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IN NORMAL AND ADENOVIRUS-INFECTED CELLS

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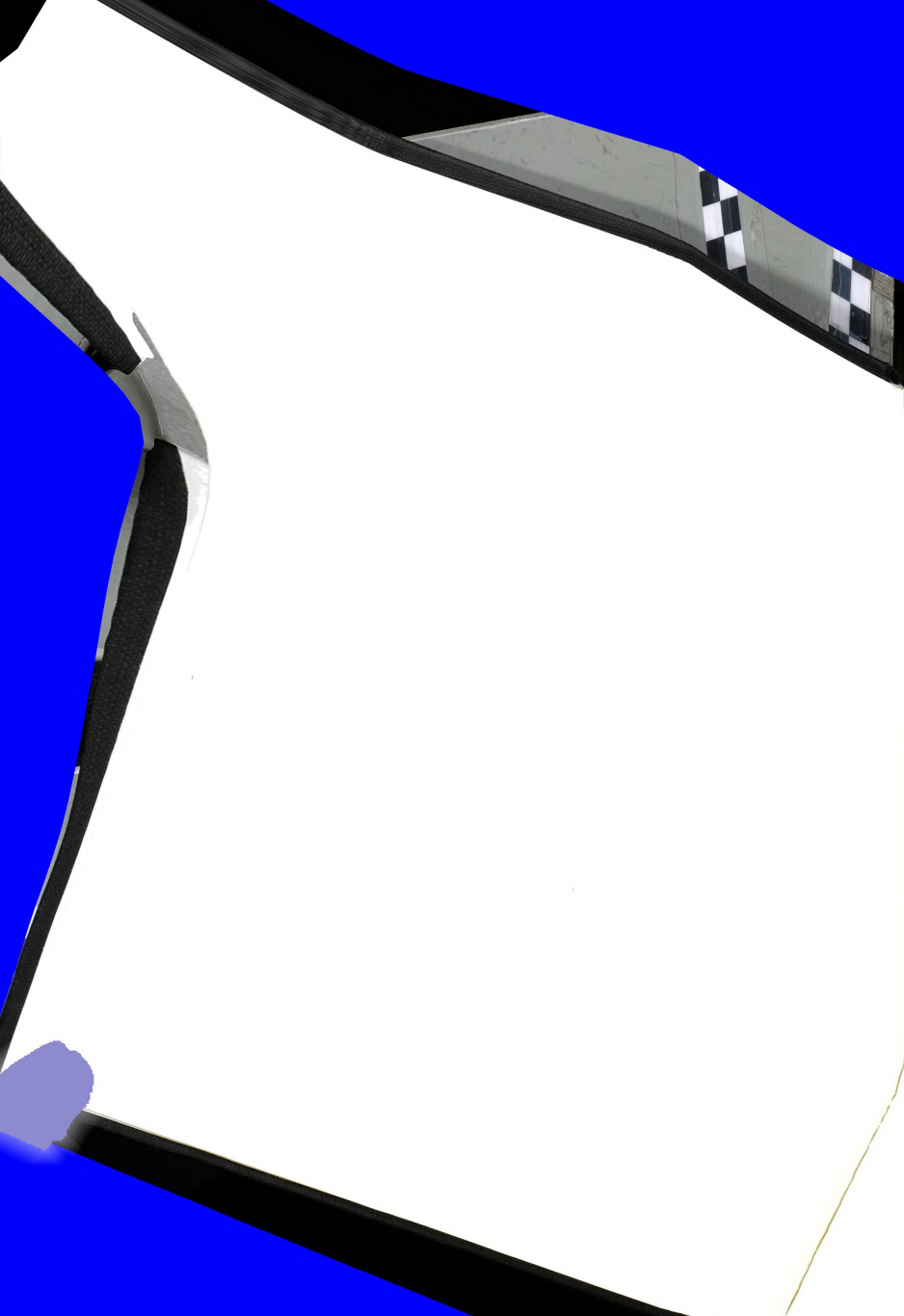

IDENTIFICATION AND CHARACTERIZATION OF THE NUCLEAR RNA TRAFFICKING PATTERN IN NORMAL AND ADENOVIRUS-INFECTED CELLS

By

Roger Martin Denome

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF THE NUCLEAR RNA TRAFFICKING PATTERN IN NORMAL AND ADENOVIRUS-INFECTED CELLS

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The study of nuclear partitioning of RNA has received very little attention as a possible area of regulation of gene activity. The physical location of RNA transcription and processing has been ascribed to the nuclear matrix, which is a salt-insoluble proteinaceous network that fills the nuclear space and is contiguous with the lamina and pore complexes. Described here are experiments that determine the fate of nuclear RNA after it has completed these matrix-associated processing steps. Pulse-chase and continuous label experiments indicate that after this RNA is processed it changes its state of attachment in the nucleus such that it is now removed from the nucleus in the high salt extraction step of matrix isolation. It is this salt-extractable RNA that will be transported to the cytoplasm. Late in adenovirus infection, when transport (but not transcription or processing) of cellular sequences is decreased, these sequences do not make the transition from the matrix-associated to the salt-extractable nuclear pool. The implication of these data on the regulation of gene expression in both virus infected and normal cells is discussed.

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INTRODUCTION

RNA metabolism has received a great deal of attention. Studies investigating transcription, various processing steps, and translation have revealed the complexity in these processes and have advanced our understanding of the basis of gene regulation to a point where a great many regulatory phenomena can be understood on the molecular level. One area of RNA metabolism that has not received much attention is the actual movement of RNA within the cell and the effect of this movement on gene expression. Described here is a set of experiments designed to determine if there is indeed some organized partitioning of RNA within the nucleus of a eukaryotic cell, and if so, whether this partitioning is used as an additional level for regulation of RNA metabolism.

The idea of gene regulation at the level of RNA partitioning is not new. Messenger RNAs are sequestered in the cytoplasm of amphibian oocytes. These RNAs are not available to the translational machinery, thus allowing for the storage of the large amount of mRNA required for the early stages of development of the organism.

The work described here addresses two questions that have not been addressed directly before:

1. Is there some physical partitioning of nuclear RNA that separates this RNA into functionally different groups?
2. If there is some physical partitioning of RNA within the nucleus, is this partitioning used as a means of regulating gene expression?

The importance of this work, regardless of its conclusions, in the advancement of our knowledge of gene regulation cannot be estimated. This study attempts to determine whether the potential for gene regulation at this level exists. This work examines the possible role of regulation at this level in the massive effects on cellular RNA metabolism caused by adenovirus infection. Further, more subtle uses of these mechanisms for regulation of cellular phenotype and differentiation await both more sensitive techniques for the analysis of nuclear RNA populations and a great deal more basic research into the structure of the nucleus and its relationship to RNA metabolism. From this point of view, this research is just a first step into a field that holds great potential for the understanding of gene regulation and cellular differentiation.

LITERATURE REVIEW

Intranuclear RNA movement is a subject about which almost nothing is known. A great deal of work has been done on characterization of RNA transcription, processing, and translation; the association of RNA with other macromolecules during these processes has also been described in some detail. RNA transport from the nucleus has received less attention, although some advances have been made in the area of defining *in vivo* and *in vitro* model systems that can be used to study this phenomenon. Information from studies in all of these areas bears on the subject of intranuclear RNA trafficking.

This review is an attempt to pull together all of the pertinent data bearing on the subject of intranuclear RNA partitioning and movement from all of these fields. RNA processing, nuclear architecture, and nucleocytoplasmic RNA transport will be reviewed here. This is not an attempt to cover these subjects in depth; rather the information in these general areas that bears upon intranuclear RNA movement will be described. Where possible, major reviews of the general subject discussed will be cited, since much of this work has been reviewed in much more depth than will be used here.

RNA processing

Eukaryotic organisms usually synthesize a primary RNA transcript that requires several processing steps to produce a functional RNA. These modifications vary from transcript to transcript, and in fact may be different for the same primary transcript made at different times or in different cell types. For example, primary transcripts of the immunoglobulin μ heavy chain genes are processed differently depending on whether the protein produced is to be membrane bound or secreted (4, 49, 67). The murine α-amylase mRNA exhibits tissue-specific polyadenylation, which may result in either a differential stability or differential affinity for translational machinery (95).

Primary transcripts produced by a given RNA polymerase seem to share common processing pathways. Ribosomal RNA undergoes a series of modifications that produces three mature rRNAs (5.8S, 18S, and 28S) from a 45S primary transcript (31). This processing involves the removal of external and internal transcribed spacers from this primary transcript, plus internal methylation.

Messenger RNAs are produced by a more variable processing pathway. Primary transcripts are "capped" by the addition of a methyl guanosine in the structure $m^7G(5')ppp(5')X$ where "X" is the 5' most nucleotide of the transcript (reviewed by Shatkin, 75). This

structure, or a modification of it, has been found on every mRNA examined from eukaryotic cells. Capping occurs extremely quickly after the initiation of RNA transcription, and probably occurs on the nascent chain (7, 69).

Precursors to mRNAs also undergo endonucleolytic cleavage at their 3' end, followed by the addition of polyadenylic acid at a point near this incision (reviewed by Brawerman, 21). This processing step does not seem to be as universal as the production of the 5'cap structure and the frequency of polyadenylation seems to be both species and RNA sequence dependent. For example, histone mRNAs are rarely polyadenylated; a large minority of mRNA in Drosophila (about 25%) is not polyadenylated, whereas all yeast messages examined are polyadenylated. The length of the polyadenylic acid (poly(A)) tail also varies from RNA to RNA.

Precursors to mRNAs are also spliced to remove nucleotide sequences that are not present in the mature mRNA (reviewed by Breathnach and Chambon, 22). The function(s) of these "introns" that are removed is not known, and not all primary transcripts contain them.

Finally, mRNAs are methylated at internal nucleotides, usually at adenosines in the sequences Gpm^{6}ApC or Apm^{6}ApC. This methylation usually occurs in the nucleus and is conserved during the processing of precursor RNA (43). Inhibition of methylation in SV40-infected

cells with cycloleucine inhibits the production of mRNA, but not the synthesis of hnRNA (32). Similar results suggesting that methylation is important for processing and nucleocytoplasmic RNA transport of cellular mRNA have been reported (25).

RNA polymerase III products (small nuclear RNAs (snRNA), tRNAs) are also processed from primary transcripts that are longer than the mature RNA. The processing of the snRNAs will be discussed in the section on RNA transport since their processing occurs after the RNA is transported to the cytoplasm. Transfer RNAs are produced by endonucleolytic scission of long primary transcripts to produce a mature-sized tRNA. Further base modification of this product is required before the RNA is transported and functional (45, 97).

All of the modifications described above, with the exception of those occuring on snRNAs, occur in the nucleus and are presumably required for normal RNA transport and function. There are some RNA species that are restricted to the nucleus (see 73 and 74 for examples); the function of these is unknown, although some snRNAs have been implicated as structural elements in the nuclear matrix (86, see below). There does not seem to be any consistent differences between transported RNAs and nuclear restricted ones, nor does there seem to be a common biochemical characteristic of all RNAs that are transported.

Nuclear architecture

The study of intranuclear RNA movement must take into account information supplied by ultrastructural studies of the nucleus. Without some physical framework to accompany any biochemical descriptions of RNA processing and transport, the description of RNA traffic within the nucleus will never be complete. Fortunately, the picture that has emerged from a broad range of studies on nuclei from many species is one of suprising consistency in nuclear structure. Because of this consistency, generalizations using data obtained from species in a broad phylogenetic spectrum can be considered.

If the nucleus of a cell is stripped of its membranes, digested with DNase, and extracted with high salt concentrations, the resulting insoluble structure looks very much the same as the original nucleus (13, 76). It retains the same shape, size, and organization of nucleoli as are observed in the original nucleus. This DNA-depleted salt-stable nuclear structure, the nuclear matrix, has been found in rat liver (48), Tetrahymena macronucleus (91), Friend erythroleukemia cells (51), HeLa cells (17, 84), BSC-1 cells (12), chicken liver (10, 20) and ventral prostate (10), murine 3T3 cells (40), chicken oviduct cells (28), and chicken erythroblasts and erythrocytes (68). Its structural constituents after DNase digestion are mainly protein and RNA. Extraction of membrane-free DNA-free nuclei with high salt concentrations removes a large portion of the RNA but leaves the

structure morphologically unchanged. Digestion of the matrix with protease destroys it morphologically. Digestion of the matrix with RNase has left the matrix structurally intact in most cases. There is one report of RNase digestion destroying the integrity of the matrix (26); whether this was an artifact of the system being used or whether there are in fact some structural RNAs in the matrix is unknown. There is also one report suggesting that a small nuclear ribonucleoprotein particle (snRNP) containing snRNA is a structural constituent of the matrix. This class of particles was detected using immunofluorescense techniques and antibodies specific for a subgroup of the snRNPs (88). Again, it is impossible to confirm or deny this from the available literature.

The matrix structural proteins fall into two classes. The lamina proteins comprise about 40-60% of the matrix proteins (13, 38). The remaining proteins are of heterogeneous size. Actin and vimentin are present, probably as components of the matrix that correspond to thin and intermediate cytoskeletal elements (26). Electronmicroscopic analysis of the matrix shows a network of heterogeneous-sized fibers filling the nuclear space and contiguous with the peripheral lamina. The nucleolus is seen as a darkly-staining body imbedded within this network.

A number of properties have been attributed to the matrix. All are consistent with the idea that the matrix is used as a "workbench" for

nuclear functions. DNA binding has been ascribed to the matrix, as a way of holding chromatin in the 40-60 kilobase pair loops that are found in eukaryotic nuclei (57), as a site of DNA replication (63), as a method of aligning interphase chromatin (2), and as the site of transcription (see below). The site of attachment of the DNA to the matrix of murine 3T3 cells (40) and Drosophila Kc cells (57) is DNA sequence dependent, and the proteins involved in the attachment of HeLa DNA to the matrix seem to share extensive homology with other such proteins found in a wide variety of organisms (17, 60). Electron microscopic autoradiography of nuclei labelled for 1 minute with ^{3}H-thymidine in vivo shows that 85% of the newly-labelled DNA is associated with the interior of the nucleus. Similarly labelled nuclei that were fractionated into either matrix or lamina suggested a similar distribution of newly-synthesized DNA. Assays of the nuclear matrix and lamina for DNA polymerase activity found DNA polymerase α activity specifically associated with the interior of the matrix, consistent with its role in normal cellular DNA replication. DNA polymerase β activity copurified with the lamina portion of the nucleus. DNA polymerasee β activity, which is involved in repair synthesis, has been localized to the perilaminar nuclear space by autoradiography (77). The genomes of SV40 (1), adenovirus (94), and influenza (42, 44) viruses are matrix-associated in infected cells, as are newly-synthesized RNAs from adenovirus (54, 55), SV40 (12), influenza (44) and host cell (86) genomes. Estradiol and dihydrotestosterone bind specifically to the nuclear matrix of

liver and prostate of chickens competent to bind these hormones. These hormones did not bind to tissues that were not normally target tissues for these hormones. Isolated nuclear matrix from these target tissues was also able to specifically bind these hormones (10). This indicates that the matrix may be the point of action for their modulation of transcription. Indirect immunofluorescense using antibodies specific for adenovirus $E1A_a$ protein (30) and SV40 T antigen (98), which are know to modulate transcription of cellular and viral sequences (18, 30, 37, 46, 62, 87, 98), has localized these proteins to the matrix in infected cells. There is some disagreement in the literature as to whether or not actively transcribed DNA is preferentially associated with and protected from nuclease digestion by the matrix. Robinson et al. (66) found that the chicken ovalbumin gene was closely associated with the matrix after DNase I digestion in oviduct cells but not in chicken liver, suggesting that the matrix association does indeed offer some nuclease protection to actively transcribed genes. However, Ross et al. (68) found no such protection of the β-globin gene in a similar study in erythroblasts.

High molecular weight hnRNA is associated with the matrix (55, 86), and when individual transcripts are examined, both mature- and precursor-sized molecules are found in association with the matrix. This has been shown in chicken oviduct cells using probes for ovomucoid and ovalbumin mRNA (28), chicken erythrocytes and

erythroblasts using probes for globin mRNA (68), and Tetrahymena macronuclei using probes for rRNA and its precursors (41).

All of these data argue that the matrix plays a central role in nuclear macromolecular metabolism. What other structure(s) may be involved in RNA transport is unknown. The fact that most of the studies on matrices involve extraction of DNA-depleted nuclei with high salt concentrations makes analysis of other less stable nuclear structures difficult. There is some controversy over the exact in vivo disposition of the matrix. (See references 20 and 96 for example.) Some of this uncertainty may be the result of the sensitivity of the matrix to variations in isolation procedure. Changing the order of DNase and salt washes or leaving out protease inhibitors during isolations may have lead to poor matrix isolation that in turn leads to a variety of misinterpretations (48). One example of this is probably the work by Berezney and Coffey (13) on protein components of the matrix. They found that the matrix was composed of three proteins of 60-70 kilodaltons. These are in fact the sizes of the lamina proteins (38), indicating that the preparation of matrices was not performed correctly.

Only two publications examine the RNA extracted by the high salt washes. Ciejek et al. (28), working with chicken oviduct nuclei, separated this RNA electrophoretically on denaturing agarose gels, and then transfered it to nitrocellulose. This was then probed with

nick-translated DNA probes for ovalbumin and ovomucoid mRNA. Only mature-sized RNA was found. A similar study by Ross et al. (68) found equivalent results using chicken erythroblasts and erythrocytes and probes for globin RNA. These data, along with the fact that unprocessed precursors to mRNA and rRNA are always associated with the matrix, argue for some key role for the matrix in RNA processing.

RNA transport

RNA transport from the nucleus has been studied using a variety of approaches, all of which have their disadvantages. Several groups have worked with isolated nuclei in attempts to develop *in vitro* systems. These have met with only dubious success, mainly because of the lack of proper controls on the types of RNA transported. One of the main conclusions to be drawn from the data resulting from several of these papers is that RNA is in fact transported from the nucleus, and that the double membrane surrounding the nucleus has little to do with this process. Immunoglobulin κ light chain mRNA (82) and rRNA (39) are transported with the same efficiency from nuclei, regardless of whether they are membrane bound. In *Tetrahymena*, the amount of rRNA transport is directly proportional to the temperature of the medium in which the cells are grown (39, 89). This regulation has been shown to be a property of some intranuclear structure, and is believed to involve some "temperature sensitive domain of the nuclear matrix" (90).

Others have worked with developmental systems where expression of a given RNA is controlled over time by some internal or environmental stimulus. The major system for this work has been the modulation of transport of cellular RNAs in adenovirus infection, with some additional information coming from work on the "heat shock response" in Drosophila, CHO and HeLa cells. A third area of study is the characterization of RNA-associated macromolecules. Work on both the nuclear and cytoplasmic RNA-associated proteins and RNAs has led to the description of a large group of associations which may or may not have something to do with RNA transport.

Infection of HeLa cells with adenovirus brings about a set of massive changes in cellular metabolism. These changes all result in the eventual takeover of the cellular machinery to produce virus. Adenoviral infection is divided into early and late periods, with the delineation between the two being the onset of viral DNA replication. Early in infection, cellular metabolism is effected only slightly (27). Cellular RNAs are synthesized and transported to the cytoplasm at normal rates, and translation of cellular mRNA occurs normally. During this time, the expression of heat shock (46, 62) and β-tubulin (79)genes is increased by the protein coded for by early region 1A (E1A) of the virus. Whether this altered gene expression is responsible for events seen later in the infection is unknown, although the transcription of heat shock genes is back to preinfection levels by the time viral DNA synthesis begins (62).

Late in infection, transcription from the late viral promoter results in a large increase in the amount of viral RNA in the nucleus and cytoplasm (3, 11, 27). The processing of this adenovirus-encoded RNA seems to follow the same pathway as cellular RNAs (3, 7, 14, 53, 55, 61, 98). The transport of cellular polyadenylated RNA (6, 11) and rRNA (27) to the cytoplasm is reduced to almost undetectable levels, even though the transcription (6) and processing (34) of these sequences seems to occur at normal rates. Very late in infection transcription and processing of ribosomal RNA is inhibited (27), although this may just be due to the lack of ribosomal proteins that are required for transport of rRNA from the nucleus.

Several investigators have looked for the block to cellular RNA transport, either by looking at total cellular hnRNA and rRNA, or at specific cellular and viral RNA sequences late in infection. Nuclear viral RNAs seem to be associated with the same proteins as cellular RNA molecules (16, 59, 83). Both total cellular poly-A-containing RNA (11) and rRNA (27) transport is reduced to almost zero, although transport of rRNA continues later in infection than that of mRNA (27). Examination of individual RNA sequences late in infection has revealed a slightly more complex picture. The transport of some cellular sequences, such as the mRNA coding for dihydrofolate reductase (DHFR), is inhibited only 4 fold (93). Furthermore, about half of the reduction seen in DHFR mRNA transport can be accounted for by a reduction in the nuclear stability, but not the

transcription rate, of the transcripts late in infection (92). (Adenovirus causes an analogous loss of stability of the cytoplasmic heat shock RNAs late in infection (62).) Other RNAs are not transported at all in late infection, and the transcription of histone mRNA is inhibited late in infection (35). These investigations have not yielded either a consistent cause for the loss of cellular RNA transport nor have they localized the block in RNA transport to some subnuclear processing step. The inhibition of cellular RNA transport is not due simply to a "swamping" of the transport system by large amounts of adenoviral RNA, since about 50% of the RNA in the nucleus late in infection is cellular in origin (11). Very little is known about the viral functions that cause the loss in cellular RNA transport. Babiss and Ginsberg (8) have described an adenovirus mutant that is missing the protein coded by the early region 1b gene and does not inhibit host cell RNA transport late in infection. Similar results with a deletion mutation affecting the smallest early region 4 protein product were found by Sarnow et al. (70). Interestingly, this protein is normally associated with the matrix late in infection.

The linkage of the template for a given RNA seems to have a some effect on whether or not that RNA will be transported late in infection. When the entire human α-globin gene was inserted into the adenovirus genome, it was transcribed and transported to the cytoplasm late in infection (71). These data indicate that physical

linkage of the template for transcription to the viral genome is enough to allow RNA transport. Consistent with this hypothesis is a report that influenza virus transcripts are transported in cells doubly infected with adenovirus and influenza (47). These data are at odds with the information on DHFR mRNA presented above, since linkage of that gene to the cellular genome does not seem to result in inhibition of RNA transport. In an experiment analogous to the work on α-globin described above, 293 cells, which have a copy of the adenovirus E1 gene integrated into the cellular genome, were infected with an adenovirus mutant deleted for the E1 cistron (78). Late in infection, the cellular copy of the E1 gene was transcribed and transported to the cytoplasm, indicating that something beyond simple linkage is controlling RNA transport.

Work with heat-shocked Drosophila, CHO, and HeLa cells has led to contradictory information on the effects of physiological stress on RNA transport. In Drosophila, heat shock results in the inhibition of normal cellular RNA synthesis (33), the inhibition of rRNA processing, and the enhanced transcription and translation of a set of RNAs coding for "heat shock proteins" (56). The amount of protein associated with the nuclear RNA after heat shock is reduced by a factor of 20 (56). However, in these experiments there was no preferential association of the heat shock RNA with the protein that is still associated with RNA. Heat shock of CHO cells results in essentially the same effect as heat shock in Drosophila (19).

Furthermore, processing of rRNA is aberrant and the resulting "misprocessed" rRNA is never transported to the cytoplasm, even after normal RNA processing and transport is resumed at normal temperatures. In HeLa cells, the amount of protein associated with nuclear RNA during heat shock is reduced compared to normal levels (56), similar to what is seen in Drosophila. Hela cells continue to transcribe RNA coding for non-heat shock proteins during heat shock, but most of the RNA that is transported to the cytoplasm is that coding for heat shock proteins. The association of heat shock with the loss of RNA transport from the nucleus is a tenuous one. As mentioned above, adenovirus infection induces the production of heat shock protein early in infection. During this period cellular RNAs are transcribed and transported at normal rates.

It has been suggested that the association of various macromolecules with RNA in the nucleus and cytoplasm could be used as signals or mechanisms for RNA transport. The proteins that are associated with the RNA at various times during the transcription and transport process have not been assigned any functions. Nuclear RNA seems to become associated with proteins over its entire length while it is being transcribed (15), and these proteins seem to have some sequence specificity. Electronmicrographs of transcription complexes show a regular pattern of proteins associated along the length of the RNA. Nuclease digestion studies of polyoma virus RNA suggest that the late polyoma virus RNAs are also completely covered with proteins; some

sequences seem to be extremely resistant to RNase digestion (80). Similar studies on nuclear adenovirus RNA came to the same conslusion (59, 81). Studies by Augenlicht (5) have found that the oligonucleotides protected by protein associations do not have a unique sequence but do seem to be enriched for guanosine and cytosine residues and the sequences AGC, AGGC, and GAGC. There is a 78 kilodalton poly-A binding protein that is associated with the poly-A-tails of nuclear RNA (72). A 60 kilodalton protein seems to be associated at regular intervals along the poly-A tails of cytoplasmic RNA (9, 14, 72). Nuclear ribonucleoprotein particles have been isolated. There are 6 proteins (28-40 kilodaltons) associated with the 30S "monoparticles" and the multimeric forms of this basic particle (16, 50, 59, 64, 65, 79). In contrast, three proteins (120, 76, and 52 kilodalton) are bound to cytoplasmic polysomal poly-A-containing RNA (50). In addition, most of the investigations have described extra, higher molecular weight RNA-associated proteins that are present in low abundance. Several groups have looked for differences between the RNA-associated proteins in normal and adenovirus-infected HeLa cells (16, 83, 86). No major differences have been found, although there is one report of a protein that is preferentially, but not exclusively, associated with adenovirus mRNA in the nucleus late in infection (85).

It has been suggested that snRNAs might play a role similar to the one suggested for RNA-associated proteins. There is good evidence for a role for the snRNAs in RNA splicing (23, 24, 36, 58); whether these or some other RNA(s) are used as a signal for transport is not known.

The processing and transport of tRNA has been studied in some detail. The final RNA is clipped from a large precursor (45) and transported to the cytoplasm by what is probably a carrier mediated transport system (97). Inhibition of the initial processing events inhibits both base modification and transport.

The nucleocytoplasmic transport of snRNA has been studied and can be used as an example of the variety that may exist in mechanisms of RNA transport. These RNAs are processsed from a large transcript and transported to the cytoplasm. There they become associated with a specific set of proteins, and an additional 1-8 nucleotides are removed from the 3′ end of the molecules. The RNA-protein complex is then transported back to the nucleus where additional nucleotides may be removed from the 3′ end (52). This processing pathway must be vastly different than that used by other cellular RNAs, but it serves as a reminder that the "RNA transport system" may not be one or even a few systems.

Conclusion

Several conclusions can be drawn regarding RNA transport from the nucleus and the types of processing that must take place before transport can occur. Both mRNA and rRNA must be completely processed before they can be transported to the cytoplasm; intact precursor-sized molecules are not found in the cytoplasm, although there are reports of free introns (29) and external transcribed spacers from rRNA primary transcripts (31) in the cytoplasm. Although nuclear RNA stability may be used as a means of controlling transport in some cases (as in the case of DHFR mRNA), this does not seem to be a general phenomenon. The same can be said about the use of transcriptional modulation as a means of controlling transport. The site of RNA transcription and processing is almost certainly the nuclear matrix; other areas of nuclear architecture involved in RNA trafficking and transport have not been described. Because some RNAs are nuclear restricted, there must be some form of sequence-specific RNA transport regulation, but to what extent this and other factors control transport is unknown. Adenovirus infection obviously presents an excellent system for perturbing nuclear trafficking of transcripts; it also has led to a genetic system that might be useful in describing both the factors that are normally involved in this process and how this process might be taken over during adenovirus infection. Although the snRNAs and tRNAs obviously have a mechanism

of processing different from that of mRNA and rRNA, the fact that mRNA and rRNA seem to react similarly to adenovirus infection suggests that, although there is certainly not a single RNA transport system, there may be only a few such systems.

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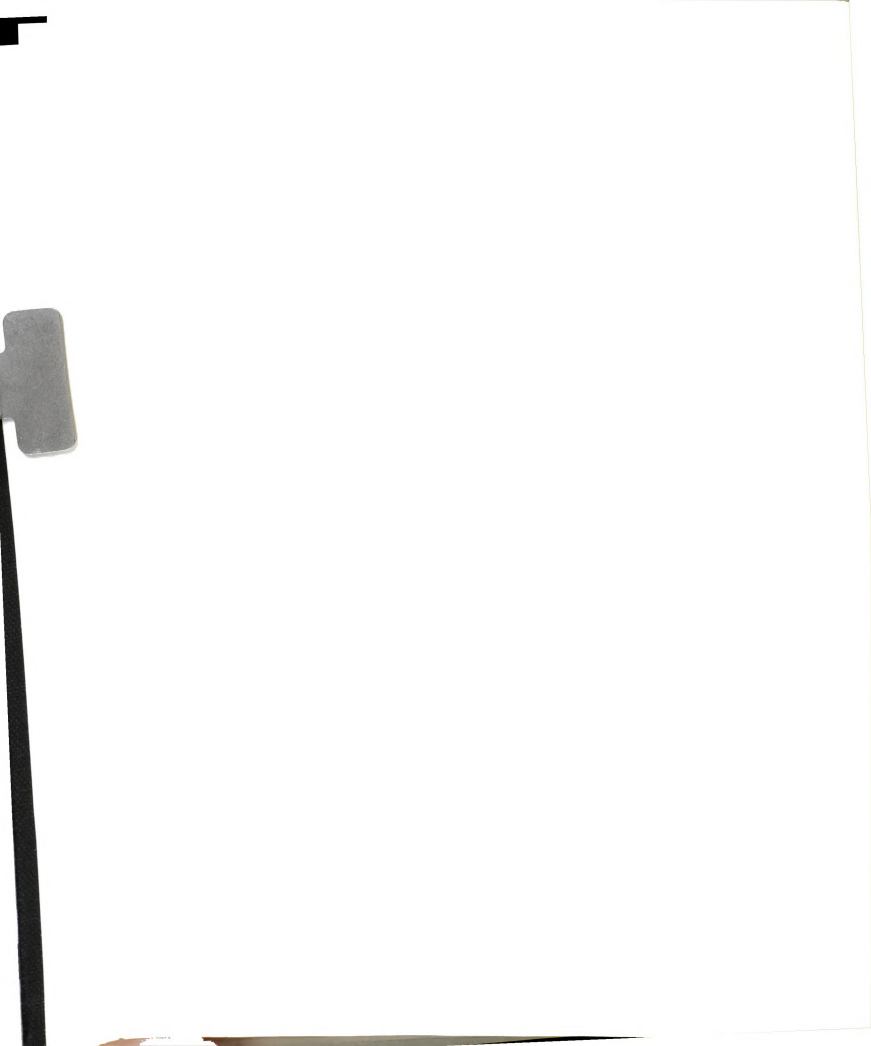

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INTRANUCLEAR RNA TRAFFICKING

INTRODUCTION

Maturation of eukaryotic ribosomal and messenger RNA involves a variety of processing steps. Production of a cap structure (41), endonucleolytic cleavage and addition of polyadenylic acid (9), internal methylation (24), and removal of intervening sequences from mRNA precursors (10) or transcribed spacers from rRNA primary transcripts (15) are required for the production of a functional RNA. All of these processes are intranuclear; obviously, the additional step of transporting the RNA out of the nucleus is required before the mRNA or rRNA can be functional *in vivo*. This process of nucleocytoplasmic RNA transport is poorly understood. The completion of the above mentioned maturational events is almost certainly not the rate-limiting step in RNA transport, since in many instances (11, 16, 20, 23, 36) the amount of mature-sized RNA present in the nucleus far exceeds the amount of precursors for that RNA. This indicates that something other than the completion of RNA processing is required for transport.

To attack the problem of what, besides "normal" RNA processing, is required for RNA transport, we have used HeLa cells infected with

adenovirus as a model system in which RNA transport has been perturbed. Late in adenovirus infection, host cell-specific RNA species are transcribed (2, 16), polyadenylated and processed normally (16). However, these sequences are not transported to the cytoplasm (2, 4, 11, 16). Adenovirus-encoded RNA, which undergoes essentially the same processing steps as cellular RNA (6, 30, 35, 48), is transported in large quantities at this time (4).

Where is this block to cellular RNA transport? Is it at the point of exit from the nucleus, or is there a block at some other step between the completion of processing and the final departure from the nucleus? Obviously, an understanding of the physical and functional relationships between precursor and mature RNAs within the nucleus is required before a complete understanding of the transport process is possible.

The physical location of transcription of both rRNA and mRNA (cellular (12, 38, 39) and adenoviral (31)) has been ascribed to an intranuclear proteinaceous network called the nuclear matrix. The matrix and its associated RNA can be isolated from membrane-denuded, DNA-depleted nuclei by extraction with high salt concentrations (5, 42). The RNA in the salt-insoluble fraction (i.e. matrix-associated RNA) consists of both mature and precursor-sized molecules for mRNA (12, 31, 39, 49) and rRNA (23). The salt-soluble fraction (i.e. salt-extractable RNA) has been

analyzed less thoroughly. In the salt-extractable RNA from chicken oviduct nuclei, Ciejek et al. (12) found 18S and 28S rRNA and mature-sized mRNA hybridizing to probes specific for ovomucoid and ovalbumin RNA but no precursor-sized molecules for these species. Similarly, Ross et al. (39) found only mature-sized globin RNA when the salt-extracted material from erythroblast and erythrocyte nuclei was probed with a globin cDNA. These observations suggest that RNA processing occurs in association with the matrix.

In this report we describe experiments designed to:

1. determine the functional relationship between the salt-extractable and matrix-associated nuclear RNA pools, and

2. identify the major block to the transport of cellular RNAs late in adenovirus infecton.

We will show that mRNA and rRNA precursors are processed to mature-sized molecules in association with the nuclear matrix. This RNA then changes its state of attachment in the nucleus such that it can now be removed with 0.5 M NaCl. It is the RNA in this salt-extractable pool that will be transported to the cytoplasm. Furthermore, we will show that the block to the transport of cellular RNA late in adenovirus infection is at or before the movement of cellular sequences from the matrix-associated to the salt-extractable pool.

MATERIALS AND METHODS

Cell growth and labelling conditions P_3K myeloma cells were grown in Dulbecco's minimal essential medium plus antibiotics and 10% horse serum. HeLa cells were grown in Eagle's minimal essential medium plus 2 mM glutamine, non-essential amino acids, antibiotics, and 10% newborn calf serum. Both cell types were grown in spinner flasks at 37°C in a 5% CO_2 humidified incubator. For labelling, cells were concentrated 10 fold (to 5-10 X 10^6 cells/mL) and incubated with 100-200 µCi/mL ^{3}H-uridine in normal growth medium for the indicated times.

Cell fractionation and RNA isolation Cell lysis and nuclear fractionation were essentially the same as those of Ciejek et al. (12). Cells were pelleted and washed 1-2 times in RSB (10 mM NaCl, 10 mM Tris HCl, pH 7.4, 3 mM MgCl, 50 µg/mL phenylmethylsulfonyl fluoride, Sigma), lysed either by Dounce homogenization or treatment with 1% Nonidet P-40 (NP-40), and the nuclei were pelleted at 700 X g for 4 minutes. The supernatant was treated as total cytoplasm. Nuclei were washed in RSB, stripped of their membranes with 1% NP-40 if isolated by Dounce homogenization, and then treated with 0.2 mg/mL RNase-free DNase I (32) for 30 minutes at 0°C. Nuclei were pelleted as above, resuspended in RSB, and brought to 0.5 M NaCl with the gradual addition of 5 M NaCl while vortexing slowly. Nuclei were then incubated at 0°C for 15 minutes

and pelleted. The supernatant was treated as the salt-extractable pool. The pellet (i.e. the matrix fraction) was resuspended in RSB, and cytoplasmic, salt-extractable, and matrix pools were brought to 5% sarkosyl. RNA was isolated as described by Fetherston et al. (15).

Adenovirus isolation and infection conditions Adenovirus was isolated using the procedure of Munroe (34). Cells were infected essentially as described by Beltz and Flint (4). Briefly, cells were pelleted, and resuspended in medium at 1 X 10^7 cells/ml. Adenovirus was added and cells were incubated at 37° C for 1 hour with stirring. Cells were diluted to 5-10 X 10^5 cells/mL and harvested at times indicated. Adenovirus infections were performed at virus concentrations that gave complete inhibition of actin RNA transport to the cytoplasm 20 hours post-infection.

Pulse-chase conditions Pulse-label conditions were essentially those of Beltz and Flint (4). Briefly, cells were pretreated in medium with 15 mM glucosamine for 45 minutes, and 15 mM glucosamine plus 0.08 µg/mL actinomycin D for 15 minutes. Cells were pelleted, resuspended at 1-2 X 10^7 cells/mL in medium plus 0.08 µg/mL actinomycin D and 0.2-0.5 mCi/mL ^{3}H-uridine. At the end of the pulse, the cells were added to 5 volumes of cold medium, pelleted, and resuspended at 1 X 10^6 cells/mL in warm medium plus 15 mM glucosamine, 0.08 µg/ml actinomycin D, 10 mM uridine and

cytidine. Cells were removed at indicated times, added to 2-3 volumes ice cold RSB, and pelleted.

Plasmid preparation and filter hybridizations Plasmid DNAs were isolated by the method of Garger et al. (18), using a 50 Ti Beckman rotor at 40,000 rpm at 15°C for 15 hr. DNA was immobilized on filters and pretreated as described by Fetherston et al. (15). Hybridizations were in 4 X SSC, 0.1% SDS, 10 X Denhardt's, 10 µg/mL yeast tRNA at 68°C for 48 hours. Filters were washed, treated with RNase T_1 and RNase A and processed as described by Schibler et al. (40). Total counts in each hybridization were determined by TCA precipitation of an aliquot of the hybridization mix. Specific RNA hybridized is expressed as parts per million (ppm) of this total. Hybridizations over a broad range of RNA concentrations were performed to insure probe (DNA) excess.

Northern hybridizations, dot blots, and nick translations Formaldehyde agarose gel electrophoresis was performed as described by Maniatis et al. (29), and blots were transferred and probed as described by Thomas (46). Dot blots were performed using a Beckman dot blot "minifold" and hybridizations were performed as described by Thomas (46).

Probes The following probes were used in northern blot, dot blot and filter hybridizations.

i) pL21-1: cDNA clone specific for immunoglobulin kappa light chain mRNA ($mRNA_\kappa$, 45, obtained from W. Salzer, U.C.L.A.).

ii) pIS_6: a subclone we constructed of pEC_κ (13), provided by B. van Ness (Fox Chase Cancer Center). The 0.95 kbp BglII-HindIII fragment of pEC_k, which is part of the 3.3 kbp intron separating the variable and constant regions of the $mRNA_\kappa$ precursor, was inserted into the BamHI-HindIII sites of pBR322.

iii) pλrDNA: pBR322 clone derived by inserting the 6.7 kbp murine rDNA in λgtWES MR100 (22, provided by P. Leder, Harvard University) into the EcoRI site of pBR322. pλrDNA contains the 3′ terminal 181 bp of 18S rDNA, both internal transcribed spacers, 5.8S and 28S rDNA (47).

iv) pλ18S: a subclone we constructed from λgtWES MR100. The insert in pBR322 contains the 3′-terminal 181 bp of 18S rDNA, internal transcribed $spacer_1$, 5.8S rDNA and the 5′ terminal half of internal transcribed $spacer_2$.

v) p5′Sal: almost the entire 5′ external transcribed spacer from the murine rDNA cloned into the SalI site of pBR322 (33, provided by R. Reeder, Fred Hutchinson Cancer Research Center).

vi) pAdcos: a cosmid clone of all but the left 10% of the Ad-2 genome constructed by E. Werner (manuscript in preparation).

vii) pActin: pBR322 subclone of LK121 clone (supplied by L. Kedes, Stanford University), which is a human actin pseudogene clone (14). pActin consists of the 2.0kbp BglII fragment of LK121 inserted into the BamHI site of pBR322.

viii) pHe7: cDNA clone of an poly-A containing RNA of unknown specificity, isolated from a HeLa cDNA library (2, supplied by A. Babich, Rockefeller University).

DATA

Nuclear Salt-extractable RNA represents an in vivo Nuclear RNA pool. Nuclei from HeLa or myeloma cells were isolated either by Dounce homogenization or treatment with 1% NP-40 in RSB. Nuclei were washed, stripped of membranes with NP-40 if isolated by homogenization, and then treated with 0.2 mg/mL DNase I for 30 min at 0°C. The resulting membrane-free, DNA-depleted nuclei were then extracted with 0.5 M NaCl in RSB. The salt-extractable fraction, the insoluble matrix fraction which remained, and the cytoplasm were brought to 5% sarkosyl and layered onto 5.7 M CsCl cushions. The RNA was then isolated as described by Glisin et al. (21). The average yields for this fractionation are given in table 1. RNA

Table 1. RNA recovery after cellular fractionation[a]

		RNA recovered ($\mu g/10^6$ cells)
MOCK INFECTED	Cytoplasm	5.61
	Salt-Extr	1.05
	Matrix	2.00
AD-2 INFECTED	Cytoplasm	13.6
	Salt-Extr	2.17
	Matrix	1.49

[a] Average recovery of RNA (in $\mu g/10^6$ cells) from three separate experiments. Cytoplasmic RNA (cytoplasm), salt-extractable RNA (Salt-Extr), and matrix-associated RNA (Matrix) was isolated from mock- and adenovirus-infected cells 20 hours after infection.

removed during DNase treatment and the following wash amounted to less than 1% of the total and was not analyzed further. Fractionations performed on cells prelabelled with ^{3}H-thymidine for at least two cell generations indicate that less than 0.5% of the DNA copurifies with the RNA in this procedure. Use of non-RNase-free DNase, the lack of protease inhibitor during RNA isolation, or any change in the order of individual steps within this protocol resulted in RNA fractions that had characteristics similar to total nuclear RNA, as has been described by Kaufman et al. (27).

One of the obvious caveats that must be considered when separating components of a biological system is that the components must represent biologically significant classes. To insure that the salt-extractable RNA was present as an <u>in vivo</u> nuclear pool and was not just the result of cytoplasmic contamination that occured during fractionation, mixing experiments were performed. Myeloma cells were labeled with ^{3}H-uridine for 4 hours and the cytoplasm was isolated as described in methods. The labelled cytoplasm was then mixed with an equivalent number of HeLa cells, which were then fractionated into cytoplasmic, salt-extractable and matrix-associated RNA. Aliquots were removed and TCA-precipitable counts were determined for the three fractions. The remaining RNA was used in dot blots, starting with equal cell equivalents of RNA at the highest concentration followed by serial two-fold

dilutions. Blots containing equivalent amounts of nuclear fractions isolated from myeloma cells were used for comparison. These blots were then probed with nick-translated pL21-1 DNA, which is specific for immunoglobulin kappa light chain RNA ($mRNA_{\kappa}$). The autoradiograph of this dot blot is shown in figure 1. There is at least a 64-fold difference in the hybridization intensity of the myeloma fractions compared to the HeLa fractions, indicating 1-2% contamination of the nuclear fractions with added myeloma cytoplasm. Contamination of the Hela nuclear fractions by the myeloma cytoplasm, as estimated by the amount of ^{3}H recovered, is about 1%.

A second, more direct way of determining the amount of cytoplasmic contamination of the nuclear RNA fractions is to determine the amount of "cap-2" structures in the nuclear fractions. These structures are only found in cytoplasmic RNA (41). Using the same isolation procedure described here, Mary Edmonds (personal comunication) has found no cap-2 structures in the poly-A-containing RNA from either of the nuclear populations. The level of sensitivity of this assay is on the order of 5%. These data argue strongly for the nuclear fractions being essentially free of cytoplasmic contamination.

Nuclear Salt-extractable pool contains only mature RNA We have performed experiments to analyze size and sequence specificity of

Figure 1. Dot blot analysis of salt-extractable and matrix-associated RNA. RNA isolated from Hela cells mixed with cytoplasm from an equivalent number of myeloma cells (lanes 1 and 3), or from myeloma cells alone (lanes 2 and 4) was diluted serially (two-fold) (rows A-G) and applied to nitrocellulose. Row H contains 10µg yeast tRNA. The probe was nick-translated pL21-1 DNA. Lanes 1 and 2 are salt-extractable RNA; lanes 3 and 4 are matrix-associated RNA.

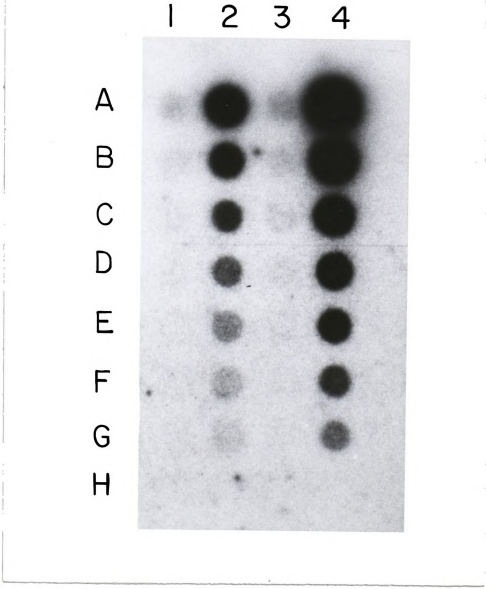

Figure 1

the RNA in the various fractions. Hela and myeloma RNA from cytoplasmic, salt-extractable and matrix-associated pools was electrophoretically separated on 1% agarose-formaldehyde gels and stained with ethidium bromide (figure 2a). The RNA from both cell types shows 28S and 18S rRNA in all three fractions. Only the matrix-associated pool contains prominent bands with mobilities slower than 28S rRNA. Similar gels were run, blotted and hybridized to nick-translated probes specific for rRNA (figure 2b). Only matrix-associated RNA contained hybridizing bands with relative mobilities of 45S, 37S, 34S, and 32S, the mobilities of the known precursors to rRNA (15). Similar blots of myeloma RNA probed with nick-translated pL21-1 DNA revealed precursor-sized molecules for $mRNA_{\kappa}$ only in the matrix-associated RNA (figure 2c).

Because northern blots give no accurate measure of the amount of a given RNA species, quantitative dot blots were performed using serial dilutions of salt-extractable and matrix-associated RNA from myeloma cells. These were probed with nick-translated DNA specific for intron (pIS_6) or exon (pL21-1) sequences from the immunoglobulin kappa light chain gene, or for the external transcribed spacer (p5'Sal) or 18S and 5.8S (pλ18S) sequences from the rRNA cistrons. Densitometric scans of the autoradiograms of these dot blots (table 2) indicate that greater than 94% of the pIS_6-specific sequences and 95% of the p5'Sal-specific sequences

Figure 2. Size analysis of HeLa and myeloma RNA fractions. HeLa and myeloma cells were fractionated into cytoplasmic, salt-extractable, and matrix-associated RNA, and the RNA was separated on 1% agarose-formaldehyde gels. Gels were stained (panel A), or RNA was transferred to nitrocellulose and probed with nick-translated pλrDNA (panel B) or pL21-1 (panel C) DNA. Lanes: a, HeLa cytoplasmic RNA; b, HeLa salt-extractable RNA; c, HeLa matrix-associated RNA; d, myeloma cytoplasmic RNA; e, myeloma salt-extractable RNA; f, myeloma matrix-associated RNA.

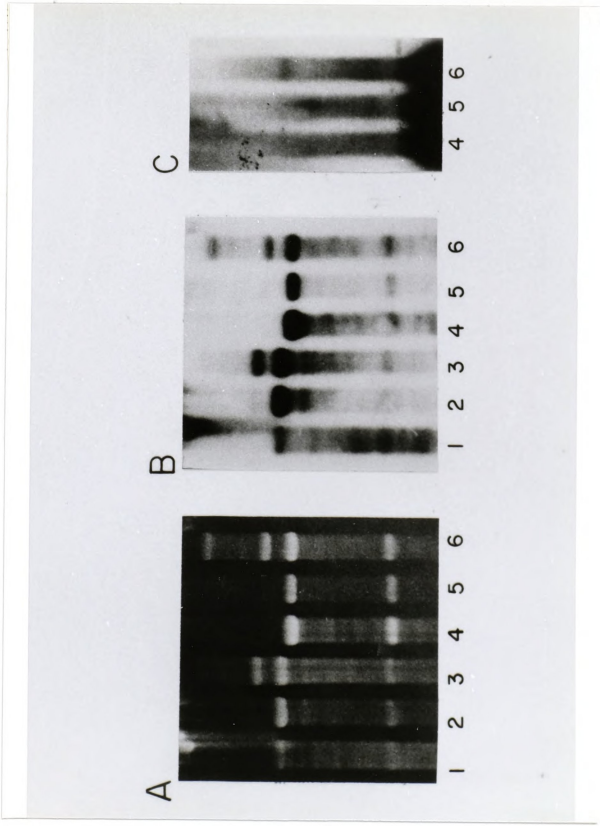
A
1 2 3 4 5 6
B
1 2 3 4 5 6
C
4 5 6

Table 2. Relative distribution of introns and exons in nuclear RNA pools[a]

	Probe DNA			
	p5'Sal	pλ18S	pIS_6	pL21-1
Salt-extr	4.9%	32.5%	5.9%	50.7%
Matrix	95.1%	67.5%	94.1%	49.3%

[a] Relative distributions of intron and exon sequences in the nuclear pools were determined from densitometry scans of autoradiographs of dot blots. RNA from the salt-extractable (Salt-extr) and matrix pools from myeloma cells was dot blotted and probed with p5'Sal (specific for 5' external transcribed spacer from rRNA primary transcript), pλ18S (specific for internal transcribed spacers, 18S and 5.8S rRNA), pIS_6 (specific for intron from mRNA precursor), and pL21-1 (specific for mRNA exons). Values given are in percent of the total nuclear hybridization.

are found associated with the matrix. Work with chicken oviduct cells using probes for ovomucoid and ovalbumin mRNAs and rRNA (12), with erythroblasts and erythrocytes using probes for globin mRNA (39), and with adenovirus-infected HeLa cells and probes for adenovirus-encoded RNA (31) found precursors for these RNAs only in association with the matrix. Ciejek et al. (12) examined the salt-extractable RNA pool of oviduct cells and found only mature-sized RNA. These findings, in conjunction with our observations, suggest that precursors to mRNAs and rRNAs are found only in association with the matrix. Mature-sized RNAs are found in both nuclear fractions.

Nuclear Salt-extractable RNA behaves with kinetics expected for an intermediate in RNA transport. If the salt-extractable RNA is the pool from which RNA is transported to the cytoplasm, cells continuously labelled with ^{3}H-uridine should show distinct labelling kinetics for cytoplasmic, salt-extractable and matrix-associated RNA pools. Matrix-associated RNA should be labelled rapidly and should be the first of the three pools to reach a steady-state specific activity. Salt-extractable RNA should become labelled after a short lag period (equivalent to the briefest processing time for matrix-associated RNAs). Cytoplasmic RNA should be labelled only after a longer lag, corresponding to nuclear RNA processing time. In addition, the specific activity of the salt-extractable RNA should reach the same level as the matrix-

associated RNA, whereas the cytoplasmic RNA should not reach this level in any reasonable time because of the presence of long-lived species such as rRNA. The data from two such continuous labelling experiment are shown in figure 3; the parallel between the data and the scheme described above is obvious.

Pulse-chase experiments were also performed to insure that there was in fact a one-directional flow of RNA from the matrix to the salt-extractable pool, then to the cytoplasm. If there is a "precursor-product" relationship between these fractions, newly-labelled RNA should be found on the matrix at short times, in the salt-extractable pool at "intermediate" times, and in the cytoplasm at long times. Myeloma cells were pretreated with glucosamine (15 mM) and actinomysin D (0.08 µg/ml) and labelled in the presence of actinomycin D for 8 minutes. They were then chased for 2 hours in the presence of actinomycin D, glucosamine, and excess cold uridine and cytidine. At various times, 6×10^7 cells were removed and fractionated. Aliquots of the isolated RNA were removed for TCA-precipitation, and the remaining RNA was hybridized to pL21-1 DNA immobilized on nitrocellulose filters. Figure 4a shows the data for total counts from one such experiment. Matrix-associated counts decreased with time. Salt-extractable counts increased for a period of about 30 minutes and then decreased. Cytoplasmic counts increased slowly. Figure 4b shows the data from hybridizations of the RNA in figure 4a to

Figure 3. Continuous label kinetics of RNA populations. Myeloma cells were labelled continuously with ^{3}H-uridine, aliquots were fractionated into cytoplasmic, salt-extractable and matrix-associated RNA at various times, and specific activities determined. ●——● , cytoplasmic RNA; O——O, salt-extractable RNA; ■——■ , matrix-associated RNA.

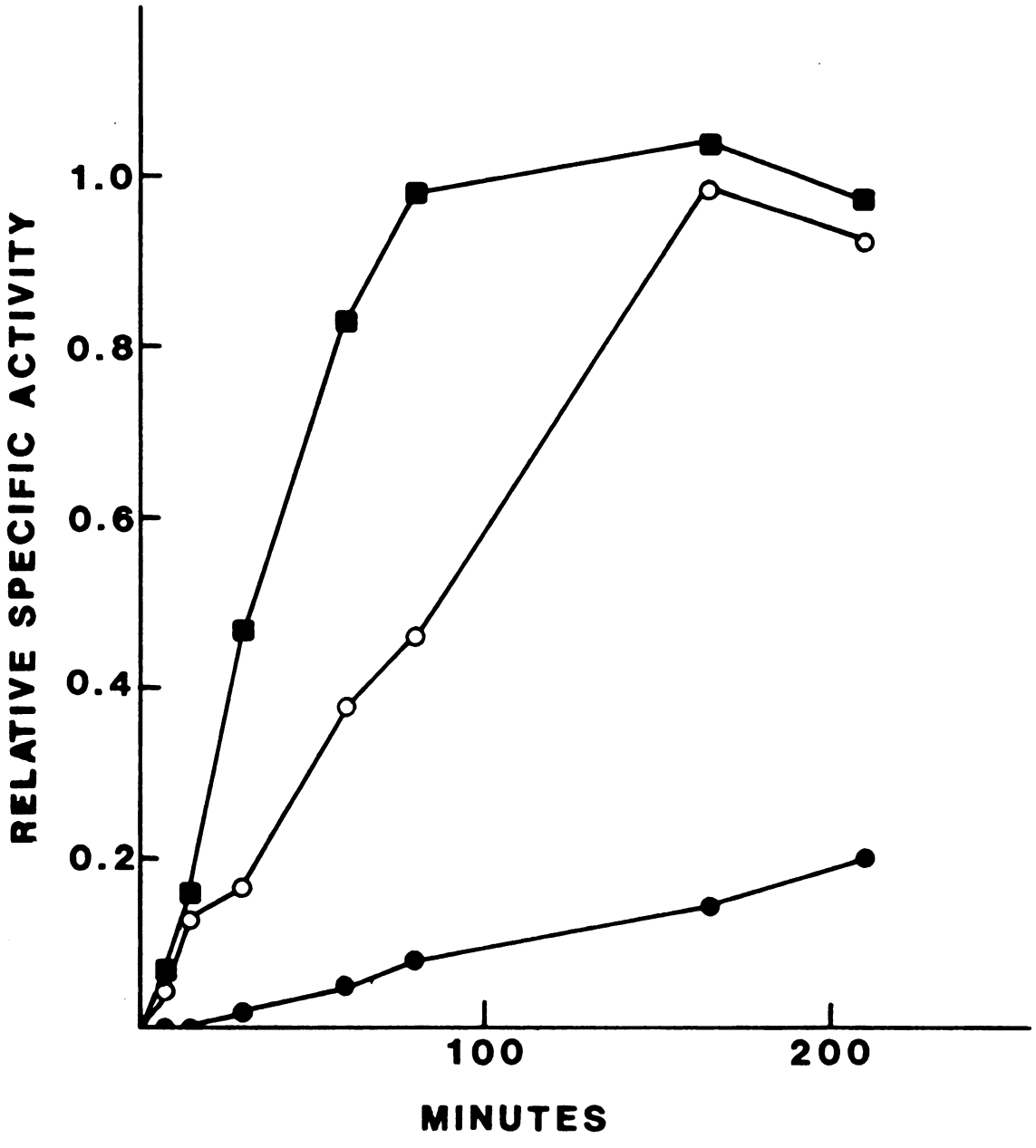

Figure 3

Figure 4. Pulse-chase kinetics of labeling of RNA populations. Myeloma cells were pulsed with ^{3}H-uridine for 8 minutes and chased as described in the text. Cells were fractionated at various times after the initiation of the chase, and the RNA was used for TCA precipitation (for total counts, panel A) and hybridization to pL21-1 DNA immobilized on nitrocellulose filters (panel B). ●——● , cytoplasmic RNA; ○——○ , salt-extractable RNA; ■——■ , matrix-associated RNA.

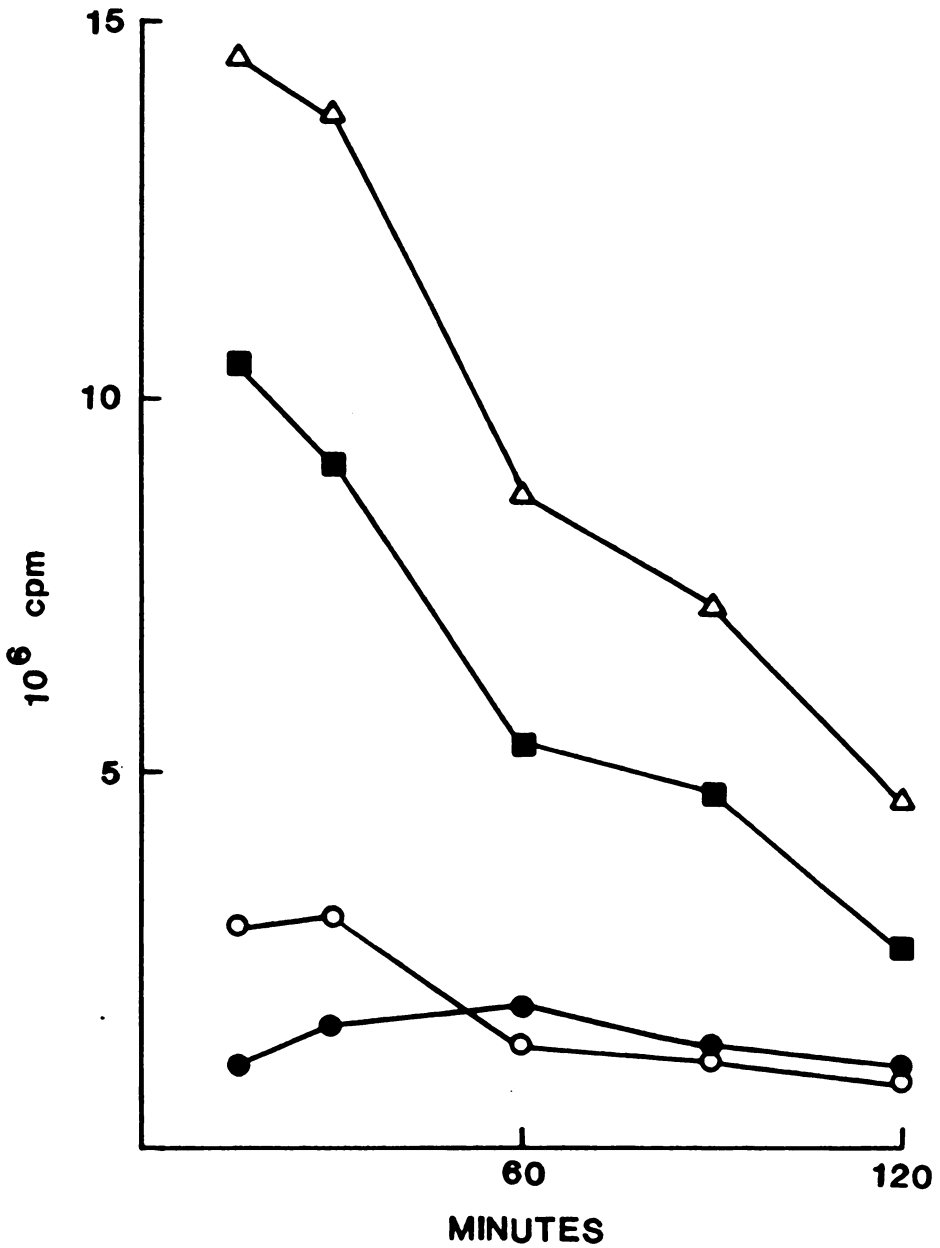

Figure 4
Panel A

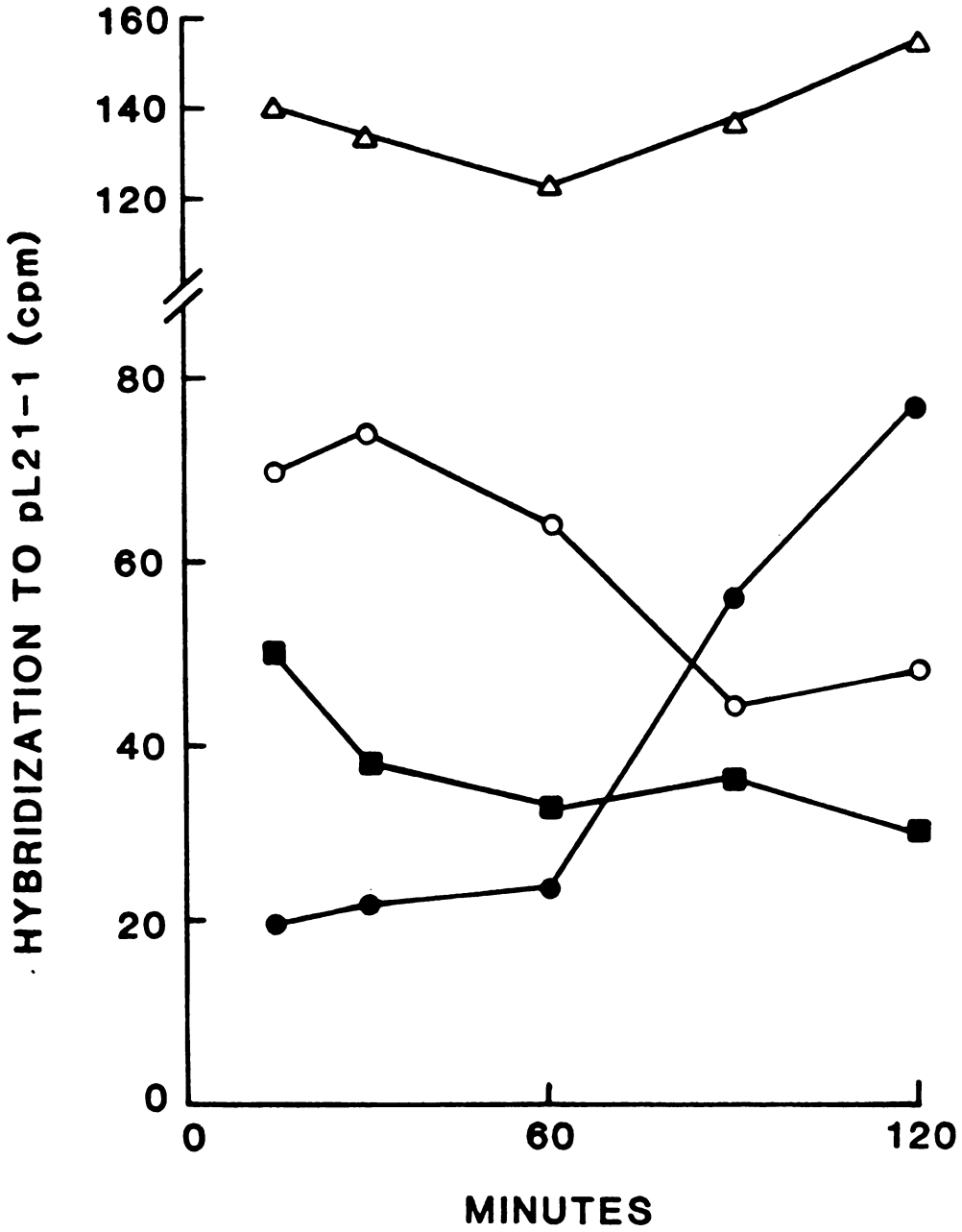

Figure 4
Panel B

pL21-1 DNA. This probe is specific for exon regions of the kappa light chain gene, and because the filters were treated with RNase after hybridization, only exon-specific sequences are detected in this analysis. The data resemble those in figure 4a. Matrix counts decreased with time, while salt-extractable counts increased and then decreased. After a 60 min lag, the cytoplasmic counts increased. The total counts hybridized was relatively constant throughout the chase, indicating that most of the matrix-associated exon sequences present at the earliest time point were eventually transported to the cytoplasm. This is in agreement with Gilmore-Hebert and Wall (20), who found there was no major loss of kappa light chain exons during pulse-chase experiments in which myeloma cells were fractionated into nuclear and cytoplasmic RNA.

These data, along with our observation that the salt-extractable pool contains only mature, non-cytoplasmic RNA, point to the salt-extractable RNA pool as the intermediate between the matrix-associated RNA and the cytoplasmic RNA.

Adenovirus infection inhibits the movement of cellular sequences from the matrix-associated pool to the salt-extractable pool. It has been reported that late in adenovirus infection, cellular sequences are not transported from the nucleus to the cytoplasm, whereas adenovirus mRNA is transported (4). Late in Ad-2 infection, HeLa cells were labelled with ^{3}H-uridine for 2 hours and fractionated into cytoplasmic, salt-extractable and

matrix-associated RNA. These fractions (and analogous fractions from mock-infected cells) were hybridized to filter-immobilized probes specific for cellular mRNAs (actin and pHe7 mRNA), rRNA, and adenovirus RNA. The data from these hybridization are given in Table 3. Late in infection, transport of newly-synthesized actin and pHe7 RNAs to the cytoplasm was greatly reduced. These RNAs were detectable at near normal levels in the matrix-associated fraction, indicating that transcription rates for these species are not vastly changed from normal levels, as described by Babich et al. (2). Ribosomal RNA transport was also reduced by a factor of 10, as described by Castiglia and Flint (11). As expected, adenovirus RNA was transported in large quantities. When the salt-extractable RNA from these cells was hybridized to these probes, the amount of newly-labelled cellular RNA detected was reduced by 8-20 fold relative to control values.

To insure that the loss of cellular sequences in the salt-extractable pool was not due to some secondary effect of infection, cells at intermediate times in infection were subjected to the same experimental procedures. At the intermediate times examined, the amount of actin RNA transported to the cytoplasm was reduced 2-3 fold while pHe7 and ribosomal RNA transport was unaffected. If movement of RNA from the matrix to the salt-extractable pool is the point of inhibition to transport during infection, then at intermediate times only sequences with

Table 3. Levels of cellular and viral RNAs late in Ad-2 infection[a]

RNA source		RNA HYBRIDIZED Actin ppm hybridized	pHe7 ppm hybridized	rRNA ppm hybridized	AD-2 ppm hybridized
MOCK INFECTED	Cytoplasm	146	60	202,000	
	Salt-extr	72	23	221,000	
	Matrix	8.3	5.3	213,000	
AD-2 INFECTED	Cytoplasm	0	13	18,100	479,000
	Salt-extr	3.6	2.8	16,900	432,000
	Matrix	4.6	4.6	76,100	366,000
CYTOPLASM AD-2 / CYTOPLASM MOCK[b]		0	0.22	0.09	
SALT-EXTR AD-2 / SALT-EXTR MOCK[c]		0.05	0.12	0.08	

[a] *In vivo* labelled RNA from mock- and adenovirus-infected HeLa cells was hybridized to DNA probes immobilized on nitrocellulose filters. Values for hybridization to pActin (Actin) and pHe7 are averages of duplicate hybridizations from two independent experiments. Values for hybridization to pλrDNA (Ribosomal RNA) and pAdcos (AD-2) represent values from a single experiment where a wide range of RNA concentrations was used for each probe to insure probe excess. In both cases, similar experiments gave qualitatively similar results.

[b] CYTOPLASM AD-2 / CYTOPLASM MOCK represents the of amount of a given sequence transported late in infection relative to mock values, and indicates the loss in nucleocytoplasmic transport of that sequence.

[c] Salt-extr AD-2 / Salt-extr MOCK represents the of amount of a given sequence present in salt-extractable nuclear pool in infected cells relative to mock infected cells.

reduced transport will show lower levels of that RNA in the salt-extractable pool. Data from these experiments (table 4) show exactly this trend.

These data indicate that the inhibition of cellular RNA transport by adenovirus infection occurs at or before the movement of these sequences from the matrix-associated pool to the salt-extractable pool. Furthermore, these results argue convincingly that the movement of RNA from the matrix to the salt-extractable pool reflects the in vivo intranuclear trafficking, since loss of transport is always accompanied by loss of movement between nuclear pools.

Table 4. Levels of cellular RNAs at intermediate times in Ad-2 infection[a]

RNA source		RNA Hybridized: Actin ppm hybridized	pHe7 ppm hybridized	Ribosomal RNA ppm hybridized
MOCK INFECTED	Cytoplasm	176	62	58,400
	Salt-extr	38	13	96,000
	Matrix	8.4	3.4	145,800
AD-2 INFECTED	Cytoplasm	63	60	44,000
	Salt-extr	11	11	86,900
	Matrix	12	11	167,400
CYTOPLASM AD-2 / CYTOPLASM MOCK		0.36	0.97	0.75
SALT-EXTR AD-2 / SALT-EXTR MOCK		0.29	0.85	0.90

[a] Levels of cellular RNAs at intermediate times in Ad-2 infection were determined as in Table 3, except that cells were harvested at a time when actin RNA hybridization in the cytoplasm from adenovirus infected cells was reduced 3 fold relative to controls. Probes and conditions are described in the legend to Table 3. Values represent those of a single experiment. Similar experiments gave analogous results.

DISCUSSION

In this report, we describe experiments designed to characterize the nature and function of two nuclear RNA populations that are separable by salt-extraction of membrane-free, DNA-depleted nuclei. Further, we have examined the effect of adenovirus infection on the movement of RNA between these pools and from the nucleus to the cytoplasm. Two major conclusions can be drawn.

1. RNA is transcribed and processed in association with the salt-insoluble (i.e. matrix-associated) nuclear pool. Mature RNA then moves to the salt-extractable pool, and subsequently is transported to the cytoplasm.
2. Late in adenovirus infection, the movement of cellular sequences is inhibited at or before the movement of this RNA from the matrix-associated pool to the salt-extractable pool. This block is sufficient to explain the inhibition of nucleocytoplasmic transport of cellular sequences late in adenovirus infection.

Nuclear RNA partitioning The RNAs associated with the two nuclear fractions described are different in sequence composition, size, and labelling kinetics. These differences all argue for the first conclusion stated above. The fractionation procedure used

here results in very little contamination of nuclear RNA fractions with cytoplasmic RNA. This was assessed using mixing experiments and by analysis of the cap structure of the RNA fractions. Both analyses yielded estimates of contamination of less than 5%. All of this contamination could be explained by the presence of mitotic cells (which should be present in logarithmically growing myeloma and HeLa cells at a frequency of about 5%) or by a low level of nuclear disruption during fractionation (28). Furthermore, the contamination of the salt-extractable pool by the matrix-associated RNA is minimal (see below). Regardless of its origin, the slight contamination observed does not alter the basic conclusions.

The composition of the two nuclear populations is markedly different. The matrix-associated transcripts consist of precursors and mature species of the RNAs examined. In addition, hybridizations using probes specific for an intron from the immunoglobulin kappa light chain gene and the transcribed spacer from rRNA detected these sequences almost exclusively in the matrix-associated fraction. In contrast, the salt-extractable fraction contained only mature-sized RNAs for all of the sequences examined by us or others (12, 39).

The labeling kinetics of these nuclear populations are different and are consistent with a precursor-product relationship between the two. In continuous label experiments, the specific activity of

the RNA in the two pools reached the same level at steady-state. Labelling of the salt-extractable pool showed a slight lag period before beginning to be labelled, consistent with the idea that this pool receives its RNA, after processing, from some other source. Labelling of the matrix-associated pool showed almost no lag period, consistent with previous reports that transcription occurs in association with the matrix. Results of pulse-chase experiments extend these observations. Data for total newly-synthesized RNA and RNA of an individual sequence (kappa light chain exon) showed that there is a flow of RNA from the matrix-associated pool to the salt-extractable pool, then to the cytoplasm. Although the total amount of newly labelled RNA decreases with time, the amount of exon $mRNA_{\kappa}$ stays reasonably constant.

Throughout this report we have used the term "matrix-associated RNA" with a meaning equivalent to the term "salt-insoluble nuclear RNA". There is some degree of uncertainty in the literature as to the extent, nature and function of the nuclear matrix (see (8) for instance). The fact that the only way to isolate the nuclear matrix is by extraction with high salt concentrations makes it difficult to determine how much of the structure seen after extraction represents <u>in vivo</u> architecture. Along with transcription and RNA splicing, the sites of DNA replication (37), steroid hormone binding (3), and DNA attachment (7, 43) have been ascribed to the matrix. Further evidence for the existence of some

internal nuclear structure comes from the regular spacial arrangment of polytene chromosomes in Drosophila salivary gland nuclei during interphase (1). This nuclear RNA fractionation procedure results in functionally distinct and biologically significant RNA populations. Since this is essentially the same fractionation used to isolate matrices, this offers further indication that these structures are present in vivo.

The matrix-associated and salt-extractable populations both contain mature-sized RNAs. This does not preclude the possiblity that there are some other less obvious biochemical differences between these two pools. It is possible that the matrix-associated mature-sized RNA still retains some covalently linked remnants of the splicing process. If these remnants were relatively small, our analysis would not have detected them. Regardless of these possibilities, our data define functionally distinct subpopulations of nuclear RNA.

Adenovirus-induced modulation of intranuclear trafficking The effect of adenovirus infection on the transport of cellular RNA has been described in detail (2, 4, 11, 16). Late in infection, the transport of cellular RNA to the cytoplasm is essentially stopped, while adenoviral RNA is transported in large quantities. We have found that at the time cellular sequences are not transported to the cytoplasm, they are not moved from the matrix-associated to the

salt-extractable pool. Transcription of cellular sequences at this time occurs at near normal levels (2), eliminating this as a possible cause for the loss of transport. Furthermore, at intermediate times in infection, when some cellular RNA sequences are transported and some are not, only those RNAs that are being transported are found in large amounts in the salt-extractable pool. Preliminary evidence from several experiments in which the transport of various RNAs was inhibited to varying degrees suggests that there is a linear correlation between the amount of nucleocytoplasmic transport and the amount of RNA in the salt-extractable pool (data not shown). This is further evidence that the salt-extractable pool is indeed the intermediate between the matrix-associated pool and the cytoplasm. It also suggests that the block to cellular RNA transport takes place coincidentally with or before this movement takes place. It is possible that the cellular RNA makes this transition between the nuclear pools and is degraded quickly after entering the salt-extractable pool. There are two reports of a decrease in the half-life (by a factor of 2-4) of cellular sequences in the nucleus late in infection (16, 50). The authors of these reports acknowledge that this change in half life is not sufficient to explain the reduction in cellular RNA transport and suggest that there is some other specific block to the transport of these sequences. We believe that we have described this block.

Different types of RNA are affected differently by adenovirus infection. Transcription of certain cellular sequences increases early in infection, although their transcription rates are reduced to pre-infection levels by late times (19). Histone RNA transcription is repressed late in infection, consistent with its cell cycle dependency (17). Similarly, the transport of different RNA species to the cytoplasm is inhibited at different rates. The transport of poly-A containing RNA is reduced before an effect on rRNA is observed (4). Our data suggest that there are even some differences in the rate of inhibition within the poly-A containing RNA, since actin mRNA transport to the cytoplasm is reduced 3 fold before the transport of pHe7 RNA to the cytoplasm is affected. The reasons for these differences are unknown; however, the effect on the salt-extractable pool always mirrors the change in transport.

RNA intranuclear trafficking and transport to the cytoplasm The mechanism that selectively inhibits the intranuclear movement of cellular sequences while allowing adenoviral RNAs to make this change remains enigmatic. There seem to be no structural differences between the adenoviral and cellular RNAs. In fact, RNA transcribed from an entire α-globin gene inserted into the adenovirus genome is treated as an "adenovirus message" and is transported late in infection (Schneider, R.J., C. Weinberger, and T. Shenk. Abstr. 1984 Tumor Virus Meeting on SV40, Polyoma, and Adenoviruses, p. 125). This suggests that the linkage of the

sequences coding for the RNA in question is more important than the sequence of that RNA. RNA need not be transcribed from the adenovirus genome to be transported late in infection. Transcripts from the influenza genome (which are made in association with the nuclear matrix (25, 26)) are transported late in adenovirus infection (26). Work by Spector et al. (44), who found that RNA transcribed from the E1 gene of Adenovirus type 5 integrated into the genome of 293 cells was transported to the cytoplasm late in adenovirus infection, suggests that RNA sequence may also be involved.

One possible explanation for this selective transport is that adenoviral transcription is physically associated with the pool of a "matrix release factor" that is required for movement of the RNA between the two nuclear pools. Because of this "position effect", adenovirus transcripts are preferentially associated with this signal to the exclusion of all other RNA. Potential "matrix release factors" are the nuclear RNA-associated proteins and the small nuclear RNAs. Another possibility is that the salt-extractable RNA is that which is "in transit" between the site of processing and the nuclear pore. Obviously, some physical movement is necessary to accomplish this. The matrix-associated RNA would then represent a "traffic jam" of RNA waiting to use a "transportation system" of limited capacity. Adenovirus RNA would then inhibit intranuclear movement of cellular RNA by usurping the

transport system. These models require that a great deal more be understood about transport in general before they can be tested.

We have characterized a previously overlooked intranuclear RNA trafficking pathway that explains normal RNA transport and its disruption by adenovirus infection. The existance of the salt-extractable nuclear pool as an intermediate between the matrix-associated and the cytoplasmic RNA pools adds another level of complexity to nuclear RNA processing, and offers possibilities for previously unappreciated levels of gene regulation. Only when the biochemical entities involved in this intranuclear movement are elucidated will the extent of gene regulation at this level be understood.

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APPENDIX

APPENDIX

INTRODUCTION

The following material represents work performed in the laboratory of Dr. Jack Silver at Michigan State University between January, 1983 and January, 1984. The three sections have been published as individual reports in the journals NATURE and Science, and I would like to thank the editors of those journals for allowing me to reprint these articles here. I would also like to thank my coauthors on these papers, and especially Dr. Silver for giving me the opportunity to do this research.

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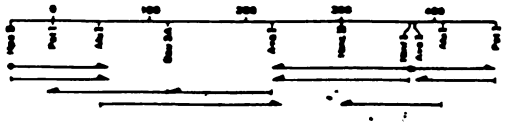

Fig. 1 Partial restriction map and strategy for sequencing Thy-1 cDNA clone, pT64. The entire cDNA clone was cleaved at selected sites with restriction endonucleases and fragments corresponding to the insert were purified by acrylamide gel electrophoresis and electroelution. Fragments were treated with calf intestinal or bacterial alkaline phosphatase and ^{32}P-labelled at both 5' ends with T4 polynucleotide kinase as described[13]. Alternatively, some fragments were ^{32}P-labelled at both 3' ends with DNA polymerase[26]. Double-end-labelled fragments were separated by acrylamide gel electrophoresis, electro-eluted and subjected to partial chemical degradation sequence analysis as described by Maxam and Gilbert[13]. Partially cleaved fragments were separated on 8, 15 and 20% acrylamide gels. The extent of sequence determined from each fragment is indicated by the length of the arrow. The closed circles and vertical lines at one end of each arrow indicate labelling at the 3' end or 5' end, respectively. The sequencing strategy used here allowed us to sequence both DNA strands completely.

Thy-1 cDNA sequence suggests a novel regulatory mechanism

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Thy-1 was originally defined in mice as a cell-surface alloantigen of thymus and brain with two allelic forms, Thy-1.1 and Thy-1.2 (ref. 1). Subsequently, the Thy-1.1 alloantigenic determinant was identified in rats[2]. In both species, Thy-1 is present in large amounts on thymus and brain cells[3] and in smaller quantities on fibroblasts[4], epidermal cells[5], mammary glands[6] and immature skeletal muscle[7]. In many of these tissues the level of Thy-1 expression changes dramatically during cell differentiation. The molecules expressing the Thy-1 antigenic determinant have been isolated from rat and mouse brain cells and have been shown to have a molecular weight of 17,500 (ref. 8). One-third of the Thy-1 molecule is carbohydrate and the remainder is a polypeptide of 111 amino acids whose sequence has been fully determined[9]. We report here the isolation and characterization of a cDNA clone encoding the rat thymus Thy-1 antigen but find that the DNA sequence ends prematurely at a position corresponding to amino acid 103. It appears to be a complete transcript, however, as the last codon is followed directly by a poly(A) tract.

Poly(A) containing RNA was isolated from W/Fu rat thymocytes, and cDNA was synthesized by reverse transcription using oligo(dT)$_{12-18}$ primer. Double-stranded cDNA was synthesized as described previously[10]. A cloned cDNA library was constructed by inserting the total cDNA population into the *Pst*I site of pBR322 using poly(dG).poly(dC) homopolymeric extensions[11]. Colonies containing cDNA were screened[12] with a synthetic ^{32}P-labelled oligodeoxynucleotide (17-mer) mixture composed of all 32 possible sequence permutations corresponding to amino acids 82–87 of Thy-1. Two hybridization-positive clones were isolated from ~10,000 colonies. One clone (pT64) contained cDNA encoding the Thy-1 antigen and its entire sequence was determined by the procedure of Maxam and Gilbert[13]. The overall structure of the cDNA clone pT64 and the sequencing strategy used are shown in Fig. 1.

The DNA sequence contains a 45-base 5'-untranslated region followed by 57 bases coding for a presumptive leader peptide of 19 amino acids starting at the first methionine codon (numbered −19 in Fig. 2). The leader sequence exhibits several features characteristic of leader peptides present at the amino terminus of membrane bound proteins[14]; it contains many hydrophobic amino acids (11 non-polar residues) and terminates in a residue with a small neutral side chain (glycine). The remainder of the predicted amino acid sequence is in agreement with the published amino acid sequence for rat brain Thy-1[9]. There are, however, several unusual features at the 3' end of the Thy-1 cDNA clone. The DNA sequence ends prematurely at a position corresponding to amino acid 103, 8 amino acids earlier than predicted from the protein sequence, but is followed directly by a poly(A) tract. There is, however, no termination codon and, furthermore, a presumptive polyadenylation signal, 5'-AATAAA-3' (ref. 15), which is part of the coding sequence, is found 12 nucleotides upsteam from the poly(A) tract. Two alternative explanations for these unusual features at the 3' end can be proposed: (1) a deletion of the sequence encoding the C-terminal peptide occurred during the cloning procedure while retaining the sequence corresponding to the poly(A) tract, or (2) the AATAAA sequence at positions 293–298 was recognized as a polyadenylation signal *in vivo* and position 310, which is 12 nucleotides downstream from the AATAAA sequence, served as a polyadenylation site.

The first explanation cannot be excluded although such a cloning artefact has not been described before. The second explanation is a very attractive model for a mechanism regulating rapid changes of Thy-1 gene expression during cell differentiation and is similar to that observed in other systems. For example, during differentiation, B lymphocytes undergo a shift from expression of membrane-bound IgM to secreted IgM. It has been shown that the transcription unit for secreted and membrane-bound IgM heavy chains contains two separate poly(A) addition sites. The basis for selection between these

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```
                                                    -19
                                                    met asn pro val
GTGGAAGCTAGCGGAGCGCAGACCCAGGACGGAGCTATTGCCACC      ATG AAC CCA GTC

                                                                -1   1
ile ser ile thr leu leu leu ser val leu gln met ser arg gly gln
ATC AGC ATC ACT CTC CTG CTT TCA GTC TTG CAG ATG TCC CGA GGA CAG

                                    10
arg val ile ser leu thr ala cys leu val asn gln asn leu arg leu
AGG GTG ATC AGC CTG ACA GCC TGC CTG GTC AAC CAG AAC CTT CGA CTG

        20                                          30
asp cys arg his glu asn asn thr asn leu pro ile gln his glu phe
GAC TGC CGT CAT GAG AAT AAC ACC AAC TTG CCC ATC CAG CAT GAG TTC

                                40
ser leu thr arg glu lys lys lys his val leu ser gly thr leu gly
AGC CTG ACC CGA GAG AAG AAG AAG CAC GTG CTG TCA GGC ACC CTG GGC

 50                                         60
val pro glu his thr tyr arg ser arg val asn leu phe ser asp arg
GTT CCC GAG CAC ACT TAC CGC TCC CGC GTC AAC CTT TTC AGT GAC CGC

                70                                          80
phe ile lys val leu thr leu ala asn phe thr thr lys asp glu gly
TTT ATC AAG GTC CTT ACT CTA GCC AAC TTC ACC ACC AAG GAT GAG GGC

                                        90
asp tyr met cys glu leu arg val ser gly gln asn pro thr ser ser
GAC TAC ATG TGT GAA CTT CGA GTC TCG GGC CAG AAT CCC ACA AGC TCC

        100         103
asn lys thr ile asn val
AAT AAA ACT ATC AAT GTG (A)24
```

Fig. 2 Primary structure and predicted amino acid sequence for pT64. Amino acids are numbered relative to the amino-terminus of the protein sequence as determined by Campbell *et al.*[9]. The 19-amino acid leader sequence 5′ to the codon for the first amino acid is numbered −19 to −1. The hexanucleotide sequence, AATAAA, at the 3′ end is underlined. A 24-base poly(A) tail directly follows codon 103.

two gene products apparently resides in the choice of poly-adenylation site of the precursor RNA followed by differential 'splicing out' of introns[16,17]. Expression of the late adenovirus transcription unit is also controlled through differential poly(A) site selection[18]. A similar mechanism may be responsible for regulating Thy-1 gene expression. Differential poly(A) site selection may control the expression of the C-terminal coding segment, which is apparently the region of Thy-1 responsible for membrane integration[9] and, in the presence of differentiation-inducing factors such as thymic hormones, complete expression of cell-surface Thy-1 may be induced. Komuro and Boyse demonstrated that Thy-1 negative precursor cells in the bone marrow and spleens of mice can be induced to differentiate *in vitro* into Thy-1 positive T lymphocytes within 2 h of incubation with crude thymus extract[19]. Our hypothesis might explain the mechanism responsible for this rapid induction of Thy-1 on the cell surface.

DNA and amino acid sequence analyses have revealed homology among the major histocompatibility antigens, β_2-microglobulin and immunoglobulins, and suggests that all three have evolved from a common ancestral gene encoding a primitive domain[20–22]. Homology between Thy-1, which has a domain-like structure including a disulphide loop of the appropriate size, and immunoglobulins has been noted at the amino acid sequence level[23]. We have compared the nucleotide sequences of Thy-1 cDNA and the mouse λ_1 light chain variable region[24] which was shown to have the highest degree of amino acid sequence homology to Thy-1[23]. When the sequences are aligned as shown in Fig. 3, overall homology between the nucleotide sequence of Thy-1 and the variable region is 36%. This sequence homology supports the hypothesis that Thy-1 and immunoglobulin have evolved from a common ancestral gene. Furthermore, our observations concerning the Thy-1 cDNA clone, pT64, suggests that the Thy-1 gene may even have inherited a remnant of a mechanism involved in regulating immunoglobulin gene expression, namely differential poly-adenylation. Additional experiments aimed at defining the genetic organization and regulating elements of Thy-1 expression are in progress. Thus Thy-1, which has long been used primarily as a marker for T lymphocyte differentiation[25], may itself represent an intriguing system for the study of gene expression.

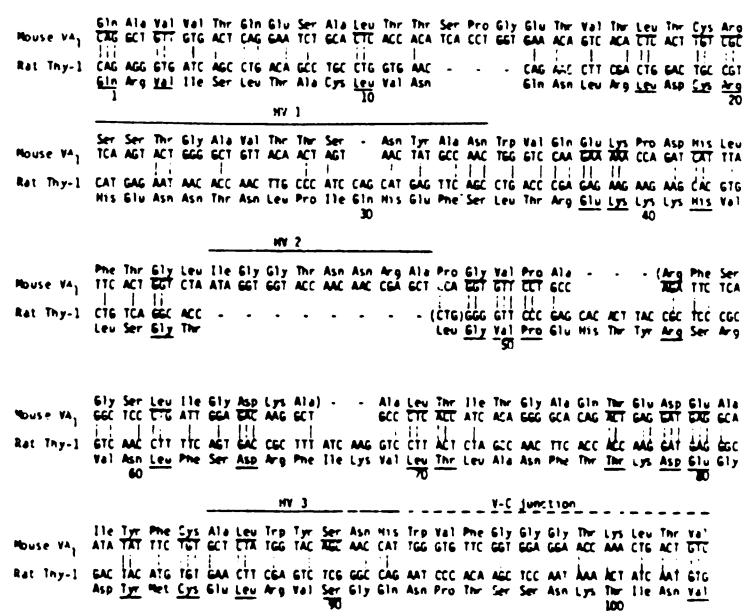

Fig. 3 Comparison of the DNA sequences of mouse $V_{\lambda 1}$ (MOPC 104E) and rat Thy-1. DNA sequences were aligned with the amino acid sequence alignment of Williams *et al.*[23], except for certain regions, indicated by parentheses, that were realigned to increase homology. Identical amino acid residues are underlined and nucleotide sequence homologies are indicated by vertical lines. Gaps inserted to maximize homology are indicated by dashes. The hypervariable regions (HV1, HV2 and HV3) are lined above the amino acid sequence and the variable–constant (*V–C*) junction (*J* region) is indicated by a dotted line. It is interesting that although the overall nucleotide sequence homology between Thy-1 and $V_{\lambda 1}$ is 36%, comparisons of small segments reveal considerable variation in the degree of homology. The homology is 41% in positions 1–20, 19% in positions 21–34 (sequences corresponding to the first hypervariable region 1), 41% in positions 35–85, 38% in positions 86–92 (HV3) and 24% in positions 93–103 (sequences corresponding to the *J* region).

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A Hydrophobic Transmembrane Segment at the Carboxyl Terminus of Thy-1

Abstract. *The mode of integration of the glycoprotein thy-1 within the cell membrane has been controversial due to an apparent lack of a transmembrane hydrophobic segment. Rat and mouse complementary DNA and genomic clones encoding the thy-1 molecule have been isolated and sequenced. These studies have enabled us to determine the intron-exon organization of the thy-1 gene. Furthermore, they have revealed the existence of a sequence which would encode an extra segment (31 amino acids) at the carboxyl terminus of the thy-1 molecule. These extra amino acids include a 20-amino acid hydrophobic segment which may be responsible for integration of thy-1 within the plasma membrane.*

Thy-1 is a membrane glycoprotein found predominantly on the cell surface of thymocytes and brain cells (*1*, *2*). Originally identified in mice, proteins similar to thy-1 are present in many species (*3*–*6*) although the distribution of thy-1 among hematopoietic cells seems to vary (*7*, *8*). Thy-1 proteins isolated from rat and mouse brains have been sequenced and consist of a protein moiety of 111 and 112 amino acids, respectively (*9*, *10*). These sequences were lacking a hydrophobic segment, which is necessary for integration of thy-1 within the lipid bilayer of the membrane. This has prompted speculation that thy-1 is covalently linked to some hydrophobic component such as glycolipid which anchors the thy-1 molecule to the membrane. The DNA sequence analyses re-

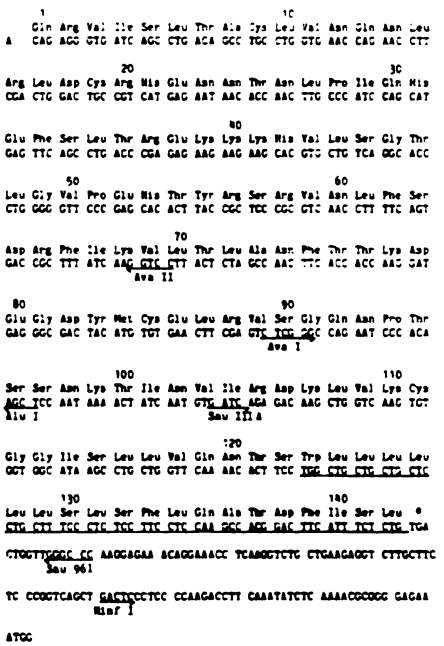

Fig. 1. The nucleotide sequence of the cDNA insert of the thy-1 cDNA clone pT86 and the predicted amino acid sequence of the complete rat thy-1 antigen. The DNA of pT86 was cleaved with restriction endonucleases (BRL) and fragments corresponding to the insert were purified by polyacrylamide gel electrophoresis and electroelution. Purified fragments were treated with calf intestinal alkaline phosphatase (Boehringer), labeled at both 5′ ends with T4 polynucleotide kinase plus [γ-^{32}P]ATP (*11*), and cleaved secondarily to generate subfragments with only one labeled end. The restriction enzyme sites shown and both Pst I sites are those that were labeled at the 5′ end. The subfragments were separated by acrylamide gel electrophoresis, electroeluted, and subjected to partial chemical degradation sequence analysis (*15*). Both strands of the insert cDNA were sequenced. The hydrophobic 20-amino acid segment is underlined and the termination codon is indicated by an asterisk.

ported here have revealed a hydrophobic region (amino acids 124 to 143) which probably represents the transmembrane segment responsible for anchoring thy-1 to the cell.

We previously isolated a rat complementary DNA (cDNA) clone, pT64, which encodes part of the thy-1 molecule (*11*). Analysis of this clone allowed us to define a signal peptide of 19 amino acids as well as part of the 5′ untranslated region of thy-1. Unfortunately, this clone terminated prematurely at amino acid 103 which prevented us from defining the carboxyl end of the molecule. After screening a second rat thymocyte cDNA library using the entire Pst I insert of pT64 as a probe, we obtained an additional thy-1 cDNA clone, pT86. The sequence of the insert from this clone (Fig. 1) started at amino acid 1 and was in agreement with the DNA sequence of pT64. Furthermore, it encoded the entire sequence of 111 amino acids of the thy-1 molecule which was previously obtained by conventional protein sequencing methods. The reading frame encoding this sequence continued on for an additional 31 amino acids before a termination codon, TGA (T, thymine; G, guanine; A, adenine), was encountered. This codon was followed by a 3′ untranslated region of 119 nucleotides before the deoxyguanylate-deoxycytidylate (dG-dC) homopolymeric tail was observed. Within these 31 amino acids there was an extremely hydrophobic stretch of 20 amino acids (including six consecutive leucine residues), which strongly resembled the transmembrane segments found in other membrane proteins. In order to determine whether these extra 31 amino acids were also present in the normal thy-1 gene, rat and mouse genomic libraries were screened with the thy-1 cDNA clone, and positive clones were analyzed in terms of restriction enzyme digestion products and DNA sequence. The coding sequence of the mouse thy-1 gene (Fig. 2) was distributed among three exons; the first one shown (actually the second exon of the gene since an intron is present within the 5′ untranslated part of the gene) encoded part of the 5′ untranslated region and the first 12 amino acids of the signal peptide. This was followed by an intron of 590 nucleotides and then by another exon encoding the remainder of the signal peptide and amino acids 1 to 106 of the mature thy-1 protein. After this exon, there was an additional intron of 386 nucleotides and an additional exon encoding amino acids 107 to 143 plus the termination codon, TGA. A polyadenylation signal, AATAAA, is located 1110 nucleotides downstream from the termination codon. The organization of the rat thy-1 gene is like that of the mouse gene, including the presence of an extra 31 amino acids in the third coding exon (*12*).

Hybridization analyses of rat and mouse DNA and RNA ruled out the possibility of a second thy-1 gene encoding a molecule of 112 amino acids or,

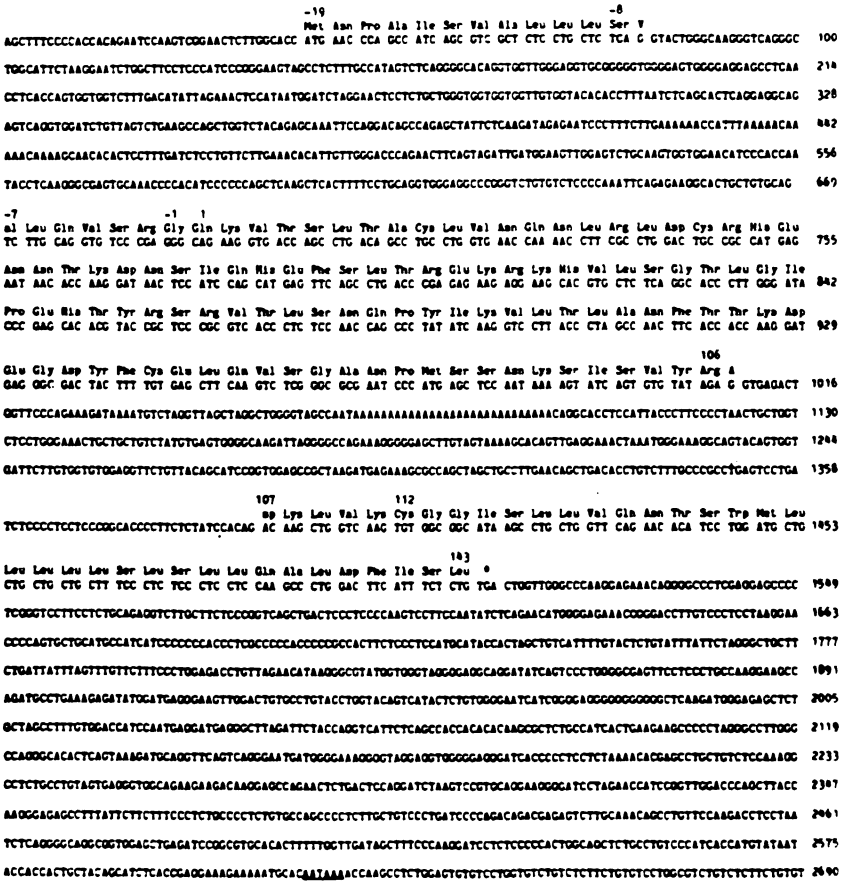

Fig. 2. Nucleotide sequence of the mouse thy-1 gene. A genomic library was prepared in the cosmid vector c2RB (*16*) with mouse (C57BL/6) DNA partially digested with Sau IIIA. The genomic library was screened with a nick-translated thy-1 cDNA probe and two thy-1 genomic clones were obtained. The thy-1 gene was subsequently subcloned into the Eco RI site of pBR322 and sequenced (*15*). The termination codon is indicated by an asterisk.

alternatively, differential processing of a thy-1 nuclear transcript giving rise to a second messenger RNA (mRNA) encoding a thy-1 molecule of 112 amino acids. Only a single thy-1 gene was observed in both the rat and mouse genome and only a single thy-1 mRNA species of approximately 1.85 kilobases (kb) in brain and thymus tissue was detected (*12*). This mRNA hybridized to a nick-translated DNA fragment corresponding to amino acids 107 to 143 of the thy-1 molecule (*12*) and thus included the extra 31 amino acids observed in the cDNA and genomic clones. There is no evidence for a second smaller mRNA encoding a shorter thy-1 molecule.

The discrepancy between the DNA sequence data and the protein sequences of Williams and Gagnon (*10*) may be explained in either of two ways. One possibility is that, although the purified thy-1 molecule is 143 amino acids long, the hydrophobic peptide containing the extra 31 amino acids was lost during the preparation and purification of the peptide fragments that were used for sequencing. Alternatively, the discrepancy between the DNA and protein data may reflect a processing step in which the newly synthesized thy-1 molecules are cleaved to yield mature molecules of a different size. To further explore this possibility, "pulse-chase" experiments were performed as follows. Cells from a murine thymoma, BW5147, which expresses thy-1 on the cell surface were labeled with [^{35}S]methionine for 5 minutes, followed by incubation with unlabeled methionine for various periods of time. Included in these experiments was the compound deoxymannojirimycin which inhibits the cleavage of mannose residues from *N*-linked glycans of thy-1 and consequently simplifies the patterns observed (*13*). Immunoprecipitation of the thy-1 molecule, with a rabbit antiserum to rat thy-1 antibody (*14*) that cross-reacts with murine thy-1, revealed that thy-1 was initially present in two forms of different molecular weights and that within 10 minutes the larger form was converted to a smaller one (Fig. 3). To determine whether this conversion was due to cleavage of the carboxyl terminal 31 amino acids from the thy-1 molecule, pulse-chase experiments were performed with [^{3}H]tryptophan. Since tryptophan is present only in the carboxyl terminal 31 amino acids (at position 124) and absent from the rest of the molecule, cleavage of this extra 31 amino acid stretch would result in the production of an unlabeled thy-1 molecule. Although incorporation of [^{3}H]tryptophan is low, both molecular weight forms were visible when thy-1 was labeled with [^{3}H]tryptophan (Fig. 3); furthermore, the thy-1 molecule was visible even after labeling was followed by an extensive chase (45 minutes in the presence of unlabeled tryptophan) indicating that the mature thy-1 molecule extends beyond the 112 amino acids proposed by Campbell *et al.* (*9*) and at least to amino acid 124. Our inability to detect any other conversion step even after a 90-minute chase (data not shown) suggests that the mature thy-1 molecule has a size consistent with that predicted from the cDNA and genomic data and that its mode of integration in the membrane is via the hydrophobic stretch of 20 amino acids present at the carboxyl terminus.

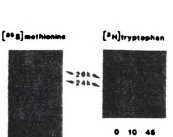

Fig. 3. Biosynthesis of thy-1 in the BW5147 murine thymoma cell line. BW5147 cells were transferred to methionine- or tryptophan-free medium (5×10^6 cells per microliter), incubated for 60 minutes, and exposed to [^{35}S]methionine or [^{3}H]tryptophan at a final concentration of 100 and 250 μCi/ml, respectively. After 5 minutes, nonradioactive amino acid was added to a concentration of 1 m*M* (zero time point). The oligosaccharide cleavage inhibitor deoxymannojirimycin was included during the preincubation period and was continuously present thereafter. Samples (5×10^6 cells) were withdrawn at the time points indicated and processed for immunoprecipitation. Immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrilamide gel electrophoresis (12.5 percent gel).

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Structural organization of the rat *thy-1* gene

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Thy-1 is a differentiation marker expressed predominantly on thymocytes, T cells and brain tissue[1]. Its presence on murine peripheral T cells but not B cells has long been used to distinguish between these two populations of lymphocytes[2]. Although analogues of Thy-1 have been described in several mammalian species[3-6], its tissue distribution in different species varies widely[7,8], precluding its use as T-cell-specific marker. The Thy-1 molecule is a cell-surface glycoprotein of relative molecular mass 18,000, one-third of which represents carbohydrate[9]; the protein moieties of the rat and murine Thy-1 molecules[10] have been sequenced and found to consist of 111 and 112 amino acids, respectively. An unusual aspect of Thy-1 is the apparent absence of a hydrophobic segment comparable to that observed in other membrane glycoproteins which would allow integration of Thy-1 within the membrane lipid bilayer. This has prompted speculation that Thy-1 is anchored to the cell surface by some other hydrophobic component such as glycolipid. Here we report the structure of *thy-1* complementary DNA and genomic clones and describe the exon–intron organization of the gene. More importantly, our data indicate that Thy-1 is initially synthesized as a molecule of 142 amino acids, 31 amino acids longer at the carboxyl end than the Thy-1 molecule isolated and characterized by Campbell *et al*[11]. An extremely hydrophobic region of 20 amino acids lies within this 31-amino acid stretch and may represent the transmembrane segment responsible for anchoring Thy-1 to the cell membrane.

We have previously isolated a rat *thy-1* cDNA clone, pT64, which terminates prematurely at amino acid 103, preventing us from defining the carboxyl end of the molecule[12]. Screening of a second rat thymocyte cDNA library using pT64 as a probe yielded an additional *thy-1* cDNA clone, pT86. The DNA sequence of the insert (Fig. 1) encodes the entire 111 amino acids of the Thy-1 molecule previously obtained by conventional protein sequencing methods. Surprisingly, however, the reading frame encoding this sequence continues for an additional 31 amino acids before reaching a termination codon (TGA). These 31 amino acids contain an extremely hydrophobic stretch of 20 amino acids (note the six consecutive leucine residues), which strongly resembles segments found in other membrane proteins. This unexpected observation prompted us to determine whether these extra 31 amino acids are also present in the normal *thy-1* gene.

A rat genomic library prepared in λ Charon 30 was screened with the *thy-1* cDNA clone, and of several positive clones obtained, one was selected for further structural analysis. Its DNA sequence was determined starting ~100 nucleotides 5′ to the initiation codon of the gene. By comparing the genomic sequence with the sequences of the two cDNA clones and performing 3′ S_1 nuclease mapping (data not shown), we deduced the intron–exon organization of the rat *thy-1* gene.

The coding sequence is distributed among three exons (Fig. 2) the first one shown (actually the second exon of the gene, because an intron is present in the 5′-untranslated part of the gene) encodes part of the 5′-untranslated region and the first 12 amino acids of the signal peptide. This is followed by an intron of 667 nucleotides, then another exon encoding the remainder of the signal peptide and amino acids 1–105 of the mature Thy-1 protein. Beyond this, there is then an additional intron of 402 nucleotides and an additional exon encoding amino acids 106–

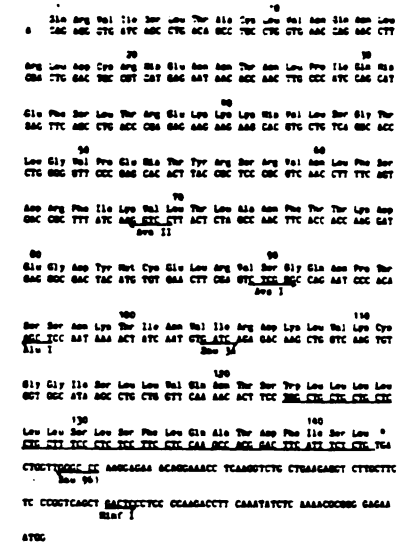

Fig. 1 Nucleotide sequence of the cDNA insert of pT86 and the predicted amino acid sequence of the complete rat Thy-1 antigen. The cDNA library was constructed using mRNA from W/Fu rat thymocytes as described previously[12]. One hybridization-positive clone (pT86) was isolated from ~10,000 colonies using the entire *Pst*I insert of the first *thy-1* clone, pT64, as a probe. A restriction map was constructed based on the size of DNA fragments obtained after restriction endonuclease digestion. The entire DNA of pT86 was cleaved at selected sites with restriction endonucleases (BRL) and fragments corresponding to the insert were purified by acrylamide gel electrophoresis and electroelution. Purified fragments were treated with calf intestinal alkaline phosphatase (Boehringer), ^{32}P-labelled at both 5′ ends with T4 polynucleotide kinase plus [γ-^{32}P]ATP as described previously[12] and cleaved secondarily to generate subfragments with only one labelled end. The restriction enzyme sites shown and both *Pst*I sites are those that were labelled at the 5′ end. The subfragments were separated by acrylamide gel electrophoresis, electroeluted and subjected to partial chemical degradation sequence analysis as described by Maxam and Gilbert[13]. Both strands of the insert cDNA were sequenced. The hydrophobic 20-amino acid segment is underlined and the termination codon is indicated by an asterisk.

142 plus the termination codon, TGA. Two polyadenylation signals, AAUAAA, are located 569 and 1,055 nucleotides, respectively, downstream from the termination codon, although only the latter one is actually used for polyadenylation. Thus, the sequence of the rat *thy-1* gene is perfectly consistent with the cDNA sequence and strongly suggests that rat Thy-1 is synthesized initially as a polypeptide of 142 amino acids rather than 111 amino acids. We have recently isolated and sequenced the mouse and human *thy-1* genes and find that both also contain an additional 31 amino acids at the carboxyl end (T.S. *et al.*, manuscripts in preparation).

To eliminate the possibility of a second *thy-1* gene encoding a molecule of 111 amino acids or differential processing of a *thy-1* nuclear transcript which would give rise to a second messenger RNA encoding a Thy-1 molecule of 111 acids, Southern and Northern blots were performed. The Southern blots (Fig. 3) indicate the presence of a single *thy-1* gene in both the rat and mouse genome. Similarly, Northern blots (Fig. 4) demonstrate the existence of a single mRNA species of ~1.85 kilobases (kb) in brain and thymus tissue. This mRNA hybridizes to a nick-translated DNA fragment corresponding to amino acids 106–142 of the Thy-1 molecule (data not shown) and thus includes the extra 31 amino acids observed in the cDNA and genomic clones. There is no evidence for a second smaller

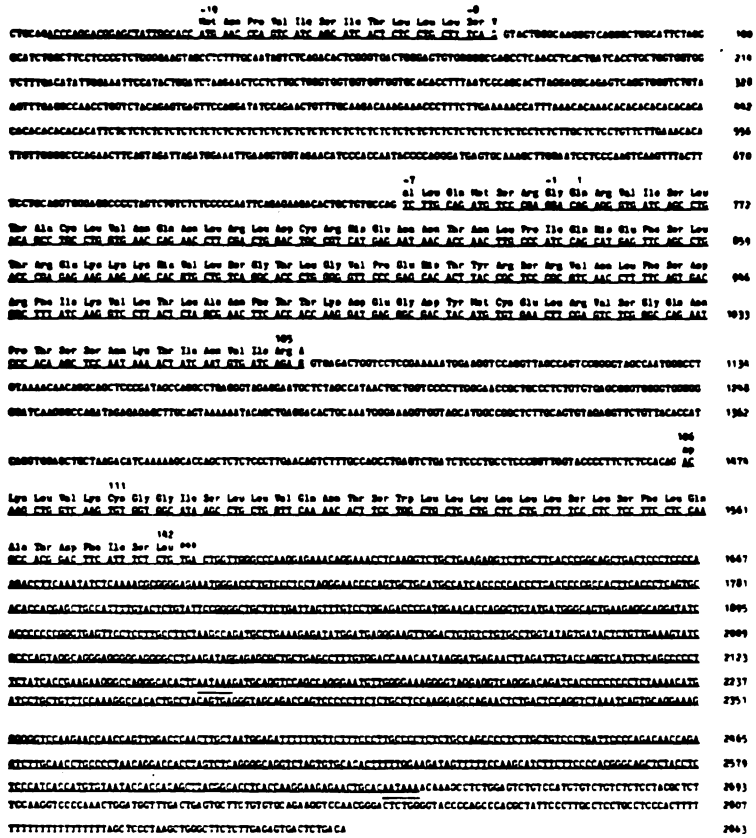

Fig. 2 Nucleotide sequence of the rat *thy-1* gene. A genomic library was prepared in the λ vector Charon 30 using a partial *Mbo*I digest of rat thymus DNA. The isolated gene was sequenced according to Maxam and Gilbert[13]. The underlined portions represent the exons deduced from the cDNA sequences and the 3'S_1 nuclease mapping. The protein sequence is numbered from −19 to −1 (signal peptide) and 1–142. The two polyadenylation signals at nucleotide positions 2,154 and 2,641 are indicated by double lines. Asterisks indicate the termination codon.

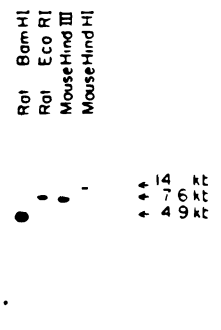

Fig. 3 Southern blotting of DNA from rat and mouse thymus. DNA of high relative molecular mass, isolated from rat and mouse thymus, was digested with restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII. Each of the digests (10 μg) was electrophoresed through a 0.7% agarose gel and transferred to a nitrocellulose filter. The *Pst*I fragment of pT64 was nick-translated and used as a probe. Hybridization was carried out in 5 × Denhardt's and 6 × SSC, at 65°C.

Fig. 4 Northern blotting of RNA from rat brain, spleen and thymus. Total cellular RNA was isolated from rat brain, spleen and thymus using the guanidinium isothiocyanate/caesium chloride method[14]. RNA (10 μg) were electrophoresed through 1% agarose gels containing formaldehyde. RNA was then transferred to a nitrocellulose filter and subsequently hybridized with a nick-translated *Pst*I fragment of pT64. The position of rRNA was visualized by staining the gel with acridine orange and *Hin*dIII fragments of λ DNA ^{32}P-labelled at the 5' end were used as size markers.

mRNA encoding a shorter Thy-1 molecule. S_1 nuclease mapping (data not shown) indicates that the large size of the mRNA is the result of a large 3' untranslated region (1,062 nucleotides) extending from the end of the coding region to the second polyadenylation signal.

There are two possible reasons for the discrepancy between the cDNA and genomic data and the protein sequence data of Williams and Gagnon[10]. First although the purified Thy-1 molecule is indeed 142 amino acids long, the hydrophobic peptide containing the extra 31 amino acids may have been lost during the preparation and purification of the peptide fragments used for sequencing. Alternatively, there may be a processing step in which the newly synthesized Thy-1 molecules are cleaved to yield mature molecules of a different size. Pulse-chase experiments to explore the latter possibility further failed to reveal any post-translational proteolytic processing. Furthermore, when ^{3}H-tryptophan is used for incorporation, the mature Thy-1 molecule is visible even after an extensive (30 min) chase (T.S. *et al.*, manuscript in preparation). Thus, the mature Thy-1 molecule extends beyond the 111 amino acids proposed by Campbell *et al.*[11] and up to at least amino acid 123, the position of the sole tryptophan residue. These data suggest strongly that Thy-1 is anchored to the cell surface by a conventional hydrophobic transmembrane segment. It is intriguing, however, that the intracytoplasmic segment described for other membrane glycoproteins is absent and the functional significance of this remains unknown.

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