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PROCESSING AND TRANSPORT OF IgG_{κ} mRNA IN ISOLATED MYELOMA NUCLEI

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ABSTRACT

PROCESSING AND TRANSPORT OF IgG_K mRNA IN
ISOLATED MYELOMA NUCLEI

By

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In vitro requirements for the translocation of RNA from isolated nuclei of MOPC-21 (IgG_K) murine myeloma cells is studied in a transport system that supports synthesis and processing of RNA. Intact cells are labeled and the transport of prelabeled RNA from isolated nuclei is monitored. Nuclei are purified by the most stringent procedures to avoid cytoplasmic contamination and nuclear damage. The dependence of RNA transport on cytosol, spermidine, rNTPs and energy is evaluated. The energy-dependence of transport of RNA from isolated nuclei is determined by studying the effect of inhibitors of cellular NTPases.

We have determined the Poly (A) content and size of intranuclear and transported RNA isolated from nuclei incubated under different rNTP conditions; from this study we determine that the differences in the RNA transported are only quantitative.

Comparison of cytoplasmic RNA isolated from cells prelabeled for 35, 45 and 60 minutes with the RNA transported from isolated nuclei incubated *in vitro* a further 30 minutes demonstrates that processing of RNA occurs in the isolated nuclei and that non-specific leakage of RNA does not occur. This point is further established by hybridization

studies. Intranuclear and transported RNA are hybridized to a κ light chain cDNA probe. Both the mature and the precursor molecules of the transcript are found intranuclearly; however, only the mature 13-14S mRNA is transported from the nucleus. The absence of hybridization of <13S species proves that degradation of this RNA does not occur in this system.

DEDICATION

To my parents, who nurtured and stimulated my desire for learning, and to Miguel and Esther, whose support and self-denial made this project possible.

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INTRODUCTION

Eukaryotic messenger RNAs undergo extensive post-transcriptional modifications. All messengers studied to date have some or all of the following modifications: internal and 5'terminus methylation, polyadenylation at the 3'terminus, binding of specific proteins, and splicing of specific internal sequences (intervening sequences, IVS). The prevalence of these modifications through evolution indicates their importance in the modulation of gene expression. Little is known about the role of these post-transcriptional modifications. The 5'cap structure seems to be involved in the recognition and/or stabilization of the 40S-mRNA initiation complex of the translational machinery. Polyadenylation is probably involved in the transport of RNA to the cytoplasm and/or stabilization of cytoplasmic mRNA. The role of the internal methylations is totally unknown. The presence of intervening sequences in DNA may be required for the termination of transcription. In newly synthesized RNA IVS are putatively required for normal processing and stabilization of transcripts in the nucleus.

Evidence of confinement of specific mRNAs to the nucleus of some cells indicates that this may be another mechanism involved in the regulation of gene expression, and may be due to nucleocytoplasmic interactions.

Different systems using isolated nuclei have been devised to study the requirements for regulated translocation of RNA from the nucleus under conditions that would mimic *in vivo* occurrences. The cell systems and the conditions of each system differ widely, and it is difficult to draw firm conclusions from the evidence available. It is apparent that nuclei isolated from normal and tumor cells have different requirements for a source of exogenous energy for transport of RNA and for the maintenance of nuclear stability. Systems that employ conditions which allow RNA synthesis in the isolated nuclei transport mRNA-sized messengers.

In this dissertation we study the processing and translocation of RNA and the specific messenger for the light chain of the immunoglobulin molecule ($mRNA_K$) from isolated nuclei of murine myeloma cells MOPC-21, under conditions that permit *in vitro* RNA synthesis and processing.

LITERATURE REVIEW

Nuclear Structure and Its Relationship to RNA Biogenesis

The eukaryotic nucleus is surrounded by an envelope consisting of an inner and an outer membrane, its intracisternal space being in direct continuity with the endoplasmic reticulum cisterna. The outer membrane is studded with ribosomes; the inner membrane is found under electron microscopy examination contiguous to heterochromatin and fibrous layers. Electron microscopic studies reveal the octagonal nuclear pore complexes regularly spaced throughout the nuclear envelope. The nuclear pore complex consists of eight globular or tubular subunits surrounding a central dense disk that penetrates the nucleoplasm and cytoplasm, passing through both nuclear membranes. The number of nuclear pore complexes is raised in metabolically active nuclei and is reduced in near-dormant nuclei as those of mature avian erythrocytes (reviewed by Harris, 1978).

In synchronized HeLa cells the number of pore complexes is highest within the first four hours after cell division and is not affected by the inhibition of protein synthesis during this period, indicating that the nuclear pore complex is assembled from preexisting macromolecules. Structures greatly resembling nuclear pore complexes are detected attached to chromosomes during cell division (Maul, 1977). Evidence from different groups indicates that during cell division the chromosomes carry fragments of the nuclear envelope which will form

at least part of the envelope of the daughter cell nuclei (Harris, 1978).

Deoxyribonuclease and high salt treatment of nuclei removes the chromatin and nucleoplasm, but the nucleus preserves its shape and shows a sponge-like network known as the nuclear matrix or skeleton (Riley and Keller, 1978; Harris, 1978). This network is connected to the nucleoli, the inner membrane and the nuclear pore complexes. Under DNase treatment the nuclei retain almost 100% of the protein and heterogeneous nuclear ribonucleic acid (HnRNA), provided a proteolytic inhibitor is added. This indicates the important role of protein-ribonucleic acid interactions in the nuclei. HnRNA added to DNase-treated nuclei does not fortuitously bind or pellet with nuclei (Miller et al., 1978; Herman et al., 1978).

The nuclear pore complex and the nuclear matrix are resistant to detergent treatment that dissolves more than 98% of the nuclear envelope; in these nuclei RNA and DNA are totally restricted to the nuclei. Ten percent of the protein is lost and is shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to be almost exclusively of nuclear envelope origin (Aaronson and Blobel, 1974).

The current belief is that the nuclear matrix is the structure primarily responsible for the maintenance of nuclear integrity and that most, if not all, nuclear components are somehow attached to it. The role of the nuclear envelope and more specifically of the nuclear pore complex is ascribed to the regulation of nucleocytoplasmic exchanges (Harris, 1978; Clawson et al., 1979; Agutter and McCaldin, 1979; Hamer and Leder, 1979).

Post-Transcriptional Regulation of Gene Expression

The full understanding of gene transcription and expression is extremely complex and far from being totally comprehended. It is known that there exist regulatory mechanisms at the levels of DNA recombination, transcription, post-transcription, translation and post-translation of genetic material.

The phenomenon of post-transcriptional processing of tRNA, rRNA and mRNA both in prokaryotes and eukaryotes has been known for many years. RNAs are transcribed as larger precursor molecules which undergo base and/or ribose methylations, and cleavage and/or modification at both 5' and 3' termini. Very recently it has been discovered that many RNAs also undergo internal cleavages or splicing. Among the many extensive reviews of these processes are those of Lewin (1974), Perry (1976), Revel and Groner (1978) and Abelson (1979), to which we will refer repeatedly in this review.

i. Post-transcriptional processing of tRNA

tRNA species are transcribed as larger molecules which undergo methylation and other very specific base modifications and are cleaved and/or modified at both ends of the molecule. The nature of these modifications is tRNA species specific. Recently it was reported that a short intervening sequence within the molecule is removed in some species of yeast tRNA (Goodman et al., 1977) but does not exist in others (Abelson, 1979; Wolfe, 1979), and that there exist temperature-sensitive mutants deficient in this splicing process. Using this tRNA as substrate, Abelson's group has partially purified a specific endonuclease and ligase which make and rejoin a 3'-phosphate and 5'-hydroxy group (Knapp et al., 1979). Precursor yeast tRNA is

correctly spliced and translated in *Xenopus laevis* oocytes, indicating the evolutionary preservation of this cellular mechanism (De Robertis and Olson, 1979; Abelson, 1979).

ii. Post-transcriptional processing of rRNA

rRNA is transcribed as one large 30S or 45S precursor molecule in prokaryotes and eukaryotes, respectively. This molecule contains the sequences of the mature ribosomal species separated by large spacers that are excised in a series of specific steps after previous methylation of the molecule. Intervening sequences are reported for *Drosophila melanogaster,* yeast mitochondrial and *Chlamydomonas reinhardii* rRNA (reviewed by Abelson, 1979).

iii. Post-transcriptional processing of mRNA

Processing of mRNA in prokaryotes is not very well known. Some bacterial mRNAs are polyadenylated at their 3' end (Edmonds and Kopp, 1970), and T7 early mRNA is transcribed as a large molcule which is cleaved into five mature mRNAs (Lewin, 1974). In contrast, post-transcriptional processing has been thoroughly investigated in eukaryotes.

In 1966 Penman reported that short-pulse labelled nuclear RNA showed a very heterodisperse profile, peaking around 45S (molecules much larger than polyribosomal mRNA), and that the profiles shifted to smaller species with longer labelling periods. Only about 10-30% of these nuclear sequences reach the cytoplasm. The nature of this HnRNA has been the focus of controversy for many years. Many authors proposed it as the precursor molecule to mRNA, but others contended it simply represented aggregates of RNA molecules formed during the isolation procedure (Lewin, 1975a,b).

Goldberg's group report that the UV-target size of Adenovirus-2 mRNA is approximately three times larger than that of the mRNA itself, indicating its processing from a molecule that many times larger (Goldberg et al., 1976).

Today, making use of RNA and DNA sequencing methods, and totally denaturing conditions in electron microscopy and hydroxymethyl mercury agarose gels, paired to recombinant DNA and hybridization technology, many authors have demonstrated large nuclear precursors for a variety of mRNAs, such as ovalbumin, immunoglobulin, globin, Adenovirus and SV-40 (reviewed by Abelson, 1979).

Most mRNAs are methylated internally and at the 5'terminus (Perry and Kelley, 1974); the latter is termed the "cap" structure (Rottman et al., 1974). Both modifications are also found in the HnRNA molecule, and the kinetic studies of Perry's group indicate that the HnRNA cap and most of the internal methylations are conserved during processing (Perry, 1976; Perry and Kelley, 1976). There are functional messengers which lack the cap structure, such as picornavirus, or the internal methylations, such as globin mRNA (reviewed by Shatkin, 1976; Rottman, 1976). These findings and others in which the extent of inhibition of *in vitro* translation of capped mRNAs by cap analogs ranges from none to total indicate this structure's function and requirement may differ with each system. The cap may play an important role in stabilizing the first mRNA-40S subunit complex of the translation machinery (Rottman, 1976; Kaempfer et al., 1978; Revel and Groner, 1978).

Confirmed reports of capped but non-polyadenylated HnRNA molecules led researchers to affirm that either capping occurs first, which would not be very surprising, as transcription starts at that same end

of the molecule, or that it was at the 5' end where the coding sequences lay in the precursor molecule (Perry and Kelley, 1974; Perry, 1976). With the discovery of splicing, this point of contention has been settled.

A polyadenylate tract of 180 to 200 bases that is added sequentially by two different polymerases is found at the 3'terminus of many HnRNA molecules (Edmonds et al., 1971; Lewin, 1974). The same length poly A tract is found in many cytoplasmic and polyribosomal mRNA molecules. Kinetic and pulse-chase studies by some groups indicate that the poly A tract is conserved from HnRNA to mRNA (Darnell et al., 1971). Other reports, though, indicate that nuclear poly A tracts are not quantitatively converted to cytoplasmic poly A tracts (Perry et al., 1974).

The role of this poly A modification of the molecules seems to be involved in the transport of mRNA to the cytoplasm, as studies with cordycepin, an inhibitor of polyadenylate polymerase, indicate. This drug inhibits polyadenylation but not synthesis of HnRNA and drastically reduces the appearance of mature mRNA in polyribosomes (Adesnik et al., 1972; Abelson and Penman, 1972). But the existence of non-polyadenylated messenger molecules such as histones (Sonensheim et al., 1976; Adesnik et al., 1972) indicates the necessity of at least another mechanism of nucleocytoplasmic transport.

The shortening of the poly A tract with age has been widely reported (Greenberg, 1975; Perry, 1976). The group of Huez contends that this shortening reduces the translation efficiency and stability of globin mRNA injected into *Xenopus laevis* oocytes (Marbaix et al., 1975), while readdition of the poly A tail restores both functions (Huez et al., 1978). Other reports show that this shortening does not

affect either function of the mRNA of globin or HeLa and L cells when studied *in vitro* in the homologous system (Lewin, 1975a; Revel and Groner, 1978).

Heywood has proposed that the base pairing of short oligo(U) RNAs, termed translational control RNA (tcRNA), with the poly A tract inhibits the translation of mRNAs, thus modulating gene expression of mature and functional messengers (Bester et al., 1975).

It is amply documented that the poly A tract of polyribosomal mRNA has a 78,000 MW protein associated with it (Blobel, 1973) and that a protein of the same molecular weight is bound to the poly A tail of HnRNA and free cytoplasmic mRNA. Its role could be in the nucleocytoplasmic transport and/or preservation of the polyadenylate tract in the cytoplasm.

HnRNA, polysomal and free cytoplasmic mRNA are associated with other specific proteins, different for each of the three RNA stages (Perry and Kelley, 1968; Spirin, 1969; Blobel, 1970; Olsnes, 1970; Blobel, 1973; Pederson, 1974; Lewin, 1974; Liautard et al., 1976). Although their function is still unknown, the proposed role is as recognition and/or modification signals at different stages of mRNA biogenesis. The change of proteins from HnRNP to mRNP is proposed to occur just before or at the time of release of mRNA from the nucleus, and that this new set of protein signals the maturity of the mRNA and/or has an active role in its transport to the cytoplasm. The different proteins in polysomal and free cytoplasmic RNA might indicate when a messenger should be translated, or give it protection while it is free in the cytoplasm (reviewed by Revel and Groner, 1978).

In 1977 many groups, such as those of Chow and Klessig (Chow et al., 1977; Klessig, 1977), reported that Adenovirus-2 mRNA was not absolutely colinear with its DNA but that close to its 5' end a large segment was missing as if it had been looped out during transcription. Reports of this phenomenon appeared in quick succession for other systems, such as SV-40 (Aloni et al., 1977), ovalbumin (Lai et al., 1978; Breathnach et al., 1977), globin (Jeffrey and Flavell, 1977) and immunoglobulin (Brack and Tonegawa, 1977; Tonegawa et al., 1978). Using recombinant DNA technology with different hybridization techniques, it was soon discovered that the splicing out of sequences was mostly a post-transcriptional event, which could entail up to seven excision events, as is the case in ovalbumin transcripts (Dugaiczyk et al., 1978; Mandel et al., 1978).

HnRNA is effectively a precursor molecule at least for some mRNAs; and large sequences, specific both in number and location, and most within the coding sequences, are removed. These are called intervening sequences (IVS) or introns and the conserved coding sequences exons.

In the case of the immunoglobulin gene Tonegawa's group (Hozumi and Tonegawa, 1976; Brack and Tonegawa, 1977) and others have demonstrated that there also exist recombination events at the DNA level. The constant and variable regions of the molecule are more widely separated in embryonic than in adult cells.

Sequencing studies of the IVS have shown that, although their length within a mRNA species is very constant, the sequence itself has diverged widely, except for the flanking sequences at the junction of intron and exon. All junctions, except for tRNAs, follow Chambon's rule of -(GT.....AG)- base composition as the flanking sequences.

This seems to indicate the requirement for a given secondary structure of the IVS and a precise sequence at the site of splicing. As already mentioned, yeast tRNA is spliced correctly in frog oocytes. The preservation of this machinery through evolution indicates the importance of this mechanism in gene expression.

Khoury's group, studying the inability of SV-40 to transform undifferentiated F-9 murine teratocarcinoma cells, have found that the cells support normal viral RNA transcription but not the splicing of the precursor molecule (Segal et al., 1979). F-9 cells can be induced to differentiate by treatment with retinoic acid, whereupon spliced RNA is detected and cell transformation occurs (Segal and Khoury, 1979). This clearly indicated the cell has all the necessary information for splicing, but it is somehow suppressed in the undifferentiated state.

This same group has constructed a whole genome cDNA in which the DNA for the early 16S mRNA does not contain the intervening sequence. By extensive sequencing and restriction analysis, they could determine to have a faithful copy of the remainder of the genome. After transcription and translation of this cDNA, they cannot detect mature 16S mRNA or the protein it codes, VPI. In this preliminary report they conclude that the IVS is required for the transcription and/or expression of this messenger (Gruss et al., 1979).

A similar conclusion is obtained using mouse globin-SV-40 hybrid virus for the study of regulation of transcription. Leder's group has constructed hybrids with none or one or more splice junctions, either from the homologous SV-40 or the heterologous globin sequences. Stable transcripts are only detected when at least one splice junction from either species is present in the hybrid genome. All the recombinant

genomes retain the SV-40 late promoter. *In vitro* elongation of *in vivo* initiated transcripts yields the hybrid transcripts. The authors conclude that the IVS are not required for transcription, but for stability of nuclear RNA species. They offer a model by which unspliced precursors are processed at the nuclear envelope and transferred to the cytoplasm; unspliced molecules are rapidly degraded in the nucleus (Hamer and Leder, 1979).

In Adenovirus-2 infected HeLa cells, 16 hours after infection, only viral-specific mRNA sequences reach the cytoplasm. Utilizing hybridization techniques, Beltz and Flint (Beltz and Flint, 1979) have found that the transcription levels, complexity and polyadenylation of host HnRNA are the same as in mock-infected cells. This narrows down the regulation of nucleocytoplasmic translocation in this system to a defect in capping, splicing or another as yet unknown mechanism. In the sea urchin a subset of mRNA sequences found in the polyribosomes in the blastulae is present in the nucleus but not in the cytoplasm in adult tissue (Wold et al., 1978).

iv. Nucleocytoplasmic translocation of RNA: *in vitro* studies

The role of transport of nuclear RNA to the cytoplasm as a modulator of gene expression has been suspected for many years, but unequivocal evidence supporting this role is not available. Schneider first studied this problem in isolated rat liver nuclei that had been labelled *in vivo*. He found transport to be temperature-dependent, stimulated by citrate, ATP and AMP, but not by adenosine, and inhibited by high Mg^{2+} concentrations. He postulated the stimulatory effect of ATP was due to its chelating effect of Mg^{2+} ions and suggested the RNA was released as a ribonucleoprotein (RNP) particle (Schneider, 1959).

From this time on the controversy over whether ATP is or is not required for transport, and whether its effect is as a chelator, an energy-giving system or as a recognizable structure, has not been settled.

Chatterjee and Weissbach (1973) in a broader study agree with Schneider's conclusions. The four ribonucleoside triphosphates (rNTP), the two methylene derivatives of ATP, pyrophosphate and EDTA (known cation chelators) stimulate transport, while inorganic phosphate and AMP do not. In their system 15mM Mg^{2+} totally inhibits transport.

In the HeLa-Adenovirus-2 system (Raskas et al., 1973), the two methylene derivatives of ATP stimulate transport, but the chelators pyrophosphate and EDTA do not. Maximum transport is achieved at 6mM ATP or 1mM ATP plus phosphoenolpyruvate (PEP) and phophocreatine kinase (PCK). The authors propose that ATP plays a role similar to that in muscle cells, where the hydrolysis of ATP is not what drives the reaction but that ATP binds to and relaxes or dissociates some nuclear structure responsible for retaining RNA in the nucleus. This structure may require the hydrolysis of ATP to regain its functional structure after the translocation event.

In regenerating rat liver nuclei the role of ATP is as an energy generator (Yu et al., 1972). GTP and α-β methylene ATP are just as effective, but β-γ methylene ATP is not. Transport is increased by 77% with 2.0mM ATP, 2.5mM PEP and 6.4 units of PCK. At 36°C release plateaus after five to ten minutes of incubation but keeps increasing linearly when the energy source is added at five minute intervals. At these levels of ATP and energy source the nuclei lyse or clump; the authors find this is inhibited by the addition of 5mM spermidine and a high speed supernatant fraction of the cytoplasm called cytosol

(we will further refer to these two additions to the system later on).

In the mouse brain system 1.0mM ATP gives optimum release; nuclear lysis does not occur without further additions (Weck and Johnson, 1978).

Racevskis and Webb report that ATP is required for the *in vitro* processing of the 45S rRNA precursor in rat liver nuclei. Working with this same system, Ishikawa's group (Ishikawa et al., 1969) find the same ATP requirement for a 20% release of RNA at 20°C or 30% at 36°C, but they do not look at the state of the nuclei after the incubation. We may infer they did find lysis, as they have now modified their assay system to contain cytosol and spermidine (Ishikawa et al., 1978). In the new system they find the non-hydrolyzable ATP analogs and AMP+PP to be equally effective; GTP is about two-thirds, UTP very slightly, and CTP, EDTA and AMP are not effective in RNA release. This again favors a conformational function of ATP similar to that proposed by Raskas.

RNA release in isolated myeloma nuclei is ATP independent, and at a concentration of 4mM ATP nuclear lysis occurs, as demonstrated by microscopic examination and release of labelled DNA (Stuart et al., 1975, 1977).

Smuckler's group, having observed that in rat tumor cells RNAs normally restricted to the nucleus are found in the cytoplasm, conducted the following experiment. They induced tumors in rats by feeding them the carcinogens dimethyl-amino-benzene and acetylaminofluorene, then labelled the cells *in vivo* and studied the release of RNA *in vitro* under the standard transport conditions. They found that nuclear RNAs previously restricted to the nucleus were transported now, and that this release was ATP-independent (Smuckler and Koplitz,

1973). The change could be ascribed to a property of tumor cells and not some other action of the carcinogens, as the structural analog aminobenzene did not induce this change in nuclear behavior. In a recent report this same group reports that, although the essence of their previous work holds true, ATP does increase further the already elevated release of RNA from isolated nuclei of carcinogen-treated rats (Clawson et al., 1980).

This observation and that in myeloma nuclei hinted that normal and tumor cells might have different *in vitro* requirements for the release of RNA which might indicate *in vivo* differences.

Schumm and Webb (1975) studied the different ATP requirements of rat liver, rat hepatoma 513D and rat Novikoff hepatoma for the transport of RNA in isolated nuclei. They found that, while normal rat liver nuclear RNA transport was totally ATP-dependent, Novikoff hepatoma was totally independent and 513D hepatoma was 25% dependent. We must bear in mind that the ATP concentration of tumor cells has been reported to be two times higher than in normal cells (Smuckler and Koplitz, 1972). We have indirect evidence of a high ATP concentration in MOPC-21 myeloma nuclei. These nuclei can synthesize *in vitro* 20% of control RNA in the absence of ATP, but synthesis is zero when any of the other three rNTPs is omitted (W. Eschenfeldt, personal communication).

Since ATP is required for transport of mRNA-sized species at least in normal cells, many groups in the field are studying its mode of action and are interested in its possible interaction with a nuclear envelope NTPase. This nuclear Mg^{2+}-dependent NTPase has been reported both in the proximity of the nuclear pore complex and throughout the nuclear envelope. This discrepancy is probably due to technical

problems with the lead based histochemical assay but is now generally accepted to be at the nuclear pore (Harris, 1978). Schumm and Webb (1975) report that beryllium nitrate, which inhibits a nuclear pore phosphatase, also inhibits the transport of RNA in isolated nuclei of normal rat liver, 513D hepatoma and Novikoff hepatoma cells to the same extent that ATP is required in each system. Agutter et al. (1976), using isolated nuclear envelopes as their source of the NTPase, find that oligomycin (an inhibitor of Mg^{2+}-dependent ATPase), quercetin (an inhibitor of hexose transport) and ATPγS inhibit the enzymatic activity to the same extent as they inhibit release of RNA in isolated nuclei. The authors do not explain how they solubilize quercetin, a highly insoluble compound soluble only in ethanol or at pH 13 but that is only active at a pH range of 7 to 8 (Salter et al., 1978). In a more recent publication this group finds that the thiol blocking reagents p-chloromercuribenzenesulfonate, p-hydroxymercuribenzoate, phenyl arsenoxide, Atoxyl and Melarsen also inhibit the enzymatic activity. Some of these compounds are only soluble in ethanol, and the final ethanol concentration in the transport assay is of 7%, which the authors contend has no effect on their assay.

Colchicine at the very high concentration of 4mM inhibits 50% of NTPase activity (Agutter et al., 1979). This compound has been reported to inhibit RNA transport of rat liver nuclei (Schumm and Webb, 1974). Colchicine is an inhibitor of microtubule assembly but has also been reported as a nucleoside transport inhibitor in HeLa cells.

Schuppe et al. (1974) find that 5mM spermidine, the level that Webb's and Ishikawa's groups used to inhibit nuclear lysis, absolutely inhibits Mg^{2+}-dependent nuclear ATPase activity in rat liver nuclei.

The other major point of contention concerning *in vitro* RNA release systems is that over the requirement for spermidine and cytosol. Cytosol is a post-microsomal high speed supernatant which needs to be dialyzed against water or low salt buffer to prevent it from clumping nuclei (Yu et al., 1972). The reason for this effect has not been investigated, to our knowledge, but by removing the microsomes the cytosol may become enriched in dialyzable peptides, ions, or other factors responsible for the clumping.

As already mentioned, Webb's group found that in their system the nuclei lysed and this lysis could be inhibited by cytosol and 5mM spermidine. The protein concentration in their cytosol fraction is of up to 36 $mg \cdot ml^{-1}$, which is totally non-physiologic compared to the number of nuclei. In their study, they find that cytosol also has a stimulatory effect on RNA release from normal rat liver nuclei. This same effect is seen in Novikoff hepatoma nuclei, although in these it is not required for the maintenance of nuclear integrity (Schumm and Webb, 1975). No such stimulatory effect is found in myeloma nuclei (Stuart et al., 1977) or in mouse brain nuclei, where unfractionated cytosol has an inhibitory effect on RNA transport (Weck and Johnson, 1978) and is not required to prevent lysis.

Shchuppe et al. (1974) also report a 50% inhibitory effect of cytosol on RNA release in the rat liver system; they do do not give evidence of the stability of their nuclei, so no conclusions can be drawn from these studies.

Ishikawa's group had earlier reported a system for the release of RNA from rat liver nuclei of sucrose, low salt and 2mM ATP (similar to that of Webb's) to give 20% release and refuted the allegations by other groups of nuclear lysis in the system. This latter system

incorporates cytosol, spermidine and an energy generating sytem. It is totally ATP dependent, increases linearly with regular addition of PEP and PCK, has overall less RNA released and, although they show no data, indicates the RNA is bigger than in the previous system. Webb's group, in a study of cytosol dependent release of RNA, find this release is linear throughout the concentration. They test up to 36 $mg \cdot ml^{-1}$, at which point it reaches the amount released in the absence of cytosol. However, they do not look at nuclear integrity (Schumm et al., 1973). In a later report, having changed their assay temperature from 36°C to 30°C, they find a sigmoidal concentration dependence curve and argue that the temperature shift must account for these different results. Taking into account the discrepancies and lack of information on nuclear lysis, it is difficult to draw any conclusions.

Finding that the cytosol had some regulatory function over RNA release, Webb's group investigated the effect of homologous versus heterologous cytosol on RNA release using normal, regenerating and 513D hepatoma rat liver cells. In each case the autologous cytosol gave the highest release (although the differences are extremely small and may not be significant) (Schumm et al., 1973). They also report differences in the species of RNA released as assayed by hybridization competition studies and sucrose gradients. Again, the differences in the gradients are extremely small and neither samples nor gradients are given any denaturing treatment. The authors conclude that the protein component in each cytosol specifically favors the release of selected RNA species.

Following this tract, Webb's group partially fractionated cytosol from normal rat liver and studied the effect of different fractions

on RNA release. They find that a fraction that binds to DEAE-cellulose at pH 8.5 specifically favors release of ribosomal RNA (McNamara et al., 1975), whereas the unabsorbed fraction, specifically if the chromatography is done at a pH of 7.5, induces release of 4-10S mRNA species. Both fractions inhibit nuclear lysis and are extremely heat labile and sensitive to freeze-thawing. Because of their heat lability, the responsible factors are regarded as proteins, but not ribosomal proteins. A purified preparation of ribosomal proteins does not alter rRNA release and slightly inhibits that of the 4-10S species. The authors speculate that free initiation factors might be a signal *in vivo* for further release of rRNA and these are the factors in the cytosol fraction stimulating its release. To our knowledge no group has pursued this interesting line of work by studying release of rRNA with purified initiation factors.

In a later study, Webb's group reports the existence of two modulators of mRNA release: a DEAE-cellulose unabsorbed fraction at pH 7.5 which is five times more stimulatory than unfractionated cytosol and extremely labile to freeze-thawing, and an inhibitory DEAE-absorbed fraction (Racevskis and Webb, 1974). A similar report comes from a group working on mouse brain nuclei that had previously reported the slight inhibitory effect of unfractionated cytosol on release of RNA (Weck and Johnson, 1978). Now the cytosol is fractionated on a DEAE-cellulose column after filtration over G-100 Sephadex, and a heat labile stimulatory and a more resistant inhibitory fraction are found. They speculate that nucleocytoplasmic transport may be modulated by a balance between these two fractions *in vivo*. The system used permits RNA synthesis; optimal RNA release is at 2mM Mg^{2+} and at 1mM ATP.

Searching for a possible interaction between cytosol and nuclear envelope in nucleocytoplasmic release, Weck and Johnson treat the nuclei with detergent to dissolve the membranes. They find an enhanced release in the absence of cytosol, equaling that of the cytosol stimulated release in the untreated nuclei, which is impervious to further increase by the addition of cytosol. They postulate that the cytosol modified the envelope, allowing it to release RNA that it was retaining in the nucleus.

Other studies also give the nuclear envelope some role in nucleocytoplasmic modulation. In *Tetrahymena pyriformis* nuclei, a biphasic release with a discontinuity at 17 degrees, at which temperature the envelope fluidity suffers the same discontinuity, is reported (Herlan et al., 1979). No such discontinuity is found in myeloma nuclei (Patterson et al., 1980), nor in rat liver nuclei (Clawson and Smuckler, 1978).

In isolated myeloma nuclei the extent and size distribution of RNA released at 37°C is the same in intact and membrane-denuded nuclei, leading the authors to postulate that the nuclear envelope has no role in the restriction of RNA (Stuart et al., 1977). In later studies conducted at 25°C, a role in nucleocytoplasmic regulation is found for the nuclear envelope (Patterson et al., 1980).

All the studies on the subject confirm that the RNA is released as a ribonucleoprotein (RNP) and that a certain percentage is polyadenylated. After two hours *in vivo* labeling some RNPs are ribosomal subunits that band in cesium chloride at their specific buoyant density of 1.5 $g \cdot cm^{-3}$ and show the 18S and 28S RNA species upon deproteinization (Yu et al., 1972). Shorter labelling periods from thirty to sixty minutes yield mostly messenger ribonucleoprotein (mRNP).

These band at their characteristic buoyant density of 1.41-1.43 $g \cdot cm^{-3}$ in cesium chloride and show mRNA profiles on sucrose gradients and SDS-PAGE electrophoresis (Worofka and Sauerman, 1978; Roy et al., 1979; Raskas et al., 1973).

In myeloma nuclei 16-20% of released RNA is polyadenylated (Stuart et al., 1977). Gross and Ringler (1979), studying release of RNA synthesized *in vitro*, report 3-5% is polyadenylated. Roy et al. (1979), also in an *in vitro* synthesized RNA system, find that 3.5% of the released RNA is polyadenylated.

Webb et al. (1975) find the size of released RNA after a 30 to 60 minute *in vivo* labeling of rat liver nuclei is 4-10S, and although they cite this as confirmation of a reliable transport system, this is small for mRNA. Either their system supports extensive leakage of small RNA or the RNA is degraded during handling. Other groups agree that the RNA released in the absence of ATP is of small size of 4-7S, and of mRNA size, 10-28S, in the presence of ATP (Ishikawa et al., 1978; Agutter and McCaldin, 1979; Brummer and Raskas, 1972). In HeLa cells release of RNA from isolated nuclei at 2mM ATP gives a profile of 6-28S (Chatterjee and Weissboch, 1973). A similar RNA profile is found in myeloma nuclei in the absence of ATP (Stuart et al., 1977).

The bulk of the transport studies are done in a surrogate cytoplasm incapable of sustaining RNA synthesis. We believe that synthesis, processing and RNA release are probably interrelated and accordingly have changed our transport conditions to those of standard synthesis conditions as described by Marzluff et al. (1973). Under these conditions, initiation of RNA synthesis occurs as demonstrated by Smith et al. (1978) and Ganguly and Banerjee (1978), by using 5-γ-S labelled GTP or CTP, or 5-γ-Hg GTP or CTP. The transcripts initiated with a

substituted nucleotide are specifically retained on affinity mercury-sepharose or SH-sepharose columns, respectively. These transcripts show a 77% sensitivity to α-amanitin, the specific polymerase II inhibitor; 17% of this RNA is polyadenylated, and this polyA(+)RNA sediments as mRNA-sized with a peak between 16-17S in denaturing 99% formamide gradients. The non-adenylated species show a profile of smaller species, peaking at 15S. This *in vitro* synthesized RNA is also capped (Manley et al., 1979). Considering the reliability of the above system in RNA synthesis and processing, we believe it is a better one to study nucleocytoplasmic transport of RNA.

The phenomenon of splicing of IVS has given rise to questioning their significance and role in gene expression. The overwhelming evidence to date is that only mature-sized RNAs are ever transported to the cytoplasm (reviewed by Abelson, 1979).

In MOPC-21 myeloma cells a 40S and 24S precursor and the 13S mature mRNA of the κ light chain are detected in the nucleus of these cells by recombinant DNA technology. Only the mature 13S mRNA molecule can be detected in the cytoplasm (Gilmore-Hebert and Wall, 1978). Similarly, in myeloma MPC-11 cells two larger precursor molecules are found for the three immunoglobulin gene products, the γ_{2b} heavy chain, the κ light chain, and the κ light chain fragment. Only the mature species are found in the cytoplasm (Schibler et al., 1979).

We have already mentioned that Hamer and Leder have proposed that splicing of the IVS takes place in the nuclear membrane, and any unspliced species of RNA does not get translocated to the cytoplasm, being rapidly degraded in the nucleus. Their current unpublished results further support this hypothesis (Leder, personal communication). The only evidence refuting this hypothesis was reported in SV-40

infected F-9 teratocarcinoma cells. Polyadenylated unspliced transcripts were detected in the nucleus and in the cytoplasm of the infected cells (Segal et al., 1979). More recent reports from this group contradict their own report, only finding the unspliced RNA in the nucleus; unfortunately, they offer no explanation for this contradiction, making it very difficult to draw any firm conclusions for this system.

A similar study searching for precursor and mature mRNA in the transport system is lacking, and that is what we propose to do in our MOPC-21 myeloma system. We will look for precursor and mature molecules of mRNA in the nucleus and in the surrogate cytoplasm of our *in vitro* transport system.

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ARTICLE 1

TRANSPORT OF RNA FROM ISOLATED NUCLEI DUPLICATES *IN VIVO* PROCESSES

By

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ABBREVIATIONS: Tris-HCl, Tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetate; 2ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; Me_2SO_4, dimethylsulfoxide.

ABSTRACT

Transport of prelabeled RNA from isolated nuclei is studied under conditions permitting RNA synthesis. Cytosol and spermidine are not required to maintain nuclear stability, and have an inhibitory effect on transport. ATP and GTP increase release of RNA by 25%. This stimulatory effect is not derived from the energy released by the hydrolysis of the triphosphates, since addition of the Mg^{2+}-ATPase inhibitor quercetin has no effect on the quantity of RNA transported. The RNA transported is identical in size and percentage of poly A(-) and poly A(+) species in the presence or absence of ATP. The size distribution of intranuclear and released RNA corresponds to the profiles of comparable RNA isolated from intact cells.

INTRODUCTION

The most widely used system for *in vitro* studies of nucleocytoplasmic transport employs sucrose and non-physiologic ion concentrations [1-5]. Under these conditions it has been reported that nuclei lyse and/or clump in the presence of ATP or an energy source, and that spermidine and/or cytosol are required to inhibit lysis and maintain nuclear stability [1,4]. Rat liver nuclei have been reported to require ATP, cytosol and spermidine for optimum and regulated RNA transport [1,2,6]. Using the same rat liver nuclei system, Smuckler's group [7] finds only a requirement for ATP. Other groups find that

unfractionated cytosol is inhibitory [8,9] or has no effect on RNA transport [10]. Fractionated cytosol contains an extremely heat-labile stimulatory and an inhibitory fraction that affect RNA release [6,8,11].

Tumor cells have different requirements for "faithful" release of RNA. Hepatoma and MOPC-21 myeloma nuclei do not require spermidine or cytosol to maintain nuclear integrity [5,12]. Hepatoma 5123D cell nuclei are only dependent on exogenous ATP for 25% of their regulated release and Novikoff hepatoma nuclei are totally ATP-independent [12], as are myeloma nuclei when assayed in the sucrose-low salt transport system [10]. Transport of RNA from liver cell nuclei isolated from carcinogen-treated rats was reported previously to be ATP-independent [3]. A recent report from the same group finds enhanced RNA release in the presence of ATP [13].

The RNA released from rat liver nuclei in the absence of ATP is small, sedimenting between 4S and 10S. With the addition of ATP the species released are of mRNA size [2,14,15]. In myeloma nuclei the RNA released is mRNA-like in the absence of ATP [10].

The mode of action of ATP is under debate. Some groups find that the energy derived from ATP hydrolysis is required to drive nucleo-cytoplasmic transport and that transport is suppressed by inhibitors of the nuclear membrane NTPase [4,7,11-13,15].

Other ribonucleoside triphosphates and non-hydrolyzable analogs have been reported to enhance RNA release. These latter data suggest that rNTPs or structural analogs are recognized and bound to a nuclear membrane or matrix structure, resulting in an alteration which allows the release of mature RNA from the nuclei [2,16].

Our present system to study nucleocytoplasmic exchange is a modification of that used in our previous studies [5,10] and supports prolonged RNA synthesis [17], initiation [18], processing [17,18], polyadenylation [17,19] and capping of RNA [20]. We show that myeloma nuclei release mRNA-sized RNA species in the presence or absence of ATP. Only the magnitude of the release is enhanced by ATP. Further, putative inhibitors of a nuclear membrane NTPase do not modulate the amount of RNA transported.

METHODS

MOPC-21 mouse myeloma tissue culture cells were maintained and pulsed with radiolabeled precursors as previously described [5,10]. For studies of RNA synthesis *in vitro*, isolated nuclei were incubated with 10 μCi ^{3}H-GTP/ml (16.7 Ci/mmol, ICN). *In vitro* RNA synthesis is supported for up to 2 h using this system (data not shown).

Cytosol was prepared and its protein content measured as described [5]. Nuclei were purified by a modification of already described methods [21,22]. Briefly, nuclei were isolated by Dounce homogenization and pelleted through 2.4 M sucrose, 1 mM $MgCl_2$ in an SW50·1 rotor of the Beckman L5-50 ultracentrifuge for 1 h at 50,000x g. The nuclei were resuspended in 0.32 M sucrose, 25 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, examined, counted and pelleted by centrifugation for 5 min at 900x g. Nuclei were resuspended at 1 x 10^7/ml in nuclear synthesis buffer (12.5% glycerol, 25 mM Tris HCl [pH 8.0], 5 mM $MgCl_2$, 150 mM KCl, 0.1 mM EDTA, 10 mM 2ME, 0.4 mM ATP, GTP, UTP, CTP [17]).

Release of prelabeled RNA was monitored for 30 min at 25°C unless indicated otherwise. Aliquots were taken at the indicated time points

and added to 0.8 ml of ice-cold synthesis buffer. The nuclei were pelleted and the post-nuclear supernatant withdrawn. RNA release was calculated either as the percentage of acid-precipitable radioactivity in released RNA compared to the total at 0 time, or as the percentage of the sum of the acid-precipitable radioactivity in the released and intranuclear fraction at each time point. This second method will be used for all calculations of RNA release. Using this latter procedure we consistently find a 30% higher release. This can be explained partially by the sticking of pelleted nuclei to the test tube, even when these are siliconized. Of more significance is the finding that the intranuclear acid-precipitable radioactivity decreases steadily after slightly increasing from 0 time to 10 min.

RNA was extracted and ethanol-precipitated as described [23] and resuspended in RNase-free double distilled water or buffer as indicated. Poly A(+) RNA was fractionated by two purifications over oligo d(T)-cellulose (generous gift from F. M. Rottman) as described [24]. RNA was analyzed on 5-20% or 15-30% sucrose-SDS gradients in the following buffer: 30 mM Tris (pH 7.4), 5 mM EDTA, 0.5% SDS, after heating the samples in the same buffer 4 min at 80°C and quick chilling, or treating with 75% Me_2SO_4 [25]. The same profiles were obtained with either treatment, as well as when samples were treated with and analyzed on 70% formamide 5-25% sucrose gradients [26].

RESULTS

Nuclear integrity

Nuclei are free of cytoplasmic contamination and retain the double membrane and normal appearance after our standard incubation, as demonstrated by electron microscopy (data not shown). Lysis is

monitored by release of prelabeled DNA and amounts to a maximum of 1.7% after 30 min incubation. This value is similar to the percentage of DNA in mitotic cells and may represent loss of nuclear integrity at this cell cycle stage.

Kinetics of RNA release

As shown in Figure 1, RNA is released linearly for 20 min, and then at decreasing rates, leveling off by 45 min. Twenty percent of the total RNA is released under control conditions. Release of RNA is 75 ± 2% of control when ATP or GTP are omitted, 95 ± 1% when CTP or UTP are omitted, 80% of control at 0.2 mM ATP, GTP, CTP, UTP and 59 ± 1% from 0.1 mM each to zero ribonucleoside triphosphates (Figure 1).

Effect of inhibitors on transport of RNA

The effect of inhibitors on transport of RNA is shown in Table I. Quercetin is a known inhibitor of hexose transport [27] and has been reported to inhibit release of RNA [4,15]. We have tested this compound dissolved either in KOH (pH 8.3) or in 0.5% and 1% ethanol. In all cases it did not inhibit transport, or inhibited transport to the same extent as the solvent control. The Na^+-K^+-ATPase inhibitors ouabain and ethacrynic acid did not affect transport. Schumm and Webb have reported the inhibition of transport by colchicine at 4 mM [28]. We find that with 100 μM colchicine, or its analog colcemid, RNA release is decreased by 20%, but the intranuclear TCA-precipitable radioactivity is decreased by 60%. Colchicine is an inhibitor of microtubule assembly and it is also reported to inhibit transport of nucleosides. α-Amanitin, an RNA polymerase II inhibitor, has no effect on the amount of prelabeled RNA released.

Effect of cytosol and spermidine

As cytosol and spermidine have been reported to be necessary for regulated and maximal transport of RNA, we tested their effect in our system. The results are summarized in Table II. Spermidine at 5 mM completely inhibits release; cytosol at 5 mg/ml inhibits release by 25%. This inhibition is overcome by increasing the concentration of ATP to 2 mM, or adding an energy source. Both additions give levels of release equal to our control. Resuspension of nuclei in the corresponding amount of unlabeled cytoplasm gives the same release.

Characterization of RNA

In our system we obtain release of messenger-sized RNA in the absence of cytosol and spermidine. Shown in Figure 2A are the profiles of poly A(+) RNA isolated from the released and nuclear-retained fractions after a 30 min transport assay, following a 35 min prelabeling period in intact cells. Figure 2B shows these same fractions when the pulse is 60 min. Comparing the profiles of cytoplasmic RNA labeled for 35 min, 60 min and under steady state conditions with released RNA after a further 30 min incubation in our assay system, we see a progression in the quantity of mature 18S and 28S RNAs in released RNA as the 4S and mRNA fractions decline with longer labeling periods (Figure 3). When we compare the profiles of released RNA obtained under different ribonucleotide conditions we find the difference is quantitative but not qualitative, as shown in Figure 4.

Quantitation of poly A(+) RNA

The percentage of poly A(+) RNA of the intranuclear fractions after 30 min release assay is 9.5 ± 0.5% for 35 min prelabeling and 2 ± 0.5% for 60 min prelabeling (Table III). The percentage of

poly A(+) RNA in the cytoplasmic RNA is 18 ± 1% and 15 ± 2%, respectively, and for released RNA the values are 21 ± 2% and 17 ± 1%, respectively. The same values are obtained when one or more ribonucleotide triphosphates are omitted from the incubation mixture, as shown in Table IV.

DISCUSSION

We have defined a transport system for myeloma nuclei which permits continued synthesis and processing of intranuclear RNA. Synthesized RNA is mRNA-sized and processed with longer labeling times [17,18,29]. The translocation of RNA is increased by ribonucleotides but does not require spermidine or cytosol for maintenance of nuclear integrity or regulated release. The RNA released shows a profile consistent with the length of the pulse and the release assay. These profiles and the fact that only 13S RNA hybridizes to a MOPC-21 K-cDNA probe (C. Otegui and R. J. Patterson, manuscript in preparation) indicates we neither have aggregation nor degradation of released RNA. These data indicate that the loss of TCA-insoluble radioactivity from the intranuclear RNA after 10 min incubation (see Materials and Methods) does not represent degradation by exogenous RNase, although this intranuclear RNA turnover may not totally reflect *in vivo* occurrences.

Inhibition of *in vitro* RNA synthesis by α-amanitin does not alter the amount of prelabeled RNA released. This may indicate that, at least for short periods, continued synthesis is not required for translocation of prelabeled RNA; however, we cannot rule out other explanations. The RNA released might have been processed prior to the initiation of the release assay containing α-amanitin. We have not

determined poly A content or size of this RNA, and qualitative differences with control RNA could exist.

From our studies it can be inferred that the inhibitory action of colchicine and colcemid in myeloma nuclei is due to an effect on nuclear RNA stability and not on release per se. It has been established that nuclear RNA is bound to and retained by a nuclear matrix in chromatin-depleted and membrane-denuded nuclei [30,31]. Colchicine may disrupt the nuclear matrix causing rapid degradation of nuclear RNA.

The controversial results obtained by different groups by the addition of cytosol to the system may be explained by the accumulating evidence of the extreme lability of the stimulatory fraction [6,8,11]. Storage of cytosol at -20°C and -80°C has been routinely employed. In tissue culture systems, such as ours, high protein concentration of cytosol can only be obtained by precipitation or lyophilization of cytosol. We do not know the effect of these procedures on the stability of the stimulatory fraction.

Shchuppe et al. [9] report that 5 mM spermidine totally inhibits the nuclear Mg^{+}-ATPase. The systems that incorporate spermidine may require higher ATP concentrations to overcome this inhibition. Leake et al. [32] have reported that spermidine causes nuclear shrinkage and changes in chromatin structure. Systems that utilize *in vitro* RNA synthesis conditions with 10-12.5% glycerol and 80-150 mM KCl do not require spermidine or cytosol to maintain nuclear integrity [8,19]. Glycerol is required for and enhances RNA synthesis [17-20,33]. The mode of action of glycerol in stabilizing nuclei is not comparable to that of spermidine. It does not alter the nuclear morphology, and our data show that spermidine totally inhibits the release that is

observed at a concentration of 12.5% glycerol in our control system.

Evidence is accumulating that the nuclear envelope regulates nucleocytoplasmic RNA translocation [4,7,11,13,15]. Contrary to our previous report in which myeloma nuclei were assayed at 37°C [5], we find that at 25°C the nuclear membrane is involved in the regulation of transport [34]. It is widely accepted that a nuclear envelope NTPase is located in the immediate vicinity of the pore complex [4,11,15,35]; studies with isolated nuclear membranes locate a Mg^{2+}-dependent ATPase in this structure. Inhibition of its activity parallels the inhibition of RNA release in isolated nuclei [13,36].

The energy released on the hydrolysis of nucleoside triphosphates by a nuclear envelope nucleoside triphosphate is required for RNA transport in nuclei from rat liver [1,5,7,11], SV-40-3T3 [4], and thioacetamide-treated rat liver cells [13]. Other systems, although requiring nucleoside triphosphates or nonhydrolyzable analogs, show an energy independent release of RNA [2,14,16]. In our system this is also the case; the rNTPs increase release, but this is not affected by quercetin as reported in energy-requiring systems [4,15]. The ATP concentration of rat tumor cells has been reported to be 2.5 times higher than in normal cells [37]. We have indirect evidence of high intranuclear ATP concentration in MOPC-21 myeloma cells: *in vitro* RNA synthesis is 20% of control when ATP is omitted from the assay, but zero when any of the three other rNTPs are omitted [29]. In these tumor systems the requirement for exogenous ATP is probably reduced, and in fact elevated concentrations may cause lysis due to the Mg^{2+} chelating effect of ATP.

The data presented in this communication and our ongoing studies utilizing hybridization of intranuclear and released RNA to specific

cDNA probes demonstrate that in our system we observe only the translocation of properly processed RNA, proving non-specific RNA "leakage" does not occur. Having ascertained this, we plan to investigate the mechanisms that regulate nucleocytoplasmic translocation of RNA.

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Table I. Effect of inhibitors on RNA release

Inhibitor	Solvent	Percent RNA Released[a]
None (control)	---	20.0
Quercetin (30 µg/ml)	EtOH (1%)	19.2
None	EtOH (1%)	15.7
Quercetin (30 µg/ml)	EtOH (0.5%)	22.3
None	EtOH (0.5%)	17.7
Quercetin (30 µg/ml)	KOH (5 mM at pH 8.3)	22.6
None	KOH (5 mM at pH 8.3)	22.9
Ethacrynic acid (10^{-4} M)	EtOH (0.5%)	18.0
Ethacrynic acid (10^{-3} M)	EtOH (0.5%)	17.4
Ouabain (10^{-3} M)	H_2O	20.8
Ouabain (10^{-4} M)	H_2O	20.7
Ouabain (10^{-6} M)	H_2O	19.7
α-Amanitin (1 g/ml)	H_2O	19.3

[a]Percent RNA released assayed as indicated in Materials and Methods following 30 min incubation at 25°C in transport system.

Table II. Effect of spermidine and cytosol on release of prelabeled RNA

rNTPs (0.4 mM)	Additions to the Incubation Mixture: Spermidine (5 mM)	Cytosol (5 mg/ml)	PEP+PCK	Percent RNA Released[a]
+ (control)	-	-	-	20.0
-	-	-	-	12.3
-	+	-	-	4.0
+	+	-	-	4.0
+	+	+	-	14.5
+	-	+	-	14.8
+ (2 mM ATP)	+	+	-	21.6
+	+	+	+	22.1
Nuclei resuspended in unlabeled cytoplasm				20.3
Above plus 2 mM ATP				23.0

[a]Percent RNA released assayed as indicated in Materials and Methods.

Table III. Poly A(+) content of PNS, released and intranuclear RNA

Pulse (min)	Percent poly A(+) RNA[a] PNS	Released	Intranuclear[b]
35	18 ± 1	21 ± 2	9.5 ± 0.5
45	N.D.[c]	18 ± 1	6.0 ± 0.5
60	15 ± 2	17 ± 1	2.0 ± 0.5

[a]Percent poly A(+) content assayed as indicated in Materials and Methods.

[b]Assayed after 30 min transport incubation at 25°C.

[c]Not determined.

Table IV. Poly A(+) content of released and intranuclear RNA under different rNTP conditions

	Percent poly A(+) RNA	
	Released	Intranuclear
Control	21 ± 2	10.0 ± 0.5
Minus ATP	21 ± 2	10.1 ± 0.5
Minus GTP	19 ± 2	N.D.
Minus 4 rNTPs	17 ± 2	N.D.

Cells were pulse-labeled for 35 min. RNA isolated from nuclei incubated 30 min at 25°C in the transport system.

Figure 1. Release of RNA in isolated myeloma nuclei. (—●—) control - 0.4 mM rNTPs; (—⊟—) minus ATP; (—○—) minus GTP; (—▲—) minus rNTPs.

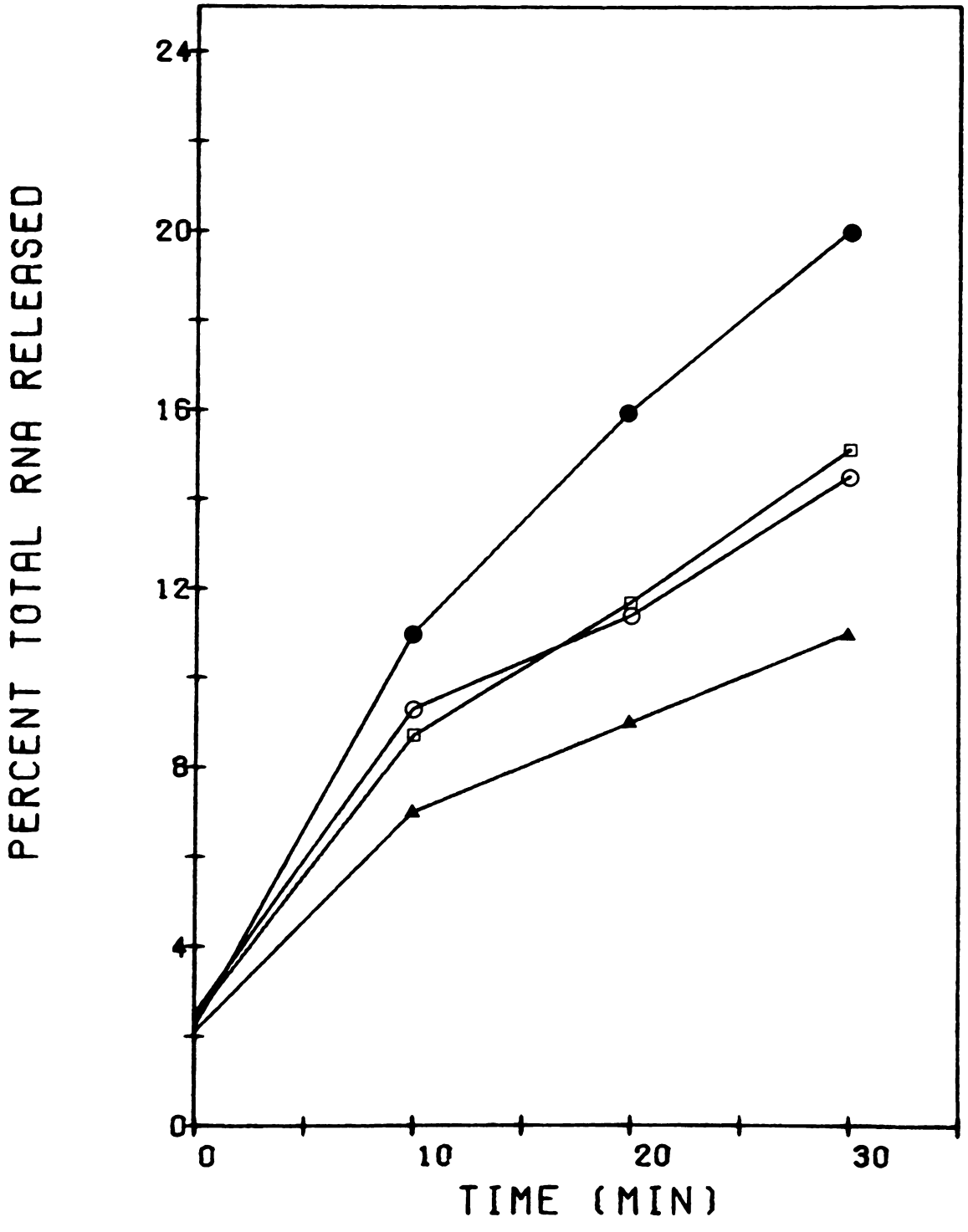

Figure 1

Figure 2. RNA profiles of poly A(+) intranuclear and released RNA. Cells were prelabeled 35 or 60 min with ^{3}H-uridine, and intranuclear and released poly A(+) RNA isolated after incubation of isolated nuclei for 30 min at 25°C. Released RNA was analyzed on 5-20% sucrose-SDS gradients, intranuclear RNA was analyzed on 15-30% sucrose-SDS gradients in SW50.1 rotor, 3.5 h, 190,000x g. Figures are a composite of the gradient profiles. A) Cells prelabeled 35 min. (—○—) intranuclear RNA; (—●—) released RNA. B) Cells prelabeled 60 min. (—○—) intranuclear RNA; (—●—) released RNA. Direction of sedimentation is from left to right. Arrows indicate 18, 28 and 45S markers.

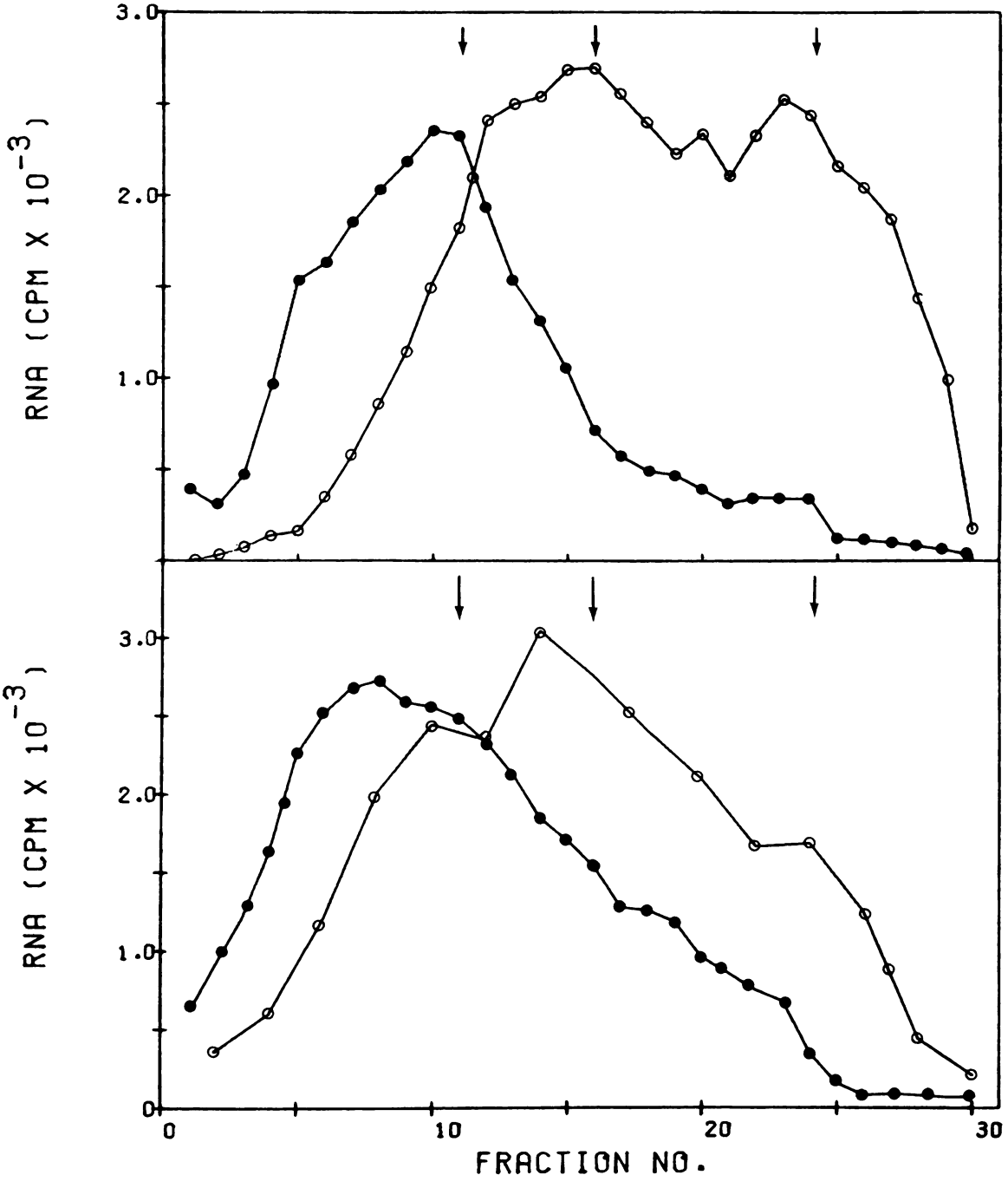
RNA (CPM X 10^{-3})
3.0
2.0
1.0
RNA (CPM X 10^{-3})
3.0
2.0
1.0
0
0
10
20
30
FRACTION NO.

Figure 2

Figure 3. RNA profiles of total cytoplasmic and released RNA following different labeling conditions. Samples analyzed as in Figure 2. A) Cells prelabeled 35 min. (—○—) PNS; (—●—) released RNA after 30 min transport assay. B) Cells prelabeled 60 min. (—○—) PNS; (—●—) released RNA, after 30 min transport assay. Arrows indicate 4, 18 and 28S markers.

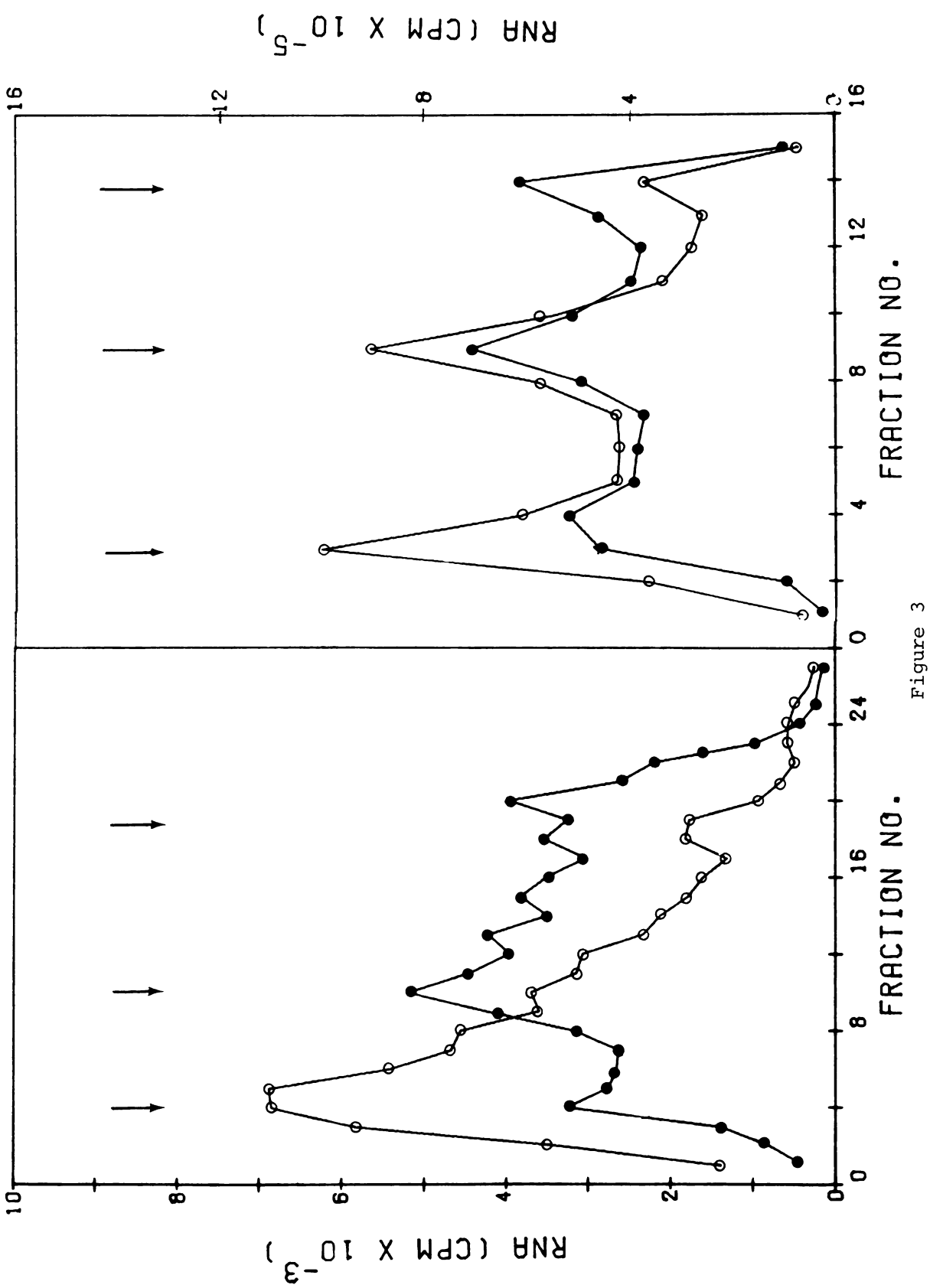

Figure 3

Figure 4. Poly A(+) RNA released under different rNTP conditions. Gradients analyzed as in Figure 2. (—△—) control; (—✱—) minus ATP; (—□—) minus rNTPs. Arrows indicate 18 and 28S markers.

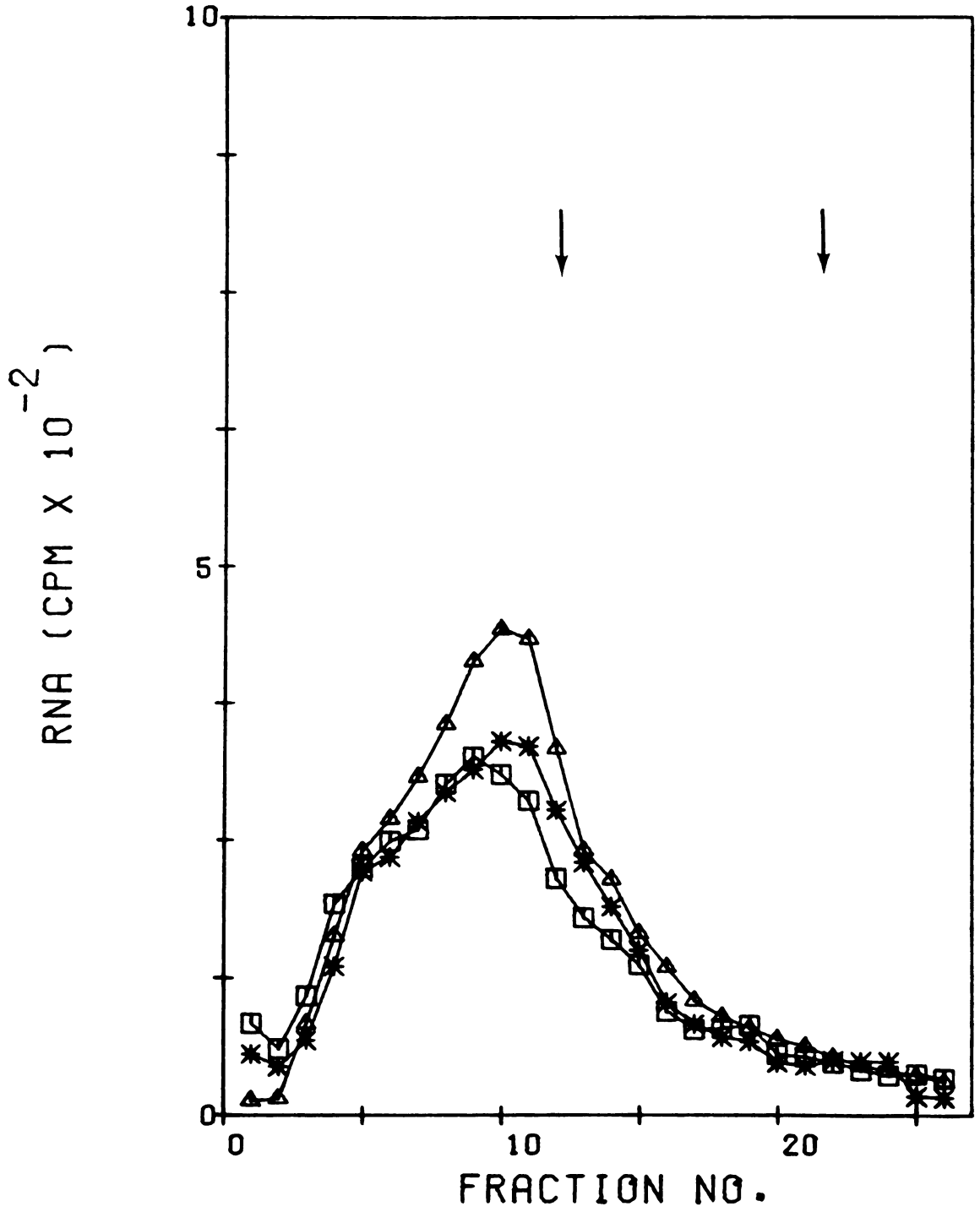

Figure 4

ARTICLE 2

TRANSPORT OF AUTHENTIC IMMUNOGLOBULIN LIGHT CHAIN mRNA FROM ISOLATED NUCLEI

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ABBREVIATIONS: Me_2SO_4, dimethyl sulfoxide; IVS, intervening sequences; NSB, nuclear synthesis buffer; AEV, avian erythroblastosis virus; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein; HnRNA, heterogeneous nuclear RNA.

ABSTRACT

A specific recombinant DNA clone is used to study translocation of immunoglobulin κ light chain mRNA from isolated nuclei. As *in vivo*, only mature 13-14S mRNA is detected in the released fraction. Two classes of RNA molecules are detected in the nucleus, the authentic mRNA-sized species and a larger precursor.

INTRODUCTION

Most eukaryotic mRNAs that have been studied undergo extensive post-transcriptional modifications. These include internal methylations, 5' "capping", 3' polyadenylation, binding by specific proteins and splicing or cleavage of specific sequences (intervening sequences) from a larger precursor transcript.

The significance of these modifications in the regulation of gene expression is largely unknown. Almost nothing is known about the biological role of internal methylations or the RNA-associated proteins. There is mounting evidence that capping stabilizes the initial 40S-mRNA complex, although there are reports debating this point (5,6).

The poly (A) tract is required for the appearance of polyadenylated sequences in polyribosomes (4,7). However, it is not known whether the poly A tract is required for the transport of the transcript across the nuclear envelope, to confer stability to the untranslated messenger in the cytoplasm, or for other unknown functions (1,2).

The role of splicing of IVS is as yet unknown. Leder's group has postulated that the intervening sequence junctions are required for the elongation of transcripts (8,9). Khoury's group found that intervening sequences are required for the stability of RNA nuclear precursors (10,11).

Nucleocytoplasmic translocation of RNA has been studied by many groups under conditions that do not support transcription (12-16) and by other groups under conditions that support *in vitro* RNA synthesis (17-21). Putative cytoplasmic modulators of RNA translocation have been described (20,22).

In the study of the translocation of prelabeled RNA from isolated nuclei into a surrogate cytoplasm we employ conditions of *in vitro* RNA synthesis that allow initiation, elongation and processing of RNA transcripts (18,21,23-25). We demonstrate the release of authentic κ light chain mRNA sequences. Only the mature 13-14S molecule is transported from the nucleus, while both this molecule and a larger precursor are detected in the nucleus.

MATERIALS AND METHODS

Maintenance of cells

MOPC-21 murine myeloma tumor cells adapted to tissue culture (P3) were maintained as described (15,16).

Isotopic labeling

Steady state isotopic labeling of cells was achieved by incubation of cells at an initial cell density of 2.3-2.8 x 10^5/ml with 20 μCi/ml of ^{3}H-UdR (Schwarz-Mann 33 Ci/mM). For 45 and 60 min pulses 10^8 cells at 4.0-4.5 x 10^5/ml were concentrated ten-fold. Cells were

pelleted, resuspended in prewarmed media and allowed to equilibrate 30 min before labeling with 10 mCi of ^{3}H-UdR.

Isolation of nuclei

Cells were lysed by Dounce homogenization as described (15). Nuclei were purified by a combination of the methods described by Blobel and Potter (26) and Banks and Johnson (27). After cell lysis nuclei were pelleted 7 min at 900x g, resuspended in 5 ml of 2.4 M sucrose, 1 mM $MgCl_2$ and centrifuged 1 h at 50,000x g in an SW50·1 rotor of a Beckman L5-50 ultracentrifuge. Nuclei were gently resuspended with a Kontes pestle in 0.32 M sucrose, 25 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, and repelleted 7 min at 900x g. Nuclei were resuspended in NSB: 12.5% glycerol, 25 mM Tris (pH 8.0), 5 mM $MgCl_2$, 150 mM KCl, 1 mM EDTA, 10 mM 2ME, 0.4 mM ATP, GTP, UTP, CTP (23).

Using this isolation procedure the nuclei have an intact double membrane nuclear envelope and have no cytoplasmic contamination as verified by electron microscopy at 0 time and after 30 min transport assay (data not shown).

Transport assay

Nuclei were resuspended in NSB at a concentration of 1 x 10^{7}/ml and incubated 30 min at 25°C. Aliquots were taken at various intervals and the reaction stopped by addition of 0.8 ml ice-cold NSB. Nuclei were pelleted 10 min at 900x g and the supernatant used to determine RNA transported. This is quantitated as the percentage of acid-insoluble radioactivity in the supernatant to that in the supernatant plus the resuspended nuclei.

Extraction of RNA

RNA was extracted from the cytoplasmic, intranuclear and released RNA as described (28). DNase at 40 µg/ml was used in the extraction of intranuclear RNA. Proteinase K at 200 µg/ml was used in all the fractions.

Oligo d(T)-cellulose purification

Oligo d(T)-cellulose was a generous gift from F. M. Rottman. Purification of poly(A)-containing RNA was done twice, as described by Purchio et al. (29).

Analysis of RNA and filter hybridization

Ethanol precipitates of RNA were resuspended in double-distilled RNase-free H_2O or the corresponding buffer for analysis on gradients. When analyzed on sucrose-SDS gradients, the resuspension and gradient buffer used was 30 mM Tris (7.4), 5 mM EDTA, 0.5% SDS. Samples were either heated 4 min at 80°C and quick chilled or treated with 75% Me_2SO_4 (30). For analysis on 70% formamide gradients, the method described by Suzuki et al. (31) was used. The same results were obtained with formamide and sucrose-SDS gradient analysis. Each gradient fraction was hybridized to 5 µg of pL21-1 DNA bound to nitrocellulose filter. The hybridization was performed in the gradient buffer made 0.6 M NaCl, at 65°C for 24 h using sucrose-SDS gradients. For hybridization of intranuclear RNA the solution was made 10x Denhardt's; for released and cytoplasmic RNA 1x Denhardt's was used. The fractions from formamide gradients were made 0.6 M NaCl, the formamide concentration reduced to 50% and Denhardt's solution added as above; hybridization was complete after 4 days at 37°C. The same results were obtained with both methods. A filter with 5 µg of *S.*

typhimurium DNA (generous gift from L. Snyder) was included in each reaction and subtracted as background hybridization. The possible effect of increasing sucrose concentration on hybridization was evaluated; up to 30% sucrose had no effect on hybridization (data not shown).

Isolation of plasmid DNA

Immunoglobulin light chain κ mRNA clone pL21-1 was obtained from W. Salser and R. Wall. Plasmid DNA was purified on CsCl-EtBr buoyant density gradients and bound to Schleicher and Schuell (S&S) BA85 nitrocellulose filters. The purity and identity of the plasmid were verified by restriction analysis with Hae III, Hpa II and Hind II (BRL) following the instructions of the manufacturer.

RESULTS

In vitro RNA synthesis in MOPC-21 isolated nuclei was maintained for at least 2 h (25; and our own results, data not shown).

Transport of prelabeled RNA from isolated nuclei under *in vitro* synthesis conditions has been characterized for MOPC-21 cells (manuscript in preparation). Briefly, RNA is released linearly for 15-20 min, and then with decreasing rates for 25 min (Figure 1). RNA profiles of released and intranuclear RNA after 35, 45 and 60 min pulses in whole cells and 30 min release assay demonstrate that processing of prelabeled RNA occurs in this system; only processed RNA (as determined by the release of 18S and 28S mRNA markers) is transported to the surrogate cytoplasm (manuscript in preparation).

Purity of the plasmid DNA was ascertained by restriction analysis with Hpa II, Hae III and Hind II. Only the specific fragments reported for pL21-1 (32,33) were found with the three analyses. Figure 2 shows

the restriction analysis of pL21-1 with Hind II. As published (32), three fragments not found in the parent pMB9 are evident. The 168bp fragment has been mapped within the constant region of κ light chain.

To establish our hybridization conditions, steady state cytoplasmic RNA was hybridized as described. As shown in Figure 3, a distinct peak at 13-14S hybridizes to the light chain DNA probe. The intranuclear RNA after a 45 min prelabeling and 30 min release assay shows hybridization to the mature 13S mRNA κ species; a shoulder at 24S is also evident and a small peak at 40S (4a). The released RNA hybridizes only to the mature 13S mRNA κ. We did not detect hybridization to any precursor molecules in the transported RNA (Figure 4b). When prelabeling was for 60 min, only the 13S mature species was found in the poly A(+) intranuclear fraction (Figure 5). Poly A(-) intranuclear and released RNA showed no hybridization to the cDNA probe (data not shown).

DISCUSSION

Most systems devised to study the transport of prelabeled RNA from isolated nuclei employ conditions that do not support RNA synthesis (12-16). Other groups have incompletely characterized the release of RNA labeled *in vitro* from isolated nuclei (17-21). To our knowledge, only one group (20) has studied transport of prelabeled RNA from isolated nuclei under *in vitro* RNA synthesis conditions. The major criticism of transport studies has been that the RNA released into the surrogate cytoplasm represents non-specific leakage from nuclei. This was evidently the case in the early reports in which the RNA released was of 4-7S (12,34).

Our study characterizing the system (manuscript in preparation) provides evidence of processing in isolated nuclei and transport of mature RNAs only. Recent reports have determined the existence of precursor molecules of IgG_{κ} light chain in the nuclei of MOPC-21 cells (35) and of IgG_{κ} and IgG_{2b} mRNAs in MPC-11 cells (36). Only mature transcripts are detected in the cytoplasm.

Using a specific MOPC-21 κ light chain recombinant DNA probe we show evidence for the first time of the transport of a specific mature mRNA from isolated nuclei. A 24S precursor molecule is found intranuclearly and is not released to the surrogate cytoplasm.

In Adenovirus-2 infected HeLa cells polyadenylated host mRNA sequences are confined to the nucleus late in infection (37). The total synthesis and polyadenylation of host RNA is at comparable levels as those in mock-infected cells. The host RNA has not been characterized further. The confinement of host sequences to the nucleus could be due to differences in methylation, capping, splicing or to another unknown regulatory mechanism. It is possible that the translocation of processed mature RNA is prevented by a virus-induced mechanism, or by a viral-induced depletion of a cellular mechanism required for translocation of transcripts from nucleus to cytoplasm.

In the adult sea urchin, RNA sequences that are expressed in the blastulae are transcribed and retained in the nucleus (38). Similarly, globin transcripts are confined to the nucleus in non-erythroid cells (39) and in AEV-transformed chicken erythroblasts (40). In these studies the RNA has not been characterized and the retention of RNA in the nucleus could be due to one of many different mechanisms.

Khoury's group has reported that unspliced SV-40 polyadenylated mRNA is found in the cytoplasm of SV-40 infected undifferentiated F-9

teratocarcinoma cells (41); these cells are resistant to transformation by SV-40. F-9 cells can be induced to differentiate by treatment with retinoic acid. Following differentiation they become susceptible to transformation by SV-40 and the mature viral late 16S mRNA is now present in the cytoplasm of the host cell (11).

SV-40 deletion mutants in the IVS of late 16S mRNA fail to produce stable transcripts or the gene product it codes for, VPI (10). Initiation of transcription is not impaired, and the authors suggest, although they show no data supporting their hypothesis, that transcription is not affected by the absence of IVS in the DNA. They postulate that transcripts lacking the intervening sequence, and thus unable to undergo splicing, are unstable. Leder's group, utilizing SV-40-mouse β globin hybrids (8,9), have determined the absolute requirement of at least one intron-exon junction for termination of transcription. They postulate that the splicing of the IVS takes place at the nuclear envelope; properly processed transcripts are translocated to the cytoplasm, unspliced transcripts are rapidly degraded within the nucleus (9, personal communication).

A recent study suggests the role of snRNP in the splicing of HnRNA (42). The 5' end of snRNA shows extensive complementarity to the 3' and 5' preferred or consensus splice junction sequence of 26 IVS studied. A model is proposed by which the snRNP interacts with the 3' and 5' ends of the intron simultaneously and aligns the splice junctions; the snRNP could contain or be the splicing enzyme.

We have ascertained that only processed IgG_K mRNA is translocated from isolated myeloma nuclei. We are confident that this system does not result in non-specific leakage of RNA and that it can be used to study the regulation of nucleocytoplasmic interactions. In work

already in progress we are determining whether unspliced rRNA and the IVS of ribosomal and IgG_{κ} mRNA are found in the intranuclear and released fractions of our isolated nuclei. We plan to investigate the possible regulatory control of RNA translocation by specific cellular molecules.

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Figure 1. Transport of RNA from isolated myeloma nuclei. Transport determined as described in Materials and Methods.

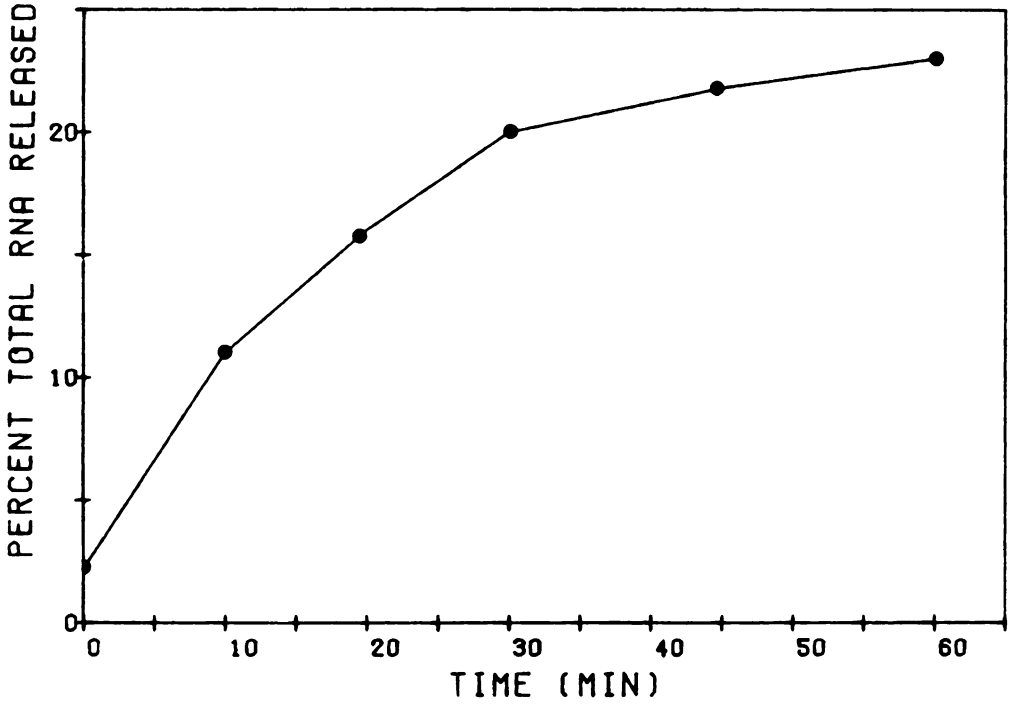

Figure 1

Figure 2. Restriction analysis of pL21-1 digested with Hind II. Two micrograms of plasmid DNA (Lanes 2 and 3) were digested following the instructions of the manufacturer. The sample was analyzed on a 5% acrylamide gel, run 75 min at 200V. The gel was stained with 1 µg/ml Et-Br. Hae III-digested ϕX174 serves as marker (Lanes 1 and 4).

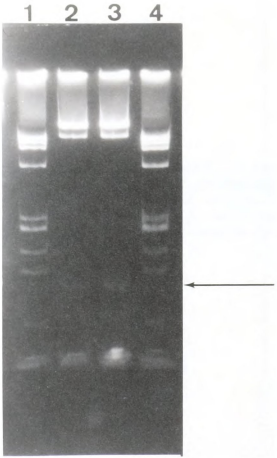

Figure 2

Figure 3. Hybridization of steady state cytoplasmic RNA with pL21-1 recombinant DNA. Cells (10^7) were labeled with 20 μCi/ml of [^{3}H]-uridine overnight. Total cytoplasmic RNA was fractionated as described in Materials and Methods and each fraction exhaustively hybridized to 5 μg of plasmid DNA.

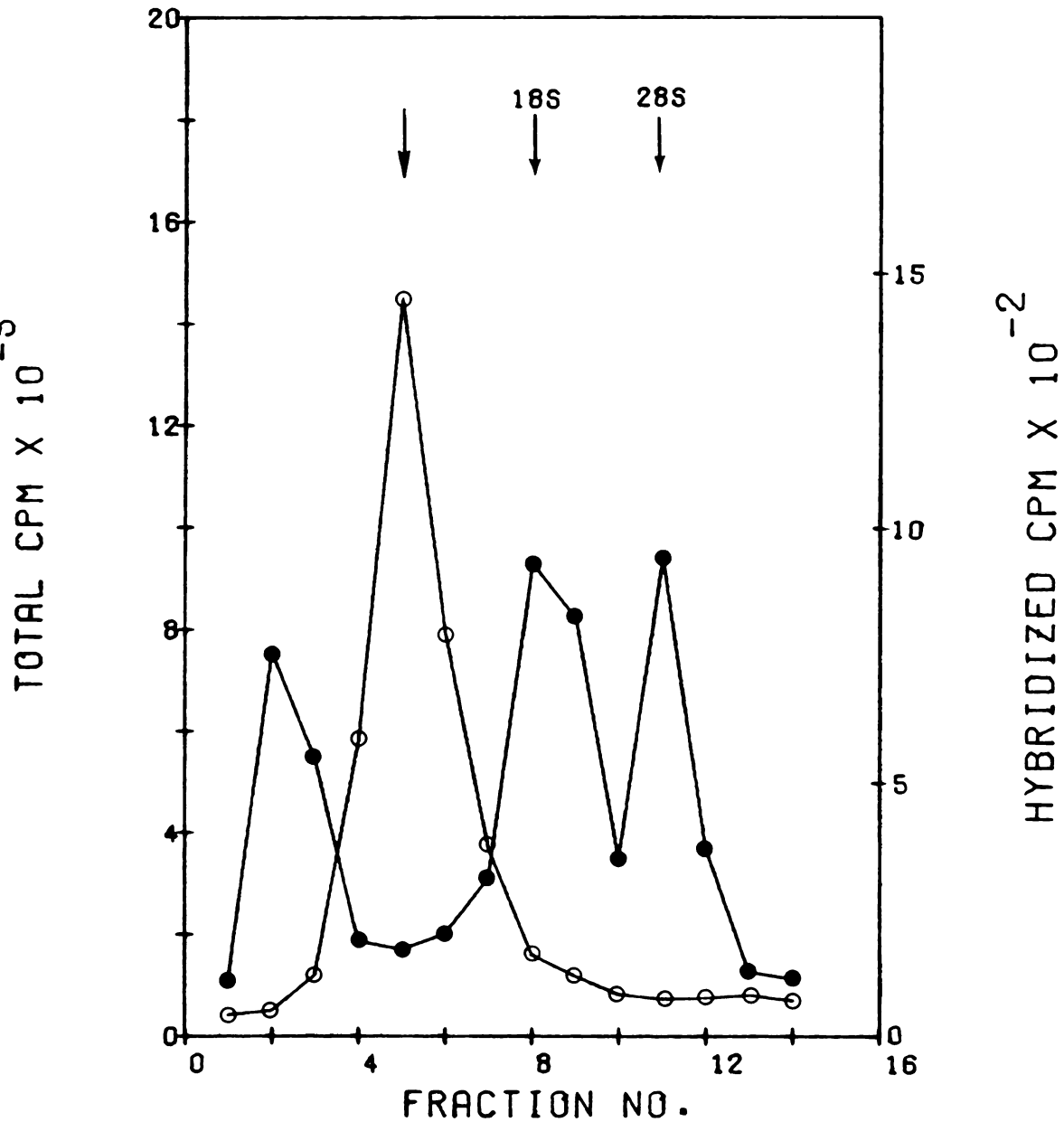
18S
28S
TOTAL CPM X 10^-5
HYBRIDIZED CPM X 10^-2
FRACTION NO.
0
4
8
12
16
20
15
10
5

Figure 3

Figure 4. Hybridization of intranuclear and transported RNA with recombinant DNA. Cells (10^8) were prelabeled for 45 min. Nuclei were isolated as indicated in Materials and Methods and incubated 30 min at 25°C in the transport assay. RNA was isolated from the intranuclear and the transported fractions and analyzed on sucrose-SDS gradients as described. Individual fractions were exhaustively hybridized with recombinant plasmid DNA. A) Total intranuclear RNA. B) Total transported RNA.

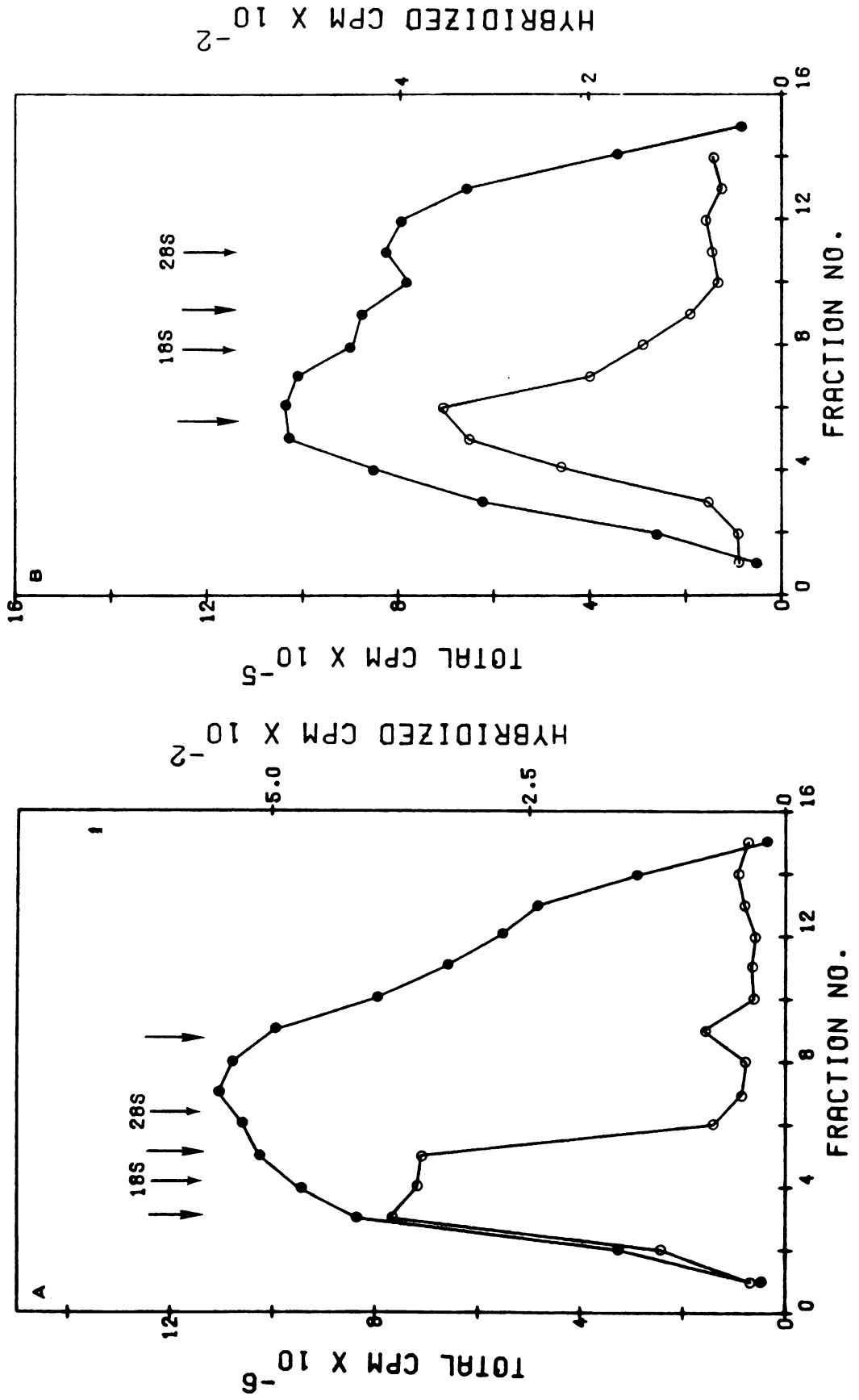
A
B
18S
28S
FRACTION NO.
TOTAL CPM X 10^-6
HYBRIDIZED CPM X 10^-2
TOTAL CPM X 10^-5
HYBRIDIZED CPM X 10^-2

Figure 4

Figure 5. Hybridization of poly A(+) intranuclear RNA with recombinant DNA. Cells (10^8) were prelabeled for 60 min. Nuclei isolated as indicated in Materials and Methods and incubated 30 min at 25°C in the transport assay. Intranuclear RNA was isolated and purified over oligo d(T)-cellulose. The poly A(+) fraction was analyzed on sucrose-SDS gradients as described. Individual fractions were exhaustively hybridized with recombinant plasmid DNA.

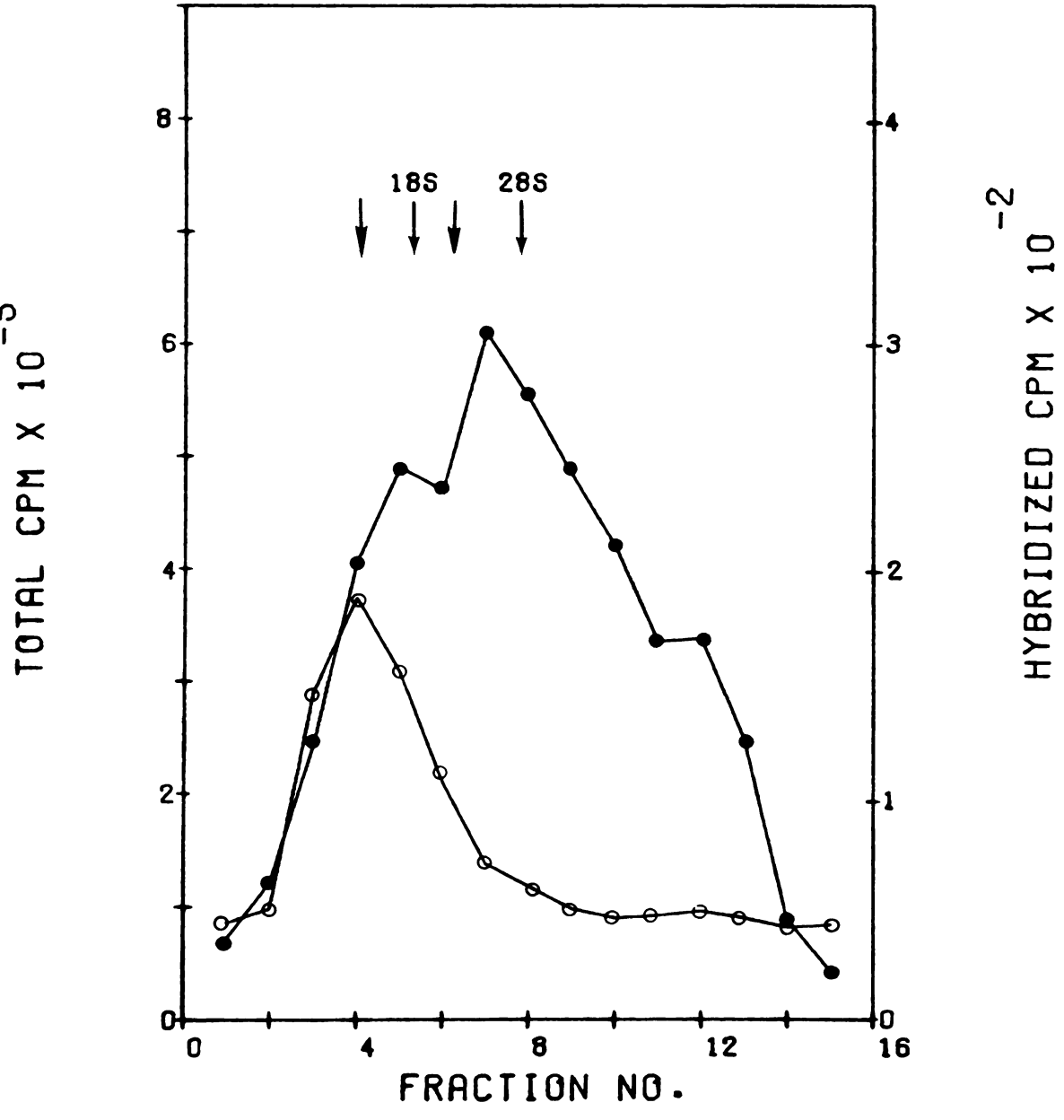
18S
28S
TOTAL CPM X 10^-5
HYBRIDIZED CPM X 10^-2
FRACTION NO.
0
2
4
6
8
0
1
2
3
4
0
4
8
12
16

Figure 5