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5.1 **Role of cellular proteins in RNA virus replication:**

Viruses are obligate intracellular parasites that utilize the host cell machinery for their replication. The viral genomes have the elements required for their replication and they are equipped with the essentials that might not be available from the host. The replication of the DNA viruses is well understood in terms of the requirements derived from the host cell. Thus, the virus for its gene expression and genome replication utilizes cellular transcription factors and cellular DNA-dependent DNA polymerase. Availability of a polymerase capable of using RNA as a template governs the difference in requirements of a RNA virus. All RNA viruses thus encode an RNA polymerase since cellular machinery for replication of RNA doesn't exist in the host cells. RNA viruses typically have a small genome and must, therefore, rely more on the cell to assist in viral replication. There is increasing evidence that the RNA viruses frequently subvert cellular factors for replication and transcription of viral RNAs. The participation of cellular factors in viral RNA-dependent RNA synthesis follows two modes. In the first, cellular proteins are present as part of the RNA-dependent-RNA polymerase (RdRp) holoenzyme; in the second, they bind directly to the RNA template, and then directing the RdRp to the template. However, these two modes are not mutually exclusive. In fact, some factors may possess both functions and thus serve as a bridge between viral RdRp and the viral RNA template.

The proteins subverted by the virus for its replication need not necessarily serve the same function as in the host. Identifying the cellular proteins associating with the replication complexes of the viruses, the essential functions performed by these cellular proteins during the viral replication, the role of these proteins in virus-host interactions, their influence on the host range and virulence of the virus are some of the interests which have led to intense pursuits in the area of viral RNA-protein interactions in recent years.

The flavivirus genome is ~11 kb long single-stranded, plus-sense RNA. It serves as the only viral mRNA and encodes a single open reading frame of about
10 kb. A large polyprotein is translated from this open reading frame, which is subsequently cleaved by both host and viral protease to three structural and seven non-structural proteins. The functions of the non-structural proteins are not understood clearly, however, both NS3 and NS5 proteins are likely to be components of the viral RNA replicase involved in the genome replication (Rice et al., 1985; Bartholomeusz and Wright, 1993). The 95 bases 5'-NCR and 585 bases 3'-NCR flank the coding region of the genome. The terminal nucleotides of the 3'-NCR and the 5'-NCR form highly conserved secondary structures, which have been postulated to contain the cis-acting elements required for the synthesis of other strand of RNA. Computer folding predictions and RNase cleavage studies have indicated that the 3'-terminal nucleotides of the flavivirus genome form a stable stem-loop (SL) structure. Deletions introduced into the 3'-NCR of flaviviruses like DEN4 (Bray et al., 1996), TBE (Mandl et al., 1998) and subgenomic replicons of KUN (Khromykh and Westaway, 1997) altered the infectivity of the mutants and reduced the efficiency of RNA replication. Chen et al. (1997) demonstrated that the non-structural proteins of JEV, NS3 and NS5 bind specifically to the putative SL structure present within the 3'-NCR of JEV plus-strand. Both these proteins could interact with each other and form protein-protein complex in vivo. This interaction between the NS3 and NS5 substantiated the postulates of the non-structural proteins as a component of the putative viral replicase (Rice et al., 1985; Bartholomeusz and Wright, 1993). The notion was proposed on the basis of the presence of conserved motifs of an RNA helicase in NS3 and the G-D-D motif in NS5, a characteristic of RdRp. The putative RdRp of JEV was thought to mediate the specific interaction in the infected cell extracts by the uncharacterized cellular proteins binding to the plus-strand 3'-NCR (Chen et al., 1997). In case of the flavivirus WNV, Blackwell and Brinton (1995) reported three BHK cellular proteins with apparent molecular masses of 56, 84, 105 kDa that specifically bound to the in vitro synthesized RNA containing the SL sequence. One of these cellular proteins was identified as translation elongation factor EF-1 which also interacted with other flaviviruses such as YF and DEN viruses (Blackwell and Brinton, 1997). It was proposed that the interaction of the 3'-SL with cellular protein was related to the initiation of negative-strand
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synthesis. Previous studies in our lab identified at least three proteins of apparent molecular masses of 32, 35 and 50 kDa binding to the 3′-NCR of JEV genome. One of these proteins was identified as the 36-kDa murine Mov34 protein. The murine Mov34 belongs to a family of proteins whose members have been shown to be involved in RNA transcription and translation. Murine Mov34 has been proposed to be a component of the 26S proteasome involved in degradation of the ubiquitinated proteins in an ATP-dependent reaction (Ciechanover, 1994; Rechsteiner et al., 1995; Hershko et al., 1989). There is no experimental evidence for the role of Mov34 in JEV replication; however, the protein may direct the transport and localization of JEV RNA to a cellular microenvironment suitable for virus replication (Ta and Vrati, 2000). Besides, Mov34 is a key protein for cell cycle progression and in the absence of this protein the cell growth is arrested (Mahalingam et al., 1998). It has been observed in our lab that PS cells infected with JEV show a cell cycle arrest at 24 h post-infection. Ta and Vrati (2000), thus, postulated that Mov34 binding with JEV RNA might be responsible for the cell cycle arrest that would allow the cellular resources for the virus to grow.

The replicase complex of JEV has not been well characterized. The virus replication involves the synthesis of the minus-sense antigenome, which then acts as the template for the synthesis of a large number of plus-sense genomic RNA molecules. As in other flaviviruses, there is disproportionate synthesis of the plus- and the minus-sense RNA molecules during JEV replication (Uchil and Satchidanandam, 2003). It is proposed that the difference in the levels of the two RNAs could be due the difference in the efficiency of the replication complexes binding to the plus- or the minus-strand RNA. Equally possible is that different replication complexes bind to the two strands. The viral RdRp and few other non-structural proteins playing a role in replication are indispensable components of the replicase complex (Uchil and Satchidanandam, 2003). The cellular proteins subverted for the benefit of the viral replication could thus be a major contributor to the complexity of the existing viral replication machinery. This, together with the previous evidence of cellular protein interaction with JEV 3′-NCR (Ta and Vrati, 2000) formed the basis for investigations into cellular proteins binding to
the 5'-NCR of JEV genome and the 3'-end of the antigenome. In the present work, I have identified two proteins, alpha II spectrin/brain fodrin and an ATPase inhibitor, that bind to the 5'-NCR of JEV. I have also presented evidence for the binding of SV2A protein with JEV 3'-NCR sequence.

5.2 Cellular proteins bind to JEV RNA sequences:

To determine if cellular proteins bind to JEV genome and the antigenome, gel mobility shift assays were carried out. In these assays, short stretches of genomic or the antigenomic RNA, likely to be involved in virus replication, were synthesized in vitro as a radiolabelled probe and these were allowed to interact with the cellular proteins from mouse brain. These proteins had earlier been incubated with non-specific RNAs such as the yeast t-RNA, poly(I)-poly(C) or poly(A)-poly(C)-poly(U). Binding of cellular proteins with radiolabelled RNA probe thus indicated a specific RNA-protein interaction. These studies showed that indeed proteins from the mouse brain bind to the non-coding regions of JEV genome and the antigenome. These assays aren't very informative as resolution of the RNA–protein complexes on the gels is poor. And hence, very little can be ascertained from these assays about the number of proteins in the total cell lysate that interact with the RNA or their sizes.

UV-cross-linking assays and North-western blottings were done to characterize the RNA binding proteins in terms of their numbers and size. Four proteins from mice brain cells bound to the 5'-NCR of JEV genome in the UV-cross-linking assay. These proteins had an apparent molecular mass of 35, 33, 28 and 23 kDa. In the North-western blotting experiment, however, eight proteins of apparent molecular masses of 209, 161, 77, 54, 42, 38, 36, and 32 kDa showed binding with the 5P RNA representing the 5'-NCR of JEV. Similarly, five proteins from the mouse brain cytoplasmic extract bound to the 3'-end of JEV antigenome in the UV-cross-linking experiment. These proteins had an apparent molecular mass of 85, 80, 62, 53 and 36 kDa. The North-western blotting experiment,
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however, showed that these five proteins had molecular masses of 76, 70, 60, 38 and 30 kDa.

It is to be noted that while both UV-cross-linking assay and the North-western blotting identified proteins of similar size as binding to the 3N RNA representing the 3'-end of JEV antigenome, there were significant differences in the number and size of proteins identified as binding to the 5'-NCR using the two methods. As to which of the two methodologies used above is more reliable in terms of giving information on the proteins that bind to the RNA is debatable. In fact, it can be envisaged that the two methodologies need not necessarily give an identical answer to the number and size of RNA interacting proteins. UV-cross-linking relies on the interaction of proteins with RNA in an aqueous phase. In such a scenario the interaction is not purely on a one-to-one basis. The chances that a protein binds to RNA in presence of some other protein, as a facilitator of the interaction with RNA, are very high. Similarly, presence of a certain protein may interfere with the interaction of RNA with another protein in the aqueous phase, or higher amounts and higher affinity binding of a certain protein with RNA may preclude the chances of other protein/s binding with it. Also, the UV-cross-linking followed by RNase treatment removes all those regions of RNA not bound to protein. Therefore, the chances of weak interactions sustaining the RNase treatment are less. Whereas in a North-western blotting assay proteins in the lysate are resolved on a denaturing gel and transferred to a solid support. Following denaturation, each protein interacts individually with the radiolabelled RNA probe in presence of non-specific competitors. Thus chances of comparatively weaker interactions showing in a North-western blotting are more than those in the UV-cross-linking assay.

A mouse brain cDNA expression library was screened with radiolabelled RNA to identify proteins interacting with JEV RNA segments. Two of the cellular proteins that bound to the 5'-NCR of JEV RNA were identified. These were a 12-kDa mitochondrial ATPase inhibitor and a 240-kDa alpha II spectrin or brain fodrin. Similarly, an 80-kDa SV2A protein was identified as binding with
the 3'-NCR of JEV. This method, however, failed to identify protein/s binding to
the 3'-end of the antigenome. The reasons are more likely to be of technical
nature and do not necessarily represent any limitation of the methodology.

Of the three proteins identified in the present work as binding to JEV RNA
sequences, SV2A was studied in further details. The protein was synthesized in E.
coli and the recombinant SV2A was shown to bind the 3'-NCR RNA by UV-
cross-linking assay, North-western blotting and gel mobility super-shift assay.

5.3 The synaptic vesicle protein :

The Synaptic vesicle protein 2 or SV2 was one of the first proteins that
were localized to synaptic vesicles of the nervous system; it is an integral
membrane glycoprotein present in all synaptic vesicles (Bajjalieh et al., 1996).
SV2 does not have a homolog in yeast, which suggests that it is not a component
of the basic membrane trafficking mechanism. The SV2 protein has three
isoforms referred to as SV2A, SV2B, and SV2C (Bajjalieh et al., 1992; Feany et
al., 1992; Bajjalieh et al., 1993; Janz et al., 1998). The expression of these
various isoforms is co-regulated (Ascady et al., 1998). SV2B shows 65%
sequence identity while SV2C has 62% identity with SV2A (Janz and Sudhof,
1999).

The SV2A is a glycosylated protein, which is expressed in all neurons, and
is the only form expressed in most γ-aminobutyric acid (GABA)ergic and
glutamatergic neurons (Ascady et al., 1998). The expression levels in brain have
been well studied. SV2A is expressed in high levels in the subcortex i.e. basal
ganglia and thalamus while SV2B is expressed in high levels in the hippocampus
and cortex. SV2C is only present in a small subset of neurons in the basal
forebrain and brainstem (Janz et al., 1999). In spite of differential expression
these proteins are present throughout suggesting that they might have similar
activities although they may not have identical functions (Bajjalieh et al., 1993).


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The SV2 protein has 12 putative membrane-spanning domains called trans-membrane regions (TMRs) with cytoplasmic C- and the N-terminus (Fig. 5.1). The N-terminus of SV2s contains a long sequence that varies among three SV2 proteins but includes a short conserved region (Bajjalieh et al., 1992; Janz and Sudhof, 1999). Most cytoplasmic loops connecting the TMRs are also short except for the extended sequence between the TMRs 6 and 7 that are highly conserved among SV2s. Most intravesicular loops connecting the TMRs are short except for the sequence between TMRs 7 and 8 that are highly glycosylated (Buckley and Kelly, 1985). Comparisons between SV2 isoforms show that the TMRs and the cytoplasmic loops connecting the TMRs are highly conserved, while the N-terminal cytoplasmic sequence and all of the intravesicular loops between TMRs and the cytoplasmic loops connecting TMRs exhibit little homology (Janz and Sudhof, 1999).

The synaptic vesicles contain another protein called SVOP (Janz et al., 1999). This protein has a similar trans-membrane structure as SV2s with 12 putative TMRs and the cytoplasmic N- and C- termini. However, SVOP does not have the epitope for the SV2 monoclonal antibody and is expressed in a single isoform. Moreover it lacks the long cytoplasmic loop between TMRs 6 and 7 and the large intravesicular loop between the TMRs. It is thought that SVOP may be an evolutionary precursor of SV2s and that SV2s are an evolutionarily late acquisition that is specific for vertebrates (Sudhof et al., 1999).

The SV2 proteins show significant homology to a large family of transport proteins such as proton co-transporters, sugar transporter, citrate transporter. In fact, the SV2A and SV2B show conservation of sequences for the sugar transporter motifs (Maiden et al., 1987). This similarity to a large class of transport proteins suggested SV2 might be a neurotransmitter transporter specific to synaptic vesicles (Maycox et al., 1990; Bajjalieh et al., 1993). Similarly, both SV2A and SV2B show predominance of acidic residues in the N-terminal region, a characteristic of calcium binding proteins (Geppert et al., 1994; Lebrere et al., 1989).
SV2A knockout studies in mice showed that it is not required for vesicle formation or vesicle fusion and stability. SV2A is not required for the embryonic development but is crucial for normal neurotransmission and survival. Animals homozygous for the SV2A gene disruption appear normal at birth but fail to grow and experience severe motor seizures. These mice eventually die by the third week of their life as these high seizure incidences prevent them from feeding and drinking properly (Janz et al., 1999). In the SV2A homozygous knockout mice, the onset of the development of motor seizures parallels the developmental conversion of GABA from an excitatory to an inhibitory neurotransmitter in the central nervous system (CNS) (Cherubini et al., 1991; Rivera et al., 1999). The generalized nature of seizures is indicative of widespread hyperexcitability. The heterozygote mutants are of normal size indicating that a single copy of SV2A is sufficient to support growth. Interestingly, heterogygous mice show higher frequency of seizures than the wild type mice. The seizure phenotype is interesting in light of their viability and normal size. This suggests that CNS functioning is especially sensitive to SV2A expression levels (Crowder et al., 1999). The SV2B deficient mice displayed no apparent morbidity or premature mortality, were fully fertile and fully capable of caring for their offspring (Janz et al., 1999).

Electrophysiological studies of spontaneous inhibitory neurotransmission in the CA3 region of the hippocampus of SV2A knockout mice revealed that loss of SV2A leads to a reduction in action potential-dependent GABAergic neurotransmission. The gene disruption studies showed no change in the synapse density or synapse morphology, neither there was any effect on establishment or pruning of the synapse. Similarly, there were no developmental aberrations or differences in the formation of characteristic areas of brain. Thus the absence of SV2A in the glutamatergic and GABAergic neurons underlies the seizure phenotype and loss of SV2A reduces GABA secretion (Crowder et al., 1999).

Deletion of both SV2A and SV2B was no more deleterious than a single deletion of SV2A. These studies were confirmed by working with the
Figure 5.2: Transmembrane domain structure of SV2s.

Shown above is the proposed transmembrane domain structure of SV2s (Sudhof et al., 1999). Circles indicate amino acids in the primary sequence of SV2s, with 12 potential TMRs predicted by sequence analyses. Residues that are conserved in SV2A, SV2B and SV2C are shown in black, residues that are identical in at least two of the three isoforms in gray, and non-conserved residues in white. Coding exons are numbered 1 through 12 and positions of introns are indicated by lines crossing the peptide chain. Branched lines show positions of N-linked carbohydrates. The N- and C-termini are identified by letters.
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electrophysiological phenotypes of the hippocampal cultures where the double knockouts showed severe synaptic depression. The reason for this is that all neurons express SV2A where as SV2B is co-expressed with SV2A in only a subset of neurons, including the hippocampal cells (Bajjalieh et al., 1999). Thus the lethality of SV2A is caused by the functional changes in neurons expressing the SV2A because there are very few neurons that express only SV2B. The recruitment of granules to the readily releasable pool is reduced significantly in the absence of SV2A. This decrease in exocytosis occurred despite the upregulation in SV2C expression, suggesting that SV2A and SV2C expression do not perform entirely redundant functions. This means that SV2C does not compensate completely for loss of SV2A (Xu and Bajjalieh, 2001).

The function of the SV2s remains unknown despite efforts. Studies have been undertaken to elucidate the possible functions it might play by identifying the cofactors required for its activity and studying the protein interactions of SV2 with the other synaptic proteins. SV2s are thought to regulate exocytosis of neurotransmitter, a process regulated by calcium. Formation of SNARE complexes is one of the last events before exocytosis. The process of membrane fusion requires the formation of protein complex called SNARE complex. The components of the SNARE complex at the synapse include a vesicle protein (VAMP/synaptobrevin) and two plasma membrane proteins (syntaxin and synaptosome associated protein; SNAP-25). The effect of SV2 on SNARE complex formation was studied. The brain tissue from SV2A knockout mice contained smaller proportion of the target-SNARE (t-SNARE) Syntaxin in SDS-resistant complexes, which means that SV2 modulates secretion by regulating the progression of the basic membrane-trafficking components to a fusion-competent state. Thus SV2 acts before the final stages of fusion that is, before the formation of SNARE complexes. The lower proportions of these complexes were not due to the lower concentrations of syntaxin (Buckley et al., 1992; Bajjalieh et al., 1993).

SV2 interacts with the synaptic vesicle protein synaptotagmin. This interaction is mediated by the cytosolic, amino terminus of SV2 and the second
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protein kinase C homology (C2B) domain of synaptotagmin. This interaction is isoform specific i.e. SV2A not SV2B interacts with synaptotagmin I. The C2B also contains the C2-key calcium-binding motif (Sprang et al., 1995) and mediate homo-oligomerisation of synaptotagmin at high calcium concentrations (Sudhof et al., 1996). Mice in which the synaptotagmin C2B domain has been disrupted lack the low affinity component of regulated secretion (Sudhof et al., 1994) suggesting that the C2 domain may mediate synaptotagmin’s role as a low affinity calcium sensor (Bajjalieh et al., 1996). Since SV2 and synaptotagmin interact, it is possible that it helps in providing calcium-regulated modulation of one or both proteins’ action. Thus, synaptotagmin via its interactions with SNARE proteins regulate targeting of SV2 to its counterpart in the plasma membrane. Synaptotagmin seems to function as calcium-regulated inhibitor of synaptic transmission (Rand et al., 1993; Kaang et al., 1995; Poo et al., 1995). Equally possible is that SV2 inhibits the availability of synaptotagmin to form protein complexes that mediate synaptic vesicle fusion until calcium concentrations rise in response to membrane depolarization (Bajjalieh et al., 1996). Similarly it is possible that the SV2 proteins prevent residual calcium build up. In the absence of the SV2 protein this calcium build up would lead to heightened release of excitatory neurotransmitter coupled with no release of inhibitory neurotransmitter and thus would result in hyperactivity or over-excitation leading to seizures. In a nutshell, one can say that SV2A acts as a modulator of calcium-stimulated exocytosis rather than as effector. Still the data available so far has not been conclusive enough to assign a role to the SV2 proteins.

The SV2A protein was analyzed for the presence of RNA binding motifs in Motifscan feature of SWISSPROT database. None of the known RNA binding motifs was found, although a glycine-rich region is present at the N-terminus of the protein between the amino acids 64 to 137 (Fig. 5.2). Certain glycine-rich plant proteins have RNA-binding property although precise role of glycine-rich sequences in RNA-protein interaction is not clear (Alba and Pages, 1998). It may be noted that bacteriophage clone 2FA1, that contained the cDNA insert encoding amino acid 555-742 of SV2A protein, interacted with the radiolabelled RNA

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Figure 5.2: Amino acid sequence of the *Mus musculus* SV2A protein.

Shown above is the amino acid sequence of SV2A containing 742 amino acids. The SV2A protein contains a motif common to all transporters including the motifs specific for the subfamily of sugar transporters (Buckley *et. al.*, 1993). Underlined is the stretch showing the sugar transporter protein signature pattern. The protein also contains a glycine-rich motif (shown in box) between residues 64-137. It may be noted that the 73 amino acid glycine-rich motif contains 20 glycine residues (~27%) whereas the 742 amino acid protein contains a total of 76 glycine residues (~10%).
representing the 3'-NCR of JEV. It would, therefore, appear that JEV RNA binding site of SV2A might be localized between amino acids 555 and 742 of the full-length protein.

5.4 Implications of SV2A interaction with JEV RNA:

JEV is a neurotropic virus that actively replicates in the brain leading to encephalitis. The clinical symptoms of the disease include motor seizures leading to death. Studies summarized above have established that SV2A is necessary for the normal neurotransmission. The work reported in this thesis shows that the 3'-NCR of the JEV genome is capable of binding to the SV2A protein from the mice brain. As to what role this interaction may be playing in virus replication is not clear but it is quite tempting to speculate that this interaction could lead to symptoms of seizure during JEV infection. During the course of JEV replication in the neurons, binding of the SV2A protein to JEV RNA may bring down the levels of free SV2A available for its functions related to neurotransmission. This non-availability or low availability of SV2A could lead to no or low secretion of the GABA or an inhibitory neurotransmitter. Thus in response to a stimulus there are chances of neuronal hyperactivity or overexcitement. Excessive stimulation in the absence of any control by the inhibitory neurotransmitter would lead to seizures. It may be noted that the interaction of SV2A with JEV RNA is unlikely to be relevant for virus replication as JEV replicates actively in cells that lack SV2A.

5.5 Binding of spectrin and ATPase inhibitor with JEV RNA:

Besides the evidence for SV2A binding to JEV 3'-NCR, preliminary evidence is presented in this thesis for two proteins from the mouse brain which bind to the 5'-NCR of JEV genome. These are a 240-kDa alpha II spectrin also known as brain fodrin and a 12-kDa ATPase inhibitor.
Spectrins are plasma membrane associated cytoskeletal proteins with a molecular mass of 240 kDa. This protein constitutes 2-3% of total protein in the brain (Davis and Bennett, 1983; Bennett and Gilligan, 1993; Broadie et al., 2001). In the neurons, spectrin is often preferentially localized to both central and peripheral synapses (Bloch and Morrow, 1989; Daniels, 1990; Bewick et al., 1996; Kordeli, 2000) suggesting a role for spectrin at the synaptic membrane. Proteins, which play an important synaptic role, have all been shown to bind to spectrins e.g. calmodulin, sodium channels, munc-13 and synapsin I (Srinivasan et al., 1988; Steiner et al., 1989; Dubreuil et al., 1991; Sikorski and Goodman, 1991; Iga et al., 1997; Sakaguchi et al., 1998; Wood and Slater, 1998). Proposed roles for synaptic spectrins include capture and tethering via synapsin I of synaptic vesicles near active zone (Landis, 1988; Goodman et al., 1995; Sikorski et al., 2000), initiation of SNARE vesicle fusion by dimpling the cell membrane (Goodman, 1999) and anchoring of glutamate and/or acetylcholine receptors within the postsynaptic density (Bloch and Morrow, 1989; Daniels, 1990; Bloch et al., 1997; Wechsler and Teichberg, 1998; Hirai and Matsuda, 1999; Featherstone et al., 2001). The α- and β-spectrin mutant studies in the Drosophila neuromuscular junction showed that there was no alteration in morphological synaptogenesis, no change in ultrastructural localization of presynaptic vesicles or in the postglutamate receptor field function. However the subcellular localization of numerous synaptic proteins is disrupted. This could result in inappropriate assembly, transport or localization of proteins required for synaptic function (Featherstone et al., 2001).

Alpha II spectrin has been shown to be devoid of any inhibitory activity. Some undescribed proteolytic activities have been shown to remove the first 25 amino acids to yield the N-terminus of the Inhibitory Protein Factor or IPF and another to yield a C-terminus of the IPF. IPF refers to three distinct proteins IPF-α, -β, and -γ. IPF-α derived from fodrin, was shown to inhibit ATP-dependent glutamate uptake into purified synaptic vesicles. In addition it was shown to inhibit uptake of GABA into synaptic vesicle. Because this protein acts on glutamatergic and GABAergic system, most probably IPF-α could be a novel
component of a presynaptic regulatory system functioning in modulation of neurotransmitter accumulation into synaptic vesicles and thus regulate the overall efficacy of neurotransmission (Ueda et al., 1997). These properties of spectrin suggest that its interaction with JEV RNA may have implications for the control of neurotransmission during the viral infection. It is known that JEV replication inside the host cell takes place in the membraneous packets (Uchil and Satchidanandam, 2003). Since spectrin is a cytoskeletal protein it is possible that it’s binding with JEV RNA helps in tethering or anchoring the viral genome to membraneous structures so that the rest of the replication complex can then assemble.

The other protein that binds to the 5’-NCR of JEV genome is the 12-kDa ATPase inhibitor. In most cell types the ATP synthase-ATPase complex is the primary enzyme responsible for ATP synthesis. This complex is located in the inner mitochondrial membrane of all eucahyotic cells and functions by coupling the energy of a proton electrochemical gradient to the synthesis of ATP. The ATP-synthase-ATPase complex can be separated into two distinct moieties. The $F_0$ which is a membrane proton channel and $F_1$, the moiety that catalyses the synthesis of ATP (Lebowitz and Pedersen, 1996). ATPase inhibitor is a natural inhibitor peptide of the mitochondrial ATP synthase-ATPase complex (Isobe et al., 1997). The protein has been purified from bovine heart tissue, rat liver, yeast, plant and buffalo (Isobe et al., 1997). The mitochondrial ATPase inhibitor prevents the enzyme $F_1$ ATPase from switching to ATP hydrolysis during the collapse of the electrochemical gradient e.g. during oxygen deprivation (Walker et al., 1996). Thus, under normal conditions this 12-kDa protein serves as a brake for ATP hydrolysis before the ATP pool is exhausted (Isobe et al., 1997). Most of the processes of neurotransmission are ATP-dependent and so are the transcription and translation processes that are necessary parts of JEV genome replication. It is, thus, possible that during the virus replication the interaction of ATPase inhibitor with the viral RNA ensures ready availability of energy generated by enhanced ATP hydrolysis to help its replication process.
5.6 Concluding remarks:

Studies described in the present work were aimed at identifying cellular proteins that may be involved in JEV replication. To this end, attempts were made to identify cellular proteins interacting with sections of JEV genome and antigenome likely to be involved in replication. The three proteins that were identified during this work don't appear to have a direct role in RNA replication. However, proteins such as the spectrin and the ATPase inhibitor may play an indirect role in virus replication by providing anchorage or energy as discussed above although evidence for such activities remains to be established. An interesting outcome of the present study is the finding that SV2A protein interaction with JEV genome may be responsible for the clinical symptoms of seizure during the course of JEV infection. Attempts are underway in our lab to show interaction of SV2A with JEV genome in vivo.