CHAPTER V

DISCUSSION

The RNA isolated from BHK cell culture as well as canine brain was prepared by single-step extraction of the postnuclear cytoplasmic lysate with an urea-SDS-phenol-chloroform (USPC) mixture. This is highly indicative of high quality RNA. To further assess the efficiency of the procedure, the RNA samples were subjected to agarose electrophoresis using formaldehyde as a denaturant. Results such as those shown in Figure 8 were routinely obtained. This finding, combined with data shown in Figure 9 suggest that the method is suitable for rabies RNA extraction. we were able to detect all five intact rabies RNA species which suggest that the method is also highly qualitative. The ratio of UV absorption at $A_{260/280}$ =1.75-1.8 was consistantly obtained when fresh samples were used. The RNA from canine brain was also prepared by utilizing the USPC extraction protocol. The yield was approximately 1.5-2 μg/mg canine brain which is comparable to that reported by Ermine *et al.*(1988). RNA extracts from canine brain also showed a similar, if not, identical result on formaldehyde-agarose gel using the same extraction procedure (Figure 8). The rabies transcripts could also be detected as individual distinct bands similar to that shown in Figure 9. Thus, high quality of RNA used in the current study could be obtained using this extraction protocol. The USPC extraction method is quantitative, rapid and result in RNA of high quality and integrity. Large numbers of samples could be accomodated within a single day. The method is also simple and rapid, thus minimizing contamination of laboratory personnel with dangerous viral pathogens. Intact RNA obtained is the prerequisite to subsequent work where cloning of intact fragments were required.

Construction of rabies gene-specific probes were achieved by amplification of rabies gene sequences via specific primer designs in PCR. The oligonucleotide primer pairs were custom synthesized using the published nucleotide sequence of whole rabies viral genome (PV strain) as a reference. These gene portions were selected from specific and conserved regions (Tordo *et al.*, 1986; Tordo *et al.*, 1988). Five individual segments of rabies viral genome coresponding to five rabies structural genes were PCR-amplified and subloned into PGEM-3Z plasmid vector. The recombinant plasmids were isolated and used as rabies cDNA probes for detection of rabies specific RNA. Five rabies cDNA probes were constructed and designated as pRAB-N, pRAB-NS, pRAB-M, pRAB-G

and pRAB-L derived from the portions of N-, NS-, M-, G- and L gene, respectively (Figure 15). These gene portions were confirmed as authentic rabies viral genome specific using DNA homology search program against the database (Genebank). In addition, no cross-hybridization could be detected with the genome of either DNA or RNA from various sources such as herpes simplex virus, papilloma virus, *E. coli*, human DNA and canine brain (Figure 10-11, 16-20). Thus, we were successful in cloning ragies specific probes for subsequent use in in the determination of transcriptional events.

Another utility of the cloned probes is in detection of rabies virus presented in biological samples. Dot hybridization technique was employed in the determination of sensitivity of the probes. The entire recombinant plasmids were non-isotopically labelled and used as probes by using ECL-random prime system. As little as 0.1-1 μg of RNA from infected BHK cells was able to detected by chemiluminescent-labelled probes (Figure 16-20). These probes were able to self-hybridize to as little as 0.2-1.5 ng rabies cDNA as shown in Figure 16-20. These indicated that all five rabies cDNA probes are sensitive and specific for rabies RNA. Sensitivity of our cloned probes for target sequences could be further enhanced by conversion of the cloned cDNA portion to antisense RNA probes thus increasing the hybridization efficiency. Sensitivity of RNA probes has been reported to be up to ten fold higher than DNA probes in detection of herpes simplex viral nucleic acid (Chantratita, 1989). In the current study, our PCR derived rabies cDNA fragments were subcloned into pGEM-3Z plasmid containing both SP6 and T7 RNA polymerase promoters. Therefore, large amount of single stranded RNA probe could be produced by *in vitro* transcription. There are some inherent advantages of using RNA probes in hybridization experiments. They offer high specificity since they do not contain vector-specific sequence as prepared by either nick translation or random prime DNA labelling system. Since the RNA probe is single stranded, there is no loss of hybridization activity by reannealing. However, the sensitivity of detection gained is significantly compromised by extraneous steps required and the high susceptibility to ribonuclease degradation of the probes. In addition, extra care is required for probe handling to minimize degradation to degree that RNA approach is only feasible when cDNA counterpart is inadequate in term of sensitivity. This approach is also not suitable for routine diagnostic work due to aforementioned reasons. In the current study, the detection sensitivity was sufficient for our purposes. Therefore, no extra steps to improve detection limit was attempted.

All five rabies probes: pRAB-N, pRAB-G, pRAB-NS, pRAB-M and pRAB-L, showed promising results on DNA-RNA dot hybridization (Figure 21-25). Out of 39 canine brain samples, all 29 positive by FA was proven positive by dot hybridization, and 10 negative by either FA or MI was found negative by dot hybridization. The positive and negtive results by dot hybridization were consistently correlated to the FA and the MI. The sensitivity and the specificity of dot hybridization was 100% when compared to FA and MI which were used as gold standard. In addition, it was found that sequence homolgy appears to exist between laboratory strain (CVS) and street rabies strains under this experimental conditions. Thus, the cloned cDNA probes offers great utilities in the diagnosis of rabies virus in biological samples.

In Thailand, routine diagnosis of rabies virus has been performed by FA technique and confirmed the FA negative result by mouse inoculation (MI). Tissue culture technique is not widely used in ordinary diagnostic laboratories since it requires expert technician and tissue culture facilities. PCR technologies have been noted for the sensitivity and specificity in rabies virus detection (Ermine *et al.*, 1990; Sacramento *et al.*, 1991; Kamolvarin *et al.*, 1993). In fact, the amount of virus, i.e. nucleocapsid protein and RNA, is maintained in nanogram level which can be easily detected. Therefore, the high sensitive technique for detection the viral RNA in picogram level as PCR is not usually required. So far, there is no suitable technique for rabies diagnosis. The FA technique requires expertise and expensive fluorescent microscope for examination whereas the MI is time consuming and requires the animal technique facilities. At Pastuer Institute, Ermine and his coworkers (1989) developed the dot hybridization technique for rabies diagnosis. The technique showed potentials for utilization in routine diagnosis. However, Ermine's method requires the use of radioactive-labelled probes and the rabies cDNA probes were derived from M13 which has been shown cross-detection with mammalian nucleic acid owing to partial sequence homology present. Moreover, using the ^{32}P-labelled probes in dot hybridization requires several days for autoradiography. In this current study, modification and improvement of dot hybridization demonstrated a promising results for the diagnosis of rabies from canine brain. All five rabies probes, pRAB-N, pRAB-G, pRAB-NS, pRAB-M and pRAB-L were able to detect rabies specific RNA from infected canine. Utilization of ECL non-radioactive system offers many advantages in this application. Labelled probe can be stored for several months and no purification is required. Exposure time on film is reduced to 1-15 min. The cost per test is approximately 25 baht (1

US$). In contrast, the cost per PCR and FA test are 450 and 30 baht, respectively (18 and 1.2US$ respectively). Nevertheless, it should be noted that the cost of equipments (PCR or fluorescent microscope) and their maintenance cost are not included. In conclusion, non-radioactive dot hybridization technique is easy, rapid (1-2 day), sophisticated equipment is not needed and cost effectiveness. In addition, it was found that the decomposition of canine brain tissue does not affect the sensitivity of dot hybridization (Ermine *et al*., 1989). These potential possibilities provide an alternative method for diagnosis of rabies virus, in particular, when fluorescent microscope is not available.

Multiple sampling of events in a single cell is difficult or imposible, necessitating the use of many cells in each investigation. Risk of variation among individual subpopulation of cells in the total population must be taken into consideration. Furthermore, interpretation of results derived from such approach are subjected to batch variations.To circumvent the above mentioned complications, the G_1 arrest of BHK cells was performed as described in materials and methods. Synchronization of BHK cells was determined by examination of the kinetics of ^{3}H-uridine uptake and cell growth according to the studies of Tobey and Ley (1970), Petersen *et al*. (1969) and Tobey *et al*. (1988). ^{3}H-uridine incorporation as a function of time exhibits a positive exponential relationship while cell growth was still in the lag phase, and ^{3}H-uridine uptake was at saturated level prior to cell growth entering the log phase. Incorporation of ^{3}H-uridine does accurately reflect the rate of RNA synthesis upon releasing of a culture in stationary phase. The apparent incorporation curve is typical of resting cells being stimulated to enter the active cell cycle. RNA synthesis was linearly increased in first two hours and with a constant rate thereafter. This indicate that synchrony-induced cells are prompted in RNA synthesis, and cells were indeed synchronized.

The transcriptional events of rabies virus was studied under low input multiplicity (m.o.i. = 0.01). The quantity of infecting virion was kept a limiting factor such that the advent of transcription of each rabies virus genes could be differentiated. In addition, the infectivity of viral particle and the *in vivo* primary transcription of rabies virus were maximized by host cells saturation. Transcriptional events was determined after viral adsorption process. Viral poly(A)$^+$ transcripts were bound by oligo(dT) and copied to cDNA utilizing reverse transcriptase. Minute amount of cDNA was subsequently amplified by PCR. The order of primary transcription of rabies virus in current study was preceeded from the N to L, NS, M and G, respectively. The transcription rate of L increased during 3-5 hpi. In

contrast, the RNA transcripts of NS and M were detected at 3 hpi and the accumulation of both transcripts was maintained during 3-7 hpi of observation. The accumulation of L transcript apparently decreased concomitant with the decrease in the steady state level of G transcript at 6 hpi. The steady state level of newly synthesized G transcript dramatically increased during 5-7 hpi. The results suggested that the fluctuation of L transcript might play a vital role in regulating of expression of a majority of rabies structural genes. In addition, the data is also highly suggestive of relationship between steady state levels of L and G newly synthesized mRNA species.

The pattern of transcriptional order of rabies structural genes: N, L and NS are related to the function of N, L and NS protein in the viral RNA synthesis. Data from the study of L protein of VSV suggested that purified L protein contains a specific protein kinase which is different from cellular kinases (Barik and Banerjee, 1991, 1992). The L-associated kinase and cellular kinase specifically mediate the phosphorylation of the NS protein. These two kinases act sequentially in catalyzing a cascade phosphorylation pathway. Three forms of NS protein was classified according to the degrees of phosphorylation, i.e. native NS protein (P_0), semiphosporylated intermediate (P_1) and a fully phosphorylated form (P_2). N protein and RNA template is transcriptionally active only when the fully phosphorylated form (P_2) is associated with L polymerase. In addition, it was belived that transcription of VSV was controlled by the degree of phosphorylation of NS protein (Banerjee and Barik, 1992). The possible functional domains of NS protein of VSV which has been mapped to a hypervariable region between amino acid residues 137 to 214 may act as a spacer or hinge to provide accurate and correct arrangement of the active domains which, in turn, plays an important regulatory roles in transcription (Chattopadhyay and Banerjee, 1987). Such a spacer-like regions have also been reported in transcriptional activators of DNA-dependent RNA polymerase of yeast GAL4 and GCN4 (Hope and Struhl, 1986; Ma and Ptashne, 1987). Our data showed that steady state level of L transcript at 6 hpi decreased. This could be as result of regulatory effect of phosphorylated NS protein. Moyer and his coworkers found that negatively charged tubulin protein was a factor necessary for the RNA synthesis of both Sendai and VSV, however, the exact binding site and the control mechnisms remain unknown (Moyer *et al.*, 1986). Thus, there may be cellular factors, such as transcriptional factor or activator, which regulate the transcription of rabies virus. To date, it has not been clarified whether the regulation of rabies viral transcription is regulated by virus NS protein itself or by cellular

factors such as cellular kinase, tubulin, other protein factors, or may be from virus and host cells interaction or the combination of both.

All five rabies RNA transcripts encoding the message of structural gene N, G, NS, L and M was detectable using RT-PCR during primary transcription measurement (0-6 hpi). All transcribed species were detectable after 5 hpi. By comparison, the transcription process of VSV is complete by 3 hr postinfection (Flamand and Bishop, 1973). This rate was estimated to be 5 time slower for rabies virus (Ermine and Flamand, 1977). It has been shown that transcription rate and mRNA accumulation of rabies were much lower than that of VSV. RNA synthesis rate of rabies virus in BHK-21 has been found to be 10% that of VSV (Ermine and Flamand, 1977). This most likely reflects the low specific activity of the key enzyme rabies virion transcriptase which has been shown to be 1/150 that of VSV-Indiana (Kawai,1977). These detected transcripts had already been polyadenylated since they could bind to oligo(dT) in RT-PCR step. This finding was later confirmed by the work of Holloway and Objeski (1980) in which separation of the RNA species from infected BHK cell extracts into three classes with sedimentation value of 42S, 30S and 12S to 16S was reported. The smallest class 12S to 16S, corresponding to transcripts of N, NS, M and G, were detected as early as 6 hpi. In contrast, the 30S or L-transcript were detected by 9 hpi. The 42S virion-size RNA was observed only at a much later time in growth cycle. The RNA isolated from virus polysomes contained adenylate-rich sequences. Ermine and Flamand (1977), were able to classified rabies RNA in BHK cell culture into 3 categories: 1) the short (+) molecules sedimentint between 8 and 25S, and possibly at 30S; 2) (+) and (-) double stranded structures sedimenting between 25 and 35S; 3) (+) and (-) molecules of the genome length. The viral RNA synthesis was detectable from the first 4 hpi till at least 20 hpi. The maximum RNA synthesis was between 8 and 12 hpi. In current study, the synthesis of primary transcripts occured between 4 and 5 hpi for gene N, NS and L whereas that of the G occured at 6 hpi (Figure 27). These results suggested that cultural condition is major critical parameter for studying the viral transcription. It should also be noted that the studies of Ermine and Flamand as well as that of Holloway and Objeski was performed in continuous presence of actinomycin D (2μg/ ml cultural medium), the inhibitor for host cellular RNA and protein synthesis. Thus the presence of actinomycin D might interfere with the reported results as. In additon, their experiments were carried out in non-synchronous system and fractionation of the viral life cycle into four hour periods was done on arbitary bases.

The Genome of RNA viruses, such as poliovirus, is transcribed and translated into a single giant polyprotein that is subsequently cleaved to yield the individual gene products. Whereas influenza virus, have each gene located on a separate RNA segment such that transcription automatically yields a monocistronic mRNA. Rhabdoviruses contain multiple genes in their genome and require more than one gene product for their reproduction cycle. Ultraviolet inactivation studies have shown that VSV transcription follows the order of genes on viral genome. In addition, there exists a gradient in the quantity of the transcripts. It is still not clear whether this gradient results from a single initiation at the 3' end of the RNA genome followed by a 'stop-start' synthesis (Emerson, 1982) or is due to internal initiations coupled with a 'cascade mode' of RNA synthesis (Testa *et al.*, 1980). So far, the transcription and replication mechanisms of rabies virus have never been reported and the quantity of each mRNA species is still obscure. The gene order of primary RNA synthesis in our findings was not follow the order of the genes on viral genome. This suggests that the mRNA synthesis of rabies virus initiate at internal genes. Solubilized VSV virions initiate transcription at internal gene *in vitro*, suggested that each gene may have its own polymerase entry site (Chanda and Banerjee, 1981; Naeve and Summer, 1981). The *in vitro* reconstitution of purified nucleocapsid template with solubilized transcriptase was performed in the transcription products in the absence of UTP and GTP (Emerson, 1982). Only leader gene product was synthesized, the mRNA oligonucleotides (pentamer) were detected after transcription of full-length of leader RNA was permitted. However, the native virion synthesized leader gene and mRNA pentamer in partial conditions. Since all five mRNAs appear to start with the same pentameric sequence, the order of transcription could not be clearly identified. In addition, It should be noted that at initial RNA synthesis *in vivo*, polymerases are already located at internal during virus packaging and are able to initiate transcription at those internal sites.

The primary transcriptional order of rabies virus in the current study suggested that initiation was preceeded from N-gene. This result correlated well with the finding in VSV (Banerjee, 1987) and the role of N protein in viral multiplication. N protein is closely associated with viral RNA polymerase and full-length genome RNA forming an active complex that synthesize the virion size RNA in replication process. In addition, encapsidation of genome size RNA by N protein protect the RNA from ribonulcease and extreme environments. In summary, these informations suggest that N-gene is the most promising candidate for inhibition of the viral multiplication in further study. One of the methods used to interrupt the virus multiplication is to regulate the gene expression by anti-sense

RNA. We do hope that these informations coupled with other attempts may lead to disrupt the life cycle of rabies virus or may be success at antiviral therapy.