AN ABSTRACT OF THE THESIS OF

Richard Yue-Leung To for the degree of Doctor of Philosophy

in Biochemistry/Biophysics presented on June 8, 1981

Title: STUDIES ON THE TRANSFORMING GENE OF AVIAN MYELOBLASTOSIS VIRUS

Abstract approved: Redacted for privacy

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[^{3}H]cDNA complementary to genomic RNA from avian myeloblastosis (AMV) virus was fractionated by exhaustive hybridization against RNAs from an endogenous virus RAV-0 as well as from an exogenous virus Pr-RSV. The cDNA fractions that show no homology to RAV-0 or Pr-RSV were further tested by hybridizing to RNAs from SR-RSV, AEV, and MC29, each of which represents a separate class of oncogenic avian retroviruses. The results demonstrated that the AMV genome contains its own unique sequence that may represent the AMV oncogene which is different from other classes of oncogenes.

The AMV-specific (oncogene) cDNA probes were shown to have homologous sequences in the normal cell genomes from five different avian species in addition to chicken, as well as from calf thymus. The non-AMV-specific (virogene) cDNA probes were shown to have no homology to normal cellular DNAs except those from chicken and pheasant. These results reinforce the concept that the specific viral oncogenes are probably of cellular origin whereas the non-specific viral structural and replicative genes are of an exogenous nature.

Hybridizations of the fractionated cDNA probes to cellular DNAs

extracted from myeloblasts and erythrocytes infected with AMV, as well as those from uninfected normal erythrocytes indicate that AMV oncogenes do integrate into the genomes of non-target cells (erythrocytes) as well as into target cells (myeloblasts).

Quantitative studies on the number of viral copies integrated into target and non-target chicken cells were performed by hybridizations using excess [^{3}H]cDNA probes whose specific activities were accurately determined by the kinetic method. Each normal chicken erythrocyte was found to contain about 15 sequence equivalents homologous to the AMV virogenes. Infected erythrocytes showed about 19 copies of the virogenes per cell and about three copies per cell of the oncogenes. Infected myeloblasts presented about 24 copies per cell virogenes and seven copies of the oncogenes per cell. The implications of these results were discussed in a model of AMV infection.

Studies on the Transforming Gene of Avian Myeloblastosis Virus

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed June 1981

Commencement June 1982

APPROVED

Redacted for privacy

Professor of Biochemistry
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Date thesis is presented June 8, 1981

Typed by Kitty Dougherty for Richard Yue-Leung To

To my wife, Lily

for her ineffable endurance and sacrifice

To our parents

for their confidences

ACKNOWLEDGEMENTS

I wish to thank Dr. George Beaudreau for his exuberant optimism, adamant patience and inspiring guidance throughout this project. Technical advices provided by Tim Herman, Don Jump and Margot Pearson are deeply appreciated. I also treasure the many hortatory and creative insights from George Rohrmann, Dick Cheney, Mark Galinski and Doug Leisy.

Special thanks to Kitty Dougherty for her skillful typing and fervid assistance in the final preparation of this manuscript.

" The ultimate contribution of virology to the cancer problem may not come from the discovery of a particular cause of cancer but from the understanding of cell function and control mechanisms which will emerge from the study of integrated viral genomes. "

W. P. Rowe, 1973

Cancer Research 33: 3061.

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INTRODUCTION

CLASSIFICATION OF AVIAN RETROVIRUSES (ARV)

The study of avian retroviruses (often referred to as avian RNA tumor viruses or avian leukosis-sarcoma viruses) has revealed a remarkable phenomenon in that an extremely broad spectrum of pathogenesis and oncogenesis can be induced by a group of seemingly closely related viruses. While resembling all retroviruses of other animal species, the ARVs as a group share a very similar morphology; a set of group-specific structural proteins; a virion-associated reverse transcriptase; a dimeric single-stranded RNA genome with relatively consistent gene ordering; a mode of intracellular replication that directs the transfer of genetic information from genome RNA to proviral DNA which integrates into host DNAs; and synthesis of mRNA or progeny RNA from integrated viral DNA (1). Although subspectra from the vast array (or disarray) of neoplastic manifestations can be organized from distinct responses to subgroups of cloned viruses, etiological classification has never been easy or resolute. In a recent review, J. W. Beard (2) points out that the infectious activity of a virus changes spontaneously as it undergoes laboratory passages. An extreme example is the Rous sarcoma virus (RSV), which has been altered from having an early restricted fowl host to becoming infectious to and causing transformation in tissue cultures of many other species of

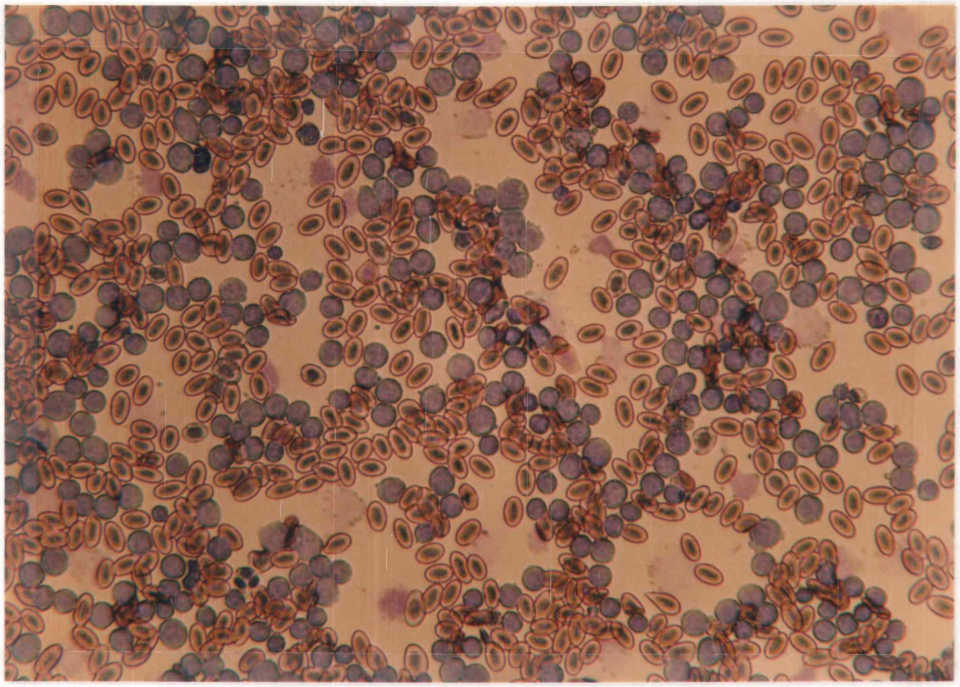


Figure 1. Blood smear at a magnification of 160 X. Peripheral blood was withdrawn from Avian Myeloblastosis virus infected chicken about 15 days post-infection. Cells were stained with Wright's stain to show the purple nuclei and blue cytoplasm of proliferating myeloblasts.

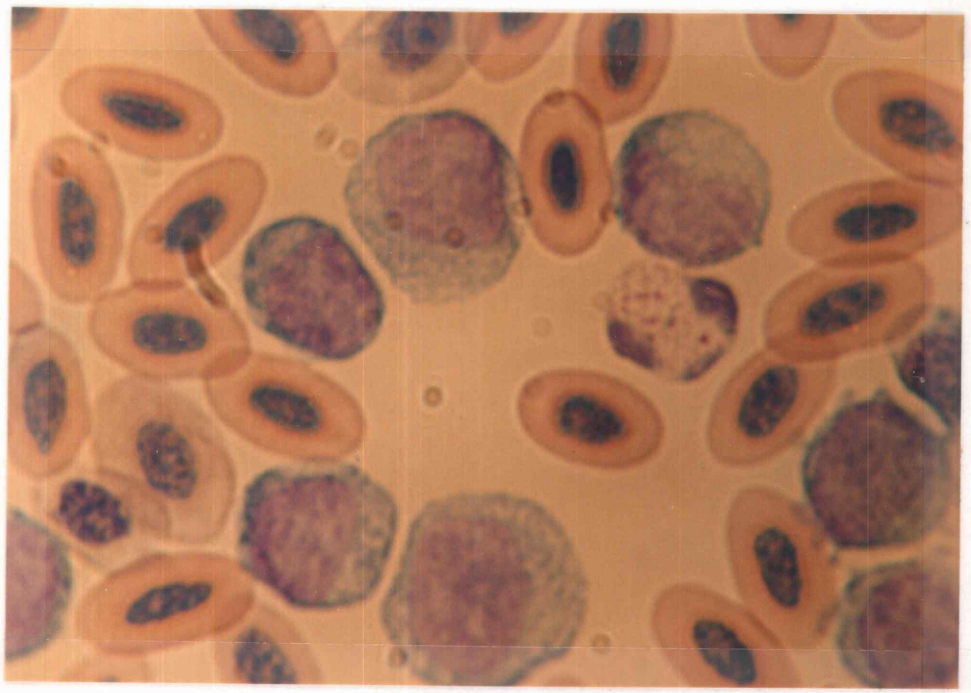


Figure 2. Blood smear at a magnification of 1000 X. Cells and staining were the same as in figure 1.

TABLE 1. CLASSIFICATION OF AVIAN RETROVIRUS STRAINS

Virus Groups and Subgroups	Predominant Neoplasms	Oncogenecity on CEF	Hemopoietic Target Cells in Culture
Avian Sarcoma (ASV)	Sarcoma	Strongly transforming	None
Defective leukemis (DLV)			
AEV	Erythroblastosis Sarcoma	Weakly transforming	Erythroblast type
MC29	Myelocytomatosis Monocytic leukemia	Transforming	Macrophage type
AMV	Myeloblastosis	Nontransforming	Early myeloid type
Lymphatic leukemia (LLV)	Lymphatic leukemia Osteopetrosis Nephroblastoma Endothelioma Erythroblastosis Sarcoma	Nontransforming	None

reptiles, mammals, as well as humans. Graf and Beug (3) have also described many cases of spontaneous disappearance and appearance of viral strains during passages of an original field isolate. Such exhibitions of viral alterations, coupled with fluctuations in the multiplicity of host responses, make for a dynamic system whose classification is a challenging endeavor.

Nevertheless, avian leukosis-sarcoma viruses have been assigned to various groups based on the type of neoplasms they induce (2), and on the differentiation phenotypes of chicken embryo fibroblast (CEF) and hemopoietic cells transformed in vitro by these viruses (3), or on their relative transforming ability on host cells in vivo and in vitro (4).

Elaborating on the classification proposed by Graf and Beug (3), ALSVs can be divided into three major groups (Table 1) depending on the major neoplasms manifested in their hosts, on their relative oncogenicity on CEF culture, and also on the types of hemopoietic cells that they can transform in vitro. The first group, the avian sarcoma viruses (ASV), includes the original Rous sarcoma virus isolate (RSV), plus all the nondefective derivatives and recent isolates of such strains as Praque (Pr), Mill-Hill (MH), Fujinami (FSV), Bratislava (B77), and Harris (H). All strains induce sarcoma as the major neoplasms in host animals and they can also cause a number of other solid tumors such as chondroma, osteochondroma and endothelioma (2), but there is no evidence that any of the ASVs can induce leukemia in vivo. ASVs also transform CEF readily in vitro but are unable to

transform any hemopoietic cells in culture.

The second major group, the defective leukemia viruses (DLV) are so termed because of their inability to replicate in the host cells by themselves. These viruses require nondefective helper viruses for their propagation. However, replicating or not, the DLVs can rapidly induce different kinds of leukemia within two to four weeks post-infection, and hence, are sometimes also classified as acute leukemia virus (5). All DLVs transform one kind or another of hemopoietic cells *in vitro*, but their abilities to transform CEF in culture vary with each subgroup. Seven independent field isolates of DLVs have been assigned to three subgroups according to the types of distinctly predominant leukemia that they induce and also to their genetic structures responsible for oncogenicity (6). These three subgroups are: 1) avian erythroblastosis virus (AEV), which includes strains R and ES4 that are probably identical (7), induces erythroblastosis *in vivo* and transforms erythroblast type hemopoietic cells in culture; 2) avian myelocytomatosis-type virus (also as MC29 subgroup) including strains of MC29, MH2, CMII, and OK10, causes myelocytomatosis *in vivo* and transforms macrophage-like cells or cells of late myeloid lineage *in vitro*; 3) avian myeloblastosis type viruses (strains BAI-A and E26) induce myeloblastosis *in vivo* and transforms myeloblasts or cells of early myeloid lineage in culture. All three subgroups can also occasionally induce such solid tumors in animals as sarcoma, endothelioma, renal and hepatic tumors, and epithelioma (skin carcinoma). In addition, both AEV subgroup and MC29 subgroup mildly transform CEF *in vitro*

whereas AMV subgroup does not. Figures 1 and 2 show AMV target cells.

The third major group of ARVs, the lymphatic leukemia viruses, is characterized by the following: 1) ability to induce leukemia and related diseases after a long period of latency; 2) replication competence; and 3) the inability of transforming CEF or hemopoietic cells in culture. These viruses are often called lymphatic leukemia viruses for the reason that they cause lymphatic leukemia as the predominant disease, but they have also been reported at various times to be quite capable of inducing a host of other diseases such as osteopetrosis, nephroblastoma, endothelioma, erythroblastosis and also sarcoma. Most of the viruses included in this group are either independent field isolates or associated with DLVs as helper viruses. Some of the transformation-defective mutants of avian sarcoma viruses (td ASV) are included in this group because there are evidences that these viruses also contain oncogenic potentials very similar to those of leukosis viruses as reported by Biggs and colleagues (8). Endogenous virus RAV-0 may also be loosely included into this group on the grounds that RAV-0 is genetically very close to other associated viruses, but the oncogenicity of RAV-0 has not been well defined.

In view of the multipotential with respect to each viral group's oncogenicity, it is evident that a large number of factors must be at work independently and in consortment to bring about the large array of perimeters defining cellular transformation. Some of these factors, such as basic genetic structures, integration modes, genetic variation among individuals, as well as target cell specificities are no doubt

prominent for investigations. We shall briefly survey the recent progress in these aspects and report our own investigation in these areas by using [^{3}H]cDNA sequences complementary to avian myeloblastosis virus genome RNA as probes.

MOLECULAR STRUCTURES AND GENETIC MAPPING OF ARV

The genetic structures of retroviruses in general and of avian RNA tumor viruses in particular have been extensively reviewed by Bishop (1), Vogt and Hu (9), Wang (10), and Coffin (11). The genome RNA extracted from virions by sodium dodecyl sulfate (SDS) and phenol contains a 60-70s component of molecular weight ranging from 4.5×10^6 to 7×10^6 daltons depending on virus and the methods of determination. This 60-70s RNA can be readily dissociated by heat or by dimethylsulfoxide treatment into two 30-40s subunits of molecular weight between 2.2×10^6 and 3.5×10^6 daltons. The lower molecular weights are usually associated with defective virus strains representing partial deletions in their genomes. The high values are normally found with nondefective sarcoma viruses which represent the largest size class of genome RNAs. The two 30-40s RNA subunits are generally believed to be linked by hydrogen bonding at their 5' ends. These 5' ends are capped with a 7-methyl guanosine linked 5' to 5' via a triphosphate to a second 2' O-methylated guanosine residue, giving a structure represented by $m^7G^{5'}ppp^{5'}Gm$. All genome RNAs also contain poly(A) sequences of approximately 200 nucleotides long at their 3' ends. These 5' and 3' common end structures resemble those found in eukaryotic mRNAs after

post-transcriptional modifications, and in fact, viral genome RNAs have been shown to serve as messengers.

Nucleotide sequence determinations (11) have verified that the 5' and 3' regions of ALSV RNAs are terminally redundant. As shown in Fig. 3, the terminal region from 5' end inward contains a 5' copy of this redundant sequence (designated as R_5) of about 20 nucleotides; a sequence of about 80-100 nucleotides unique to the 5' end (U_5); and the primer binding site (P) for the attachment of tryptophan tRNA. The terminal region from the 3' end inward excluding the poly(A) contains the 3' equivalent of the redundant sequence (R_3) and a sequence unique to the 3' end (U_3). These terminal sequences are highly conserved among different strains of ARVs, not only in their genome RNAs, but also in different species of subgenomic viral mRNAs derived from the 3' half of the viral genome (*env* and *src* mRNAs). This indicates that the 5' terminal sequences have to be spliced onto subgenomic mRNAs in very much the same way as do the leader sequences in eukaryotic mRNAs (12), and it has consequently been implied that these terminal redundant sequences are essential for viral replications.

The physical mapping of specific coding regions within the ARV genome can be accomplished by several methods, all of which involve the use of deletion or temperature-sensitive mutants of one kind or another. In one method, the heteroduplex analyses by electronmicroscopy, complementary DNA (cDNA) is usually prepared from nondefective virus and is used to form heteroduplexes with the RNA of a deletion mutant. The orientation of a heteroduplex is determined by tagging the 3' poly(A)

of the RNA with SV40 relaxed circular DNA to which poly(dT) has been linked by terminal transferase. The deletion loop can then be located with reference to the 3' end (13). A second method utilizes cDNA complementary to various regions of a nondefective virus. These specific cDNAs are then hybridized to various lengths of poly(A)-tagged fragments of a virus RNA under investigation and the gene order is deduced from the relative percentages of each size class RNA fragments that can be hybridized by each species of the cDNAs (14).

In the third and most commonly used mapping technique, radioactive genome RNAs are partially fragmented by alkali treatment. Only those fragments containing the 3' end poly(A) are then selected and digested with RNase T_1 which cleaves at the G residue. The resultant T_1 oligonucleotides are separated by 2-dimensional gel electrophoresis and the relative positions of these oligonucleotides with respect to the 3' end of the genome can then be calculated by the relative intensities of radioactivity from each spot compared to some internal standards. Those oligonucleotides showing the highest radioactivities are closest to the 3' end because of their higher frequencies of occurrence. A genetic map in the region of the 3' end can thus be deduced by comparing such T_1 fingerprints from various deletion mutants.

Figure 3 summarizes the genetic mapping of ALSVs from various sources, such as RSV genome map from Coffin and Belleter (15) and Wang (10), and leukemia viruses from Stehelin et al. (6) and from Bister and Duesberg (5). In general, nondefective Rous sarcoma virus (RSV) has been most thoroughly studied and can be used as the prototype of ARV

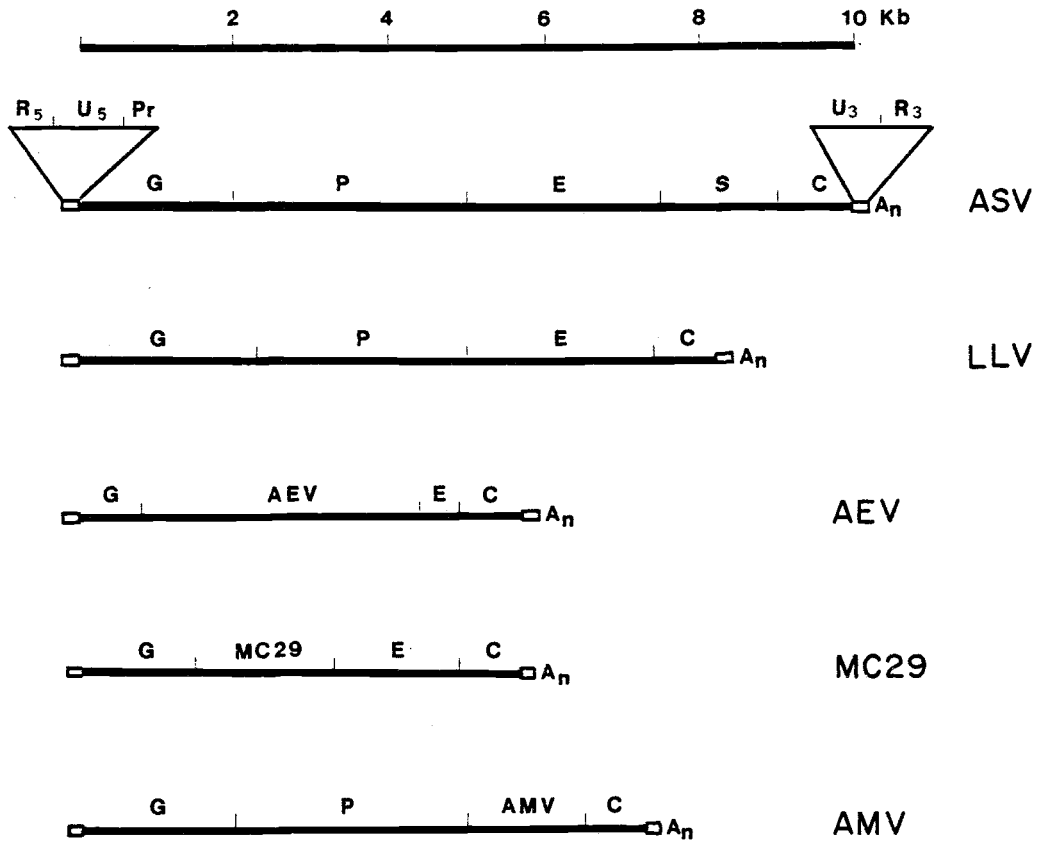


Figure 3. Comparative genetic maps of the RNAs of avian retrovirus. Representatives are the Avian sarcoma virus (ASV) group, the lymphatic leukemia virus (LLV) group, and the AEV, MC29 and AMV subgroups of the defective leukosis viruses (DLV). Scale in kilo-base pairs is presented at the top. The genetic regions are indicated as (G) for gag gene, (P) for pol gene, (E) for env gene, (C) for common C gene, and (S) for src gene of the sarcoma viruses. The different oncogene regions for the DLVs are labeled correspondingly. (R_3) and (R_5) are the terminal repeat regions, and together with the unique regions (U_3) and (U_5) make up the long terminal repeats (LTR). (Pr) is the primer site.

genetic maps. The RSV genetic regions occupy about 10,000 nucleotides in length and are arranged from 5' to 3' as gag-pol-env-src-c. Gag gene consists of about 2000 nucleotides and is located immediately adjacent to the 5' terminal sequence. It codes for the internal group specific proteins of the virion and its sequence is highly conserved among different strains of ARVs as indicated by both hybridization data and peptide analyses of its translational products. Pol gene consists of about 3000 nucleotides and codes for the reverse transcriptase which is characteristic of all retroviruses and therefore the pol sequence is also highly conserved. Env gene has about 2500 nucleotides and codes for the viral glycoproteins which are responsible for subgroup host range specifications. From hybridization experiments, evidence has shown that the env sequences of subgroup A and C show nearly 100% homology and those of subgroups B, D and E are only slightly different from A and C subgroups (in the comparative range of 80 to 90%), whereas, env genes from subgroup F show marked difference to the other subgroups (16,17). Src gene is the oncogene of RSV and codes for a phosphoprotein $pp60^{src}$ which exhibits activity for tyrosine phosphorylation and is thought to be responsible for transformation. C region is a common sequence of about 700-800 nucleotides that are highly conserved among exogenous viruses but is absent in endogenous RAV-0 virus. Tsichlis and Coffin recently have found that RAV-0 viruses that acquired this C region from exogenous viruses through recombination showed an enhanced growth rate. They therefore proposed that the common C region may be a promotor for RNA synthesis (18).

The gene structures of nondefective lymphatic leukemia viruses (LLV) are very similar to that of the RSV except that the src genes are missing (5). There is no evidence at the present time to show whether some of the sequences in nondefective lymphatic leukemia virus have oncogenic potential and no specific oncogene has been identified from these viruses yet.

The gene mapping of the defective leukemia viruses (DLV) are not yet conclusive at this time due to the fact that suitable mutants are difficult to obtain, that the DLVs themselves are difficult to isolate free from their associated helper viruses, and that their extensive defectiveness in all three replication genes, gag, pol, and env, render T_1 oligonucleotide analyses difficult. Recently, attempts are being made to clone the DLV DNAs and then to rescue those infectious DNAs by transfection in order to obtain undisputed genome representations of the DLVs (19). The structures shown in Fig. 3 are only tentative and their gene orders are mainly deduced with reference to the nondefective leukemia viruses or from the ordering of transcriptional products (6,70). Nevertheless, DLV specific sequences (which represent 20-40% of their genome RNA) are believed to be flanked at 5' and 3' ends by sequences homologous to their respective helper viruses (5). The specific sequences from the three DLV subgroups appear to be different from each other and bear no homology to the src gene of RSV (21). In fact, these DLV specific sequences may well represent three independent classes of cellular oncogenes since Stehelin et al. (6) were able to demonstrate by hybridization that all three sequence classes have

homologies in normal uninfected chicken.

The information pertaining to the genetic structures of AMV subgroup is particularly lacking, mainly because replication-defective leukemogenic component of AMV stock has not yet been isolated free of helper viruses which are usually present in large excess in any AMV preparations (22). However, three lines of evidence indicate that there may be a specific AMV sequence different from all other DLVs or helper viruses: 1) Duesberg et al. (23) have been able to isolate a defective viral particle (DVP) from AMV-transformed myeloblasts that are nonproducer cells for the helper viruses, and both hybridization and T_1 fingerprint data indicated that the DVP RNA contained a 1.5 kb sequence unrelated to other classes of avian tumor viruses. 2) At about the same time, Souza and colleagues (24,25) utilized heteroduplex analyses to identify from AMV a cloned provirus segment of about 900 nucleotides that showed no homology to the helper viruses. 3) Chen et al. (26) and Roussel et al. (27) used the technique of selection by exhaustive hybridization to isolate from AMV cDNA an AMV unique sequence that had no homology to other classes of DLVs. It is apparent, then, that there exists an AMV specific sequence in the AMV stock, despite the discrepencies concerning sizes, complexities and location of this sequence in the AMV genome deduced by different researchers. All the information concludes that the specific sequences from ASV, AEV, MC29 and quite possible AMV, make up four distinct classes of ALSV transforming genes, each of which is capable of inducing a distinct type of neoplasm. In this report, we shall demonstrate by exhaustive

hybridization the selection of an AMV unique cDNA sequence with no homology to ASV, AEV or MC29 and demonstrate that this cDNA probe can be used for further investigations.

PROVIRUS INTEGRATION

That retrovirus RNA is reverse transcribed into DNA provirus during replication and that both the unintegrated linear form and integrated form of this provirus are co-extensive as well as co-linear are well established (1,11). Gilboa et al. (28) have described in detail a model of provirus synthesis. Briefly stated, transcription of the nascent minus DNA strand starts near and proceeds towards the 5' end of the positive RNA template while using a $tRNA^{Trp}$ as primer. The minus DNA strand, in some manner, bridges over to the 3' end of either the same or another RNA template. Continuous transcription towards the 5' end of this second RNA template results in a DNA strand that has at each of its end's an identical-long-terminal-repeat (LTR) sequence homologous to the terminal redundant sequences as well as both the 3' and 5' terminal unique sequences of the RNA template (for details see ref. 11 and 29). The positive DNA strand is known to be transcribed from the minus strand even before the transcription of the latter is completed (30). As a result, the provirus DNA is longer than the viral genomic RNA by about 300 nucleotides.

Three configurations of unintegrated double-stranded ASV DNA are identified in the infected cell: 1) the linear form with one LTR at each end; 2) a circular form with two LTRs arranged and joined in

tandem; and 3) a circular form with only one LTR (30). Although the actual precursor of the integrated provirus is still under investigation, it is known that the structure of the integrated proviral DNA is identical to that of unintegrated linear DNA (that is cell DNA-3'-5'------3'-5'-cell DNA), and that subgenomic viral DNAs with intact LTRs at both ends can also be integrated (32). In fact, Copeland and colleagues (33) have shown that at least in transfection experiments, even subgenomic RSV DNA fragments lacking 5' terminal sequences could integrate and transform NIH 3T3 mouse cells, albeit at 100-1000 fold lower transformation efficiency than transfection with intact RSV DNAs.

However, the relationship between provirus integration and viral gene expression is still unclear at the present. On one hand, there is evidence which indicates that transformation may not occur even with integration of the viral oncogene. By using restriction fragment analyses on RSV DNA transformed mammalian cells, Collins et al. (34) revealed that the integrated RSV proviruses detected in transformed parental cells as well as in morphologic revertant cells were practically indistinguishable as far as the proviral gene structures and arrangements with flanking cellular sequences were concerned. Our results will further investigate this point. On the other hand, there is also evidence indicating that transformation may occur without provirus integration. Taylor et al. (35) discovered that when cells were transformed by replication-defective viruses, unique integration sites could be detected within a cell clone, but when cells were transformed

by nondefective viruses, such unique integration sites could be detected only if the provirus integrates before clonal development under conditions whereby progeny viruses could not spread for reinfection. In cases where a single transformed cell had divided for several generations before provirus integration, multiple sites as well as seemingly random integration sites were observed. By using temperature-sensitive mutant viruses, Taylor and his colleagues were also able to detect persistent unintegrated proviruses under various conditions. In fact, in ASV-transformed duck cells, unintegrated viral DNA can account for 20-70% of all the detectable viral DNAs (37). These results prompted Taylor to suggest that these presistent unintegrated proviruses might represent some terminal products of a synthesis reaction that had proceeded beyond the stage when normal integration would have occurred.

Furthermore, Cooper and Okenquist (36) have found that when chick cells were transfected by RSV DNA, colonies of transformed cells could be obtained only under conditions that allowed secondary infection and no transformation could be detected under conditions restricting viral proliferation, even though intact viruses were being produced by the recipient cells. They, therefore, suggested that transfection proceeds with the synthesis of progeny viruses from the primary recipient cells. These progeny viruses then infect and transform secondary cells. These results indicate that at least in transfection, expression of transformation competent viruses by the primary cell may not result in its own transformation.

With the evidences that transformation may not depend on the mere

integration of viral DNA into host genome, the next question to ask is whether the quantity and location of integration can affect cell transformation. In normal chicken, the amount of endogenous RAV-0 proviruses per cell ranges from one to two genome equivalents (38) to about ten genome equivalents (39) depending on individual chicken as well as individual investigator. At least one healthy adult chicken has been found with no endogenous RAV-0 sequences in its genome (39), implying that endogenous viral sequence is not essential for chicken survival. Although it is possible to infect chicken with exogenous RAV-0, and it is quite probable to have autoinfection by individual chickens, the number of genome equivalents per cell in exo-RAV-0 infected chickens does not change considerably from the above values (40,41), hence suggesting that all chickens are probably in certain states of being autoinfected and that there are finite numbers of integration events for each kind of virus per cell.

The work by Astrin and colleagues (42,43) disclosed not only that endogenous RAV-0 genes could be located at any of a number of defined sites in different chicken chromosomes, but also that proviruses located at different sites could produce different transcriptional products, implying proviral structural differences at different loci. As a matter of fact, Hughes et al. (44) had verified the presence of defective endogenous proviruses. As a result of these differences, the phenotype of viral genes expressed by an individual chicken depended on which integration site was occupied by the endogenous provirus. It is still unresolved as to whether or not the transcriptional control of

each integration site depends on its adjacent cellular sequences as proposed by Cooper and Silverman (45) who reported that by shearing cellular DNA to liberate the endogenous viral sequences from neighboring cell DNA, the resultant DNA preparation could have an increased efficiency of transfection by endogenous viral DNA.

Moreover, Jenkins and Cooper (46) found that in contrast to the specific finite sites of endogenous viral integration, exogenous RAV-0 could integrate at numerous locations. They also showed that these integrated exogenous RAV-0 sequences were 10^4 fold more infectious in transfection assays than the endogenous sequences, thereby supporting the hypothesis that most of the endogenous RAV-0 proviruses are defective and may be under the inhibitory control of flanking cellular sequences.

Anyway, as in the case of exogenous RAV-0 integration, other exogenous viruses are also found to be integrated at multiple sites. Examples are given by sarcoma viruses strains PrA and B77 (47,48), by a nondefective leukemia virus MAV-2 (49), and by the defective leukemia virus AMV and its helper viruses (50,51). In the study by Souza et al. (50), the replication-defective AMV was rescued from nonproducer myeloblasts by a nontransforming but replication competent helper virus tdB77 RSV. While tdB77 could be recovered from all the cell clones transformed by this AMV preparation, restriction analyses of these clones revealed that with the exception of three clones, no specific tdB77 integration sites could be detected whereas specific integration sites for AMV could be observed. This phenomenon indicated that under

most conditions, tdB77 proviruses did not integrate into cell genomes until after the host cell had been transformed by AMV and had undergone cell division several times, thus reinforcing the observations made by Taylor et al. as described earlier. The fact that AMV was integrated at the time when helper viruses were not leads us to another interesting facet of provirus integration, namely, the competition for integration sites.

The fact that exogenous viral infection leads to a definite number of proviruses being integrated per cell is well established, although various copy numbers have been reported even for a single virus type. AMV infected myleoblasts have been reported to contain from nine to 20 genome equivalents per leukemia cell (52,53,54,55), but an upper limit definitely exists. Khoury and Hanafusa (56) have even performed an investigation on the integration of RAV-2 at various multiplicities of infection (MOI) and found that when normal cells contained about 14 genome equivalents of RAV-2 per cell, infection would increase this figure to about 20 regardless of the initial MOI, clearly demonstrating a limit on stable integration events. Chattopadhyay et al. (57) has reached a similar conclusion about the limitation of exogenous proviral integration into mammalian cells. Since the integration events are limited it would be interesting to see if different viruses could compete for the integration sites.

Akiyama and Vogt (58) approached this problem by first double-infecting cells with two different viruses. Then after shearing the infected cell DNA to a size twice as big as the proviral sequences, they

transfected sensitive cells with this sheared DNA and found that only one kind of virus could be recovered per transformed focus, thereby establishing that co-infecting viruses did not integrate in tandem. They then proceeded to double-infect susceptible cells with two different viruses (RAV-1 and B77) in two different fashions: 1) simultaneous infection with one virus at a higher MOI than the other; and 2) sequential introduction of the two viruses at the same MOI. The results showed that the virus introduced at higher MOI or at first always interfered with the integration of the other virus, rendering it less efficient in transfection assays. The fact that the second virus was never completely blocked from integration indicated that: 1) there are strain specific integration sites in addition to common integration sites; 2) the first virus may exit from some preoccupied sites; or 3) interference may not be site specific but actually involve the mechanism of integration by influencing the efficiency of integration by the second virus (even of the same type).

Copeland and Cooper (59) demonstrated that upon transfection, NIH 3T3 cells also demonstrated preferences toward MC29 and AEV proviruses but not their helper provirus MCAV-A. In any event, knowing that proviruses do compete for seemingly limited integrations, we should examine the significance of occupying more than one integration site by a single type of infecting virus.

When infecting cloned rat cells with Moloney murine leukemia virus, Steffen and Weinberg (60) showed that infected clones which were actively producing viruses exhibited multiple proviruses per cell, whereas,

nonproducing clones contained on the average only one exogenous provirus per cell, hence illustrating one probable effect of multiple integration, namely for effective viral replication.

Wyke and Quade (61) discovered that when rat cells were infected at low MOI (0.01) and at high (7.5) with ASV, those cell clones from high MOI infections usually attained more integrated provirus (three to six per cell) than those from low MOI infections (one per cell). Also, high MOI infected rat cells in general produce ten fold more rescuable viruses than low MOI infected cells, although one low MOI infected clone also showed high viral production. These results suggest that expression of a single provirus depends on integration site. Multiple proviruses may either supplement each other or have a higher chance of integrating at the correct site for expression.

We shall report our own investigation on the quantitative differences exhibited by AMV specific sequences integrated into target and nontarget homopoietic cells and deduce how this difference can relate to manifestation of oncogeneity in target cells.

THE ONCOGENES

In speculating the origin of retroviruses, the oncogene hypothesis proposed by Todaro and Huebner (62) stated that both virogenes (responsible for viral replication) and oncogenes (responsible for inducing neoplasms) were normally repressed intrinsic parts of the natural genetic fabric of all vertebrate cells and that upon derepression, whether wholly or partly, these genes would induce a spectrum of host responses

ranging from viral antigen expression to neoplastic manifestation. Moreover, Temin and others (63,64) have suggested that retroviruses might have originated from cellular transposable elements based on the structural resemblances between the proviral LTRs and the end sequences of the moveable elements, and based on the similar ways these two entities integrate into multiple locations within the cell genomes (64).

Current evidences indicate that while the endogenous counterparts of viral oncogenes exhibit the traditional characteristics of cellular genes, the endogenous virogenes do not. In the traditional sense, a cellular gene occupies a definite site in the cell genome, is transmitted vertically and can be detected across phylogenetically related host species. Let us first examine these properties in the virogenes, namely, the endogenous RAV-0 sequences which are homologous to avian leukosis virus genomes. First, when definite potential sites are available within the chicken genome for the integration of endogenous RAV-0 proviruses, the actual occupied sites vary among individuals and in this limited sense, resemble the integrations of exogenous proviruses during infection (43). Second, although most normal chickens carry in their genomes five to ten genome equivalents of the vertically transmitted RAV-0, the one exceptional chicken which has been shown lacking the endogenous sequences (39) well indicates that these virogenes are non-essential for the development of a normal chicken. Third, the distribution of endogenous RAV-0 among Galliform birds has been found to be sporadic (65), inferring that the spread of the RAV-0 from domestic chickens to pheasants is probably through horizontal

infection rather than through evolution. Lastly, there is at the present no evidence that the presence of RAV-0 has any significance in carcinogen-induced neoplasms in the natural populations. In fact, the RAV-0 resembles a foreign agent which has been able to establish itself within the host genome merely because of its lack of influence on the host.

On the other hand, the viral oncogenes do seem to have been derived from cellular genes. Since the sarcoma gene (src) of ASV has been the most studied oncogene, we shall review the evidences indicating its cellular origin. Foremost, the existence in uninfected chicken cells of a sequence homologous to the ASV src was detected by molecular hybridization and has since been termed the endogenous sarc gene (66). Hybridization data indicating the wide spread presence in vertebrate cells of sequences homologous to sarc suggested that most of these sequences have been conserved over a long period of evolution. Thus, if infection was the source of cellular sarc genes, the event must have taken place very early in the evolution of the vertebrates. Second, in contrast to the multiple sites of RAV-0 sequences in the chicken genome, the sarc sequences are mostly situated at unique loci within the genome of any given species (67) and these loci are different from those occupied by RAV-0 (68). The sarc sequences therefore resemble other unique cellular genes. In addition, the phosphoprotein $pp60^{src}$ encoded by the ASV src gene has a structurally and functionally similar endogenous counterpart $pp60^{sarc}$ in uninfected avian and mammalian cells (69,70). Both $pp60^{src}$ and $pp60^{sarc}$ phosphorylate tyrosine (which is a

rare happening in normal cells) but the level of $pp60^{src}$ in a transformed cell is 50 times higher than the level of $pp60^{sarc}$ in an uninfected cell (71). It therefore is tempting to conclude that this quantitative difference in protein kinase might be responsible for transformation. But there is no evidence that varying the level of this kinase is going to cause transformation. As a matter of fact, transformation might be the result of other cellular genes (such as the globin genes) being activated by the infection of RSV, MC29, as well as AEV (67,72) and it has been suggested that the exogenous proviral LTRs might be introducing novel transcriptional promotors to cellular genes situated downstream to the integrated provirus. This suggestion tends to explain how the lymphatic leukemia virus can induce neoplasms when their oncogenes are largely deleted. Cooper and Neiman (73) have demonstrated that when DNA extracted from NIH 3T3 mouse cells transformed by RAV-2 transfection was used for a second transfection of mouse cells, some activated cellular genes could be responsible for secondary transformation, yet these cell genes were not linked to the integrated RAV-2 provirus in the first transfection. We should at the same time acknowledge that transfection may be different from natural infection (74).

If the transforming ARV originated from recombination between cellular oncogenes and nontransforming viral replicative genes, the mechanism of such a recombination has never been elucidated.

Even though the acute oncogenic viruses may be true recombinants, their isolation usually involves long periods of continued passages

in vivo, thus rendering difficult direct observations of the recombination process. Nevertheless, Rapp and Todaro have recently proposed a rapid method of generating oncogenic mouse type C virus *in vitro* that may provide insight to the mechanism of recombination (75). On the contrary, the viral src genes in ASV undergo non-conditional and temperature-sensitive mutations at high frequencies involving variable amounts of deletion at various locations along the sequence (76,77) and thus produce full or partial transformation-defective mutants (td or ptd ASVs). Some of these ptd ASVs can occasionally induce tumors in chicken as well as in quail cells, and the sarcoma viruses that can be recovered from these tumors are designated as rASVs. rASVs contain sarcoma sequences slightly different from parental ASVs and therefore are thought to be the recombinants between ASV and endogenous sarc genes (78). Careful studies by Vigne et al. (79) revealed that the src sequences from these rASVs were polymorphic among each other as well as with wild-type ASV src. Thus, if rASVs are indeed recombinants, either the cellular sequences are genetically variable or different cellular junctures are being incorporated into the recombinants.

Another example of recombination is the acquisition by RAV-0 virus of the exogenous common C region from defective leukosis virus MC29 during double infection (80). Moreover, the subgroup E (an endogenous envelope subgroup) oncogenic virus RAV-60 could very well represent a recombinant between endogenous provirus and exogenous ALSVs (81,82). Other varieties of recombinations between endogenous and exogenous sequences have been discussed by Eisenman et al. recently (83) whereas

recombinations among exogenous viruses, including crosses within the env genes have also been described (84).

In contrast to the src gene of ASV, the oncogenes of DLVs are not as well defined. Since the DLVs can transform fibroblast in culture (with the exception of AMV) nonproducing transformed cell clones free of replicating helper viruses can be isolated for the purification of DLV genomes. All DLVs were found to contain distinctive oncogenes corresponding to the types of specific neoplasms that they induce (6, 27,85). MC29-transformed cells contain a gag-gene related protein of 110,000 molecular weight, labeled p110, which probably is coded by gag-gene and the oncogene for MC29. MC29 deletion mutants, which produce smaller proteins, are much less efficient in transforming hematopoietic cells although their abilities to transform fibroblasts are retained (86). In contrast, two size classes of viral specific mRNAs can be isolated from AEV transformed erythroblasts. The larger genome size mRNA appears to code for an AEV polyprotein, when no protein has been identified as the product of the smaller subgenomic mRNA (87). Graf and Beaug (88) have described a temperature-sensitive mutant in AEV that could not transform erythroblasts at the nonpremissive temperatures thereby indicating a transforming protein. Even though none of these DLV mutations have been mapped, they nevertheless implicate the specific transforming products of oncogenes in DLVs.

However, the inability of AMV to transform fibroblasts greatly hampered the verification of a transforming gene in AMV. AMV specific protein has yet to be identified in transformed myeloblasts. Attempts

have been made to identify the defective AMV genome by methods such as isolating specific cDNAs (26,27), by cloning proviruses (25,50), and by probing the myeloblast intracellular RNAs (23,23,89). Gonda et al. (89) recently has identified in both AMV virions and transformed myeloblasts two classes of RNAs that are smaller than the helper viral RNAs. They therefore suggested that a 7.2 kb RNA may be the true genome AMV RNA and the smaller 2.3 kb entity may code for the transforming proteins. But, until an AMV specific protein can be isolated, the best we can do is to speculate on circumstantial evidences that depend on the nonhomology between AMV specific sequences and their helper sequences.

MATERIALS AND METHODS

EQUIPMENT AND REAGENT TREATMENT

All equipment and glassware employed in the following procedures were either autoclaved at 200°F or dry heat sterilized at 300°F. Ninety-five percent ethanol for nucleic acid precipitation was passed aseptically through sterilized glass filters. Phenol for protein extraction was distilled, stabilized by 8-hydroxyquinoline and cresol, and equilibrated with 0.01 M Tris or TNE buffer. Wherever applicable, reagents and buffers were either filtered through nitrocellulose membrane filters (Schleicher and Schuell, Keene, N. H.), or autoclaved 15 min at 200°F, or both.

VIRUS AND CELL COLLECTION

Avian Myeloblastosis Virus and AMV-infected Cells

One day old chicks were inoculated intravenously with 10^{10} to 10^{11} AMV particles. After nine to 15 days, blood was collected by cardiac puncture from chicks that showed at least 50% of their peripheral blood cells as immature leukocytes (myeloblasts), using heparinized conical centrifuge tubes. After centrifugation at 3,000 X g for 10 min, the blood separated into three distinct layers, with the plasma fraction layered on top, the dense erythrocytes settled at the bottom, and the white myeloblasts sandwiched in between. The plasma portion was pipetted off and stored at -60°C as a source for AMV particles. The myeloblasts were separated from the infected erythrocytes and both

were washed three times with 1 X SSC buffer to minimize contamination with plasma virus, or mixing of the cell fractions. This technique could effectively separate erythrocytes from myeloblasts with less than 0.5% cross mixing. Normal chicken peripheral blood does not contain myeloblasts, therefore all myeloblasts collected this way were infected myeloblasts. The cells were frozen in liquid nitrogen and stored at -60°C until needed. AMV containing plasma was also obtained from J. W. Beard.

Normal Chicken Erythrocytes and Avian Fibroblasts

Normal erythrocytes were collected by bleeding healthy adult chickens (Babcock hens) by cardiac puncture into heparinized conical centrifuge tubes, centrifuged at 3000 X g, separated from plasma and white cell buffy coat, washed twice with 1 X SSC and frozen for storage.

Pheasant and quail 12 day-old embryos were obtained from E. E. Wilson Game Reserve at Corvallis, Oregon. Turkey, duck and goose embryos, along with healthy adult chickens were supplied by the Department of Poultry Science, Agricultural Experimental Station at Oregon State University. All embryos were decapitated, eviscerated, deboned, skinned, washed in 1 X SSC and stored at -60°C.

Avian Erythroblastosis Virus

Two to four-week old chicks (Jenk's Cornish Cross) were inoculated intravenously with about 10^9 viral particles (Strain R stock from Dr. J. W. Beard's collection) obtained from AEV-infected chicken liver

homogenate. Eight to ten days later, 80% to 90% of the inoculated chicks came down with acute symptoms of the disease. Blood was collected from those chicks showing at least 30% to 40% of their peripheral blood cells as immature blast cells (erythroblasts). The blood was then processed as with AMV-infected blood and the AEV containing plasma so obtained was stored at -60°C.

Viruses from Tissue Cultures

All virus-containing tissue culture fluids were collected at six hour intervals from confluent chicken embryo fibroblast monolayers maintained in sterile disposable plastic 100 X 15 mm culture dishes (Falcon), each containing 8-10 ml Hams F10 medium supplemented with 5% calf serum. Rous Associated Virus-0 (RAV-0) was obtained from Line 100 X 7 chicken embryo fibroblasts that are known to be spontaneous producers of the endogenous virus. Myeloblastosis Associated Virus (MAV-1 and MAV-2) were isolated from culture fluids collected from fibroblast cultures infected with the viruses. Rous Sarcoma Virus (Pr-RSV) was purified from infected tissue culture fluid provided by Dr. Ursula Heine from NCI at NIH, Maryland. Before purification procedures, virus particles in culture fluids were concentrated by pelleting at 55,000 X g for two hrs at 4°C with FA21 rotor, resuspending in small amounts of tissue culture media. RNAs from MC29 and Schmidt Ruppin D-RSV were extracted by Don Jump in our laboratory.

ISOLATION OF NUCLEI

Isolation of Nuclei from Myeloblasts

Sixteen ml of packed and frozen myeloblasts were thawed in a total volume of 30 ml 1 X SSC (pH 6.8) at a cell concentration of no more than 7 X 10^8 cells per ml. Each 5 ml of the cell suspension was put into a 30 ml round bottomed Corex centrifuge tube (Corning) and an equal volume of a lysis buffer containing 0.25% Nonidet P-40 in 1 X SSC was added. The cells were gently pipetted with a wide bored Pasteur pipet at room temperature for about five min. The solution was immediately centrifuged at 1600 X g for four min, and the supernatant containing cell debris and detergent was promptly removed from the nuclei pellet to prevent further detergent action on the nuclei, which was then resuspended gently but quickly in 1 X SSC buffer to avoid clumping, and was washed once in the same buffer. Ca^{++}, 0.01 M was added to the wash buffer to stabilize the nuclear membrane in cases where the nuclei were being further handled before nucleic acid extraction.

Isolation of Nuclei from Normal and Infected Erthrocytes

Cells were thawed in 1 X SSC (pH 6.8) to a concentration of about 3 X 10^9 cells per ml with no more than 5 ml being put into a 30 ml Corex centrifuge tube. Equal volume of a lysis buffer consisting of 0.25% Nonident P-40 in 1 X SSC was added to the cells and the mixture was vigorously pipetted with a wide bored Pasteur pipet for 15 min at room temperature. The lysate was centrifuged at 10,000 X g for eight min. The pelleted nuclei was resuspended in 1 X SSC and washed twice

with the same buffer in the same manner. At this stage, most of the cells were free of extracellular viral materials, but individual nuclei were still being enveloped by collapsed cell membranes which required light shearing to be removed. The nuclei were resuspended in 1 X SSC in a Virtis 45 homogenizing flask, slightly homogenized at low speed setting for 20 min to strip off the cell membranes, and pelleted at 500 X g for five min. The fragile nuclei were then washed and handled the same way as with the myeloblast nuclei described above.

EXTRACTION AND FRAGMENTATION OF CELLULAR DNA

Hirt Extraction of Chicken DNA's

In order to avoid contamination of the extracted cellular DNA by endogenous or exogenous viral nucleic acids, the method of DNA extraction first proposed by Hirt was employed (90). The procedure is based on the preferential precipitation of undergraded high molecular weight cellular DNA with high salt from contaminating viral nucleic acids which are of relatively small molecular weights.

Freshly prepared cell nuclei were suspended in TNE to a concentration of about 1 X 10^7 nuclei per ml. Each 2 ml aliquot of the nuclei was put into a polycarbonate FA21 centrifuge tube (Beckman) with a 60 ml capacity. A volume of 14 ml of TNE_{10} containing 0.1 mg per ml Proteinase K (E. Merck, Darmstadt, Germany) and 0.6% SDS was added. The nuclei were lysed at 37°C for 1.5 hr with constant but gentle agitation. An extra 44 ml of TNE_{10} with 1.36 M NaCl was then added

to the tube to make the final NaCl concentration to about 1.1 M. The tube was then sealed and gently inverted at least ten times to insure sufficient mixing. The mixture was stored at 4°C for 12 hrs, at which time it was centrifuged at 18,000 rpm in a FA21 rotor (45,000 X g) for eight hrs at the same temperature. The gel-like pellet, which consisted of SDS, protein and nucleic acid, was then suspended in a total of 40 ml TNE, transferred to a Virtis cup and briefly sheared with a Virtis 45 homogenizer for three min at the maximum speed of 45,000 rpm to facilitate easier handling of the viscous nucleic acid solution. The sheared nucleic acid was then phenol and ether extracted three times each. The residual ether was removed by passing dry N_2 through the solution.

To further shear the nucleic acid into smaller fragments, glycerol was used to increase the viscosity of the solution. One volume of the extracted nucleic acid was mixed with two volumes of sterilized 100% glycerol in a 50 ml Virtis homogenizing cup, and then chilled in a dry ice-ethanol bath. This cold viscous nucleic acid solution was homogenized for 15 min at maximum speed with the Virtis 45 homogenizer. The sheared nucleic acid was precipitated overnight at -20°C from the glycerol by the addition of 0.05 X volume 4 M NaCl and 2 X volume of 95% ethanol.

The precipitated nucleic acid was pelleted from ethanol by centrifugation at 10,000 X g for 15 min and resuspended in TNE. Previously heated (80°C for 20 min) RNase T_1 and Pancreatic RNase A were added to concentrations of five units per ml and 10 µg per ml respectively, and

the solution was incubated at 37°C for 40 min to digest the RNA strands. Proteinase K was then added to a concentration of 0.1 mg per ml and the solution was further incubated for another 15 min to destroy the RNases. The resultant DNA was extracted again with phenol and ether and was ethanol precipitated. All final DNA products were dissolved in 1 X SSC; concentrations determined by OD_{260} and OD_{280}, and stored at -20°C.

Marmur Extraction of Avian Fibroblast DNA

Prepared embryos (91) (see cell collection methods) were thawed, washed, and suspended with TNE_{10} in a Virtis 45 homogenizer cup. After being blended at maximum speed (45,000 rpm) for ten min, the homogenate was immediately added with SDS to a concentration of 2% and with previously self digested pronase to 0.1 mg per ml. The mixture was incubated at 37°C for one hr with constant stirring, extracted three times with phenol and twice with ether before being precipitated with ethanol at -20°C for ten hr. After being pelleted from the ethanol and resuspended in TNE, the nucleic acid was sheared in glycerol with a Virtis homogenizer and reprecipitated in ethanol as previously described. The fragmented nucleic acid was again pelleted, resuspended in TNE and digested with RNase A and RNase T_1. Proteinase K was then added to digest the enzymes and the whole mixture was deproteinized by phenol and ether extractions. The final DNA product was once again ethanol precipitated, resuspended in 1 X SSC and stored at -20°C.

Agarose Gel Electrophoresis

The size of cell DNA fragmented by mechanical shearing in glycerol was determined by agarose gel electrophoresis. One percent agarose gel was prepared with 1 X E buffer (0.04 M Tris, 0.02 M Na acetate, 0.005 M EDTA) containing 0.5 mg per ml ethidium bromide, and was put into 30 X 0.64 cm (inside diameter) lucite tubes. Fifty µl sample of cell DNA (0.3 mg per ml in 10% glycerol) was added to the top of the gel which was then run for six hr at 50 volts. A separate gel with Hae III restriction enzyme digested PM2 (a pseudomonas phage) DNA fragments was run in parallel to serve as size markers. The DNA bands were observed under UV lights.

EXTRACTION OF VIRAL RNA

Purification of Viruses

Viral suspensions containing 10^9 to 10^{10} viral particles per ml were clarified by centrifugation at about 4,000 X g for five min at 4°C with small amounts of cellite to pellet cells and debris. The viral particles were then pelleted through 15 ml of 20% sucrose-TN buffer onto a 1 ml cushion of 70% sucrose-TN in a SW27 centrifuge tube (Beckman) by centrifuging at 25,000 rpm (about 100,000 X g) for two hr at 4°C. The virus pellet was pipetted off the 70% sucrose pad and was diluted with sufficient amount of TN buffer to attain a final sucrose concentration of no more than 10%. These partially purified virus particles were then banded by zonal centrifugation in a 20-60% sucrose-TN

linear gradient at 100,000 X g for 14 hr to 16 hr at 4°C (at 26,000 rpm in a SW27 rotor). In gradients where the virus bands (density of about 1.16 gm per ml) were not readily visible, reverse transcriptase assays were performed on 5 µl aliquots of the gradient fractions in order to locate the virus. The viral fractions were then pooled and diluted to less than 20% sucrose with TN buffer and pelleted by centrifugation at 100,000 X g for 90 min at 4°C.

Reverse Transcriptase Assay

Each 5 µl virus aliquot was lysed for 15 min at 37°C in a 10 µl solution containing 0.25% nonidet P40, 0.075 M dithiothreitol, 0.025 M Tris-hydrochloride (pH 8.3), 0.01 M $MgCl_2$, 0.15 M KCl, 0.005 M EDTA and 10% glycerol (52). To the activated virus was added 30 µl of an assay mixture containing dATP, dCTP, and dGTP at 0.002 M each, 1 µCi [^{3}H]TTP (1 mCi per ml, from Schwarz-Mann), 3 mg per ml DNase-digested calf thymus DNA primer, 0.04 M Tris-HCl (pH 8.3), 0.006 M $MgCl_2$, and incubated at 37°C for at least 30 min. The reaction was terminated by the addition of ice-cold 10% trichloroacetic acid. The precipitates containing incorporated [^{3}H]TTP were collected on nitrocellulose filters (Schleicher and Schuell, Keene, N. H.), dried in oven and counted by liquid scintillation.

RNA Extraction

Freshly purified virus was pelleted at 100,000 X g for 1.5 hr at 4°C. The virus pellet was resuspended in 5.5 ml TNE and gently

homogenized to disperse virus aggregates by a Dounce-type homogenizer in an ice bath. Proteinase K was added to a final concentration of 2 mg per ml and SDS was added to a final 1% concentration. The mixture was incubated at 37°C for 15 min and phenol extracted three times, followed by ether extraction twice. The viral RNA in the aqueous phase was precipitated at -20°C by the addition of 2 X volume 95% ethanol and 0.05 X volume 4 M NaCl. This viral RNA precipitate was pelleted from the ethanol by centrifugation at 10,000 X g and redissolved in TNE. The RNA was subsequently fractionated by zonal centrifugation in a 10-30% glycerol-TNE linear gradient at 200,000 X g for 90 min at 4°C. RNA sizes in different fractions from the gradient were determined by E. coli rRNA markers fractionated under exactly the same conditions. Fractions containing 60-70s genome size viral RNA were pooled and ethanol precipitated overnight. The precipitate was pelleted again by centrifugation and redissolved in 1 X SSC buffer. RNA concentrations were then determined by OD_{260}.

PREPARATION OF [^{3}H] cDNA COMPLEMENTARY TO AMV GENOME RNA

AMV [^{3}H]cDNA was prepared by a modified endogenous reaction in which purified virus was disrupted with a limited amount of a neutral detergent. In the presence of deoxyribonucleotide triphosphate, the detergent-released RNA served as template for DNA synthesis by the liberated viral reverse transcriptase. Calf thymus DNA, digested to small fragments by DNase I, was added as short primer sequences for additional reaction initiation sites on the templates. Actinomycin D

was used in the reaction to inhibit DNA-dependent DNA synthesis so that double-stranded DNA-DNA formation was minimized.

Endogenous Reaction

Avian myeloblastosis virus was purified as previously described from a 45 ml portion of AMV-infected chicken plasma containing about 5×10^{11} viral particles per ml (supplied by J. W. Beard). Purified virus particles were disrupted for five hrs at 0°C in a 1 ml solution of 0.25% Nonidet-P40, 0.075 M dithiothreitol, 0.025 M Tris-HCl (pH 8.3), 0.01 M $MgCl_2$, 0.15 M KCl, 0.005 M EDTA and 10% glycerol. To the virus lysate was added a 0.5 ml solution of 30 mg per ml calf thymus DNA primer, and the mixture was incubated at 37°C for ten min. To the virus-primer solution was then added a reaction mixture consisting of: 0.5 ml modified basal medium (0.4 M Tris-HCl, pH 8.3, 0.06 M $MgCl_2$); 0.5 ml actinomycin D (Sigma) of 2 mg per ml; a 0.5 ml mixture of dATP, dGTP, and dCTP at 2.2 mM each; 2.5 ml of 2 miCi [^{3}H]TTP in H_2O (Schwarz-Mann). The reaction mixture was further incubated at 37°C. One µl aliquots were removed at 15 min intervals, TCA precipitated onto nitrocellulose filters, dried, and liquid scintillation counted to monitor the incorporation of [^{3}H]TTP into acid precipitable components. The reaction was terminated by the addition of 0.5 ml 0.4 M EDTA at pH 7.3 after 1.5 hr when the rate of [^{3}H] incorporation reached a plateau level. The solution was phenol extracted twice to remove viral and enzyme proteins, ether extracted twice and finally ethanol precipitated at -20°C for eight hr.

Purification by Sephadex G-75 Column

This resultant ethanol precipitate was a mixture of viral RNA, RNA-[^{3}H]-labeled DNA hybrid, single- and double-stranded [^{3}H]-labeled DNA, and double-stranded small calf thymus DNA fragments. After being pelleted from the ethanol (12,000 X g for 10 min) and redissolved in 2 ml of TN buffer, the nucleic acid mixture was passed through a TN buffer equilibrated Sephadex G-75 column (20 X 2 cm) to remove the small calf thymus DNA fragments. Radioactive fractions containing single-stranded, double-stranded and hybrid [^{3}H]cDNA were pooled and diluted with 0.01 M sodium phosphate buffer (pH 7.2) to a final NaCl concentration of 0.06 M.

Hydroxylapatite Chromatography

The nucleic acid mixture was adsorbed onto a 10 ml bed volume hydroxylapatite (Bio-Rad) jacketed column (described later in detail) maintained at 57°C and was washed with up to 20 ml of 0.01 M sodium phosphate buffer containing 0.3 M NaCl. The separation of nucleic acid molecules in states of different strandedness was achieved by stepwise elution with 50 to 60 ml each of 0.13 M phosphate buffer containing 0.3 M NaCl for single strands, 0.2 M phosphate buffer for hybrids, and 0.4 M phosphate buffer for double strands, successively.

Alkaline Hydrolysis

The [^{3}H]cDNA-RNA hybrids eluted in the 0.2 M phosphate buffer were dialysed against H_2O, lyophilized to dryness, and redissolved in

6 ml H_2O. NaOH was added to the hybrid solution to give a final concentration of 0.4 M NaOH. The solution was boiled for one min and incubated at 37°C for three hrs to hydrolyze the RNA component. After being neutralized with HCl, the AMV [^{3}H]cDNA single strands were ethanol precipitated at -20°C for 12 hrs, pelleted by centrifugation at 12,000 X g for 20 min and redissolved in 2 X SSC buffer. The specific activity of the resultant [^{3}H]cDNA was calculated to be about 2 X 10^7 cpm per µg by using the specific activity of [^{3}H]-TTP provided by Schwarz-Mann (64 Ci per mole). However, when optical density was used, the specific activity was determined to be about 6 X 10^6 cpm per µg. A kinetic method was thus used to determine a more accurate specific activity.

PURIFICATION OF [^{3}H]cDNA BY HYBRIDIZATION WITH LIMITED AMOUNTS OF AMV RNA

The [^{3}H]cDNA produced in the endogenous reverse transcriptase reaction was obtained in such a fashion that some viral sequences were being transcribed more often than others, resulting in multiple copies of some portions of the genome. In order to obtain a more even cDNA representation of all viral sequences, the [^{3}H]cDNA copies were hybridized to genome AMV RNA in 1:1 mass ratio. Those cDNA that were present in DNA-RNA hybrids, fractionated by hydroxylapatite chromatography, were extracted as purified [^{3}H]cDNA in the following procedure.

Hybridization

A 1 ml reaction mixture containing about 5.5 X 10^7 cpm [^{3}H]cDNA, 7 µg of 35s AMV RNA, 40% formamide, 0.41 M NaCl and 0.2 M Na Citrate

was sealed in a 2 ml serum bottle, boiled for four min in a 98°C water bath, and incubated at 50°C for 24 hrs, at which time the $C_o t$ value was approximately 4.5 mole·sec/litre.

S_1 Nuclease Assay

At the end of incubation, triplicate 1 µl samples were removed from the reaction mixture for assaying by S_1 nuclease the degree of hybridization. Each 1 µl sample was put into 100 µl of a S_1 assaying reaction mixture at pH 4.5 containing 0.03 M K acetate, 0.37 M NaCl, 0.006 M $ZnSO_4$, 62.5 µg per ml double-stranded and 12.5 µg per ml single-stranded calf thymus DNA. The solution was then divided accurately into two 50 µl aliquots. To one aliquot was added 4 µl solution of 2 IU per ml S_1 nuclease which had been diluted in 2 X SSC from a 40 IU per ml stock S_1 nuclease (Cal Biochem, San Diego, CA). To the other aliquot, which served as a control, was added 4 µl of 2 X SSC. Both aliquots were incubated at 42°C for 45 min. S_1 resistant nucleic acid was precipitated by the addition of ice-cold 10% trichloroacetic acid and was collected on BA85 nitrocellulose membrane filters (Schleicher and Schuell, Keene, N. H.). After subtraction of background counts, the number of counts per minute on the filter from the S_1 treated aliquot was compared to those from the non-treated control aliquot to determine the fraction of [^{3}H]cDNA hybridized.

Hydroxylapatite Fractionation

The remainder of the [^{3}H]cDNA-RNA reaction solution was added to

10 ml of 0.01 M sodium phosphate buffer with 0.3 M NaCl for hydroxylapatite chromatography. A column having a 10 ml bed volume prepared from previously boiled hydroxylapatite suspension (Bio-Rad, Richmond, CA) was maintained at 57°C in a jacketed column and equillibrated by passage of 100 ml 0.01 M phosphate buffer at pH 7.3. The nucleic acid was adsorbed onto the hydroxylapatite by slowly passing the reaction solution through the column at a flow rate of no more than 0.5 ml per min. The column was rinsed with about 15 ml 0.01 M phosphate buffer containing 0.3 M NaCl to remove any unadsorbed materials. The nucleic acids were fractionated according to their state of strandedness by stepwise elution from the column, using successively 50 ml each of 0.13 M phosphate buffer containing 0.3 M NaCl for DNA single-strand molecules, 0.2 M phosphate buffer for RNA/DNA hybrid molecules, and 0.4 M phosphate for DNA double-strand molecules. The 0.3 M NaCl in the phosphate buffer was added to protect the double-strands and hybrids from dissociation under the relatively high temperature and low salt conditions. The [^{3}H]cDNA that were eluted in hybrid fractions were pooled, dialysed against water, lyophilized to dryness and resuspended in 6 ml of water. The nucleic acid solution was then subjected to the same treatment as in [^{3}H]cDNA preparation, whereby the RNA component was removed by alkaline hydrolysis, and the remaining purified [^{3}H]cDNA was neutralized, ethanol precipitated, redissolved in 1 X SSC and stored at -60°C.

ISOLATION OF $cDNA_{mye}$(A) THAT EXCLUDED ENDOGENOUS SEQUENCES AND $cDNA_{mye}$(S) THAT EXCLUDED COMMON EXOGENOUS SEQUENCES

[^{3}H]cDNA complementary to the part of AMV RNA sequences that were non-homologous to endogenous sequences was designated as $cDNA_{mye}$(A) and was isolated by exhaustive hybridization with excessive RAV-0 RNA (an endogenous virus) to remove the endogenous sequences. [^{3}H]cDNA complementary to unique AMV RNA sequences was designated as $cDNA_{mye}$(S) and was prepared by hybridizing total cDNA with excessive RSV RNA (an exogenous virus) to remove common exogenous sequences.

In both cases, approximately 1 X 10^7 cpm of the [^{3}H]cDNA was mixed with at least a ten-fold mass excess of either RAV-0 or RSV RNA in a 0.5 to 0.6 ml reaction solution containing 40-45% formamide, 0.46 M NaCl, 0.02 M Na citrate at pH 7.2. The mixture was sealed in a capped polypropylene tube (Sarstedt, Princeton, N.J.), boiled at 89°C for four min and incubated in a 50°C water bath for 25 hrs (C_rt = 12 mole·sec/litre). At the end of incubation, triplicate 1 µl samples were removed and the degrees of hybridization were assayed by S_1 nuclease on the samples as previously described.

The remainder of the reaction mixture was added to 5 ml of 0.01 M sodium phosphate buffer containing 0.2 M NaCl at pH 7.2. The nucleic acid was then fractionated on a hydroxylapatite column following the same procedures as described in the purification of [^{3}H]cDNA. Single-stranded cDNA fractions ($cDNA_{mye}$) that were eluted with 0.13 M phosphate buffer, and hybrid fractions that were eluted with 0.4 M phosphate buffer, were pooled separately, dialysed against H_2O for at least

12 hrs, lyophilized to dryness and resuspended in small amounts of H_2O.

The single-stranded $cDNA_{mye}$ (A or S) portions from the column was then subjected to a second purification by hybridizing with limited excess of AMV genome RNA at a 7:1 mass ratio (equivalent to 1.4:1 sequence ratio). The portion of $cDNA_{mye}$ (A) or $cDNA_{mye}$ (S) that hybridized with AMV RNA was isolated by a second hydroxylapatite chromatography with phosphate buffer, dialysed, lyophilized and resuspended in H_2O. After alkaline hydrolysis with added NaOH to 0.4 M and neutralized with HCL, the purified cLeuk probes were again dialysed, lyophilized and resuspended in 1 X SSC and stored in capped polypropylene tubes at -60°C.

In order to isolate cDNA corresponding to the endogenous viral RNA sequences (gag, pol and env regions) the pooled hybrid fractions from the first HA column were alkaline hydrolysed for four hrs to remove the RNA as described previously, neutralized, and ethanol precipitated overnight. The cDNA precipitate was redissolved in 1 X SSC.

[^{3}H] PROBE EXCESS HYBRIDIZATION

Hybridization of AMV RNA with Excess [^{3}H]cDNA

A reaction mixture was prepared with about 5.2 X 10^3 cpm per µl purified [^{3}H]cDNA, 27.8 picogram per µl of AMV RNA, 48% formamide, 0.45 M NaCl, and 0.02 M Na citrate at pH 7.2. Seven µl aliquots were sealed into capillary tubes, boiled for three min and incubated in a 50°C water bath for various lengths of time. A parallel reaction

excluding the AMV RNA was performed as a background control to assay the degree of S_1 resistance by the cDNA alone. At designated times, three capillaries from each reaction were removed from the water bath and immediately frozen in a -60°C ethanol bath to terminate the reactions. To assay the amount of hybridized cDNA in each capillary, the content was expelled onto a piece of parafilm and exactly 5 µl was quantitatively transferred to a 100 µl S_1 assay mixture at pH 4.5 (0.03 M K acetate, 0.37 M NaCl, 0.006 M $ZnSO_4$, 62.5 µg per ml and 12.5 µg per ml of double-stranded and single-stranded calf thymus DNA respectively); 0.024 IU S_1 nuclease (Cal Biochem, San Diego, CA) was added, and the mixture was incubated at 42°C for one hr. The S_1 resistant [^{3}H]cDNA was precipitated by the addition of 10% ice-cold trichloroacetic acid and was collected on BA 85 nitrocellulose membrane filter to be counted by liquid scintillation.

Hybridization of Cellular DNA with Excess $cDNA_{mye}$(S)

Reaction mixtures were prepared with 1.2 X 10^3 cpm per µl of cLeuk S (about 0.17 nanogram per µl), approximately 30% formamide, 0.46 M NaCl, and 0.012 M Na citrate at pH 7.2. Cellular DNA was added at 0.6 µg per µl for myeloblast DNA and 0.89 µg per µl for infected or normal erythrocyte DNA's. Eight to 9 µl aliquots were sealed in capillary tubes, boiled for eight min at 98°C and incubated at 50°C for various lengths of time for two to 32 hrs. Three capillaries from each reaction were removed at each time interval and immediately frozen until assayed. To assay for $cDNA_{mye}$(S) hybridized to cellular DNA, content

From each capillary was expelled onto a piece of parafilm and seven or 8 µl exact aliquot was quantitatively transferred to 100 µl of S_1 assay mixture. The solution was S_1 digested as previously described and S_1 resistant $cDNA_{mye}(S)$ was trichloroacetic acid precipitated onto nitrocellulose filter to be counted.

Hybridization of Cellular DNA with Excess [^{3}H]cDNA Sequences Common to Exogenous Viruses $cDNA_{exo}(S)$

Reaction mixtures were prepared with 3.2×10^3 cpm per µl of $cDNA_{exo}(S)$ isolated as common to exogenous viruses, 33% formamide, 0.48 M NaCl, 0.03 Na citrate at pH 7.2. To each reaction mixture was added 0.6 µg per µl of DNA from myeloblast, infected erythrocyte or normal erythrocytes. Approximately 8 µl aliquots were sealed in capillary tubes, boiled for eight min and incubated at 50°C for various time lengths. Aliquots of exactly 7 µl from each capillary were S_1 digested at 42°C for one hr, trichloroacetic acid precipitated and the S_1 resistant precipitates were collected on nitrocellulose filters to be counted by liquid scintillation.

SATURATION HYBRIDIZATION OF [^{3}H]cDNA WITH VARYING AMOUNTS OF AMV RNA

To decide on the amount of excess AMV RNA required to obtain the maximum hybridization with [^{3}H]cDNA, and to determine the degree of sequence representativeness of the prepared cDNA, a series of hybridization reactions were performed with varying AMV RNA:cDNA mass ratios ranging from a low of 0.07 nanogram/nanogram to a high of 242

nanogram/nanogram.

All hybridization reactions here and in subsequent procedures were performed in triplicates to obtain average results.

A reaction mixture, six to 70 µl in volume, contained at least 1 X 10^3 cpm [^{3}H]cDNA and a known amount of AMV RNA. The mass ratio was calculated by the known concentration of a stock AMV RNA as determined by optical densities and by the specific activities of the cDNA as determined by a kinetic method. All reactions were performed at 48% formamide in 3 X SSC.

The reactions were sealed in capillaries, denatured by boiling in 98°C for three min and incubated at 50°C for different lengths of time that gave C_rt values of around 1 mole·sec/litre, at which time the hybridizations should have all been completed.

The extents of hybridization were determined by dividing the capillary content into two exact aliquots and treating one aliquot with S_1 nuclease as described earlier.

KINETIC HYBRIDIZATION OF [^{3}H]-LABELED PROBES WITH VARIOUS VIRAL RNAs IN EXCESS

Hybridizations of the three types of [^{3}H]-labeled probes, namely the total cDNA, $cDNA_{mye}$(A) and $cDNA_{mye}$(S) with various types of viral RNAs in excess were carried out under relatively stringent conditions of 48% formamide, 0.45 M NaCl, 0.04 M Na citrate at pH 7.2. Each reaction aliquot, about ten to 20 µl in volume, contained approximately 1 X 10^3 cpm of a specific probe plus at least 100 to 200 fold excess in mass of the viral RNA under investigation at a concentration of between

three to six nanograms per µl. The reaction solutions were sealed in appropriate size capillary tubes, boiled for three min and incubated at 50°C for various lengths of time, giving C_rt values of ranging from 2×10^{-5} mole·sec to 1 mole·sec. Triplicate reactions for any one C_rt value were assayed by treating exactly half of the content in a single capillary with S_1 nuclease. The content from each capillary was expelled into 210 µl of S_1 assay mixture, which was then divided quantitatively into two exactly equal aliquots. S_1 nuclease was added, as previously described, to one aliquot and both aliquots were incubated at 42°C for one hr. S_1 resistant [^{3}H] probes were trichloroacetic acid precipitated onto nitrocellulose filters. The counts from S_1 treated half of the reaction were compared to those from the non-treated half as the percent of input of [^{3}H] probes hybridized to the viral RNA at a certain time during the course of the reaction.

SATURATION HYBRIDIZATION OF [^{3}H]cDNA WITH VARYING AMOUNTS OF CELLULAR DNA

Hybridizations were performed in about 42% formamide, 0.43 M NaCl, and 0.04 M Na citrate at pH 7.2. Reaction mixtures contained a minimum of 500 cpm [^{3}H]cDNA and varying amounts of cellular DNAs, giving cell DNA:cDNA mass ratios that ranged from 0.035 to 3.7 mg/ng for myeloblasts and infected erythrocytes, and from 0.035 to 8 mg/ng for normal erythrocytes. To maintain relatively consistent total DNA concentrations of between one to 8 mg per ml through all reactions, it was necessary to vary from reaction to reaction the reaction volumes as well as the actual amounts of cDNA. All reactions were performed in

capillaries of appropriate sizes, boiled for eight min to denature cell DNAs, and incubated for varying lengths of times, ranging from 110 hrs to 235 hrs, in order to bring the hybridizations to completion according to DNA concentrations. Extents of hybridization were again assayed by S_1 nuclease.

KINETIC HYBRIDIZATION OF [^{3}H]-LABELED PROBES WITH CELLULAR DNA'S

The [^{3}H]-labeled probes used in these reactions were total cDNA, $cDNA_{mye}$(A) and $cDNA_{mye}$(S). Cellular DNAs included those from chicken myeloblasts and erythrocytes (normal and infected) and those from fibroblasts of other avian species.

The reactions were performed in 3 X SSC with 30% formamide. Cellular DNAs were present in excess to [^{3}H] probes ranging in mass ratio from three to 6 mg of cell DNA per ng probe. Reaction volumes, about 80 µl each, were adjusted so the DNA concentrations stayed within six to 12 mg per ml. All reactions were sealed in capillaries, denatured at 98°C for eight min and incubated at 50°C for various times. For analysis, content in each capillary was transferred into 1.1 ml S_1 assay mixture, which was then divided into two exact aliquots of 500 µl each. One aliquot was treated with 0.12 IU per ml S_1 nuclease for 45 min at 42°C. Both aliquots were trichloroacetic acid precipitated. The fraction of [^{3}H] probes hybridized was determined by comparing the amount of [^{3}H] precipitated from the two aliquots.

RESIDUAL HYBRIDIZATIONS

Detection of AMV Unique Sequences in $cDNA_{mye}(A)$

To detect non-homology between AMV and another exogenous virus such as RSV, $cDNA_{mye}(A)$ was first being exhaustively hybridized with an excess of RSV RNA. The residual $cDNA_{mye}(A)$ that remained as single strands were then hybridized to an excess of AMV RNA, or to excess cellular DNAs from either myeloblasts or normal erythrocytes.

Approximately 3×10^4 cpm of $cDNA_{mye}(A)$ was mixed with 780 nanograms of RSV RNA (RNA:cDNA mass ratio in excess of 200:1) in a 200 µl solution containing 46% formamide, 0.45 M NaCl, and 0.04 M Na citrate. Aliquots of 8 µl each were sealed in capillaries and the remainder of the solution was sealed in a capped polypropylene tube (Sarstedt, Princeton, N.J.). All samples were boiled for three min and incubated at 50°C. Triplicate capillaries were incubated at various times ranging from six min to 24 hrs, and stored at -60°C until being assayed. At 24 hrs of incubation (C_rt of about mole•sec) 525 ng of AMV RNA or 3 mg of single-strand cellular DNA was added to the solution in polypropylene tube which now contained RNA cDNA hybrids, single-strands RSV RNA, and single-stranded $cDNA_{mye}(A)$. Formamide and salt concentrations were readjusted back to their respective starting levels. The mixture was then divided into 12 aliquots, sealed in capillaries and reincubated at 50°C.

In the case where AMV RNA was added to the residual $cDNA_{mye}(A)$, triplicate capillaries were removed and frozen at periods ranging from

six min to 26 hrs (a C_rt value of about 1 mole·sec with respect to AMV RNA concentrations). In the cases where cellular DNAs were added, capillaries were removed at periods ranging from 15 min to 96 hrs (a C_ot value of 10^4 mole·sec with respect to cellular DNA concentrations). All samples were S_1 assayed for the percent of hybridization to establish C_rt and C_ot curves.

Detection of Sequences in $cDNA_{mye}$(A) Common to Exogenous Viruses

Three X 10^4 cpm of $cDNA_{mye}$(A) was mixed with 780 ng of RSV RNA in 200 µl solution of 46% formamide, 0.45 M NaCl, and 0.04 M Na citrate. The reaction was carried out as described above with aliquots sealed in capillaries for establishing C_rt curve and the remainder of the solution sealed in a capped polypropylene tube.

After 24 hrs of incubation, 205 ng of MC29 RNA was added to the reaction in the polypropylene tube containing the residual $cDNA_{mye}$(A), divided into aliquots in capillaries and further incubated as described earlier. All samples were eventually assayed by S_1 nuclease.

A reverse reaction was also performed with similar procedures, except that 3 X 10^4 cpm of $cDNA_{mye}$(A) was first hybridized with 684 ng of MC29 RNA. The residual $cDNA_{mye}$(A) single-strands were then hybridized to 300 ng of added RSV RNA.

PREPARATION OF [^{125}I] AMV RNA

The iodination procedure was a slight modification of that described by Tereba and McCarthy (92). Approximately 8.7 µg AMV RNA

was sealed in a capillary tube with a 51 µl solution of 0.12 M Na acetate (pH 4.9), 0.7 mM $TlCl_3$, and 2 miCi [^{125}I] (ICN). After 15 min of incubation at 60°C, the mixture was applied to a Sephadex G-75 column (20 X 0.7 cm) equilibrated with 0.015 M Na acetate and 1.5 mM Na citrate, pH 7.2. The iodinated RNA was eluted with the void volume ahead of the free [^{125}I]. The [^{125}I] labeled RNA fractions were pooled into a test tube which was then stoppered and incubated for another 15 min at 55°C to liberate [^{125}I] bound to an unstable intermediate (5-iodo-6-hydroxy-hydrocytidylic acid) formed in the previous incubation (103). The RNA, iodinated at the C-5 position on cytosine residues, was purified once more by passing through a second Sephadex G-75 column. This [^{125}I] RNA was then concentrated by lyophilization and was fractionated on a 10-30% glycerol-TNE gradient by zonal centrifugation at 200,000 X g for 1.5 hrs. Fractions containing [^{125}I] RNA in the size range of 8-15s were pooled and ethanol precipitated. The precipitate was resuspended in distilled water and stored at -60°C. The specific activity of the iodinated RNA was about 7.3 X 10^6 cpm per µg.

SATURATION HYBRIDIZATION OF [^{125}I] RNA WITH AMV [^{3}H]cDNA AT VARYING DNA/RNA RATIOS

To test the representation of viral sequences in prepared cDNA, [^{125}I] RNA was hybridized to cDNA at varying ratios of cDNA RNA. Reactions were performed with 3 X SSC containing about 43% formamide in volumes ranging from ten to 35 µl in order to maintain relatively consistant nucleic acid concentrations of from 0.1 to 0.3 µg per ml.

Also, 0.86 ng or 3.4 ng [^{125}I] RNA (specific activity of about 7.3 X 10^6 cpm per μg) were used in each reaction to hybridize with zero to 33 ng of AMV [^{3}H]cDNA with DNA/RNA ratios ranging from zero to 38 ng/ng. The reaction mixtures were sealed in capillaries, boiled for seven min and incubated at 50°C for about six to seven days to bring the $C_o t$ values in all reactions to about 0.14 mole·sec. The content of each capillary was divided into two portions. To one portion was added five units per ml RNase T_1 and 10 μg per ml of RNase A. The trichloroacetic acid precipitated nucleic acids from both portions were collected on nitrocellulose filters. The [^{125}I] radioactivities from both portions were counted with a Packard gamma counter and the RNase resistant RNA was expressed as the fraction of input [^{125}I] RNA hybridized.

RESULTS

DETERMINATION OF THE SPECIFIC ACTIVITY OF [^{3}H] cDNA PROBE

Accurate determination of the specific activity of [^{3}H]cDNA probe was important to the interpretation of our results, especially those pertaining to the estimation of the number of integrated proviral copies in the host genomes. However, the small quantity of available labeled probe rendered such a determination by optical procedures highly inaccurate if not impossible. Also, indirect calculations using specific activity of the labeled deoxyribonucleoside triphosphate ([^{3}H]TTP) supplied by the manufacturer did not provide any guarantee on the accuracy of the specific activity of the final product (52). Contamination of any of the unlabeled deoxyribonucleoside triphosphates (which were used in excess in the cDNA preparations) could contribute greatly to the inaccuracy.

To overcome these problems, a direct calibration of [^{3}H]cDNA against known quantities of viral RNA was used to determine the specific activity by kinetic analysis of RNA-DNA hybrid formation (52).

Figure 4 shows the results of hybridizing excess AMV [^{3}H]cDNA to a constant amount of 0.21 ng AMV RNA per reaction under standard conditions for various lengths of time. A control reaction was performed with the exact conditions but without the viral RNA and acid precipitable background counts were subtracted from the experimental results when presented in Fig. 4.

When the [^{3}H]cDNA in a reaction was present in about 27 fold

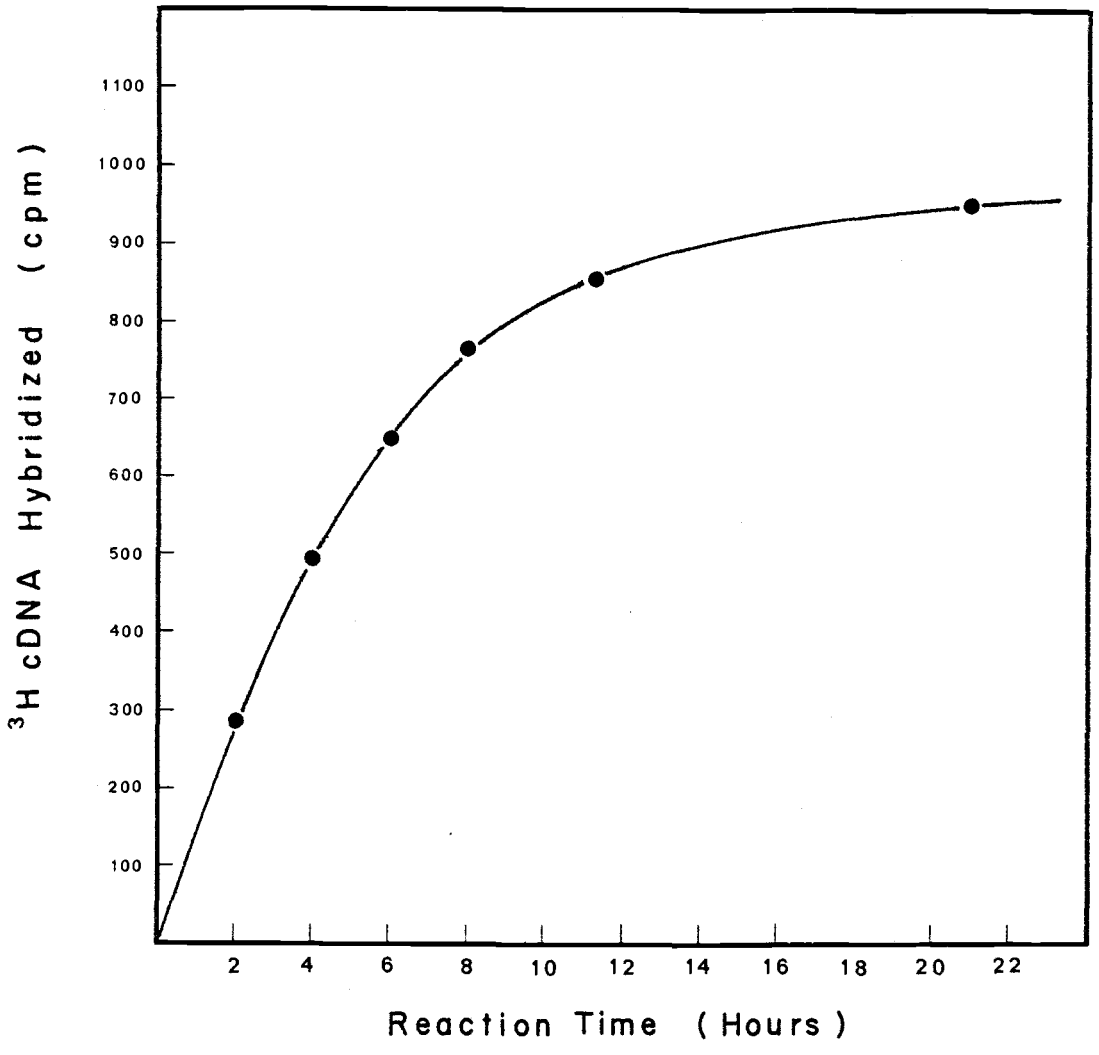


Figure 4. Kinetic hybridization of excess total [^{3}H]cDNA to AMV RNA. Each reaction contain about 3.6 X 10^4 cpm cDNA and 0.2 µg AMV RNA. The amount of cDNA hybridized was assayed by acid precipitation after digesting the non-hybridized cDNAs with S_1 nuclease.

excess to the viral RNA, the concentration of the reactable driver [^{3}H]cDNA remained relatively constant throughout the reaction and determined the rate of hybridization of the tracer RNA. The amount of [^{3}H]cDNA in acid precipitable hybrid form after S_1 endonuclease treatment reached a saturation plateau between 18 to 24 hr. The reaction rate of hybrid formation followed pseudo-first-order kinetics (93) which can be described by the following equation:

$$\frac{1}{C_{DNA}} \cdot \ln \left(\frac{C_{RNA}}{C_{RNA} - C_{hybrid}} \right) = kt$$

where C_{DNA} is the initial [^{3}H]cDNA molar concentration, C_{RNA} is the initial AMV RNA concentration determined by optical densities, C_{hybrid} is the concentration of [^{3}H]cDNA in hybrid form at time t, and k is the rate constant for the hybridization reaction. A plot of the left hand side of this equation against t will give a straight line with a slope of k.

When a series of different specific activities were assigned to the [^{3}H]cDNA, such that arbitrary values of C_{DNA} and C_{hybrid} could be obtained, the hybridization data in Fig. 4 were analyzed with the above pseudo-first-order rate equation. A sample of the calculated values is shown in Table 2.

Figure 5 shows the plots from six of such specific activity assignments. The assignment of 6.9×10^3 cpm per ng gave the best linear fit for the data from Fig. 4 and was represented by a solid line in Fig. 5. Both assignments of 6.7×10^3 and 7×10^3 cpm per ng gave non-linear

TABLE 2. CALCULATED DATA ON PSEUDO-FIRST-ORDER RATE EQUATION WITH ASSIGNED SPECIFIC ACTIVITIES TO cDNA

	$\frac{1}{C_{DNA}} \cdot \ln \frac{C_{RNA}}{C_{DNA} - C_{hybrid}} \cdot 10^{-5}$[a] at time intervals of:										
Time (hours)	1	2	3	4	5	6	8	10	12	14	21
cDNA in hybrid (cpm)	142	280	400	500	584	652	760	825	865	890	945
Specific activity (cpm/ng)	C_{DNA} conc.[b] ($M \times 10^6$)										
6.5×10^3	2.47	0.69	1.50	2.36	3.26	4.21	5.18	7.45	9.90	12.8	17.1	-
6.7×10^3	2.39	0.69	1.49	2.34	3.21	4.12	5.03	7.07	9.06	11.0	13.0	-
6.9×10^3	2.32	0.69	1.48	2.32	3.16	4.03	4.88	6.73	8.40	9.90	11.2	17.5
7.0×10^3	2.29	0.69	1.48	2.31	3.14	4.00	4.84	6.62	8.21	9.59	10.7	15.5
7.2×10^3	2.23	0.69	1.47	2.29	3.10	3.93	4.73	6.39	7.80	8.96	9.88	12.9
8.0×10^3	2.00	0.68	1.45	2.21	2.98	3.71	4.40	5.73	6.75	7.49	8.03	9.45

[a] C_{RNA} was determined by optical procedures and C_{hybrid} values were calculated from experimental hybrid activities and assigned specific activities.

[b] Concentrations of DNA were calculated on assigned specific activities.

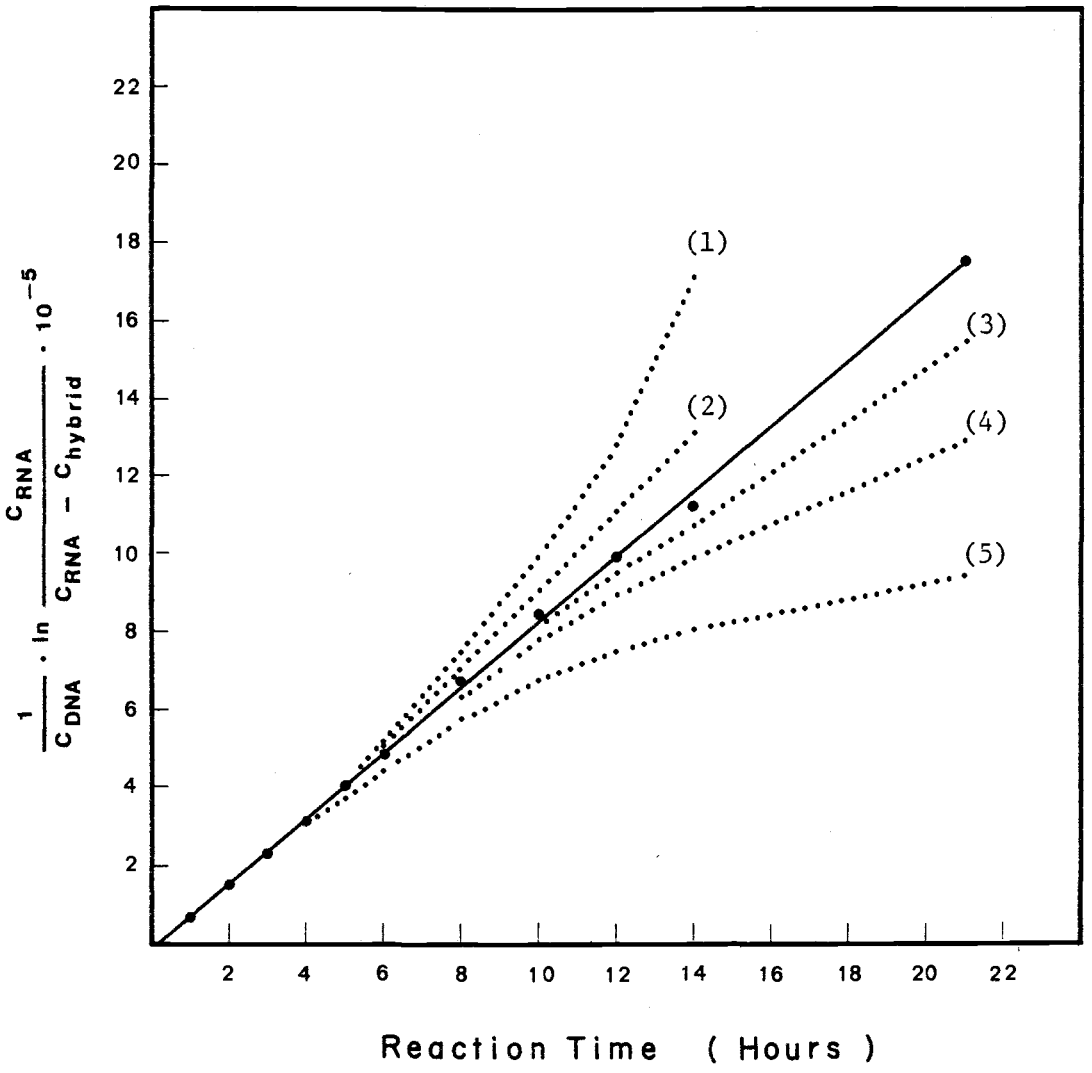


Figure 5. Analysis of the specific activity of the [^{3}H]cDNA probe. Data from figure 4 were calculated with different specific activities assigned to the cDNA as in Table 2. Value from Table 2 are plotted. Dotted lines are: (1) 6.5 X 10^3cpm/ng; (2) 6.7 X 10^3cpm/ng; (3) 7.0 X 10^3cpm/ng; (4) 7.2 X 10^3cpm/ng; and (5) 8.0 X 10^3cpm/ng. Solid line represents the linear plot obtained with a specific activity of 6.9 X 10^3cpm/ng.

plots because of the errors produced in the natural log term (C_{hybrid}) in the rate equation.

This specific activity of the [^{3}H]cDNA probe was determined using probe preparations derived from the whole virus genome but should be representative of any [^{3}H]cDNA probe sequences fractionated from the total and representing individual genes.

CHARACTERIZATION OF [^{3}H]cDNA PROBE

Endogenous reverse transcriptase reactions with detergent-disrupted AMV particles were used in the presence of actinomycin D to synthesize [^{3}H]-thymidine labeled complementary DNA. After fractionation on hydroxylapatite colum, about 70% of the [^{3}H] labeled product was obtained in the DNA-RNA hybrid form. Alkaline treatment would yield the desired single-stranded [^{3}H]cDNA which could be sedimented at about 6s to 10s in glycerol gradients, had specific activities in the range of 0.6×10^4 to 1.0×10^4 cpm per ng as determined by kinetic analysis, and was highly uneven in the representation of AMV RNA sequences due to transcription of viral RNA containing deleted regions. To improve this representation of viral sequences, the labeled cDNA was hybridized to purified 70s viral RNA in a 1:1 molar ratio. About 50% of the input [^{3}H]cDNA hybridized with the 70s RNA and recovered as purified single-stranded [^{3}H]cDNA probes.

To determine if this cDNA could be used effectively as a probe for our experiments it was necessary to demonstrate that: 1) it retained the ability to hybridize to RNA; 2) it contained most if not all of the

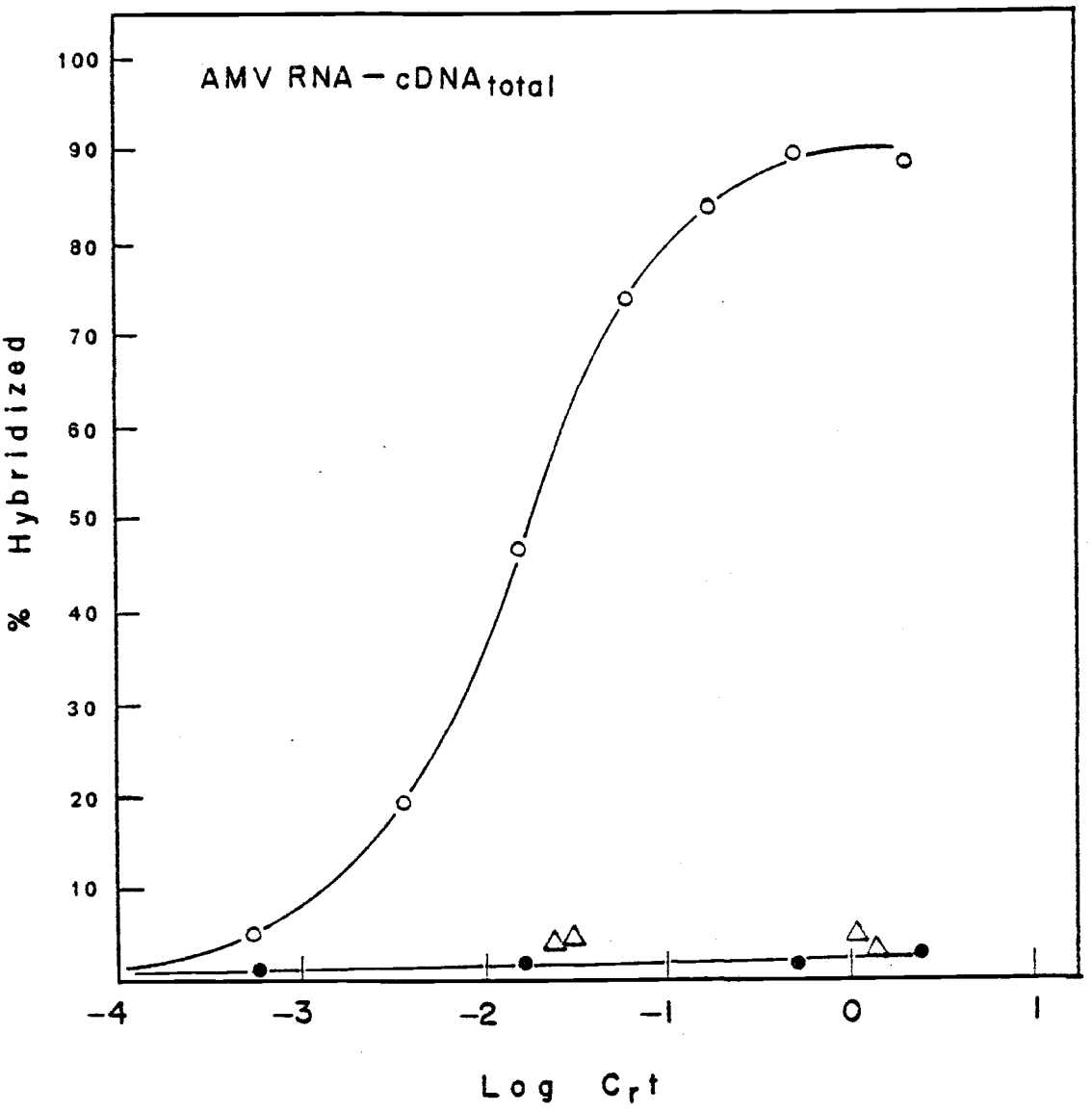


Figure 6. Kinetics of hybridizing excess AMV RNA to [^{3}H]cDNA. The reactions were carried out in 0.45M NaCl and 50% Formamide at 55°C (○). cDNAs isolated by hydroxylapatite with 0.2M phosphate buffer (△), and with 0.4M phosphate buffer (●) were also incubated under similar conditions but without AMV RNA.

sequences represented in the viral genome; and 3) the sequences it contained were in proportions similar to their content in 70s RNA.

To demonstrate hybridizing ability with AMV RNA, the cDNA probe was reacted with about 260 fold excess AMV RNA under relatively stringent hybridization conditions of 0.4 M NaCl, 48% formamide and 50° C. The reaction kinetics are shown in Fig. 6 as a plot of percent input cDNA hybridized to RNA against the value of log $C_r t$ where C_r is the molar concentration of AMV RNA and t is the reaction time in seconds. The data presented in Fig. 6 were not adjusted and represented actual experimental averages of at least triplicate results, all of which were within 3% to 5% of the average values. About 2% to 5% of the [^{3}H]cDNA probe consistently showed resistance to S_1 endonuclease digestion even in the absence of RNA or after boiling for up to eight min. However, this is a phenomenon widely observed and has been attributed to self-annealing (94). At a RNA concentration of about 9 µg per ml, the [^{3}H]cDNA reached a plateau value of about 90% in hybrid form after about eight to ten hr of reactions in sealed capillary tubes. When half of the cDNA had hybridized the half $C_r t$ showed a value of around 0.016 to 0.02 mole·sec/litre which corresponded to a relative genome complexity of about 2.56×10^6 to 3.2×10^6. These values were roughly consistent with values published under similar reaction conditions (95). The linear portion of this kinetic curve, when extrapolated to 0 and 100% hybridization, covers close to two logs of $C_r t$ which would have been the situation for an ideal second order reaction.

To determine if the cDNA probe contained most of the sequences in

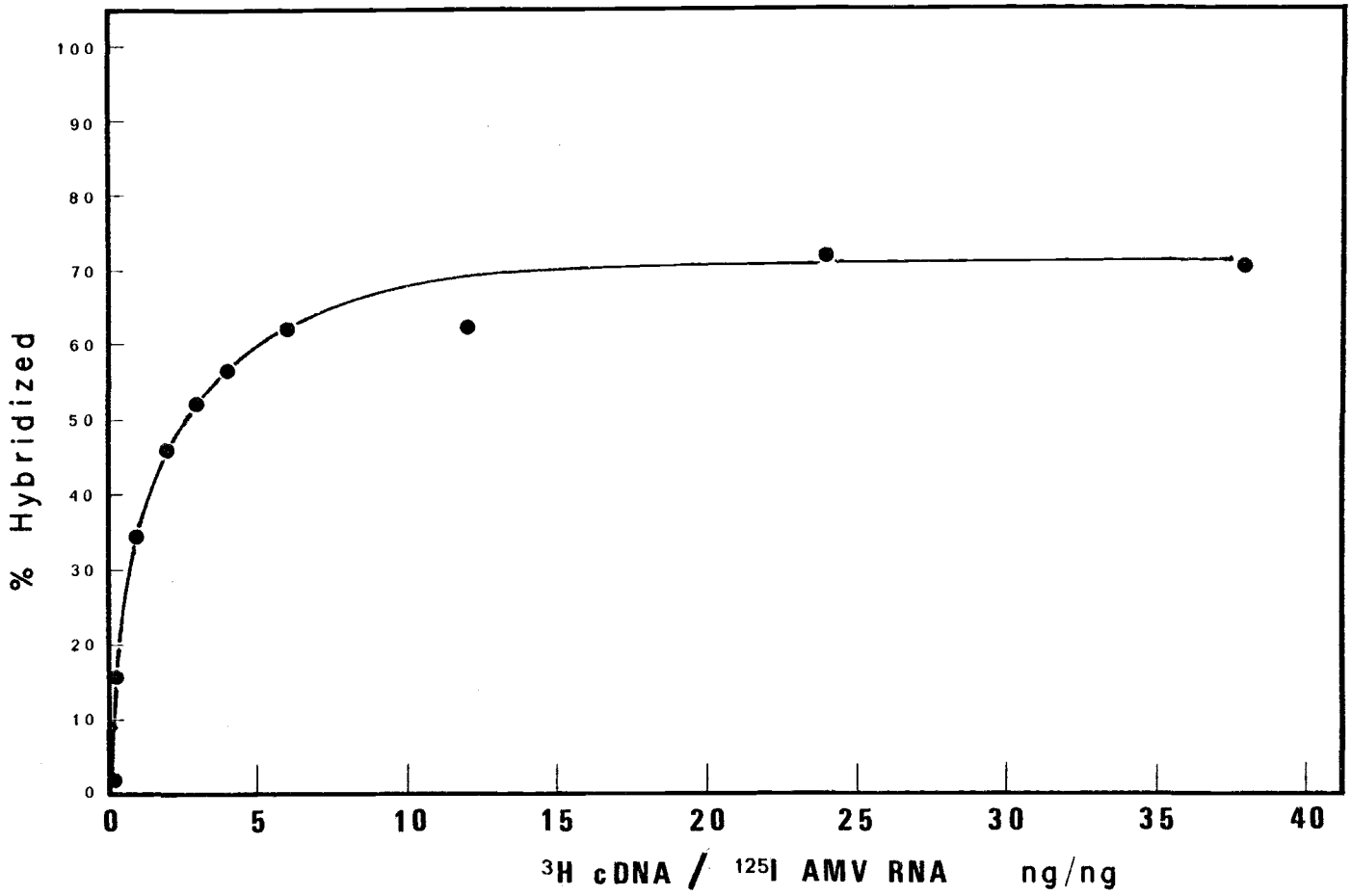


Figure 7. Hybridization of [^{125}I]RNA from AMV with increasing amounts of [^{3}H]cDNA. The mass ratio of [^{3}H]cDNA to [^{125}I]RNA is indicated on the x-axis. The percent of [^{125}I]-RNA protected from ribonuclease digestion at each of the cDNA:RNA ratio is indicated on the y-axis. All points represent saturation levels of hybridizations.

viral genome [^{3}H]cDNA was hybridized to [^{125}I] labeled AMV RNA at varying mass ratios ranging from below 0.5 to over 30. These protection reactions were performed under identical conditions and were carried out to a $C_o t$ value that allowed sufficient reaction time for maximum hybridization. Fig. 7 shows a plot of the maximum percent of input [^{125}I] RNA hybridized against various cDNA-RNA ratios. At a cDNA-RNA ratio of about 7.0 the percent of [^{125}I] RNA hybridized approached a plateau value of about 70% and any higher ratio did not improve significantly the protection of AMV RNA by the [^{3}H]cDNA probe. This result was comparable to published data (96,97,98) and indicated that the cDNA probe contained at least 70% of the viral sequences.

It has been reported that RNA preparations labeled by [^{125}I] to greater than 10^{7} cpm per μg retained only 75% acid precipitability of the isotope with complete loss of hybridization capacity after storage of ten weeks (99). This reduction in hybridizability appeared to be attributed to two main reasons: 1) the presence of iodine in position 5 suppressing the base property of the amine groups and consequently altering the pairing characteristics of the nucleotide (100); and 2) the fragmentation of the majority of the 70s AMV RNA by iodination into labeled products of 4s or less, rendering some of the labeled RNA too small to form hybrids. RNA fragmentation could be a result of several factors: 1) high termperature during iodination procedures; 2) chain scissions caused by molecular disintegration as a result of areas of high coulombic charges created by auger electrons produced during the decay of the isotope; and 3) deamination catalysed by the reducing

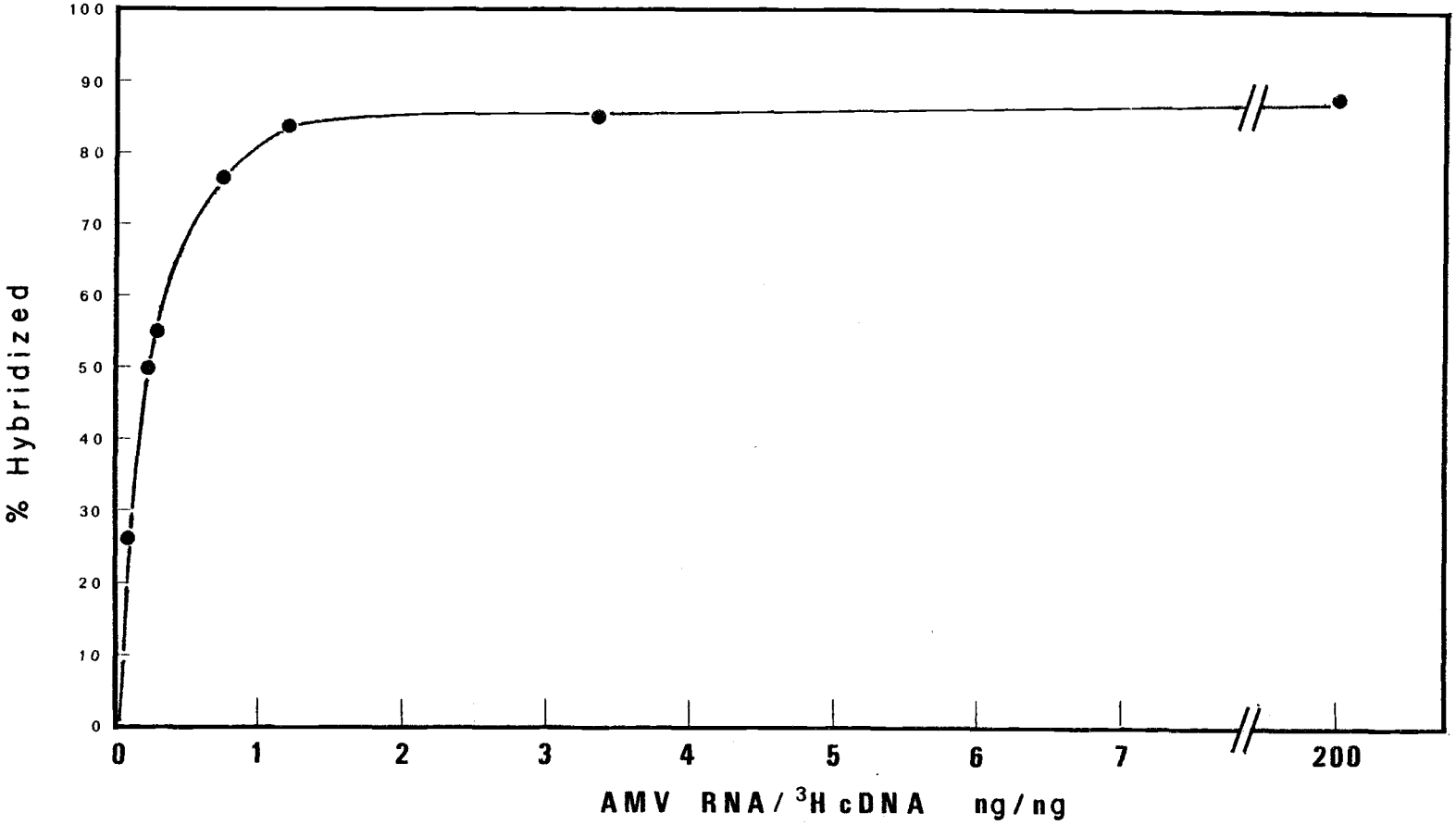


Figure 8. Hybridization of total [^{3}H]cDNA with increasing amounts of unlabeled AMV RNA. The mass ratios and percent hybridizations are presented as in Figure 7. The percent of [^{3}H]cDNA hybridized at each RNA:cDNA ratio was assayed by the fraction of cDNA resistant to S_1-nuclease digestion at saturation hybridization.

agent sodium bisulphite at a pH below 7 during iodination or as a contaminant after iodination (101). In any event, the less than complete protection of AMV RNA by cDNA probe appeared to be the result of iodination of the RNA rather than the lack of viral sequence representation in the probe. However, this problem was to be further resolved by the following experiment.

To further detect the complete representation of viral sequences in the cDNA probe and to test if these sequences were in proportions similar to those in the viral genome, purified 70s AMV RNA was hybridized to [^{3}H]cDNA at varying RNA:cDNA mass ratios in protection reactions similar to those described previously in [^{3}H]cDNA -[^{125}I]RNA hybridization. Figure 8 shows the plot of maximum percent of [^{3}H]cDNA hybridized against various RNA:cDNA ratios ranging from 0.1 to 200. At a RNA:cDNA ratio of about 1.5 the maximum percent of [^{3}H]cDNA in hybrid reached a plateau value of between 85% to 90%. This value was maintained even at a RNA:cDNA ratio of 200 and was consistent with results presented in Fig. 6. The fact that even at a RNA:cDNA ratio of just one, the [^{3}H]cDNA was protected by AMV RNA to over 80%. This indicated not only most of the viral sequences were present in the cDNA probe but that these sequences were indeed in the same proportions as in the viral genome RNA. If either of these two conditions were not true there would have been some over-abundant sequences in the probe that would require more than an equal molar amount of AMV RNA to protect, and the RNA:cDNA ratio would have to be much higher than one to reach the hybridization plateau.

The results of these experiments verified that the [^{3}H]cDNA probe was a nearly true complement to the viral genome RNA and that it can be used effectively in further hybridization experiments.

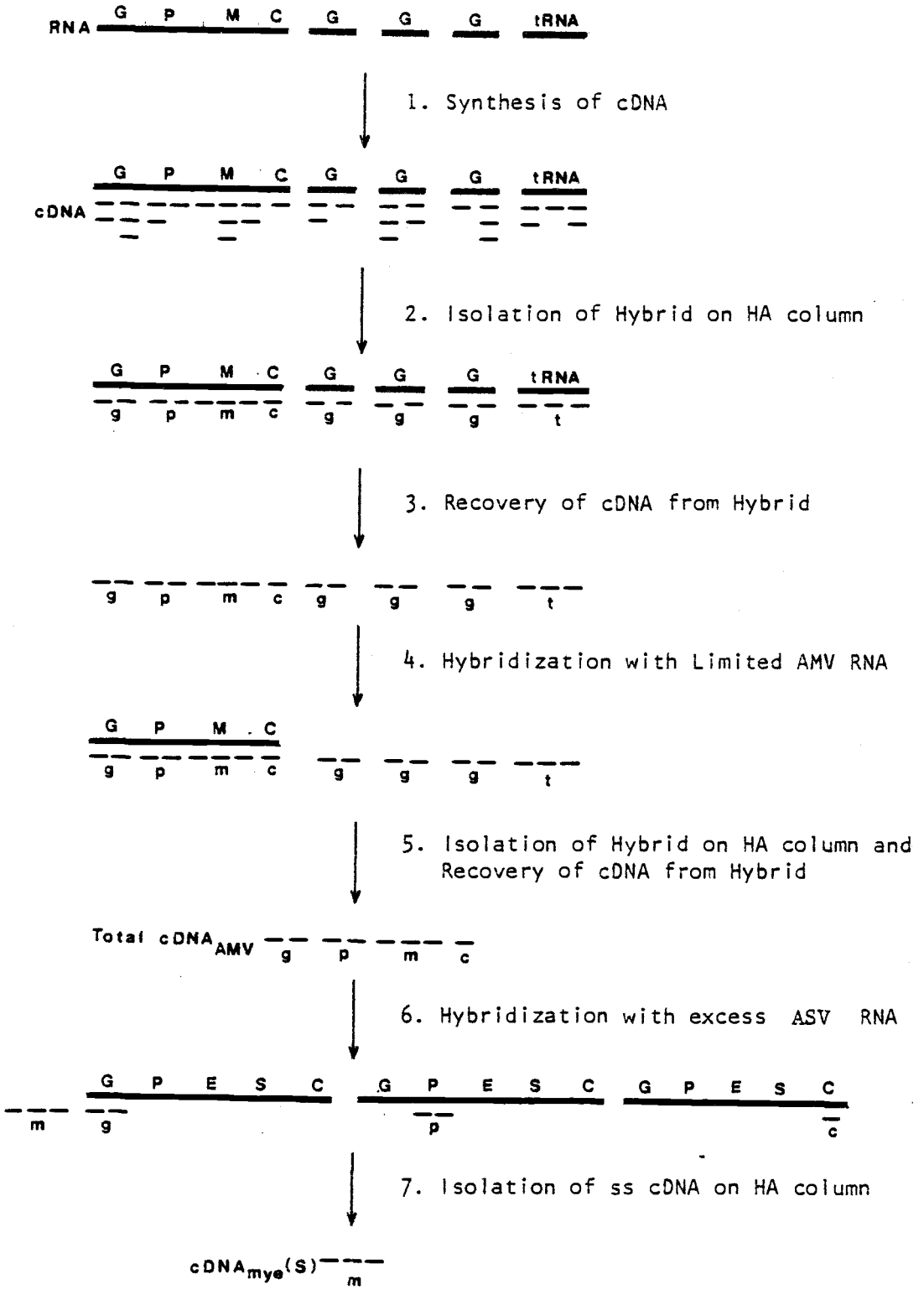
ISOLATION OF $cDNA_{mye}$(A) PROBES

General Strategy

$cDNA_{mye}$ probe was isolated from AMV RNA through hybridization with RAV-0 RNA (an endogenous virus) or RSV RNA (an exogenous virus) to remove sequences homologous to genomes of either endogenous viruses or exogenous viruses correspondingly. Those cDNAs that remained unannealed after hybridization should represent sequences unique to AMV. Where hybridization is carried out with RAV-0 RNA, the cDNA corresponding to the C-region of the viral genome will be included in this fraction.

Figure 9 illustrates the general strategy for the isolation with some step variations also being employed in the actual experiment as described later. Purified AMV particles contained a mixture of RNAs that included genome sized RNA molecules, defective RNAs (as indicated by the gag gene fragments in Fig. 9), tRNAs and quite possibly other host RNAs as well. When these RNAs were used as template for cDNA synthesis (step one) by endogenous reverse transcriptase reactions on detergent-disrupted viral particles, both desired but uneven and undesired cDNA molecules were obtained. The resultant cDNA included RNA-cDNA hybrids as well as single-stranded and double-stranded DNA molecules that needed to be separated on hydorxylapaptite column (step two).

Figure 9. Strategy for the preparation and isolation of $cDNA_{mye}(S)$. [^{3}H]cDNA complementary to the genome of AMV was synthesized with detergent disrupted virions (Step 1). cDNA molecules in association with virion RNA templates were selected by hydroxylapatite chromatography (Step 2). The cDNAs were then released from their templates by alkaline hydrolysis (Step 3) and rehybridized to limited amount of purified AMV RNA (Step 4). Those probes that hybridized to the AMV RNA were selected by hydroxylapatite chromatography and released from the AMV RNA by alkaline hydrolysis as before (Step 5). Such purified total cDNA (or $cDNA_{AMV}$) were fractionated by hybridizing to excess ASV RNA (Step 6) and isolated by hydroxylapatite (Step 7). The cDNA that did not anneal to ASV RNA were labeled as $cDNA_{mye}(S)$, and those that did anneal to ASV RNA were designated as $cDNA_{exo}(S)$. Abbreviations used to indicate different genetic regions are the same as those used in Figure 3. Since the env gene is considered to be deleted from AMV, it is not shown in the diagram, but the env portion in the cDNA complementary to MAV RNAs would have been removed by hybridizing to the ASV RNAs.

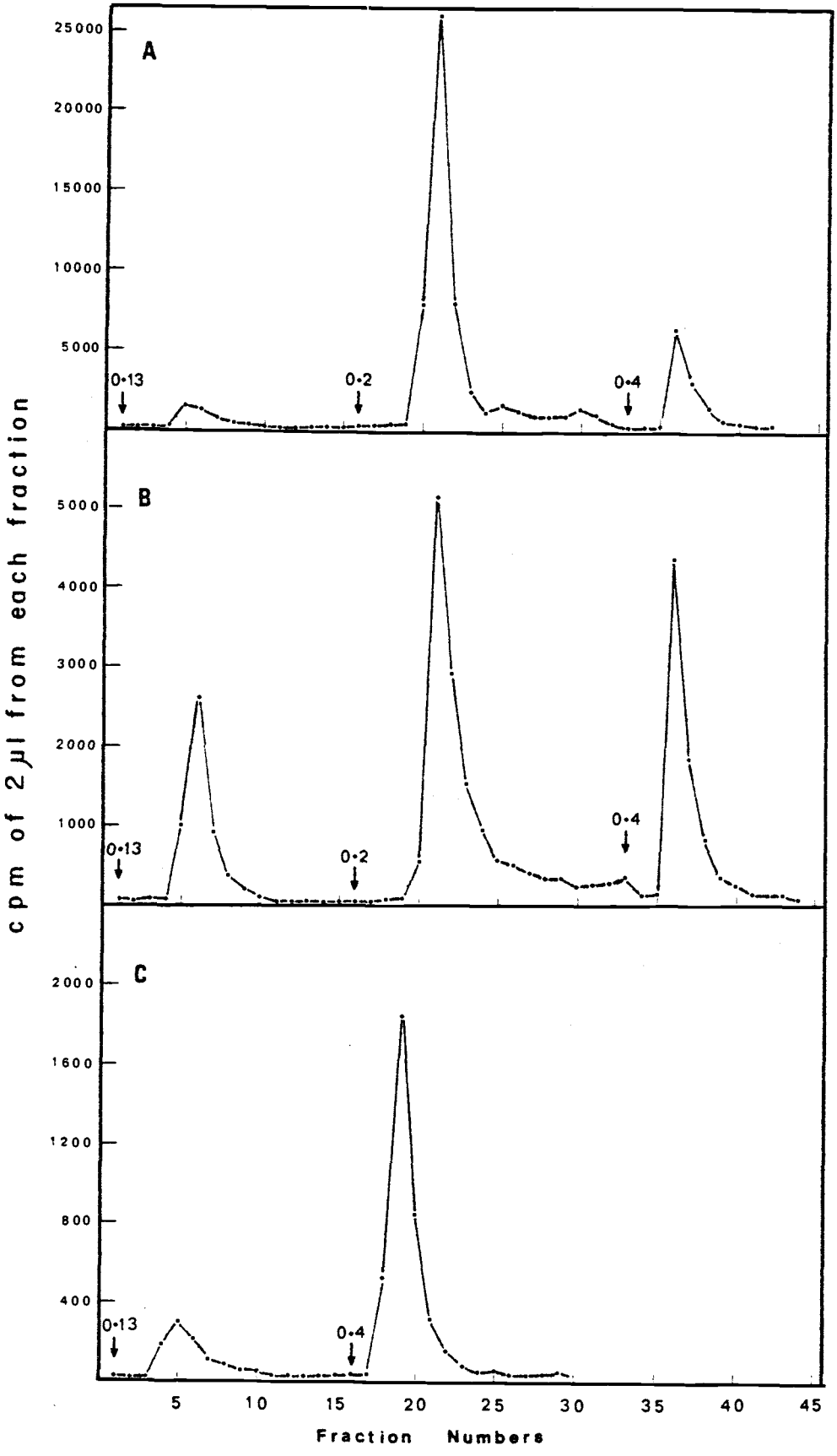

RNA
G P M C G G G tRNA
1. Synthesis of cDNA
cDNA
G P M C G G G tRNA
2. Isolation of Hybrid on HA column
G P M C G G G tRNA
g p m c g g g t
3. Recovery of cDNA from Hybrid
g p m c g g g t
4. Hybridization with Limited AMV RNA
G P M C
g p m c g g g t
5. Isolation of Hybrid on HA column and
Recovery of cDNA from Hybrid
Total cDNA AMV g p m c
6. Hybridization with excess ASV RNA
G P E S C G P E S C G P E S C
m g p c
7. Isolation of ss cDNA on HA column
cDNA mye (S) m

This mixture of nucleic acids, after being loaded onto a hydroxylapaptite column in 0.01 M phosphate buffer, was then eluted with a stepwise increase in molarities of the phosphate buffer. As shown in the elution profile in Fig. 10 panel A, single-stranded molecules were eluted with 0. 13 M phosphate buffer in the first peak, When 0.2 M phosphate buffer was added to the column a major second peak consisting of RNA-cDNA hybrids followed. The third peak containing double-stranded molecules was finally eluted with 0.4 M phosphate buffer.

The RNA-cDNA hybrids obtained in the 0.2 M phosphate peak from step two were pooled and recovered from the phosphate buffer through extensive dialysis and lyophilization. The strands of the hybrid molecules were separated by alkaline which also hydrolysed the RNA portion (step three). The recovered cDNA represented at approximately 1:1 ratio to the RNA sequences obtainable from viral particles, but not necessarily at the same ratio to 70s genome RNA, because of the presence of undesired sequences homologous to both host RNAs and deleted AMV RNAS. To remove these undesirable sequences, the cDNA was hybridized to a limited amount of purified 70s AMV RNA at a ratio of no more than one (step four).

The RNA-cDNA hybrids from step four were then separated from the unannealed cDNA by hydroxylapaptite chromatography as before (step five). The elution profile in Fig. 7 panel B, indicates that although the majority of the RNA-cDNA hybrids were eluted by 0.2 M phosphate buffer in the second peak, a large portion of these hybrids trailed behind and had to be eluted with either an excessive amount of 0.2 M

Figure 10. Fractionation of cDNA by hydroxylapatite chromatography. Nucleic acids were loaded onto the hydroxylapatite column in 0.01M phosphate buffer at 50°C, and then were eluted stepwisely by 0.13M, 0.2M, and 0.4M phosphate buffers as indicated by the arrows in the figure. 4 ml fractions were collected and their radioactivities were monitored by 2µl aliquots. Samples loaded at each panel: A. total nucleic acids after endogenous reverse transcriptase reaction; B. cDNA isolated from the 0.2M phosphate peak in A and hybridized to limited AMV RNA; C. cDNA isolated from 0.2M phosphate peak in B and hybridized to excess of ASV RNA. cDNA isolated from 0.13M phosphate peak in C corresponded to $cDNA_{mye}(S)$, and cDNA isolated from 0.4M phosphate peak corresponded to $cDNA_{exo}(S)$.


A
B
C
0·13
0·2
0·4
cpm of 2µl from each fraction
Fraction Numbers
25000
20000
15000
10000
5000
4000
3000
2000
1000
1600
1200
800
400
5
10
15
20
25
30
35
40
45

phosphate buffer or 0.4 M phosphate buffer as a third peak. When the cDNA, recovered from this 0.4 M phosphate, was subjected to self-annealing tests it showed no greater amount of self-annealing than those cDNA recovered from the 0.2 M phosphate fraction (Fig. 6). Therefore, the cDNA from the third peak could not have been double-stranded. Such false peaks have been explained by G. Bernardi (102) as the result of substances having strongly curved adsorption isotherms, thereby extending their elution range and therefore could not be eluted by a solvent of constant composition without trailing. Each new molarity step of the eluent would release an additional amount of the same substance. Such an adsorption isotherm could be due to the different GC and AT contents of the cDNA fragments. Nevertheless, the cDNA recovered from step five represented roughly 1:1 ratio to sequences in the 70s genome RNA as discussed earlier in figures 7 and 8.

To isolate cDNA sequences unique to AMV, cDNA isolated from step five was hybridized to at least ten-fold excess of either RAV-0 RNA or ASV RNA (step six). Figure 9 describes the hybridization with ASV RNA, and the cDNA that remained unannealed after hybridization represented sequences absent in exogenous viruses as indicated by the m region (for myeloblastosis) in the figure (94). These cDNA regions were subsequently designated as $cDNA_{mye}(S)$ (sarcoma virus isolated).

When RAV-0 RNA was hybridized in excess to cDNA from step five the m region and common c region of the cDNA remained unannealed, representing sequences in the AMV genome that were not shared by endogenous viruses and was designated as $cDNA_{mye}(A)$ for associated virus

isolated.

Both $cDNA_{mye}$(A) and $cDNA_{mye}$(S) were separated from annealed cDNA in step six by a third hydroxylapatite chromatography (step seven) by elution with 0.13 M phosphate buffers as single strands (Fig. 10 panel C). Those cDNA that remained annealed to viral RNA molecules were then eluted with 0.4 M phosphate buffer to eliminate trailing peaks and subsequently recovered as structural cDNA representing AMV sequences that were shared by either endogenous or exogenous viruses.

Because of the extensive manipulation involved in each isolation step, samples of the cDNA recovered from each hydroxylapatite procedure has been tested for their fidelity by hybridizing to AMV RNA before the start of a next step. One variation from this general procedure was to reverse steps six and seven in Fig. 9 with steps four and five, thereby isolating the $cDNA_{mye}$ first before purifying them through hybridization with limited amounts of AMV RNA. This variation had the advantage of conserving AMV RNA in the purification step but risked the loss of a large part of the $cDNA_{mye}$ isolated. Another variation called for an additional purification step by hybridization with a limited amount of AMV RNA after the $cDNA_{mye}$ had been isolated by the general procedure to ensure the fidelity of the $cDNA_{mye}$. However, neither variation was effective in improving the specificities of the $cDNA_{mye}$ isolated by the general procedure and therefore deemed unnecessary.

Table 3 lists examples of the percent yield for the desired products obtained in each procedure during the isolation of $cDNA_{mye}$(S).

TABLE 3. cDNA YIELD FROM HYDROXYLAPATITE CHROMATOGRAPHY AT EACH STEP OF ISOLATION FOR $cDNA_{mye}(S)$

Procedural steps and materials to be chromatographed	Starting cDNA (cpm)	cDNA separated on HAP (cpm) as:			Recovered cDNA (%)	Cumulative recovered cDNA (%)
		ss	Hybrid	ds		
1) Total cDNA synthesized by endogenous reverse transcriptase	1.47×10^8	9.30×10^6	1.03×10^8	2.56×10^7	70	70
2) cDNA from hybrid in step 1 hybridized to limited amount of AMV RNA	5.86×10^7	1.07×10^7	2.86×10^7	1.73×10^7	48.8	34.2
3) cDNA from hybrid in step 2 hybridized to excess amount of RSV RNA	6.35×10^6	1.52×10^6	5.15×10^6	-	23.9	8.17
4) $cDNA_{mye}(S)$ hybridized[1] to limited amount of AMV RNA for repurification	2.25×10^6	1.36×10^6	1.04×10^6	-	46	-

[1] $cDNA_{mye}(S)$ were obtained from another set of experiments in which an additional step of purification had been employed.

The listed yields were for cDNA directly eluted from hydroxylapatite columns. Subsequent recovery and purification procedures rendered the actual yield in each step less than those listed. The final $cDNA_{mye}$ product was usually obtained as about 5% of the starting labeled probes.

SPECIFICITY OF $cDNA_{mye}$(A)

The specificity of $cDNA_{mye}$(A) was examined by kinetic hybridizations with vast excess of 70s AMV RNA (Fig. 11) and with vast excess of 70s RAV-0 RNA (Fig. 12). Theoretically, if the driver viral RNA is in vast excess its concentrations should dictate the rate of reaction regardless of the concentration and complexity of the tracer molecules. A comparison between Fig. 11 and Fig. 12 deomonstrates that indeed both $cDNA_{mye}$(A) (Fig. 11) and total [^{3}H]cDNA AMV (Fig. 6) show very similar hybridization kinetics when AMV RNA was used in excess in both cases. The fact that at a C_rt value of about 1 mole·sec/litre, 90% of both $cDNA_{mye}$(A) and total purified [^{3}H]cDNA were hybridized to AMV RNA indicated that for all practical purposes, the entire sequences in $cDNA_{mye}$(A) had retained their ability to hybridize to viral counterparts as well as total cDNA despite all the isolation procedures. This was an important character for the $cDNA_{mye}$(A) which was used later as a probe to detect viral sequences in cellular DNA.

Figure 12 shows that when over 50% of the total purified AMV cDNA hybridized to an excess of RAV-o RNA, practically none of the $cDNA_{mye}$(A) had any homology with RAV-0 RNA. This indicates that our purpose of removing endogenous sequences from the total purified AMV[^{3}H]cDNA had

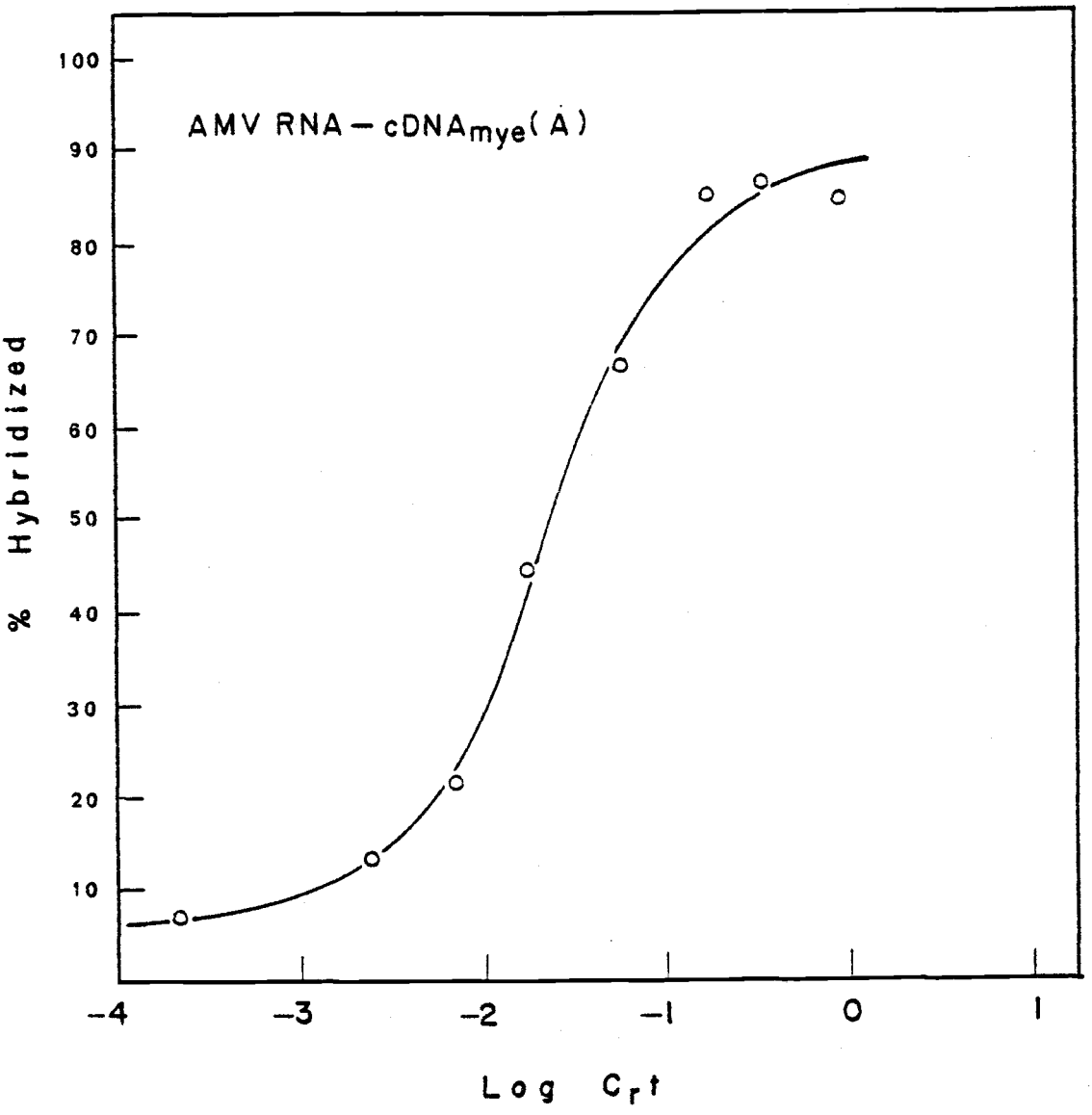


Figure 11. Kinetics of hybridizing $cDNA_{mye}$(A) to excess unlabeled AMV RNA. Reaction conditions were the same as in Figure 6. Percent hybridization was determined by the fraction of $cDNA_{mye}$(A) resistant to S_1-nuclease digestion. C_rt is in $mole.sec.litre^{-1}$.

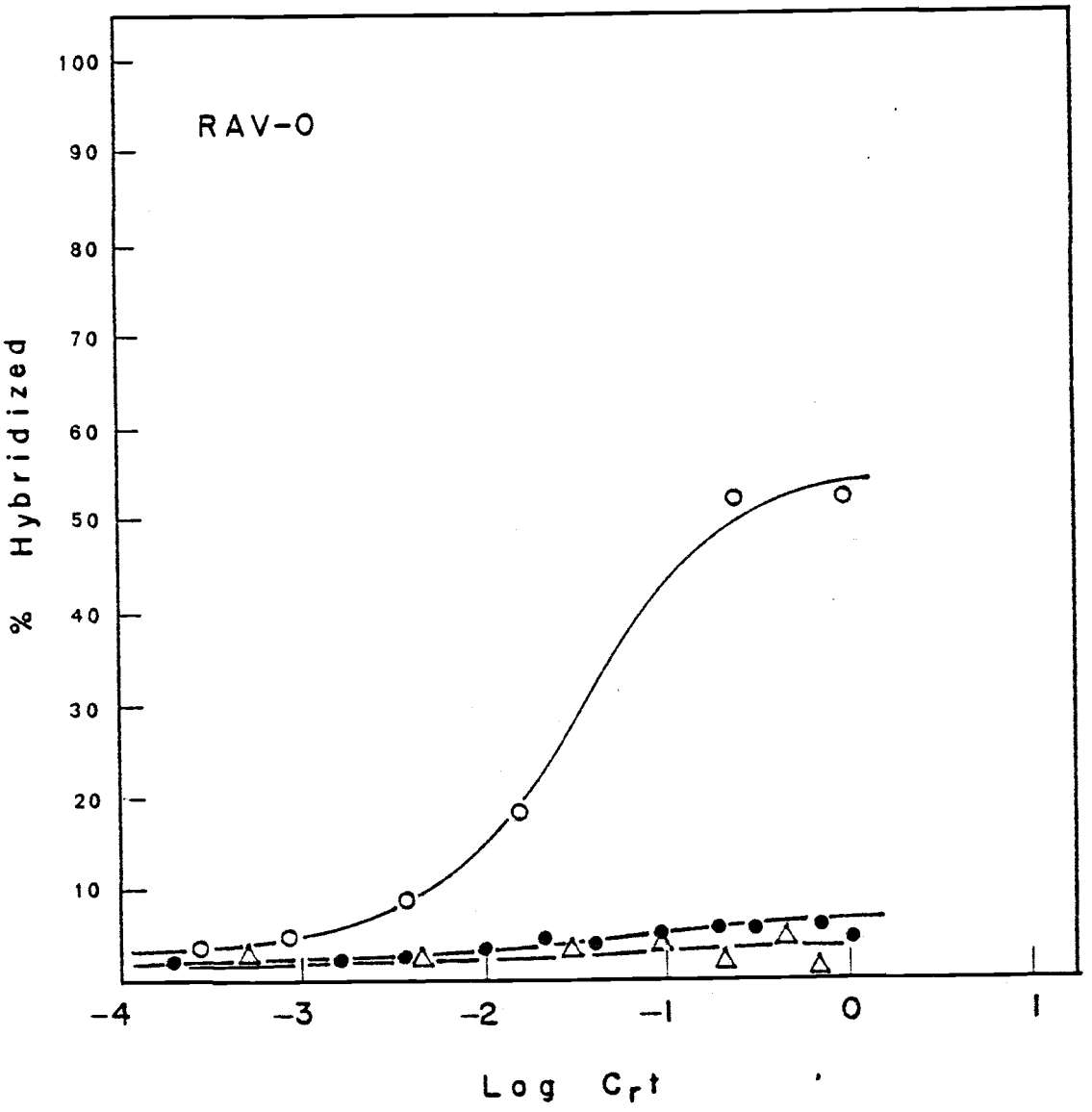


Figure 12. Kinetics of hybridizing excess RAV-0 RNA to various fractionated cDNA probes. (○) total $cDNA_{AMV}$; (●) $cDNA_{mye}$(A); (△) $cDNA_{mye}$(S). Reaction conditions and assaying were the same as Figure 6 and Figure 11.

been accomplished by the procedure employed and that the $cDNA_{mye}(A)$ probe was indeed devoid of sequences homologous to RAV-0 genome RNA.

RELATIONSHIP BETWEEN $cDNA_{mye}(A)$ AND EXOGENOUS VIRAL RNA

Although $cDNA_{mye}(A)$ has been proven to lack nucleotide sequences homologous to RNA of an endogenous RAV-0 virus, it nevertheless might still harbor sequences homologous to the common c region generally believed to be present in the genome RNA of all exogenous avian oncornaviruses (94). To obtain a $cDNA_{mye}$ probe that was truly unique to AMV, it was necessary to remove this c region from cDNA through hybridization with RNA from an exogenous virus other than AMV. However, in order to justify the elaborate isolation procedures, it was important to prove beyond a reasonable doubt that first, the $cDNA_{mye}(A)$ did indeed contain sequences hybridizable to RNA from other exogenous viruses; and second, there would be a sufficient proportion of the cDNA remaining unannealed to facilitate isolation after hybridization with an exogenous viral RNA other than those from AMV.

To verify the above two points, a series of residual hybridizations were performed. In the first reaction, $cDNA_{mye}(A)$ was hybridized with an excessive amount of 35s RSV RNA to a saturation C_rt of about 1 mole•sec/litre. Then an excessive amount of AMV RNA was added to the same reaction mixture so as to hybridize with whatever $cDNA_{mye}(A)$ sequences that remained unannealed after initially reacting with RSV RNA. Figure 13 shows that in the initial reaction with RSV RNA about 30% of

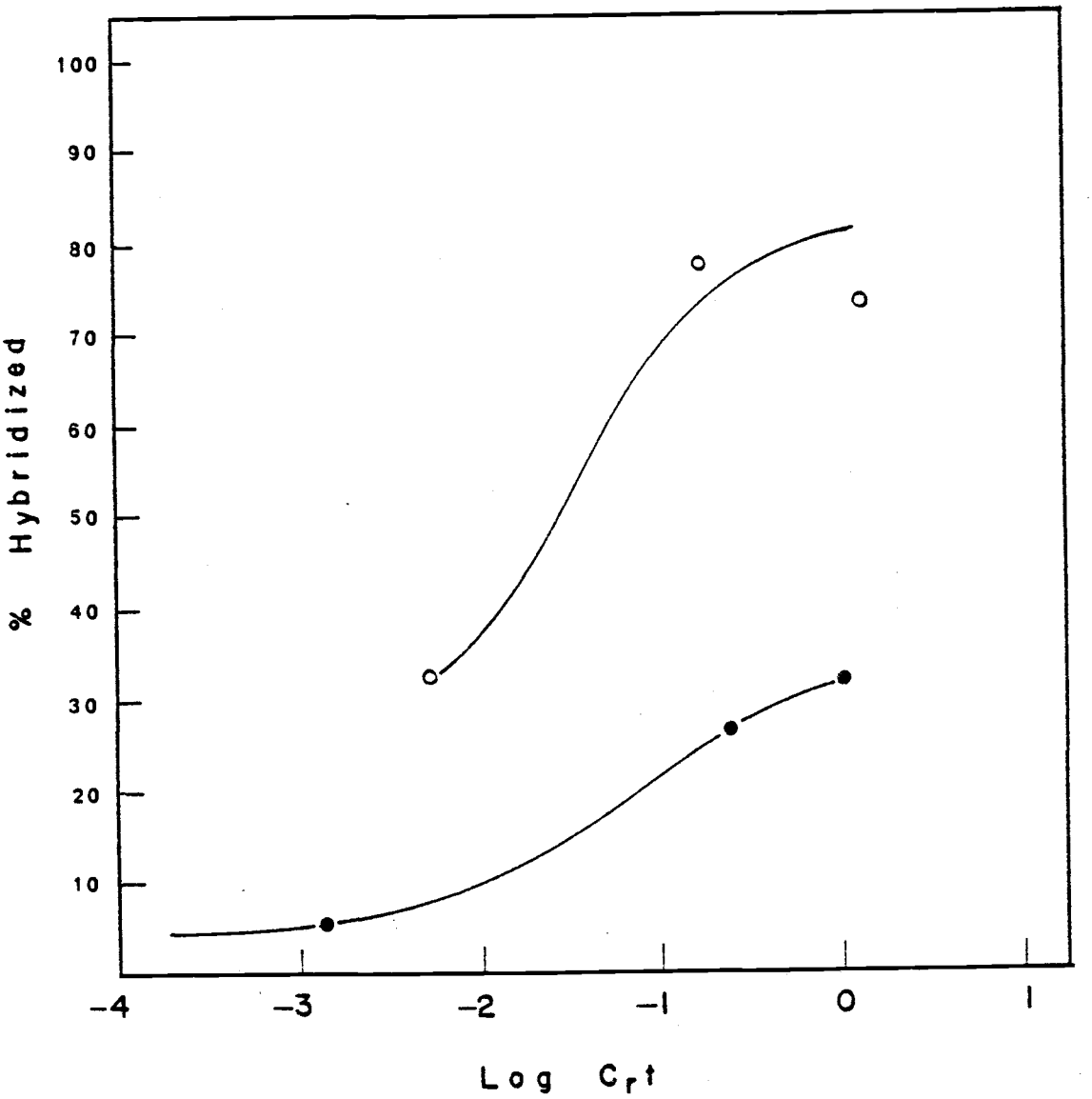


Figure 13. Kinetics of hybridizing excess AMV RNA to residual $cDNA_{mye}$(A) after hybridization to ASV RNA. $cDNA_{mye}$(A) was initially hybridized to saturation with excess Pr-ASV RNA (●). Residual $cDNA_{mye}$(A) was hybridized to excess AMV RNA (○).

the probe hybridized, indicating that there was indeed $cDNA_{mye}(A)$ sequences homologous to RSV RNA. In the residual reaction with added AMV RNA an additional 50% of the $cDNA_{mye}(A)$ hybridized at a new saturation. This clearly demonstrated that there were significant amounts of AMV sequences left in the $cDNA_{mye}(A)$ probe that would not be represented in the RSV RNA and that these sequences could be isolated by our procedure.

To examine the specificity of the region in $cDNA_{mye}(A)$ that hybridized with RSV RNA, a second experiment was performed in which $cDNA_{mye}(A)$ was again hybridized to excess RSV RNA to saturation in an initial reaction. Then excess MC29 RNA was added to the reaction to hybridize with residual probe to a new saturation C_rt value. Figure 14 shows that when 30% of the $cDNA_{mye}(A)$ again hybridized with RSV RNA, practically none of the residual probe reacted with MC29 at all. To show that this result was not due to the inability of MC29 RNA to react with the probe, a reverse experiment was performed. $cDNA_{mye}(A)$ was initially hybridized to excess MC29 RNA, followed by an addition of excess RSV RNA to the reaction mixture. Figure 15 shows that MC29 did hybridize with at least 30% of the $cDNA_{mye}(A)$ sequences at saturation and that addition of RSV RNA did not increase the percentage of probe hybridized. This proved that the portion of $cDNA_{mye}(A)$ that hybridized to RNA of one exogenous virus (RSV) also hybridized with the RNA of at least one other exogenous virus (MC29).

In another experiment, $cDNA_{mye}(A)$ was hybridized to excess RSV RNA in the initial reaction. But the residual reaction was performed by

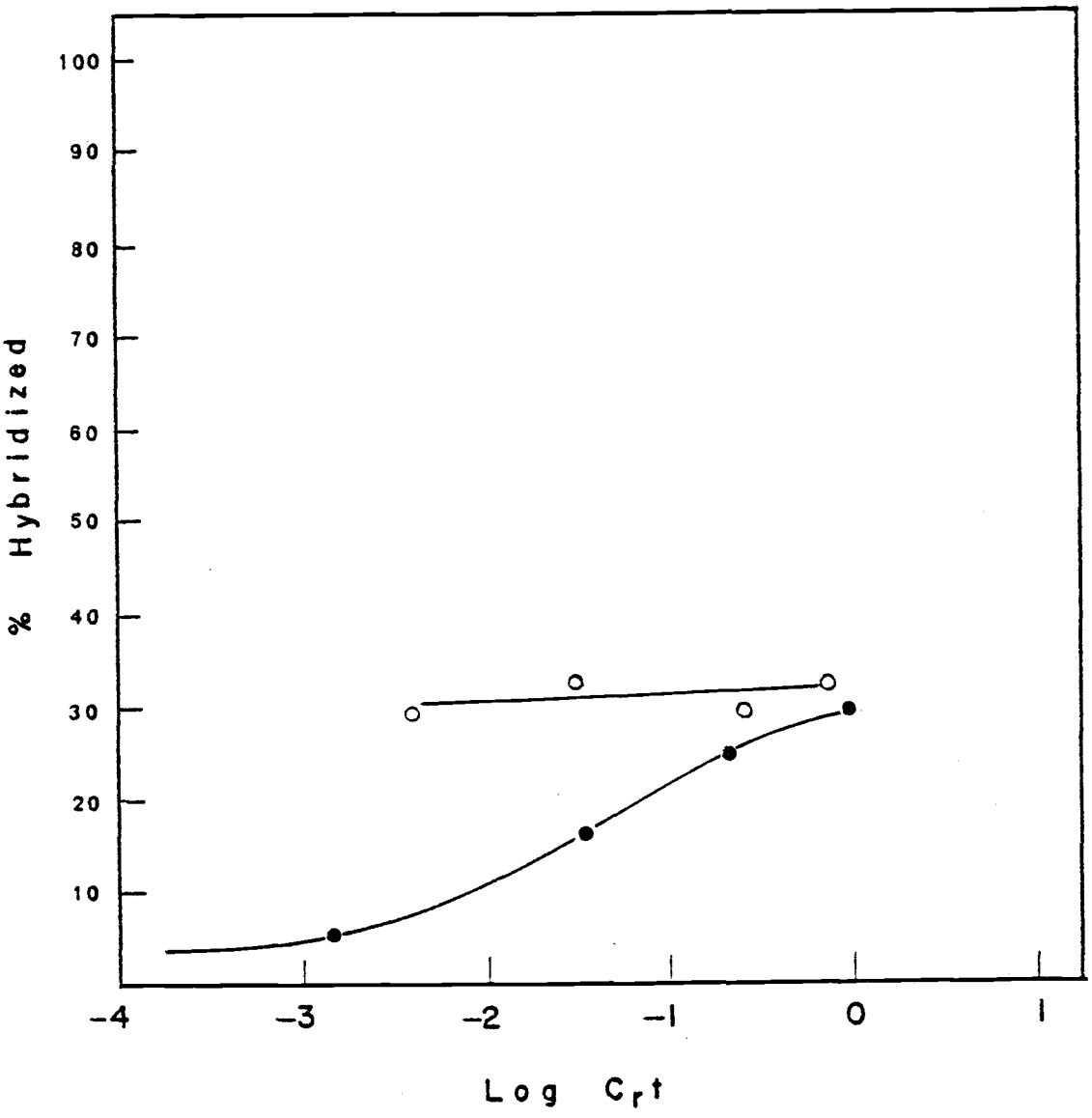


Figure 14. Kinetics of hybridizing excess MC29 RNA to residual $cDNA_{mye}$(A) after hybridization to ASV RNA. $cDNA_{mye}$(A) was initially hybridized to saturation with excess Pr- ASV RNA (●). Residual $cDNA_{mye}$(A) was hybridized to excess MC29 RNA (O).

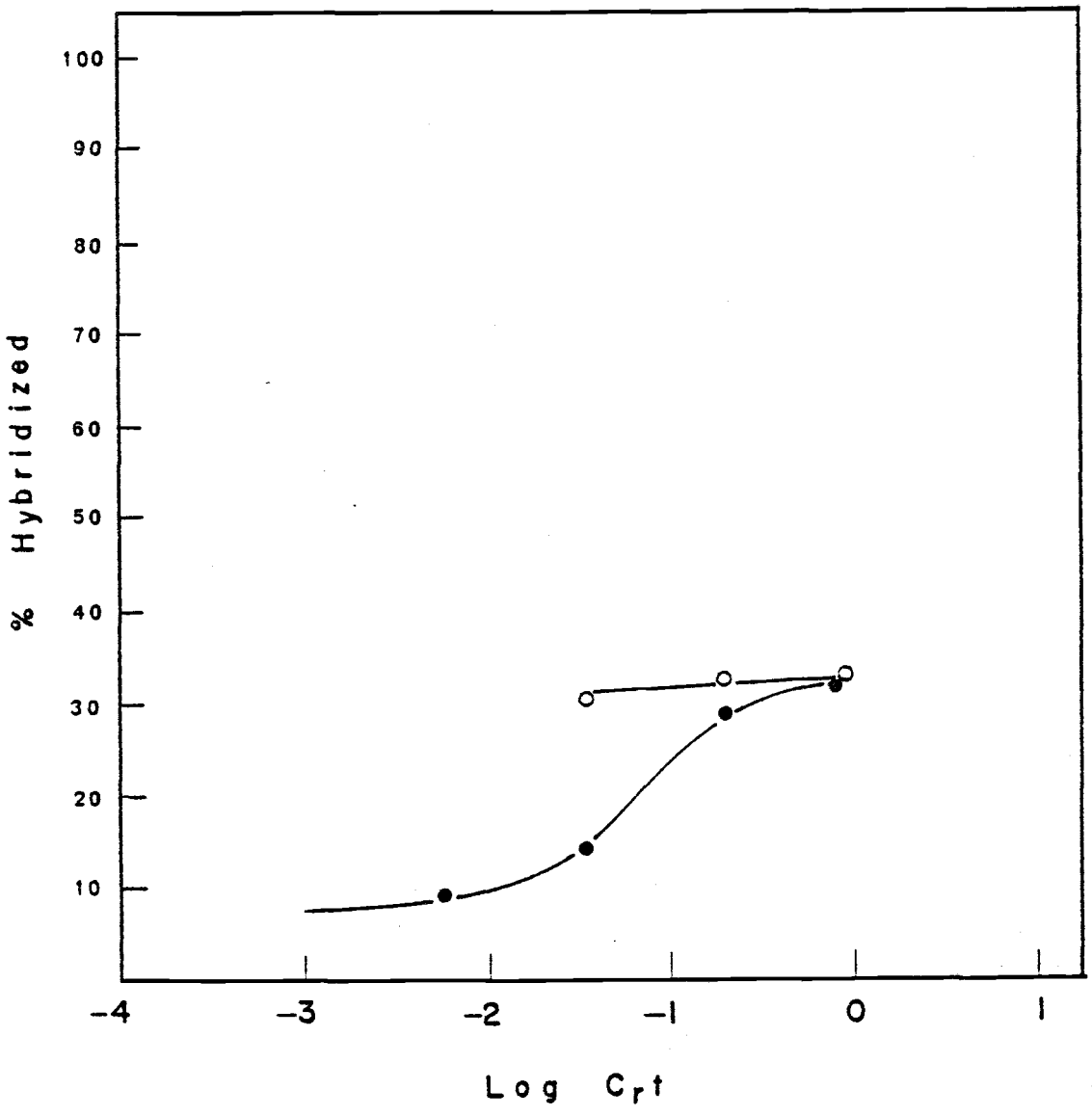


Figure 15. Kinetics of hybridizing excess ASV RNA to residual $cDNA_{mye}$(A) after hybridization to MC29 RNA. $cDNA_{mye}$(A) was initially hybridized to saturation with excess MC29 RNA (●). Residual $cDNA_{mye}$(A) was then hybridized to excess Pr-ASV RNA (○).

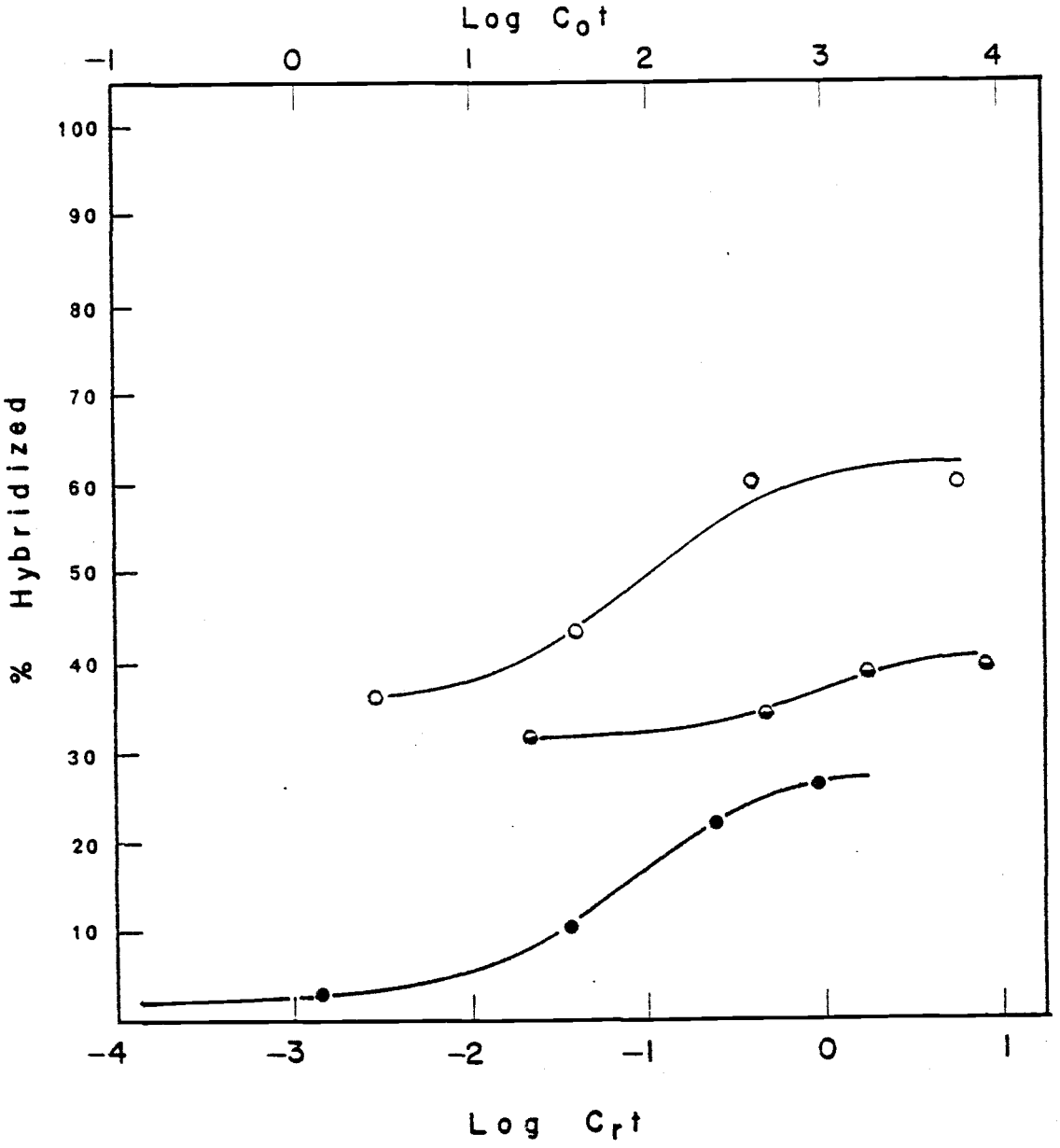


Figure 16. Kinetics of hybridizing cellular DNAs to residual $cDNA_{mye}$(A) after hybridization to ASV RNA. $cDNA_{mye}$(A) was initially hybridized to saturation with excess Pr-ASV RNA (●). Residual $cDNA_{mye}$(A) was then hybridized to cellular DNAs from either infected myeloblasts (O), or from uninfected normal erythrocytes in (◒) in chicken.

the addition of either excess myeloblast DNA or excess normal chicken erythrocyte DNA to the reaction mixture. Figure 16 illustrates that after 30% of the probe has reacted with RSV RNA, an addition of normal erythrocyte DNA increased the amount of hybridized probe by only about 10%, whereas, when myeloblast DNA was added to the residual probe, an additional 35% of the $cDNA_{mye}$(A) hybridized. This was an additional evidence to show that a $cDNA_{mye}$ probe which would be isolated by hybridization with RSV RNA could be used effectively to detect viral sequences in cellular nucleic acids.

Table 4 summarizes the results from Fig. 10 to Fig. 13 for the series of residual hybridizations.

TABLE 4. HYBRIDIZATION OF $cDNA_{mye}$(A) TO REACTANTS IN RESIDUAL EXPERIMENTS

Reactants and % hybridization in initial reaction	% of $cDNA_{mye}$(A) hybridized				
	AMV RNA	RSV RNA	MC29 RNA	Normal RBC DNA	Myeloblast DNA
RSV RNA 30%	80	-	30	40	65
MC29 RNA 30%	-	30	-	-	-

SPECIFICITY OF $cDNA_{mye}$(S)

The specificity of $cDNA_{mye}$(S) was examined by kinetic hybridizations with at least a 200-fold excess of AMV RNA (Fig. 17), RSV RNA

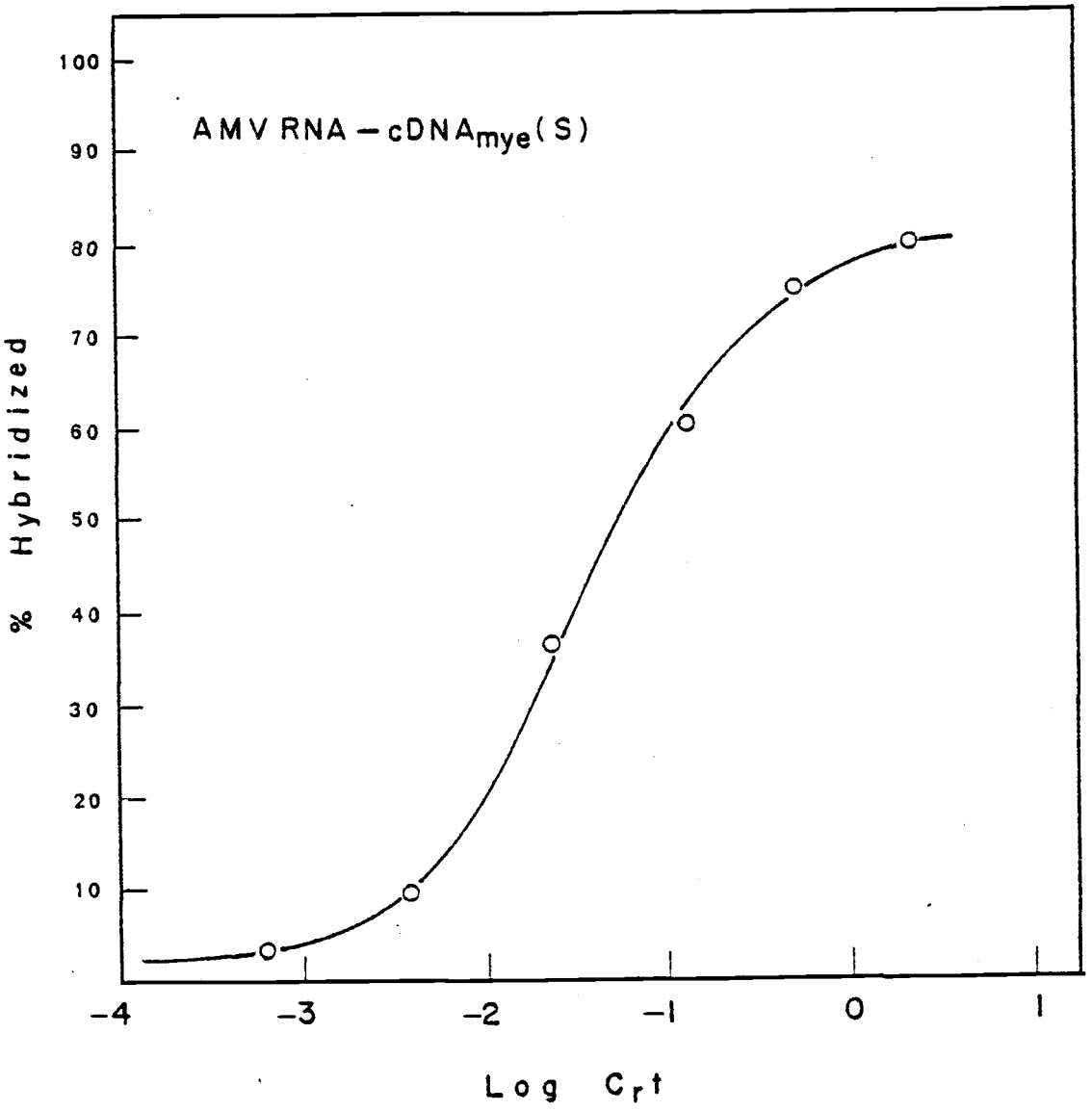


Figure 17. Kinetics of hybridizing $cDNA_{mye}$(S) with excess AMV RNA. Reaction and assay conditions were the same as in Figure 6 and in Figure 11.

(Fig. 18), and RAV-0 RNA (Fig. 12). As has been previously described in the case of $cDNA_{mye}(A)$, the hybridization kinetics of $cDNA_{mye}(S)$ and of total [^{3}H]cDNA should be identical when both were reacted to the same vast excess of driver AMV RNA. A comparison between Fig. 3 and 14 indicates that the two hybridization kinetic curves were very similar as expected and that over 80% of the $cDNA_{mye}(S)$ were hybridizable to AMV RNA at saturation.

Figure 18 shows the kinetic hybridizations of RSV RNA to total [^{3}H]cDNA, to $cDNA_{mye}(S)$, and $cDNA_{mye}(A)$. At saturation, 65% of the total cDNA was hybridized. Since the maximum percentage hybridization that could be experimentally obtained between completely homologous sequences was only about 90-95%, the 65% hybridized total cDNA therefore corresponded to a sequence homology between RSV and AMV of around 70% which was comparable to the generally accepted figure. When $cDNA_{mye}(A)$ hybridized to at least 30% with RSV RNA (as shown in Fig. 13), $cDNA_{mye}(S)$ showed no sequence homology with RSV RNA at all. This demonstrated that in the isolation of $cDNA_{mye}(S)$, the region in cDNA that was homologous to exogenous viral RNA had been removed.

To verify that $cDNA_{mye}(S)$ did not contain any sequence homologous to an endogenous viral RNA, the probe was reacted with excess RAV-0 RNA. Figure 12 shows that RAV-0 RNA was unable to hybridize $cDNA_{mye}(S)$ to any extent.

To prove that $cDNA_{mye}(S)$ truly represented sequences unique to AMV, the probe was hybridized to RNAs from three different viruses, namely MC29, AEV and SR-D RSV, each was believed to belong to its own

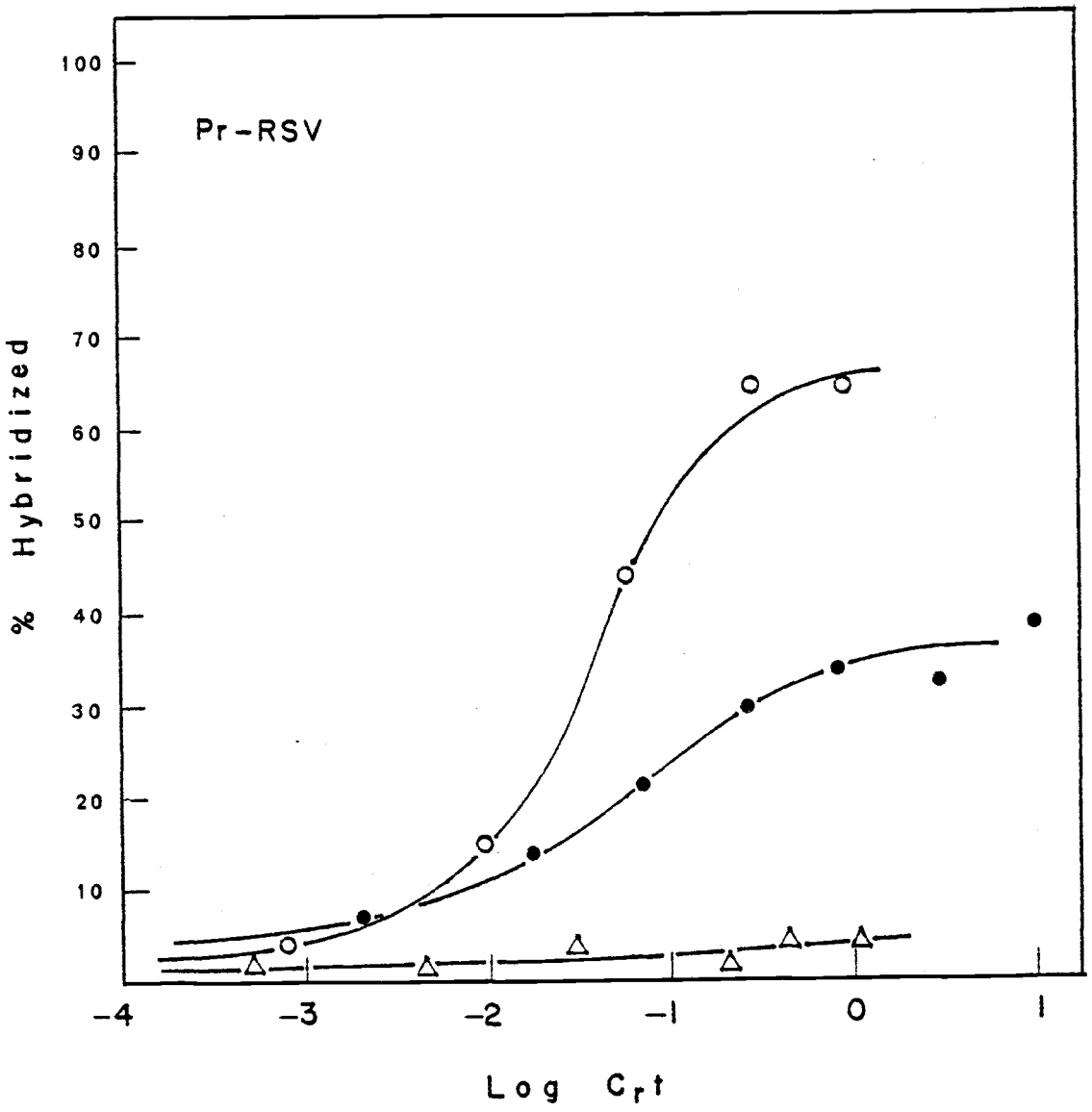


Figure 18. Kinetics of hybridizing excess Pr-ASV RNA to various fractionated cDNA probes. (O) total $cDNA_{AMV}$; (●) $cDNA_{mye}$(A); (△) $cDNA_{mye}$(S). Reaction and assay conditions were the same as in Figure 6 and Figure 11.

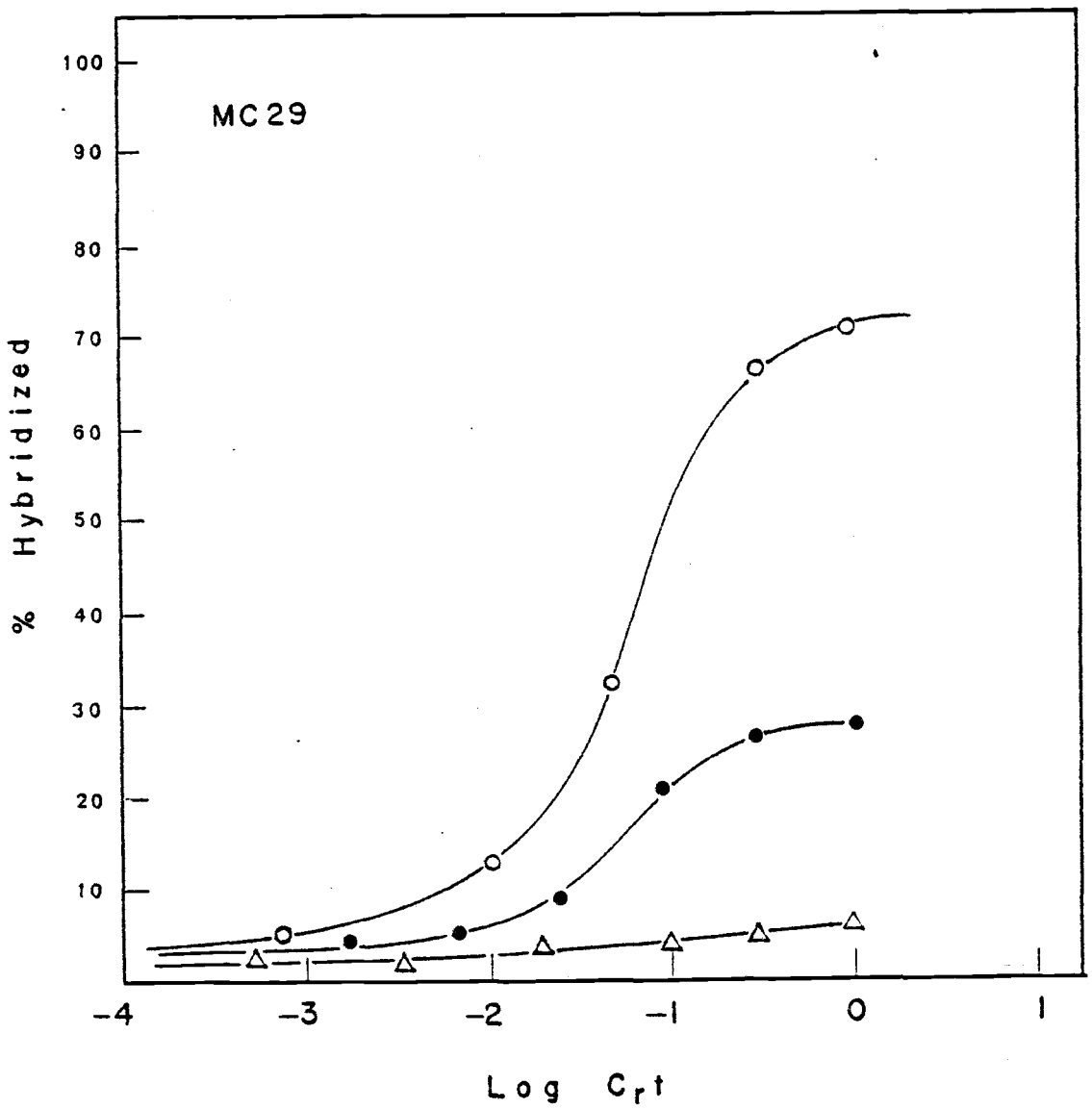


Figure 19. Kinetics of hybridizing excess MC29 RNA to various fractionated cDNA probes. (○) total unfractionated $cDNA_{AMV}$; (●) $cDNA_{mye}$(A); (△) $cDNA_{mye}$(S). Reaction and assay conditions were the same as in Figure 6 and Figure 11.

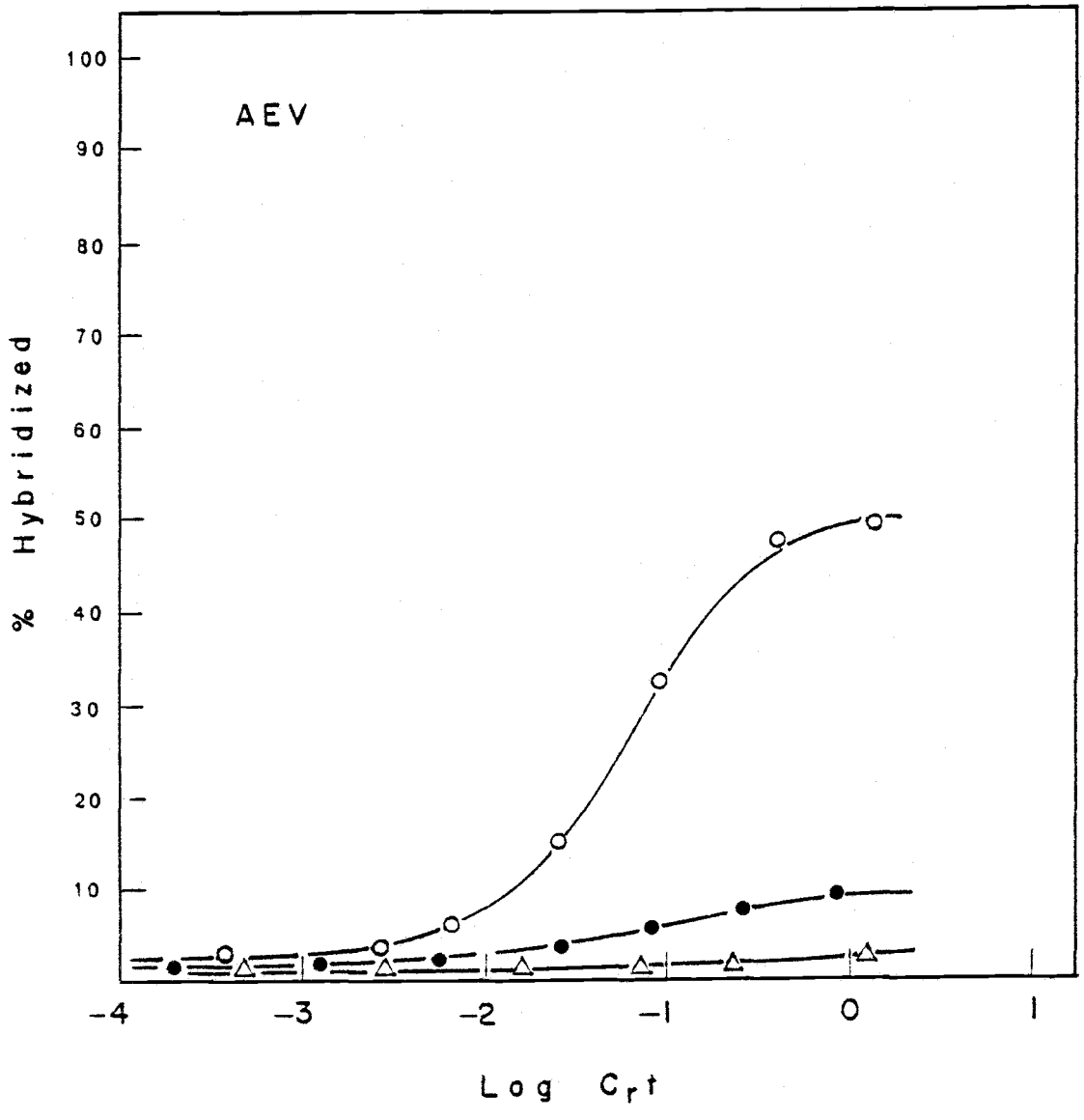


Figure 20. Kinetics of hybridizing excess AEV RNA to various fractionated cDNA probes. (○) total unfractionated $cDNA_{AMV}$; (●) $cDNA_{mye}$(A); (△) $cDNA_{mye}$(S). Reaction and assay conditions were the same as in Figure 6 and Figure 11.

distinct class of avian oncornavirus. The results were also compared to hybridization of total cDNA and $cDNA_{mye}$(A) with the same RNAs.

Figure 19 is the result of kinetic hybridizations with MC29 RNA to the three different probes. MC29 RNA hybridized about 75% of the total cDNA, corresponding to a sequence homology between MC29 and AMV of about 80%, which was 10% more than the homology between RSV and AMV. This result was quite expected since both MC29 and AMV belong to the leukosis group whereas RSV belongs to the sarcoma group. MC29 was also believed to be closer related to AMV than any other avian oncornavirus. However, MC29 RNA was unable to hybridize any of the $cDNA_{mye}$(S) probe, indicating that there was no homology between MC29 RNA and that part of the AMV genome represented by $cDNA_{mye}$(S).

Figure 20 represents the kinetic hybridization between cDNA probes and RNA of AEV, another leukosis virus but one that does not infect endoderm or ectoderm as do AMV and MC29. AEV RNA only hybridized 50% of the total cDNA, corresponding to a sequence homology between AMV and AEV of about 52-55%, which was considerably less than that between RSV and AMV. AEV RNA hybridized $cDNA_{mye}$(A) to a surprisingly low 10%, indicating that AEV strain R might be a very distinctive virus from AMV, and suggesting that the c region between AMV and AEV might also be different. As might be expected none of the $cDNA_{mye}$(S) sequences hybridized with AEV, again demonstrating the uniqueness of $cDNA_{mye}$(S).

Figure 21 represents the reactions between the probes and the RNA from SRD RSV, which was another strain of RSV different from the Prague strain as shown in Fig. 18.

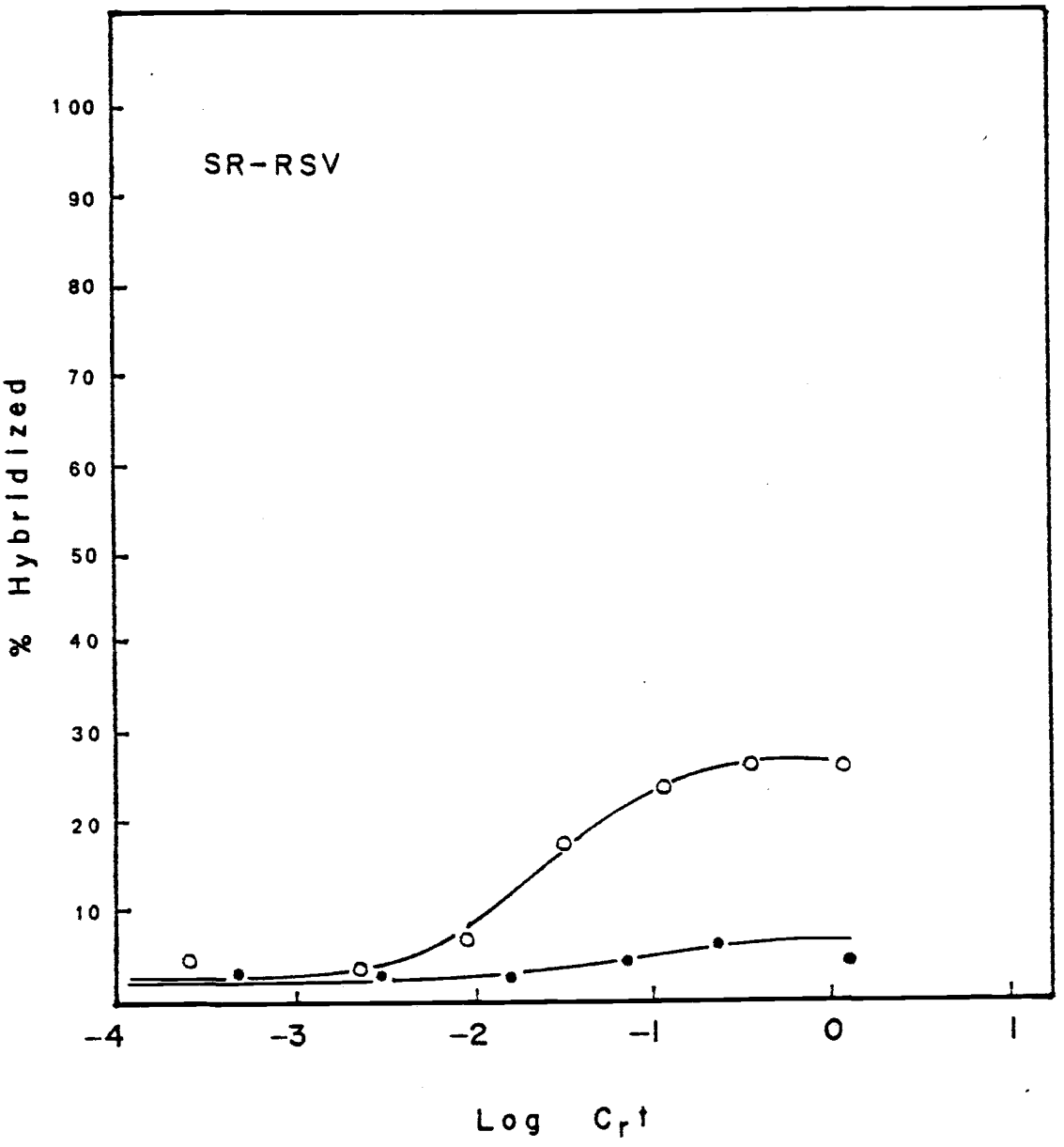


Figure 21. Kinetics of hybridizing excess SR-D RSV RNA to various fractionated cDNA probes. (O) $cDNA_{mye}$(A); (●) $cDNA_{mye}$(S). Reaction and assay conditions were the same as in Figure 6 and in Figure 11.

TABLE 5. SUMMARY OF HYBRIDIZATIONS BETWEEN DIFFERENT [^{3}H]cDNA PROBES AND VARIOUS VIRAL RNAs

RNA	Percent of [^{3}H]cDNA Probes Hybridized by Viral DNA[a]				
	Total cDNA	$cDNA_{mye}$(A)	$cDNA_{mye}$(S)	$cDNA_{endo}$(A)	$cDNA_{exo}$(S)
AMV	90	90	85	82	92
RSV (Pr)	65	32	2-3	67	-
RSV (SR-D)	-	30	2-5	-	-
MC29	70	25	2-5	61	-
AEV	52	10	2-3	63	-
RAV-0	55	5-6	2-3	70	-

[a]All hybridizations were carried to saturation at C_rt values of about 1 mole·sec/litre. All percent values were unadjusted and those below 5% were actually representing background resistance to the cDNA probes to S_1 nuclease digestions.

Thus it has been demonstrated that $cDNA_{mye}(S)$ indeed represented AMV sequences with no homology to RNA from other avian oncornaviruses and the results are summarized in Table 5.

Specificity of Structural cDNA Probes

$cDNA_{endo}(A)$. The portion of [^{3}H]cDNA that represented the structural genes gag, pol and env in the AMV genome was recovered from hybrids with RAV-0 RNA during the isolation of $cDNA_{mye}(A)$ and was subsequently designated as $cDNA_{endo}(A)$ (for endogenous sequences isolated by associated viral RNA). $cDNA_{endo}(A)$ should theoretically be able to hybridize completely with AMV and RAV-0 molecules, and thus, could be used as a probe to test the comparative homology among structural genes of different viruses. RNAs from AMV, RSV, MC29, AEV and RAV-0 were hybridized in excess to $cDNA_{endo}(A)$ and the results are summarized in Table 5. Neither AMV RNA nor RAV-0 RNA could hybridize more than 82% of the $cDNA_{endo}(A)$ tested, indicating that some partial degradation of the probe might have taken place during the isolation procedure, rendering portions of the probe long enough to be precipitated by acid but not long enough for hybridization. However, the relatively high levels of hybridization with RNAs from all viruses tested clearly demonstrated the relatively high degree of homology among the structural genes of these viruses. The results also showed that $cDNA_{endo}(A)$ could be used to detect endogenous sequences in cellular DNA.

$cDNA_{exo}(S)$. The portion of cDNA that represented the AMV structural genes plus the common c region was recovered from hybrids with

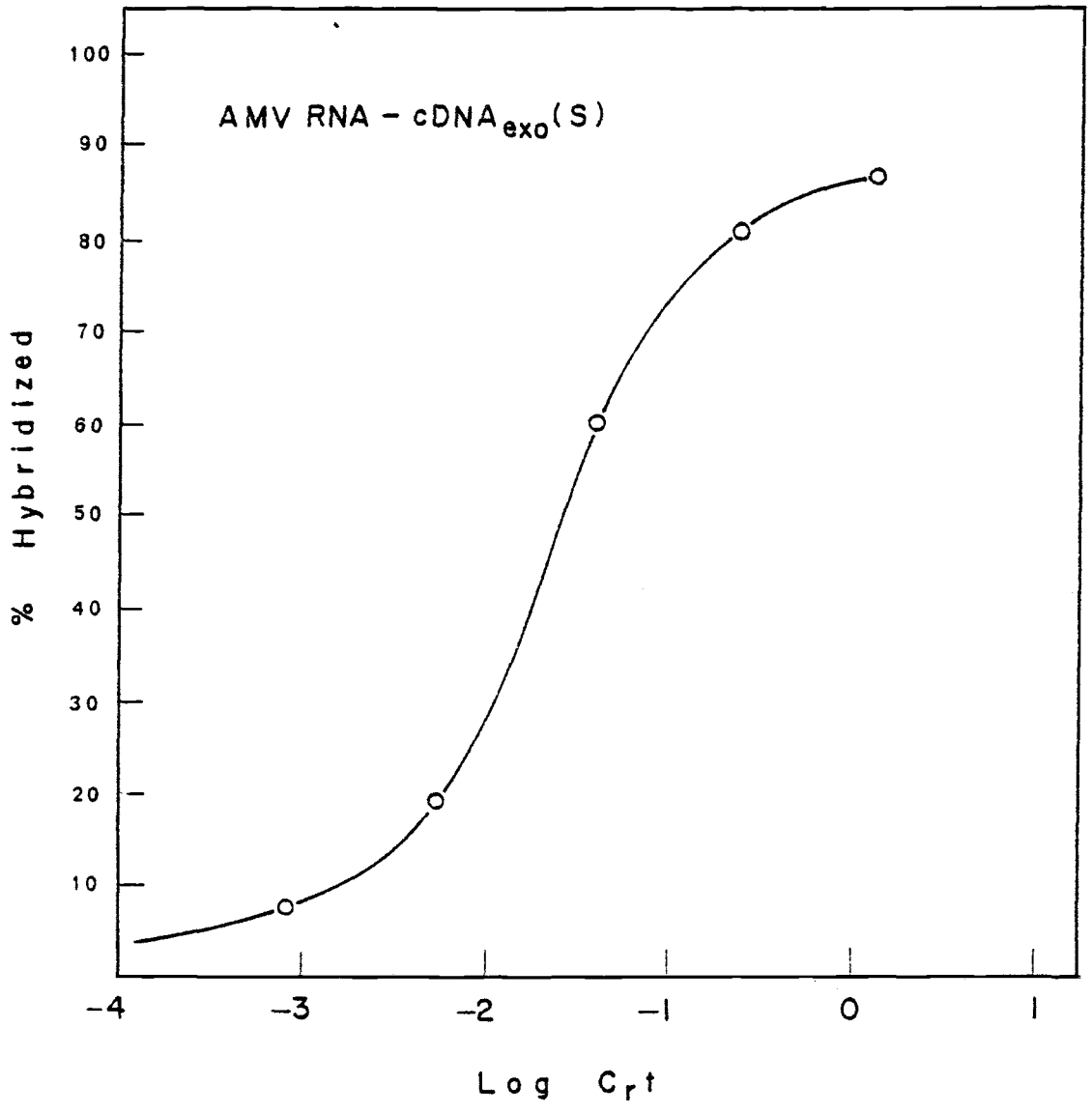


Figure 22. Kinetics of hybridizing excess AMV RNA to cDNA$_{exo}$(S). Reaction and assay conditions were the same as in Figure 6 and in Figure 11.

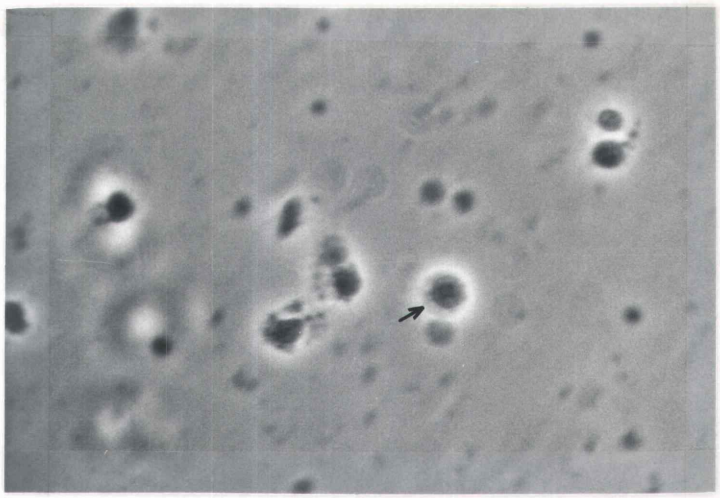
RSV RNA during isolation of $cDNA_{mye}(S)$ (Fig. 9, step six) and was designated as $cDNA_{exo}(S)$ (for sequences common to exogenous viruses isolated by sarcoma viral RNA). The specificity of this probe was examined by kinetic hybridization with excess AMV RNA and the results are presented in Fig. 22 and Table 5. Over 90% of the $cDNA_{exo}(S)$ probe hybridized to AMV RNA with a reaction kinetic very similar to that exhibited by total [^{3}H]cDNA (Fig. 6) indicating the integrity of the probe. $cDNA_{exo}(S)$ was also to be used to determine copy number of viral structural sequences in chicken cellular DNA in later experiments.

PREPARATION OF CELLULAR DNA FROM CHICKEN

Nuclei Isolation

To avoid contamination of cellular DNA from viral nucleic acids in infected or non-infected cell cytoplasm, all chicken cellular DNAs were isolated from purified nuclei preparations. Myeloblast nuclei preparation simply required the dissolution of plasma membrane and cytoplasmic contents by mild detergent action of 0.12% Nonidet P-40. The NaCl-Na citrate buffer was maintained at pH 6.8 since nuclear membrane was unstable at alkaline pH in the absence of Ca^{++}. The major problems encountered in this procedure were the fragility of the nuclear membrane and the high tendencies for the liberated nuclei to adhere together, resulting in clumps that were difficult for further processing. Three measures have been proven effective to overcome these problems. First, the cells and nuclei were kept in the solution at a fairly low concentration of no more than 10^9 per ml. Second, centrifugation to

(A)



(B)

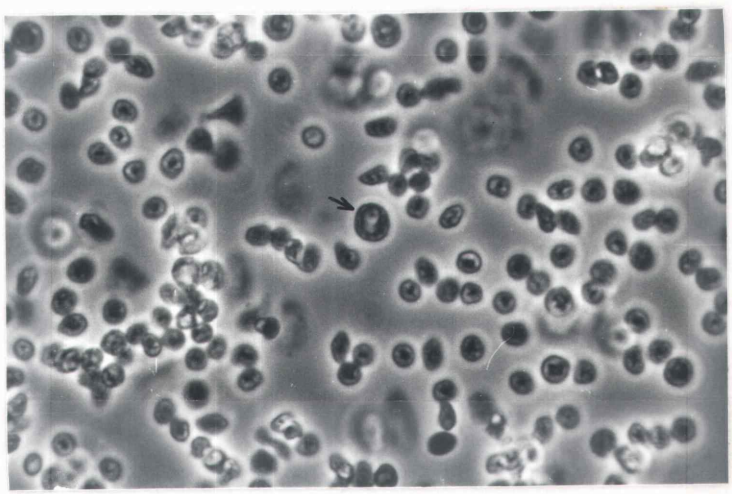
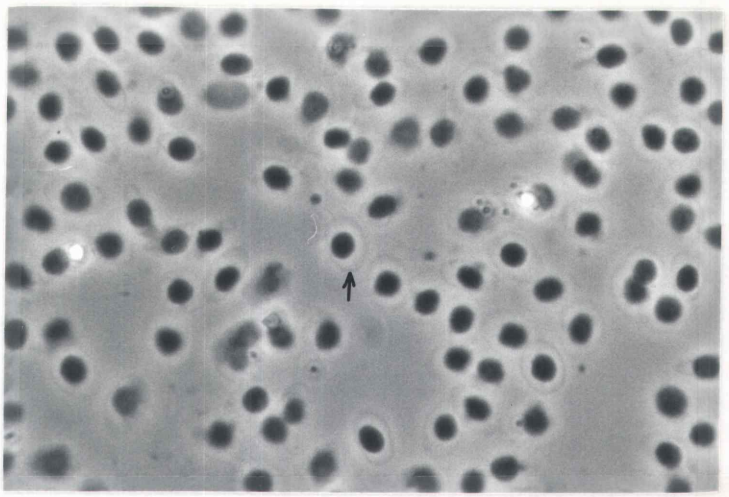


Figure 23. Isolation of myeloblast nuclei. Phase contrast microscopy at 400 X magnification. (A) Cells were suspended in SSC buffer, and arrow indicates cell membrane. (B) Cells were lysed in SSC + 0.25% Nonidet-P40 detergent; nuclei were clearly liberated and showed slight clumping. Arrow indicates a still intact myeloblast surrounded by naked nuclei.

separate nuclei from solubilized cell debris was kept to no more than 1500 x g for five min or less. The number of nuclei to be centrifuged in a 30 ml Corex tube was limited to 5×10^9. These measures primarily minimized crowding and overpacking of the nuclei. When these precautions were exercised together with swift but gentle handling, myeloblast nuclei were isolated with excellent results. Figure 23, panel A showed a myeloblast before detergent treatment with the plasma membrane clearly visible under phase contrast microscopy. Panel B showed nuclei liberated by detergent; slight clumping was already evidenced in the picture.

Nuclei preparations from infected or uninfected chicken erythrocytes presented an additional problem. The erythrocyte cell membrane was much more rigid and more resistant to detergent action than that of the myeloblast. Figure 24, panel A shows the picture of erythrocytes after detergent treatment. The plasma membranes were still very much intact although much of the hemoglobin had already been released from the cells. Controlled mechanical shearing as described in methods was employed to strip the cell membranes physically off the nuclei. Figure 24, panel B shows the liberated nuclei after brief mechanical homogenization. Cytoplasmic debris and strands of collapsed cell membranes were clearly separated from the nuclei. Once the nuclei were rid of the cell membranes, they were as fragile as myeloblast nuclei and needed to be handled with the same precautions and care.

(A)



(B)

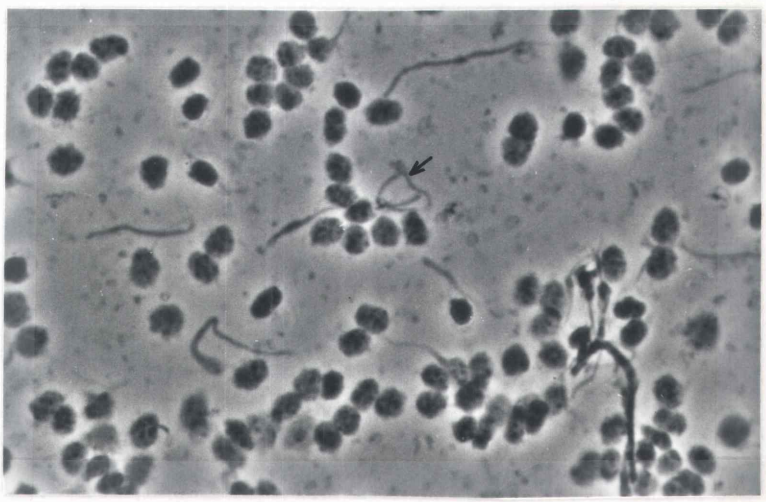


Figure 24. Isolation of erythrocyte nuclei. Phase contrast microscopy at 400 X magnification. (A) Cells had been treated with 0.25% Non-idet-P40, but the cell membranes (arrow) were still intact. (B) After detergent treatment, the cells were subjected to light mechanical shearing and the cell membranes (arrow) were stripped off the nuclei.

Hirt Isolation of Chicken Nuclear DNA

As an extra precaution against contamination of cellular DNA by exogenous or endogenous viral nucleic acids, the Hirt method was employed to preferentially precipitate high molecular weight undegraded cellular DNA with high salt concentration.

For testing the effectiveness of the procedure in separating viral and cellular DNA, about 6000 cpm of ^{32}P labeled SV 40 DNA was mixed with about 10^9 nuclei. After the nuclei had been lysed by SDS-proteinase treatment, the mixture was divided into two aliquots. NaCl was added to final concentrations of about 1.2 M in the two samples and both were incubated at 0° C overnight. The samples were then centrifuged at 45,000 X g in a FA21 rotor for either four or six hr. After centrifugation, aliquots from both supernatants were precipitated by cold trichloroacetic acid. Acid precipitable ^{32}P activities were measured and compared with input activities as the percent recovery of SV 40 DNA from the viral cell DNA mixture. Centrifugation for four hr resulted in 80.5% of the input SV 40 DNA being recovered from the supernatant, whereas centrifugation for six hr enhanced the recovery to 89.9%. When considering the fact that the SV 40 DNA in the supernatants might not have been totally acid precipitable and that centrifugation in the actual DNA isolation procedure was usually carried out for seven to eight hr, the resultant salt precipitated cellular DNA should be free of over 90% of whatever contaminating viral nuclei acids. If there had been ten copies of unintegrated viral DNA per nucleus (the actual figure should be much less since most unintegrated

viral DNAs were found outside of the nuclei), contaminating unintegrated provirus in nuclear DNA would have been less than one copy per nucleus.

DETECTION OF INTEGRATED VIRAL SEQUENCES IN CHICKEN DNAs

The detection of viral sequences integrated into cellular DNA was carried out by kinetic hybridization of tracer [^{3}H]cDNA probes with excess cellular DNA. The problem involved in such hybridization was three-fold. First, size differences between cDNA molecules and cellular DNA molecules may result in highly different reaction rate between reassociation and hybridization and this is difficult to control. Second, since the number of integrated viral sequences in cellular DNA has not been well established and has varied in the literature by as much as ten-fold (53,104,105,116), the amount of cellular DNA corresponding to an actual excess of integrated viral DNA sequences needed to be determined. Third, the large excess of cellular DNA required for hybridization may interfere with the determination of results in two ways: quenching of probe radioactivity and restraint of DNA molecule mobility by the viscosity of the DNA solution.

Since cell DNA has a size at least a million-fold greater than that of the viral cDNA probe, reassociation of the separated cell DNA strands would occur at a much faster rate than hybridization of complementary sequences with cDNA probe. To solve this problem, the cell DNA was mechanically sheared in glycerol at a low temperature maintained by a dry ice-alcohol bath. The resultant DNA was in the range of eight

to 10s, or about 300 to 600 base pairs in length as determined by agarose gel electrophoresis. Although this cell DNA size was still much larger than the four to 6s size of the cDNA probes, at least the cDNA molecules had a competing chance. The second and third problems were solved by saturation hybridization as discussed below.

Saturation Hybridizations of Total [^{3}H]cDNA$_{AMV}$ with Varying Amounts of Chicken DNAs

Saturation hybridization of total cDNA$_{AMV}$ with chicken DNA at various cell DNA to probe ratios were performed to determine an optimal ratio at a manageable DNA concentration and volume to attain the maximum hybridization with the minimum amount of probe.

Preliminary experiments had determined that at least 500 cpm of [^{3}H]cDNA per reaction was the minimum requirement to withstand quenching by the million-fold excess of cell DNA and that DNA concentrations not exceeding 12 mg per ml were sufficient to obtain reasonable cell DNA/cDNA ratios when, at the same time, maintaining manageable reaction volumes. Figure 25 summarizes the hybridization results from chicken DNAs of viral-transformed myeloblasts, erythrocytes from infected birds, and uninfected erythrocytes. All reactions were carried to C_rt values of at least 10^4 mole·sec/litre with respect to cell DNA which ranged from less than 0.1 mg per ng of probe to over 30 mg per ng of probe. Both myeloblast DNA and erythrocyte DNA from infected chickens hybridized total cDNA$_{AMV}$ to plateau values of 70% and 62% respectively at mass ratio of about 2 mg/ng. Normal erythrocyte DNA required a mass

ratio in excess of 4 mg/ng to reach a maximum hybridization of about 55%. Clearly, there are nucleotide sequences in normal chick DNA as well as DNA from infected birds that show homologies to cDNA copies of the viral genome. Assuming that the chicken genome is 2.4×10^{-6} µg, whereas AMV genome is 3×10^{6} daltons, one DNA provirus would represent 0.0004% of chicken genome.

HYBRIDIZATION KINETICS OF [^{3}H]cDNA PROBES WITH CELLULAR DNA

In accordance to the optimal DNA ratios obtained in saturation hybridizations as presented in Fig. 25, all kinetic hybridizations involving cellular DNAs were carried out at cell DNA/cDNA ratios of between three to 6 mg/ng with cell DNA concentrations ranging from six to 12 mg per ml. Each reaction carried a minimum probe of 500 to 600 cpm. To minimize nonspecific binding of the probes, all reactions were performed under relatively stringent but identical conditions (66) of 0.45 M NaCl, 30% formamide at 50° C. Since reactions were all identical, salt concentration corrections were not necessary for the kinetic curves and since all data were presented for the purpose of comparisons, normalization for 0 and 100% hybridizations were not made.

Hybridization with Chicken DNAs

Figure 26 shows that when a maximum 70% of the total $cDNA_{AMV}$ hybridized with myeloblast DNA, only 65% and 55% of the probe hybridized with DNAs from infected and uninfected erythrocytes respectively. While these results were consistent with those shown in saturation

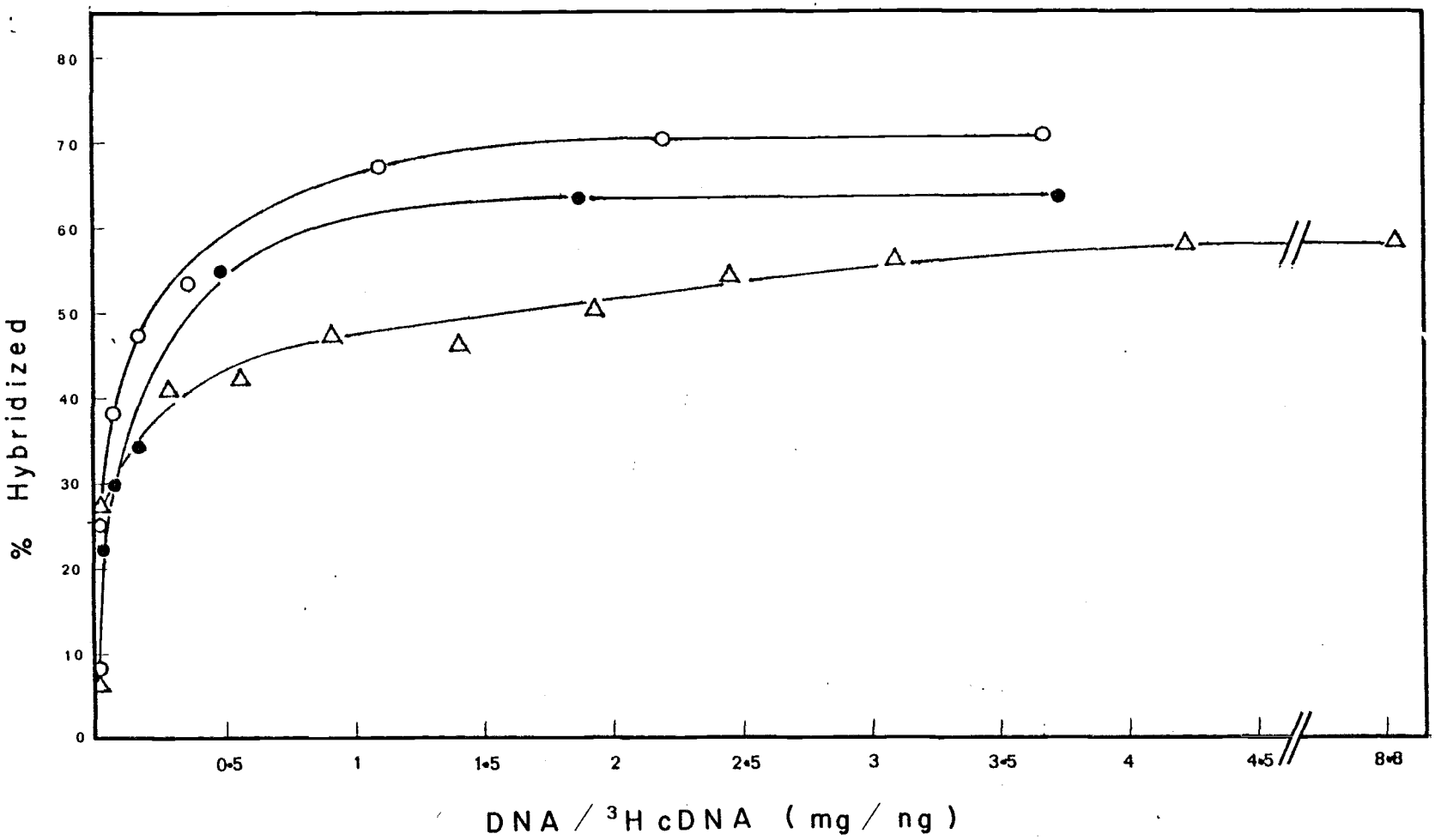


Figure 25. Hybridizations of total [^{3}H]cDNA with increasing amounts of cellular DNAs. Each reaction contained at least 500 cpm [^{3}H]cDNA in 0.4M Na^+, 42% formamide. DNA concentrations were relatively constant. Percent hybridization at saturations were assayed with S_1-nuclease. (O) infected myeloblast DNA. (●) infected erythrocyte DNA. (△) normal erythrocyte DNA.

hybridizations (Fig. 25), the maximum extents of hybridization were significantly lower than those obtained in AMV RNA-cDNA reaction (90%, Fig. 6). Three factors undoubtedly are responsible for these low final levels of reactions (106,107,108).

First, although the cell DNA had been sheared to lengths of 300 to 600 base pairs, the probe was only about 150 to 200 nucleotides long. Since reaction rate is proportional to the square root of reactant length, the second-order rate constant for probe hybridization could still be two to three times smaller than that the cell DNA reassociation (107).

Second, a low concentration of viral integrated sequences in cell DNA also reduced the level of probe hybridization (108). It has been calculated that with a rate constant for probe hybridization threefold less than that for cell DNA reannealing and with a two to fourfold molar excess of integrated sequences, a final probe hybridization of 30 to 50% could be expected (107). At a cell DNA to cDNA ratio of 4 mg/ng, with chicken genome at 2.4×10^{-6} µg and AMV genome (35s RNA) at 5.0×10^{-12} µg, it can be calculated that the integrated viral sequences in chicken cells range from 16 fold excess for single copy integration to 320-fold excess for 20 integrated copies. This vast difference in integrated sequence excess could contribute to the different maximum hybridization levels exhibited by the three types of chicken DNAs.

The third reason that could account for the difference in hybridization levels would be qualitative differences in the extent of

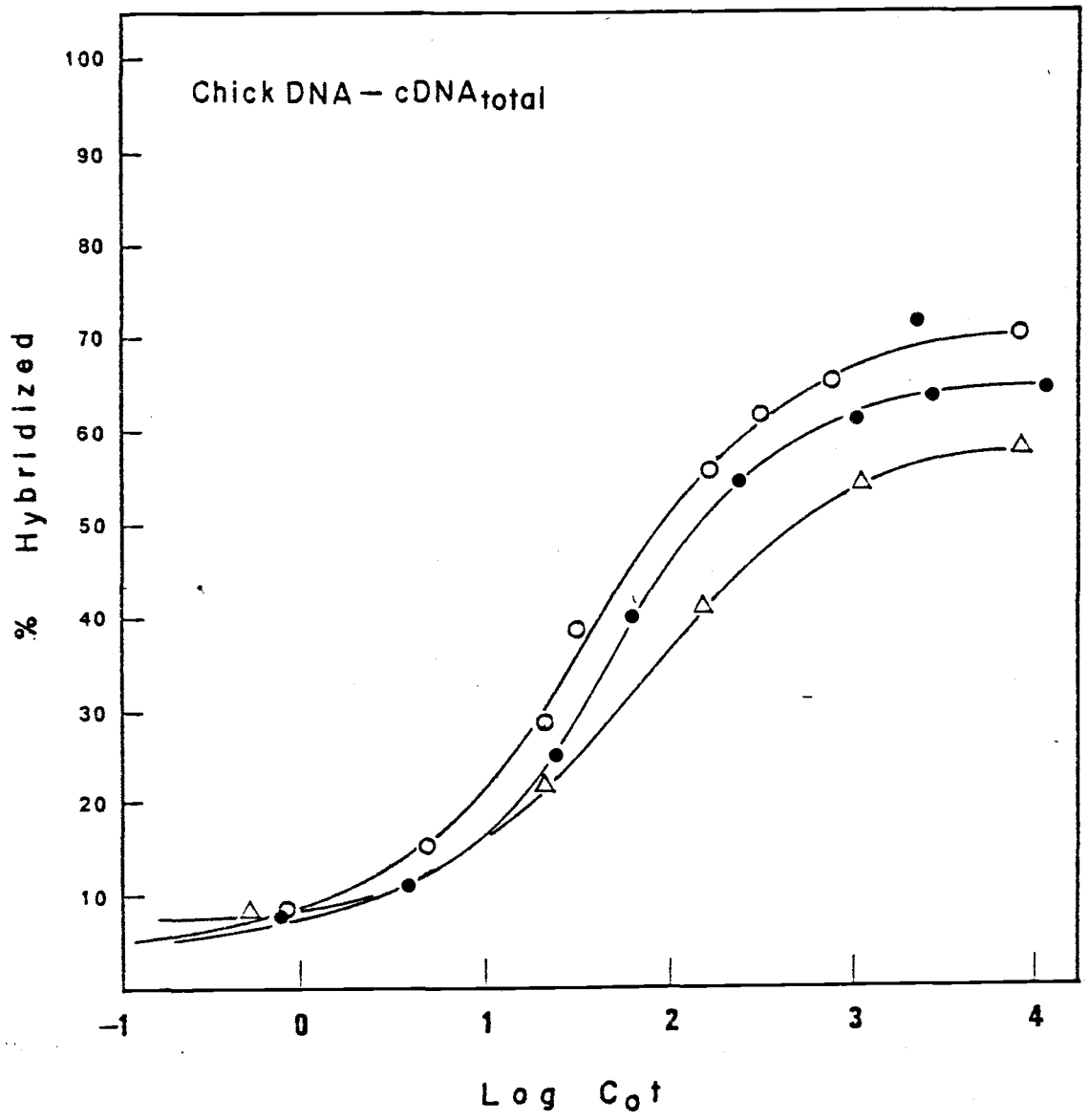


Figure 26. Kinetics of hybridizing total unfractionated [^{3}H]cDNA to different chicken cellular DNA. Reactions are performed at 0.45M Na^+, and 30% formamide. Cellular DNA: cDNA ratios were between 3 to 6 mg/ng. Cell DNA concentrations were 6 to 12 mg/ml. Percent hybridizations assayed by S_1-nuclease digestion. (○) infected myeloblast DNA. (●) infected erythrocyte DNA. (△) Normal erythrocyte DNA.

homology between the cDNA probe and the cell DNAs with an exception for the DNA from transformed myeloblast (97,109,110). Since mature intact viral particles are being replicated from transformed myeloblasts, it is unlikely that myeloblast DNA doe not contain the complete viral informations. If the 70% hybridization obtained by myeloblast DNA represented complete viral sequences, then the infected and uninfected erythrocyte DNAs (Fig. 26) should represent about 85% and 75% homology respectively of the AMV sequences.

Since the exact effects of the above factors on hybridization kinetics have not been well established, attempts to normalize these kinetic curves in Fig. 26 with respect to published reassociation kinetics of unique chicken sequences (108,109) would involve extensive assumptions and manipulations of data, thus rendering it difficult to obtain any accurate estimation of the copy number for integrated viral sequences in the three types of chicken cells. However, when compared to published half $C_o t$ values for chicken DNA of about 310 mole•sec/litre (109) the results in Fig. 26 indicate that all three types of chicken cells contain more than single copy of integrated viral sequences in their DNAs. It can be concluded from the data that myeloblast DNA contain more integrated AMV sequences than either infected or uninfected erythrocyte DNAs. Quantitative estimation of viral genome copies being integrated was to be performed by probe excess hybridizations and will be presented in a later section.

The extent of total cDNA hybridization by myeloblast DNA was comparable to the 72% published by Shoyab and Baluda (110) who utilized

cell DNA excess-AMV RNA hybridizations. But their homology between normal chicken fibroblast DNA and AMV was about 20% less than those shown in Fig. 26 and had a higher half $C_o t$. This discrepancy can probably be attributed to the fact that in DNA-RNA hybridizations the rate of reaction by the RNA probe is much less than that by the DNA reassociation and therefore the level of DNA-RNA hybridization may not reflect exactly the extent of homology as well as DNA-cDNA hybridizations.

Figure 27 shows the kinetics of hybridizing $cDNA_{mye}(A)$ with DNAs from myeloblasts as well as infected and uninfected erythrocytes. While myeloblast DNA hybridized 55% of $cDNA_{mye}(A)$, infected erythrocyte DNA hybridized about 45% of the probe and uninfected erythrocyte DNA hybridized only about 20% to 22%. The lowering of maximum hybridization levels compared with total cDNA may be explained by the fact that $cDNA_{mye}(A)$ represented less than 30% of the total $cDNA_{AMV}$ and therefore the integrated viral sequences in cell DNA would be at lowered excesses when the same cell DNA to cDNA ratios were maintained. This lowered excess effect was also reflected by the comparative percentage drop in maximum hybridizations exhibited by the DNAs from the three cell types. Myeloblast DNA had the most integrated sequences (as demonstrated in Fig. 26) and showed the least drop in hybridization extent of about 15%, whereas infected erythrocyte DNA dropped about 20% and normal erythrocyte DNA showed a drastic difference of over 30%. With these low levels of maximum hybridization levels, it was impractical from a kinetic standpoint to estimate the accurate number of viral

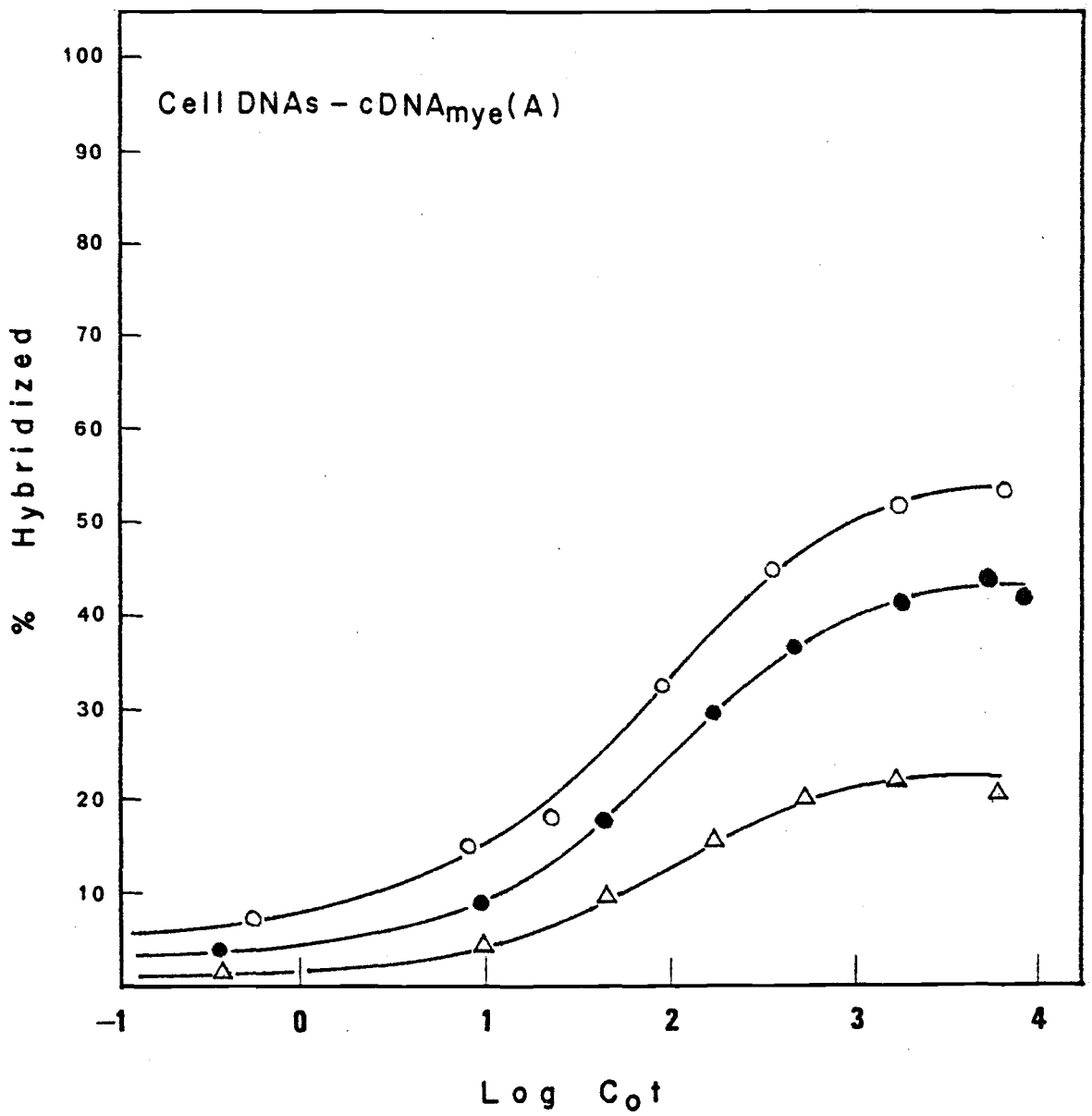


Figure 27. Kinetics of hybridizing $cDNA_{mye}$(A) to excess different chicken cellular DNAs. Reaction and assay conditions were the same as in Figure 26. Chicken DNAs were from infected myeloblasts (○), infected erythrocytes (●), and from normal erythrocytes (△).

equivalents in cell DNAs.

Although the changes in hybridization reflected the quantitative differences of integrated viral sequences in the three cell types, they could not rule out the possibility of qualitative differences among integrated sequences. While the majority of the total $cDNA_{AMV}$ was hybridized by the endogenous RAV-0 sequences in the normal erythrocytes, none of the $cDNA_{mye}$(A) should be hybridized by any of the same endogenous sequences. The fact that over 20% of the $cDNA_{mye}$(A) was hybridized indicates that in normal chicken DNA there are sequences homologous to unique portions of the genome of exogenous viruses.

The presence of AMV unique sequences in target hemopoietic tissues was further investigated by kinetic hybridizations of $cDNA_{mye}$(S) with DNAs from infected and normal blood cells. Figure 28 shows that when both myeloblast DNA and leukemic erythrocyte DNA hybridized about 45% of $cDNA_{mye}$(S), normal erythrocyte DNA hybridized only about 25% of the probe. This clearly supported the results obtained with $cDNA_{mye}$(A) (Fig. 27) that there are differences in AMV specific sequences between infected and noninfected cells. This difference may be due to the number of copies or sequence homology.

However, since $cDNA_{mye}$(S) was presumed to be about two-thirds the size of $cDNA_{mye}$(A) (14), the reduced sequence excess effect discussed above should further reduce the maximum hybridizations with $cDNA_{mye}$(S) from those obtained with $cDNA_{mye}$(A). But a closer examination of data presented in Fig. 27 and Fig. 28 revealed that only myeloblast DNA showed a 10% reduction in level of hybridization. Neither leukemic

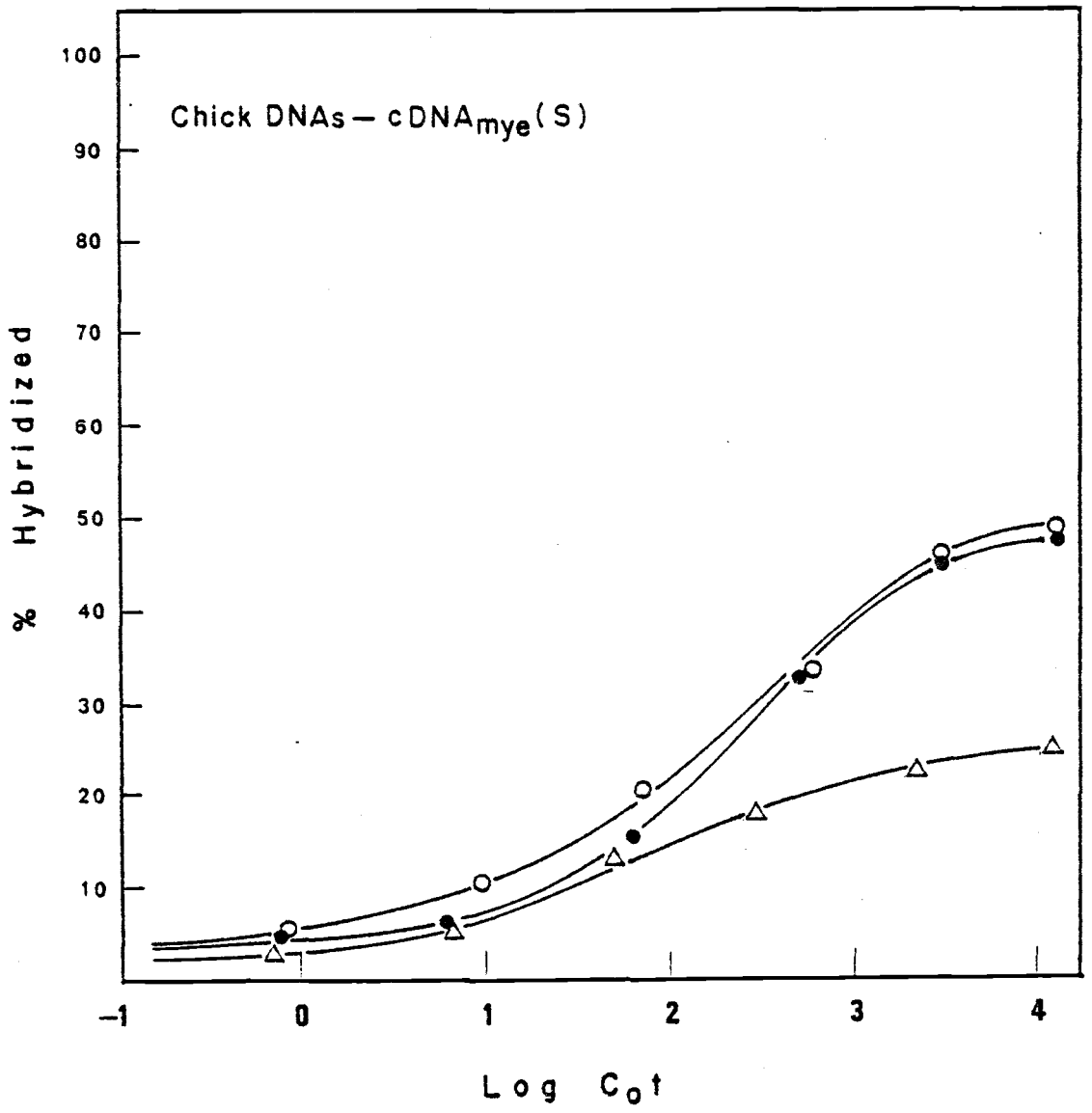


Figure 28. Kinetics of hybridizing $cDNA_{mye}$(S) to excess different chicken cellular DNAs. Reaction and assay conditions were the same as in Figure 26. Chicken DNAs were from infected myeloblasts (○), infected erythrocytes (●) and from normal erythrocytes (△).

nor normal erythrocyte DNAs showed any reduction. One possible explanation is perhaps that the integrated sequences in erythrocytes (infected or normal) lacked homology to a region in $cDNA_{mye}(A)$ that was not found in $cDNA_{mye}(S)$, for example, the common c region. Therefore, when erythrocyte DNAs hybridized $cDNA_{mye}(S)$ instead of $cDNA_{mye}(A)$ the effect of reduced sequence excess was balanced by the effects of better homologies, thus rendering no change to the final extents of hybridization. But in the case of myeloblast DNA, which contained all sequences in both $cDNA_{mye}(A)$ and $cDNA_{mye}(S)$, the reduced sequence excess effect for the smaller $cDNA_{mye}(S)$ prevailed and lowered the final hybridization. This interpretation would imply distinct qualitative as well as quantitative differences between AMV sequences integrated into myeloblasts and erythrocytes.

DNA from both infected cells (erythrocytes and myeloblasts) appeared to have enhanced AMV sequence integration when compared to normal erythrocyte DNA. Verification of this by probe excess hybridization will be presented later.

To demonstrate that the lowering in hybridization extents observed with both $cDNA_{mye}(A)$ and $cDNA_{mye}(S)$ when compared with total cDNA was not due to breakdown of the probes during isolation, and to gain further insight into sequence homologies between myeloblasts and erythrocytes, $cDNA_{endo}(A)$ and $cDNA_{exo}(S)$ were hybridized to chicken blood cell DNAs. The results of $cDNA_{endo}(A)$ hybridizations are presented in Table 6, which shows that DNA from myeloblasts hybridized 53% of the probe and that both infected and uninfected erythrocytes hybridized

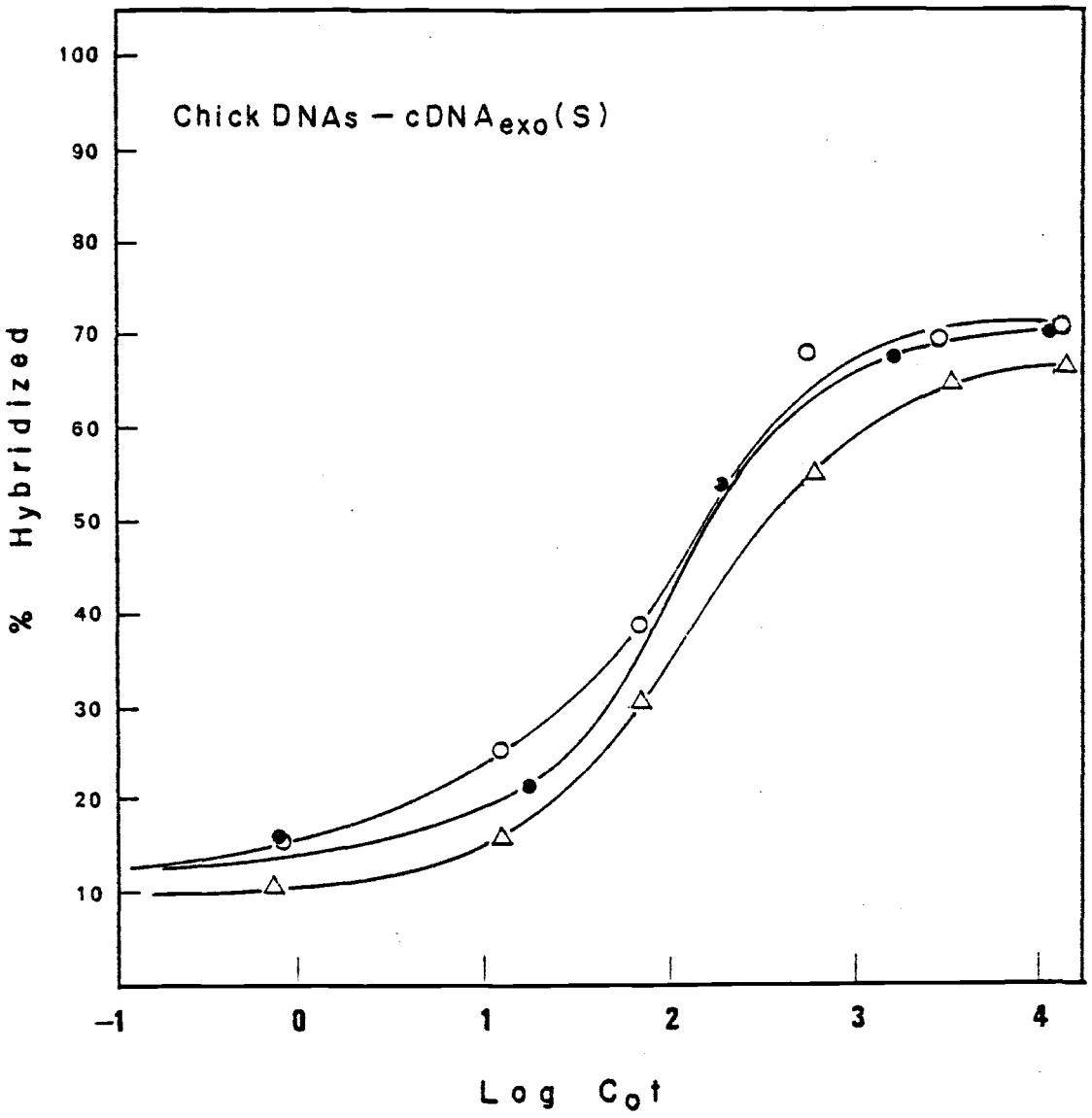


Figure 29. Kinetics of hybridizing cDNA$_{exo}$(S) to excess different chicken cellular DNAs. Reaction and assay conditions were the same as in Figure 26. Chicken DNAs were from infected myeloblasts (○), infected erythrocytes (●), and from normal erythrocytes (△).

about the same levels of 46 to 47%. The results of kinetic hybridizations with $cDNA_{exo}(S)$, given in Fig. 29, show much higher final hybridization levels; both myeloblasts and leukemic erythrocytes hybridized about 70% of the probe, whereas normal erythrocytes hybridized about 65%. This demonstrated not only that the probes retained their ability for hybridization after isolation but that by eliminating AMV unique sequences from total cDNA, the resultant enhanced homologies between erythrocyte DNAs and $cDNA_{exo}(S)$ actually elevated the final hybridization level by 30%, despite the fact that the complexity of $cDNA_{exo}(S)$ was smaller than that of total cDNA.

These results demonstrate that although normal erythrocytes had homology with total AMV sequences of about 55%, which was consistent with published values (111), they actually had over 65% homology with the structural portion of the AMV genome that was common to other exogenous viruses and had only 20% homology with unique AMV sequences. Although AMV sequences integrated into both myeloblasts and leukemic erythrocytes during infection, there were apparent quantitative and quite possibly qualitative differences between the sequences integrated into the two different cell types.

Detection of AMV Sequences in Other Avian Species

Sequences homologies between the endogenous virus RAV-0 and avian species (105,109,110,111,112) have been reported to be 55-70% with normal chicken, 10-20% with pheasant, 2-20% with quail, 3-10% with turkey, and less than 1% with duck under moderately stringent

conditions of hybridizations. AMV has also been reported (110,111) to have sequence homologies of about 10% with pheasant, 2-3% with quail and turkey, and less than 1% with duck. But since AMV shares about 60-70% homology with RAV-0, the specificity of homologies between AMV and avian species could not be determined by the above levels of hybridization with total viral sequences.

Hybridizations of $cDNA_{mye}$(A) and $cDNA_{mye}$(S) with any avian DNA would indicate homology between unique AMV sequences and that of avian species, whereas hybridizations with $cDNA_{endo}$(A) would indicate homology between avian DNA and the portion of AMV genome that shares common sequences with endogenous viruses. The results of kinetic hybridizations between the $cDNA_{mye}$ probes and fibroblast DNAs extracted from embryos of pheasant, quail, turkey, goose and duck are presented in Fig. 30 through Fig. 34 and are also summarized in Table 6. $cDNA_{endo}$(A) hybridizations were assayed only at saturation $C_o t$ values and the results are presented in Table 6 also.

Pheasant DNA hybridized 15% of the total cDNA (Fig. 30) and 10% of $cDNA_{endo}$(A) (Table 6). Both of these results agreed with published values listed above. But the fact that pheasant also hybridized 18% $cDNA_{mye}$(A) and 17% $cDNA_{mye}$(S) indicates that there is a considerable amount of homology between pheasant and the unique AMV sequences.

All other tested avian species, quail (Fig. 31), turkey (Fig. 32), goose (Fig. 33), and duck (Fig. 34) hybridized about 10% of $cDNA_{mye}$(A), indicating significant homologies between avian DNAs and unique AMV sequences. Further verification of these relationships were provided

TABLE 6. SUMMARY OF HYBRIDIZATIONS BETWEEN DIFFERENT [^{3}H]cDNA PROBES AND VARIOUS CELL DNAs

DNAs[b]	Percent of [^{3}H]cDNA Probes Hybridized by Each Cell DNA[a]				
	Total cDNA	$cDNA_{mye}$(A)	$cDNA_{mye}$(S)	$cDNA_{endo}$(A)	$cDNA_{exo}$(S)
Chicken					
Myeloblast	70	55	50	53	72
Leukemic erythrocyte	65	45	48	46	70
Normal erythrocyte	55	20	23	47	66
Pheasant	15	18	17	10	-
Quail	-	10	15	4	-
Turkey	-	10	20	3	-
Goose	-	10	11	3	-
Duck	-	10	10	5	-
Calf thymus	-	8-9	-	2-3	-

[a]These values were summarized from maximum hybridization levels presented from Fig. 26 to Fig. 34, plus other individual experiments. Each value represents the average of at least three determinations.

[b]Cell DNA/cDNA ratios were 3-6 mg/ng and DNA concentrations were 6-12 mg/ml.

by hybridizations of $cDNA_{mye}$(S) with the avian DNAs. Fifteen percent of this probe hybridized to quail, 20% to turkey, 11% to goose, and 10% to duck, thereby confirming the homologies obtained with $cDNA_{mye}$(A).

The hybridization levels of $cDNA_{endo}$(A) to 53%, 46%, and 47% by myeloblasts, leukemic and normal erythrocytes correspondingly (Table 6) were all lower than those obtained with $cDNA_{exo}$(S) (Fig. 29). This lowering of hybridizations may be due to either a lower cell DNA excess because of the smaller $cDNA_{endo}$(A) (by not having the common c region), or a loss of reactivity by the breakdown of $cDNA_{endo}$(A), as evidenced by only 82% of this probe being hybridized by excess AMV RNA (Table 6) instead of the expected 90% or above. But the results did show that myeloblast DNA hybridized $cDNA_{endo}$(A) to a higher level than either leukemic or normal erythrocytes.

Pheasant DNA, which reportedly could have 10-20% homology with RAV-0, hybridized 10% of $cDNA_{endo}$(A) (Table 6). This homology was to be expected since both pheasant and domestic chicken belong to the same subfamily Phasianinae within the order of Galliformes. Also, Chen and Vogt (113) have reported isolation from normal pheasant of an endogenous leukosis virus that had helper activities with Bryan high titer strain of Rous sarcoma virus, suggesting the close relationship between pheasant and chicken endogenous viruses.

The DNAs from the rest of the tested avian species (quail, turkey, goose and duck) reportedly can hybridize RAV-0 probes from one to 20%. Our results indicated that none of these DNAs hybridized more than 5% of $cDNA_{endo}$(A) (Table 6). Calf thymus DNA hybridized $cDNA_{endo}$(A) to

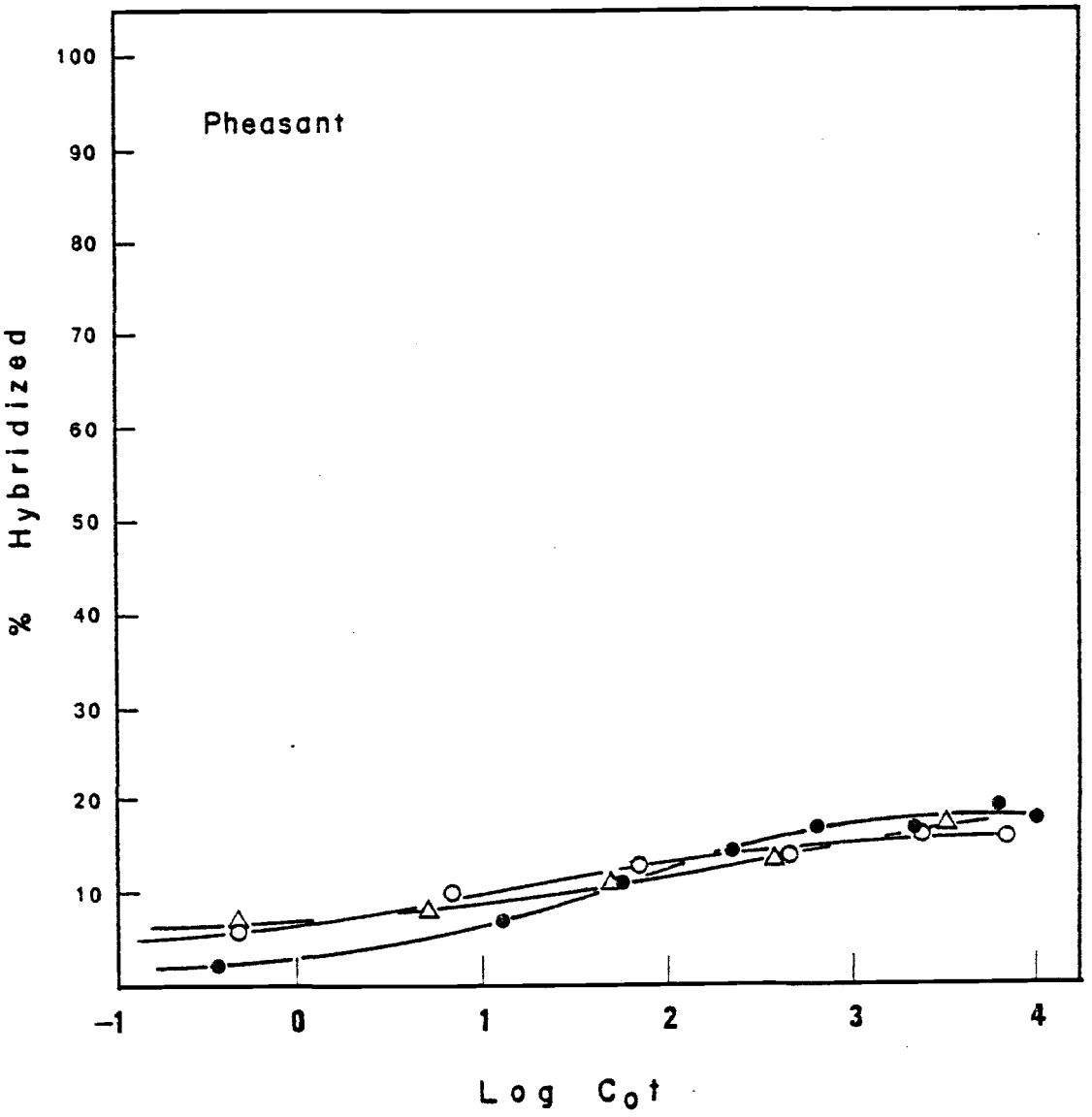


Figure 30. Kinetics of hybridizing excess pheasant DNA to different cDNA probes. Reaction and assay conditions were the same as in Figure 26. [^{3}H]cDNA probes were total unfractionated cDNA (○), $cDNA_{mye}$ (A) (●), and $cDNA_{mye}$ (S) (△).

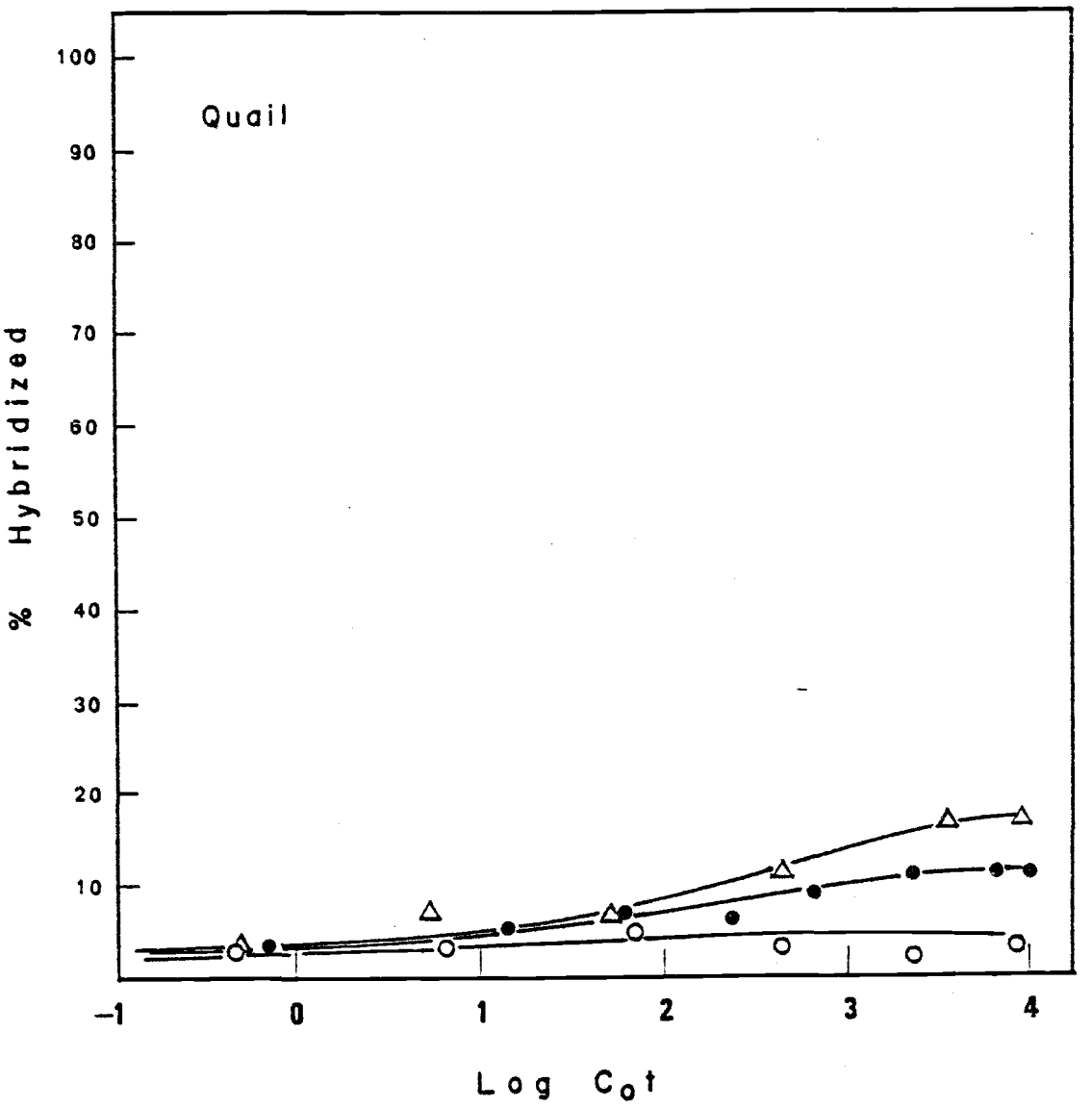


Figure 31. Kinetics of hybridizing excess quail DNA to different cDNA probes. Reaction and assay conditions were the same as in Figure 26. [^{3}H]cDNA probes used were total unfractionated cDNA (○), $cDNA_{mye}$ (A) (●), and $cDNA_{mye}$ (S) (△).

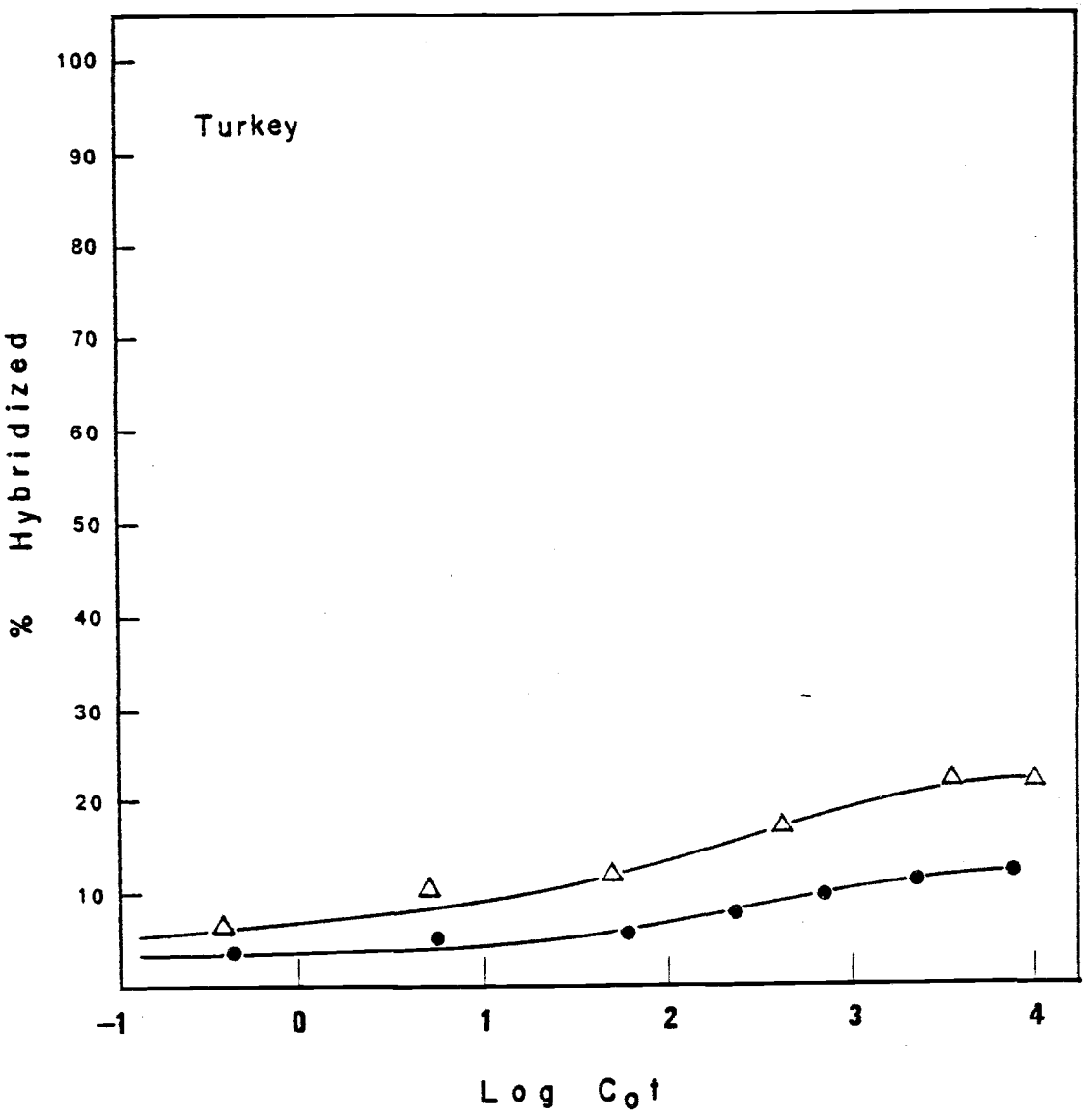


Figure 32. Kinetics of hybridizing excess turkey DNA to two different cDNA probes. Reaction and assay conditions were the same as in Figure 26. [^{3}H]cDNA probes used were $cDNA_{mye}$(A) (●) and $cDNA_{mye}$(S) (△).

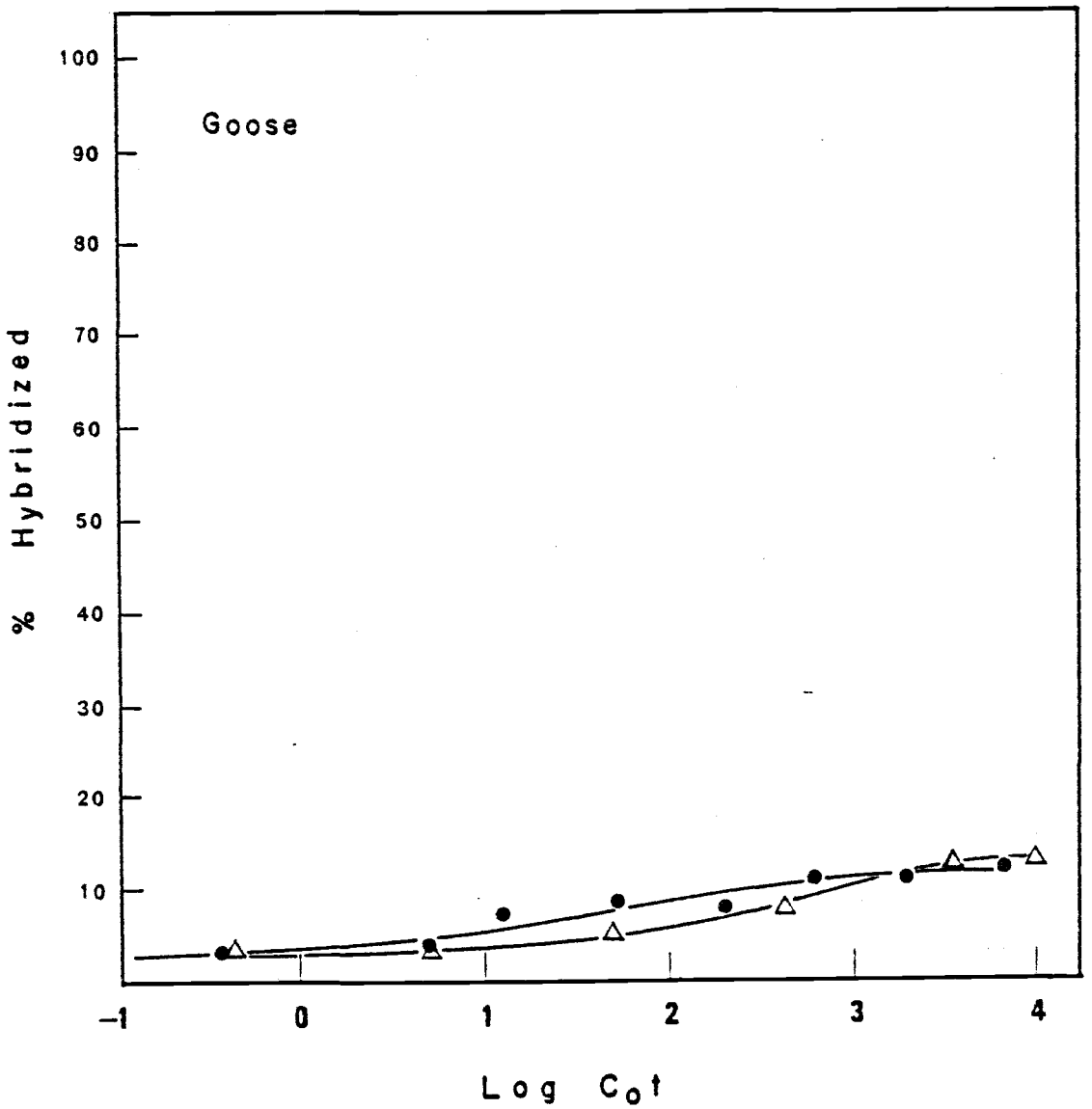


Figure 33. Kinetics of hybridizing excess goose DNA to two different cDNA probes. Reaction and assay conditions were the same as in Figure 26. [^{3}H]cDNA probes used were $cDNA_{mye}$(A) (●) and $cDNA_{mye}$(S) (△).

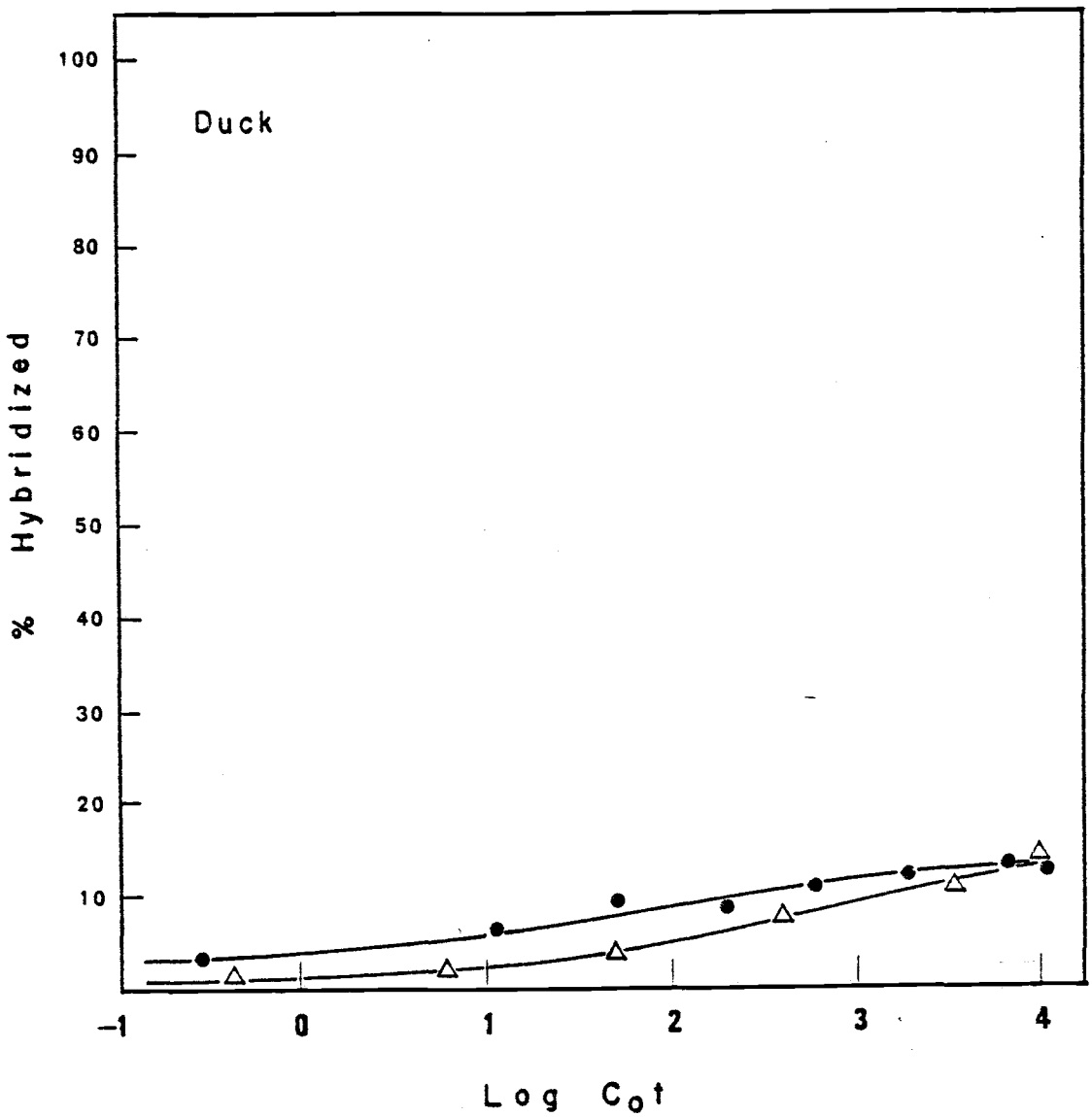


Figure 34. Kinetics of hybridizing excess duck DNA to two different cDNA probes. Reaction and assay conditions were the same as in Figure 26. [^{3}H]cDNA probes used were $cDNA_{mye}$(A) (●) and $cDNA_{mye}$(S) (△).

only 2-3%, which was significantly lower than the 8-9% obtainable with $cDNA_{mye}$(S). These low levels of hybridizations may be attributable to the stringent reaction conditions but they do support the low homologies reported by Shoyab and Baluda (110) under similar conditions. The wide range of reported homologies between RAV-0 and quail (2-20%) and turkey (3-10%) may reflect actual sequence differences among individual avians. Humphreis et al. (14) have shown restriction site evidences demonstrating that the exogenous RAV-0 virus, obtained as a result of self-infection, did have sequences different from endogenous RAV-0. These different exogenous RAV-0 sequences therefore could contribute to the differences among individual animals.

Our results show that when the portion of AMV genome that is shared by all other tested exogenous viruses demonstrates no homology with other avian and calf thymus DNAs tested, except members of the subfamily phasianinae, the part of the genome that is unique to AMV shares homology with all avians tested as well as calf thymus. Published evidences indicate that each of the distinctive transforming genes from ASV (66,109,114), MC29 (115), and AEV (87) may have been derived from a separate genetic determinant in the avian genome as well as from other vertebrate genomes, whereas part of these viral genomes are highly conserved among viruses only. Our results with AMV closely parallel these findings and definitely support the theory that the putative progenitor of oncogenes were not structurally related to one another.

QUANTITATION OF PROVIAL SEQUENCES IN CHICKEN CELLS

The published determinations of integrated viral genome equivalents in cell DNAs usually utilize two major methods. In the first method, tracer amount of viral probe, either in the form of labeled cDNA or labeled RNA, is hybridized in liquid to cell DNA which is in an amount vast enough to provide an excess of integrated sequences with respect to the probe. The concentration of integrated sequences in the reaction is then estimated by comparing the hybridization rate of the probe with the reassociation rate of unique cellular DNA sequences (104,108,109). The limitations of this type of reaction has been reviewed by Neiman et al. (108) and generally involves factors such as the rate difference between hybridization and reassociation, the size difference between cell DNA fragments and probe, and the inadequate excess of proviral sequences in the cellular DNAs. Uneven representation of the whole viral genome by the probe and probe degradation during the long incubation period (five to seven days), plus the fact that the negative probe is competing with negative cell DNA single-strand sequences for the same positive proviral site often lead to less than complete reactions. The million-fold excess of total cell DNA with respect to the probe also causes problems in handling difficulties and in quenching the probe radio-activities. All these factors contribute to substantial uncertainties with regard to the reaction kinetics and subsequently render the estimation of integrated sequences inaccurate. It is because of all these limitations that this method is not used in this report even though the kinetic data with cell DNA

hybridizations are all available from Fig. 26 through Fig. 29.

In the second method, denatured cell DNA is trapped on nitrocellulose filter and a vast amount of viral probes are added to the reaction (41,54,116). Those viral probes that hybridize with integrated proviral sequences in the trapped cell DNA will remain on the filter after treatment with single-strand specific nucleases. The amount of viral probes hybridized on the filter should then be equivalent to the amount of proviral sequences in the cell DNA on the same filter. This method eliminated most of the problems encountered in kinetic hybridizations involving the probes such as probe size, reaction rate, degradation, and incomplete representation of viral genome. Since the cell DNA strands are immobilized on the filter, competition with probe is also avoided. However, the major difficulties with this method are the calibration of cell DNAs trapped on the filters and the requirement of large amounts of viral probes. Because of the small amounts of AMV specific probes available, this method also has not been employed in this report.

The quantitative analysis of integrated viral specific sequences in chicken cells was then performed by a third method as published by Heilman et al. (52). The general strategy involves hybridizing a small but known amount of cell DNA with an excess quantity of viral probes in a liquid medium. When all the available proviral sites in the cell DNA have been saturated by the probes, the unhybridized excess probes are removed by S_1 digestion and the actual amount of hybridized probe is calculated from the acid precipitable radioactivities

by using a specific activity that has been estimated with pseudo-first-order kinetic analysis as described previously. The concentration of cell DNA in a stock reaction mixture can be pre-determined by optical procedures and the actual amount of cell DNA in each hybridization reaction depends only on the conveniently chosen reaction volume that can be measured accurately. Since the amount of hybridized probes reflect the equivalent quantity of cell DNA proviral sequences in the reaction just as in the second method above, and by knowing the exact mass of cell DNA in the reaction, the genome equivalent of integrated viral sequences per cell can then be calculated.

The advantages of this method over the first two methods are as follows: 1) it eliminates most of the hybridization kinetic problems just as the second method would; 2) the minimal amount of cell DNA required per reaction reduces quenching and handling difficulties; 3) competition for the proviral DNA strands is minimized because the cell DNA sites are greatly diluted by the excess probes available; 4) the actual amount of cell DNA per reaction can be determined more accurately than in the second method; and 5) the small reaction volumes reduce the amount of probe required.

Figure 35 shows the amounts of radioactivities from $cDNA_{exo}(S)$ hybridized to equal amounts of myeloblast DNA, leukemic erythrocyte DNA and normal erythrocyte DNA at various time intervals during the course of the reaction. Each data point represents the average result from at least three determinations. A 7 µl reaction contained 4.3 µg cell DNA and about 2.2×10^3 cpm $cDNA_{exo}(S)$ which corresponded

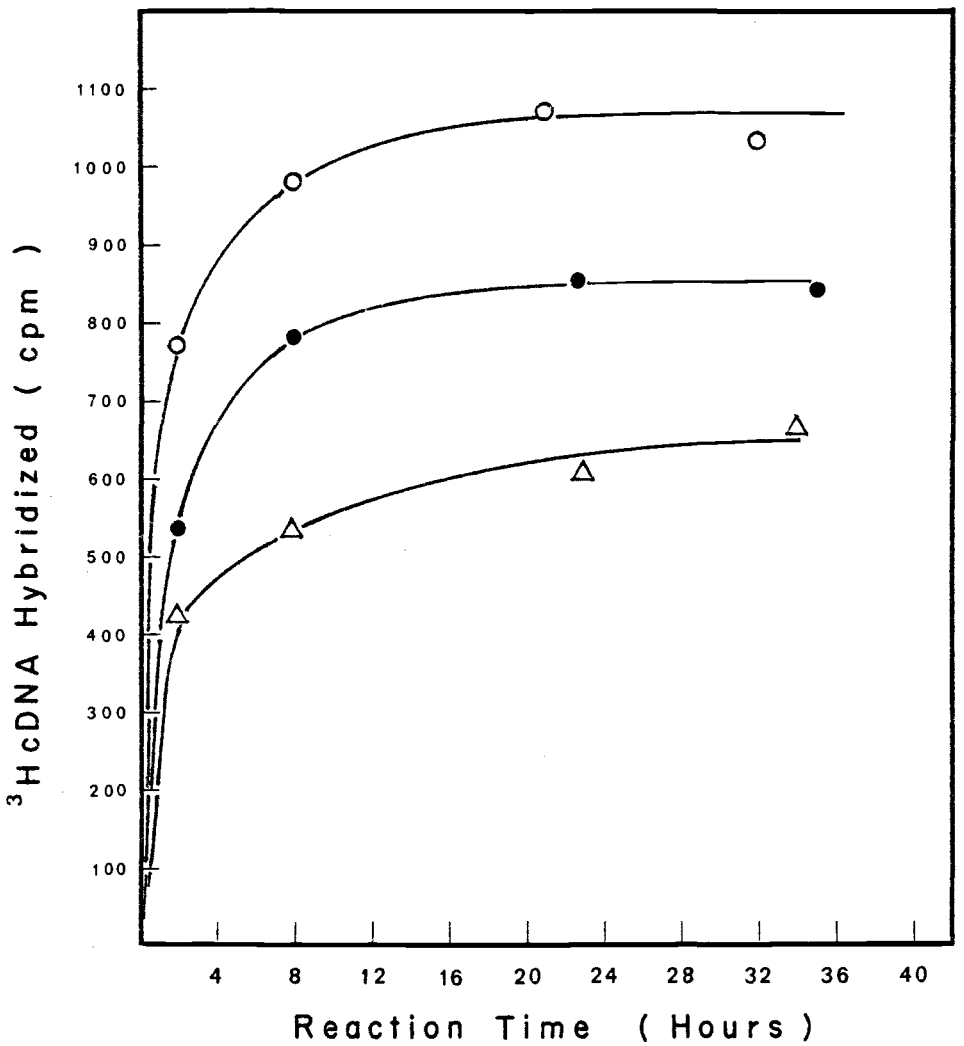


Figure 35. Kinetics of annealing excess $cDNA_{exo}$(S) with chicken cellular DNAs. Each reaction contained about 2.2 X 10^4 cpm [^{3}H]-probe and about 4.2 µg of infected myeloblast DNA (○), or infected erythrocyte DNA (●), or normal erythrocyte DNA (Δ). [^{3}H]$cDNA_{exo}$(S) hybridized were assayed as the amount resistant to S_1-nuclease digestion after anealing.

to 3.18 ng with a specific activity of 6.92×10^3 cpm per ng as determined by kinetic analysis (Fig. 5). Since 2-5% of the probe showed consistent S_1 resistance that has been attributed to self-annealing, control reactions were carried out without cell DNA but with the same amount of probes and under the same conditions as the actual hybridization reactions. These background S_1 resistent activities were subtracted from the hybridized probe activities when the data were presented in Fig. 35. As shown in the figure, all three types of cell DNAs hybridized $cDNA_{exo}(S)$ to saturation levels between 20 to 24 hr. At saturation, myeloblast DNA hybridized a maximum average of 1051 cpm per reaction, leukemic erythrocyte DNA hybridized 847 cpm and normal erythrocyte DNA hybridized an average of 675 cpm.

To calculate the genome equivalents incorporated into cell DNAs, several assumptions were followed: 1) AMV genome has molecular weight of 3.0×10^6 daltons; 2) the $cDNA_{exo}(S)$ represents 72% of total AMV genome (as determined by the hybridization of AMV total cDNA by RSV RNA to 65% out of the possible 90%); and 3) $cDNA_{mye}(S)$ represents 28% of AMV genome. With a specific activity of the probe at 6.92×10^3 cpm per ng and an estimated chicken genome of 2.4×10^{-6} μg, $cDNA_{exo}(S)$ equivalents in myeloblast were calculated to be about 24 copies per cell or 12 copies per haploid genome. Leukemic erythrocyte has a calculated value of 19.4 copies per cell or 9.7 copies per haploid genome and normal erythrocyte contained 15.5 equivalents per cell or 7.7 per haploid genome. These results are summarized in Table 7.

Figure 36 illustrates similar probe excess hybridizations in which

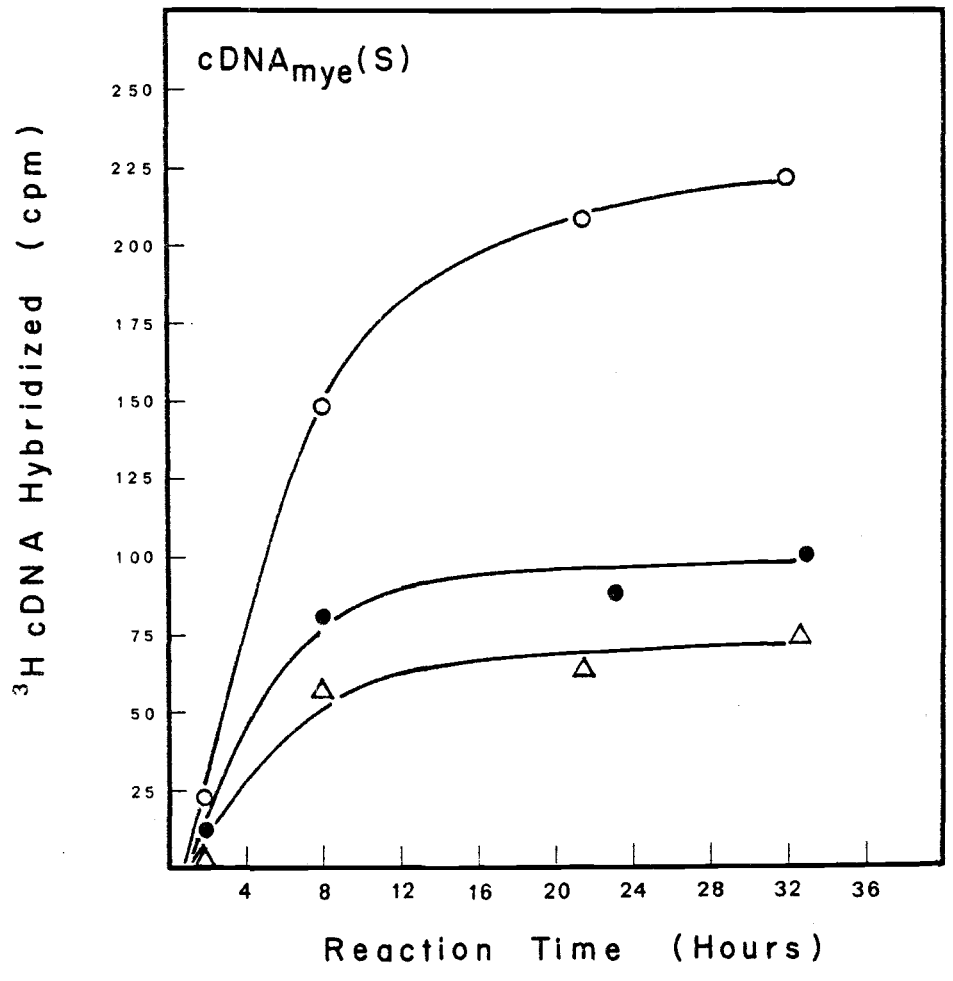


Figure 36. Kinetics of hybridizing excess $cDNA_{mye}$(S) to chicken cellular DNAs. Each reaction contained about 1 X 10^4 cpm [^{3}H]-probe and about 4.8 to 7.1 µg of infected myeloblast DNA (○), or infected erythrocyte DNA (●), or normal erythrocyte DNA (△). [^{3}H]$cDNA_{mye}$(S) hybridized were assayed as the amount of cDNA resistant to S_1-nuclease digestion after hybridization at designated time.

the amount of $cDNA_{mye}(S)$ activities hybridized to the three types of chicken cell DNAs are plotted against reaction times. As in Fig. 35, the maximum levels of probe hybridizations reached plateau values at between 20 and 24 hr. Each 7 µl reaction contained 8.09×10^3 cpm $cDNA_{mye}(S)$, and 4.23 µg cell DNA in the case of myeloblasts, whereas 9.08×10^3 cpm of the probe was hybridized to about 7.15 µg cell DNA in the cases of leukemic and normal erythrocytes. Hybridization conditions, control background measurements and assay procedures were all similar to those performed in Fig. 35. The data presented in Fig. 36 are all corrected to represent 7.15 µg cell DNAs for the purpose of comparisons. Myeloblast DNA hybridized a maximum average of 222.9 cpm which was calculated to be about 7.8 equivalents of $cDNA_{mye}(S)$ per cell or 3.9 equivalents per haploid genome. Leukemic erythrocyte hybridized 100.5 cpm that corresponded to 3.5 probe equivalents per cell or 1.8 equivalents per haploid genome. Normal erythrocyte hybridized an average of 74.5 cpm that represented about 2.6 $cDNA_{mye}(S)$ equivalents per cell or 1.3 equivalent per haploid genome.

The summarized data in Table 7 reveals that although the increase in integrated viral sequences between leukemic and normal erythrocytes is significant, the ratio of integrated equivalents between $cDNA_{exo}(S)$ and $cDNA_{mye}(S)$ in leukemic erythrocyte is not much different from that in normal erythrocyte. But this $cDNA_{exo}(S)$:$cDNA_{mye}(S)$ ratio in myeloblast is significantly less than those in both types of erythrocytes. This smaller ratio in myeloblast indicates that during infection the frequency of integration of sequences unique to AMV in myeloblast is

TABLE 7. SUMMARY OF PROBE EXCESS-CELL DNA HYBRIDIZATIONS TO DETERMINE VIRAL SEQUENCE EQUIVALENTS INTEGRATED INTO THE THREE TYPES OF CHICKEN CELLS.

	Sequence Equivalents per Haploid Genome Determined by Each Probe				
	$cDNA_{exo}(S)$		$cDNA_{mye}(S)$		Ratio
Cell DNA	cpm hybridized	sequence equivalent	cpm hybridized	sequence equivalent	$cDNA_{exo}(S)/cDNA_{mye}(S)$
Leukemic Myeloblast	1051	12.0 (9)[a.]	223	3.9 (5)	3.1
Leukemic Erythrocyte	848	9.7 (4)	101	1.8 (1)	5.4
Normal Erythrocyte	675	7.7	75	1.3	5.9

a. Figures within parentheses represent exogenously acquired sequence equivalents per diploid cell in excess of endogenous sequences in normal erythrocytes.

higher than the frequency of integration of common viral sequences as compared with those integration frequencies in infected erythrocytes.

RELATIONSHIPS BETWEEN AMV AND ITS HELPER VIRUSES

Since a subgroup A virus and a subgroup B virus have been isolated from standard BAI stock of AMV by Smith and Moscovici (117), these two helper viruses, which are subsequently designated as MAV-1 and MAV-2 respectively, have been shown to be capable of rescuing the replicative defective avian myeloblastosis virus from transformed nonproducer cells (22,118). It has been reported that when both MAV-1 and MAV-2 were able to induce nephroblastoma and osteopetrosis in chicken, they were not able to cause myeloblastosis as did a standard stock AMV (51,117). Therefore, a leukemogenic agent distinct from both MAVs is apparently coexisting with its helper viruses within the standard AMV stock, although such an agent free of MAVs has never been isolated. While working on isolating linear viral DNA intermediates from infected cells, Bergamann et al. (51) discovered that cells infected with standard AMV contain a DNA intermediate not present in cells infected with only MAV-1 and MAV-2. Furthermore, Duesberg et al. (23) was able to recover a defective viral particle (DVP) from myeloblasts transformed by AMV in the absence of nondefective helper viruses. Oligonucleotide analysis revealed that RNA from this DVP contained regions homologous to the AMV genes of gag, pol, and common c but not homologous to the env gene. Instead, DVP RNA contains between the pol and the c regions a specific sequence that has no equivalent part in the MAV genomes. All these

evidences indicate that a unique AMV sequence responsible for myeloblastosis could at least be partially absent from the helper MAV viruses. Since a standard AMV stock normally contains more helper viruses than transforming viruses (26) and the defective AMV is very difficult to isolate free from its helper viruses, it is of interest to investigate the relatioship between the unique AMV transforming sequences and the helper viruses MAV-1 and MAV-2.

In our first approach, an attempt was made to isolate AMV specific complementary DNA that had no homolgous counterparts in both MAV-1 and MAV-2 by a negative selection procedure similar to those employed in the isolation of $cDNA_{mye}(A)$ and $cDNA_{mye}(S)$. Total AMV cDNA was prepared by an endogenous reverse transcriptase reaction on virions purified from a standard BAI AMV stock as described previously. The total cDNA was then subjected to exhaustive hybridizations by excessive amounts (20-fold excess in mass) of a combination of purified 60-70s MAV-1 and MAV-2 RNAs. Hybridized and unhybridized cDNAs were separated by hydroxylapatite chromatography and were subsequently recovered from the RNAs. After two cycles of such selection procedures, the unhybridizable cDNAs were designated as $cDNA_{mye}(MAV)$ (as cDNA complementary to meyloblastosis oncogene as isolated by MAV RNAs), and those cDNAs that hybridized to MAV RNAs were recovered and designated as $cDNA_{exo}(MAV)$ in accordance to our previous designation schemes.

The isolation procedure was tested by hybridizing the resultant probes with excess amounts of RNAs from AMV, RAV-0 and MAV-1,2 to C_rt values in excess of 1 mole•sec/litre. The results are compared to

equivalent hybridizations of total AMV cDNA with the same RNAs as presented in Table 7. The 79% of $cDNA_{mye}$(MAV) and 78% of $cDNA_{mye}$(MAV) hybridized by AMV RNA were expected and show the fidelity of the probes. The 80% of $cDNA_{exo}$(MAV) hybridizing with MAV RNAs are also expected since they were isolated from hybrids originally. But the 65% $cDNA_{mye}$(MAV) that hybridized to MAV-1 and MAV-2 are unexpectedly high. It indicates that although some MAV homologous sequences had been eliminated in $cDNA_{mye}$(MAV) the isolation procedure did not produce AMV sequences free of MAV homologies. It also showed that although MAV RNAs and AMV RNAs have a distinct 10% difference in homology when both were hybridized to total AMV cDNAs (83% with MAV RNAs versus 93% with AMV RNA), this difference was not sufficient to allow a distinct resolution of AMV unique sequences from MAV sequences by our isolation procedures.

In our second approach $cDNA_{mye}$(A) isolated by RAV-0 RNA and $cDNA_{mye}$(S) isolated by Pc RSV RNA were hybridized with excess amounts of MAV-1,2 RNAs. The results presented in Table 8 show that while 40% of $cDNA_{mye}$(A) could still be hybridized by MAV RNAs, only about 27% of $cDNA_{mye}$(S) shows hybridization with the same RNAs. This difference can be accounted for by the presence of common c sequences in $cDNA_{mye}$(A) but not in $cDNA_{mye}$(S). In both cases, the MAV homologous sequences had been significantly reduced from the cDNA probes, although never completely eliminated.

Since $cDNA_{mye}$(S) was isolated with Pr RSV RNA which is mainly subgroup C specific, and since the helper viruses MAV-1 and MAV-2 in the

TABLE 8. HOMOLOGIES BETWEEN VARIOUS cDNA PROBES AND RNAs FROM MAV-1 AND MAV-2 IN COMPARISON WITH THOSE FROM AMV AND RAV-0

RNAs	Percent of [^{3}H]cDNA Probes Hybridized by Various RNAs				
	Total $cDNA_{AMV}$	$cDNA_{mye}$(MAV)	$cDNA_{exo}$(MAV)	$cDNA_{mye}$(A)	$cDNA_{mye}$(S)
MAV-1,2	80-83	65	80	40	27
AMV	90-95	79	78	90	85
RAV-0	55-60	43	63	< 5	< 5

AMV stock belonged to subgroup A and subgroup B respectively, it may be argued that residual cDNA sequences complementary to the env genes of MAV-1 and MAV-2 might account for the 26% $cDNA_{mye}(S)$ hybridizable by the MAV RNAs. However, Tal et al. (16) and Hayward et al. (17) have clearly demonstrated that while subgroup A and subgroup C viruses showed nearly 100% homologies in their env genes, subgroup B and subgroup C viruses had env gene homologies in excess of 80%. This slight difference in env genes between Pc RSV and MAV-2 would account for only a small fraction of the 26-27% $cDNA_{mye}(S)$ hybridized by the MAV RNAs. Besides if residual env gene sequences were actually present in $cDNA_{mye}(S)$, they would have been hybridized by the helper virus RNAs in hybridizations with MC29 and AEV RNAs which also belong to subgroups A and B as in Fig. 19 and Fig. 20. All these evidences assure us that the envelope gene sequence has been removed from the cDNA.

One possible explanation for this homology between MAV RNAs and $cDNA_{mye}(S)$ is that Mav-1 and MAV-2 RNAs do contain sequences homolgous to about 26% of the AMV oncogene region. In view of recombinations occurring in RAV-0 (80) and RAV-60 (81,82) between defective leukosis viruses and their helper viruses, and between transformation defective sarcoma viruses and cellular oncogenes (119) plus other evidences of recombinations as described by Eisenman et al. (83), it would be difficult to believe that helper viruses in avian myeloblastosis stocks do not contain at least partial sequences of the oncogene regions. After all, MAV-1 and MAV-2 isolates have been reported to cause osteopetrosis and nephroblastoma, as well as lymphoid leukemia (117,120) in

chickens in the absence of the defective myeloblastosis inducing agent.

DISCUSSIONS

PREPARATION OF PROBES

It has been well established that the retrovirus genome consists of three relatively conserved genes, the gag, the pol, and the env, which are all involved in coding for viral structural and replicative proteins. Also, the avian sarcoma viruses (ASV) and the acute or defective leukosis viruses (DLV) have been shown to possess cellularly derived oncogenes unique to each subgroup of the transforming retroviruses. In fact, oncogene-coded proteins believed to have transformation roles have been isolated from viruses belonging to the AEV group, the MC29 group as well as many subgroups of the sarcoma viruses. However, a definitive oncogene from AMV still eludes isolation mainly because of three major difficulties: the inability of AMV to transform fibroblasts, the poor growth in vitro of the AMV target myeloblasts, and the lack of AMV-specific proteins. Current researches aiming at isolating the AMV oncogene generally employ two approaches. In the first approach, AMV transformed myeloblasts are cultured and non-producer cells are selected to avoid contamination with the helper viruses that are always present in the original infecting AMV stocks. RNAs extracted from these non-producer myeloblasts are then tested for AMV specific sequences (23, 121). In the second approach, DNAs from AMV transformed myeloblasts are extracted and cloned by recombinant phages, and the AMV-specific recombinants are then selected from the recombinant library (24,25,26). In both cases, the detection of such

presumptive oncogenes require AMV-specific probes which are usually radioactive labeled cDNAs negatively selected to remove non-AMV-specific sequences.

The availability of cDNA sequences specific to different regions of the viral genomes has played a valuable role in the elucidation of the genetic structures as well as the functioning of the retroviruses. However, major criticisms of such a cDNA probe have consistently been its purity and its full representation of different viral genetic regions in the same proportion as they occur in the viral genome. In the early stage of the work presented in this report, the preparation of AMV-specific cDNA probes involved much effort in purifying and characterising the products.

The results of annealing 90% total cDNA with AMV RNA under various RNA:cDNA ratios demonstrate that our total cDNA probe has nearly full homology with viral RNA. The fact that maximum level of hybridization was attained at a 1:1 ratio indicates that the total cDNA represents all regions of the RNA in closely the same proportions as they appear in the genome. These conclusions are made possible by the accurate determination of the cDNA specific activities with the probe excess-hybridization kinetic methos of analysis. This analysis is based on the pseudo-first-order reaction expected when trace amounts of (+)strand RNA is reacted with excess (-)strand cDNA. Under these conditions, the excess (-) strand cDNAs cannot react with itself and therefore the concentration of the driver cDNA remains constant as described by Galau et al. (93) instead of the usual second-order ki-

netics for nucleic acid reassociations as described by Britten and Kohne. Figure 5 shows that even a 3% deviation from the determined specific activity will result in an obvious non-linear plot, and this 3% deviation is well within the overall experimental error (52). Therefore our reported copy number of viral genome per cell would not have been distorted by the use of an erroneous specific activity.

The 90% hybridizations obtained from annealing all isolated specific probes ($cDNA_{mye}(A)$, $cDNA_{mye}(S)$, $cDNA_{endo}(A)$, and $cDNA_{exo}(S)$) back to AMV RNA show that the isolation procedures by exhaustive hybridization and subsequent purifications did not dampen the abilities of the fractionated specific cDNA sequences to react with AMV RNA. $cDNA_{mye}(A)$ was prepared from total cDNA by removing sequences homologous to RNA of an endogenous virus RAV-0. When $cDNA_{mye}(A)$ was annealed back to RAV-0 RNA, the resultant level of hybridization was no more than background, thereby proving the effectiveness of the isolation procedure in fractionating the total probe.

ISOLATION OF cDNA PROBES HOMOLOGOUS TO UNIQUE AMV SEQUENCES

In the experiments described by Figures 13 through 16, two considerations have been successfully accounted for. First, it was demonstrated that $cDNA_{mye}(A)$ contained common regions homologous to exogenous viruses Pr-RSV and MC29. Second, the removal of these regions from $cDNA_{mye}(A)$ did not impair the hybridization of the remaining cDNA with AMV RNA or with AMV sequences integrated in cellular DNAS. These results therefore justified the isolation of $cDNA_{mye}(S)$ by

exhaustively hybridizing total AMV cDNA against Pr-RSV RNA. When $cDNA_{mye}(S)$ was reacted with RNAs from Pr-RSV (Figure 18) and also from RAV-0 (Figurel2), low background levels of hybridization indicated that $cDNA_{mye}(S)$ was indeed free of homologies from endogenous as well as exogenous viruses. Such characterisations of our probes are especially important for the reliable quantitative studies of AMV integration during infection.

To further demonstrate the specificity of the AMV unique sequences in $cDNA_{mye}(S)$, the probe was hybridized to RNAs from other exogenous viruses, SR-RSV, AEV, and MC29, each of which represents a separate subgroup of ARV with distinctively identified oncogenes. Data from Figure 19 through Figure 21 illustrate that while all these exogenous viruses show extensive homologies with the total AMV cDNA, none of them has any homology with $cDNA_{mye}(S)$, therefore reinforcing the specificity of $cDNA_{mye}(S)$. Although the implication of $cDNA_{mye}(S)$ representing a probe for AMV specific oncogene is still circumstantial at best, it nevertheless strengthens the belief that subgroups of avian acute leukemis viruses (DLVs) and sarcoma viruses (ASVs) contain different classes of distinctive oncogenes.

Further evidence for different classes of oncogenes have been provided by the discoveries of transforming proteins as oncogene-coded products from a Feline sarcoma virus (FeSV), a Fujinami sarcoma (FSV) virus, as well as from the Abelson murine leukemia virus (A-MuLV) in addition to the well studied $pp60^{src}$ coded by the oncogene of ASV. These proteins are all protein kinases that phosphorylate tyrosine

residues, and yet they are all structurally very different. For example, the p120 protein encoded by A-MuLV oncogene and $pp60^{src}$ encoded by ASV both phosphorylate tyrosines in vinculin (a protein believed to be associated with cytoskeleton for cell adhesion) and in a cellular p36 protein substrate (122). Most of the different oncogenes studied thus far show endogenous counterparts not only in normal hosts but also in other vertebrate species (69, 70). In addition, Chinkers and Cohen (123) have identified an epidermal growth factor (EGF)-associated protein kinase in normal cells that also phosphorylates tyrosine, yet this EGF-associated kinase is at least antigenically different from $pp60^{src}$ and from endogenous $pp60^{sarc}$. If all the viral oncogenes are indeed derived from cellular genes, then apparently the normal vertebrate genome can code for a host of structurally different proteins with similar functions which occur only rarely in the normal cells.

It is still a mystery as to how these protein kinases bring on transformation in their target cells. Anderson et al. (124) studied the cellular effects of different partial mutants of RSV with different deletions in the oncogenes and discovered that these partial mutants produced different combinations of phenotypic changes, thereby suggesting that rather than interacting with one single primary target substrate in the host cell and subsequently inducing a cascading effect, $pp60^{src}$ probably acts on several different primary target substrates. Furthermore, Parry et al.(125) found that when cells infected with temperature-sensitive mutants of ASV were arrested in the

G_1 stationary phase by serum starvation at 41°C (nonpermissive temperature), the cells behaved differently from similarly arrested uninfected cells even though the ts-pp60^{src} was supposed to be nonfunctional at this temperature. These results suggest that other than the apparent effect of the specific oncogenes, viral infection may turn on other abnormal cellular functions, a point to be further substantiated.

One evidence in support of the above observation comes from the studies of AEV transforming genes by Beug et al.(126). Upon infection AEV synthesizes a p75 protein which is the combination of an AEV oncogene (erb) encoded product and a partial gag gene product. A transformation mutant with deletion in the erb region of p75 fails to transform erythroblast in vivo, but is still capable of transforming fibroblasts. This result indicates that while tissue specific transformation may depend on the expression of an intact specific oncogene, other transformation phenotypes may not require the entire oncogene sequence. In fact, as will be discussed later, lymphatic leukosis viruses induce transformation without the help of any known viral oncogenes. The fact that AMV is able to transform myeloblast type hemopoietic cells in culture but not fibroblast (see Table 1) may then be explained by the possible lacking of certain sequences in the mye gene (myeloblast oncogene) required for fibroblast transformation but not for myeloblast transformation. This informations lead us into our next area of investigation, namely, the integration of AMV oncogenes in target and non-target tissue cells.

TARGET CELL STUDIES

Since it is known that viral oncogenes have endogenous counterparts not only in their normal hosts, but also in other species, we decided to investigate the presence of AMV oncogene sequences in other uninfected avian species. AMV has been shown by earlier reports to have from 1% to 10% homologies in different normal avian species (27,30). But these early studies did not differentiate between AMV-specific oncogene sequences and sequences of the endogenous virus which have also been shown to be present in various avian species, especially in pheasant and turkey. Our results of hybridizing fractionated AMV cDNA probes to cellular DNAs from five avian species in addition to normal chicken and to calf thymus DNA indicate that indeed the AMV-specific sequences appear to be present in all cell DNAs tested at a higher homology levels than the non-AMV-specific sequences ($cDNA_{endo}(A)$) (Table 6). Chen (127) has also shown that AMV-specific RNAs can be detected in different uninfected chicken tissues. The significance of these findings is twofold: 1) the data support the hypothesis that oncogenes are derived from cellular origin, whereas the virogenes (the non-AMV-specific sequences) are derived from extracellular sources; and 2) the percent homologies between AMV oncogene sequences and those endogenous oncogene sequences ocurring in different avian species roughly correspond to their purported phylogenic relationships, which assumes that chicken, pheasant, turkey and quail are evolutionarily closer related to each other than to goose

and duck (128).

The actual determination of the specific target cells for each of the three subgroups of defective leukosis viruses (DLVs) encounters a major difficulty in that pure populations of target cells are difficult to isolate, since DLVs usually transform less than 1% of the bone marrow cells in vitro. However, it has been shown that bone marrow cells transformed by AEV,AMV, and MC29 in culture resemble erythroblasts, myeloblasts, and macrophages correspondingly (129). Boettiger and Durban (130) earlier had shown evidences indicating that AMV target cells exihibit markers characteristic of differentiated cells and subsequently suggested that even though DLV infections may occur at an undifferentiated progenitor cell, transformation could be triggered at a later stage depending on normal differentiation. Graf, von Kirchbach, and Beug (131) recently demonstrated that bone marrow cells separated on the bases of such characteristics as substrate adherence, phagocytic activities, and surface antigens corresponding to erythroblasts, myeloblasts, and macrophages, showed different susceptibilities towards different DLVs, therefore indicating different separable populations of target cells. By using specific antisera against each type of the purported target cells, they were able to eliminate from bone marrow cells the specific targets of the corresponding DLVs. Graf and his co-workers therefore concluded that DLVs specifically transform different types of bone marrow cells already committed to erythroid or myeloid differentiation, and thereby supporting the observations made by Boettiger and Durban. We then

investigated the possibilities of DLVs infecting non-target cells in the absence of apparent transformations.

Hybridizations of $cDNA_{mye}(S)$ to chicken cellular DNAs from infected myeloblasts, from infected erythrocytes as well as from uninfected erythrocytes clearly indicate that AMV oncogenes have been integrated into both infected target cells (myeloblasts) and infected non-target cells (erythrocytes) (Figure 28). DNAs from both infected cell types hybridized the $cDNA_{mye}$ probes to considerably higher levels than those obtainable from uninfected erythrocytes. One can argue that the sequences detected in infected erythrocytes may be the integrated proviruses of the helper viruses MAV-1 and MAV-2 which are always present in an AMV stock. But since RNAs form MAV-1 and MAV-2 can only hybridize about 27% of $cDNA_{mye}(S)$ (Table 8), and if the 50% $cDNA_{mye}(S)$ hybridized by infected myeloblast DNA represents the maximum hybridization level experimentally attainable under these conditions, then MAV-1 and MAV-2 proviruses integrated into infected erythrocytes should only hybridize a maximum of about 15% of $cDNA_{mye}(S)$. Therefore, the 48% $cDNA_{mye}(S)$ hybridized by infected erythrocyte DNA provides unequivocal evidence for the presence of integrated AMV oncogenes in non-target cells. Hybridizations of non-AMV-specific sequences ($cDNA_{exo}(S)$) by the DNAs of the three chicken cell types (Figure 29) then illustrate the presence of integrated AMV replicative genes and integrated helper proviruses in infected cells, as well as endogenous proviruses present in both the uninfected and infected cells. Since we were working with myeloblasts and mature erythrocytes

that can be easily separated from peripheral blood collections with less than 0.5% cross contamination, our data are relatively free from the contamination problems encountered by other workers who attempt to separate target blast cells from bone marrow extractions.

Other evidences supporting our observation come from the works of Graf, Beug, and Hayman (132). These workers have demonstrated that AMV and MC29 can superinfect as well as replicate in erythroblasts already transformed by AEV. Likewise, MC29 can replicate in myeloblasts already transformed by AMV in culture. These findings indicate that DLVs can indeed infect non-target cells. Furthermore, these same workers were able to show that when erythroblasts were transformed by temperature-sensitive mutant AEV at 35°C, and then were superinfected with AMV or MC29, the subsequent erythroblast differentiation to mature erythrocytes at 41°C (which inhibits the functioning of the AEV transforming protein) is not interfered by the replication of the superinfecting nonhomologous DLVs, and thus reinforcing the observation that when DLVs infect non-target cells, their transforming abilities are not expressed.

Souza and Baluda (133) recently have used AMV-specific probes to detect the presence of integrated AMV proviruses in various infected chicken tissue cells. They hybridized the probes to filter-bound cellular DNA restriction fragments separated by gel electrophoresis and showed that AMV can also integrate into chick embryo fibroblasts without inducing transformation. They were also able to detect quite readily helper virus sequences in erythrocytes from leukemic chicken,

but were only able to detect very faint bands of AMV-specific sequence from the same DNAs. Our results indicate that their difficulty in detecting AMV oncogenes in infected erythrocytes by the Southern transfer and filter hybridization method may be of a quantitative nature rather than a qualitative nature, as illustrated by the results of our next investigation.

QUANTITATIVE STUDIES ON INTEGRATED AMV SEQUENCES

As have been described in an earlier section of this report, the amount of AMV sequences integrated into infected cells has been reported as ranging from nine to twenty genome equivalents per cell. Shoyab et al. (54) had earlier reported that each AMV transformed myeloblast contained about twenty AMV genome equivalents, which is about twice as much as those detected in a single uninfected cell (nine to ten copies per normal cell). By using probe excess hybridizations, Heilman et al. (52) later also detected about twenty AMV equivalent copies per transformed myeloblast and as much as 12 copies per erythrocyte from AMV infected chicken, but they were able to detect only five to six copies per uninfected cell. Since these workers were using unfractionated AMV total cDNA as probes, they were not able to distinguish between the sequences of endogenous proviurses which are homologous to the replicative genes of most ARVs and the sequences of cellular oncogenes (proto-oncogenes or endogenous oncogenes) which are homologous to the exogenous AMV oncogenes. When we used the fractionated AMV cDNA specific sequences as probes, we were able to

gain better insights into the quantitative aspects of AMV infection.

Summarized data in Table 7 show that an average diploid normal chicken cell contains about 15 copies of sequences homologous to the AMV replicative genes (detected by the $cDNA_{exo}$(S) probe). This value reflects the abundance of endogenous proviruses in this particular line of Cornish Cross chicken which has more endogenous proviruses than the usual White Leghorn chickens used by some other workers (Maxine Linial, personnel communication). Our results also show that a normal chicken cell contains only two copies of the endogenous sequences homologous to the AMV oncogene. This difference in copy numbers between endogenous provirus and cellular endogenous oncogene might have contributed to the lower estimates of endogenous provirus in normal cells reported by other workers such as Heilmann et al. and Shoyab et al. who used total cDNA probes. Khoury and Hanafusa (56) in a later report, used the cDNA probe from a lymphatic leukosis virus RAV-2 (which has better homology to endogenous virus than AMV) and detected about 14 copies per cell of the endogenous proviruses in normal cells. This value is in agreement with the one detected by our $cDNA_{exo}$(S) probe.

In the case of the infected erythrocytes, we found about 19 copies of the replicative genes per cell, and about three copies per cell of the oncogene sequences. By subtracting from these values the copy numbers of the endogenous provirus and the endogenous oncogene from normal cell, we obtained four copies of exogenous replicative gene equivalents and only one copy of exogenous AMV oncogene equivalent

integrated into each cell by infection. Since the exogenous replicative genes can be derived from the MAV helper viruses as well as the AMV, we concluded that for an average erythrocyte infected with AMV stock, one AMV provirus integrates along with three helper proviruses, and this phenomenon may explain the difficulty encountered by Souza and Baluda who had trouble detecting the exogenous oncogenes in AMV-infected erythrocytes as described earlier.

In the case of the transformed myeloblasts, approximately 24 copies of the replicative sequences can be detected per cell, and this value is equivalent to nine copies of exogenous replicative genes per cell acquired by infection. About seven copies of oncogenes can be found in each cell, and likewise five of these should correspond to the integrated exogenous AMV oncogenes. These data indicate that AMV infection results in the integration into a myeloblast on the average five AMV proviruses along with four helper proviruses. Since the number of helper proviruses integrated into myeloblasts and erythrocytes are approximately equivalent, and since AMV has been shown to be able to replicate in erythrocytes, our results suggest that myeloblasts may be able to integrate AMV proviruses more efficiently than erythroblasts, and that transformation may depend on the provirus integration site as well as the number of such sites being occupied by the appropriate provirus.

We can now summarize the current findings concerning the integrations and transformations of the avian retroviruses. For ARVs in general, the pertinent informations can be summarized as follow:

1) cell division is required for viral expression regardless of whether or not the cell is already producing a similar virus (134); 2)the number of integrations per cell for each type of virus appears to be limited (our data), and excessive integrations may induce cytopathic effects on the host cell (135); 3) different types of virus do exihibit certain degrees of integration interference (58); 4) multiple integrations show higher efficiency of viral expression than single integration (59); and 5) viral long terminal repeat (LTR) sequences integrated in the proximity of a cellular gene may induce the expression of that particular gene (downstream promotion) (136,137) and thus has been postulated to be the mechanisms by which ARVs lacking the oncogenes induce neoplasms.

For the acute leukosis-sarcoma viruses (ASVs and DLVs) in particular, the following general characters prevail; 1) early in infection viral genes in the cytoplasm can be expressed and can subsequently induce transformation even before the integration of the transforming proviruses (35,138) or of the associated helper proviruses (50); 2) oncogenes can integrate and replicate in non-target cells or revertant cells without inducing any transformed phenotypes in the host (34,132 and our data); and 3) in revertant cell, the oncogenic provirus can be rescued by helper viruses and regain its transforming ability upon second infection, therefore implying that cellular regulations can subpress oncogene expression even in target cells (139). With this information, we can attempt to speculate on the events of AMV infection.

According to a recent model of hemopoietic cell differentiation as described by Till and McCulloch (140), differentiation from a progenitor cell proceeds via a large array of parallel pathways, which are assembled from a large number of genetically or microenvironmentally directed components. Only certain such assemblies yield licit programs for the generation of a particular differentiation end product. A progenitor cell committed to the myeloid lineage therefore has functioning assemblies of pathways quite different from those of a progenitor cell committed to the erythroid lineage. Transformation arises if enough of the legitimate assemblies are disrupted and the cell loses its direction of differentiation. If only a fraction of these licit assemblies are disrupted, then partial transformation results, and if the disrupting conditions are reversed, then the cell will regain its control of differentiation.

To apply this model to AMV transformation, we propose that at the early stage in AMV infection of a myeloid destined progenitor, the AMV oncogene encoded transforming protein probably disrupts enough licit pathway assemblies that the cell loses its differentiating capability and is arrested at the myeloblast stage. Either due to the effect of this disruptive action by the virus, or due to the licit pathways of a committed program, the cell allows multiple integrations of the AMV proviruses into its genome. The resultant excess of integrated AMV proviruses will then serve to maintain the transformed state of the cell by providing more AMV sequences to withstand mutational pressures during the clonal evolution of the original infected cell. The effect

of multiple integration is probably threefold. First, the AMV proviruses will be more efficiently expressed. Second, the AMV proviruses may have more chances of integrating near a susceptible cellular gene and induce its expression by neighbourhood promotion, thereby causing a spectrum of neoplasms characteristic of the long term diseases induced by the lymphatic leukosis viruses which lack the oncogenes, or resulting in the frequent tumors in survivors of acute leukosis virus. And third, by occupying more integration sites, the AMV may be able to interfere with the integration of other types of ARVs, thereby limiting the transforming potentials of other oncogenes. The end result of all these will be more stably transformed cells.

If the infected cell is a progenitor cell committed to the erythroid lineage, then the AMV transforming protein either has no effect at all or is only effective to a small fraction of the pathway assemblies ligitimate for erythroid development, and the cell is not transformed. Either because of the property of the cell, or because of the ineffectiveness of the AMV transforming protein in a non-target cell, AMV provirus integration is limited, and the cell can easily overcome whatever adverse effect the provirus can induce. As a result, the AMV provirus is perfectly capable of directing the synthesis of progeny viruses, but the cell is never under any real threat of transformation.

Verification of this model of AMV infection will depend on the further investigation of the action of AMV transforming proteins, the comparison of integration sites of AMV proviruses into target and

non-target cells as well as revertant cells, and the elucidation of the proviral integration mechanism.

At the mean time, to finally close this discussion, it is only appropriate to again quote W. P. Rowe one more time as he answered in 1973 to the question of whether C type viruses cause human cancer, " the answer to this question is now, ' It doesn't matter. ' That is, the C type virus is so integral a part of the cell (and this almost certainly will be found for human cells as well) that its expression in malignant cells is in itself sufficiently important and potentially exploitable that whether the viral genomes are the cause, in a formal or semantic sense, is of little additional practical importance. "

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APPENDIX

APPENDIX

The abbreviations and nomenclatures used in this report are as follows:

AEV	Avian Erythroblastosis Virus
ALV	Avian Leukosis Virus
AMV	Avian Myeloblastosis Virus
ARV	Avian retrovirus
ASV	Avian sarcoma virus
cDNA or total cDNA	Total unfractionated cDNA complementary to AMV RNA
$cDNA^{mye}$ (A)	AMV-specific cDNA fractionated by RAV-0 RNA
$cDNA^{endo}$ (A)	Non-AMV-specific cDNA fractionated by RAV-0 RNA
$cDNA^{mye}$ (S)	AMV-specific cDNA fractionated by Pr-RSV RNA
$cDNA^{mye}_{exo}$ (S)	Non-AMV-specific cDNA fractionated by Pr-RSV RNA
DLV	Avian defective leukosis virus
LLV	Lymphatic Leukosis virus
MC29	Myelocytomatosis Virus Strain 29
MuLV	Murine Leukosis Virus
RSV (Pr)	Rous Sarcoma Virus Prague Strain
RSV (SR-D)	Rous Sarcoma Virus Schmidt-Ruppin Strain-subgroup D
RAV	Rous Associated Virus (belongs to the LLV group)
RAV-0	Endogenous RAV
MAV	Myeloblastosis Associated Virus
AEAV	Erythroblastosis Associated Virus
MCAV	Myelocytomatosis Associated Virus
td ASV	Transformation Defective Avian Sarcoma Virus
ts ASV	Temperature-sensitive Mutant of Avian Sarcoma Virus
gag (G)	Viral gene encoding the structural proteins of virion core
pol (P)	Viral gene encoding reverse transcriptase
env (E)	Viral gene encoding virion envelope proteins
src (S)	Transforming gene (oncogene) of sarcoma virus
sarc	Cellular equivalent of viral src gene
mye (M)	Transforming gene of AMV
Myeloblast	Myeloblasts used in the experiments reported here were all collected from peripheral blood of leukemic chicken
Erythrocyte	Infected erythrocytes were collected from peripheral blood of chicken infected with AMV
TNE	0.01M Tris-(hydroxymethyl)aminomethane, 0.15M NaCl, 0.01M ethylenedinitrilo-tetraacetic acid (EDTA).
TN	TNE without EDTA
E buffer	Electophoresis buffer: 0.04M Tris(hydroxymethyl)amino-methane, 0.001M EDTA, 0.02M Na Acetate, pH7.2.