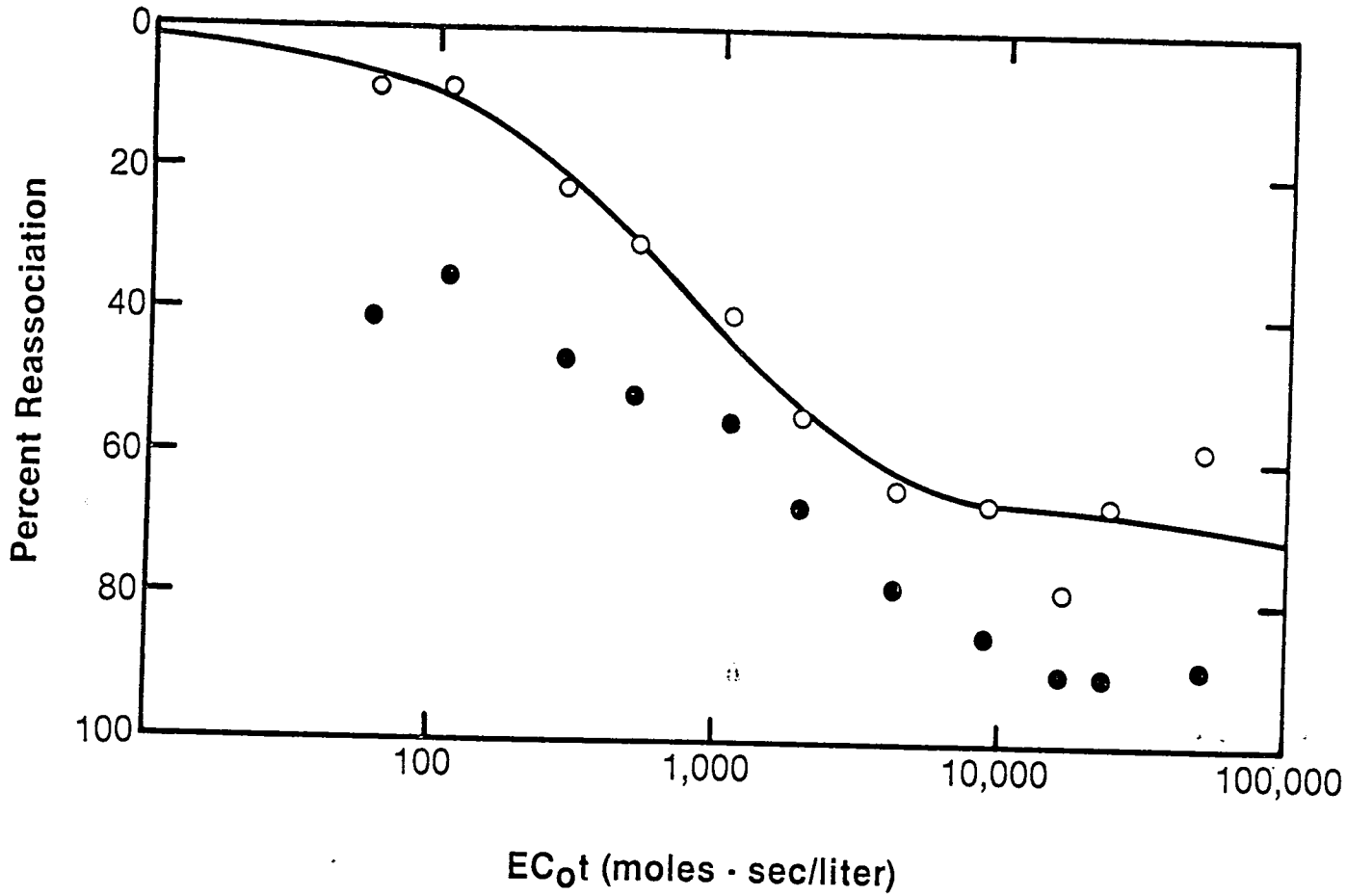
point the cellular DNA being tested has not reassociated appropriately, duplex formation involving the [^{3}H] viral probe also becomes suspect. Therefore, the reassociation of cellular DNA was routinely monitored in all reactions conducted during this study.

An example of the reassociation pattern of total cellular DNA consistently obtained from hybrid detection on HAP column chromatography is presented in Figure 8. The M7[^{3}H]cDNA viral probe was hybrdized to 8155 (M7) denatured cellular DNA. As shown in Figure 8, approximately 40% of the total 8155(M7) cellular DNA has reassociated by C_0t 100. The remaining frequency classes of DNA show an approximate $C_0t_{1/2}$ of 2000. The final extent of total cellular DNA reassociation achieved was 90% at C_0t 16,000. The final extent of total cellular DNA reassociation for all C_0t curves shown in this study was greater than 80%. These values are quite consistent with the DNA reassociation data previously mentioned for a variety of mammalian DNAs, and furthermore suggest that the association scored between the M7[^{3}H]cDNA viral probe and the 8155(M7) cellular DNA proceeded appropriately.

Detection of baboon endogenous virus-related sequences in DNA from exogenously infected cell lines. The results reported earlier for the A204(M7) cellular DNA indicate that there exists a quantitative difference in the extent of proviral information contained in an exogenously infected cell line vs. an endogenous cell system. In order to determine if similar results could be obtained with other exogenously infected cell lines, a variety of cellular DNAs were screened by hybridization to the M7 viral probe. The association kinetics of the M7[^{3}H]cDNA probe with two additional human DNAs from cells exogenously infected with and producing a baboon endogenous virus are depicted in Figure 9. The A204(M7) C_0t curve

Figure 8. Association kinetics of the M7 viral [^{3}H]cDNA probe with DNA from 8155(M7) infected cells. Hybridization conditions and hybrid detection were identical to those described in Materials and Methods. Each reaction point on the C_0t curve contained an average of 0.01 ng (350 cpm) of M7 [^{3}H]cDNA probe incubated with an average of 310 μg of denatured DNA (average size 540 bases). Cellular DNA self-association was monitored by measurement of optical density (absorbance at 260 nm) at each reaction point on the C_0t curve. Monitoring the cell DNA reassociation served as an internal control on the validity of the hybridization reaction. (o——o) M7[^{3}H]cDNA reassociation with 8155(M7)DNA yielded an $EC_0t_{1/2}$ of 645. ●, percent reassociation of total cellular DNA.

FIGURE 8
CELLULAR DNA REASSOCIATION TIME COURSE



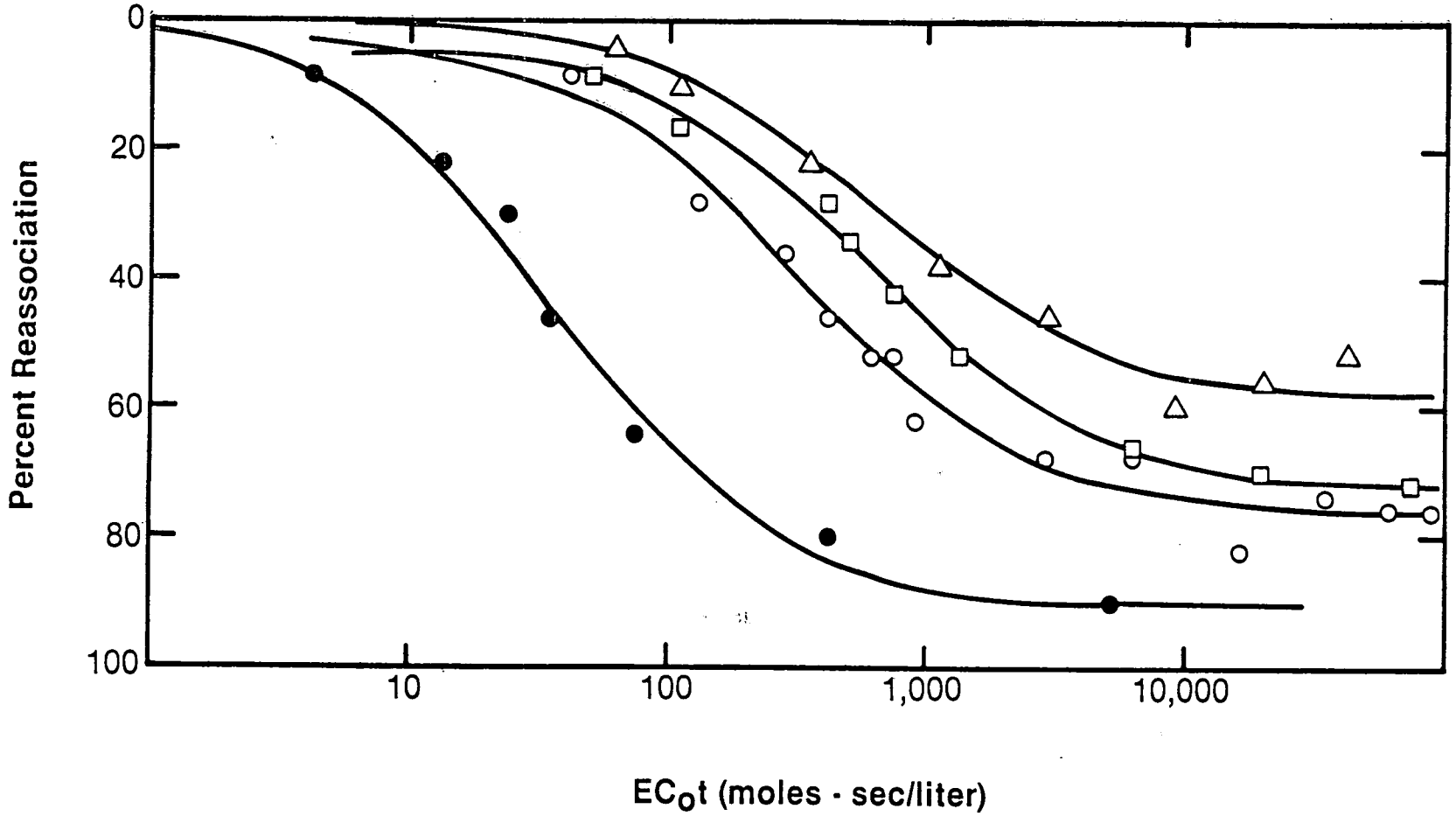
has been included for reference. The rate of association of the viral probe with these human DNA preparations HOS(M7) and A673(BAB455-K), is distinctly different than that observed with BEF-2 DNA (reference C_0t curve). The $C_0t_{1/2}$ values observed, approximately 600 for both the HOS(M7) and A673(BAB455-K), indicate that these exogenously infected human cell lines contain fewer copies of viral-related sequences (5 or 6) as compared to the 95 copies detected in the BEF-2 endogenous system. These results confirmed those obtained with the A204(M7)DNA preparation and indicate that human cells exogenously infected with the baboon endogenous virus contain 10 to 20 times fewer viral-related sequences per haploid genome than baboon cells.

A difference in the final extent of reassociation is also apparent in the association kinetics shown in Figure 9. The difference, approximately 15%, between the final extent of reassociation reached with BEF-2 cellular DNA vs. that obtained with the A204(M7) cellular DNA preparations is a reflection of the extent of proviral representation contained in these cellular DNA preparations. That is, since fewer viral-related sequences are present in the human cellular DNA preparations, an increased amount of cellular DNA would have to be present in the reaction mixture in order to achieve a final extent of reaction equal to that observed with BEF-2 cellular DNA. Therefore, the 15% difference observed in the final extent of reassociation might have been eliminated in part or in full by increasing the amount of human cellular DNA in the hybridization reaction mix.

A much greater difference in the final extent of reassociation, approximately 30%, was observed with the A673(BAB455-K) human cellular DNA association with the M7[^{3}H]cDNA probe (Figure 9). The association

Figure 9. Hybridization of the baboon M7[^{3}H]cDNA viral probe to DNAs isolated from baboon virus producing, exogenously infected human cell lines. DNA isolation methods, hybridization conditions and hybrid detection procedures were as previously described in Materials and Methods. (●——●), the C_0t curve representing reassociation kinetics of the M7 viral probe with BEF-2 cellular DNA was included for reference. (o——o), the reassociation kinetics curve of M7[^{3}H]cDNA with A204(M7) infected cellular DNA. Approximately 320 μg of A204(M7) denatured cellular DNA was incubated with 0.01 ng of viral probe for each reaction point indicated on the curve. (□—□), the M7 viral probe's reassociation pattern with HOS(M7) infected cellular DNA. Reaction mixtures contained 0.01 ng of M7[^{3}H]cDNA incubated with an average 300 μg of denatured HOS(M7) cellular DNA. (Δ——Δ), the C_0t curve representing reassociation kinetics of A673(BAB455-K) infected cellular DNA with the M7[^{3}H]cDNA viral probe. An average of 210 μg of A673(BAB455-K) denatured cellular DNA was incubated with 0.01 ng of M7 viral probe. Note that the A673(BAB455-K) $EC_0t_{1/2}$ 592 is quite similar to the HOS(M7) $EC_0t_{1/2}$ 632, but that their final extents of reassociation to the M7 viral probe differ by approximately 11%.

FIGURE 9
HYBRIDIZATION KINETICS OF M7cDNA WITH DNA FROM INFECTED HUMAN CELLS



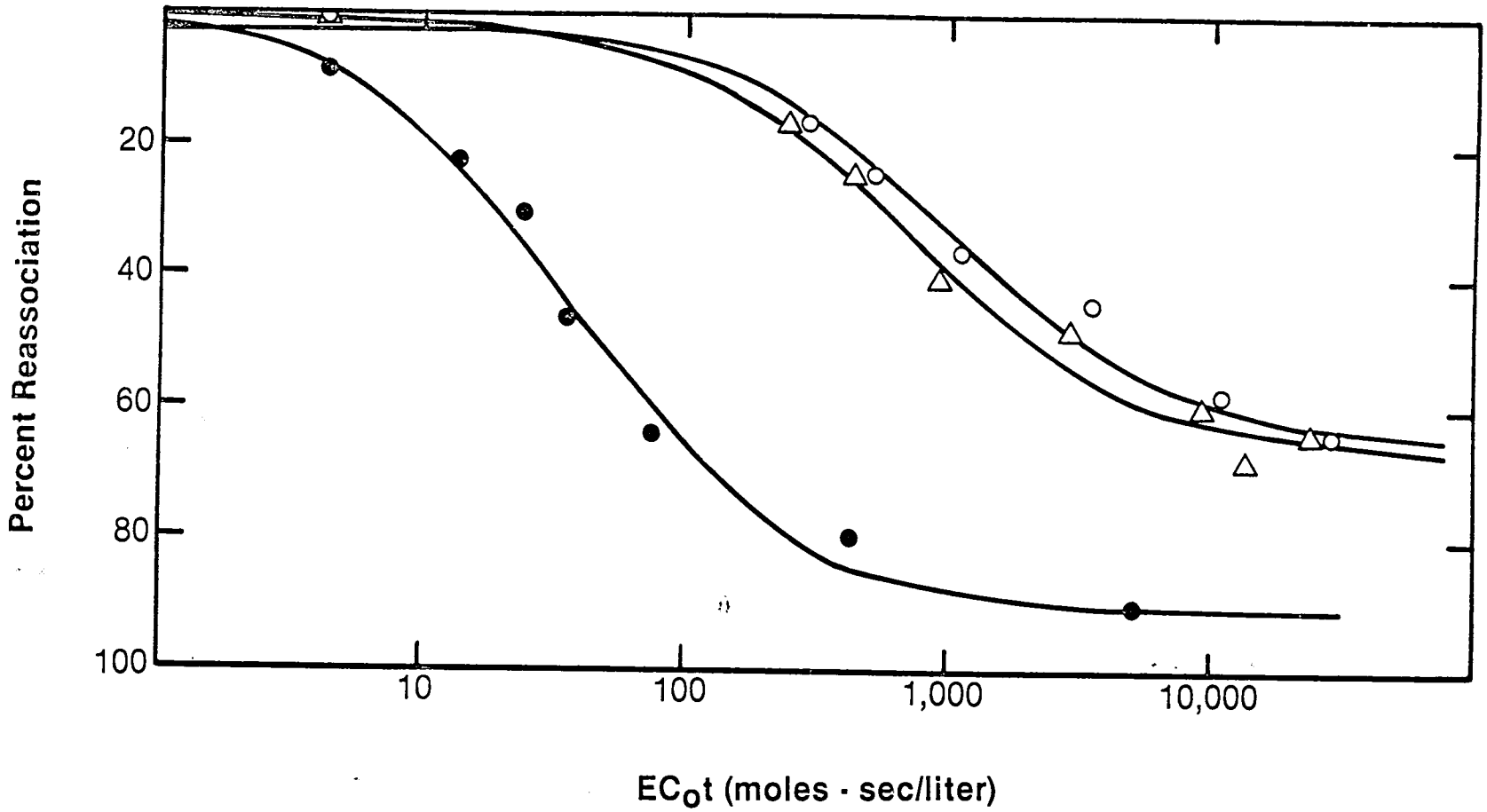
kinetics of this human cellular DNA preparation with the M7 viral probe also indicated the presence of approximately 6 copies of viral-related sequences per haploid genome. Although a portion of the 30% difference in final extent of reassociation might be eliminated by increasing the total amount of cellular DNA present in the reaction mix, the remaining difference in final extent of reaction might be attributed to the specificity of the M7[^{3}H]cDNA viral probe. That is, although the BAB(455-K) baboon endogenous virus (P. anubis kidney isolate) is related to the M7 (P. cynocephalus placental isolate), they are not identical (Haseltine and Kleid, 1978). The slight differences between these two baboon endogenous virus isolates may be greatly magnified due to their propagation in heterologous human cell lines, and the specific endogenous baboon viral sequences represented in the M7[^{3}H]cDNA viral probe.

Additional hybridization reactions conducted between the M7[^{3}H] viral probe and denatured cellular DNAs obtained from RD(BAB8-K) and D17(BAB8-K) producing cell lines yielded a similar pattern of association kinetics (Figure 10). Both the number of viral-related sequences detected in their cellular DNAs (3 to 5), and the difference in final extents observed with these reactants, 25 to 28% as compared to the BEF-2 C_0t curve, correlated with the earlier data reported. Since these exogenously infected cell lines were producing the BAB8-K kidney isolate (P. cynocephalus), the differences observed in final reaction extents may again be attributed to a slight difference between the two baboon endogenous virus isolates, and the amount of cellular DNA present in hybridization reaction mixtures.

The results of the hybridization reactions described above confirm the initial data reported for the A204(M7) cellular DNA association kinetics with the M7[^{3}H]cDNA viral probe. Based on a direct comparison

Figure 10. Hybridization of the baboon M7[^{3}H]cDNA viral probe to DNAs isolated from cell lines exogenously infected with and producing the BAB8-K endogenous baboon virus isolate. DNA isolation methods, hybridization conditions and hybrid detection procedures were as described in Materials and Methods. (●——●), the C_0t curve representing reassociation kinetics of the [^{3}H]thymidine labeled viral probe with BEF-2 cellular DNA is included as a reference point. (Δ——Δ), a C_0t curve representative of the reassociation kinetics of the M7 viral probe with RD(BAB8-K) infected cellular DNA. Approximately 350 μg of RD(BAB8-K) denatured cellular DNA was incubated with 0.01 ng of the M7 [^{3}H]cDNA probe. (o——o), the C_0t curve indicative of reassociation kinetics between the M7 viral probe and D17(BAB8-K) cellular DNA. Each reaction point represents 0.01 ng of M7[^{3}H]cDNA incubated with an average of 335 μg of D17(BAB8-K) denatured cellular DNA. Note that the differences in final extents of reassociation of RD(BAB8-K) and D17(BAB8-K) with M7[^{3}H]cDNA, and that observed for reassociation of BEF-2 cellular DNA with the viral probe range from 25-28%.

FIGURE 10
HYBRIDIZATION KINETICS OF M7cDNA WITH DNA FROM INFECTED CELLS



of the reassociation kinetics of human unique sequence DNAs, the M7[^{3}H] cDNA viral probe associates with cellular DNA of baboon origin (endogenous system) with kinetics indicating the presence of 95 copies of viral-related sequences present per haploid genome. Association kinetics of the M7[^{3}H] viral probe with DNAs from a variety of cell lines exogenously infected with a baboon endogenous virus isolate indicate that only 3 to 11 copies of viral-related sequences are present per haploid genome. Therefore, exogenously infected cell systems contain 10 to 20 times fewer viral-related sequences per haploid genome than baboon cells.

Quality of M7[^{3}H]cDNA hybrid formation with DNA extracted from an exogenously infected human cell line. Since a difference was observed in the final extent of reactions with some DNAs isolated from human cell lines exogenously infected with baboon endogenous virus, a series of thermal elutions was conducted on representative M7[^{3}H]cDNA-human cellular DNA hybrids to check their degree of thermal stability. Well-matched or complementary duplexes dissociate at higher temperatures (approximately half-eluted by 80°C) than hybrids having a greater degree of mismatching. A thermal elution profile representative of this series of melts involves the [^{3}H] viral probe and RD (BAB8-K) human cellular DNA. As shown in Figure 11, 50% of the bound [^{3}H]cDNA had eluted by 82°C. The temperature at which 50% of the bound [^{14}C]HeLa native DNA had eluted was 87.2°C. Again the [^{14}C]HeLa native DNA preparation was included as representative of a well-matched duplex and served as a marker on column performance. The difference in melting temperature (ΔTm) observed between these two sets of duplex molecules, [^{3}H] and [^{14}C], was only 5.2°C. The small ΔTm value achieved indicated a high degree of complementarity between the [^{3}H] viral probe and the specific viral-related sequences which it

Figure 11. Thermal elution profile of the hybrid formed between M7[^{3}H] cDNA and RD(BAB8-K) DNA. A reaction mixture containing 1.8 x 10^{-2} ng (600 cpm) of M7[^{3}H]cDNA was incubated with 496 μg of RD(BAB8-K) denatured cellular DNA to a C_0t of 14,600 and applied to an HAP column at 50°C. A total of 56.5% of the ^{3}H counts per minute bound. A 50 μl sample of native HeLa[^{14}C]DNA was applied to the column to serve as a marker for total cellular DNA dissociation. The material bound to the column was eluted during a linear temperature gradient (0.8°C per fraction). Each fraction was measured for absorbance at 260 nm, and was assayed for radioactivity after addition of two volumes of Aquasol II. The inset contains an integral plot of the data. Temperature at which 50% of bound ^{3}H eluted = 82°C; temperature at which 50% of bound ^{14}C eluted = 87.2°C. o——o, ^{3}H counts per minute in each fraction; Δ——Δ, ^{14}C counts per minute per fraction, o——o, O.D.$_{260}$ nm.

FIGURE 11
THERMAL STABILITY OF THE M7cDNA-RD(BAB8-K)
DNA HYBRID

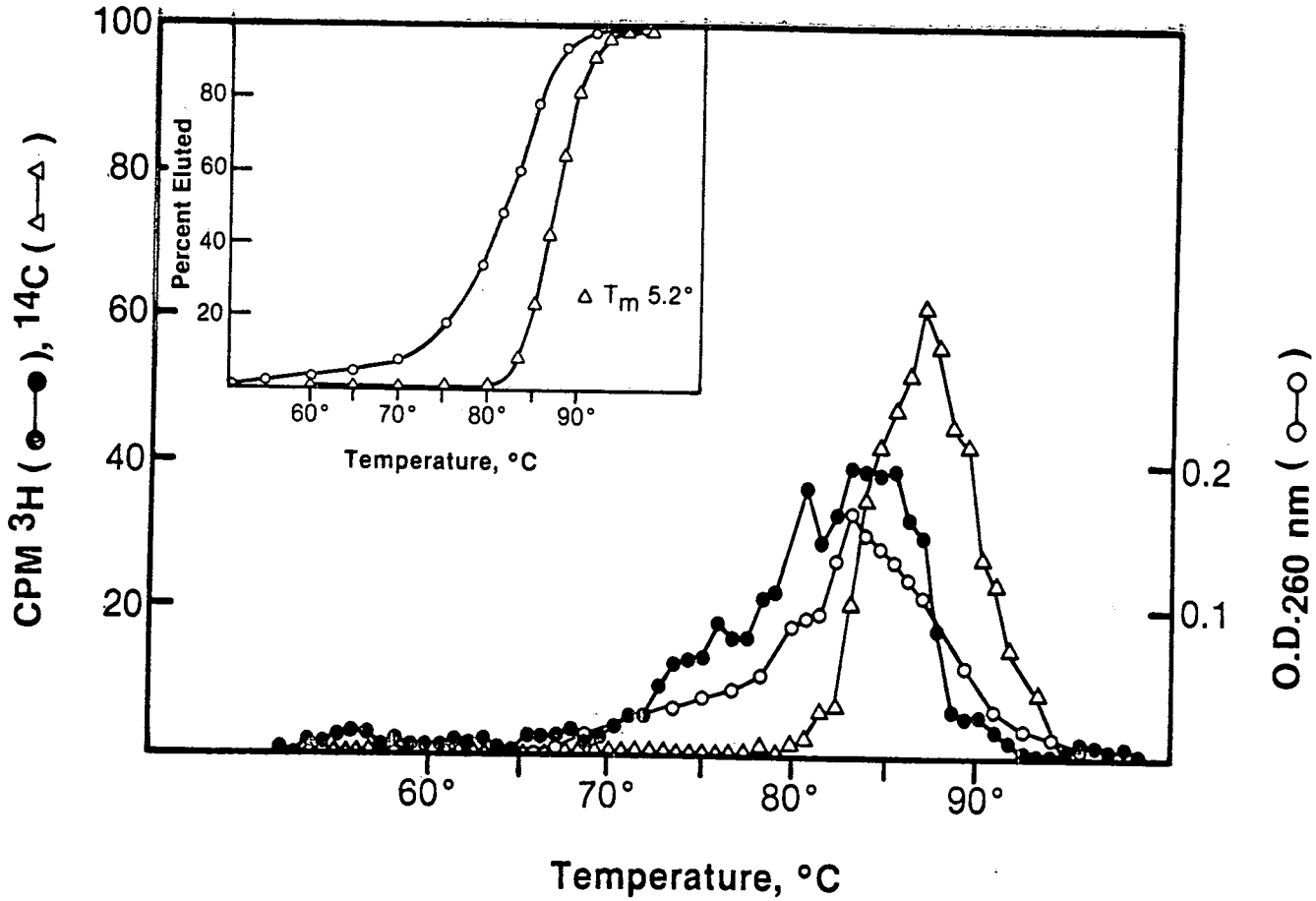


TABLE 1

THERMAL ELUTION DATA ON M7cDNA HYBRIDS

DNA Source[a]	EC_0t	$[^3H]Tem_{50}$[b]	$[^{14}C]Tem_{50}$[c]	ΔT_m[d]
BEF-2	1,600	83.7°C	88.7°C	5°C
BEF-1	4,000	79.0°	86.5°	7.5°
P. papio	28,500	80.4°	88.0°	7.6°
A204(M7)	39,300	77.9°	85.7°	7.8°
HOS(M7)	34,000	79.7°	86.9°	7.2°
RD(BAB8-K)	19,500	82.0°	87.2°	5.2°
A673(BAB455-K)	13,700	73.0°	85.5°	12.5°

[a]DNA was extracted from these cells as described in Materials and Methods.

[b]The M7[^{3}H]cDNA probe was hybridized to these cellular DNAs as described in Materials and Methods. Each reaction mix contained from 900 to 2,000 [^{3}H]cpm per assay point, and from 100 μg to 1.0 mg of cellular DNA. Hybridizations were conducted in 1.0 M phosphate buffer, and incubated at 63°C to the EC_0t values shown above. The temperature at which 50% of the hybridized [^{3}H]cDNA dissociated is reported as Tem_{50}.

[c]Column operation and elution conditions were monitored via a native HeLa [^{14}C]DNA marker.

[d]The ΔT_m is the difference in Tem_{50} between the [^{14}C]HeLa native DNA marker and the M7[^{3}H]cDNA-cellular DNA hybrids.

detected in the human cellular DNA preparation. Also, the ΔTm generated by this melt varies by only 0.2°C from the ΔTm previously reported for the endogenous system involving the M7[^{3}H] viral probe and BEF-2 cellular DNA.

A series of thermal elution analyses were conducted on selected M7 [^{3}H]cDNA-cellular DNA hybrids (Table 1). The [^{14}C]HeLa native DNA marker was half-eluted by 85.5°C or above in all columns used for hybrid Tm analysis. Respective ΔTm values of 7.2°C and 7.8°C were obtained from hybrid melts involving HOS(M7) and A204(M7) cellular DNAs. The ΔTm values obtained by thermal elution of hybrids involving the baboon cellular DNAs, BEF-1 and P. papio, were approximately 7.5°C. These results are comparable to those reported for the A204(M7) and HOS(M7) cellular DNA hybrids. A much larger ΔTm of 12.5°C was observed in the melt involving the A673 (BAB455-K) human cellular DNA. If comparisons were to be made based on the ΔTm data obtained from these hybrid thermal elution profiles, one might conclude that: (1) the binding observed between the M7[^{3}H]cDNA viral probe and cellular DNAs from M7-producing human cell lines was specific since comparable ΔTm values were achieved with baboon cellular DNAs from BEF-1 and P. papio; (2) the ΔTm values obtained from BEF-2 (P. cynocephalus) and P. papio liver DNA hybrid melts show a 2.5°C difference which may be a reflection of a difference in baboon species; and (3) the large ΔTm value observed in the hybrid melt involving the A673(BAB455-K) human cellular DNA preparation may be a reflection of the slight differences known to exist in the genomes of baboon endogenous virus isolates.

Expression and detection of viral-related sequences in various baboon DNAs. One of the original questions posed in this thesis, i.e., of a correlation between active virus production and the number of viral-related

TABLE 2

SUMMARY OF HYBRIDIZATION DATA WITH M7cDNA M7[^{3}H]cDNA PROBE

	% Hybridization to Cell DNA	$EC_0t_{1/2}$	Number of copies of viral-related sequences/ haploid genome+
Virus Producing Cells			
Human - A204(M7)	75*	300	11
HOS(M7)	70*	630	5
A673(BAB455-K)	59*	590	6
RD(BAB8-K)	66*	710	5
Canine - 8155(M7)	69*	640	5
D17(BAB8-K)	63*	1000	3
Baboon - *P. cynocephalus*			
BEF-2 (low-producer)	91*	35	95
BEF-3 (high-producer)	80	~53	~65
Non-Virus Producing Cells			
Human - A204	4	NT	<1
Baboon - *P. cynocephalus* (?)			
BEF-1	71*	330	10
Tissues			
Baboon - *P. cynocephalus*			
Placenta #1129	54	~51	~65
Placenta #2027	71	~80	~40
Spleen #1589	70†	~56	~60
P. cynocephalus (?)			
Liver #1570	69†	~120	~30
P. papio			
Liver	68*	250	13-14
Mouse - *Mus musculus*			
Liver	4	NT	<1

Legend - Table 2

*The % reassociation shown for these hybrids is the final extent of reaction as taken from the best fit curve generated by least squares with equal weight for each measured value (Britten et al., 1974). All other values reported are the actual final extents of reassociation reached with hybridization reactions carried out to an extreme C_0t.

†The M7[^{3}H]cDNA probe used in these hybridization reactions was not the original probe used in the bulk of this study. Synthesis and purification of this cDNA probe was conducted similar to that described in Materials and Methods. Under identical reaction conditions, this M7 cDNA reassociated from 85 to 90% with both its original genomic RNA template and cellular DNA of baboon origin (BEF-2).

+Calculations on the number of copies of viral-related sequences present per haploid genome were made as previously described in the legend to Figure 6.

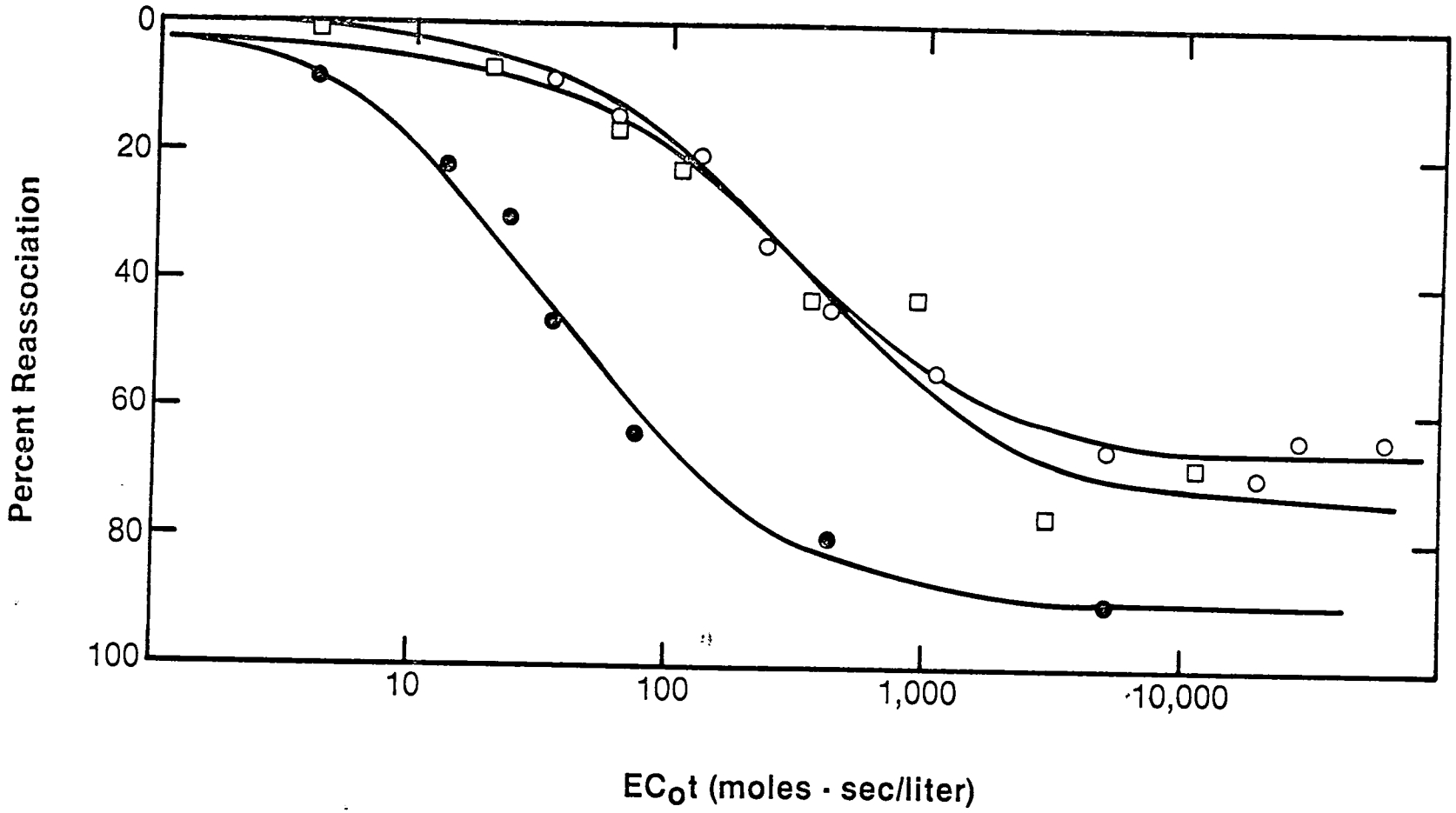
~Approximate number based on comparative association kinetics with BEF-2.

sequences detected in endogenous cellular DNA, was addressed by screening a variety of tissues and cell lines of baboon origin. The results obtained from this set of hybridization reactions are reported in Table 2. The DNAs extracted from placenta #1129 and #2027, from spleen #1589, and from a virus-producing cell line (BEF-3) all achieved an $EC_0t_{1/2}$ approximating that observed with the low level virus-producing cell line, BEF-2. The number of viral-related sequences present in these P. cynocephalus DNAs range from 40 to 80 copies per haploid genome as compared to the 95 copies observed in BEF-2 DNA. Since both placentas proved capable of releasing infectious virus upon co-cultivation with D17 cells (McAllister, personal communication), and there appeared to be no difference in the amount of proviral sequences contained in the DNAs from low vs. high virus producing baboon cell lines, one might conclude that there is no definitive correlation between the level of expression of the baboon endogenous virus and the number of viral-related sequences present in the DNA of cells transmitting endogenous viral information.

Also presented in Table 2 are the results obtained with a definite non-virus producing cell line of baboon origin, BEF-1. Association kinetics of hybridization reactions involving this cellular DNA (Figure 12) indicate that only 10 copies of viral-related sequences per haploid genome were detected using the M7[^{3}H]cDNA probe. Similar results were obtained with one additional baboon tissue tested, liver #1570, in which 30 copies per haploid genome were detected. Both this BEF-1 cell line and liver #1570 lacked any detectable p30 antigens, showed no virus release upon cocultivation with heterologous cell lines, and resisted virus induction by treatment with bromodeoxyuridine (McAllister, personal communication). Although both of these cellular DNAs are supposedly of P. cynocephalus

Figure 12. Hybridization of the baboon M7[^{3}H]cDNA viral probe to various baboon DNAs. The [^{3}H]thymidine labeled cDNA probe was synthesized in an endogenous reaction via the virion-coded reverse transcriptase as previously described in Materials and Methods. Cellular DNA was extracted from tissue and cell lines also as previously described. (o——●), the C_0t curve representing reassociation kinetics of the M7[^{3}H]cDNA probe with BEF-2 cellular DNA. C_0t points reported here were achieved by incubating 0.01 ng (410 cpm) of viral probe with an average of 175 μg of BEF-2 (P. cynocephalus) denatured DNA. (o——o), a C_0t curve representative of the reassociation kinetics of the M7 viral probe with P. papio liver DNA. Reaction mixtures contained 0.01 ng M7[^{3}H]cDNA and 220 μg of denatured liver DNA. (□——□), the M7[^{3}H]cDNA probe's reassociation pattern with BEF-1 DNA were incubated with 0.01 ng of the M7[^{3}H]cDNA probe. Although these DNAs were of baboon origin, differences in their rates of reassociation with the M7 viral probe were reflected by their equivalent half C_0ts: BEF-2 $EC_0t_{1/2}$ 35, BEF-1 $EC_0t_{1/2}$ 330, and P. papio $EC_0t_{1/2}$ 246. Note also the difference observed in the final extent of reassociations among these baboon DNAs.

FIGURE 12
HYBRIDIZATION KINETICS OF M7cDNA WITH BABOON DNA

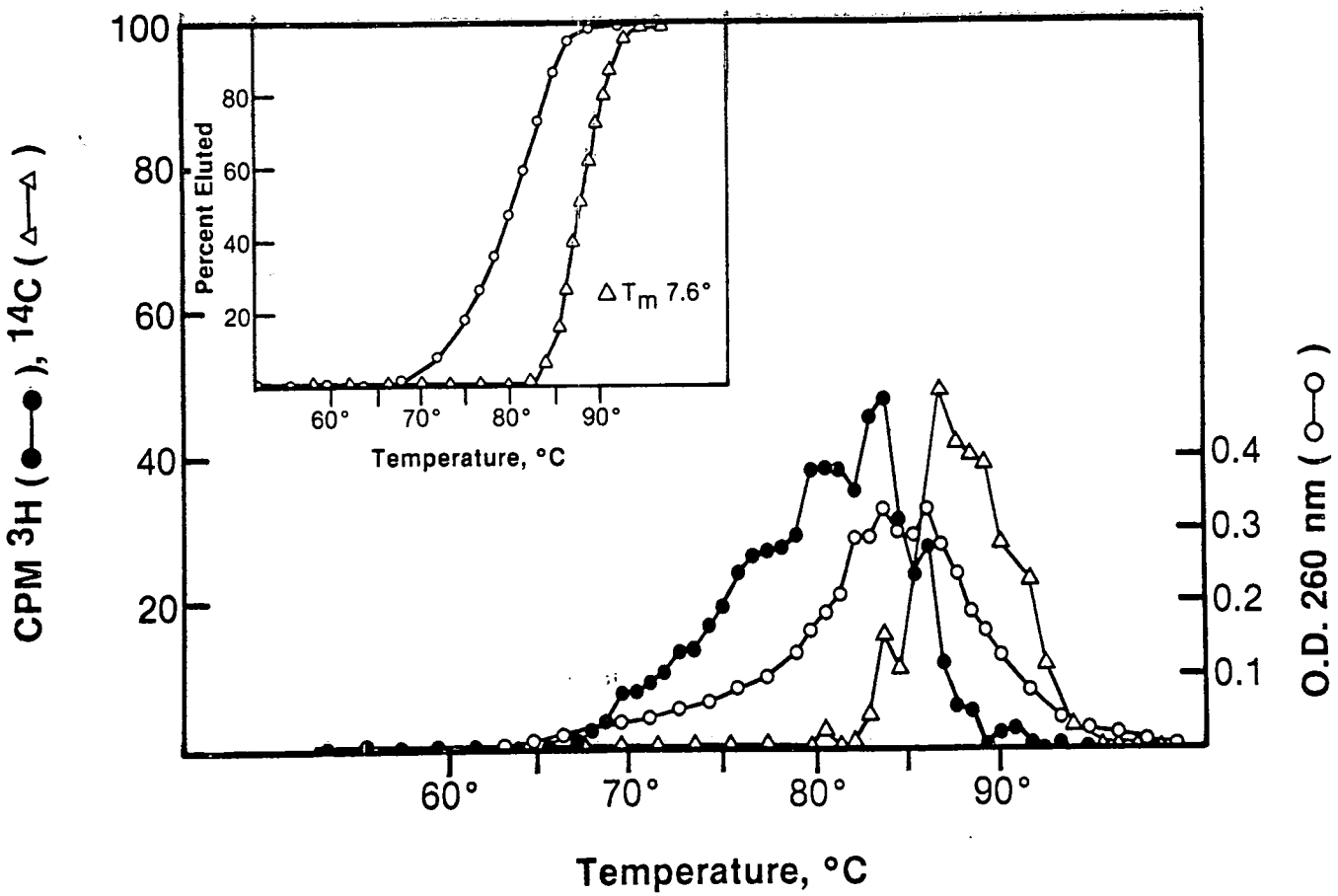


origin, their association kinetics with the M7[^{3}H]cDNA viral probe suggest they behave differently than all other P. cynocephalus DNAs tested.

In an effort to determine if the difference observed with the BEF-1 cellular DNA could be related to the level of virus expression in an endogenous cell system, or might be the result of subtle differences in endogenous viral information as carried in baboon species and detected by the [^{3}H] viral probe, an additional set of hybridization reactions were conducted employing another baboon (P. papio) liver DNA. The hybridization kinetics of this C_0t curve (Figure 12) suggested 13 to 14 copies of viral-related sequences were present per haploid genome in this baboon liver DNA preparation. Also, the final extent of reaction achieved with this DNA preparation was reduced by approximately 22% from that reached with the BEF-2 (P. cynocephalus) cellular DNA. When this M7[^{3}H]cDNA P. papio hybrid was checked for thermal stability by thermal elution on an hydroxylapatite column (Figure 13), a Tem_{50} of 80.4°C and a ΔTm of 7.6°C was recorded. The ΔTm recorded showed a 2.6°C reduction as compared to the ΔTm observed in the thermal elution profile with BEF-2. Thermal elution analysis of the M7[^{3}H]cDNA-BEF-1 hybrid molecules resulted in a Tem_{50} of 79°C and a 2.5°C reduction in ΔTm as compared to BEF-2. These results may indicate that the viral-related sequences detected in BEF-1 and P. papio cellular DNAs by this viral probe might be related to but not identical with the sequences present in the M7[^{3}H]cDNA viral probe. Evidence favoring this conclusion is also provided by the reduction in [^{3}H]Tem_{50} observed in the thermal elution profile of hybrids formed between the M7[^{3}H]cDNA and A673(BAB455-K) cellular DNA. The 7.5°C increase in ΔTm over that seen for BEF-2 cellular DNA might be due in part to differences between the M7 (P. cynocephalus) and BAB455-K (P. anubis) baboon endogenous

Figure 13. Thermal elution profile of the hybrid formed between M7[^{3}H] cDNA and P. papio liver DNA. A reaction mixture containing 1.8 x 10^{-2} ng (600 cpm) of M7[^{3}H]cDNA with an average size of 160 nucleotides, and 890 μg of P. papio denatured liver DNA with an average size of 450 nucleotides was incubated to C_0t 21,000 and applied to an HAP column at 50°C. A total of 59.5% of the ^{3}H counts per minute bound. A 50 μl sample of native HeLa[^{14}C]DNA was applied to the sample prior to column loading as a control on column performance. The material bound to the column was eluted during a linear temperature gradient (0.8°C per fraction). Fractions were measured for absorbance at 260 nm as a check on appropriate dissociation of cellular DNA, and were assayed for radioactivity after addition of two volumes of Aquasol II. The inset presents an integral plot of the data. Temperature at which 50% of bound ^{3}H eluted = 80.4°C; temperature at which 50% of bound ^{14}C eluted = 88.°C. o——o, ^{3}H counts per minute in each fraction; Δ——Δ, ^{14}C counts per minute per fraction; o——o, $O.D._{260}$ nm.

FIGURE 13
THERMAL STABILITY OF THE M7cDNA-P. papio DNA HYBRID



virus isolates. Therefore, a possibility exists that the BEF-1 and liver #1570 DNA preparations are really not of P. cynocephalus origin, but rather are representatives of another baboon species.

Up to this point, all of the hybridization data gathered indicates that approximately 10 to 20 times more viral-related sequences are present in DNA of baboon origin than present in cell lines exogenously infected with a baboon virus isolate. In addition, within cell lines of baboon origin, there exists no clear correlation between the number of viral-related sequences present in cellular DNA and the actual level of virus production demonstrated by the cell line. The M7[^{3}H]cDNA probe used in these studies also demonstrated the ability to discriminate the various baboon species studied. Presumably this differentiation was based on slight differences in the endogenous viral information contained in the cellular DNAs tested. No conclusions can be made regarding the extent of proviral information contained in baboon cellular DNA obtained from non-virus-producing sources because those sources investigated herein (BEF-1 and liver #1570) may be of dubious origin.

Extent of nucleic acid homology between human DNA and the M7[^{3}H]cDNA viral probe. To determine if sequences homologous to the M7[^{3}H]cDNA could also be detected in the DNAs extracted from tissues of human origin, the viral probe was reacted with normal human and leukemic human DNA preparations. The limits of detection for the M7 viral probe as previously defined, dictated that 1.0 μg of cellular DNA per microliter probe would be required for detection of one copy of viral-related sequences per haploid genome to reach 80% of its final extent of reaction. Table 3 lists the various tissue DNAs examined for nucleic acid sequence homology to the M7 viral probe. The M7 viral probe was 81% reassociated to its

TABLE 3

M7cDNA - LACK OF HOMOLOGY WITH HUMAN DNAs

	C_0t	% Reassociation [^{3}H]	% Reassociation O.D.	Total O.D. (mg) DNA
M7[^{3}H]cDNA	NA	5.5	NT	NT
M7 70S viral RNA[a]	5	81.2	NT	NT
Mouse Liver DNA[b]	1.23×10^5	2.9	94.3	0.7
	1.24×10^5	4.9	92.3	1.0
Human (normal) DNA	2.0×10^4	10.1	83.4	0.3
Human (leukemic) DNA[c]	9.3×10^4	8.9	90.2	0.2
	9.0×10^4	5.7	80.4	0.6
	8.6×10^4	8.2	92.7	0.8
P. papio liver DNA	6.7×10^3	57.8	86.0	0.1

NA - Not applicable.

NT - Not tested.

[a]Approximately 0.3 nanograms of M7 70S RNA was reassociated with the [^{3}H] cDNA viral probe.

[b]*Mus musculus* liver DNA was prepared as previously described in Materials and Methods.

[c]DNA was extracted from cells obtained from a patient (#119118) having acute myelogenous leukemia.

original RNA template at a C_rt of 5. Incubation of the M7 viral probe alone for an extended period of time yielded a background level for association of only 5%. Reaction of the M7 endogenous baboon viral probe with baboon (P. papio) liver DNA resulted in 58% reassociation at a C_0t value less than 10,000. Although the majority of mouse cellular DNA sequences reassociated to greater than 90%, less than a 5% association with the M7[^{3}H]cDNA was achieved at C_0t values exceeding 100,000. All human cellular DNA sequences reassociated to 80% or greater at C_0t values ranging from 20,000 to 90,000. Association with the M7 viral probe reached 10% with normal human DNA and approximated 9% with cellular DNAs obtained from leukemic human patients. Since the inclusion of increasing amounts of leukemic human cellular DNA under reaction conditions designed to detect one copy of viral-related sequences per haploid genome did not indicate any increase in association with the M7 viral probe, the level of viral-related sequences present must be less than one copy per haploid genome.

Summary of proviral representation in various cellular DNAs. Table 2 includes all of the information gathered regarding the number of viral-related sequences detected in the cellular DNAs screened in this study. Hybridizations of the M7[^{3}H]cDNA viral probe to DNAs extracted from an uninfected human cell line (A204) and from mouse (Mus musculus) liver detected less than one copy of viral-related sequences per haploid genome. Cellular DNAs extracted from cell lines exogenously infected with and producing a baboon endogenous virus isolate, regardless of species of origin, contained eleven or less viral-related sequences per haploid genome. That is, approximately 10 to 20 times less endogenous viral information was contained in cellular DNA from exogenously-infected cell lines than was contained in DNA of baboon origin.

Hybridizations conducted with baboon (P. cynocephalus) cellular DNAs obtained from established cell lines and baboon tissues detected approximately 40 to 95 copies of viral-related sequences per haploid genome. The one exception noted within the tissue of P. cynocephalus origin was that obtained with DNA from baboon liver #1570 which only contained approximately 30 copies of viral-related sequences per haploid genome. Although the amount of proviral information exhibited in this preparation is reduced in comparison to the rest of the P. cynocephalus group tested, it represents approximately 6 times the amount of information contained in the exogenously infected cellular DNAs mentioned earlier. This may also be of interest since, in spite of numerous attempts conducted in another laboratory (McAllister, personal communication), no infectious virus has ever been isolated from this tissue.

Results obtained from hybridization reactions employing cellular DNA from a different baboon species, P. papio, also indicated a decrease in the number of M7 viral-related sequences detected per haploid genome. Similar results were obtained with the BEF-1 cellular DNA preparation. Although the BEF-1 cell line is reportedly of P. cynocephalus origin, both its association kinetics and thermal elution profile suggest that it may be more closely related to the P. papio species. As previously mentioned, the decrease in viral-related sequences detected by the M7 viral probe may be a reflection of slight differences in endogenous baboon viral information contained in this species as differentiated by this M7 viral probe.

Summary of thermal elution analysis of M7 hybrids. Table 1 contains the information gathered from thermal elution analysis of selected M7cDNA-cellular DNA hybrids. If a comparison is made using the $[^3H]$-$[^{14}C]$ ΔTm data obtained from this analysis, three distinct groupings for hybrids

become apparent. The first group contains the BEF-2 and RD(BAB8-K) with a ΔTm of 5°C and 5.2°C, respectively. A second group is composed of P. papio, BEF-1, and all of the exogenously infected cellular DNAs [with the exception of A673(BAB455-K)] with ΔTm ranging from 7.2°C to 7.8°C. The third and last group contains the A673(B455-K) cellular DNA with a ΔTm of 12.5°C. This grouping allows a distinct separation of baboon species, i.e., BEF-2 (P. cynocephalus), and BEF-1 - P. papio, as discriminated by the M7 viral probe. A second distinct separation involving human cell lines infected with and producing three different baboon endogenous virus isolates also became apparent, i.e., RD(BAB8-K), HOS(M7), A204(M7), and A673(BAB455-K). Therefore, it appears that subtle differences in endogenous baboon viral information as contained in DNA of various baboon species, as well as slight differences between actual baboon endogenous viral isolates expressed in exogenously infected human cell lines, were detected and differentiated by the M7[^{3}H]cDNA viral probe utilized in this study.

V. Discussion

M7, the endogenous baboon virus (placental) isolate used for these studies, was propagated in and recovered from cultures of an A204(M7) infected cell line. The budding process and morphology of the M7 virions produced by this exogenously infected human cell line are typical of C-type retraviruses. Preparations of [^{3}H]-labeled M7 virions, produced by A204(M7) monolayer cultures post exposure to a pulse of radioactively-labeled nucleotides, banded at 1.14-1.16 g/cm^3 when subjected to isopycnic centrifugation on sucrose gradients. The [^{3}H]-labeled nucleic acid isolated from these band-purified virions sedimented as 70S RNA upon velocity sedimentation centrifugation through neutral sucrose. SPAGE analysis of the M7 viral proteins generated a gel profile similar to that of a well-documented murine C-type retravirus (RLV). Both the major envelope glycoprotein (gp70) and the major internal core shell protein (p30) were evident in both viral protein gel profiles. The presence of a virion-associated reverse transcriptase activity was documented by synthesis, via an endogenous reaction, of a DNA strand complementary to the viral genomic RNA. The extent of reassociation achieved with this M7[^{3}H]cDNA viral probe in reactions containing DNA from baboon tissues which lacked any detectable level of virus expression (BEF-1 and liver #1570) suggests that M7 is an endogenous baboon virus isolate. Other investigators have previously reported data identical to that summarized above, and have concluded that the baboon endogenous virus isolates comprise a distinct group of C-type retraviruses (Benveniste _et al_., 1974a; Sherr _et al_., 1974; Todaro _et al_., 1974).

The majority of the studies presented here were conducted to detect sequences homologous to the M7[^{3}H]cDNA probe in cellular DNAs of various

origin. Since the exact number of viral-related sequences detected in cellular DNAs have been known to vary with the type of viral probe employed (Baluda, 1972; Neiman, 1972; Varmus et al., 1973b), the M7[^{3}H]cDNA probe was initially characterized with respect to both specificity of binding and actual genomic representation. Although synthesized in the absence of actinomycin D, the M7[^{3}H]cDNA probe proved to be more than 96% single-stranded as measured by HAP column chromatography. The M7cDNA probe also proved to be at least 81% complementary to the M7 virion 70S RNA. Its average size, as determined by centrifugation in an alkaline sucrose gradient, was about 160 nucleotides.

Experiments designed to determine the complexity of the M7cDNA suggested that it represented less than about 5% of the viral genome (N. Rice, personal communication). It is possible, therefore, that the predominant species in this probe is the so-called "strong-stop" DNA, the copy of the approximately 120 nucleotides (Haseltine and Kleid, 1978) located between the tRNA primer and the 5' end of the viral genome. If this is so, and if M7 viral RNA exhibits the terminal redundancy reported to date for Moloney leukemia virus (Coffin et al., 1978), then this cDNA may have two binding sites per genome, and by extension, two binding sites per provirus. Thus, finding 10 viral related sequences per haploid cellular genome may in fact reflect only 5 proviral sequences. For this reason, all calculations in the text are expressed as "number of viral-related sequences" instead of the more definitive "number of proviral copies."

The M7cDNA viral probe described above was reassociated with various cellular DNAs under reaction conditions designed to allow the annealing of even distantly related nucleotide sequences (1.0 M phosphate buffer, 63°C). Under these reaction conditions this M7cDNA probe consistently

detected more proviral information in DNAs of baboon origin than in DNAs from exogenously infected heterologous cell lines. As shown in Table 2, a range of 30 to 95 copies of viral-related sequences per haploid genome were detected in the baboon cellular DNAs tested while only 3 to 11 copies were present in the DNA of exogenously infected heterologous cell lines. These results agree with data previously reported on the copy frequency of endogenous virogenes in mammalian cells. Benveniste and Todaro (1974) examined the reiteration frequency of mouse, rat, pig, cat, and baboon viral nucleic acid sequences in tissues and cell cultures of the species of viral origin and in exogenously infected cells of heterologous species. Their data indicated that endogenous viral-related sequences are present at about 5 to 15 copies per haploid genome. Heterologous cell lines exogenously infected with and producing various virus isolates were found to contain fewer copies (1 to 2) per haploid genome. This difference between endogenous and exogenously infected cells has also been reported by Okabe et al. (1978), who found approximately 20 to 25 genome copies of RD-114 related sequences in the DNA of various cat cells but only 1 to 5 genome equivalents in an RD-114 infected human cellular DNA preparation.

Data collected from the association kinetics of reactions involving baboon DNAs from both high virus-producing and low virus-producing cell lines clearly indicates no difference in the number of viral-related sequences present in their cellular DNAs. A range of 40 to 85 copies of proviral information was found in baboon tissues expressing an active level of virus production. The specific comparisons made involved the low virus-producer cell line, BEF-2, and the high virus-producer line, BEF-3, which respectively contained 95 and 65 copies of viral-related sequences per haploid genome. Recently, an additional high-producer

BEF-2 cell line was checked for frequency of proviral sequences. In a side by side reaction, the DNA representative of the high virus-producing cell line demonstrated virtually identical association kinetics as those obtained with the original low producer BEF-2 DNA preparation. Also, the association kinetics observed for these BEF-2 DNAs were similar to the juvenile spleen and baboon placenta DNAs tested, both of which demonstrated low levels of virus production.

The original question of a possible change in copy number from non-producer to producer status cannot be addressed. Only two baboon cellular DNAs tested proved incapable of any form of viral expression. These DNAs, BEF-1 and liver #1570, had lower numbers of viral-related sequences then all the virus producing cells of baboon origin tested. However, since the copy number was also lower in the other baboon species tested (*P. papio*), and since a possibility exists that BEF-1 and liver #1570 were not of *P. cynocephalus* origin at all, the question of extent of proviral information in a non-producer endogenous cell system remains unanswered.

In a collaborative effort with Dr. Robert McAllister (Childrens Hospital of Los Angeles), an attempt was made to correlate the number of viral-related sequences, viral expression, and transfection potential of the various cellular DNAs screened in this study (Table 4). A major difference between endogenous type-C virogenes present in the DNA of cells spontaneously releasing virus (usually a heterologous system) and those not producing detectable levels of virus (usually an endogenous system) is that the viral sequences present in the chromosomal DNAs of heterologous systems prove to be transfective (capable of infecting) permissive cell lines (Cooper and Temin, 1976; Nicolson *et al.*, 1978). The transfection potential of the exogenously infected heterologous cell

lines, RD and D17, confirm the earlier data reported. Although only a few copies of viral related sequences are present in their cellular DNAs, both have proven capable of transfecting permissive cells. Similarly, DNA proviral sequences present in the chromosomal DNA of baboon cells which demonstrate active levels of virus production have also been proven capable of transfecting permissive cells. Usually, higher doses of these baboon DNAs are required to observe transfection. The data also suggests that baboon cellular DNA of nonvirus expressing origin (BEF-1 and liver #1570) lacks transfection capabilities as compared to the DNA of virus-expressing origin (placentas #1129, 2027, and BEF-3). This indicates that natural host sequences are only infectious if obtained from cells producing virus. In addition, the baboon cellular DNA obtained from the low-producer line (BEF-2) was also found incapable of transfecting permissive cells at the dosage levels tested. This result is in contrast to the earlier data reported for other mammalian species, and the reason for it remains unknown.

Although proviral sequences related to baboon endogenous virus have been reported in DNA of human leukemia tissues (Wong-Staal _et al._, 1976), an obvious lack of homology between the M7[^{3}H]cDNA probe and cellular DNAs of normal and leukemic human origin was indicated by the reassociation kinetics observed in these studies. The original work reported utilized baboon [^{125}I]-viral RNA as a probe to detect related sequences in human DNA preparations. Since the M7[^{3}H]cDNA viral probe used in the studies reported here represented <5% of the genomic RNA, it is possible that baboon viral-related sequences other than those represented by this cDNA probe may have been detected under hybridization conditions employing the entire viral genome. However, numerous other attempts have also failed to detect baboon viral-related sequences in human DNAs (Benveniste _et_

TABLE 4

CORRELATION BETWEEN EXTENT OF PROVIRAL REPRESENTATION, VIRAL EXPRESSION, AND TRANSFECTION POTENTIAL†

	P_{30} Antigen[a]	Virus Production[b]	Transfection[c]	Number Viral-related sequences/haploid genome
Tissues				
Placenta (1129)	+	+	+	~65
Placenta (2027)	+	+	+	~50
Spleen (1589)	+	+	-	~60
Liver (1570)	-	-	-	~30
Baboon Cell Strains				
Embryo fibroblast				
BEF-1 (7503551)	-	-	-	10
BEF-2 (2162)	+	+	-	95
BEF-3 (1129)	+	+	+	~65
Exogenously Infected Cell Lines				
D17 (BAB8-K)	+	+	+	3
RD (BAB8-K)	+	+	+	5

[a]Placentas were tested by RIA. All others were tested by CF test. (-) CF titer is equivalent to <2000 ng/ml P_{30} antigen.

[b]Assayed by cocultivation.

[c]Transfection studies were conducted as previously reported (McAllister *et al.*, 1978).

†The data presented in columns a, b, and c was obtained from a pre-print written by Dr. Robert M. McAllister on work conducted as part of a collaborative study on the infectivity of endogenous baboon type C viral genes.

al., 1974a; Benveniste et al., 1975; Sherr et al., 1974). Although the cDNA viral probe used here represented less than 5% of the viral genomic RNA, less than one copy of the sequences which it does represent were present in the human DNAs tested.

Literature Cited

Aaronson, S.A., W.P. Rowe. Nonproducer clones of murine sarcoma virus transformed BALB/3T3 cells. Virology 42:9-19, 1970.

Bader, J.P. The requirement for DNA synthesis in the growth of Rous sarcoma and Rous associated viruses. Virology 26:253-261, 1965.

Bader, J.P. Metabolic requirements for infection by Rous sarcoma virus. I. The transient requirement for DNA synthesis. Virology 29:444-451, 1966.

Bader, J.P. A method for the propagation of large amounts of Rous sarcoma virus. Virology 36:140-142, 1968.

Bader, J.P. Reproduction of RNA tumor viruses. In Comprehensive Virology, Vol. 4, H. Frankel-Conrat, R. Wagner, eds., Plenum Press, New York, pp. 253-332, 1975.

Baltimore, D. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature 226:1209-1211, 1970.

Baltimore, D., R. McCaffrey, D.F. Smoler. Properties of reverse transcriptases. In Second ICN-UCLA Symposium on Molecular Biology. C.F. Fox, W.S. Robinson, eds., Academic Press, New York, pp. 51-59, 1973.

Baluda, M.A. Widespread presence, in chickens, of DNA complementary to the RNA genome of avian leukosis virus. Proc. Natl. Acad. Sci. U.S.A. 69:576-580, 1972.

Benveniste, R.E., G.J. Todaro. Homology between type C viruses of various species as determined by molecular hybridization. Proc Natl. Acad. Sci. U.S.A. 70:3316-3320, 1973.

Benveniste, R.E., R. Heinemann, G.L. Wilson, R. Callahan, G.J. Todaro. Detection of baboon type C viral sequences in various primate tissues by molecular hybridization. J. Virol. 14:56-67, 1974a.

Benveniste, R.E., M.M. Lieber, D.M. Livingston, C.J. Sherr, G. J. Todaro, S.S. Kalter. Infectious C-type virus isolated from a baboon placenta. Nature 248:17-20, 1974b.

Benveniste, R.E., G.J. Todaro. Multiple divergent copies of endogenous C-type virogenes in mammalian cells. Nature 252:170-173, 1974.

Benveniste, R.E., C.J. Sherr, M.M. Lieber, R. Callahan, G.J. Todaro. Evolution of primate type-C viral genes. In Fundamental Aspects of Neoplasia, A.A. Gottlieb, O.J. Plescia, D.H.L. Bishop, eds., Springer-Verlag, New York, pp. 29-53, 1975.

Bernhard, W. The detection and study of tumor viruses with the electron microscope. Cancer Res. 20:712-727, 1960.

Bishop, J.M., W.E. Levinson, N. Quintrell, D. Sullivan, L. Fanshier, J. Jackson. The low molecular weight RNAs of Rous sarcoma virus. I. The 4S RNA. Virology 42:182-195, 1970a.

Bishop, J.M., W.E. Levinson, D. Sullivan, L. Fanshier, N. Quintrell, J. Jackson. The low molecular weight RNAs of Rous sarcoma virus. II. The 7S RNA. Virology 42:927-937, 1970b.

Biswal, N., B. McCain, M. Benyesh-Melnick. The DNA of murine sarcoma-leukemia virus. Virology 45:697-706, 1971.

Bittner, J.J. Milk influence of breast tumors in mice. Science 95:462-463, 1942.

Bolognesi, D.P., R.C. Montelaro, H. Frank, W. Schäfer. Assembly of type C oncornaviruses: A model. Science 199:183-186, 1978.

Bonar, R.A., J.W. Beard. Virus of avian myeloblastosis. XII. Chemical Constitution. J. Natl. Cancer Inst. 23:183-188, 1959.

Britten, R.J., D.E. Kohne. Repeated sequences in DNA. Science 161:529-540, 1968.

Britten, R.J., J.S. Smith. A bovine genome. Carnegie Inst., Wash. Yearb. 68:378-386, 1970.

Britten, R.J., D.E. Graham, B.R. Nuefeld. Analysis of repeating DNA sequences by reassociation methods. Enzymol. 29:363-418, 1974.

Clark, J.M., J.P. Bader, Inhibition of development of transformation by Rous sarcoma virus in cultures of high cell density. J. Cell Physiol. 83:203-210, 1974.

Coffin, J.M., H.M. Temin. Comparison of Rous sarcoma virus-specific deoxyribonucleic acid polymerases in virions of Rous sarcoma virus and in Rous sarcoma virus-infected chicken cells. J. Virol. 74: 625-634, 1971.

Coffin, J.M., W. Haseltine. Terminal redundancy and the origin of replication of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. 74:1908-1912, 1977.

Coffin, J.M., T.C. Hageman, A.M. Maxam, W.A. Haseltine. Structure of the genome of Moloney murine leukemia virus: A terminally redundant sequence. Cell 13:761-773, 1978.

Cooper, G.M., H.M. Temin. Lack of infectivity of the endogenous avian leukosis virus-related genes in the DNA of uninfected chicken cells. J. Virol. 17:422-430, 1976.

Dahlberg, J.E., R.C. Sawyer, J.M. Taylor, A.J. Faras, W.E. Levinson, H.M. Goodman, J.M. Bishop. Transcription of DNA from the 70S RNA of Rous sarcoma virus. I. Identification of a specific 4S RNA which serves as a primer. J. Virol. 13:1126-1133, 1974.

Dales, S., H. Hanafusa. Penetration and intracellular release of genomes of avian RNA tumor viruses. Virology 50:440-458, 1972.

Dalton, A.J., U.I. Heine, J.L. Melnick. Symposium: Characterization of oncornaviruses and related viruses - a report. J. Natl. Cancer Inst. 55:941-943, 1975.

de Harven, E. Morphology of murine leukemia viruses. In Experimental Leukemia. M.A. Rich, ed., Appleton-Century-Crofts, New York, pp. 97-129, 1968.

Duesberg, P.H. Physical properties of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. 59:930-936, 1968.

Ellerman, V., O. Bang. Experimentelle Leukamie bei Huhnern. Zentr. Bakteriol. Abt. 1. 46:595-609, 1908.

Emanoil-Ravicovitch, R., C.J. Larsen, M. Bazilier, J. Rabin, J. Peries, M. Bairon. Low-molecular-weight RNAs of murine sarcoma virus: Comparative studies of free and 70S RNA-associated components. J. Virol. 12:1625-1627, 1973.

Erikson, E., R.L. Erikson. Isolation of amino acid acceptor RNA from purified avian myeloblastosis virus. J. Mol. Biol. 52:387-390, 1970.

Erikson, E., R.L. Erikson. Association of 4S ribonucleic acid with oncornavirus ribonucleic acids. J. Virol. 8:254-256, 1971.

Gallagher, R.E., R.C. Gallo. Type-C RNA tumor virus isolated from cultured human acute myelogenous leukemia cells. Science 197:350-353, 1974.

Gallagher, R.E., S.Z. Salahuddin, W.T. Hall, K.B. McCredie, R.C. Gallo. Growth and differentiation in culture of leukemic leukocytes from a patient with acute myelogenous leukemia and re-identification of type-C virus. Proc. Natl. Acad. U.S.A. 72:4137-4141, 1975.

Garapin, A.C., H.E. Varmus, A.J. Faras, W.E. Levinson, J.M. Bishop. RNA-directed DNA synthesis by virions of Rous sarcoma virus: further characterization of the templates and the extent of their transcription. Virology 52:264-274, 1973.

Gianni, A.M., D. Smotkin, R.A. Weinberg. Murine leukemia virus: Detection of unintegrated double-stranded DNA forms of the provirus. Proc. Natl. Acad. Sci. U.S.A. 77:447-451, 1975.

Giard, D.J., S.A. Aaronson, G.J. Todaro, P. Arnstein, J.H. Kersey, H. Dosik, W.P. Parks. In vitro cultivation of human tumors: Establishment of cell lives derived from a series of solid tumors. J. Natl. Cancer Inst. 51:1417-1423, 1973.

Gibbons, J.R., A.V. Grimstone. On flagellar structure in certain flagellates. J. Biophys. Biochem. Cytol. 7:697-716, 1960.

Gilden, R.V., S. Oroszlan, M. Hatanaka. Comparison and evolution of RNA tumor virus components. In Viruses, Evolution and Cancer. E. Kurstak, K. Maramorosch, eds., Academic Press, New York, pp. 235-257, 1974.

Gilden, R.V. Interrelationships among RNA tumor viruses and host cells. In Advances in Cancer Research, Vol. 22, G. Klein, S. Weinhouse, eds., Academic Press, New York, pp. 157-202, 1975.

Gilden, R.V. Biology of RNA tumor viruses. In Molecular Biology of Animal Viruses, Vol. 1, D.P. Nayak, ed., Marcel Dekker, Inc., New York, pp. 435-542, 1977.

Gillespie, D.S., S. Gillespie, R.C. Gallo, J.L. East, L. Dmochowski. Genetic origin of RD114 and other RNA tumor viruses assayed by molecular hybridization. Nature 244:51-54, 1973.

Gillespie, D., W.C. Saxinger, R.C. Gallo. Information transfer in cells infected by RNA tumor viruses and extension to human neoplasia. In Progress in Nucleic Acid Research and Molecular Biology, Vol. 15, W. Cohn, ed., Academic Press, New York, pp. 1-8, 1975.

Gillespie, D., S. Gillespie, F. Wong-Staal. RNA-DNA-hybridization applied to cancer research: Special reference to RNA tumor viruses. In Methods in Cancer Research, Vol. XI, H. Busch, ed., Academic Press, New York, pp. 205-245, 1976.

Goulian, M., Z.J. Lucas, A. Kornberg. Enzymatic synthesis of DNA. XXV. Purification and properties of DNA polymerase induced by infection with phage $T4^+$. J. Biol. Chem. 243:627-636, 1968.

Grandgenett, D., G. Gerard, M. Green. A single subunit from avian myeloblastosis virus with both RNA-directed DNA polymerase and ribonuclease H activity. Proc. Natl. Acad. Sci. U.S.A. 70:230-234, 1973.

Gross, L. Spontaneous leukemia developing in C_3H mice following inoculation in infancy with Ak-leukemic extracts or Ak-embryos. Proc. Natl. Soc. Exptl. Biol. Med. 76:27-32, 1951.

Gross, L. Oncogenic Viruses. Pergamon Press, New York, 1970.

Guntaka, R.V., O.C. Richards, P.R. Shank, H.J. Kung, N. Davidson, E. Fritsch, J.M. Bishop, H.E. Varmus. Covalently closed circular DNA of avian sarcoma virus: Purification from nuclei of infected quail tumor cells and measurement by electron microscopy and gel electrophoresis. J. Mol. Biol. 106:337-357, 1976.

Hanafusa, H., T. Hanafusa. Noninfectious RSV deficient in DNA polymerase. Virology 43:313-316, 1971.

Hartley, J.W., W.P. Rowe. Production of altered cell foci in tissue culture by defective Moloney sarcoma virus particles. Proc. Natl. Acad. Sci. U.S.A. 55:780-785, 1966.

Haseltine, W.A., D. Baltimore. In vitro replication of RNA tumor viruses. In ICN-UCLA Symposium on Molecular and Cellular Biology, Vol. 4, Animal Virology. D. Baltimore, A.S. Huang, C.F. Fox, eds., Academic Press, New York, pp. 175-213, 1976.

Haseltine, W.A., D.G. Kleid. A method for classification of 5' termini of retroviruses. Nature 273:358-364, 1978.

Hatanaka, M., T. Kakefuda, R.V. Gilden, E.A.O. Callan. Cytoplasmic DNA synthesis induced by RNA tumor viruses. Proc. Nat. Acad. Sci. U.S.A. 68:1844-1847, 1971.

Hirt, B. Selective extraction of polyoma DNA from infected mouse cell culture. J. Mol. Biol. 26:365-369, 1967.

Hoyer, B.H., N.R. Rice, N.W. van de Velde. Sonication of DNA to produce fragments suitable for reassociation experiments. Carnegie Inst. Washington Yearb. 72:214-217, 1973.

Huebner, R.J., G.J. Todaro. Oncogenes of RNA tumor viruses as determinants of cancer. Proc. Natl. Acad. Sci. U.S.A. 64:1087-1094, 1969.

Hurwitz, J., J.P. Leis. RNA-dependent DNA polymerase activity of RNA tumor viruses. I. Directing influence of DNA in the reaction. J. Virol. 9:116-129, 1972.

Johnson, G.S., R.M. Friedman, I. Pastan. Analysis of the fusion of XC cells induced by homogenates of murine leukemia virus-infected cells and by purified murine leukemia virus. J. Virol 7:753-758, 1971.

Johnson, R.W., A. Perry, O.R. Robinson, Jr., G.P. Shibley. Method of reproducible large volume production and purification of Rauscher murine leukemia virus. Appl. Microbiol. 31:182-188, 1976.

Kacian, D.C., K.F. Watson, A. Burny, S. Spiegelman. Pruification of the DNA polymerase of avian myeloblastosis virus. Biochim. Biophys. Acta. 246:365-383, 1971.

Kakefuda, T., G.G. Lovinger, R.V. Gilden, M. Hatanaka. Electron microscopic studies of circular DNA in mouse embryo fibroblasts infected by Rauscher leukemia virus. J. Virol. 21:792-795, 1977.

Kalter, S.S., R.J. Helmke, M. Panigel, et al. Observations of apparent C-type particles in baboon (Papio cynocephalus) placentas. Science 179:1332-1333, 1973.

Klement, V., W.P. Rowe, J.W. Hartley, W.E. Pugh. Mixed culture cytopathogenicity: A new test for growth of murine leukemia viruses in tissue culture. Proc. Natl. Acad. Sci. U.S.A. 63:753-758, 1969.

Kohne, D.E. Evaluation of higher organism DNA. Q. Rev. Biophys. 33:327-375, 1970.

Kohne, D.E., R.T. Britten. Hydroxylapatite techniques for nucleic acid reassociation. In Methods in Nucleic Acid Research, Vol. 2, G.L. Cantani, D.R. Davies, eds., Harper and Row, New York, pp. 500-532, 1971.

Kung, H.J., N.D. Bailey, M.O. Nicholson, R.M. McAllister. Structure, composition, and molecular weight of RD-114 RNA. J. Virol. 16:397-411, 1975.

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 1970.

Levinson, W.E., J.M. Bishop, N. Quintrell, J. Jackson. Presence of DNA in Rous sarcoma virus. Nature 227:1023-1025, 1970.

Lieber, M.M., R.E. Benveniste, D.M. Livingston, G.J. Todaro. Mammalian cells in culture frequently release type C viruses. Science 182: 56-59, 1973.

Lieber, M.M., G.J. Todaro. Mammalian type C RNA viruses. In Cancer: A Comprehensive Treatise. F. Becker, ed., Plenum Press, New York, pp. 91-133, 1975.

Linial, M., W.A. Mason. Characterization of two conditional early mutants of Rous sarcoma virus. Virology 53:258-273, 1973.

Loni, M.C., M. Green. (In press).

Maizel, J.V., Jr. Polyacrylamide gel electrophoresis of viral proteins. In Methods in Virology, Vol. V., K. Maranorsh, H. Kaplowski, eds., Academic Press, New York, pp. 179-246, 1971.

Manly, K., D.F. Smoler, E. Bromfeld, D. Baltimore. Forms of DNA produced by virions of the RNA tumor viruses. J. Virol. 7:106-111, 1971.

Markham, P.D., M.A. Baluda. Integrated state of oncornavirus DNA in normal chicken cells transformed by avian myeloblastosis virus. J. Virol. 12:721-732, 1973.

Marmur, J. A procedure for the isolation of DNA from microorganisms. J. Mol. Biol. 3:208-218, 1961.

McAllister, R.M., J. Melnyk, J.Z. Finklestein, E.C. Adams, Jr., M.B. Gardner. Cultivation in vitro of cells derived from a human rhabdomyosarcoma. Cancer 24:520-526, 1969.

McAllister, R.M., M.O. Nicholson, M.B. Gardner, R.W. Rongey, S. Rasheed, P.S. Sarma, R.J. Huebner, M. Hatanaka, S. Oroszlan, R.V. Gilden, A. Kabigting, L. Vernon. C-type virus released from cultured human rhabdomyosarcoma cells. Nature 235:3-6, 1972.

McAllister, R.N., M.O. Nicholson, R. Heberling, H. Charman, N. Rice, R.V. Gilden. Infectivity of endogenous baboon type C virus related genes. In Proceedings of VIII International Symposium on Comparative Research on Leukemia and Related Diseases, Amsterdam, pp. 135-138, 1978.

Miyamoto, K., R.V. Gilden. Electron microscopic studies of tumor viruses. I. Entry of murine leukemia virus into mouse embryo fibroblasts. J. Virol. 7:395-406, 1971.

Mizutani, S., H.M. Temin. Enzymes and nucleotides in virions of Rous sarcoma virus. J. Virol. 8:409-416, 1971.

Mölling, R., D.P. Bolognesi, H. Bauer, N. Busen, H.W. Plassmann, P. Hausen. Association of the viral reverse transcriptase with an enzyme degrading the RNA moiety of RNA-DNA hybrids. Nature 234:240-243, 1971.

Nakata, Y., J.P. Bader. Studies on the fixation and development of cellular transformation by Rous sarcoma virus. Virology 36:401-410, 1968.

Neiman, P.E. Rous sarcoma virus nucleotide sequences in cellular DNA: Measured by RNA-DNA hybridization. Science 178:750-753, 1972.

Nermut, M.V., H. Frank, W. Schäfer. Properties of mouse leukemia viruses. III. Electron microscipic appearance as revealed after conventional preparation techniques as well as freeze-drying and freeze-etching. Virology 49:345-358, 1972.

Nicolson, M.O., R.V. Gilden, H. Okabe, R.M. McAllister. Infectivity of endogenous RD-114 virus-related genes in cat cells. Proceedings of VIII International Symposium on Comparative Research on Leukemia and Related Diseases, Amsterdam, pp. 139-140, 1978.

Okabe, H., R.V. Gilden, M. Hatanaka, J.R. Stephenson, R.E. Gallagher, R.C. Gallo, S.R. Tronick, S.A. Aaronson. Immunological and biochemical characterization of type C viruses isolated from cultured human AML cells. Nature 260:264-266, 1976.

Okabe, H., J. DuBuy, M. Hatanaka, R.V. Gilden. Reiteration frequency of feline type C viral genomes in homologous and heterologous host cell DNA. Intervirology 9:253-260, 1978.

Oroszlan, S., C. Foreman, G. Kelloff, R.V. Gilden. The group-specific antigen and other structural proteins of hamster and mouse C-type viruses. Virology 43:665-674, 1971.

Parks, W.P., R.V. Gilden, A.F. Bykovsky, G.G. Miller, M.V. Zhdanov, V.D. Soloviev, E.M. Scolnick. Mason-Pfizer virus characterization: A similar virus in an human amniotic cell line. J. Virol. 12:1540-1547, 1973.

Quigley, J.P., D.B. Rifkin, E. Reich. Phospholipid composition of Rous sarcoma virus, host cell membranes and other enveloped RNA viruses. Virology 46:106-116, 1971.

Rand, K., J. Davis, R.V. Gilden, S. Oroszlan, C. Long. Fusion inhibition bioassay of a type C viral protein. Virology 64:63-74, 1975.

Reich E., R.M. Franklin, A.J. Shotkin, E.L. Tatum. The effect of Actinomycin on cellular nucleic acid synthesis and virus production. Science 134:556-557, 1961.

Reitz, M.S., N.R. Miller, F. Wong-Staal, R.E. Gallagher, R.C. Gallo, D.H. Gillespie. Primate type-C virus nucleic acid sequences (woolly monkey and baboon types) in tissues from a patient with acute myelogenous leukemia and in viruses isolated from cultured cells of the same patient. Proc. Natl. Acad. Sci. U.S.A. 73:2113-2117, 1976.

Reynolds, E.S. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17:208-212, 1963.

Rhim, J.S., H.Y. Cho, R.J. Huebner. Nonproducer human cells induced by murine sarcoma virus. Int. J. Cancer 15:23-29, 1975.

Riggs, J.L., R.M. McAllister, E.H. Lennette. Immunofluorescent studies of RD-114 virus replication in cell culture. J. Gen. Virol. 25:21-29, 1974.

Rinan, J., G.S. Beaudreau. Viral DNA-dependent DNA polymerase and the properties of thymidine labeled material in virions of an oncogenic RNA virus. Nature 228:427-430, 1970.

Ringold, G.M., K.R. Yamamoto, P.R. Shank, H.E. Varmus. MMTV DNA in infected rat cells: Characterization of unintegrated forms. Cell 10:19-26, 1977.

Robin, M.S., S.Salzberg, M. Green. Cytoplasmic synthesis of viral DNA early during infection and cell transformation by the murine sarcoma leukemia virus. Intervirol. 4:268-278, 1974.

Robinson, W.S., A. Pitkanen, H. Rubin. The nucleic acid of the Bryan strain of Rous sarcoma virus: Purification of the virus and isolation of the nucleic acid. Proc. Natl. Acad. Sci. U.S.A. 54:137-141, 1965.

Robinson, W.S., H.L. Robinson, P.H. Duesbery,. Tumor virus RNAs. Proc. Natl. Acad. Sci. U.S.A. 58:825-829, 1967.

Rokutanda, M., H. Rokutanda, M. Green, K. Fujinaga, R.K. Ray, C. Gurgo. Formation of viral RNA-DNA hybrid molecules by the DNA polymerase of sarcoma-leukemia viruses. Nature 227:1026-1028, 1970.

Ross, J., E.M. Scolnick, G.J. Todaro, S.A. Aaronson. Separation of murine cellular and murine leukemia virus DNA polymerases. Nature 231:163-167, 1971.

Rous, P.A. Sarcoma of the fowl: Transmissable by an agent separable from the tumor cells. J. Exptl. Med. 13:397-411, 1911.

Rowe, W.R., W.E. Pugh, J.W. Hartley. Plague assay techniques for murine leukemia viruses. Virology 42:1136-1139, 1970.

Rowe, W.P. The kinetics of rescue of the murine sarcoma virus genome from a nonproducer line of transformed mouse cells. Virology 46: 369-374, 1971.

Sarkar, N.H., E.Y. Lasfargues, D.H. Moore. Attachment and penetration of mouse mammary tumor virus in mouse embryo cells. J. Microsc. (Paris) 9:477-484, 1970.

Sarkar, N.H., D.H. Moore, R.C. Nowinski. Symmetry of the nucleocapsid of the oncornaviruses. In RNA Viruses and Host Genomes in Oncogenesis. P. Emmeldt, P. Bentvelzen, eds., North-Holland, Amsterdam, pp. 71-79, 1972.

Schidlovsky, G. Structure of RNA tumor viruses. In Recent Advances in Cancer Research: Cell Biology, Molecular Biology, and Tumor Virology, Vol. 1, R.C. Gallo, ed., CRC Press, Cleveland, Ohio, pp. 190-245, 1978.

Schwartz, D.E., P.C. Zomecnik, H.L. Weith. Rous sarcoma virus genome is terminally redundant: The 3' sequence. Proc. Natl. Acad. Sci. U.S.A. 74:994-998, 1977.

Scolnick, E., E. Rands, G.J. Todaro, S. Aaronson. RNA-dependent DNA polymerase activity in five RNA viruses: Divalent cation requirements. Proc. Natl. Acad. Sci. U.S.A. 67:1789-1796, 1970.

Sherr, C.J., M.M. Lieber, R.E. Benveniste, G.J. Todaro. Endogenous baboon type C virus (M7): Biochemical and immunologic characterization. Virology 58:492-503, 1974.

Sherr, C.J., G.J. Todaro. Radioimmunoassay of the major group specific protein of endogenous baboon type C viruses: Relation to the RD-114/ ccc group and detection of antigen in normal baboon tissues. Virology 61:168-181, 1974a.

Sherr, C.J., G.J. Todaro. Type C viral antigens in man. I. Antigens related to endogenous primate virus in human tumors. Proc. Natl. Acad. Sci. U.S.A. 71:4703-4704, 1974b.

Smoler, I., D. Molineux, D. Baltimore. Direction of polymerization by the avian myeloblastosis virus deoxyribonucleic acid polymerase. J. Bio. Chem. 246:7697-7700, 1971.

Spiegelman, S., A. Burny, M.R. Das, J. Keydar, J. Schlom, M. Travnicek, K. Watson. Characterization of the products of RNA directed DNA polymerase in oncogenic RNA viruses. Nature 227:563-567, 1970.

Strand, M., J.T. August. Type-C RNA virus gene expression in human tissue. J. Virol. 14:1584-1595, 1974.

Studier, F.W. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. 11:373-390, 1965.

Taylor, J.M., A.J. Faras, H.E. Varmur, W.E. Levinson, J.M. Bishop. J. Biochem. 11:2343-2351, 1972.

Taylor, J.M., R. Illmensee. Site on the RNA of an avian sarcoma virus at which primer is bound. J. Virol. 16:553-558, 1975.

Teich, N.M., R.A. Weiss, S.Z. Salahuddin, R.E. Gallagher, D.H. Gillespie, R.C. Gallo. Infective transmission and characterization of a C-type virus released by cultured human myeloid leukemia cells. Nature 256:551-555, 1975.

Temin, H.M., H. Rubin. Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture. Virology 6:669-688, 1958.

Temin, H.M., H. Rubin. A kinetic study of infection of chick embryo cells in vitro by Rous sarcoma virus. Virology 8:209-222, 1959.

Temin, H.M. The effects of Actinomycin D on growth of Rous sarcoma virus _in vitro_. Virology 20:557-582, 1963.

Temin, H.M. The nature of the provirus of Rous sarcoma. Natl. Cancer Inst. Monograph. 17:557-568, 1964a.

Temin, H.M. The participation of DNA in Rous sarcoma virus production. Virology 23:486-494, 1964b.

Temin, H.M. Carcinogenesis by avian sarcoma viruses. Cancer Res. 28: 1835-1838, 1968.

Temin, H.M. S. Mizutani. RNA-dependent DNA polymerase activity in virions of Rous sarcoma virus. Nature 226:211-213, 1970.

Temin, H.M., D. Baltimore. RNA-directed DNA-synthesis and RNA-tumor viruses. Adv. Virus Res. 17:129-186, 1972.

Todaro, G.J., R.J. Huebner. The viral oncogene hypothesis: New evidence. Proc. Natl. Acad. Sci. U.S.A. 69:1009-1015, 1972.

Todaro, G.J., R.C. Gallo. Immunological relationship of DNA polymerase from human acute leukemia cells and primate and mouse leukemia virus reverse transcriptase. Nature 244:206-209, 1973.

Todaro, G.J., C.J. Sherr, R.E. Benveniste, M.M. Leiber, J.L. Melnick. Type C viruses of baboons: Isolation from normal cell cultures. Cell 2:55-61, 1974.

Todaro, G.J. Type C virogenes: Genetic transfer and interspecies transfer. _In_ Tumor Virus Infections and Immunity. R.L. Crowell, H. Friedman, J.E. Prier, eds., University Park Press, Baltimore, pp. 35-44, 1976.

Todaro, G.J., R.E. Benveniste, S.A. Sherwin, C.J. Sherr. MAC-1, a new genetically transmitted type C virus of primates: "Low frequency" activation from stumptail monkey cell cultures. Cell 13:775-782, 1978.

Tooze, J. The molecular biology of tumor viruses. Cold Spring Harbor Laboratory, New York, 1973.

Tronick, S.R., E.M. Scolnick, W.P. Parks. Reversible inactivation of the deoxyribonucleic acid polymerase of Rauscher leukemia virus. J. Virol. 10:885-888, 1972.

Valentine, A.F., J.P. Bader. Production of virus by mammalian cells transformed by Rous sarcoma and murine sarcoma viruses. J. Virol. 2:224-237, 1968.

Varmus, H.E., W.E. Levinson, J.M. Bishop. Extent of transcription by the RNA dependent DNA polymerase of Rous sarcoma virus. Nature 233:19-23, 1971.

Varmus, H.E., P.K. Vogt, J.M. Bishop. Integration of deoxyribonucleic acid specific for Rous sarcoma virus after infection of permissive and nonpermissive hosts. Proc. Natl. Acad. Sci. U.S.A. 70:3067-3070, 1973a.

Varmus, H.E., C.B. Hansen, E. Medeiros, C.T. Deng, J.M. Bishop. Detection and characterization of RNA tumor virus-specific nucleotide sequences in cell DNA. Le petit Symposium, 1973b.

Varmus, H.E., R.V. Guntaka, W. Fan, S. Heasley, J.M. Bishop. Synthesis of viral DNA in the cytoplasm of duck embryo fibroblasts and in enucleated cells after infection by avian sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 71:3874-3878, 1974.

Varmus, H.E., R.V. Guntaka, C.T. Deng, J.M. Bishop. Synthesis, structure, and function of avian sarcoma virus-specific DNA in permissive and non-permissive cells. Cold Spring Harbor Symp. Quant. Biol. 39: 987-996, 1975.

Verma, D.M., N.L. Meuth, E. Bromfeld, K.F. Manly, D. Baltimore. A covalently linked RNA-DNA molecule as the initial product of the RNA tumor virus DNA polymerase. Nature 233:131-134, 1971.

Vigier, P., A. Golde. Effects of Actinomycin D and of Mitomycin C on the development of Rous sarcoma virus. Virology 23:511-519, 1964.

Weinberg, R.A. Structure of the intermediates leading to the integrated provirus. Biochem. Biophys. Acta. 473:39-55, 1977.

Weissbach, A., A. Bolden, R. Muller, H. Hanafusa, T. Hanafusa. Deoxyribonucleic acid polymerase activities in normal and leukovirus infected chicken embryo cells. J. Virology 33:754-761, 1972.

Wetmur, J.C., N. Davidson. Kinetics of renaturation of DNA. J. Mol. Biol. 31:349-370, 1968.

Wilson, R.G., J.P. Bader. Viral ribonucleic acid polymerase: Chick embryo cells infected with vesicular stomatitis virus or Rous associated virus. Biochem. Biophys. Acta. 103:549-555, 1965.

Wong-Staal, F., D. Gillespie, R.C. Gallo. Proviral sequences of baboon endogenous type C RNA virus in DNA of human leukemic tissues. Nature 262:190-195, 1976.