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Purification and characterization of brain-specific small cytoplasmic BC1 and BC200 RNPs

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Purification and Characterization of Brain-specific Small Cytoplasmic BC1 and BC200 RNPs

by

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Abstract

Purification and Characterization of Brain-specific Small Cytoplasmic BC1 and BC200 RNPs

By

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The brain-specific small cytoplasmic BC1 and BC200 RNA contain many interesting features. They are first known RNA polymerase III transcripts with neuronal specificity. The expression of BC RNA is regionally and developmentally regulated. Furthermore, it is one of few RNAs with is actively transported into dendrites. Two type of BC small RNAs have been identified which is conserved in different animal order, i.e., BC1 RNA in the rodents and BC200 RNA in the primates. The role of BC RNAs is unknown, however, they may be involved in translational regulation and RNA sorting. Characterization and purification of those scRNPs is used to approach their physiological function.

Several piece evidence support the notion that BC1 and BC200 RNA exists as an RNP in cytoplasm of neuron. First, the BC RNAs in the brain extract have lighter buoyant density than theirs naked RNA have. Second, the BC RNAs in the brain extract (BC1 RNP ~ 8.6 S; BC200 RNP ~ 11.2 S) have different S value from their naked RNA (BC1 RNA ~ 6 S; BC200 RNA ~ 7.6 S). Further studies suggest that other observation such as the behavior of BC RNP in the electrophoresis field and the proteinase K and RNase A treatment also support the same idea.

Much progress have been made toward the goal of purification of BC1 RNP. It has been studied the behavior of BC1 RNP with different fractionation methods and integrated BC1 RNP purification scheme. Although it has been reached 2500 fold enrichment starting from brain crude extract, there are high risk for BC1 RNP falling apart and contamination. It have been solved most of difficulties during BC1 RNP purification. In addition to conventional biochemical approach, several new strategies and new methods also have been applied to facilitate the identification and purification of BC1 RNA assorted proteins.

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Chapter I

Introduction and Background

General concepts of BC RNA genes and their transcripts

BC1 RNA and BC200 RNA belong to the group of small RNAs and are present in the cytoplasm of specific neurons. In its 152 base transcript, BC1 RNA contains a portion that is homologous to ID repetitive elements and a unique region connected through a punctuated adenosine-rich bridge [1]. BC200 RNA has a similar domain arrangement; however, it features an Alu repetitive element rather than an ID element at its 5' end and a stretch of cytosine residues at the 3' end of the unique region [2]. Being transcriptional units like tRNA and 5S RNA genes, the genes of BC1 RNA and BC200 RNA are transcribed by RNA polymerase III. The functions of BC1 and BC200 RNA remain to be elucidated. The expression of BC1 RNA is developmentally regulated [3] and may coincide with synaptogenesis [V. Liu and H. Tiedge, in preparation]. The subcellular location of BC1 RNA is mainly in the soma and dendrites which is very similar to BC200 RNA [2].

BC1/200 genes

The evolutionary concept

There are at least three different short dispersed sequences (SINEs) in the mammalian genome sharing common features. The Alu family in primates, the B family and ID element in rodents may have been generated from an RNA intermediate and spread within the genome by reverse transcription followed by random insertion [4].

This mechanism also has been suggested for the generation of processed pseudogenes and small nuclear RNA pseudogenes [5]. Many hypotheses concerning the physiological functions have been suggested for those short interspersed sequences, for example, the Alu element may serve as an origin of DNA replication [6] and the B family may be involved in gene regulation [7]. However, concerning their extreme heterogeneity in evolution, the more persuading explanation for those repetitive elements is that they are "selfish-genes" which have no function but only propagate in the genome [4, 5, 8]. A more recent and reasonable proposal suggests that retroposition is one of several evolutionary strategies to generate diversity of genes and their expression. In this shotgun approach some of the retroposons may be recruited or exapted as new genes or regulatary elements [9-11].

There are 1 X 10^5 copies of "identifier" elements [or R. dre. 1 (rat dispersed repetitive element 1) which were first identified as an insert in α-tubulin pseudogenes [Lemischka, 1982 #82] or B3 by new classification] in the rat genome. They consist of a 75 bp. sequence that also can be found in the 5' domain of BC1 RNA [12, 13]. ID elements are proposed to be generated by retroposition of BC1 RNA. There are several reasons to support this notion. First, comparing the nucleotide sequence of the original tRNA founder (it has been suggested that the BC1 gene came from $tRNA^{Ala}$ [14]), the sequence of the ID element within the BC1 gene has a greater percentage of homology to tRNA than other ID elements. Second, there are ID elements that look like truncated versions of BC1 gene at different portions. This may indicate that ID elements are generated by BC1 RNA self-priming in different regions. Third, there is one active BC1 gene in every rodent studied, suggesting that the BC1 gene has been in existence for at least 55 million years [15]. However, the numbers of ID elements vary in different species. Fourth, BC1 RNA is also transcribed in the germ line which is consistent with the notion that it is a master gene for ID retroposon propagation [4, 8]. Like their progenitor, the ID elements contain the intragenic promoter of box A and box B [16]. However, only few if any transcriptional units have been identified so far. Most ID elements became silent as pseudogenes, they may be transcribed by RNA polymerase II as introns in hnRNA, untranslatable regions in mRNA, or rarely in exons [17].

The numbers of ID elements vary among different species, demonstrating that there is very little selection pressure on their existence; although we can not rule out the preservation of small portions of DNA sequence within repetitive elements. On the other hand, the BC1 genes including coding regions and some flanking regions (presumably containing regulatory elements) are conserved in all rodents that have been studied (rat, mouse, Chinese hamster and guinea pig), and their transcripts are all brain specific. Based on this observation, the BC1 gene served as a marker to trace the evolutionary relationship among rodents. [15]

In primates, no repetitive sequences similar to ID elements were found, however, an analogue to the BC1 gene and transcript was found in humans. Human BC200 RNA has surprisingly similar characteristics to the BC1 gene, such as neuron-specific expression and dendritic localization [2]. The probable origin of the BC200 gene is from an Alu element that was originally derived from SRP RNA. BC200 gene consists of an Alu monomer (Alu Lm) as well as a unique

region which is connected by an A-rich sequence. There are at least four other BC200 gene copies in the human genome all of which are probably pseudogenes. Two pseudogenes have also been cloned. From the comparison of cDNA and genomic DNA sequence, only one of these three genes generates all BC200 RNA. On the basis of this finding, this active gene is assigned to BC200 α gene, and the two pseudogenes are named β and γ gene, respectively. It must be emphasized that between BC200 α and β gene there are only three nucleotide differences in the whole coding sequence, indicating that the β pseudogene has arisen recently [18].

Dimeric Alu elements consist of two similar monomers, Alu Lm and Alu Rm, to form a ~ 300 nucleotide long DNA sequence [19]. They occupy nearly 5% of the human genome, such that almost ever 5000 nucleotides may contain an Alu element. The generation of Alu elements from SRP RNA can be dated back to the mammalian radiation. Since then, propagation of Alu elements has been independent in mammal orders. It reached its peak in human during primate evolution, and the mouse in rodents [6]. Alu elements may not always form a dimeric structure in the genome. There are also monomeric Alu elements which are believed to be older. [19, 20]

Using the flanking sequence of the BC200 α gene, BC200 genes in other primates have been cloned using a PCR based strategy. In analogy to the BC1 gene in rodents, the BC200 gene and some of its flanking regions are also conserved during primate evolution. It has been cloned from human, apes, old world monkeys (Rhesus monkey), and new world monkeys (Owl monkey), which indicates that the BC200 gene is at least 35 - 55 million years old. The neuron-specific expression BC200 RNA has also been in conserved in the above species. [B. Skryabin & J. Brosius, in preparation.].

Here we have an interesting example of two genes that have been independently recruited in different mammalian orders. The BC1 gene can only be found in rodents and is conserved in all studies species. The BC200 gene is conserved in all studied primates. They clearly have different origins but their RNAs share features of neuron specific transcription and dendritic localization. Since the BC200 gene was cloned by screening a human brain cDNA library with the BC1 unique region, both molecules may interact with similar RNAs and/or proteins in the two different orders. This convergent mechanism may provide similar physiological functions regardless of origin (crystallins of eye lens are a good example [21]). In terms of RNA function, the secondary structure may be

more important than the primary sequence. For example, the RNA part of telomerase is not conserved in different studied species [22]. However, it provides the same functions as the backbone and template for the catalytic part of telomerase [23]. At this point, we can propose that BC200 RNA is the analogue of BC1 RNA in primates.

Transcriptional regulation

BC1 RNA is a small transcript in rodents, which is transcribed by RNA polymerase III (review for pol III transcript see [24]). BC1 RNA is one of the few identified RNA polymerase III transcripts with tissue specificity. Unlike other developmentally regulated or tissue specific Pol III transcripts (i.e., 5S RNA gene in oocyte development of *Xenopus* [25] and tRNA-alanine in secretory gland of silkworm [26]), there is only one copy of BC1 gene in the rat genome responding to tissue specificity, developmental regulation and transcriptional efficiency [27-29]. The BC1 gene contains a perfect match of box A and one mismatch of box B within the gene that can support a low level of transcription in an *in vitro* transcription system. To get maximum transcripts in this system, flanking regions such as a TATA box and PSE (proximal sequence element) of BC1 gene are also required [J. A. Martignetti & J. Brosius, in preparation]. However, in the *in vitro* transcription systems studied, extracts from different species or organs can equally support the transcription of the BC1 gene. This indicates that BC1 gene may be negatively regulated in an *in vitro* system as well as in cell lines [30, 31]. Using transgenic animal models, our lab (W. Chen, unpublished results) studies those cis-acting or trans-acting elements that are responsible for the developmental as well as tissue specific regulation *in vivo*. A 1.4 bp construct, which includes ~400 bp of 5' upstream and ~800 bp of 3' down stream sequences, contains all the necessary information for efficient transcription and brain specific regulation. Further 5' deletion up to the PSE of the BC1 gene still maintains tissue-specific expression, although the expression level is dramatically reduced.

Between box A and box B, there are some repeat sequences that may bind a zinc-finger protein. In gel-shift data, it appears that there are proteins which can bind to this region and be competed out by single stranded DNA [32]. These results are not yet convincing and may represent an artifact. First, once TFIIIB and TFIIIC (both are the components of basic transcriptional machinery) bind to their target DNA sequence (box A and box B), they will cover the binding site between box A and box B [24]. Second, only low amounts of non-specific

competitors (dI:dC) were applied in every binding reaction. However, if this idea is true, there may be a trans- acting factor involved in BC1 gene regulation. Since those shifted bands can be competed out by single strand DNA, this trans-acting factor may interact with RNA as well [33-35]. In a later experiment, the same group approached this question, and identified a 30 kDa protein factor which can be identified by UV cross-linking to the radiolabeled DNA probe [32]. The protein, named BP - 1, has the ability to bind to BC1 DNA as well as RNA. The location of BP - 1 protein is developmentally regulated its transcript translocated from the nucleus to the cytoplasm [36]. The behavior of BP-1 protein is unusual both for an RNA binding protein and DNA binding protein. It can bind BC1 gene at conditions with less than 0.5 μg of dI:dC (a non-specific competitor) in the DNA gel-shift assay. It separates from the BC1 RNA peak after heparin column chromatography, which suggests the binding to BC1 RNA is also very weak and questions whether there is any physiological significance. Since this protein has never been purified to homogeneity, those questions need to be further examined. However, the possible function of BP-1 protein may act in analogy to TFIIIA which is responsible for 5S gene transcription, 5S RNA translocation from nucleus to cytoplasm or the storage of 5S RNA in the cytosol [33, 34].

The primate BC 200 gene is also transcribed by RNA polymerase III *in vitro*. Likewise, it contains a TATA box in the appropriate position. Since the BC200 α gene and BC200 β pseudogene differ at only 3 nucleotides within their coding region, the flanking control regions must be important for active and tissue specific expression [18].

ID element and BC1 gene

Several observations have been made : First, the ID containing transcript of BC1 gene is expressed specifically in the brain. Second, there are many brain-specific genes containing ID elements. Third, those ID elements share a stretch of sequence with the 72 bases enhancer element of SV40 virus. It has been proposed that those repetitive elements are responsible for tissue specific gene expression in brain, therefore they were given the name of "identifier." [37, 38]. Later studies showed there are ID-containing Pol II transcripts in liver and kidney as well as other tissues. This hypothesis turned out to be wrong [39]. However, this idea is certainly not the only hypothesis that tries to propose the physiological role of ID elements and the relationship between the BC1 RNA gene and ID elements.

In a transfection experiment [40], it has been shown that a reporter gene construct with an ID element has 2 - 4 fold increased expression levels when compared to those without the ID element. The same construct that was transfected into a cell line with BC1 RNA expression has higher expression levels than that of a primary culture without expression of BC1 gene. It suggests that the ID elements can function in "cis" to stimulate Pol II gene's expression, act as a regulatory unit with position effect or provides an environment to facilitate the transcription of Pol II. It also suggests that BC1 transcripts contribute such expression enhancement. This idea is very interesting, however, with weak evidence, since the difference between cell line and primary cell culture lies not only in the absence or presence of BC1 RNA expression. A better control group in this experiment, would be a cell line where the BC1 gene is disrupted instead of a primary cell culture which does not express BC1 RNA.

Some other experiments concern the functions of ID elements which were inserted into a particular genes [41, 42]. Those experiments suggest that the variation between different ID elements are very large. A special ID element in the rat growth hormone gene is capable of influent the expression of this gene in the neuronal cell line compared to non-neuronal cell line. It also has been attempted to understand the function of *Alu* elements. A series of deletion experiment were designed to assay the cis-acting elements which are required for the expression of keratin 18 gene in the transgenic mice. The result suggests that Alu sequence can "insulate" this gene expression into a copy number dependent manner [7].

RNA as a functional unit

Some interesting features of RNAs

Recent studies show evidence that RNAs are not only a link between information storage (DNA) and functional execution (protein) but also can act in either capacity [6]. Many studies find that the RNA can act as a primer during DNA replication [6]. After transcription, RNAs play a major role in RNA processing and regulation [6, 43-45]. In addition to their major functions in translation, tRNAs are also involved in replication of retroviral genomes [46], in the ubiquitin pathway to degrade protein [47] and in the first step of chlorophyll synthesis [48]. Another interesting observation is that mRNA of aminoacyl-tRNA synthetase shares homologous secondary structure with its corresponding tRNA. Thus both RNAs compete for a same protein [49]. This type of interaction

provides an auto-regulation loop to balance the available source of charged-tRNA for regulation of protein synthesis rate.

Almost 1% of transcripts are small RNAs that were classified by means of caps on their 5' ends and by their subcellular localization [50]. The major small nuclear RNAs (snRNA) and small nucleolar RNAs (snoRNA) are involved in the processing of other RNA [44, 51]. It has also been suggested that minor sn(sno)RNA which include other U RNAs and 7SK RNA may be responsible for nuclear events (i.e., replication or transcriptional regulation [28, 29]). The best known small cytoplasmic RNA (scRNA), 7SL RNA, is the backbone of the signal recognition particle which facilitates ribosome docking at the endoplasmic reticulum. A set of scRNAs like tcRNA may also participate in translational events [52]. The functions of most scRNAs are still unknown [44]. Some small RNAs like BC1 RNA, are transcribed as a single gene unit rather than being processed from other larger transcripts; some small RNAs are encoded within a intron [53].

RNA can be an enzyme, or genetic material for viroids and viruses. RNA can also be an intermediate in Ty element transposition in yeast [54], in hepatitis B virus replication [55] and in the formation of processed genes, pseudogenes and repetitive elements [5, 8]. On the transcriptional level of regulation, X chromosome inactivation can be executed through an RNA molecule [56]. TFIIIR in the silk worm may be a transcription factor [57]. The transposase transcribed in insertion element IS10 is regulated by an antisense RNA against the enzyme coding region [58]. There may be additional RNA-mediated events that need to be elucidated.

The possible functions for BC1 RNA

In the aforementioned functions, RNA may play the following roles. First, functioning as a catalytic unit which can participate in enzymatic reactions. Second, in order to provide enough affinity and specificity during macromolecular interactions such as spliceosome formation. RNA can form hetero- or homo-duplexes between DNA-RNA or RNA-RNA. Third, the flexible structure of RNA with the possibility of conformational changes can facilitate or modulate various reactions including protein synthesis on ribosomes [59]. In the case of 7SL RNA, the original thinking for the functions of its RNA portion was only to serve as a scaffold to hold different proteins together [60]. It was later thought that the Alu domain of 7SL RNA which somewhat resembles a tRNA

may lead to translation arrest by competing for the A site within a ribosome [61]. However, more evidence has shown that RNA - RNA interaction between nucleotides 97-110 region of 7SL RNA and a region of 18S rRNA, and another region of 7SL RNA may compete for the 5S rRNA binding site on rRNA [62]. Those interactions may account for the SRP binding to free ribosomes as well as for translation arrest. The conformational change in 7SL RNA during the SRP cycle also provides the flexibility to facilitate functions of the protein components of SRP [62]. The question arises to what extent BC RNAs are involved in any of those functions?

There are three distinct portions in BC1 RNA, namely, the ID portion, the poly A stretch, and the unique region [1]. The ID portion of BC1 RNA may form an RNA-RNA duplex with hnRNAs or mRNAs that contain a complimentary strand of ID sequence, and compete for some factors with other transcripts which include the same strand of ID sequence. By this type of interaction, the BC1 RNAs may be involved in the RNA transport, transcription regulation, RNA stability, and translation regulation for a special subset of genes or mRNAs. The poly A stretch is shared by most mRNAs and some small RNAs. Poly A-binding protein (PABP) or other related proteins can be considered a candidate to bind this region [63]. An area with part of the A-rich region and the unique region is the region with sequence similarity between BC1 and BC200. It will be interesting to establish whether there is a shared functional significance.

BC200 may have the same possible role as BC1 RNA. As with the ID element, Alu elements also can be transcribed into hnRNA. Most of the short Alu transcripts are restricted in the nucleus. Only a few can be targeted out of nucleus. This suggests that most of Alu containing transcripts (hnRNA) splice out their Alu containing RNA before being exported to the cytoplasm. B1 transcripts and BC200 RNA are the few Alu-like RNAs that can be exported to the cytoplasm [64]. It is an interesting question why so many short Alu transcripts (mainly of intron origin) are restricted to the nucleus and rapidly degraded, while B1 transcripts and BC200 RNA have such a different fate [6]. BC200 RNA shares the Alu domain with 7SL RNA. Since this domain in 7SL RNA is responsible for translation arrest in the function of SRP, the Alu domain in BC200 RNA may also serve a similar function [61] in dendritic protein biosynthesis.

BC1 and BC200 as a RNP

Nearly all of the RNA within the cell may exist as ribonucleoprotein particles. Four ribosomal RNAs form a ribosome [43]; mRNAs either combine with ribosomes or form the informosomes [65], sn-URNAs form the snRNPs and cooperate with other snRNPs and introns to form spliceosomes [66]; 7SL RNA forms the signal recognition particle [60]; and M1 RNA associates with C5 protein to become RNase P [44, 67]. In some cases, proteins may protect the RNA core from degradation or enhance the efficiency of function of RNA. In other cases, proteins have their own physiological function and cooperate with RNA to complete complicated reactions.

In the field of RNP studies, the main focus is on small RNPs in the nucleus that concerns RNA maturation [44]. There are fewer studies on scRNPs. Since BC1 RNA is a cytoplasmic RNA, we only focus in the following on this type of RNP. The scRNPs can be divided into organelle associated (sorRNP) and cytoplasmic RNP groups [44].

Organelle associated RNP

The organelles of a eukaryotic cell are derived from ancient prokaryotic cells though endosymbiosis [6]. The function of small RNP in those organelles resembles more that of snRNP than scRNPs. A very interesting observation concerning those RNPs is that the protein component of RNP is usually nuclear encoded. Two or more small RNPs have been studied: RNase P in mitochondria and chloroplasts and MRP in mitochondria [67, 68].

Cytoplasmic RNPs

There are several scRNPs which have been described within the past decade. Most of them are thought to be involved in translational control [69]. Some of the scRNAs in chicken embryonic muscles have been suggested to be involved in translation regulation and are named for tcRNA (translational control RNA) or RNP [52]. One example is the 3' end of tcRNA 102 which is complimentary to the 5' untranslatable region of fast myosin heavy chain mRNA [70]. It has been proposed that tcRNA102 is a natural antisense RNA for regulation of specific muscle mRNAs. The tcRNA are very heterogeneous RNAs ranging from 70 to 90 nucleotides. The protein components of tcRNP exhibit nearly 45 different peptides by resolution of two-dimensional gels [71]. Since those proteins have no effect on translation, it is suggested that the function of those proteins is mainly for protection of tcRNA from RNase degradation. However, this family of RNPs is too complicated for a detailed study, and most functional assays were done using *in vitro* translation systems. Although it has

been shown that those RNAs can interact with specific mRNAs, associated with or without mRNP, and activate or inhibit translational events, it is very hard to define what is real or artificial at this stage. Other scRNAs from human placenta may play a role in the regulation of mRNP metabolism [72]. With a very strong effect on the translation initiation step, the RNA increases its inhibition effects especially after protein association.

With a very good functional assay system, the signal recognition particle has been purified a decade ago [73]. It can be a model system scRNPs studies [60, 74]. SRP contains 300 nucleotides of RNA and can be cleaved by micrococcal nuclease into two subparticles that are responsible for different protein binding as well as different functions [61]. The SRP(S) subparticle, which participates in protein translocation into ER, contains four peptides, SRP 54 for signal recognition, SRP 68/72 for protein translocation, and SRP 19 with unknown function [75]. The other subparticle that contains an Alu domain has two protein components, 9/14, that function as a dimer [76, 77]. This subparticle is thought to be involved in translational arrest, although this is still contested [78]. There are many SRP(S) counterparts that persist in prokaryotic cells. The 4.5 S RNA or scRNA in eubacteria and 7S RNA in archaebacteria share common structure features with 7SL RNA [79]. The SRP p54 may also exist in the *E. coli.* with similar function [80]. In the concept of evolution, there must have been a convergent (different origin) or divergent (common ancestor) mechanism to cause the RNA structure to be different with stretches of common sequence [81].

Another scRNA like vault has its own special features. Vault is very large particle with a mass of 8 MDa. There are at least 55 copies of the major vault proteins, p104, and some other minor proteins like p210, p192 and p54 [82, 83] in a vault. Those protein components form 2 flower-like wheel structures with 8 petals each and these two flower-like structures combine to form a kernel-like structure [84]. There is a species of 140 base long RNA within each petal that provides the backbone of such conformation. From the data of molecular weight and the arrangement of this macromolecule, vault may have some degree of connection with nuclear pores or the plug of nuclear pores. Vaults are very conserved in lower and higher eukaryotic cells [85]. The purification conditions for maintaining those polymers is the same as basket-like chlathrin polymers. Therefore, the *in vivo* situation may somewhat differ. Besides the very elegant EM work to figuring out the architecture of this polymer, and its role as a molecular marker in tracing the cell lineage of glia cells [86], there is no further

information concerning the functions of the RNA and protein portions. Recently, the vault RNA has been cloned from rat and bullfrog [87]. The vRNA in rat consists 141 nt. and frog has two vRNAs of 89 and 94 nt. Despite the difference in length, the secondary structures of vRNA in rat and bullfrog are related. Like BC1 RNA, vRNA is also transcribed by RNA polymerase III from a single copy gene. It is unclear whether the RNA portions have evolved for a function or only transitionally participate in the formation of the whole complex and to be later discharged after maturation.

A similar puzzle has bothered researcher working on prosomes. Prosomes are a 19 - 20 S particle with a tube or cylindrical shape with proposed functions including tRNA synthetase activity, pre-tRNA 5' end processing, and mRNA repression [88-90]. By EM, immunological reactions, RNA identification and proteinase assay, it has been shown that another particle, multiple catalytic proteinase [89, 91], may be identical with the prosome. So they were renamed from prosome and MCP to proteosome [92]. However, in the highest purified preparation, the particle maintains its proteinase activity with no detectable RNA portion. Since the function of proteosome (MCP), which plays a major pathway of nonlysosomal intracellular protein degradation and antigen presentation, is different from those proposed functions of prosome, it indicates that prosomes or proteosomes belong to a group of particles which share some but not all features [93, 94].

BC1 RNA is also present in the cytoplasm of neurons as ribonucleoprotein particle. There are several pieces of evidence to support this notion. First, by running the extract in CsCl or Cs_2SO_4 equilibrium gradient, the buoyant density of BC1 is consistent with an RNA-protein complex. Second, in sucrose gradients, the S value of the BC1 particle from cytosol is larger than that of "naked" BC1 RNA. Third, certain portions of BC1 RNAs are protected by some factors and are more RNase resistant. Fourth, the BC1 RNA-protein complexes which are generated by UV cross-linking have the same S value as the native form [95]. Thus far, we lack further information regarding those proteins that may bind the BC1 RNA. Candidates include the poly (A) binding protein and a zinc finger protein which has been mentioned earlier. Another candidate is the dimer of 9/14 kDa SRP protein. The ID element domain of BC1 RNA shares some homology with the conserved box 1 and box 2 sequence which may be the target for 9/14 protein binding. As for BC200 RNA binding proteins, it has been

suggested that its Alu domain (also present in SRP RNA) may bind to the 9/14 kDa dimer as 7SL RNA does.

Purification of the BC1 particle and determination of its protein component will be an important prerequisite to reveal the function of BC1 RNA. It is also important to understand the relationship between BC1 and BC200 RNA. Some of these factors that bind to BC1 RNA or associate with BC1 RNP particle may also belong to the RNA sorting machinery. Based on this idea, studying the BC1 RNA binding protein may help us understand the molecular basis of RNA sorting events in the neuron.

Dendritic transport of BC1 RNA

Neuronal polarity

The polarity of a neuron is not only to establish different morphological cell domains, but it also correlates with its function [96]. Neurons usually have many short, branched dendrites that receive the signal input and one long, less-branched axon that mediates the signal output. Not so different from the epithelial cell [97-99], the polarity of a neuron has been suggested to come from the microtubule filaments which have - to + orientations in axons and both orientations in dendrites [100]. This arrangement which depends on the orientation of microtubule filaments can explain those differences between dendrites and axons including the difference in morphology, in RNAs or protein components, in the transmitter vesicle transport, in the synaptic polarity, and in the capacity of protein synthesis *in situ*. [101-103]. However, other cytoskeleton components also have different distributions between axons and dendrites. It has been shown that some phosphorylated forms of neurofilaments are predominant in the dendritic fields [104].

RNA sorting within neurons

The RNA sorting phenomenon is another way to establish neuronal polarity (for review see [99, 105]). The uneven distribution of mRNAs is a significant force in the development of *Xenopus* oocytes and *Drosophila* embryos. This kind of mRNA gradient acts as morphogen which determines the protein concentration and then the polarity of embryo and cell fate. Other important examples for sorting come from the studies of myelin basic protein (MBP) mRNA in oligodendrites and vimentin mRNA in the muscle cell, suggesting a spatial control of macromolecular assembly. This mechanism can also separate different

isotypes of β-actin in different cytosol compartments. In neurons, this RNA sorting mechanism is very important in the *in situ* (local) protein synthesis and maintenance of the polarity of neurons [106-108].

Depending on the subcellular localization, the sorted RNA in the neuron can be divided into dendritic RNA (such as the mRNA of MAP II (HMW), calcium / calmodulin dependent protein kinase II α-subunit and BC RNA) [2, 109-111], and axonal RNA (such as mRNA of tau or certain peptide hormones [112, 113]). Dendritic mRNA may serve as a trans-synaptic signal effector and can synthesize proteins more efficiently in exact sites. The function of axonal mRNA is still unknown, since protein synthesis machinery is not detected in axonal terminal beside growth cone. It may serve as a retrograde signal or modulator in signal transmission.

Based on the study of MBP in oligodendrites [114], it has been suggested that there are several steps during mRNA transportation. Those include the assembly of RNP particle, translocation of RNP particle, and anchoring of particle to the cytoskeleton and translation of localized mRNAs. All mRNAs can form a mRNP with sets of hnRNPs proteins. However, to determine wether an RNA can or can not be sorted may depend on a signal sequence (patch) that is on a specific RNA that can bind to special proteins. This mRNP then docks with cytoskeleton associated motors and is transported into the final compartment for anchoring or translation. RNAs are selectively transported through the cytoskeleton into dendrites and are energy-dependent with the rate of 11-25 mm per hr [115].

Most BC1 RNA is detected in the soma and dendrites [111]. Since BC1 RNA is relatively simple compared to other mRNAs and may share a common RNA transportation mechanism, it may reveal the molecular basis of RNA sorting mechanism. BC1 RNA may be involved in the RNA sorting machinery as follows. It may involve the step of docking of mRNA onto a cytoskeleton track or anchoring the mRNA in dendritic field. It may also inactivate the mRNPs until it reaches the adequate compartment.

***In situ* protein synthesis**

The idea of protein synthesis in dendrites arose from observations that polyribosomes were located in dendrites and labeled amino acids were incorporated into protein in detached dendritic fragments [116]. Polyribosomes persist in spine-bearing and aspiny dendrites. In the spine-bearing neurons, they

are selectively located at the base of spines, where nearly 50% of polyribosomes are associated with ER-like structures [117, 118]. Free polyribosomes are also found on top of the spine as well as in other dendritic regions. The difference in the locations of polyribosomes may indicate translation of different subsets of mRNAs [119]. Quantitative studies have shown that the activity of the protein-synthesizing machinery is correlated with synaptogenesis and reinnervation [120]. The next question is what messenger is translated by those extra-somal ribosomes. To date, there are only few mRNAs known to be selectively transported into dendrites. They include MAP-2 (microtubule associated protein-2, high molecular weight) which is thought to be important in the regulation of dendritic development and plasticity [109, 110, 121] and the α subunit of Cam kinase II (Ca^{++}/calmodulin dependent protein kinase II) which is involved in the cascade of calcium signals [107]. Some critical proteins translated *in situ* may be involved in very important regulation steps in response to local demands during dendritic development and synaptogenesis. Studies of expression of BC1 during development have shown spatial and temporal correlation with the onset of synaptogenesis, suggesting that the BC1 particle plays a role in these localized translation events as well.

Perspective

From RNA world to neuronal circuitry

During the turn of last century, Ramon y Cajal made his famous postulate that learning may produce prolonged morphological changes in the effectiveness of the synaptic connections between nerve cells and those changes may serve as the mechanism of memory. Studies in long term potentiation in the hippocampal formation in rat and gill-withdrawal behavior in *Aplysia* have elicited the relationship between synaptic events and behavior. It is very important to study these mechanisms at the molecular level. The signal input (stimulation), signal transduction and gene expression all have some kinds of macromolecules involved. Studying the molecular components in each step is a fundamental approach to clarify those events. Concerning the behavior of BC1 RNA , it seems that this small RNA may participate in the post-synaptic response as well as plastic change in some neuronal circuitry.

There are three steps of evolution taking place to build up life on earth. The first step is the pre-life world, where materials interact randomly to create some kind of order which could form, grow, propagate, replicate itself and

degrade. The RNA world may be the first system that shows most, if not all of the characteristics of life. When those macromolecules assembled together and the first cells were formed, the era of biological evolution began. This is the second step. During this period, RNA was not only mediating many fundamental metabolic reactions but was also involved in building up the (genomic) complexity of creatures. Since neurons were constructed into higher and higher architecture, this became an evolution in parallel with life [122]. Once the neuronal network can handle abstract information and open the world of imagination, it can provide the necessary power to interpret and alter the material world, and the final step, evolution turns into civilization via humanity. From the RNA world to neuronal circuitry, it is very interesting to follow a molecule from its origin to present day and then into the future. BC1 and BC200 particles may serve as an excellent subject to provide some overall insight about the evolution of life.

Chapter II

Identification and Characterization of BC1 RNP Particles

Introduction

Brain-specific cytoplasmic RNA (BC1 RNA) belongs to a group of small non-messenger RNA and is present in the cytoplasm of a subset of neurons [1, 111]. There is only one single active gene encoding the BC1 RNA. Like 5S RNA and tRNA genes, it is transcribed by RNA polymerase III. However, it is one of few pol III transcripts that is regulated temporally and spatially.

BC1 RNA contains 152 nucleotide with a typical polymerase III termination of several uridines. BC1 RNA can be divided into three parts which may serve as functional domains as well [1]. From its 5' end to 3' end, the first 75 nucleotides is a repetitive element called identifier (ID) which is dispersed in the rodent genome at up to 100,000 copies. The 32 nucleotides at the 3' end may form a stem loop called the unique region. Using this sequence as a probe to perform genomic Southern blotting can only identified a single band under high stringency conditions. Connecting these two domains is stretch A with few other bases and named A-rich region (**figure 1**).

The function of BC1 RNA is still unknown. Since part of BC1 RNA (ID region) is derived from tRNA which may have a similar secondary structure, it is suggested that BC1 RNA may interact with ribosomes and be involved in translation regulation. Combined with the results of *in situ* hybridization which show dendritic location of BC1 RNA and the synaptogenesis-dependent expression of BC1 transcripts, this RNA may play a role in the protein synthesis in dendritic compartment and respond to synaptic activity [118]. According to this hypothesis, it is also suggested that dendritic RNA, such as the mRNA of MAP II (HMW), CamKinase α subunit and/or some other RNAs, may be involved in the reinforcement of the connection between pre- and post-synapse where the LTP is taken place [106, 123]. It is also possible that BC1 RNA/RNP may play a role in dendritic RNA sorting or transportation. Recently it has been shown that BC1 RNA coexists with the mRNA of vasopressin, oxytocin and neurofilament in the axonal terminals of hypothalamus-pituitary tract neurons [113]. Since the BC1 RNA is the only RNA sorted both into dendrites and axons, it suggests that BC1 RNA itself may be involved in RNA sorting. The simplicity of BC1 RNA may serve as a good model to study this phenomena in neurons as well.

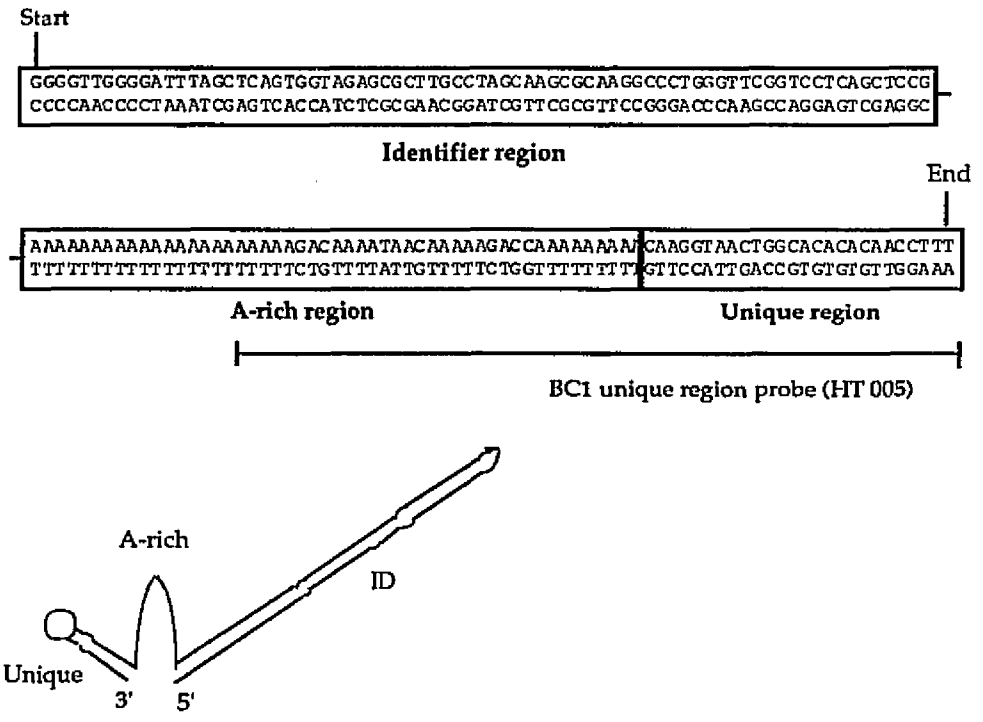


Figure 1. BC1 gene sequence, RNA domains, detected probe (HT005) and predicted RNA secondary structure (GCG program)

In order to understand the function of BC1 RNA, one approach is to study its binding proteins. Like many other small RNAs such as 7SL or U RNAs [73, 124], RNA itself provides a specific interaction with other RNAs as well as serves as a backbone to form a particle with multiple functions. In this chapter, evidence is presented to support the idea that BC1 RNA forms an RNP in the cytosol of neuron. In the meantime, its also possible to elucidate some physical characteristic of BC1 RNP before starting purification efforts.

Results and Discussion

BC1 RNA forms an RNP

Several lines of evidence support that BC1 RNA exists as an RNP in the brain. A summary of results follows:

-After challenge with high concentration of cesium salt, the behavior of BC1 RNA in brain extract is consistent with the characterization of an RNA-

protein complex and demonstrates a smaller buoyant density than its RNA both in Cesium Sulfate and Cesium Chloride gradient.

-The distribution of BC1 RNA signal in CsCl gradient can be altered by the treatment of proteinase.

-Compared with its "naked" RNA, BC1 RNA signal in the brain extract significantly retards its mobility in a electrophoretic field such as agarose gel or polyacrylamide gel.

- On sucrose gradient, the BC1 RNA in the extract has a larger S value than its "naked" RNA.

Cesium salt gradient [125, 126]

CsCl and Cs_2SO_4 gradient were used to identify whether there are any proteins binding to BC1 RNA. There are two established criteria in justifying an RNP particle with this method of cesium salt gradient. First, the interaction between protein and RNA must be very strong (high affinity) in order to be stable in the 4 M high salt environment. Second, since the densities of the protein-only, RNA-protein particle and RNA-only are different, the cesium salt can form a series of density cushions which can counteract the movement of a particle in the ultra-centrifugation field. For this reason, one can estimate the density of target particle by measuring the density of surrounding cesium salt.
Using "naked" total brain RNAs as a control, brain extract was run into cesium salt gradients. To check whether the protocol (100K Xg, 36 hr.) of ultra-centrifugation is sufficient to form a continuous gradient of cesium salt, the Refraction Index (RI) of every 30 fractions from 4 different tubes were measured. The result of the RI profile with the loading of different material within a standard run were plotted against the fraction numbers (**figure 2a and 3a**). As one might expect, they can form a good gradient after ultra-centrifugation both in the Cs_2SO_4 and CsCl run. However, the curves of slope (density gradient) are different between Cs_2SO_4 and CsCl. In the Cs_2SO_4 run, the slope is a straight line. Even one tube (diamond marker) which was damaged after ultracentrifugation still maintained its gradient linearity. The density curve of CsCl gradient, however, has two slopes, the density of first 1/3 of tube increases only slightly, then follows with a deeper density gradient than Cs_2SO_4 gradient. For testing the RNP, both cesium salts serve equally well. However, the CsCl salt may have more capacity per tube for the material with low density as well as more stringent than Cs_2SO_4 gradient .

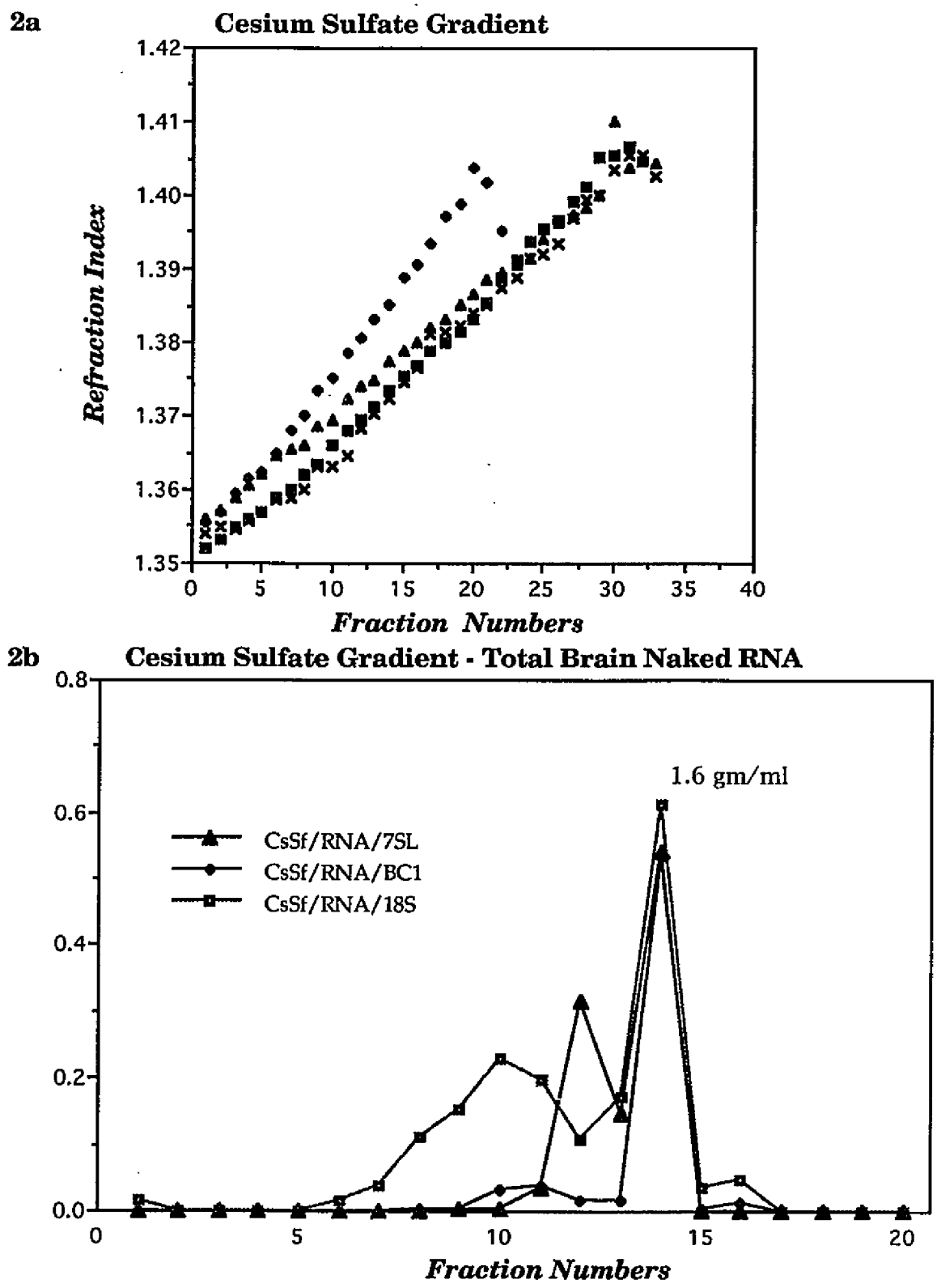
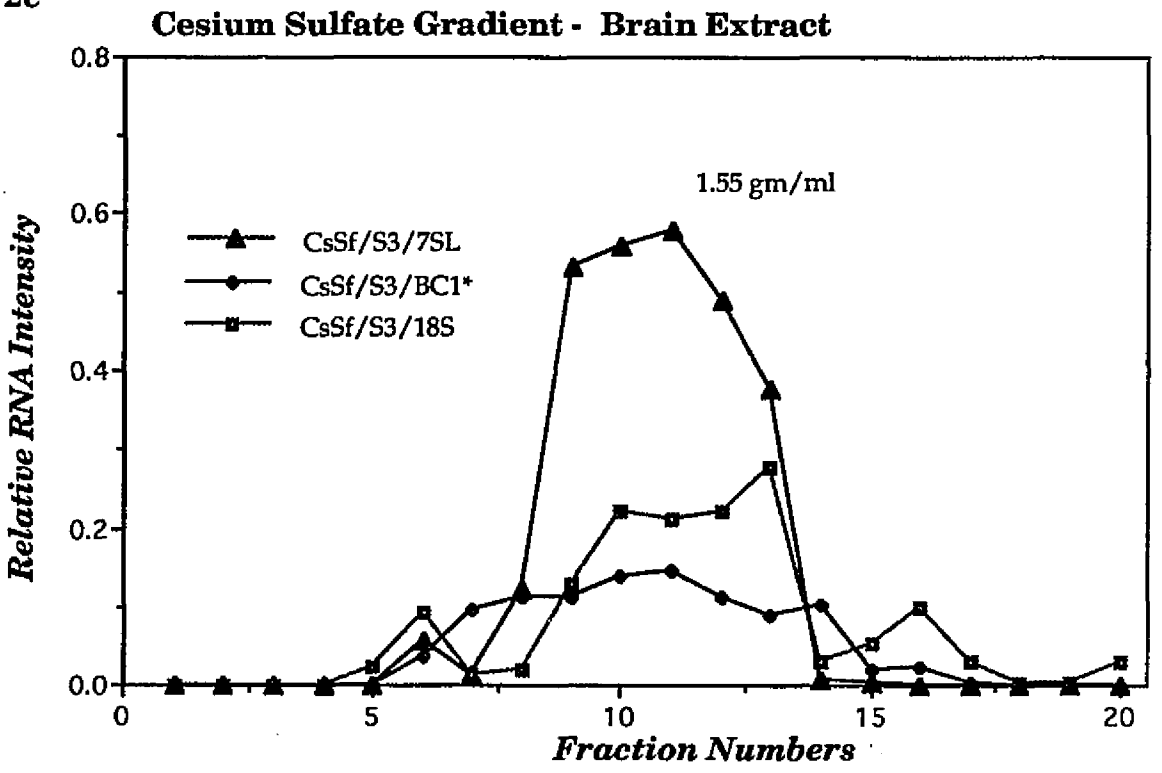
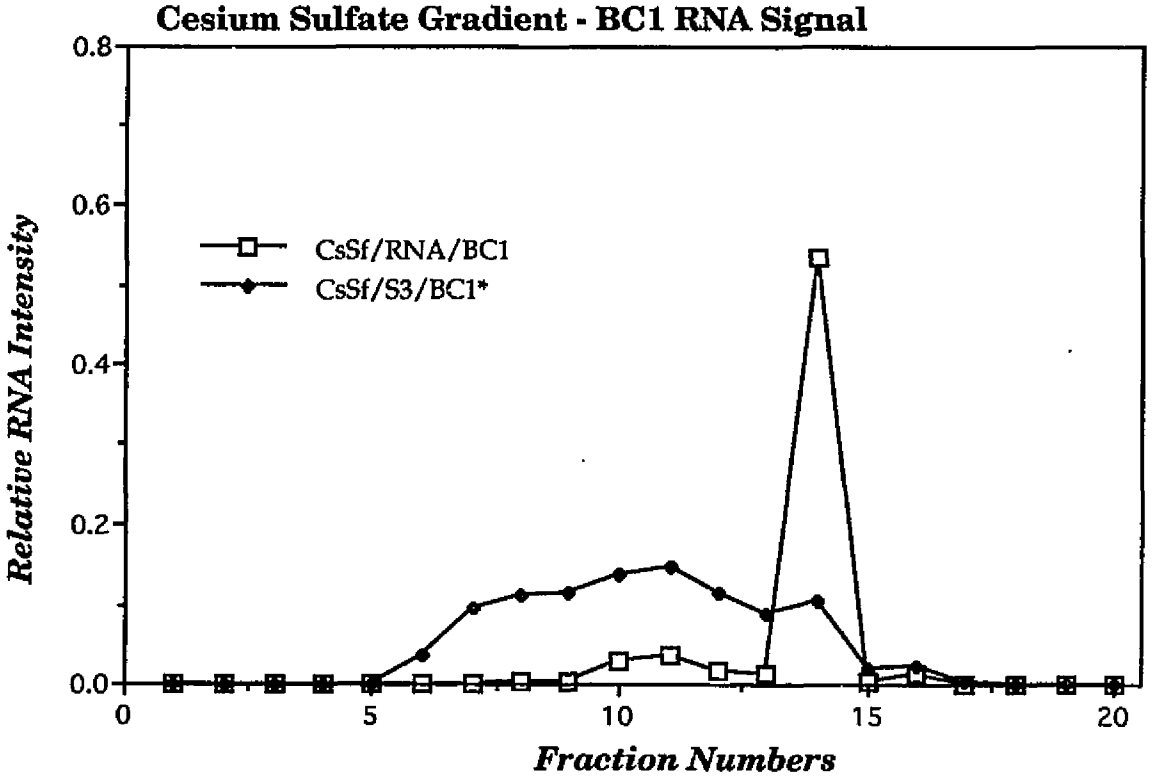


Figure 2. BC1 RNA elution profile in Cs2SO4 gradients. (2a) 4 seperate runs to test gradient linearity. (2b).RNA profile of "naked" RNAs. (2c) RNA profile of brain extract. (2d) Superposition of the BC1 RNA from (b) and (c).

2c


Cesium Sulfate Gradient - Brain Extract
1.55 gm/ml
CsSf/S3/7SL
CsSf/S3/BC1*
CsSf/S3/18S
Relative RNA Intensity
Fraction Numbers
0.0
0.2
0.4
0.6
0.8
0
5
10
15
20

2d


Cesium Sulfate Gradient - BC1 RNA Signal
CsSf/RNA/BC1
CsSf/S3/BC1*
Relative RNA Intensity
Fraction Numbers
0.0
0.2
0.4
0.6
0.8
0
5
10
15
20

The result of RNA signal distribution within centrifugation tubes, and the plot of the strength of RNA hybridization signal versus fraction numbers is shown in **figure 2b**. The "naked" RNA peak locates in the cesium sulfate fraction with a refractive index of 1.3826 which is the same as the 18S rRNA, 7SL RNA, and BC1 RNA; however, the RNA signals in the tubes of brain extract (**figure 2c**) have higher position along the tube than from the tubes of total brain RNAs, which means the density of RNA is getting lighter in the tube of brain extract. It is an indication that there is low density material which can interact with those RNAs. All the RNP signals locate at the range of R.I. from 1.36 to 1.378, and the peak signal of BC1 RNP is close to SRP and 18S RNP. The mean of BC1 and 7SL RNA signal is in the fraction with a R.I. = 1.3662 and 18S RNA are in the fraction of a R.I. = 1.3782. Since both SRP and ribosomes are well known RNP particles, it is apparent that BC1 RNA which has similar behavior in the Cs_2SO_4 gradient also forms an RNA-protein complex. Compared to their "naked" RNA signal which are banded, all tested RNPs in the Cs_2SO_4 gradient, however, forms a wide range of distribution. A possible explanation for a wide range of densities of one specific RNP is due to the different lengths of the RNA or to different protein components. The RNase activity seems to be eliminated in such high salt conditions, therefore it is plausible that the protein composition somehow is different at different densities. Taking these finding together, it supports that BC1 RNA is as SRP RNA and 18 S rRNA, which forms an RNP with several proteins binding to it. To transfer the R.I. of cesium sulfate into density , the following formula was used

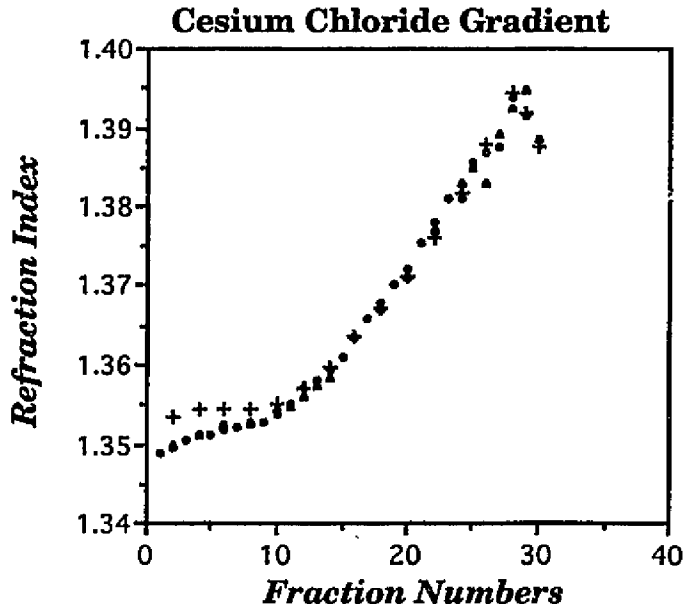
$$r_{25^o} = 0.9954 + 11.1066(\eta-\eta_0) + 26.4460(\eta-\eta_0)^2,$$

η = measured R.I., ηo = PI of water = 1.3330*

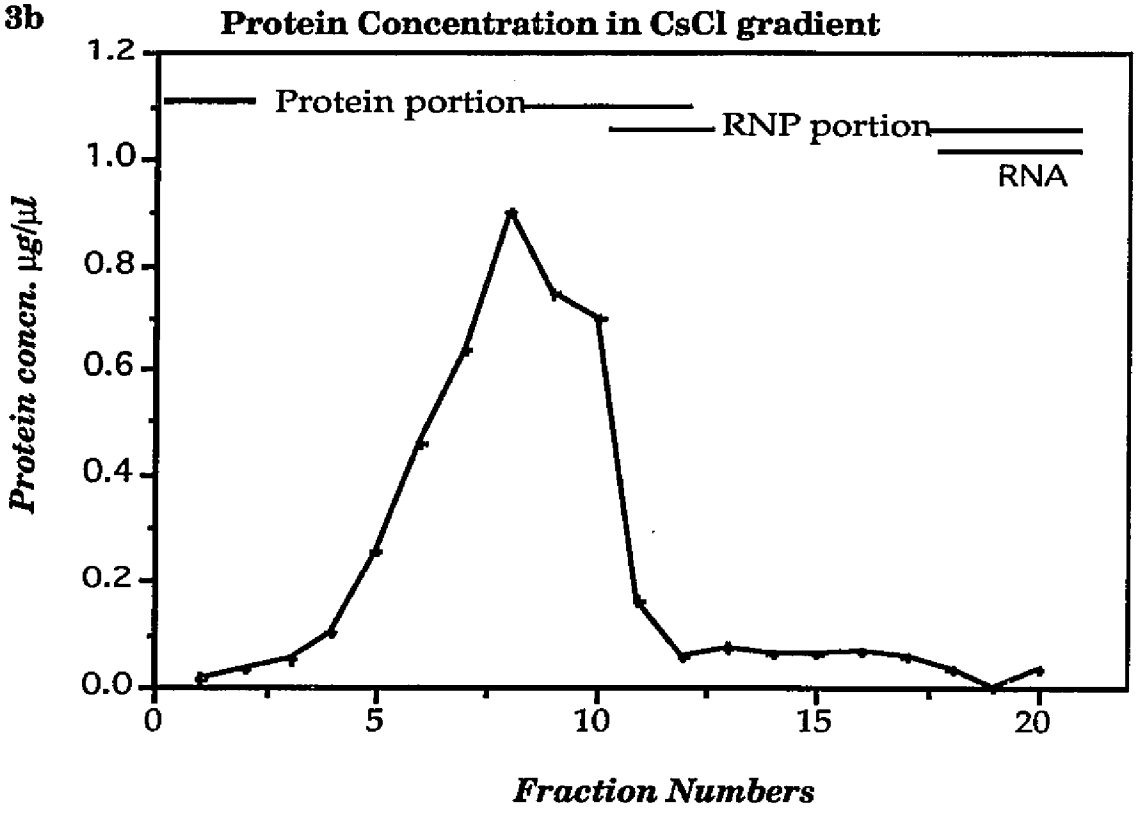
The buoyant density of BC1 RNP in cesium sulfate gradient is in the range of 1.4 to 1.55 gm/ml which is close to the buoyant density of SRP and ribosome (or small ribosomal subunit). Their RNAs have the same buoyant density of 1.6 gm/ml.

In terms of the RNA-protein interaction, CsCl is more stringent than Cs_2SO_4. Following the Cs_2SO_4 experiment, the CsCl was used to perform the same procedures. The protein concentration was also measured in different fractions. It demonstrates that the protein concentration is dramatically reduced in the region with higher density (RNP region) (**figure 3b**). Most of RNA signals are precipitated on the bottom which is the characteristic of CsCl gradients. Compared to the run of total brain RNA (**figure 3c**), brain extract shows two

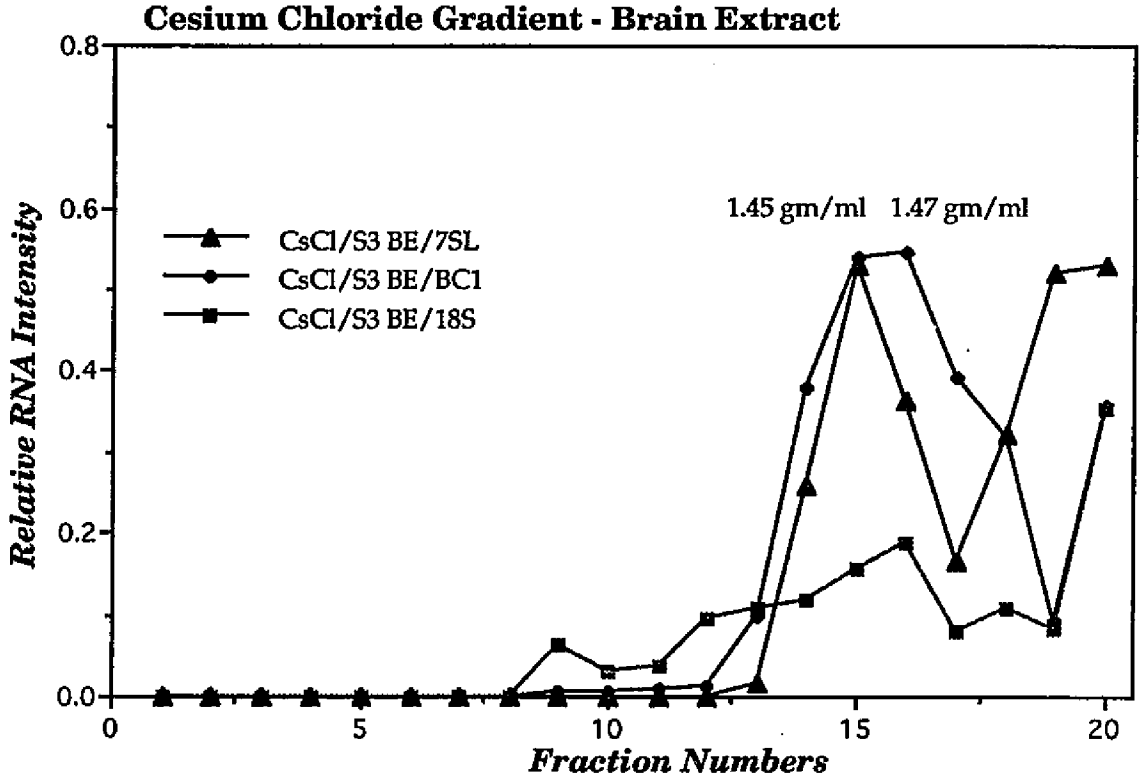
3a


Cesium Chloride Gradient
Refraction Index
1.40
1.39
1.38
1.37
1.36
1.35
1.34
0
10
20
30
40
Fraction Numbers

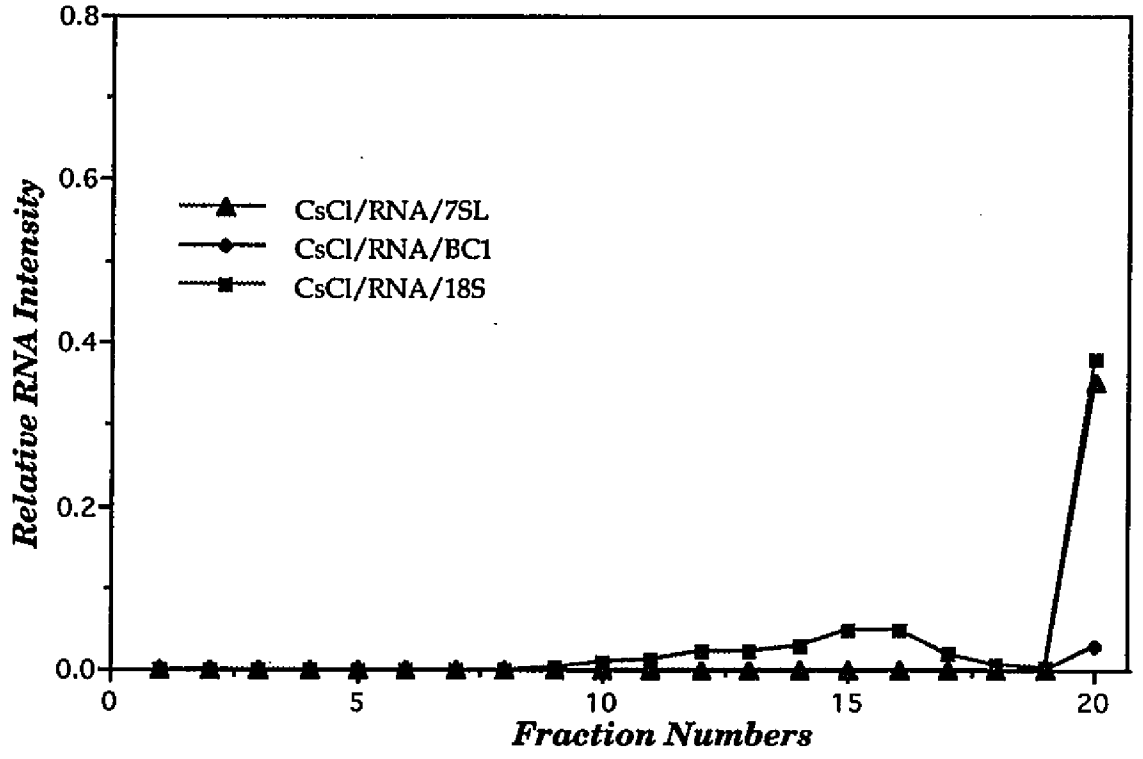
3b


Protein Concentration in CsCl gradient
Protein portion
RNP portion
RNA
Protein concn. µg/µl
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
Fraction Numbers

3c


Cesium Chloride Gradient - Brain Extract
0.8
0.6
0.4
0.2
0.0
Relative RNA Intensity
1.45 gm/ml
1.47 gm/ml
CsCl/S3 BE/7SL
CsCl/S3 BE/BC1
CsCl/S3 BE/18S
0
5
10
15
20
Fraction Numbers

3d


Cesium Chloride Gradient - Total Brain Naked RNA
0.8
0.6
0.4
0.2
0.0
Relative RNA Intensity
CsCl/RNA/7SL
CsCl/RNA/BC1
CsCl/RNA/18S
0
5
10
15
20
Fraction Numbers

3e

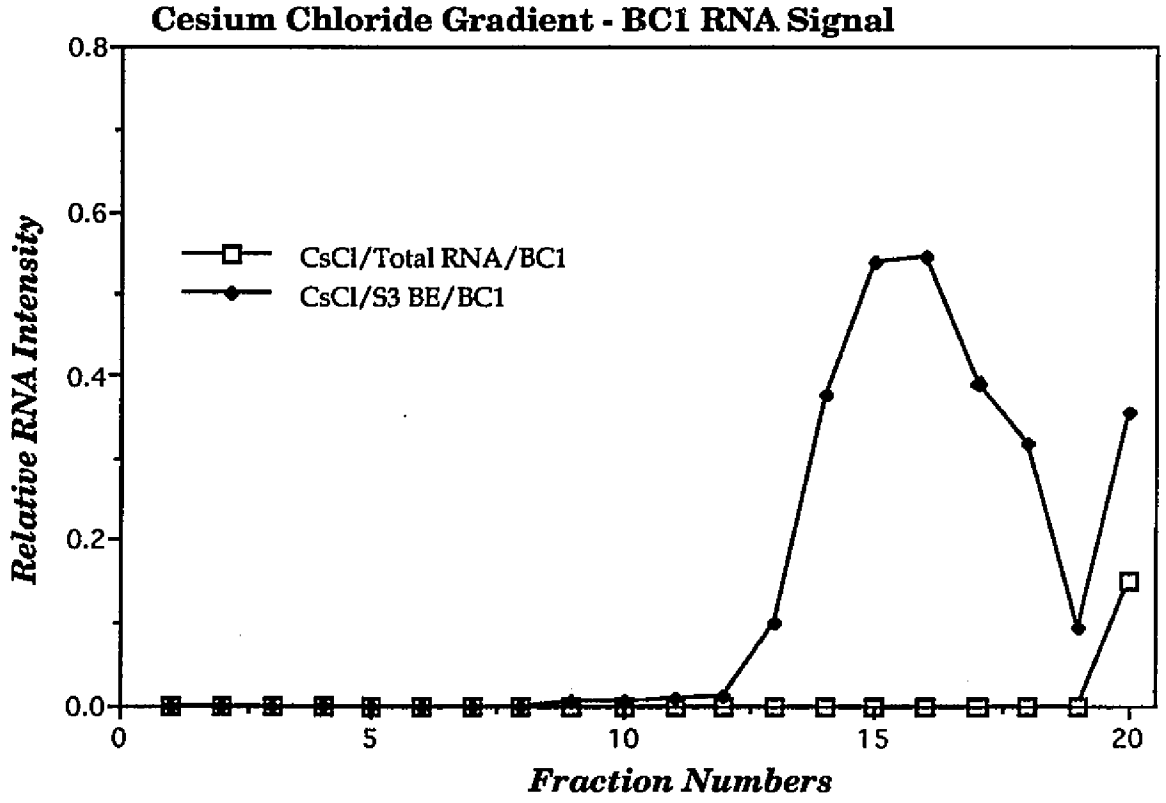


Figure 3. BC1 RNA elution profile in CsCl gradients. (3a) Gradient profile. (3b) Protein concentration within the CsCl gradient. (3c) RNA profile of "naked" RNAs. (3d) RNA profile of brain extract. (3e) Superimposition of BC1 RNA from (c) and (d). (BE = Brain Extract)

peaks of RNA signals for any one of RNA species (**figure 3d**). It is suspected that the bottom one is free RNA or RNA with very few protein binding. Another peak around R.I. = 1.472 is considered in the RNA-protein complex range (RNP portions). The signal distribution is very similar between 7SL and BC1 RNP. The majority of signals of 18S RNA are on the bottom with an expanded weaker signal along the low half of tube. The intact 18S RNA containing particle does not survive in the CsCl gradient and sediments at the bottom of tube. It may also due to the size of 18S RNA which requires a high ratio of binding protein to maintain a lighter density. After interpreting the refractive index into density ($r25^{o}$ = 10.8601R.I.-11.1066), it was shown that the buoyant density of SRP in CsCl

solution is 1.47 gm/cm^3, and BC1 particle is 1.45 gm/cm^3. The different buoyant density of BC1 RNP between Cs_2SO_4 and CsCl gradient may be caused by the different hydration degree or different components of associated proteins. The result of buoyant density in CsCl gradient can be used to estimate the percentage of protein within an RNP particle. SRP particle contains 68.5% of protein and BC1 particle contains 73.28% of protein as determined by the following formula:

$$\% \text{ protein} = 1.87 - \rho \ / \ 0.004\rho \ , \ \rho = \text{buoyant density in CsCl solution.}$$

It also provides an indirect method to predict the molecular weight of those binding proteins. SRP RNA has 300 nucleotides which is nearly a M.W. of 99 kDa. There are six associated proteins in the SRP from canine pancreases with a total molecular weight of 236 kDa. This suggests that the protein percentage of SRP is 70%, which are very close to the result of the prediction from the buoyant density of SRP in CsCl gradient. Since the M.W. of BC1 RNA is around 50 kDa, it may suggest that the sum of BC1 binding proteins M.W. may be close to 138 kDa.

Sucrose gradient [127]

By using the "zonal method" and following equation:

Sx/Sm = distance (x) / distance (m)

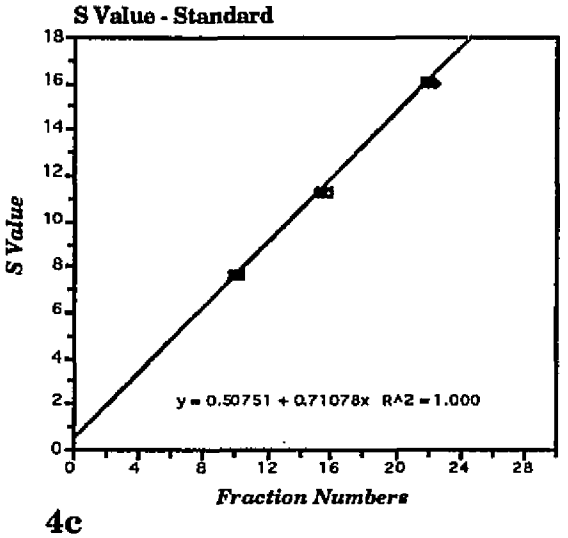
= fraction number (x) / fraction number(m)

x = unknown S value; m = standard marker's S value

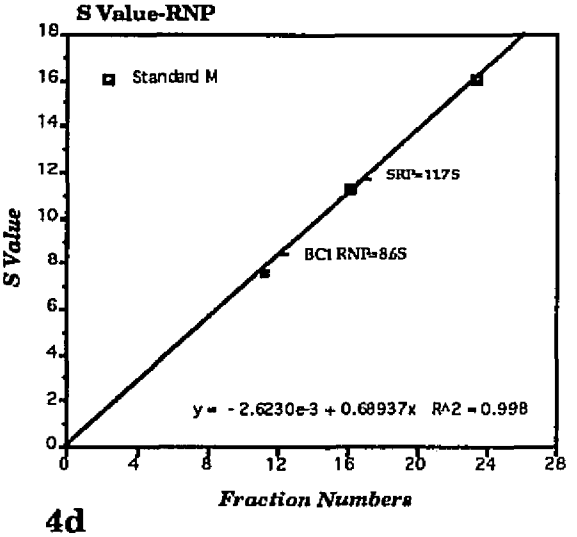
the S value of BC1 RNP has been determined. Three standard markers were applied, alcohol dehydrogenase (7.61S), catalase (11.3S) and β-galatosidase (16S). To approach the S value of BC1 particle, BC1 RNA and BC1 particle after CsCl gradient run. In each run, SRP and 7SL RNA act as reference groups. After those enzyme activity were assayed, the normal distribution of each enzyme activity was obtained by using graphic software (Igor). The mean of normal distribution which represents the major location of each enzyme along the sucrose gradient was calculated and plotted against the S value. The first trial tests the consistency of this system. The result is shown in **figure 4a.** Three individual runs show good consistency and precision. The same protocol was applied to the run of brain extract along the sucrose gradient. The S value of different testing groups have been calculated as follows (**figure 4b, 4c, 4d,**). The S value of BC1 particles from brain extract is nearly 8.6S, and that of the SRP is close to 11.7S. The S value of BC1 RNAs which come from total brain RNA is nearly 6.4S, and 7SL ~= 8.8S. The BC1 particles which came from the CsCl gradient (called af Cs BC1) is 5.7S, and SRP which came from same preparation is nearly 8.3S.

Figure 4. S values of different RNPs in sucrose gradients. (4a) Sucrose gradient calibration.(4b) S value of RNPs. (4c) S value of "naked" RNA. (4d) The S value of RNPs after CsCl run. (4e) S value of in vitro synthesized RNAs. (4f) S value of "naked" RNA.

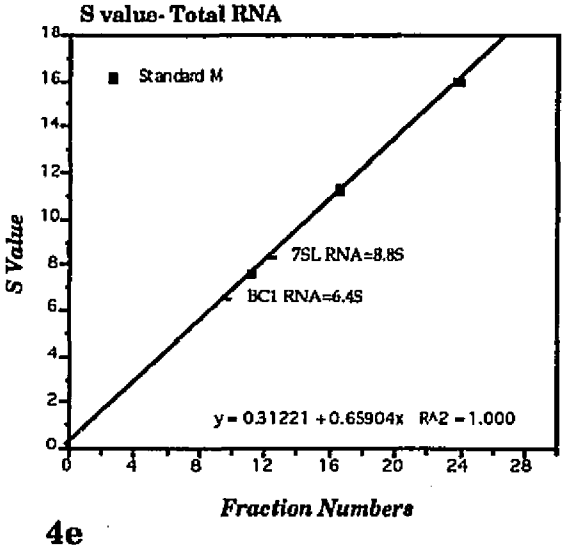
4a



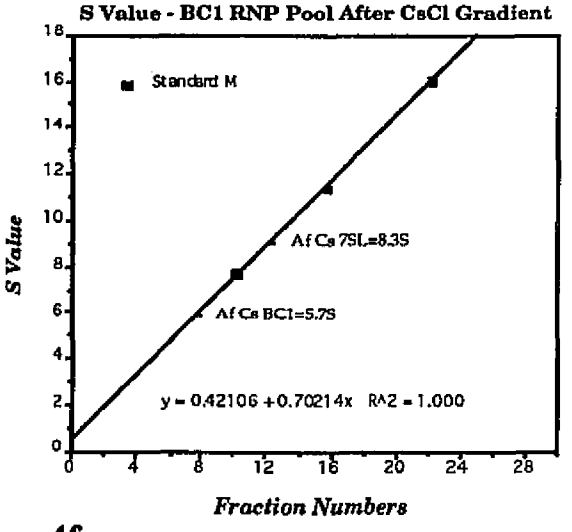
4b



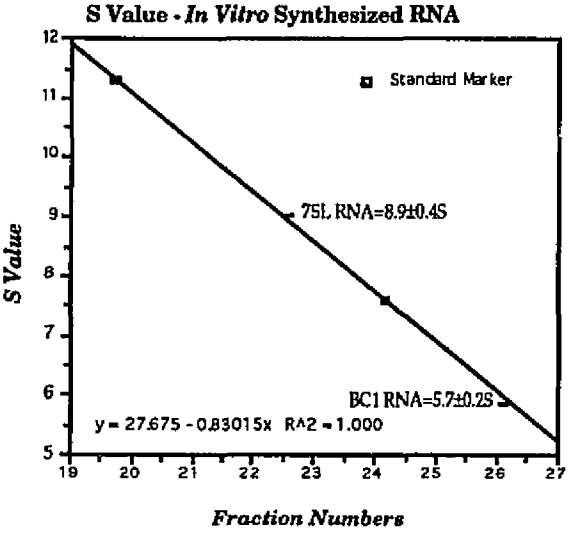
4c



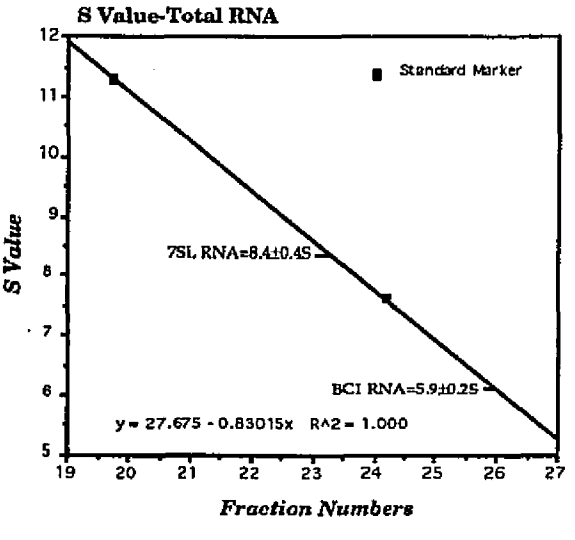
4d



4e



4f



The S value of SRP (11.7S) is not so different from the published data, however, the S value of BC1 particle is different from the 10S which was suggested by another group [95]. Although we studied different species, the BC1 RNA sequence in rat and mouse are nearly identical. There are several explanations for such a different result. First, the preparation of the loading material may be different. We used the diluted brain extract which is a post-microsomal fraction (100K X g) and Kobayashi's group [95] use the post-mitochondria extract (15K X g). Second, the gradient material is different. Sucrose gradient was used in our measurements and Kobayashi's group used glycerol gradient. Third, the marker is different. They use pure RNA marker such as 18S RNA, 5.8S RNA and tRNA (4S). On the contrary, we used well characterized protein markers and applied them both exogenously and endogenously. A problem for those RNA markers is that their S value has not been determined in the glycerol gradient.

Another discrepancy in lies the S value of the particles after cesium chloride with the S value of the "naked" RNAs. The S values of BC1 particles and SRP from the CsCl run are smaller than the ones of "naked" RNA which was prepared from total brain. In fact, already the S value of BC1 (6.4S) and 7SL RNA (8.8S) are larger than one would expect. This problem which is mentioned above may simply be caused by the over-estimation of the S values of those RNAs. In the study of small RNA, the size of small RNAs are usually estimated from the relative migration distance in relation to other small RNAs such as 5S RNA in a denatured condition. Unlike at denaturing conditions, RNAs form a very complex secondary structure in solution. The density and shape of a particle has great influence on its migration in the sucrose gradient. To confirm the S value of BC1 and 7SL RNA, *in vitro* synthesized BC1 and 7SL RNA (**figure 4e**) as well as total RNA from another source (**figure 4f**) has been examined for the integrity of BC1 RNA in terms of RNA length. Before running the sucrose gradient, two other precautions were taken. One was to change the slope of the sucrose gradient; another was to run the standard markers exogeneously to avoid RNase contamination. In both results, BC1 RNA has smaller but close S values (6S) than the previously runs, and 7SL RNA has the same S value (8.8 S). Although there is some variation in different runs, it is clear that BC1 and 7SL RNA have larger S values (6.4S & 8.8S) in sucrose gradient than the S value which were estimated from the denaturing gel (5.7S & 7S). It should be mentioned that BC1 RNA and

7SL RNA have another peak with higher S value in the run of total RNA but not in the run of *in vitro* synthesized RNAs. One explanation is that the BC1 RNA and 7SL RNA may associate with other RNAs or some contaminant and then increase their respective S value. An alternative explanation is that there are two conformations of RNAs existing in the total brain RNA which can also be seen in the run of native gel. Going back to the original puzzle that the S value of BC1 and 7SL RNP after CsCl centrifugation are smaller than the "naked" RNAs. One explanation is the RNP which are prepared from the pool of CsCl gradients have been stripped off all their binding proteins during the steps of desalting and concentration. This idea may be confirmed by immunological methods with the antibody against different protein components of SRP. The result may indicate not only the existence of 7SL binding protein but also the species of proteins after CsCl gradient and following preparation steps. It is also possible that the RNA conformation is changed by its binding protein. The predicted secondary structure of BC1 RNA is one long stem-loop and one small stem loop (**figure 1**) which are connected by a single strand RNA bridge. It may also form a tRNA-like tertiary structure with possible additional 3′ structure (s). It is difficult to predict the influence of RNA conformation after protein binding. The answer awaits the availability of purified protein components.

Native gel electrophoresis

Further evidence to support the notion that BC1 exists as an RNP came from electrophoresis data (**figure 5**). Agarose gel and polyacrylamide gel have been used to separate snRNPs and the spliceosome previously [128, 129]. In such a dilute and charge separated condition, it may require some degree of affinity to maintain the association of different components within a particle. The hybridization results are shown in the **figure 5**. The movement of BC1 and SRP RNAs which came from brain extract are significantly slower than those that originated from "naked" brain RNA. None of those RNA signals formed a sharp band. This may be a indication for particles with complicated conformations. Since the charge of particle is more important than its size in determining the migration distance in the agarose gel system, it is hard to predict the behavior of a particle with a size less than the diameter of the agarose pore. However, if we only compare within one RNA species such as BC1 RNA, it is shown that the native charge of RNA is compensated by other associated molecules. The result is the mobility change between RNA and RNP.

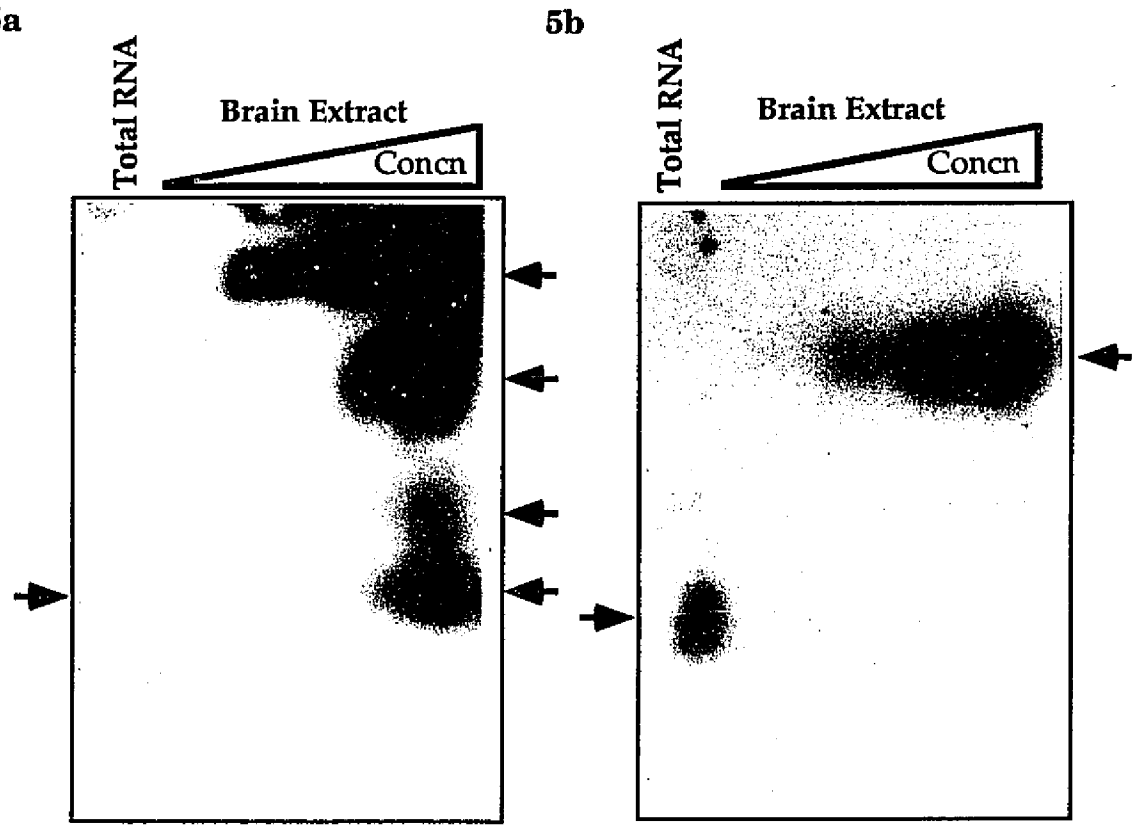


Figure 5. The mobility difference of "naked" BC1 RNA and BC1 RNP in 1% of agarose gel electrophoresis. (5a) RNA blot probing for 7SL RNA; (5b) RNA blot probing for BC1 RNA. (Arrows indicate the RNA siginal.)

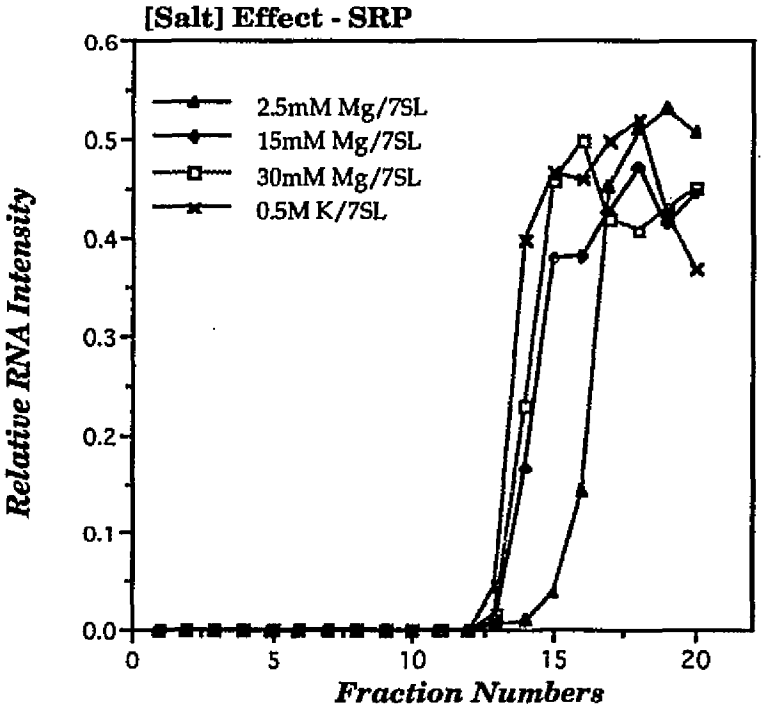
Characterization of BC1 RNP particles

Taking advantage of RNA precipitation and high stringency, CsCl gradient was used to test the distribution of BC1 and SRP particles after different kind of treatment.

Salt concentration effect

It has been reported that assembly or stability of an RNP is influenced by the salt concentration [130, 131]. In the case of SRP, its assembly requires the existence of Mg^{++} ions. Once deprived of all the salt in the solvent, SRP will disassemble. One ought to control the salt concentration to dis- or re-assemble the SRP *in vitro*. Based on this reason, the effect of salt on integrity of BC1 RNP particles has been tested. A further application of this study is try to find conditions under which one can disassemble or reassemble BC1 RNP.

6a



6b

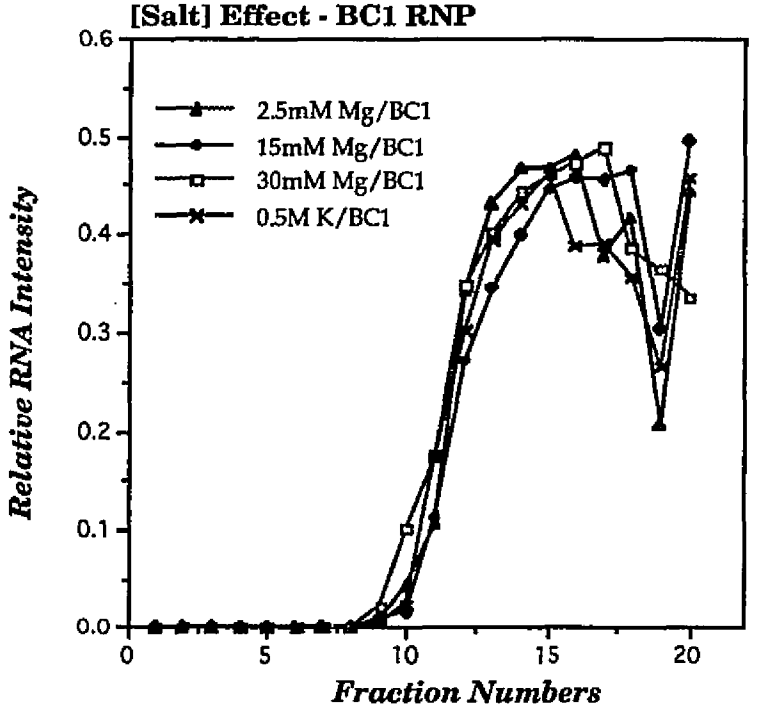


Figure 6. The effect of salt concentration in the distribution of BC1 RNA (6a) and 7SL RNA (6b) in CsCl gradients.

During the preparation of brain extract, the magnesium and potassium concentrations in homogenization buffer were altered. There are four testing groups, 2.5, 15, 30 mM $MgCl_2$ with 100 mM KCl and 15 mM $MgCl_2$ with 500 mM KCl. The signal of BC1 RNA did not change in each group; however, the signal of 7SL RNA shifted down (to bottom) once the concentration of Mg^{+2} was lower (**figure 6a**). This suggests that SRP requires higher [Mg^{+2}] for assembly and sheds some of its binding protein in response to the low magnesium concentration. On the contrary, BC1 RNP does not experience such an effect of magnesium (**figure 6b**). Some other divalent cations such as Ca^{++} or Zn^{++} may be required for the BC1 RNP formation. Since both elements function in the μM level, Other assay systems must be used.

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High KCl concentration did not alter the distribution of BC1 and SRP RNA signal dramatically. The common role that monovalent cations such as potassium or sodium in the cytosol provide is ionic strength. As the salt concentration is lowered, the protein-protein interaction increases; if it is too high, the weaker the interaction between macromolecule will be and the stronger the hydrophobic force will be. Since increasing the KCl up to 5 times does not change the buoyant density and the distribution of RNA signal, it may suggest a small effect of [KCl] concentration. The explanation of such results may come from the effect of cesium salt. In the portion of RNP region in CsCl gradient, the concentration of cesium chloride already reaches 4 M high salt. In this condition, most protein-protein interaction is eliminated. Adding more potassium will not have any effect in this assay system. Since the protein-protein interaction is decreased in a high salt environment, it may also suggest that BC1 particle with a

buoyant density of 1.45 gm/cm^3 represent the core particle which is organized by the RNA and its binding protein rather than a physiological form of particles.

Other treatments

The brain extracts were treated with RNase A (10 mg/ml, 5 µl), proteinase K (20 mg/ml, 5 µl) and heat (70°C) for 30 min. before loading onto CsCl gradient (**figure 7a1, a2**). The purpose of RNase treatment is to address the protection of BC1 RNA by its binding proteins. This may provide some information for the purification of BC1 RNP. Proteinase K treatment is to provide further evidence to support the existence of BC1 RNP. As it has been shown previously, there are differences in buoyant density and the mobility between BC1 particle and "naked" BC1 RNA. Proteinase K treatment can confirm that such change is caused by protein factors. Heat treatment also provides information about the stability of BC1 RNP.

7a1 **7a2**

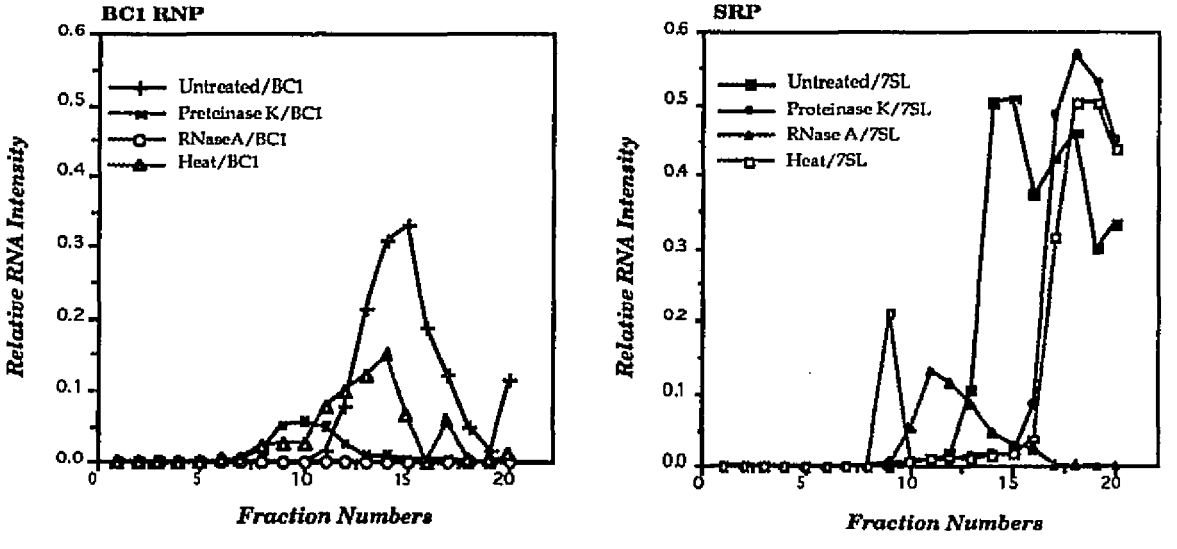
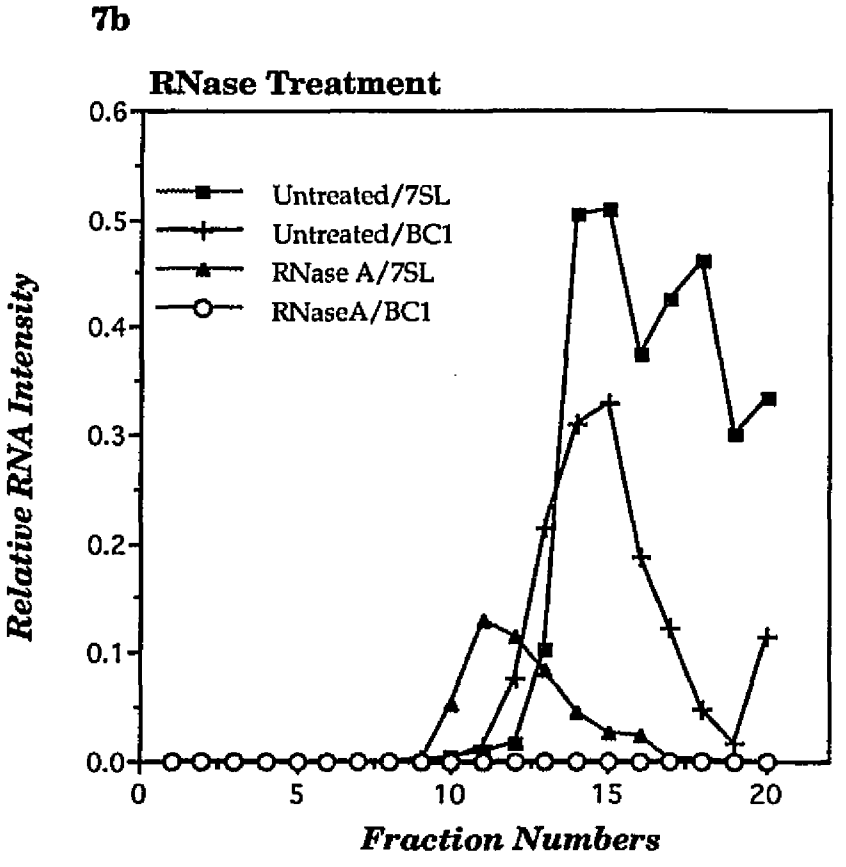


Figure 7. The distribution of (7a1) BC1 RNA and (7a2) 7SL RNA in CsCl gradient after: (7b) RNase treatment. (7c) Proteinase K treatment. (7d) Heat treatment.

The signals of BC1 and 7SL RNA in treated groups were compared to the signal of those groups without treatment. Both the BC1 RNA signal in RNA fractions and RNP fractions disappear after RNase A treatment, and the signal of 7SL RNA declines dramatically (**figure 7b**). The differential RNase resistance

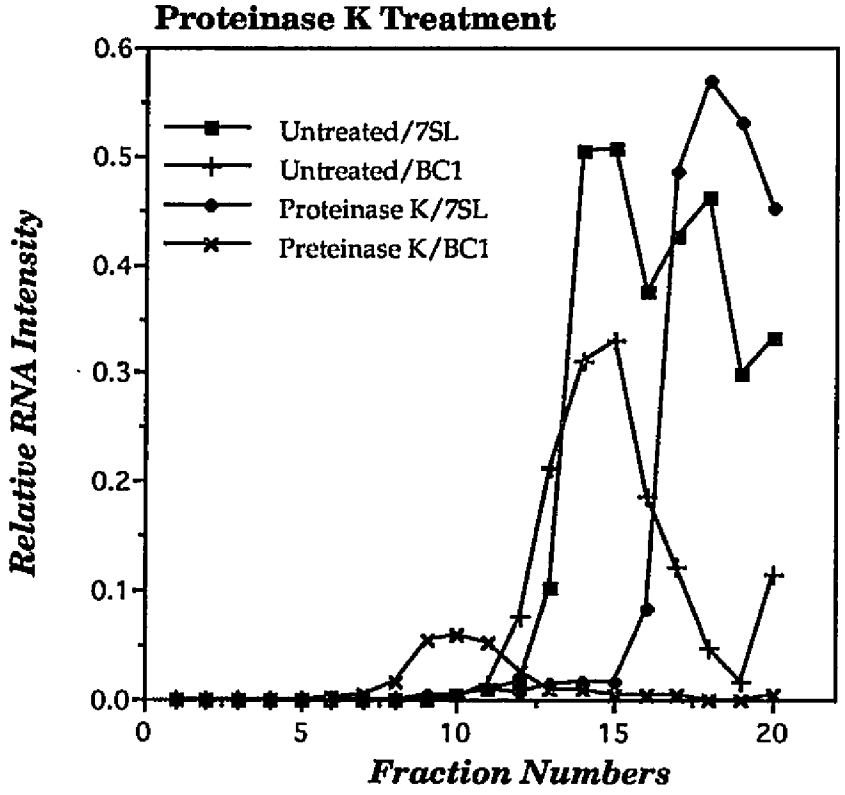
between 7SL and BC1 RNA may result from the original amount of 7SL and BC1 RNAs in the brain extract. However, BC1 RNP may also be more sensitive to the RNase than the SRP, at least in the portion of RNA which is complimentary to the DNA oligomer (probe). The up-shift of the signal of SRP RNA may be explained by a piece of SRP RNA rather than a intact RNA which was protected by the proteins from RNase degradation. When a technique called RNA footprinting with splint labeling method was applied and performed with crude extract [132](see next section), it showed that the portion of BC1 RNA which is more sensitive to RNase is also the target of hybridization probe in the RNA dot blot assay.



After proteinase K treatment (**figure 7c**), the signal of SRP RNA shifted down without changing its total signal intensity; however, the BC1 RNA signal shifted up with a weaker signal. This opposite movement may suggest that 7SL RNA loses its binding proteins and BC1 RNA adds more proteins. Since the signal of BC1 RNA declines, it may indicate that RNase also plays a role in this change. The shift-up signal may be explained by a protein-protein interaction for

protecting a BC1 RNA fragment from further degradation. As nothing but proteinase K in the extract has been added which was predigested to get ride of RNase activity, it not only provides further evidence that BC1 RNA relies on proteins factors to protect it from RNase degradation, but it also suggests that specific ribonuclease play a role in degrading the BC1 RNA but not the SRP RNA. From this experiment as well as previous studies. RNase, both from exogenous and endogenous, can be hazard to the integrity of BC1 RNA or RNP. This complicates purification of this RNP.

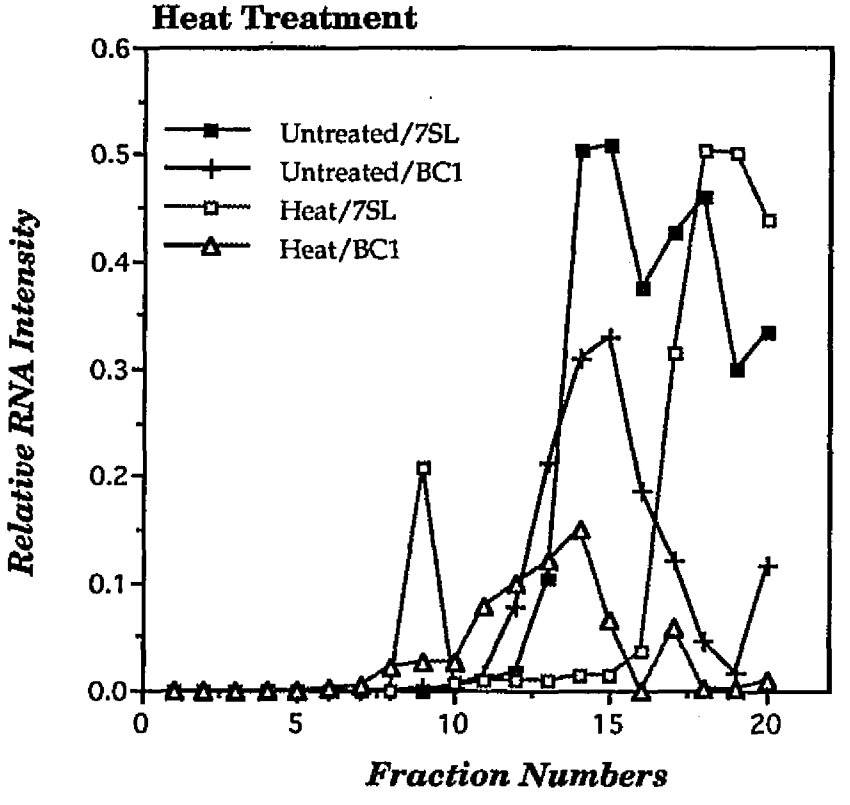
7c



The BC1 particle is more heat stable than SRP (**figure 7d**). After heat denaturing, the signal of BC1 RNA got weaker but still maintained its RNP position. Whether the proteins on BC1 RNA is more heat resistant (cytoskeleton components?) or BC1 RNP reassembles very fast, is a interesting observation which may provide some ideas concerning the function of BC1 RNP. As in other experiments, once the BC1 particle was altered, the BC1 RNA is a good target for RNase. Thus, one can not make use of heat stability of BC1 RNP as an advantage

to be integrated it into purification scheme. The signal of SRP only changes its distribution but not its strength. Most of SRP RNA signal goes to bottom which suggests the RNA binding protein lost its activity during heat treatment. Minor signal shifts up to very high fractions may represents the forming of large protein-RNA complexes which presumably result from the aggregation of denatured proteins.

7d



In summary, like the SRP, BC1 RNA is present as an RNP in the brain extract. BC1 RNP has an S value of 8.6 and its buoyant density is 1.4 - 1.55 g/ml in Cs_2SO_4 gradient and 1.45 g/ml in CsCl gradient. Unlike SRP, the assembly of BC1 RNP core particle is insensitive to Mg^{++} ions and is more heat stable. The BC1 RNP is sensitive to RNase which may suggest that RNase may play a role in the regulation of the function of BC1 RNP in the brain. For the sake of purification, reducing the RNase activity is a major task.

BC1 RNA footprinting with the splint method

To obtain further information on how the proteins interact with BC1 RNA, a well established technique that use a new labeling method has been used [132]. This so called RNA footprinting by the splint labeling method can tell which portion of BC1 RNA is covered by protein which renders it resistant to RNase degradation (**figure 8**).

Even without adding any RNase, the portion of BC1 RNA 3' end up to the ID domain of BC1 RNA already becomes the target of endogenous RNase (**figure 8**, lanes without RNase). This sensitive region includes a small part of the ID domain, A-rich region and unique region in which the target of the antisense probe is routinely used to detect the BC1 RNA. This RNA degradation increases after 10 min. incubation at 37°C without adding RNase (**figure 8a**, Brain extract without RNase). After adding small amount of RNase, the "naked" RNA and brain extract show different results. The "naked" RNA shows degradation in other part of ID domain and the level of intact BC1 RNA decreases (**figure 8a**, Brain RNA with RNase). On the contrary, the RNA from brain extract showed the protection in the small portion of unique region as well as two discrete fragments in the ID domain (**figure 8a**, Brain extract with RNase). Once increasing the RNase, BC1 RNAs disappears which suggests multiple cleavage events. Those protected fragments may be interpreted as the domains which are covered by their binding proteins. Summary of those RNase sensitivity sites is diagrammed in the **figure 8b**. Both "naked" brain RNA and the *in vitro* synthesized RNA show no protected region, suggesting this protection is not caused by RNA structure. Those sites not only provide the possible clues towards the secondary structure of BC1 RNA, but also give an indication of the change of RNA structure after protein binding. From this study, it is suggested that intact BC1 RNP is very sensitive to the RNase which may contribute to significant loses of BC1 RNA signal during purification.

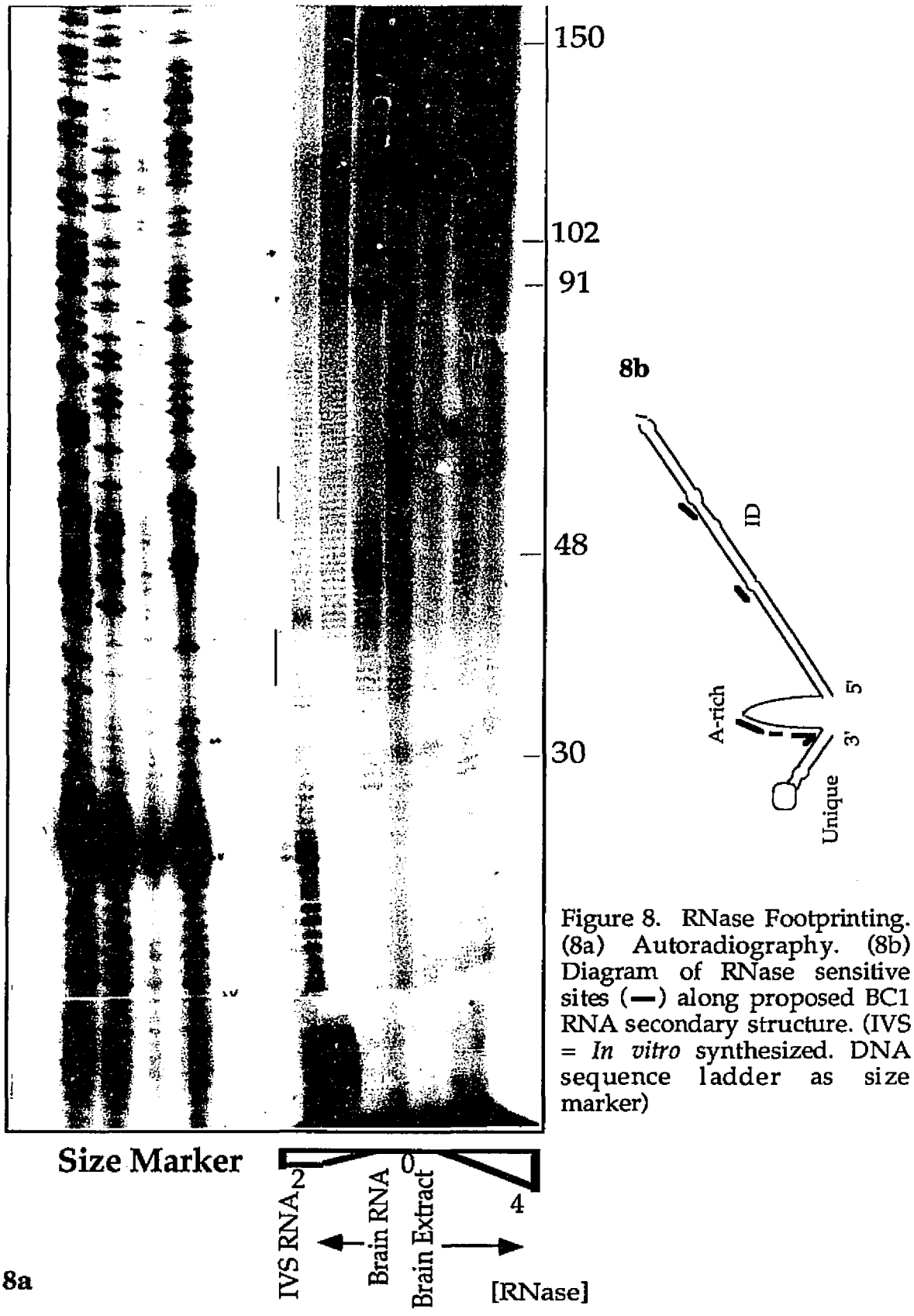
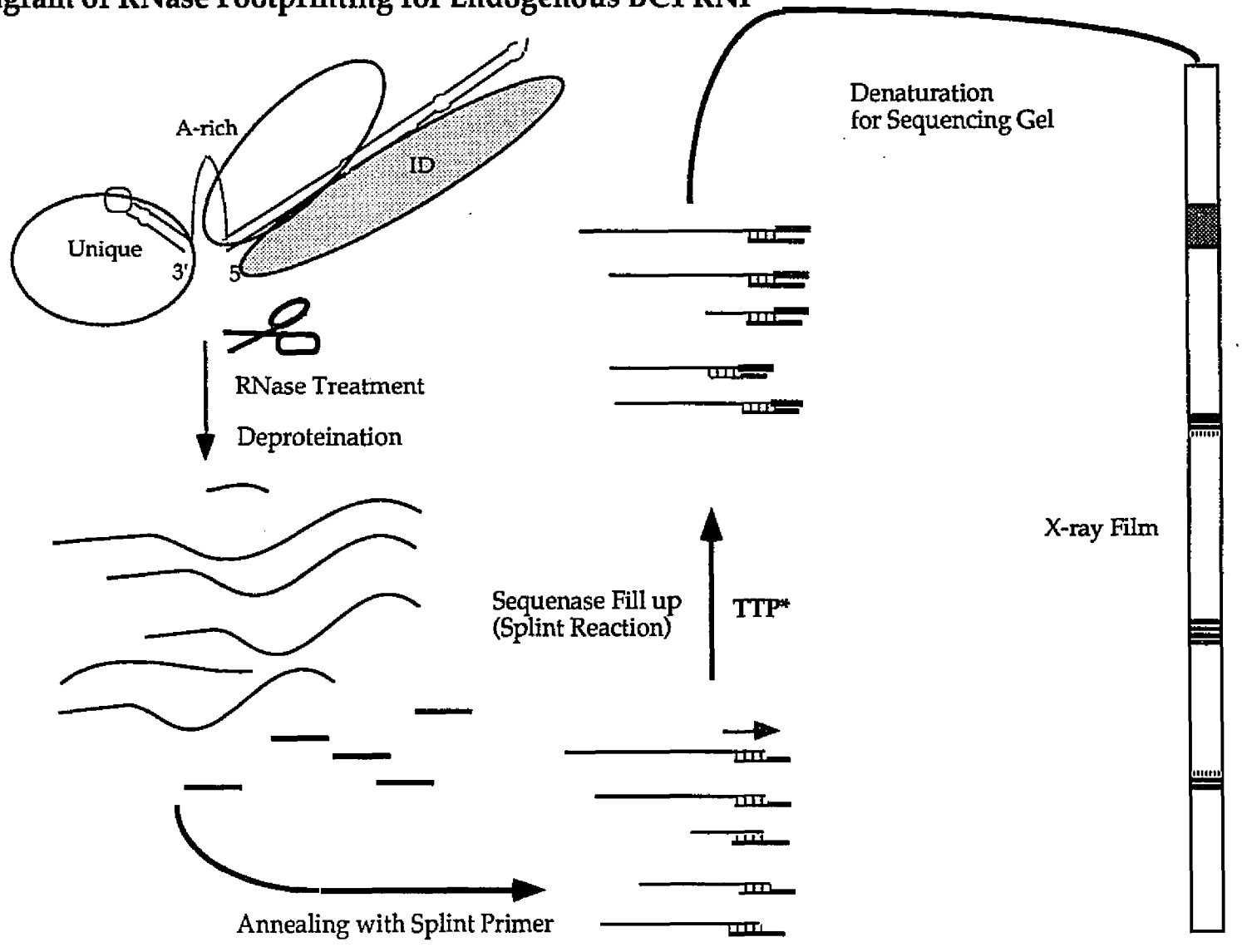


Figure 8. RNase Footprinting. (8a) Autoradiography. (8b) Diagram of RNase sensitive sites (—) along proposed BC1 RNA secondary structure. (IVS = *In vitro* synthesized. DNA sequence ladder as size marker)

Diagram of RNase Footprinting for Endogenous BC1 RNP



Chapter III

Purification of BC1 RNP particles

Introduction

The RNAs within a cell can be classified into different groups based on their metabolic roles: namely, ribosomal RNA, messenger RNA and transfer RNA [6]. However, nearly 1% of total RNA belongs to a group of small RNA with a size less than 8S as well as several large RNAs which do not fit the traditional classification [44]. Depending on their subcellular distribution, small RNA can be further classified into small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small cytoplasm RNA (scRNA) and small organelle RNA (sorRNA). The snRNA and snoRNA usually play a role in pre-mRNA and pre-rRNA processing [133, 134]. The MRP is involved in DNA replication of mitochondria [68]. Besides SRP (signal recognition particle) which plays a role in the translocation of nascent peptide chain into the ER lumen, the functions of other scRNAs are still obscure [60].

BC1 RNA is a 152 nt. small RNA which is present predominately in the cytoplasmic fraction of brain extracts and trace amounts in ovary and testes [1, 3]. Using *in situ* hybridization analysis, it has been shown that BC1 RNA is specifically transcribed in neurons [111]. The expression level of this RNA varies in different subsets of neurons. One interesting finding is the subcellular localization of BC1 RNA which is in the soma as well as in the proximal and distal part of dendrites. The gene of BC1 RNA is conserved among all studied rodent species; however, no homologous counterpart of this small RNA was found in other mammalian orders such as artiodactyls (bovine) and primates. There is one analogue small RNA, BC200 in the primate brain, which shares common features with BC1 RNA [2].

The function of BC1 RNA is still unknown; however, there are several possibilities based on various observations. BC1 RNP may be involved in the translational regulation or it is involved in the RNA sorting machinery. From sequence comparison, BC1 RNA has originated from $tRNA^{Ala}$ [14]. In theory, it may also preserve a tRNA-like secondary structure and therefore it would not be surprising that BC1 RNA interacts with ribosomes and regulates protein synthesis [43, 111]. Another clue comes from its dendritic location. There are only few known mRNAs that are sorted into the dendritic compartment of neurons (for review see [105]. The activity-dependent protein synthesis is thought to be

important in the reinforcement of the connections between the pre- and post-synaptic sites. Dendritic RNAs may be an important target in response to this local demand of activity input [109, 118, 120]. Since BC1 RNA is a non-messenger RNA and is enriched in dendritic spines [135], it may suggest that BC1 RNA plays a role in such *in situ* protein synthesis events [111]. Recent findings suggest that BC1 RNA is the only RNA that is colocalized both in dendritic mRNA and axonal mRNA [113]. Since the axonal terminal usually excludes the protein synthetic machinery, it would suggest BC1 RNA plays a role other than translational regulation. However, as the BC1 RNA has several domains, it may also exert different functions.

To understand the physiological role of BC1 RNA, it is important to characterize its binding proteins. Since the ID domain shares some sequence similarities with the Alu domain of SRP RNA, the Alu binding 9/14 kDa proteins [77] may be a component of BC1 RNP particle. The results of filter binding assays, however, rules out this possibility. It has also been suggested that a zinc finger containing protein, named BP-1, may bind to BC1 RNA as well as BC1 DNA at the ID domain between box A and box B. The 30 kDa protein was identified by UV cross-linking to a radio-labeled DNA probe [36, 95]. Not only is this protein associated with BC1 RNA only with low affinity, but the purification of this factor is still far from homogeneity. Further studies are necessary to confirm that Bp-1 protein binds to BC1 RNA.

Despite the instability and heterogeneity of BC1 RNP, and without a functional assay, a significant progress have been made toward the purification of BC1 RNP. Furthermore, a great deal has been learned about the nature of BC1 RNP. In this chapter, I will show the behavior of BC1 RNP during various fractionation methods and their integration into a purification scheme. Although the purification of BC1 RNP is not complete, new approaches have been provided to understand the functional aspects both in the RNA binding and protein association of BC1 RNP.

Results and discussion

Conventional biochemical purification relies on established chromatography techniques which can process large amounts of material with reproducible results. However, different purification targets can cause different problems. Purification procedures have to be uniquely tailored for one's targeted

particle. Although there are some principles to follow, most of the time it is trial and errorapproach [136, 137].

Towards the goal of purification of the BC1 particle, it became a great challenge to purify a particle which is unstable, without assayable function and with complexity. I have solved many associated problems, and reached a step that can identify BC1 RNA binding protein indirectly. It will require further work to get enough material for protein microsequencing or for immunological methodologies. In this chapter, I will divide the effort of BC1 RNP purification into several parts. Starting from evaluating various fractionation methods, practical methods were integrated into a complete scheme, followed by refinement of purification procedures and finally additional means were provided to facilitate the purification.

Evaluation of the purification method

Starting

Important parameters for purification of a macromolecule in the beginning are as follows: First, what are the characteristics of the targeted particle that can be monitored during the purification procedures? Second, what is the starting material? Third, what is the buffer system that can maintain the particle intact and has the flexibility to be employed for subsequential steps [138].

For BC1 RNP purification, an initial buffer which contains Tris and KCl was adequate to process brain tissue for extract without changing the RNP. It is very difficult to judge the buffer system without indication of any functional assay. At least, there are no drawbacks during all purification procedures. A spectrum of the proteinase inhibitors were also included in the preparation of brain extract to avoid potential protein degradation.

There were some considerations about the source of BC1 RNP. It is known that BC1 RNA is expressed in neurons as well as some cell lines [30]. When we used brain as starting material, it contains both neurons and non-neuronal cells. It is the natural source of BC1 RNP but with a lot of dilution caused by other cell types. Neuronal cell lines may provide a cleaner source of BC1 RNP than brain but with the risk of the abnormality of BC1 RNP. Because of these practical reasons, we chose rat cerebrum to start with.

Since we do not know yet the function of BC1 RNP, we are unable to monitor the purification of BC1 RNP by functional assay(s). However, the RNA portion of BC1 RNP provides a way to track the distribution of BC1 RNP

indirectly. In conjunction with RNA dot blots, this assay method has an acceptable speed, high sensitivity (nonogram level) and short handling time (96 samples within one hour). The major drawback of this assay method is that the signal of BC1 RNA is not necessarily equivalent to the signal of BC1 RNP. In addition, it does not provide any information about the protein components of BC1 RNP or the integrity of this particle. The signal that we are tracking, in fact, is the 3' end of BC1 RNA. In order to overcome those problems, I established an additional protocol to assess BC1 RNP. Combining a denaturing urea gel and RNA blotting, one can test the integrity of BC1 RNA. In addition, native gel systems can assess integrity of BC1 RNP. Both assay methods may require 4 to 7 days to obtain results. They are more useful in pilot experiments to exam the intactness of BC1 RNA or RNA after a given set of manipulations.

Fractionation technique

Purification is the separation of target particles from other unwanted particles. The strategies that one usually applies involve is following: concentration and precipitation, centrifugation, chromatography and electrophoresis. All the fractionation techniques that I have evaluated are shown in **table 1.** It provides information that may apply not only to the purification of BC1 RNP but also the purification of other RNP particles.

Table 1: Test of Fractionation Methods

- Centrifugation
  - Differential
    - Separate the BC1 RNP from other large organelles
  - Sedimentation Velocity
    - Preparative Sucrose/Glycerol Gradient
  - Sedimentation Equilibrium
    - Preparative Cesium Sulfate Gradient
- Concentration
  - Ultrafiltration
  - Precipitation
    - Ammonium Sulfate
    - Polyethylene Glycol
    - Potamine Sulfate
- Chromatography
  - Gel Filtration
    - Sephacryl High Resolution S 300 (Pharmacia)
    - Superdex 200 (Pharmacia)
  - Ions Exchanger

Anion exchanger
Q Sepharose Fast Flow (Pharmacia)
Mono Q (Pharmacia)
Cation exchanger
Sepharose S Fast Flow (Pharmacia)
Hydroxylapatite matrix
Hydroxylapatite, High Resolution (M.W. 1004) (Calbiochem)
Hydrophobic Interaction
Phenyl Sepharose Fast Flow (Pharmacia)
Affinity interaction
Dyes
Affi-Gel Blue Gel (Bio-Rad)
Heparin Gel
Econo-Pac Heparin Cartridge (Bio-Rad)
Polynucleotide matrix
Oligo dT, dC Cellulose (InVitrogen; Collab. Research))
Poly (U) Sepharose 4B (Pharmacia)
ω-Aminoalkyl agarose
Resin set (Sigma)
Chromatofocusing
Polybuffer Exchanger (pH 7.5 -5) (Pharmacia)

Electrophoresis
Native Gel
Polyacryamide
Agarose
Prep-Cell (Bio-Rad)

Concentration and Precipitation

During a buffer exchange or desalting step, a dialysis step is required. In other cases, reducing the volume is also necessary for subsequential steps or for analytical purpose. There is a risk for BC1 RNP containing solution to be exposed to dialysis buffer for a long time. It always results in signal loss, despite of precautions. Several fractionation methods can reduce the volume and change the salt contents. Those methods that I have tested are shown in the **table 2.** which include ultrafiltration and precipitation.

Precipitation is a very important method for purification and concentration. However, to purify a complex particle such as BC1 RNP, I experienced some difficulties. Three common methods which are based on different principles have been tested.

The "salt out" effect which is accomplished with ammonium sulfate is the most popular method in enzyme purification. The major advantage so called "shearing" (increasing the concentration of ammonium sulfate stepwise) can

roughly separate the proteins depending on their special "salt out" concentration of Ammonium Sulfate. Although I could precipitate the BC1 RNA signal at 25% and 60% of ammonium sulfate (**figure 1a**), the procedure is lengthy and one has the risk of the BC1 RNP falling apart. The fact that there are two "salt out" concentrations of BC1 RNP, may suggest the heterogeneous nature of BC1 RNP (see also the test of phenyl sepharose column).

Table 2: Summary of concentration and precipitation purification

Method	BC1 RNA Fractionation	Time needed	Risk	Note
Ultrafiltration Centricon, Centriprep	In the retention fraction	2-4 hr	-	Desalt Concen. volume limited
Ammonium Sulfate	Ppt in the 25% and 60% of $(NH_4)_2SO_4$	12-24 hr	Protein denaturing	Volume unlimited Aggregation, Undissolved
Polyethylene Glycol 8000 & 400	Ppt with 25% PEG	4-8 hr	Ppt incomplete RNase contamination	Increase volume Dissolving takes long time
Potamine Sulfate	No frationation	-	-	-

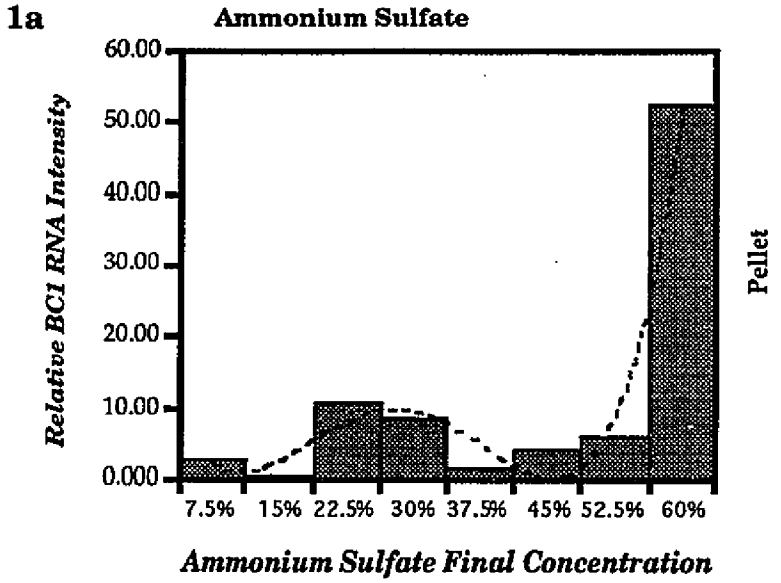
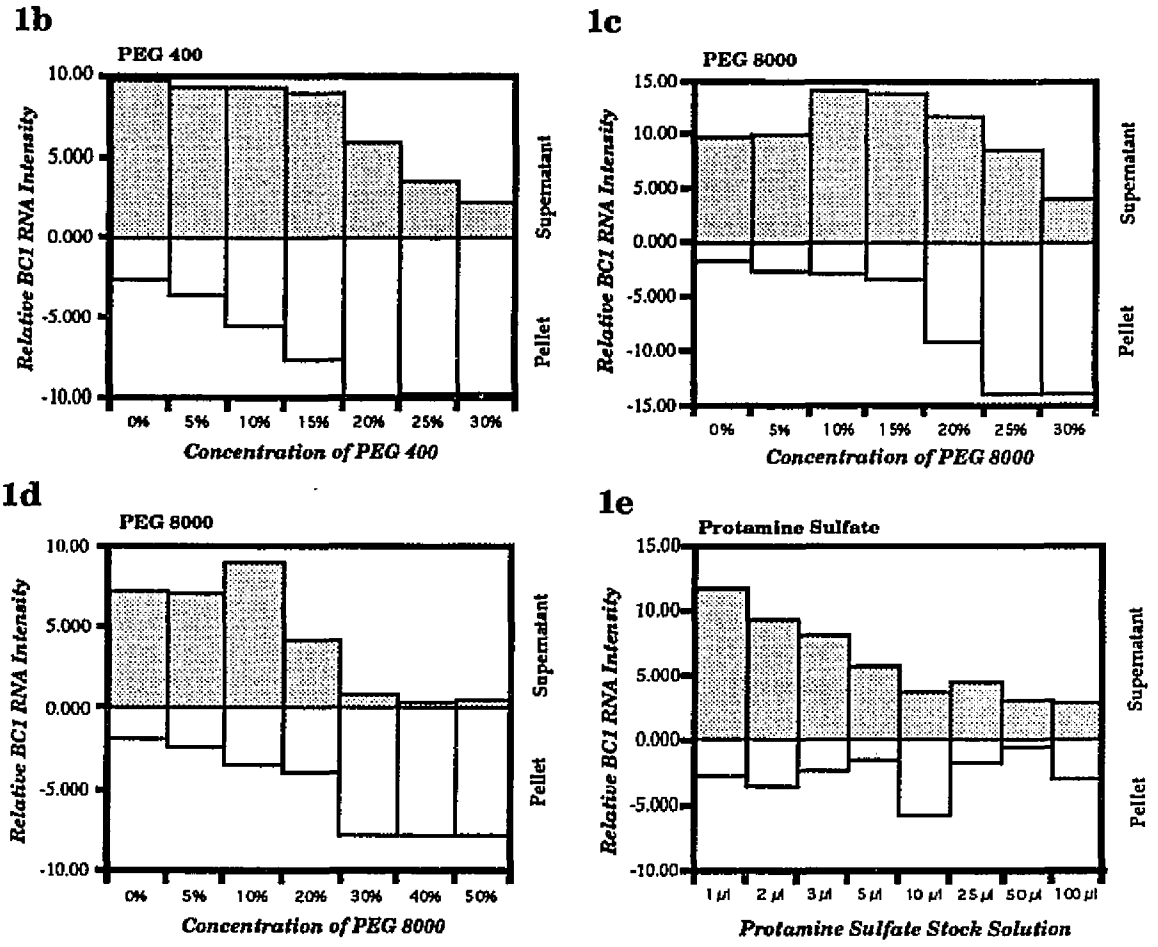


Figure 1. RNP precipitation with various reagents.(1a) Ammonium Sulfate. (1b) PEG 400 (0% - 30%). (1c) PEG 8000 (0% -30%). (1d) PEG 8000 (0% - 50%). (1e) Protamine Sulfate.



Polyethylene glycol is an alternative method which can trap particles with minimal risk of protein denaturation. Since different molecular weight PEGs have different effects, I tested the effect of two types (400, 8000) of PEG (**figure 1b, 1c, 1d**). There is a point where the BC1 RNA signal switches from supernatant to the pellet fraction while increasing the concentration of PEG. Although this switch point (30% of PEG in both 400, and 8000) is reproducible in a large volume, it always results in loss of BC1 RNA signal. It is speculated that RNase contamination as well as incomplete precipitation are the possible explanation.

The precipitation manner of protamine sulfate is dependent on ionic force interaction between protamine and highly negative charged molecules such as nucleic acids. Unfortunately, no concentration of protamine sulfate could precipitate the BC1 RNA signal completely (**figure 1f**).

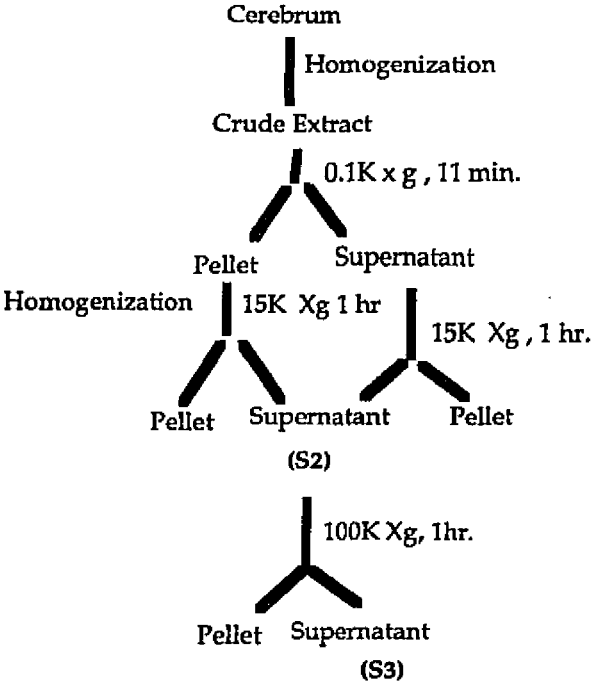
The only method successfully used to concentrate and desalt the BC1 RNP pool is ultrafiltration. Using selective membranes with defined M.W. cut off, water and small molecules are forced to flow through membrane by

centrifugation or by air pressure but retain the large molecules. I routinely used M.W. cut off of 30 kDa and 10 kDa. This kind of device (centricon or centriprep, Amicon) is convenient and rapid. The only problem is the limitation in processing volume (15 ml to 25 ml each run). As it is the most expensive concentration method, it can not be applied without any restriction. For large volumes with more than 50 ml, retention column chromatography is applied. BC1 RNP can bind to the column matrix until eluted (see section of chromatography) [138].

Table 3: Differential centrifugation of brain extract

Brain Extract Preparation	Volume (ml)	Total Protein (mg)	BC1 RNA Intensity (OD)	Recovery (%)	RNA / Protein Ratio (OD/mg)	Enrichment (fold)
Crude Extract	27.8	6244.3	83.4	100	1.33×10^{-2}	1
S2	35	378.2	49	58.7	1.29×10^{-1}	9.7
S3	31	342.3	37.5	44.6	1.08×10^{-1}	0.83

Flow Chart of Table 3



Centrifugation

Depending on the S value or the buoyant density, some degree of separation can be achieved through centrifugation. Differential centrifugation is a

common procedure that processes tissue extracts to a degree which can then be handled by chromatography. The relative BC1 RNA signal to protein concentration after differential centrifugation is shown in **table 3.** More than 50% of the BC1 RNA signal is lost after this preparation. Nevertheless, it can achieve a more than 10 fold enrichment of BC1 RNP. The final fraction (S3 fraction) is equivalent to the S100 fraction (after 100K xg spinning) which is thought to be free from nuclei and cell debris (S1 fraction), mitochondria (S2 fraction) and microsomes (S3 fraction). What may account for the loss of BC1 RNA signal from the S1 to S3 fraction? There are at least three possibilities. Intact neurons and BC1 RNA within the nucleus may be responsible for the loss of BC1 RNA during the first step of the separation. It has been tried to eliminate the unbroken neuron during preparation by re-homogenizing the pellet of the S1 step. However, there may still be portions of BC1 RNAs that are lost with the nuclei. Because BC1 RNA may not form a mature RNP in the nucleus, as well as to reduce the contamination of snRNP, it is necessary that one use a Dounce B homogenizer to keep the nucleus intact. The loss of BC1 RNA in steps 2 and 3 may have different explanations. From the experiment done by a visiting fellow in our laboratory who measured association of BC1 RNA with the cytoskeleton preparations [N. Schuhin, unpublished result], and the recent experiment of immunoprecipitation with antibodies against cytoskeleton components (see later section), it appears that BC1 RNA or BC1 RNP is associated with the cytoskeleton. There is a great deal of cytoskeleton that is associated with membrane or forms a filamentous network structure and could be removed from the extract through high speed centrifugation [119, 139]. Another reason for loss of BC1 RNA is the location where BC1 RNP may play a role in the physiological events. It has been reported that BC1 RNA is enriched in the synaptosome preparation [135]. Similar to microsomes, synaptosomes can be precipitated with 100 xg centrifugation. On one hand is the increase in yield of the mature form of BC1 RNP (in synaptosomes), on the other hand is added the proteinase contamination (in microsomes). The decision is a very difficult one to make. From the point of reality, we don't have any good information to tell us whether an extra procedure such as adding detergent will alter the conformation of BC1 RNP or not. We don't have too much choice but to suffer the loss of BC1 RNA signal.

Two kinds of centrifugation methods have been used to fractionate the BC1 RNP from other particles. One is sedimentation velocity centrifugation, the other is sedimentation equilibrium centrifugation. Two runs of preparatory

sucrose gradient (35 ml) results are shown in **figure 2** (see also the section of additional methods). The absorbance at OD 280 nm has been calculated, and there is an 8 fold enrichment of the BC1 RNP. When S value standard markers are run in parallel, it shows that the majority of BC1 RNA signal is in a range of less than 11.3S (catalase marker). The only concern about the sucrose gradient is long processing time (22 hr) and RNase contamination. A preparatory glycerol gradient was also tested and the performance is similar to that of the sucrose gradient.

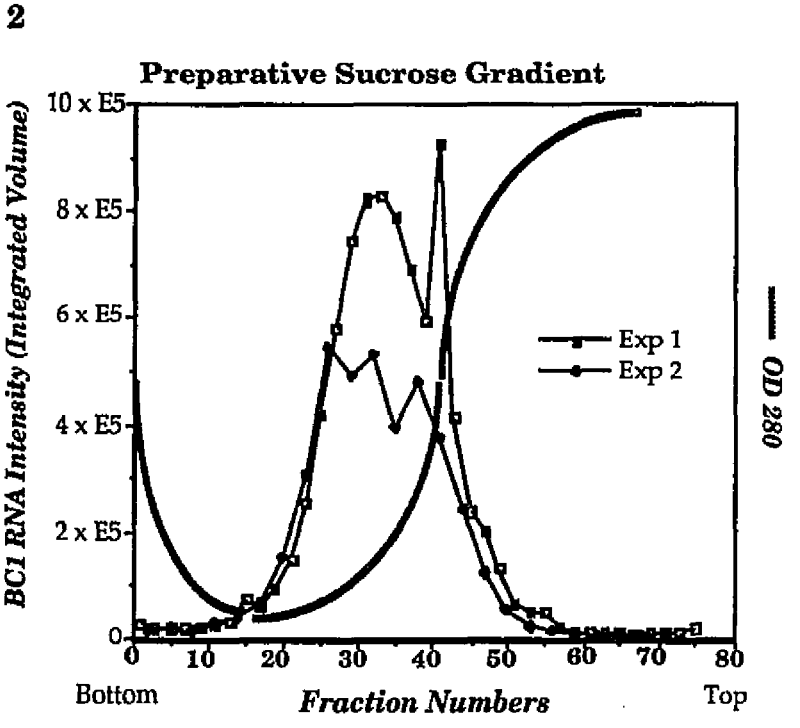


Figure 2. BC1 RNP distribution in the preparative sucrose gradient

Another centrifugation method is cesium salt gradients. Although cesium sulfate or cesium chloride gradients have been used to study the BC1 RNP (chapter 2), there was not a lot of effort invested in these fractionation methods. It is known that there are two separate signal peaks of BC1 RNA in the CsCl gradient and the presence of a smearing distribution of BC1 RNA signal in the Cesium Sulfate gradient (chapter 2). In addition to the result of poor banding, the small capacity (0.5 - 1 ml) and very high salt condition required (2 - 4 M) are the other major disadvantage of these methods. Nevertheless, the ability to separate

RNP from protein by CsCl gradient may have the potential to reduce the protein contamination and facilitate the purification of RNA binding proteins.

Chromatography

To establish the fractionation of BC1 RNP via chromatography, different principles were applied to separate BC1 RNP from other particles. Separation principles included size, shape, surface charge, hydrophobicity, specific interaction with other material and pI value of BC1 particles. In addition to applying these separation principles, choosing the column material was another issue. There are many different commercial column matrices was available. The correct choice of column material is dependent on other's past experience, convenience of testing, economic considerations and trial and error. Mono Q, Blue agarose and ω–aminoalkyl agarose have been used to purify other snRNP or scRNP [73, 124]. Superdex 200, Mono Q and Polybuffer exchanger are all available in this institute (Dr. Kohanski and Dr. Probst). All other columns were personally packed and tested according to the manufacturer's instructions. Several criteria have been applied to evaluate the column performance. The major criterion is the elution profile of BC1 RNA in a test run. This elution profile of BC1 RNA signal also has direct influence on enrichment and the collection pool. During the testing of different columns, the BC1 RNA signal to protein ratio has been used as the indication rather than the "specific activity" for enzyme purification. The risk in this, of course, is the non-parallel relationship between BC1 RNA and BC1 RNP. However, several observations can help to make a good judgment including the behavior of the BC1 RNA signal in the column or in the electrophoresis system.

The distribution of BC1 RNA in various columns with different fractionation principles is shown in **table 4**. Nearly all the columns with negative charges, such as FFS, Heparin, and some of the ω–aminoalkyl agarose with low carbon number (C2, C3), the BC1 RNA signal is in the flow through portion and thus does not interact with the column material. Some of the matrix including Phenyl Sepharose, oligo dC, Blue agarose, Polybuffer exchanger and ω–aminoalkyl agarose (C4) have a dispersed signal, appearing in both the flow through fraction and bound fraction. The signal of BC1 RNA in Phenyl Sepharose has a very interesting pattern. One half of the BC1 signal is in the flow through portion, while the other half is in the early elution fractions, suggesting that BC1 RNP are separated into two distinct portions, with different degrees of

hydrophobicity under the influence of 0.8 M ammonium sulfate. Because signal separation is suggestive of bad separation and/or particle alteration, these columns were abandoned without further testing or evaluation.

Table 4: BC1 RNA distribution with different fractionation methods

Flow though	Continuous Peak	Split Peaks	Smearing	Signal Retained
Most	**Wide Peak**	**2 Peaks with Tail**	**Smearing**	**Partial**
FFS	Superdex 200	FFQ	Blue Agarose	C8-ω-aa agarose
Heparin column	S300 HR	Mono Q		Oligo dC cellulose
C2-ω-aa agarose	Phenyl	Hydroxylapatite		
C3-ω-aa agarose	Sepharose*	Cs_2SO_4 gradient		**Most**
	C4-ω-aa agarose*	Prep-cell		Oligo dT cellulose
Partial		Native gel		Poly(U) agarose
Phenyl Sepharose*	**Peak with Tail**			C10-ω-aa agarose
C4-ω-aa agarose*	Sucrose gradient	**Multiple Peaks**		C12-ω-aa agarose
Polybuffer exch.*	C5-ω-aa agarose	Polybuffer exch.*		
Oligo dC cellulose	C6-ω-aa agarose			
	DEAE cellulose			

(*) indicates a same fractionation method in two different categories.

The BC1 RNP can interact with anion exchangers, hydroxylapaptite, oligo dT cellulose, Poly(U) sepharose and ω–aminoalkyl agarose (> 5C), however, none of them give a good peak and sometimes have a tailing signal. The BC1 RNA signal can not be eluted out under normal conditions, in neither the polynucleotide column (dT & poly (U)) nor ω–aminoalkyl agarose with high carbon number (C> 10). One can try to elute the BC1 RNA from the column with urea or GuHCl, however, with the consequence of protein denaturation. In the FFQ column, BC1 RNP forms two identical peaks with a long tailing until high salt is used. In the Mono Q and hydroxylapatite column, these two peaks are unequal and may be dependent upon the elution profile of counter ions. The tailing of signal is another problem. It may account for less than 10% of total BC1 RNA signal in the Mono Q and hydroxylapatite column, but it is more than 30 % in the ω–aminoalkyl agarose column (C6) with a continuous gradient (see below). The explanations for this abnormal signal distribution of BC1 RNA in these columns possible include the following: 1. the BC1 RNP may fall apart in this column due to salt concentration, 2. BC1 RNPs are very heterogeneous particles, 3. the amount of charge of column material is too high for BC1 RNP and causes the dissociation of associated proteins. Clear evidence to support these ideas is not available yet. Recently, some results have suggested that BC1 RNP turns out to be a very complex particle (see later section). Although use of

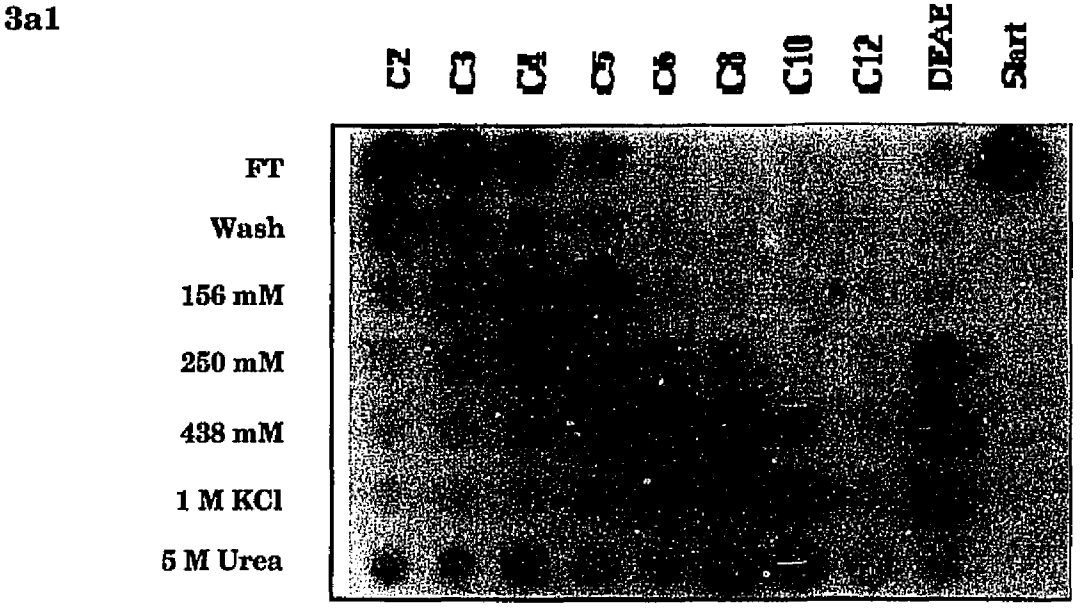
these columns (Mono Q and hydroxylapatite) to fractionate BC1 RNP have minor disadvantages, they are the major tools for fractionation and concentration.

The ω–aminoalkyl agarose matrix can be a very good example of how to test the property of column material (**figure 3**). The same amount of column matrix and same procedures were applied to handle all 8 different matrices as well as a positive control of DEAE matrix. The buffer is manually applied and flow out with gravity. After application of the same amount of material (Superdex 200 pool), the columns were washed with loading buffer and eluted with steps salt gradient. The result of OD 280 nm (proteins), OD 260 nm (nucleic acids) and BC1 RNA (dot blot) was shown in **figures 3a1, 3a2, 3b** and **3c**. From the result of the RNA dot blot, we can see that there is a relationship between carbon number and BC1 RNA signal elution profile. The higher the carbon number of column matrix, the higher the required salt concentration to elute BC1 RNP. The BC1 RNA signal as well as most of the OD 260 nm and 280 nm absorbance material is in the flow through portion of the C2 and C3 columns. The C4 column can bind BC1 RNP but not completely. Once the carbon length is more than 8, BC1 RNA(P) becomes increasingly difficult to elute. The most likely candidates for BC1 RNP purification are the C5 and C6 columns. When the ratio of 280 and 260 absorbance were calculated (accumulated absorbance value of fractions without BC1 signal is divided by total absorbance), it was shown that the C5 column may get rid of 87% of the 280 absorbance material and 78% of the 260 absorbance material, while the C6 column may get rid of 64% and 60%, respectively. However, since the total absorbance is higher in the C5 column, it suggests that there is some material not eluted out from the C6 column. The performance of the C6 column with a larger column dimension (10 ml) in FPLC system was evaluated. The result of elution profile of BC1 RNA shows the serious tailing problem with a continuous gradient. It suggests that an analytic comparison with a selection of columns can screen for the most useful column material very efficiently, however, the real performance must be tested under full scale conditions.

Electrophoresis

Gel electrophoresis is a very powerful tool to fractionate macromolecules, although its small capacity is a major limitation. For this reason, electrophoresis is often applied to analytical rather than preparative purposes. However, in conjunction with microsequencing as well as other detection methods,

3a1



3a2

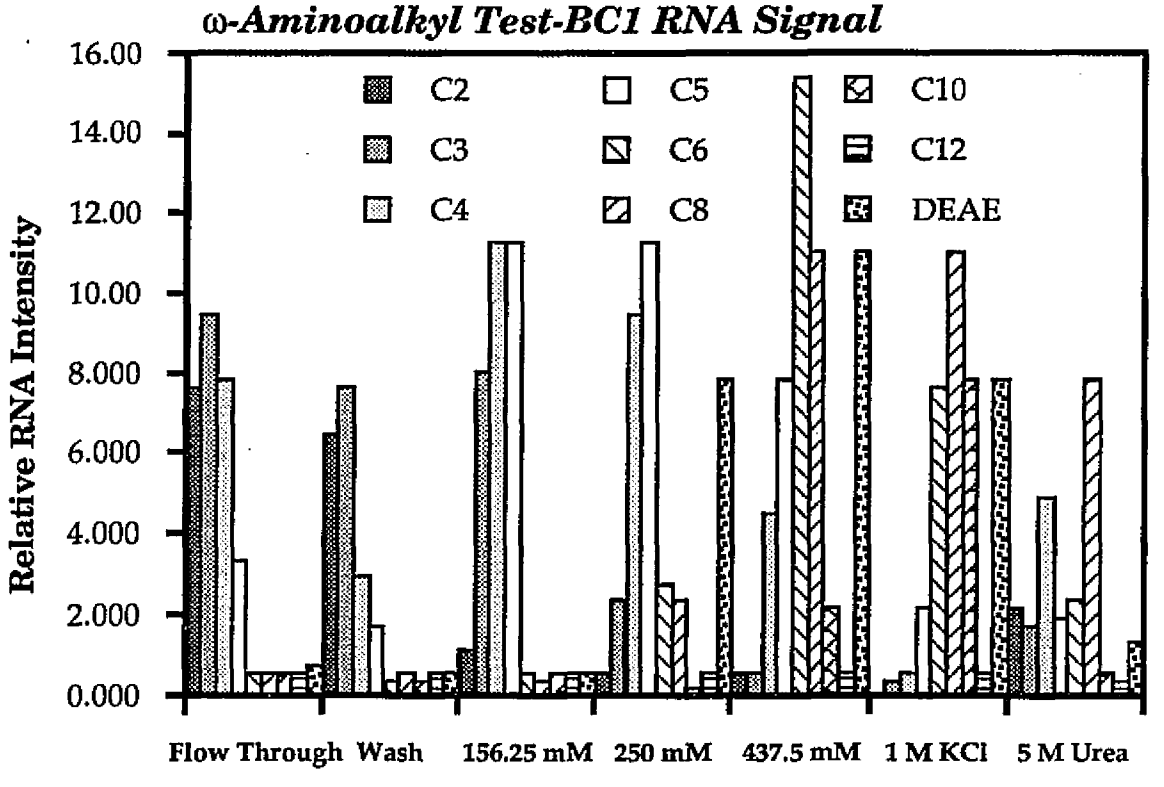
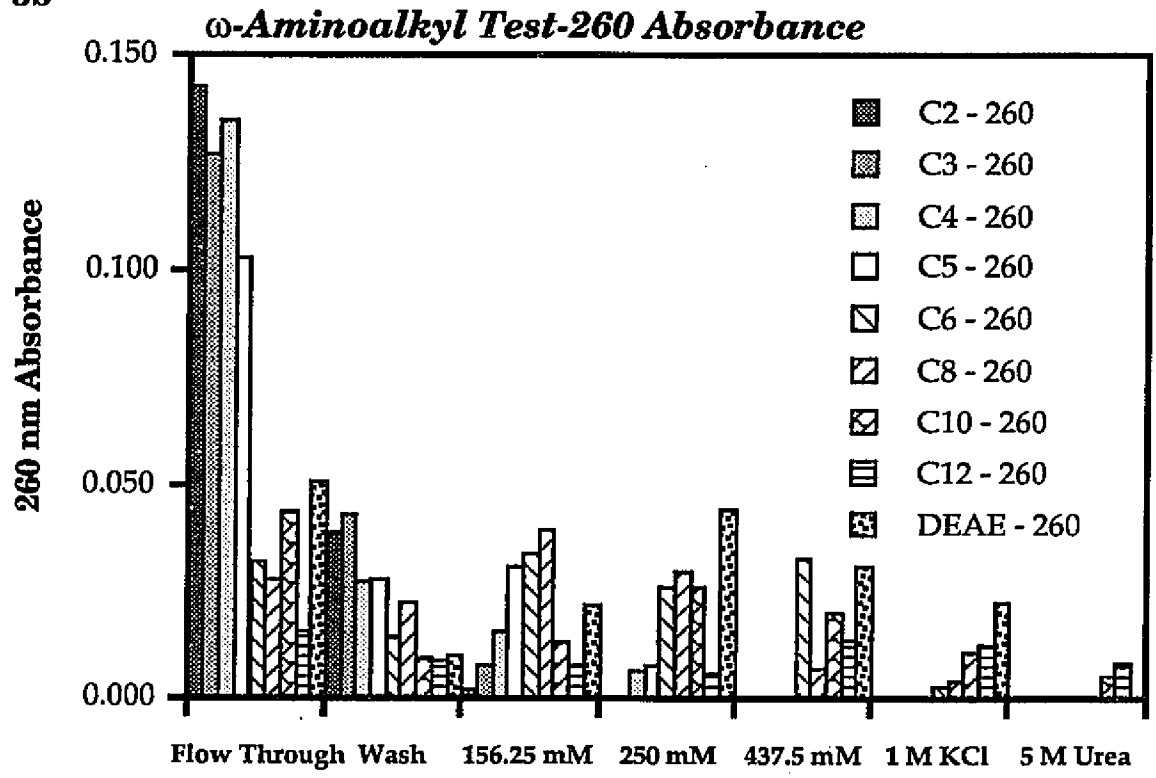
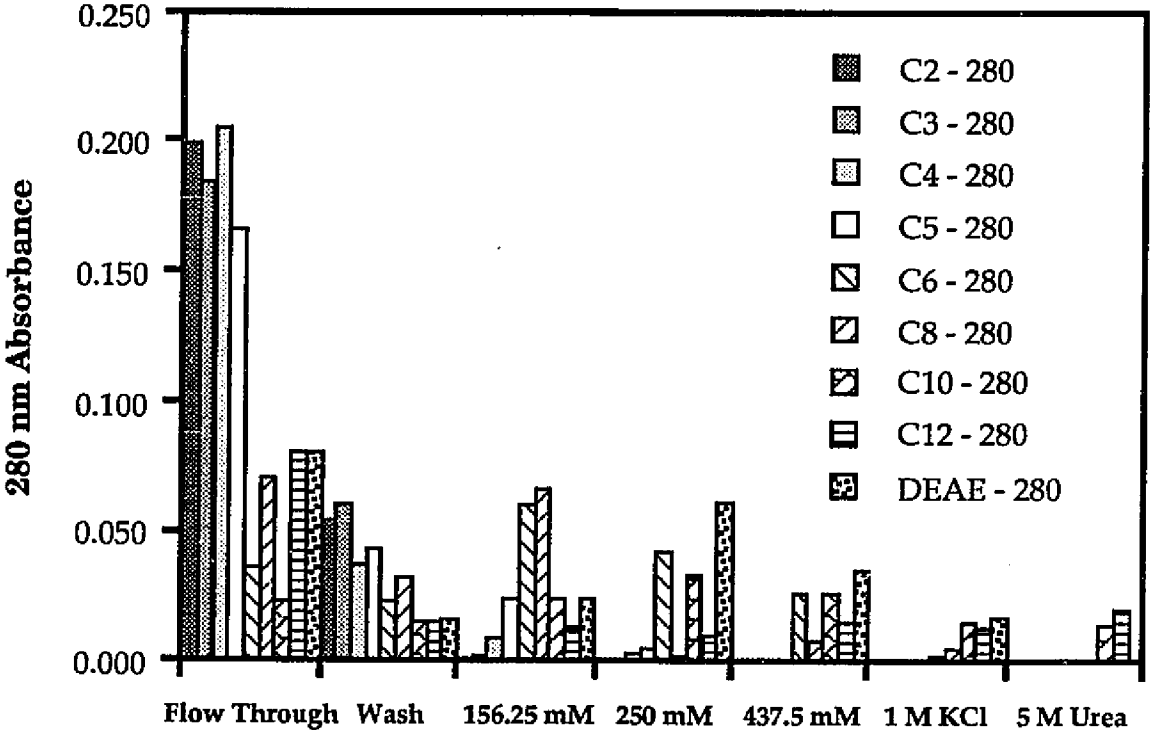


Figure 3. ω-aminoalkyl agarose resin set. BC1 RNA signal detected by the RNA dot blot (3a 1-2) and nucleic acid and protein by absorbance at 260 nm (3b) and 280 nm (3c) with various fractions.

3b


ω-Aminoalkyl Test-260 Absorbance
260 nm Absorbance
0.150
0.100
0.050
0.000
C2 - 260
C3 - 260
C4 - 260
C5 - 260
C6 - 260
C8 - 260
C10 - 260
C12 - 260
DEAE - 260
Flow Through
Wash
156.25 mM
250 mM
437.5 mM
1 M KCl
5 M Urea

3c


ω-Aminoalkyl Test -280 Absorbance
280 nm Absorbance
0.250
0.200
0.150
0.100
0.050
0.000
C2 - 280
C3 - 280
C4 - 280
C5 - 280
C6 - 280
C8 - 280
C10 - 280
C12 - 280
DEAE - 280
Flow Through
Wash
156.25 mM
250 mM
437.5 mM
1 M KCl
5 M Urea

electrophoresis becomes more and more popular in the characterization of an unknown protein. As for BC1 RNP purification, considerable effect has been put in to use gel electrophoresis in the purification scheme. This method has been successfully applied to characterize the integrity of BC1 RNP and the size of BC1 RNA from different preparations. However, in terms of BC1 RNP purification, such a system can only provide a limited contribution. One of its major limitation is the low recovery of proteins from both agarose and acrylamide gels; another is the signal distribution of BC1 RNP in these gels.

Two types of gel media have been tested for the separation of BC1 RNA. Agarose gel has a larger pore size and separates macromolecules based on charge; the polyacrylamide gel, however, has much smaller sieve size and separates through both charge and size of molecule. The buffer systems which provide the pH and counter ions also influence the separation of proteins and particles. Agarose gel has been used to purify the vault RNP and the native polyacrylamide gel has been used to purify a multiple protein complex called proteosomes.

To test the best separation conditions of agarose gel, the buffer pH, agarose percentage, and choice of buffer were altered. Results indicate that the mobility of BC1 RNP particle is pH dependent which has slower mobility in pH 6.5 Mes-Glycine buffer than in pH 8.8 Tris-Glycine buffer. It is suggested that the BC1 RNP in the brain extract has an isoelectrofocusing point (pI) around the weak acid range. Tris-glycine buffer has a better banding pattern of BC1 RNA signal than Tris-Boric acid buffer. The best result of agarose gel separation is shown in **figure 7b**. Very good separation can be achieved for either the different forms of BC1 RNP or the naked RNA from RNP. In this system, the RNA co-migrates with the tracking dyes of bromophenol blue and phenol red, and BC1 RNP co-migrates with cresyl green. To overcome the capacity limitation as well as the low particle recovery, the prep-cell (Bio-Rad) has been tested for such a purpose. Agarose lacks the strength to support itself in the cylinder of the prep-cell. Attempts to change the consistency of agarose by mixing in polyacrylamide or using multiple layers of agarose could not improve the performance. Finally, the only agarose that can fit in the prep-cell is the Gold Agarose from FMC. This finding provides an alternative method to fractionate organelles [82] but not for BC1 RNP purification. The results of BC1 RNP distribution is shown in **figure 5**. There not only is a wide peak of BC1 RNA signal but the requirement of considerable time to complete a run.

5

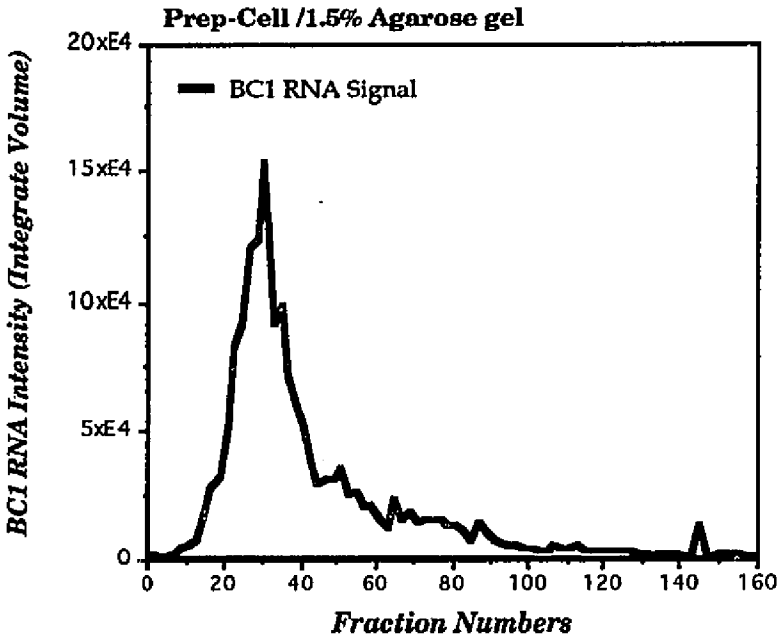


Figure 5. BC1 RNP distribution in preparative 1.5% agarose gel electrophoresis.

The polyacrylamide gel in a non-detergent buffer (native gel) is another major focus. The percentage of gel, the degree of cross-linking (acrylamide to bisacrylamide ratio), running buffer and gel dimension were tested. Because of several available "zone sharpening techniques" to improve the banding, the stack gel format, gradient pore as well as the polymerization catalytic reagent were also tested [138]. To summarize, the polyacrylamide gel gives a more smeared result than the agarose gel. This smeared result becomes worse in both the high percentage of gel as well as with a high degree of cross-link. Although very good banding has been achieved at 7.5% gel in Tris-Glycine buffer (**figure 7a**), it is misleading, as the BC1 RNP leaves the stacking gel and enters the top of the separating gel in these experimental conditions. After many times of trying to achieve the best conditions for fractionating the BC1 RNP, it is realized that BC1 RNP itself has very a high degree of heterogeneity. However, the naked RNA has highly negative charges, it can be separated from RNP by a great distance. In these gel conditions, the "naked" RNA co-migrates with the front of ions and

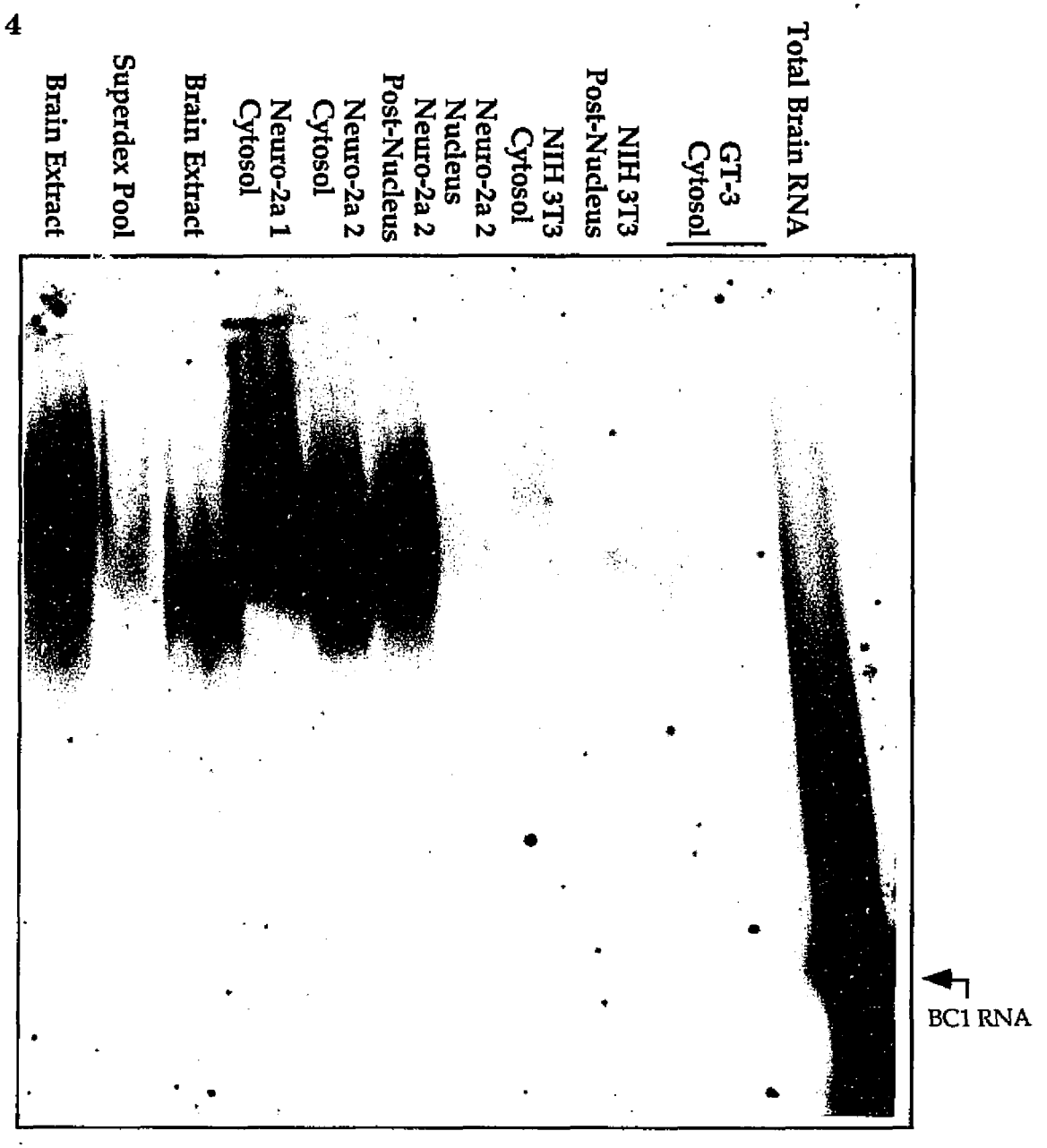


Figure 4. BC1 RNA distribution from various cell lines in native polyacrylamide gradient gels (4% -10%, acryl: bis = 37.5 :1).

leading dye. **Figure 4** is a summary result, which shows the migration pattern of BC1 RNP from brain extract and several different extracts of cell lines. In this 4 - 10% gradient gel (acryl:bis = 37.5 :1) with 3.3% stacking gel (acryl:bis = 20:1) and Tris-Glycine buffer, BC1 RNP signal is not a band but a smear. The signal of BC1

RNA came from total brain RNA and migrated to the end of the gel in two distinct bands. Since there is only one major band with several weak degradation products in the run of urea gel, it suggests that there are two conformations of BC1 RNA in the native gel system. (It needs to be noted that although BC1 RNA is expressed in both neuronal cell lines (Neuro-2a and GT-3) and in a fibroblast cell line (NIH 3T3), the latter signal is relatively very weak. The polyacrylamide gel in the prep-cell has also been tested, the only condition under which enough signal can be detected using 4 ml of brain extract directly loaded into the prep-cell.

Integration of purification procedures.

Column selection

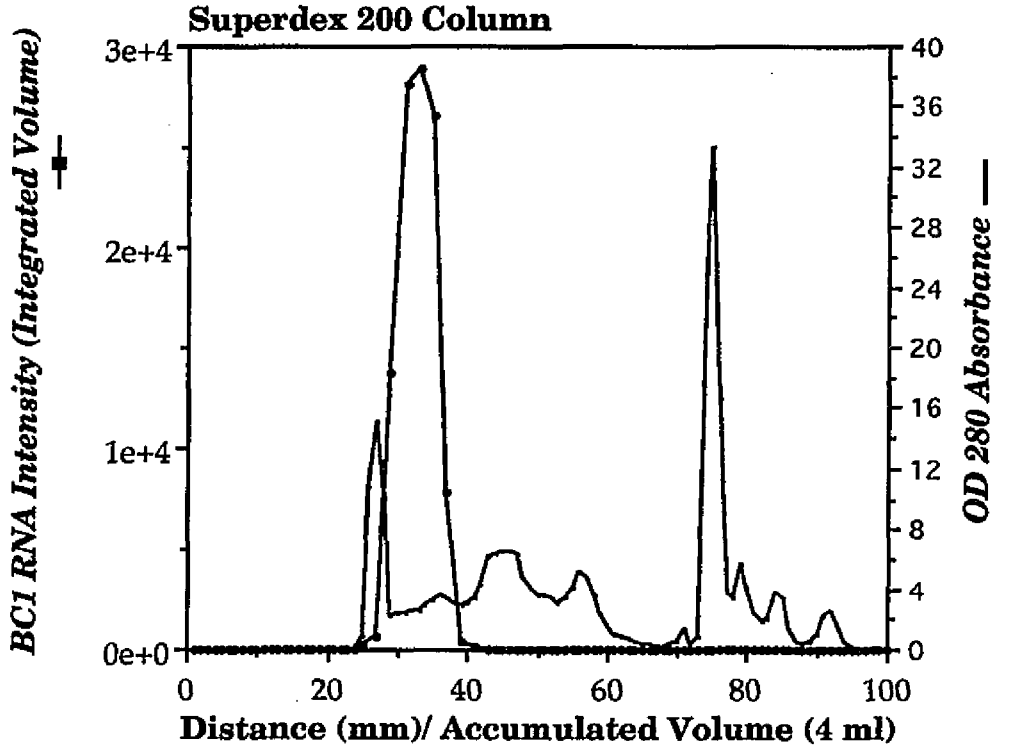
Following the testing of different column materials, the next challenge was how to connect the possible candidate columns to establish an efficient and reproducible procedure. Several criteria have been considered in order to integrate these columns. The first one is the elution profile of BC1 RNP which determines whether a column can be used or not. It also provides two other important factors, the enrichment and the collection volume (dilution factors). The running time and loading capacity (protein concentration and volume) of column will influence the arrangement of the different columns. Finally, the salt concentration in the collection pool also influences the subsequential steps. Based on the characterization of BC1 RNA signal in **table 3**, the columns have been considered to be candidate as follows: FFS, heparin column, Superdex 200, S300 HR, sucrose gradient, C5-ω–aminoalkyl agarose, C6-ω–aminoalkyl agarose, DEAE cellulose, FFQ, Mono Q, hydroxylapatite, Cs_2SO_4 gradient, prep-cell and native gel.

Sucrose gradients and native gels which have small amounts of loading capacity have to be considered as later steps following the chromatography. Among the gel filtration columns, Superdex 200 was chosen material as it has a larger loading capacity and shorter processing time than the HR300 column does. Among the anion exchangers, Mono Q has a better separation and a shorter running time. As mentioned previously in this chapter, the ω–aminoalkyl agarose (C6) has a very long tail with the continuous gradient, which reduces its performance. Those two columns (C5, C6) can serve as a retention column or one can use a smaller dimension with the elution of step salt gradients. Up to this

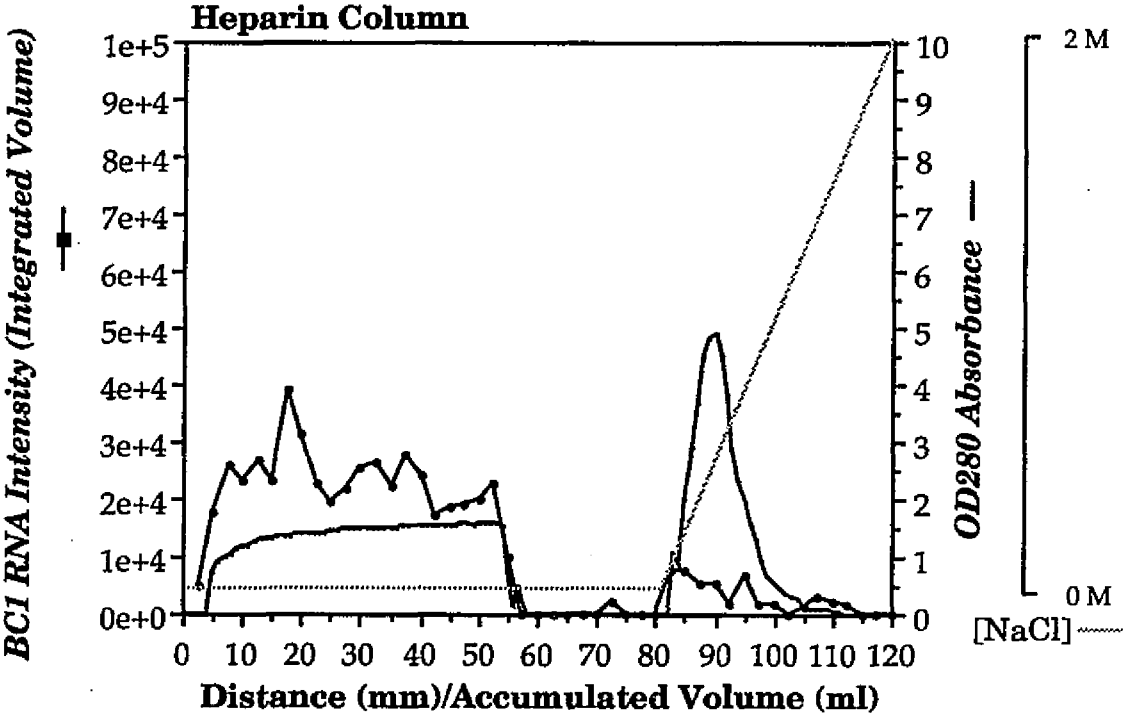
point, it was decided to use Superdex 200, FFS, heparin, hydroxylapatite and Mono Q columns to fractionate the BC1 RNP. The following step discusses how to connect these columns in proper order, reducing the time consumed and to avoid buffer exchange, desalting and concentration steps.

Since the gel filtration has mild separation conditions and generates a very large collection volume, it was incorporated as the first column despite its medium loading capacity. Anion exchange columns can be loaded with very large volumes. However, their collection fractions have a salt concentration of 0. 2 to 0.35 M KCl. Since the BC1 RNP will not interact with FFS and heparin matrices, their loading volume and collected volume are similar. However, to achieve optimal fractionation, these columns cannot be the subsequential step of those columns which use a high salt concentration for elution. For this reason, they directly follow the Superdex 200 column. To use the advantage that heparin can retain free proteins that interact with nucleic acid interaction proteins as well as RNase, this column was chosen as the second column. To arrange the order of the hydroxylapatite and Mono Q columns caused some difficulty, since their collection pools contain sodium phosphate and sodium chloride, respectively. After loading the hydroxylapatite collection pool directly into the Mono Q column, there was an unexpected finding that the original OD 280 profile of Mono Q had been split into two peaks. The BC1 RNP signal neither goes to the flow through portion nor keeps the same elution salt conditions but goes to the late elution OD 280 peak. As this shifting of signal is sodium phosphate concentration dependent, it is believed that the phosphate group plays an important role here. It is speculated that phosphate transfers the Mono Q column temporarily into a phosphate column. After this effect is overcome by a higher concentration of KCl, then BC1 RNP as well as other proteins with similar properties start to be eluted. This effect not only provides an added two-fold enrichment, but also makes the Mono Q as a bifunctional column. These results of different runs as well as the enrichment is shown in **figure 6 a-f** and **table 5**. It shows that the above column arrangement can achieve a 345-fold enrichment starting from S3 brain extract. It suggests that, starting from brain tissue until the end of the Mono Q pool, there is a 2500-fold enrichment of BC1 RNP. The majority of BC1 RNA signal loss is in the hydroxylapatite column but with good cleaning of protein contamination. The questions arises now are how pure the BC1 RNP is and what is the size of BC1 RNP in the last step.

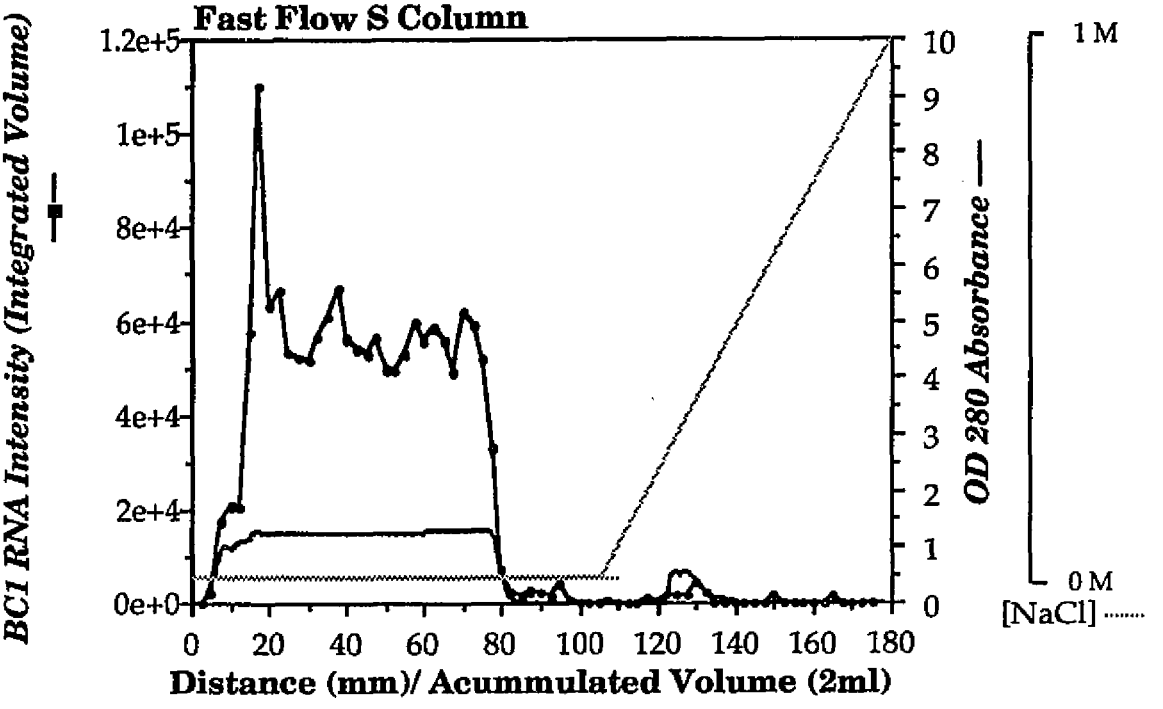
6a


Superdex 200 Column
BC1 RNA Intensity (Integrated Volume)
OD 280 Absorbance
3e+4
2e+4
1e+4
0e+0
40
36
32
28
24
20
16
12
8
4
0
0
20
40
60
80
100
Distance (mm)/ Accumulated Volume (4 ml)

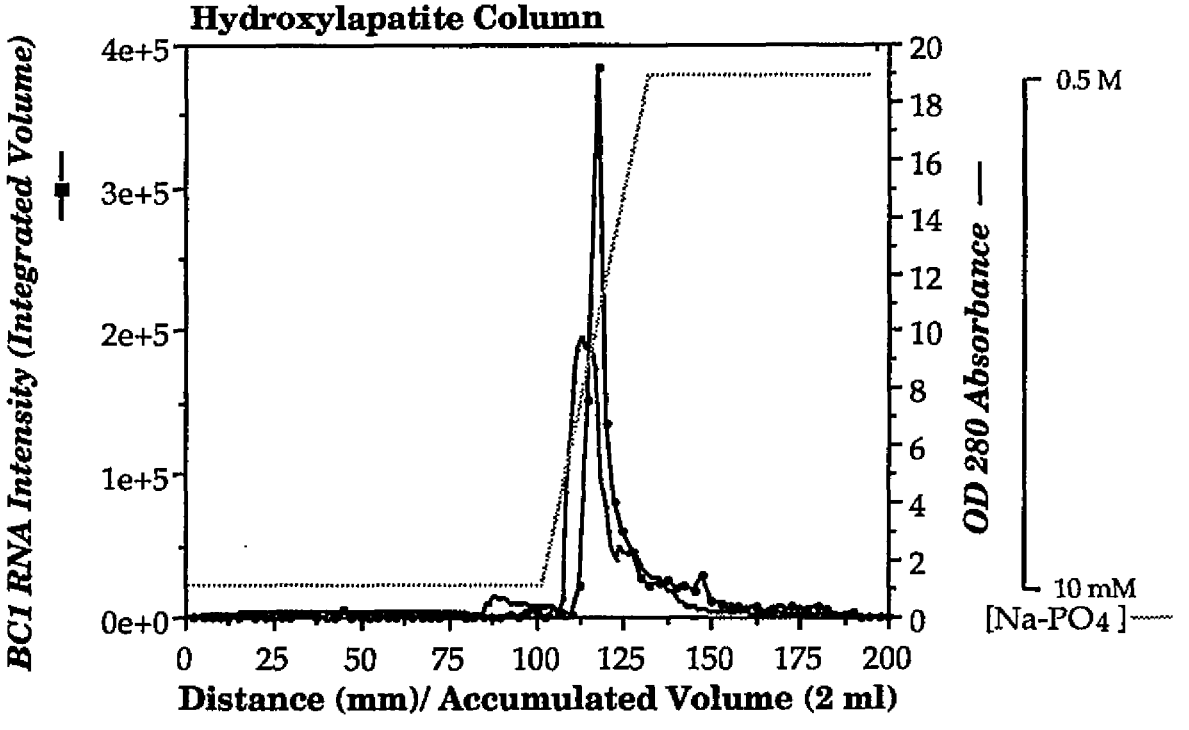
6b


Heparin Column
BC1 RNA Intensity (Integrated Volume)
OD280 Absorbance
1e+5
9e+4
8e+4
7e+4
6e+4
5e+4
4e+4
3e+4
2e+4
1e+4
0e+0
10
9
8
7
6
5
4
3
2
1
0
2 M
0 M
[NaCl]
0 10 20 30 40 50 60 70 80 90 100 110 120
Distance (mm)/Accumulated Volume (ml)

6c


Fast Flow S Column
BC1 RNA Intensity (Integrated Volume)
1.2e+5
1e+5
8e+4
6e+4
4e+4
2e+4
0e+0
0 20 40 60 80 100 120 140 160 180
Distance (mm)/ Acummulated Volume (2ml)
OD 280 Absorbance
10 9 8 7 6 5 4 3 2 1 0
1 M
0 M
[NaCl]

6d


Hydroxylapatite Column
BC1 RNA Intensity (Integrated Volume)
4e+5
3e+5
2e+5
1e+5
0e+0
0 25 50 75 100 125 150 175 200
Distance (mm)/ Accumulated Volume (2 ml)
OD 280 Absorbance
20 18 16 14 12 10 8 6 4 2 0
0.5 M
10 mM
[Na-PO4]

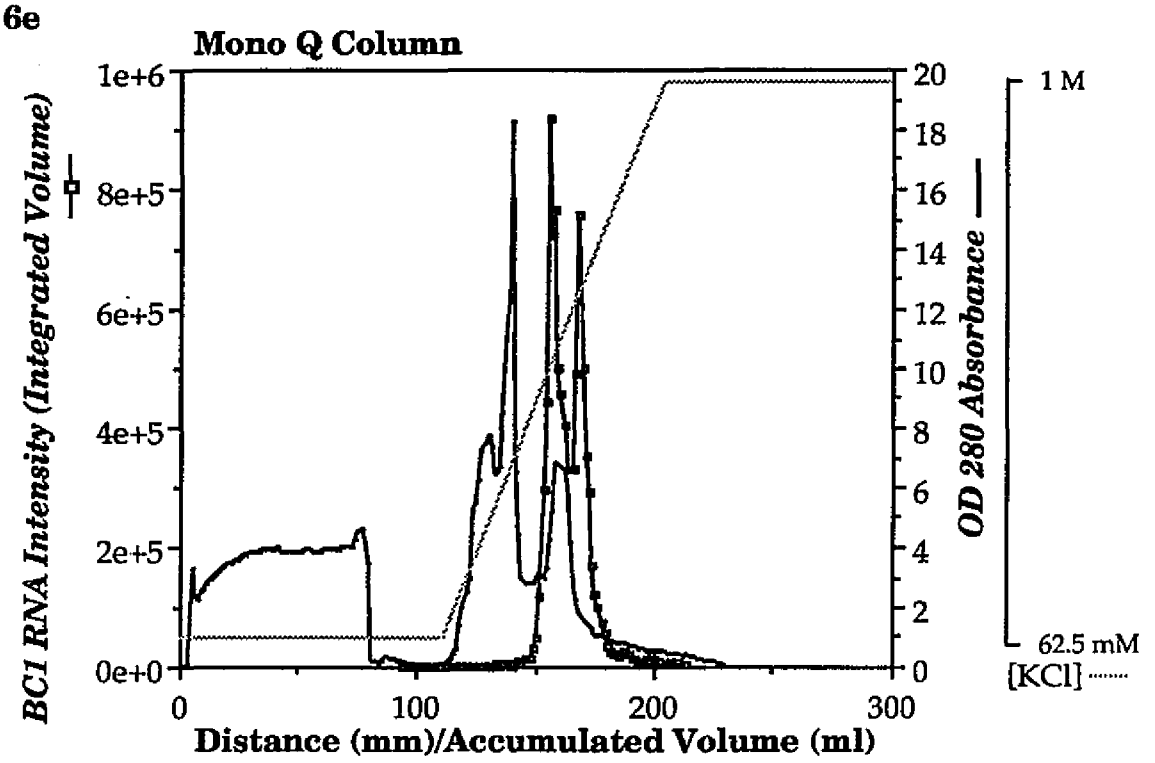
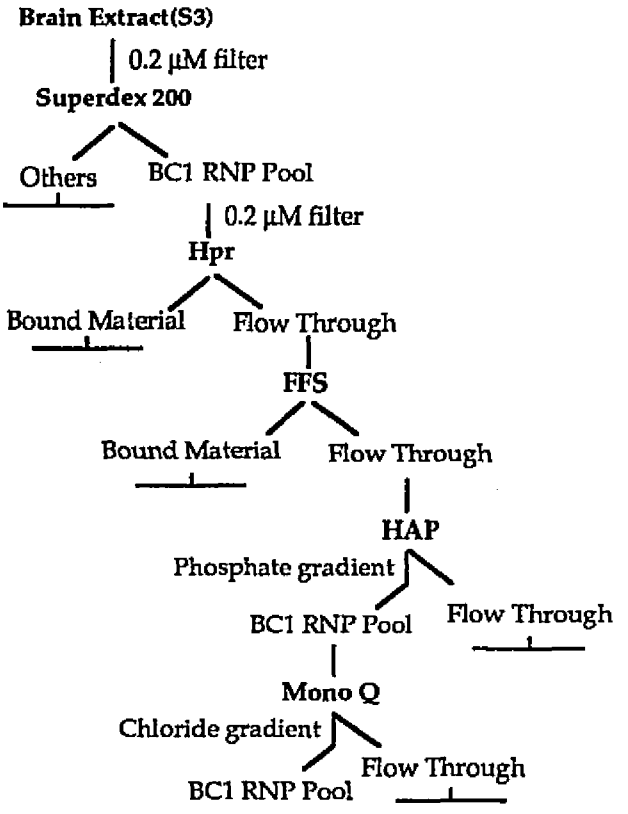


Figure 6. Purification profile of BC1 RNP with (6a) Superdex 200. (6b) Heparin. (6c) Fast flow S. (6d) Hydroxylapatite. (6e) Mono Q.

Table 5: The Enrichment of BC1 RNA

Steps of Purification	Volume	Total Proteins (mg)	BC1 RNA Intensity ($X 10^6$)	Recovery (%)	RNA/Protein Ratio (Intensity/mg)	Cumulative Enrichment (fold)
Brain Extract	15	68.7	98.2	100	1.4	1
Superdex 200	148	3.7	95.9	97	25.9	18.1
Heparin	148	1.9	93.7	95.5	50.4	35.3
FFS	150	1.3	90.1	91.7	67.6	47.3
HAP	72	0.12	21.2	21.6	184.4	129
Mono Q	13	0.04	19.2	19.6	492.6	345

Flow Chart of Table 5



Integrity of BC1 RNP

To answer the question of the size of the BC1 RNP after running on a different column, the native gel system was applied to analyze the integrity of BC1 RNP. In this assay system, it is assumed that the BC1 RNP in the crude extract reflects the intact form of BC1 RNP. If we compare the size of the particles from different pools with the size of the particle in brain extract, it may provide an idea of the effect of column conditions on BC1 RNP.

In the first test, the polyacrylamide native gel system was applied to assay the integrity of BC1 RNP (**figure 7a1**). Using brain extract as an indication of intact BC1 RNP, the migration of BC1 RNA signal in different pools were determined by RNA blotting. Except for the Mono Q pool, the signals of BC1 RNP particles coming from Superdex 200 (SD), Heparin (Hpr), Fast flow S (FFS) and hydroxylapaptite (HAP) have a similar migration distance to the particle from brain extract. Although the signal is not as sharp as the lane with brain extract, they still maintain fairly intact as BC1 particles. However, the signal of BC1 RNA disintegrates after the run on Mono Q (MQ), it suggests that the intact BC1 RNP falls apart into different forms. To rule out the possibility that this

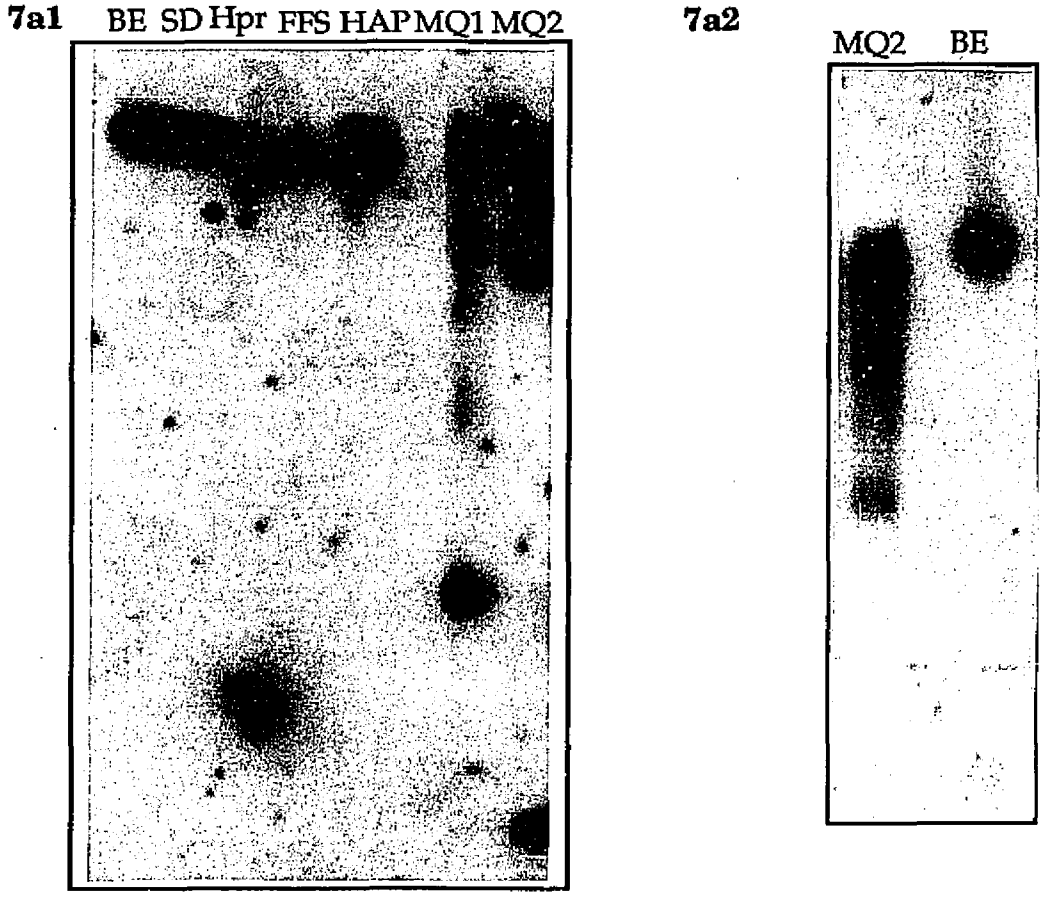
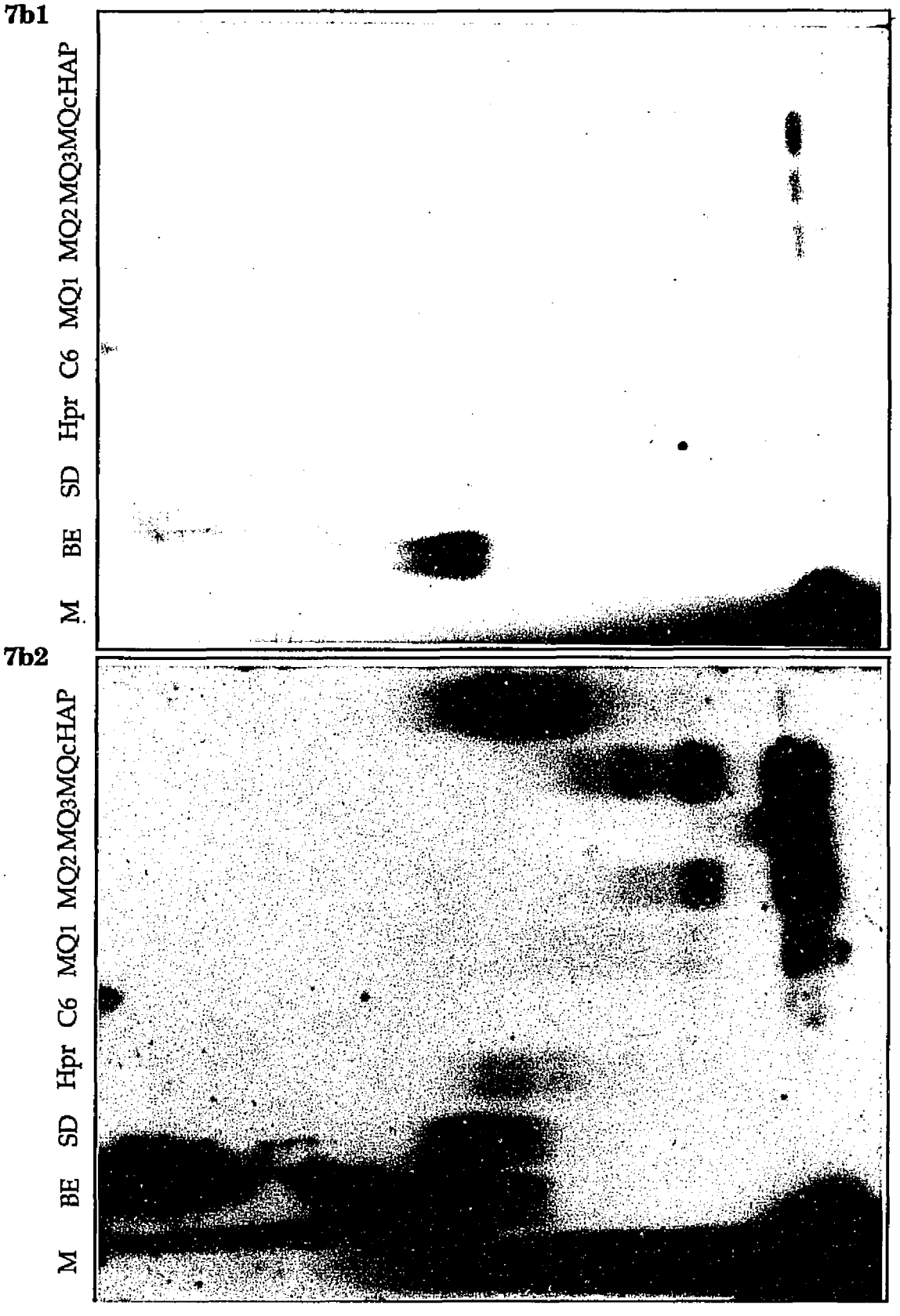


Figure 7. The integrity of BC1 RNP after different steps. (7a) 7.5% polyacrylamide gel (acryl:bis = 19:1). (7b) 1% agarose gel electrophoresis with short times (overnight) (b1) and long times (6 days) (b2) exposure. (BE=Brain Extract, SD=Superdex 200, Hpr=Heparin, FFS=Fast Flow S, HAP=Hydroxylapatite, MQ=Mono Q, M=total brain RNA as Marker)

smearing effect is not caused by the gel system, another run using lower voltage and riboflavin as cross-linking agent has been tested (**figure 7a2**). The result was identical. In this buffer system, "naked" RNA co-migrates with the front of ions, which indicates that all the bands in the Mono Q pool are still RNPs. Later studies showed that once the percentage of the gel was lowered from 7.5% to 5%, or run for a longer time, the sharp bands which were generated from the different pools smeared as well (**figure 4** as an example). This misleading observation is caused by the migration of BC1 RNP, which just leaves the stacking gel and travels into the top of the separation gel in the 7.5% gel system.


7b1
M
BE
SD
Hpr
C6
MQ1
MQ2
MQ3
MQcHAP
7b2
M
BE
SD
Hpr
C6
MQ1
MQ2
MQ3
MQcHAP

This suggests that BC1 RNP may be a very heterogeneous RNP with variation in its protein components.

To reduce the heterogeneous distribution of the BC1 RNP signal, The agarose gel which can tolerate small variations among the RNP was used. The result is shown in **figure 7b1**. The migration of BC1 RNP in the lanes of SD and HAP are faster than that in the lane of brain extract which suggests that an additional molecule is associated with BC1 RNP in the brain extract. When the solution of brain extract BC1 RNP was diluted with buffer, it became a slightly smaller complex. The size of particles from the Mono Qc pool (c stand for a concentrated pool) are quite different. In a short time exposure (24 hr), there are two bands of BC1 RNA signal in the Mono Qc lane. The migration distance of the lower band is very close to the band of "naked" RNA. It is speculated that this band may correspond to either the smallest BC1 RNP or "naked" RNA. However, the same sample loaded in the native polyacrylamide gel system has a different migration distance between the lowest signal band and the "naked" RNA band. It supports that the lowest band of MQc in agarose gel is an RNP rather than naked RNA. After a long time exposure (7 days), additional bands can be identified. In the Mono Qc lane, there are two new bands with shorter migration distance. Combining with previous two bands, they form a ladder-like pattern. It suggests that there are several forms of BC1 RNPs generated after the run of the anion exchange column. This may be caused by the sequential loss of protein(s). It also suggests that BC1 RNP is a multiple protein complex. These multiple forms of BC1 RNP do not necessarily correspond to the salt concentration for elution. The lanes of MQ1, MQ2 and MQ3 represent the division of the BC1 RNP eluted peak from the Mono Q column into front, middle and tail portions. Aside from the slow migration complexes of the middle portion of the eluted peak, there is no difference of the BC1 RNP bands between the front and tail portion of the eluted peak. In addition to those previous pools, the BC1 RNP pool from C6 column was included in this agarose gel. It shows a very low band as the Mono Q pool does in a long time exposure

In conclusion, several anion exchange columns will separate the BC1 RNP into different forms and/or disrupt the integrity of BC1 RNP. However, it is very hard to define a particle size once it undergoes a conformational change or becomes associated with different targets. In the case of SRP, the defined particle is the core particle and not necessarily the true conformation of SRP in *in vivo* conditions. It has been shown that SRP can maintain its integrity after the run of

ω–aminoalkyl agarose (C5) and falls apart during Mono Q chromatography [73]. Because it is a functional unit and can be defined by an *in vitro* assay system, one accepts that SRP particle contains 6 proteins with an RNA. In contrast, we do not have an assay system to ask whether a special form of BC1 RNP still maintains its activity.

Purity of BC1 RNP

Another question that arises from the above study is whether the different forms of BC1 RNP have the same length of BC1 RNA. An RNA denaturing gel (6% urea gel, acryl : bis = 19 : 1) method has been applied to answer this question. Along these lines, after achieving 2500-fold enrichment, the question of purity and amount of BC1 RNP arises. Although we have tracked the BC1 RNA signal from the beginning of purification, enrichment sufficient enough to separate BC1 RNP from other RNPs or proteins. Since we can limit protein contamination by several methods, other RNAs or RNPs become the major concerning source of contamination. After separating the RNA contents from different preparations, they are applied to the denaturing gel. In a gel stained with EthBr (**figure 8a**), one can not see the BC1 RNA but a smaller piece of RNA, approximately 80 nucleotides in size is visible. The sensitivity of EthBr staining for RNA is two to five times weaker as for DNA. More than 50 ng of BC1 RNA may be needed in order to be detected by these methods. Having used a BC1 RNA sense probe to estimate the amount of BC1 RNA, it seems that a working range of a hundred nanogram level has a discernible signal in the dot blot assay. The staining result is different from what we might expect. It suggests a possible error in choosing oligonucleotides as the quantitative control.

After using BC1 unique probes and then ID probes, one can see that BC1 RNA, some degradation products and some tRNAs can be identified (**figure 8b**). Since the BC1 RNA from the MQ pool is mostly intact, it rules out the possibility that the different forms of BC1 RNP in the previous study are generated by different lengths of BC1 RNA. The same volume of solution from different preparations were loaded in this experiment, and not surprisingly one can not detect any signal from BC1 RNA in the lanes of SD, Hpr, HAP and C6 during a short time exposure. As in other preparations, there are fewer intact BC1 RNA remaining in the brain extract than the ID-containing small transcripts. This suggests that inappropriate handling of those samples at room temperature or experiencing several cycles of freezing/thawing will degrade BC1 RNA in a distinct pattern. It

also provides an explanation of why BC2 and BC3 RNAs were detected in previous reports [12] and a suggestion of the relatively high RNase resistance for the ID domain of BC1 RNP. A 4.5 S RNA [140] and a 7SL RNA probe were also used to examine the purity of the Mono Q pool. Both probes indicated there is 7SL RNA signal but none of the signal of 4.5 S RNA can be detect in the last pool. Having applied pCp and RNA ligase to examine the RNA population within different preparations, it appears that there are very few large RNAs (larger than BC1 RNA) remaining in the Mono Q pool. However, there are some small pieces of RNAs which may be tRNA or degradation products.

8a

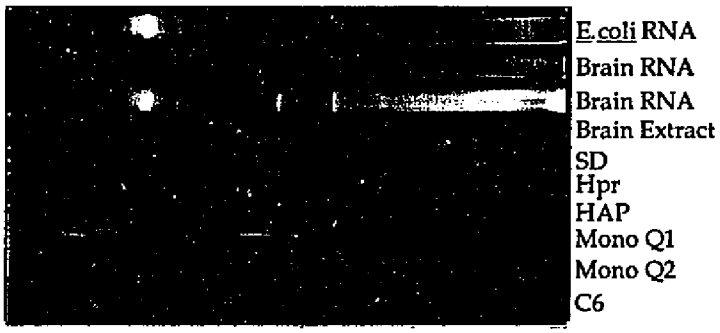


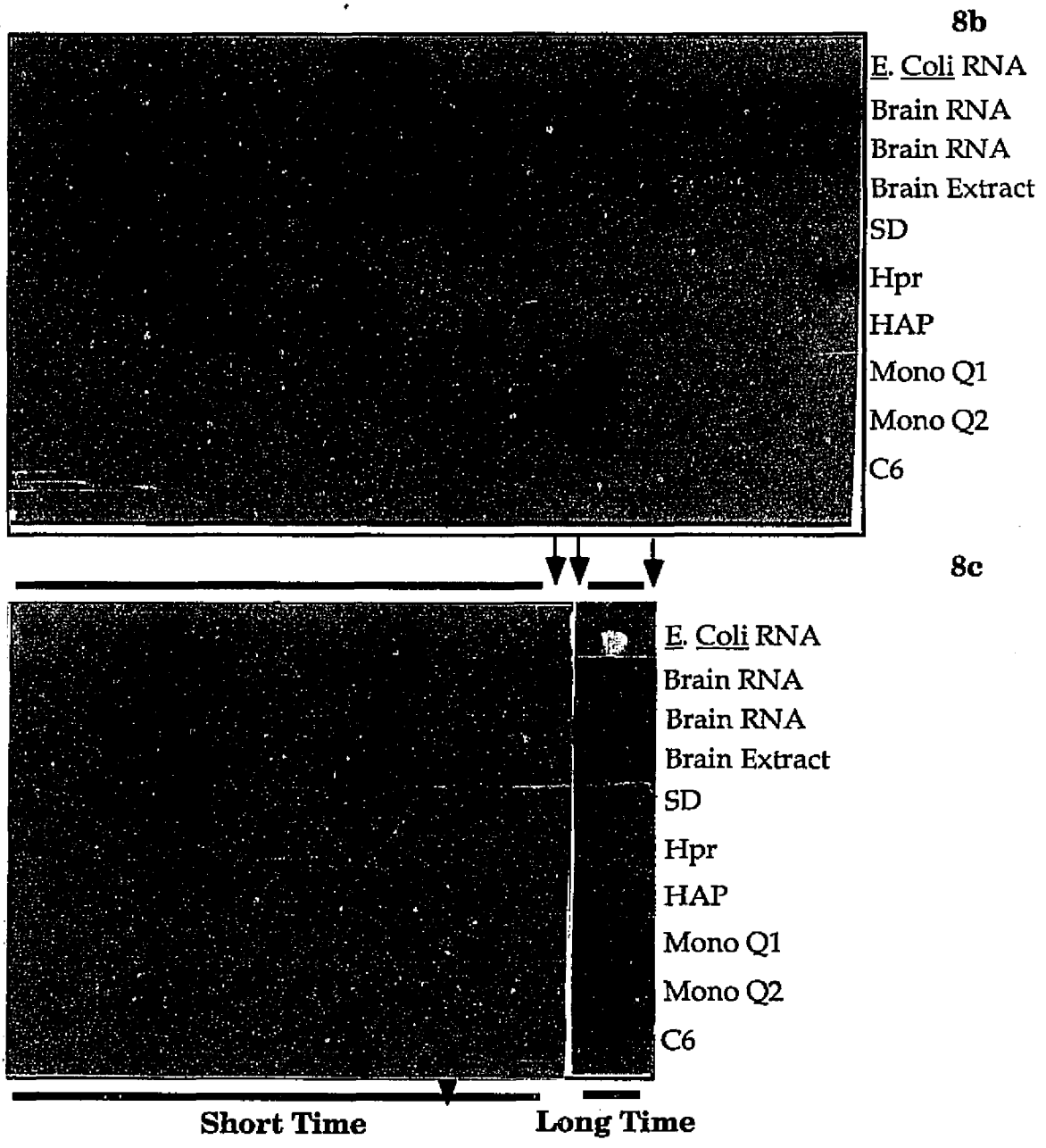
Figure 8. Homogeneity of BC1 RNP. (8a) 6% Urea gel with EthBr staining (acryl:bis=19:1). (8b) RNA blot with BC1 unique region probe and IDprobe (Overnight exposure). (8c) Different exposure time of (8b) (Longtime =7 days, Short time = 2 hr, Arrow and bar indicate the corresponding region for exposure; SD=Superdex, Hpr= Heparin, HAP=Hydroxylapatite, C6 = ω-aminoalkyl agarose, Carbon=6)

The signal of BC1 RNA as attained by using 3' end labeling method is weaker than the hybridization signal. It may suggest that BC1 RNA is not a good substrate for RNA ligase [141]. There are two strong bands in the size range of tRNA which can be picked up by the rat ID probe. As there is sequence homology between this ID probe and $tRNA^{Ala}$, it is plausible these two bands are real tRNAs which are also enriched in the brain extract.

Refinement of the purification

Problems with BC1 RNP purification

After testing many methods to further purify the BC1 RNP without getting further separation, it is realized there are uncertainties when we track the



BC1 RNP by its RNA colocalization. First, quantitation of BC1 RNA(P) is a problem. It is necessary to handle enough material to be detected in a general staining system for RNA or RNP, and then we can address the issue of contamination by other proteins and RNP simply by analyzing the banding pattern. As long as we can get a high signal to noise ratio, we are obtaining encouraging results. The lack of such a system is the second problem. Third, is the quality of BC1 RNP. It seems the BC1 RNP has signal separation in a variety

of columns. How can we maintain the particle intact and obtain a homogeneous form of BC1 RNP to work on. Fourth, the degradation of BC1 RNA and RNP is a serious problem. Although all precautions have been taken before and during the column procedures, BC1 RNA degradation still occurs. For solving those problems and to reach a good purification outcome, modification of purification strategies were made.

BC1 RNA standard curve

We now take the actual amount of BC1 RNA rather than the relative intensity of BC1 RNA as an indication of purification. During this approach, enough material can be applied to detect BC1 RNA as well as other RNA contamination. Large amounts of BC1 RNA have been synthesized *in vitro* and quantified by UV absorbance. To generate the standard curve, BC1 RNA in a series of dilution has been applied to the RNA dot blot assay. After calculating the relationship between BC1 RNA amount and the hybridization signal, the following formula is obtained (**figure 9a**) :

$Y = 7.898 + 0.84887\,X$,

X= log (μg of BC1 RNA);

Y= log (BC1 RNA intensity = Integration Volume Value)

Since the hybridization signal of BC1 may vary among different experiments and depends on the strength of radioisotope and hybridization conditions, one standard approach has been used. We assumed that the relative signal strength (slope) is maintained within different runs of hybridization. Once we put an RNA sample with known concentration in every hybridization blot, itcan become a reference to correct above formula. This standard is 0.1 μg of *in vitro* synthesized RNA, and the formula correction is as follows:

Y(shift) = Y(measure intensity, standard) - [7.898+ 0.84887 log (amount of BC1 RNA. standard)].

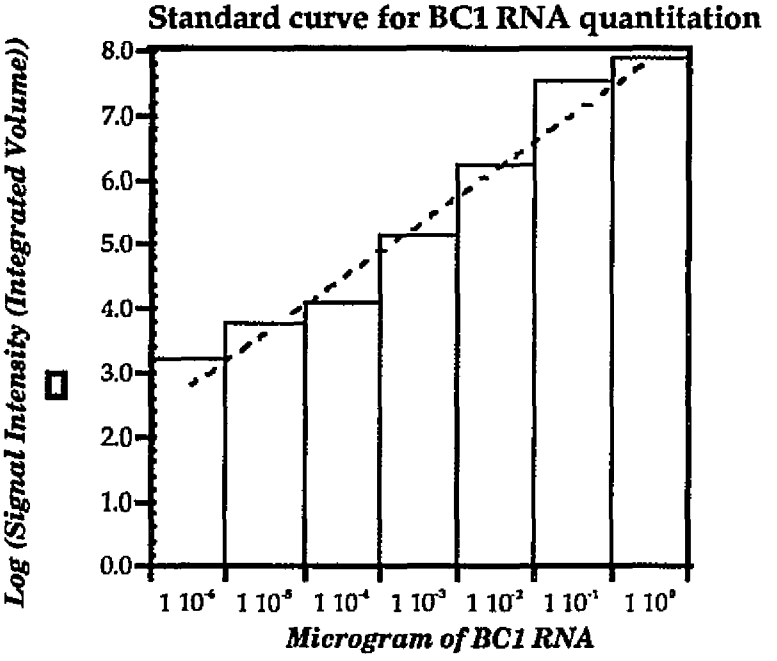
After measuring the RNA intensity of 0.1 μg of BC1 RNA standard, one can get the value of alternation in each individual experiment. Using the following formula, one can calculate the BC1 RNA concentration of an unknown signal.

Y(measure ,unknown)-Y(shift) = 7.898 + 0.84887X

Using this quantitative method, the amount of BC1 RNA in the rat brain was calculated. It suggests that every gram of brain can yield a maximum of 0.3 to 0.4 μg of BC1 RNA. Eight gram of brain was used and processed it into brain extract (S3), and there are nearly 1.5 μg of BC1 RNP to start with. If we subtract

the amount due to loss and degradation of BC1 RNA during purification, there is certainly not enough material to work with. Reduction of purification steps, processing time and loss of BC1 RNA signal are very important issues for the success of BC1 RNP purification.

9a



--- y = 1.9559 + 0.84887x R= 0.98775

There are several methods that we can apply to purify RNP in a short and limited step manner with high yield. The affinity purification is a efficient method to fractionate the target particles. Since we do not have an antibody against the BC1 RNP available to perform immunopurification, the only possibility is to use nucleotide interaction. Unfortunately, the structure of BC1 RNA does not allow many portions to be used for affinity purification. If it does bind to BC1 RNP, as oligo dT column does, the strong interaction and high background bring out other problems in need to be solved. The approach has therefore been to streamline the original column procedures.

Modification of chromatography steps

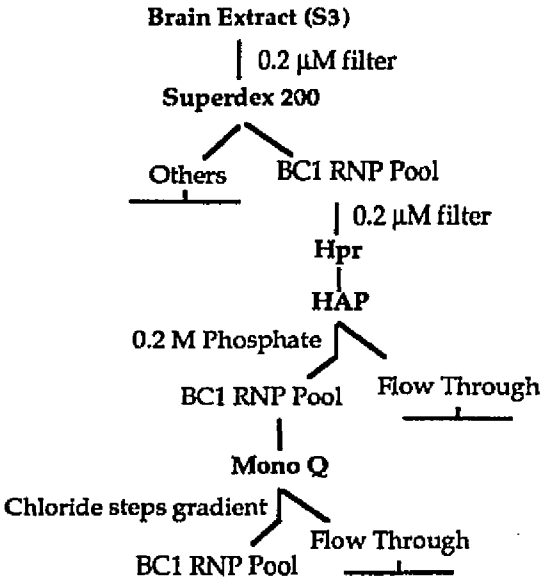
Starting from the five column purification scheme, the column set up has been modified as follows. First, to reduce the buffer exchange between two column steps, a buffer identical to the gel filtration (LS buffer) and 1 M KCl Mono Q buffer (HS buffer) has been used throughout all steps. Therefore, it not only avoid the dialysis step but also simplify the purification scheme. Second, the

FFS column seems superfluous, thus it can be eliminated without much effect on the purification scheme. Third, the flow through material from Hpr column can be directly applied to the HAP column without any delay. Fourth, a high concentration of phosphate buffer causes precipitation once it runs into a buffer containing Mg^{++}. For this consideration, a 0.2 M phosphate buffer has been replaced in the original gradient to elute out the bound material. It may reduce the performance of the HAP column but serves as a retention column to change the buffer into a phosphate-containing buffer. The elution salt gradient of Mono Q has been changed and tested to reduce the running time, to eliminate the BC1 RNP separation and to solve the tailing problem. The final procedure of this "abbreviated" chromatography is shown in **table 6** and the new Mono Q profile in **figure 9b**. Comparing this enrichment with the previous protocol (**table 5**), there is a slight loss of performance (18 fold to 16 fold). However, the result is a 65-fold concentration to a very small volume. It can detect the BC1 RNA signal in an EthBr staining gel (**figure 10 a**). In this particular run, BC1 RNA can be seen in fraction 19 or 20 with a concentration of 0.5 μg/ml. In another run, BC1 RNA is more concentrated at fraction 19 with several other RNA species (**figure 10b1**). Besides BC1 RNA, there are other RNAs that have been enriched by this method. Although we don't know identity of these RNAs, this procedure definitely provides an opportunity to discover and purify other small RNPs. An RNA blot has been used to confirm the location of BC1 RNA (**figure 10b2**). It not only shows a highly concentrated state of BC1 RNA in fraction 19, but also reveals a smaller form of BC1 RNA that is 10 to 20 nucleotides shorter. Using this highly concentrated Mono Q pool, some new possibilities were considered and new methods were used.

Table 6: The enrichment of abbreviated column purification scheme

Steps of Purification	Volume (ml)	Total Protein (mg)	BC1 RNA (μg)	Recovery (%)	RNA/Protein Ratio (μg/mg)	Enrichment (fold)
Superdex 200	125	128.1	1.325	100	1×10^{-2}	1
Heparin/ HAP	30	96.1	0.544	41	5.6×10^{-3}	0.56
Mono Q	2	3.1	0.526	39.6	1.6×10^{-1}	16

Flow Chart of Table 6:



Additional steps

A sucrose gradient may be the best subsequential procedure to follow the new Mono Q pool, since it has a limited capacity, mild conditions and it can tolerate a wide range of salt conditions. The separation result of a sucrose gradient is shown in **figure 11a**. From an EthBr stained gel, one can detect good separation of BC1 RNA from small (top) and large (bottom) RNAs. There are only a few major species of RNA coexisting within the BC1 RNA containing fractions. However, the effort that correlates the protein to RNA signal has been less rewarding. An S value standard (catalase) is always run side by side to monitor the sucrose gradient (see chapter 2). It seems that the peak of BC1 RNA is fairly consistent with previous result. However, there is also BC1 RNA signal with large S values. It may suggest that BC1 RNP is also associated with other large molecule even after some degree of purification.

After the sucrose gradient, native gel electrophoresis was used to get further separation of BC1 RNP [129]. With 2D gel in mind, I would like to establish a system to further characterize this RNP. It is known that one can not get a sharp band of BC1 RNP in the native gel system, however, taking advantage of good separation of RNA to RNP and other particles, native gel may provide some degree of separation. Two approaches have been tested: One is to desalt/concentrate the sample, another is to directly use the concentrated Mono Q pool with electrophoreses starting at low current. Otherwise, all other parameters, such as the gel component, running time or the thickness of the gel,

are identical. The separation result are somehow different with each run. There are several defined bands with the desalted sample but two diffuse bands in the non-desalting sample. Since the Centricon filtration system with 10 kDa M.W. cut off was used to desalt/concentrate a low molecular weight factor may possibly be responsible for the inter- or intra-molecular association of BC1 RNP. A old staining method (Stains-all) which can stain protein as well as nucleic acid with limited sensitivity was used (**figure 12a3**) in parallel with Coomassie Blue staining (**figure 12a1**). Since the protein and nucleic acids have a different staining color, it may indicate the location of RNP in conjunction with other staining methods. Both staining result are shown in **figure 12 a1, a2** along with the hybridization signal of BC1 RNP (**figure 12a2**). A phosphate-containing blue band in Stains-all stained gels co-migrates with the peak of BC1 RNP signal. However, the interpretation of this result becomes complicated when the band is as diffuse as BC1 RNP (**figure 12a2**). Once run another piece of gel slice into SDS-

9b

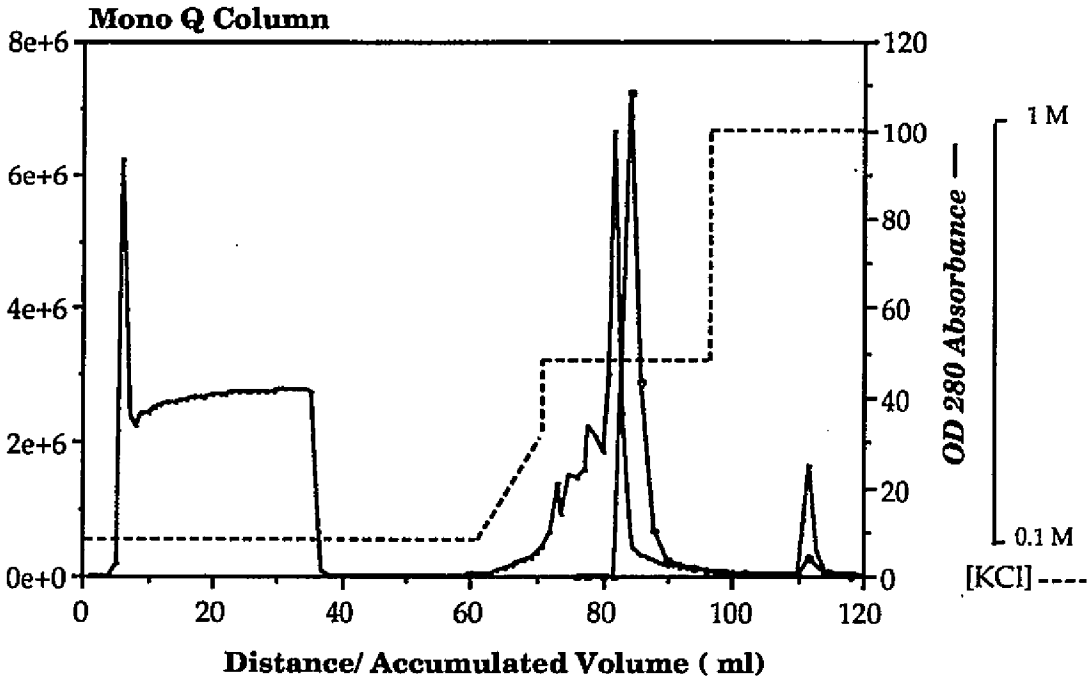
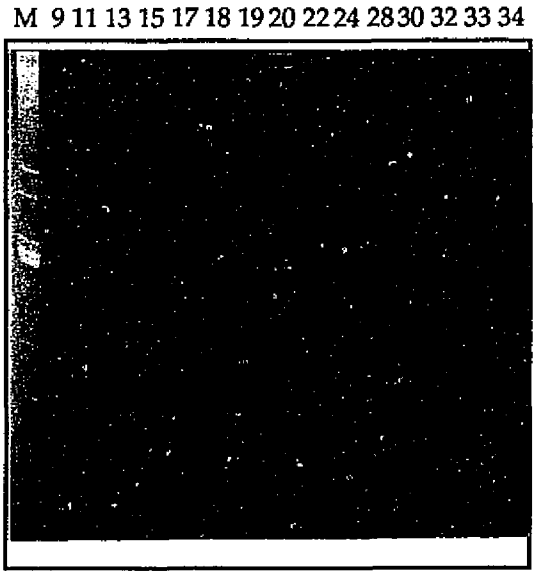


Figure 9. Optimized BC1 RNP purification. (9a) BC1 RNA standard curve (9b) Elution profile of modified Mono Q column.

PAGE, it reveals some interesting bands within the signal peak of BC1 RNP (**figure 12b**).

10a



10b1 **10b2**

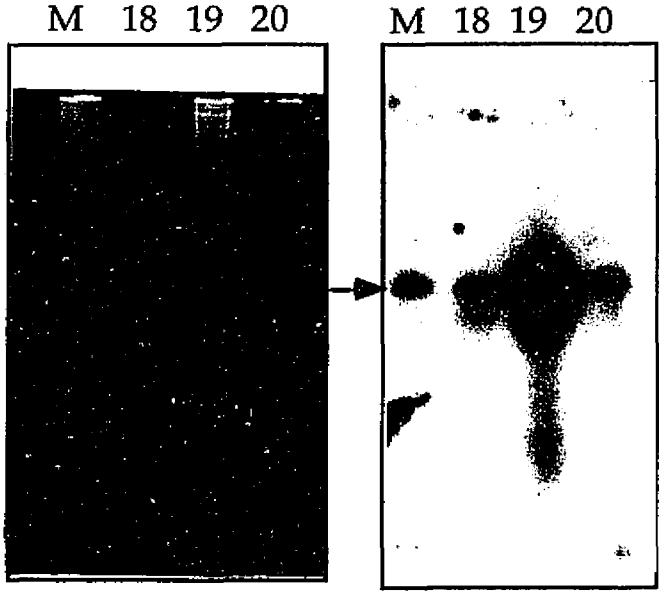


Figure 10. BC1 RNA from Mono Q fractions in (10a) 6% urea gel (acryl : bis = 19:1). (10b1) Selected fractions of (10a). (10b2) RNA blotting of (10b1) probing for BC1 RNA.

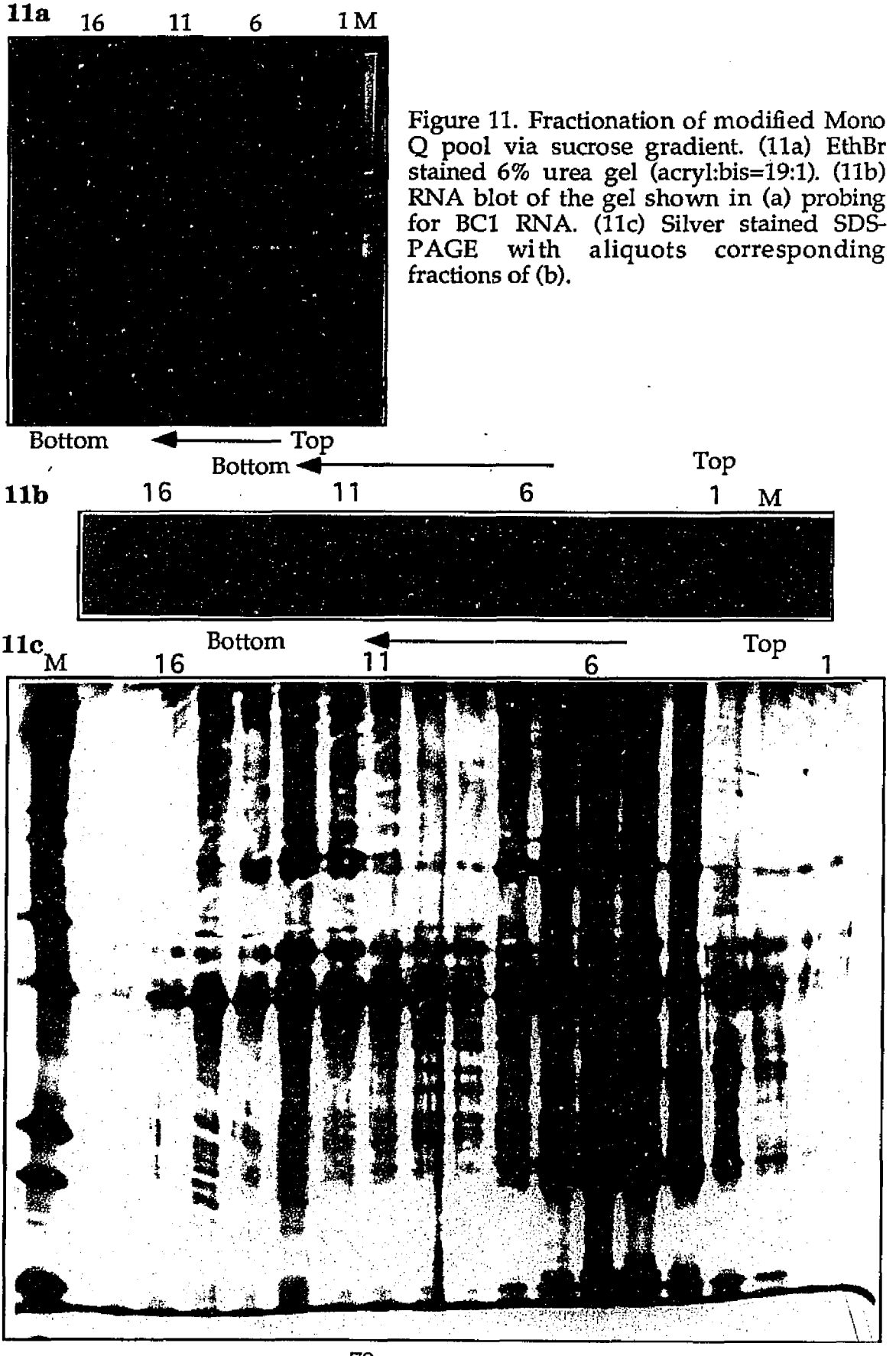


Figure 11. Fractionation of modified Mono Q pool via sucrose gradient. (11a) EthBr stained 6% urea gel (acryl:bis=19:1). (11b) RNA blot of the gel shown in (a) probing for BC1 RNA. (11c) Silver stained SDS-PAGE with aliquots corresponding fractions of (b).

Other methods to obtain the BC1 RNP particle.

Identification of the BC1 RNA binding proteins

Once the BC1 RNP is the dominant RNP in the preparation, alternative methods can be used to identify BC1 RNA binding proteins indirectly. The following attempts have been made. Since one can use UV radiation to cross-link an RNA binding protein to its cognate RNA and combine it with RNase treatment to reduce the noise of non-specific binding, it is possible to identify RNA-binding proteins from the epitope of cross-linked polynucleotide fragments (RNA tagged) [142, 143]. This highly sensitive method based on RNA-binding ability may be a good way to study a complicated RNP such as BC1 RNP. The resulting autoradiograph and its protein gel with silver staining is shown in **figure 13b, a,** respectively. The lane of ATP* is a control group to check the protein kinase activity within the preparation. Both polynucleotide kinase and RNA ligase were used to identify the RNA-containing proteins [141]. This result suggests that one can identify three strong bands both in the kinase group and ligase group. Two of those bands comigrate around 55.4 kDa protein marker, and a higher band close to 90 kDa is identified. There are other weaker bands as well, which may be caused by cross-linking efficiency or contamination with other RNP. In conjunction with a 2D gel system, one can identify or purify those radioactive spots. Because of sample shortage, a control where a sample without UV radiation was included could not performed. It needs to be included in further studies.

Identification BC1 RNP associated proteins

Other methods can be applied after achieving high enrichment of BC1 RNP. One is to screen a bank of sera from patients with autoimmune disease,since one can find high titer of anti-sera against small RNPs such as anti-Sm, anti-Ro, and anti-La, etc. [144]. Another way is to screen protein candidates by using an immunoprecipitation method to determine the possibility of a protein bound or associated with BC1 RNP. Both methods have been tested and the results are as follows.

After screening a set of antisera from patients with autoimmune disease, one autoimmune serum has shown a strong ability to immunoprecipitate both BC1 and BC200 RNA. Further studies suggest that some of its binding activity iscaused by the interaction with RNA directly. This anti-RNA antiserum

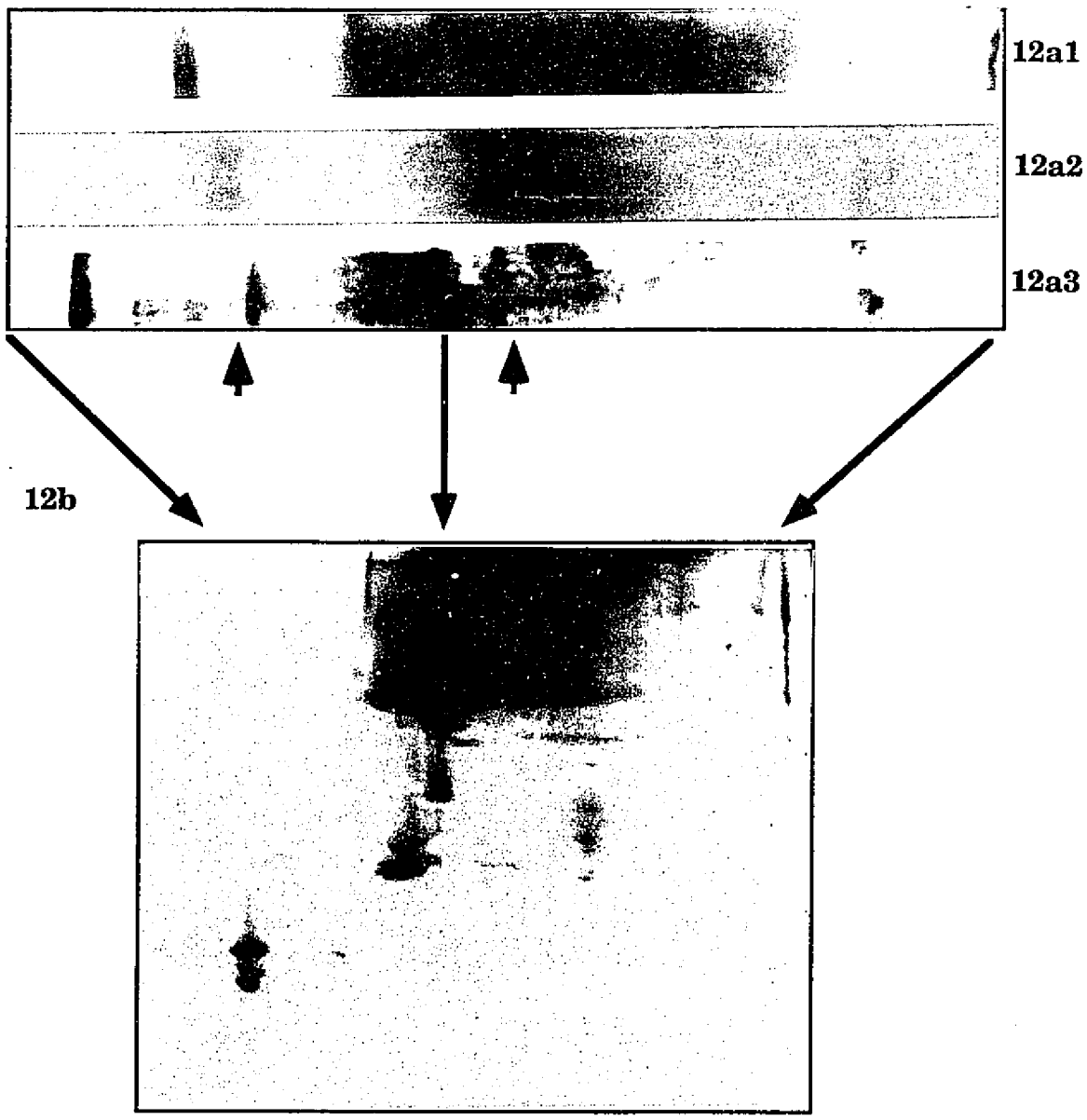


Figure 12. Fractionation of BC1 RNP through 4% - 10% native gel (acryl : bis = 37.5 :1) (12a). Neighbouring gel slices stained with Commassie blue (12a1) and Stains-all (12a3). Distribution of BC1 RNA by RNA blotting. (12a2) A representative gel slice was run for a second dimension into SDS-PAGE and stained for proteins with Commassie blue (12b). (Short arrows indicate the BC1 RNA; long arrows indicate the corresponding position between the gel slice and the SDS-PAGE)

can immunoprecipitate a subset of RNAs including 7SL, BC1 and BC200 RNA but not tRNAs or some other RNAs (**figure 14a,** lane 1, supernatant) [145]. Because of this multiple RNA binding activity and low titer, this antiserum (α–

3b) could only be used in later steps of the BC1 RNP purification or act as positive control in the immunoprecipitation test. The interaction between antiserum and BC1 RNA can be blocked by adding BC200 RNA, suggesting that BC1 and BC200 share a common epitope.

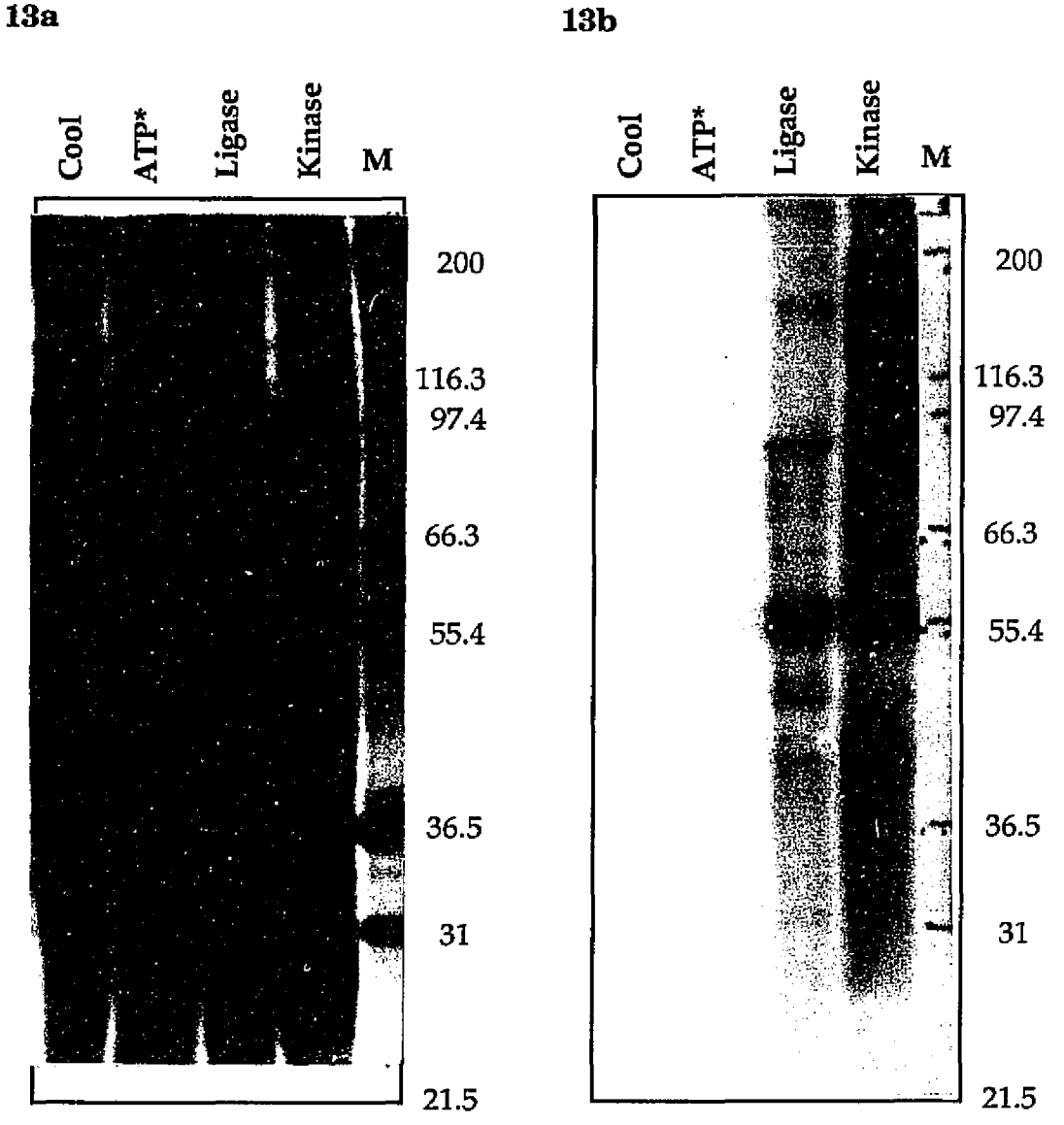
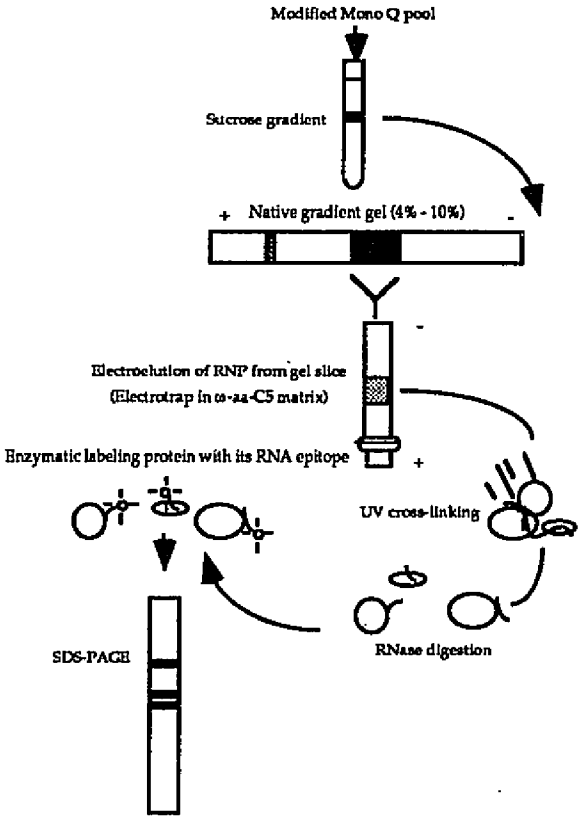


Figure 13. Identification the BC1 RNA binding factors by RNA tagging method. (13a) Tagged proteins. (13b) Corresponding gel with silver stain. Cool = No P^{32}-ATP and enzyme added. ATP= Add P^{32}-ATP. Ligase=labelwith RNA ligase and pCp. Kinase=label with polynucleotide kinase with ATP.

In the first set of experiments, the association between BC1 RNP and cytoskeleton components was tested. An antibody against synaptophysin acts as

a negative control, as this protein is in axonal terminals which is a different compartment where BC1 RNP is found. The results, shown in the pellet portion of **figure 14a2**, demonstrate the possible association of BC1 RNP with two cytoskeleton components, α–tubulin and neurofilaments. This is the first evidence to support the notion that BC1 RNP is associated with the cytoskeleton. It may bring up a lot of interesting possibilities about BC1 RNA transport and docking in dendrites. Because only 1 of 100 of BC1 RNP was brought down by these two antibodies, it suggests a low affinity or indirect interaction of BC1 RNP to the cytoskeleton.

Diagram of RNA tagging method



In the second set of experiments, some candidate proteins as well as some antibodies against proteins from the post-synaptic density (PSD) (α–2 = α–Bip/Hsp 70 like protein; α–3 = α–internexin) [146] were tested. A further question is whether the poly A binding protein can interact with the A-rich region of BC1 RNA. This possibility is ruled out by the lack of immunoprecipitation of BC1 RNP with an antibody against the poly A binding protein (**figure 14b2**, lane 5, pellet). One of antibodies which is against α–

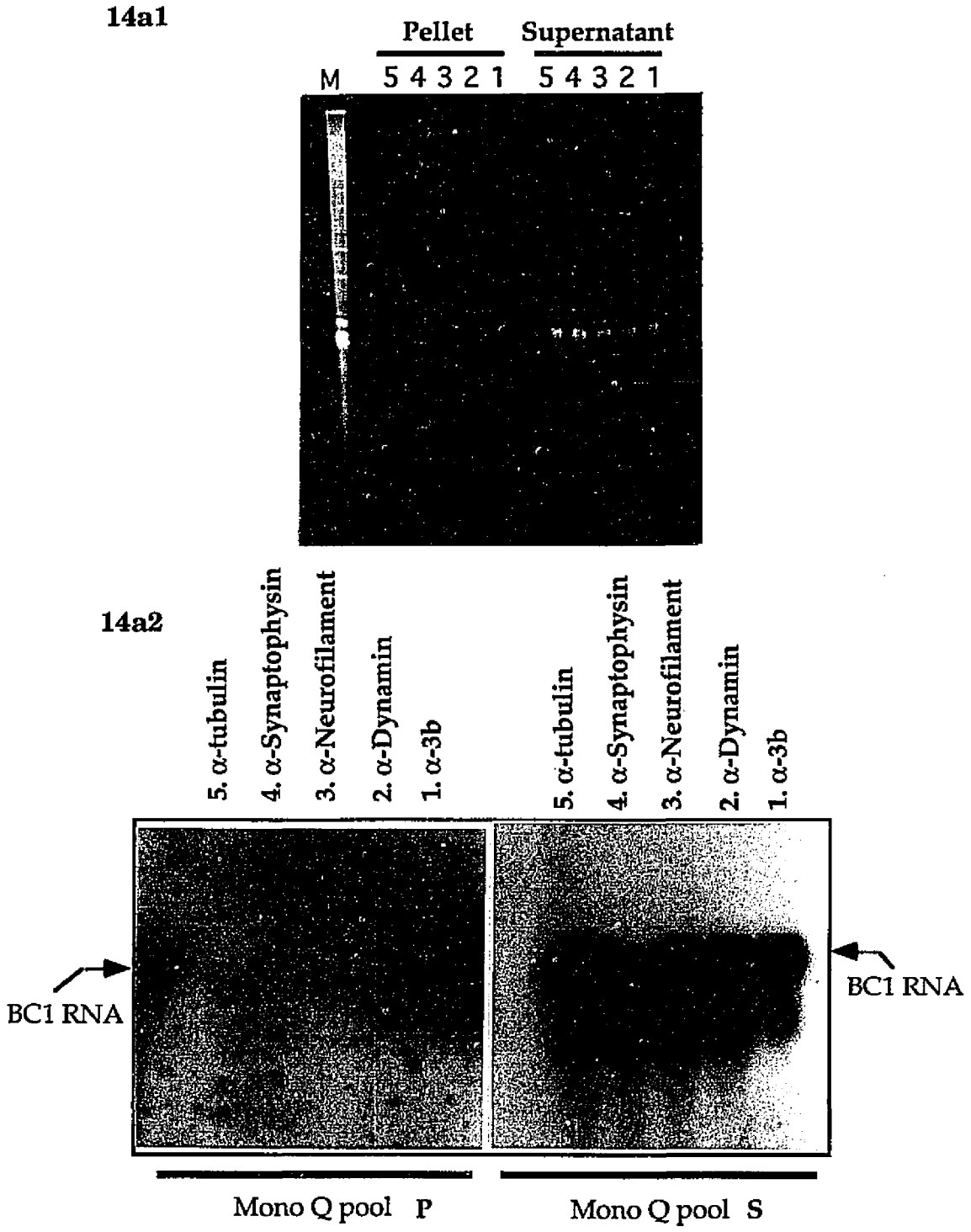


Figure 14a. Immunoprecipitation of the Mono Q fraction of rat brain extract with various antibodies against cytoskeleton components. (14a1) RNA gel to reveal BC1 RNA. (14a2) Detection of BC1 RNA by RNA blotting. (P=pellet, S = supernatant.)

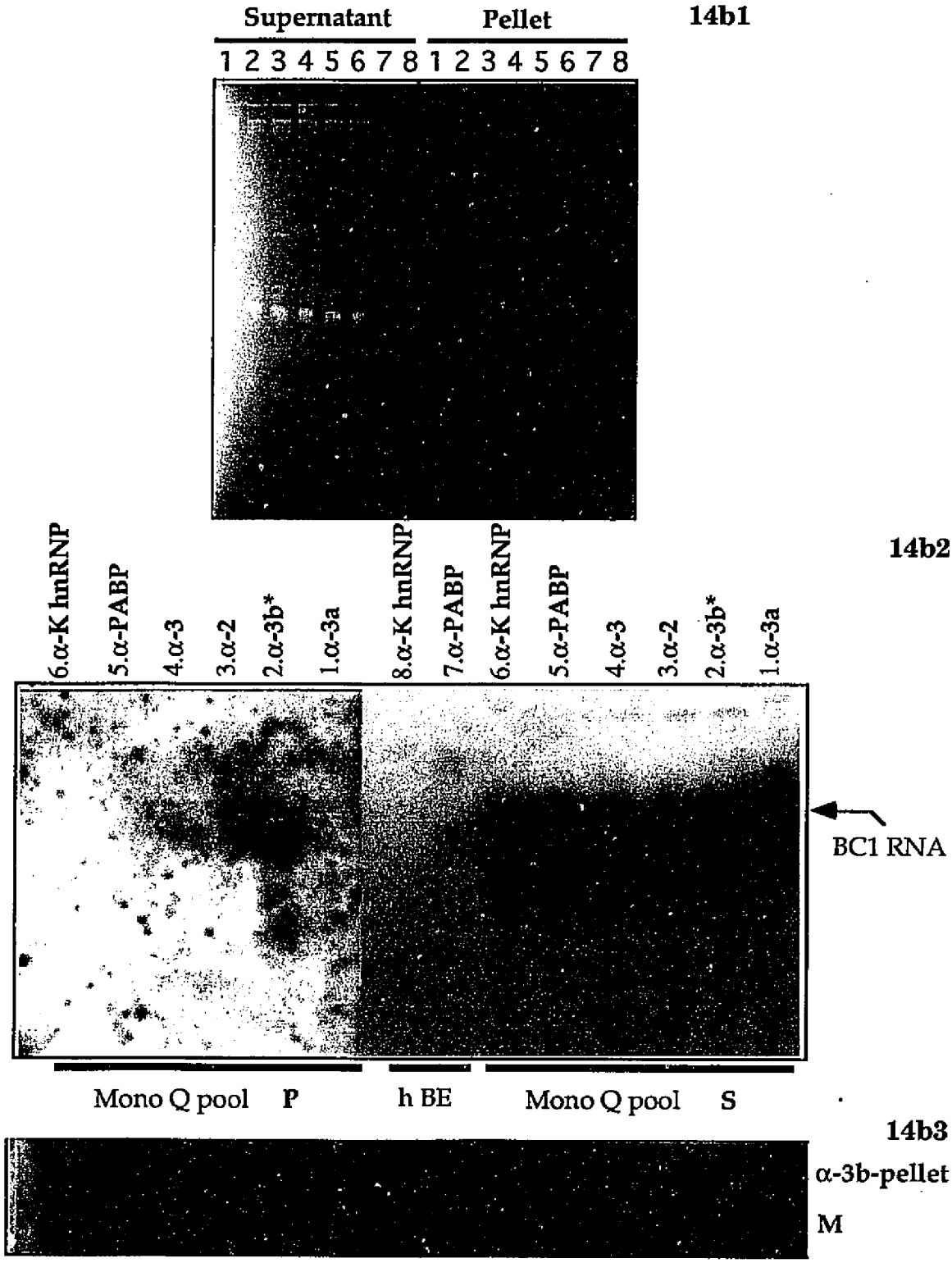


Figure 14b. Immunoprecipitation with various antibodies. (14b1) RNA gel to reveal RNA. (14b2) Detection of BC1 RNA by RNA blotting. (P=pellet, S=supernatant). (14b3) The protein profile of pellet portion of α-3b-antibody test. (M = M.W. marker)

internexin (α–3) has a very weak signal. This signal has been confirmed using a highly sensitive method (phosphor-image). Because of low titer of this antiserum, it may require another source of antibody to study the interaction between BC1 RNP and α–internexin. However, it may also suggest a low binding affinity between BC1 RNP and this protein. A–internexin belongs also to the neurofilament family with early expression on a set neurons after migration. The function of α–internexin is still obscure, especially in dendritic neurofilaments .

Although the purification of BC1 RNP is not complete, both biochemical and molecular biological methods have been used to enrich the BC1 RNP. New strategies and methods have been developed. The results not only shed more light on the function of BC1 RNP but also provide important information for the further purification of BC1 RNP.

Chapter IV

In Vitro Study of BC1 RNA-Binding Proteins

Introduction

RNA-binding proteins play a role in RNA stability, RNA translocation as well as RNA metabolism [147]. They can be an effector or serve as a bridge to link other macromolecules. The protein portion of telomerase acts as an RNA-directed DNA polymerase [22]. The iron-response element (IRE) binding protein influences the mRNA stability [148]. The glyceralaldehyde 3-phosphate dehydrogenase (GAPDH) may interact with a subset of tRNA and lose its cofactor binding as well as enzyme activity [149]. In most cases, the RNA-binding protein without significant enzyme activity, may either serve as a cofactor of the RNA molecule or integrate into larger machinery such as ribosome or spliceosome.

BC1 RNA is a neuron-specific small cytoplasmic RNA whose function remains to be elucidated. Its tissue-specific transcription as well as dendritic location points to interesting physiological roles [3, 111]. BC1 RNA can be divided into three portions which may serve different functional domains as well [1]. At 5' end is identifier (ID) domain which is a short intersperse sequence (SINE). Like SRP RNA is an ancestor of Alu elements in primates, BC1 RNA is the founder of ID elements. As a result, the ID domain also exists in other transcripts, which leads to complexities in studying the BC1 RNA-binding proteins. The 3' end of BC1 RNA may form a stem loop. This region was defined as unique region, since it can only identify one band in genomic Southern hybridization. It may also suggest that if there is any function assigned in this region, it will served a unique function in the neuron. Connecting ID domain and unique region is the A-rich region with two GAC interrupted. This A-rich region may not interact with poly A binding protein (PABP), for it may be covered by other two domains and shorten the length of poly A stretch by the GAC insertion [63]. Recently, the trial that uses PABP antibody to bring down the BC1 RNP by immunoprecipitation has failed, which provides strong evidence against the association of PABP with BC1 RNA. However, it can not rule out that other proteins may interact with this region.

Searching the BC1 RNA-binding proteins *in vitro* may serve as a parallel method to characterize the BC1 RNP particles. If one tracks the BC1 RNA starting from its transcription to its final destiny, there are many procedures involved. The BC1 RNA must be moved from its synthesis site to a nucleus pore complex,

then the translocation takes place. Once BC1 RNA goes into the cytosol, it must be selectively transported from the soma to the end of neuronal processes. It is not known whether the function of BC1 RNA must be exerted in a right compartment or not, one may suspected that different stages of BC1 RNA may have some common or different proteins to associate with.

For an *in vitro* study, those binding proteins may not be the components of mature BC1 RNP except those with high affinity to the BC1 RNA. Nevertheless, it will provide an opportunity to survey those different stages of BC1 RNP. In the case of BP-1 protein [95] ,which was identified by an *in vitro* study, it may function similar to what TFIIIA is to the 5S rRNA [33, 35]. Both TFIIIA and BP-1 can bind DNA (the gene) as well as RNA (the gene's transcript). There are multiple functions of TFIIIA. Other than a transcription factor, it also provides an alternative pathway for translocation as well as the storage of 5S rRNA. BP-1 protein may play similar roles. However, because of its low affinity to BC1 RNA, whose binding can be displaced by heparin, BP-1 may not be a component of BC1 RNP.

Results and discussion

Assay the binding activity via RNA protection and gel-shift assay

The methodology of studying the protein and nucleic acid interaction has been developed extensively [150]. One standard method is the mobility shift assay. Since the protein-nucleic acid complex will migrate slower than the nucleic acid in a native gel system, which is caused by the neutralization of the negative charge and increases of the molecular mass, one can use it to assay the activity of proteins binding to nucleic acid. However, there are a lot of factors in a crude extract which can interact less specifically with nucleic acid, one must include some sort of blocks to reduce the non-specific binding. In the study of protein-RNA interaction, random RNA or heparin can serve this purpose. As for increasing the stringency, RNase degradation and the RNA structure alternation has been used to examine the affinity between the RNA-protein complex [151]. RNase treatment may be the best method to study the protein and RNA interaction with a complicate RNA or without knowing the protein binding site.

RNase titration and isotope test

To test whether there are any factors which can bind BC1 RNA strong enough to resist RNase degradation and cause a mobility shift. RNase protection and gel shift assay was performed in a standard manner [150] (**figure 1**). Comparing the lane of probe-only to the probe plus extract, one can see the radiolabeled RNA with brain extract migrating slower than the RNA-only. This suggests that there are some factors can bind BC1 RNA despite the presence of 0.5 μg yeast tRNA in each reaction. Both lanes form a smear pattern, which may be caused by the RNA probe degradation before experiment, the buffer system for running the gel or interact with non-specific factors. Once adding the RNase into the binding reaction, one can distinguish three shifted bands both in the G labeled or U labeled RNA probes. Although the intensity of bands is varied in different labeled probes, the migration distance is nearly the same. One can titrate the RNase amount to test the RNase resistance of different bands. In both case of G labeled and U labeled RNA, band one (high) and three (low) are relatively resistant to RNase up to very high concentration (thousand unit of RNAse Ti and 25 μg of RNase A). On the contrary, band two (middle) can't resist very high RNase concentration. It is also noted that in the U-labeled shifted band one the gel-shift complex becomes smaller when RNase amount increasing. This may suggest there are some sort of 'breathing' between the end of RNA and its binding proteins.

There are other bands that migrate as fast as "naked" RNA. Since those bands can be generated by RNA probe only after digestion with RNase, they may be considered as degraded RNAs. However, this can't rule out the possibility that some protein-RNA complexes can't be resolved from RNA-only in this gel system. It may need further study to answer this question. It also suggests that this assay system may only provide a window to study some degree of affinity between the RNA and its binding factors. Different gel system and different gel composition may generate different shifted-band as well as different resolution (chapter 5).

Domain specific binding test

In addition to full length BC1 RNA, two truncated BC1 RNAs were synthesized to investigate which domain can cause those shifted bands. The results (**figure 2**) indicated that the ID portion alone can cause all three shifted bands. The RNA with unique domain only gives two very light bands in the range of shifted band 2 and shifted band 3. Since we don't have the construct to

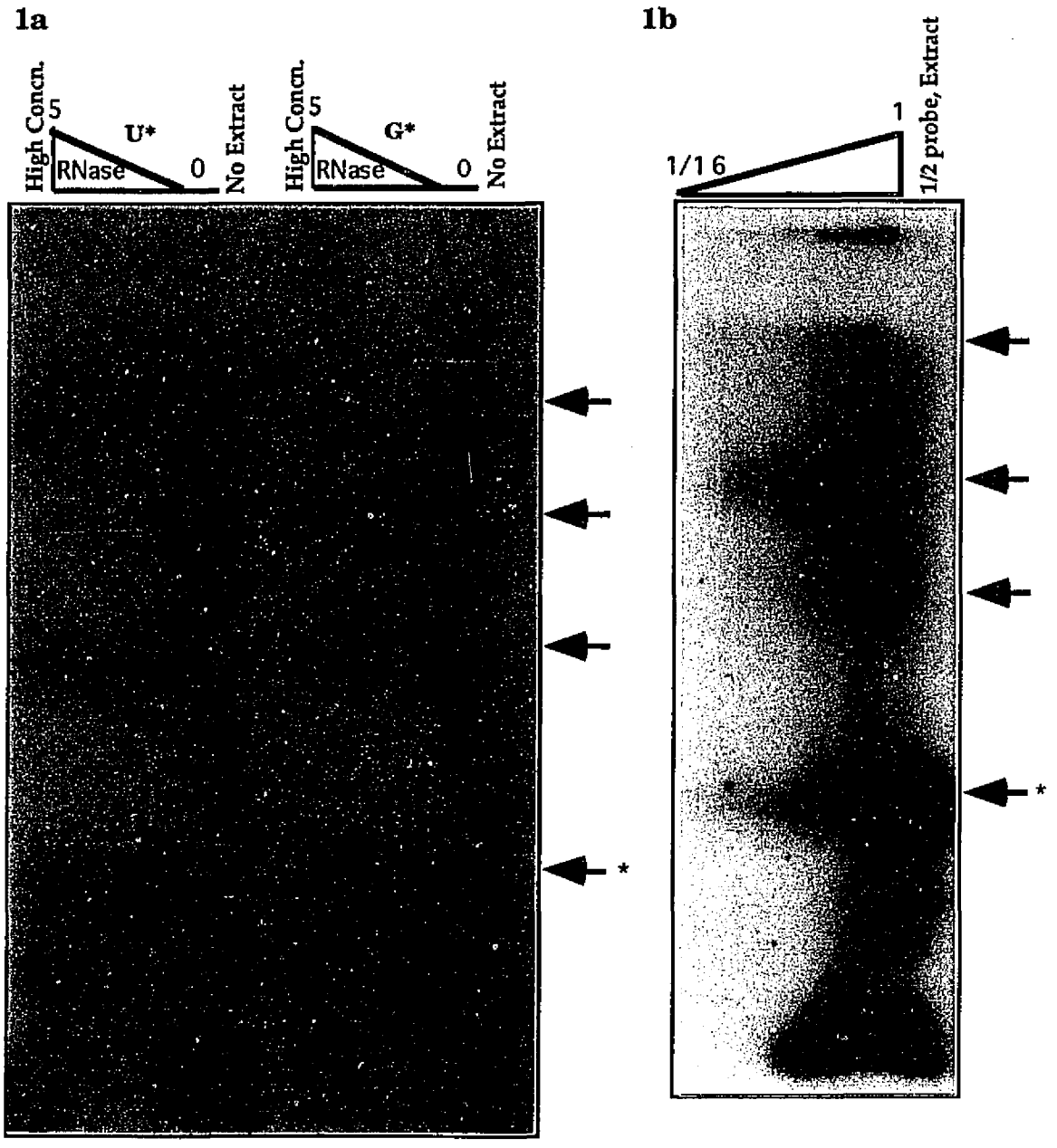


Figure 1. RNase protection gel-shift assay. (1a) Titration of RNase and assay of radioactive nucleotide (G*. GTP labeled probe; U*. UTP labeled probe). (1b) Titration of probe concentration Arrows indicate the shifted bands, Arrows * indicate the free probes)

produce an RNA corresponding to the A-rich region, the contribution of this domain to those shifted bands is unknown. However, the band-shift pattern of whole BC1 RNA can be mimicked by ID portion only, this result may suggest

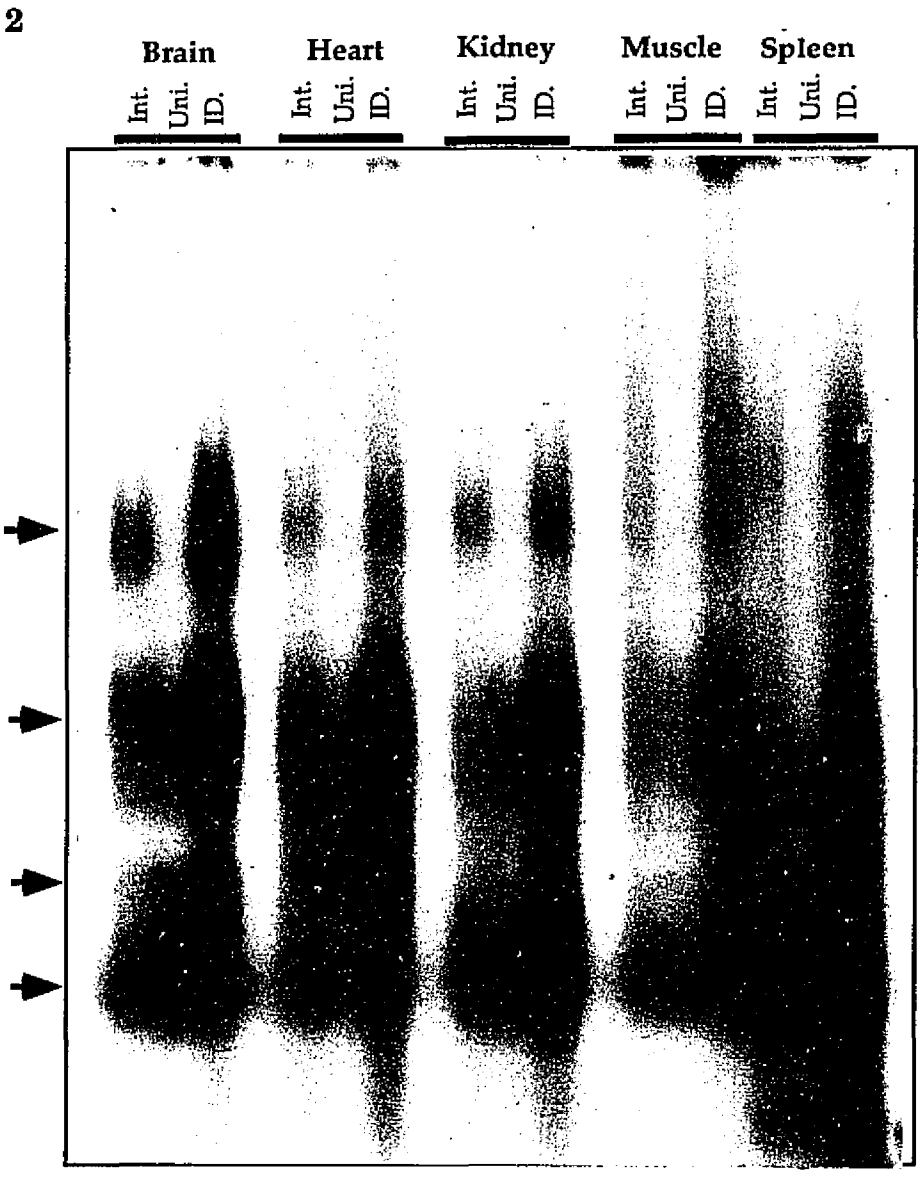


Figure 2. BC1 RNA domain interactions with binding factors in different tissue extracts. (Int.= Full length RNA, Uni. = Unique region RNA, ID = ID domain RNA; Arrows indicate the shifted bands.)

that the A-rich region may not cause additional shifted bands. If there are any shifted bands, they must be covered by the bands generated by ID and unique region. There are several possibility that one domain of RNA can generate different shifted bands. The length of RNA may be different or these protein components are various in different shifted bands. Further study suggested that both possibility contribute more or less to those migration distances. However, protein-protein interaction may also play a role to cause the higher shifted bands.

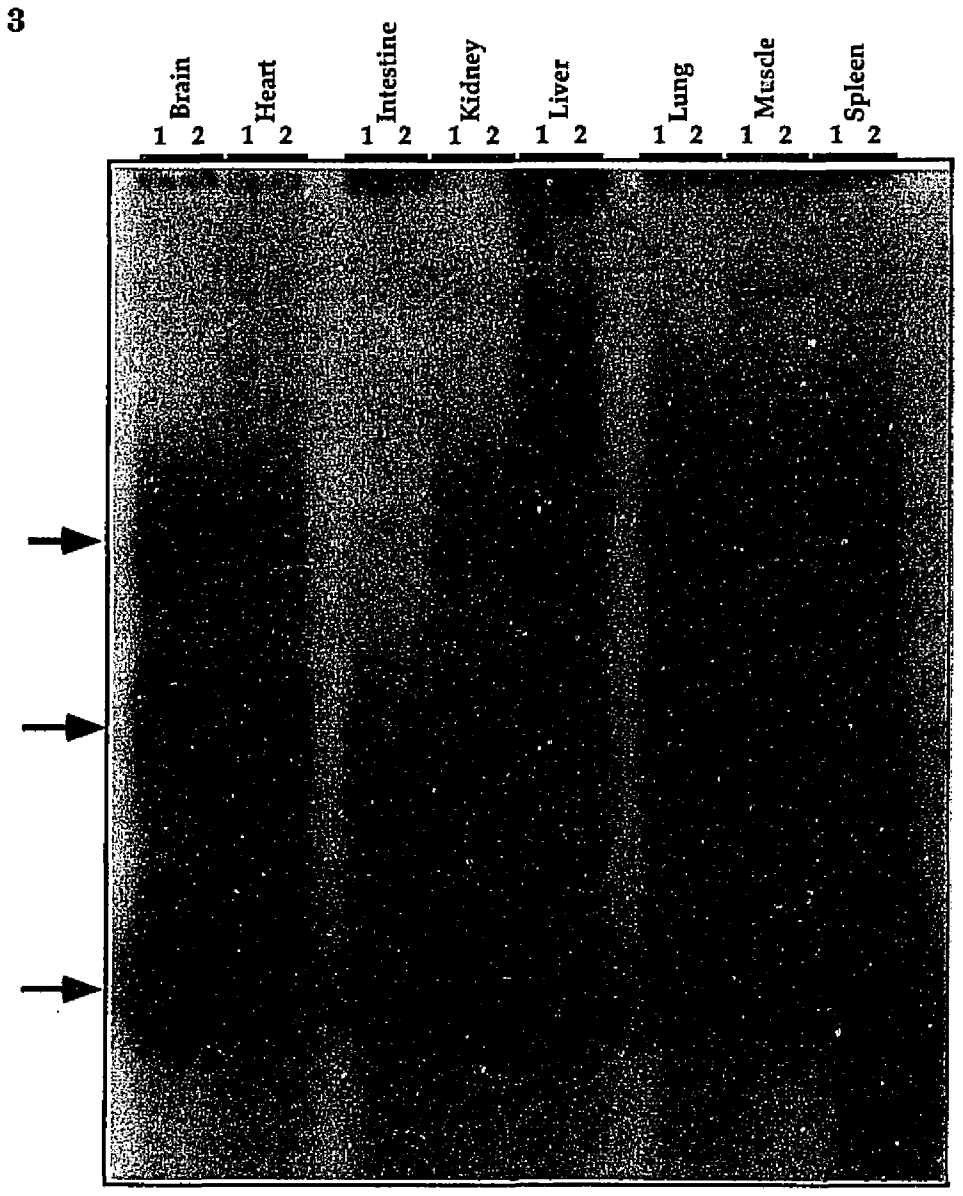


Figure 3. Binding factor activity to BC1 RNA with different tissue extracts. (Arrows indicate the shifted bands)

Tissue specific binding test

Using the same assay system, one can ask a general question whether the BC1 RNA binding proteins are brain specific. The same amount of protein from different tissues which has been prepared by the same method as the brain extract is performed in a parallel manner with the same amount of radiolabeled RNA (**figure 3**). Although brain extract gives the strongest shifted bands in all of

those reactions, there is BC1 RNA binding activity in other tissue extracts as well. Except in intestine and muscle extract, these RNA binding activity can be found in heart, kidney, liver, lung and spleen. The liver always give a complete smear pattern and never can generate a shifted band with RNase digestion. It is possible there are some other material bound to RNA very strong and prohibit the RNase activity in the liver extract. It needs to be noted that those shifted bands somehow different from each other both in migration distance and the resistance of RNase degradation. For example, the shifted band one in brain extract is lower than the corresponding bands in heart, kidney and lung extracts. On the other hand, band two in the brain is higher than its corresponding bands in all other tissue extracts. The second shifted band in the lung extract gets weaker when adding double amount of RNase. This may suggest that the factors bind to BC1 RNA or the protein-RNA complex somehow differ in different tissues. It has been tested which domains of BC1 RNA contribute to those shifted bands in other tissue (**figure 2**). Like with the brain extract, ID domain provides the most shifted bands in other tissues, and unique region only gives very faint shifted band. The shifted bands which are generated from the interaction between unique domain and its binding factors were examined further (**figure 4**). It shows that the brain extract has higher activity to bind the unique domain of BC1 RNA than other tissues do. Based on the fact that ID transcripts are not restricted to the brain tissue, it would not surprise there are BC1 RNA binding activity in other tissues. Although it is very hard to make conclusion from those *in vitro* assay results, it may suggest that the BC1 RNA or BC1-like RNA may have more additional physiological functions in some of the other tissues. Since the brain extract provides a stronger binding ability to BC1 RNA and has different migration pattern in this assay system compared to other tissues, one may also speculate that there are some things unique about BC1 RNP complex in the brain.

No other lab has been using this assay system to study the interaction between a complex RNA (contain at least two stem loop and a flexible hinge) and its binding proteins before. From this study, it shows that the power of such assay to study a more complex system without losing its simplicity and speed. One can apply this method to ask some fundamental questions such as the Kd of RNA-protein interaction as well as the influence of different treatments. All of those information can provide the hint of regulation pathway of the RNP and the conditions of purification.

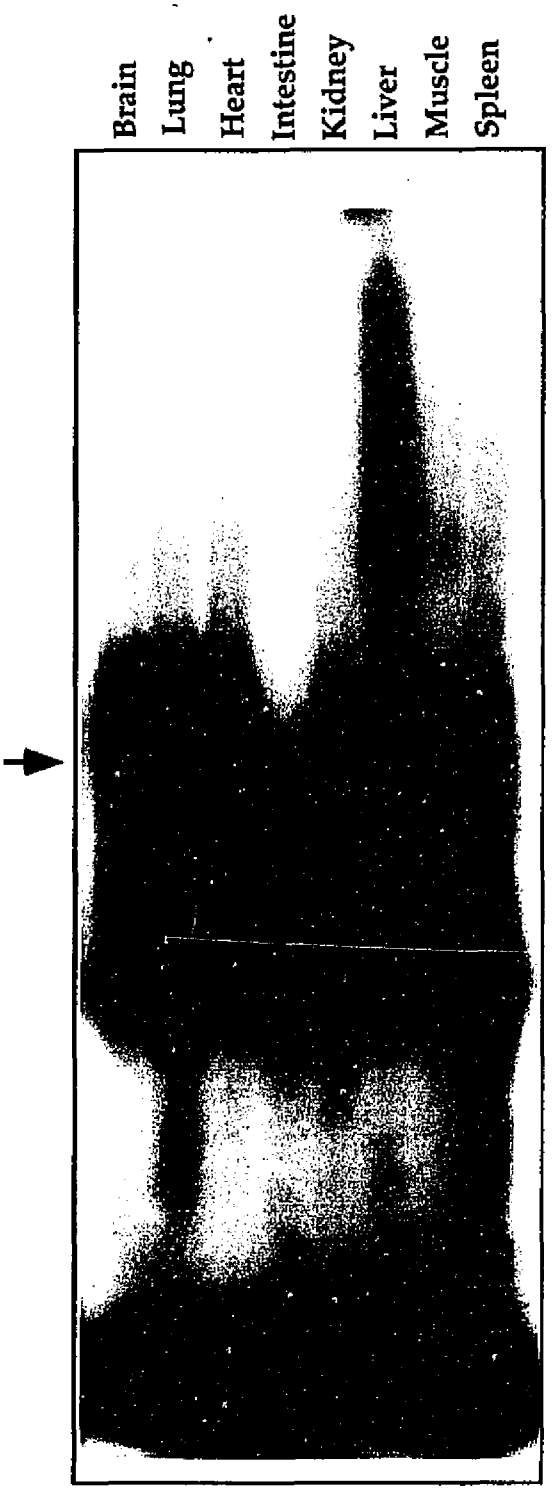


Figure 4. Binding efficiency of protein factors in different tissue extracts interacting with the unique domain of BC1 RNA. (Arrow indicate the major shifted bands)

Identifying the BC1 RNA binding proteins by nucleotide transfer

It is possible to acquire the estimated molecular weight of nucleic acid binding protein by a method called nucleotide transfer or labeling transfer experiment [152]. This method uses UV cross-linking without adding any cross-linking agent [142, 143]. Since the length of free radicals on the nucleic acid, which is activated by UV light, is very short, it is believed that the distance between the free radical to its target must be very close. It also suggests that not only the distance but the spatial conformation are important to form a new covalent bond between protein and nucleic acid. However, there are two major drawbacks with this method for estimating the molecular weight. One is the nucleotide (or polynucleotide) epitope on a protein will dramatically alter its migration behavior in the SDS gel system. Another is the non-specific interaction between denatured protein and nucleic acid. Combining the RNA protection and gel shift assay with the nucleotide transfer method, a protocol has been integrated that can avoid or reduce such problems and provides high efficiency and specificity.

Since the RNA binding proteins may interact with BC1 RNA in different regions, it will be expected there are multiple protein bands identified by the nucleotide transfer method. The experimental result is shown in the **figure 5**. As mentioned before, brain extract can generate 3 shifted bands from the RNase protection and gel-shift assay. Before loading those UV cross-liked band-shift complexes into SDS-PAGE, another dose of RNase was added to clean up the non-specific binding as well as shorten the polynucleotide tag. There are two strong bands and two weak bands can be labeled specifically in the shifted band one. The two strong bands are estimated close to 67.8 and 58.5 kDa, and two very weak bands are 133 kDa and more than 200 kDa. In the shifted band 2, there are protein bands close to 68.5, 56.4 and 29.4 kDa, and two weak bands with 38, and 31.7 kDa. The 56.4 kDa band has the strongest labeling intensity among all other bands. The band of 31.7 kDa consists of several bands together and gradually mixes with the stronger band of 29.4 kDa. The 62.1 kDa band and 29.4 kDa band are the strongest bands generated in the gel shifted band 3. In a short exposure, the 62.1 kDa band reveals a doublet and combines with two other weak bands of 59 and 56.4 kDa becoming a continuous ladder pattern.

There are several possibilities that can contribute to the different labeling strength among different bands. One is the number of radiolabeled uridine contained in the protected and cross-linked RNA fragments. This may be

examined by using another radiolabeled nucleotide such as GTP. Another possibility is the distance and arrangement between RNA and protein may cause different degree to be cross-linked through this method. The other alternative is, the band shift complexes are too large or difficult to be eluted out from the gel slice, especially those high molecular weight proteins in the shifted band 1. The length of cross-linked RNA on the same protein may also generate some effects; however, this effect may cause a difference both in the intensity of radiolabeled and migration distance. The continuous pattern in the 29.4 kDa (shifted band 2) and 62.1 kDa (shifted band 3) region may result from this. The high M.W protein bands from shifted band 1 are consistent with the migration distance of shifted band 1. The proteins with such high molecular weight may be the components of cytoskeleton. However, it needs further study to support this idea.

If we don't count the small variation less than 3 kDa, it can simplify the protein band pattern quite well. It suggests that the RNA binding proteins from different shifted bands may share some common proteins. One can propose a core particle of BC1 RNP which contains three major proteins, 68.5 kDa, 56.4 kDa and 29.4 kDa. It is true that those bands can also be seen when using same method to label the protein from a shifted-band which was generated by using the Mono Q pool as protein source. It must be emphasized that in the Mono Q pool, BC1 RNA is 200 times more concentrated than brain extract and there are probably no or few free BC1 RNA binding proteins. One weak shifted bands can be detected by adding small amount of RNase mixture. The protein components generating such shifted band is shown in **figure 5a**. The top bands composite with two strong bands and two weak bands among the range of 92 to 88 kDa range. The second band is in the 54.3 kDa range which are very close to the one of core proteins (56.4 kDa) of BC1 RNP. Another one is 19.4 kDa which may also be an RNA band only. There are some weak bands corresponding to the bands of the hypothetical core BC1 RNP and some others don't. It is a little unexpected that the protein in Mono Q pool can generate such a complex pattern. Since small amount of RNase was applied in this assay, it may suggest that the complicated radioactivity protein bands may cause by the lower stringency. On the other hand, post-translational modification of proteins may also contribute some degree of variation. However, when increasing the RNase amount after cross-linking reaction, all protein bands disappear. Considering there is less RNA and less protein in Mono Q pool which may not influence the RNase function like crude extract, it is not surprising that all labeled RNA was degrade by RNase. It

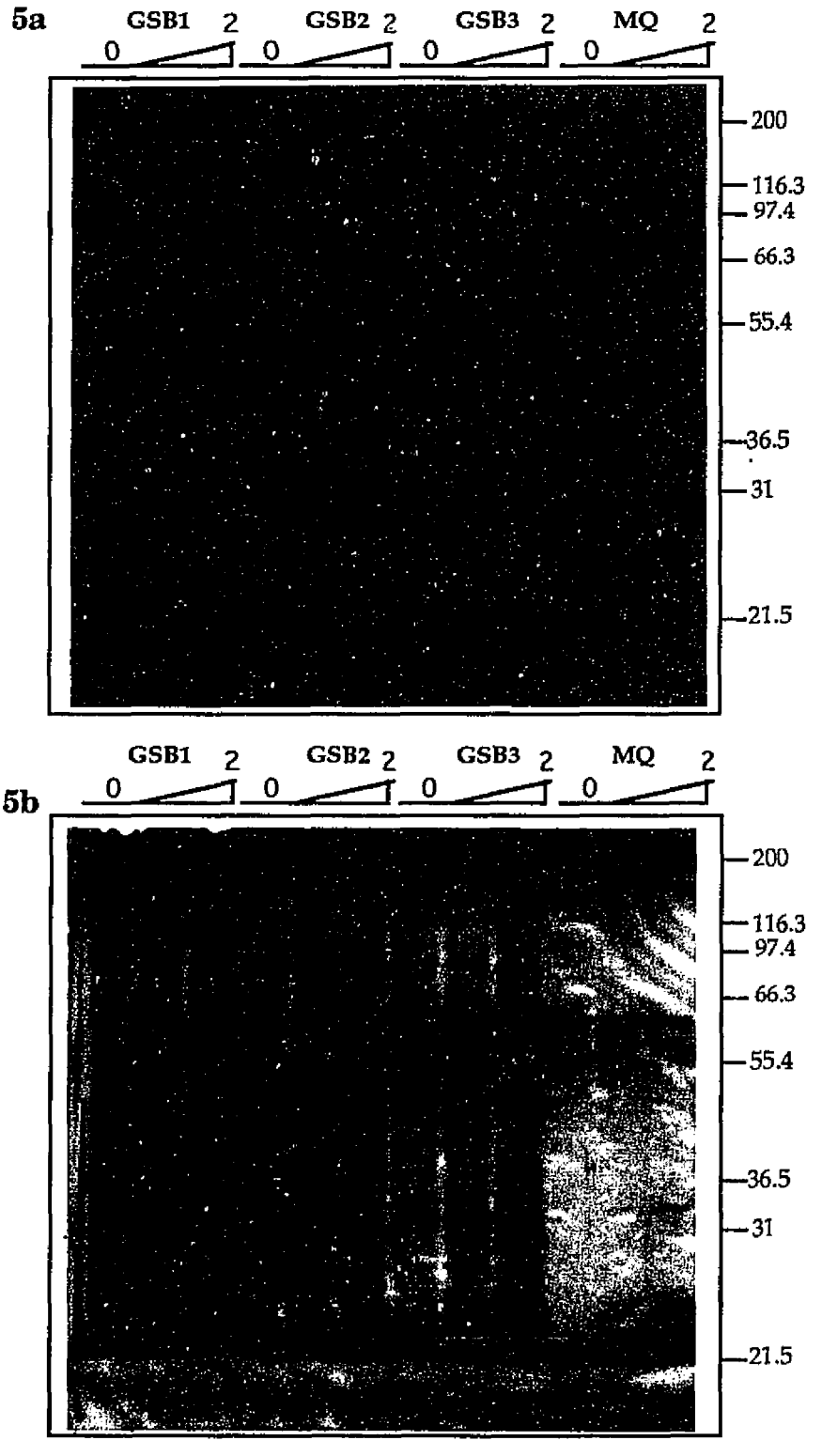


Figure 5. Nucleotide transfer experiment using brain extract (GSB. Gel shifted bands, 1-3) and Mono Q pool (MQ). (5a). SDS-PAGE autoradiography. (5b). SDS-PAGE with Silver Staining.

may need a titration to narrow down the best concentration of RNase to maintain both specificity and signal. This result also suggests that radiolabeled U is not directly linked to the protein, otherwise it can resist the digestion of RNase.

Affinity purification of BC1 RNA binding factors

Based on the results that BC1 RNA can form an RNP complex *in vitro* , it is possible to up-scale and purify those RNA binding proteins through their RNA binding ability. In fact, all previous *in vitro* characterizations are preparation to purify those RNA binding proteins. One can assay the RNA binding ability to track and quantitate the purification of RNA binding protein. After established the purification scheme, I have some interesting finding about the BC1 RNA binding factors. Although the purification result is not very good, it can facilitate the progressing of further purification of BC1 RNA binding proteins.

This kind of affinity purification method, usually requires pre-cleaning steps to reduce non-specific interaction. Using the protocol for the purification of iron-response element binding protein [148], the purification procedures were set up as follows.

Superdex 200 column chromotography

A gel filtration column was used as first step to separate the BC1 RNA binding factors from rat brain extract. The column profile is same as profile of BC1 RNP purification but using RNase protection gel-shift assay to track BC1 RNA binding activity (**figure 6**). It suggests there are at least three peaks of BC1 RNA binding activity in the fractions of the Superdex 200 run. The first peak co-elute with BC1 RNP, the second peak has a late elution time suggesting a smaller molecule size, and the third peak is only represent one single fraction with high activity. Since the peak 1 contains a greater percentage of endogenous BC1 RNA, it is difficult to judge the total BC1 RNA binding activity in this pool.

There are 4 to 5 shifted bands can be seen in the gel. There are one fraction with strongest signal of all 5 bands in the peak 1 which also corresponds to the peak signal of BC1 RNP. It suggests that the formation of shifted band is a equilibrium between endogenous RNA and radiolabeled RNA. Peak 3 behaves totally different. If we rule out this is an artifact, the possibility that 5 bands are all strong in a single fraction can happen only under the following conditions in gel filtration column. First, those 5 bands are generated from a different length of RNA with one or a few proteins which have a close size range. Second, the

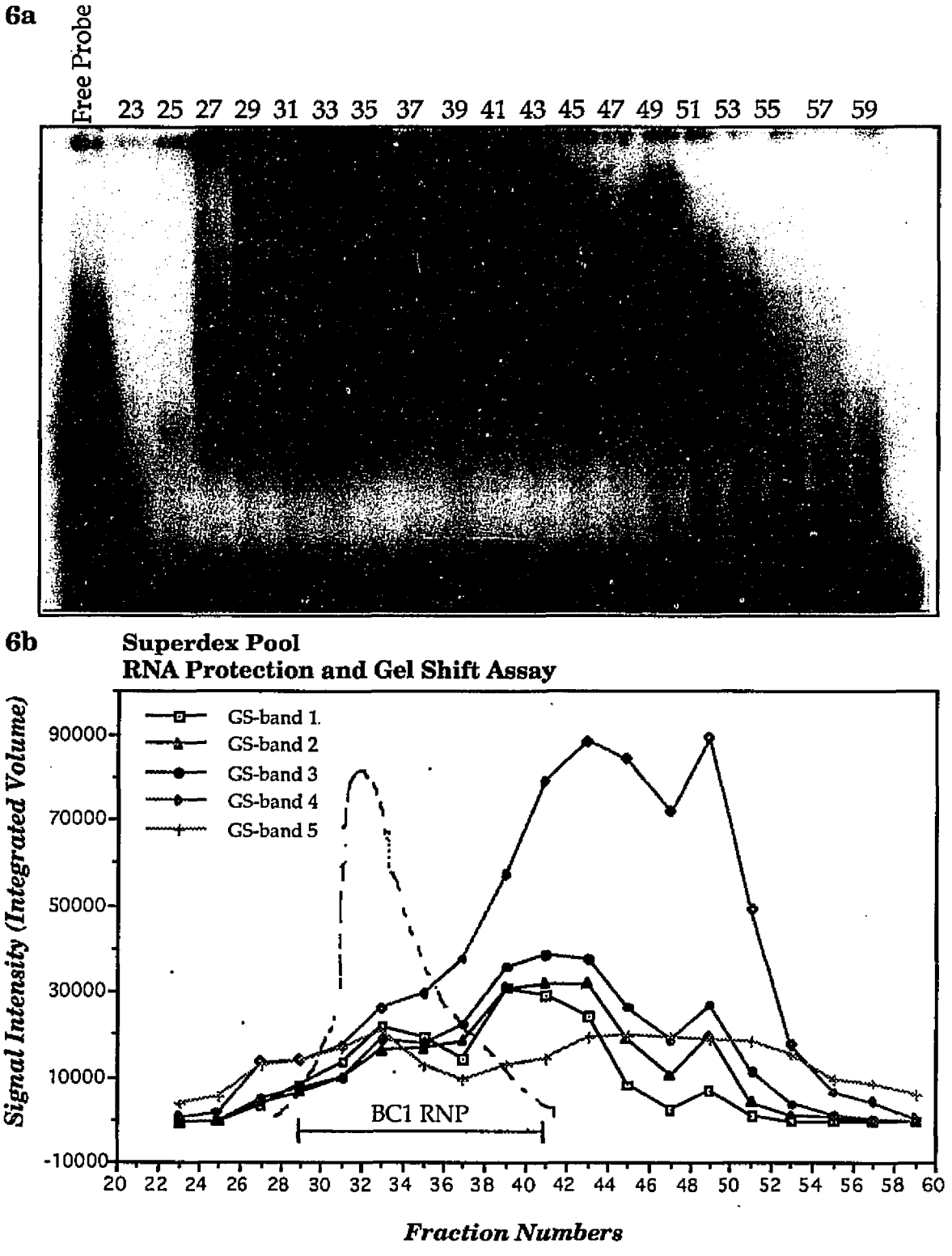


Figure 6. The BC1 RNA binding activity with Superdex 200 column chromatography. (6a) Autoradiography of RNase protection gel-shift assay from different fractions. (6b) Intensity of individual bands in different fractions. (----) indicative of BC1 RNP distribution.

formation of BC1 RNP complex is limited by one or few proteins. Such limiting factor(s) may just be an accessory factor in which facilitates the formation of BC1 RNP but not the component of BC1 RNA binding factors. Third, those binding factors form a complex before forming the RNP. Under the assumption that all BC1 RNA binding factors are same in different peaks, one may examine those possibilities from the distribution of gel-shifted signal in peak 2. There are different signal peaks for different shifted bands. Band 1 is in the fraction 39, band 2 and 3 are in the fraction 41, and band 4 are in the fraction 43, which suggest there are at least 3 different size of factors play a role in the formation of RNP complex. It indicates that a single protein BC1 RNP complex may not be true.

For the following steps, the Superdex pool is divided into two parts. Peak 1 contains the fraction of 29 - 40 and peak 2 contains fraction of 41- 53. Each pool has 90 ml of solution to start with. Before going to the next step, both pools have been tested for the best binding conditions. One idea is to test whether RNase can release the RNA binding proteins from endogenous RNA. Another is to test if the reducing agent can increase the binding activity as in the case of iron-response-element binding proteins [132]. Both results were negative. The result of RNase treatment is a surprise. The control group with water added instead of RNase into the reaction but handling in the same manner has the higher binding activity, suggests the decline of binding activity after RNase treatment is not caused by the incubation at 37°C for 30 min. Either RNase A/T1 mixture can not disrupt the protein/RNA association very efficiently or they can influence subsequential binding reaction. The influence of [Ca^{++}] in this binding reaction also has been shown. It seems that it will alter the Kd of RNP formation (data not shown). Since no other treatments can alter BC1 RNP and release its binding proteins easily without the cost of protein denaturation (chapter 2), the two Superdex pools were loaded into following column without any treatment.

Heparin column chromotography

The elution profile of BC1 RNA binding factors activity using step salt gradient is shown in the **figure 7a**. The most activity was eluted by 0.5 - 1 M KCl salt. After adjusted by elution volume and the volume for activity assay, the total activity in different fractions is shown in the **figure 7b**. Although there is some activity which can't bind to the heparin matrix and some of them were eluted with 250 mM KCl, the majority of binding activity (95%) is associated with the

two high salt fractions. The only surprise is the high affinity between BC1 RNA binding factors to the heparin. In the case of iron-response element binding protein, 0.2 M KCl will release this protein from heparin column [148]. In aspects of purification, this high salt condition may have very good purifying effects, as other RNA binding proteins have been eliminated and will not contaminate the later steps. On the other hand, high salt solution need to be reduced before further purification or analysis, which increases the time needs and the risk of protein denaturation. An interesting question is why the BC1 binding factors have such high affinity to heparin.

7a **7b**

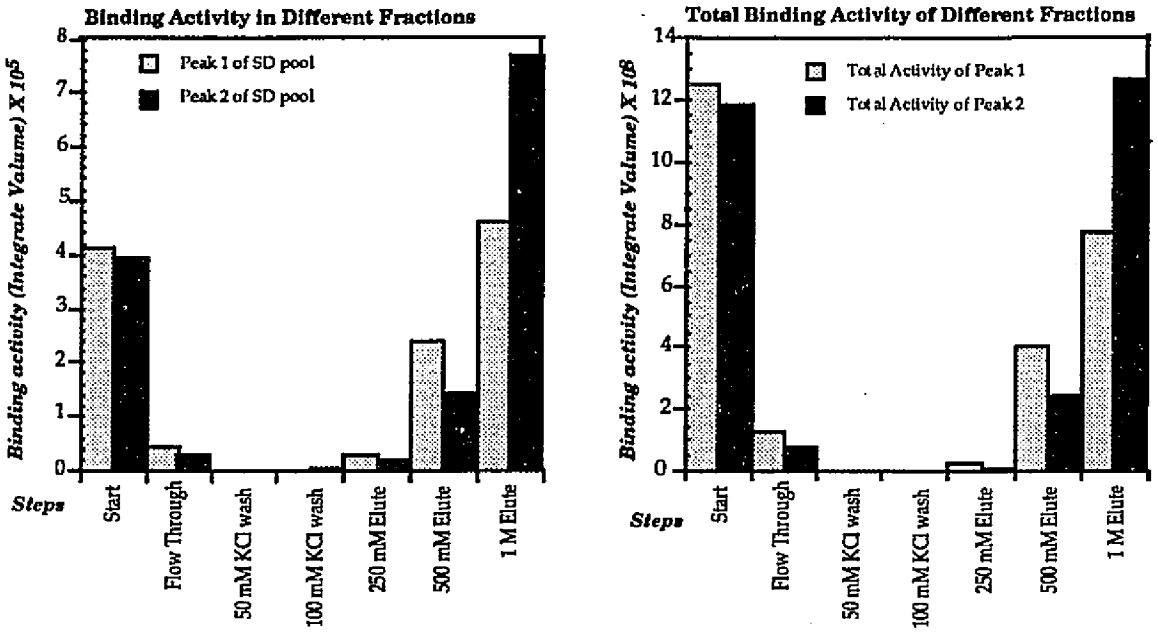


Figure 7. BC1 RNA binding activity of different Heparin column fractions. (7a) Relative binding activity. (7b) Total binding activity. (SD = Superdex 200).

Heparin matrix has been used to purify coagulation proteins, some plasma proteins, lipoproteins, steroid-type receptors and proteins or enzymes that act on nucleic acids such as RNA polymerase or restriction endonuclease [153]. Some of them are binding factors to heparin or heparin sulfate *in vivo*, and some of them have a high positive charge to interact with nucleic acid. The latter is the major reason that one can use the heparin column to clean up the protein source before loading into a DNA or RNA affinity column. Despite the fact that BC1 RNA is in the cytosol and heparin or heparin sulfate is in the extracellular matrix, there is high affinity between heparin and BC1 RNA binding proteins.

BC1 RNA binding proteins are probably not the natural receptors for heparin, so the only possibility is the BC1 binding proteins contain highly positive charge on the surface or the recognition site for sugar component (D-glucuronic acid, N-acetyl-D-glucosamine, D-galactose and D-xylose). The next step will involve a heparin washing step, which will elucidate the relative affinity of BC1 RNA binding factors to BC1 RNA and heparin.

BC1 RNA column chromotography

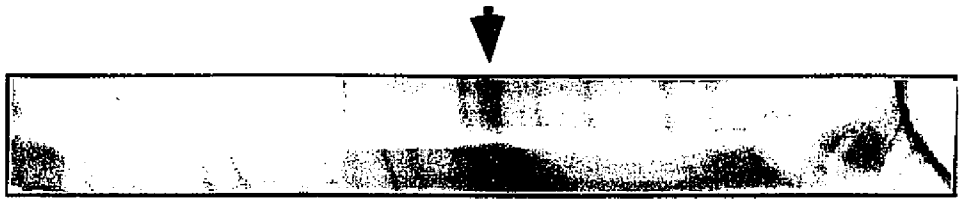
Taking advantage of the fact that BC1 RNA contains a poly A stretch, we can anneal the BC1 RNA to poly (U) sepharose beads. I have tested the matrix of oligo dT cellulose and poly(U) agarose for affinity purification. The capacity of oligo dT cellulose column is better than that of the poly(U) sepharose column. Theoretically, this is an unlikely result, as the polynucleotide chain of poly U (300 nt.) are longer than dT (12-18 nt.) column and the affinity between poly A to poly U is higher than poly A to oligo dT. Once the oligo dC cellulose was used to check the specificity of oligo dT cellulose, it is suggested that the cellulose itself plus oligo dC also can retain some BC1 RNA. Because of spatial hindrance considerations of spatial hindrance as well as specificity, the poly (U) agarose was chosen for setting up a BC1 RNA column. A small amount (10^6 cpm) radiolabeled BC1 RNA was loaded together with 200 μg of BC1 RNA. After monitoring the radioactivity from different fractions, there are only 40% to 50% of BC1 RNA associated with this column. After subtracting the possible degraded radiolabeled probe, there may be 100 - 120 μg BC1 RNA on this column.

After desalting and concentrating, the peak 1 and peak 2 activities after the heparin column were loaded onto BC1 RNA column. To avoid the non-specific binding and keep RNA intact, heparin, tRNA, rRNA and RNasin were included in this loading solution. Following the loading, the column was washed with heparin in low salt buffer, and then eluted the bound material with salt step gradient up to 2 M KCl. Once different fractions were assayed, none of them contain the BC1 RNA binding activity. All efforts such as changing the gel condition, omitting the RNase treatment after the binding assay (gel shift assay without RNase) and increasing the protein concentration , the results are still the same. What is the fate of the binding activity? Is it lost during desalting and concentration? Are heparin, tRNA and rRNA too strong to compete out all the BC1 RNA binding activity? Except for rRNA, heparin and tRNA have been

included in the RNase protection gel shift assay without any effect in those shifted bands which are generated from BC1 RNA with brain extract. The flow through fractions during concentration was checked, and the results were still the same.

One possibility is that the binding activity may still be associated with the column and can't be eluted with 2 M salt. This was checked by running an aliquot of agarose beads of the BC1 RNA column directly into SDS-PAGE. The results are shown in the **figure 8a**, suggesting that after competing with heparin,

8a



other RNAs as well as washing with high salt, there are some proteins still associated with the column material. Do those proteins belong to BC1 RNA binding factors or do they bind to RNA or agarose beads nonspecifically? Since BC1 RNP can remain intact in the 4M concentration of cesium salt solution, the high salt may be not efficient enough to elute the proteins out. It is known that Guanidine HCl and some other reagent can achieve it by denaturing proteins. In further experiments such proteins could be renatured and tested for their ability to bind specifically to BC1 RNA.

In order to identify those proteins, a new method using the property of RNase specificity and UV cross-linking was performed. Since RNase T1 cleaves RNA after G residue and RNase A acts on pyrimidine (U and C), one can use such specificity to distinguish BC1 RNA from poly (U) RNA. First, RNase treatment can release proteins with BC1 RNA (both RNase T1 and RNase A) or poly (U) (RNase A but not RNase T1) fragments. Second, UV cross-linking can tag the proteins with the fragment of RNA they are binding to. Third, enzyme labeling can light up those proteins with RNA motif. These result was shown in **figure 8b.** To avoid the contamination of other RNAs and their binding proteins, only the distinct strong bands and the band unique to RNase T1 or shared with RNase A treatment are counted, as BC1 RNA and poly(U) are the major RNA species within the column. Two different enzymes were used to label the RNA fragments, polynucleotide kinase and RNA ligase [141]. It needs to be noted that each enzyme acts on different ends of RNA and may compensate the variation of substrate preference.

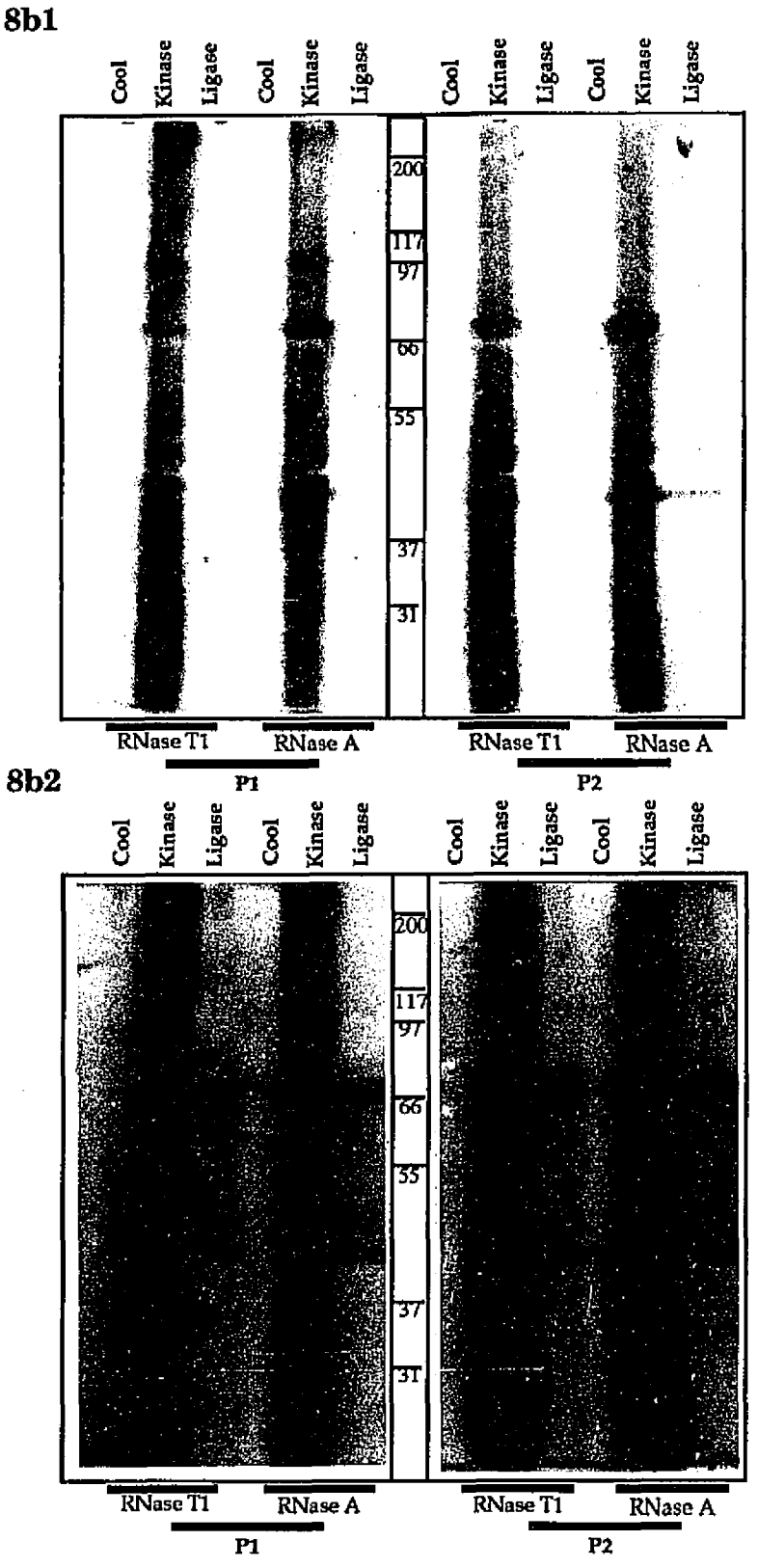


Figure 8. Identification of BC1 RNA binding factors within BC1 RNA column. (8a) Bound material in column matrix. (8b) RNA tagged proteins with kinase or ligase. (b1) Short time (overnight). (b2) Long time (3 days) exposure.

Besides one major band, the pattern of protein bands are similar between the peak 1 and peak 2 pool. This unique band in peak 1 corresponding to the 100kDa protein is very close to the bands (92 - 88 kDa) which were identified in nucleotide transfer experiment using the Mono Q pool as protein source. This band is also seen to be a little different in migration distance between RNase T1 group and RNase A group. It may be caused by the length of tagged RNA. Below this band, there is another weak band (95.3 kDa) also unique in the peak 1 pool. It has a similar behavior to the above stronger band which also runs faster in the RNase A treated group. Based on its behavior and the signal ratio between strong and weak bands, suggesting that both protein bands are caused by the same protein factor with different length of RNA attached. Other common major bands are 82.2 and 44.4 kDa bands and three small proteins ranging from 27 kDa, 24.6 kDa to 22.5 kDa. RNA ligase can only light up 82.2 and 44.4 kDa bands and an extra band with M.W. 48.2 kDa. It is a problem that compares M.W. of those proteins from this study with the result of nucleotide transfer experiment, as the RNase applied is different in two experiments. The protein bands of 82.2 and 44.4 kDa are not like typical proteins bands in the SDS-PAGE, they are wider. However, they present in both RNase treated groups with close migration distance, and this may rule out the possibility that those bands are generated by nucleic acid only. Thus needs to be studied further to established their origin.

In summary, there are proteins tightly associated with the BC1 RNA / poly(U) column, which may belong to BC1 RNA binding factors. It suggests that BC1 RNA binding factors have high affinity to their targets and have extraordinary heparin association ability.

Chapter V

Identification and characterization of BC200 RNP particles

Introduction

BC200 RNA is a brain-specific small cytoplasmic RNA, which was first identified in the monkey brain. It was first cloned in the human by screening a specially tailed RNA brain cDNA library with probes complementary to the unique region of BC1 RNA [2]. However, it is clear that the BC200 gene does not share the same evolutionary origin with the BC1 gene as shown by sequence discrepancy of the repetitive domain between these two genes. The BC200 gene is well conserved among several primate species, including human, apes and monkeys [Skryabin & Jurgen, in preparation]. Interestingly, like the BC1 RNA of the rodent, BC200 RNA can also serve as an evolutionary marker for the identification of the onset of primate divergence [15].

Despite the fact that these two BC RNAs have arisen from different origins, BC200 RNA shares several features similar to the BC1 RNA. First, like the BC1 RNA, BC200 RNA can also be divided into 3 domains: the repetitive element, an A-rich region, and a unique region. Unlike the BC1 RNA, the repetitive element of BC200 RNA is derived from a Alu element. Along with no detectable sequence similarities in the flanking region, this is the major evidence suggesting the different origins of the BC1 and BC200 RNA. Second, BC200 RNA shares similar tissue-specific distribution with the BC1 RNA; it is expressed exclusively in neuronal tissue except the ovary and testes. Third, and the most interesting feature BC200 RNA shares with BC1 RNA is its dendritic localization. Since BC200 RNA shares so many features with BC1 RNA, it is possible that BC200 RNA may play similar roles in the brain of the primates as does BC1 RNA in the rodent. Therefore, it is reasonable to treat the BC200 RNA as an analogue rather than a homologue of the BC1 RNA [2].

All Alu containing transcripts such as SRP RNA, BC200 RNA, and B1 transcript share a similar secondary structure as well as conserved sequences which can interact with the 9/14 kDa proteins from the SRP (**figure 1**) [76]. The function of Alu element containing transcripts is not well-defined, although it has been proposed that it may be responsible for translational arrest as well as the nuclear-cytoplasmic translocation of these Alu containing transcripts. Since the Alu element portion of BC200 RNA can also bind to the 9/14 kDa proteins suggests that the Alu domain of the BC200 may share similar functions as in the SRP RNA [D. Zorf, unpublished data]. However, a recently study has shown that Alu binding proteins, at least in their sizes, may vary depending on the

developmental stages or tissues origins [K. Strub, unpublished result]. Alu-domain binding proteins other than 9/14 kDa proteins have been identified with *in vitro* studies [154]. The A-rich and unique region of BC200 share about 70% sequence similarity to BC1 RNA. This may explain why BC200 could be cloned by using the probes containing BC1 RNA 3' antisense sequences. Whether this sequence homology contributes to the functional similarity between BC1 and BC200 needs to be elucidated.

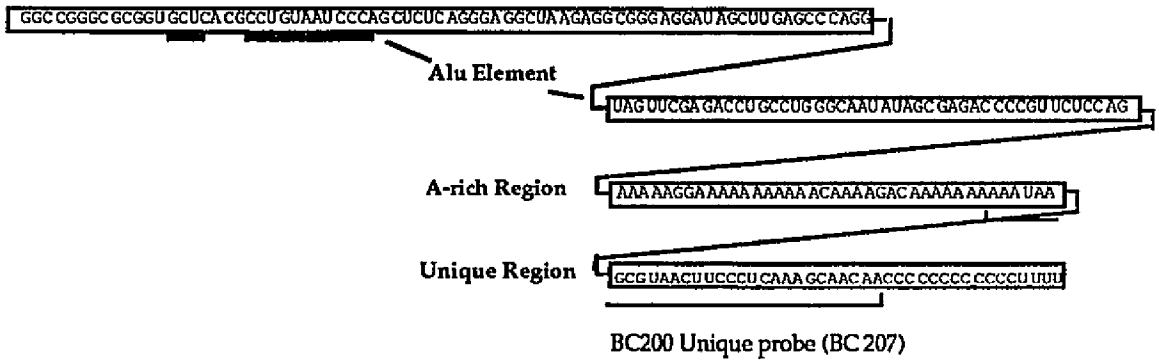


Figure 1. Contiguous sequence of BC200 RNA and its domains. Conserved sequence of box 1 and box2 for binding to 9/14 kDa protein dimer is indicated by underlining. The position of the probe (BC 207) complementary to BC200 RNA is indicated.

In this chapter, several lines of evidence are provided to suggest that BC200 RNA exists in the brain extract as an RNP particle. I have also shown that there are some specific factors in the human brain that interact with BC200 RNA. However, unlike BC1 RNP, nearly 30% of BC200 RNP is associated with other large macromolecules to form an even larger complex. Since the BC200 RNA contains a Alu domain, it is quite possible that it may also associate with ribosomes and thus be involved in the translational regulation.

Results and Discussion

Dendritic location of BC200 RNA in the Brain of rhesus monkey

Our laboratory began to use the monkey system as a model system to study the expression of BC200 RNA during the developmental period as well as other experimental manipulation. After the monkey BC200 RNA gene had been

cloned and its unique region was subcloned in a vector that generates both sense and antisense RNA respectively, I has studied the expression of BC200 RNA in the hippocampus as well as other cortical regions in the monkey.

Distribution of BC200 RNA in monkey brain

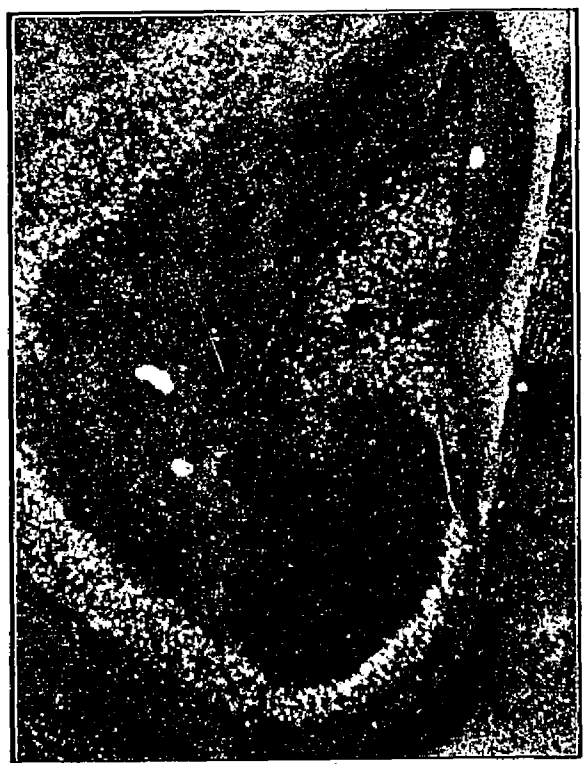
In situ hybridization was used to elucidate the distribution of BC200 RNA in monkey brain sections, 7SL RNA was used as control. Monkey brain slices (*Mecaca sp.*) were kindly provided by Dr. J. Morrison. In a laminated brain region such as hippocampus, the signal of BC1 RNA as well as that of 7SL RNA have very distinguishable patterns (**figure 2a-d**). The 7SL signal was very strong in the hilus field with a continuous belt through all CA (Cornus Ammonis) regions. This signal seems to be restricted within the pyramidal cell layer. This is consistent with the higher expression and somatic location of 7SL RNA in the pyramidal cells. Compared to the 7SL RNA signal, the signal of BC200 RNA seems to mark the margin of 7SL RNA signal region. In the dentate gyrus, 7SL RNA is expressed in the granular cell and polymorphic layer (hilus), however, the intense signal of BC200 RNA is in the proximal portion of granular cell dendrited within the molecular layer. This region, the dendritic field of granular cells in the dentate gyrus, receives the axonal innervation from the perforant path. In the CA field, BC200 RNA signal is divided into two bands along the pyramidal cell layer, where the 7SL RNA has stronger signal. Those two bands are along the fields of basal dendrites (stratum oriens) and the field of apical dendrites (stratum radiatum).

The restriction of an intense signal of BC200 RNA in the dendritic fields within different regions suggests that BC200, like BC1 RNA is also a dendritic RNA. Compared to the human *in situ* hybridization result, the signal ratio between soma to their dendritic field are different in human and monkey. In the monkey study, the signal of dendritic field is stronger than the signal in soma; in human, it is about equal [2]. However, unlike the 7SL RNA which shows 4 strong bands along the gray matter of the neocortex, BC200 RNA is expressed intensely in the surface layer and deep layer (layer 5). Another interesting observation is that there are slight individual variations in expression patterns among all studied monkeys. Further investigation needs to be conducted to examine the layered expression pattern of BC200 RNA.

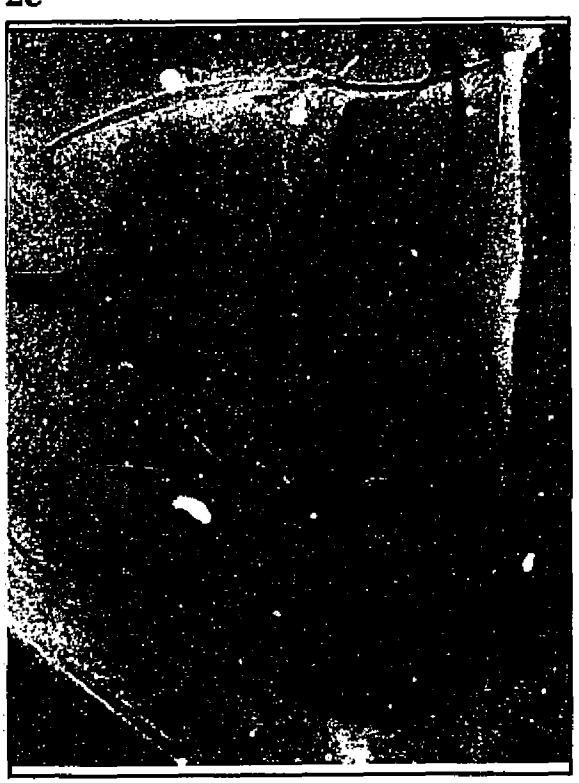
Figure 2. In situ hybridization of monkey hippocampus. (2a) BC200 RNA. (2b) 7SL RNA. (2c) BC200 sense control. (2d) Diagram of hippocampal formation. (sub=subiculum, EC=entorhinal cortex, pp=perforant path, DML=dentate gyrus molecular layer,GLC=granular layer cell, H=hilus, MF=mossy fiber, HF=hippocampal fissure, SL=stratum lucidum, F/F= fimbria/fornix, LV=lateral ventricle.)

2a

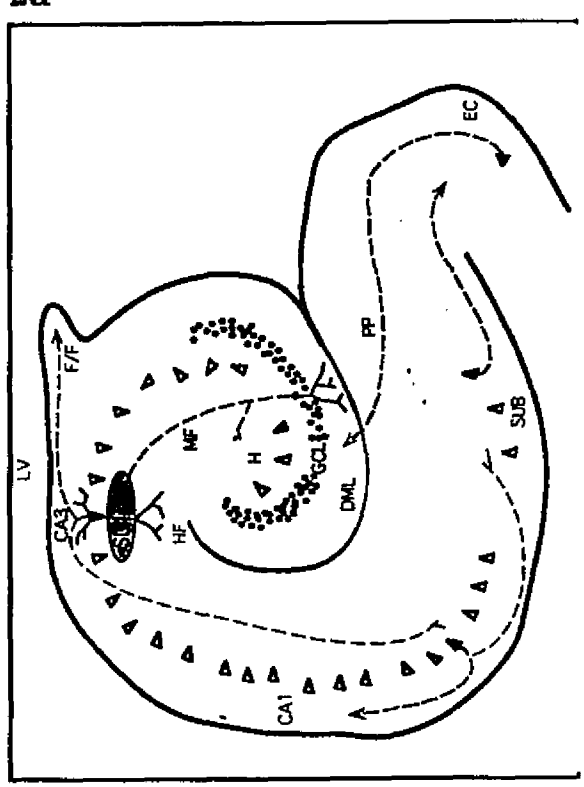
2b



2c



2d



BC200 is present as an RNP in the human brain extract.

The BC200 RNA signal has been examined from several primate brain sources to find a good source for further characterization. Due to the fixation method and tissue freshness, several monkey and human brain samples lack any BC200 RNA signal. The degradation of BC200 RNA in postmortem tissue and the non-frozen fixation method which cross-links the BC200 RNA to tissues, make those tissues poor sources for BC200 RNA for further studies using biochemical approaches. This sets limitation on performing the experiments such as the electrophoresis. However, with the limitation of brain sources, I have been able to show that BC200 RNA exists in the human brain as an RNP complex by using standard cesium salt and sucrose gradient centrifugation.

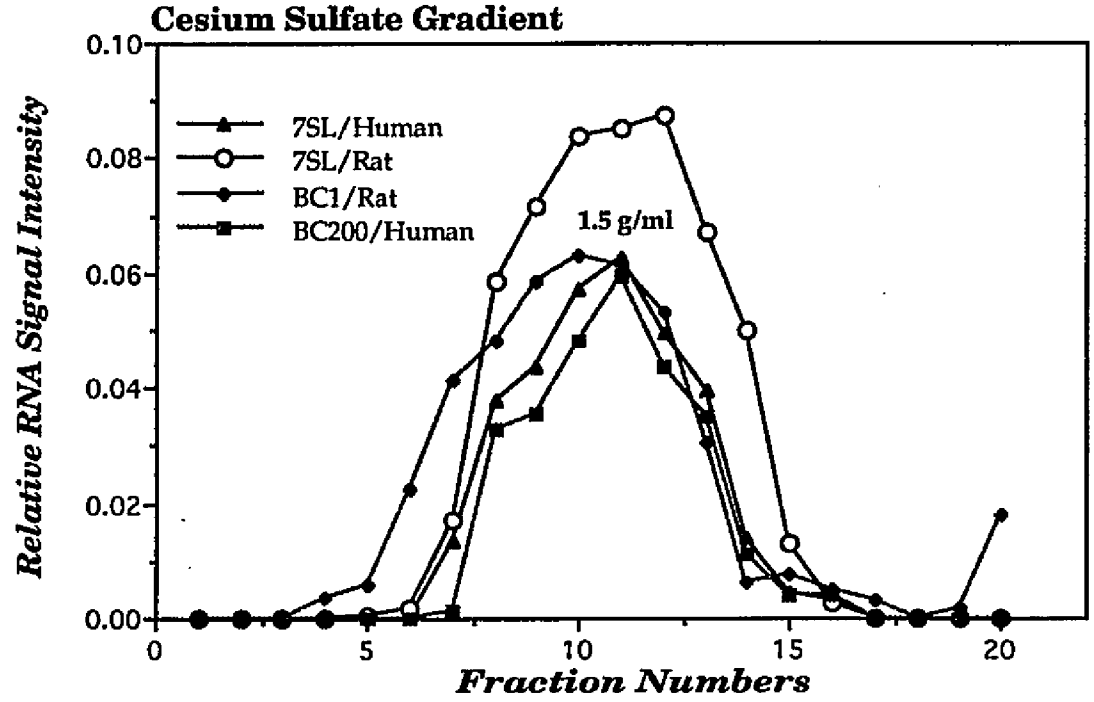
Cesium Salt Gradient

Following the protocol which has been applied to study the BC1 RNP complex, human brain extract was run into the cesium salt gradient. The endogenous SRP was used as a reference and rat brain extract (BC1 RNP and rat SRP) was run in parallel with human brain sample. The signal of BC200 was identified by RNA dot blot analysis (**figure 3a, 3b**)

Cesium sulfate gradient

The signal distribution of BC200 RNA within different fractions is consistent with the range of other known RNPs in Cs_2SO_4 gradient. Not only the distribution of RNA signal but also the peak of RNA signal are identical both in 7SL/human and BC200 RNA. This is the first indication that BC200 RNA survives in the Cs_2SO_4 solution and may form an RNP. The estimated density in the peak of RNA signal, based on the reference density of BC1 RNP and SRP/rat, is 1.5 g/ml. The signal of BC1 RNP shows lower buoyant density than the other three RNPs. It is in consistent with previous studies. Interestingly, the buoyant density of SRP/rat (1.55 g/ml) is larger than the same particle in the human brain (1.5 g/ml) in this particular experiment. This is probably due to experimental variation caused by the fractionation. After checking the result of 7SL RNA signal from six samples, it is possible that the variation within one or two fractions is common in-between two parallel runs. For this reason, the results are more reliable when compared in a same tube or a general signal distribution. However, one can not rule out the possibility that there are variations in the protein factors. The first reports of 7SL RNA binding proteins came from the

3a



3b

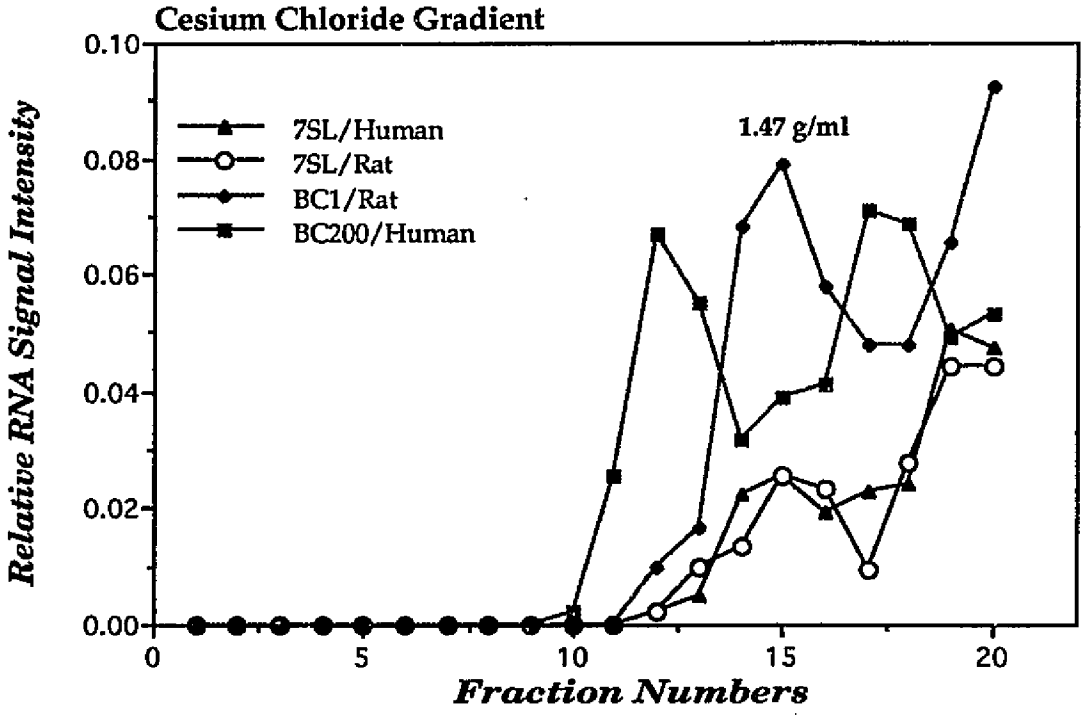


Figure 3. BC200 RNA in the Cs_2SO_4 gradient (3a) and CsCl gradient (3b).

study of the canine pancreas. Little attention has been put into small changes or modifications of those RNA-binding proteins in different species or tissues. The functional demands of SRP in different tissues may be somehow different, as one will expect that regulation can take place in the 7SL RNA level as well as in the behavior of the RNA binding proteins.

Cesium chloride gradient

CsCl gradient is also performed to further characterize the BC200 RNP [107]. Like the BC1 RNP, the SRP in the rat and the SRP in human, the signal of BC200 forms two peaks in the density range as other RNPs. The second peak is presumably the "naked" RNA, as the RNA is usually precipitated in CsCl gradient. Since high salt condition will disrupt protein-protein interactions, the first peak is likely to be the core RNP which contains only the RNA and its binding proteins. The overall signal of BC200 and SRP/human are lower than the BC1 and SRP/rat. Because both the RNPs which comes from human brain extract have lower signal, it may simply reflect the lower level of RNP or intact RNA in this preparation. The signal distribution of BC200 RNP is very close to the human and rat SRP. A major difference among SRP/rat, SRP/human and BC200 RNP is the signal ratio of peak 1 and peak 2. This may suggest that not only the BC200 RNP and SRP/human are less abundant but also the binding activity of their binding proteins are weaker. Part of BC1 RNA signals is with a lighter density than previous experiments. This phenomena has also been observed in the RNase A or proteinase K treated brain extract. The best explanation is the brain extract which was loaded into this CsCl gradient run has some degree of BC1 RNA degradation. The BC200 RNP and SRP/human have the same buoyant density as SRP/rat in the CsCl gradient, which is equal to 1.47 g/ml. This suggests that BC200 RNP has 68.5% of proteins associated with the RNA. Since the BC200 RNA has the molecule weight of about 66 kDa, its binding proteins have the sum of molecular weight equal to 170 kDa. When the 9/14 kDa dimer which might be associated with BC200 RNA *in vivo* is subtracted, it leaves another 140 - 150 kDa proteins which are present in the BC200 RNP.

S value

Sucrose gradients were used to characterize the S value of BC200 RNP as well as the human SRP. Due to the problems with signal detection, some modifications have been made from the original protocol for studying the S value

of BC1 RNP. In particular the volume of extract loaded, and standard marker applied. There are some restrictions to the concentration of protein solution which can be applied to sucrose gradient to determinate the S value. Once the protein concentration gets too high, inter-particle association is likely to occur. However, twice as much of human brain extract has to be used to detect the BC200 RNA as compare the rat brain extract for BC1 RNA. Although it is 2/3 less than the amount which was applied to cesium salt gradient, the BC200 RNA signal in sucrose gradient is far less than the signal in cesium salt gradient. Since the sucrose gradient, unlike the cesium salt gradient which has the ability to inhibit RNase activity, it is believed that RNase may play some roles here. Because it is not know whether RNase inhibitors will change the property of the particle, nothing is done to the undiluted brain extract. For the same reason, the addition of standard marker also reduces the available signal. Thus the calculation of S Value can only be based on the standard maker from different tubes.

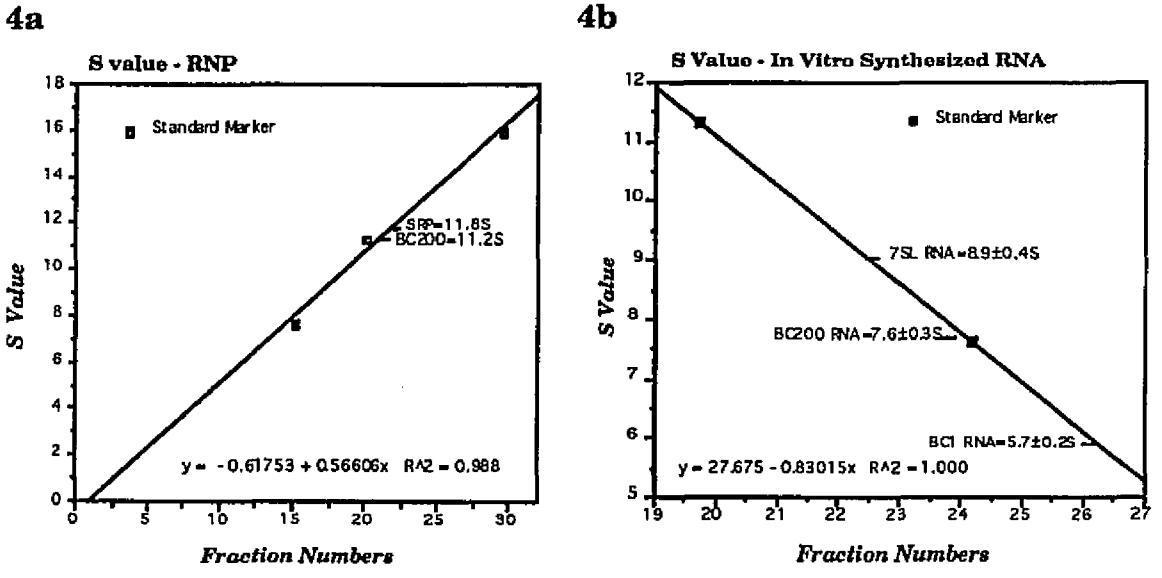
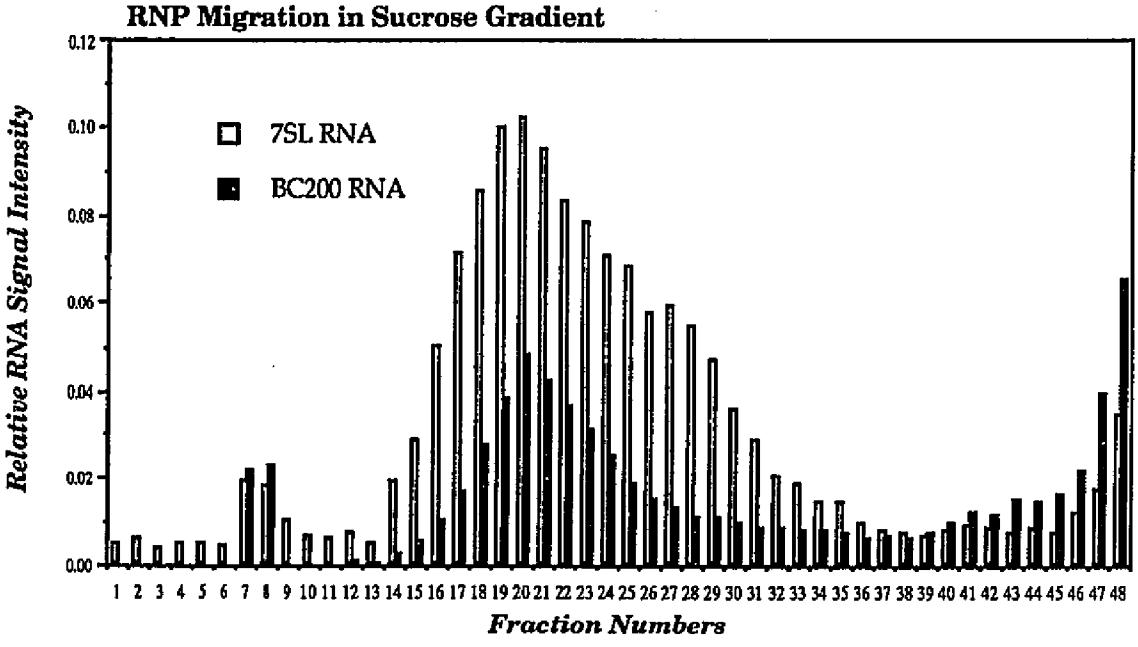


Figure 4. The signal of BC200 RNA in 5% - 20% sucrose gradients. (4a) S values of RNP. (4b) The S value of *in vitro* synthesized RNA. (4c) The signal distribution of BC200 and7SL RNA in the sucrose gradient.

It is not surprising that the signal of BC200 RNP, like SRP, has three peaks (figure 4c). The middle peak which contains most of BC200 RNA or SRP signal

reveals the expected S value. There are two lines of evidence which support this idea. First, after the corresponding S value in the middle peak is calculated, it shows that SRP has a S value 11.8S which is very close to the S value in original report as well as that of rat SRP. Second, the S value of the first peak is smaller than the S value of "naked" RNA synthesized *in vitro*, however, the bottom peak has S value higher than 20S. It is possible that the first peak is a degradation product. The bottom peak may have interesting features. It is well known that SRP has a low affinity to free ribosomes and once the signal peptide emerges from the ribosome, the SRP associates with ribosome with high affinity through the interactions between SRP p54 and the signal peptide [75]. Two regions in the 7SL RNA sequence may interact with ribosomal RNA. One region (97-110 nt.) which could interact with 18S rRNA is also conserved in the BC200 RNA [2]. It is possible that the high S value pellet may represent the association between ribosomes and BC200 RNP. Although more evidence is required to support this idea, this would support that BC200 RNP may play a role in translational regulation. It is also possible that BC200 RNP may associate with other macromolecules such as cytoskeletal components, as it can be transported into neuronal dendritic compartments.

4c



The calculated S value of BC200 RNP is 11.2 S which is very close to that of SRP (**figure 4a**). In fact, they are so close that it is very hard to find out any significant difference other than the experimental variation without using of normal distribution. Fortunately, these two particles were identified in a same tube with duplicate RNA blot. The total relative signal intensity of BC200 RNA is only half of the 7SL RNA has. However, BC200 RNA has twice more signal in the bottom portion than 7SL RNA does (**figure 4c**). This suggests that BC200 have higher affinity to large molecule than SRP does. The similarity of signal distribution between BC200 RNP and SRP is not only restricted in the sucrose gradient but is also found in cesium salt gradient. It has to be noted that BC200 RNA (200 nt.) is only 2/3 the length of 7SL RNA (300 nt.), and therefore 60% of BC200 RNA (Alu domain) only equals 40% of 7SL RNA (Alu domain.). It is not surprising that BC200 RNA shares some common property with 7SL RNA with the Alu domains. But another 40% of the BC200 RNA (80 nt.), which is unrelated to 7SL RNA, can bring the BC200 RNP to nearly the same S value and the same buoyant density as SRP in both CsCl and Cs_2SO_4 gradients. It will be very interesting to find out these underlying reasons. The S values of *in vitro* synthesized BC200 RNA, BC1 RNA and 7SL RNA were also studied (**figure 4b**). The S value of BC200 RNA (7.6 ±0.3S) and BC1 RNA (5.7±0.2S) have the same ratio to their length, suggesting that BC200 RNA has some similarity in shape with the BC1 RNA. On the contrary, 7SL RNA (8.9±0.4S) does not have this proportional relationship between S value and the length of RNA. All the S values of those RNAs that were calculated from exterior standard makers are larger than the usual S values. This may be due to the different methods used to determine the S values. It is possible that the S value is over-estimated. However, the S value of 7SL RNA is consistent with the S value which is acquired from the total rat brain RNA (8.4± 0.4S) in a parallel run. This makes the argument that over-estimation plays an important factor in here. However, the relative S values are still correct, as those three RNAs are run under the same condition and are from same tube.

Study the activity that binds to BC200 RNA

As the quantity and quality of samples are limited, *in vitro* analysis becomes the only possible method to characterize the BC200 RNA binding proteins and maybe the method for the purification of these RNA binding proteins. In the following study, those BC200 RNA binding proteins are

characterized by 2 different buffer systems. As expected, different buffers give different banding with a same RNA/extract reaction, this may provide more information for further identification of these RNA-binding proteins.

TBE buffer system

0.5X TBE buffer with 4% polyacrylamide gel (40 : 1 = acrylamide : bis-acrylamide) was used to study the characteristics of the BC1 RNA binding proteins. I have examined whether the same protocol of RNase protection gel shift assay, may also be applied to BC200 RNA as well as other RNA binding proteins as a general method. Using BC1 RNA as the reference, I studied the shifted bands which are generated by the association between BC200 RNA as well as 7SL RNA to their binding factors. In the same experiment, the tissue and species specificity have also been examined (**figure 5**). The shifted bands which were generated by adding BC1 RNA into rat brain extract reveals the same pattern as previous study (chapter 4). There are three to four bands from top to the middle of the gel. The shifted bands one (top) is always weaker with the UTP labeled BC1 RNA. The previous finding that when BC1 RNA is incubated with liver extract that good shifted bands can not be obtained using 4 units of RNase in the mixture is also reproduced here. The interesting feature is that the difference in the shifted bands pattern from rat brain extract and human brain extract. The BC1 RNA can also be shifted when the human brain extract is used, although two out of three bands have different migration distance. Both the first band and third band migrated faster than the corresponding bands from the rat brain extract, suggesting that there are some proteins that can interact with BC1 RNA in human brain extract. Until the availability of antibodies against BC1 or BC200 binding protein, one can not determine whether these are the same proteins. The migration pattern also suggests that there are different proteins associated with different BC1 RNA fragments within those shifted bands. It has to be noted that the second shifted bands share the same properties in both the migration distance and intensity using both rat and human brain extract. Although BC1 RNA is not expressed in the primate brain, its unique region shares about 70% similarity of identity to that of the BC200 RNA unique region and can be cross-hybridized to each other. It has been characterized a serum from a human patient with autoimmune disease. This serum can specifically bring down BC1 RNA (P) in a immunoprecipitation assay. Since this binding can be blocked by BC200 RNA, it suggests that this antiserum recognizes a common

epitope in BC200 and BC1 RNP. Based on these observations, it will not be surprising to find that indeed BC1 and BC200 RNA share some common binding factors that are conserved both in human and in the rat.

A reciprocal experiment using BC200 RNA incubated with rat brain and human brain extract was performed (**figure 5**). Although the major band-shift patterns are different between rat brain extract (3 bands) and human brain extract (2 bands), the first band from both extract still has very close migration distance. It is difficult to conclude from the available data without further experiments, as the resolution power of this gel system is limited. However, it is reasonable to make the assumption that there may be factors in the rat brain extract as well as human brain extract which can interact with BC200 RNA. The shifted-bands of 7SL RNA in the human brain and rat brain are same, suggesting that the 7SL binding factors in the human and rat brain are identical. Considering that there are 99% homology between the human and mouse 7SL RNA, this result is not surprising. This observation does not agree completely to the result of buoyant density from Cesium salt gradient. Although it is improper to compare the result from two different systems, this result of gel shift assay may support the experimental variation in the run of Cesium Sulfate gradient.

The band-shift patterns are different when 7SL RNA was incubated with the rat liver extract. There are 4 continuous bands formed in this extract, two of them are close to the two bands which are generated from both brain extracts and the other two are absent from both brain extracts. One possible explanation is that there is higher binding activity in the liver extract than brain extracts. Although the protein concentration in liver extract is 1.3 times higher than brain extracts, which can account for the increased strength in signal but can not account for the additional shifted bands. Another explanation is the SRP in the liver are somehow different from the SRP in the brain in the sense of binding factors or different modifications. The tissue specificity or developmental regulation of SRP conformation is also a very interesting field to be unveiled. The continuous 4-band pattern is also seen in the BC200 RNA with rat liver extract. Since BC200 RNA shares only homology in the Alu domain with 7SL RNA, one can speculate that these bands are generated from the Alu domain in both RNAs. It is well known that 9/14 kDa protein can interact with the Alu domain [77]. In footprinting study, these two proteins only bind in a discrete RNA portion of Alu domain [76]. It is known that the RNA will not be protected from RNase degradation other than the region covered by these two proteins and then will

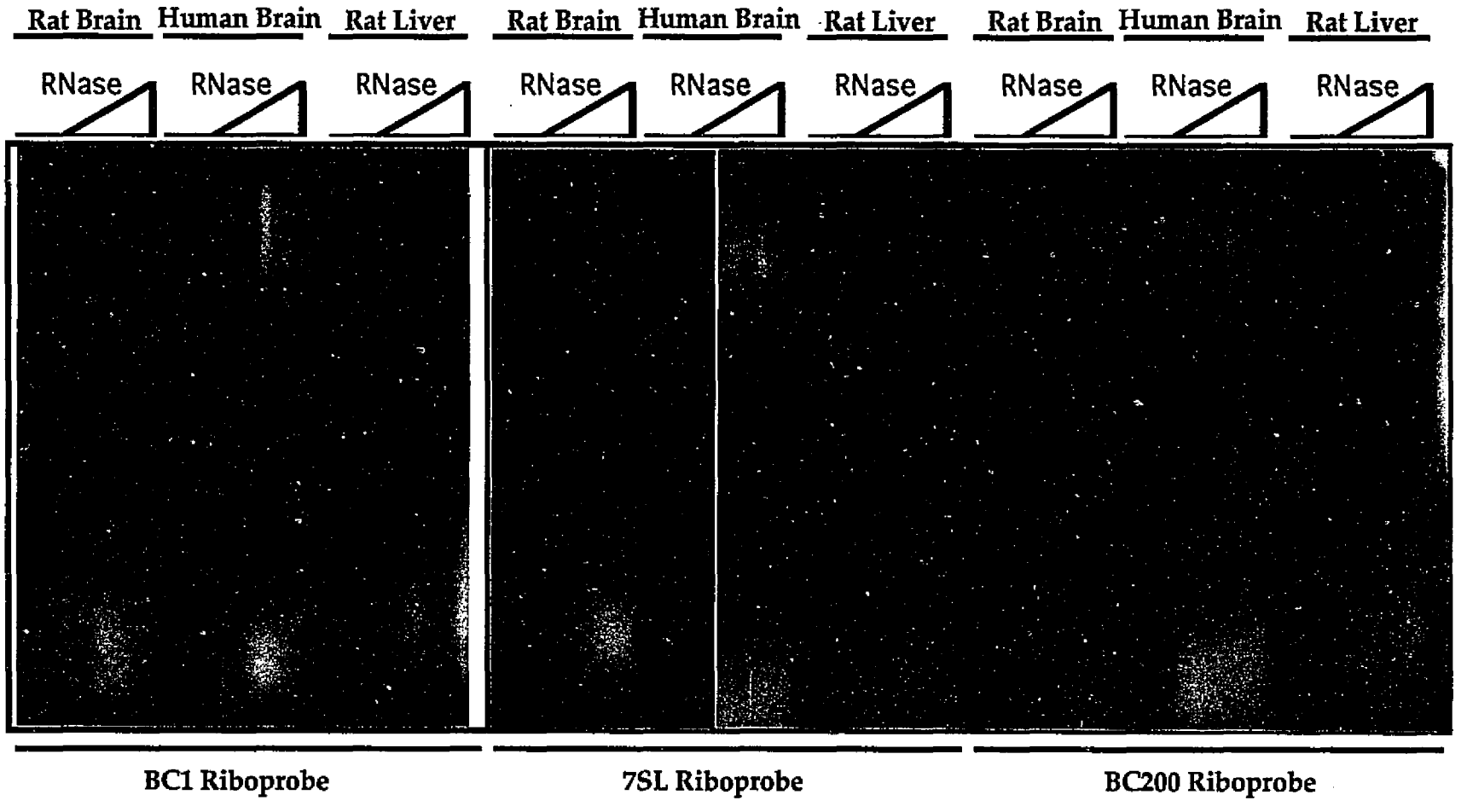


Figure 5. RNase protection gel-shift assay in TBE buffer system with different extracts and different probes

generate multiple shifted bands. One of the novel B1-Alu RNA binding proteins, which was identified in a human Hela cell and a murine erythroleukemia (MEL) cell lines, is also capable of binded Alu containing RNA in different regions. This similar band shift result between BC200 and 7SL RNA suggests that BC200 RNA has the potential to interact with Alu RNA binding protein *in vitro* as 7SL RNA. This is consistent with the result of filter binding assay using *in vitro* synthesized RNA and purified SRP 9/14 kDa proteins (Dr. Zorf unpublished result) It also implies that we may not know the complete picture of SRP which have been study for the past 11 years. BC200 RNA, although it is shorter than 7SL RNA, may also have a very complex structure. It is very important and challenging to understand the function of BC200 RNP in the neuron. These results also suggest that the RNase protection gel shift assay has a potential to provide a convenient tool for studying the RNP. In conjunction with other biochemical tools, one should be able to track, RNP in a more complex system.

Tris-glycine buffer system

To further study those band-shift patterns, the RNA protection gel-shift assay can be modified using another buffer system. Using the same preparation, those reaction mixtures are loaded in the same order but with Tris-Glycine buffer (**figure 6**). The Tris-glycine buffer has a different effect on the shifted bands. First, the band-shift patterns are different from TBE buffer. Second, the free probes co-migrate with the leading ions (loading dye), as the RNAs have high negative charges. Third, the background is lighter than that of TBE gel. Fourth, the signals of shifted bands are more defined but weaker than in the TBE gel. Without the addition of any RNase, the band-shift patterns can already be distinguished. These results also can also be obtained in the TBE gel system although the background is much higher. It is possible that endogenous RNase activity as well as radiolysis can cause the degradation of RNA probe. Another possibility is those bands which are generated from the full length probe associated with different protein components before adding RNase. However, when RNase is introduced into the reaction the signal in shifted bands and the trapped signal in the well are all decreased without altering the migration distance of those bands. This suggests that those gel-shift complexes are already in a solid conformation.. If we compare this gel with the TBE gel, it seems that RNase has more profound effect in the band-shift complex in Tris-glycine system than that of TBE buffer.

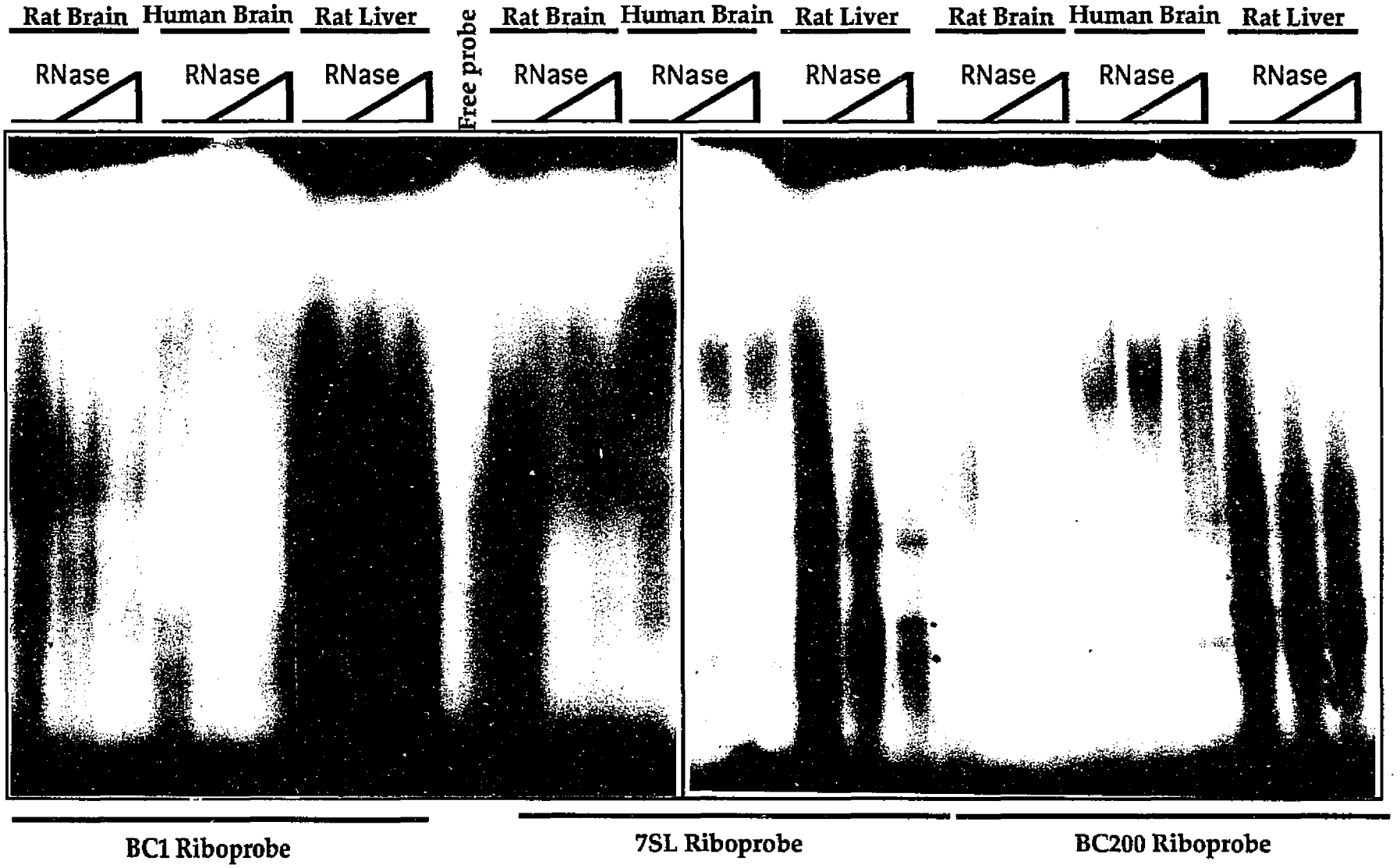


Figure 6. RNase protection gel-shift assay in Tris-Glycine buffer system with different extracts and different probes.

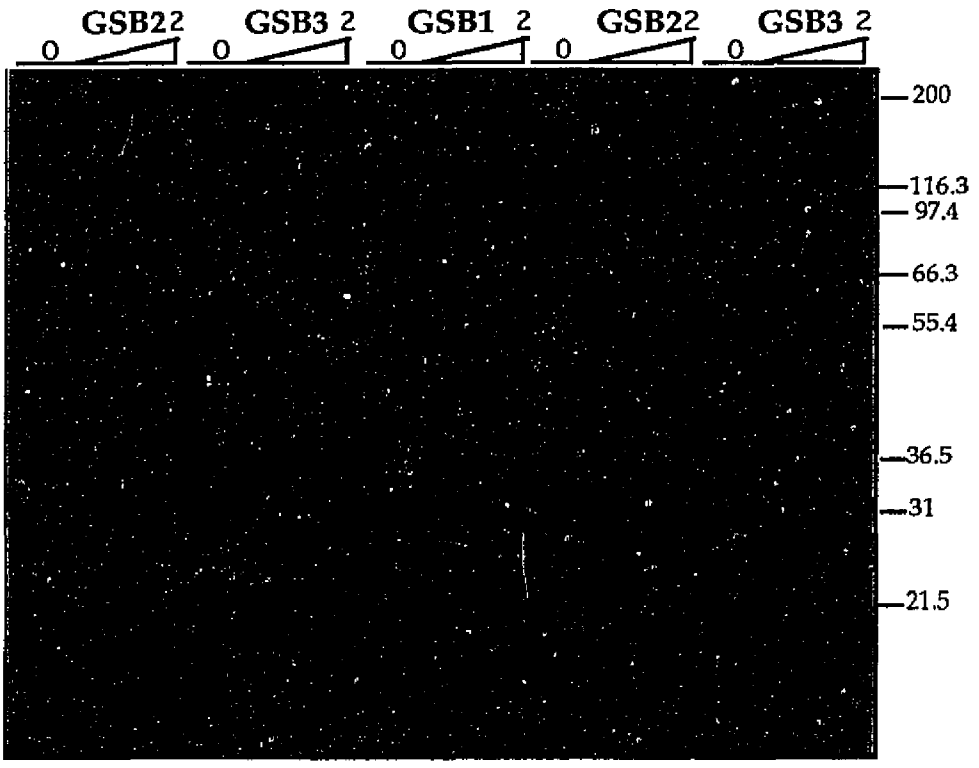
The band-shift pattern of BC1 RNA in the rat brain extract contains only one strong band followed with two weaker and faster migrating bands (**figure 6**). This pattern is different from the pattern with the human brain extract. This result does not correlate completely with the observation which made from that of TBE gel. It needs to be further studied to clarify this difference. The same phenomenon is observed when BC1 RNA is incubated with liver extract, that there is not any shifted bands but complete smears.

The result obtained from BC200 RNA experiment is somehow different (**figure 6**). There is one fast migrating band present using the rat brain, human brain and rat liver extracts. Another band is present only with the rat brain and liver extracts. The shifted bands from rat brain are less resistant to RNase; they still exist after treatment with 2 unit of RNase mixture. Other than one of three shifted bands which is shared with other extracts, BC200 RNA has other two bands which are unique in the human brain extract. However, when compared to the band-shift pattern of 7SL RNA in the same human extract, it seems that it is identical to the BC200 RNA pattern. This suggests that Alu domain may be responsible for generating these band-shifts. This phenomenon is also found when comparing the band-shift pattern of BC200 RNA and 7SL RNA using liver extract. Except one band in this gel is specific for BC200 RNA, all other three bands can find their counterpart in 7SL RNA group. Both results indicate that those identical shifted-bands are generated from the same RNA domains but with some tissue specificity. A very weak signal in 7SL RNA group was also found using rat brain extract. Either the rat brain extract has lost its 7SL RNA binding activity or this RNA-protein complex is very difficult to survive in this gel system.

Identification the binding factors to BC200 RNA

A nucleotide transfer experiment in conjunction with RNA protection gel shift assay are used to identify factors binding to the BC200 RNA [143, 152]. The TBE gel system is chosen because it gives stronger signals. In a parallel reaction, 7SL RNA also receives same treatment as the BC200 RNA using human brain extract. Silver-staining protein gel and its autoradiograph are shown in the **figure 7a, 7b**. The three strongest shifted bands (1 to 3) are chosen to perform nucleotide transfer experiment. Beside the shifted-band 3, other two shifted bands did not give any labeled proteins in the BC200 RNA group. As for the reaction with 7SL RNA, only shifted-band 3 did not reveal any protein bands by this method.

7a



7b

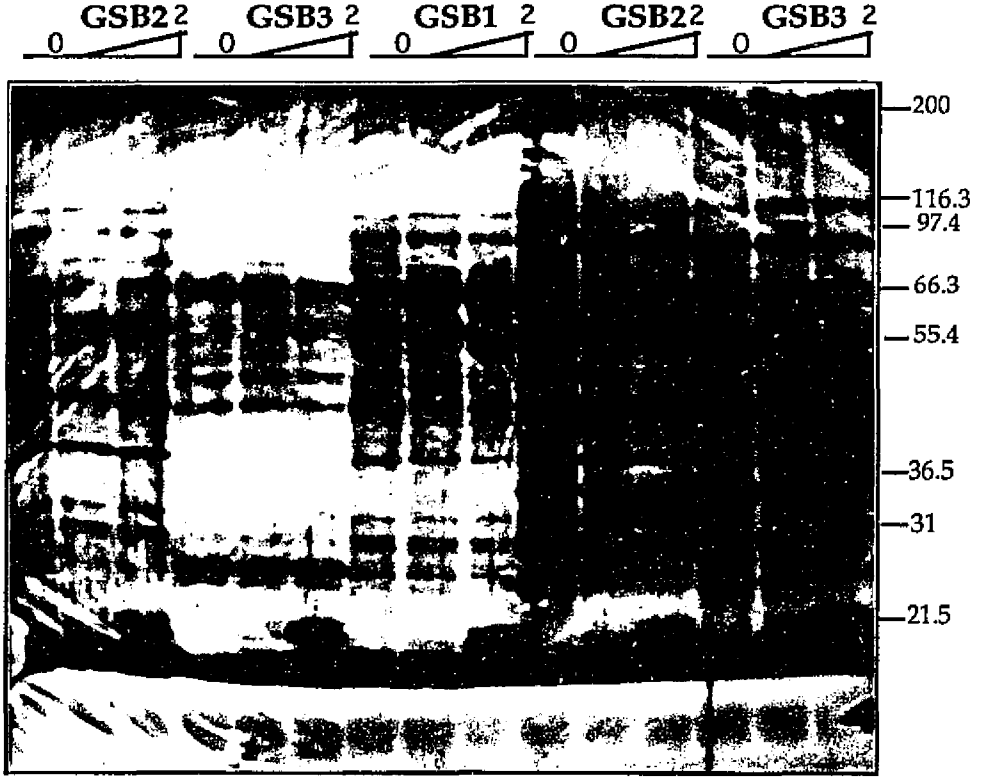


Figure 7. Nucleotide transfer experiment using BC200 and 7SL probes with human brain extract. (7a) SDS-PAGE autoradiography. (7b) SDS-PAGE with Silver Staining.

Although the strength of shifted bands of BC200 and 7SL RNA is less than half of that of BC1 RNA, the protein factors which are labeled by this method are far weaker (nearly 10 fold) than the experiment with BC1 RNA (chapter 3). The two protein bands from shifted-band 3 with BC200 RNA have a M.W. close to 52.1 and 42.8 kDa. Additional two protein bands which can't resist to RNase challenge are from the shifted-band 3 (56 kDa) and shifted-band 1 (14.9 kDa). The 7SL RNA group have a common labeled protein band (24.1 kDa) from both shifted-band 1 and 2; however, there is another protein (27.2 kDa) from shifted-band 2 higher than this common one.

There are several reasons which can contribute to the weakness or absence of radiolabeled protein signal in certain shifted bands. First, the different RNA binding activities of brain extract may account for the different signal strengths between BC1 RNA and BC200/7SL RNA shifted-bands. Second, there are less radiolabeled uridine incorporated in the protected fragments. Third, the RNA fragment which can cross-link to protein are not connected with radiolabeled uridine. Fourth, the shifted-band complexes are too large or can be less efficiency to be eluted out from the gel slice. When the cpm of gel-slice was counted before or after elution, there is at least 50% radioactivity still retained in the gel slice. This has been further elucidated by embedding the gel slice into stacking gel and run its contents into SDS-PAGE without heating and RNase treatment. The overall signal of BC200 and 7SL RNA group are already low in the gel slice and some additional labeled protein bands within those slice are present, suggesting that there are some other protein factors can be cross-linked to RNA. However, without heat-denaturing as well as RNase treatment, there is possibility of non-specific association to RNA probe and the migration change of non-complete denatured proteins.

The SRP core particle contains 6 proteins with the sizes of 54, 68/72, 19, and 9/14 kDa. Two proteins within the range of 24.1 and 27.2 kDa has been identified from above experiment. If we subtract the effect of polynucleotide on proteins, the most close proteins are 19 and 14 kDa. In the case of protein phosphorylation, the migration pattern in the SDS-PAGE change in a unpredictable manner, since the phosphate groups will increase the negative charge and expel the association of SDS. The same scenario is also true when the polynucleotide is cross-linked to a protein. Other methods such as protein blotting are required to confirm the identity of those proteins.

Chapter VI

Conclusion

Prospective the function BC1 RNP

Junk RNA?

Alu elements and ID elements are considered "Selfish DNA", which implies they possess no function but to propagate themselves within the genome. The tissue-specific transcripts studied in this laboratory were generated by a reorganization of one those elements (juxtaposition) in the genomic DNA that generated a neuronal specific promoter. One scenario is that these two RNAs have no function but are chance products. Although a function for either BC1 RNA or BC200 has not yet been established, there are several lines of evidence to reject the concept of "junk RNA". First, BC1 and BC200 exist as an independent transcription unit. Gene regulation as well as expression may require numerous other factors to participate. Second, these two RNAs can be transported out of nucleus. On the contrary, 90% of Alu-containing RNA is degraded in nucleus and only few of them are capable to exit the nucleus. This suggests that BC RNA can participate in the nucleus transportation machinery. Third, only a handful of known neuronal RNA species are sorted into the dendritic processes. However, BC RNAs can be transported even into distal parts of dendrites. Transport out of those RNAs into far distance doesn't not have any benefit for enhancing its "selfish" property. Fourth, both genes have existed at least 40 million years and the coding region is far more conserved than the flanking region. Clearly, this supports that there is selective pressure on the RNA portion. If there was no functional role for these RNAs, the cell would not spend a lot of energy on a useless molecule.

Translation Regulation

It has been shown that the protein synthesis machinery exists in the dendrites (see introduction). Based on two observations, BC1 RNA may play a role in translation regulation. One is the origin of BC1 RNA, another is its dendritic location. The ID element in the BC1 RNA has high homology to its tRNA founder. This tRNA-like sequence may suggest a tRNA-like secondary structure of the 5′ domain of BC1 RNA and compete for tRNA function either at the ribosome or with other molecules involved in the translation.

The physiological function of dendritic mRNA may be responding to the local demand of protein synthesis in a regulated manner. BC1 RNAs are not

translatable mRNAs but colocalized with those mRNAs. It suggests that BC1 RNA may be involved in regulation of local protein synthesis events. As for the BC200 RNA, the dendritic location is very similar to that of BC1 RNA; however, the translational regulation may have a different aspect. The 5′ Alu element domain of BC200 RNA shares high sequence similarity with another Alu element containing small RNA, 7SL RNA, and can interact with the SRP 9/14 kDa proteins *in vitro*. The Alu domain of 7SL RNA is thought to be involved in translational arrest. BC200 RNA may thus exhibit a similar property during regulation of translation in dendrites.

RNP Sorting

When BC1 RNA was discovered as a member of dendritic RNAs, the possibility arose is that it may directly participate in the RNA sorting machinery. Recent findings show that BC1 RNA is also sorted into the axonal terminals of neurons in SON with other mRNAs such as vasopression, oxytocin, dynorphin and neurofilament. It became the only RNA that can be sorted into the two different neuronal processes [113]. Since axonal terminals usually lack protein synthesis machinery, BC1 RNA may play a role other than regulating translation. It is also speculated that multiple protein particles such as SRP or chaperon p60 participate in the initial steps of protein translocation or transportation. BC1 RNP may thus be involved in the docking or anchoring of mRNA to cytoskeleton components. Another piece of evidence comes from the experimental indication that BC1 RNA can associate with two different cytoskeleton components. It may simply suggest that BC1 RNA itself may depend on the microtubules (transport ?) and neurofilament network (docking?) for sorting [112]. However, it also suggests that BC1 RNP is capable to interact with cytoskeleton proteins which fits very well with the requirement of mRNA docking.

Both hypothetical roles of BC1 RNP in translation regulation or RNA sorting, suggest that BC1 RNP may be a mediator (chaperone) which can interact with other macromolecules, alter their conformation, make a proper connection to other molecules or receive a signal and then release its association. For example, in the case of SRP, it interacts with newly synthesized signal peptide and causes the translational arrest of the associated protein synthesis complex. Not until the SRP receptor on ER membrane is reached and the whole complex docked in a proper order, will the SRP unblock protein synthesis complex [78]. This kind of mechanism may also exist for non-secretory protein. BC1 and BC200

may be one of those particles involve in the control of mRNA of non-membrane bound protein to be translated according to demand,

Dendritic Spines Signaling Event

The down regulation of BC1 RNA signal in the hippocampus pyramidal cell after estrogen application is consistent with the loss of dendritic spines in those neurons. This indicates that the regulation of BC1 RNA in the dendrite may be controlled by endonuclease. This location of BC1 RNA in dendritic spine as well as the estrogen dependent BC1 RNA decline may suggest it plays a role in the signaling events within the dendritic spines [123, 135, 156]. A preliminary observation from a *in vitro* binding assays gives a hint that the Ca^{++} concentration may influence the association between BC1 RNA and its binding proteins. This supports the idea that BC1 RNP may be a target molecule in dendritic spines during calcium influx [157].

Transcription Regulation

Bp-1 is a protein that has recently been reported to bind BC1 gene as well as BC1 RNA. This sort of bifunctional binding behavior has only been demonstrated clearly with TFIIIA with the 5S RNA gene. If the Bp-1 has similar functions as TFIIIA, it may suggest Bp-1 can control BC1 RNA transcription, nucleus output and storage [33, 35]. However, in the study of transcription regulation of BC1 RNA gene by using transgenic mice, it shows the increase of gene dosage of BC1 RNA does not have any effect on the expression level of endogenous BC1 RNA. This suggests that if Bp-1 is associated with BC1 RNA, either it is not sequestered by large amount of BC1 RNA or it is not the factor essential for BC1 RNA synthesis. Alternatively, a large excess of protein would be necessary to compensate the increasing of BC1 RNA.

It has been shown that Alu as well as ID elements exhibit the potential of enhancer activity to regulate nearby genes [7, 42]. If a factor binding to BC1 RNA can also interact with DNA in the ID region, the expression level of BC1 RNA may have an influence on expression of such genes. This so called sequester effect may decrease the available pool of suppresser or activator, and alter the gene expression.

Some other aspects of BC1 RNA

RNA function

RNA itself can have the following functions. RNA may have enzymatic activity [43, 158], its can act as template or primer, it may serve as a backbone in multiple subunit complex and it provides specific interactions with other RNAs or proteins. Since BC1 RNA does not exhibit any secondary structure resembling well defined ribozymes, it likely does not contain this kind of enzymatic activity [159]. While, it has been suggested that the cleavage activity for pre-mRNA splicing reside on snRNAs. They do not exhibit similarities to the structures of ribozyme. There are other types of enzymatic activities in RNA besides the endonuclease [43]. Whether BC1 RNA contains any enzymatic activity awaits further experimentation. Since in this thesis it has been shown clearly that BC1 RNA can form an RNP complex, its role as backbone is minimal function assigned to it. The role of RNA/RNA interaction is very difficult to determine, as the protein cofactors may prevent or facilitate this interaction. Sense/antisense interactions are well studied in pre-mRNA splicing and rRNA processing; however, this kind of interaction with scRNA has not been demonstrate yet. The likely domain of BC1 RNA which can interact with other RNAs is its 5′ ID domain, as there are numerous mRNA and hnRNA transcripts that contain this repetitive element in the opposite orientation. However, if the ID domain of BC1 RNA forms a long stem loop, it could limit the possibility of inter-molecule interaction. Both in the sucrose gradient and native gel, there are two locations of BC1 RNA signal. Either BC1 RNA has different length or secondary structure or it can bind to other RNAs / RNPs at lease *in vitro*.

In the study of BC1 RNA integrity and footprinting, there are indications hot spots within the BC1 RNA which are easily attacked by RNase. It has been shown that the degradation of BC1 RNA has a discrete pattern with or without protein association. Therefore, BC1 RNA may be the target to sequester or regulate the presence and function as a consequence of BC1 RNP. From characterization of BC1 RNP, it is clear that there are endogenous RNases that favor degradation of BC1 RNA but not 7SL RNA.

RNA usage

Beside studying the function of BC1 RNA(P), BC1 RNA as well as BC200 RNA and their respective genes can serve as evolutionary marker for species relationship. Gene conservation and the neuronal expression of BC1 RNA have

been used to support rodent monophyly. It also possible to use BC200 to study the evolutionary relationships in primates.

Because of the relative simplicity of BC1 RNA compared to other mRNA, this molecule is first choose to study RNA sorting phenomena. By using the microinjection technique, it may determine the essential domain of BC1 RNA which is required for the RNA sorting in neuronal processes. After defining such domains, the protein factors which are involved in the RNA transportation and docking can be identified in continued biochemical studies. In addition to the model system to study the RNA sorting within neurons, BC1 RNA may also act as signal sequence to target other non-dendritic RNA into the dendritic field.

Since expression of BC1 and BC200 RNA are de-regulated in the cell lines and certain tumors, the molecules may also serve as a tumor markers and have some clinical application for cancer diagnosis.

BC1 RNP

Comparing the S values of "naked" RNA and RNP, suggests that BC1 RNP like SRP forms a relatively flat and rod-like structure. This prediction would be consistence with large particle with a smaller S value. In the case of SRP, its conformation changes during it functional cycle which suggests it has a very flexible conformation or shape. The actual size of BC1 RNP is difficult to predict. In comparison to the SRP, BC1 RNP has a fast migration in electrophoretic field and a later elution time. This may suggest BC1 RNP is smaller than SRP. Even if the BC1 core RNP is small, it does not necessarily mean that it is a simple particle. From the multiple band pattern in native agarose gels after enrichment of BC1 RNP on Mono Q pool, it is conceivable that some of BC1 RNP associated proteins may exhibit only weak interactions with BC1 RNP and BC1 RNP can undergo structural changes at different condition. When the Mono Q pool is separated by gel filtration again, it still maintain a same elution behavior. This also the case when the Mono Q pool is applied to a sucrose gradient. The different result between agarose gel and the gel filtration or sucrose gradient may be in their resolution power and fractionation principle. There is a smear signal distribution of BC1 RNP in polyacrylamide native gels after many steps of purification, which may suggest the surface charge of BC1 RNA binding proteins has a great deal of variation. These kind of heterogeneity and conformation different both in RNP and RNA-binding protein level may reflect the function of BC1 RNP, by interacting with numerous other macromolecules.

New purification approach

What is a possible solution to achieve the high purification of BC1 RNP? The problems of the affinity purification strategy have been discussed extensively [160, 161]. There are clears limits due to the size and structure of BC1 RNA. Beginning from the highly concentrated and enriched of Mono Q q pool, there are several route can be taken. One approach is based on the study of hnRNA binding protein [65, 162, 163], another has some other modification to fit the need of small RNP. However, both approaches require a step of UV cross-linking. In the study of hnRNA binding proteins which are also very complex, the partial purified and UV cross-linked hnRNP is used to generate the monoclonal antibodies. After acquisition of the necessary antibodies, the purification scheme can add an affinity step which is based on the interaction of antibody and antigen. [164] The purification of BC1 RNP can fit in this procedure quite well. After sucrose gradient or one more step (native gel or other), the final enriched pool can act as the antigen to generate monoclonal antibody through a immunosuppressor protocol. To screen antibodies may based on the well established immunoprecipitation procedure or a slight modification procedure which is used the radioactivity *in vitro* synthesized BC1 RNA to form a RNP *in vitro* ,which can increase the sensitivity and reduce the assay time [144]. Those antibodies can also use to screen the expression cDNA library [165, 166]].

Another approach is use the CsCl gradient and denaturing gel to separate the UV cross-linked BC1 RNP from other contamination after Mono Q pool. Depending on the yields, one may either continue to immunological approach or microsequencing steps. The basic concept of the above approaches are based on UV-cross-linking to stabilize the interaction between RNA and protein, and then more stringent separation methods can be applied. The result may solely depend on the efficiency of UV cross-linking to generate the covalent bonds between nucleic acid and protein. In a study of U1 RNA to A protein interaction, the cross-linked efficiency may only reach 5% of the total amount [143]. According to the result of using UV light cross-linked in both endogenous or exogenous BC1 RNA to its binding protein, the efficiency looks very promising. It needs to be tested to understand how efficient this approach will be. If the efficiency is very

low, a short arm cross-linking agent may be applied to increase the cross-linked RNP population.

Those approaches that I have been applied may still serve as a candidate procedures for purification of BC1 RNP. One is the RNA tagging method, another is to screen autoimmune antibody. The essential requirement for successful purification of BC1 RNA binding protein is that the amount of BC1 RNP must be higher than other RNP contamination. The autoimmune antiserum, however, must have high specificity and high titer. The antiserum that I have used can not serve as first two steps of enrichment. However, later step may enough for characterization the protein component of BC1 RNP. Nevertheless, this antibody or other antibodies which can interact with BC1 RNA or RNP themselves has been more biomedical interests beside being as a tool for BC1 RNA purification.

Chapter VII

Experimental Methods

Starting

Brain Extract

Fresh cerebra were taken from nearly 10 weeks old rats (strain D.S.) after paralyzing them with CO_2. These brains were homogenized with 10 ml ice cold homogenization buffer (100 mM KCl, 15 mM $MgCl_2$, 10 mM Tris pH 7.5, 1 mM DTT, 0.32 M Sucrose, 0.2 mM PMSF, 5 mg/l leupetin, chymostatin, peptatin, antipain) for each brain with Dounce type B homogenizer. This crude extract went though differential centrifugation as following. Crude extract was spun at low speed (SS-34, Sorvall RC5, 3K rpm) for 11 min. to get rid of debris and intact cells. Supernatant (S1) was spun again at higher speed (SS-34, 12K rpm) for 1 hr to precipitate mitochondria. Applying supernatant (S2) to quick seal tubes and then spin with ultra-speed (T1270, Sorvall RC70, 36.5K rpm) for another 1 hour. This microsome-free supernatant (S3) was used to determine the S value of BC1 RNP and apply to cesium salt gradient. However, for conventional purification, concentrated brain extract was used. There are two modifications for above procedure. One is to use 2.5 ml of homogenization buffer per gram of brain for homogenization; the other is to rehomogenize the pellet from low speed spinning and directly bring this crude extract into high speed centrifugation. Both supernatant portions were combined (S2) and submitted went to ultracentrifugation. The same procedures are also applied to prepare other tissue extracts as well as human brain extracts. For small amount of tissue, small scale preparation is applied. Except the variation in tube dimension and centrifuge, depending on the working volume, the time and force (xG) are always kept constant.

Tracking BC RNA signal

To track the BC1 RNP signal for purification or other experiments, RNA dot blot was used. In order to monitor the protein composition during purification, conventional discontinuous SDS PAGE is used.

RNA dot blot

Same volume of denaturing solution (0.4 portion of 40% formaldehyde and 0.6 portion of 20 X SSC) is added to each fraction which came from different preparations. These mixtures are incubated for 1 - 2 hours at room temperature (optional) and heated at 65°C for 15 min, and then put into ice immediately. Before loading these fractions into the dot blot apparatus (Schleicher & Schuell),

SSC was brought to the final concentration of 10X. In the meantime, two sheets of 3M paper and one sheet blotting filter (Genescreen, Dupont) were soaked at 10 X SSC for at least 15 min. After loading all fractions into wells and following with gentle suction, 300 µl of 10 X SSC was added to wash each well. The filters were sit under UV light for 13 min. to cross-link the RNA to the filters. These filters were ready for hybridization or storage.

Hybridization

The procedures for detecting the RNA signal are the same for both RNA dot blot and Northern transfer [2]. DNA oligonucleotide probes (HT005-BC1, 35B-7SL or ID probe) were labeled by kinase reaction and double strands DNA (ribosomal DNA or other) was labeled by nick translation. Before hybridization, filters were rinsed with 5 X SSC and placed into a container. After draining residual solution, five milliliter pre-hybridization solution (10 X Denhardt's solution, 0.5 M Tris-HCl, pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, and yeast tRNA (0.1 mg/ml)) was added and prehybrided for 30 min. The probes were then added directly into pre-hybridization solution (10^6 cpm/ml). Hybridization reaction is usually overnight (6 - 12 hr). Hybridization temperatures are vary which depend on the length and GC content of individual probes, however, usually it is 55°C. After pouring out hybridization solution, filters were rinsed with washing solution (2X SSC, 0.1% SDS) and washed three times with 60 ml of washing solution at the same temperature of hybridization for 40, 30, 20 min, respectively. Without drying, the filters were wrapped with Saran wrap, and put into a cassette for autoradiography.

Protein detection

For detecting the protein composition for different purposes, SDS-PAGE was used [167]. Depending on the working volume or salt concentration, the loading dye (4 X) was added directly or the solution was precipitated with 10% trichloroacetic acid first. After heating at 80°C for 10 min, samples were put on ice and loaded into 13% or 10% SDS polyacrylamide gel. The gel was run under 15 mA constant current for 4 hr. The protein gel was fixed in fixation solution (50% methanol, 12 % acetic acid) for at least 2 hr before staining.

Coomassie Blue Staining

After fixation, the gel was put into Coomassie Blue solution (50% methanol, 0.05% Coomassie Brilliant Blue R, 10% acetic acid) and agitated for another 4 hr. The gel was washed briefly with fixing solution and then destained with destained solution (5% methanol, 7% acetic acid) for 2 hr. Destaining

solution was replaced several times until blue bands and a clear background were obtained [168].

Silver staining

After fixation, the following steps were performed:

1, The gel was incubated in heavy metal solution (50% Methanol, 12% TCA, 2% CuCl2, 1% $ZnCl_2$) for 15 min. with gentle agitation. 2, Washed with propanol solution (10% 2-propanol, 5% glacial acetic acid) for another 15 min. 3, Reacted with oxidation solution (0.01% $KMnO_4$) for 15 min. 4, Washing the gel with one time propanol solution and two times water for 10 min. each. 5, The gel was placed into silver nitrate solution (0.15% $AgNO_3$) for 15 min. with vigorous shaking, and then washed with water briefly. 6, The gel was alkalinized by incubating with 10% Na_2CO_3 for 50 sec. 7, Protein bands were visualized with developing solution (2% K_2CO_3 plus ~0.02% Formaldehyde) for 4 - 6 min. or up to 20 min. Finally, the developing reaction was stopped by 1% glacial acetic acid. The stained gel was photographed or dried with a gel dryer within 2 sheets of cellophane.

Fractionation Methods [138, 169]

Centrifugation

CsCl and Cs_2SO_4 gradients [126]

The stock CsCl (or Cs_2SO_4) solution in 0.01 M Tris-HCl with different densities (r= 1.7 ~, r= 1.4 ~ and r= 1.2 ~ for CsCl and 3M, 2M and 1 M for Cs_2SO_4) were prepared. Before loading into ultracentrifuge tubes, cesium salt solution is brought into buffer which contains 20 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$ and 1 mM DTT as final concentration. Five different densities of cesium salt solution for step gradient were prepared before used. Samples (brain extract, total brain RNA, *in vitro* synthesized RNA) were laid onto the top of cesium salt step gradient and spun with high speed (AH 629, Sorvall RC70, 28.5K rpm) at 20°C. After spinning for 44 hr, solution in the tubes was aliquoted with pipet aid (from top to bottom) or pump (bottom first) into 20 or 40 fractions.

Sucrose Gradient and S Value [170]

Sucrose stock solutions (5% and 20%) are prepared in the same ionic strength as the homogenization buffer (100 mM KCl, 15 mM $MgCl_2$, 10 mM Tris pH 7.5). Both sucrose solutions were loaded separately into each gradient mixer cambers. A continuous flow is generated by peristaltic pump with the flow rate 1 ml/min, and deliver the mixture solution into a centrifugation tube (Dupont, No.

03126, PA 17 ml). This sucrose gradient can maintain for several hours at 4°C before sample loading. S value is approached by the "zonal method" [127]. Protein solution (brain extract) is layered onto the top of sucrose density gradient and followed with centrifugation. A standard run is handled by the rotor AH 629 (Sorvall RC-70 ultracentrifuge, 28.5K rpm, brake on) for 20 hr at 4°C. Each tube was loaded with 0.3 ml of sample or 2.5% sucrose solution with standard marker. Three different standard markers are used for interior or exterior (parallel run) to determine S values, alcohol dehydrogenase (Yeast, 0.2 mg/ml in 50 mM Tris-HCl buffer, pH 7.5. S value = 7.61), catalase (Bovine heart, 0.7 mg/ml in 50 mM Tris-HCl buffer, pH 7.5. S value = 11.3) and β-galactosidase (E. coli, 1 mg/ml in 50 mM Tris-HCl, pH 7.3. S value = 16). After centrifugation, each tube was divided into 40 fractions. It is usually used 50 µl from each fraction for tracking the RNAs signal and 10 µl for the enzyme assay.

Preparatory sucrose/glycerol gradient

Those procedures are similar to the methods mentioned above. The only different is the volume and loading capacity. It use a 35 ml centrifugation tube (Dupont,) with 25 ml of 5 - 20% sucrose or glycerol gradient solution. A same rotor and same parameter was used as described previously.

Enzyme assay

Assay methods for the relative enzyme activity were adopted from different sources with some modification. Alcohol dehydrogenase is measured by following the increasing of absorbance at OD 340 nm at 20 sec later. This reaction is taken place in the quartz cuvette with 1 ml reaction mixture (3M Ethanol, 0.06M Sodium Pyrophosphate, 1.5 mM DPN (β-nicotinamide adenine dinucleotide). Catalase with an olive-green color can be seen on the blotting filter. Measurement of catalase activity can be done by various methods, however, Pyrogallol test is the most convenient one. Each Eppendorf tube contains 200 µl of 0.2M Pyrogallol, 20 µl of 0.05% hydrogen peroxide and 20 µl of Na-phosphate buffer (0.1 M, pH 7), and then water is added up to 1 ml. After adding 10 µl of enzyme solution into each tube, reaction is taken place at room temperature for 5 min. The reaction is stopped by adding 10 µl of 5N H_2SO_4 and measured the absorbance at OD 430 nm. Assay for β-galactosidase is handled at 96 wells assay plate. After adding 10 µl of enzyme solution, a 190 µl of the reaction mixture (170 µl of 0.1 M sodium phosphate buffer, pH 7.0, 7 µl of 3.36 M 2-mercaptoethanol, 13 µl of 0.03M $MgCl_2 \bullet 6H_2O$) to each well. Before starting

reaction, a 10 µl substrate (0.068 M ONPG (o-Nitropheny-β-D-Galatopyranoside) was added. Absorbance was measured at OD 430 nm.

Chromatography

For purification of the protein components of BC1 RNP, two scales of purification have been tested. The main purpose of analytical purification is to identify the protein component of BC1 particle; of the preparative purification, however, is to get a large enough amount of particles to work on.

Gel filtration chromatography -

Superdex 200 and S 300 HR chromatography

Two different matrices have been used, Sephacryl 300 HR (Pharmacia) for low pressure column and Superdex 200 (26/40) for FPLC (Pharmacia) [171]. For convenience and loading capacity, Superdex 200 column with FPLC are major gel filtration column to be used for BC1 RNP purification. Samples form S3 brain extract as well as other preparations were applied to this column. After loading 5 - 7 ml of sample, the column was run with elution buffer (100 mM KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose). The flow rate for each operation is 2 ml/min. and a fraction of 4.25 ml was collected for 95 fractions. The BC1 and 7SL RNA signal were detected by RNA dot blot, and the peak fractions corresponding to the BC1 RNA signal were collected for following procedures.

Ion exchange chromatography

Mono Q chromatography

Mono Q (5/5) or (10/10) anion exchanger column with FPLC (Pharmacia) was carried on after Hydroxylapatite column or other column. The sample was diluted to 100 mM [Cl^-] with elution buffer without KCl. After binding the particles onto column matrix, the column was washed with starting solution (62.5 mM KCl, 15 mM MgCl2, 10 mM Tris-HCl, pH. 7.5, 2.5 M Sucrose). Bound material was eluded with salt with a gradient from 62.5 mM to 1M KCl. The flow rate was of 1 ml/min. and a fraction of 1 ml was collected for 100 (40) fractions. In the late experiments, there are several modification took place. First, without dialysis or dilution, the HAP pool was directly loaded in to Mono Q column. Second, the starting solution was changed to the elution buffer (100 mM KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose) that was used for gel filtration column. Third, the elution profile of salt gradient was changed upon demanding.

Fast flow Q sepharose (FFQ) chromatography

Two dimension of FFQ columns were used, The small one (16 X 10 cm) for the pool from gel filtration column and large one (2.6 x 20 cm) for the S3 brain extract. The buffer as well as parameter are similar to that of Mono Q, however, the flow rate is either 1 ml/min. (small column) or 2 ml/ml (large column) and fractions size is 2 or 4 - 5 ml /min. respectively.

Fast flow S sepharose (FFS) chromatography

Fast Flow S matrix (pharmacia) was packed into 1.5 cm diameter column (Bio-Rad) with a final dimension of 15 X 9.4 cm. For running this anions exchange column, phosphate buffer was used and NaCl serves as counter-ions. After re-equilibrium the column with low salt buffer (10 mM MgCl2, 50 mM Na-Phosphate-pH 7, 0.25 M Sucrose), 50 ml of preparation which came from Superdex 200 pool was loaded with a flow rate of 0.5 ml/min. Under same flow rate, the column was washed with 5 volume of low salt buffer and eluted the bound material with 8 volume of 0 - 1 M NaCl gradient. Every 5 ml fraction was collected and part of solution was taken for tracking the BC1 RNA.

Hydroxylapatite chromatography [172]

Five milliliter of hydroxylapatite (Calbiochem) were used in a 1.5 cm diameter column (Bio-Rad). The procedure is somehow similar to other ion exchange columns. Samples came from FFQ, gel-filtration or FFS pools. After loading samples and washing the column, bound material was eluted with 0 to 0.5 M phosphate gradient (in 2.5 M Sucrose). Flow rate is 1 ml/min. and the fraction size is 2 ml per fraction. In the late experiments, the bound material was directly eluted by 0.2 M phosphate buffer (0.2 M Na-PO_4, 100 mM KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose) up to 35 ml for a 5 ml column under the monitor of a UV detector.

Hydrophobic interaction column

Phenyl sepharose [173]

Phenyl sepharose matrix was packed into 1.5 cm diameter column (Biorad) with a final dimension of 15 X 3.5 cm. After re-equilibrium the column with starting buffer (100 mM KCl, 10 mM $MgCl_2$, 50 mM Na-Phosphate-pH 7, 0.25 M Sucrose, 0.8 M $(NH_4)_2SO_4$), The Superdex 200 pool (10 ml) was adjusted to 0.8 M $(NH_4)_2SO_4$ with saturated $(NH_4)_2SO_4$ and loaded into this column. Under a same flow rate (0.5 ml/min.), the column was washed with 5 volume of starting buffer and eluted the bound material with 8 volume 0.8 M to 0 M Ammonia Sulfate declining gradient. Every 1 ml fraction was collected and part of the solution was taken for tracking the BC1 RNA.

Affinity Interaction Chromatography

Dyes column

Blue agarose came from Bio-Rad (Affi-Gel Blue Gel) and packed into 10 ml disposable column with a final volume of 1 ml. After equilibrium the column with L.S. buffer (100 mM KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose), the SD pool was loaded into this column though gravity. After washing with 5 volume L.S. buffer, the bound material was eluted by the 1 ml step salt gradient (0.1 to 1 M KCl at 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose). Eluted solution was collected individually and measured the BC1 RNA content.

Heparin Column [153]

Pre-pack heparin column (Econo-Pac Heparin Cartridge, Bio-Rad) is connected to the FPLC system via a FPLC fitting kit (Bio-Rad). The test run is similar to a standard ions exchanger matrix procedure and use NaCl up to 2 M as counter ions. Both flow through and bound fractions were collected and detected the signal of BC1 RNA. Since the signal of BC1 RNA is in the flow through portion, it has been simplified those procedures to four steps in the late experiments: loading sample, washing column, and cleaning up the column with H.S. solution (1 M KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose) and then re-equilibrium column with L.S. solution (100 mM KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose).

Polynucleotide column

Polynucleotide matrices (cellulose or sepharose) were swollen in DEPC water for 6-7 hr and then applied to disposable columns for packing. After equilibrium the columns with H.S. buffer (1 M KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose), 6 ml of Mono Q pool plus 2 ml 4M KCl is loaded equally to three polynucleotide columns (dT, dC, poly (U)). Flow through portions were reloaded for another two times, and the columns were washed with 5 volume (5 ml) of H.S. buffer and the same amount of 2M buffer H.S. solution (2 M KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose). Bound material is eluted with 3.5 ml 0.1 M buffer (100 mM KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose) plus 6M GuHCl or with same amount of 8M Urea at room temperature. Each fraction was collected individually and detected the BC1 RNA by RNA dot blot.

ω-Aminoalkyl agarose [60]

A kit of Agarose $NH[CH_2]_xNH_2$ was came from Sigma with 1 ml each. The X equals to 2, 3, 4, 5, 6, 8, 10 and 12 carbon chain. After packing into disposable columns, they were washed with 5 ml of milli Q water and then with 5 ml of 2M KCl. After another 10 ml DEPC (diethyl pyrocarbonate treated) water washing, the columns were equilibrated with 5 ml of starting buffer (same as LS buffer of IEC column). The SD pool (5 ml) was loaded into individual column. After washing with 5 ml LS buffer (100 mM KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose), bound material was eluted with 5 ml each of 156.5 mM, 250 mM, 437 mM and 1 M KCl (Beside KCl, all with 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 2.5 M Sucrose), respectively. Finally, it is used 5 ml of 5 M Urea for cleaning bound material from the column. RNA dot blot was applied to detect the distribution of BC1 signal. Large scale columns (10 ml) were also applied, the procedures are similar to the anion exchanger column .

Chromatofocusing [Giri, 1990 #125]

Polybuffer Exchanger

Mono Q pool has been went through the Polybuffer exchanger (Pharmacia, 0.5 X 10 cm) and eluted the bound material with Polybuffer pH 7.5 - 5. All the procedures were handled as manufacturer recommended. Starting from the flow through portion till the end of low pH. elution, all fractions (1 ml each) have been collected to study their BC1 RNA content.

Electrophoresis

Native gel [128, 129]

Both agarose gels and polyacrylamide gels have been used to study BC1 RNP separation. It was used 0.5 to 1 % agarose with the buffer of TBE (89 mM Tris-borate, 89 mM boric acid) or Tris-glycine (25 mM Tris, 0.2 M glycine) under a constant current 10 mA at 4°C. For polyacrylamide gel, it was use same buffer system and a 4 - 10% gradient or 5%/7.5% non-gradient gel (acryl : bis = 37.5 : 1) with a 10 mA current at 4°C to study BC1 RNP. Once the leading dye (bromophenol blue) reaches the edge of gel, those gels are the subject of blotting for detecting the BC1 RNA location. Beside the detection of BC1 RNA signal in the gel, different slices of polyacrylamide gel may go for different uses. Some of them were stained with Coomassie Blue or Stains-all, and some of them were placed on second dimension and separated by SDS-PAGE.

Electroblotting

Electroblotting is a routine method to be used to detect BC1 RNA signal or protein within the gel. After gel and blotting membrane (Genescreen (Dupont), for RNA, nitrocellulose (Schleicher & Schuell) for protein) was soaked in the transfer buffer for 10-15 min, the transblotting apparatus (Bio-Rad) and the gel - membrane - filter-paper sandwich was assembled as the manufacturer suggestion. Different gels use different buffer to transfer the contents of gel. Urea gel for RNA separation uses NaoAc buffer (0.1M Tris, (pH 7.8), 0.05 M NaoAc, 5 mM EDTA) as transfer buffer with 200 mA constant current at 4°C for 16 hr. Native gel uses the same buffer for gel separation with 150 mA at 4°C for O/N. The same parameter was applied to SDS-PAGE where the native gel buffer contained 0.1 % SDS [168].

Prep Cell

The Prep Cell (Bio-Rad) is a preparative level of electrophoresis device with a continue elution chamber which provides the collectable elution solution. The 1% Gold-agarose (FMC) was packed into the cylinder chamber with 10 cm high. After agarose gel is polymerized, the Prep cell was assembled as manufacture suggested. Other conditions are same as the run of smaller scale agarose gel with 15 mA constant current. Once the leading dye reaches the edge of gel, one starts to collect the eluting solution with 0.5 ml per min. flow rate and 4 ml per tube for 80 fractions.

Immunoprecipitation

The procedures of immunoprecipitation are nearly the same as reported earlier [144]. After protein A sepharose (PSA) was swollen in NET-2 buffer (Tris pH 7.5, 150 mM NaCl, 0.05% NP-40), every 0.5 ml of solution (2.5 mg PAS) was aliquoted into 1.5 ml Eppendorf tube. Antiserum was added to the swollen PAS. (monoclonal antibody use 3 (α-neurofilament) - 5 (α-poly A binding protein & α-hn RNP protein C), antoimmunoe sera and polyclonal antibody (α-tubulin, α-synaptophysin, α-Dynamin, α-α-internexin and α-Bip-like protein) was 10 µl). Reaction mixture was placed on a rotary shaker for two hours at cold room. Antibody-bound PAS was washed with spin-down method (200 rpm for 3 min.) for 3 times with 1 ml NET-2 buffer for every washing. In the final washed pellet, it was added 200 µl Mono Q pool with NET-2 buffer to a volume of 0.5 ml and incubated this mixture for another 2 hr at 4°C. The immunoprecipited complexes were washed again as before for at least 4 times. The supernatant before the washing step and the pellet after washing were collected and separate the protein

from RNA portion using phenol/chloroform extract. Those RNAs were detected by EthBr staining and hybridization after seperating with 6% urea gel (acryl : bis = 19 : 1), and the proteins were separated via SDS-PAGE.

RNA-footprinting

The protection regions of BC1 RNA from RNase degradation have been studied by RNA footprinting. Same amount of S3 brain extract (100 µl) is treated as following. Two samples were deproteinized first and then add 0 or 1 unit of RNase mixture (1.25 µg/µl RNase I and 2.5 units of RNase T1), and three other samples were incubated with 0, 1, 2 units of RNase mixture at 37°C for 10 min. and then were deproteinized. The RNA which came from different treated samples were subjected to label the BC1 RNA by the splint methods [132]. In the RNA pellet, 0.4 µg of splint primer (oligomer with 15 mer of complementary sequence of BC1 RNA 3' end plus 12 dT and one dG at end), 1 µl of 10 X annealing buffer (40 mM Tris, 20 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT), and water to a final volume of 10 µl were added. Annealing the primer with RNA at 70°C for 10 min and followed by setting on ice for 2 min, the reaction mixture was added 2 mM S^{35}-TTP, 40 unit of RNasin, and 26 units Sequenase in every reaction mixture and then incubating at 37°C for half hour. Those reaction samples were added loading buffer (100% formamide, 0.01% bromophenol blue) and subjected to separate different RNA fragments though 6% sequencing gel (acryl : bis = 19 : 1). A sequencing mixture was run parallel to identify the molecular weight of those RNA-DNA fragments [174].

***In situ* hybridization**

Monkey brain sections were generously provide by other investigators [155]. The procedures for *in situ* hybridization followed previous work [111].

***In Vitro* Study of BC1 RNA Binding Proteins**

RNA protection gel shift assay

This protocol was based on the published work [150] with a minor modification. In the Eppendorf tube or 96 wells assay plate (Falcon), it was added 10 µl brain extract (as well as other tissue extract), 1 µl *in vitro* synthesis GTP-labeled RNA (1X 10^5 - 1X10^6 cpm) and binding buffer (same as brain extract buffer) for a total volume of 20µl. Those reaction mixtures were incubated at

room temperature for 15 min (binding reaction). and then added 2μl RNase mixture (2.5U RNase T1 and 1.25 μg RNase A (Pharmacia)) into every samples for another 10 min. Those reactions were loaded into 4 % native polyacrylamide gel (acryl : bis = 39 : 1) with the running buffer of 0.5 X TBE (45 mM Tris-borate, 45 mM boric acid). Once the fast blue reaches the 3/4 length of gel, the current was stopped and the gel was dried at gel-dryer (Bio-Rad) before exposing against X -ray film. In every reaction, it contains 0.1 μg of tRNA as non-specific binding competitor (in the later experiments such as nucleotide transfer experiment, tRNA was increased to 1 μg/μl concentration). Other competitors such as heparin or cold BC1 RNA domain or or conditions such as 0.01% NP-40 or 500 mM NaCl have been tested. Since those results except the addition of cool BC1 RNA have no effect on shifted bands, they did not included in late experiments.

Nucleotide (labeling) transfer experiment [143, 152]

Following the RNA protection and gel-shift assay, the gel was exposed in the germicidal lamp with the energy approximately $6.5X10^3$ ergs/mm^2 for 13 min. The gel was exposed against the X-ray film for O/N to locate the shifted bands. The gel-shifted bands were excised and divided into 5 equal pieces. To the gel slices, RNase mixture were added (0 to 3 units) and incubated for 10 min. at room temperature. Protein gel loading buffer (for SDS-PAGE) was added into the tube with gel slice to a 1X concentration and boiled for 3 min. The solution in each tube was loaded into the well of 10 % SDS-PAGE to analysis the proteins contents. Radioactivity protein bands were detected by X-ray film.

Affinity purification

The procedure of affinity purification is based on the published works [148]. RNA protection gel shift assay was used to track the BC1 RNA-binding activity through out all procedures. After brain extract was run through the Superdex 200 column, different fractions were subjected to assay its BC1 RNA binding activity. Two high binding activity peaks were pooled out and incubated with heparin sepharose (Pharmacia) with a ratio of 1 mg protein/g beads. After incubated those mixture solution at room temperature for O/N, the solution with heparin sepharose was poured into a 10 ml disposable column and subjected to the washing and elution steps. Every washing steps used 5 times column volume with 50 mM and 100 mM KCl in the Tris-Mg-sucrose buffer (20 mM Tris, pH 7.5, 15 mM $MgCl_2$, 1 mM DTT and 0.25 M sucrose). Different salt concentrations

(0.25, 0.5 and 1 M) in the TMS buffer were used to elute the bound material. The fractions contain the highest activity were loaded into BC1 RNA column. BC1 RNA column was made by using 200 μg of BC1 RNA annealing with 0.2 g poly (U) sepharose. The annealing efficiency was tracked with 10^6 cpm radiolabeled BC1 RNA. The heparin pool with BC1 RNA binding activity was concentrated and reduce the salt concentration and then added to a final concentration of 10 μg/ml yeast tRNA, 5 mg/ml Heparin and 35 μg/ml rRNA and 30 unit/ml RNasin to prevent RNase activity and non-specific binding. After loading those mixture pool into BC1 RNA column for 2 times, the column was washed with 0.1 M TMS buffer with 5 mg/ml heparin (10 volume) and 0.1 M TMS buffer only (20 volume). The bound material was eluted by 0.25, 0.5, 1 and 2 M KCl in TMS buffer for 5 ml each. Those beads after final eluted were treated with RNase (A or T, respectively), 8 M urea or loading buffer (SDS-PAGE) to exam the protein contents, respectively.

RNA tagging [143, 152]

BC1 RNA was cross-linked to its binding protein with UV light (germicidal lamp with the energy approximately $6.5X10^3$ ergs/mm^2 for 13 min). RNase solution (RNase mixture 1.25 μg RNase A and 2.5 units RNase T1 or same amount of RNase A or RNase T1 only) was added to trim the RNA epitope on cross-linked proteins. Oligonucleotide kinase and RNA ligase were used to identify those proteins with RNA tag. A standard kinase reaction was carried with 2 μl enzyme (T4 polynucleotide kinase 30unit/μl, BRL), 1X kinase buffer and 30 μcuri of γ-P^{32}-ATP. After incubated for 1hr at 37°C, the reaction was stopped with protein loading buffer and subjected for SDS-PAGE. For RNA ligase reaction, there were added 2 μl enzyme (RNA ligase 20unit/μl, BRL), 5 μl pCp mixture (10 μM with ~ 120 Ci/mmole), 1 μl 0.1 M ATP, 2 μl of 10 X buffer (50 mM HEPES, pH. 7.5, 20 mM MgCl2, 3.3 mM DTT, 10 μg/ml BSA), 3 μl of 10 % (v/v) dimethy sulfoxidein and 1 μl RNasin (40 unit/μl) in every reaction. Reaction mixtures were incubated at 37°C for 1 hr and detected the radiolabeled protein by 10% SDS-PAGE and autoradiography.

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