METHYLATION OF FELINE LEUKEMIA VIRUS VIRION AND INTRACELLULAR RNA

By

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ABSTRACT

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Several methods for the extraction of RNA from the Rickard strain of feline leukemia virus (R-FeLV) were compared, and the method which resulted in the highest yields of viral high molecular weight RNA was selected for use in subsequent experiments. Two major size classes of native RNA were detected in R-FeLV, one sedimenting at 50-60S and the other at 4S. When native 50-60S RNA was denatured with dimethylsulfoxide (Me_2SO) and centrifuged through sucrose gradients in 99% Me_2SO, subunits of approximately 28S were obtained. It was shown that the subunits obtained from such gradients migrated more slowly during polyacrylamide gel electrophoresis than 28S ribosomal RNA. The native 4S RNA obtained from sucrose gradients was resolved by electrophoresis in 7% polyacrylamide gels into four distinct classes. Two of these low molecular weight RNAs co-migrated with cellular 4S and 5S RNA while the other two slower-migrating RNAs were probably forms of the 7-8S RNA reported in other oncornaviruses.

The high molecular weight subunit RNA of R-FeLV was analyzed for the presence of methyl groups. Following purification of native 50-60S F-FeLV RNA on nondenaturing aqueous sucrose density gradients,

R-FeLV 28S subunit RNA, doubly labeled with (^{14}C) uridine and (methyl-^{3}H) methionine, was isolated by centrifugation through denaturing sucrose density gradients in dimethylsulfoxide. As calculated from their respective ^{3}H/^{14}C ratios, R-FeLV 28S RNA was methylated to the same degree as host cell poly A^{+} mRNA. When the 28S R-FeLV RNA was hydrolyzed to completion with RNAase T2 or alkali all of the methyl-^{3}H chromatographed with mononucleotides on Pellionex-WAX, a weak anion exchanger. The methyl-labeled material co-chromatographed with 6-methyladenosine if the mononucleotide fraction obtained by Pellionex-WAX chromatography was hydrolyzed to nucleosides by bacterial alkaline phosphatase or with 6-methyladenine if purine bases were released from the mononucleotides by acid hydrolysis. In another experiment in which R-FeLV 28S RNA uniformly labeled with ^{32}P was hydrolyzed and then analyzed by Pellionex-WAX chromatography, all of the ^{32}P label again co-chromatographed with mononucleotides. Thus R-FeLV 28S RNA does not appear to contain a 5' structure, either methylated or nonmethylated, similar to those recently reported for cellular and some animal virus mRNAs.

It was found that purification of a polynucleotide by hybridization to its mercurated complementary sequence and chromatography on sulfhydryl-agarose (SH-agarose) was unsatisfactory, due to the instability of SH-agarose prepared by the conventional cyanogen bromide technique. This technique was improved by the use of SH-agarose in which the sulfhydryl group is attached to the agarose through a stable ether linkage. In addition, a method was developed whereby hybridized RNA or DNA could be recovered from the SH-agarose column separately from the mercurated probe. This

methodology was applied to the purification of R-FeLV intracellular RNA from host cell RNA.

The F-422 line of feline thymus tumor cells, chronically infected with R-FeLV was labeled with ^{32}p and the total cytoplasmic RNA was isolated. The RNA was centrifuged through sucrose gradients and R-FeLV virus-specific RNA (vRNA) was located by hybridization of portions of the gradient fractions to R-FeLV complementary DNA (cDNA). Virus-specific RNA classes with average sedimentation coefficients of approximately 36S, 28S, 23S, and 15S were identified. Each class of RNA was recovered and hybridized with R-FeLV mercurated cDNA, and the hybrids were chromatographed on columns of sulfhydryl-Sepharose to separate them from unhybridized cellular RNA. While insufficient amounts of 36S and 28S vRNA were obtained for further analysis, the 23S and 15S vRNA classes were analyzed to determine the nature of their 5' termini. Each of these vRNA classes was found to contain cap structures in amounts sufficient to account for approximately one cap per molecule. The structure of the 23S vRNA cap was found to be $m^7G^{5'}ppp^{5'}GmpAp$ while that of the 15S vRNA cap was $m^7G^{5'}ppp^{5'}GmpGp$. The possible relationship between R-FeLV and other defective RNA tumor viruses is discussed.

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PART IV

LIST OF ABBREVIATIONS

Am	2'-O-methyladenosine
cDNA	complementary deoxyribonucleic acid
Cm	2'-O-methylcytidine
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
EDTA	ethylenediaminotetraacetate
FeSV	feline sarcoma virus
FLF	feline lung fibroblasts
Gm	2'-O-methylguanosine
m^6A	6-methyladenosine
Me_2SO	dimethylsulfoxide
m^7G	7-methylguanosine
mRNA	messenger ribonucleic acid
MuLV	murine leukemia virus
Oligo (dT)	oligodeoxythymidine
Poly A	polyadenylic acid
R-FeLV	Rickard strain of feline leulemia virus
RAV	Rous-associated virus
RNA	ribonucleic acid
RNAase	ribonuclease
rRNA	Ribosomal ribonucleic acid
RSV	Rous sarcoma virus

SDS	sodium dodecyl sulfate
SH-agarose	sulfhydryl-agarose
tRNA	transfer ribonucleic acid
Um	2'-O-methyluridine
vRNA	viral ribonucleic acid

LITERATURE REVIEW

ONCORNAVIRUSES

Definition and Classification

RNA-containing viruses capable of inducing leukemias or solid tumors have been isolated from a number of vertebrate species. These viruses possess common morphological and biochemical properties but can be distinguished on the basis of their immunological and biological characteristics. They are referred to as RNA tumor viruses, oncornaviruses, or "C-type" or "B-type" particles. The first two terms reflect the most outstanding attribute of this class of viruses, i.e., their ability to induce malignant transformation of the cells they infect. In many cases a viral isolate may possess morphological and biochemical properties similar to known RNA tumor viruses yet not have been shown to be oncogenic. These viruses are usually just referred to as "C-type" viruses, a name applied to RNA viruses with certain morphological features as visualized under the electron microscope; "B-type" viruses have a related but somewhat different structure and are only rarely isolated. C-type viruses have been isolated from chickens, mice, rats, hamsters, guinea pigs, cats, snakes, cattle, and several primates including man. Some common properties of oncornaviruses have been described by Nowinski *et al.* (99).

The known oncogenic C-type viruses are divided into the leukemia viruses, which cause malignancies of the reticuloendothelial system in susceptible animals, and the sarcoma viruses which induce

solid tumors of connective tissues (145). More importantly from an experimental point of view, sarcoma viruses transform fibroblasts in tissue culture (144) whereas leukemia viruses do not. Most avian sarcoma viruses, and all known mammalian sarcoma viruses, are replication-defective (145); i.e., they are unable to replicate in the absence of a helper virus. All infectious stocks of mammalian sarcoma viruses are actually mixtures of pseudotype sarcoma virus and helper leukemia virus. The sarcoma virus is referred to as a pseudotype since it carries the envelope proteins specified by the helper virus. Sarcoma viruses can be shown to be capable of transforming cells in the absence of helper virus, but such cells produce no infectious virus. Infectious tumor virus can be recovered from these so-called transformed non-producer cells by superinfection with helper virus (64).

Leukemia viruses are generally considered to be replication positive, transformation negative (r+t-), meaning that they can replicate in the absence of a helper virus and do not transform fibroblasts in culture. However, the Abelson leukemia virus which was produced by passage of a strain of murine leukemia virus in prednisolone-treated BALB/c mice (136) can transform fibroblasts in culture (116, 123, 133) but produces B-cell and null cell leukemias, not sarcomas, when injected into mice (123, 126). Abelson virus is also replication defective (123). In addition, the attenuation of some leukemia viruses in tissue culture (132, 163) suggests that some leukemia viruses may become replication defective. Thus the division between leukemia viruses and sarcoma viruses may not be as clear as previously thought.

Composition

The RNA tumor viruses have similar overall chemical compositions, being composed of 60-70% protein, 20-30% lipid, 2% carbohydrate, and about 1% RNA (8). Much if not all of the protein is virus-coded. The virus-coded proteins can be separated into three groups: (a) the low molecular weight (core) structural proteins, (b) the envelope glycoproteins, and (c) the RNA dependent DNA polymerase, located in the viral core. These proteins have been extensively characterized for avian (10, 45, 46, 69), murine (63, 98), and feline (61, 63) oncornaviruses. All of the lipids found in the coats of oncornaviruses are also found in uninfected cells, indicating that they are of cellular origin (110).

When native (non-denatured) RNA is isolated from oncornaviruses and subjected to sedimentation velocity analysis, two general size classes are detected (4, 13, 31, 39, 72, 87). The larger RNA, corresponding to the viral genome, sediments at about 50-70S. The smaller RNA class sediments at about 4S. Treatment of the 50-70S RNA with heat or other hydrogen bond-disrupting agents converts the 50-70S RNA to 28S-40S with the release of smaller RNAs (see below).

Replication

To account for data which indicated that DNA synthesis was an obligatory step for successful oncornavirus replication following cell infection (142), Temin (141) proposed that an early step following infection was the synthesis of a DNA copy of the viral RNA. This unpopular hypothesis gained credibility with the discovery in 1970 of a viral enzyme, RNA dependent DNA polymerase (reverse transcriptase), which catalyzes the synthesis of DNA on an RNA template (5, 143).

Since that time much more has been learned about the life cycle of RNA tumor viruses. The first step in infection of the host cell is the binding of the virus to appropriate cell surface receptors, after which the virion is internalized by phagocytosis and rapidly transported to the nucleus in vacuoles (23). DNA complementary to the viral RNA is apparently synthesized in the cytoplasm (151, 152) by the reverse transcriptase during transport to the nucleus. The unintegrated DNA found in infected cells includes linear (135) and closed-circular (57) double-stranded forms; both are infectious in appropriate host cells (134, 135). The viral DNA subsequently becomes integrated into the host cell genome (90, 130, 153) where it is replicated along with the cellular DNA and vertically transmitted from cell generation to generation. Integrated viral DNA then serves as the template for synthesis of new virion RNA and virus-specific mRNA (62, 103).

Virion RNA

As mentioned earlier, the RNA found in the virions of oncornaviruses can be separated into two major size classes by sedimentation through sucrose gradients. The low molecular weight RNA contains transfer RNA which is capable of being aminoacylated with a variety of amino acids (36, 56, 146, 157). The minor base composition of this RNA class was found to be similar to cellular tRNA (111). Analysis of the isoaccepting species of tRNA with the seven greatest amino acid acceptance capacities in avian myeloblastosis virus revealed no entirely new or missing tRNAs as compared to normal chicken cells (56). One of the primary questions concerning oncornavirus tRNA is whether these molecules are incorporated into the virion fortuitously or by

viral direction. The most direct evidence concerning this question comes from the work of Wang et al. (157). Two strains of Rous sarcoma virus (RSV) were used in this study. One of them, the Bryan strain, requires the presence of a helper virus (RAV) for a productive infection, and is referred to as RSV(RAV). The other strain of RSV, Schmidt-Ruppin, is not defective and requires no helper virus. When RSV was grown alone it had a tRNA population very different from RAV grown alone in the same type of cells. However, the tRNA population of RSV was very similar to that of RSV(RAV) even though RAV is present in a ten-fold excess in the latter case. The authors conclude that in the RSV(RAV) mixed infection, it is the RSV that controls which tRNAs will be selected for inclusion in the virions.

Analysis of low molecular weight oncornavirus RNA by polyacrylamide gel electrophoresis or chromatography on methylated albumin kieselguhr allows the detection of several small RNAs other than tRNA which are not resolved by sedimentation in sucrose gradients (9, 111). One of these small RNAs is 5S RNA, identical to cellular ribosomal 5S RNA. Another small RNA is about 7S or 8S in size and had not previously been detected in cells. This RNA is single stranded, with a molecular weight of approximately 80,000, constitutes 3-5% of the RNA in RSV, and is not detectably methylated (9). It can exist in two configurations, the "a" and "b" forms, which are separable by gel electrophoresis (114). A subsequent analysis of uninfected cells revealed the presence of a 7S RNA identical to that contained in oncornaviruses (38). Although the function of the 7S RNA is unknown, it is found intracellularly associated with polyribosomes at a level of approximately one molecule of 7S RNA per molecule of messenger

RNA (156).

When the 50-70S RNA of C-type viruses is subjected to heat, dimethylsulfoxide, formamide, or other denaturants, it is irreversibly converted to 28-40S subunits with the concomitant release of associated small RNA molecules (4, 37, 92). The small molecules consist of 4S, 5S, and 7-8S RNAs similar to those which exist free in virions (43, 96). All of these small RNAs except one component of the 4S RNA are released at a temperature lower than or equal to the temperature at which 50-70S RNA is converted to its 28S-40S subunits (22). The one 4S RNA which is dissociated from the 28-40S RNA at a higher temperature serves as primer for the initiation of DNA synthesis by reverse transcriptase (22); it has been identified as tryptophan tRNA in avian oncornaviruses (65) and proline tRNA in murine oncornaviruses (109).

For a long time the number of 28-40S RNA subunits in the 50-60S RNA complex was in doubt, with most authors favoring 3-4 subunits. In addition, whether or not the subunits were different (resulting in a haploid genome) or identical (yielding a polyploid genome) was an often debated question. Results in the last few years have convincingly demonstrated that there are two identical subunits in the 50-70S RNA complex (30, 33, 79, 82, 83, 113, 161). Especially noteworthy was the elegant work in Davidson's lab (30, 82, 83) in which a technique was devised which allowed spreading of the 50-70S RNA for electron microscopy without extensive dissociation into its subunits. This work showed that the high molecular weight RNA molecules are dimers of the subunits non-covalently attached near their 5' ends. Models were suggested (83) for the pairing of two identical subunits

at their homologous, rather than opposite, ends; two of these models would also account for the observation that denatured 50-70S RNA cannot be renatured *in vitro*.

The size of the subunits of the 50-70S RNA appears to be variable, since their sedimentation coefficients have been reported to range from 28S for the Rickard strain of feline leukemia virus (R-FeLV) (13, 35), the Soehner-Dmochowski strain of murine sarcoma virus (34), and RD-114 virus (35) to 35-44S for Rauscher murine leukemia virus (4). For the mammalian viruses there seems to be a correlation between the size of the subunits and the classification of the virus as a leukemia or a sarcoma virus. Except for R-FeLV (13) and RD-114 (35), mammalian leukemia virus RNA subunits usually are reported to sediment at 35-40S (88, 112), whereas those from mammalian sarcoma viruses are smaller (28-30S) (34, 88, 89, 112, 148). The significance of this difference is not known at the present time.

The 28-40S subunits of oncornaviruses contain a sequence of polyadenylic acid (poly (A) at their 3' ends (60), and the subunits from at least some oncornavirus 28-40S RNA can be translated *in vitro* in cell-free protein synthesis systems (59, 78, 121, 122, 131). However, the only virus-specific protein synthesized under these conditions is the precursor to the low molecular weight structural proteins. There are at least three other genes in 28-40S RNA -- *env*, coding for the envelope proteins; *pol*, coding for the DNA polymerase; and *src* or *onc*, the products of which are unknown (6). The order of these genes on the avian sarcoma virus subunit RNA is 5'-gag-pol-env-src-3' (6); the *gag* gene codes for the precursor to the structural low molecular weight proteins. A recent report (74) claims to have identified two

polypeptides synthesized *in vitro* by nondefective RSV subunit RNA, which are not synthesized in the presence of transformation-defective RSV subunit RNA; this observation has not been confirmed.

Intracellular Viral RNA

The observation that *in vitro* translation of virion 28-40S subunit RNA yields only the precursor to the low molecular weight structural proteins leaves the question open as to the mechanism of synthesis of other virus-specific proteins. When virus-infected cells are examined for the presence of virus-specific RNA, several size classes are usually found (25, 40, 58, 147, 149; Conley and Velicer, in press). These RNAs are also found on polysomes and are classified as messenger RNA on the basis of their release from polysomes upon treatment with EDTA (40, 41, 154). The viral subunit-size RNA in infected cells is translated into *gag* protein precursors (104, 150). A 22S virus-specific RNA from Rauscher murine leukemia virus-infected cells directs the synthesis of a precursor to viral envelope proteins when injected into *Xenopus laevis* oocytes (150). A 20-28S RNA from Rous sarcoma virus-infected cells also directed the synthesis of an *env*-gene product *in vitro* (104). These findings have led to a model for translation of oncornavirus-specific mRNA in which the 28-40S RNA has several translation initiation sites, of which only the most 5' terminal is active or "open" in the intact subunit RNA (104). Viral proteins other than the product of the *gag*-gene would then be synthesized from smaller mRNAs which are processed from the 28-40S RNA. Shanmugan (127) has identified an activity on microsomes which specifically cleaves murine leukemia virus 28-40S RNA; whether or not this may be a processing enzyme is unknown.

Synthesis of Viral DNA

The synthesis of complementary DNA from the oncornaviral 50-70S RNA by reverse transcriptase has been studied *in vitro* in endogenous reactions. In these reactions, virions are partially disrupted with a non-ionic detergent in the presence of magnesium and deoxynucleotide triphosphates, one or more of which is usually radioactively labeled. In early experiments it was found that the average size of the DNA synthesized was much smaller than genome length (42, 48, 91), sedimenting at about 4-10S. In addition, the viral RNA sequences were unequally represented in the DNA product (32, 139), suggesting that portions of the RNA template were preferentially transcribed. It was found that most of the DNA synthesized in such reactions is complementary to the 5' terminal 200 nucleotides of the RNA template (16, 66). The DNA products are covalently attached to an RNA primer (47, 155). The discovery that the RNA primer for DNA synthesis is bound to the template near the 5' end (117) provided an explanation for the observation of small DNA products complementary to the 5' end of the template. However, since DNA synthesis proceeds in a 3' to 5' direction with respect to the template, the question was raised as to how a complete DNA copy of the oncornavirus genomic RNA could be synthesized.

Recent studies have shown that under certain circumstances -- i.e., in the presence of high levels of deoxynucleotide triphosphates or limiting concentrations of magnesium -- full length DNA copies of oncornavirus RNA can be synthesized in endogenous reactions (117, 118). These "tricks" for obtaining complete DNA copies of oncornavirus RNA probably do not accurately reflect the situation *in vivo*. Nevertheless,

it is obvious that complete DNA copies are synthesized in vivo, since the genomic information must be preserved during replication. Therefore it is necessary to develop a model which allows complete transcription of the oncornavirus genome RNA even though synthesis begins only 100-200 nucleotides from the 5' end of the template. In order for this to occur, reverse transcription must involve "jumping" over a gap at the 5' end of the template, either to the 3' end (neglecting the poly A) of the other 28-40S subunit in the 50-70S RNA complex or of the same subunit. Continuing synthesis on the other subunit is the more attractive hypothesis, for two reasons: (1) continued synthesis by jumping to the 3' end of the same subunit would result in the loss of some genetic information (see below), and (2) continued synthesis on the other subunit provides an explanation for the existence of two identical RNA subunits in RNA tumor viruses.

The recent demonstration (17, 18, 67, 73, 125) that the 28-40S RNA subunits of RNA tumor viruses are terminally redundant has provided evidence which allows the development of detailed models for the in vivo transcription of oncornavirus RNA into DNA. In one model, DNA synthesis begins at the primer RNA and proceeds to the 5' end of the template, in the process copying the redundant portion at the 5' section of the template (Fig. 1). Ribonuclease H then degrades the RNA template in the RNA:DNA hybrid from the 5' end to the primer; RNAase H will not degrade double-stranded RNA, so the product now consists of single-stranded DNA (containing the redundant sequence) attached to the template by its RNA primer. If the RNA template circularizes, the free single-stranded DNA can pair with the redundant sequence immediately adjacent to the poly (A) at the 3' end and DNA

synthesis can be continued. Alternatively, the free single-stranded DNA may pair with the 3' sequence of the other 28-40S RNA subunit and continue DNA synthesis on that template. By the first mechanism, some genomic information would be lost; the redundant sequence would be contained at only one end of the DNA copy. The second mechanism would allow complete recovery of the genomic information in the DNA product, with the redundant sequence at both ends.

METHYLATION OF mRNA

Since excellent reviews on the methylation of mRNA have appeared recently (14, 119, 128), this topic will be covered only briefly here.

In 1974 Perry and Kelley (105) reported the existence of a low level of methylation in L cell mRNA. Some of the methyls were in alkali-resistant oligonucleotides which had to be derived either from the 5' terminus or from an internal run of 2'-O-methylnucleotides. This data together with results from Rottman's laboratory concerning Novikoff hepatoma mRNA methylation (26) and from Shatkin's group on reovirus mRNA led Rottman *et al.* to postulate the existence of what has come to be known as "caps" at the 5' termini of eucaryotic mRNAs (120). Caps have the general structure $m^7G(5')ppp(5')N'(m)pN''(m)pN'''p$, where 7-methylguanosine is connected through an inverted (i.e., 5'-5') pyronphosphate linkage to a nucleotide N' which may or may not be 2'-O-methylated; N' is in turn connected through a conventional 3'-5' phosphodiester bond to the next nucleotide N", which also may or may not be 2'-O-methylated. Caps in which N' is not methylated

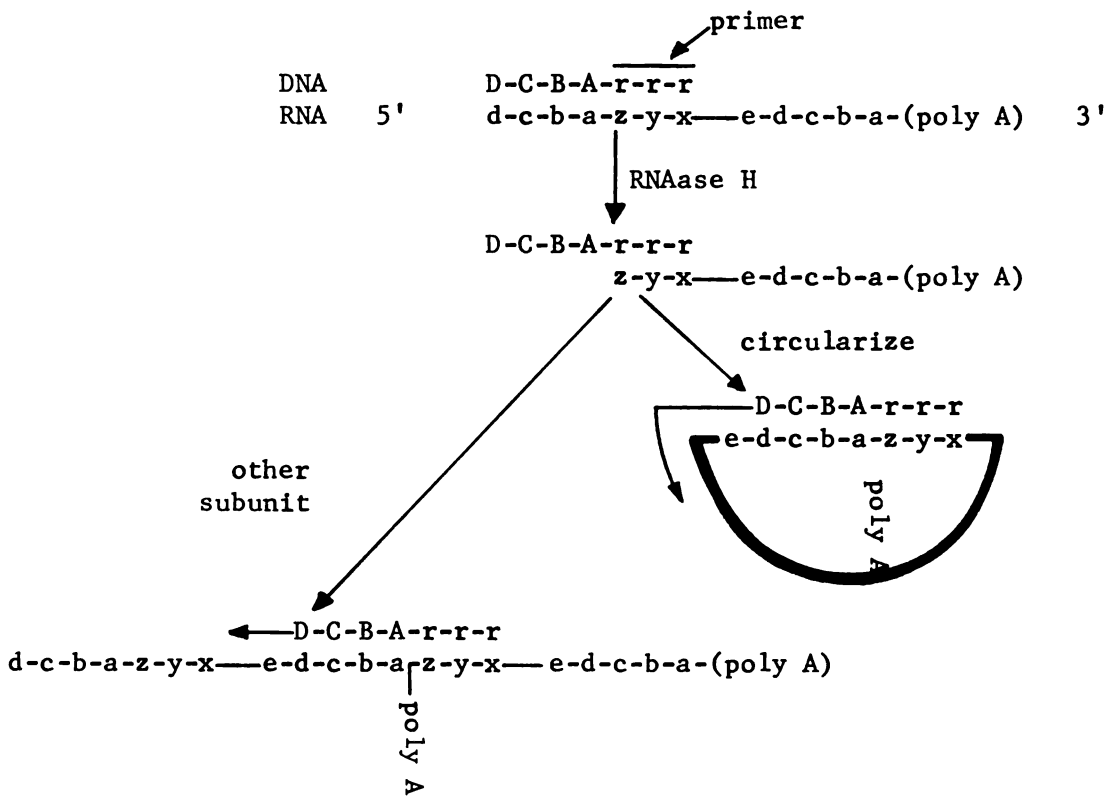

Figure 1. Models for the transcription of oncornavirus RNA into DNA.

are referred to as "cap zero", while caps in which N' and N" are methylated are called "cap 1" and "cap 2", respectively.

Distribution of Caps

Since the original discovery of methylated nucleotides in eucaryotic mRNA, some or all of which are found in caps, many eucaryotic cellular and viral mRNAs have been shown to be capped. Caps have been found in the mRNA of mammalian cells (3, 21, 27, 52, 84, 86, 93, 101, 107, 158, 159), sea urchins (44), insects (75, 164), birds (108), slime molds (29), and yeast (138). The mRNA of many eucaryotic viruses is also capped, including reovirus (51), cytoplasmic polyhedrosis virus (50), brome mosaic virus (24), tobacco mosaic virus (165), Sindbis virus (68), vesicular stomatitis virus

(1), Newcastle disease virus (20), influenza virus (81), avian sarcoma virus (55, 76, 140), Moloney murine leukemia virus (11, 115), vaccinia virus (160), simian virus 40 (84), adenovirus (137), and herpes virus (7) mRNAs. Picornavirus (97) and satellite tobacco necrosis virus (162) RNAs are not capped; poliovirus RNA is instead blocked at the 5' end by a covalently linked protein (85). Mammalian cellular mRNAs and mammalian viral mRNAs synthesized in vivo contain both cap 1 and cap 2 structures, while mammalian viral mRNAs synthesized in vitro by virion-associated enzymes contain only cap 1. The degree of methylation of the cap tends to increase as one moves up the evolutionary scale. Yeast mRNA contains exclusively cap zero, slime mold mRNA contains mostly cap zero and some cap 1, brine shrimp larvae (94) and sea urchin embryo mRNAs contain only cap 1, and mammalian mRNA contains both cap 1 and cap 2. In viral mRNA N' is always a purine, but cellular mRNA may contain either a purine or a pyrimidine at this position. It has been suggested that mRNAs in which N' is a purine are derived from the 5' end of the primary transcript while those with a pyrimidine at this position are derived by internal cleavage of the primary transcript (124).

Properties of Caps

The existence of caps in viral and cellular RNA was first suspected because the 5' ends of these RNAs were resistant to phosphorylation by polynucleotide kinase, even if the RNA was first treated with phosphomonoesterase to remove any pre-existing phosphate groups. We know now that in fact no free 5' hydroxyl exists which could serve as a substrate for polynucleotide kinase in these RNAs, and the phosphates within the cap are protected from

phosphomonoesterase by the inverted 7-methylguanosine. The free 2' and 3' hydroxyls of the 7-methylguanosine form a cis,diol which provides the basis for several chemical manipulations of caps. The cis,diol interacts with borate ions, thus allowing the purification of caps by chromatography on columns of dihydroxyboryl-cellulose (55). The cis,diol can also be oxidized to the dialdehyde form with periodate, and then either labeled by reduction with ^{3}H-borohydride (53) or treated with aniline to remove the 7-methylguanosine by elimination (160). Caps are not degraded by alkali or by ribonucleases A, T1, T2 or nuclease P1; however, P1 will remove nucleotides in the N" and N''' position even if N' and N" are 2'-O-methylated. Phosphodiesterases degrade caps to yield $pm^{7}G$, pN(m), and p. The positive charge on the $m^{7}G$ partially neutralizes the negative phosphate groups in the triphosphate bridge; the interaction of this positive charge with the phosphates apparently results in a rigid structure that may be preferred for initiation of mRNA translation (Hickey et al., quoted in reference 128). The positive charge on $m^{7}G$ is lost under alkaline conditions where $m^{7}G$ is converted to a ring-opened derivative.

Synthesis and Degradation of Caps

Study of the mechanism of cap synthesis has so far been limited almost entirely to viral systems. These systems are easier to study than eucaryotic cells because purified virions contain all the enzymes necessary for cap formation but lack the myriad unrelated proteins found in cells. Two types of synthetic mechanisms have been found, one in vaccinia virus and reovirus and the other in vesicular stomatitis virus. In reovirus, pppG is condensed with pppC to yield

pppGpC (54). A virion phosphohydrolase removes the terminal phosphate from this structure to give ppGpC. Guanylyl transferase then condenses $ppp\overset{*}{G}$ with the ppGpC, yielding $\overset{*}{G}pppGpC$. In the presence of S-adenosylmethionine this structure becomes methylated at the 7 position of the first G and at the 2' position of the second G to give $m^7GpppGmpCp$. While no further methylation occurs *in vitro*, the cytosine residue is sometimes 2'-O-methylated *in vivo*, presumably by a cellular enzyme.

The mechanism of cap synthesis by disrupted vesicular stomatitis virions proceeds by a somewhat different mechanism (2, 19). In this reaction $ppp\overset{**}{G}$ is condensed with pApN...to yield $\overset{**}{G}pppApN...$; this structure is subsequently methylated in the presence of a methyl donor. A similar mechanism may be operative for cellular mRNAs derived by internal cleavage from a larger precursor (128).

Evidence has been presented indicating that caps in mammalian cellular mRNA are degraded at the same rate as the rest of the mRNA molecule (102, 106). Enzymes specific for the degradation of caps have been found in extracts of tobacco (129) and HeLa (100) cells. The Hela enzyme cleaves m^7GpppX to yield pm^7G and ppX. It does not degrade caps which are in long polynucleotides such as mRNA, indicating that its probable function is to eliminate residual caps after the remainder of the mRNA has been degraded.

Biological Function of Caps

Both *et al.* (12) first demonstrated the importance of caps for the translation of mRNA. They found that reovirus and vesicular stomatitis virus mRNAs required 5' terminal m^7G in caps for efficient translation in a wheat germ, cell-free protein synthesis system. The

m^7G of the cap is important since its removal from various mRNAs resulted in decreased translation in cell-free protein synthesis systems (77, 93). When pm^7G was added to a cell-free system, the translation of capped mRNAs was markedly inhibited (70, 71); uncapped mRNAs were not affected. The 3'monophosphate of m^7G or the nucleoside were not inhibitory. In another study (15) cap analogues containing m^7G were inhibitory but GpppGm was not. When capped reovirus mRNAs were incubated with ribosomes, the ribosomes protected fragments of the mRNAs including the caps from digestion by ribonuclease (80). This result indicates that caps are involved in ribosome binding by mRNA. However, the requirement of caps for translation in the reticulocyte cell-free system was not as stringent as in the wheat germ system (95). In addition, uncapped polio virus or encephalomyocarditis virus mRNAs were translated efficiently in similar cell extracts. Thus, it appears that caps facilitate the translation of mRNA but are not an absolute requirement.

Caps may also play a role in the stabilization of mRNA against degradation by exonucleases (120). Recently it was reported that capped reovirus mRNA is more stable in Xenopus laevis oocytes or in cell-free protein synthesis extracts of wheat germ or L cells than the corresponding uncapped RNA (49). Further study will be necessary to more precisely define the role of caps in mRNA stabilization.

Internal Methylation of mRNA

When the methylnucleoside composition of Novikoff hepatoma cell mRNA was examined, it was found that about 50% were in base-methylated nucleosides other than m^7G (26). The base-methylated nucleosides consisted entirely of 6-methyladenosine (m^6A), in an amount

sufficient to account for about three m^6A residues per average mRNA molecule. None of the m^6A was in the poly (A) section of the mRNA (27, 107). Many cellular mRNAs have since been reported to contain internal m^6A, but with the exception of adenovirus mRNA (84) and oncornavirus RNA (11, 140), viral RNAs have not been reported to contain this modification. In addition, globin mRNA does not contain m^6A (108).

It is interesting to note that in HeLa cell mRNA (159) and avian sarcoma virus RNA (28), m^6A occurs in only two sequences: Gpm^6ApC and Apm^6ApC. The studies on HeLa cell mRNA were performed on a sample containing hundreds or thousands of different mRNA molecules, rendering the finding of this simple pattern especially remarkable. Although the function of the m^6A residues in mRNA is completely unknown, the specificity of the sequences in which it occurs implies that it may have a specific role in the expression of genetic information.

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PART I

PRELIMINARY CHARACTERIZATION OF FELINE LEUKEMIA VIRUS RNA

INTRODUCTION

Before detailed analysis of the RNA of R-FeLV could be undertaken, it was essential to define conditions that would allow isolation of the RNA in undegraded form. Since at the beginning of this study no published work existed on the RNA of the Rickard Strain of FeLV it was also necessary to determine the kinds and sizes of RNA present within the virion. It had been shown that the FeLV-5 strain of FeLV contained 74S, 37S and 4-10S RNA classes, and that upon denaturation the 74S RNA was converted to species sedimenting at 37S and 4S (1). The first objective of this research was to determine if similar RNA classes and/or others exist in R-FeLV.

METHODS

Cells and Virus

R-FeLV, propagated in the feline thymus tumor cell line F-422 and isotopically labeled with ^{3}H-uridine, was obtained from David Brian in the laboratory of Dr. L. F. Velicer, Department of Microbiology and Public Health, Michigan State University.

F-422 cytoplasmic RNA was isolated as described (2) from (^{3}H) or (^{14}C) uridine labeled F-422 cells obtained from David Brian. Novikoff hepatoma cytoplasmic poly A^{+} RNA labeled with (^{3}H) uridine, was a kind gift from Ronald Desrosiers of our laboratory.

Viral RNA Extraction

Method 1: The pelleted virus was suspended in 0.5 ml of TNE-7 (0.01 M Tris-HCl, pH 7.3; 0.1 M NaCl; 1 mM EDTA) containing 1%

dithiothreitol, 10% phenol, 0.1% (w/v) bentonite, and 0.2% diethylpyrocarbonate. The mixture was stirred for 20 min at 0°C with an equal volume of phenol saturated with TNE-7. One volume of chloroform : isoamyl alcohol (99:1) was added and the mixture was stirred for an additional 10 min. The extract was centrifuged for 30 min at 12,000 x g at 4°C. The aqueous phase was removed and mixed for 5 min with an equal volume of TNE-7 saturated phenol and again centrifuged. The aqueous layer was stored at -80°C until further analysis.

Method 2: The pelleted virus was suspended in 0.5 ml of a solution containing 0.5% SDS, 0.5% sodium deoxycholate, 0.6 mg/ml bentonite, 0.01% 8-hydroxyquinoline and 0.001 M Tris-HCl (pH 8.8) and mixed with an equal volume of phenol containing 0.1% 8-hydroxyquinoline for 5 min at 4°C. The aqueous phase was separated by low-speed centrifugation for 10 min and extracted again with an equal volume of phenol containing 0.1% 8-hydroxyquinoline. After centrifugation, the aqueous phase was stored at -80°C until further analysis.

Method 3: The pelleted virus was suspended in .5 ml of TNE-9 (0.01 M Tris-HCl, pH 9.0; 0.1 M NaCl; 1 mM EDTA) containing 1% SDS and mixed with one volume of TNE-9 saturated phenol and one volume of chloroform : isoamyl alcohol (24:1) for 5 min at room temperature. The phases were separated by centrifugation for 10 min and the aqueous layer was extracted again with phenol and chloroform : isoamyl alcohol. After phase separation by centrifugation, the aqueous layer was removed and stored at -80°C until further analysis.

Method 4: This method was the same as method 3 except that chloroform : isoamyl alcohol was not included.

Method 5: The pelleted virus was suspended in 1 ml TNE-9 and then made 1% SDS and proteinase K (E. M. Laboratories) was added to a final concentration of 250 ug/ml. After incubation at 37°C for 10 min, the RNA was extracted as in method 4. Carrier E. coli tRNA was added to a concentration of 250 ug/ml and precipitated by the addition of 2 volumes of ethanol.

Centrifugation in Aqueous Sucrose Gradients

Analysis of viral native 50-60% RNA or the subunits of this RNA was performed in aqueous sucrose gradients with an SW 50.1 rotor as described in the figure legends.

Centrifugation in Sucrose Gradients in Me_2SO

When RNA was to be centrifuged under denaturing conditions, the RNA was dissolved in 0.02 ml .1% SDS and 0.2 ml of 99% dimethylsulfoxide (Me_2SO) containing 10 mM LiCl, 1 mM EDTA was added. The sample was heated to 54°C for 2 min, cooled, and centrifuged through a 4.6 ml 5-20% sucrose gradient in 99% Me_2SO, 10mM LiCl, 1 mM EDTA in a Beckman SW 50.1 rotor at 45,000 rpm and 25°C for 14 h. Fractions of approximately 0.2 ml were collected and counted in a scintillation counter.

Polyacrylamide Gel Electrophoresis

RNA samples were characterized by polyacrylamide gel electrophoresis in an EC Model 470 vertical slab gel cell (E. C. Apparatus Corp., Philadelphia) according to the procedure described by Dingman and Peacock (3). Native high molecular weight RNA was electrophoresed at 200V in 2.0% acrylamide-2.0% agarose gels for 2.5-3 h. Gel tracks

were fractionated with a stacked razor blade apparatus and 1 mm slices were digested with 0.5 ml NCS (Amersham/Searle) : H_2O (9:1) for 2 h at 50°C. The reaction mixtures were neutralized with glacial acetic acid, and 0.5 ml H_2O and scintillation fluid were added. The scintillation vials were heated to 55°C for 1 h and cooled, to reduce background counts due to chemiluminescence. Low molecular weight RNA was analyzed in a similar manner except that electrophoresis was through a 7% acrylamide gel for 3.5 h.

RESULTS

Comparison of Methods for Viral RNA Extraction

Four different procedures for the extraction of viral RNA were simultaneously compared to determine which method results in the greatest yield of intact high molecular weight RNA. These were all variations of methods that already existed in the literature. The extractions were performed on aliquots of the same virus sample so that the results were directly comparable. Of the approximately 70,000 ^3H cpm present in each of the viral aliquots before extraction, 24,700 cpm was recovered in the aqueous phase after extraction by Method 1; 62,880 cpm by Method 2; 26,650 cpm by Method 3; and 43,000 cpm by Method 4. This represents 35%, 90%, 38% and 61% recoveries, respectively. The remainder of the radioactivity was found in the phenol phase and the interphase.

To determine which of the four methods produces the greatest proportion of intact high molecular weight RNA, aliquots of each sample were analyzed by polyacrylamide gel electrophoresis. As can

be seen in Figure 1, three major classes of RNA are detected by this procedure after extraction by Methods 1, 3 and 4, with an additional RNA class being detected after extraction by Method 2. Table 1 shows the proportions of the various RNA classes in each case. Viral RNA purified by Method 4 contained the largest percentage migrating in peak I with 51%. The only method in which peak II RNA was produced was Method 2; this class of RNA probably represents the subunits of the native RNA in peak I. Since viral RNA extracted by Method 4 contained the highest proportion of RNA in native 50-60S RNA (peak I), and since RNA extracted by Method 4 was recovered in the second highest yield, it was concluded that of the four methods for viral RNA extraction Method 4 was the best.

Table 1

Distribution of Radioactivity in R-FeLV RNA Extracted by Various Methods

Extraction Method	% of Total Radioactivity			
	Peak I	Peak II	Peak III	Peak IV
1	34	0	17	49
2	33	29	14	24
3	29	0	23	48
4	51	0	19	30

Figure 1. Polyacrylamide gel electrophoresis of R-FeLV RNA extracted by Methods 1-4.

(a) RNA (2350 cpm) extracted by Method 1 was electrophoresed as described in Materials and Methods for 2.5 h.

(b) RNA (5000 cpm) extracted by Method 2; electrophoresis in a 2% polyacrylamide/0.5% agarose gel.

(c) RNA (2550 cpm) extracted by Method 3.

(d) RNA (4175 cpm) extracted by Method 4.

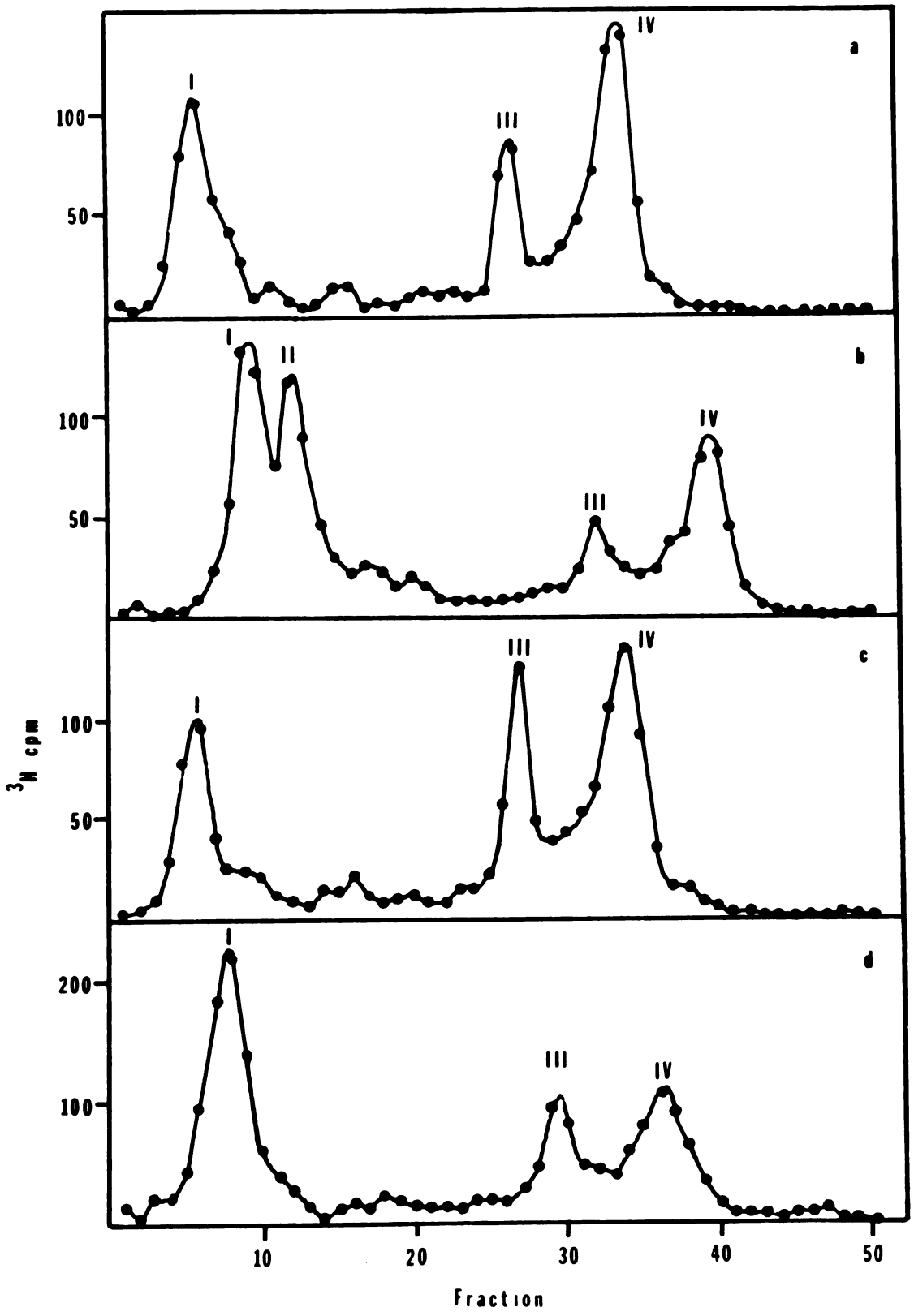

Figure 1. Polyacrylamide gel electrophoresis of R-FeLV RNA extracted by Methods 1-4.

In later experiements a modification of Method 4 was employed. In this technique (Method 5) the viral pellet was first suspended in a larger volume (1 ml instead of 0.5 ml) of TNE-9 and then made 1% in SDS and incubated with proteinase K before phenol extraction. This procedure produced viral RNA in which 50-60S RNA represented greater than 50% of the RNA more consistently than when RNA was extracted by Method 4 (Figure 2).

Size Analysis of R-FeLV RNA

When native R-FeLV RNA was centrifuged through non-denaturing sucrose gradients, two major classes of RNA were detected (Figure 2). The larger RNA class sedimented in a broad peak in the 50-60S region; the peak fraction was approximately 52S as determined by comparison to 28S ribosomal RNA (rRNA) run on a parallel gradient. The smaller RNA class co-sedimented with cellular 4S RNA. In different viral RNA preparations, the 50-60S RNA represented 40-60% of the total RNA; about 50% was average.

The native 50-60S RNA could be denatured by heating in an aqueous buffer to 100°C for 2 min or by dissolving in 90% Me_2SO and heating to 55°C for 2 min, to yield high molecular weight RNA subunits and smaller RNAs. However, heating the RNA to high temperatures in an aqueous buffer was found to result in extensive degradation. Therefore, to determine the size of the subunits of native 50-60S RNA the RNA was denatured in 90% Me_2SO and centrifuged through a denaturing sucrose gradient in 99% Me_2SO. Figure 3 shows the results of one such analysis. Under these conditions, 70-80% of the RNA co-sedimented with 28S rRNA. This smaller-than-expected size was not a result observed only with gradients containing Me_2SO;

Figure 2. Sedimentation analysis of R-FeLV native RNA extracted by Method 5.

R-FeLV RNA labeled with (^{14}C) uridine was centrifuged through a 4.8-ml 5 to 20% sucrose gradient in TNE-7/0.1% SDS in a Beckman SW 50.1 rotor for 40 min at 45,000 rpm and 23°C. Fractions of 0.2 ml were collected and assayed by scintillation counting. F-422 cellular 28S rRNA was run as a marker on a parallel gradient.

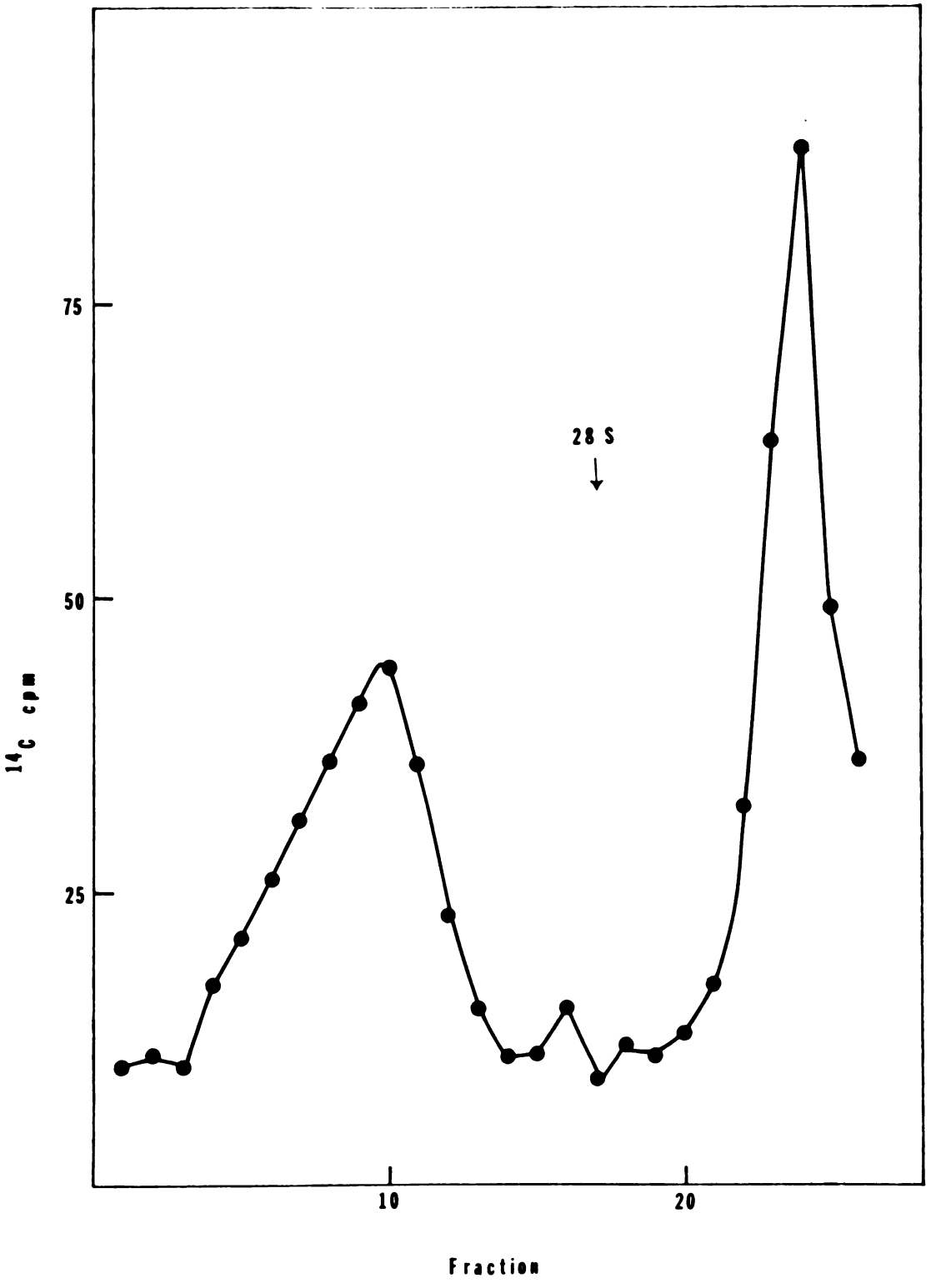

Figure 2. Sedimentation analysis of R-FeLV native RNA extracted by Method 5.

Figure 3. Analysis of R-FeLV high molecular weight subunit RNA by sedimentation in a denaturing sucrose gradient.

R-FeLV 50-60S RNA, isolated as in Figure 2, was denatured and centrifuged through a sucrose gradient containing 99% Me_2SO as described in Materials and Methods. F-422 cytoplasmic RNA was run as marker on a parallel gradient.

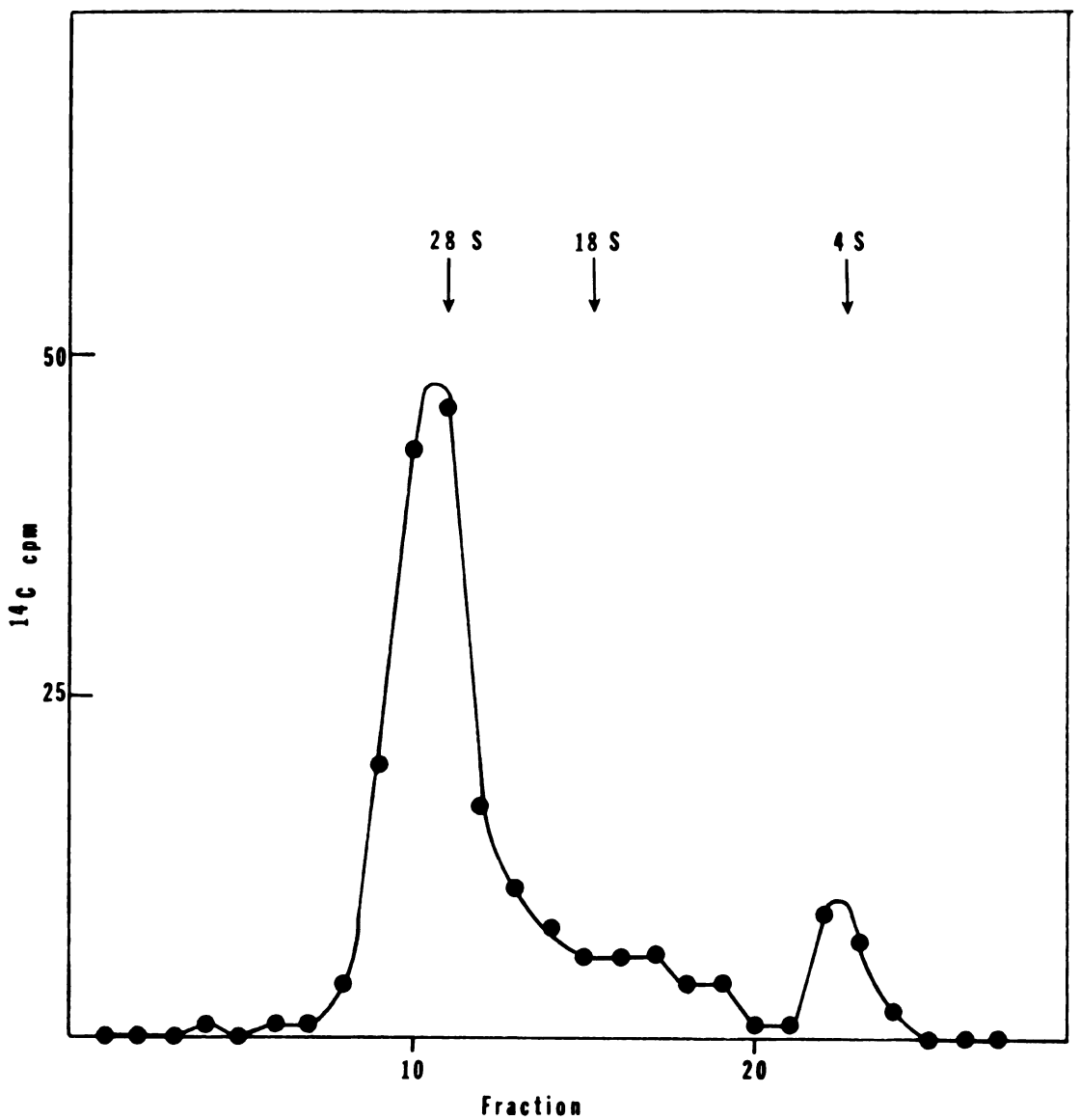

Figure 3. Analysis of R-FeLV high molecular weight subunit RNA by sedimentation in a denaturing sucrose gradient.

Figure 4 shows that when viral RNA sedimenting at 28S on Me_2SO gradients is re-run on an aqueous sucrose gradient, it continues to co-sediment with 28S rRNA. When analyzed under a variety of centrifugation conditions R-FeLV high molecular weight subunit RNA was always observed to sediment at about 28S.

Despite the fact that viral subunit RNA co-sediments with 28S rRNA in sucrose gradients it nevertheless migrates more slowly than the same marker when subjected to polyacrylamide gel electrophoresis. Figure 5 shows an example in which R-FeLV high molecular weight subunit RNA, labeled with (^{14}C) uridine, was mixed with (^{3}H) uridine-labelled ribosomal RNA. The RNA was purified by centrifugation through a sucrose gradient and electrophoresed through a 2.0% polyacrylamide/1.5% agarose gel. From this analysis it would be concluded that R-FeLV subunit RNA has a considerably greater molecular weight than 28S rRNA. It should be noted, however, that the gel electrophoresis was not conducted under conditions in which RNA is denatured. In addition, it appears that viral subunit RNA is not the only RNA which co-sediments with 28S rRNA in sucrose gradients but migrates more slowly than that marker during gel electrophoresis. In Figure 6, uninfected Novikoff cell poly (A)-containing RNA, which had previously been isolated from the 28S region of a sucrose gradient, was analyzed by gel electrophoresis. A large fraction of this RNA migrated more slowly than 28S rRNA.

In order to achieve better resolution of R-FeLV low molecular weight RNAs, viral RNA was electrophoresed through a gel containing a higher concentration of acrylamide (Figure 7). Four major low molecular weight RNA classes were detected; one of the RNA classes

Figure 4. Analysis of R-FeLV high molecular weight subunit RNA by sedimentation in a non-denaturing sucrose gradient.

R-FeLV 28S RNA isolated from a sucrose gradient containing Me_2SO as in Figure 3 was ethanol precipitated and centrifuged through a 4.8-ml 10-30% sucrose gradient in TNE-7/0.1% SDS for 140 min at 45,000 rpm and 23°C. Novikoff hepatoma cell cytoplasmic RNA was run on an identical parallel gradient. The sedimentation profiles are superimposed. o—o , R-FeLV RNA; •—• , Novikoff RNA.

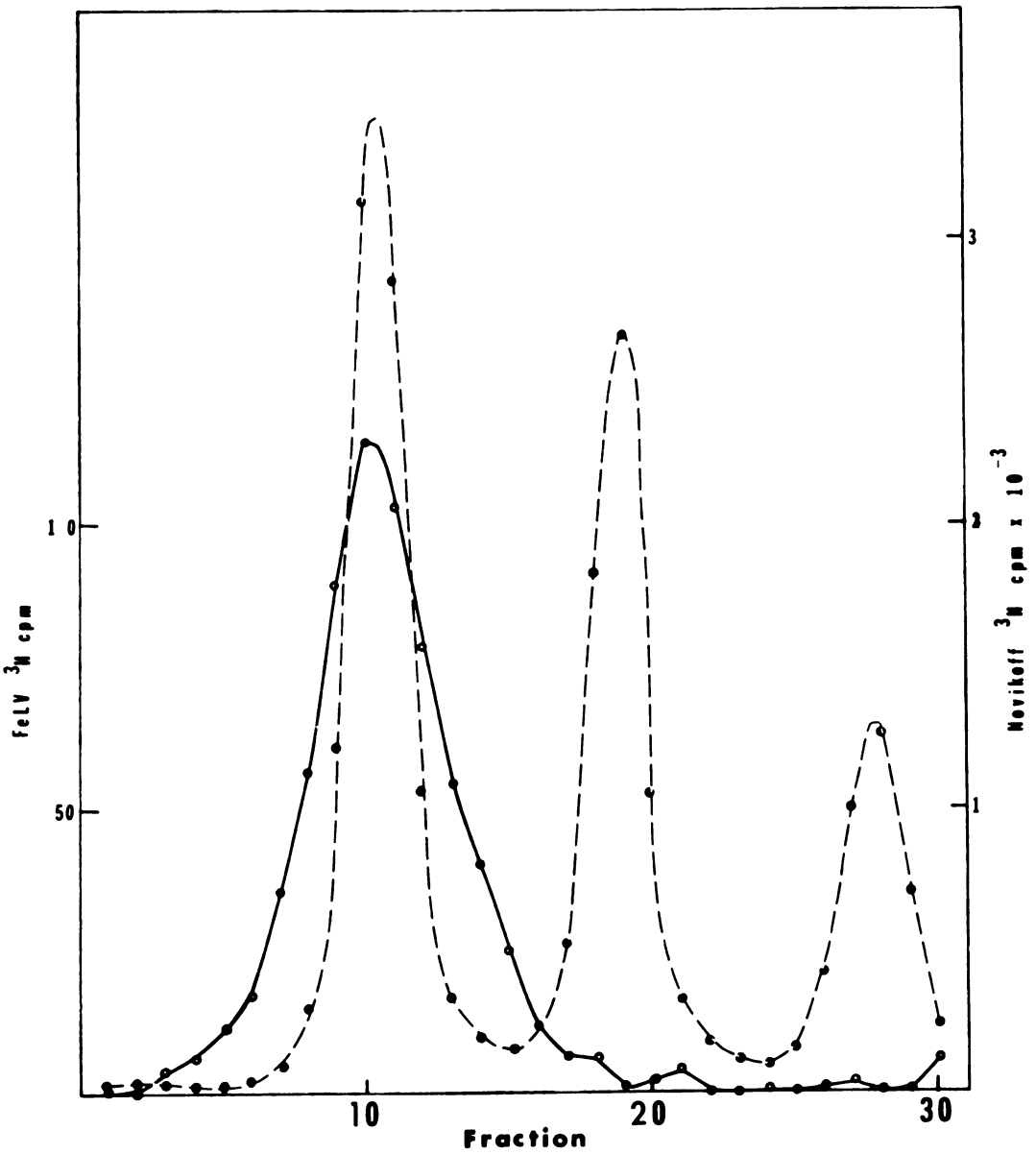

Figure 4. Analysis of R-FeLV high molecular weight subunit RNA by sedimentation in a non-denaturing sucrose gradient.

Figure 5. Polyacrylamide gel co-electrophoresis of R-FeLV high molecular weight subunit RNA and 28S rRNA.

R-FeLV 50-60S RNA, labeled with (^{14}C) uridine, was mixed with (^{3}H) uridine-labeled Novikoff 28S rRNA and centrifuged through a sucrose gradient in Me_2SO as described for Figure 3. The 28S peak was recovered, ethanol precipitated, and electrophoresced in a 2.0% acrylamide/1.5% agarose gel for 160 min as described in Materials and Methods. Electrophoresis was from left to right.

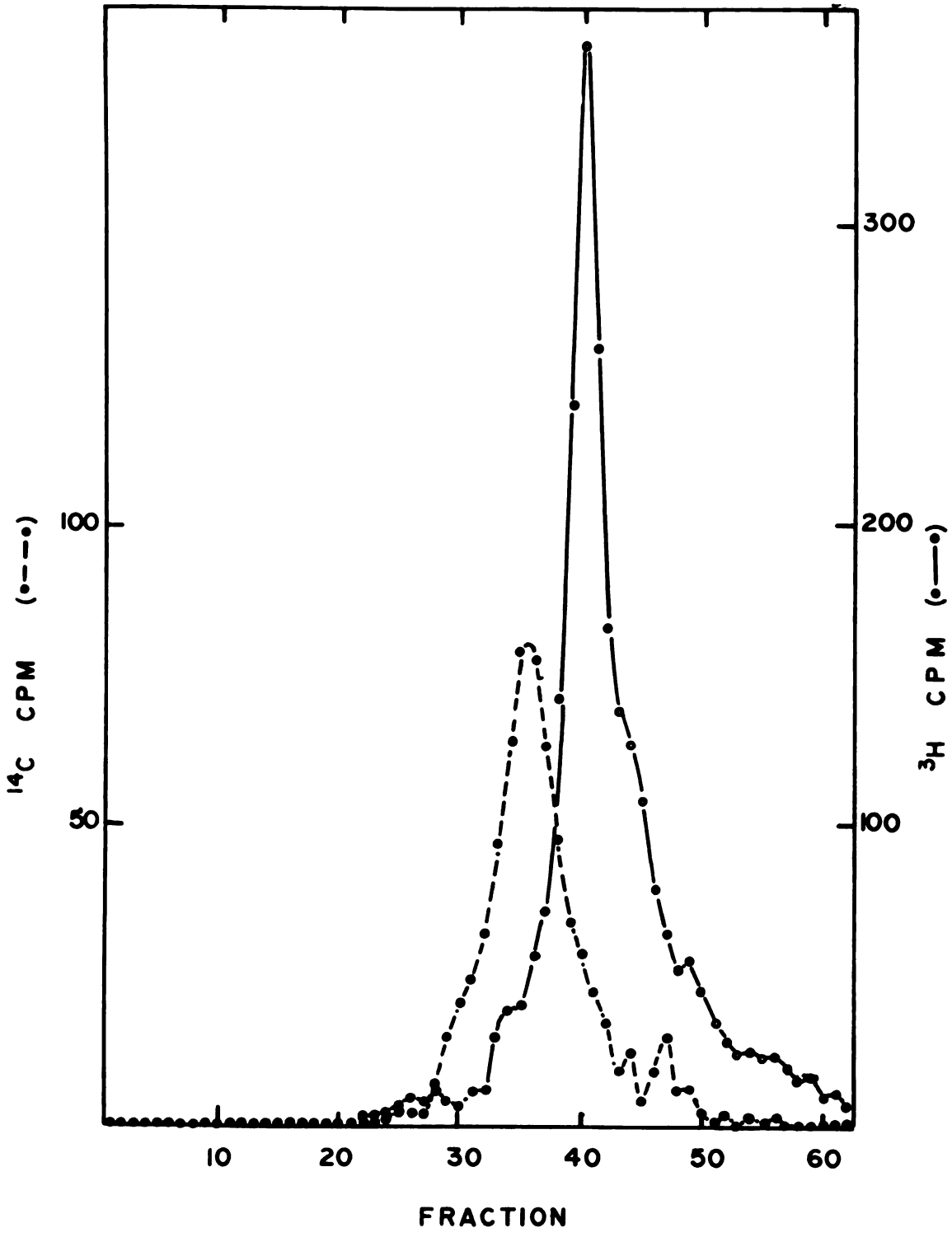

Figure 5. Polyacrylamide gel co-electrophoresis of R-FeLV high molecular weight subunit RNA and 28S rRNA.

Figure 6. Polyacrylamide gel co-electrophoresis of Novikoff cytoplasmic poly (A)-containing 28S RNA and 28S rRNA.

Novikoff cytoplasmic poly (A)-containing RNA, labeled with (^{3}H) uridine, was mixed with (^{14}C) uridine-labeled F-422 28S rRNA and centrifuged through a sucrose gradient in Me_2SO as described for Figure 3. The RNA sedimenting at 28S was recovered by ethanol precipitation and electrophoresed as described for Figure 5.

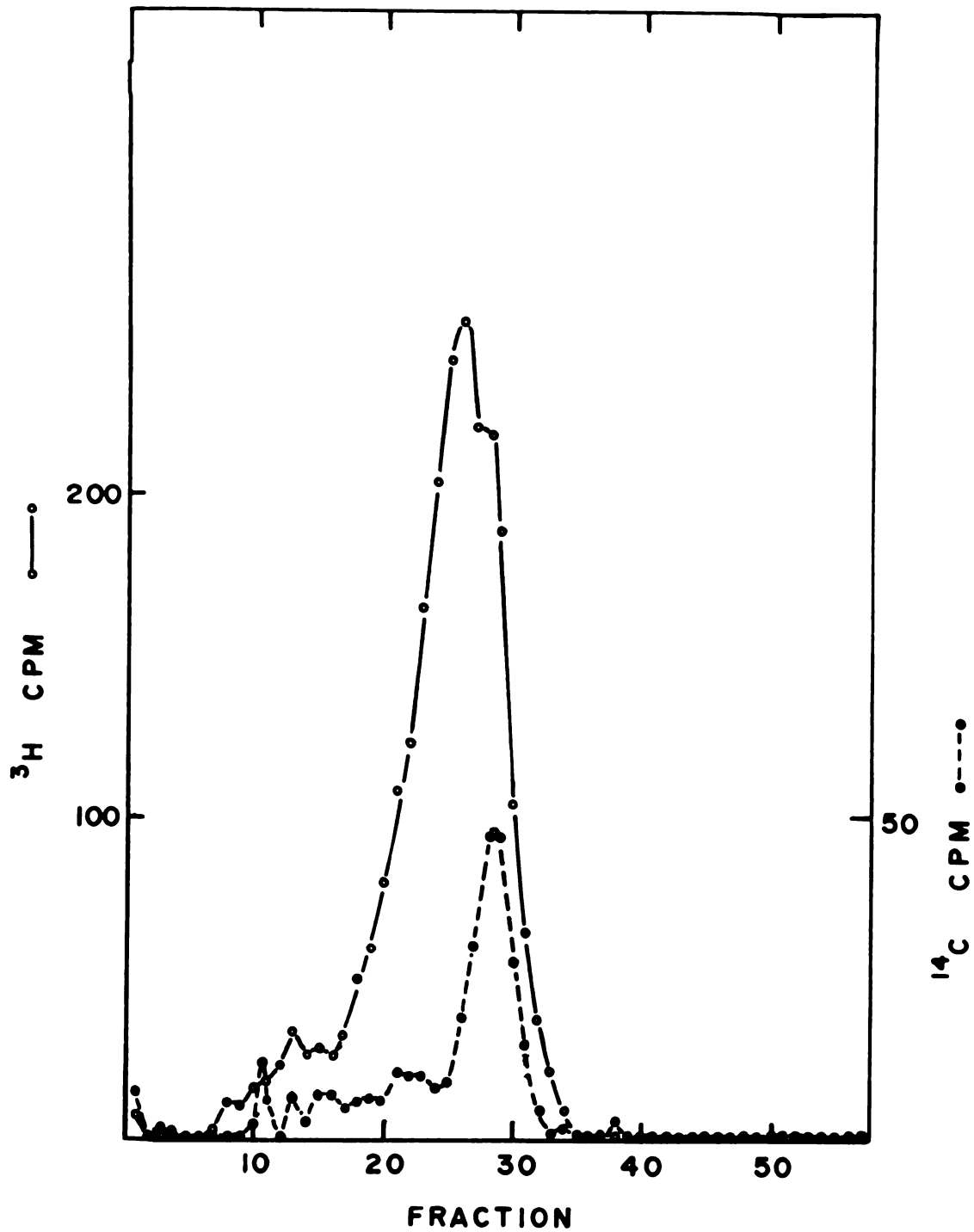

Figure 6. Polyacrylamide gel co-electrophoresis of Novikoff cytoplasmic poly (A)-containing 28S RNA and 28S rRNA.

Figure 7. Analysis of R-FeLV low molecular weight RNA by electrophoresis in a 7% polyacrylamide gel.

R-FeLV total RNA was centrifuged through a sucrose gradient in Me_2SO as described in Figure 3. The low molecular weight RNA was recovered by ethanol precipitation and electrophoresed with unlabeled F-422 cytoplasmic RNA on a 7% acrylamide gel as described in Materials and Methods. The positions of cellular 5S and 4S RNA were determined by staining of the gel.

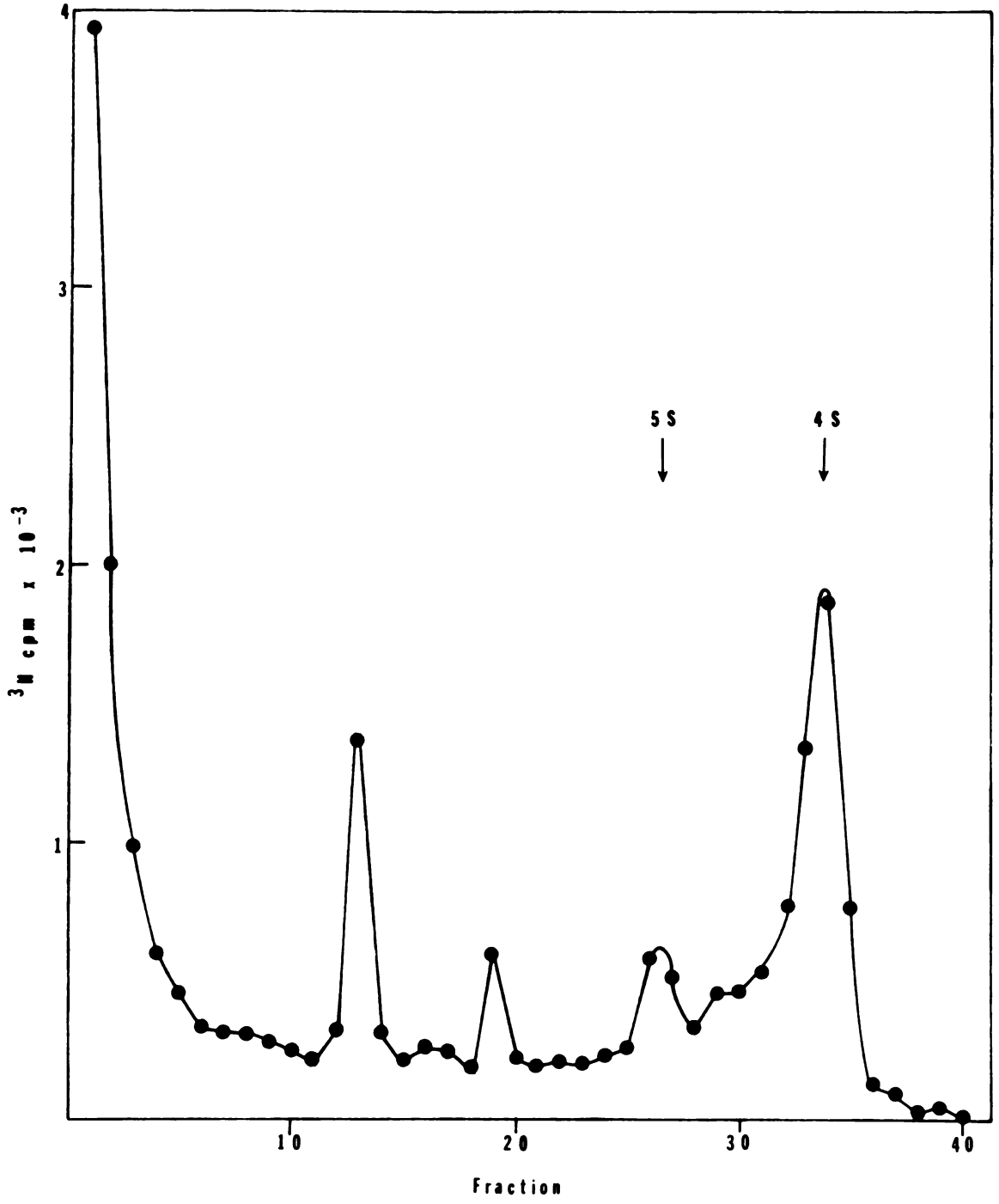

Figure 7. Analysis of R-FeLV low molecular weight RNA by electrophoresis in a 7% polyacrylamide gel.

co-migrated with cellular 4S RNA, another migrated with cellular 5S RNA, and the other two classes migrated more slowly.

DISCUSSION

By comparison of several methods for extraction of RNA from R-FeLV it was determined that extraction at pH 9.0 with SDS and phenol, in the absence of chloroform, produced the highest yields of high molecular weight RNA. By increasing the extraction volume to 1 ml instead of 0.5 ml, and by digesting viral proteins with proteinase K prior to the phenol step, the method was improved to the point where high molecular weight RNA consistently represented greater than 50% of the RNA. It was also found that extraction of RNA from virions immediately after virus purification was preferable to extraction from virus that had first been frozen at -80°C, even for a relatively short time (1-2 days). Occasionally it was possible to extract intact high molecular weight RNA from virus that had been frozen and thawed, but usually such RNA was almost completely degraded (data not shown).

When native R-FeLV RNA was analyzed on aqueous sucrose gradients, two major size classes were observed (Figure 2.). The larger RNA class sedimented in a heterogeneous peak in the 50-60S region of the gradient, with an average size of about 52S. This size is smaller than that observed for most other mammalian leukemia viruses, including the FeLV-5 strain of FeLV (1); it is similar to the size observed for mammalian sarcoma viruses (4). The reason for this unusually small size of R-FeLV genomic RNA is unknown. The

smaller RNA class, usually constituting almost 50% of R-FeLV RNA, sedimented with cellular 4S RNA on sucrose gradients.

Analysis of native R-FeLV RNA on low percentage polyacrylamide gels demonstrated the presence of at least two low molecular weight RNA species which were not resolved by sedimentation in sucrose gradients (Figure 1). These RNAs probably correspond to the 7-8S and 4S RNA found in other oncornaviruses (5). In these cases the 4S RNA has been found to be mostly transfer RNA, of cellular origin (6). The function of the 7-8S RNA is unknown, but it is also found in uninfected cells (7). Better resolution of R-FeLV low molecular weight RNAs was achieved by electrophoresis in a 7% polyacrylamide gel (Figure 7). The 4S and 5S RNAs probably correspond to the analogous cellular RNAs, while the two slower migrating RNAs are probably the "a" and "b" form of 7-8S RNA noted by others (8).

R-FeLV high molecular weight subunit RNA is also of a smaller size than usually observed for mammalian leukemia viruses, sedimenting in sucrose gradients under a variety of conditions with 28S rRNA. As noted for the native 50-60S RNA, this value is more similar to that observed for mammalian sarcoma viruses (4, 9, 10) than for leukemia viruses. However, R-FeLV high molecular weight subunit RNA migrates during polyacrylamide gel electrophoresis as if it is substantially larger than 28S rRNA (Figure 5). This phenomenon is also observed for some cellular poly (A)-containing RNAs (Figure 6). Thus the true molecular weight of R-FeLV subunit RNA is in doubt, since the contribution of its secondary structure to its sedimentation coefficient in sucrose gradients or its migration in polyacrylamide gels is unknown. Measurement of the length of R-FeLV subunit RNA by

electron microscopy would probably be the most unambiguous method for resolving this uncertainty.

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PART II

METHYLATION OF THE HIGH MOLECULAR WEIGHT SUBUNIT RNA OF FELINE LEUKEMIA VIRUS

INTRODUCTION

In order to gain a better understanding of how RNA tumor viruses function much study has been directed in recent years towards elucidating the nature of the virion RNA. That work, most of which was done with avian viruses, has shown that oncornavirus RNA consists of a high molecular weight species and several smaller ones (14). The high molecular weight RNA (usually designated 60-70S RNA) can be dissociated into high molecular weight subunit RNA (usually designated 30-40S RNA) plus smaller RNAs by heat or other hydrogen bond-disrupting agents. A previous report describes the size and kinetics of appearance of the RNA in a mammalian oncornavirus, the Rickard strain of feline leukemia virus (R-FeLV) (5). This communication describes the methylation of the high molecular weight subunits of R-FeLV RNA.

The high molecular weight subunit RNA of oncornaviruses resembles cellular messenger RNAs in several ways. Both are single-stranded RNA molecules that can be translated in a cell-free protein synthesis system (28). Cellular mRNAs contain a post-transcriptionally added stretch of adenosine residues at their 3' ends and oncornavirus 30-40S RNA has been found to contain the same modification (13).

Recently it has been found that cellular mRNA (6, 23) and some animal virus RNAs (21, 27, 30) contain methylated nucleotides as an additional modification. It was originally postulated that structures containing 5' terminal 7-methylguanosine and a 5'-5' pyrophosphate linkage might be a general feature of all mRNA molecules (26). Such "cap" structures containing three adjacent phosphates have been

found at the 5' ends of mRNAs from several types of cells (2, 7, 24, 29) and from reovirus (11), vesicular stomatitis virus (1), vaccinia virus (31), cytoplasmic polyhedrosis virus (10), SV40 (17), and two strains of an avian sarcoma virus (12, 15). In addition, internally located residues of 6-methyladenosine (m^6A) have been reported to be in cellular mRNAs (6) and in SV40 specific mRNA (17) but appear to be absent in viral mRNAs synthesized in vitro (1, 10, 11, 30) or VSV mRNA synthesized in vivo (22). The physiological functions of either the 5' caps or the m^6A residues are unclear but recent evidence suggests that the caps may be necessary for efficient translation of some viral mRNAs in vitro (4).

Erikson (9) first studied the methylation of oncornavirus RNA by growing AMV-producing cells in the presence of (methyl-^3H) methionine. It was found that AMV 4S RNA was highly methylated and while the 60-70S RNA contained a low level of methylation it was not determined whether this was due to methyl groups in the 30-40S RNA or in the 60-70S associated 4S RNA. Others have looked for methyl groups in the high molecular weight RNA of Rous sarcoma virus (3) and of murine sarcoma virus/murine leukemia virus (20) and have not found them. While the present study was in progress two strains of Rous sarcoma virus, RSV-Prague (15) and ASV-B77 (12), were reported to contain methylated nucleotides, including a 5' cap structure, in their 30-40S RNA. We report here that the high molecular weight subunit RNA of R-FeLV, a mammalian leukemia virus, is methylated and we describe its unique pattern of methylation.

MATERIALS AND METHODS

Virus

The Rickard strain of feline leukemia virus was purified from the culture fluid of feline thymus tumor cell line F-422 cells which were propagated as previously reported (5).

The Prague strain of Rous Sarcoma Virus, type C, clone S312, was propagated in chick embryo fibroblasts (CEF) obtained from SPAFAS, Inc., embryos. The cells were grown in roller bottles in the standard Medium 199 and nutrient mixture F-10 combination essentially as previously reported for duck embryo fibroblasts (18) except that 5% calf serum was used. Cells were infected in the presence of 1 ug/ml DEAE-dextran at a multiplicity of 0.01 FFU per cell at the time of cell seeding or within 24 h of seeding. The cells were allowed to grow to confluency and then maintained in the same medium with 1% calf serum for 3 to 4 days until extensive transformation occurred, at which time the cells were used for radioactive labeling of RNA. The original virus stock and all primary CEF cells were kindly supplied by Dr. Eugene J. Smith at the USDA Regional Poultry Research Laboratory, East Lansing, Michigan.

Isotopic Labeling of RNA

For labeling R-FeLV RNA simultaneously with L-(methyl-^{3}H) methionine and (^{14}C) uridine, F-422 cells in mid-logarithmic growth phase were pelleted and resuspended to 2.5 x 10^{6}/ml in 400 ml of methionine-free medium, which, in addition to 15% fetal calf serum, contained 20 mM sodium formate, 20 uM each of adenosine and guanosine,

25 uCi/ml of L-(Methyl-^{3}H) methionine (5-15 Ci/mmol from New England Nuclear), and 0.03 uCi/ml of (^{14}C) uridine (50 mCi/mmol from New England Nuclear). Cells were incubated for 4.5 h at which time they were pelleted and resuspended in 400 ml of normal medium and incubated for a second 4.5 h period. Virus was immediately purified from the tissue culture fluids and RNA was extracted from pooled virus as soon as purification of virus from the second 4.5 h labeling interval was completed.

To label R-FeLV RNA with ^{32}P, F-422 cells at 2.5 x 10^{6}/ml were incubated in 200 ml of phosphate-free medium containing 10% fetal calf serum and 10 mCi of ^{32}P as orthophosphate (26 Ci/mg P from Amersham/Searle) for 4.5 h at which time the cells were pelleted and resuspended in 200 ml of normal medium and incubated for a second 4.5 h period. Virus was purified immediately from the tissue culture fluids, pooled, and the RNA was extracted immediately.

When labeling R-FeLV RNA with (^{14}C) uridine alone for use in the RSV mixing experiment, F-422 cells were handled essentially as above except that the cells were resuspended to 2.5 x 10^{6}/ml in 200 ml of normal medium containing 0.15 uCi/ml of (^{14}C) uridine (50 mCi/mmol from New England Nuclear).

For labeling RSV RNA a large roller bottle of confluent, transformed CEF cells was incubated with 10 ml of medium containing 200 uCi/ml of (^{3}H) uridine (40-50 Ci/mmol from New England Nuclear). Cells were incubated for 12 h at which time the labeling medium was removed and the cells were incubated for a second 12 h period in normal medium. Virus was purified from each supernatant fluid immediately after harvest and the RNA was immediately extracted as described for

R-FeLV.

Virus Purification

Feline leukemia virus was purified from F-422 cell tissue culture fluids clarified as previously described (5). Virus was sedimented onto a 4 ml cushion of 45% sucrose (w/w) made up in TNE (0.02 M Tris HCl, pH 7.5; 0.1 M NaCl; 0.001 M EDTA) at 25,000 rpm, 4°C, for 2 h in a Beckman SW27 rotor. The viral band, carefully collected from above to avoid any pellet, was diluted with TNE and the virus was sedimented to an interface of 45% sucrose (w/w) (4 ml) and 20% sucrose (w/w) (20 ml). The viral band was collected from above, diluted with TNE, and pelleted through a barrier of 20% sucrose (w/w).

Rous sarcoma virus was purified in a similar manner from the culture fluid of transformed CEF cells.

Purification of RNA

The viral pellet was resuspended in 0.5 ml of TNE and the suspension was made 1.0% sodium dodecyl sulfate (SDS) by adding 10% SDS in TNE. Approximately 0.2 mg of proteinase K (E. M. Laboratories) was added and the solution was incubated 5 min at 37°C before being extracted three times with an equal volume of TNE-saturated phenol. The RNA was precipitated by addition of carrier RNA (5 A_{260} units /ml, Torula grade B RNA, Calbiochem) and 2 vol ethanol.

The RNA precipitate was dissolved in 0.1-0.2 ml TNE, 0.1% SDS, layered onto a 4.8 ml 5-20% sucrose gradient in TNE, 0.1% SDS, and centrifuged in a Beckman SW50.1 rotor for 40 min at 45,000 rpm and 23°C. Fractions of approximately 0.2 ml were collected and aliquots were counted in a scintillation counter. The fractions containing 50-60S RNA were pooled and the RNA was precipitated by addition of

carrier RNA and 2 vol ethanol.

To obtain R-FeLV subunit RNA the 50-60S RNA was dissolved in 0.02 ml TNE, 0.1% SDS and 0.2 ml of 99% Me_2SO, 1 mM EDTA, 10 mM LiCl. The solution was heated to 60°C for 2 min, quickly cooled to room temperature, and layered onto a 4.8 ml 5-20% sucrose gradient in 99% Me_2SO, 1 mM EDTA, 10 mM LiCl. Centrifugation was in a Beckman SW50.1 rotor for 14 h at 45,000 rpm and 25°C. The gradients were fractionated, aliquots were counted, and the fractions containing R-FeLV RNA were pooled and the RNA precipitated by the addition of carrier RNA (5 A_{260} units/ml), 0.1 vol 1 M sodium acetate pH 5.1, and 2 vol ethanol.

For isolation of cellular RNA, F-422 cells were pelleted and washed once with a balanced salt solution. After swelling on ice for 8 min in hypotonic buffer (10 mM Tris HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$) the cells were broken by dounce homogenization. Nuclei were pelleted by centrifugation at 800g for 2 min and mitochondria were removed by centrifugation at 10,000g for 7 min. The supernatant was made 0.1 M NaCl, 0.01 M EDTA, 0.5% SDS and incubated with proteinase K, after which the RNA was isolated by phenol-chloroform extraction as described (6). The poly A-containing cytoplasmic RNA was isolated as also described (7).

Hydrolysis of RNA

RNA was alkaline hydrolyzed in a total volume of 0.5 ml of 0.4 N KOH for 14-18 h at 37°C. The solution was neutralized with perchloric acid on ice and the $KClO_4$ was removed by centrifugation. The supernatant was lyophilized before being dissolved in 0.005 M sodium phosphate pH 7.8, 7 M urea, for analysis by high speed liquid

chromatography on Pellionex-WAX (see below).

RNAase T2 digestion of RNA was performed by incubating 2 units enzyme per A_{260} unit of RNA in the presence of 0.15 M sodium acetate pH 4.5, 0.9 M NaCl, and 0.01 M EDTA for 2 h at 37°C. The solution was neutralized with KOH before analysis by Pellionex-WAX chromatography.

Acid hydrolysis of mononucleotides was performed as described (7).

Chromatography Systems

The mono- and/or oligonucleotide products of alkaline or RNAase T2 hydrolysis of RNA were analyzed by Pellionex-WAX (Reeve Angel) high speed liquid chromatography as described (7) except that a gradient of 80 ml was used. When the mononucleotides from this column were to be recovered, the appropriate fractions were desalted by DEAE-cellulose (carbonate form) chromatography and subsequently lyophilized. The mononucleotides were then hydrolyzed to nucleosides by treatment with bacterial alkaline phosphatase (Worthington) and analyzed by high speed liquid chromatography on Aminex A-5 (Biorad) as previously reported (25). Purine bases (plus pyrimidine nucleotides) obtained by acid hydrolysis of the mononucleotide fraction from Pellionex-WAX were also analyzed on Aminex A-5 as described (7).

RESULTS

Size of Methyl Labeled RNA

Feline leukemia virus RNA that had been doubly labeled with (methyl-^{3}H) methionine and (^{14}C) uridine yielded two peaks after centrifugation through a non-denaturing aqueous sucrose gradient, one

at about 4S and the other at about 52S (Figure 1). These values are similar to those we have previously reported for ^{3}H-uridine labeled R-FeLV RNA (5). About 17% of the ^{3}H and 48% of the ^{14}C were in the high molecular weight peak. The absence of significant amounts of material sedimenting between the two peaks suggested that very little nonspecific degradation of R-FeLV high molecular weight RNA had occurred.

When the R-FeLV 50-60S RNA thus purified was subjected to centrifugation under denaturing conditions in 99% Me_2SO the majority of the ^{14}C sedimented at 28S (Figure 2), again similar to our results with ^{3}H-uridine labeled R-FeLV RNA (5). Most of the methyl-^{3}H sedimented at 4S. This result would be expected if most of the ^{3}H were contained in 50-60S associated 4S RNA which has been shown to contain large amounts of methylated nucleosides (9). However, a significant amount (22-27%) of the ^{3}H sedimented at 28S, indicating that this RNA species also contains methylated nucleosides. As shown in Table 1, the $^{3}H/^{14}C$ ratios of R-FeLV RNA obtained in several experiments were the same as the respective $^{3}H/^{14}C$ ratios of the host cellular mRNA. Thus R-FeLV 28S RNA and cellular poly A^{+} mRNA are methylated to the same degree on the basis of methylated nucleosides/uridine.

In a control experiment native high molecular weight RSV RNA which had been labeled with (^{3}H) uridine was mixed with (^{14}C) uridine-labeled R-FeLV prior to extraction and purification of RNA from the latter virus. While the ^{14}C labeled R-FeLV subunit RNA sedimented at 28S on Me_2SO gradients the majority of the ^{3}H-labeled RSV subunit RNA was not reduced from its usual size of 33S (on Me_2SO gradients)

Figure 1. Isolation of R-FeLV native high molecular weight RNA by centrifugation through aqueous sucrose gradients.

R-FeLV RNA was sedimented through a 5-20% aqueous sucrose gradient as described in Materials and Methods. F-422 cellular 28S rRNA was run as a marker on a parallel gradient. Fractions denoted by the bars were pooled and the RNA was ethanol precipitated.

(a) RNA labeled with (methyl-^{3}H) methionine and (^{14}C) uridine.

(b) RNA labeled with $^{32}PO_4$.

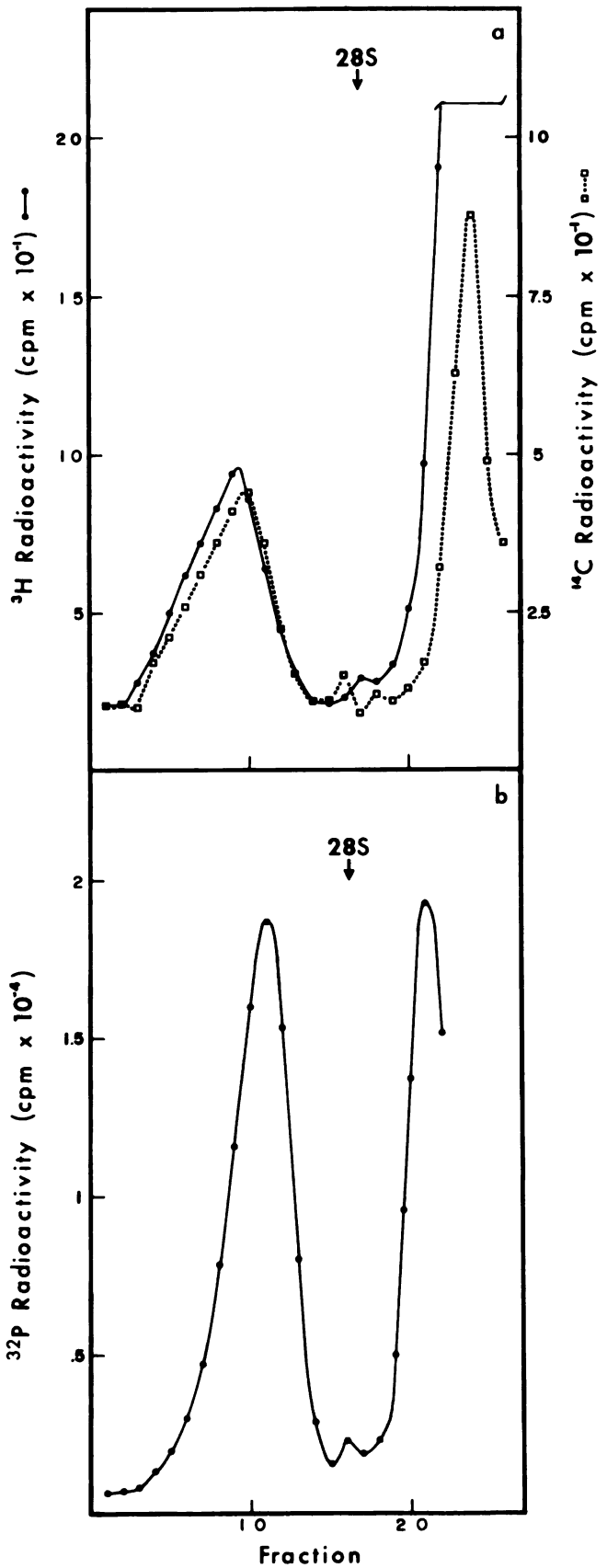

Figure 1. Isolation of R-FeLV native high molecular weight RNA by centrifugation through aqueous sucrose gradients.

Figure 2. Isolation of R-FeLV high molecular weight subunit RNA by centrifugation through sucrose gradients in Me_2SO.

R-FeLV high molecular weight RNA obtained as in Figure 1 was sedimented through a 5-20% sucrose gradient in 99% Me_2SO as described in Materials and Methods. F-422 cellular 28S rRNA was run on a parallel gradient. Fractions denoted by the bars were pooled and the RNA was ethanol precipitated and saved for further analysis.

(a) RNA labeled with (methyl-^3H) methionine and (^{14}C) uridine.

(b) RNA labeled with $^{32}PO_4$.

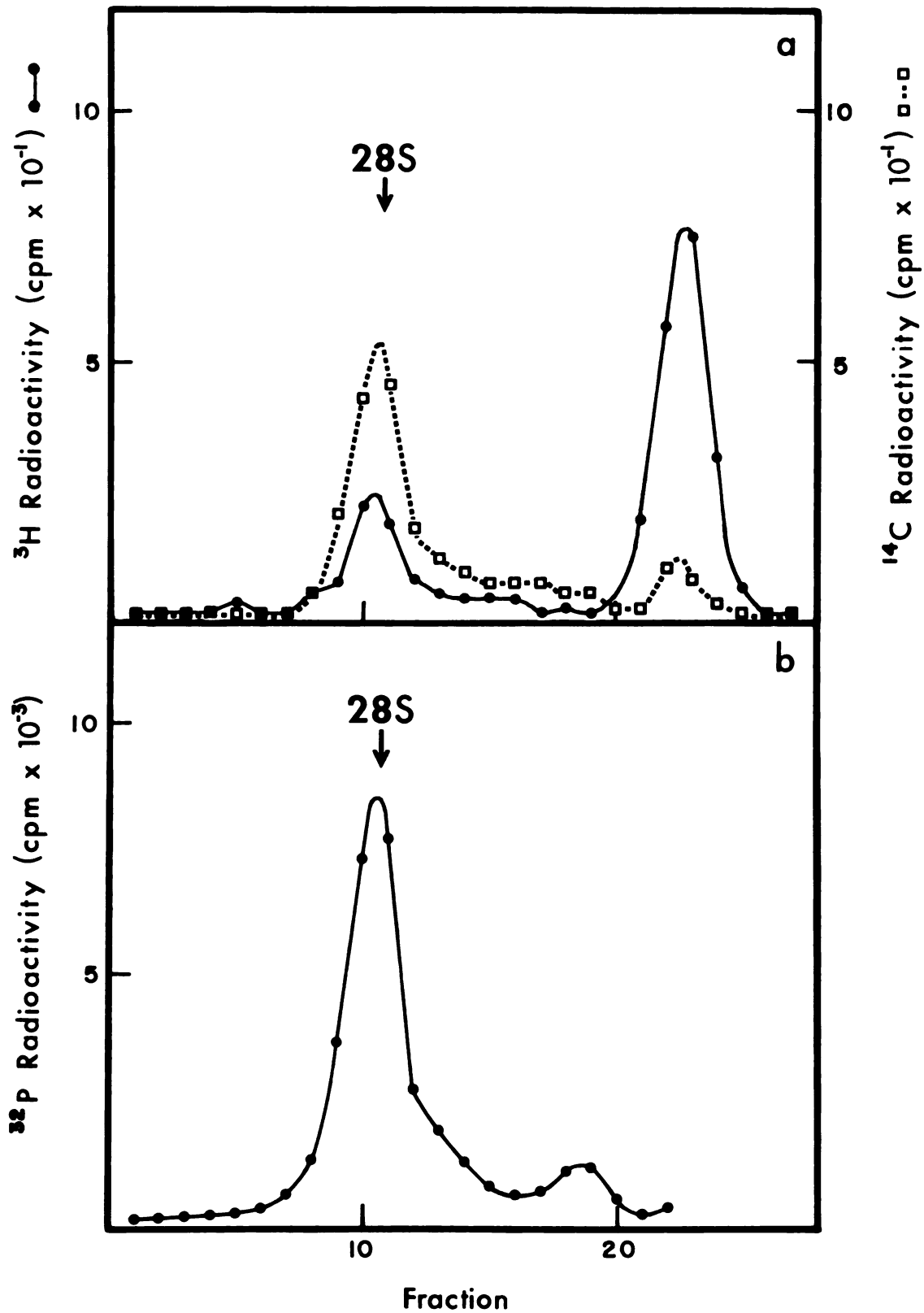

Figure 2. Isolation of R-FeLV high molecular weight subunit RNA by centrifugation through sucrose gradients in Me_2SO.

Table 1

Extent of Methylation of R-FeLV 28S RNA
Relative to F-422 Cellular Poly A+ mRNA.[a]

Experiment	$\frac{^3H \text{ cpm}/^{14}C \text{ cpm FeLV 28S RNA}}{^3H \text{ cpm}/^{14}C \text{ cpm F-422 poly A+ mRNA}}$
1	0.91
2	1.04
3	1.00
4	1.04
Average	1.00

a R-FeLV 28S RNA labeled with (methyl-^3H) methionine and (^{14}C) uridine was purified as described in Figures 1 and 2. The $^3H/^{14}C$ ratio was determined by liquid scintillation counting and was compared to the $^3H/^{14}C$ ratio of F-422 cellular mRNA obtained by oligo (dT)-cellulose chromatography in the same experiment.

Figure 3. Me_2SO/sucrose gradient analysis of RSV RNA added to R-FeLV prior to RNA extraction.

RSV native high molecular weight RNA labeled with (^3H) uridine was added to (^{14}C) uridine-labeled R-FeLV and the RNA was immediately extracted as described in Materials and Methods. The native high molecular weight RNA was purified by aqueous sucrose gradient centrifugation and then centrifuged through a sucrose gradient in Me_2SO as described in Figure 2. Ribosomal 28S RNA was run as a marker on a parallel gradient.

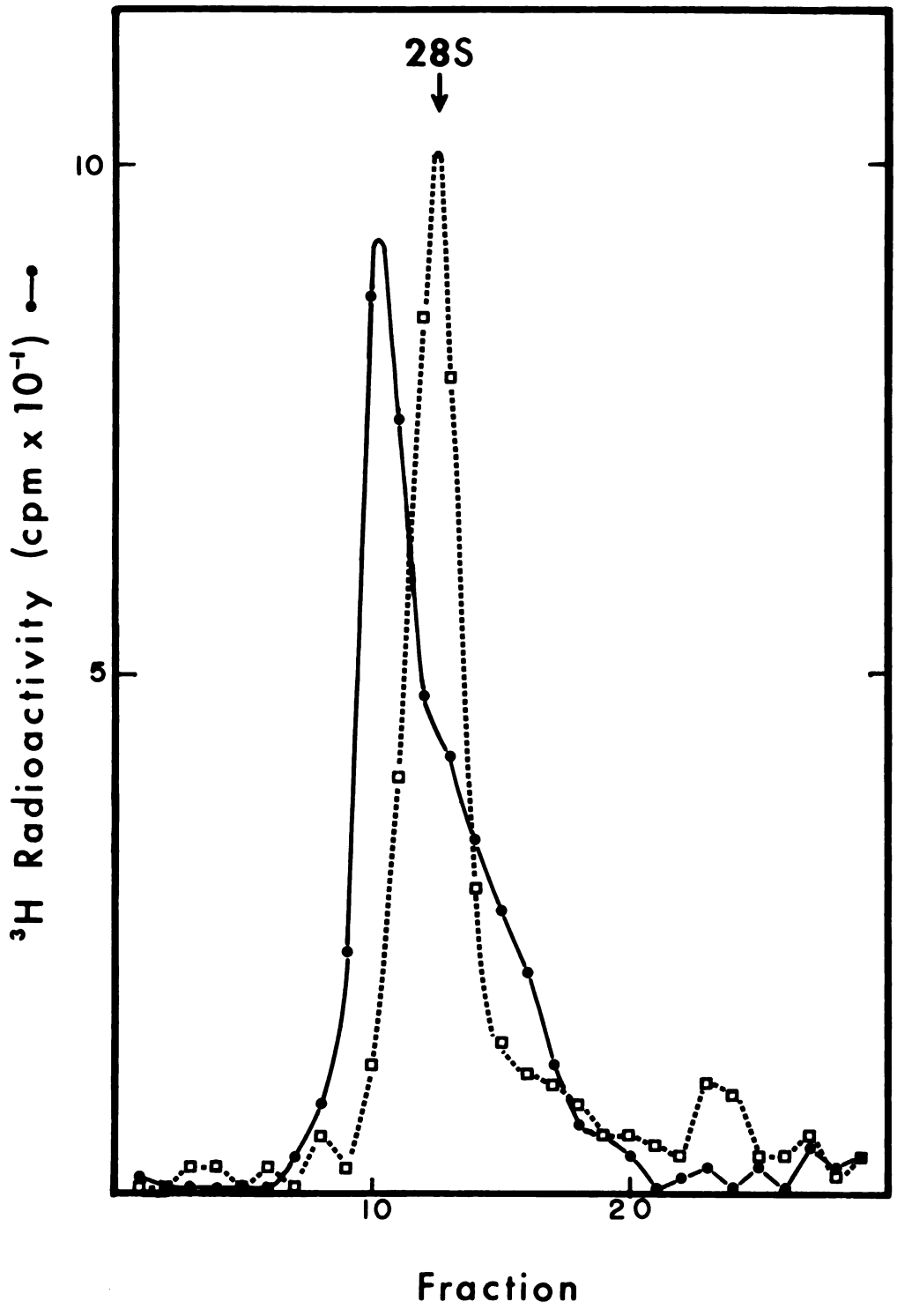

Figure 3. Me_2SO/sucrose gradient analysis of RSV RNA added to R-FeLV prior to RNA extraction.

(Figure 3). Therefore it seems unlikely that the 28S RNA we observe from R-FeLV is derived from a larger 30-40S molecule, similar to the high molecular weight subunit RNA of avian oncornaviruses, by degradation during RNA purification. Furthermore it is unlikely that the R-FeLV 28S RNA is derived from a large precursor that is cleaved within the virus as a function of time, since virus labeled for a short time (60 min) with (^{3}H) uridine also yields RNA sedimenting at 28S (unpublished results).

Absence of Methylated Caps in 28S RNA

Within the last year several animal virus RNAs (1, 10, 11, 12, 15, 17, 31) and eukaryotic cellular mRNAs (2, 7, 24, 29) have been reported to contain at their 5' ends "caps" of the general type $m^7G^{5'}ppp^{5'}Nmp_{(1-2)}Np$. It was therefore of interest to determine if R-FeLV 28S RNA contains a similar structure. Methyl labeled 50-60S R-FeLV RNA was first obtained by centrifugation through aqueous sucrose gradients as in Figure 1a to reduce or eliminate possible contamination by host cell mRNA (see below). The 28S R-FeLV RNA was then obtained by centrifuging the 50-60S through a sucrose gradient in Me_2SO as shown in Figure 2a. The R-FeLV 28S RNA thus purified was hydrolyzed to completion with alkali or RNAase T2. As shown in Figure 4a no resistant structures larger than mononucleotides were found in R-FeLV 28S RNA by Pellionex-WAX chromatography. When analyzed by the same procedures poly A^+ mRNA obtained from the cytoplasm of host cells in the same experiments contained oligonucleotides migrating between $(U_p)_5$ and $(U_p)_7$ markers (Figure 4b), as expected for structures such as described above. Thus R-FeLV 28S RNA does not contain a detectable quantity of methylated caps at its

Figure 4. Analysis of the RNAase T2 digestion products of methyl labeled RNA.

RNA labeled with (methyl-^{3}H) methionine and (^{14}C) uridine was hydrolyzed to completion with RNAase T2 as described in Materials and Methods. Mixtures of oligomers of U, containing $(Up)_3$ through $(Up)_7$ (e.g., $(Up)_3$ is UpUpUp), were mixed with the sample and quickly applied to a Pellionex-WAX high speed liquid chromatography column equilibrated with 0.005 M sodium phosphate, pH 7.8, 7 M urea. The column was developed with a linear gradient of 0.0 M $(NH_4)_2SO_4$ (40 ml) to 0.2 M $(NH_4)_2SO_4$ (40 ml) in 0.005 M sodium phosphate, pH 7.8, 7 M urea. Markers were monitored by UV absorbance and radioactivity in the collected fractions was determined by scintillation counting of aliquots.

(a) R-FeLV 28S RNA.

(b) F-422 cellular poly A^+ mRNA.

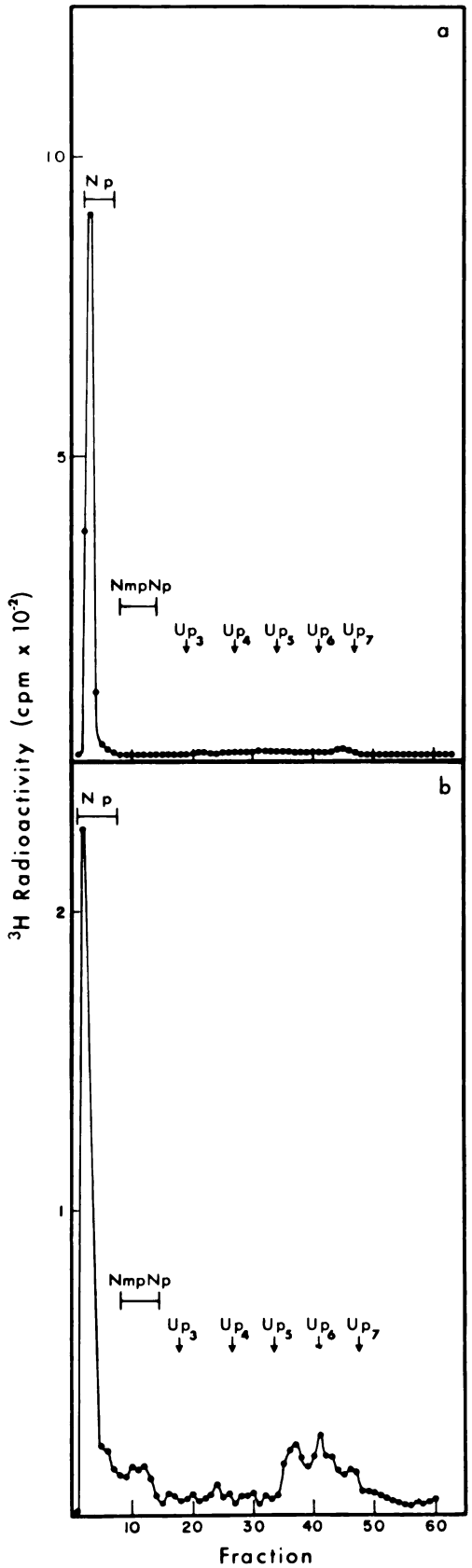

Figure 4. Analysis of the RNAase T2 digestion products of methyl labeled RNA.

5' ends nor does it contain internal nucleosides which are methylated at the 2' position, since such structures would be resistant to these hydrolysis procedures and would migrate with dinucleotide or larger markers. It was found that if the aqueous sucrose gradient centrifugation step for the isolation of native 50-60S R-FeLV RNA was omitted (i.e., if total viral RNA was applied directly to the Me_2SO sucrose gradient) then variable amounts of 2'-O-methylated nucleosides appeared in the final Aminex high speed liquid chromatographic analysis of R-FeLV 28S RNA. These 2'-O-methylated nucleosides may have been derived from contaminating cellular mRNA since chromatography of the RNA on oligo(dT)-cellulose did not result in their removal. However, when the aqueous sucrose gradient centrifugation step was included no 2'-O-methylated nucleosides were found in R-FeLV 28S RNA.

m^6A Is the Only Methylated Nucleoside in 28S RNA

Since after alkaline or RNAase T2 hydrolysis all of the methyl labeled nucleotides co-chromatographed with mononucleotide standards it was concluded that only base methylated nucleotides are present in R-FeLV 28S RNA. The mononucleotides derived from R-FeLV 28S RNA and purified by high speed liquid chromatography (Pellionex-WAX) were desalted by adsorption to DEAE-cellulose (carbonate form) and elution with 1 M ammonium carbonate. After removing the ammonium carbonate by lyophilization the mononucleotides were dephosphorylated with bacterial alkaline phosphatase and chromatographed on Aminex A-5, a cation exchanger which separates nucleosides. As shown in Figure 5a virtually all (90%) of the methyl-^3H co-chromatographed with 6-methyladenosine standard. In another experiment, purine bases were

Figure 5. Determination of the methylated components of R-FeLV 28S RNA.

(a) Mononucleotides obtained as in Figure 4a were desalted and treated with alkaline phosphatase. The nucleosides thus generated were analyzed by Aminex high speed liquid chromatography as described in Materials and Methods. Standards are uridine (U), guanosine (G), adenosine (A), and 6-methyladenosine (m^6A).

(b) Mononucleotides obtained as in Figure 4a were desalted and purine bases were released by acid hydrolysis as described in Materials and Methods. The sample was analyzed by Aminex high speed liquid chromatography. Standards are guanine (Gua), adenine (Ade), and 6-methyladenine (m^6Ade).

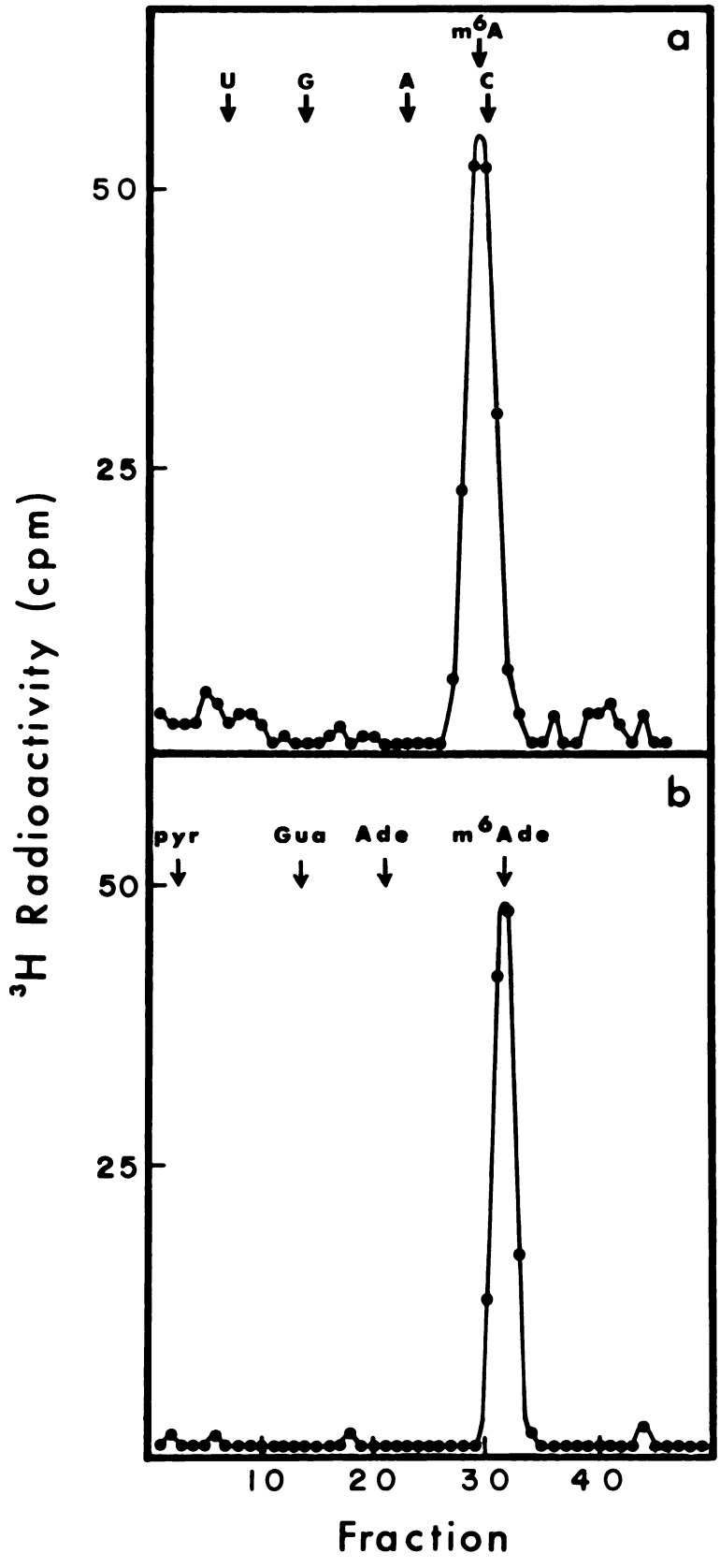

Figure 5. Determination of the methylated components of R-FeLV 28S RNA.

removed from the mononucleotides by acid hydrolysis and analyzed by high speed liquid chromatography on Aminex A-5. More than 96% of the methyl-^{3}H co-chromatographed with authentic 6-methyladenine (Figure 5b). The absence of any methyl-^{3}H radioactivity chromatographing in the position of adenine or guanine confirms that no ring-labeling of purine bases occurred. Thus m^6A is the only methylated nucleoside in R-FeLV 28S RNA.

Absence of nonmethylated Caps in 28S RNA

Although R-FeLV 28S RNA did not contain a methylated 5' cap, we considered the possibility that the RNA of feline leukemia virus (grown in F-422 cells) might differ from other viral and cellular mRNAs by containing a nonmethylated cap of the type $N^{5'}ppp^{5'}Np$. We would not have detected such a structure in our experiments with methyl-labeled RNA. Therefore we examined R-FeLV RNA which had been uniformly labeled with $^{32}PO_4$. RNA purified as in Figures 1b and 2b was hydrolyzed with alkali and examined by pellionex-WAX chromatography. As shown in Figure 6a no ^{32}P-labeled material eluted in the position of the $(Up)_4$ marker as would be expected for a structure such as above. A structure with 4 phosphates should have contained 0.05 to 0.08% of the total cpm (600,000 cpm in the experiment shown in Figure 6a), well within our limits of detection. The small peak eluting near the $(Up)_6$ marker represents only 40 cpm and was not reproducible. Again in control experiments with host cell poly A+ mRNA, alkali-resistant ^{32}P-labeled structures from 300,000 cpm of hydrolyzed RNA eluted between $(Up)_5$ and $(Up)_7$ markers on Pellionex-WAX (Figure 6b). Therefore R-FeLV 28S RNA lacks nonmethylated as well as methylated 5' caps. We have not, however, been able to determine what

Figure 6. Analysis of the alkaline digestion products of RNA labeled with $^{32}PO_4$.

RNA labeled with $^{32}PO_4$ was hydrolyzed to completion with alkali and analyzed by Pellionex-WAX high speed liquid chromatography as in Figure 4.

(a) R-FeLV 28S RNA.

(b) F-422 cellular poly A^+ mRNA.

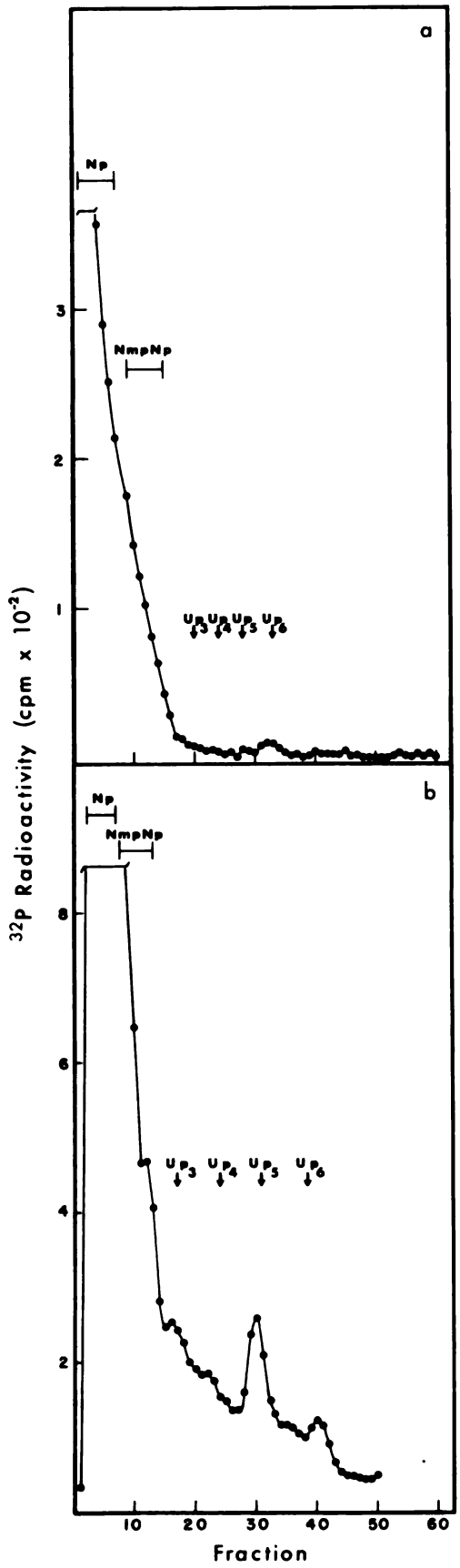

Figure 6. Analysis of the alkaline digestion products of RNA labeled with $^{32}PO_4$.

is at the 5' end of R-FeLV 28S RNA since no structures corresponding to pNp, ppNp, or pppNp were detected in $^{32}PO_4$ labeled RNA and our attempts to label the 5' end of R-FeLV RNA using polynucleotide kinase have been unsuccessful.

DISCUSSION

The results presented here show that R-FeLV 28S subunit RNA does contain methylated nucleosides. The possibility that these methylated components derive from contaminating 50-60S associated 4S RNA seems very unlikely since the R-FeLV 28S RNA was purified by centrifugation through a denaturing density gradient, and since the methylation pattern of R-FeLV 28S RNA differs completely from that of 4S RNA (6). Furthermore it is unlikely that the R-FeLV 28S RNA was contaminated with cellular 28S rRNA since methylation of the latter molecule occurs principally on the 2' position of ribose (6), whereas the only methylated nucleoside in R-FeLV 28S RNA is a derivative of adenosine methylated at the N-6 position. This was demonstrated both by analysis of the nucleosides produced by phosphomonoesterase digestion of the RNAase T2-generated mononucleotides and by analysis of the bases released after acid hydrolysis of the methyl-labeled mononucleotides. Since R-FeLV 28S RNA is methylated to the same degree, on a per uridine basis, as cellular poly A^+ mRNA (Table 1) the number of m^6A residues per R-FeLV 28S RNA molecule can be calculated if the total number of nucleotides in the latter RNA is known. If the molecular weight of R-FeLV 28S RNA is the same as that of 28S rRNA, i.e., 1.8×10^6, then there are about 5,000 total nucleotides and

about 10 m^6A residues per R-FeLV RNA molecule. However, if the molecular weight of R-FeLV 28S RNA is 2.8 to 3.2 x 10^6 (see below) then there are 8,700 to 10,000 total nucleotides and about 17 to 20 m^6A residues per R-FeLV 28S RNA molecule. These calculations use the value of 0.2% methylation given by Perry and Kelly (23) for cellular mRNA. The error due to differences in the base compositions of R-FeLV 28S RNA and cellular poly A^+ mRNA is assumed to be small and is neglected.

Our results also indicate that R-FeLV 28S RNA contains no detectable "cap" (that is, a structure with an inverted 5'-5' pyrophosphate linkage (26)), either methylated or nonmethylated, at its 5' end. However, the amounts of radioactive R-FeLV 28S RNA used in these studies place limits on the conclusions that can be reached. Thus it can be concluded that less than one in ten R-FeLV 28S RNA contains a cap. Cap structures have been found recently in cellular mRNAs (2, 7, 24, 29) and in animal virus mRNAs (1, 10, 11, 17, 31) including those from two strains of an RNA tumor virus (12, 15). The latter reports both dealt with an avian sarcoma virus whereas this report concerns a mammalian leukemia virus. Whether or not this fact is important to the differences reported remains to be determined.

There are several possible explanations for the unexpected absence of a cap structure at the 5' end of R-FeLV 28S RNA. One possibility is that a 5' cap structure is present in an earlier transcript of R-FeLV high molecular weight subunit RNA, but that after packaging in the virus the 5' cap is removed either naturally or as an artifact by endonucleolytic hydrolysis of the RNA molecule. Since the R-FeLV subunit RNA isolated in our laboratories cosediments with 28S ribosomal RNA (both on aqueous and Me_2SO gradients), whereas the

usually accepted value for RNA tumor virus subunit RNA is about 35S, it might be argued that degradation may have occurred during RNA isolation. While this possibility cannot be rigorously excluded, the control experiment, in which RSV 30-40S RNA added to R-FeLV before RNA extraction was not reduced in size, argues against it. In addition such degradation would have to result from a very specific nick since we always observe R-FeLV RNA subunit molecules that are 28S and since very little material sediments between the 28S and 4S peaks (Figure 2). Furthermore, it does not appear that R-FeLV 28S RNA results from breakdown of RNA within the virion as a function of time, since R-FeLV subunit RNA labeled for as short a time as 1 h still sediments at 28S. That R-FeLV and other mammalian RNA tumor virus subunit RNAs can have a sedimentation coefficient of about 28S is supported by the results obtained by East et al. (8) for FeLV (Rickard), FeSV (Rickard), RD-114, and Crandell virus, and by Manning et al. (19) for MuSV (Moloney). Whether the 28S value represents a difference in chain length or conformation remains to be determined, but it is interesting to note that although R-FeLV subunit RNA cosediments with 28S ribosomal RNA it nevertheless migrates more slowly than the same marker during polyacrylamide gel electrophoresis (with an estimated molecular weight of about 3.2×10^6) (5). In this regard RD-114 subunit RNA, which also cosediments with 28S rRNA (8), has a molecular weight of 2.8×10^6 as determined by electron microscopy (16). Therefore R-FeLV 28S RNA could have a molecular weight in the 2.8 to 3.2×10^6 range.

Another possible explanation for the absence of a 5' cap in R-FeLV 28S RNA is that the R-FeLV high molecular weight RNA destined for packaging into virions is not methylated by the normal post-

transcriptional modification enzymes or that a methylated 5' end is removed from the RNA before packaging. This possibility cannot be eliminated, and in fact the finding of no caps in packaged viral RNA does not preclude their presence in similar polysomal viral RNA species. Studies to determine if polysomal R-FeLV RNA does indeed contain a 5' cap structure are being actively pursued. Recent preliminary results in our laboratories indicate, however, that our stocks of feline leukemia virus (Rickard), produced by F-422 feline thymus tumor cells, have only a low level of infectivity. Therefore the present results on R-FeLV RNA methylation may pertain mainly to defective particles. Experiments are in progress to determine if this is indeed the case. If so, it will be interesting to examine the relationship between the absence of a cap structure in R-FeLV 28S RNA and the defective nature of the virus.

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PART III

A METHOD FOR PURIFICATION OF MERCURATED NUCLEIC ACID HYBRIDS ON ETHER-LINKED SULFHYDRYL-AGAROSE

INTRODUCTION

The importance of purification of nucleic acid sequences from mixtures in which the sequence of interest represents only a fraction of the sample has stimulated the development of a number of techniques for this purpose. Isolation of specific sequences on the basis of size or base composition has been practical in only a few unusual cases. Purification schemes which rely on the base sequence itself for specificity are potentially much more powerful techniques. One of the oldest of such methods involves hybridization of a nucleic acid probe to a mixture of sequences and subsequent separation of single-stranded and double-stranded nucleic acids on hydroxyapatite (1). A limitation of this method is that double-stranded regions in extraneous sequences may co-purify with the hybrid. Purification has also been achieved by hybridization of a nucleic acid mixture to a probe covalently bound to a solid support (2-4). While these techniques are very useful, they have some disadvantages. Coupling of the probe to the support is often an inefficient process, making it difficult to achieve high concentrations of the probe. In addition, hybridization to a probe bound to a solid support does not proceed as rapidly or go as far toward completion as hybridization in solution (5, 6).

Recently Dale and Ward (7) have described a technique in which mercurated polynucleotides can be separated from their unmercurated counterparts by chromatography on columns of sulfhydryl-agarose (SH-agarose). Using their technique a mercurated nucleic acid probe can be annealed in solution to a complementary component in a nucleic

acid mixture and the resulting hybrid can be bound to SH-agarose. After removal of unhybridized nucleic acid by extensive washing, the hybrid is recovered by elution with a reducing agent such as 2-mercaptoethanol. In practice, we have found that the instability of the bond linking the sulfhydryl group to the agarose, when the SH-agarose is synthesized by the conventional cyanogen bromide technique (8), can be a serious disadvantage. This is especially true when the nucleic acid sequence to be purified constitutes only a small fraction of the sample. In this case, the extensive washing of the SH-agarose required to remove unhybridized sequences can result in the loss of the hybrid due to breakage of sulfhydryl-agarose bonds. We describe here the use of this method with SH-agarose in which the sulfhydryl group is bound to the agarose *via* a stable ether linkage. In addition, we report conditions under which hybridized nucleic acid can be recovered from the column separately from the mercurated probe, allowing the probe to be re-used in subsequent hybridizations.

MATERIALS AND METHODS

SH-agarose with the sulfhydryl group bound to the agarose support through an ether linkage was synthesized essentially as described by Axen *et al.* (9). Cross-linked Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden) was washed extensively with distilled water and freed of interstitial water by vacuum filtration. The agarose (6 g) was suspended in 4.8 ml 1*N* NaOH and 0.6 ml of epichlorohydrin (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added slowly with stirring at room temperature. The temperature was

raised to 60°C and stirring was continued for 2 h after which the gel was washed extensively with distilled water and then with 200 ml of 0.5 M phosphate buffer (8.2g $NaH_2PO_4 \cdot H_2O$ + 8.44g$Na_2HPO_4 \cdot 7H_2O$ dissolved in 200 ml distilled water; pH = 6.3). After suspending the agarose in 12 ml of the same buffer, 6 ml of 2 M sodium thiosulfate was added and the mixture was shaken at room temperature for 6 h. The gel was again washed by vacuum filtration with distilled water and suspended in 0.1 M sodium bicarbonate to a final volume of 12 ml. The S-alkyl-thiosulfate gel was reduced by addition of 6 ml of a solution of dithiothreitol (200 mg/ml) in 1mM EDTA for 30 min at room temperature. Finally, the product was washed with 60 ml of 0.1 M sodium bicarbonate, 1M NaCl, 1 mM EDTA, and then with 200 ml 1 mM EDTA.

Synthesis of SH-agarose by the cyanogen bromide technique was as described (8). The sulfhydryl content of the SH-agarose was determined as described by Ellman (10), in which thiol groups are titrated colorimetrically with dithionitrobenzene. SH-agarose synthesized as described here contained 2.6 umol SH/ml gel.

Complementary ^{3}H-DNA (^{3}H-cDNA) was synthesized with AMV DNA polymerase using bovine 18S ribosomal RNA (rRNA) for a template as described (A. Thomason et al., submitted for publication).

Bovine pituitary ribosomal RNA was a gift from John Nilson. Novikoff cell ribosomal RNA, labeled with (^{3}H) adenosine, was a gift from Ronald Desrosiers.

RNA was mercurated as described by Dale and Ward (7) in a reaction containing 0.05 M sodium acetate, pH 6.0, 0.02 M mercuric acetate, and 60 mg/ml RNA incubated at 65°C for 20 min. The reaction was stopped by addition of 0.4 vol cold quench buffer (0.01 M Tris·HCl,

pH 7.5, 1 M NaCl, 0.1 M EDTA) and unreacted mercuric acetate was removed by chromatography on a column of Sephadex G-50.

Hybridization conditions and chromatography on SH-agarose are described in the figure legends.

RESULTS

When SH-agarose synthesized by cyanogen bromide activation (8) was used for affinity chromatography of mercurated polynucleotides at room temperature, it was found that the capacity of the column decreased progressively over a period of several months. Figure 1 shows that this decrease in capacity was accompanied by a steady loss of sulfhydryl groups from the agarose. After 113 days the sulfhydryl content of the gel (0.55 umol SH/ml packed agarose) was less than one-fourth of the original level (2.58 umol/ml). SH-agarose stored at -4°C underwent a slower loss of sulfhydryl groups (data not shown). Under comparable conditions, SH-agarose with the sulfhydryl group attached through an ether linkage did not undergo any decrease in sulfhydryl content (Figure 1). The capacity of this gel, which contained approximately 2.60 umol SH/ml, was high. When ^{3}H-labeled cDNA in which all of the thymidine residues were replaced by mercurated uridine was mixed with 200 ug of mercurated calf thymus DNA and passed over a 3- ml column of ether-linked SH-agarose, 92% of the radioactivity was retained by the column (data not shown). Thus the capacity of the column was not exceeded by this amount of mercurated nucleic acid.

In order to develop a method in which a mercurated polynucleotide can be removed from the SH-agarose separately from its hybridized

Figure 1. Stability of SH-agarose prepared with an ether linkage and by cyanogen bromide activation.

Each of the SH-agarose preparations was in use in a chromatography column at room temperature during the time span in which measurements were made. In order to check the sulfhydryl content, a portion of the gel was removed from the column and incubated with 0.15 M 2-mercaptoethanol for 30 min to reduce all of the sulfhydryl groups. The gel was washed extensively with distilled water and interstitial water was removed by vacuum filtration. The sulfhydryl content of 0.1 g portions of the gel was determined by procedure of Ellman (10). SH-agarose prepared by the cyanogen bromide technique, open circles; ether-linked SH-agarose, closed circle.

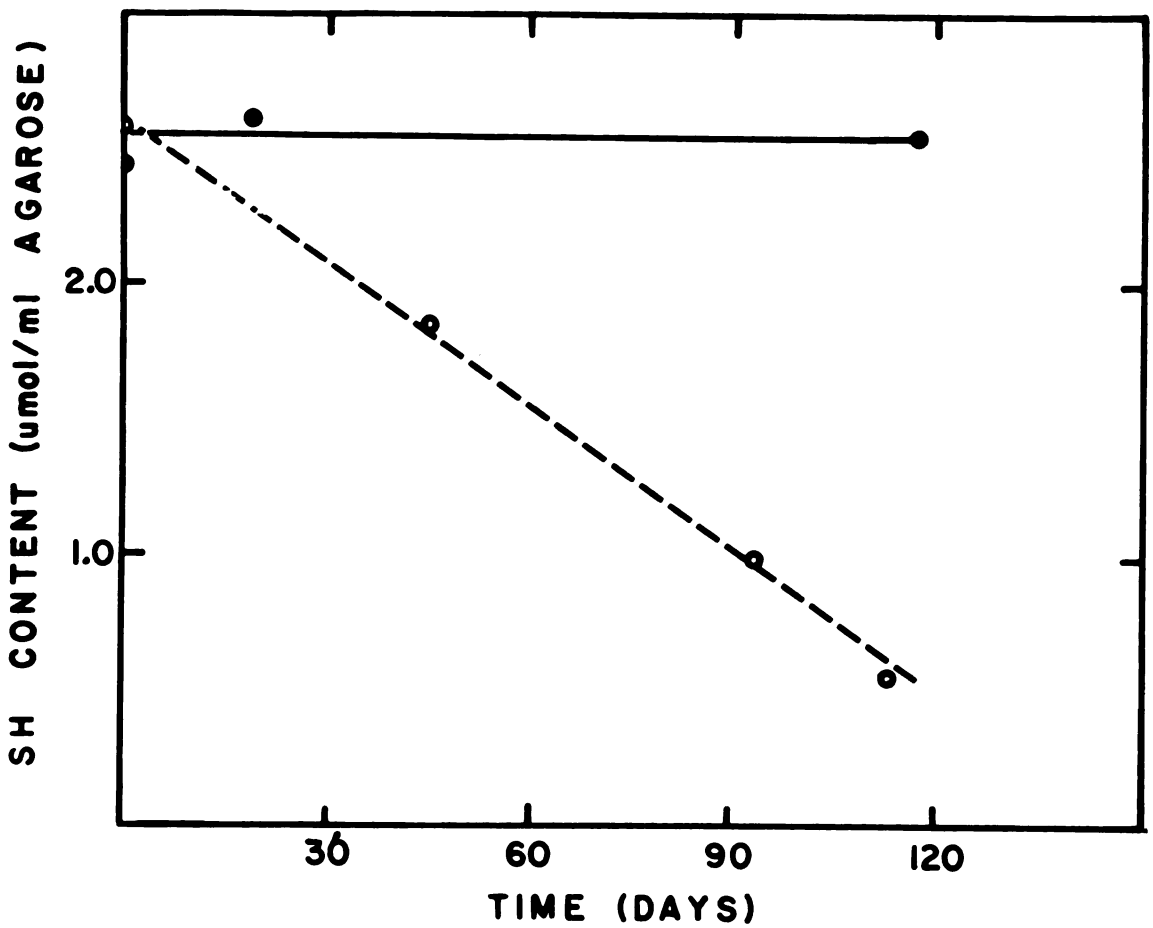

Figure 1. Stability of SH-agarose prepared with an ether linkage and by cyanogen bromide activation.

complementary sequence, experiments were undertaken to determine if the complementary sequence can be eluted with formamide and heat without removing the mercurated polynucleotide. Figure 2 shows an experiment in which unlabeled, mercurated bovine ribosomal RNA (rRNA) was hybridized to unmercurated ^{3}H-labeled cDNA complementary to 18S rRNA and chromatographed on ether-linked SH-agarose. Approximately 67% of the ^{3}H-cDNA was bound to the column. The column was then washed successively with 99% formamide, 0.25 mM EDTA, 0.2% SDS at room temperature (24°C), 40°C, 50°C, 65°C, and 75°C; finally, the mercurated RNA (along with any ^{3}H-cDNA remaining hybridized) was eluted with 2-mercaptoethanol. It was necessary to elute the column with starting buffer after each formamide and heat treatment in order to remove all of the released ^{3}H-cDNA; the reason for this is unknown, but it may be that the NaCl in the starting buffer displaces nucleic acid which becomes attached to positively charged groups on the agarose. The T_m (temperature at which 50% of the hybrid is dissociated) of the ^{3}H-cDNA$_{18S\ rRNA}$:Hg-rRNA complex was found to be about 33°C under these conditions; greater than 95% of the bound ^{3}H-cDNA was released at 65°C, and this temperature was used for dissociation of hybrids in subsequent experiments.

As shown in Figure 3, only 0.15% of the ^{3}H-cDNA$_{18S\ rRNA}$ was bound to the column and released by formamide at 65°C when chromatographed in the absence of complementary Hg-RNA. Chromatography of a variety of unmercurated nucleic acids, both DNA and RNA, indicated that non-specific binding to the column under these conditions is in the range of 0.1 to 0.3% (data not shown).

Figure 4 demonstrates that little mercurated RNA is released from

Figure 2. Determination of conditions for the dissociation of a SH-agarose bound DNA:Hg-RNA hybrid.

^{3}H-cDNA, synthesized with AMV DNA polymerase from a bovine 18S rRNA template, was hybridized with a large excess of bovine Hg-rRNA in 0.2 ml 50% formamide, 0.05 M HEPES (pH 6.8), 0.5 M NaCl, 0.2% SDS for 2 h at 43°C. The reaction was diluted into 0.8 ml starting buffer (0.01 M Tris-HCl (pH 7.5), 0.3 M NaCl, 0.2% SDS) and applied to a 0.9 x 3.0 cm column of ether-linked SH-agarose at a flow rate of 6.3 ml/h. Fractions of 1.25 ml were collected. At the times indicated by F, one column volume of 98% formamide, 0.25 mM EDTA, 0.2% SDS was run onto the column. The eluant flow was stopped and the temperature was raised by pumping heated water through a water jacket surrounding the column for 5 min. Starting buffer was immediately run through the column at the position marked by the second arrow in each set. 2-mercaptoethanol (0.15 M) was run through the column at the time marked by ME. Recovery of ^{3}H-cDNA was 100%.

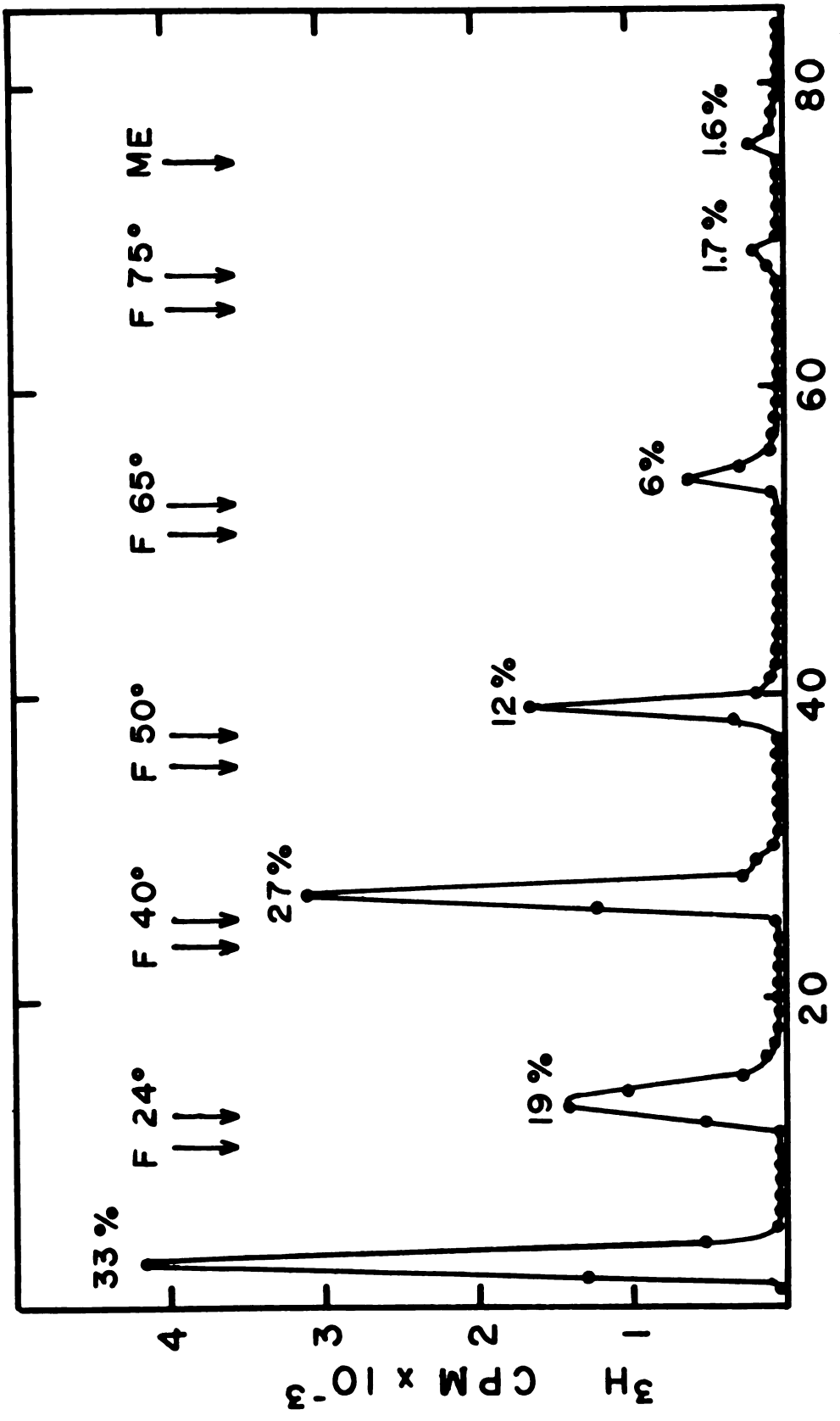

Figure 2. Determination of conditions for the dissociation of a SH-agarose bound DNA:Hg-RNA hybrid.

Figure 3. Determination of the level of non-specific adsorption of cDNA to ether-linked SH-agarose.

Approximately 49,000 cpm of ^{3}H-cDNA was applied to the column as in Figure 2, but mercurated RNA was not included. At the point marked by F, 99% formamide, 0.25 mM EDTA, 0.2% SDS was run onto the column and the temperature was raised to 65°C for 5 min. Starting buffer was run through the column beginning with the position marked by S, and starting buffer containing 2-mercaptoethanol was begun at the position denoted by ME. Recovery of ^{3}H-cDNA was 96%.

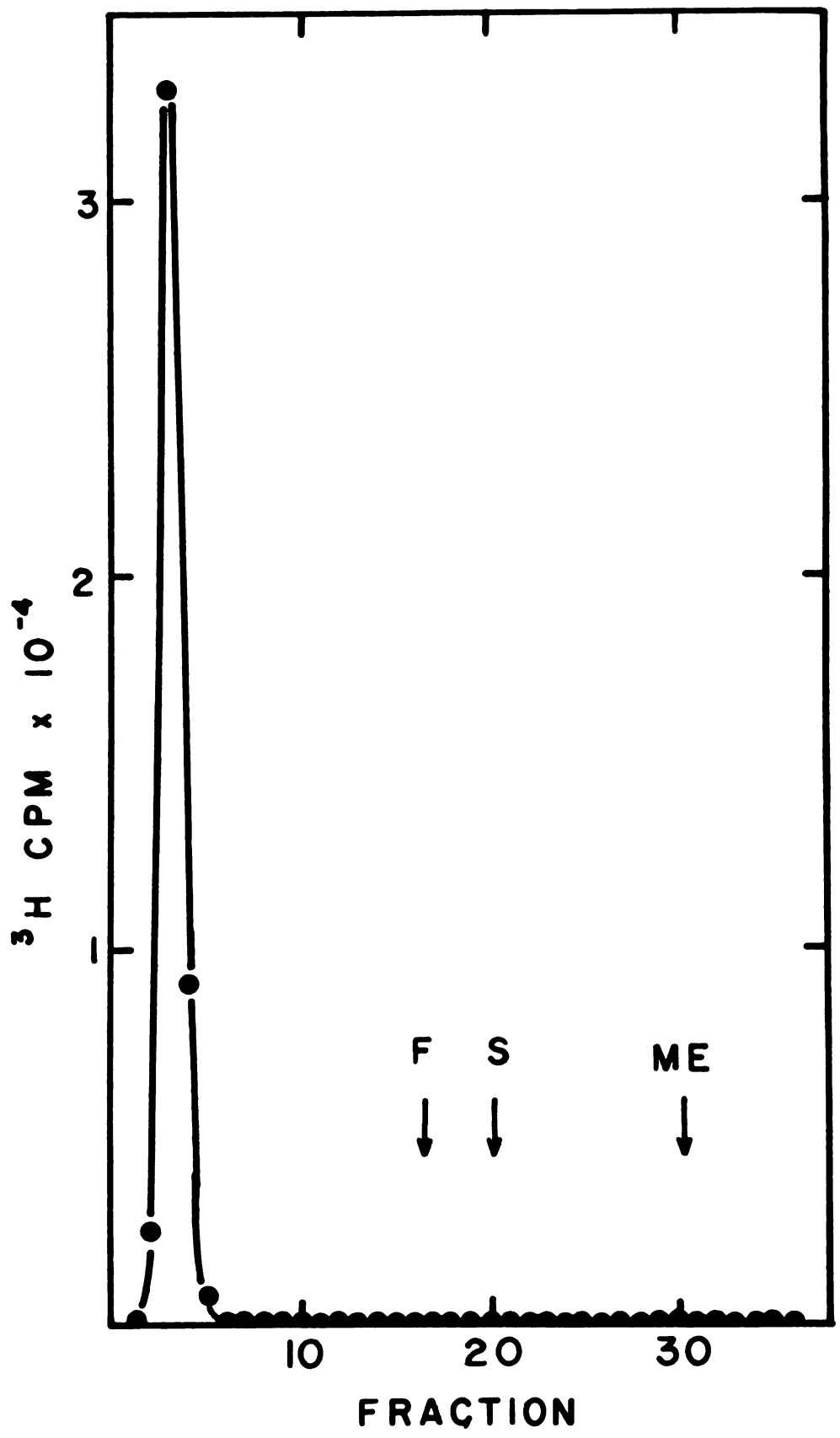

Figure 3. Determination of the level of non-specific adsorption of cDNA to ether-linked SH-agarose.

Figure 4. Determination of the amount of mercurated RNA released from ether-linked SH-agarose by formamide and 65°C.

Novikoff 28S rRNA labeled with (^{3}H) adenosine and mercurated as described in Materials and Methods, was chromatographed on ether-linked SH-agarose as described in Figure 3. Recovery of ^{3}H-(Hg)-RNA was 94%.

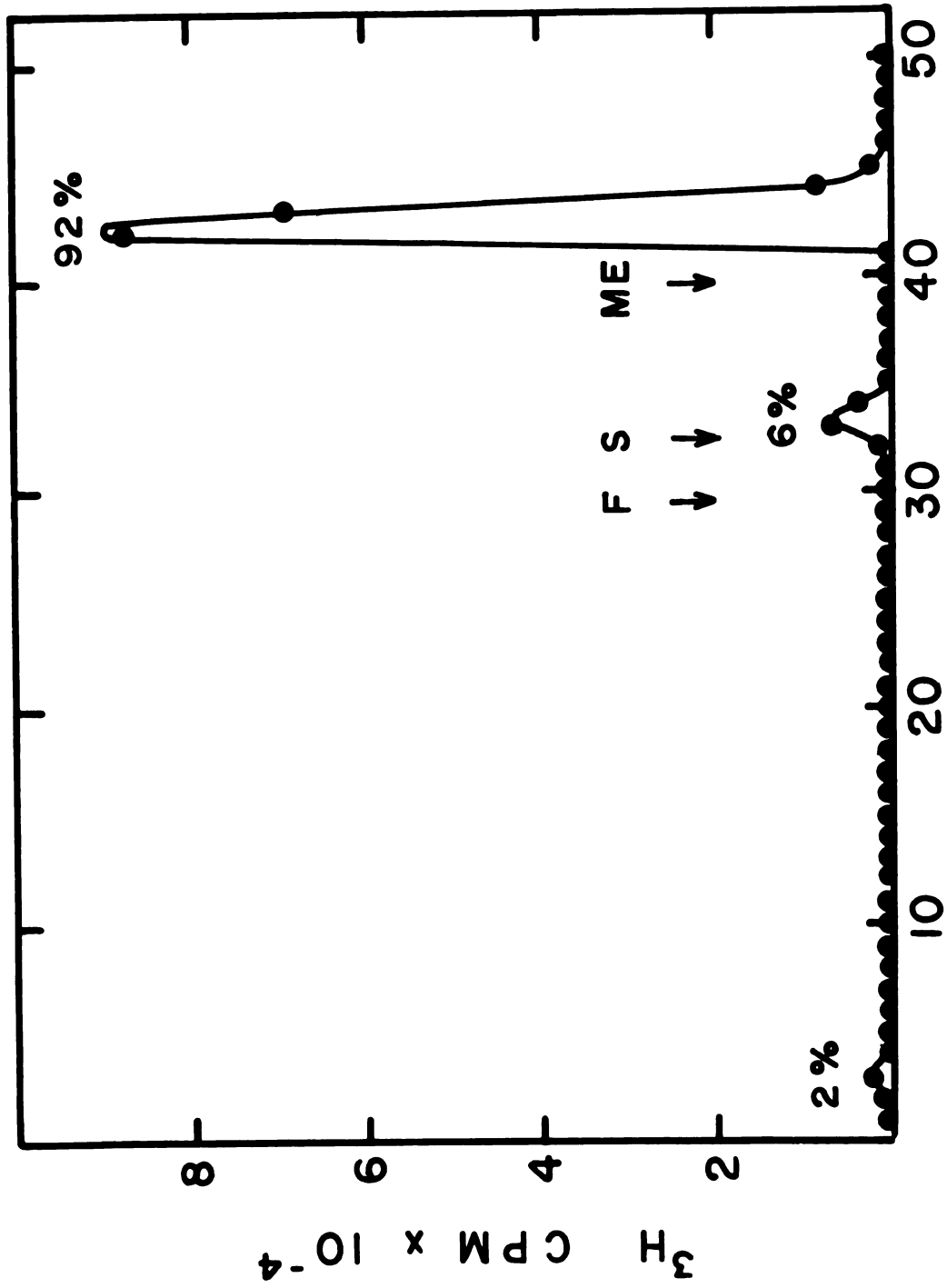

Figure 4. Determination of the amount of mercurated RNA released from ether-linked SH-agarose by formamide and 65°C.

the column by formamide and 65°C; approximately 6% of mercurated Novikoff 28S ^{3}H-rRNA was eluted under these conditions. In other experiments with both mercurated RNA and DNA, 1-10% of the mercurated polynucleotide was released by formamide and 65°C.

DISCUSSION.

In initial experiments in which we attempted to purify an RNA sequence from a mixture by hybridization to its mercurated cDNA followed by chromatography on SH-agarose, we found that extensive washing of the column was necessary to remove what was then considered to be residual unhybridized RNA. However, when it was found that the capacity of the column as well as its sulfhydryl group content was steadily decreasing, we suspected that the RNA that continued to elute from the column was actually RNA hybridized to cDNA; the release of the hybrid would result from breakage of the sulfhydryl-agarose bonds. This problem was overcome by the use of SH-agarose in which the SH-group is attached to the agarose through a stable ether linkage.

The data presented here demonstrate that purification of a nucleic acid sequence by hybridization to a mercurated complementary probe, followed by separation of the hybrid from other nucleic acid sequences on SH-agarose, can be improved by the use of ether-linked SH-agarose. Synthesis of this material is somewhat easier than preparation of SH-agarose by cyanogen bromide activation (8). It is also less time-consuming; synthesis can be completed in a day, whereas two days are necessary when the cyanogen bromide method is

used.

In addition, we have described conditions whereby hybridized RNA or DNA can be recovered from the SH-agarose column separately from the mercurated probe. The mercurated probe can then be re-used in additional hybridizations; this consideration can be important when only small amounts of the probe are available and it is desirable to recycle the probe. The low level of non-specific binding of nucleic acid to the ether-linked SH-agarose permits purification of RNA sequences which comprise only a small fraction of a mixture. For example, we have used this method to purify intracellular oncornavirus RNA sequences, which represent only 1-2% of the cellular RNA (A. Thomason et al., manuscript in preparation).

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PART IV

PURIFICATION OF INTRACELLULAR FELINE LEUKEMIA VIRUS-SPECIFIC RNA AND ANALYSIS OF THE 5' TERMINI

INTRODUCTION

Within the last two years a variety of eucaryotic mRNAs, hnRNAs, and viral RNAs have been shown to contain blocked "cap" structures of the type $m^7G^{5'}ppp^{5'}N''(m)p$ at their 5' termini. These include the high-molecular-weight RNA subunits of two non-defective strains of an avian sarcoma virus (ASV) (13, 16) and of a mammalian leukemia virus, the Moloney strain of murine leukemia virus (MuLV) (2, 20). In a previous communication (28) we reported unexpected results indicating that the subunit RNA of the Rickard strain of feline leukemia virus (R-FeLV) does not contain a 5' terminal cap structure. The RNA subunits of this virus are also unusual in that their sedimentation coefficient, 28S (3), is smaller that that observed for the RNA subunits of most other mammalian leukemia viruses (35-40S). As noted earlier (28), these properties may possibly be related to the replication defectiveness of this virus, since the infectivity of our stock of R-FeLV is very low. Nevertheless, it was possible that intracellular virus-specific RNA acting as messenger RNA (mRNA) might be capped even though virion subunit RNA was not capped. This possibility was strengthened by the results of others (see 22 for references) indicating that caps may be important for the translation of some mRNAs. In the present communication we describe the purification of intracellular R-FeLV virus-specific RNA by hybridization to mercurated complementary DNA (cDNA) and chromatography on sulfhydryl-Sepharose (SH-Sepharose), and the analysis of this RNA for 5'-terminal cap structures.

MATERIALS AND METHODS

Cells and Virus

R-FeLV was purified from the culture fluid of F-422 feline thymus tumor cells propagated as described (3). For large scale production of R-FeLV, 3 l of cells at 2 x 10^6/ml were incubated for 12 h with 0.33 uCi/ml of (^{3}H) uridine as a tracer to follow viral RNA purification. Cells were removed from the culture fluid and the supernatant was clarified as previously reported (3). The supernatant was concentrated to approximately 200 ml with a Pellicon filter apparatus (Millipore) at 0-4°C and virus was purified by centrifugation as described (28).

For labeling F-422 cell RNA with ^{32}P, cells grown to 1 x 10^6/ml in normal medium were concentrated to 2.5 x 10^6/ml and incubated in 200 ml of Swimm's phosphate-free medium containing 5% fetal calf serum for 3 h, at which time 20 mCi of carrier-free ^{32}P as orthophosphate (Amersham/Searle) was added. After an additional 12 h the cells were pelleted and washed twice with phosphate-buffered saline (PBS).

Feline lung fibroblast (FLF-3)cells were grown to near confluency in Leibowitz-McCoys medium containing 15% heat-inactivated fetal calf serum in three 1380 cm^2 roller bottles and the medium was replaced with 5 ml of Swimm's phosphate-free medium containing 5% fetal calf serum. After a 3 h pre-incubation period, 1 mCi of carrier-free ^{32}P as orthophosphate was added and the incubation was continued for 12 h. The medium was removed and the cells were scraped from the bottle, washed with PBS, and extracted as described below for F-422

cells.

RNA Isolation

F-422 cell cytoplasmic RNA was isolated by a modification of the procedure of Cory and Adams (4). Cells were suspended in 25 ml ice-cold hypotonic buffer (0.01 M Tris-hydrochloride, pH 7.5; 0.01 M NaCl; 1.5 mM $MgCl_2$) and swelled on ice for 10 min. The cells were broken by 20 passes with a B pestle in a Dounce homogenizer. Nuclei were removed by centrifugation at 800 x g for 2 min, and mitochondria were pelleted at 10,000 x g for 7 min. One volume of SDS buffer (2% SDS; 15 mM KCl; 40 mM Tris·HCl, pH 7.5; 3.5 mM $MgCl_2$; 20 mM EDTA) along with 0.5 ml of a solution of pre-incubated proteinase K (E. M. Laboratories) (48 mg/ml) was added, and the mixture was incubated for 20 min at room temperature. The digest was adjusted to 0.1 M Tris HCl, pH 9.0, 0.1 M NaCl, and extracted three times with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1). RNA was precipitated from the aqueous phase by the addition of 2 vol ethanol. The pelleted RNA was dissolved in a buffer containing SDS and incubated with proteinase K at 250 ug/ml for 30 min at 37°C, and then either re-extracted with phenol/chloroform/isoamyl alcohol or applied directly to sucrose gradients. This second treatment with proteinase K is essential for removing the last traces of ribonuclease, and is superior to any other method for nuclease inactivation that we have tried.

R-FeLV RNA was purified from virions as previously described (3), except that the extraction was performed at pH 9.0 and a second incubation with proteinase K was included after the initial phenol extration and ethanol precipitation.

Poly (A)-containing RNA was isolated as described (7).

Preparation of cDNA

The conditions for cDNA synthesis from R-FeLV 28S subunit RNA were optimized for maximum yield of the cDNA product. High specific activity ^{3}H-cDNA, for use in analytical hybridizations to sucrose gradient fractions, was synthesized in a reaction containing 0.05 M Tris·HCl, pH 8.3; 8 mM $MgCl_2$; 50 mM KCl; 20 mM 2-mercaptoethanol; 1 mM each dATP, dCTP, and dTTP; 0.1 mM ^{3}H-dGTP, specific activity 15 Ci/mmol (Amersham/Searle); 2.5 mg/ml calf thumus DNA fragments, as primer (26); 50 ug/ml RNA template; and 750 units/ml AMV reverse transcriptase (1 unit incorporates 1 nmol of dTMP into an acid-insoluble product in 10 min at 37°C). Synthesis was at 37°C for 3 h. The reaction mixture was phenol extracted and precipitated with ethanol after the addition of carrier RNA. The precipitate was incubated in 0.6 N NaOH at 37°C for 2 h, after which the cDNA was purified by chromatography on Sephadex G-50.

cDNA to be mercurated was synthesized in a similar reaction except that dTTP was replaced by dUTP, dGTP was 1 mM and ^{3}H-dGTP was added in an amount sufficient to yield 4-6 x 10^{5}/cpm in the product. In initial experiments a mixture of Hg-dUTP and dUTP was used in the reaction, but the enzyme was found to preferentially incorporate the unmercurated nucleotide. The yields of cDNA using DNA fragments as primer were approximately 20-fold greater than in similar reactions using oligo (dT) as primer. The DNA fragment-primed reactions with low concentrations of dGTP yielded 1-2 ug cDNA/10 ug RNA template and those with 1 mM dGTP yielded 4-6 ug cDNA/10 ug RNA template. Except for the use of the DNA fragments as primer, the conditions employed here for cDNA synthesis were similar to those described by

Efstratiadis et al. (8). In agreement with their results, we find that cDNA synthesized under these conditions is substantially single-stranded (90%) even in the absence of actinomycin D, as determined by digestion with S1 nuclease.

Mercuration of cDNA (5) was in 55 mM sodium acetate, pH 6.0, 20 mM mercuric acetate, and 20-100 ug/ml cDNA at 65°C for 20 min. The reaction was stopped by addition of 0.4 vol of ice cold quench buffer (0.01 M Tris·HCl, pH 7.5; 1.0 M NaCl; 0.1 M EDTA) and the mercurated cDNA (Hg-cDNA) was passed over a Sephadex G-50 column to remove unreacted mercuric acetate.

Analytical Hybridization

Hybridization of the high specific activity ^{3}H-cDNA probe made from R-FeLV RNA (^{3}H-cDNA$_{FeLV}$) to sucrose gradient fractions was essentially as described (9; A. Conley and L. Velicer, submitted for publication). Hybridization reactions, conducted in duplicate, were in sealed 10 ul capillary tubes each containing approximately 3000 cpm ^{3}H-cDNA. Reactions were stopped by freezing in a dry ice and ethanol bath, and reactions were kept frozen on dry ice until they were assayed by digestion with S1 nuclease (Miles Laboratories, Inc.).

Preparative Hybridization to Hg-cDNA and Sulfhydryl-Sepharose Chromatography

Hg-cDNA was hybridized to RNA in 25 mM HEPES, pH 6.8, 0.5 M NaCl, 0.2% SDS, 50% formamide (purified by re-crystallization (27)) at 43°C for times long enough to ensure that the reaction went to completion (1-5 h) in sealed, siliconized glass tubes. The reaction mixture was diluted into 5-6 vol of (SH-Sepharose) starting buffer (0.01 M Tris·HCl, pH 7.5; 0.2 M NaCl; 0.2% SDS) and applied to a

0.9 x 2.5 cm column of SH-Sepharose with a peristaltic pump at a flow rate of 5-7 ml/h.

In initial experiments, the Hg-cDNA:RNA hybrid was applied to the column and washed extensively with starting buffer; the hybridized RNA was then released by the addition of 99% formamide, 0.2% SDS, heating to 70°C, and immediately following with starting buffer. The wash with starting buffer, containing 0.2 M NaCl, was necessary to remove all of the RNA from the column. Bound Hg-cDNA was then eluted from the column with starting buffer containing 0.15 M 2-mercaptoethanol; the 2-mercaptoethanol was removed by chromatography on Sephadex G-50. Removal of 2-mercaptoethanol by Sephadex G-50 chromatography is faster than removal by dialysis as described by Dale and Ward (5), but it does result in dilution of the sample.

In later experiments the bound Hg-cDNA:RNA hybrid was eluted intact from the column with 2-mercaptoethanol, freed of 2-mercaptoethanol by Sephadex G-50 chromatography, ethanol precipitated with carrier RNA and re-applied to the SH-Sepharose column to remove residual non-hybridized RNA. The hybrid again was eluted with 2-mercaptoethanol and purified as after the first pass over the column. Finally, the hybrid was dissociated by dissolving in 99% formamide, 0.01 M Tris·HCl (pH 6.9), 0.2% SDS, 0.25 mM EDTA, and heating to 70°C for 4 min. The sample was diluted with 2 vol SH-Sepharose starting buffer and passed over the SH-Sepharose column the third time. RNA was excluded from the column in the void volume and collected by ethanol precipitation; Hg-cDNA was recovered from the column by elution with 2-mercaptoethanol. This latter procedure, although more laborious, reduced the amount of cDNA and non-specifically bound RNA that eluted with the hybridized

RNA.

SH-Sepharose, in which the Sulfhydryl group is attached to agarose via an ether linkage, was prepared as described (A. Thomason and F. Rottman, submitted for publication). SH-Sepharose prepared in this manner is much more stable and less subject to non-specific binding of RNA than SH-Sepharose prepared by conventional cyanogen bromide activation of Sepharose.

Analysis of RNA for 5' End Groups

RNA labeled with ^{32}P was digested with RNAase T2 as described (28) or with a combination of RNAase T2 (10 units A_{260}unit RNA), RNAase T1 (1 unit/A_{260}unit RNA), and RNAase A (5 ug/A_{260}unit RNA) for 6 h at 37°C. In some cases reovirus RNA labeled in vitro with (^{3}H) S-adenosylmethionine (a gift from Dr. A. Shatkin) was mixed with the ^{32}P RNA before digestion. The reaction products were separated by chromatography on DEAE-Sephadex in the presence of 7 M urea as previously reported (10).

Ribonuclease-resistant oligonucleotides recovered from the DEAE-Sephadex columns were freed of urea and salt by chromatography either on Biogel P-2 (10) in the presence of ammonium bicarbonate or on DEAE-cellulose. The DEAE-cellulose column was washed with 0.02 M ammonium acetate after the sample was bound, and the oligonucleotide was then eluted with 0.5 M ammonium acetate. Ammonium acetate was removed by flash evaporation and lyophilization. The DEAE-cellulose procedure was adopted since chromatography on Biogel P-2 sometimes resulted in degradation of cap structures and/or incomplete removal of urea.

Chromatography on acetylated dihydroxyborylaminoethyl-cellulose

(DBAE-cellulose) (Collaborative Research, Inc.) was as described by McCutchan et al. (17), except that the composition of the application buffer was 0.05 M Tris HCl, 0.6 M KCl, 0.01 $MgCl_2$, 20% ethanol, pH 7.7. Cap structures can be degraded to nucleosides and free phosphate in the presence of impure morpholine (A. Thomason, unpublished observation); this problem is not experienced when Tris·HCl replaces morpholine, and the DBAE-cellulose still binds cap structures under these conditions. Bound material was eluted from DBAE-cellulose with Buffer G (17) containing 1 M sorbitol. Salts and sorbitol were removed by chromatography on DEAE-cellulose in the presence of ammonium acetate.

Digestion of caps with nuclease P1 and analysis of the products by high speed liquid chromatography on Partisil-SAX have been described (M. Kaehler et al., in press).

RESULTS

Synthesis and Mercuration of cDNA

The cDNA used as a probe in these experiments was synthesized in an exogenous reaction using AMV reverse transcriptase and purified R-FeLV 28S subunit RNA as template. It was felt that cDNA prepared from an RNA template that had been purified through two sucrose gradient centrifugations (28) would contain fewer non-viral sequences than cDNA prepared in an endogenous reaction. The use of DNA fragments, generated by DNAase I digestion of calf thymus DNA, as primer for cDNA synthesis (26) allowed the yield of cDNA to be greatly increased as compared to the use of oligo $(dT)_{12-18}$ as primer; yields of up to 60% of the input RNA were obtained. In addition, cDNA

prepared in this manner is a more representative transcript than cDNA prepared in endogenous reactions or with oligo (dT) as primer (6). The average size of the cDNA, as measured by sedimentation through a denaturing sucrose gradient (Figure 1), was about 12S; this corresponds to an average length of about 800-1000 nucleotides.

For synthesis of cDNA that was to be mercurated, dTTP was replaced in the reaction mixture with dUTP; thus, during mercuration both deoxyuridine and deoxycytidine residues in the cDNA were mercurated instead of just deoxycytidine, as would have been the case if dUTP had not been included in the reaction. Mercurated derivatives of uridine are less subject to demercuration than similar derivatives of cytidine (5), a factor to be considered when a mercurated polymer is to be re-used and exposed several times to heat and 2-mercaptoethanol. AMV reverse transcriptase accepts dUTP as well as dTTP at the 1 mM level, as judged by the yields of the cDNA product.

When cDNA is mercurated under the conditions described in Materials and Methods, the resulting Hg-cDNA is capable of forming stable hybrids with its complementary RNA. The level of mercuration is sufficient to allow essentially complete binding to SH-Sepharose, but is not so high that removal from the support is difficult with 2-mercaptoethanol. When cDNA is mercurated as in Materials and Methods but in 20 mM sodium acetate instead of 55 mM, 20-50% of the cDNA binds so tightly to SH-Sepharose that it is difficult to release with 2-mercaptoethanol.

Isolation of F-422 Cell Virus-Specific RNA

F-422 cell total cytoplasmic RNA labeled with ^{32}P was isolated, denatured, and centrifuged through sucrose gradients in a SW27 rotor

Figure 1. Determination of the size of cDNA synthesized in the exogenous polymerase reaction.

cDNA was synthesized using purified R-FeLV 28S subunit RNA as template and calf thymus DNA fragments as primer as described in Materials and Methods. dTTP was replaced by dUTP and all four dNTPs were 1 m$\underline{M}$. After purification, the cDNA (10,000 cpm) was dissolved in 220 ul of 90% Me_2SO, 1 m$\underline{M}$ EDTA, 10 m$\underline{M}$ LiCl, heated to 60°C for 2 min, and centrifuged through a 4.8 ml 5-20% sucrose gradient in 99% Me_2SO, 1m$\underline{M}$ EDTA, 10 m$\underline{M}$ LiCl in a Beckman SW50.1 rotor for 22 h at 45,000 rpm and 25°C. F-422 cell cytoplasmic RNA was run as marker on a parallel gradient. The gradients were fractionated and fractions were assayed for radioactivity by scintillation counting.

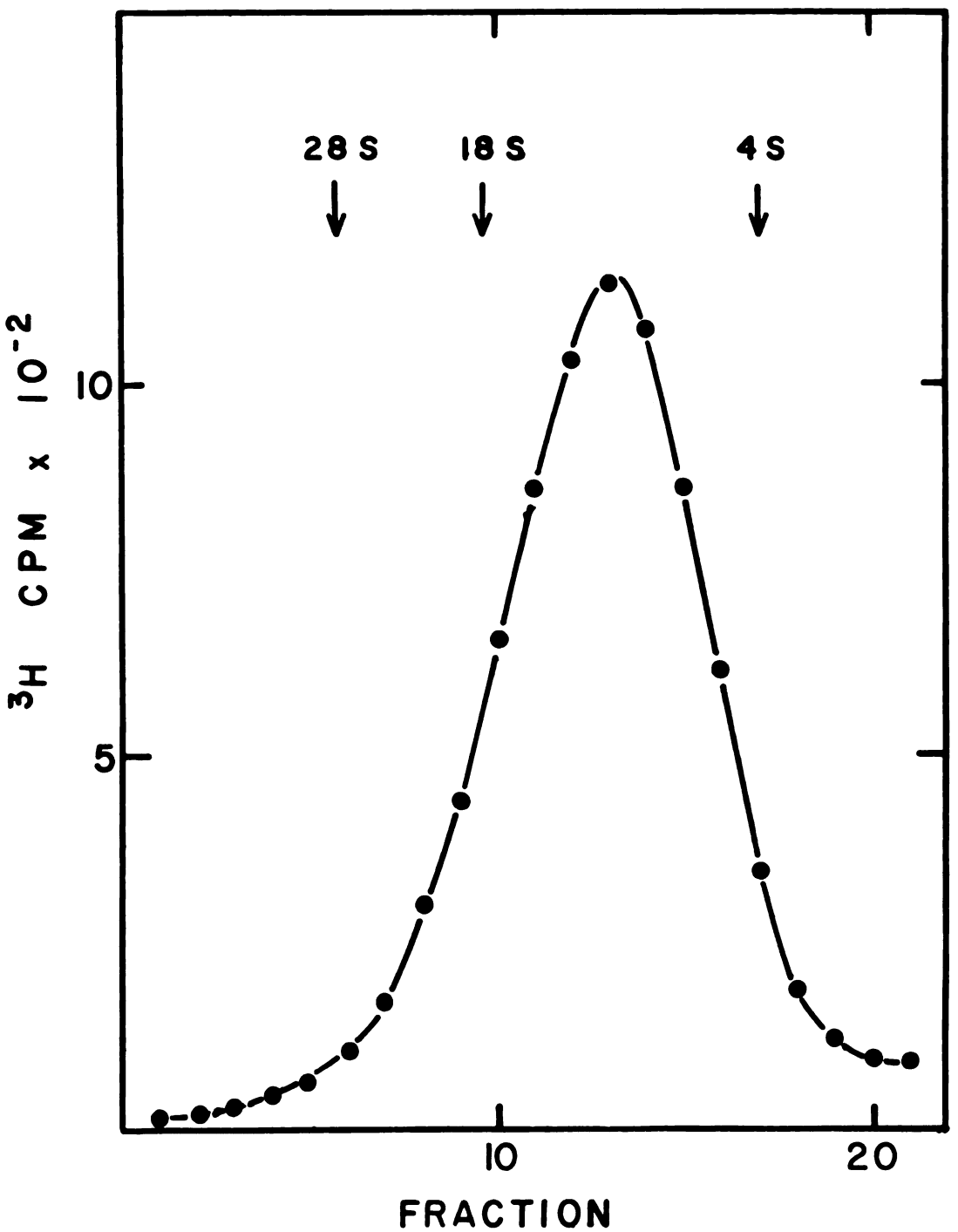

Figure 1. Determination of the size of cDNA synthesized in the exogenous polymerase reaction.

to separate RNA species on the basis of size. After fractionation of the gradients, the positions of viral-specific RNA were determined by hybridization of $^{3}H\text{-}cDNA_{FeLV}$ to aliquots of each fraction. The results are shown in Figure 2a. The predominant viral-specific RNAs detected with this probe sedimented at approximately 23S and 4-18S (average 15S), with smaller peaks at 36S and 28S. This analysis detected lesser amounts of the intracellular 36S and 28S RNAs than in a previous study (A. Conley and L. Velicer, submitted for publication) (see Discussion). One possible explanation for this is that the RNA examined here might be degraded. However, the sedimentation profile of a small amount of the F-422 cell cytoplasmic RNA, centrifuged in a SW50.1 rotor for a time sufficient to put 28S RNA near the bottom of the gradient, indicated that the RNA was essentially intact (Figure 2b).

Fractions from the gradients containing 36S, 28S, 23S, and "15S" RNA (peaks I-IV in Figure 2a) were pooled separately and ethanol precipitated. Each class of RNA was hybridized with $Hg\text{-}cDNA_{FeLV}$ and subjected to chromatography on SH-Sepharose as depicted for 23S RNA in Figure 3. The amounts of 36S and 28S viral-specific RNA (vRNA) obtained in this manner were insufficient for additional characterization and these RNA species were not further analyzed. Less than 0.02% of RNA non-complementary to the Hg-cDNA probe co-purifies with complementary RNA when chromatography is conducted as in Figure 3. Even when less rigorous purification procedures are used, such as those described for control experiments in Figure 4, only 0.05-0.2% of non-specifically bound RNA is released with hybridized RNA. Nevertheless, this amount of contamination can be significant when the RNA to be purified constitutes only 0.2-2.0% of the sample. In addition,

Figure 2. Sucrose gradient sedimentation of F-422 ^{32}P-labeled cytoplasmic RNA and location of R-FeLV virus-specific RNA. (a) RNA (2 x 10^9 cpm; approximately 100 A_{260} units) labeled with ^{32}P and isolated as described in Materials and Methods was dissolved in 0.18 ml 0.01 M Tris·HCl, pH 7.5, 1 mM EDTA, 0.2% SDS and 1 ul was removed for the analysis described in (b). To the remainder, four volumes of 99% Me_2SO, 1 mM EDTA, 10 mM LiCl was added and after heating to 60°C for 2 min the sample was diluted with 9 vol of 0.01 M sodium acetate, pH 5.0, 0.1 mM EDTA, 0.2% SDS, to a total volume of 9 ml. Portions of 1.5 ml were layered onto each of six 36- ml 5-20% sucrose gradients in 0.01 M sodium acetate, pH 5.0, 0.1 mM EDTA, 0.2% SDS and centrifuged in a Beckman SW27 rotor for 10 h at 22,500 rpm and 23°C. Aliquots of the collected fractions were hybridized with ^3H-cDNA and assayed by digestion with S1 nuclease. Hybridization values were converted to relative virus-specific RNA (9).

(b) The 1 ul aliquot of the ^{32}P-labeled RNA removed from the sample before centrifugation on SW27 gradients in (a) was mixed with 1 ml of 0.01 M Tris·HCl, pH 7.5, 0.12 M 18 ul 0.01 M Tris·HCl, pH 7.5, 1 mM EDTA, 0.2% SDS, and 0.2 ml 99% Me_2SO, 1 mM EDTA, 10 mM LiCl. The sample was centrifuged through a sucrose gradient in Me_2SO as described for Figure 1, except that centrifugation was for 18 h.

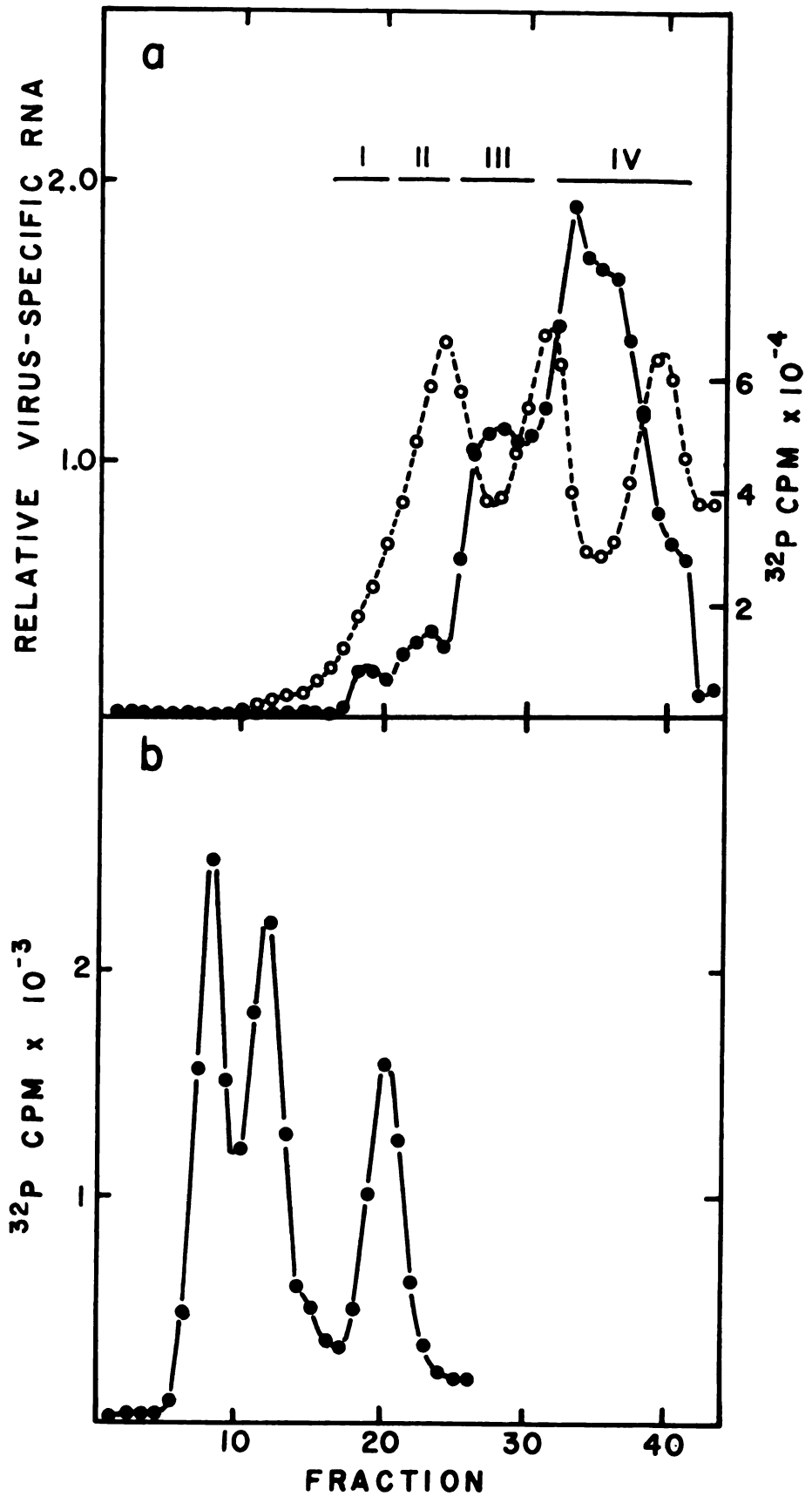

Figure 2. Sucrose gradient sedimentation of F-422 ^{32}P-labeled cytoplasmic RNA and location of R-FeLV virus-specific RNA.

Figure 3. Purification of F-422 virus-specific 23S RNA hybridized to Hg-cDNA by chromatography on SH-Sepharose.

Fractions from the sucrose gradient in Figure 2a containing 23S RNA (peak III) were ethanol precipitated, and the RNA was hybridized for 4 h in a 0.2 ml reaction volume to (^{3}H)Hg-cDNA and chromatographed on SH-Sepharose as described in Materials and Methods. At the times marked by the arrows, buffer containing 0.15 M 2-mercaptoethanol was applied to the column. Aliquots (2.5 ul) of each fraction (1.25 ml) were assayed for ^{32}P and ^{3}H by scintillation counting.

(a) SH-Sepharose chromatography of the original hybridization reaction.

(b) Re-chromatography on SH-Sepharose of the hybrid eluted by 2-mercaptoethanol in (a), after dissociation by formamide and heat as described in Materials and Methods.

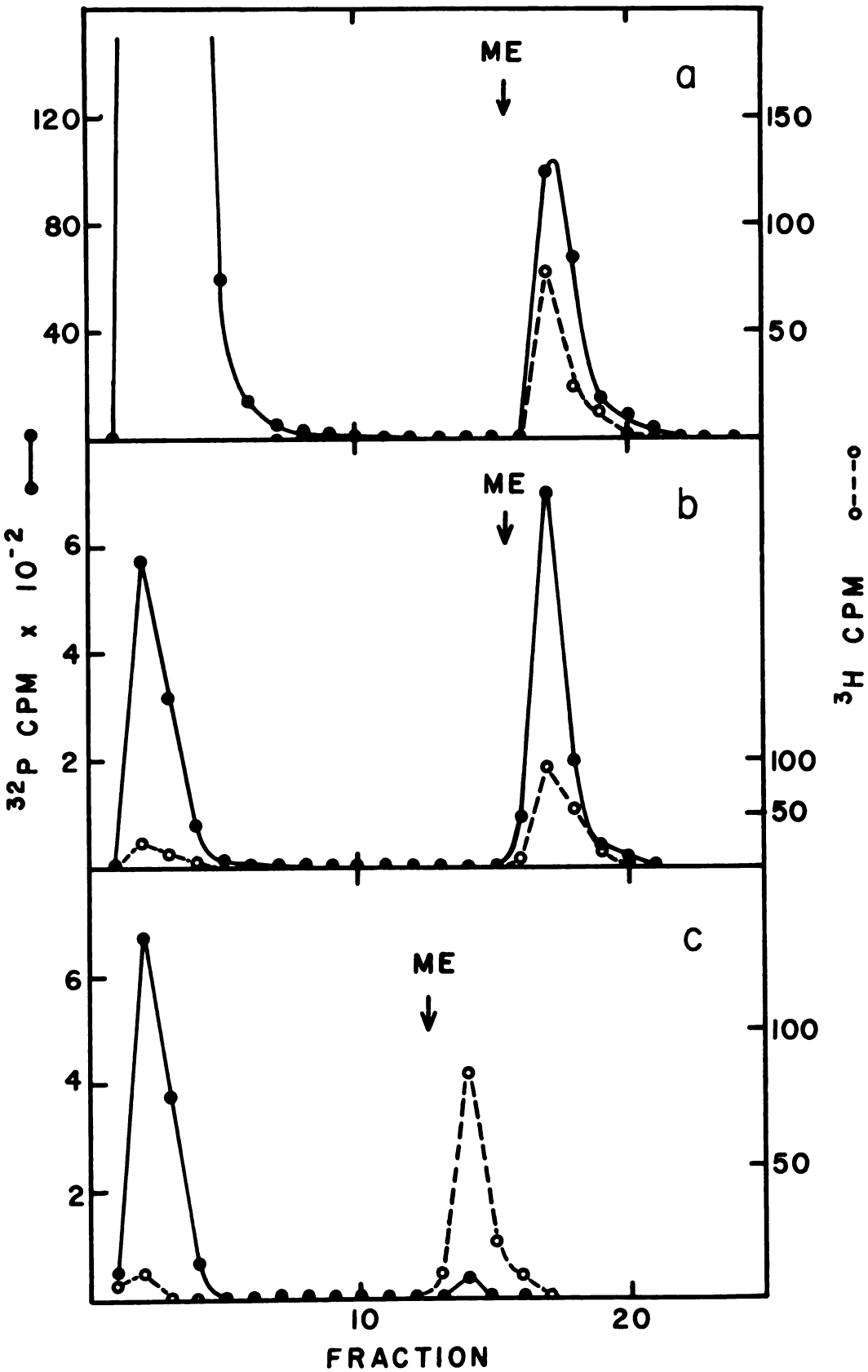

Figure 3. Purification of F-422 virus-specific 23S RNA hybridized to Hg-cDNA by chromatography on SH-Sepharose.

Figure 4. SH-Sepharose chromatography of Hg-cDNA hybridized with ^{32}P-labeled R-FeLV RNA and FLF-3 cytoplasmic RNA.

(a) (^{3}H)Hg-cDNA was hybridized with ^{32}P-labeled R-FeLV 28S subunit RNA as described in the text and chromatographed on SH-Sepharose as described in Materials and Methods.

(b) (^{3}H)Hg-cDNA was incubated with ^{32}P-labeled uninfected FLF-3 cytoplasmic RNA and chromatographed on SH-Sepharose as in (a). At the times denoted by F, one column volume of 99% formamide, 0.2% SDS was run onto the column; the temperature was raised to 70°C for 4 min; and the column was immediately washed with starting buffer. Starting buffer containing 2-mercaptoethanol was applied at the times marked by ME.

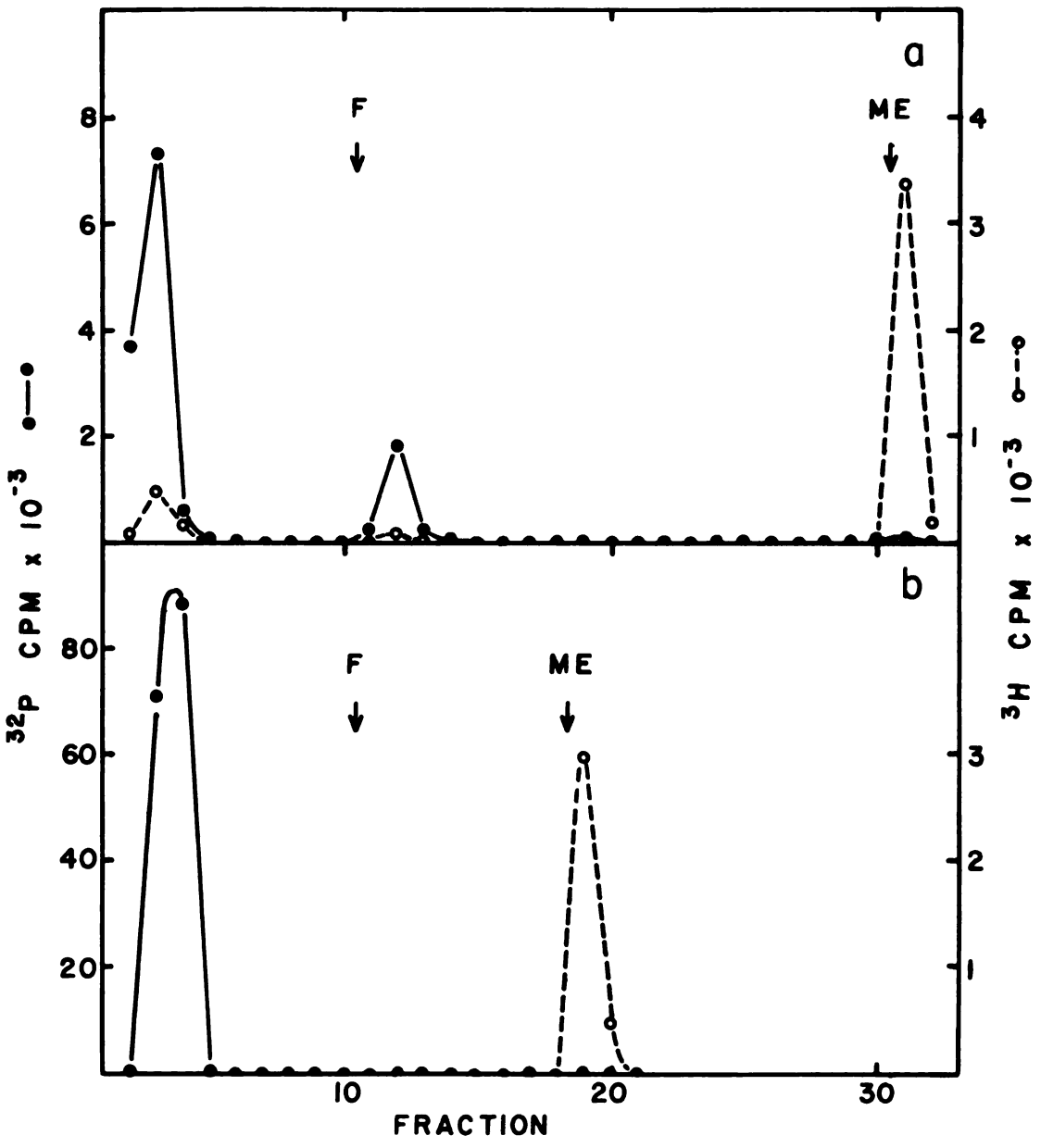

Figure 4. SH-Sepharose chromatography of Hg-cDNA hybridized with ^{32}P-labeled R-FeLV RNA and FLF-3 cytoplasmic RNA.

some of the Hg-cDNA bound to SH-Sepharose is eluted by the formamide and heat step shown in Figure 4. This problem is much reduced when the hybrid is denatured before application to the column, as in Figure 2.

In the control experiment shown in Figure 4a, 0.3 ug of Hg-cDNA was hybridized to 3.0 ug of ^{32}P-labeled R-FeLV 28S subunit RNA; 0.36 ug, or 12% of the input RNA was bound to SH-Sepharose along with the Hg-cDNA. This amount of RNA represents complete saturation of the Hg-cDNA. When 7.0 ug of ^{32}P-labeled uninfected FLF-3 total cytoplasmic RNA was hybridized to 0.3 ug of Hg-cDNA, only 4 ng or 0.06% of the RNA was retained by SH-Sepharose and eluted by formamide and heat. These results demonstrate the specificity of this purification technique, which can be improved still further by use of the modifications described in Figure 3.

Analysis of F-422 "15S" and 23S vRNA for 5' Termini

The ^{32}P-labeled "15S" and 23S vRNA species were each mixed with reovirus RNA (labeled *in vitro* with (^{3}H)S-adenosylmethionine) and digested with ribonucleases A, T1, and T2. The products were characterized by chromatography on DEAE-Sephadex, 7 M urea, as shown in Figure 5 for 23S vRNA. Essentially all of the ^{3}H migrated near the $(pUm)_5$ marker, which was expected since all of the methyl groups in reovirus RNA are in cap I structures (12). Most of the ^{32}P-labeled material eluted at the position characteristic of 3' mononucleotides. Much smaller amounts of material eluted with dinucleotides and near $(pUm)_4$ and $(pUm)_5$ markers. The material eluting with dinucleotides is presumed to be dinucleotides derived from contaminating ribosomal RNA (7). It appears that vRNA has some complementarity with rRNA, since little or no FLF-3 cell (containing no vRNA) rRNA co-purifies

Figure 5. DEAE-Sephadex column separation of RNAase A, T1, and T2 digestion products of F-422 23S virus-specific RNA.

The 23S vRNA purified as described in Figure 3 was mixed with ^{3}H-labeled reovirus mRNA and digested with RNAase A, T1, and T2 as described under Materials and Methods. The reaction mixture was diluted with 4 volumes of 7 M urea, 0.02 M Tris·HCl, pH 7.2, and applied to a 0.6 x 28 cm column of DEAE-Sephadex. A gradient (50 ml/side) of 0.1 M to 0.4 M NaCl in 0.02 M Tris·HCl, pH 6.8, 7 M urea was run through the column at a flow rate of approximately 9 ml/h. Fractions of 1.1 ml were collected and 0.11 ml aliquots were assayed for radioactivity by scintillation counting. Standandard oligonucleotides (pUm through (pUm)5) were included as markers.

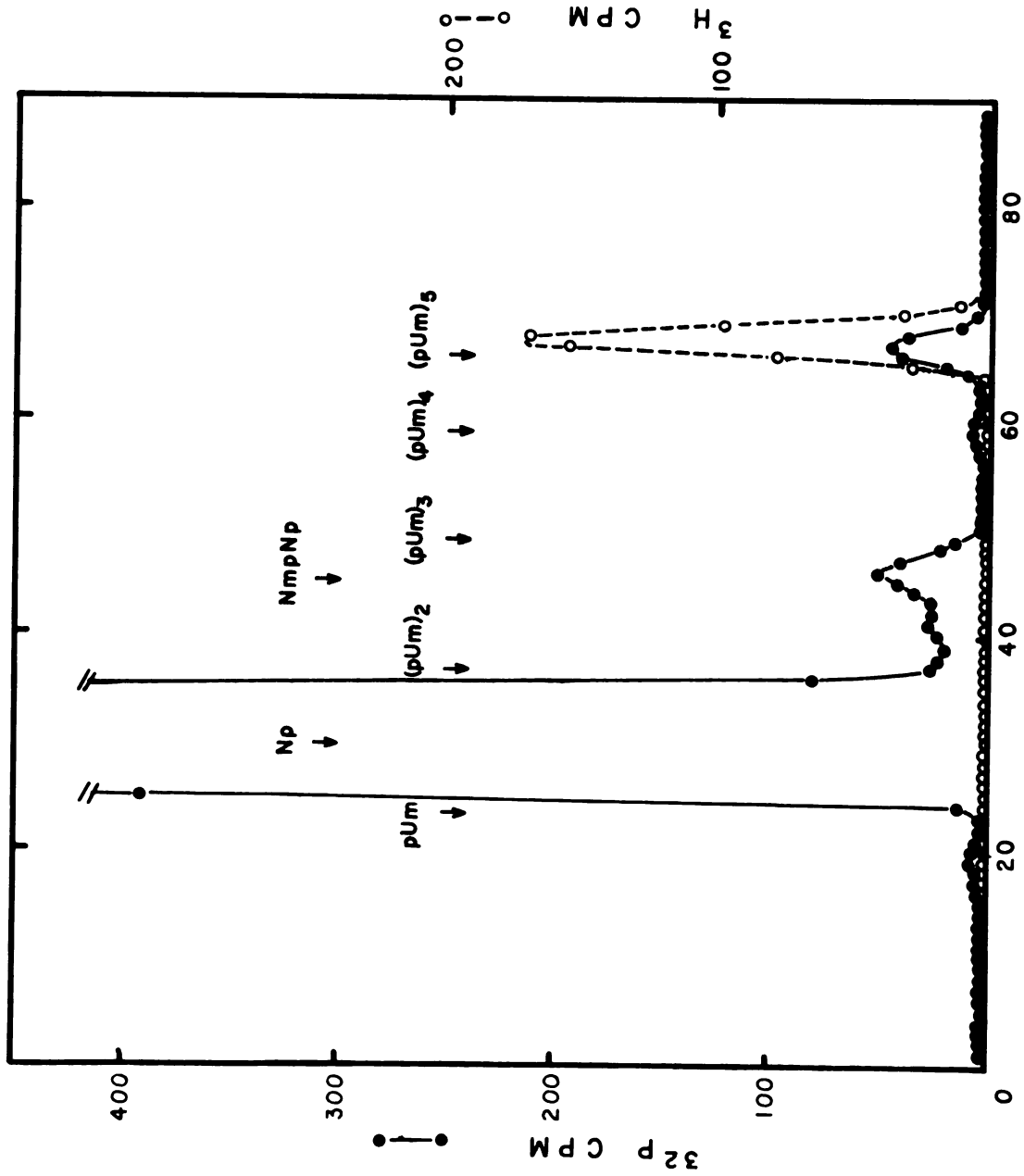

Figure 5. DEAE-Sephadex column separation of RNAase A, T1, and T2 digestion products of F-422 23S virus-specific RNA.

with Hg-cDNA$_{FeLV}$ (Figure 4b). The most likely explanation is that during hybridization of Hg-cDNA$_{FeLV}$ to F-422 cell RNA in formamide, triplex structures of the type Hg-cDNA$_{FeLV}$:vRNA:rRNA are formed. Material eluting from DEAE-Sephadex near the $(pUm)_4$ marker may be trinucleotides also derived from rRNA. If this is indeed the case, then purification of F-422 cell RNA on oligo (dT)-cellulose prior to hybridization to Hg-cDNA should remove most of the rRNA and greatly reduce the amount of material migrating with dinucleotides and $(pUm)_4$ after DEAE-Sephadex chromatography. This step was not performed since any vRNA which might not contain a poly (A) stretch would then have been excluded from our analysis.

The material eluting from the DEAE-Sephadex columns near the $(pUm)_5$ marker was presumed to represent the 5' caps of the RNA being investigated. However, in our initial experiments analysis of these structures yielded unexpectedly high amounts of free $^{32}PO_4$ (50-80%). Since some preparations of nuclease P1 will degrade nucleoside 5' diphosphates to nucleoside 5' monophosphates (A. Thomason, unpublished data), as well as removing 3' monophosphates (14), it was initially suspected that the material eluting near the $(pUm)_5$ marker from the DEAE-Sephadex columns might actually be in the form of ppNp or pppNp. Such structures co-migrate with caps on DEAE-Sephadex columns (21). When chromatographed on DBAE-cellulose (Figure 6) most (90%) of the large oligonucleotide material was bound, ruling out the possibility that it consisted of ppNp or pppNp structures since these do not contain 2' and 3' hydroxyl groups necessary for binding to DBAE-cellulose. It was subsequently found that the free PO_4 which we sometimes observed was derived from an unexplained degradation of caps

Figure 6. DBAE-cellulose chromatography of presumed cap structures from 23S vRNA.

Radioactively-labeled material eluting near the $(pUm)_5$ marker from the DEAE-Sephadex column in Figure 5 was desalted on DEAE-cellulose/ammonium acetate and chromatographed on acetylated DBAE-cellulose as described under Materials and Methods. Eluting buffer was applied after fraction 8. Fractions of 1 ml were collected and 0.1 ml portions were assayed for radioactivity. F-422 ^{32}P caps, closed circles; reovirus ^{3}H caps, open circles.

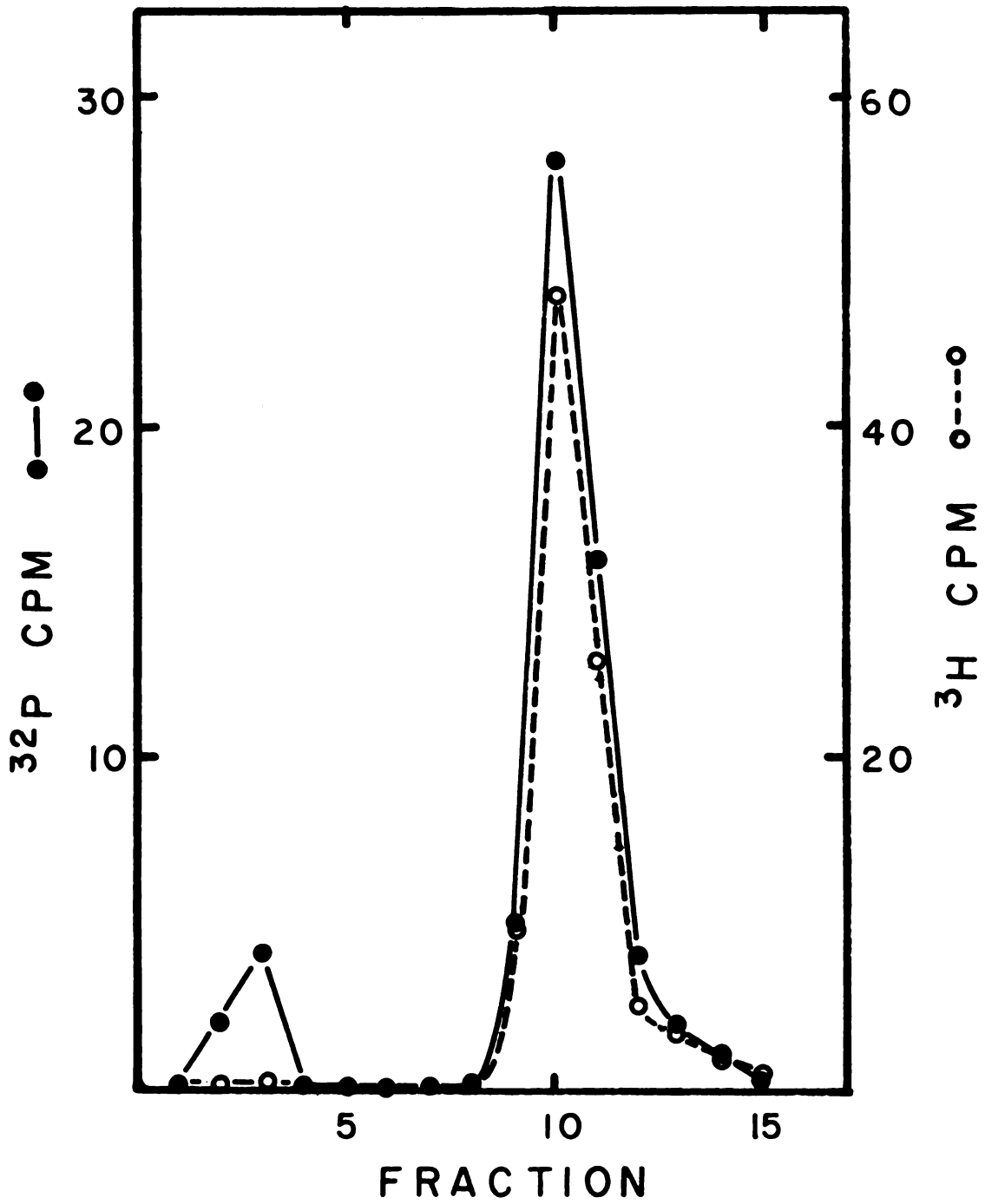

Figure 6. DBAE-cellulose chromatography of presumed cap structures from 23S vRNA.

during de-salting on Biogel P-2. This difficulty was avoided by de-salting oligonucleotides on DEAE-cellulose in the presence of ammonium acetate.

Under the conditions of labeling employed here, nearly all of the ^{32}P-labeled material in caps is in the form of cap I structures ($m^7G(5')ppp(5')N'mpN''p$). When completely digested with nuclease P1 such structures theoretically should yield 60% of the ^{32}P in "core" caps ($m^7G(5')ppp(5')N'm$), 20% in nucleotide 5' monophosphates, and 20% in free PO_4. Figures 7 and 8 show the high speed liquid chromatographic analysis on Partisil-SAX of the nuclease P1 digestion products of F-422 cell 23S and "15S" vRNA caps. A somewhat larger than expected proportion of the ^{32}P was in free PO_4 and less than the predicted amount of radioactivity was in "core" caps; this most probably reflects the partial degradation of caps during sample preparation. For both RNAs the only "core" cap detected was $m^7G(5')ppp(5')Gm$. The N'' position for the 23S vRNA was comprised of 79% pA and 21% pG; for the "15S" vRNA it was 84% pG and 16% pA. The most likely interpretation of this data is that the 23S vRNA is terminated by $m^7G(5')ppp(5')GmAp$ and the corresponding structure in the "15S" vRNA is $m^7G(5')ppp(5')GmGp$ with some cross contamination of each of the RNA classes with the other in the samples analyzed. Examination of the sucrose gradient profile of the vRNA in Figure 2a indicates that such cross contamination of 23S and "15S" vRNA is indeed probable.

Figure 9 shows a control experiment in which F-422 cytoplasmic total poly (A)-containing RNA was analyzed as described for the viral-specific RNAs. ^{32}P-labeled structures chromatographing with all five

Figure 7. Analysis of the nuclease P1 digestion products of presumed cap structures from 23S vRNA.

The material bound to DBAE-cellulose in Figure 6 was de-salted and digested with nuclease P1. Marker "core" cap standards and nucleoside 5'-monophosphates were added and the sample was subjected to high speed liquid chromatography on Partisil-SAX. After elution of nucleoside 5'-monophosphates with 0.02 M KH_2PO_4, pH 3.33, the "core" caps were eluted with a gradient composed of 50 ml 0.1 M and 50 ml 0.3 M KH_2PO_4, pH 3.55. The ^{32}P was distributed as follows: adenosine 5' monophosphate (pA) 17%; guanosine 5-monophosphate (pG), 5%; free PO_4, 25%; and $m^7G(5')ppp(5')Gm$, 53%. For the ^3H reovirus cap marker, 94% eluted with $m^7G(5')ppp(5')Gm$, with 6% being excluded from the column as nucleosides. Standards: adenosine 5'-monophosphate (pA); cytosine 5'-monophosphate (pC); guanosine 5'-monophosphate (pG); uridine 5'-monophosphate (pU); core caps containing 7-methylguanosine (m^7G) and 2'-O-methyladenosine (Am), 2'-O-methylcytosine (Cm), 2'-O-methyluridine (Um), or 6-2'-dimethyladenosine (m^6Am). At the time marked by 1 M, the column was washed with 1 M KH_2PO_4.

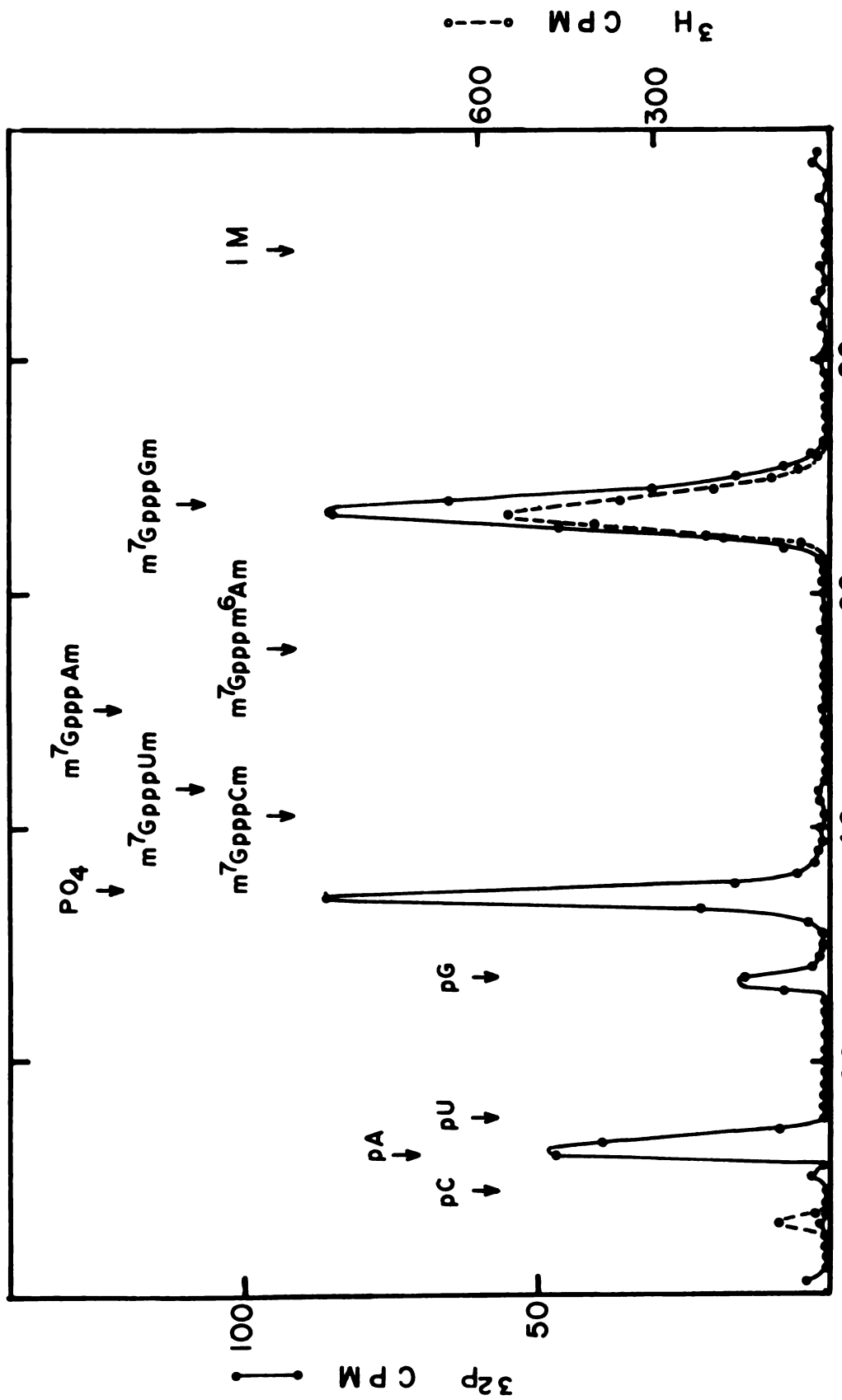

Figure 7. Analysis of the nuclease P1 digestion products of presumed cap structures from 23S vRNA.

Figure 8. Analysis of the nuclease P1 digestion products of presumed cap structures from "15S" vRNA.

Material eluting near the $(pUm)_5$ marker from a DEAE-Sephadex column similar to the one shown in Figure 5 was de-salted on DEAE-cellulose/ammonium acetate and analyzed as described in Figure 7. The ^{32}P was distributed as follows: pA, 3%; pG, 16%, free PO_4, 30%; and $m^7G(5')ppp(5')Gm$, 51%. Eighty-nine percent of the ^3H from the reovirus RNA was in $m^7G(5')ppp(5')Gm$ and 11% was in nucleosides. Markers were the same as in Figure 7.

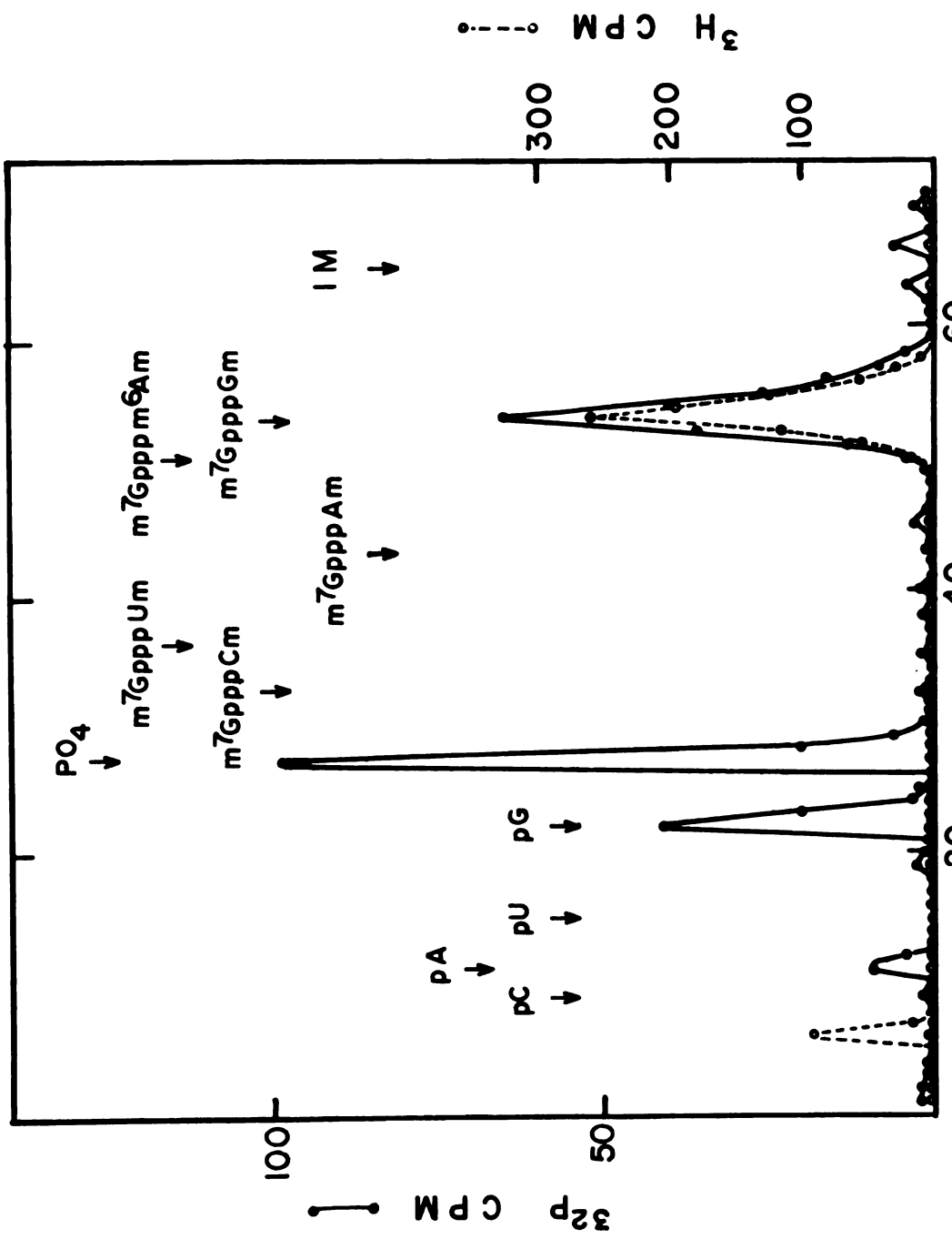

Figure 8. Analysis of the nuclease P1 digestion products of presumed cap structures from "15S" vRNA.

Figure 9. Analysis of the nuclease P1 digestion products of presumed cap structures from F-422 polysomal poly (A)-containing RNA.

The poly (A)-containing RNA was isolated from F-422 cell polysomal RNA labeled with ^{32}P by chromatography on oligo (dT)-cellulose. After digestion with ribonuclease T2 the products were separated on a DEAE-Sephadex column as in Figure 5. Labeled material eluting near the $(pUm)_5$ marker was de-salted on Biogel P-2 and analyzed by digestion with nuclease P1 and chromatography on Partisil-SAX as described in Figure 7. Nucleoside 5'-monophosphates were not completely resolved in this run. The radioactivity was distributed as follows: nucleoside 5' monophosphates, 26%; free PO_4, 40%; $m^7G(5')ppp(5')Cm$, 7%; $m^7G(5')ppp(5')Um$, 3%; $m^7G(5')ppp(5')Am$, 3%; $m^7G(5')ppp(5')^mAm$, 10%; and $m^7G(5')ppp(5')Gm$, 13%. Standards were the same as in Figure 7. Reovirus RNA was not mixed with this sample prior to analysis.

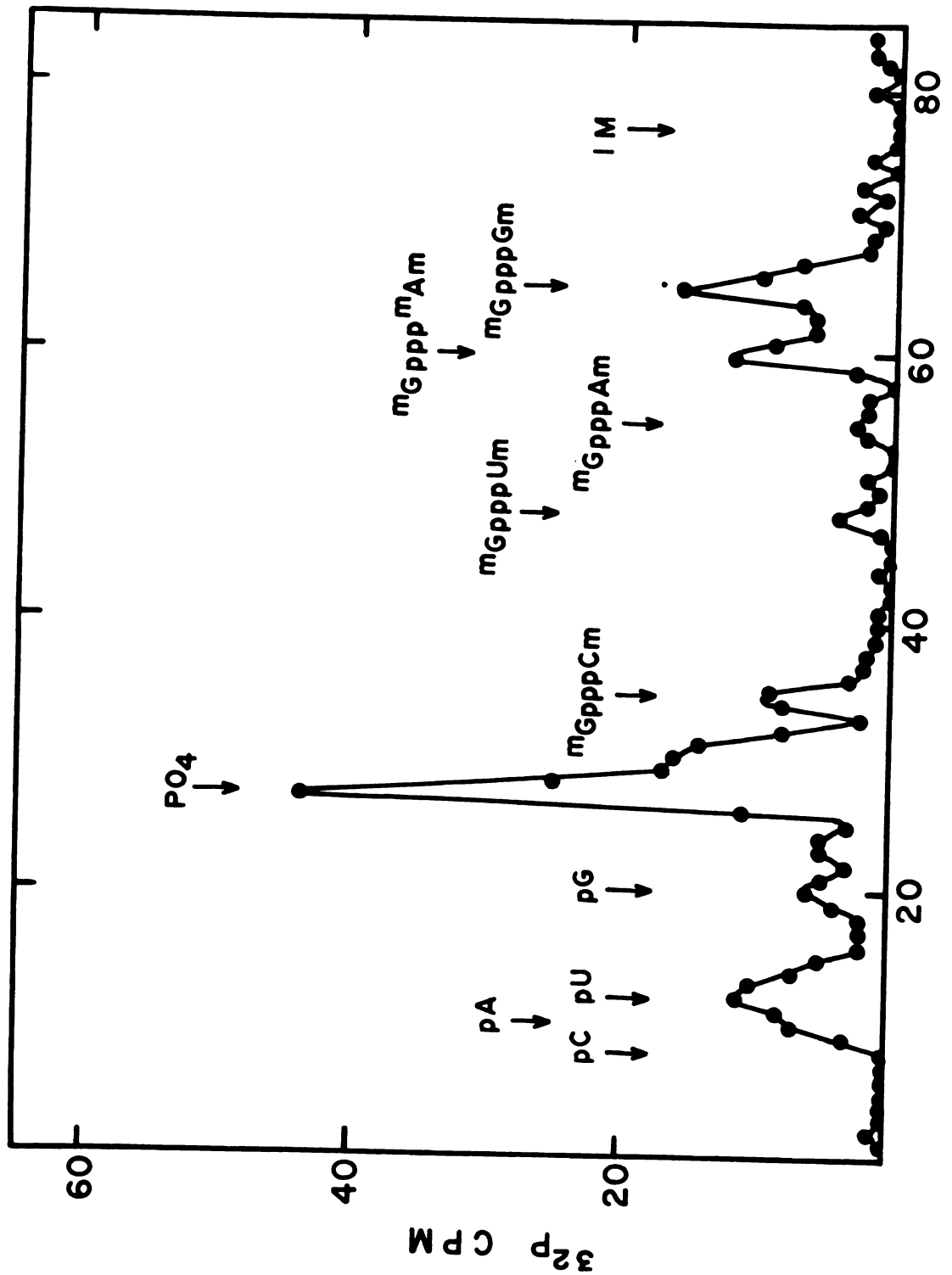

Figure 9. Analysis of the nuclease P1 digestion products of presumed cap structures from F-422 polysomal poly (A)-containing RNA.

cap standards were detected, with $m^7G(5')ppp(5')Gm$ and $m^7G(5')ppp(5')-m^6-Am$ predominating.

DISCUSSION

The results presented here demonstrate that despite the absence of caps in R-FeLV virion subunit RNA the two smaller intracellular virus-specific RNAs, which we have designated 23S and "15S" vRNA, in F-422 cells have capped 5' termini. The amounts of the ^{32}P label in cap structures (0.16% for 23S and 0.38% for "15S" vRNA) indicates that there is approximately 1 cap/3125 nucleotides in 23S vRNA and 1 cap/1315 nucleotides in "15S" vRNA. These values are close to what would be expected for capped RNA molecules sedimenting at 23S and "15S". The "core" cap structure of $m^7G(5')ppp(5')Gm$ found on both of these RNAs is the same as found in virion 35S RNA for two avian sarcoma viruses (13, 16), murine leukemia virus (2), and in the RNA of many vertebrate RNA viruses (22). However, the penultimate bases A and G found here in 23S and "15S" vRNA, respectively, differ from results with ASV and MuLV virion RNAs in which the penultimate base is a pyrimidine (2, 13, 16, 20). Since the first nucleotide (excluding m^7G) at the 5' end of both the 23S and "15S" vRNAs is G, no light is shed upon the question of whether these RNAs are primary transcripts or are processed from higher molecular weight RNA(s). If this position had been occupied by a pyrimidine, this would have suggested that these RNA molecules are probably not primary transcripts (21).

We have demonstrated that hybridization to mercurated cDNA and chromatography on SH-Sepharose can be used to purify RNA sequences

which represent only a small fraction of the cellular RNA. This technique allows hybridization to be performed in solution, a process which is faster, easier to control, and goes farther towards completion than hybridization to a probe bound to a solid support (23, 25). Synthesis of cDNA in solution results in much greater yields of the product than when cDNA is synthesized using support-bound oligo $(dT)_{12-18}$ as primer (35); and the use of DNA fragments generated by digestion of DNA with DNAase I as primer for cDNA synthesis (26) produces a more representative transcript than cDNA synthesized with oligo $(dT)_{12-18}$ as primer (6). The purification technique described here is highly specific, with only about 0.02% of non-specifically bound RNA co-purifying with RNA complementary to the Hg-cDNA probe. However, since the cDNA is not permanently bound to a solid support it must be manipulated many times during an experiment; chromatography over Sephadex G-50, ethanol precipitations, and scintillation counting of aliquots to monitor recovery result in gradual loss of the cDNA, limiting the number of times it can be re-used. In addition, extended hybridizations at high temperatures cannot be performed since the Hg-cDNA probe is de-mercurated under these conditions (5). However, this is usually not a problem since low hybridization temperatures can be employed when formamide is included in the hybridization reaction. It is important to use purified, re-crystallized (27) formamide, since impure formamide may degrade RNA and cDNA.

A smaller proportion of virus-specific 36S and 28S RNA, relative to lower molecular weight viral RNA, was detected in F-422 cellular cytoplasm in the present experiments than in a previous study (A. Conley and L. Velicer, submitted for publication). There are several

possible explanations for this difference. The most likely explanation for the variation between the data presented here and in the previous study concerns the difference in the way the cDNA probes were synthesized. In the previous study, cDNA was made in an endogenous polymerase reaction; thus the probe mainly represented sequences near the 5' end of the RNA template (11). cDNA used in the present study, synthesized with DNA fragments as primer, is more likely to be a representative transcript (6). If the smaller intracellular vRNA species lack the 5' sequences of the higher molecular weight RNA, then their relative amounts would be underestimated using a cDNA probe synthesized in an endogenous reaction. A second possibility is that in the present study the higher molecular weight RNAs have been extensively degraded to smaller sizes. This possibility seems unlikely, since cellular ribosomal RNA in the same preparations appeared to be largely intact (Figure 2b). In addition, an experiment was conducted in which radioactively labeled R-FeLV virion subunit RNA was mixed with F-422 cells immediately prior to extraction of RNA from the cells. Although the virion RNA was partially degraded by this procedure which did not include double proteinase K (data not shown), it was not sufficient to account for the difference between Figure 2a and the previous study. The fact that enough caps were found in the 23S and "15S" intracellular vRNA to account for about one cap per molecule also argues against significant degradation of the RNA. A third possibility is that under the conditions of labeling employed here less 36S and 28S vRNA was synthesized, relative to smaller vRNAs, than when cells are grown in medium containing phosphate. The fact that (.17A_{260} vs. expected .13)

as much 28S vRNA was incorporated into virions when cells were grown in phosphate-free medium as when they were grown in normal medium (data not shown) makes this explanation seem improbable. In any event, insufficient amounts of the higher molecular weight vRNAs were obtained in the present study to allow characterization of their 5' termini.

In this and a previous study (28) we have shown that while F-422 cell mRNA and some viral mRNAs are capped, most or all of the virion 28S subunit RNAs are not capped. The significance of this finding is unknown at the present time. The size of R-FeLV virion subunit RNA, 28S, is another unusual feature; most mammalian leukemia viruses have subunit RNAs that sediment at 35-40S. In addition, the infectivity of our stocks of R-FeLV is very low (A. Haberman, unpublished data). Nevertheless, 35-40S RNA containing virus-specific sequences has been found in these cells and in very low levels in the virions released from them (A. Conley and L. Velicer, submitted for publication).

It should be noted that the 28S subunit RNA found in this system is similar in size to the 27-30S subunit RNA observed in the defective mammalian sarcoma viruses (18, 29, 30, 31, 32, 33). R-FeLV also appears to have some sort of replication defect since it is not very infectious; indeed, all of the infectivity of these R-FeLV preparations may be due to a small proportion of non-defective virions. However, our stock of R-FeLV does not contain a classical sarcoma virus since it does not transform fibroblasts (A. Haberman, unpublished data). To our knowledge there are no other reports concerning the nature of the 5' end of a mammalian, defective RNA tumor virus subunit RNA.

It should also be pointed out that other mammalian leukemia viruses have been observed to lose their infectivity upon prolonged

passage in tissue culture (24, 35). The nature of the RNA subunits of these attenuated leukemia viruses has not been reported.

One possible explanation for these observations is that the replication defective mammalian RNA tumor viruses -- the sarcoma viruses and possibly R-FeLV and other attenuated leukemia viruses -- represent defective interfering particles derived from the respective non-defective viruses. Such particles are common in other viral systems (15). The Moloney strain of murine sarcoma virus (MuSV) has been shown to behave like a defective interfering particle (1). The subunit RNA of MuSV is similar in size (19) to the subunits of R-FeLV RNA; whether or not MuSV subunit RNA is capped has not been reported.

It is also possible that the defectiveness of R-FeLV is not related to the unusual size and lack of a cap in its RNA, but may be the result of other factor(s).

Further work will be needed to determine if any of these speculations are valid, but it is possible that the tendency to defectiveness of the RNA tumor viruses may have broad implications for the understanding of viral replication and oncogenicity.

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