Chapter IV

Insert size determination and sequence analysis of the immunoreactive recombinant clone.
Introduction:

An immuno-reactive recombinant phage plaque was identified by differential immunoscreening of λgt11- *P. falciparum* genomic expression library using anti-*P. yoelii* sera and sera from normal individuals residing in malaria endemic area, was chosen for further molecular characterizations. The characterization involved adequate amount of phage DNA preparation from the immunoreactive phage and restriction digestion to release the insert. The released insert was purified from the gel, subcloned into a sequencing vector and sequenced. To ascertain its origin from *P. falciparum* and its conserved nature across other species of *Plasmodium*, the released insert was radio-labeled and hybridized to *P. falciparum*, human and other *plasmodium* species DNA. The DNA sequence analysis, blast search and Clustal W alignment was carried out. The predicted function of the identified protein and its homology with other known proteins was worked out. The sequence of the KP1 insert was deposited in the Genome bank and accession number (AF256227) was obtained.

Experimental Methodology:

1. Insert size determination of the seropositive clone (KP1):

   The purified immunoreactive plaque (KP1) was further grown in-large scale for phage DNA preparation and subjected for insert size analysis. The insert size analysis was carried out by the restriction enzymatic digestion of the recombinant phage DNA to release the insert as well as by PCR amplification method. The procedure followed for phage DNA preparation and insert analysis are described below.
a. Preparation of recombinant phage DNA from the seropositive clone:

The amount and quality of phage DNA depends upon the preparation of phage lysate from the individual plaque, which can be prepared by two procedures viz. a) Liquid lysate method and b) Plate lysate method. The former method was used for the lysate preparation, followed by the DNA extraction by using Lambda DNA Kit (Qiagen, USA).

Single colony of host cell (Y1090 r'm') was inoculated into 10ml of LB medium supplemented with 10 mM MgSO₄ and 0.2% maltose. The culture was incubated at 37°C in an orbital shaker until the O. D (600 nm) reached 0.1-0.2. A single positive plaque was transferred as an agar “plug” with a sterile pasture pipette into the host cell culture. A control i.e. agar plug (without a plaque) was also included for comparison and grown along with the sero-positive plaque. The cultures were incubated in an orbital shaker incubator with vigorous shaking (200 rpm) with good aeration for 4-6 hour at 42°C. After 4-6 hours of shaking, the lysis of bacteria was evident and the medium became clear in comparison with the control (without phage) media. After complete lysis of bacterial cells, 200 µl of chloroform was added and incubated for 2 minutes at 37°C to lyses the remaining bacterial cells. The lysed culture was centrifuged (3000 rpm for 10 minutes) to remove the bacterial debris and the supernatant was transferred to a fresh tube for DNA extraction. Two-milliliter aliquots of supernatant from the lysate were stored at 4°C as a stock in sterile vial, with a drop of chloroform.

The above lysate was used for phage DNA extraction. The lysate (25ml) was added with 25 µl of Buffer L1 (300 mM NaCl; 100 mM Tris-HCl, pH 7.5; 10 mM EDTA;
0.2 mg/ml BSA; 20 mg/ml RNase A; 6 mg/ml DNase I) and the mixture was incubated at 37°C for 30 minutes. Five milliliters of ice-cold buffer L2 (30% polyethylene glycol 6000; 3 M NaCl) was added to the above mixture, mixed by inverting the tubes 2-3 times and kept in ice for 60 minutes to allow phage to precipitate. The mixture was centrifuged (8000g for 10 min), supernatant was discarded and the pellet was resuspended in 3 ml of buffer L3 (100 mM NaCl; 100 mM Tris-HCl, pH 7.5; 25 mm EDTA). Three ml of buffer L4 (4% SDS) was added and to the mixture, which was incubated at 70°C. The tube was cooled on ice; 3 ml of buffer L5 (3 M potassium acetate, pH 5.5) was added and centrifuged at 8000g for 10 minutes at 4°C. The supernatant was collected very carefully avoiding any white precipitates to a separate tube. The Qiagen-tip 25 (Qiagen, USA) was equilibrated with 3 ml of buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100) and the supernatant was passed through the column. The column was washed by passing 5 ml of buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol) and the DNA was eluted with 3 ml of buffer QF (1.25 M NaCl; 50 mM MOPS, pH 8.5; 15% ethanol). The DNA was precipitated by adding 0.7 volume of isopropanol and centrifuged at room temperature for 30 minutes. The pellet was washed with 70% ethanol, air dried and dissolved in 100 µl of sterile water. The amount of DNA and purity was determined by taking O.D at 260/280 using a spectrophotometer.

b. Restriction enzymatic digestion of recombinant phage DNA:

The genomic expression library of P. falciparum was constructed in the EcoRI cloning site of λgt-11 vector and hence the recombinant phage DNA (KP1 clone) prepared from the positive plaque was digested with EcoRI restriction enzyme to release the insert. A positive control phage DNA (with known size of insert) was also digested.
simultaneously to ensure the reaction conditions and enzymes working fine. The reaction condition is stated below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage DNA</td>
<td>10 µl</td>
</tr>
<tr>
<td>10x buffer</td>
<td>6 µl</td>
</tr>
<tr>
<td>EcoRI enzyme</td>
<td>1 µl</td>
</tr>
<tr>
<td>Sterile H2O</td>
<td>13 µl</td>
</tr>
<tr>
<td>Total</td>
<td>30 µl</td>
</tr>
</tbody>
</table>

The above mixture was mixed well, briefly centrifuged and incubated at 37°C for 4 hrs. After the reaction was completed, the enzyme was inactivated at 70°C for 10 min. The release of insert was checked by agarose gel electrophoresis. Agarose gel (1%) was prepared in 1X TBE (Tris/borate/EDTA: 89 mM Tris, 89 mM boric acid, 2 mM EDTA) electrophoresis buffer containing ethidium bromide (0.5 µg/ml). To the digested DNA, 6 µl of 6X gel loading Dye (20% Ficoll 400, 0.1 mM disodium EDTA pH 8.0, 1.0% SDS, 2.5% bromophenol blue, 2.5% xylene cyanol) was mixed and loaded in to wells of the gel along with undigested, positive control and DNA size markers (λ Hind III digested DNA). The gel was subjected to electrophoresis in 1X TBE buffer at a constant voltage (70 Volts) and when the bromophenol dye reached 3/4th of the gel, the gel was viewed on a UV transilluminator and photographed using a polaroid camera.

c. PCR amplification of insert from the positive plaque:

The other method of identification of the size of the cloned insert was PCR, in which the insert cloned in λgt 11 vectors was amplified by using the primers designed on the flanking regions on either side of the multiple cloning sites. The insert was amplified
using the phage DNA, as well as the using single positive plaque by PCR amplification and the procedure was as follow.

1. An isolated positive plaque was picked up and dissolved in SM buffer and incubated at room temperature for 1 hour for allowing the phage to elute out of the agar plug.

2. 20 μl of the lysate was taken in a 0.65 ml microfuge tube and boiled in a water bath for 10 minutes. It was centrifuged and the 2 μl of the supernatant was used for PCR amplification.

3. The PCR amplification was carried out using the following recipe and conditions.

   Template DNA: 2 μl (Boiled lysate)
   10 x PCR buffer: 5 μl
   Primer Forward: 1 μl (2 pmol)
   Primer Reverse: 1 μl (2 pmol)
   Taq DNA polymerase: 1 μl (2 units)
   Sterile H₂O: 40 μl
   Total volume: 50 μl

   The above reaction mixture was briefly centrifuged and amplification was carried out in a Thermal Cycler (Perkin Elmer, USA) with hot lid incubation option. The reaction condition was carried out with initial denaturation (95°C - 5 minute) followed by 30 cycles (95°C - 1 min, 55°C - 1 min, 72°C - 1 minutes) and final extension at 72°C for 7 minutes. The amplified product was resolved on 0.7% agarose gel as mentioned in earlier section.
II. Insert DNA purification from the agarose gel:

The insert released from the EcoR I digested recombinant phage DNA (clone 1) was purified from the agarose gel. The purified insert was used for southern blotting, northern blotting, subcloned into sequencing and expression vector. The gel purification kit (Qiagen, USA) was used to purify the insert DNA from the agarose gel slice following the manufacturer’s protocols.

III. Human and other malarial parasite DNA:

Human DNA was essential for use as a control since there are all possibilities of human DNA contamination in the parasite DNA preparation, in spite of having undertaken all precautions to avoid it during different stages of parasite material preparation. DNA from other malarial parasites was required to evaluate the conserved nature of the identified insert. Human DNA and other malarial parasite DNA (*P. cynomolgi* and *P. berghei*) was kindly provided by Dr. Pawan Malhotra, ICGEB, New Delhi, India. The DNA from other stains of *P. falciparum* (3D 7) was kindly obtained from Dr. Chetan Chitinis, Malaria Group, ICGEB, New Delhi, India.

IV. Southern hybridization:

Detection of specific DNA fragments by gel-transfer hybridization (Southern blotting) was developed by Prof. Southern in 1975. In this method the double-stranded DNA fragments are generated by restriction nuclease treatment followed by separation according to length by agarose gel electrophoresis. The separated DNA fragments are transferred to a nitrocellulose sheet by blotting. The nitrocellulose sheet containing the bound DNA fragments is placed in a sealed plastic bag or tube together with buffer
containing a radioactively labeled DNA probe specific for the required DNA sequence. The sheet is exposed for a prolonged period to the probe under conditions favoring hybridization. The sheet is removed from the bag and washed thoroughly, so that only probe molecules that have hybridized to the DNA on the paper remain attached. After autoradiography, the DNA that has hybridized to the labeled probe will show up as bands on the autoradiograph. The procedure for radiolabeling of KP1 insert followed by hybridization to enzymatic digested blot of *P. falciparum* and Human DNA are described below.

**a. Preparation of probe:**

The insert DNA (KP1) was labeled with radioisotope using random prime labeling method and used as probe for southern blotting and dot blot hybridization. Random Prime labeling was done using a kit (Gibco-BRL, USA) as follows:

**Step1: Template preparation:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert DNA (clone 1)</td>
<td>2 µl (200 ng)</td>
</tr>
<tr>
<td>Sterile H₂O</td>
<td>18 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>20 µl</td>
</tr>
</tbody>
</table>

The above mixture was briefly centrifuged, kept in boiling water bath for 10 minutes and **snap chilled on ice to denature the DNA.**
Step 2: Labeling reaction:

- dTTP: 2 μl
- dGTP: 2 μl
- dATP: 2 μl
- α<sup>32</sup>PdCTP: 2 μl (50 μCi)
- Random Priming buffer: 15 μl
- Klenow enzyme: 1 μl (3 units)
- Sterile H<sub>2</sub>O: 6 μl

**TOTAL:** 30 μl

To the above reaction mix the 20 μl of denature probe was added, briefly centrifuged and incubated at room temperature for 2 hours.

Step 3: Probe purification:

Unlabelled radioisotope molecules were removed using a PCR purification Kit (QIAGEN, USA) and the procedure is as follow:

1. To the labeled probe reaction, 250 μl of buffer PB was added and loaded on to the column. It was centrifuged for 1 minute and the supernatant was discarded.
2. The column was washed with 750 μl of PE buffer and centrifuged for 1 min and the flow through was discarded.
3. The column was loaded with 100 μl of elution buffer and centrifuged for 1 minute and the elute was collected in a fresh tube.
The eluted probe was denatured by boiling in a water bath for 10 minutes and quenched on ice. The probe is now ready to use for hybridization experiments. By following similar procedure 1 kb ladder DNA was also labeled to localize the DNA base pair markers.

b. Restriction digestion and gel electrophoresis of Genomic DNA:

In this experiment, equal amount *P. falciparum* DNA and human DNA were digested with EcoR I, subjected to electrophoresis and transferred to nitrocellulose paper for probing (Blot 1). Similarly, *P. falciparum* DNA was also blotted (blot 2) to nitrocellulose after digesting with different restriction enzymes and electrophoresis.

i. Restriction Digestion for blot 1:

<table>
<thead>
<tr>
<th></th>
<th><em>P. falciparum</em></th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>10 µl (5 µg)</td>
<td>10 µl (5 µg)</td>
</tr>
<tr>
<td>10 X EcoR I buffer</td>
<td>4 µl</td>
<td>4 µl</td>
</tr>
<tr>
<td>EcoR I enzyme</td>
<td>3 µl (60 units)</td>
<td>3 µl (60 units)</td>
</tr>
<tr>
<td>Sterile water</td>
<td>23 µl</td>
<td>23 µl</td>
</tr>
<tr>
<td>TOTAL</td>
<td>40 µl</td>
<td>40 µl</td>
</tr>
</tbody>
</table>

The above reaction mixtures were briefly centrifuged, incubated at 37°C water bath overnight to allow for complete digestion and then heat inactivated at 70°C for 10 minutes. To each tube containing the reacted mixture 8 µl of 6X DNA loading dye was added and the sample was subjected to electrophoresis along with a DNA size marker (1 kb ladder) on an agarose gel (0.7%, 1X TBE buffer). After staining with ethidium
bromide the stained gel was observed under UV transilluminator, photographed and then subjected for capillary transfer.

ii. Restriction digestion for blot 2:

*P. falciparum* DNA was digested with EcoR I, Pst I, Sma I, Mse I and Nde I restriction enzymes with their respective buffers and the digested DNA was subjected to electrophoresis as mentioned above. The capillary transfer of DNA from gel to Nitrocellulose was carried as below.

c. Capillary blotting of DNA to nitrocellulose membrane:

The enzymatically digested and resolved DNA from the both the gels was transferred to Nylon membranes by capillary transfer method (Southern, 1975). The method was as follows:

1. The gels were treated with solution I (0.25 M HCl) for 15 minute to depurinate the DNA.

2. Subsequently the gels were treated with Solution II (0.5 NaOH, 1M NaCl) for 15 minutes on a shaker.

3. The gels were neutralized with Solution III (3 NaCl, 0.5 M Tris.Cl pH 7.4) for 30 min with gentle agitation, with frequent changes of fresh solution.

4. Nylon membranes (Hybond N+, Amersham Pharmacia, USA) were cut to the size of each gel and allowed to wet in the deionized water. Both the gels were blotted separately following similar procedures.
5. A 3 mm Whatman 3 filter paper, longer and wider than the gel was placed on a glass plate and wetted with 10X SSC (Sodium chloride/sodium citrate buffer: 1.5 M NaCl, 0.15 M Na₃ citrate.2H₂O, pH 7.0). The glass plate along with the Whatman paper was placed over a tray filled with 10X SSC so that both the ends of the Whatman paper formed wicks.

6. Three pieces of 3 mm Whatman paper of the gel were dipped in 10X SSC and placed on a damp filter paper and the treated gel was placed above the filter paper stack. The air bubbles trapped in between the filter paper and gel were removed by rolling a glass rod gently.

7. The nylon membrane was placed on the gel and on the top of it three pieces of 3 mm Whatman (the size of gel prewetted in the 10X SSC) were placed. Air bubbles were removed as before. A stack of dry paper towels were placed above this setup and a weight was placed on the top of the stack. Around the gel the sides were covered with parafilm to prevent any direct contact of stack to the buffer.

8. The transfer was allowed to take place overnight, then the paper stacks were removed and positions of the gel slots and orientation were marked on the membrane. The membrane was removed and air-dried on a sheet of Whatman filter paper. The dried membrane was UV cross-linked and subjected to hybridization using hybridization oven (Techne Instrument, UK).

9. The membrane was added with 10 ml of prehybridization buffer (5X SSC, 5X Denhardt's solution, 1% SDS and 100 µg/ml of sheared salmon sperm DNA). It was blocked at 55°C for 6 hours.
10. Denatured probe (labeled insert) was added to prehybridization solution and hybridization was continued for 16 hours at 55°C.

11. Hybridized membrane was subjected to stringent washing as follows: 2x SSC, 2 times at room temperature for 5 minute each, 2X SSC, 0.1% SDS for 15 minutes at 55°C and finally with 1X SSC, 0.1% SDS for 30 minutes at 60°C.

12. Membrane was exposed to X- Omat film (Kodak, USA). Autoradiogram was developed using developer and fixer (Kodak, USA).

V. Dot blot hybridization:

Dot blot hybridization is a simple method, where the DNA is directly spotted on the nitrocellulose membrane. The blotted membrane is hybridized as southern blot with specific DNA fragment to identify the presence or absence of homologous fragments. It is useful when the amount of DNA is low and also in processing a large no. of samples to obtain quicker results.

One microgram of genomic DNA from different species of Plasmodium (P. yoelii, P. cynomolgi and P. berghei) and strains of P. falciparum (FCD 3, 3D 7) along with human DNA (-ve control) and phage recombinant DNA (+ve control) were diluted in 20 µl with distilled water, boiled for 10 min and snap chilled on ice. The nitrocellulose membrane (Hybond N +) was pre-wetted in 6x SSC and the denatured DNA was spotted on to the membrane in small aliquots (4 µl) and then allowed to dry completely. The procedure was repeated so that in a small spot all the DNA sample was spotted. The membrane was subjected for pre-hybridization, hybridization, washing and development together with other Southern blot described in the earlier section.
VII. Subcloning of the insert in pBKS + vector and sequencing:

The purified insert obtained from the seropositive clone (KP1) was subcloned into the sequencing vector by the following method:

**a. Ligation of KP 1 insert into pBlue Script vector KS+:**

The EcoR I digested vector DNA (pBKS+, Stratagene, USA) was used for ligation of insert DNA as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid vector DNA:</td>
<td>1 µl</td>
<td>(100 ng)</td>
</tr>
<tr>
<td>Insert DNA:</td>
<td>5 µl</td>
<td>(300 ng)</td>
</tr>
<tr>
<td>T4 DNA ligase:</td>
<td>1 µl</td>
<td>(5 units)</td>
</tr>
<tr>
<td>10X buffer:</td>
<td>2 µl</td>
<td></td>
</tr>
<tr>
<td>Sterile Water:</td>
<td>11 µl</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL:</strong></td>
<td>20 µl</td>
<td></td>
</tr>
</tbody>
</table>

The reaction mixture was mixed well, briefly centrifuged and incubated at 16°C for overnight. The ligated DNA materials were transformed into the competent cell of DH5α.

**b. Competent cell preparation:**

A single well-separated colony of *E. coli* (DH5α) was inoculated into 10 ml of LB and grown overnight at 37°C shaker incubator. One milliliter of this culture was inoculated into 100 ml of LB and incubated at 37°C with vigorous shaking till the O.D value reached to 0.4 at 600 nm (3 hours). Then the culture was harvested by centrifuging.
(2000g for 10 min, 4°C), supernatant was discarded and 20 ml of sterile ice-cold 0.1 M CaCl₂ was added slowly. The suspension was incubated on ice for 4-5 hours, centrifuged as before and the supernatant was discarded. The pellet was resuspended in 2 ml of 0.1 M ice-cold calcium chloride solution and aliquoted into 200μl volume into sterile microcentrifuge tubes. The aliquots were stored at -70°C until use.

c. Transformation of ligation mixture into the competent cells:

The transformation of ligated mixture from the above step was carried out following steps:

1. 10 μl of ligation mixture was added to the 200 μl of DH5α competent cells, mixed gently and incubated on ice for 40 minutes with intermittent shaking.
2. The above mixture was heat shocked at 42°C for 90 seconds and immediately transferred to ice for 2 minutes.
3. 800 μl of prewarmed SOC medium (2% Bacto Tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 0.25 mM KCl) was added to the above transformation mixture and was allowed to grow for 45 minutes at 37°C.
4. 200 μl of the above transformation mixture was plated on LB agar Plate (100 μg ampicillin/ml) supplemented with 0.5 mM IPTG and 80 μg/ml X-gal. The plates were incubated at 37°C for overnight.

d. Screening of Transformants:

Transformants were selected based on the blue white colonies among the transformants, the white colonies were picked up individually, plasmid DNA were
prepared and checked for the presence of insert by restriction digestion. The mini-
plasmid DNA preparation was done as follows:

1. A single colony was inoculated into 10 ml of LB (100 μg/ml Ampicillin) and grown
   at 37°C overnight at 200 rpm.

2. 1.5 ml of overnight culture was taken and centrifuged at 5000 rpm for 5 minutes and
   the supernatant was discarded.

3. To the pellet, 200 μl of Solution I [50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM
   EDTA and RNase (10 μg/ml)] was added and vortexed well.

4. 200 μl of Solution II (0.2 M NaOH, 1% SDS) was added to the above tube, mixed by
   inverting the tubes several times and incubated at room temperature for 5 minutes to
   lyse the cells.

5. 250 μl of chilled Solution III (3 M Sodium acetate, pH 5.2) was added to the lysate
   and the tube was inverted several times and incubated in ice for 15 to 20 minutes.

6. The lysate solution was centrifuged at 14000 rpm for 20 minutes and the supernatant
   was transferred to a fresh tube.

7. To this supernatant, equal volume of phenol, chloroform, isoamyl alcohol (25:24:1)
   was added, mixed by inverting the tubes several times and centrifuged at 10000 rpm
   for 10 minutes.

8. The supernatant was removed to a fresh tube and added with equal volume of
   chloroform, mixed and centrifuged at 5000 rpm for 5 minutes.

9. The supernatant was transferred to a fresh tube and the plasmid DNA was precipitated by
   adding 0.7 volume of isopropanol followed by centrifugation at 14000 rpm for 20
minutes. The pellet was washed with 500 μl 70% ethanol, air dried and 50 μl of sterile water was added to the pellet.

10. 10 μl of DNA was taken for restriction analysis to check the cloned insert. The DNA was digested with EcoR I restriction enzyme for 2 hours at 37°C. The digested DNA was analyzed on a 0.7% agarose gel, stained with ethidium bromide and checked for presence of the insert under a UV transilluminator. Positive colonies (containing insert) were stocked as glycerol stocks (850 μl of overnight culture added with 150 μl of sterile glycerol) and stored at -80°C.

e. Large scale recombinant plasmid (pBKS+KP 1 construct) DNA preparation:

One of the positive transformants containing the insert was grown further to prepare a midi plasmid DNA preparation for further use. For this purpose Qiagen midi preparation Kit was used as mentioned below.

1 10 μl of Glycerol stock of the positive colony was inoculated in to 100 ml of LB containing 100 μg/ml ampicillin and grown overnight at 37°C in a shaker incubator with 200 rpm.

2 The culture was harvested by centrifugation (4000 g /15 minutes at 4°C), supernatant was discarded and tube was inverted on a paper towel to drain out any remaining supernatant from the tubes.

3 The pellet was resuspended in 8 ml of P1 buffer (containing RNase 10 μg/ml) and vortexed until all the clumps were dissolved.

4 8.0 ml of P2 buffer was added to the suspension, tube was inverted slowly for 3 to 4 times and incubated at room temperature for 5 minutes.
Further, 8.0 ml of ice-cold buffer P3 was added, mixed well by inverting, placed on ice for 30 minutes and centrifuged (10,000 g for 20 minutes).

Passing 4.0 ml of buffer QBT equilibrated the Qiagen tip-100 and the supernatant from above step was loaded immediately on to it.

The Qiagen tip was washed twice with 10 ml of buffer (Buffer QC) and finally DNA was eluted with 5.0 ml of buffer QF.

The plasmid DNA was precipitated by adding 3.5 ml of isopropanol and centrifuging at 10000 g for 15 minutes. The pellet was washed with 70% ethanol, air dried and dissolved in 500 µl of sterile water.

The quality of DNA was checked by agarose gel electrophoresis and concentration and purity of was determined by reading O.D at 260/280 nm. The DNA thus prepared was used for sequencing.

VII. Sequencing of the KP1- insert and sequence analysis.

The custom DNA sequencing and synthesis facility, Iowa State University, Iowa, USA was utilized for sequencing the insert. The KS+ and M13 primers were used for initial sequencing of the cloned insert and later based on the DNA sequence of the insert, primers were designed and complete sequencing of both the strand was carried out. The insert of the recombinant phage DNA of clone 1 was also partially sequenced (single read) using the λgt11 forward primer to confirm both the sequences. The sequence was analyzed by using EDIT Sequence, Mega align and Map Draw soft wares (DNA STAR, USA)
Results:

a. The size of cloned insert:

The size of the insert in the immunoreactive recombinant clone was determined by restriction digestion and PCR amplification. The immuno-reactive recombinant phage DNA was purified and digested with restriction enzyme, EcoR I, to release the insert. The digested recombinant phage DNA exhibited the presence of an insert of approximately 1.1 kb upon electrophoresis (Fig 4.1). The PCR using the primers at the flanking regions of the cloning site also resulted in the amplification in a fragment equal in size to that released by EcoR I digestion (Fig 4.2). The insert of the immunoreactive recombinant phage was designated as KP1 insert (clone 1 insert).

b. Origin of the cloned fragment (KP 1 insert) and its copy number:

Southern blot analysis were carried out to confirm that the origin of the KP insert is from *P. falciparum* parasite and also to determine its copy number. In order to confirm its origin the clone 1 insert was labeled with radioisotope and used for probing the EcoR I digested *P. falciparum* and human DNA blotted on to nitrocellulose membrane. The insert hybridized to *P. falciparum* DNA but not to human DNA, thus confirming that the insert molecule is from *P. falciparum* (Fig 4.3 and 4.4). The probe was also hybridized to *P. falciparum* DNA digested with different restriction enzymes (EcoR I, Pst I, Sma I, Mse I and Nde I) to identify the copy numbers (Fig 4.5 and 4.6). The hybridization resulted showed stronger band in EcoR I digested DNA lane but fainter band of same size in other restriction enzyme digested lanes, which indicates that the gene may a single copy gene.
c. The cloned fragment is highly conserved:

To test the conserved nature of the isolated insert (KPI), the α dCTP $^{32}$P labeled insert was hybridized to different species of Plasmodium viz. P. yoelii, P. berghei, P. cynomolgi and two strains of P. falciparum (FCD 3, 3d7) by dot blot hybridization assay. The insert hybridized to all the species of Plasmodium with being stronger with P. falciparum and P. berghei (Fig 4.7).

d. Sub-cloning of insert DNA into sequencing vector and sequence analysis:

The insert was ligated into the sequencing vector pBKS+ (Fig 4.8), ligated mixture was transformed, and plasmid DNA was prepared from ten transformants. By EcoRI digestion of the plasmid DNA, one of the plasmid showed the insert (Fig 4.9). Both the strands of the insert were sequenced and the insert size was found to be 1132 bp. The nucleotide sequence of the cloned insert has been deposited in the Genbank (Accession number AF 256227, submitted on 15.04.2000, Fig 4.10). The A, C, G and T composition was 453 (40.0 %), 126 (11.1 %), 186 (16.4 %) and 367 (32.4 %) and thus the insert was found to be highly A+T rich (72 %), which is a typical characteristic of malarial parasites. The insert contained a complete open reading frame (ORF), which is shown graphically in Fig 4.11. The predicted amino acids sequences is presented fig 4.12. The translation product of the insert corresponds to a molecular mass of 42618.40 Daltons. Analysis of amino acid composition indicated acidic in nature (pI 4.348) of the protein with 8 Cystine residues in the insert. The insert is more hydrophilic and shows high antigenic index (Fig 4.13).
The DNA sequence of the insert was blasted in the databases [(http://www.ncbi.nlm.nih.gov), Altshul et al., 1997] and result is presented in Fig 4.14. The KP insert has showed 74 % protein homology with a small cDNA fragment (Accession No AF256227, submitted on 15th April 2000) of *P. falciparum*, which was described as hypothetical protein. However, it indicated considerable protein homology with karyopherin beta (importin beta) of other organism like Yeast, Drosophila, *C. elegans* and Human. The Clustal W alignment of the protein was presented in Fig 4.15. Three cysteine residues out of eight cysteine were found to be conserved but interestingly lysine residues were found to be more conserved among karyopherin beta of all the organisms. There are also stretch of amino acids like EQAVT (glutamic acid, glutamine, alanine, valine and threonine), KYY (lysine, tyrosine and tyrosine), RGK (arginine, glycine and lysine) and IECIS (isoleucine, glutamic acid, cystine, isoleucine and serine) are fairly conserved between karyopherin beta of all the organisms. Karyopherin beta is receptor protein well studied in yeast and human, which is involved in vital functions like nuclear and cytoplasmic transport and trafficking of proteins and other molecules. In malaria still its function yet to be discovered.

Discussion:

The cross-reactive clone identified by differential immunoscreening was found to contain an insert of 1132 bp. The restriction analysis of the recombinant phage DNA as well PCR amplification showed similar size of the insert. The southern hybridization studies using the released insert from the recombinant phage (KP1 insert) suggested that cloned insert was derived from *P. falciparum* and had high copy number in the genome. The fragment did not hybridize to human DNA, which is an important control in the
experiments. During malarial parasite DNA preparation there is every chance of human leucocytes DNA contamination, hence the southern blotting and hybridization is the first step in the molecular characterization and to prove that it is derived from *P. falciparum*. Southern blotting is one of the cornerstones of recombinant DNA analysis since its first description by Prof. E. M. Southern in 1975 and is used widely in molecular characterization. Dot-blot hybridization is an alternative to southern blotting and relatively simple technique to carry out to determine the relative abundance of target sequence in the blotted DNA preparation. The dot-bolt hybridization using KP 1 insert as a probe showed that the homologous gene is present in other species of malarial parasites as well as in two strains of *P. falciparum*. These results indicated that the gene is abundantly present and highly conserved across the species of malarial parasites. Conserved antigens are specially important, since they may contain invariant oligopeptides constituting targets of either broad range vaccine immunity (Saul et al., 1992; Tanner et al., 1995) or potent curative drugs (Land et al., 1995; Francis et al., 1994). During the present time, the availability of complete genome sequences of some of the organism and human made life easier to cross check the genome data bases and verify the source of the DNA as well as homologous genes.

*Plasmodium* parasite genome size is about 23 Mb coding approximately for 5300 genes spread in 14 chromosomes (Gardner et al., 2002). The *Plasmodium* genome is highly AT rich (Weber, 1987). According to Weber, *P. falciparum* genome had an A+ T content of about 82.0 %, which is higher than DNA of any other organisms. The coding sequence has around 69.0 %. The sequence analysis of KP 1 inset showed that it is 72 %, which is similar to the typical characteristics of *P. falciparum* nucleotide sequences. The
KP1 insert had a complete open reading frame, which suggests that it is the coding sequence of the protein. The sequence homology showed that it is a novel protein of *P. falciparum* showing significant homology with nuclear transport protein "Karyopherin" of other organisms such as yeast, including human and the accession number has been obtained from the Genbank (AF256227). Karyopherin, also called Nucleoporins, imports the proteins containing classical nuclear localizing signals (NLS) and docks these NLS proteins into the nuclear pore complex (NPC) (Nigg, 1997; Bobble *et al*., 1998, Chook and Bolbel, 1999). The Nobel laureate, Prof Blobel and his team have carried out substantial work on this protein in human and yeast but until now nothing is known about this protein in malarial parasites. It regulates many signal transduction pathways, which are necessary for the survival of any organism in its changing environment and for its growth and multiplication. The published report on the function and mechanism of karyopherin beta in transport and trafficking is presented in fig. 4.16 and 4.17.

Malaria parasite rapidly switches from one stage to other stages and multiplies very fast inside the RBC. Such a high multiplication rate must be necessitating transport and traffic of millions of molecules in and out of its system as well from host for its essential components. Information on the transport and trafficking of molecules with reference to malarial parasites is scanty. Recent discovery of utilization of host enzyme δ-aminolevulinate dehydratase (ALAD) by the parasite for heme synthesis has added a new phenomenon in the concept of parasite biology (Bonday *et al*., 2000). The understanding of protein and solute transport system, and identification and characterization of proteins involved in such systems will pay rich dividends in terms of identification of new chemoprophylactic/chemotherapeutic targets and development of
new and much needed antimalarial strategies (Opinion of the open meeting sponsored by Wellcome Trust and Novartis Foundation, 29th June 1999). The cloned *P. falciparum* insert (KP1) is conserved across different species of *Plasmodium* and is a partial coding sequence of a vital functional receptor protein "Karyopherin". Therefore we further characterized this molecule as described in next chapter.
Top:

**Figure 4.1.** Insert size determination of the immuno-reactive recombinant phage by restriction digestion. Recombinant phage DNA from the KP clone was digested with restriction enzyme to release the insert, subjected for agarose gel electrophoresis (0.8%) and stained with ethidium bromide. Lanes were loaded with Hind III digested λ DNA (Lane M), Undigested (Lane 1) recombinant phage DNA, EcoRI digested positive control phage DNA (Lane 2, known recombinant phage with 2.2 kb insert size) and EcoRI digested (Lane 3) immuno-reactive recombinant phage (KP clone) DNA.

Bottom:

**Figure 4.2.** PCR amplification of the insert from the immuno-reactive phage (KP clone) DNA and phage lysate by using λgt 11 specific primers (Forward and Reverse primer flanking the cloning sites). The amplified products were subjected for agarose gel electrophoresis (0.8%) and stained with ethidium bromide. Lanes were loaded with Hind III digested λ DNA (Lane M), amplified products of non-recombinant λgt 11 DNA (Lane 1: -ve control) and lysate (Lane 2: -ve control), amplified product of recombinant phage (KP clone) DNA (Lane 3) and its lysate (Lane 4).
A. Figure 4.3. Agarose gel electrophoresis of enzymatic digested human and parasite DNA. DNA was subjected to electrophoresis (80V) in 0.7% agarose gel and stained with ethidium bromide. Lanes were loaded with 1Kb DNA size marker (Lane M), EcoR I digested human DNA (Lane 1) and EcoR I digested *P. falciparum* DNA (Lane 2). The gel was used for Southern transfer and hybridization.

B. Fig 4.4. Autoradiogram shows the hybridization of both human and parasite DNA with radiolabelled KP insert (released by EcoR I digestions of the immunoreactive recombinant phage DNA). The DNA samples were subjected to electrophoresis, Southern transferred to the nitrocellulose membrane, hybridized with the $^{32}$P labeled KP insert, washed and exposed X-Omat Kodak film. The EcoR I digested human DNA (Lane A), EcoR I digested *P. falciparum* DNA (Lane 2) and 1 kb Maker DNA (Lane M- hybridized with 1kb ladder DNA radiolabelled probe). KP insert probe hybridized to *P. falciparum* DNA.
A. **Figure 4.5.** Agarose gel electrophoresis of *P. falciparum* DNA digested with different restriction enzymes. Digested DNA was subjected to electrophoresis in 0.7% agarose gel and stained with ethidium bromide. Lanes were loaded with Sma I (1), Nde I (4), Mse I (3), Sau III A (lane 4) and EcoR I (5) digested *P. falciparum* genomic DNA. Lane M was loaded with 1 kb DNA size marker. The agarose gel was used for Southern blotting and hybridization.

B. **Figure 4.6.** Autoradiogram showing hybridization of Southern blotted nitrocellulose membrane (*P. falciparum* DNA digested with different enzyme and southern transferred) with radiolabeled KP insert. Lanes showing binding of $^{32}$P - labeled KP insert to blots of *P. falciparum* digested with Sma I (1), Nde I (4), Mse I (3), Pst I (4) and EcoR I (5). Lane M is 1 kb DNA size marker, probed with radiolabelled – 1 kb maker DNA to highlight the size marker.
A. Fig. 4.7. Autoradiogram showing dot blot hybridization of radiolabelled KP inserts binding with DNA from different *Plasmodium* species. The DNA from different *Plasmodium* species were spotted manually on the nitrocellulose membrane, air-dried, hybridized with $^{32}$P-labeled KP insert. The hybridized bolt was washed and exposed to X-ray film. The spots were showing binding of KP insert with genomic DNA of *P. yoelii* (spot 1), 3D 7 strain of *P. falciparum* (spot 2), *P. berghei* (spot 3), FCD 3 strain of *P. falciparum* (spot 4), *P. cynomolgi* (spot 5), human DNA (-ve control spot 6) and KP insert DNA (+ve control spot 7)
Top:

Figure 4.8. Structural map of pBluescript II KS(+-) plasmid showing multiple cloning sites in the lacZ gene

Bottom:

Figure 4.9. Sub-cloning of KP insert (purified from immunoreactive recombinant phage by EcoR I digestion) into pBluescript II KS (+/-) plasmid at EcoR I cloning sites

Agarose gel electrophoresis of undigested pBKS recombinant plasmid (Lane 1), EcoRI digested recombinant pBKS plasmid (Lane 2) and 1Kb DNA size marker (Lane M) Lane 2 shows the subcloned 1 1 Kb KP insert DNA into pBKS II plasmid vector.
Figure 4.10. The nucleotide sequences of the KP insert obtained by automated DNA sequencing using the pBKS-KP recombinant plasmid DNA. The nucleotide sequences deposited in the *Plasmodium* genome databases and obtained the accession number (AF256227).
Plasmodium falciparum VCRC-MAL 1 protein (KPI) gene, partial cds.

REFERENCE
1 (bases 1 to 1132)
AUTHORS
Patra, K., Hoti, S., Malhotra, P., Das, P. and Chauhan, V.
TITLE
Cloning and sequence analysis of a conserved gene from Plasmodium falciparum
JOURNAL
Unpublished
FEATURES
organism="Plasmodium falciparum"

BASE COUNT
453 a 126 c 186 g 367 t

ORIGIN
1 gcgtttctca aattatatt ttagtttaga acaaaagcttg actgtcata taggttaattgc
61 aagttgtatt gaagaagact tttttaaaaata ttattctact gttttcctta tgtgaaaga
121 tattttacct aagggctgtt ttgtaagaga aagaaacctgc agagttagaa ccattggaatg
181 ttatttcatt attggttttat ggggtgtaaa atgattttttt atgaaagatt cttaaggtag
241 ttgatgttct cttttaaaat ttagctcttac aaaaaatttt ccggtgata tctgtaaaaaa
301 atatatttccaa aagggctgtt gcgtgtatag tagctcata ggttgatgact tttatcctat
361 tctaatcagat atagttctca gctatattatct gttttacattt accatttaatc
421 agaaggtgaa gcggatctct caaattatct ggttctagat ggcaaatgat taggatgtaa
481 aacattctta ttggtatttca aagaaaaagct tttagattta ttattatattattttagaatgt
541 attaaacatg attatatcct aacagaaacctgct agagttgctgctg ccattggaatg
601 aaattaagaa ttggctagat aaatttttca aagagttgctgctg ccattggaatg
gtaatcttgcttca aagatttttc attaaacatg aacagaaacctgc ccattggaatg
661 ttattatatg tggtaatattc tcaatgattt ccgaattatg accatgatat tctagcttctcattg
721 attaaacatg cagcatcagaaa aagtttttca aagtatttac gaaacaaatatgtagatcaca
781 ttatttttcatat atattatttct ttttcattc ttttaatcatt ttaaatttata tatccatcattc
tatttttcatat atattatttct ttttcattc ttttaatcatt ttaaatttata tatccatcattc
tatttttcatat atattatttct ttttcattc ttttaatcatt ttaaatttata tatccatcattc
tatttttcatat atattatttct ttttcattc ttttaatcatt ttaaatttata tatccatcattc
tatttttcatat atattatttct ttttcattc ttttaatcatt ttaaatttata tatccatcattc
tatttttcatat atattatttct ttttcattc ttttaatcatt ttaaatttata tatccatcattc
tatttttcatat atattatttct ttttcattc ttttaatcatt ttaaatttata tatccatcattc
tatttttcatat atattatttct ttttcattc ttttaatcatt ttaaatttata tatccatcattc
Top:

A. Figure 4.11. Graphical presentation of open reading frame KP insert in all possible six reading frame. The insert shows a completed open reading in 2nd frame.

Middle:

B. Figure 4.12. Predicted amino acid sequence based on the nucleotide sequence of the KP insert in 2nd reading frame.

Bottom:

C. Figure 4.13. It shows the hydrophilicity plot and antigenic property of the immunoreactive insert (KP insert). The plot was derived by using DNA star software using the predicted protein sequences of the KP insert.
A

NSSNYLLVRERQAVTAIAAVIGVIEEDFLKYSTVVPMMKDIIQKAVEESEERTCRGKAIECISIIGLSVGKDIFIEADEKCMNALLQISSSTKDMPDDTVEYIQEAVGIRICALGNDFYPYLSSIVPTLSVSPKPLTDEEDLTTTMVSNGQYVGLKTSLEDQEKAOLDIIIIEVLKENYKDYIQATATAVLPMNYELSDEIKQKALTAVSELIESARILSEKTDNKSMLLAILTAAAEKVLSLLETKLDDNYEIYLDVMIIIESHGLYMCQLKAGSNLVLPENTKLFFNQIFALLQYSTDRVVYNYKKNDDVDDEELLIDREEELEQNYRTNLDILGVLKHYHTQFLNTCELCIGFINNYMS

B

C

Hydrophobicity Plot - Kyte-Doolittle
Antigenic Index - Jameson-Wolf
Figure 4.14. BLAST search results of KP insert showing homology to different known proteins *Plasmodium* and other organism NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) blast search site was used to obtain the results and the immunoreactive *P. falciparum* insert (KP insert) shows homology with Karyopherin beta 3 of other organism.
## BLAST Search Results

### BLAST SEARCH RESULTS

Sequences producing significant alignments:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
<th>Score</th>
<th>E Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;emb</td>
<td>CAA06601.11</td>
<td>(AJ005572) hypothetical protein (Plasmodium...</td>
<td>146</td>
</tr>
<tr>
<td>&quot;p</td>
<td>C174177.1</td>
<td>importin beta subunit - fission yeast (Schizosaccharomyces...</td>
<td>134</td>
</tr>
<tr>
<td>&quot;p</td>
<td>CO041010181.1</td>
<td>Human IMPORTIN BETA-3 SUBUNIT (KARYOPHERNIN B...</td>
<td>132</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>NP_002262.1</td>
<td>karyopherin (importin) beta 3 &gt;gi</td>
<td>1212696</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>NP_014039.1</td>
<td>Karyopherin; Psalp &gt;gi</td>
<td>1709850</td>
</tr>
<tr>
<td>&quot;emb</td>
<td>CACA7393.1</td>
<td>(213528) PSE-1 [Saccharomyces cerevisiae...</td>
<td>127</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC14250.1</td>
<td>Ran-GTP binding protein; RanBP6 [Homo sapiens...</td>
<td>127</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AA952107.1</td>
<td>Karyopherin beta3 gene product [Drosophila...</td>
<td>122</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC036621.1</td>
<td>(AC006624) similar to other Ran_GTP binding...</td>
<td>90</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>NP_011035.1</td>
<td>Karyopherin beta 4; Kapi123p &gt;gi</td>
<td>1751502</td>
</tr>
<tr>
<td>&quot;ap</td>
<td>O005614191</td>
<td>SCHIDO HYPOTHETICAL 420.8 KD-PROTEIN CIFS.11C...</td>
<td>38</td>
</tr>
<tr>
<td>&quot;ap</td>
<td>O050100121</td>
<td>SCHIDO PUTATIVE IMPORTIN BETA-4 SUBUNIT (KARY...</td>
<td>37</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>NP_013881118</td>
<td>hypothetical protein B0261.2 - Caenorhabditis...</td>
<td>37</td>
</tr>
<tr>
<td>&quot;emb</td>
<td>CACA05691.1</td>
<td>(AJ007279) importin beta [Drosophila melanog...</td>
<td>36</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC660971</td>
<td>AF178855 1 (AF178855) Crmip [Candida albicans...</td>
<td>36</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AA238051</td>
<td>AF154120 1 (AF154120) sorting nexin 1 [Mus mus...</td>
<td>36</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC34680</td>
<td>AF122745 1 (AF222745) importin beta [Drosophili...</td>
<td>36</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>NP_014451.1</td>
<td>95 kDa structural and functional homolog o...</td>
<td>36</td>
</tr>
<tr>
<td>&quot;db</td>
<td>J492911</td>
<td>KIAA0829 protein [Homo sapiens]</td>
<td>35</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>NP_009181</td>
<td>TIP120 protein &gt;gi</td>
<td>179850</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>ID000184</td>
<td>probable phosphatidylinositol 3-kinase - fission...</td>
<td>35</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC041258</td>
<td>(AP15510) LRR F11-I interacting protein 1 [H...</td>
<td>34</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC60891</td>
<td>(AC024213) Hypothetical protein C346.1 [Cae...</td>
<td>34</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC1348</td>
<td>AF121334 1 (AF121334) TIP120 homolog [Eufolli...</td>
<td>34</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>NP_035241.1</td>
<td>leucine rich repeat (in F11) interacting ...</td>
<td>34</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>NP_003050.1</td>
<td>sorting nexin 1 &gt;gi</td>
<td>7513343</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC17182</td>
<td>(AF065483) sorting nexin 1 [Homo sapiens]</td>
<td>34</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC17183</td>
<td>(AF065484) sorting nexin 1A [Homo sapiens]</td>
<td>34</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC51448</td>
<td>(U77700) HsGCN11 [Homo sapiens]</td>
<td>34</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>NP_010515192</td>
<td>SCHIDO PUTATIVE TRANSLATIONAL ACTIVATOR C18G6...</td>
<td>34</td>
</tr>
<tr>
<td>&quot;pix</td>
<td>N122282</td>
<td>maltose ABC transporter, permease protein - Th...</td>
<td>33</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AA888235</td>
<td>(U63916) kinesin-like protein 1 [Schizosaccharodia...</td>
<td>33</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC07790</td>
<td>(AC010704 15 [AC010704] hypothetical protein [...</td>
<td>33</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>P02371</td>
<td>KINESIN-LIKE PROTEIN 1 &gt;gi</td>
<td>74921281</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AA71460</td>
<td>Similar to transcription factor g...</td>
<td>32</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC06116</td>
<td>(U49236) arginine deiminase [Giardia intestinal...</td>
<td>32</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>A4A69907</td>
<td>(M94100) S [porcine respiratory virus]</td>
<td>32</td>
</tr>
<tr>
<td>&quot;db</td>
<td>I4A99311.1</td>
<td>FctB [Staphylococcus aureus]</td>
<td>32</td>
</tr>
<tr>
<td>&quot;ap</td>
<td>P75514197</td>
<td>PRECURSOR E2 GLYCOPROTEIN PRECURSOR [SPIKE GLYCO...</td>
<td>32</td>
</tr>
<tr>
<td>&quot;ap</td>
<td>P2443197</td>
<td>PRECURSOR E2 GLYCOPROTEIN PRECURSOR [SPIKE GLYCO...</td>
<td>32</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC86765</td>
<td>ParC [Bacillus subtilis]</td>
<td>32</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>AAC86918</td>
<td>(AC004812) similar to human HsGCN1 U77700 [Por...</td>
<td>32</td>
</tr>
<tr>
<td>&quot;db</td>
<td>I4AA13729</td>
<td>(D6679) similar to Yeast translation activator...</td>
<td>32</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>A4A69906</td>
<td>(M94098) S [porcine respiratory virus]</td>
<td>32</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>A4A69905</td>
<td>(M94097) S [porcine respiratory virus]</td>
<td>32</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>A0450667</td>
<td>PARC [Homo sapiens] TOPOISOMERASE IV SUBUNIT A &gt;gi</td>
<td>7437475</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>A4A69908</td>
<td>(M94102) S [porcine respiratory virus]</td>
<td>32</td>
</tr>
<tr>
<td>&quot;ref</td>
<td>NP_101481.1</td>
<td>Contains a purine-binding domain, two hept...</td>
<td>32</td>
</tr>
<tr>
<td>&quot;gb</td>
<td>A4A69904</td>
<td>(M94096) S [porcine respiratory virus]</td>
<td>32</td>
</tr>
</tbody>
</table>
Figure 4.15. Clustal W alignment of amino acid sequence of KP insert with homologous known protein sequences viz. importin beta of Yeast (Acc no T411171), karyopherin beta of Human (Acc No 000410), karyopherin beta-3 of Yeast (Acc Nos, NP-02262.1, NP-014039.1, CAA77639.3), hypothetical protein of *P. falciparum* (Accession number AJ005572), Ran GTP binding protein (AF039023), karyopherin beta 3 gene product of Drosophila (AE003605) and Ran GTP binding protein of *C. elegans* (AC006624). The dark boxes in the alignment report indicate similar or matched amino acid sequences.
Alignment Report of 1Kb Protein align, using Clustal method with PAM250 residue weight table.

September 30, 2000 4:44 pm

Majority

KP-P.falciparum

Hypo-P.falciparum-AJ005572

IMPORTIN-Yeast-T411171

L Karyopherin-Human-000410

L Karyopherin-Beta-3- Yeast

Karyopherin-Yeast-NP-014039.1

PSE-1-Yeast-211538

Ran-GTP binding Protein-ACC14260

Karybeta3-AAP52017-Drosophila

Ran-GTP--AC006624-C. elegans

-------------------------------

VLEQAVTTIASVADAAEKKFVKYDTRLML

Majority /

KP-P.falciparum

Hypo-P.falciparum-AJ005572

IMPORTIN-Yeast-T411171

L Karyopherin-Human-000410

L Karyopherin-Beta-3- Yeast

Karyopherin-Yeast-NP-014039.1

PSE-1-Yeast-211538

Ran-GTP binding Protein-ACC14260

Karybeta3-AAP52017-Drosophila

Ran-GTP--AC006624-C. elegans

-------------------------------

LKHILQNAVQKELRLRGLGKTIECISLIGLA

Majority

KP-P.falciparum

Hypo-P.falciparum-AJ005572

IMPORTIN-Yeast-T411171

L Karyopherin-Human-000410

L Karyopherin-Beta-3- Yeast

Karyopherin-Yeast-NP-014039.1

PSE-1-Yeast-211538

Ran-GTP binding Protein-ACC14260

Karybeta3-AAP52017-Drosophila

Ran-GTP--AC006624-C. elegans
Report of 1Kb Protein align, using Clustal method with PAM250 residue weight table.

Cont
A. **Figure. 4.16:** Several of the major nucleocytoplasmic transport pathways are diagrammed over an electron micrograph of the yeast cell. Members of karyopherin β family involved directly in specific pathways in yeast and/or metazoan cells are shown in red. Blue denotes other major pathways for which the direct involvement of specific transport factors remain to be established. Abbreviations: C, cytoplasm; hnRNP, heterogeneous nuclear ribonuclear protein; N, nucleus; NES, nuclear export signal; NLS, nuclear localization signal; snRNP, small nuclear ribonuclear protein.


Bottom:

B. **Figure. 4.17:** A model for the importin-dependent nuclear protein import cycle. The initial cytoplasmic events in import is the binding of the import substrate to the importin α-β heterodimer, the α subunit provides the binding site for the NLS of the import substrate. The resulting trimeric complex then docks to the cytoplasmic periphery of NPC, and is subsequently translocated to the nuclear side of the NPC. The translocation is mediated by importin β, requires energy, which is probably provided by Ran GDP/GTP cycles, and is finally terminated at the nuclear side of the NPC by direct binding of Ran-GTP to the importin β, binding which disassembles the importin heterodimer. This disassembly appears to be a specific nuclear event because free Ran-GTP should be stable inside the nucleus only. The NLS-bearing imported protein is then released. The two importin subunits returned to the cytoplasm by different routes; importin β is probably exported as a complex with Ran-GTP, thus precluding binding of importin α to the importin β on the way out. The dotted arrow is used to differentiate importin export from import, and also to show that nothing definite is known about the export of importin to the cytoplasm. In the cytoplasm, the importin β-Ran-GTP complex needs to be dissociated before the importin α-β heterodimer can be re-form and accomplish the next round of import. The dissociation of the importin β from the Ran-GTP appears to involve the activities of RanGAP1 and ranBPI, which converts Ran-GTP into Ran-GDP. NE. Nuclear envelope, α- is importin α, β is importin β.
