TRANSCRIPTION OF THE ADENOVIRUS GENOME DURING PRODUCTIVE INFECTION OF HELA CELLS

by

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ABSTRACT

TRANSCRIPTION OF THE ADENOVIRUS 2 GENOME DURING PRODUCTIVE INFECTION OF HELA CELLS

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Richard Price

Submitted to the Department of Biology in July, 1972, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The properties of the RNA polymerase activities which transcribe the adenovirus genome during productive infection of HeLa cells have been studied. The major portion of adenovirus DNA is transcribed by an activity which closely resembles polymerase II of the host cell. This activity is inhibited by α-amanitine and stimulated by $(NH_4)_2SO_4$. Its product is high molecular weight heterogeneous RNA. The polymerase activity transcribing the bulk of both early and late viral RNA appears to be very similar.

A distinct RNA polymerase which labels low molecular weight RNA has also been identified. The products of the polymerase activity are molecules of discrete size. In nuclei from infected cells, RNA is labeled which co-migrates in gel electrophoresis with both host cell 5S RNA and the 5.5S adenovirus-associated species. The activity also labels RNA with the properties of precursor to transfer RNA.

The kinetics of labeling and the results of chase experiments suggest that this latter polymerase activity is re-initiating the synthesis of new molecules in vitro. Consistent with this hypothesis is the observation that the 5.5S virus-associated RNA molecules contain radioactive guanosine tetraphosphate which is presumed to be derived from the 5' terminus.

The polymerase activity is completely resistant to inhibition by

α-amanitine, and it is comparatively active when Mn++ is substituted for Mg++. These properties are in contrast to those of the polymerase activity transcribing the high molecular weight heterogeneous RNA from either the host or the adenovirus genome. It thus appears that the adenovirus genome is transcribed by two distinct RNA polymerases during the productive infection; one resembles closely the host cell polymerase transcribing high molecular weight heterogeneous RNA, and the other is similar to the activity responsible for the synthesis of the host cell 5S RNA and the precursor to tRNA.

Thesis Supervisor: Sheldon Penman, Professor of Biology

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ABBREVIATIONS

RSB	reticulocyte standard buffer: .01M-Tris pH 7.4, .01 M NaCl, .0015 M $MgCl_2$
SDS	sodium dodecyl sulfate
SDS-sucrose	(.100 M NaCl, .001 M EDTA, .5% SDS, .01 M Tris, pH 7.4, 15-30% w/w gradient of sucrose)
RNAse	ribonuclease (Worthington)
TCA	trichloroacetic acid
CsCl	cesium chloride (Varlacoid Chemical)
NP40	Nonidet P40 (Shell Chemical Co.)
Tris	Tris (hydroxymethyl) amino methane (Sigma)
EDTA	ethylene diamine tetra-acetic acid (Versene)
cordycepin	3' deoxyadenosine (Sigma)
NaOH	sodium hydroxide
HSB	(0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris, pH 7.4)

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INTRODUCTION

The adenovirus 2 virion contains one linear double-stranded DNA molecule with a molecular weight of approximately 22 million daltons, enclosed in a capsid composed of nine structural proteins. The virus-directed macromolecular metabolism during lytic infections resembles that of the host cell in many ways, making the virus a possible model system for elucidating the mechanisms whereby mammalian cells synthesize and process their macromolecules.

A. The Structure and Composition of the Virion

Electron microscopy has shown the adenoviruses to be structures with a diameter of 700 Å, having icosahedral symmetry with twelve vertices and twenty triangular faces [1]. The 252 capsomers can be divided into hexons, of which there are 240, and pentons. The hexons are surrounded by six capsomers, while the pentons, being located at the twelve vertices of the icosahedron, are in contact with five capsomers [2]. Hexons are composed of a single structural protein with a molecular weight of 120,000 daltons [3]. Electron microscopy and serological studies of the virion have shown the pentons to be composed of two structural proteins, a penton base and an associated fiber. The base has a molecular weight of 70,000 daltons, and the fiber a molecular weight of 62,000 daltons [4].

Electron microscopy of thin sections of virion preparations shows a dense nucleoid with a diameter of approximately 400 Å [5-7]. This structure is sensitive to both proteases and DNase, suggesting that it represents a DNA-protein complex similar to the so-called cores of other viruses. The nature of the proteins associated with this structure is unknown, although SDS polyacrylamide gel electrophoretic analysis of highly purified virions reveals the presence of at least six polypeptides in addition to the three capsid proteins [4]. These proteins, ranging from 50,000 to

10,000 in molecular weight, are synthesized in the cytoplasm of the infected cell. Several of them are assumed to be components of the nucleoid.

The genome of the adenoviruses is a linear double-stranded DNA molecule of 20-25 million daltons molecular weight [8-10]. Heat denaturation mapping of DNA from adenovirus 2 suggests that this molecule is neither circularly permuted nor terminally redundant [11]. The human adenoviruses have been classified into three main groups on the basis of the GC contents of their DNA, homology of their genomes as assayed by reciprocal DNA/DNA hybridization, and oncogenicity when injected into non-permissive species. The GC content ranges from 47 per cent for some members of group A to a high value of 57-60 per cent for the serotypes of group C. Adenovirus 2 is a member of group C, having a GC content of 59 per cent. The members of group C are not oncogenic in experimental animals, but are capable of transforming cells in culture [9,12].

B. Macromolecular Metabolism Associated with Multiplication of the Virus

1. The Productive Infection

The events associated with the lytic cycle of adenovirus 2 have been extensively described, largely by Green and co-workers and by Ginsberg and his colleagues. Adenovirus 2 grows well in either KB or HeLa cells in spinner culture, taking approximately 24 hours to complete the growth cycle. The mechanism of viral penetration of the host cell is unknown. The virion is first detectable in the cytoplasm as a structure lacking the penton [13,14]. This partly degraded particle, which is sensitive to DNase, then migrates to the nucleus where release of the cores and finally the release of naked DNA occurs [13,15]. Approximately 2 hours are required for these events.

As with a number of other viruses, it is useful to divide the viral

growth cycle into two stages. Those events which precede the onset of viral DNA replication are termed 'early,' while all subsequent events are classified as 'late.' Viral-specific RNA can be detected both in the nucleus and the cytoplasm beginning approximately two hours after infection [16,17]. (Times post-infection are given as the time following a one-hour viral adsorption period.)

Approximately 0.1% of the labeled nuclear RNA and 2% of the labeled RNA reaching the cytoplasm early in infection is viral-specific [18]. The synthesis of this RNA occurs in the presence of inhibitors of protein synthesis, suggesting that the transcription is accomplished by either a host enzyme or a virion component. The fact that no RNA polymerase has been found associated with purified virions suggests that a host enzyme is responsible for this synthesis.

Saturation hybridization of early RNA extracted from the cytoplasm suggests that this RNA represents 10 to 20 per cent of the viral genome [19]. The viral RNA found in the nucleus early in infection appears to differ from the cytoplasmic RNA in its base composition, suggesting that not all of the viral RNA which is transcribed early in infection reaches the cytoplasm.

Analysis of viral RNA found in the cytoplasm early in infection reveals peaks migrating at 23S and 16S in polyacrylamide gels. The early viral RNA found in the nucleus appears larger and more heterogeneous [18].

Nothing is known regarding the nature of the proteins synthesized early in infection. An antigen, known as the T antigen, appears in the nucleus early in infection [19,20]. It is not known whether the T antigen is coded for by the virus or the host. Two groups have recently reported the partial purification of the T antigen. This protein fraction displayed

no RNA or DNA polymerase, thymidine kinase or nuclease activity [21,23].

DNA synthesis is first detectable about 8 hours after infection and continues for approximately 12 hours [24,25]. Green has reported that the synthesis of early RNA continues after the initiation of DNA synthesis [18, 19]. However, Ginsberg has recently reported the results of competition hybridization experiments suggesting that the early sequences are not synthesized late in infection [26]. Ginsberg, whose technique includes extensive washing and RNase treatment of the hybridization filters, claims that when such procedures are omitted results similar to those reported by Green are obtained.

The onset of DNA synthesis is accompanied by the appearance of new viral RNA sequences in the cytoplasm [27]. Saturation hybridization experiments reported by Green suggest that the viral RNA sequences found in the cytoplasm late in infection account for 80-100% of the viral genome [28]. In contrast to the early RNA, late in infection the base composition of the nuclear and cytoplasmic viral RNA sequences are identical [18, 27]. However, as was seen early in infection, the viral-specific nuclear RNA is large and heterogeneous in size, sedimenting from 18S to 60S, while the adenovirus RNA extracted from either polysomes or whole cytoplasm appears to be smaller and of discrete size classes. Green has reported that viral RNA found on polysomes late in infection displays peaks at 23 and 18S when analyzed by polyacrylamide gel electrophoresis [18]. Hirsch has found that RNA extracted from either polysomes or cytoplasm late in infection falls into two classes, sedimenting at 24 and 18S in sucrose gradients and displaying electrophoretic mobilities corresponding to 28S and 23S [29]. Hirsch finds that little if any host RNA is found on polysomes late in infection, while approximately 70-80% of the HnRNA is cellular.

Darnell and co-workers have recently demonstrated the presence of a short sequence of polyadenylic acid (poly(A)) on the 3'-OH terminus of adenovirus mRNA synthesized late in infection [30]. The poly(A) appears to be added to the large nuclear viral RNA post-transcriptionally.

The synthesis of a unique species of RNA composed of 151 bases has been observed in adenovirus-infected cells [31]. This RNA, termed VA-RNA by Weissmann and co-workers, is synthesized in very large quantities, and appears rapidly in the cytoplasm. The sequence of VA-RNA has recently been determined, allowing attempts at predicting its secondary structure [32,33]. Extensive intra-molecular base pairing is possible, forming a structure closely resembling that of tRNA.

VA-RNA was reported to hybridize both with host cell and viral DNA with equal efficiency on a DNA mass basis, although the efficiency of hybridization to DNA of both types was surprisingly low [31]. While the present work was in progress, Ohe reported that the adenovirus DNA contains one copy of the VA-RNA sequence, while no detectable hybridization to host cell DNA was observed [34]. Results of the present work also suggest that the VA-RNA is a product of the viral genome.

The function of the VA-RNA is unknown. The small amount of VA-RNA associated with polyribosomes is removed by washing at moderate ionic strength [34]. Attempts to charge the VA-RNA with labeled amino acids have been unsuccessful [35].

The origin of the RNA polymerase responsible for the synthesis of late viral RNA is unknown. Green has reported that RNA polymerase extracted from E. coli or uninfected KB cells transcribes only the early RNA sequences when given adenovirus DNA as a template, suggesting that late in infection there occurs an alteration in either the enzyme or the DNA tem-

plate [36].

Late in infection, protein synthesis in the cytoplasm is almost entirely viral-directed [37]. The majority of this synthetic activity is concerned with the production of components of the virion, which are produced in approximately 100-fold excess [17,38]. The newly synthesized virion polypeptides migrate to the nucleus where mature virions are assembled by mechanisms which are not understood.

While viral macromolecular synthesis has ceased by approximately 25 hours post-infection, virion assembly continues at a rather reduced rate for another 24 hours, after which time the infected cells, filled with virions, begin to lyse.

The effects of productive adenovirus infection on host cell syntheses have been examined in detail, mainly by Green and co-workers. When actively growing suspension cultures are infected, host cell DNA synthesis is gradually shut off, so that by 18 hours post-infection only viral DNA is being produced [24]. Nucleolar RNA synthesis also gradually declines, by 16 hours after infection the rate of production of mature 28S and 18S RNA species is only 10-20% of the control level [39]. In contrast, however, host HnRNA synthesis appears to continue at the same or perhaps at even an increased rate throughout the infectious cycle.

While, at least quantitatively, host HnRNA synthesis continues unabated throughout infection, late in infection most, if not all, of the polysomal and cytoplasmic messenger-like RNA is viral [29]. This suggests that late in infection either processing of host HnRNA is faulty, or that host messenger RNA is unstable in the cytoplasm.

The effect of infection on host protein synthesis is quite similar to that just described for messenger RNA. While early in infection host pro-

tein synthesis appears to proceed at the normal rate, late in infection most, if not all, host protein synthesis has ceased [40]. This finding cannot be entirely explained by the absence of host messenger RNA synthesis late in infection, as Singer and Penman have recently measured the half-life of HeLa cell mRNA to be on the order of 24 hours [41]. Thus even in the absence of the continued flow of newly synthesized host messenger RNA to the cytoplasm, considerable host protein synthesis would be expected unless other factors are operative.

2. Abortive Infection and Transformation

The only features which all abortive infections by adenoviruses appear to have in common is the lack of production of infectious virus. However, the step in the infectious cycle at which viral replication is blocked appears to be different in a number of cell types.

The abortive infection of baby hamster kidney (BHK) cells by adenovirus 2 and 12 has been described extensively by Doerfler. While the synthesis of early RNA is detectable, no viral DNA replication is observed [42,43]. When infection of BHK by adenovirus 2 or 12 is performed with high multiplicities of [^{3}H]-thymidine-labeled DNA, there appears to be considerable integration of the viral DNA into host sequences [44]. Zur Hausan and Sokol suggested that the association of the ^{3}H label with host sequences was due to degradation of the input DNA and re-utilization of the components [49]. More recent experiments published by Doerfler strongly suggest that he is in fact observing integration [48]. The apparent integration of viral DNA into host chromosomes is accompanied by death of most of the cells, while a small fraction (0.1 per cent) of the cells are transformed.

The results of a more recent report by Doerfler and co-workers sug-

gest that the input viral DNA undergoes considerable fragmentation prior to integration, providing a possible explanation for the failure of attempts by several groups to rescue the virus by fusion of transformed with permissive cells [48]. Shortly after infection of BHK cells, the ^{3}H-adenovirus DNA is degraded into fragments sedimenting at 18S. Approximately 4 hours later viral DNA begins to be associated with rapidly sedimenting host DNA. The fact that, concomitantly with the association of viral with host DNA, the level of the 18S viral DNA fragments in the cell falls suggests that it is the fragments, rather than the input DNA, that is integrated.

A candidate for the enzymatic activity responsible for this fragmentation of viral DNA has recently been identified [45-47]. Burlingham, et al., have detected an endonuclease associated with highly purified virions. This endonuclease, which is believed to be the penton fiber, shows a preference for GC-rich regions of double-stranded DNA and more recently has been shown to be capable of converting intact adenovirus DNA into 18S fragments in vitro [49].

The infection of African green monkey kidney (AGMK) cells by adenovirus 2 also leads to the production of little or no virus [50]. In this infection the early viral functions appear to proceed normally; viral DNA synthesis is seen to occur and virion antigens are detectable in the cytoplasm. However, simultaneous infection of AGMK cells with SV40, Simian adenoviruses or adenovirus-SV40 hybrids allows the production of infectious adenovirus [51-53]. The nature of the helper function supplied by these viruses is unknown, but a recent report has suggested that in the absence of a helper virus virion proteins accumulate in the cytoplasm, but are incapable of migrating to the nucleus for virion assembly [54].

One of the criteria used to define the three classes of human adeno-

virus is their oncogenicity. All three classes are capable of transforming cells in culture, and cells transformed by all three classes can cause tumors when introduced into an appropriate host. However, only viruses of class A, and to a limited extent class B, are capable of causing tumors when injected into a non-permissive host. The reason for this difference is unknown.

Transformation of a number of types of cells by human adenoviruses in culture has been described [18,19,55,56]. The most extensively studied system is the infection of rodent cells by either adenovirus 2 or 12. The fact that the frequency of transformation in these systems rarely exceeds 0.1% has made this process difficult to study in detail. McBride and Weiner [55] first demonstrated the transformation of cells in a primary culture of baby hamster kidney cells by adenovirus 12. More recently, Green has isolated a line of rat embryo cells transformed by adenovirus 2 [18].

While cells transformed by adenoviruses have not been examined as extensively as cells transformed by the papova viruses, it appears that the two systems have much in common. Green and co-workers have demonstrated that the transformed cells contain adenovirus-specific T antigen, messenger RNA and viral DNA [18]. The results of cRNA-DNA hybridization experiments suggest that 22-60 copies of the adenovirus genome are present in the transformed cell [18]. Green has recently reported that this number may be as low as 5, on the basis of results obtained using the quantitative hybridization technique described by Martin [85]. Hybridization experiments have shown that the viral specific cytoplasmic RNA found in transformed cells accounts for about 10% of the viral genome, and appears to be a sub-class of early RNA [18]. The covalent linkage of the viral RNA

sequences found in the nucleus to host RNA has been suggested by sequential hybridization to adenovirus DNA and cellular DNA [86].

3. The RNA Polymerases of Mammalian Cells

Although some knowledge of the RNA polymerases of higher organisms exists, the molecular mechanisms employed by the mammalian cell to control the transcription of its vast genome are completely unknown. While much speculation and experimentation has been based on the patterns emerging from several well-characterized bacterial systems, there has been no indication of any similar sorts of mechanisms occurring in mammalian cells. Consideration of the enormous size of the eukaryotic genome and the need for a large amount of coordinate control leads one to suspect that, in fact, the rather simplistic mechanisms employed by bacteria and their viruses for controlling transcription probably would not be sufficient to account for this control in higher organisms.

The possibility that this control lies, at least in part, in the template specificities of a number of species of RNA polymerase has led recently to rather extensive characterization of the enzyme found in the nucleus of the mammalian cell. While RNA polymerase activity was discovered in the nucleus of the mammalian cell in 1961 by Weiss [57], its further characterization was not possible due to the lack of methods for freeing the enzyme from its natural template in an active form. This was first achieved by Roeder and Rutter, who used high salt and sonic disruption to extract the RNA polymerase from the nuclei of sea urchin embryos and rat liver [58]. Using a procedure similar to that described by Burgess for purifying RNA polymerase from E. coli, these workers were able to separate polymerase activity into three peaks on a DEAE Sephadex column, referred to as polymerases I, II, and III, in the order of their elution.

Characterization of the enzymes according to their divalent cation, ionic strength and template requirements suggested that they were in fact three unique species [58-60].

Subsequent investigations by numerous other laboratories have shown that at least two of these enzymes, polymerases I and II, are found in the nuclei of a wide variety of eukaryotic cells [61-64]. The universality of the association of polymerase III with the mammalian nucleus is less certain. While Roeder and Rudder report its association with the nucleus of sea urchin embryos and calf thymus but not of rat liver, several other groups claim that it is not associated with the nucleus, but, rather, it is a mitochondrial enzyme, found in small amounts in enzyme preparations made from nuclei due to mitochondrial contamination [65,66].

More recently, Roeder and Rutter [67] and Chambon and co-workers [68] reported that what was previously termed polymerase II may, in fact, be composed of two distinct enzymes. Analysis of the subunits of these two forms of polymerase II by SDS-polyacrylamide gel electrophoresis has shown them to differ in only one subunit. Roeder and Rutter suggest that the small enzyme may be generated by proteolytic cleavage of the larger, since prolonged storage leads to increasing amounts of the smaller at the expense of the latter, and the presence of phenylmethylsulfonylfloride in the extraction medium dramatically increases the ratio of the amount of the large to the small enzyme [67].

The properties of the three enzymes isolated from a variety of sources have been extensively characterized and can be summarized as follows. Polymerase I is most active at low ionic strength, requires either Mn^{++} or Mg^{++}, and is relatively indifferent as to whether the template is native or denatured. The activity of polymerase II shows a rather sharp

maximum at about 75 mM ammonium sulfate, and prefers Mn++ to Mg++. Polymerase III appears to be relatively indifferent to the cation present or the ionic strength.

A rather disappointing characteristic of the behavior of these enzymes when separated from their natural template is their apparent lack of specificity in the sequences they transcribe from either native or denatured DNA. Thus Blatti, et al., [69] observed no difference in the products of the three enzymes, either in the GC content or when subjected to nearest neighbor analysis, using native calf thymus DNA as a template. Similar results were reported by Tocchini-Valentini and Crippa [70], working with Xenopus laevis. The authors isolated two RNA polymerases from the oocytes, one being localized in the nucleolus, corresponding to polymerase I of Roeder and Rutter, and the other a nucleoplasmic enzyme with the properties of polymerase II. Despite the fact that polymerase I most certainly transcribes the amplified ribosomal RNA genes in vivo, both enzymes utilized highly purified ribosomal DNA with approximately equal efficiency.

As the isolated RNA polymerases show little or no template specificity in a purified state, correlations of the enzymes with the production of specific classes of RNA have been made on the basis of two less direct criteria. The intra-nuclear localization of the enzymes has led to the tentative conclusion that polymerase I is responsible for transcription of ribosomal DNA, while polymerases II and III are presumed to be responsible for the synthesis of the species of RNA of nucleoplasmic origin [59]. In a wide variety of organisms and tissue types, polymerase I has been shown to be highly enriched in the nucleolus, while the other two enzymes seem to be primarily located in the nucleoplasm [59,70].

The second type of evidence that has been used for the correlation

between isolated enzymes and their *in vivo* product has come from studies of RNA synthesis in isolated nuclei. It is assumed that one should be able to correlate incubation conditions stimulating the synthesis of specific classes of RNA with the properties of the purified RNA polymerases. The first of these studies was reported by Widnel and Tata [71]. These workers observed that under conditions of high ionic strength and in the presence of Mn++ RNA with a base composition similar to DNA was synthesized, while in the presence of Mg++ and low ionic strength the base composition of the RNA synthesized closely resembled rRNA. These experiments have been repeated by Blatti, *et al.* [69] and Zylber and Penman [72]. The results of this work supports the conclusions based on intra-nuclear localization of the enzymes, that RNA polymerase I is responsible for rRNA transcription, while polymerase II is believed to synthesize HnRNA.

The isolation by Weiland of the toxin α-amanatine from the poisonous mushroom *Amanita phalloides* has allowed further confirmation of this conclusion. While low concentrations of α-amanitine completely prevent the elongation by polymerase II, the polymerization reaction catalyzed by polymerase I is unaffected by the drug [60,72]. Zylber and Penman [72] have shown in nuclei isolated from HeLa cells that, while synthesis of nucleolar RNA is unaffected by the drug, even at high concentrations, the labeling of the majority of the HnRNA is suppressed by 0.1 μg/ml α-amanitine. The authors suggested that the HnRNA synthesized in the presence of α-amanitine was the product of polymerase III.

It thus seems reasonably certain that polymerase I catalyzes the polymerization of the ribosomal RNA. The correlation of isolated enzymes with the production of the several classes of nucleoplasmic RNA seems less conclusive. While the results of Blatti, *et al.*, and Zylber and Penman

are certainly consistent with the notion that polymerase II is responsible for the synthesis of the majority of the HnRNA, the labeling of a considerable amount of heterogeneously sedimenting nucleoplasmic RNA in the presence of α-amanitine prevents this conclusion.

Of even greater interest is the identification of the enzyme responsible for the synthesis of the class of nuclear RNA which serves as precursor to cytoplasmic messenger RNA. Evidence is accumulating that messenger RNA is derived from at least some of the HnRNA.

The present studies using nuclei isolated from adenovirus 2-infected HeLa cells were undertaken in an attempt to further correlate the synthesis of specific classes of RNA with purified enzymes. As discussed above, during the lytic infection of human cells by adenovirus 2, the macromolecular metabolism of the virus appears to be very similar to that of the host cell in many respects. It thus seemed possible that by observing the mechanisms utilized by the adenovirus to synthesize the various classes of adenovirus-specific RNA, some insight might be gained into the way the host cell carries out the analogous functions.

The results of these studies suggest that the HnRNA containing both the early and the late classes of adenovirus RNA is synthesized by RNA polymerase II and that, by inference, this enzyme is probably responsible for the synthesis of the host HnRNA that serves as precursor to cytoplasmic messenger RNA. Nuclei isolated from HeLa cells late in the lytic cycle (16-18 hours post infection) also synthesize the adenovirus-specific 5.5S RNA (VA-RNA). The polymerase activity responsible for the synthesis of this species of RNA differs in a number of respects from that labeling the HnRNA containing adenovirus sequences. While the activity labeling adenovirus HnRNA resembles polymerase II in its response to ammonium sul-

fate and its high sensitivity to α-amanitine, the activity responsible for labeling VA-RNA is completely resistant to inhibition by α-amaitine and responds differently to ammonium sulfate and Mn++. It is also shown that extensive re-initiation of VA-RNA synthesis occurs in vitro, while the activity of the enzyme synthesizing the HnRNA containing viral sequences is limited to elongation of pre-existing chains. Two other low molecular weight RNA species are labeled in the nuclei from adenovirus-infected cells. It is concluded that these represent 5S RNA and the tRNA precursor, as they are also labeled in vitro by nuclei from uninfected cells. The species tentatively identified as pre-tRNA can be converted to a species co-migrating with mature tRNA by incubation with a cytoplasmic extract.

It thus appears that the adenovirus genome is simultaneously transcribed by two distinct cellular RNA polymerase activities late in the lytic cycle. The possible significance of this finding and the usefulness as a model system for studying the specificity of RNA polymerase-template interaction are discussed.

MATERIALS AND METHODS

A. Cells and Viruses

HeLa S3 cells were grown in suspension culture at 37°C in Eagle's Medium supplemented with 7% horse serum. For adenovirus infection, cells were harvested by centrifugation and resuspended in medium at a concentration of 1-2 x 10^7 cells/ml. Adenovirus type 2 was added at a multiplicity of approximately 100 plaque-forming units/cell and the suspension was shaken gently for 60 minutes at room temperature. The culture was then diluted with fresh medium to a concentration of 3 x 10^5 cells/ml. The time of dilution was designated the beginning of infection.

B. Purification of Adenovirus

Adenovirus 2, the gift of M. Green, was purified from HeLa cells by a modification of the method described by Doerfler [79]. Between 48 and 72 hours after infection the cells were harvested, washed in Earle's saline, resuspended in RSB (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.4), and disrupted by repeated freeze-thawing. The nuclei and debris were removed by centrifugation at 2000 x g for 5 minutes. An equal volume of Freon 113 (E. I. Dupont Co.) was then added and the resulting emulsion was mixed well. The two phases were separated by centrifugation at 2000 x g for 5 minutes. The aqueous phase was removed and re-extracted with Freon until the interface was clean. The supernatant was then layered on a 15-30% RSB-sucrose gradient and centrifuged at 25,000 rpm for 1 hour in the SW27 rotor at 4°C. The opaque virus band was collected and the density was adjusted to 0.5 g/ml by adding solid CsCl. The virus was centrifuged to equilibrium in the type 50 rotor at 35,000 rpm at 4°C. The viral band was collected and banded again in CsCl.

C. Preparation of Adenovirus DNA

Adenovirus collected from the CsCl gradient was dialyzed against RSB

with 5 mM EDTA. SDS was added to 0.1% and the virus was digested with 50 µg/ml pronase (Grade B, Calbiochem Co.) at 37°C for 1 hour. The concentration of SDS was raised to 0.5% and DNA was extracted with phenol-chloroform at 56°C. An equal volume of phenol was added, followed by the addition of two volumes of chloroform made 1% in isoamyl alcohol. The emulsion was then vortexed well, heated to 56°C, and the organic and aqueous phases were separated by centrifugation. The organic phase was removed and the procedure repeated three times. Two volumes of ethanol were added to the final aqueous phase and the DNA was collected by centrifugation and resuspended in 2XSSC. Purity of the DNA was measured by the ratio of absorbences at 280 and 260 mµ.

D. In Vitro Labeling and Purification of Nuclear RNA

At 16 to 18 hours after infection, cells were treated with 0.04 µg/ml actinomycin D for 30 minutes to suppress nucleolar activity during the in vitro labeling. Cells were collected by centrifugation at 1,500 rpm for 2 minutes and washed twice with cold Earle's saline solution. The cells were resuspended in hypotonic lysis buffer (10 mM $MgCl_2$, 24 mM KCl, 10 mM Tris, pH 8.0), which was made 0.5% in NP_{40}. One tube was then vortexed for 10 seconds, after which time the nuclei were pelleted by centrifugation at 1,500 rpm for 2 minutes. The nuclei were resuspended in lysis buffer and the lysis procedure was repeated. The nuclei were then resuspended in the incubation medium, which for most experiments was 70 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 6 mM KCL, 10 mM Tris, pH 8.0, 30% (v/v) glycerol, 1 mM dithiothreitol, 40 µM of three unlabeled ribonucleoside triphosphates. ^{3}H-labeled ATP, GTP, CTP or UTP (10-20 c/mM) was then added and incubation was carried out at 32°C. In most experiments, nuclei were prepared from 3 x 10^7 cells and resuspended in 1 ml of incubation solution. The ^{3}H-labeled nucleoside

triphosphate was added to a final concentration of 25 μc/ml in all experiments, except those in which VA-RNA was being prepared for hybridization or alkaline hydrolysis, in which case 150 - 200 μc/ml of the ^{3}H-labeled triphosphation were added to the reaction mixture.

The incubation conditions described above were chosen in order to maximize total incorporation of radioactivity. Under these conditions, the reaction is essentially complete by 30 minutes. However, when the concentration of the nuclei is reduced to 3×10^{6} per ml of incubation solution, the reaction is seen to proceed linearly for over 60 minutes.

The monovalent and divalent cation concentrations in the hypotonic lysis buffer were found to be critical to the preparation of active nuclei, and the ideal concentrations depended on the source of the cells. For example, 5 mM $MgCl_2$ and 8 mM KCl were used in the preparation of nuclei from uninfected cells. However, lysis of infected cells in this buffer resulted in the clumping of nuclei and greatly reduced incorporation. 10 mM $MgCl_2$ and 24 mM KCl were found to be optimal for preparation of nuclei from infected cells.

Labeling was terminated by the addition of cold incubation solution. When RNA released during the incubation was measured, nuclei were removed from the incubation medium by centrifugation at 2000 x g for 2 minutes and the supernatant was precipitated with 2 volumes of ethanol using yeast tRNA as carrier. The precipitate was dissolved in SDS buffer (100 mM NaCl, 10 mM EDTA, 0.5% SDS, 10 mM Tris, pH 7.4) and extracted with phenol and chloroform as described previously. RNA remaining associated with the nuclei was prepared by digestion of the nuclei with DNase (20 μg/ml) in HSB (500 mM NaCl, 50 mM $MgCl_2$, 10 mM Tris, pH 7.4), followed by phenol-chloroform

extraction.

E. Acrylamide Gel Electrophoresis

RNA was analyzed by electrophoresis in 10% acrylamide gels containing 10% glycerol as described by Weinberg, et al. [80]. Analytical studies were performed with polyacrylamide gels cross-linked with bis acrylamide. RNA was eluted from 1 mm slices by incubation in 1 ml SDS buffer overnight at room temperature. The RNA was precipitated with yeast tRNA and ethanol and resuspended in SDS buffer prior to hybridization.

F. 5' and 3' End Determination of VA-RNA

VA-RNA, labeled in vitro, was eluted from acrylamide gels as described above, precipitated with yeast sRNA and ethanol and hydrolyzed in 0.3 N NaOH overnight at 37°C. The product was then passed over a 3 x 0.5 cm column of Dowex 50W-X2 charged with NH_4^+ equilibrated with 0.05 M triethylamine bicarbonate. After evaporating to dryness, the sample was resuspended in 0.05 ml distilled water and the components separated by high voltage paper electrophoresis as described by Smith [81]. After drying, the paper was cut into 1 cm strips which were placed in scintillation vials containing 0.5 ml water. 15 ml of scintillant were then added and the samples were assayed for radioactivity as described below.

G. Hybridization

DNA-RNA hybridization was carried out under conditions of DNA excess to insure the detection of all viral sequences. Adenovirus was prepared and DNA extracted as described above. Purified ^{14}C-labeled DNA was attached to nitrocellulose filters (Carl Schleicher and Schuell Co.) as described previously [82]. Two DNA filters and one blank filter were incubated in 1 ml of the hybridization solution (30% formamide, ^{2} XSSC, 5 mM EDTA, 10 mM Tris, pH 7.4) containing the ^3H-labeled RNA. Hybridization was carried

out at 45°C for 24 hours for exhaustion hybridization. The filters were then removed from the vial, washed with 50 ml of 2 XSSC on each side and added to a vial containing 10 μg/ml pancreatic RNase. Digestion was carried out for 30 minutes at 37°C. The filters were then washed and assayed for radioactivity.

H. Assay of Radioactivity

Radioactivity was quantitated in all experiments by liquid scintillation counting. The liquid scintillant used was a solution of 4 grams of Omnifluor in 600 ml toluene plus 400 ml ethylene glycol monoethyl ether.

I. Chemicals

Actinomycin D was the gift of Mercke, Sharpe and Dohme. Ribonuclease A was purchased from Worthington Biochemicals; Deoxyribonuclease from Schwartz-Mann Company; and pronase (grade B) from Calbiochem Co. [^{3}H]-uridine (20 c/mM), [^{14}C]-uridine (40-60 mc/mM), and [^{3}H]-uridine, cytidine, adenosine and guanosine triphosphates (20-50 c/mM) were purchased from New England Nuclear Company.

RESULTS

A. *In Vitro* Transcription of Nuclear Heterogeneous RNA

In all experiments, cells were exposed to 0.04 μg/ml actinomycin D for 30 minutes prior to preparation of crude nuclei. At this concentration, the antibiotic has been shown to inhibit nucleolar transcription without affecting the synthesis of other classes of RNA both *in vivo* and in isolated nuclei [72-74].

The kinetics of incorporation of [^{3}H]-UMP into acid precipitable material by nuclei isolated from both uninfected and infected HeLa cells are shown in Figure 1. The incubation conditions are identical to those used in most of the experiments described in this work. The pattern of incorporation by nuclei from both sources is very similar, incorporation being linear for approximately 15 minutes, gradually slowing and being essentially complete by 30 to 40 minutes. The incubation conditions were chosen in order to maximize the initial rate of the reaction. The termination of incorporation after 30 minutes at 32°C is not an inherent property of the nuclei, but is due to the conditions of incubation, as re-addition of the ^{3}H-labeled and three unlabeled triphosphates at 30 minutes results in a resumption of incorporation. This suggests that the cessation of incorporation results from a limiting amount of substrate. Thus the reaction can be made to continue considerably longer if the concentration of nuclei is lower or the concentrations of the nucleoside triphosphates are increased.

The results presented in Table I indicate that the [^{3}H]-UMP is incorporated into RNA. The acid precipitable product is RNase-sensitive, and the addition of unlabeled nucleoside triphosphates is required for incorporation. Actinomycin D severely inhibits the reaction.

Alkaline hydrolysates of the ^{3}H-labeled product which remained associated with the nuclei during incubation were analyzed in order to determine

Figure 1: The Kinetics of Incorporation of ^{3}H-UMP into RNA by Isolated Nuclei.

Nuclei from 1 x 10^{7} cells were incubated in 1 ml in the presence of ^{3}H-UTP as described in Materials and Methods. 0.05 ml samples were taken at the times indicated and added to 2 ml 10% TCA, 50 mM sodium pyrophosphate. Precipitable radioactivity was assayed as described in Materials and Methods. Uninfected, closed circles; infected, open circles.

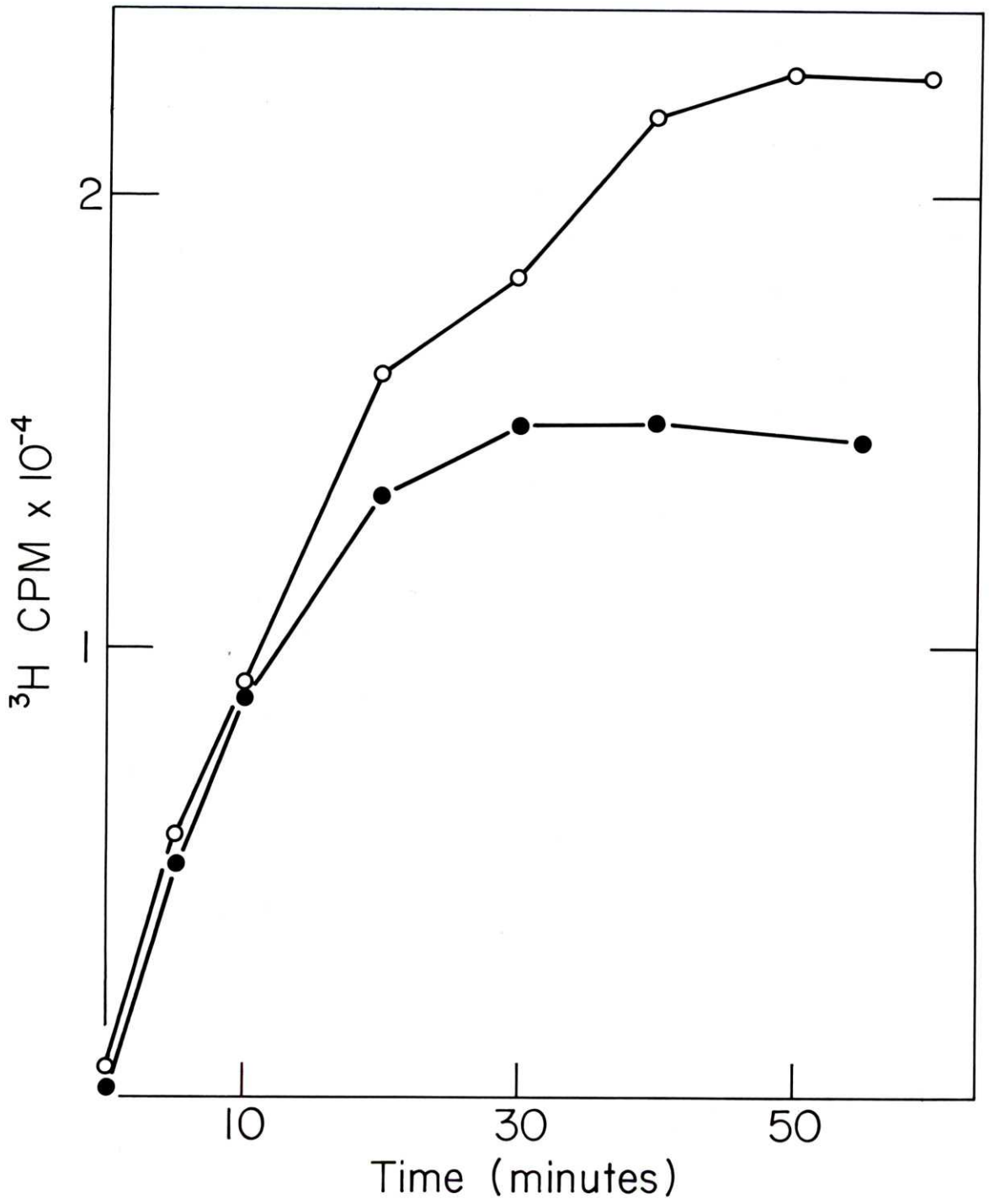

2
1
^{3}H CPM x 10^{-4}
10
30
50
Time (minutes)

TABLE I

PROPERTIES OF ^{3}H-UMP INCORPORATION BY ISOLATED NUCLEI

	Per Cent of Control ^{3}H-CMP Incorporation
Complete System	100
Minus Unlabeled ATP, GTP, CTP	6
Minus Unlabeled GTP	13
Plus 5 µg/ml Actinomycin	15
Plus RNase Digestion (20 µg/ml, 15 mins.)	4

Incubation of nuclei and determination of ^{3}H-UMP incorporation was as described in Figure 1. Incubation time was 10 minutes.

the nature of the synthetic activity. There was no evidence for the presence of nucleoside tetraphosphates when any of the four ^{3}H-labeled triphosphates was present, suggesting that no re-initiation of RNA molecules occurs during the *in vitro* incubation. Some estimate of the extent of the elongation reaction can be made by comparing the amount of nucleoside to the nucleoside monophosphate when each of the four triphosphates is ^{3}H-labeled. The results presented in Table II suggest that the elongation reaction consists of the addition of an average of 500 to 1000 bases to the nascent RNA chains that were initiated *in vivo*.

Sixteen hours after infection with adenovirus, the nucleus of the infected cell is very active in the synthesis of viral RNA. However, extensive synthesis still takes place on host cell DNA. The amount of adeno-specific RNA present in the nuclei of infected cells is measured by hybridization of the RNA to an excess of adenovirus DNA bound to cellulose nitrate filters.

The RNA from nuclei of productively infected cells labeled *in vivo* was hybridized to adenovirus DNA filters under conditions of DNA excess. The time course of hybridization is shown in Figure 2A. The amount of hybridization appears to plateau at approximately 14% of the input RNA, and this is independent of the amount of DNA present on the filters. It appears, therefore, that 14% of the RNA present in infected cell nuclei under the labeling conditions used is adeno-specific, agreeing with previous reports [18].

The RNA labeled *in vitro* in nuclei isolated from cells 18 hours after infection with adenovirus was next examined. A crude nuclear preparation was incubated under the conditions described above, in the presence of three unlabeled nucleoside triphosphates and [^{3}H]-UTP. In most experi-

TABLE II

THE PRODUCTS OF ALKALINE HYDROLYSIS OF HnRNA LABELED IN VITRO

^{3}H-Labeled Nuceloside Triphosphate	cpm ^{Np}	cpm N	cpm ^{Np}/N
ATP	48960	56	865
GTP	55740	89	626
CTP	55102	194	283
UTP	36956	88	420

Nuclei from 1 x 10^{7} cells were incubated with one ^{3}H-labeled and three unlabeled triphosphates as described in Materials and Methods. Purified RNA was hydrolyzed in 0.5 N NaOH and the products analyzed by high voltage electrophoresis.

Figure 2: The Kinetics of Hybridization of Nuclear RNA.

(A) 2×10^7 HeLa cells infected with adenovirus were labeled with 200 μc of ^{3}H-uridine for 30 minutes at 16 hours after infection. Nuclei were isolated and RNA extracted as in Materials and Methods. Hybridization was as in Materials and Methods.

(B) Nuclei from 6×10^6 infected cells at 16 hours after infection were incubated *in vitro* as in Materials and Methods. RNA was extracted and hybridized to adenovirus DNA as in Materials and Methods.

(C) Same as (B) except 0.2 μg/ml of α-amanitin was added to the incubation mixture.

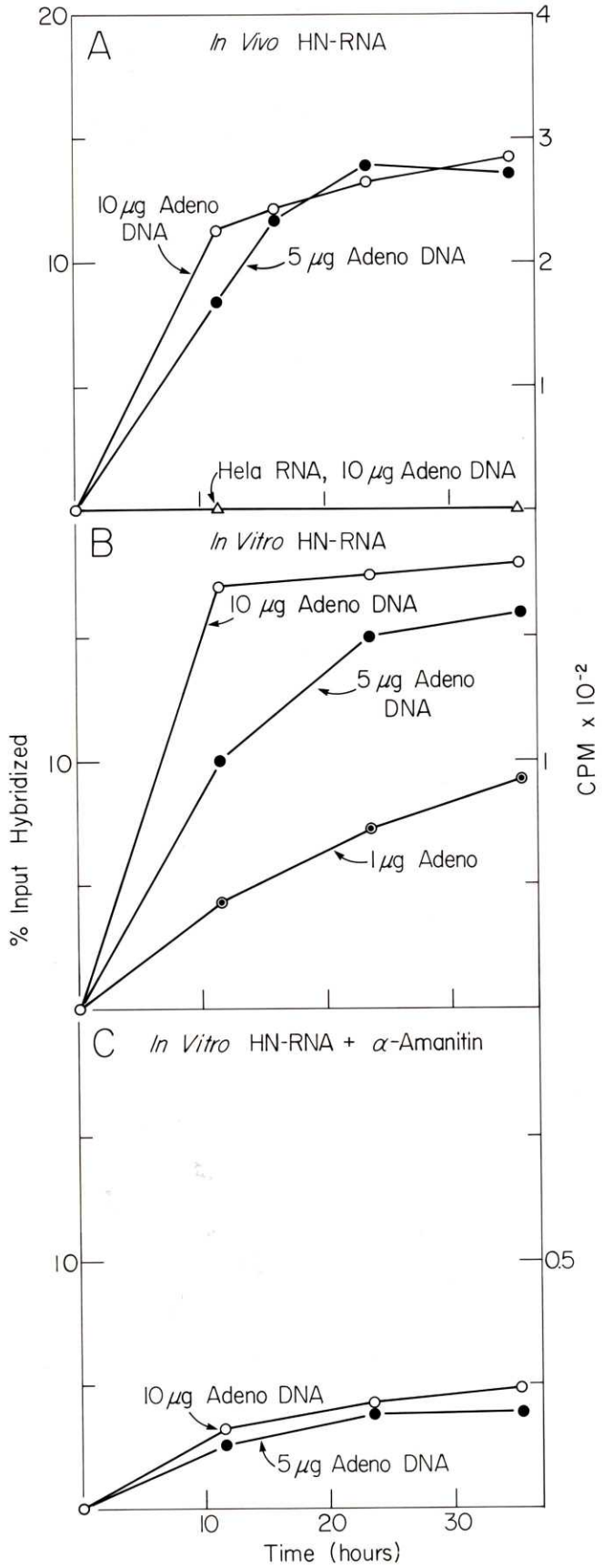
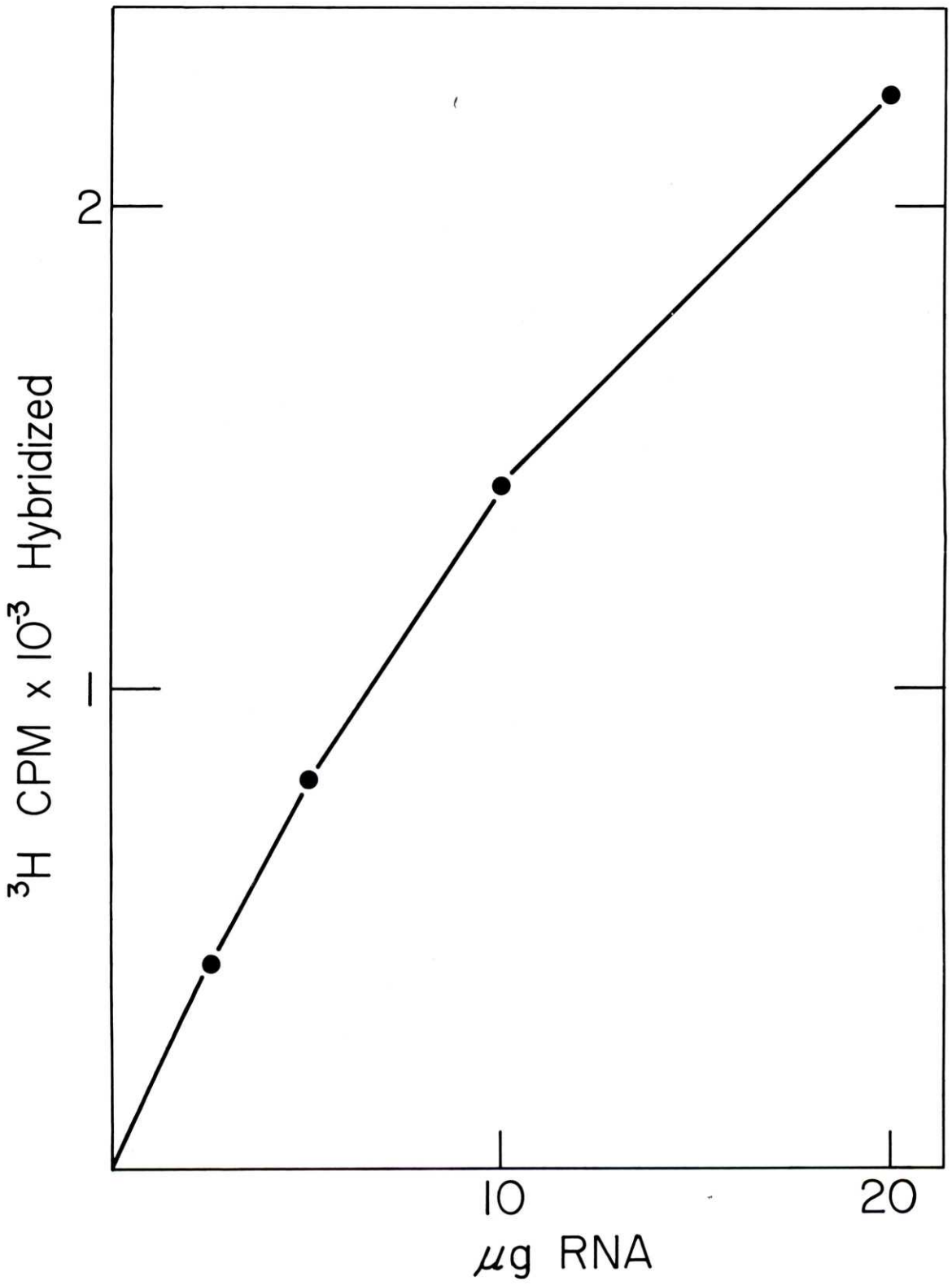

A
In Vivo HN-RNA
10 μg Adeno DNA
5 μg Adeno DNA
Hela RNA, 10 μg Adeno DNA
B
In Vitro HN-RNA
10 μg Adeno DNA
5 μg Adeno DNA
1 μg Adeno
C
In Vitro HN-RNA + α-Amanitin
10 μg Adeno DNA
5 μg Adeno DNA
% Input Hybridized
CPM x 10⁻²
Time (hours)
20
10
4
3
2
1
0.5
10
20
30

Figure 3: Hybridization of In Vitro Labeled RNA to Adenovirus DNA.

Preparation of ^{3}H-UMP-labeled RNA was as described in Figure 1. RNA was incubated in scintillation vials containing 10 µg adenovirus DNA attached to 2 nitrocellulose filters. Hybridization was carried out for 24 hours.


2
1
^{3}H CPM x 10^{-3} Hybridized
10
20
μg RNA

ments, from 1 to 5 micrograms of RNA labeled in isolated nuclei were hybridized to 10 micrograms of viral DNA immobilized on nitrocellulose filters. The results presented in Figure 3 demonstrate that the reaction is being carried out under conditions of DNA excess.

Figure 2B shows the time course of hybridization of RNA labeled *in vitro* in nuclei from infected cells. The amount of material which hybridizes to adenovirus DNA appears to plateau at approximately 18% of the total labeled RNA. Thus approximately the same fraction of the RNA labeled during the *in vitro* incubation is adenovirus-specific as during labeling *in vivo*.

The sensitivity of the activity which transcribes adenovirus DNA to α-amanitine was measured. This cyclic polypeptide has been shown to be a potent and selective inhibitor of polymerase II of the uninfected cell [59, 72]. Nuclei from infected cells were incubated in the presence of α-amanitine. The drug suppresses *in vitro* incorporation to about 15% of the level observed in the control. The small amount of labeled RNA obtained from amanitine-treated nuclei hybridizes to a limited degree with the adenovirus DNA. A plateau of approximately 6% of the input RNA labeled in the presence of α-amanitine is obtained, as shown in Figure 2C. Thus only a small percentage of the small amount of residual synthetic activity observed in the amanitine-treated nuclei is adenovirus-specific. These results are summarized in Table III. The bulk of the transcription of adenovirus DNA is clearly accomplished by an α-amanitine-sensitive polymerase. The viral-specific RNA labeled in the presence of α-amanitine is quite different from the viral RNA whose synthesis is amanitine-sensitive, as will be shown below.

The polymerase activities measured *in vitro* can be further character-

TABLE III

THE EFFECT OF α-AMANITINE ON THE LABELING OF LATE ADENOVIRUS RNA IN ISOLATED NUCLEI

	Total CPM Incorporated	CPM Per Vial	CPM Hybridized	Adeno-Specific RNA
Control	32600	1080	180	5542
Plus 0.5 μg/ml α-amanitine	4800	480	24	240

Nuclei were isolated from 6 x 10^6 cells and labeled with ^{3}H-UTP. Hybridization was as described in Materials and Methods.

ized by examining their response to altered ionic strength conditions. In particular, polymerase II is very sensitive to ionic strength, in contrast to polymerase III, which is comparatively indifferent to the ionic conditions of incubation [58,72]. The nucleoplasmic incorporation in incubated infected cell nuclei is shown in Figure 4 as a function of varying concentrations of ammonium sulfate. Both total incorporation and adenovirus-specific synthesis increase significantly with increasing ammonium sulfate, up to concentrations of 75 to 100 mM. Above this concentration, experimental results become variable, possibly due to instability of the nuclear structure at the higher ionic strengths. Thus the response of adenovirus-specific transcription to changes in ionic conditions resembles that of the total incorporation, which under these conditions is due principally to an activity which resembles polymerase II [72]. It may be noted that small but reproducible differences in response to ionic strength are noted between adeno-specific transcription and total nuclear incorporation. This suggests the possibility that the polymerase activity transcribing the adenovirus genome may in fact differ from the host enzyme in some way. It is, of course, possible that this difference in response to ionic conditions is due to effects on the rather different DNA templates, rather than reflecting differences in the polymerase.

The effect of manganese on the polymerase activities measured in vitro in nuclei is somewhat different from its effect on purified enzymes. In a previous report it was shown that the activity tentatively identified as polymerase II is inhibited by manganese. Figure 5 shows the response of total nucleoplasmic incorporation in nuclei exposed to increasing concentrations of manganese ion in the absence of magnesium. The incorporation decreases as manganese concentration increases, in agreement with previous

Figure 4: The Effect of Ionic Strength on Total and Adenovirus-Specific RNA Synthesis.

Nuclei were isolated from HeLa cells 16 hours after infection. Crude nuclei were prepared as described in Materials and Methods and resuspended in the incubation medium containing the indicated concentrations of ammonium sulfate. Nuclei from 4×10^6 cells were used in each sample. ^{3}H-UTP incorporation was allowed to proceed for 5 minutes. Adenovirus-specific RNA synthesis was determined by hybridization as described in Figure 2.

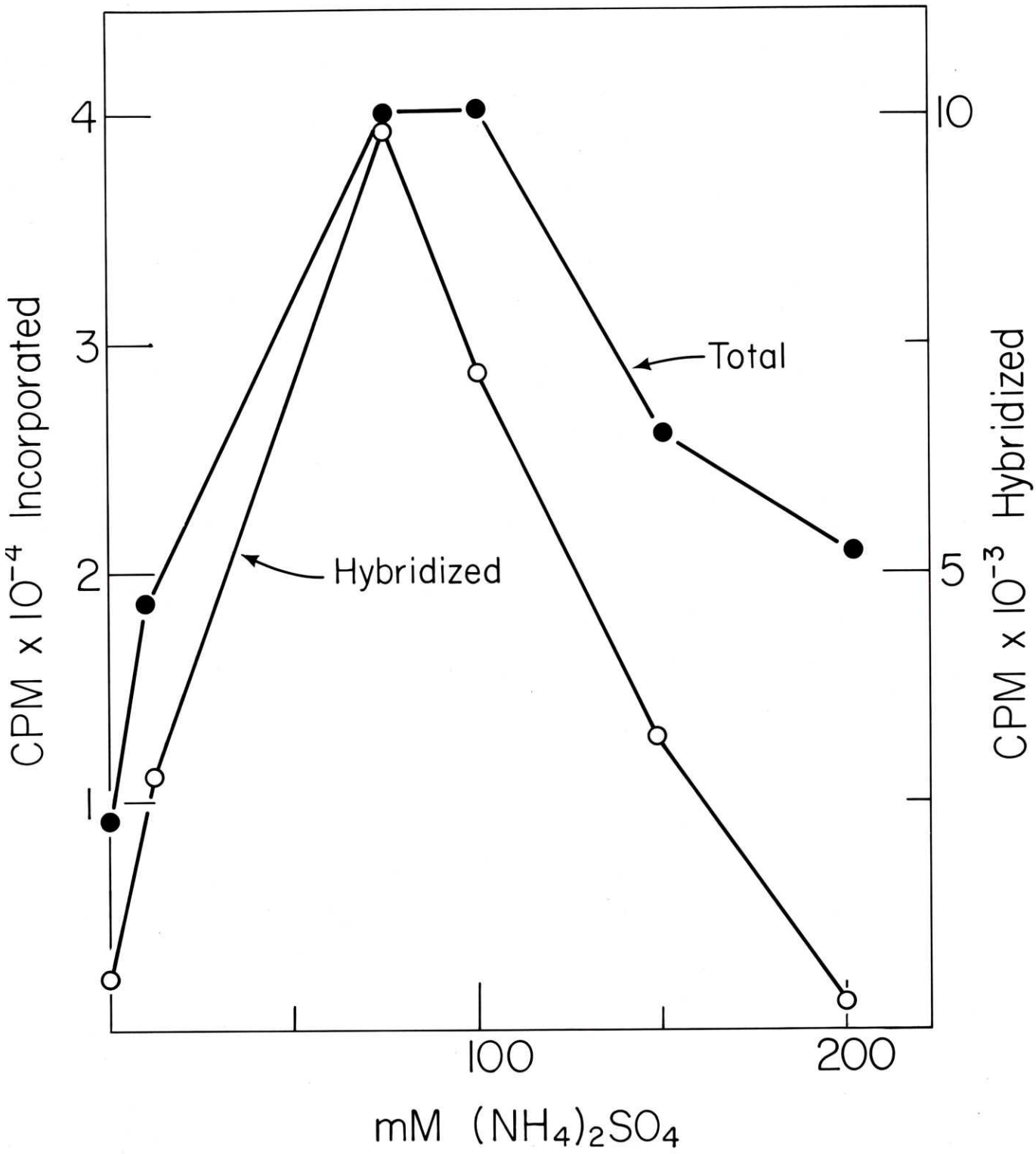

CPM x 10⁻⁴ Incorporated
CPM x 10⁻³ Hybridized
mM (NH4)2SO4
Total
Hybridized
1
2
3
4
5
10
100
200

Figure 5: The Effect of the Manganese Ion on Total and Virus-Specific RNA Synthesis.

Nuclei were isolated from HeLa cells 16 hours after infection with adenovirus. Labeling and determination of adenovirus-specific synthesis were as described in Figure 2.

CPM x 10⁻⁵ Incorporated
CPM x 10⁻⁴ Hybridized
Total
Hybridized
1
2
3
4
5
10
mM Mn⁺⁺

results. The adenovirus-specific RNA, measured by hybridization, also decreases in the presence of increasing concentrations of manganese and continues to constitute a relatively constant fraction of the total nuclear RNA. The activity responsible for most of the adenovirus transcription activity thus resembles the major host cell nucleoplasmic polymerase by this criterion.

The data in Figure 6 show the sedimentation profile of RNA labeled by nuclei obtained from uninfected cells and from cells 18 hours post-infection. The principal product labeled in both cases is large heterogeneously sedimenting RNA. Hybridization of fractions across the gradient indicate that viral-specific RNA is also heterogeneous in sedimentation values. The amanitine-resistant activity is also shown, and this consists primarily of low molecular weight RNA, which will be described below.

The adenovirus-specific RNA labeled early in infection differs in its hybridization properties from RNA produced late in infection [26,83,84]. The properties of the transcription of viral RNA early in infection were measured. Nuclei were isolated from infected cells early in the lytic cycle and incubated in vitro. The results of hybridization experiments summarized in Table IV demonstrate a small amount of adeno-specific RNA synthesis, which is almost totally inhibited by α-amanitine.

The data in Figure 7 show the response to ammonium sulfate of both the total incorporation and the adeno-specific transcription found in nuclei from early stages of infection. Here again, the response of viral-specific RNA labeling resembles that of the major cellular nucleoplasmic activity.

B. Transcription of 5.5S VA-RNA, 5S RNA and the tRNA Precursor

Much of the adenovirus RNA labeled in the isolated nuclei is large and

Figure 6: Sedimentation Profile of RNA Synthesized In Vitro.

Nuclei were isolated from 1.2 x 10^7 HeLa cells 16 hours after infection with adenovirus and labeled with ^{3}H-UTP as described in Figure 2. RNA was extracted by the phenol-SDS method and analyzed by sedimentation through a 15-30% SDS-sucrose gradient as in Materials and Methods. Centrifugation was for 16 hours at 24,000 rpm in the SW27 rotor. Fractions were collected and radioactivity measured as in Materials and Methods. Aliquots of each fraction were precipitated with ethanol, resuspended in the hybridization solution and hybridized to adenovirus DNA as described in Materials and Methods.

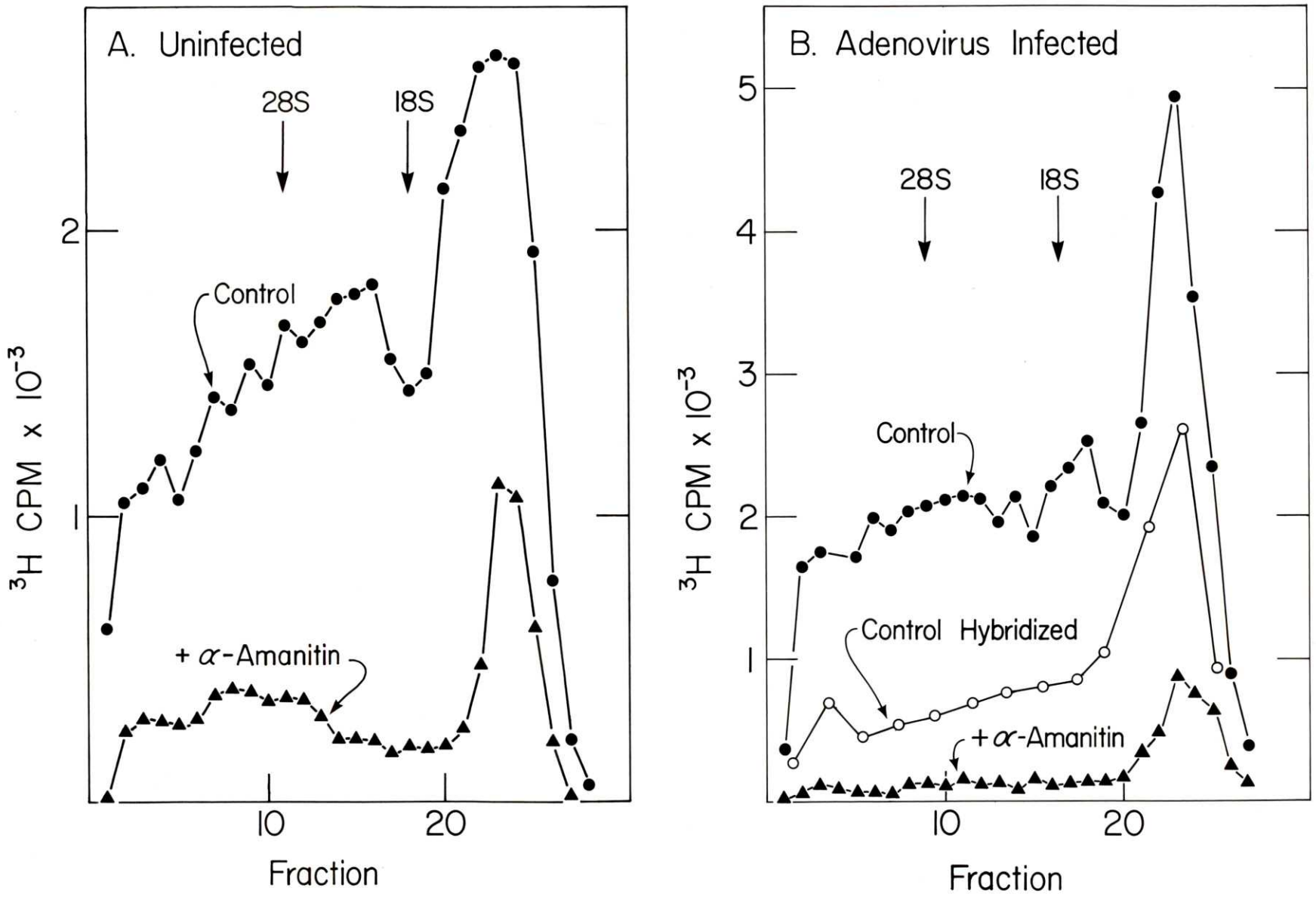

A. Uninfected
28S
18S
Control
+ α-Amanitin
^{3}H CPM x 10^{-3}
Fraction
B. Adenovirus Infected
28S
18S
Control
Control Hybridized
+ α-Amanitin
^{3}H CPM x 10^{-3}
Fraction

TABLE IV

^{3}H-UTP INCORPORATION INTO ISOLATED NUCLEI OF HELA CELLS
4 HOURS AFTER INFECTION

	Total CPM Incorporated	CPM Per Vial	CPM Hybridized	Adeno-Specific RNA
Control	126,000	24,000	320	1,675
Plus 0.5 μg/ml α-amanitine	7,600	1,600	45	212

Nuclei were isolated from cells at 4 hours after infection and incubated in vitro as in Figure 2. The RNA was extracted and hybridized as in Figure 2. The hybridization reaction was allowed to go to completion with excess adenovirus DNA.

Figure 7: The Effect of Ionic Strength on Total and Adenovirus-Specific RNA Synthesis.

Nuclei were isolated from 2.4 x 10^7 HeLa cells 4 hours after infection. One hour after virus attachment and resuspension of the cells in fresh medium, cycloheximide was added to a concentration of 25 µg/ml to prevent the synthesis of late RNA. RNA was then analyzed by hybridization for virus-specific sequences as described in Figure 2.

Total
Hybridized
100
200
mM $(NH_4)_2SO_4$
CPM x 10^{-2} Hybridized

sediments heterogeneously, but a peak of slowly sedimenting virus-specific RNA was also present, as shown in Figure 6. The drug α-amanitine inhibits the labeling of the large heterogeneous adenovirus RNA, but a significant fraction of the radioactivity remains in the slowly sedimenting peak.

Figure 8A shows the sedimentation distribution of all of the nuclear RNA (host cell plus viral-specific RNA) labeled in the presence of α-amanitine. A small amount of non-viral heterogeneously sedimenting large RNA is labeled, but most of the radioactive RNA sediments in the low molecular weight region of the gradient. The data in Figure 8A also show that practically all of the low molecular weight product is released from the nuclei during incubation, while the high molecular weight RNA remains associated with the nuclei. The data summarized in Table V show that the large, heterogeneously-sedimenting RNA whose synthesis is sensitive to α-amanitine is also retained in the nuclei. Thus it appears that all high molecular weight RNA remains associated with the chromatin of the nuclei, while the low molecular weight product is almost quantitatively released during incubation. It is not known whether this selective release of low molecular weight RNA into the incubation medium is physiologically significant, or whether it simply reflects the more rapid diffusion of the small molecules from damaged nuclei.

The low molecular weight peak labeled <u>in vitro</u> is resolved into three distinct RNA species, as shown in Figure 8B, when analyzed by gel electrophoresis. It seemed likely that one of these low molecular weight RNA species was related to the 5.5S virus-associated RNA produced in adenovirus-infected cells. RNA from the cytoplasm of infected cells labeled <u>in vivo</u> with [^{14}C]-uridine is compared to the <u>in vitro</u> [^{3}H]-UMP labeled RNA in the gel electropherogram in Figure 9. The cytoplasmic RNA peak with

Figure 8; (A) Sedimentation Profiles of RNA Labeled In Vitro Synthesized in Nuclei Isolated from Adenovirus-Infected HeLa Cells 16 Hours Post-Infection in the Presence of α-Amanitine.

Nuclei were isolated from 1.2×10^7 HeLa cells 16 hours after infection with adenovirus and labeled with ^{3}H-UTP as described in Materials and Methods. RNA was prepared from the incubation solution and the nuclei as described in Materials and Methods. Centrifugation was for 16 hours at 24,000 rpm in an SW27 rotor. Fractions were collected and radioactivity was measured as described in Materials and Methods.

(B) Gel Electropherogram of RNA Labeled In Vitro in α-Amanitine.

RNA was prepared from nuclei of infected cells as described in the legend to panel A. Following ethanol precipitation, the RNA was analyzed in a 10% polyacrylamide gel.

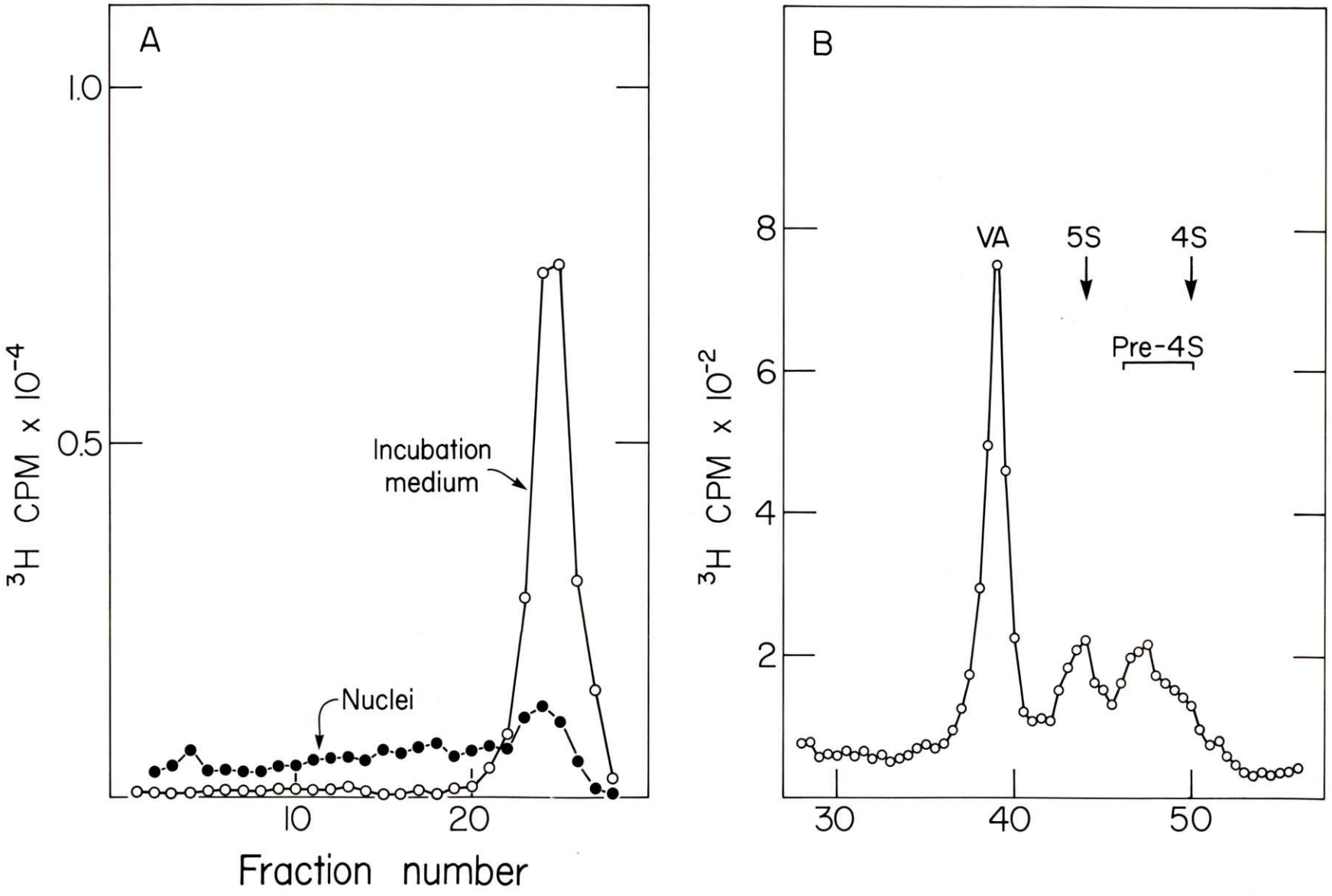

A
1.0
0.5
^{3}H CPM x 10^{-4}
Incubation medium
Nuclei
10
20
Fraction number
B
8
6
4
2
^{3}H CPM x 10^{-2}
VA
5S
4S
Pre-4S
30
40
50

TABLE V

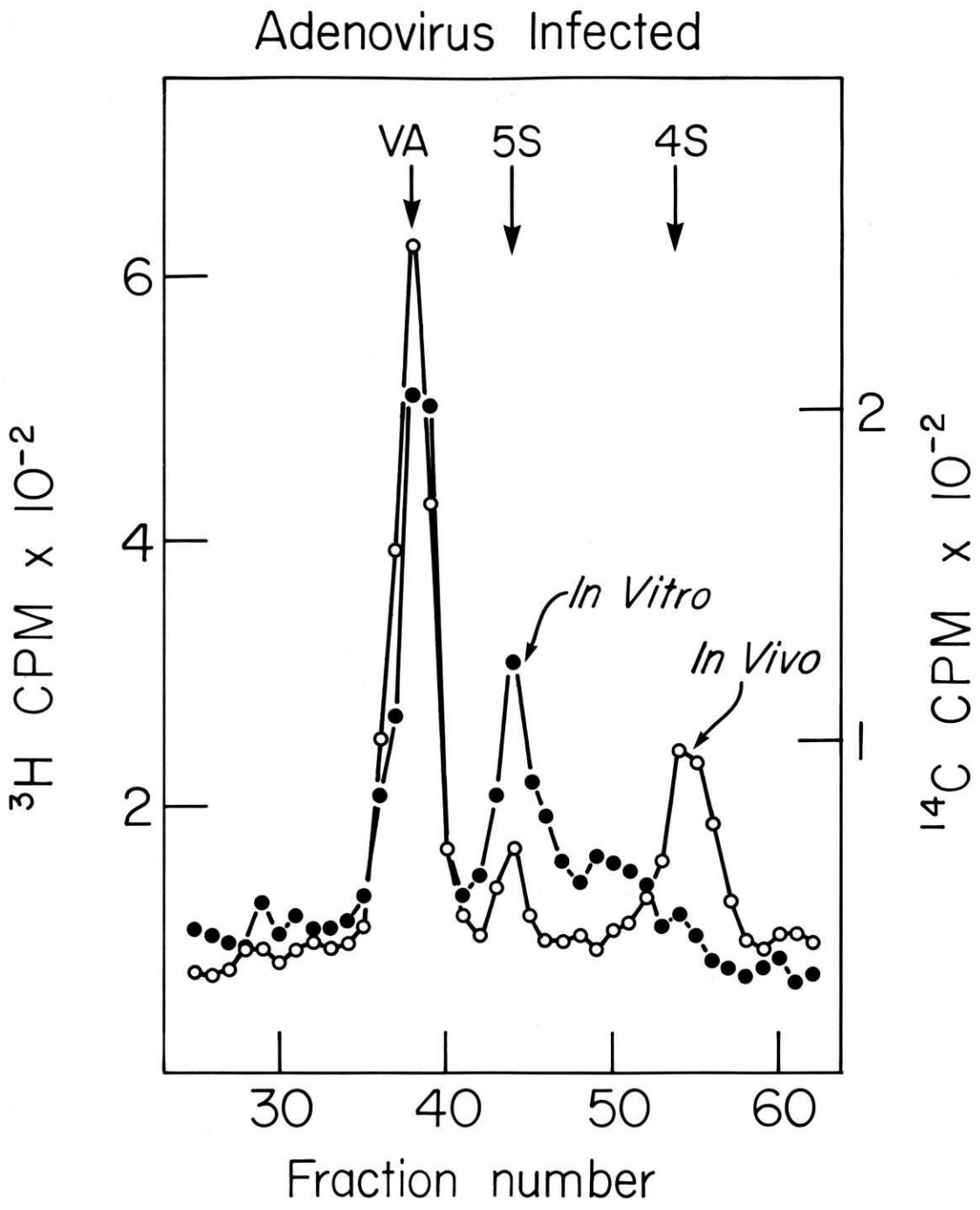
THE RELEASE FROM NUCLEI OF RNA LABELED IN VITRO

	^{3}H CPM	Per Cent Adeno-Specific	Adenovirus ^{3}H CPM
Supernatant	10,593	22	2330
Nuclear Pellet	113,096	41	43369

Nuclei were isolated from 5 x 10^{6} cells 16 hours after infection with adenovirus. After incubation for 10 minutes in the presence of ^{3}H-UTP, RNA was extracted from the nuclei and the incubation medium as described in Materials and Methods.

Figure 9: Coelectrophoresis of Low Molecular Weight RNA Synthesized In Vivo and In Vitro in HeLa Cells 16 Hours after Infection with Adenovirus.

Sixteen hours after infection with adenovirus, 2×10^7 HeLa cells were treated with 0.04 µg/ml actinomycin D for 30 minutes. 0.1 µc/ml C^{14} uridine was then added and incubation continued for 60 minutes. The cells were then washed and RNA extracted from the cytoplasm as described previously. RNA was labeled with ^3H-UTP in isolated nuclei from adenovirus-infected cells as described in Figure 1. RNA was extracted, mixed with C^{14} labeled in vivo RNA and analyzed by electrophoresis on 10% polyacrylamide gels as described in Materials and Methods. The gels were frozen, sliced and assayed for C^{14} and ^3H.


Adenovirus Infected
VA
5S
4S
6
4
2
2
1
In Vitro
In Vivo
^{3}H CPM x 10^{-2}
^{14}C CPM x 10^{-2}
30
40
50
60
Fraction number

greatest mobility corresponds to mature 4S RNA, the next slower to 5S RNA. The major peak of radioactivity corresponds to 5.5S RNA and appears only in virus-infected cells. The 5.5S RNA will be designated here as adenovirus-associated RNA (VA-RNA).

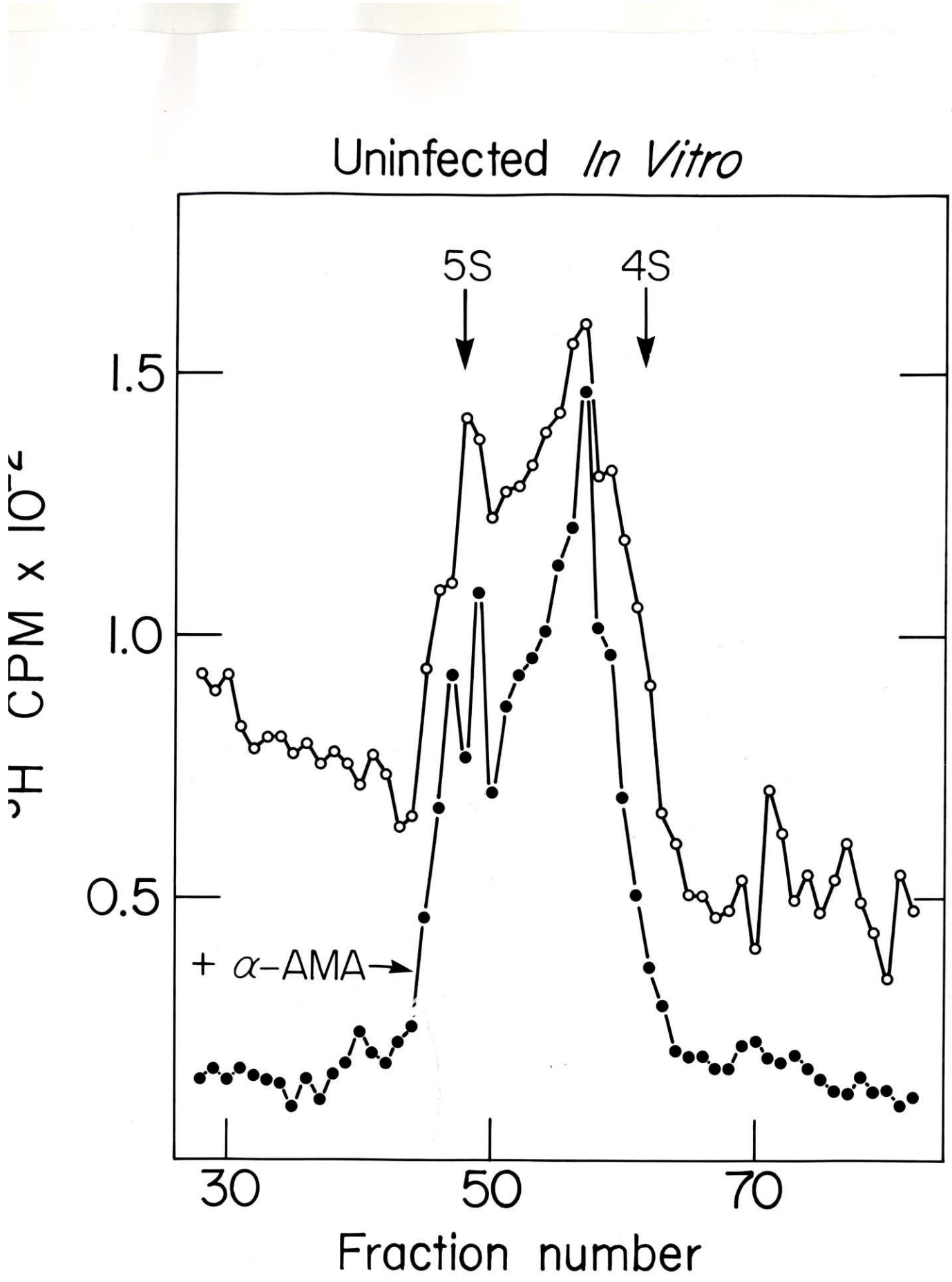
Low molecular weight RNA labeled in vitro appears quite similar to that seen in the cytoplasm of cells labeled in vivo. The 4S peak in the RNA labeled in vivo is not present in the sample labeled in vitro. There is, instead, a somewhat heterogeneous higher molecular weight distribution of more slowly migrating material. (This species is more easily seen in Figure 8B.) It has been shown that this material has the properties of transfer RNA precursor [75]. Since the primary transcription products would be expected to be labeled when nuclei are incubated in vitro, the production of labeled precursor to transfer RNA, rather than the mature species, is not surprising. A monodisperse peak corresponding to 5S RNA is apparent and an RNA species migrating with the VA-RNA is extensively labeled.

The two minor peaks tentatively identified as 5S and pre-4S labeled in vitro by nuclei from infected cells are probably the host cell species, rather than products of the viral genome. The 5 and pre-4S species are also labeled in vitro by nuclei from uninfected cells, as shown in the gel electropherogram in Figure 10, while the VA-RNA is absent. The labeling of both species is again completely resistant to inhibition by α-amanitine.

The only cellular RNA species which might be confused with VA-RNA is one found hydrogen-bonded to 28S RNA [76]. This RNA has an electrophoretic mobility similar (but not identical) to that of VA-RNA. The formation of this 28S RNA associated species is inhibited by low concentrations of actinomycin D, such as are used to pre-treat cells in these experiments.

Figure 10: Uninfected HeLa Cell Low Molecular Weight RNA Synthesized In Vitro.

Labeling, RNA extraction and gel electrophoresis were performed as described in Figure 9.


Uninfected *In Vitro*
5S
4S
1.5
1.0
0.5
^{3}H CPM x 10^{-4}
+ α-AMA
30
50
70
Fraction number

Also, the uninfected cell species remains attached to 28S during the room-temperature phenol extraction used in these experiments and thus would not appear in the low molecular weight RNA fraction. The VA-RNA is the only discrete RNA species of a size corresponding to 5.5S formed under the conditions used in these experiments.

The following experimental result shows that the VA-RNA labeled either *in vivo* or *in vitro* is indeed complementary to the adenovirus genome. RNA was extracted from the cytoplasm of cells labeled *in vivo* with [^{3}H]-uridine at 16 hours after infection. The *in vitro* labeled RNA was prepared as above. The RNA was fractionated by polyacrylamide gel electrophoresis and gel slices corresponding to the VA-RNA were eluted. The RNA obtained was hybridized with both adenovirus and whole cell DNA. The results in Table VI show that the VA-RNA hybridizes extensively with adenovirus DNA. In contrast, the amount of hybridization with whole cell DNA is negligible. However, it is not possible to conclude from this latter result whether or not sequences complementary to the VA-RNA are in fact present in the host genome, since this hybridization would be detected only if the VA-RNA sequence were significantly reiterated.

Another method of establishing the identity of the *in vivo* and *in vitro* VA-RNA is to examine the effect of blocking adenovirus DNA synthesis on their formation. Previous reports have established that the VA-RNA is formed only late in infection after viral DNA replication has begun [77]. If DNA synthesis is inhibited early in infection with cytosine arabinoside, the formation of so-called late viral RNA is prevented. Results shown in Table VII indicate that neither overall cellular RNA synthesis measured *in vivo* nor the production of 5S and 4S RNA, which are presumably synthesized from the host genome, are substantially affected by cytosine arabinoside.

TABLE VI

HYBRIDIZATION OF VA-RNA TO ADENOVIRUS AND HELA DNA

	Hybridization to 10 µg adenovirus DNA			Hybridization to 40 µg HeLa DNA		
	^{3}H CPM added	^{3}H CPM hybridized	% input hybridized	^{3}H CPM added	^{3}H CPM hybridized	% input hybridized
in vivo VA-RNA	728	448	62	3912	19	<1
in vitro VA-RNA	844	419	50	1688	23	1

VA-RNA was purified from the cytoplasm of HeLa cells labeled for 60 minutes with ^{3}H-uridine 16 hours post infection and from isolated nuclei labeled in vitro with ^{3}H-UTP as described in Figure 9. The VA-RNA was purified by acrylamide gel electrophoresis and hybridized to 10 µg adenovirus DNA and 40 µg HeLa DNA attached to nitrocellulose filters.

TABLE VII

THE EFFECT OF INHIBITION OF PRODUCTION OF ADENOVIRUS DNA ON VA-RNA SYNTHESIS

^{3}H CPM	control	plus 20 µg/ml cytosine arabinoside
in vivo		
VA-RNA	11,720	839
5S	2144	1950
4S	5701	6020
Total Incorporation	66,400	96,800
in vitro		
VA-RNA	21,678	0
Total Incorporation	185,640	203,580

Two hours after infection with adenovirus, 20 µg/ml cytosine arabinoside was added to the medium. At 16 hours post-infection, incorporation of ^{3}H-uridine into acid-precipitable material was assayed. Formation of VA-RNA in vivo was determined by labeling for 60 minutes with ^{3}H-uridine, extracting RNA from the cytoplasm and quantitating the material migrating at 5.5, 5 and 4S as described in Figure 9. Labeling of VA-RNA in isolated nuclei was determined as in Figure 9.

The labeling of VA-RNA *in vivo*, however, is decreased more than ten-fold in the presence of the drug. Essentially no VA-RNA could be detected in the *in vitro* reaction product of nuclei from cells infected in the presence of cytosine arabinoside.

That the effect of cytosine arabinoside on VA-RNA synthesis is due to its inhibition of the formation of many copies of the adenovirus genome rather than the requirement for concomitant DNA synthesis for VA-RNA transcription is shown in Table VIII. The inhibition of DNA synthesis by cytosine arabinoside for only a short time prior to the labeling at 16 hours post-infection has little effect on VA-RNA synthesis measured *in vitro*.

Thus the synthesis of VA-RNA in normal quantities, both *in vivo* and *in vitro*, is blocked by inhibiting DNA (presumably viral) synthesis during the virus growth cycle.

Surprisingly, the VA-RNA and 5S cellular RNA appear as sharp peaks in the data presented above. The *in vitro* reaction product is not comprised to any observable extent of partially complete RNA molecules which elongate to the final discrete RNA species. Nevertheless, the next experiments show that the incorporation of label into RNA molecules is indeed the result of DNA transcription.

A time course of incorporation of radioactive nucleotides into total α-amanitine-resistant nuclear RNA and into pre-4S, 5S, and VA-RNA is shown in Figure 11. The radioactivity in total RNA, pre-4S, 5S, and VA-RNA increases linearly for at least 20 minutes. The addition of actinomycin results in a prompt inhibition of all labeling, as shown in Figure 12. This result implies that labeling is accomplished by a DNA-directed RNA polymerase and is not due to an end-addition reaction. It further suggests that radioactivity is not chased into the VA-RNA peak from some higher molecular

TABLE VIII

THE LACK OF DEPENDENCE OF VA-RNA SYNTHESIS ON CONCOMITANT DNA SYNTHESIS

Sample	Total ^{3}H incorporated	Total ^{14}C incorporated	^{3}H CPM in VA-RNA	per cent control VA-RNA
IN VIVO	101,700	8421	N. D.	
IN VIVO plus cytosine arabinoside	140,466	302	N. D.	
IN VITRO	138,100		19152	100
IN VITRO plus cytosine arabinoside	132,100		16352	85

Sixteen hours after infection with adenovirus, 20 ug/ml cytosine arabinoside was added to 2 X 10^{7} HeLa cells. 30 minutes later, 10 uC/ml ^{3}H-uridine and 0.1 uC/ml ^{14}C thymidine were added and incubation continued for 60 minutes. RNA was extracted from the cytoplasm and VA-RNA synthesis determined as described in Figure 9. The labeling of VA-RNA in isolated nuclei was as described in Figure 9. N. D. = not determined.

Figure 11: The Kinetics of Incorporation of ^{3}H-UMP into 5S, 4S and VA-RNA by Isolated Nuclei.

Labeling of nuclei and determination of incorporation of ^{3}H-UMP into low molecular weight RNA species was performed as described in Figure 9.

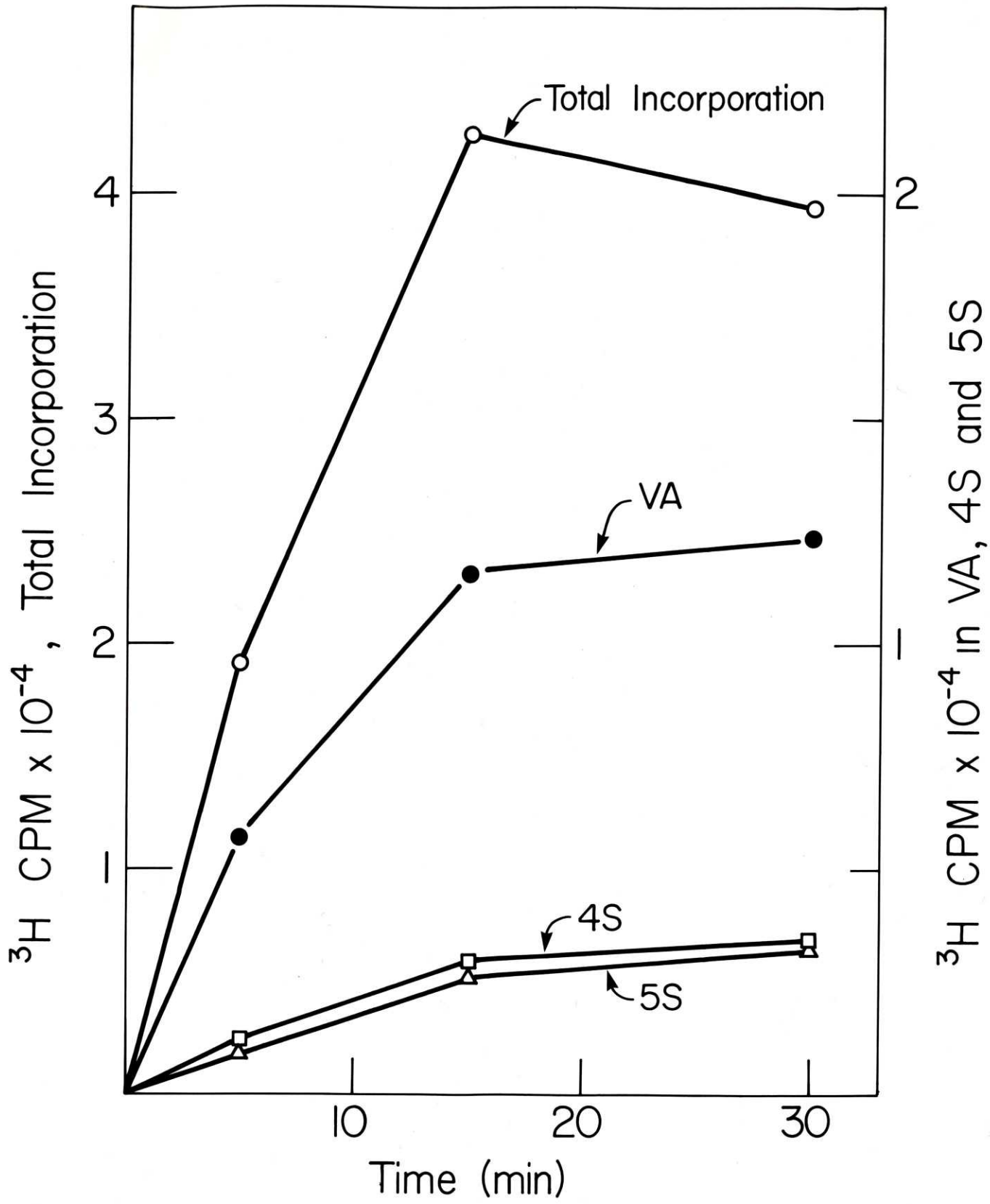
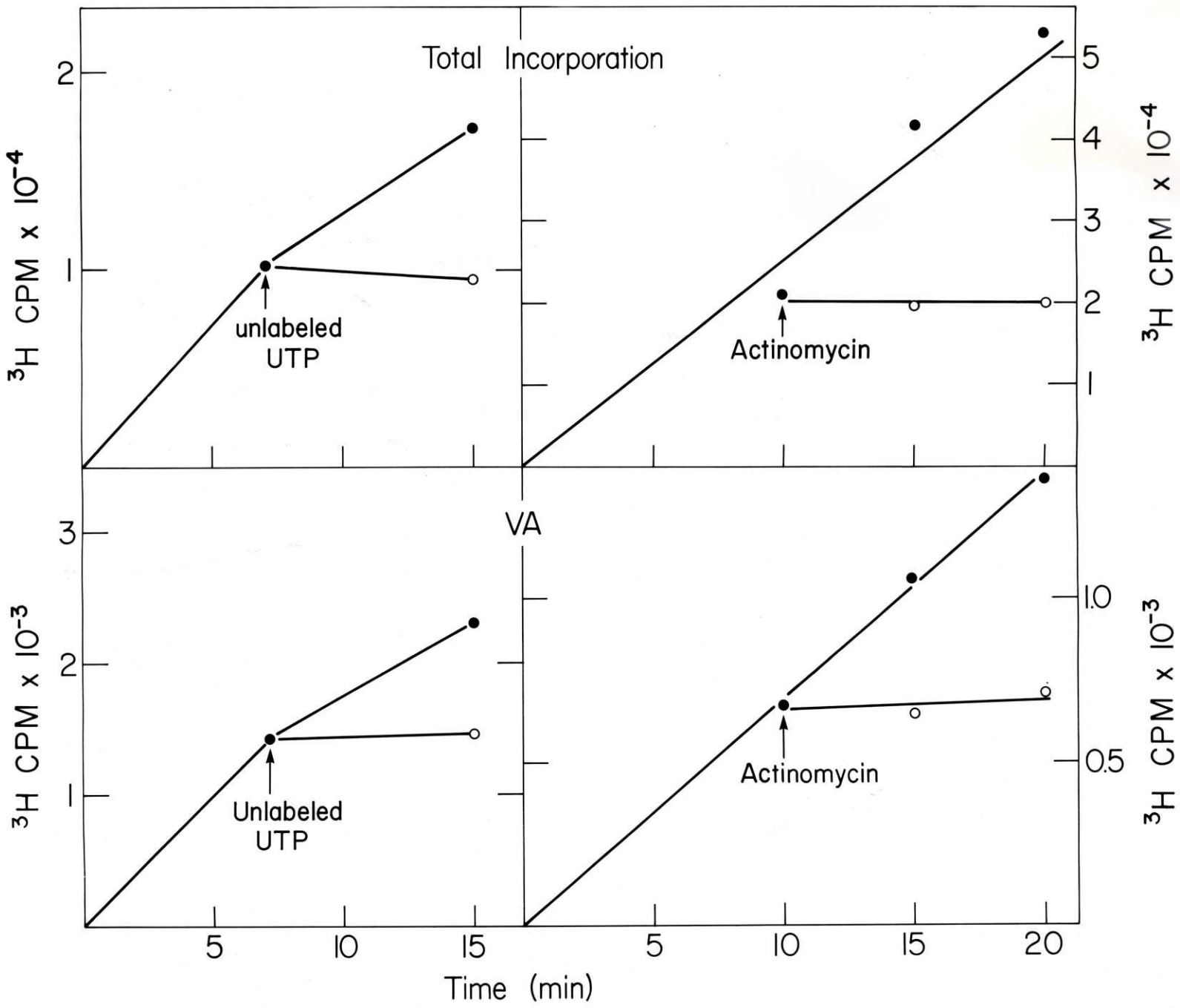

Total Incorporation
VA
4S
5S
4
3
2
1
2
1
10
20
30
Time (min)
^{3}H CPM x 10^{-4}, Total Incorporation
^{3}H CPM x 10^{-4} in VA, 4S and 5S

Figure 12: Effect of a Chase with Unlabeled UTP or Actinomycin D on ^{3}H-UMP Incorporation into VA-RNA in Isolated Nuclei.

Sixteen hours after infection with adenovirus, nuclei from 8 x 10^{7} HeLa cells were isolated and incubated *in vitro* in the presence of ^{3}H-UTP. After 7 minutes of synthesis, 20 µg/ml actinomycin or a 100-fold excess of unlabeled UTP were added to half of the incubation mixture. Labeling of VA-RNA was determined as described in Figure 9.


Total Incorporation
VA
^{3}H CPM x 10^{-4}
^{3}H CPM x 10^{-3}
unlabeled UTP
Actinomycin
Unlabeled UTP
Actinomycin
Time (min)
2
1
5
4
3
2
1
3
2
1
1.0
0.5
5
10
15
20

weight precursor molecule.

The incorporation of [^{3}H]-UMP into VA-RNA is dependent on the presence of unlabeled nucleoside triphosphates. The data presented in Table IX show no incorporation of [^{3}H]-UMP into VA-RNA in the absence of the three unlabeled triphosphates. Removal of only GTP from the complete system allows only 5 per cent of the amount of VA-RNA synthesized in the complete system. The labeling of 5S and pre-4S RNA also require the presence of all four nucleotide triphosphates.

It seemed possible that the VA-RNA molecules labeled in the *in vitro* reaction were produced by the cleavage of a higher molecular weight precursor, and this cleavage ceased after the addition of actinomycin. An experiment was performed in which a brief labeling period using radioactive UTP was followed by the addition of a large excess of unlabeled UTP. Conversion of a precursor to VA-RNA would be indicated by the entrance of radioactivity into the mature VA-RNA species during the chase period. The data shown in Figure 12 show that both total incorporation and labeling of VA-RNA cease promptly upon the addition of unlabeled uridine triphosphate. Thus there does not appear to be an appreciable amount of entry of labeled material into the VA-RNA when radioactive labeling has stopped but synthesis is allowed to proceed.

The only model consistent with the data presented above is one in which there is continued re-initiation of VA-RNA molecules, coupled with rapid RNA elongation, accurate termination and release of the finished product. The model predicts the presence of labeled 5' ends on the *in vitro* product. The products of alkaline hydrolysis of the VA-RNA synthesized *in vitro* were analyzed in order to test this model. Nuclei were incubated in the presence of the three unlabeled nucleoside triphosphates

TABLE IX

THE DEPENDENCE OF VA-RNA LABELING IN ISOLATED NUCLEI ON THE PRESENCE OF UNLABELED TRIPHOSPHATES

Sample	^{3}H-CMP CPM incorporated			
	Total	VA-RNA	5S	4S
complete system (^{3}H-CTP plus unlabeled ATP, GTP and UTP)	420,372	20,070	5850	6450
minus unlabeled ATP, GTP and CTP	27,246	171	0	0
minus GTP	58,274	540	69	360

Labeling of nuclei and determination of VA-RNA synthesis were performed as described in Figure 9. 100 µg/ml of ATP, GTP and UTP were present in the complete system.

and a ^{3}H-labeled triphosphate. Following labeling, the nuclei were removed and the VA-RNA was extracted from the incubation medium and separated on polyacrylamide gels. The VA-RNA peak was eluted from the gel, hydrolysed in 0.3 N NaOH and the products analyzed by high voltage electrophoresis on paper. Figure 13 shows the products obtained for each of the four radioactive nucleotide triphosphates. Cytosine and adenosine are present only as monophosphates, indicating that neither of these labeled bases is found in a terminal position on the VA-RNA synthesized *in vitro*. However, when UTP is the source of radioactivity, some tritium label migrates with the nucleoside, indicating that uridine is found on the 3' terminal end of the *in vitro* product. Uridine is also the 3' terminus of VA-RNA labeled *in vivo*.

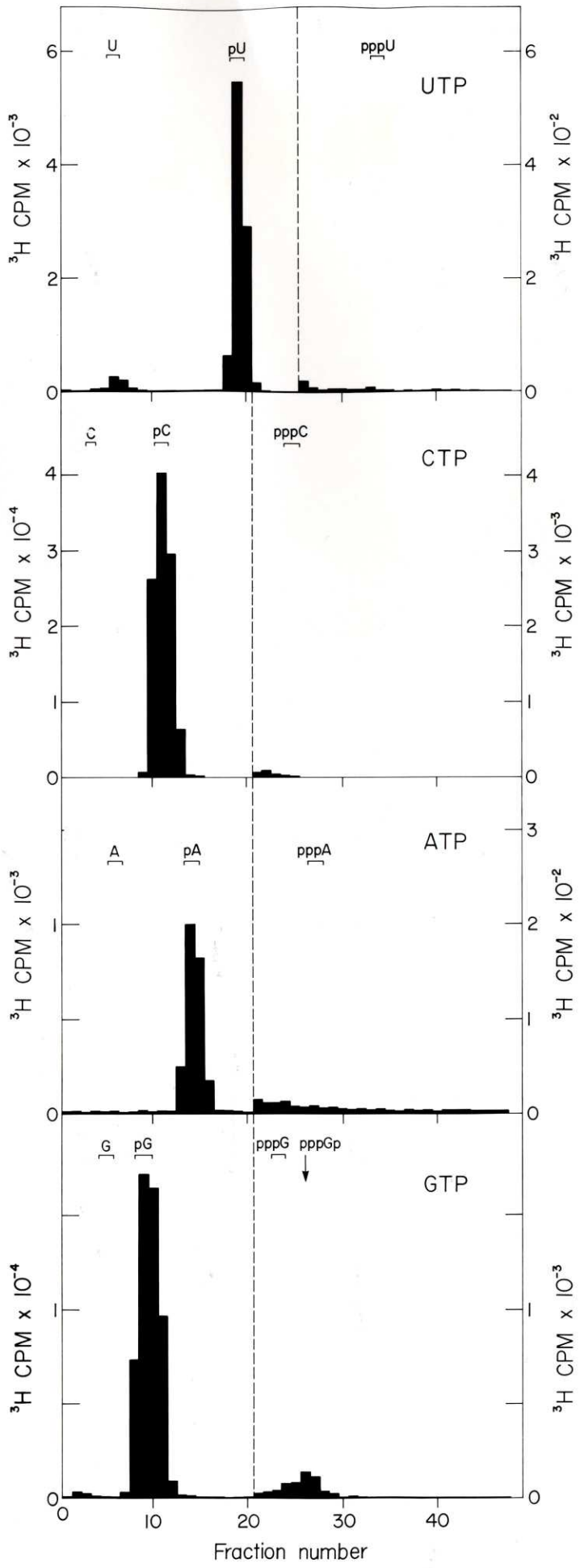
Approximately 1 per cent of the guanine label migrates slightly faster than GTP, the remainder appearing in the monophosphate. This pattern of migration suggests that it is the tetraphosphate, pppGp. The presence of this species in the hydrolysate could only occur if ^{3}H-labeled guanine is found on the 5' terminus of the *in vitro* product. The VA-RNA sequence of Ohe and Weissman [33] also shows a guanine residue in the 5' position. Thus it appears that a significant fraction of the VA-RNA made *in vitro* contains a labeled 5' end and must be initiated *de novo*.

Two determinations of the ratio of amount of Gp to presumptive pppGp gave values of 81 and 102. There are 54 G residues in the published sequence of VA-RNA. Thus it appears that at least one-half of the molecules are initiated *in vitro*. This is a lower limit, since any phosphatase activity in the crude preparations would reduce the yield of tetraphosphate.

Two determinations of the ratio of Up to U in the *in vitro* labeled VA-RNA gave values of 32 and 23. The published sequence contains 31 U residues. The approximate agreement between the predicted and measured values

Figure 13: The Products of Alkaline Hydrolysis of VA-RNA Labeled with ^{3}H-UTP, ATP, CTP and GTP In Vitro.

Nuclei were labeled and VA-RNA purified by polyacrylamide gel electrophoresis as described in Materials and Methods. After hydrolysis in 0.3 N NaOH, the products were separated by high voltage electrophoresis on paper as described in Materials and Methods. The relative migrations of the 4 samples are not comparable as electrophoresis was performed for different times.


UTP
CTP
ATP
GTP
pU
pppU
pC
pppC
pA
pppA
pG
pppG
pppGp
^{3}H CPM x 10^{-3}
^{3}H CPM x 10^{-2}
^{3}H CPM x 10^{-4}
Fraction number

indicates that the VA-RNA is labeled along its entire length, again supporting the hypothesis of extensive re-initiation.

The labeling reaction described here is distinguishable from the bulk of nuclear activity by its resistance to α-amanitine. In Figure 14 the electropherogram of nuclear RNA labeled in the presence and absence of α-amanitine shows that the labeling of VA-RNA is unaffected by the drug. The 5S and 4S RNA are somewhat difficult to observe, since they are small peaks partially obscured by heterogeneous background which is present when α-amanitine is omitted. A great deal of heterogeneous RNA is labeled in the absence of the drug and suppressed in the presence of the drug. Of course, most of the heterogeneous RNA labeled in the absence of α-amanitine is too large to enter a gel of this pore size. While labeling of the large heterogeneously-sedimenting RNA is almost completely suppressed in the presence of 0.1 μg/ml α-amanitine, concentrations of the drug as high as 3 μg/ml have no effect on the labeling of the three low molecular weight RNA species.

The ionic strength dependence of the amanitine-resistant activity is shown in Figure 15. Incorporation of [^{3}H]-UTP for a fixed labeling time during the period of linear incorporation is measured as a function of ammonium sulfate concentration. The activity synthesizing the VA-RNA is maximal at 75 to 100 mM ammonium sulfate and then falls dramatically. The decrease in incorporation of the α-amanitine-resistant activity at supra-optimal ionic strength is significantly greater than for the α-amanitine sensitive activity labeling the heterogeneous RNA, as shown in Figure 4. At lower ionic strengths, labeling of 5 and 4S RNA parallels that of virus-associated 5.5S RNA; at the higher ionic strengths, the inhibition of all incorporation is so drastic that the relatively small 5 and 4S peaks cannot

Figure 14: The Resistance of VA-RNA Synthesis to Inhibition by α-Amanitine.

Incubation, extraction of RNA and polyacrylamide gel electrophoresis were carried out as described in Figure 9.

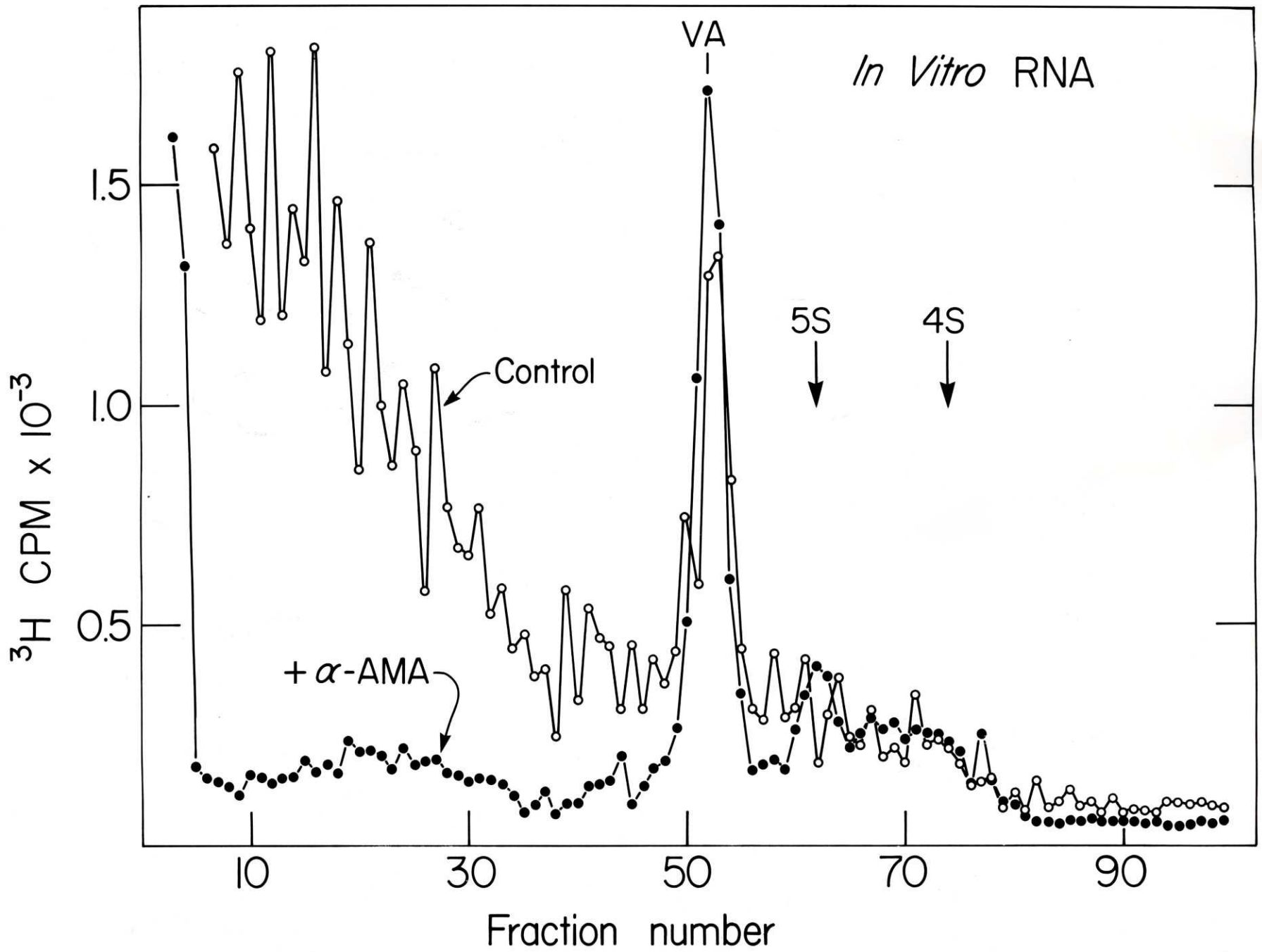
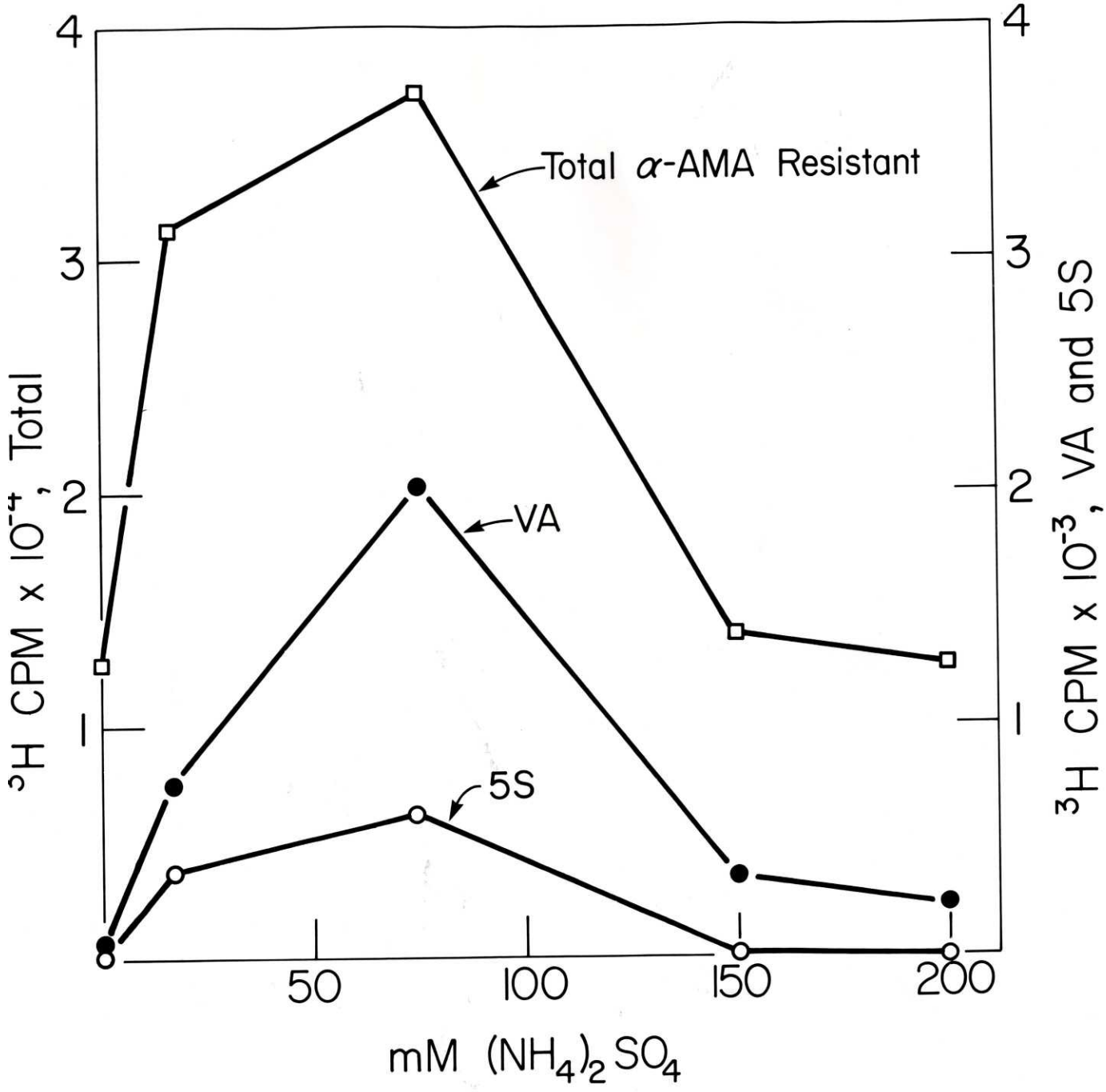

VA
In Vitro RNA
5S
4S
Control
+ α-AMA
1.5
1.0
0.5
^{3}H CPM x 10^{-3}
10
30
50
70
90
Fraction number

Figure 15: The Dependence of In Vitro Labeling of 5S, 4S and VA-RNA on Ionic Strength.

Labeling, RNA extraction and gel electrophoresis were performed as described in Figure 9. Incubation time was 10 minutes.


Total α-AMA Resistant
VA
5S
^{3}H CPM x 10^{-4}, Total
^{3}H CPM x 10^{-3}, VA and 5S
mM $(NH_4)_2SO_4$
1
2
3
4
50
100
150
200

be accurately measured.

The activity labeling the low molecular weight RNA species described here differs significantly from the major nuclear activities in its response to manganese ion. Figure 16 shows that increased manganese ion concentration severely inhibits total nuclear incorporation. However, in contrast, the labeling of the VA-RNA as well as 5S RNA increases initially with increased manganese concentration in the absence of any added magnesium. Still higher concentrations of manganese gradually reduce the amount of incorporation. Thus, also by this criterion, the synthetic systems responsible for the labeling of the large heterogeneous RNA and the low molecular weight RNA species are distinguishable.

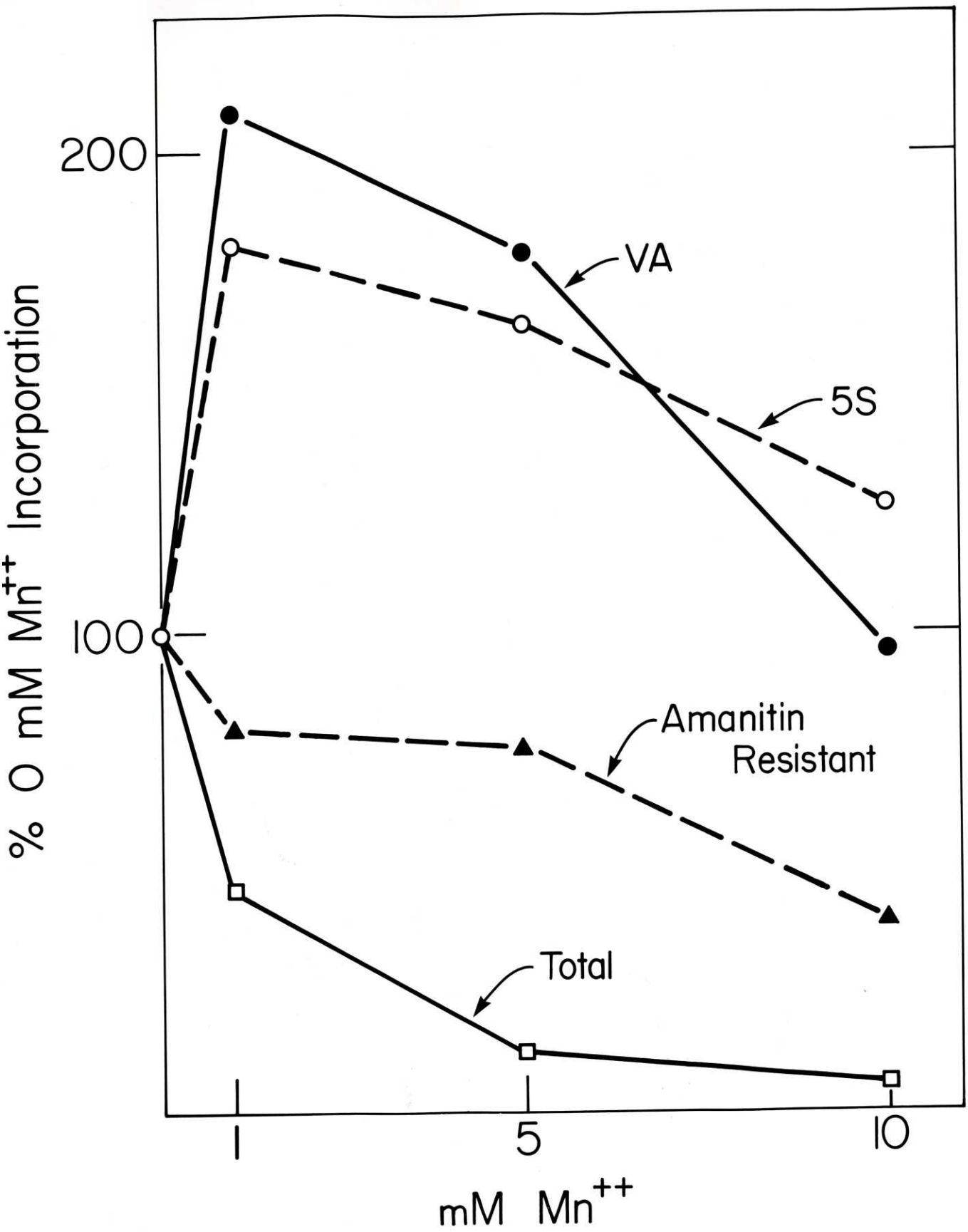
In vivo studies have shown that cordycepin (3'-deoxyadenosine) affects the various RNA synthetic systems of the mammalian cell in different ways [78]. While having no effect on HnRNA synthesis, at 20 µg/ml the drug suppresses the formation of 80 per cent of the mRNA. 45S nucleolar transcription is also severely inhibited, as is the formation of tRNA, while 5S RNA synthesis is relatively refractory to the drug. The effect of cordycepin triphosphate on the in vitro labeling of HnRNA and VA-RNA is shown in Figure 17. While the labeling of both species of RNA is affected by the drug, VA-RNA synthesis is considerably more sensitive to inhibition than is the labeling of HnRNA. A similar result is obtained when the effect of cordycepin on VA-RNA and HnRNA synthesis is examined in vivo. The results presented in Table X show that, while 20 µg/ml is sufficient to suppress nearly all of the VA-RNA labeling, HnRNA synthesis in the adenovirus-infected cell is only slightly affected.

Figure 16: The Effect of Mn^{++} on the In Vitro Labeling of 5S, VA and Total RNA.

Labeling, RNA extraction and gel electrophoresis were performed as described in Figure 9. Labeling time was 10 minutes.

Figure 17: The Effect of Cordycepin Triphosphate on the In Vitro Labeling of HnRNA and VA-RNA.

Nuclei were isolated from 1 x 10^7 cells 16 hours after infection and incubated in the presence of ^{3}H-UTP and three unlabeled triphosphates as described in Materials and Methods. The ATP concentration was 40 u M, and the ordinate is the ratio of the cordycepin triphosphate to ATP concentration. Incubation time was 10 minutes.


200
100
% O mM Mn++ Incorporation
VA
5S
Amanitin
Resistant
Total
1
5
10
mM Mn++

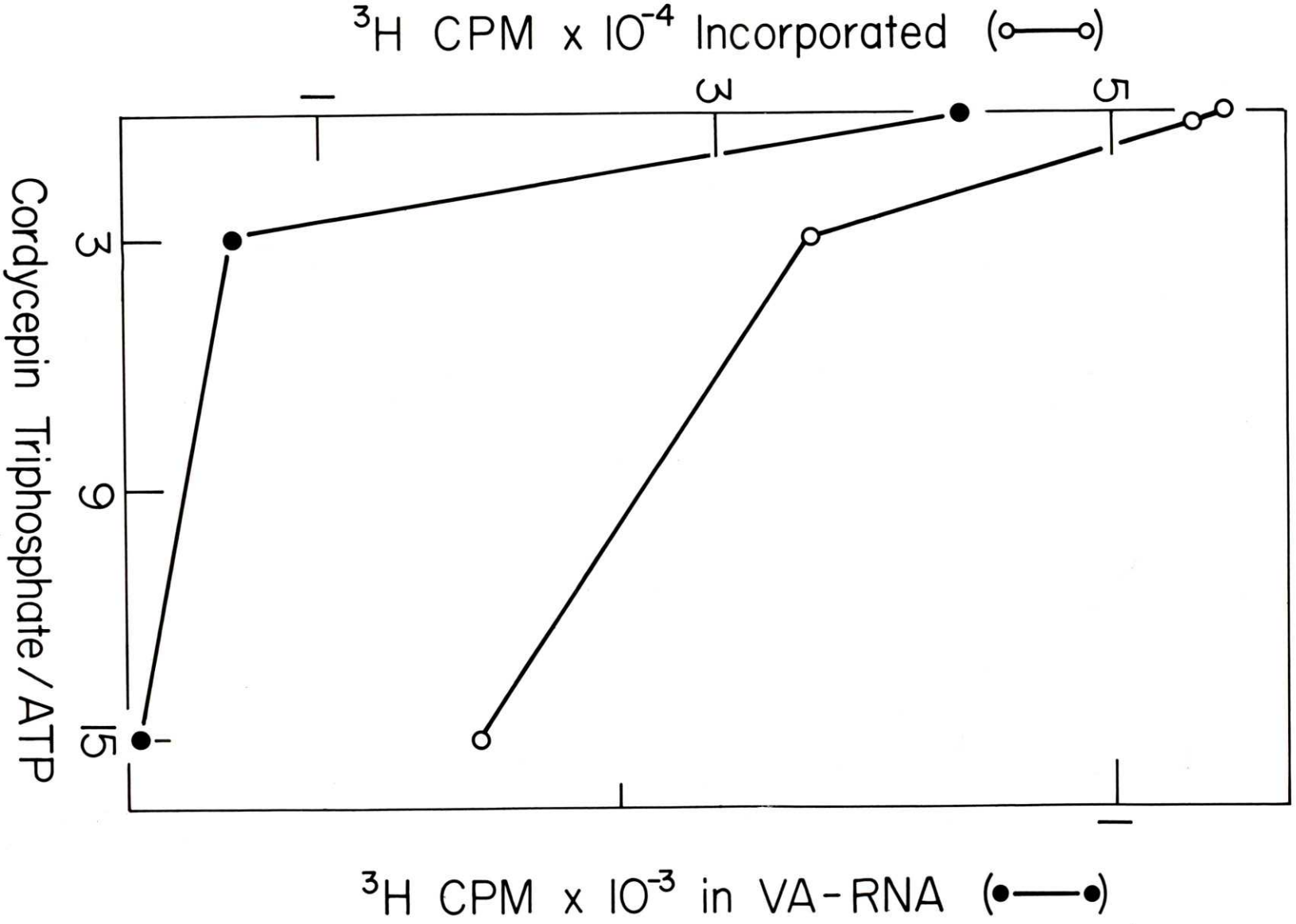

^{3}H CPM x 10^{-4} Incorporated (o—o)
3
5
Cordycepin Triphosphate/ATP
3
9
15
^{3}H CPM x 10^{-3} in VA-RNA (•—•)

TABLE X

THE EFFECT OF CORDYCEPIN ON THE IN VIVO SYNTHESIS OF VA-RNA AND HnRNA

RNA species	^{3}H CPM Incorporated Control	^{3}H incorporated +20μg/ml cordycepin	per cent inhibition
HnRNA	134,261	116,100	14
VA-RNA	12,576	2032	84

5×10^6 cells were incubated with 20 μc/ml ^{3}H-uridine for 20 minutes 16 hours after infection with adenovirus. RNA was purified from the nucleus and cytoplasm as described in Materials and Methods. VA-RNA production was determined as in Figure 2. Incorporation into HnRNA was quantitated by analysis of nuclear RNA by sedimentation through SDS-sucrose gradients as described in Figure 6.

DISCUSSION

The experimental approach used here is somewhat of a compromise between *in vivo* studies of RNA synthesis and investigations in which the properties of purified RNA polymerases are examined. The studies of purified RNA polymerases have allowed extensive characterization of the enzymes themselves. However, due to the apparent lack of template specificity of the enzymes *in vitro*, this work has yielded no information regarding the nature of the products synthesized by these enzymes in the cell. There are at least three possible explanations for this rather striking lack of template specificity displayed by these enzymes. It is quite possible that the ionic and macromolecular environment of the *in vitro* incubation is such that the enzymes are incapable of the sequence-specific recognition and interaction with DNA which they are capable of in the intact cell. Secondly, during the purification of the enzymes, specific protein factors may be lost which confer on the enzymes their ability to interact specifically with the DNA. Finally, the specificity of the enzymes may not, in fact, reside in the enzymes at all, but could be the result of the spatial and structural arrangement of the template.

Regardless of the reasons for the lack of template specificity displayed by purified RNA polymerases, it was reasoned that perhaps by studying the properties of the enzymatic activities residing in isolated nuclei, some progress could be made in attempting to identify the products of the RNA polymerases of mammalian cells. The work of Blatti, *et al.*, discussed above suggested that this approach might in fact be useful.

The results presented here suggest that the adenovirus genome is simultaneously transcribed by two distinct RNA polymerases during the productive infection of HeLa cells. The polymerase activity which synthesizes the large heterogeneous RNA containing adenovirus sequences both early and

late in the infectious cycle closely resembles the activity synthesizing cellular heterogeneous nuclear RNA both in the uninfected and infected cell. The polymerase activity synthesizing VA-RNA is distinct from this RNA polymerase in its ionic dependence and in its resistance to inhibition by α-amanitine. It resembles the activity synthesizing host cell 5S RNA and pre-4S RNA.

The conclusion that two distinct enzymes are responsible for the two synthetic activities described here must be made with some caution. Since these studies are performed on intact nuclei, the responses of enzymatic activities synthesizing the various species of RNA to certain drugs and changing ionic conditions is undoubtedly complex. It is conceivable, for example, that both the adenovirus HnRNA and the VA-RNA are synthesized by the same enzyme, but when synthesizing the small molecule the enzyme is somehow completely protected from α-amanitine. Such a possibility seems unlikely.

The behavior of the polymerase activity synthesizing host cell heterogeneous nuclear RNA, both in the uninfected and infected cell, resembles that of the enzyme responsible for transcribing large nuclear RNA containing adenovirus-specific sequences by all criteria applied in this study. Both activities are sensitive to low concentrations of α-amanitine, are greatly stimulated by increasing ionic strength, and are inhibited by Mn++. These results suggest that both cellular and adenovirus-specific HnRNA are transcribed by RNA polymerase II. However, while the activities described above are severely inhibited by Mn++, purified RNA polymerase II prefers Mn++ to Mg++. The reason for this difference is unknown.

The results presented above also lead to the tentative conclusion that the same enzyme is responsible for synthesizing the bulk of the adenovirus-

specific HnRNA both early and late in the infectious cycle. However, competitive and saturation hybridization experiments and sedimentation analyses suggest that the virus-specific sequences found in the cytoplasm early in infection differ from the late cytoplasmic viral sequences [18,19,26]. There are a number of possible explanations. It is quite possible that either the template or the polymerase undergoes some modification in the course of the infection. For example, perhaps either early or late RNA is made off of integrated viral DNA, and that as a result of integration the sequences available for transcription by polymerase II somehow differ from those available when the genome is free in the nucleoplasm. Another explanation consistent with this tentative conclusion is that the virus induces a structural or subunit change in the polymerase during infection, and that this alteration results in no change in the enzymes' response to either α-amanitine or changes in ionic conditions.

A third possibility is that the primary viral transcription products early and late in infection are identical, but that early in infection the majority of this RNA is degraded rapidly, while late in infection most of the transcribed sequences ultimately appear in the cytoplasm as messenger RNA. A precedent for such a possibility exists. Attardi and co-workers have recently reported that both strands of the mitochondrial genome are entirely transcribed, but most of one strand is rapidly degraded [86]. Studies of the fate of adenovirus RNA during pulse-chase experiments with actinomycin also support this suggestion. Preliminary evidence suggests that, while early in infection most of the viral RNA labeled during a short pulse is rapidly degraded, late in infection processing appears to be conservative [Price, unpublished observations].

In the experiments described here, the labeling of 4S and 5S RNA

closely parallels the labeling of the VA-RNA. Results of experiments performed on nuclei from uninfected cells show that the labeling of 5S and 4S RNA behaves similarly [75]. Thus it appears that the polymerase activity which labels VA-RNA *in vitro* resembles the activity which forms the cellular 5S and 4S RNA species. This conclusion is borne out by a number of drug studies performed *in vivo* which indicate that the response of the VA-RNA synthesizing system closely resembles the response of the 5S and 4S synthetic systems and is quite different from the response of the activity which forms high molecular weight nuclear RNA.

The extensive re-initiation of synthesis of VA-RNA *in vitro* poses the problem of organization of the synthetic system. At present it is not known whether re-initiation is due to sequential transcription of tandem genes or to repeated transcription of the same gene. Ohe has recently reported that the adenovirus 2 DNA contains only one copy of the VA-DNA sequence, supporting the latter interpretation.

The evidence presented above is insufficient to allow correlation of the synthetic activity labeling 5S, pre-4S and VA-RNA in isolated nuclei with an enzyme known to be located in the nucleus of the mammalian cell. It is, however, possible to draw several tentative conclusions regarding the identity of this enzyme. The evidence arguing against its being polymerase II has been outlined above. The behavior of RNA polymerase I, the nucleolar enzyme, has been studied both in isolated form and in whole nucleoli. While polymerization catalyzed by this enzyme resembles the activity synthesizing 5S, 4S, and VA-RNA in being insensitive to inhibition by α-amanitine, its responses to Mn++ and ammonium sulfate differ markedly from the activity in question. Whereas the nucleolar enzyme is strongly inhibited by Mn++, and relatively insensitive to ammonium sulfate, the ac-

tivity synthesizing the small RNA species is rather insensitive to Mn++ and greatly stimulated by ammonium sulfate. As it could be argued that these differences are due solely to effects of the ions on the organization of the rather different templates and not on the enzyme itself, it is not possible to rule out polymerase I as being responsible for the synthesis of these three small RNA species. However, the fact that polymerase I appears to be localized in the nucleolus, while the synthesis of 4S, 5S, and VA-RNA is thought to occur in the nucleoplasm,argues against the possible involvement of polymerase I in the synthesis of these small RNA molecules.

Polymerase III is the only other RNA polymerase that has been detected in association with the nuclei of mammalian cells. However, the nuclear location of the enzyme has been questioned by several groups. The uncertainty of the nuclear location of polymerase III is based on the fact that it cannot be detected in the nuclei of several tissues and is greatly enriched in mitochondrial preparations [65,66]. These experiments suggest a distinct activity associated with the nucleus responsible for the synthesis of 5S and pre-4S RNA in the uninfected cells and which is recruited by the adenovirus for the synthesis of VA-RNA during infection. The possibility that it is polymerase III is an attractive one.

It is, of course, quite possible that there are numerous other RNA polymerase species associated with the nucleus of the mammalian cell whose existence, due to instability during extraction procedures, requirement for membrane association or for other reasons, has not been detected, and that one of these enzymes is responsible for the synthetic activity in question.

While preliminary experiments have been discouraging, the potential usefulness of the adenovirus DNA and HeLa cell RNA polymerases as a model system for studying the specific interaction of RNA polymerases with DNA

is attractive. If the conclusions of this work are correct, it is possible that one has a rather small piece of DNA which contains at least two distinct promoter sites which can be specifically recognized by two different RNA polymerase species.

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