CHAPTER III

MATERIALS AND METHODS

Instruments

DNA thermal cycler 480, Perkin-Elmer, Norwalk, CT, USA.

Spectrophometer 550, Perkin-Elmer, Uberlinger, FR Germany.

pH meter, Beckman 4500, Beckman Instrument Inc., Irvine, CA, USA.

Hermle microcentrifuge Z229, B. Hermle, FR Germany.

Sorval RC-5 superspeed refrigerated centrifuge, I.E. Dupont de Nemours and Co. (Inc.), Newtown, Conn., USA.

Beckman L5-65 ultracentrifuge, Beckman Instruments International, Frankfurt, Germany.

Shaker bath CH/P 2563, Forma Scientific, Marietta, Ohio, USA.

SpeedVac SVC200, Savant Instruments Inc., Farmingdale, NY, USA.

Hybridization oven/shaker, Amersham International, UK.

Pipetman, Gilson Medical Electronics (France) SA., Villiers-le-Bel, France.

UV transilluminator Foto/Prep, Fotodyne, New Berlin, WI, USA.

FCR-10 camera, Fotodyne, Hartland, WI, USA.

-86 °C freezer C825, Forma Scientific, Marietta, Ohio, USA.

Shel-Lab CO_2 incubator 2300, Sheldon Manufacturing Inc., Cornelius, Oregon, USA.

Cytair 125, Flufrance ESI, Wissous, France.

Sterimatic autoclave, Market Forge Co., Everett, Mass., USA.

Power supply 250, BRL Life Science Technology Inc., Gaithersburg, MD, USA.

Horizon11-14, horizontal gel electrophoresis system, BRL Life Science TechnologyInc., Gaithersburg, MD, USA.

Pharmacia LKB densitometer, UltroScan XL, Sweden.

Scintillation counter, Beckman LS 6000SE , Becman Instruments, Inc., Fullerton, California, USA.

Bio-Dot microfiltration unit, Bio-Rad laboratory, Hercules, California, USA.

Thelco vacuum oven 29, GCA/Precision Scientific Co., Chicago, IL, USA.

Stovall low profile roller ROL230, Life Science Technology Inc.,

Greensboro, NC, USA.

Yankee variable speed rotator, Clay Adams, Parsippany, NJ, USA.

Radiographic cassette SA, Okamoto manufacturing Co., Ltd., Tokyo, Japan.

Stereomicroscope, Olympus SZ 11/Epi-illuminator LSGA, Olympus optical Co., Ltd., Tokyo, Japan.

Nikon/Epi-fluorescent microscope, Microphot-SA, Nikon Corp., Tokyo, Japan.

Chemical Agents

GeneAmp™ RNA PCR Kit and Taq DNA polymerase were purchased from Perkin-Elmer Cetus. The restriction endonuclease of EcoRI, PstI, HindIII and SmaI were purchased from BRL Life Science Inc.. The enzymes T_4 DNA polymerase,T_4 DNA ligase,T_4 polynucleotide kinase and calf intestinal alkaline phosphatase were obtained from New England Biolabs, Inc.

Glasgow minimum essential media, MEM non-essential amino acid solution and fetal bovine sera were purchased from Gibco BRL Life Technology, Inc.. PenicillinG, streptomycin sulfate, fungizone, gentamycin ampicillin and DEAE-dextran were purchased from Sigma Chemical Company.

ECL random prime system, ECL 3'-oligolabelling system and Hybond-N^+ were obtained from Amersham.

[5,6-^{3}H] uridine 5'-triphosphate was purchased from Amersham. Agarose (Ultra Pure), formamide (Ultra Pure) and phenol (Ultra Pure) were purchased from BRL Life Technology, Inc.. Tris-base, SDS, dithiothreitol, PMSF, NP-40, Triton X-100, ethidium bromide, MOPS, EDTA, 2-mercaptoethanol and mineral Oil were purchased from Sigma Chemical Company. The analytical grade chemical agents such as urea, magmesium chloride, potassium choride, sodium chloride, sodium citrate, absolute ethanol, isopropanol, etc. were obtained from Merck.

Virus and Cell Culture

The challenge virus standard (CVS-11), a rabies virus laboratory strain was used in this study. The virus was propagated in baby hamster kidney cells (BHK-21/S13). Briefly, a 75 cm^2 flask of confluent monolayer

of BHK-21 was grown in Glasgow minimum essential medium (GMEM) supplemented with 10% (v/v) fetal calf serum for 48 hrs. The CVS of 0.1 m.o.i. ($TCID_{50}$ /cell) was inoculated onto the monolayer cells and adsorbed for 1 hour at 37 °C. Unadsorbed viruses were washed out with medium and culture was refed with GMEM supplemented with 2% (v/v) fetal bovine serum. Virus was harvested after incubation for 72 hrs at 37 °C under 5% CO_2 atmoshere. The viral titer was determined as 50% endpoint tissue culture infectious dose ($TCID_{50}$) as described by Wiktor (1973).

The routine BHK culture was trypsinized and splited every 48 hrs. The stock of BHK and the virus seed were aliquoted and frozen at -86 °C until used. Details of medium preparation, trypsinization and virus titer determination are described in Appendix A and B.

Cytoplasmic RNA Isolation from Cell Grown in Tissue Culture

This simple procedure, described by Gough (1987), involves the isolation of cytoplasmic RNA from the postnuclear cell lysates. Up to 5 x 10^6 cells were lysed in 200 µl of buffer containing 100mM Tris-HCl (pH 7.5), 0.15M NaCl, 1,5mM $MgCl_2$, 0.65% NP-40. The nuclei pellet were discarded after centrifugation at 800*g* for 5 min. A 200 µl of extraction buffer (10mM Tris-HCl pH7.5, 7M Urea, 1% [w/v] SDS, 0.35M NaCl, 10 mM EDTA) was added to the supernatant and mixed well. Consecutively, 400 µl of phenol:chloroform:isoamylalchol (50:50:1) was added and vigorously vortexed. The aqueous and organic phases were separated by centrifugation at 10,000*g* for 5 min. The RNA was recovered from the aqueous phase by mixing with an equal volume of isopropanol and stored overnight at -20 °C. The RNA pellet was collected at 14,000*g* for 10 min., wased twice with 70% ethanol and dried *in vacuo*.

To determine the concentration, RNA was dissolved in 100 µl of pure water and measured the optical density at the appropriate dilution. One A_{260} is equivalent to 40 µg/ml RNA.

Electrophoresis of RNA through Gel Containing Formaldehyde

Cytoplasmic extract of RNA was analyzed on denaturing gel system according to method of Sambrook *et al* (1987). A 5-10 µg of RNA was dissolved in running bufer (20mM MOPS, 5mM sodium acetate, 1mM EDTA, pH 7.0) included 6.7% (v/v) formaldehyde and 50% (v/v)

formamide. After denaturation at 60 °C for 1 hr, RNA was subjected to 0.8% agarose gels in running buffer containing 2.2M formaldehyde. Electrophoresis was conducted at 4V/cm till tracking dye reached 2/3 of the gel. The gel then was stained with ethidium bromide and visualized on ultraviolet light box.

Hybridization and Detection of Rabies Nucleic Acids by Oligonucleotide probes

Detection of PCR fragments, recombinant plasmids as well as RNA on blots were conducted using enhanced chemilluminescene (ECL) system, commercially available by Amersham. ECL is associated with horseraddish peroxidase catalyzed oxidation of luminol to detect presence of oligonucleotides tailed at the 3'end with fluorescein-dUTP (Fl-dUTP) hybridized to target sequences on membrane. Terminal transferase catalyzes the tailing of Fl-dUTP onto the 3' end of an oligonucleotide probe. Hybridized probes are incubated with an anti-fluorescein horseraddish peroxidase conjugate. Enzymatic reduction of peroxide coupled to oxidation of luminol in presence of an enhancer molecule. As the luminol breaks down it passes through an excited stage and as this falls to ground state, light is emitted. The light output is detected on blue-light sensitive film providing a permanent hard copy result.

Briefly, 50 pmoles of oligonucleotide was labelled with 8 units of terminal transferase in 40 µl of cacodylate buffer (pH 7.2) containing Fl-dUTP at 37 °C for 90 min. The blot was prehybridized in buffer consisted of 5X SSC, 0.1% buffer component (supplied), 0.02% (w/v) SDS, and 0.5% (w/v) blocking agent (supplied). After prehybridization at 42 °C for 30 min, labelled oligonucletide probe was added to the buffer at 1 pmole/ml and incubated for 90 min with agitation. The blot was removed and washed with excess rinse at room temperature as follows: two 5-min washed in 5X SSC, 0.1% (w/v) SDS and two 15-min washed in 2X SSC, 0.1% (w/v) SDS. The membrane then was blocked for 30 min in buffer1 (0.15M NaCl, 0.1M Tris-HCl, pH 7.5) containing 0.5% (w/v) blocking agent. Anti-fluorescein HRP conjugate (1:1,000 dilution) was replaced and further incubated for 30 min. Non-specifically bound antibody was removed by excess washing in buffer2 (0.4M NaCl, 0.1M Tris-HCl, pH 7.5). Signal was generated by covering the blot with detection buffer (supplied) for 1 min. The membrane was drained off, wrapped and exposed to film.

Primers and Probes

Each region of rabies virus structural genes was PCR amplified by using synthesized oligonucleotide primers. Primers was desinged on the basis of the published sequences of the PV strain (Tordo, *et al.*, 1986 and Tordo, *et al.*, 1989). The position and sequences of the primers specific for each structural genes were shown in Table 2. The upstream primers contained an *Eco*RI restriction sequence (bold letters) preceed by inserted clamp nucleotides whereas the downstream primers contained a *Pst*I restriction sequence (bold letter) preceeded by inserted clamp nucleotides. It was expected that the PCR products derived from N-, G-, NS-, L-, and M-gene should be 462, 963, 628, 598, and 433 bp, respectively.

The oligonucleotide probes complementary to the inner portion of the amplified region were synthesized based on the published sequences of the PV strain. The sequences and location of the probes were described in Table 3.

PCR Amplification of Rabies Virus Genes

Amplification of RNA by the polymerase chain reaction involves a 2 step protocol. First the RNA would have to be converted to cDNA using the reverse transcriptase. This first-strand cDNA may be accomplished by extension via random hexamer, downstream primer or oligo(dT). The cDNA can then be placed directly into reaction vessel containing PCR mix and the reaction carried out as per DNA amplification.

RNA (1 µg) was hybridized to each 10 pmoles of upstream and downstream primers and reverse transcribed for 30 min at 42 °C in 10 µl reaction with 50 units of Moloney murine leukemia virus reverse transcriptase, 1mM of each deoxynucleotides, 20 units of RNasin, 10mM Tris-HCl (pH8.3), 50mM KCl and 5mM $MgCl_2$. cDNA was amplified in 50 µl reaction mixture containing 200µM each deoxynucleotides, 20µM of each primers, 10mM Tris-HCl (pH8.3), 50mM KCl and 1 unit *Taq* DNA polymerse. Amplification was performed in a thermal cycler as follows: 2 cycles of denaturation 95 °C, 60 sec; annealing 60 °C, 30 sec; elongation 72 °C, 30 sec. Annealing temperature was serially decreased every second cycle from 60 °C to 'touch down' at 50 °C followed by 20 cycles of denaturation 95 °C, 60sec; annealing 50 °C, 30 sec; elongation 72 °C, 30 sec. The amplification products were subjected to electrophoresis on 1.2%

Table 2. Primers for PCR amplification of rabies viral RNA

Rabies genes	Oligonucleotide designation (orientation)	Sequences	Position
N	TC-7 (+)	ATC.**GAA.TTC**.CAC.CTC.TAC.AAT.GGA.TGC.CG	61-80
N	TC-1 (-)	GAT.**CTG.CAG**.GCT.CAA.CCT.ATA.CAG.ACT.CA	504-523
NS	TC-13 (+)	ATC.**GAA.TTC.**ATC.CTA.GCC.TCC.TGT.TCC	1776-1793
NS	TC-14 (-)	GAT.**CTG.CAG**.CAA.GAT.GTA.TAG.CGA.TTC	2386-2404
M	TC-19 (+)	ATC.**GAA.TTC.**ATG.ACG.ATG.ACT.TGT.GGC	2574-2593
M	TC-20 (-)	GAT.**CTG.CAG**.TGG.AGT.TGA.GCC.CAT.ATG	3155-3172
G	TC-9 (+)	ATC.**GAA.TTC.**GAC.TCA.AGG.AAA.GAT.GGT.TCC	3305-3324
G	TC-3 (-)	GAT.**CTG.CAG**.CTT.AAA.TGA.CTG.AGA.CGT.CTG	4248-4267
L	TC-16 (+)	ATC.**GAA.TTC.**GTC.TAT.AGA.CCT.CGG.AGG	6989-7006
L	TC-17 (-)	GAT.**CTG.CAG**.TGA.GGT.CTG.ATC.TGT.CTG	7405-7422

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Table 3. Oligonucleotide probes for detection of rabies virus nucleic acids.

Rabies genes	Oligonucleotide designation (orientation)	Sequences	Position
N	TC-2 (+)	CTT.GAT.CCT.GAC.GAT.GTA.TG	263-282
NS	TC-15 (+)	GAG.CGA.TCT.CAG.CCT.CCA.CT	2101-2120
M	TC-21 (+)	CCA.TAG.TTG.ACC.TGC.TCT.CG	3019-3038
G	TC-4 (+)	GCT.CCA.CTA.ACC.ACG.ATT.ACA	3879-3900
L	TC-18 (+)	TCTT.GTC.ATA.GTC.AGC.GCG.TC	7158-7177

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PCR products were transfered to a nylon membrane and identified by hybridization with specific internal probes. DNA was denatured by soaking the gels in a solution of 1.5M NaCl and 0.5M NaOH for 30 min prior to neutralized by soaking in a solution of 1M Tris-HCl and 1.5M NaCl. DNA was blotted to a nylon membrane by capillary action as described by Sambrook *et al* (1989).

Development of Rabies cDNA Probes

The PCR fragments were blunt-end subcloned into *Sma*I site of pGEM-3Z. The map of pGEM-3Z is illustrated in Figure 7. PCR mixture was treated with equal volume of chloroform, and the upper aqueous phase was recovered. The amplified product was ethanol precipitated. Twenty-five µl of kinase reaction mixture, contained 500 ng DNA, 70mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 5mM DTT, 1mM ATP and 20 units polynucleotide kinase, was performed at 37 °C for 30 min. Then, the reaction was heated to 75 °C for 10 min and cooled at 4 °C. The DNA was subjected to a fill-in reaction contained 1mM dNTPs and 3 units of T_4 DNA polymerase. After 30 min at 37 °C, the DNA was extracted once with phenol:chloroform as usual procedure.

Ten µg of pGEM-3Z plasmid vector was linearized by digestion with 10 units of *Sma*I at 25 °C for 2 hours in 10 µl reaction contained 50 mM potassium acetate, 20mM Tris-acetate, 10mM magnesium acetate, 1 mM DTT, pH 7.9. Then, dephosporylated by addition of 1 unit of calf intestinal phosphatase. The reaction was allowed to proceed at 50 °C for 1 hour and heated to 75 °C for 10 min. in presence of 5mM EDTA. Protein was removed by phenol extraction per usual protocol.

The blunt-ended DNA fragments and dephosphorylated plasmid vector were loaded onto a 1.5% low melting point agarose gel in TAE buffer (40mM Tris-acetate, 1mM EDTA, pH7.6). The bands were identified and excised. These agarose slices containing purified DNA were proceeded for ligation.

Agarose slices were remelted at 65 °C for 10 min. Fifty ng of vector DNA and 10 ng of insert (2:1 molar ratio) were ligated in 50mM Tris-HCl buffer (pH 7.5) included 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 25 µg/ml BSA and 400 units T_4 DNA ligase. The reaction was carried out

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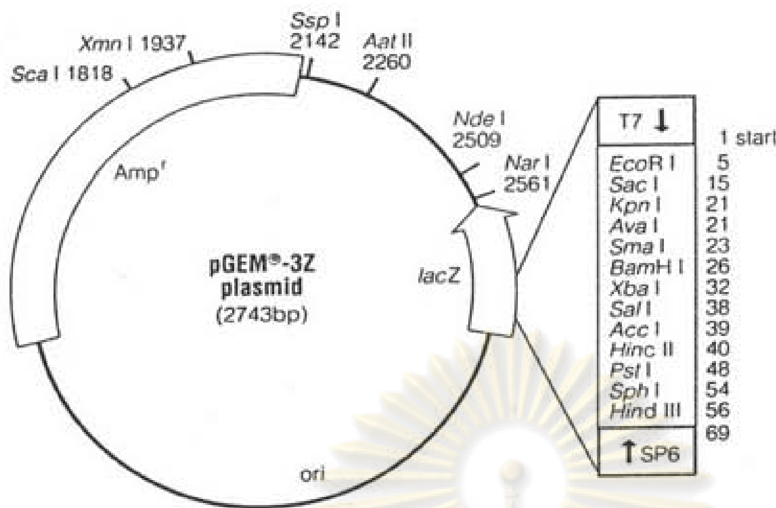

Notes:

1. Sequence reference points:
   a. T7 RNA polymerase transcription initiation site — 1
   b. SP6 RNA polymerase transcription initiation site — 69
   c. T7 RNA polymerase promoter — 2727-2743
   d. SP6 RNA polymerase promoter — 70-86
   e. multiple cloning sites — 5-61
   f. *lacZ* start codon — 108
   g. lac operon sequences — 2561-2724; 94-323
   h. *lac* operator — 128-144
   i. β-lactamase (Amp^r) coding region — 1265-2125
   j. binding site of pUC/M13 forward sequencing primer — 2686-2702
   k. binding site of pUC/M13 reverse sequencing primer — 112-128
2. Specialized applications:
   a. blue/white screening for recombinants
   b. transcription *in vitro* from dual opposed promoters
3. The pGEM-3Z vector was previously called the pGEM-Blue vector
4. The pGEM-3Z and -4Z vectors are identical except for the orientation of the SP6 and T7 promoters

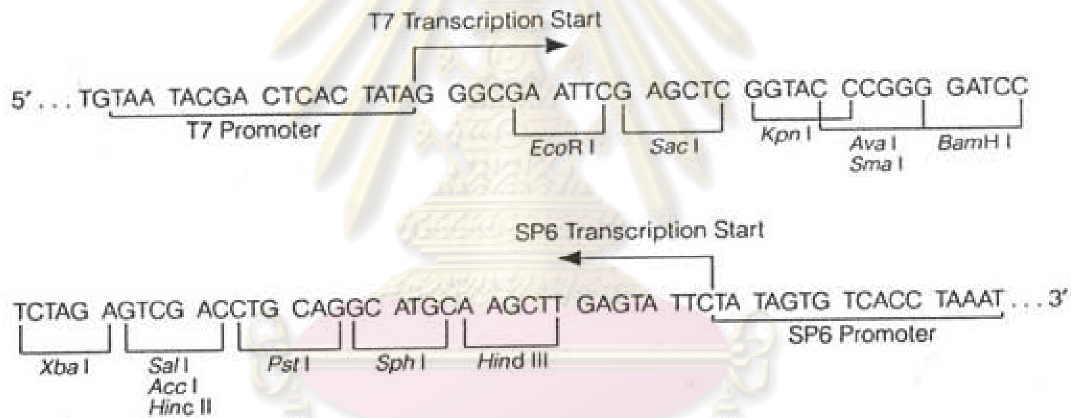

Figure 7. pGEM-3Z vector map.

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at 16 °C for 12 hrs and placed at room temperature for 2 hrs prior to heat inactivation at 65 °C for 10 min.

DNA transformation

Recombinant plasmids were transformed into *E. coli* JM109 using calcium chloride procedure as described by Hanahan (Hanahan, 1983). Briefly, 25 ml culture of *E. coli* at mid log phase (A_{600} = 0.45-0.55) was collected at 2500*g* for 10 min at 4 °C. Cells were washed once with an ice-cold 50mM $CaCl_2$ and suspended in 5 ml of same solution. Ligation reaction mixture containing 20 ng DNA was incubated with 200 µl of competent cells on ice for 30 min. After being heat shock at 42 °C for 90 sec., 800 µl SOB broth (see Appendix A) was added and placed at 37 °C with agitation for 1 hr. The cells were plated onto SOC agar (see Appendix A) containing 100 µg/ml of ampicillin, 0.5mM IPTG and 40 µg/ml X-gal. Cells were grown overnight at 37 °C. White colonies were picked up and screened for recombinant plasmids.

Screening of Transformants

A rapid PCR procedure for screening bacterial colonies for the presence of DNA sequence as described by Kurth and Bowcock (1990) was modified for screening rabies recombinant plasmids. The modification were as follows: After transformation into JM109 and growth on X-gal/IPTG plate, a bit of colony was suspended in 10 µl PCR buffer (10mM Tris-HCl, pH 8.3; 50mM KCl, 2mM $MgCl_2$). Cell suspension was boiled for 5 min. To this lysate was added 40 µl of PCR buffer containing 20µM each deoxynucleotides, 10 pmole each of the two primers corresponding to the sequence of the insert and 1 unit of *Taq* DNA polymerase. Twenty-five cycles of amplification were performed with temperature conditions as described for the initial amplification. Five microliters reaction mixture were electrophoresed on 1.2% agarose gels and stained with ethidium bromide.

Restriction Digestion of Recombinant Plasmids

Ten micrograms of plasmid was digested with 10 units of *Pst*I in buffer containing 50mM Tris-HCl, pH 8.0; 10mM $MgCl_2$, 1mM DTT and 50mM NaCl. Reaction was carried out at 37 °C for 2 hrs. Consecutively, 10 units of *Eco*RI was added and sodium chloride was raised up to 100mM

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final concentration. The reaction was further incubated at 37 °C for 2 hrs. Reaction was stopped by heat inactivation at 75 °C for 10 min.

Preparation of RNA for RNA-DNA Dot Hybridization

The samples tested consisted of infected and non-infected brains from animals sent to the laboratory for rabies diagnosis. One gram of brain material was treated as described above. Briefly, brain samples were homogenated in 2 ml of lysing buffer. The nuclei and cell debris were pelleted and discarded. The RNA supernatant was extracted with 4 ml of urea solution and 4 ml of phenol mixture. After centrifugation, the aqueous phase was precipitated with a volume of isopropanol. The pellets were washed twice in 70% ethanol, dried and resuspened in 500 μl of sterile water.

10 μg of RNA was dissolved in appropriate volume of 50% deionized formamide and 6% formaldehyde. RNA was denatured for 30 min at 55 °C, and chilled on ice. One volume of 20X SSC was added and filterd through Hybond-N^+ membrane, prewetted in 10X SSC. Membrane was air-dried and the RNA was fixed in an oven at 80 °C for 2 hr.

RNA-DNA Dot Hybridization of Rabies Probes

Rabies cDNA probes were evaluated their sensitivty and specificity for identification of rabies RNA present in canine brain specimen. These cDNA probes were labelled using the ECL random prime labelling and detection sytems (Amersham). The labelling reaction is catalyzed by Klenow DNA polymerase I. Fl-dUTP is used as the label, partially replacing TTP in the soaked reaction, so that the probe produced is labelled with fluorescein. The fluorescein residues will be subsequently detected as haptens by an enzyme-linked anti-fluorescein antibody.

In brief, 100 ng of heat-denatured cDNA was incubated at 37 °C in 50 μl reaction containing nucleotide mix, random nanomer primers and Klenow polymerase (4 units). Membranes were pre-incubated for 1 h in hybridization solution (5X SSC, 0.5% blocking agent, 0.1% SDS, 5% dextran sulfate, 100 μg/ml denatured salmon sperm DNA) and then incubated in the same solution containing 10 ng/ml labelled probe overnight at 50 °C. Membranes were washed at room temperature in 2X SSC, 0.1% (w/v) SDS, 5 min and 1X SSC, 0.1% (w/v) SDS, 15 min. Nylon

membranes were blocked, antibody-incubated, washed signal-generated and detected respectively, as standard ECL protocol mentioned above.

Preparation of the RNA-free Rabies Virus Seed

A 80 ml (eight 10-ml portions) of clarified virus suspension was centrifuged in the TY65 fixed angle rotor of Beckman centrifuge at 50,000 *g* (30,000 rpm) for 1 hr at 4°C. The supernatant fluid was carefully aspirated and the pellet was dispersed in 4 ml of buffer (50mM Tris-HCl, 0.13M NaCl, 10mM EDTA, pH 7.4). A 50 µg/ml of RNAse A was added, and incubated at 37°C for 60 minutes. The virus suspension was subjected to centrifugation at 50,000*g* as aforementioned. The virus pellet was resuspended in growth medium and kept over night at 4°C.

Synchronization of BHK-21 Cell Culture

BHK-21 cells were induced to resume traverse of the life cycle in synchrony by releasing from serum starvation according to method of Tobey and Ley (1970). A monolayer of stationary phase cells (36 hrs) was washed once with PBS and a minimal medium (GMEM, without the supplementation of glutamine, tryptose phosphate and serum) was replaced and incubated in CO_2-incubator at 37° C. After 6 hrs incubation, the cells were released by refeeding in complete medium and further incubated for 6 hrs till reached the exponential phase. Synchrony induction was directed by consideration of RNA content and cell number. Rate of incorporation of ^{3}H-uridine into BHK cells was measured as described by Tobey and Campbell (1965, see Appendix C). Cell concentrations were determined by counting aliquots of diluted cell suspension in hemocytometer (see Appendix C).

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Determination of Transcriptional Events of Rabies Virus

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The transcriptional events was examined at time intervals after the CVS rabies virus strain was adsorbed onto the surface of the synchronized BHK-21 cells. Detection of the viral transcripts from the whole cell lysates was achieved by RT-PCR technique. Resolution patterns of the amplified products was performed on 1.2% of ethidium bromide stained agarose gel.

The transcriptional events was conducted in 75 cm^2 flasks of monolayered BHK-21 cells. The cells were synchronized as mentioned above. At O time, the RNA-free viral seed was inoculated on monolayer

culture of synchronized BHK-21 cells. The viruses were allowed to adsorb onto the cell surfaces for 1 hr with occasional shaking. The unadsorbed viruses were wased out with complete medium, and cells were refed. Mocked-infection was carried out in parallel experiments. At postinfection time intervals, aliquots flask of rabies infected BHK-21 were collected to examine the rabies RNA transcripts. The RNA transcripts from whole cell lysates were detected according to slightly modified method of O'Brien *et al.*(1994). Briefly, the cells were harvested and washed twice in PBS. The cells were then resuspened in 50 μl of PBS containing 50 μg/ml yeast tRNA. The cells were lysed by heating to 90°C for 10 min and cell debris was pelleted by centrifugation. The supenatants were treated with proteinase K (1 mg/ml) for 30 min at 55°C prior to 10 min heat inactivation. These supernatants were subjected to cDNA synthesis. RT-PCR was slightly modified from above procedure. First-strand cDNA synthesis was carried out in a 10 μl reaction buffer containing 4 μl of the whole cell lysate, 1mM each dNTPs, 10 units of RNasin, 25 units of MMLV reverse transcriptase and 2.5μM oligodT. The reactions were then incubated at 42°C for 45 min followed by a 5 min incubation at 98°C to inactivate the enzymes. The PCR was conducted in 50 μl PCR buffer containing 3mM $MgCl_2$, 200μM each dNTPs, 1 unit of *Taq* polymerase and 10μM each up stream and downstream primers. The reactions were cycled 40 times at 95°C for 1 min, 55°C for 30 sec and 72°C for 30 sec in a DNA thermal cycler. PCR products were analysed by agarose gel electrophoresis.

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